Application of fragment-based drug discovery to membrane proteins

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Chapter

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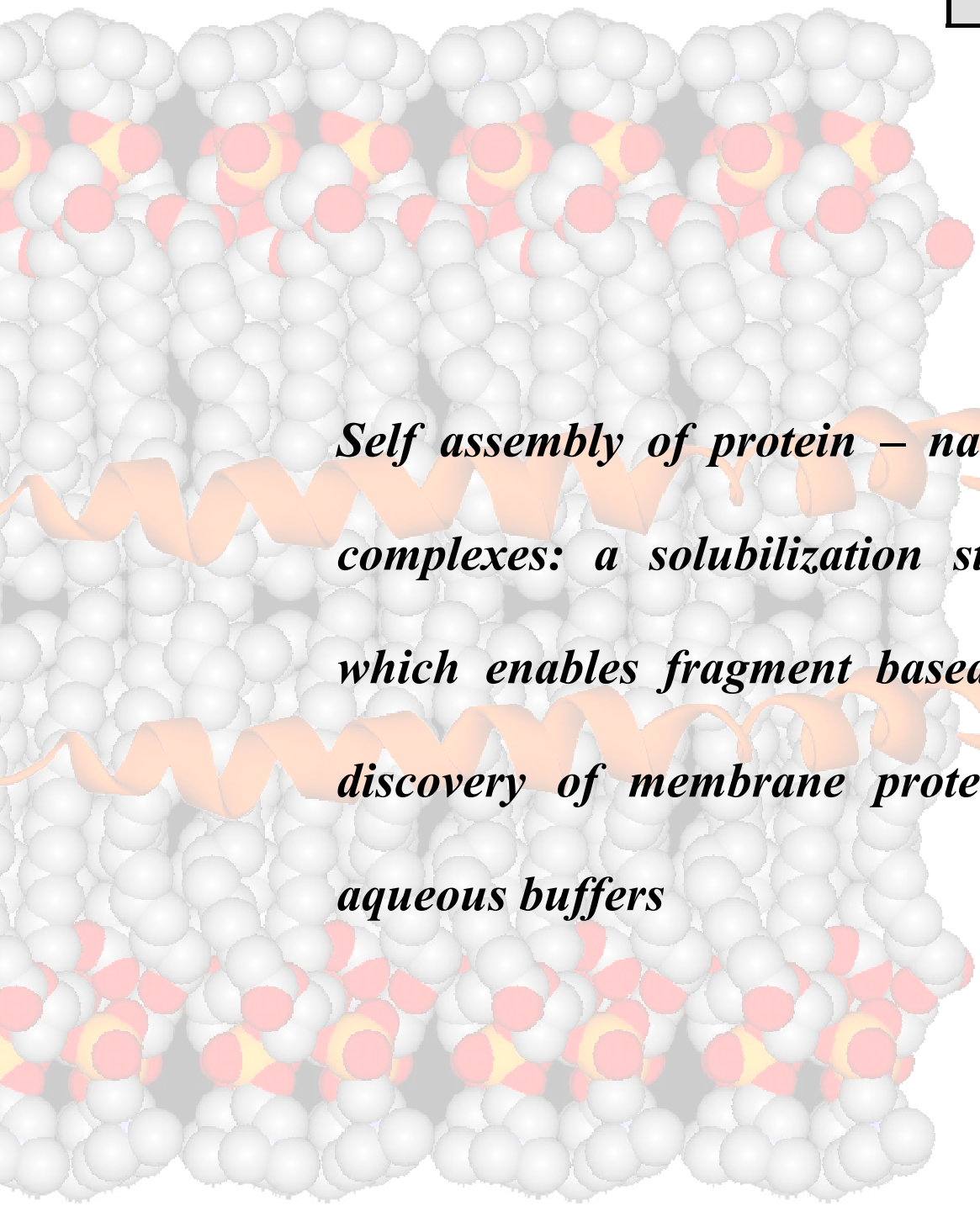

Self assembly of protein – nanodisc complexes: a solubilization strategy which enables fragment based drug discovery of membrane proteins in aqueous buffers

Present drug discovery methods for membrane bound targets typically rely on cell based assays to detect small molecules that bind to and modulate the behaviour of the target. However, these assays are not sufficiently sensitive to detect the binding of so-called drug fragments, molecules less than 300 Da in mass. It is necessary to use highly sensitive biophysical assays such as SPR, NMR or X-ray crystallography to detect binding of fragments to proteins, but the surfactants required to solubilize membrane proteins interfere with these assays. These issues have been addressed by solubilizing the target and a reference protein in nanodiscs (ND), a self-assembling lipid bilayer surrounded by an amphiphilic, helical protein. We show that the target, the Disulphide bond forming protein B (DsbB), is functional in ND, both when in solution and when immobilized on sepharose beads. Here we compare the performance of ND solubilized vs detergent solubilized DsbB in fragment screens using Target Immobilized NMR Screening (TINS). In these studies the ND solubilized and immobilized DsbB is stable during repeated cycles of fragment injection and washing in the absence of added lipid. The stability of the ND allowed us to compare the performance of empty ND vs reference protein solubilized in ND as a means to account for non-specific binding of the fragments to either protein or lipid. Our results suggest that empty ND makes a nearly ideal reference system and using this system, previously validated hits from a screen of detergent solubilized DsbB were readily detected. Our findings suggest a path to widespread application of fragment based drug discovery to membrane proteins.

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We describe the use of nanodiscs[68] (ND) as an alternative, detergent-free, membrane protein solubilization approach that enables biophysical detection of small molecule binding and is broadly applicable to membrane bound pharmaceutical targets.

Membrane proteins form the single largest class of targets for currently marketed small molecule drugs. High throughput screening has been a successful approach to discover small molecule modulators of membrane proteins, but the compounds derived from these studies often have chemical properties that are undesirable for an oral drug. Tailored, mechanism based compounds, such as kinase inhibitors, are showing great promise in the clinic with good specificity and reduced toxicity[247]. However, development of this class of drugs heavily relies on biophysical approaches such as Nuclear Magnetic Resonance (NMR), Surface Plasmon Resonance (SPR) and X-ray crystallography. Application of these techniques to soluble proteins is now widespread. In contrast, biophysical techniques, which require purified functional protein, have proven challenging to employ in studies of membrane proteins. Two particular issues that have been difficult to overcome are the necessity of solubilizing membrane proteins in a surfactant, such as in detergent micelles while maintaining protein function, and interference with the assay by the surfactant. Thus a possible solution to this bottleneck would be to employ non-detergent media to functionally solubilize membrane proteins.

The nanodisc has been developed as an alternative, surfactant free approach to solubilize membrane proteins. NDs consist of a lipid bilayer that is surrounded by a 23 kDa amphiphilic α-helical membrane scaffold protein (MSP). A variety of proteins have been functionally solubilized in NDs[248], which are much better mimics of the native membrane. However, the suitability of NDs for biophysical assays of ligand binding to membrane proteins has yet to be determined.

Target Immobilized NMR Screening (TINS) has been used to screen collections of small molecules (< 300 Da) for binding to a target[77]. TINS detects ligand binding *via* differences in the NMR spectrum of the compounds in solution recorded in the presence of an immobilized target and an immobilized non-binding reference protein. We have previously used TINS to identify inhibitors of the detergent solubilized, integral membrane protein DsbB (DsbB/DPC) using detergent solubilized OmpA (OmpA/DPC) as a reference (Chapters 4 and 5). Here we assess the

combination of TINS and NDs to a) provide a proper reference to account for non-specific binding of compounds and b) to detect known ligands in a screening assay.

We prepared empty NDs (-/ND), as well as NDs with embedded DsbB (DsbB/ND) or OmpA (OmpA/ND). Gel filtration analysis of our preparations revealed Stokes diameters of 9.63, 9.68, and 9.52 nm respectively, in accordance with literature values[68], suggesting that the complexes were well formed (Figure 1). To check for functionality, we used an enzymatic assay previously established for DsbB[99]. DsbB in *n*-dodecyl-β-D-maltoside (DDM) detergent had a substrate (Coenzyme UQ1) turnover rate of 298 ± 6 U* while DsbB/ND had a rate of 346 ± 13 U. The substrate UQ1 may have partitioned into detergent micelles effectively lowering its final concentration in solution, accounting for the apparent activity difference.

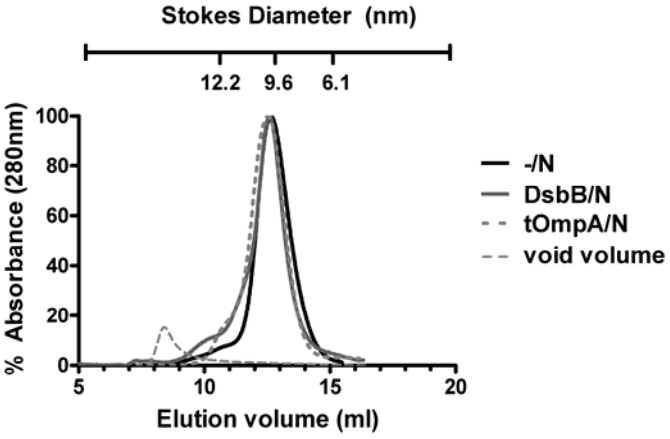

Figure 1. Characterization of empty NDs (-/N), DsbB/N and OmpA/N by gel filtration. The Stokes' Diameters were calibrated by using known proteins as standards.

After immobilization on a sepharose resin using Schiff's base chemistry, DsbB/ND had a substrate turnover rate of 329 ± 26 U strongly suggesting that it remained completely functional[23]. Interestingly, the efficiency of the immobilization reaction of DsbB/ND was 25 % higher than that of DsbB/DPC, suggesting that at least in part, immobilization involved MSP as well as DsbB. Immobilization via MSP could be a significant advantage because it is both general and avoids potential functional disruption by direct immobilization of the membrane protein.

In order to assess the suitability of the nanodisc system for ligand screening we selected 20 compound mixtures with and 20 mixtures without known ligands (a total of 183 compounds) from the screen of detergent solubilized DsbB. The influence of detergent or ND on the quality of the NMR spectra is shown in Figure 2. In both cases the compound whose spectrum is shown in

* Defined as M Q1/M DsbB-min^{-1}.

2**c** is readily identified as specifically binding to DsbB. However, the signal-to-noise ratio of the aromatic compounds (spectra **a** & **b**) in 2**e** is nearly double to that in 2**d** which enables better analysis of the aromatic peaks of the compound in 2**b,** which is now seen to bind DsbB. The reduced signal in the presence of detergent solubilized protein is likely due to non-specific partioning of 30 – 40 % of the compounds into the micelle, a result that is consistent with the biochemical data.

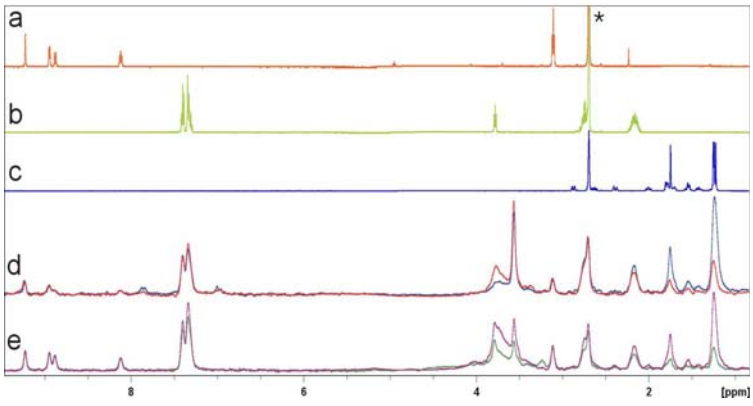

Figure 2. Effect of membrane protein solubilization system on NMR spectra of small molecule ligands. A reference NMR spectrum of each of three compounds (**a - c**). 1D ^{1}H NMR spectrum of a mix of the three compounds **a - c** in the presence of detergent solubilized OmpA (blue) or DsbB (red) (**d**) and respectively (magenta and green) the same solubilized in ND (**e**). The vertical scale in d and e is the same. The asterisk indicates the DMSO peak.

Micelles are inherently unstable, and we found it necessary to continuously reapply the detergent to maintain the functionality of immobilized, DPC solubilized DsbB[23]. In contrast, NDs appear quite stable. We monitored the integrity of DsbB/ND, as determined by binding of a known ligand, during multiple cycles of compound application and washing in lipid free buffers (Figure 3**a**). Here binding is represented by the ratio of the average peak height for each compound in the presence of Target (T) or Reference (R). Equal (non)binding of a compound to the target and reference results in a T/R of 1, while lower values indicate binding to the target and higher values indicate binding to the reference. Figure 3**a** shows that after an initial small degradation, the ligand binding capacity of DsbB remained constant, which implies that the NDs remained intact.

In TINS the reference plays an important role in balancing non-specific binding of compounds and helps to ensure a low false positive rate. The stability of the empty ND affords

the possibility to use NDs directly as a reference to account for non-specific ligand binding to the lipid bilayer and MSP instead of requiring a reference protein as such. To investigate this, we screened all 183 compounds for binding to DsbB/ND using either OmpA/ND or -/ND as a reference (Figure 3**b**).

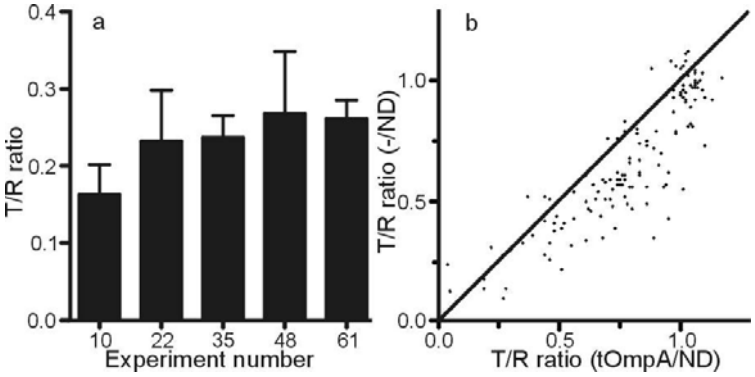

Figure 3. Stability of DsbB in nanodiscs (**a**, see text for details). Comparison of empty ND or OmpA in ND as a reference (**b**). The solid line indicates the position of a perfect correlation.

Overall there was a reasonable correlation (R^2 = 0.78, slope = 1) in ligand binding, as determined by the T/R ratio. Clearly however, the correlation is offset towards a higher T/R ratio in the presence of OmpA/ND, suggesting greater non-specific binding to this reference system.

In order to fairly evaluate the performance of NDs and the various reference systems, we assessed the biochemical activity of the hits in the screen. The compounds were tested at 250 μM using the previously described enzymatic assay. In total, 19 compounds gave significant biochemical activity (18 inhibitory and 1 stimulatory). Of these 19, 18 were detected as binding to micelle solubilized DsbB using micelle solubilized OmpA as a reference for TINS (Table 1).

	TINS Reference System		
Biochemical Hits:	OmpA/DPC	OmpA/ND	-/ND
Detected	18	5	17
Not Detected	1	14	2

Table 1. Correlation of biochemical and biophysical assays

The TINS screen of DsbB/ND using -/ND as a reference detected 17 compounds binding to the target. The screen using OmpA/ND as a reference detected only 5 of the biologically active compounds. An identical cutoff was used for all 3 screens. In general the T/R ratio was higher in the screen *vs* OmpA/ND than in the -/ND screen, as shown in Figure 3**b**. Although the overall pattern was similar in both, the T/R ratio for many compounds in the OmpA/ND screen lay over the threshold that was used to detect binders. This data suggests that OmpA/ND has a higher level of non-specific binding than -/ND. Thus empty NDs appear to perform better as a reference. Importantly, the combination of TINS with empty NDs is capable of detecting nearly 90 % of all biologically active compounds and is therefore quite useful as a tool to detect ligands that weakly interact with membrane proteins.

The reason for the higher level of non-specific binding of DsbB ligands to OmpA in NDs as compared to DPC micelles is not clear. Given the stability of OmpA, it is not likely that solubilization in the ND disrupted its structure, especially since it has been successfully solubilized in other non-detergent media[72]. One possibility is that the thickness of the POPC bilayer (46Å)[249] may not match the width of the β-barrel of OmpA (20Å), which could possibly leave exposed hydrophobic surfaces in the ND. The transmembrane portion of DsbB is about 30Å, which while less than the POPC bilayer, is considerably more than OmpA. This size mismatch may present a limitation with respect to the range of membrane proteins that can be successfully inserted into NDs. However, since NDs can be formed with different phospholipids, it should be possible to vary the thickness of the bilayer to adapt to smaller proteins.

Using the ND approach in TINS provided a more stable, biologically relevant mimic of the native membrane than detergent solubilization. Further, the partitioning of organic molecules into the hydrophobic phase appears to be significantly reduced in NDs. The empty ND forms an ideal reference system for ligand binding studies, accounting for non-specific binding of fragments to the MSP and POPC bilayer and greatly reducing false positives. These features make NDs a good choice for ligand studies using a variety of formats such as NMR. In view of the fact that a broad array of membrane proteins is compatible with NDs, it appears the path to widespread use of biophysical studies of ligand binding to membrane proteins may be open. The present protocol still requires purification and solubilization of the membrane protein. It may prove possible to

avoid both of these challenging steps by combining cell-free expression[250] with direct ND insertion of the target in order to reduce time and efforts required to solubilize membrane proteins.

Methods

Protein Purification

Protocols for gene expression and protein purification were carried out as previously reported for both OmpA[167] in *n*-octylpolyoxyethylene (C8POE) detergent micelles and DsbB[62] in *n*-dodecyl-β-maltoside (DDM) micelles. Both of these proteins have a 6x-HIS tag at the N-terminus.

Note that the OmpA used in this study is slightly different than the protein used in the ligand screening study of detergent micelle solubilized DsbB. The protein used in the current study is described in a study from the group of Popot[72]. This protein contains a 6 histidine affinity tag and three point mutation compared to wild-type, namely: K107Y, F23L and Q34K. The first mutation was deliberately introduced to enhance crystallization whereas the other two were inadvertently introduced[88]. The protein used in the previous study had 4 point mutation that were introduced to improve the quality of the NMR spectra[53]. In all cases, the mutations are on the outer face of the protein, in contact with the detergent or lipid molecules and therefore are not expected to have an influence on ligand binding.

Nanodisc self-assembly

The nanodisc self assembly procedure was repeated the same way for both OmpA and DsbB with slight adaptations from the previously reported procedures[68,251]. The reconstitution mixture contained Membrane Scaffold Protein MSP1D1(-) which lacked the HIS-tag, with mixed

micelles of POPC and cholate at a ratio of 1:65:130. This reconstitution mixture was added to the OmpA or DsbB in detergent micelles (each with 10 x the detergent CMC) in a volumetric ratio of 1:1 and left to incubate on ice for 4 hours. We always ensured a stoicheometry of MSP1D1(-) to OmpA or DsbB of 2:1. Upon addition of 0.7 mg/ml of the hydrophobic adsorbent Bio-Beads SM-2 (Biorad, Hercules, CA) and gently mixing for 4 hours at 4 ºC, the nanodiscs would undergo self-assembly. This step was limiting, whereby detergent removal below 4 hours was incomplete, but caused nanodisc complex malformation if carried out for longer (i.e 16h, data not shown). The HIS-tags of the embedded OmpA and DsbB were used to separate the empty non-tagged MSP1D1(-) complexes from the mixture by IMAC chromatography using Ni-NTA resin with buffers containing 100 mM Tris, 300 mM NaCl, and imidazole at 0 mM, 10 mM, and 100 mM for loading, washing, and elution, respectively. The assembly into a nanodisc appeared to have reduced the affinity of the proteins' HIS-tags for the nickel column. The eluted fractions were run through gel filtration (Superdex 200 10/300 from GE Healthcare) in order to remove the remaining aggregated non-embedded OmpA and DsbB, and to exchange the nanodisc-embedded proteins into Phosphate Buffered Saline (pH 7.6) for compatibility with the immobilization step required for TINS. A set of standard proteins were run through the gel filtration column in the same conditions to calibrate the Stokes' diameters of the eluted fractions

Immobilization and TINS

Fractions representing particles of 9.2 to 9.7 nm were pooled and quantified by SDS-PAGE band volume analysis (Quantity One by BioRad, USA) prior to immobilization and TINS screening, which were carried out as previously reported[23,252]. Approximately 150 nmoles of DsbB/ND or OmpA/ND were applied to 1 ml of resin (bed volume) and left to rotate gently overnight at 4 ºC in Phosphate buffered saline, pH 7.6. After this immobilization step, the supernatant was collected for quantification by centrifugation at 3000 rpm for 4 minutes at 4 ºC. The resin was further incubated for 2 hours at room temperature in 100 mM Tris, 100mM NaCl, pH 7.6 with 100 mM of reducing agent sodium cyanoborohydride in order to block the remaining unreacted aldehyde groups on the resin. Quantification of immobilized protein was carried out by

quantifying the supernatants before and after immobilization by measuring the absorbance at 280nm and by SDS-PAGE band volume analysis.

Once immobilized, the DsbB/ND and OmpA/ND were packed into two separate cells of a dual-cell sample holder[205] which enables capillary tubing to connect the cell to the autosampler Gilson 210. The dual-cell sample holder was then inserted into an 8 mm, ^{1}H selective, flow-injection probe in a 500 MHz magnet. 20 binder fragments and 20 non binder fragments, identified in a previous screen of DPC solubilized DsbB and OmpA, were present in 61 mixes, with 3 – 5 fragments per mix. The fragments were initially solubilized in stock solutions of d_6-DMSO at 100 mM, and subsequently diluted 200 fold in PBS buffer with a final DMSO concentration below 5 %. After each mix injection into the dual-cell sample holder, the pump flow was stopped and a 1D ^{1}H proton spectra of fragments in each sample could be independently acquired by spatially selective Hadamard spectroscopy[206]. Residual broad resonances from the sepharose resin were removed by a CPMG T2 filter of 80 ms. After each acquisition, PBS buffer completely void of detergents was injected for several minutes in order to wash off the mix and prepare the resin for the next mix injection. With a cycle time of 35 minutes which includes a 30 minute acquisition time and 5 minutes for sample handling, the ND screen was carried out in 1 ½ days.

Acknowledgements

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