

Lipodisks integrated with weak affinity chromatography enable fragment screening of integral membrane proteins

Duong-Thi, Minh-Dao; Bergström, Maria; Edwards, Katarina; Eriksson, Jonny; Ohlson, Sten; To Yiu Ying, Janet; Torres, Jaume; Agmo Hernández, Víctor

2016

Duong-Thi, M. D., Bergström, M., Edwards, K., Eriksson, J., Ohlson, S., To Yiu Ying, J., et al. (2016). Lipodisks integrated with weak affinity chromatography enable fragment screening of integral membrane proteins. *Analyst*, 141(3), 981-988.

<https://hdl.handle.net/10356/80267>

<https://doi.org/10.1039/C5AN02105G>

Lipodisks Integrated with Weak Affinity Chromatography Enable Fragment Screening of Integral Membrane Proteins

Minh-Dao Duong-Thi^{†,**}, Maria Bergström[†], Katarina Edwards[‡], Jonny Eriksson[‡], Sten Ohlson[§], Janet To Yiu Ying[§], Jaume Torres[§], and Víctor Agmo Hernández^{‡,*}

[†] Linnaeus University, Department of Chemistry and Biomedical Sciences, SE-39182 Kalmar (Sweden)

[‡] Uppsala University, Department of Chemistry-BMC, Box 579, SE-75123 Uppsala (Sweden)

[§] Nanyang Technological University, School of Biological Sciences, Singapore 637551 (Republic of Singapore)

* Corresponding author: victor.agmo@kemi.uu.se

** Current affiliation: Nanyang Technological University, School of Biological Sciences, Singapore 637551 (Republic of Singapore)

Abstract

Membrane proteins constitute the largest class of drug targets but they present many challenges in drug discovery. Importantly, the discovery of potential drug candidates is hampered by the limited availability of efficient methods for screening of drug-protein interactions. In this work we present a novel strategy for rapid identification of molecules capable of binding to a selected membrane protein. An integral membrane protein (human aquaporin-1) was incorporated into planar lipid bilayer disks (lipodisks), which were subsequently covalently coupled to porous derivatized silica and packed into HPLC columns. The obtained affinity columns were used in a typical protocol for fragment screening by weak affinity chromatography (WAC), in which one hit was identified out of a 200 compound collection. The lipodisk-based strategy, which ensures a stable and native-like lipid environment for the protein, is expected to work also with other membrane proteins and screening procedures.

INTRODUCTION

Membrane proteins are the by far most important targets in drug discovery and development.¹ The study of membrane proteins presents, however, challenges that are not easy to overcome. In particular, these proteins require a lipid environment mimicking their natural surroundings for optimal activity.² A variety of membrane-like structures, including self-assembled monolayers (SAMs),^{3,4} adsorbed lipid monolayers (ALMs),^{5,6} immobilized artificial membranes (IAMs),⁷ hybrid bilayer membranes (HBMs),^{8,9} supported or tethered bilayer lipid membranes (sBLMs and tBLMs),^{10,11} and proteoliposomes^{12,13} have been developed to reconstitute membrane proteins. Although useful in some cases, these structures as a rule suffer from drawbacks in terms of limited stability, restricted fluidity and/or lack of an actual lipid bilayer structure.¹⁴⁻¹⁶ As

a result, there is still a well-recognized need for the development of reliable biomimetic environments for the study of membrane protein - solute interactions. In this study, we show that lipodisks constitute a viable solution to the problems described above. We also demonstrate that proteolipodisks (i.e., lipodisks incorporating an integral membrane protein) covalently coupled to porous derivatized silica particles can be used for high throughput screening by means of weak affinity chromatography (WAC).

Lipodisks are composed of a circular planar lipid bilayer stabilized by lipids modified with a polyethyleneglycol headgroup (PEG-ylated lipids). The latter accumulate preferentially at the rim of the disks (Figure 1).¹⁷ By inclusion of functionalized PEG-ylated lipids, the lipodisks can be stably immobilized onto solid supports by covalent coupling.^{18,19} A specific advantage of lipodisks is that both sides of the lipid bilayer are exposed to the bulk solution. It is therefore possible to incorporate an integral membrane protein into the disk and always have the active site exposed to the surrounding media. Furthermore, the disks are usually immobilized perpendicular to the support,¹⁹ thus avoiding interactions between the protein and the solid surface. Also, in contrast to other planar lipid bilayer structures, such as nanodiscs²⁰ and bicelles²¹, lipodisks can be prepared with a wide range of compositions and size distributions, and they do not require the inclusion of any protein besides the one of interest. Furthermore, it has been shown that lipodisks can be prepared with lipid compositions mimicking those of actual cell membranes²². The usefulness of immobilized lipodisks is illustrated by recent reports concerning the study of lipid-peptide interactions,^{19,23} drug-membrane partitioning^{22,24-26} and the binding of the peripheral membrane protein COX-1^{18,27}. In the present report we take the lipodisk-concept one step further and demonstrate for the first time how the interactions between an integral membrane protein

and small molecules can be successfully studied using proteolipodisks covalently coupled to chromatographic media.

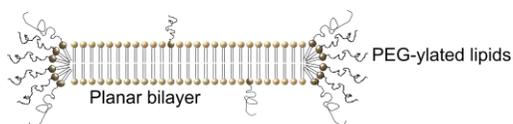


Figure 1: Cross-section of a lipodisk, showing the planar lipid bilayer stabilized by a rim of PEG-ylated lipids.

The integral membrane protein of interest in this report is the human aquaporin-1 (AQP1), a protein responsible for water transport across cellular membranes. This protein was chosen mainly because of its high robustness (it builds stable tetramers that do not denature at temperatures of up to 65 °C), and because its functionality can be easily tested by water permeability measurements²⁸. Furthermore, AQP1 plays important roles in clinical conditions such as edema, glaucoma, stroke, inflammation and tumor angiogenesis.^{29, 30} Several attempts have been made to identify and develop molecules that modulate AQP1 activity,^{29, 31, 32} but so far no approved drug for this target is available. In this work, recombinant AQP1 is reconstituted into lipodisks that have amine functionalized lipids. The material is then covalently coupled to derivatized silica particles and packed into an HPLC column. It is shown that the lipodisk-based system can be used together with WAC to detect the interaction of AQP1 with molecules in solution. WAC is a robust separation method based on reversible weak interactions (dissociation constant (K_D) in the range of 1 μ M - 10 mM) between solutes and immobilized biological molecules.³³⁻³⁶ WAC is especially useful in fragment screening; a strategy used in drug discovery to single out hits from a collection of organic substances with low molecular weight and weak affinity.³⁷ Fragment screening covers chemical space more efficiently and generates higher hit rate as compared to conventional screening of large molecules.³⁸ Fragment screening by WAC

using soluble proteins has been demonstrated using, e.g., thrombin,³⁷ cyclin G-associated kinase³⁹ and the ATPase domain of HSP90.⁴⁰ However, partly due to the lack of suitable model membranes, its application to membrane proteins has not been properly investigated. The immobilized proteolipodisks, as described in the current study, open up for the establishment of a new strategy that will allow the application of WAC in this particular niche.

EXPERIMENTAL SECTION

Chemicals

The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), N-palmitoyl-sphingosine-1-succinyl[methoxy(polyethylene glycol)2000] (Ceramide-PEG₂₀₀₀) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀ amine) were purchased from Avanti Polar Lipids (Alabaster, CA, USA). Cholesterol, octyl β -D-glucopyranoside (OG), sodium dodecyl sulphate (SDS) were purchased from Sigma (St. Louis, MO, USA). Further, 200 compounds were taken from a fragment library (PO6300-FBL-3200, TimTec LLC, Newark, DE, USA) for screening. The identity of the compounds is shown in Table S-2 in the Supplementary Information. Compound 7027127 (molecular weight: 344 g mol⁻¹, Simplified Molecular-Input Line-Entry System (SMILES):

c1cc2c(cc1CNC(=S)Nc3ccc4c(c3)nsn4)OCO2), was purchased from ChemBridge Corporation (San Diego, CA, USA)

Purification of human aquaporin-1 (AQP1)

His-tagged human AQP1 expressed from insect cells *Spodoptera frugiperda* was purified and found to be functional in a proteoliposome assay.⁴¹ The purification protocol is described briefly: cells were treated with lysis buffer (20 mM Tris-HCl pH 8, 300 mM NaCl, 2 mM β -mercaptoethanol (β -ME), 10 % glycerol, protease inhibitor cocktail, benzamide hydrochloride, 2 % OG

(Affymetrix, CA, USA)) and unsolubilized material was removed by centrifugation at $40\,000 \times g$ for 30 min at 4 °C. The supernatant containing solubilized AQP1 was purified by adsorption to Ni-NTA resin (Bio-Rad, CA, USA). After overnight protein binding at 4 °C, the resin was rinsed with washing buffer (20 mM Tris-HCl pH 8, 300 mM NaCl, 25 mM imidazole, 2 mM β -ME, 10 % glycerol) and AQP1 was released with elution buffer (20 mM Tris-HCl pH 8, 300 mM NaCl, 300 mM imidazole, 2 mM β -ME, 10 % glycerol, 1 % OG). Fractions with protein were pooled and concentrated using the Amicon® Ultra 30K centrifugal filter device (Millipore Corporation, Bedford, MA, USA) and purified with gel filtration chromatography using HiLoad 16/60 Superdex 200 preparative grade column (GE Healthcare Life Sciences, Uppsala, Sweden) at 4 °C. The used buffer was 20 mM Tris pH 8, 300 mM NaCl, 1 % OG. For weak affinity chromatography (WAC) experiments, purified proteins were buffer-exchanged to 10 mM HEPES buffer pH 7, 150 mM NaCl, 20 % glycerol, 1 % OG using the Amicon® Ultra 30K centrifugal filter device and stored at – 80 °C before use.

Lipodisk preparation

Two batches of lipodisks were prepared. The first batch (batch 1) consisted of lipodisks without protein (to be used in the control column). The second batch (batch 2) included the AQP1 protein.

For both batches, the lipids (DPPC, cholesterol, ceramide-PEG₂₀₀₀ and DSPE-PEG₂₀₀₀ amine) were first weighted to obtain a total of 10 (batch 1) and 11.8 (batch 2) μ mol lipid mixture with the molar ratio of 35:40:21:4, respectively. The weighted lipids were then dissolved in chloroform. Afterwards, the solvent was removed under a gentle nitrogen stream. The obtained lipid film was placed on a vacuum overnight in order to remove all traces of the solvent.

The films were then hydrated with, respectively, 800 μl (batch 1) and 483 μL (batch 2) of HEPES-buffer (50 mM HEPES, 50 mM NaCl, pH = 7.4), vortexed, and incubated for 20 min at room temperature ($\sim 22^\circ\text{C}$). Afterwards, the samples were subjected to three freeze-thaw cycles in liquid nitrogen and a 55°C water bath, resulting in a homogeneous suspension.

For the lipodisks without protein (batch 1), 400 μL of the corresponding suspension were taken and mixed with 357.5 μL of OG 200 mM in milliQ water. HEPES-buffer was then added in order to obtain a final volume of 1 mL. In this mixture, OG is found at a concentration of 71.5 mM, corresponding to 10 times the lipid concentration (5 mM) plus the critical micelle concentration (CMC, 21.5 mM) of the detergent. The tube was then incubated at 4°C overnight. This assures that all components are found in mixed micelles.

In the case of proteolipodisks (batch 2), 170 μL of a stock AQP1 solution (8.84 mg mL^{-1}) was added to the hydrated lipid film giving a total of 653 μl . To this mixture, a volume of 347 μL of 400 mM OG in MilliQ water was added. As in the previous case, the final concentration of OG (138.8 mM) corresponds roughly to 10 times the lipid concentration (11.8 mM) plus the CMC (21.5 mM) of the detergent. The sample was incubated overnight at 4°C to assure complete mixing of the components.

In order to produce lipodisks from the micellar solutions prepared above, gel filtration on a Sephadex G-50 column was performed. A peristaltic Pump (Minipuls 2, Gilson International, Den Haag, Netherlands) was used to control the flow rate to approximately $0.7\text{-}0.8\text{ mL min}^{-1}$. As mobile phase HEPES-buffer was used. The lipodisks and the detergent were eluted from the column as two well separated fractions detected with help of a Dual Path Monitor UV-2 (GE Healthcare, Uppsala, Sweden) operating at 254 nm. The eluted lipodisks were collected manually. In the case of proteolipodisks, the material was collected in four separate fractions in

order to be able to choose the material with the highest protein : lipid ratio. The phosphorus- and protein contents of all fractions were then determined (results are shown in Table S-1 in the Supplementary Information).

The collected samples were concentrated by ultrafiltration using a Minicon B15 concentrator (Merck Millipore, Billerica, MA, USA, cut-off value 15 000 Da). The protein- and phosphorus contents of these samples were also determined according to the protocols described in the corresponding sections. The results are summarized in Table S-1. As can be seen from the data in the table, the ultrafiltration step enhances the protein : lipid ratio of the first two fractions, suggesting that small lipid aggregates are removed. On the other hand, fractions 3 and 4 show a significant decrease of the protein : lipid ratio, suggesting, on the other hand, that protein not incorporated into the lipodisks is lost. It was therefore decided to use Fraction 1 for further experiments (higher protein : lipid ratio). Notice that the lipids after gel filtration are found mainly in the form of lipodisks.

Preparation of affinity HPLC columns

Immobilization of AQP1-lipodisks and control-lipodisks was performed through reductive amination. For immobilization of AQP1-lipodisks, 30.1 mg diol silica (10 μm diameter, 2100 \AA pore size, 18 m^2g^{-1} surface area, Kromasil, Eka Chemicals, Bohus, Sweden) was oxidized by periodic acid (100 mg mL^{-1}) in 3 h to produce aldehyde silica. The aldehyde silica was rinsed thoroughly with 3×0.2 mL water, then with 0.2 mL of 50 mM HEPES, 50 mM sodium chloride, pH 7.4. The obtained slurries were then mixed with 80 μL suspension of AQP1 lipodisks and 10 μL of 10 mg mL^{-1} sodium cyanoborohydride (final concentration of 0.67 mg mL^{-1}) in HEPES-buffer. Reaction mixtures were rotated at 4 $^\circ\text{C}$. Sodium cyanoborohydride (10 μL , 10 mg mL^{-1}) was added into the reaction mixture every 24 h. After 60 h rotating, 1 mg sodium borohydride

was added into the silica slurry followed by vortex mixing and rotating at 4 °C for 1.5 h. After that, all reactions were stopped by washings and centrifugations ($2000 \times g$, 2 min) with 4×0.2 mL HEPES-buffer. For control lipodisk immobilization, the same procedure was carried out except 90 mg diol silica and 60 μ L control lipodisk suspension was used.

After washing (4×0.2 mL HEPES buffer), the AQP1- lipodisk silica slurry was packed in HEPES-buffer into two stainless steel capillary columns (35×0.5 mm id.), control- lipodisk into one capillary by an air-driven liquid pump (Haskel, Burbank, CA, USA) at a pressure of 340 bars in 60 min. Approximately 5 mg silica (dry weight of diol silica) was needed to pack one column, of which ~ 3.2 mg stayed in the column. The remaining of silica slurries (AQP1- lipodisk and control-lipodisk) was saved for lipid and protein assays. In order to test the stability of the AQP1-lipodisks columns, test columns (35×2.1 mm id.) were prepared with not-fractionated proteolipodisks and the amount of lipids and protein immobilized on the substrate was determined. After repeated HPLC experiments, including protein inactivation experiments (see *Partial inactivation of AQP1 by acid treatment* in the Results and Discussion section), the columns were emptied and the collected silica portions were used to determine the lipid and protein contents. The same determination was done with the control-lipodisk column.

Quantitative protein analysis

Fluorescence determination

Measurements of the intrinsic protein fluorescence were performed with a SPEX fluorolog 1650 0.22-m double spectrometer (SPEX industries, Edison, NJ, USA). The excitation wavelength was set to 280 nm and the emission wavelength was 340 nm. These wavelength values correspond to the maximum absorption and emission of tryptophan respectively.

Aliquots of the samples to be studied (after solubilization, after gel filtration, and after ultrafiltration) were diluted to an expected concentration of $\sim 0.4 \mu\text{M}$ protein. A fluorimetric calibration curve was measured with six AQP1 solutions with concentration ranging between 5-400 nM. The intensity of the emission as measured for the samples was then used to calculate AQP1 concentration in the samples.

Dot-it-spot-it determination

The dot-it-spot-it protein assay (Maplestone Biotech, Knivsta, Sweden, <http://dot-it-spot-it.com>) is a newly developed method which allows quantifying protein found in small amounts and volumes. The determinations were made according to the manufacturer instructions. Shortly, 1 μL of the protein-containing sample (approx. 5 μg protein per mL) is loaded onto an analysis membrane sheet. After drying of the sample, the sheet is dipped in a solution containing carbon black particles, which bind to the proteins present. After rinsing with a washing solution, the blackness intensity of the dots is measured with an image scanner. The results are then compared with what is obtained from a series of standard solutions. Determinations performed with the samples obtained after gel filtration and after concentration provided the same results as the fluorimetric approach. The dot-it-spot-it method was then used to determine the amount of protein immobilized on the silica solely through the lipodisks (Figure 3a). For this purpose, aliquots from the slurries containing immobilized AQP1-lipodisks were mixed with equal volumes of a SDS 2 % solution and incubated at room temperature for at least 12 h. The mixtures were then centrifuged at $13\,000 \times g$ for 10 min. The supernatants were collected for dot-it-spot-it- and phosphorus analyses. The dot-it-spot-it experiments were performed in duplicates with 1 μL from the supernatants.

Quantitative lipid analysis

The concentration of lipid in the samples was calculated from their phosphorus content, assuming that 39 % of the lipid molecules contain a phosphorus atom (35 % from DPPC and 4 % from DSPE-PEG₂₀₀₀ amine according to the expected lipodisks composition). The phosphorus amount in the samples was determined by using the method developed by Murphy and Riley⁴² with the modifications described by Paraskova et al.⁴³ Briefly, a known volume of the samples to be characterized (after solubilization, after gel filtration, after ultrafiltration and after immobilization on silica followed by dissolution with SDS) was placed in heat resistant glass vials and calcinated at 550 °C for at least 4 h. When done, the samples were let to cool down to room temperature before dissolving the obtained ashes in 2 mL water. This was followed by the addition of 500 µL of a freshly prepared mixture of seven parts of 1:3:10 K(SbO)C₄H₄O₆ · 0.5H₂O (2.75 mg mL⁻¹) : (NH₄)₆Mo₇O₂₄ · 4H₂O (4 % w v⁻¹) : H₂SO₄ (2.5 M) and three parts of an ascorbic acid (0.1 M in water). After 15 minutes, the absorbance at 882 nm of the obtained solution was measured with an UV-Vis spectrometer (HP 8453, Agilent Technologies, Santa Clara, CA, USA). The concentration of phosphorus was calculated with the help of a standard curve prepared from different volumes of a phosphorus standard solution (0.65 mM, Sigma Aldrich, St. Louis, MO, USA) treated as described above.

Lipodisk characterization

After the ultrafiltration step, the lipodisks were characterized with Cryo-TEM and Dynamic Light Scattering (DLS)

Cryo-TEM

Cryo-TEM studies were performed with a Zeiss TEM Libra 120 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany) operating at 80 kV and in zero-loss bright-

field mode. Digital images were recorded under low-dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan, Scheuring, Germany). The method for sample preparation has been described in detail by Almgren et al.⁴⁴

Dynamic light scattering

The size distribution of the lipodisk suspensions was monitored by means of dynamic light scattering. The light source used was a Uniphase He-Ne laser emitting vertically polarised light at a wavelength of 638.2 nm and operating at 25 mW. Data were collected at 25 °C and scattering angle 90° using a PerkinElmer (Quebec, Canada) diode detector connected to an ALV-5000 multiple digital autocorrelator (ALV-laser GmbH, Germany).

Weak affinity chromatography

Sample preparation

200 compounds were arbitrarily selected from a fragment library (TimTec LLC, Newark, DE, USA). The library (PO6300-FBL-3200) included 3200 fragments with molecular weights (MW) from 75 to 289 (average 216); cLogP values were from -4.24 to 2.52; and number of heavy atoms was from 5 to 21. Compounds in the library were delivered at a concentration of 1.5 mM in dimethyl sulfoxide (DMSO), distributed in 40 Greiner 96-well plates with 80 compounds in each plate. Each mixture was obtained by mixing 10 compounds with distinctive MW together, then diluted 20 times with water to a final concentration of 7.5 µM for each compound in 5 % DMSO. In total, 200 compounds from TimTec library were selected and distributed into 20 mixtures. The compounds are listed in Table S-2 in the Supplementary Information.

Affinity screening

Affinity screenings were performed on an Agilent LC-MS system series 1200 (Agilent Technologies, Waldbronn, Germany). Samples were injected (0.2 µL) at 22 °C as mixtures of 10

compounds (see Table S-2). The mobile phase was 20 mM ammonium acetate, pH 6.9 and the flow rate was 15 μLmin^{-1} . The samples were detected by a single quadrupole mass spectrometer employing atmospheric pressure electrospray ionization. The spray chamber used a nebulizer pressure of 45 psig; drying nitrogen gas was at 9 Lmin^{-1} , 350 °C. Capillary voltages were set at 2800 V in positive and 2300 V in negative mode. Fragmentor voltage was 100 V in both modes, and quadrupole temperature was 100 °C.

The detection mode was selected ion monitoring on sample target masses, with alternating positive and negative ionization. Injections were duplicated and retention times were measured as peak apexes. The results from these experiments are summarized in Table S-3.

RESULTS AND DISCUSSION

Preparation and immobilization of proteolipodisks

The reconstitution of AQP1 into the lipodisks was achieved via a preparation method based on detergent depletion coupled with fractionation by size exclusion. The whole process was carried out at room temperature using aqueous buffers at physiological pH. The formation of lipodisks was confirmed by means of cryo-transmission electron microscopy (Cryo-TEM, Figure 2). The protein : lipid molar ratio in the prepared lipodisks was determined to be 1:267 (see Table S-1 in the Supplementary Information). The proteolipodisks were immobilized by reductive amination chemistry using diol-derivatized silica particles (10 μm in diameter, 2100 Å pore size). The immobilization capacity of the silica particles was determined to be ~22.4 nmol lipid (in the form of lipodisks) per mg silica.

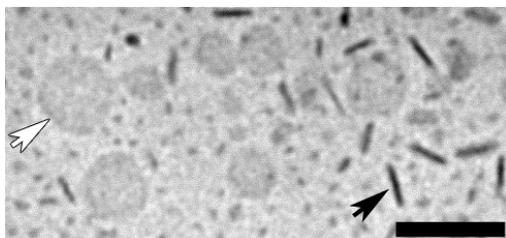


Figure 2: Cryo-TEM picture of the lipodisk suspension obtained after detergent depletion. The formation of lipodisks is confirmed by the appearance of “edge on” (black arrow) and “planar face on” (white arrow) images as expected from the different possible orientations of the lipodisks in suspension. The bar represents 100 nm. Note that the observed sample consists of a mixture of all fractions collected during preparation (see Figure S-1 in the Supplementary Information).

The protein found in the silica material can be either immobilized indirectly (i.e., solely via the lipodisks) or coupled directly to the silica via amine moieties present in the protein, as illustrated in Figure 3. Directly bound protein is most likely incorporated into lipodisks as shown in Figure 3b. The presence of detergent-stabilized protein (Figure 3c, also referred to as “bare” protein) is unlikely given the preparation method. Dynamic light scattering (DLS) experiments (see Figure S-1 in the Supplementary Information) confirmed that the samples used for immobilization were dominated by lipodisks and contained very few, if any, structures with a size small enough to be compatible with “bare” protein. It was moreover shown that the amount of immobilized material remained constant after column packing and performing repeated HPLC experiments, confirming that the lipodisks were well able to resist the experimental conditions (Table S-1, Supplementary Information).

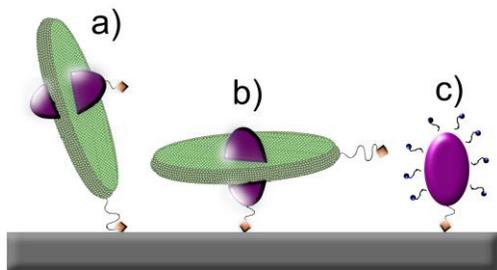


Figure 3: Representation of the possible forms in which AQP1 (represented as ovals) can be immobilized onto the silica material. Amine moieties are shown as squares: a) indirectly bound (solely via the lipodisks), b) directly bound reconstituted protein, and c) directly bound “bare” protein (surrounded by detergent molecules).

Weak affinity chromatography experiments

For the WAC experiments, the silica particles containing AQP1-loaded lipodisks were packed into an affinity HPLC column of 6.9 μl total volume (35 x 0.5 mm, *AQP1-lipodisk column*). The stationary phase in the column included silica (~ 3.2 mg), lipids (~ 72 nmol), and AQP1. The methods used to determine the protein content in the column allowed only determining the amount of indirectly immobilized protein. This was determined to be 160.3 pmol, meaning that more than half of the total protein (expected to be ~ 270 pmol according to the data obtained before immobilization) is found in structures resembling Figure 3a, while the rest is likely to be found in structures similar to those shown in Figure 3b. To characterize AQP1-specific interactions, control columns were packed with silica containing immobilized lipodisks without AQP1 (*control-lipodisk column*, ~ 34 nmol lipid), as well as with pure diol-silica treated in the same way as for the immobilization of AQP1 (*blank column*).

The AQP1-lipodisk and control-lipodisk columns were used to screen 200 compounds arbitrarily selected from a commercial fragment library (TimTec). A complete list of the compounds screened is found in Table S-2 in the Supplementary Information. Given that the tested

compounds may interact not only with the reconstituted protein, but also with the silica material and/or the lipodisks themselves, a clear definition of what can be considered as a specific AQP1 hit needs to be established. An ideal way of identifying specific AQP1 binders would be to compare the retention times of all compounds in the AQP1-lipodisk column with the retention times obtained in the same column after, somehow, inactivating the protein or blocking its active sites. Due to the currently rather limited knowledge about the AQP1 binding sites, and the lack of comprehensively characterized binders, this strategy poses, however, a difficult challenge. Therefore, we chose a different approach in our analysis and limited the hits to compounds that bind significantly more to the AQP1-lipodisk column than to the control-lipodisk column. Although this strategy is likely to generate false negatives (see discussion below), it completely eliminates the risk of false positives.

Hits were thus picked out by subtracting the retention time on the control-lipodisk column from the retention time on the AQP1-lipodisk column after normalizing the retention according to the lipid amount (i.e, correcting the retention time on the control column by considering the differences in lipid content between the control and AQP1 columns) to obtain an AQP1 *specific retention time*. Experimental values for all compounds are summarized in Table S-3 in the Supplementary Information. Compounds for which the specific retention time exceeded 0.72 min were considered as hits. The chosen threshold corresponds to 20 times the average standard deviation of duplicated injection of all compounds on all columns. Using this approach only one compound in the collection, ST013868, was identified as a hit, with an average specific retention time of 3.413 min (Figure 4). Noteworthy, ST013868 was not retained on the blank column, which means that its interaction with the silica matrix was negligible.

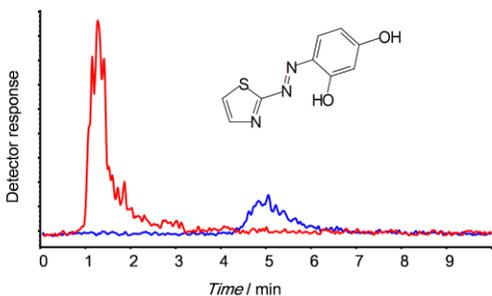


Figure 4. Extracted-ion chromatograms of ST013868 on AQP1-lipodisk column (blue) and on control-lipodisk column (red).

The affinity of ST013868 for AQP1 can be estimated according to equation 1:³⁶

$$K_D = B_{tot}/(t_{R\ AQP1} \times F) \quad (\text{Equation 1})$$

in which K_D is the dissociation constant, B_{tot} is the estimated number of AQP1 monomers in the affinity column (assuming that the 1:267 protein:lipid ratio of the original preparation holds, i.e., $B_{tot} = 270$ pmol), F is the flow rate of the mobile phase ($15 \mu\text{L min}^{-1}$), and $t_{R\ AQP1}$ is the AQP1 specific retention time (3.413 min). The value of K_D for the ST013868-AQP1 interaction thus calculated is $5.3 \mu\text{M}$.

It should be pointed out that the K_D value calculated for ST013868 should be regarded as the maximum possible, rather than the “true”, dissociation constant. There are several reasons for this. First, Equation 1 assumes that the interactions of the compounds with the lipodisks themselves are the same in the AQP1-lipodisk and control-lipodisk columns. However, the fact that most compounds showed a negative specific retention time (see Table S-3 in the Supplementary Information) suggests that the interaction with the lipodisks is decreased when the protein is present. We have at present no detailed explanation for this observation. It appears, however, that a significant proportion of the lipids in the proteolipodisks are unavailable for interactions with the compounds. It can be speculated that this situation arises because the lipids

are either covered by the protein, or engaged in more specific lipid-protein interactions. Second, it is possible that the amount of protein in the AQP1-lipodisk column is lower than determined from the protein:lipid ratio of the proteolipodisks prior to immobilization. Finally, the strategy used in the present study does not distinguish between the indirectly bound AQP1 (as illustrated in Figure 3a) and directly bound AQP1 (Figure 3b), although these are likely to present different affinities for the compound.

Partial inactivation of AQP1 by acid treatment

As mentioned above, the definition of hits used in the present study can lead to false negatives, i.e., several actual hits could be missed. In fact, we performed experiments with a proposed AQP1 inhibitor (compound 7027127 from the ChemBridge library³¹). The compound interacted significantly with both columns, resulting in negative specific retention times (see last row in Table S-3). This compound would, therefore, be overlooked by the current approach. As a possible way to circumvent this problem, we briefly tested an approach based on in-situ inactivation of the protein in the AQP1-lipodisk by acid treatment. The hit ST013868 and compound 7027127 from the ChemBridge library were selected for investigation by this method. AQP1-lipodisk and control-lipodisk columns (micro-columns, 35 x 2.1 mm) were treated briefly with 100 μ L of 25 mM glycine, pH 2.4 during 2 minutes. As expected, the retention time of ST013868 determined with the AQP1-lipodisk column was significantly reduced by the acid treatment (from 7.02 to 1.80 min) while it was virtually unchanged on the control-lipodisk column. This further supports that ST013868 is a true hit. Concerning compound 7027127, the retention time in the AQP1-lipodisk column was reduced from 24.548 to 21.264 min, a significant decrease, as expected assuming that the compound interacts with AQP1. No considerable change in retention was observed in the control column. However, as this approach

to determine specific binding of compounds cannot be validated using AQP1 (due to the lack of well characterized binders), we cannot make any definitive conclusions and it remains to be seen how this method can be adopted to determine specific binding in complex systems.

CONCLUSIONS

From the results in this report, it is clear that the lipodisk-WAC method represents a promising approach for the detection and characterization of drug- membrane protein specific interactions.

It is particularly noteworthy that a specific AQP1 hit could be unequivocally identified in the relatively small fragment library and despite the high complexity of the interactions studied.

Further, observations made in the current study indicate that promising compounds can be identified even if they also interact with the lipodisk bilayer membrane. There is, however, still some room for improvement. As determined by the protein assays, the amount of protein in the AQP1-lipodisk column was one to two orders of magnitude lower than what can be achieved with soluble proteins.^{34, 37, 45} With this amount of AQP1 in the column, only compounds with K_D values in the low μM range can be detected. In order to detect weaker interactions, a higher protein density is needed. Although no such attempts were made in the current study, it is conceivable that the protein content could be increased by isolating the proteolipodisks from empty lipodisks via, e.g., sucrose density gradient centrifugation⁹ prior to immobilization on the silica particles. Moreover, the lipodisks-based WAC method could be further improved by the use of alternative means for immobilization of the lipodisks. In particular, strategies that avoid the complications brought about by the possibility of direct coupling of protein to the matrix would increase the accuracy of the results.

Even though the lipodisk-WAC method has not been fully optimized, the encouraging results, as presented in this study, will motivate and provide the basis for further development of lipodisks-

based methods for the characterization and screening of interactions of molecular libraries with membrane proteins.

Acknowledgements

EKA (Bohus, Sweden) is acknowledged for kindly providing the silica particles. S. Ohlson and K. Edwards acknowledge funding by the Swedish Research Council (Vetenskapsrådet, VR). J. Torres acknowledges a pellet of *Spodoptera frugiperda* cells expressing AQP1 obtained from the NTU Protein Production Platform, and the funding of the National Research Foundation grant NRF-CRP4-2008-02 and MOE Tier 1 grant RG150/14.

Notes

The authors declare no competing financial interest.

References

1. C. J. Zheng, L. Y. Han, C. W. Yap, B. Xie and Y. Z. Chen, *Drug Discov. Today*, 2006, **11**, 412-420.
2. D. E. Warschawski, A. A. Arnold, M. Beaugrand, A. Gravel, É. Chartrand and I. Marcotte, *BBA-Biomembranes*, 2011, **1808**, 1957-1974.
3. M. Ornatska, *Organic Self-assembled Monolayers for Reconstitution of Ion Channels on Single Crystal Silicon*, Iowa State University, 2003.
4. F. Schreiber, *Journal of Physics-Condensed Matter*, 2004, **16**, R881-R900.
5. A. Nelson, *Biophys. J.*, 2001, **80**, 2694-2703.
6. C. Dietrich, Z. N. Volovyk, M. Levi, N. L. Thompson and K. Jacobson, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 10642-10647.
7. C. Pidgeon, J. Stevens, S. Otto, C. Jefcoate and C. Marcus, *Anal. Biochem.*, 1991, **194**, 163-173.
8. A. L. Plant, *Langmuir*, 1999, **15**, 5128-5135.
9. W. Ma, D. W. Li, T. C. Sutherland, Y. Li, Y. T. Long and H. Y. Chen, *J. Am. Chem. Soc.*, 2011, **133**, 12366-12369.
10. X. Y. Zhang, W. Y. Fu, C. G. Palivan and W. Meier, *Sci. Rep.*, 2013, **3**, 2196.
11. S. Gritsch, P. Nollert, F. Jähnig and E. Sackmann, *Langmuir*, 1998, **14**, 3118-3125.
12. K. Olesen, R. Karlsson, U. Lind, M. Davidson, A. Blomberg and A. Karlsson, *J. Chromatogr. B*, 2013, **931**, 84-89.
13. P. Seelheim and H. J. Galla, *Biochem. Biophys. Res. Commun.*, 2013, **431**, 519-523.
14. H. H. Shen, T. Lithgow and L. L. Martin, *Int. J. Mol. Sci.*, 2013, **14**, 1589-1607.
15. E. S. Gallagher, E. Mansfield and C. A. Aspinwall, *Anal. Bioanal. Chem.*, 2014, **406**, 2223-2229.
16. A. Czogalla, M. Grzybek, W. Jones and Ü. Coskun, *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, 2014, **1841**, 1049-1059.
17. A. Lundquist, P. Wessman, A. R. Rennie and K. Edwards, *BBA-Biomembranes*, 2008, **1778**, 2210-2216.

18. A. Lundquist, S. B. Hansen, H. Nordström, U. H. Danielson and K. Edwards, *Anal. Biochem.*, 2010, **405**, 153-159.
19. V. A. Hernández, K. Reijmar and K. Edwards, *Anal. Chem.*, 2013, **85**, 7377-7384.
20. T. K. Ritchie, Y. V. Grinkova, T. H. Bayburt, I. G. Denisov, J. K. Zolnerciks, W. M. Atkins and S. G. Sligar, in *Methods Enzymol.*, ed. D. Nejat, Academic Press, 2009, vol. 464, pp. 211-231.
21. E. A. Morrison and K. A. Henzler-Wildman, *BBA-Biomembranes*, 2012, **1818**, 814-820.
22. E. Johansson, A. Lundquist, S. S. Zuo and K. Edwards, *BBA-Biomembranes*, 2007, **1768**, 1518-1525.
23. K. Vainikka, K. Reijmar, G. Yohannes, J. Samuelsson, K. Edwards, M. Jussila and M. L. Riekkola, *Anal. Biochem.*, 2011, **414**, 117-124.
24. E. Boija, A. Lundquist, K. Edwards and G. Johansson, *Anal. Biochem.*, 2007, **364**, 145-152.
25. E. Boija, A. Lundquist, M. Nilsson, K. Edwards, R. Isaksson and G. Johansson, *Electrophoresis*, 2008, **29**, 3377-3383.
26. E. Johansson, C. Engvall, M. Arfvidsson, P. Lundahl and K. Edwards, *Biophys. Chem.*, 2005, **113**, 183-192.
27. E. Meiby, M. M. Zetterberg, S. Ohlson, V. A. Hernández and K. Edwards, *Anal. Bioanal. Chem.*, 2013, **405**, 4859-4869.
28. J. To and J. Torres, *Membranes*, 2015, **5**, 352-368.
29. A. S. Verkman, M. O. Anderson and M. C. Papadopoulos, *Nat. Rev. Drug Discov.*, 2014, **13**, 259-277.
30. S. Nielsen, J. Frokiaer, D. Marples, T. H. Kwon, P. Agre and M. A. Knepper, *Physiol. Rev.*, 2002, **82**, 205-244.
31. D. Seeliger, C. Zapater, D. Krenc, R. Haddoub, S. Flitsch, E. Beitz, J. Cerdà and B. L. de Groot, *ACS Chem. Biol.*, 2013, **8**, 249-256.
32. A. J. Yool, E. A. Brown and G. A. Flynn, *Clin. Exp. Pharmacol. Physiol.*, 2010, **37**, 403-409.
33. D. Zopf and S. Ohlson, *Nature*, 1990, **346**, 87-88.
34. M. Bergström, A. Lundblad, P. Pålsson and S. Ohlson, *J. Mol. Recognit.*, 1998, **11**, 110-113.
35. H. A. Engström, P. O. Andersson, K. Gregorius and S. Ohlson, *J. Immunol. Methods*, 2008, **333**, 107-114.
36. M. Bergström, S. Liu, L. Kiick Kristi and S. Ohlson, *Chem. Biol. Drug Des.*, 2009, **73**, 132-141.
37. M. D. Duong-Thi, E. Meiby, M. Bergström, T. Fex, R. Isaksson and S. Ohlson, *Anal. Biochem.*, 2011, **414**, 138-146.
38. M. M. Hann, A. R. Leach and G. Harper, *J. Chem. Inf. Comput. Sci.*, 2001, **41**, 856-864.
39. E. Meiby, S. Knapp, J. M. Elkins and S. Ohlson, *Anal. Bioanal. Chem.*, 2012, **404**, 2417-2425.
40. E. Meiby, H. Simmonite, L. le Strat, B. Davis, N. Matassova, J. D. Moore, M. Mrosek, J. Murray, R. E. Hubbard and S. Ohlson, *Anal. Chem.*, 2013, **85**, 6756-6766.
41. D. R. Carrillo, J. T. Y. Ying, D. Darwis, C. H. Soon, T. Cornvik, J. Torres and J. Lescar, *Acta Crystallogr. F*, 2014, **70**, 1657-1663.
42. J. Murphy and J. P. Riley, *Anal. Chim. Acta*, 1962, **26**, 31-36.
43. J. V. Paraskova, E. Rydin and P. J. R. Sjöberg, *Talanta*, 2013, **115**, 336-341.
44. M. Almgren, K. Edwards and G. Karlsson, *Colloid Surface A*, 2000, **174**, 3-21.
45. M. D. Duong-Thi, M. Bergström, T. Fex, R. Isaksson and S. Ohlson, *J. Biomol. Screen.*, 2013, **18**, 160-171.

Supplementary Information

Characterization of the structure, size, and lipid and protein content of the proteolipodisks; list of compounds screened by WAC, and the results from the chromatography experiments.