Application of fragment-based drug discovery to membrane proteins

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"A chemist walks into a pharmacy and asks the pharmacist,
"Do you have any acetylsalicylic acid?"
"You mean aspirin?" asked the pharmacist.
"That's it, I can never remember that word."

Q: if both a bear in Yosemite and one in Alaska
fall into the water
which one dissolves faster?
A: The one in Alaska because it is Polar.

To my family:
Who taught me that laughter is always the best medicine!

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Abbreviations

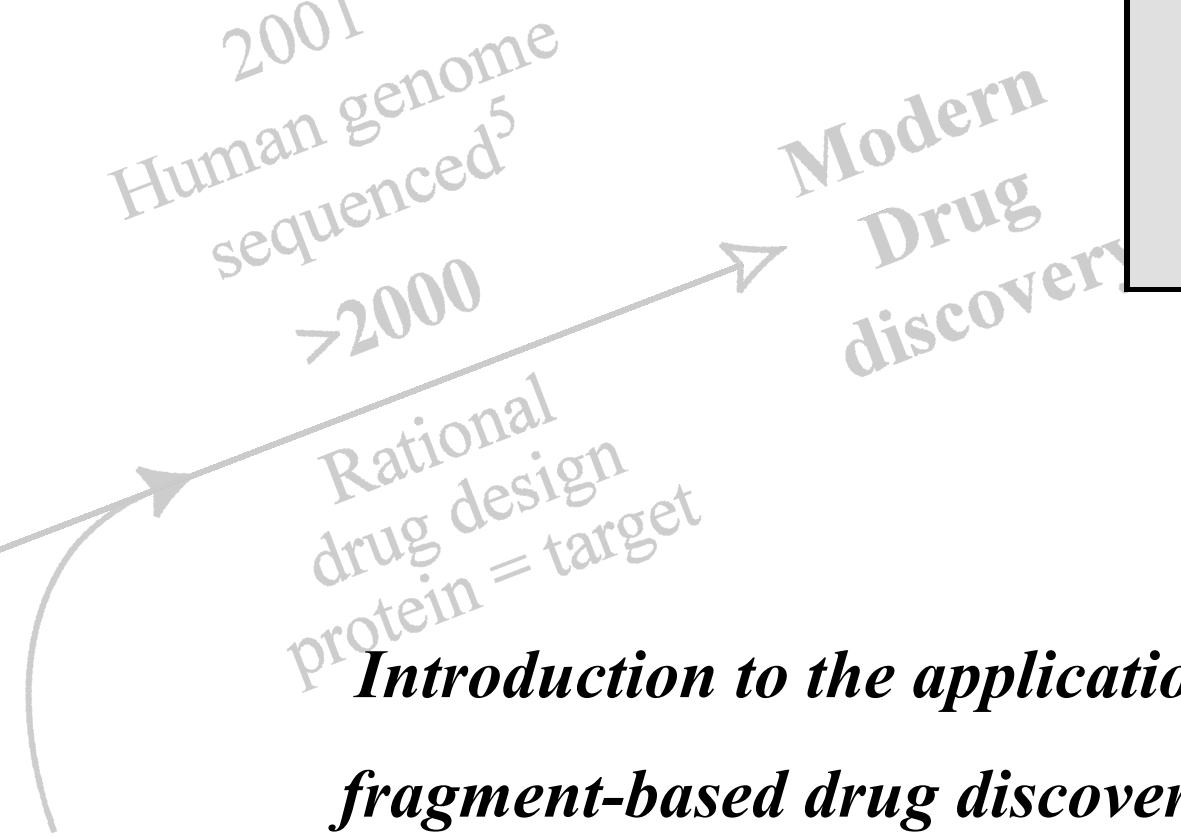
5-HT_3R	serotonin-gated mouse ion channel 5-hydroxytryptamine type-3 receptor
8-CPT	8-cyclopentyl-1,3-dimethylxanthin
ADA	adenosine deaminase
ATP	adenosine triphosphate
AMPPNP	adenyl-5'-yl imidodiphosphate
CB	cytochalasin B
CcO	cytochrome c oxidase
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHS	cholesteryl hemisuccinate
cLogP	logarithm of the partition coefficient between *n*-octanol and water
CMC	critical micellar concentration
CPA	N^6-cyclopentyladenosine
CTAC	etyltrimethylammonium chloride
CTAB	etyltrimethylammonium bromide
DDM	dodecyl-*n*-β-maltoside
DHPC	dihexanoylphosphatidylcholine
DMPC	dimyristylphoshatidylcholine
DMPE	1,2-dimyristoyl-sn-glycero-phosphatidylethanolamine
DMSO	dimethyl sulfoxide
DPC	dodecylphosphocholine
DPGPC	1, 2-diphytanoyl-*sn*-glycero-3-phosphocholine
DPPA	dipalmitoyl L-α-phosphatidic acid
DPPC	dipalmitoyl phosphatidylcholine
DPPE	1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
DsbB	disulphide bond forming protein B
FBDD	fragment based drug discovery

FID	free induction decay
FPMSMA	4-fluorophenyl)methylsulfanylmethanimidamide
FRAP	fluorescence after photobleaching
GAPS	γ-aminopropylsilane
GPCR	G-protein coupled receptor
GR	1,2,3,9-tetrahydro-3-[(5-methyl-1H-imidazol-4-yl)methyl]-9-(3-amino-(N fluoresceinthiocarbamoyl)propyl)-4H-carbazol-4-one
HA-hH_1R-HIS	heamaglutinin and 6-his tagged human histamine H_1 receptor
hA_1R	human adenosine A_1 receptor
HTA	ω-hydroxy-undecanethiol
IMAC	immobilized metal affinity chromatography
KcsA	K^+ channel from *Streptomyces lividans*
LB	langmuir–blodgett
LPC	lysophosphatidylcholine
N0840	N^6-cyclopentyl-9-methyladenine
NMR	nuclear magnetic resonance
NTA	nitriolotriacetic acid
OG	octylglucoside
OmpA	outer membrane protein B
PC	phosphatidylcholine
POPC	palmitoyl-oleoyl-phosphatidylcholine
POPG	palmitoyl-oleoyl-phosphatidylglycerol
PBS	phosphate buffered saline
PEEK	polyetheretherketones
PSLB	planar supported lipid bilayers
PWR	plasmon-waveguide resonance
QSAR	quantitative structure activity relationship
SAM	self-assembled monolayer
SCA	scaffold-based classification approach

SDS	sodium dodecyl sulfate
SERIAS	surface-enhanced infrared reflection absorption spectroscopy
SPA	scintillation proximity assay
SPFS	surface plasmon enhanced fluorescence spectroscopy
SPR	surface plasmon resonance
STD	saturation transfer difference
tBLM	tethered bilayer lipid membrane
TIFR	total internal reflection fluorescence microscopy
TINS	target immobilized NMR screening
TMA	tetramethylammonium chloride
TSP	trimethylsilyl-2,2,3,3-tetradeuteropropionic acid

Chapter

1



Introduction to the application of fragment-based drug discovery to membrane proteins

An overview of drug discovery

The need to relieve pain and suffering by medication has been with us since the dawn of humanity. Spiritual healing, accompanied by herbal medicines was passed on from ancestors who treated the symptoms of the soul. From then, a myriad of information and technological advances have contributed to each step leading to modern day drug discovery (Figure 1), where treatments are developed to treat the body.

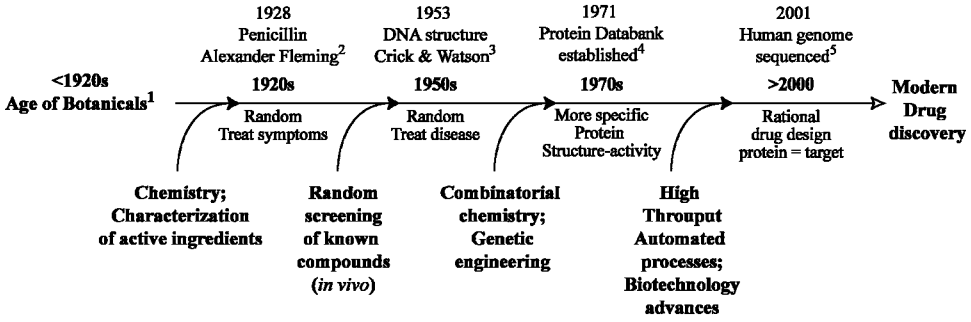


Figure 1. Historical overview of the drug discovery process.

The age of botanicals[2] was defined by the application of ancestral herbal medicines after trial and error determined which treatment was best for a particular ailment. During those days, each plant was believed to be designed to heal a particular symptom. It was not until the 19th century that active ingredients were isolated and characterized with the developing science of chemistry, such as the South American poison arrow curare by Claude Bernard[3]. From then, drug discovery was still very random with accidental discoveries such as Penicillin by Alexander Fleming in 1926[4], among many others. Developments in the 1950s led the way to serendipity in drug discovery, owing its name to the random screening of a variety of known compounds on animal models, with successful emergence of many drugs[5]. With breakthroughs such as the elucidation of the DNA structure by Watson and Crick[6], we started having a better idea of human biology. The low diversity of available molecules and the common usage of animal models with limited knowledge of drug mechanisms however, were still limiting the discovery of drugs with high specificity and low toxicity for the human protein targets. Finally, with emerging biotechnology and high throughput automated processes, the last 50 years have seen a boom in drug discovery strategies owing to our increasing knowledge related to handling and analysing biological

material. The emergence of genetic engineering and the protein data bank[7] provided us with the possibility of zooming into tissues, isolating and studying single protein targets to better understand the relationship between protein and drug structures. Combinatorial chemistry would enable an extremely wide variety of chemicals to be screened on a protein target rather than in an in vivo animal model, with potential specific changes to be made on different parts of the molecule to enhance qualities or decrease toxicity[5]. The sequencing of the human genome by the International Human Genome Sequencing Consortium has estimated that our genome contains 20000 to 25000 genes[8]. 10 – 15 % of these have been estimated to code for drug targets[9], exposing our bodies to modern day rational drug design.

Naturally, increasing knowledge of animal and human biology, developing technologies, and market considerations have altered the way the drug discovery process occurs. Large corporations of pharmaceutical companies for example are incorporating rational drug design strategies, in order to maximise the chances of success. The basic steps of modern rational drug discovery processes, which start by identifying a target and pave the way to identifying drug leads for clinical trials, are highlighted in Figure 2.

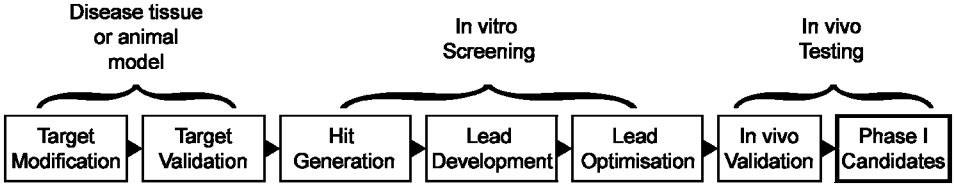


Figure 2. Basic steps in modern drug discovery.

There are increasingly perceptive methods for each of the steps involved, ranging from virtual to experimental ones. The central theme revolves around obtaining valuable information related to structure-activity relationships between the target and hit compounds in order to develop lead compounds which have an advanced trade off between better affinities and specificities, and better drug-like ADME (Absorption, Distribution, Metabolism, and Excretion) properties, as predicted by Lipinski's rule of "5" [10] (Figure 3**a**).

a

Rule of "5"
< 500 Da
< 5 H donors
< 10 H acceptors
Log P < 5

b

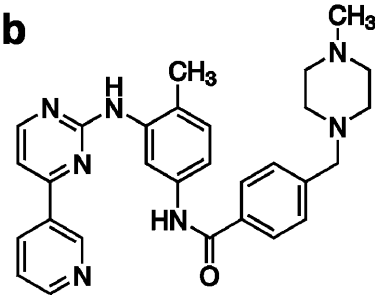


Figure 3. Predicting what makes a compound a good bioavailable drug. Lipinski's rule of "5"[10] defines the prerequisites for a good orally available drug (**a**) such as imatinib mesylate (Gleevec) (**b**), used as a drug to treat leukemia[11].

Before sensitive molecular methods had evolved, the effect of a compound on a target could only be measured by detecting an effect on target function. In order to detect such effects the compounds had to interact strongly with the target, often leading to the necessity of screening fairly large compounds in functionality based assays (Figure 3**b**). These large compounds, however, were often difficult to chemically elaborate without violating the rule of 5 due to increased hydrophobicity upon chemical elaboration. This type of screening, now coined traditional High Throughput Screening (HTS), was widespread in the last decades but was not always successful for some targets[12]. With emerging molecular methods such as Nuclear magnetic resonance (NMR), crystallography, and surface-plasmon resonance (SPR), fragment-based drug discovery (FBDD) was developed to detect hits for those targets for which HTS and other methods failed, and soon, the advantages of this strategy became more apparent.

Smaller is better: Why use a fragment approach to drug discovery?

As can be seen in Figure 4**a** with the example of a complex binding site, the increasing size of a molecule increases the probabilities of detecting it, but the probability of finding a unique match between ligand and target has an optimum at a very low complexity level. There is therefore a trade-off between the probability of detecting binding and the probability of finding a good match between ligand and target. When screening is carried out with smaller molecules, there is a better chance of finding a unique binding mode, but the binding affinity is very low

compared to larger molecules. Molecular methods are therefore required to detect the weak interactions between a target and a fragment.

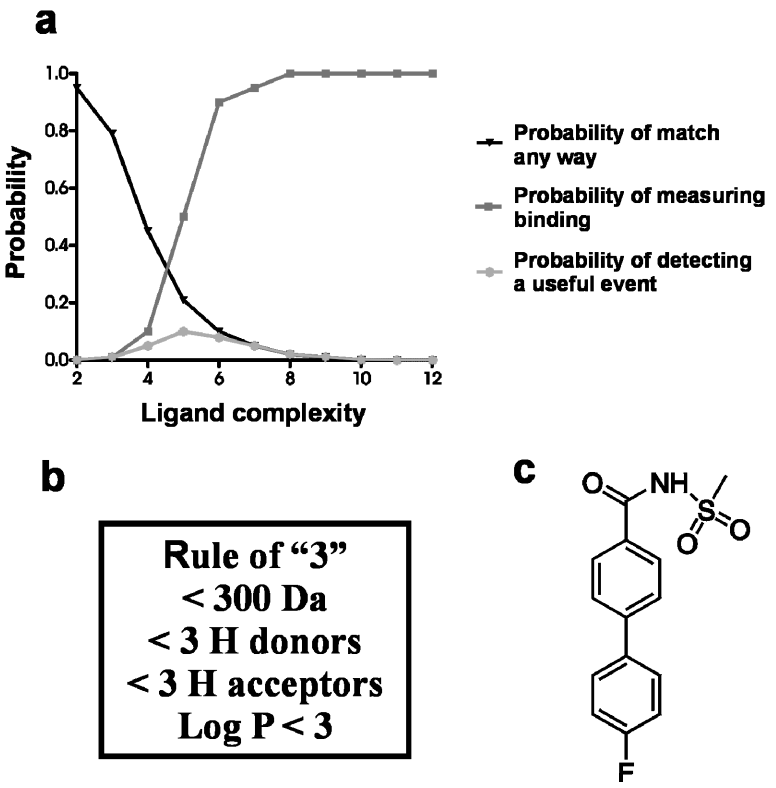


Figure 4. Using fragments in drug discovery. The success landscape for a binding site of high complexity, adapted with permission from reference 13. Copyright 2001 American Chemical Society (**a**). The rule of "3" (**b**) can be applied to predict the bioavailability of a fragment such as the one used as inhibitor of BclxL, a membrane protein involved in the survival of cancer cells[14] (**c**).

Using biophysical methods which detect interactions at the atomic level can now enable screening of fragments which were otherwise undetected by HTS methods. The high probability of finding a good match between a target binding site and a fragment is not the only advantage of FBDD. By definition, fragments are molecules which are smaller, simpler, and more soluble than drug-like compounds. This brings the Lipinski's rule of 5 down to a rule of 3[15] (Figure 4**b**) where there remains a larger margin to elaborate or link the fragment into a more potent drug which remains bioavailable. An example of a fragment is shown in Figure 4**c**.

Small – With molecular weights ranging from 160 - 300 Da, these fragments are usually easier to synthesize than the larger HTS compounds. The maximum amount of fragments that can possibly be synthesized are estimated at 10^7, as opposed to 10^{60} larger HTS compounds[16]. Furthermore, as can be seen in

Figure 5, these smaller structures are much more efficient at probing key binding areas in a target where larger compounds may sterically hinder access to these areas[17]. Clearly, the advantage of using fragment libraries lies in the fact that fragments can be smaller and yet more efficient at probing the available structure pool! For comparison, a million compounds are often screened for HTS strategies, compared to 1000 fragments in FBDD[18].

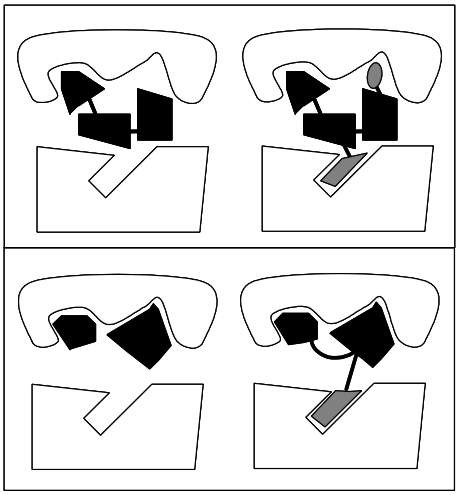


Figure 5: Capacity of an HTS compound (**top, black**) or fragments (**bottom, black**) to probe the key binding areas of a protein binding site (**white**). Fragments are better starting points and can be elaborated into larger compounds (**bottom, grey**) with better specificity and bioavailability than HTS compound (**top, grey**) without easily violating Lipinski's rule of 5.

Simple – Fragments are simple structures which tend to be void of reactive groups that can lead to toxicity[19] or metabolic instability[12]. Screening fragments is amenable to finding lead products with better starting bioavailability profiles[20], thereby reducing the often laborious chemistry efforts required to remove initial unwanted functional groups.

Soluble – As previously mentioned, previous research has established that elaborating compounds by modifying or adding chemical groups often leads to more hydrophobic compounds which easily violate Lipinski's rule of 5. FBDD is a good alternative approach to HTS because the more soluble fragments are better starting points for optimisation as a variety of chemical elaborations can be carried out with less failure in developing drug-like molecules with good ADME properties[13].

As explained, fragment screening relies on the detection of fragments which bind weakly to target proteins. In most cases, these fragments need to be elaborated into larger compounds with higher affinity. Lead optimization can be carried out in a variety of ways, but often depends on having a high resolution target structure available. This

enables one to identify which chemical groups on the fragment to modify in order to have more potent and specific affinities with the protein target binding site. Computational chemistry and informatics therefore play a key role. The initial idea was to link fragments together, whereby linking small weak fragments would lead to higher potency compounds. Although this was successful in many cases[20,21,22], linking chemistries are not always successful and can be replaced by elaboration of single or overlapping fragments by addition/removal of groups until the desired outcome is produced[14], or by screening focused libraries around a central theme scaffold of previously identified hits[12].

Requirements for fragment-based drug discovery

Interactions between proteins and fragments range from 2 μM to 5000 μM affinity values[23], hence the requirement to apply molecular methods such as NMR, crystallography, or SPR to detect hits in fragment based screens. These sensitive methods which generate invaluable information regarding target-fragment interactions at the molecular level, however, come with some stringent requirements when it comes to sample preparation and assay development.

Clearly, to obtain high resolution information at the molecular level, the target protein sample has to be highly concentrated and purified from its environment in order to increase signal to noise levels. Naturally, individual applications have their additional requirements, such as the need for high resolution crystals and functionally immobilized proteins in crystallography and SPR, respectively. Developing individual protocols for each target protein sample preparation and assay development can be extremely time consuming and often cause large amounts of proteins to be needed in the course of action. Furthermore, large amounts of fragments are often also required.

Crystallography requires 10 – 50 mg of protein, often with purity higher than 95 %, and the resulting crystals do not always diffract appropriately to provide high resolution structural information[24]. Screening involves either co-crystallizing targets with fragment cocktails, or soaking target crystals in fragment cocktails, with individual fragment concentrations ranging between 25 – 100 mM[24]. Nonetheless, there have been numerous success stories with soluble

protein targets at the heart of cancer, including kinases[25,26] and AIDS, such as reverse HIV transcriptase[27].

NMR-based fragment screening is carried out in solution and involves detecting changes in ^{1}H, ^{13}C, or $^{1}H^{15}N$ correlation signals of the protein target or the ligand. Target-based screening such as 'SAR (structure-activity relationships) by NMR' developed by Abbott laboratories enabled identification of inhibitors for cancer target tyrosine phosphatases[28] and tissue target matrix metalloproteases[20]. It is reported that 50 – 200 mg of isotopically labelled protein, soluble within the range of 0.1 - 1 mM, are required for such experimentations. These high quantities are necessary in order to screen one fragment at a time on individual protein samples, due to the inherent system which only identifies structural, albeit valuable, information regarding the molecular effects on the protein and not the ligand. Not surprisingly, the availability of a high resolution structure and assignments for the labelled protein is a prerequisite to establish the structural effects upon fragment binding and to identify the binding site for future chemical elaboration of hits into leads. There are alternative strategies which require smaller amounts of unlabelled protein such as ligand-based screening methods where changes in the magnetisation environment of the ligand are monitored, either by magnetisation saturation transfer (STD)[29] or by proton relaxation differences[30]. These systems enable higher screening throughput due to the smaller amounts of unlabelled protein required (down to μM concentrations) and the possibility of identifying hits within a cocktail of fragments. The changes are often monitored by comparing fragment spectra in the presence and absence of the target and therefore, ligand libraries must be designed to allow appropriate signal deconvolution, or isolation of signals[31]. These NMR techniques can be carried out with off-the-shelf materials and rarely need as much effort in assay development as for crystallography and SPR techniques.

Although SPR techniques were only used for secondary screening due to limitations in throughput capacities, new advances in microarray immobilization and SPR imaging techniques are providing platforms which can now enable SPR-based primary screening of fragment libraries[32]. SPR measures changes in surface electromagnetic waves refraction indices upon adsorption of an analyte to a particular surface, such as with or without an immobilized target protein. Although this was limited to detect only differences caused by adsorption of large

biomolecules, such as target proteins or DNA, advanced techniques now enable fragment binding to an immobilized target. SPR requires high efforts of assay development for each target protein, in order to enable monitoring and to control the stability of the surface, the levels of functional protein immobilized, the level of unbound protein leakage, and levels of non-specific binding of fragments to the surfaces[33]. Nonetheless, once the appropriate assay is developed, very little amounts of protein are required with down-scaling of surface to several squared nanometers in size. Furthermore, labelled analytes or proteins are not required.

Why are membrane proteins such important drug targets?

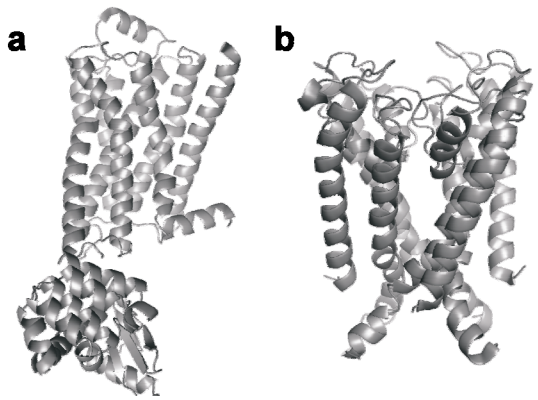


Figure 6. Structures of GPCRs and ion channels. The human GPCR β_2-adrenergic receptor (**a**) and the bacterial potassium channel KcsA which has close homology with human ion channels (**b**) have α-helical transmembrane domains. The structures are modified from pdb IDs 2rh1[34] and 2kb1[35] respectively.

60 % of today's drugs target membrane proteins[36]. As their name indicates, these proteins are located within the native cell membrane and are therefore involved in a multitude of cellular processes related to signalling, transportation, energy production, metabolism, and homeostasis. Clearly, if any of these proteins within these complex networks malfunctions, onsets of a variety of symptoms and diseases can occur.

Two major groups of membrane proteins are currently targeted due to their extremely important roles in maintaining healthy cellular processes. These are G protein-coupled receptors (GPCRs) and ion channels (Figure 6).

GPCRs are proteins which span the membrane with 7 transmembrane α-helices (Figure 6**a**) and are involved in translating an external stimulus (e.g., light, hormones, or neurotransmitters) into an internal signal in the cell. This internal signal is regulated by a signalling cascade, starting with the G protein to which the GPCRs are coupled to on the inside of the cell, down to complex

networks of other effector proteins such as kinases, ion channels, transporters, and enzymes. As such, GPCRs are at the heart of almost all physiological processes and are targeted by 30 - 40 % of drugs on the market today[37]. Faulty GPCR signalling, often due to lack of or surplus of neurotransmitters or hormones, leads to many common human disorders associated with the central nervous system, the cardiovascular system, vision, asthma, allergies, and the immune system[38], to name but a few. Targeting GPCRs in drug discovery is far from simple, for the pharmacology mechanisms are complex and diverse. The active, inactive, or self-activating (constitutive activity) states of GPCRs is determined by the type of ligand and where it binds on the GPCR. Agonists and antagonist ligands will therefore activate or inhibit the receptor, respectively. However, research has revealed constitutive activity of some GPCRs, which translates to a basal activity independent of ligand binding. Inverse agonists are therefore becoming interesting drugs as they block this type of signalling cascade and may at times be more effective than (neutral) antagonists[39]. To make matters more complex, small molecules called allosteric modulators have been noted to modulate the way these ligands bind into stronger stimulation or inhibition of activity[40].

GPCRs which are targeted by drug discovery include the Class A (rhodopsin-like) receptors and bind a range of molecules from small molecules to large peptides[41]. Some of these Class A receptors such as the adenosine receptors, histamine receptors, and the β-adrenergic receptors are good candidates for small molecule drug discovery because of the small sizes of their endogenous ligands[9], which bind to the receptors with much higher affinities than fragment interactions. Other classes of GPCRs targeted by drug discovery, such as the Class C GPCRs, have complex mechanisms such as the involvement of large N-terminal domains (Class C GPCRs) which act as gates to ligand binding only upon dimerization of the GPCR and its N-terminal domain[9]. Although it is beyond the scope of this introduction to discuss all the types of GPCR mechanisms which are targeted by drug discovery, it is clear that GPCRs are a crucial class of proteins to modulate in order to alleviate diseases. Unfortunately, to date, there are only 4 GPCRs for which complete structures are available, including the crystal structures of bovine rhodopsin[42], the β_2-adrenergic receptor[43] (Figure 6**a**), the β_1-adrenergic receptor[44], and the adenosine A_{2A} receptor[45]. Although the drug discovery process has enabled the successful

identification of ligands with mutagenesis, computational methods, and functionality based assays, the structural information of the GPCRs has provided new insights on important key properties of each individual GPCR binding site. Therefore, there is a need to obtain more structural information for other members of this class of proteins to understand where ligands bind, how to evolve them into more specific drugs, and how to modulate GPCR activity in the context of complex signalling interactions.

The second important class of membrane proteins targeted by drugs, the ion channels, are pore-forming proteins which are involved in the transport of sodium, potassium, calcium, and chloride ions through the cellular membrane in order to modulate the ionic potential between the outside and inside of the cell. These ion channels can be 'gated' by different processes such as ligand binding, temperature, mechanical tension in the membrane, and voltage, all of which are often concerted by other proteins in the cell, such as GPCRs[9]. Other molecules which can block ion channels include the deadly snake venom which consists of protein and peptide toxins that physically block the opening of the ion channels, thereby blocking all further transport of ions. Different venoms are being studied now in the hope to modulate this ion channel blockage in diseases such as multiple sclerosis[46]. Needless to state therefore how complex it is to target ion channels without creating undesirable secondary effects. Nonetheless, current drugs on the market are alleviating diseases caused by mutations in these ion channels, such as high blood pressure and muscle dystrophy, faulty acidification levels which may lead to diarrhoea or kidney stones[47], and naturally many diseases linked to the central nervous system such as migraines, seizures, and autism[48]. This was just a short list of examples, which are mostly studied by mutagenesis and 'macroscopic' assays including cell-based, radioligand, or fluorescent-based assays instead of by molecular methods due to the limited amount of complete ion channel structures currently available. Some of the complete structures that exist include the crystal structure of the potassium channel from the gram-positive bacterium *Streptomyces lividans*[49], as shown in figure 6**b**.

Clearly, with GPCRs and ion channels being key proteins of important signalling cascades, current drugs often have high levels of secondary effects. To make matters more complicated, many GPCRs and ion channels work by forming complexes of proteins by homo or hetero-

multimerization[9,50] in order to proceed with certain functions. Research is now unveiling different parcels of complex networks involved in cellular processes regulated by these important membrane proteins, along with important interactions with other components of the cell, such as molecules within the native membrane itself. The dawn of biophysical molecular methods will eventually lead to a better understanding of how to specifically target a membrane protein in a specific state and how to improve drug interactions for a particular system rather than a single protein target. As explained below, there are many reasons why membrane proteins are still difficult to study at the structural level, limiting therefore our possibilities to develop drugs which are more specific in the aim to limit side effects and understand new relationships between disease and target.

Why are membrane proteins generally excluded from fragment based drug discovery?

As previously defined, molecular approaches such as NMR, crystallography, and SPR require high yields of concentrated samples of protein target in order to yield high resolution data regarding molecular interactions. Furthermore, the sensitivity of these methods is such that extensive effort goes into preparing protein samples with the highest level of purity, so as to avoid unwanted interactions with, or signals from, other molecules.

Applying such molecular methods to membrane proteins becomes challenging because of the difficulties in meeting the above requirements, as explained below. It is rarely possible to produce membrane proteins in sufficient quantities, and even when enough is produced, the proteins must be purified, concentrated, and solubilized in a hydrophobic environment which leads to loss of protein stability and an increase in unwanted signals and interactions.

Limitations in membrane protein production

There is no general solution for production of membrane proteins in high quantities and the choice of vector sources and host cells for overexpression and production has to be defined for each new target. Such decisions may be based on the source of the original gene, the protein's requirements for optimal folding, and for posttranslational modifications. Although prokaryotic cells are the most productive hosts for soluble proteins, they often do not possess the machinery required to produce membrane proteins in the correct conformation with suitable posttranslational modifications[51]. Low success rates with prokaryotic cells are also often caused by toxicity induced by overexpressed levels of foreign membrane proteins[52]. Although there have been success stories where inclusion bodies were used to produce high levels of membrane proteins in *E.coli*[53] and cell-free *E.coli* based expression systems[54], there is an extensive amount of work required to find the correct conditions for refolding membrane proteins into stable and functional conformations.

Recombinant expression of human genes in eukaryotic mammalian cells is therefore a more appropriate system to use, but the yields generally obtained remain too low for studies involving molecular methods. GPCRs for example can be obtained in ranges from 10 - 100 fmol/mg of tissue[55], and the lack of purification and solubilization techniques makes it difficult to purify them in abundant amounts from natural sources[56]. Alternative eukaryotic cells which can produce sufficient amounts of membrane proteins for molecular methods include baculovirus infected insect Sf9[57] and yeast[58] cells. The latter was quite a novelty because it proved that, against old beliefs, some GPCRs can be fully functional without glycosylation which is incompatible with crystal formation, and could be produced in high enough levels to be put through crystallization trials.

There is clearly much effort toward overexpression of membrane proteins in high quantities, but the challenge is far from over, for mimicking the native membrane qualities in vitro is the most challenging of all, and has to be faced in each step of the process from membrane extraction to protein solubilization, and, if required by the molecular method, functional and stable immobilization.

Membrane mimics for in vitro handing of membrane proteins

The native membrane which surrounds each living cell is constituted of a lipid bilayer and an array of proteins, either transmembrane or associated with the membrane, which function in concert to create, organize, and complete cellular processes. The membrane does not only enable compartmentalisation of the cell's inner processes and components, but it is actively involved in regulating these processes by interacting with the associated proteins[59]. Although we have begun to understand this important role, elucidating each complex interaction between the different components of the membrane and individual membrane proteins is an extremely difficult task at hand, for the membrane is a complex system. For example, lipid rafts are dynamic arrangements of lipids and cholesterol which have recently been identified as important players in the regulation of membrane receptor activity and localization within the membrane[59]. To study a membrane protein at the molecular level by NMR, crystallography, or SPR, clearly, its isolation from this intricate system is required in order to have a pure sample of the protein of interest.

A membrane protein has a large amount of hydrophobic residues which are in contact with the phospholipids in the native membrane and it is estimated that the free enthalpy cost of solubilizing a membrane protein in water would be in the range of 150 – 200 kcal/mol[60]. Thus, when a membrane protein is removed from its native environment, the hydrophobic domains which were stabilized by phospholipids will be attracted to each other, causing the protein to collapse and precipitate. This is why it is crucial that all steps leading from initial extraction from the membrane to purified protein is meticulously carried out so that the target protein is reconstituted in a synthetic lipid environment which mimics the characteristics of the native one as closely as possible[61]. Which strategies to use depends on the individual protein: some may remain stable only in the native membrane, while others may be reconstituted in the simplest synthetic forms.

Approaches for solubilizing membrane proteins in vitro require the presence of amphipathic molecules which mimic the membrane phospholipid properties by presenting hydrophilic head groups to the aqueous buffer, while maintaining contacts to the proteins' hydrophobic residues with the hydrophobic tail groups. These approaches range from simple addition of high

concentrations of ionic or zwitterionic surfactants such as detergents or lipids in water to create micellar or bicellar vesicles in all handling steps[61] to very complex protocols involving the fusion of various mixtures of detergents/lipids to form a more stable and better mimic of the membrane in lipid bilayers for example. The most popular detergents include alkyl glucosides and maltosides, polyoxyethylenes, alkyldimethylamines, and cholate derivatives such dodecylphosphocholine (DPC)[52] with which micelles are formed when the surfactant is in a higher concentration than its critical micellar concentration (CMC). Below the CMC, the equilibrium shifts from micellar to monomeric forms of detergent, thereby causing loss of protein conformation and functionality as the proteins precipitate in the absence of stabilizing micellar formations. Often, detergents are used to extract membrane proteins from the native membrane, because they are good at dissociating lipids from proteins[60]. While some proteins can continue to be handled in these detergents throughout the purification procedure until the final sample for crystallization, NMR, or immobilization for SPR, others require a more stable lipidic environment in bicelles (micelles formed of lipid bilayers) or bilayers. Molecular methods have therefore been difficult to apply to membrane proteins because the environment which keeps a protein stable may not be compatible with the application we one wishes to use to study the protein.

Detergent micelles often lead to undesirable effects such as low stability or even denaturation, aggregation, and separation of subunits from multimeric formations[52]. This low stability is clearly incompatible with NMR and crystallography as the dynamic processes lead to difficulties in obtaining high resolution NMR data and loss of the initial 'true' protein structure, and prevents crystals from forming. Furthermore, detergent micelles can be too large upon solubilization of membrane proteins to successfully apply NMR[61]. The high levels of detergents necessary to maintain protein conformation can lead to unwanted signals and interactions, such as non-specific binding to the compounds used in drug discovery during SPR or solution NMR screening applications.

Although detergent micelles have been used successfully in applying NMR to the structural determination of the outer membrane protein A in DPC[53] and the Disulphide bond forming protein B[62], the use of more stable lipid formations such as lipid bicelles[63] and lipidic cubic

phases[64,65] are required for crystallization. Strategies are also evolving to include immobilization of detergent or lipid solubilized membrane proteins onto solid supports for SPR[66] or solid state NMR[67] applications. However, although these applications have enabled structural determination of membrane proteins, they are limited to proteins which are obtainable in high amounts, such as bacterial membrane proteins, or require great efforts into finding the appropriate solubilization condition which is both appropriate for protein fold and functionality, but also compatible with the method. When drug discovery is involved however, more difficult targets such as GPCRs and ion channels are involved, which often call for delicate dimerizaton states or the presence of other players in the membrane (G proteins) for a full read out of the activity. Furthermore, the synthetic membrane mimics often lead to surfaces to which fragments bind non-specifically. For all these reasons, to date, there have been no successful applications of fragment based drug discovery to membrane proteins.

New alternative solubilization strategies for membrane proteins in aqueous buffers

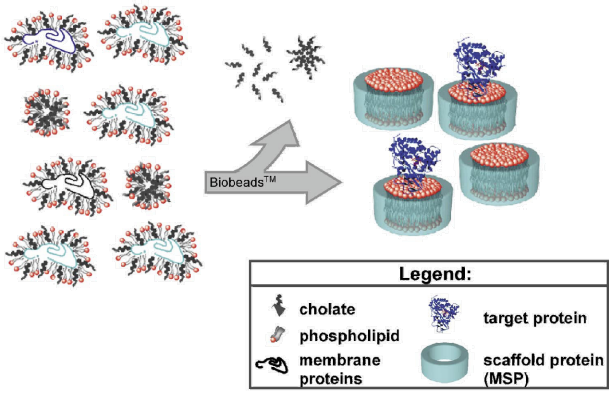


Figure 7. Self-assembly of Nanodisc complexes, reproduced from reference 68 with permission. Copyright 2003 Biotechniques. The detergent micelles of the target protein are replaced by a stable lipid bilayer surrounded by the amphiphilic MSP upon detergent removal. The resulting nanodisc complex is fully soluble in aqueous buffers.

Although there have been important developments contributing to more stable membrane proteins in vitro (better control of the expression systems[69], mutagenesis of key residues for higher protein stability[70] and better solubilization techniques[71]), these all require the presence of detergent. The self-assembly of membrane proteins in new solubilization alternatives such as the amphipols[72] or the Nanodisc[68] complex (Figure 7), however, enables handling of membrane proteins in aqueous buffers without needing to add surfactants to maintain a hydrophobically stable environment.

The Nanodisc procedure consists of mixing lipids which are solubilized in mixed micelles with cholate, and a 23 kDa amphiphilic α-helical membrane scaffold protein (MSP) to the targeted detergent-solubilized membrane protein to be incorporated into the complex. When these entities are mixed together, the lipids, detergents, and cholate form mixed micelles around the MSP and the target protein.

Upon removal of detergents, by dialysis or the addition of detergent adsorbing bio-beads, the target membrane protein, the lipids, and the MSP hydrophobic residues self-assemble into the nanodisc formation. When the appropriate conditions are met, which are different for each target membrane protein to be incorporated, the target protein is solubilized in the center of a lipid bilayer which is consequently stabilized by two monomers of MSP with the hydrophobic residues

making contact with the lipids. Due to the amphiphilic nature of the nanodisc, the final complex can be handled in aqueous buffers without causing conformation or functional loss of the embedded target protein and without requiring any presence of surfactant in the buffer. This enables easy purification and has the potential to be applied to fragment based drug discovery without causing non-specific binding of ligands to the nanodisc complex.

The nanodisc technology has been successful in embedding a variety of membrane proteins such as Bacteriorhodopsin[73], the GPCR β-adrenergic receptor[74], and the metabolically important cytochrome P450 (CYP)[75]. The nanodisc system was also previously used to measure redox potentials on CYP3A4 in the absence and presence of substrates at the active site[76]. Although the procedure of incorporation depends on every target protein and the initial solubilization state of the target protein, the resulting homogenous and standard preparations, void of any surfactant and stable in aqueous buffers, may bridge the gaps which prevent molecular methods from being applied to membrane proteins.

How can Target Immobilized NMR Screening address limitations posed by membrane proteins?

Target Immobilized NMR Screening is, as its name indicates, is a FBDD method which screens an immobilized target for fragment binding using solution NMR. The method involves immobilization of a target protein and a reference protein on an aldehyde resin with a mild Schiff's base chemistry between the primary amines of the protein and the aldehyde groups present on the commercially available resin. A flow-injection, dual sample holder can be placed in the magnet to enable flow-mediated screening of fragments by automated injection of mixtures containing upto 8 fragments over the immobilized proteins. This method enables fragment based drug discovery on membrane proteins for the following reasons.

Immobilization allows efficient use of the target protein

With the target and reference protein immobilized, a single screen of 1000 fragments can be carried out on one single sample of protein. Naturally, because a full screen can last approximately 5 days, the sample may need to be replaced if the protein shows signs of low stability. However, the amounts required are fairly low (50 – 100 nmoles). This procedure is generally applicable to other membrane proteins because of the simple and mild Schiff's base chemistry used to immobilize the protein N-terminus to the aldehyde groups on the resin[23], and because it is compatible with the use of detergents.

NMR detection of fragment signals rather than protein signals

TINS focuses on the ^{1}H 1D spectra of fragments in solution[77]. The difference in intensities of these fragments' peaks between the presence of an immobilized target protein and the presence of an immobilized reference protein allows one to identify if a fragment has bound to the target. The fact that the method directly observes differences in fragment signals in solution means that there is no necessity to produce isotopically labeled proteins and there is no *a priori* requirement for the protein structures to be well resolved, as is necessary in other NMR target-based screening methods. Furthermore, compared to other ligand based screening methods, there is no need to deconvolute the signals, because mixtures are designed to be composed of ligands with a minimum of overlapping peaks.

The reference system accounts for non specific binding of fragments to detergents

When TINS is applied to soluble proteins, the level of non-specific binding to the target protein can be accounted for by simultaneously screening a reference protein of a relatively similar size which has limited small molecule binding properties. With membrane proteins however, the presence of detergent leads to non-specific interactions with the fragments both in solution and upon contact with the immobilized, detergent solubilized protein. It is easy to predict that the

amphipathic nature of the detergents can partially solubilize the fragments (which are relatively soluble yet also composed of hydrophobic moieties) out of solution and temporarily into micelles. To minimize such interactions, the appropriate trade-off can be met, by adding detergent into the wash buffer but not into the independent fragment mixtures, provided the fragment mixture injection does not dilute the effective micelle concentration below local CMC. As stated, this minimizes interactions between fragments and detergents, but can not be eradicated due to the loss of protein functionality which would follow. Therefore, there remains a substantial amount of partial solubilization of fragments into the detergent micelles, with the effect of reducing their effective concentrations and hence, their final intensities in the NMR spectra. The reference membrane protein must therefore not only have minimal small molecule binding properties, but it must be refolded in the same detergent conditions as the target protein in order to obtain a reliable reference system. In our case the outer membrane protein A (OmpA) (Figure 8**a**) which can be folded properly in a variety of detergents[72,78,79], was found to have minimal small molecule binding. This reference system is theoretically applicable to membrane proteins and removes signals from fragments which are only non-specifically binding to the detergent micelles. To minimize signals from detergents with a high CMC, deuterated forms can be used, provided they are available.

Alternative immobilization and solubilization methods can be easily adapted

TINS can be easily adapted to different immobilization chemistries and membrane protein solubilization strategies, provided the appropriate resin is used. In our case, the sepharose aldehyde resin has proven to be compatible with the system by having a minimal line broadening effect on the fragment ^{1}H 1D spectra. A variety of chemical linkers exist which can be used to tailor the immobilization chemistry based on the requirements for functional immobilization of the target protein, such as targeting the C-terminus when the N-terminus is involved in protein functionality. Naturally, the immobilization chemistry has to be compatible with the detergents or alternative solubilization materials used to maintain the correct fold and functionality of the membrane protein. nanodiscs, as an alternative solubilization strategy to DPC micelles, have

potential to be immobilized to the same resin through the N-termini of the scaffold proteins. We envisaged therefore that TINS would be compatible with screening membrane proteins embedded in nanodiscs in the absence of detergents, providing a more stable environment for the target protein without the disadvantages of non-specific binding interactions of fragments with detergent micelles.

The aim of this thesis:

To develop methodologies which enable the application of molecular methods for drug discovery on membrane proteins.

With increasingly perceptive methods for solubilizing and immobilizing membrane proteins in functional ways, the different direct or indirect, random or oriented immobilization strategies are reviewed in **Chapter 2**. Attention is paid to a variety of applications such as chromatography, fluorescent and radioligand based applications, as well as molecular methods such as NMR and SPR.

Prior to starting a TINS screen, we first wanted to test the functionality of an important class of membrane proteins, the GPCRs. Knowing how important the native membrane is for GPCR functionality, we aimed at testing functional immobilization of the human histamine H_1 receptor and the human adenosine A_1 receptor within the native membranes from stable cell lines overexpressing these receptors. Radioligand binding studies of the receptors in solution were compared to the receptors upon immobilization within their native membrane vesicles in the presence of physiologically relevant G proteins, as described in **Chapter 3.**

The H_1 receptor mediates a variety of cellular processes which, upon faulty signalling, are at the heart of many diseases affecting children and adults today. These include immunological responses[80] such as allergies and asthma, as well as eating disorders such as anorexia[81]. Furthermore, antihistamines which are used as potent blockers of histamine induced allergic reactions provoke drowsiness due to the additional role of the H_1 receptor in regulating sleeping behaviour[82]. The A_1 receptor is just as important a pharmaceutical target as the H_1 receptor, because of its role in the central nervous system as a regulator of adenosine and hence, the physiology of many tissues. The adenosine A_1 receptor is ubiquitously found in the whole body and is at the heart of processes leading to asthma, chronic inflammatory problems, and heart failure[83] in addition to, when located in the central nervous system, many types of

neurodegenerative diseases[84]. It has also been reported to form dimers with itself or the dopamine receptor[85]. Both receptors belong to the Class A of GPCRs and generally bind small ligand molecules, thereby making them *a priori* appropriate targets for fragment based drug discovery. Although the structures for these particular proteins do not exist, the homology with other known structures currently enables *in silico* studies of ligand binding interactions[86].

Currently TINS has been explored on soluble proteins. We wished however to prove the concept that detergent solubilized membrane proteins could practically be screened by the TINS methodology as well. In **Chapter 4**, we carried out an initial pre-screen on a bacterial ion channel, the potassium ion channel KcsA, and a bacterial membrane enzyme, the Disulphide Bond Forming protein B (DsbB) with, as a reference, the immobilized bacterial Outer Membrane Protein (OmpA) also solubilized in DPC.

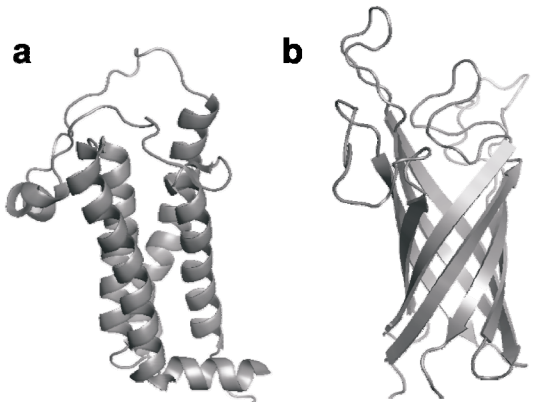


Figure 8. Structures of the target and reference proteins used in the TINS screen. DsbB has α-helical transmembrane domains (**a**) while OmpA has a β-barrel transmembrane domain (**b**). The structures are modified from pdb IDs 2k73[62] and 1g90[53] respectively.

The reference protein, located in the outer membrane of bacteria, is a β-barrel transmembrane protein (Figure 8**b**), which has a variety of roles leading to pore formation, adhesion, invasion, formation of biofilms, and as a receptor for several types of bacteriophages[87]. However, these functions are related to the extracellular domain of the protein. We believed that the transmembrane domain, which has also been studied by NMR[53,72] and crystallography[88,89], however, has minimal small ligand binding and could act as a reliable reference protein to account for non-specific binding of fragments to the detergent micelles due to its similar size and appropriate fold in DPC micelles.

Both target proteins for this TINS pre-screen are from bacterial origin, enabling us to obtain sufficient amounts for initial development of the protocol needed to adapt the TINS procedures to membrane proteins. Furthermore, the crystal and NMR structures of KcsA[90,91] and DsbB[62,92] are available, enabling future studies of protein-ligand interactions on these pharmaceutically important proteins. DsbB is a bacterial membrane protein target (Figure 8**a**) which has pharmaceutical importance due to its role in disulfide bond formation and subsequent regulation of protein toxin folding leading to the virulence of Gram negative bacteria such as *E.coli*[93] and *Bordetella pertussis*[94]. KcsA is a model protein with high homology with human potassium channels which are important pharmaceutical targets due to their role in regulating potassium ion transport through the cell membrane. As such, human potassium channels, are targeted by anaesthetics and drugs which partially alleviate autoimmune and neurodegenerative diseases[95]. On the other hand, ion channels are often non-specifically and unexpectedly targeted or blocked by drugs, causing severe secondary side effects. An example of such a challenging protein is the human ether-a-go-go related gene (hERG) potassium channel which regulates the potassium potential in cardiac myocytes[96]. Drugs not related to the cardiovascular system have been removed from the market because they either blocked the hERG channel activity by non-specifically binding to the large inner binding pocket, or by blocking its traffic to the cell surface, both leading to irregular (and potentially fatal) repolarization of the cardiac muscle[97,98]. It would therefore also be valuable to carry out parallel screens on liable targets such as the hERG channel in order to predict and avoid future drug candidates' interactions.

With the proof of principle validated on membrane proteins, we carried out a full screen on the pharmaceutically important DsbB with OmpA as a reference, in DPC micelles. The identification and validation of the hits on the membrane enzyme DsbB were possible due to an existing robust enzymatic assay which relies on the detection of substrate oxidation or reduction[99]. A soluble partner protein DsbA becomes oxidized in the presence of DsbB and the synthetic DsbB cofactor ubiquinone (UQ1). The enzymatic turn-over rate of DsbB can therefore be compared in the presence of fragment hits in order to validate their inhibitory effect on this target protein. The

results of the enzymatic assays, along with confirmation of the binding modes of fragments by orthogonal biophysical assays (by solution NMR Heteronuclear Single Quantum Correlation) are described in **Chapter 5**.

The possibility of carrying out TINS on DsbB in aqueous buffer completely void of surfactants was tested using the nanodisc system, whereby the membrane proteins can be solubilized in a lipid bilayer stabilized by a belt of two amphiphilic membrane scaffold proteins. The results of a short screen on nanodisc-solubilized DsbB was tested using two references, nanodisc-solubilized OmpA and empty nanodiscs, containing only lipid bilayers and the amphiphilic scaffold proteins. The results are described in **Chapter 6**.

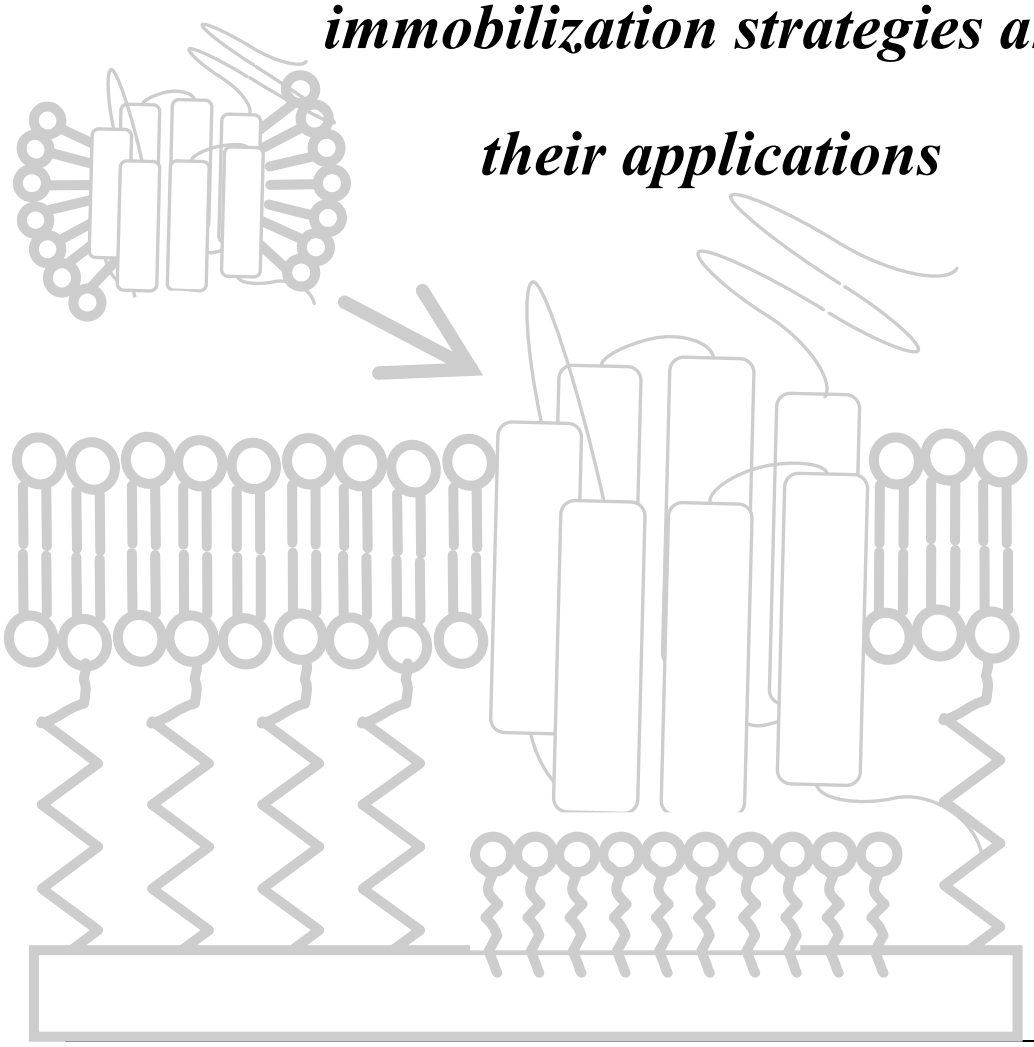
Finally, a conclusion section is presented in **Chapter 7**, including perspectives regarding the future of membrane protein research when applying biomolecular methods in drug discovery.

Chapter

2

How to catch a membrane protein in action:

A review of functional membrane protein immobilization strategies and their applications



Biological and pharmaceutical studies of proteins often require their immobilization on surfaces, and although techniques exist for soluble proteins, such applications to membrane proteins present challenges. This review focuses on immobilization procedures which have been successful in coupling membrane proteins in a functional state. The review follows a technological approach. We focus on how the protein is immobilized to the surface, with descriptions of the various linkers used, and how specific the chemistry is. We cover a range of applications including whole cell assays, radioligand binding, frontal chromatography and electrochemistry, as well as biophysical assays including impedance spectroscopy, plasmon-waveguide resonance spectroscopy, nuclear magnetic resonance and surface plasmon resonance with special attention to which surfaces are compatible with a given technique. Also included are fluorescence based assays, such as total internal reflection fluorescence and fluorescence microscopy. A brief description of the studies allowed by each immobilization strategy follows each section, including important results that demonstrate the feasibility.

This chapter has been recently submitted as a review: Virginie Früh, Ad. P. IJzerman, and Gregg Siegal. How to catch a membrane protein in action: A review of functional membrane protein immobilization strategies and their applications. *Chemical Reviews. Submitted 2009.*

Analysis of the results from the human proteome project suggests more than 30 % of proteins are membrane-bound[67]. Membrane proteins (MPs) are in the core group responsible for signal transduction, including the G-protein coupled receptors (GPCRs), which represent 30 – 40 % of marketable drug targets today[100]. It is needless to stress, therefore, the great commercial, industrial, and research value of MPs. There is growing interest in immobilizing MPs to various surfaces to create new biosensors or platforms enabling the study of their biological functions and identification of new leads for drug discovery. Soluble proteins have been readily immobilized through cross-linking aldehydes or thiols with protein amines or carboxyl groups for applications such as micro-arraying by printing[101,102,103,104]. Functional immobilization of MPs requires consideration of their physiological needs, often dictated by the quality and components of the natural hydrophobic environment surrounding this class of proteins. The aim of this review is to focus on the immobilization chemistry applied to MPs and how these have enabled ever more complex assays from simple whole cell analyses to purified proteins on a chip, from the 1990s to 2008.

With MPs, often the challenge is not the immobilization *per se*, but rather the requirements of the analysis and the protein itself with regards to protein availability and the type of lipid environment needed. Earlier studies of MPs, due to the difficulty in purifying them, involved whole cell or membrane fragment immobilization where native biological responses could be followed by 'macroscopic' methods such as frontal chromatography (Section 1). In these studies immobilization was accomplished by simple techniques such as incorporation of membrane fragments upon swelling of gel beads during freeze-thawing[105,106] or by coating beads with various cell-adhesive substances[107]. Although these systems allow one to study the native environment, the immobilization itself is random and relatively unstable. Furthermore, reliable signal detection is often limited by high levels of non-specific binding to the environment around the target MP.

Solubilized MPs may be either used as such in detergent micelles, or reconstituted in various types of detergent or lipid formations[108,109,110]. With recent advances in the development of mimics of the native membrane however, the stability and formation of various lipid assemblies

is becoming less problematic. However, one can not emphasize enough how challenging these steps are. Removing the protein from its hydrophobic environment and reconstituting it into another is a difficult process that may damage the protein or result in less than optimal functionality[68,107]. Furthermore, increasing complexity arises with increasing numbers of transmembrane segments[111]. The most successful stories are currently based on the nicotinic acetylcholine receptor and rhodopsin, due to the availability of these MPs in large quantities and relatively good stabilities. Newer membrane mimics have allowed direct adsorption of appropriately prepared MPs to flat surfaces or chips made of glass, platinum-glass, and platinum-silicon to create monolayers of lipids which hold the protein onto the surface (Section 2). Subsequent strategies have evolved to create even more stable lipid bilayers, as opposed to less stable monolayers, by fusing various types of solubilized MPs to lipid monolayers on surfaces. These methods provide more robust immobilization, applicable to more sensitive studies, provided fluorescently or radioactively labeled components are available to specifically monitor MP activity. As a result, the highly sensitive detection of such signals does not require large amounts of solubilized MP. As these methods were applied to increasing numbers of MPs, it became clear that immobilizing membranes or mimics too close to a surface may restrict diffusion of solutes below the membrane, and sterically hinder extramembranous protein dynamics. As a result, so-called solid-supported membranes have been developed, where the space below has been filled with hydrophilic polymers, combined with various linkers, to immobilize the MP in a manner that accommodates dynamic behavior. Solid-supported membranes are a better mimic of the natural environment because they create additional membrane fluidity and allow for mobility of large extramembranous protein domains[112] (Section 3). Many of these techniques have evolved in such a way that solubilization and purification of the protein may not be a prerequisite, but when this is the case, the solubilization from crude cell lysates or lipid reconstitution may be carried out *in situ* (Section 4).

On the detection side, earlier assays primarily used biological readouts. Although these techniques were sufficient for high throughput or high content assays of, for example, ligand binding and cell signalling activation, they typically required labelling of a protein or ligand to produce a signal. Recently newer, so-called label-free technologies, have emerged, such as SPR

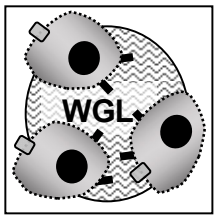
and NMR[77], that are able to directly detect ligand binding thereby rendering use of chemically, biologically, or radioactively labeled protein or ligands redundant. These technologies are sensitive to intermolecular interactions and capable of detecting weak binding affinities in the mM range (for NMR), provided the target protein is available in sufficient quantities and functionally solubilized. As opposed to soluble proteins, MP interactions with ligands, lipids, or other proteins are often specifically located on one side of the cellular membrane. Some strategies have therefore been taken one step further by using high-affinity interactions for specifically orienting MPs in the immobilized membrane on chips (Sections 5-7). Techniques involving *in vitro* expression in the presence of the tethered lipid membrane in which the protein will be integrated directly upon expression[113] are an elegant way to achieve oriented, immobilized MPs.

As strategies to couple MPs become increasingly perceptive, a thorough summary of the numerous accomplishments is useful. Here, we present such a summary in the form of text, tables, and a glossary (see p. 42 for table of contents), which focus on the chemistries of various immobilization types, with descriptions of their applications and results.

Table of Contents:

1. Non-covalent interactions with the whole cell

Via wheat germ lectin-agarose for whole cell immobilization –MP: Glucose transporter Glut1



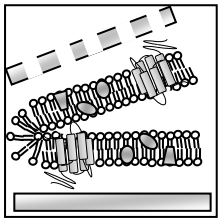
Frontal chromatography can be used to study interactions between a mobile phase and a stationary phase by regression analysis of the retention volumes. Furthermore, when whole cells are immobilized, this technique can allow the study of binding affinities of soluble ligands to MPs in their native environment. Immobilization can be achieved by adhering whole cells to agarose gel beads via wheat germ lectin (WGL)[107,114].

WGL is a derivative of wheat germ agglutinin and binds to N-acetylglucosamine and to N-acetylneuraminic acid on the extracellular face of red blood cell membranes[115]. The Glut1 transporter targeted for these assays was indirectly immobilized to Sepharose 4B gel beads prederivatized with WGL by simply incubating cells overexpressing the receptor with gel beads[115]. The interaction of Glut1 with D-glucose and cytochalasin B (CB, a fungal alkaloid that inhibits glucose transport) was measured by frontal chromatography. The K_d values of Glut1 binding of CB and D-glucose were comparable to values previously reported from studies on whole cells and membrane vesicles. These immobilization methods also allowed one to compare the K_d of Glut1 for CB and D-glucose for different types of preparations of the reconstituted receptor, without any effects on the affinity. The advantage of this technique was that cells remained intact, in contrast to electrostatic adsorption which causes hemolysis[114]. The gel beads could be stored for one month, but lost 40 % of their capacity to bind cells. It was reported that it was not cell integrity but rather the capacity of cells to bind to the gel (and therefore the remaining functionality and density of membrane glycophorins) which determined the stability of the column, along with physical and chemical properties of the storage environment. Another advantage was that protein purification was not necessary, and manipulations involving column preparation and cell immobilization were simple. Nevertheless, the immobilization was sensitive to pressure changes, and gentle washing induced detachment of the cells, causing loss in

reproducibility. Using this immobilization strategy with other applications will depend on conditions for cell homeostasis and functionality, as this technique was only optimized with respect to haematocyte cell lines.

2. Non-covalent interactions (adsorption) with the membrane

2.1 Via interactions of the native cell membrane with poly-Lysine: GPCRs and ion channels



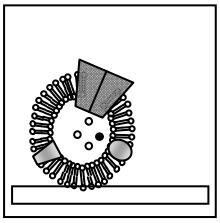
Studying whole cells by fluorescence often leads to high background signals from the cytoplasm. This issue, combined with limited access to the intracellular compartment, obscures many of the dynamic processes between the membrane leaflets. To circumvent this, supported cell-membrane sheets allow one to observe target proteins from the intracellular side when both leaflets of a cell membrane are physically pulled apart[116,117].

The ligand-gated ion channel 5-HT_3 receptor and the α_{1B}-adrenergic receptor were both overexpressed in HEK-293 cells which were grown on poly-L-lysine (PLL) coated plastic. Another PLL surface was pressed down onto the cells and removed after several minutes of contact, ripping apart the cells and exposing the membrane's intracellular face with endogenous lipids and proteins. To visualize the target lipids and proteins, the latter were expressed as fusion constructs with fluorescent proteins and the former were colocalized with fluorescent lipid markers. The supported poly-lysine membrane sheets have also been modified to allow access to the extracellular side by pressing down Silicon nitride surfaces into which arrays have been cut out[118].

Labeling with fluoresecent probes from different sides of the membranes enabled the authors to study the mobility of lipids, GPCRs and G proteins within and between the leaflets[117]. Following the dynamics of heterotrimeric G protein partitioning into lipid anchor microdomains was also possible along with the mobility regimes of glycosylated transmembrane proteins. Competition binding studies using fluorescent ligands confirmed the functionality of the proteins studied and the success of the technique.

In comparison to whole cell analyses, the advantages of supported cell-membrane sheets are numerous. The mechanical stability is excellent, minimizing unwanted movement of the observed membrane upon application of stimuli, which can be problematic for e.g. imaging applications. Although labeling is required, specific targets can be observed in their native surroundings even maintaining the appropriate orientation, while all the time monitoring cellular processes such as signaling and protein translocation. Following populations of labeled proteins was therefore possible as a direct result of removing the intrinsically highly fluorescent cytosol during the preparation of these membrane sheets. This strategy is generally applicable to all cell membranes, but is restricted to the use of labeled ligands or proteins. Adapting the immobilization to metallic surfaces may allow the strategy to be applied to surface plasmon resonance applications.

2.2 Via hydrophobic interactions of attoliter size native vesicles with glass slides: Ion channel 5-HT$_3$ receptor



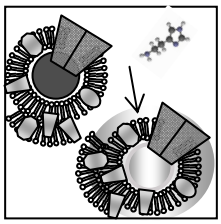
Obtaining large amounts of MPs is often limiting and therefore immobilizing miniaturized vesicles containing the target protein can be useful for studying receptor-mediated cellular responses. In this particular case, the authors have been able to produce vesicles straight from the native membrane of mammalian cells, with unprecedented attoliter volumes[119]. The vesicles can be immobilized in different manners, either through direct lipid adsorption to glass slides or *via* biotinylation of membrane components or target proteins[120,121].

Attoliter vesicles can be obtained in one step, without changes in receptor function, orientation, or localization in the membrane. Vesicle formation is a simple process induced by incubating cells, in this case expressing the serotonin-gated mouse ion channel 5-hydroxytryptamine type-3 receptor (5-HT$_3$R), with CB. This causes cells to produce tubular extensions which bud off when agitated. Native vesicles were thus formed that could be separated from whole cells by centrifugation. Usefully, the vesicles could be stored at 20 °C for weeks without causing loss of receptor function. Co-expression of cytosolic green fluorescent protein resulted in the possibility

to monitor and characterize vesicle formation and the presence of cytosolic components. Specific binding of fluorescently labeled ligands could be used to locate the receptor in the vesicles, and radioligand binding assays revealed their appropriate orientation and function. Furthermore, agonist and antagonist ligands showed the same behavior with vesicles as with detergent-solubilized receptors. Finally, Ca^{2+} signaling within the vesicles upon agonist activation could also be detected, suggesting the functionality of the complete signaling pathway.

These micrometer-sized vesicles are novel miniaturized reaction centers where receptor-based assays can be carried out in physiological conditions. These experiments not only revealed the native function of the receptors within these vesicles, but also proved that other important players such as ion pumps are still present and functional. Vesicle formation yielded native behavior when tested with CHO or HEK293 cells, suggesting this procedure can be generally applied to other cell strains. When fluorescent ligands are available, these miniaturized and stabilized compartments can provide the basis for high throughput assays where patterns of immobilization can reveal receptor function and binding affinities depending on various tailored reactive groups.

2.3 Via membrane hydrophobic interactions on beads – nicotinic Acetylcholine receptor



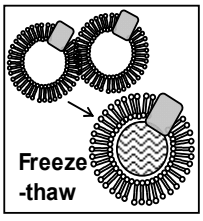
Radioligand binding assays are often used to quantify binding affinities of ligands to MPs because only picomolar amounts of protein are required. However, this requires separation of bound from unbound ligands. To circumvent this, the scintillation proximity assay system (SPA)[122,123] was developed, where receptors are immobilized on solid microspheres that contain scintillation fluid. This homogenous assay provides specific information on radiolabelled ligand binding without the need for separation from unbound ligand, as scintillation is only stimulated by radioligand in close proximity to the microspheres[124,125].

As a first attempt to apply SPA to MPs, membranes containing acetylcholine receptor were isolated from the electric organ of *Torpedo californica* and directly added to polyvinyltoluene fluorophore microbeads previously suspended in Triton X- 100. Under these conditions, the membrane preparations spontaneously adsorbed to the beads. These beads contained scintillation

fluid and upon binding of the radiolabelled ligand to the receptor, the close proximity of receptor led to photon emission proportional to the amounts of immobilized protein. In addition to obviating the requirement for separation of unbound from bound ligand, this method has less non-specific binding than usually encountered in radioligand binding assays. Radioligand binding assays of ^{125}I-α-bungarotoxin to the acetylcholine receptor enabled the study of various parameters such as dose response of acetylcholine and suberyldicholine with respect to radiolabeled α-bungarotoxin binding, providing apparent K_i values. The effects of different parameters such as concentrations of detergents and salts on the activity of the receptor were also studied, along with the detection of specific antigens by antibody binding.

SPA has a number of other advantages for MPs. Due to the great sensitivity of scintillation counting, as little as 1 ng of receptor can be detected. Although SPA requires prior radiolabelling and modification of the protein or ligand, many receptor-SPA beads are available commercially. The method has been used in a broad range of studies, varying from identification and quantification of PCR products[126] to high throughput screening of inhibitors of DNA binding proteins[127,128] to studying interactions between antibodies and antigens[129]. Finally, due to the simplicity of the immobilization procedure, it should be generally applicable to all MPs.

2.4 *Via steric trapping of membrane vesicles – MP: Glucose transporter Glut1*

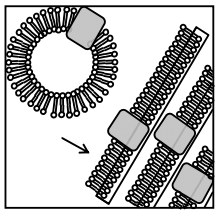


Quantitative affinity chromatography used to be limited to studying interactions between soluble substances. By immobilizing a membrane protein on gel beads, however, binding of soluble substances to MPs could be quantified by regression analysis of the retention volumes for different concentrations of ligands. In order to study interactions between soluble substances and the glucose transporter Glut1, membrane vesicles containing the latter were immobilized onto gel beads[105,106]. Membrane vesicles were obtained by stripping red blood cells of peripheral proteins and partially solubilizing them with octylglucoside (OG). In some cases, the vesicles were purified by ion-exchange chromatography for reconstitution of the Glut1 transporter into proteoliposomes[130]. It has been reported that freeze-thawing[131] and freeze-drying with subsequent

rehydration[132] can cause vesicles or proteoliposomes to fuse together and form larger particles. This approach was used to sterically trap vesicles after two cycles of freezing in ethanol/CO_2 and thawing in the presence of gel beads[105]. Using the sterically trapped liposomes, specific interactions between Glut1 and CB, and between Glut1 and D-glucose[133] were observed. Ligand binding affinities and kinetics were easily studied with such a set up due to lower non-specific binding to the lipids in vesicles compared to whole cells. Immobilized membrane vesicles had 4 times more transport inhibitor (CB) binding capacity than proteoliposome gel beads, showing that the membrane vesicles contained a high density of Glut1. In the presence of dithioerythritol (DTE), the binding capacity of CB was more than 80 % stable after 20 - 40 days in all types of columns studied. Furthermore, the activity could be controlled by inhibiting Glut1 with $HgCl_2$ and restoring Glut1 activity by adding DTE, which has an effect of partially reversing the Hg^{2+} inactivation.

Steric trapping should enable other applications such as studies of effects of ionic strength, pH, temperature, and lipid composition on ligand binding characteristics. This methodology has the potential, as do other whole cell or membrane fragment-based applications, of enabling studies of interactions occurring within the native membrane because of the presence of other components within the membrane. However, applications of this immobilization strategy remain limited to the use of gel beads for steric trapping of the swelled vesicles, where high amounts of protein per mg of membrane are required for sensitive read outs. Furthermore, the non-covalent immobilization leads to degradation during use, and, clearly, the protein to be studied needs to be stable to multiple freeze-thaw cycles.

2.5 Via direct lipid distribution on glass cover slides -MP: fd coat protein and FXYD proteins.

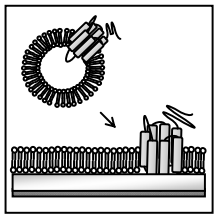


The biological role of the protein coating filamentous fd bacteriophages (fd coat protein), involves interacting with the host's cellular membrane. Three-dimensional structure determination is a powerful method to determine molecular mechanisms of protein function. However, in many cases solution

NMR studies of an MP in detergent micelles will not provide sufficient information because the dynamics and orientation of the protein in detergent micelles may be very different than in a membrane bilayer. Therefore, the fd coat protein was immobilized onto glass slides in phospholipid bilayers with one simple step for structural studies using solid-state NMR[67].

For these studies ^{15}N-labeled fd coat protein was produced in *E. coli* and solubilized in detergent micelles. The solubilized fd coat protein was then reconstituted into phospholipid vesicles consisting of a mixture of POPC/POPG[134]. Glass slides were covered with the vesicle suspension containing the reconstituted protein and the bulk water was left to evaporate. The slides were then stacked and bathed in saturated ammonium phosphate solution. Finally the slides were placed in thin polyethylene tubing and inserted into a rotor for NMR studies which allowed complete resolution of the amine resonances in a 3D magic angle spinning (MAS) NMR correlation spectrum. The choice of lipids determines the level of bilayer orientation on the slides and allows control over the horizontal and vertical spacing between phospholipids[109]. The presence of unsaturated chains leads to more fluid bilayers, and larger vertical spaces between bilayers can be obtained by incorporating negatively charged lipids[109]. The non-covalent, hydrophobic interactions with the glass were strong enough to hold the vesicles immobile on the substrate for the duration of an NMR experiment, despite the considerable shear forces generated by spinning the sample. No details were given on the protein stability or functionality, but it was fully incorporated into phospholipid bilayers onto the glass support. This immobilization strategy allows a wider range of MPs to be studied by solid-state NMR, and may be used in other applications compatible with glass surfaces. The extensive vesicle preparation was a complicated process however, and may not be generally applicable to other MPs. Although fd coat protein could be obtained in relatively large quantities, this is rarely the case with other MPs. Further, it may prove difficult to adapt this method of orienting the protein to new, small volume MAS rotors.

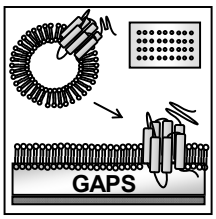
2.6 Via liposome fusion into lipid bilayers on platinum/glass or silicon slides – Bacteriorhodopsin



In order for an immobilized MP to remain functional, the fluid lipid or detergent environment must not become too rigid upon immobilization or it would impede the MPs dynamic behavior. Lipid rigidification can be minimized when proteoliposomes are immobilized by fusion with monolayers on glass surfaces to form bilayers without any chemical cross-linking. To demonstrate the approach, bacteriorhodopsin from purple membranes of *Halobacterium halobium* was added to a lipid, cholesterol, and octylglucoside mixture, and upon removal of octylglucoside during gel filtration, proteoliposomes were formed by the so-called detergent depletion technique[135]. LB lipid monolayers[136] were used to treat platinum/glass surfaces, and subsequently converted into bilayers when fused with the bacteriorhodopsin proteoliposomes[137]. Bacteriorhodopsin photoactivity in the reconstituted bilayers, monitored by electrochemistry, was comparable to natural membrane fragments containing bacteriorhodpsin on platinum surfaces. Three other MPs were also studied, including acetylcholinesterase from bovine brain, cytochrome oxidase from bovine heart and the nicotinic acetylcholine receptor from the electric organ of *Torpedo*. The purification and incorporation into proteoliposomes of the three MPs varied depending on suitable detergents, but they were all incorporated into stable lipid bilayers following the same protocol. Acetylcholinesterase enzymatic activity was easily studied by fluorescence measurements despite low immobilization yields, but only semi-quantitative data was available for Cytochrome C oxidase, while studies of the nicotinic acetylcholine receptor with its ligand ^{125}I-α-bungarotoxin, were hindered by issues of non-specific binding.

Although this technique provided a surface with a fluid membrane environment, meeting criteria for efficient molecular assays and potentially providing access to printing arrays, the usual challenge of obtaining functional protein in proteoliposomes remains. The bilayers did remain immobilized to the supports for prolonged periods of time after storage, but no information was provided on the stability of the immobilized MPs.

2.7 Via microarrayed γ-aminopropylsilane (GAPS)-derivatized surfaces –GPCR: adrenergic receptor, neurotensin receptor and dopamine receptor



Reducing the demand for protein and reagents has been one of the strategies used to address the limitations faced when MP purification and solubilization result in low yields. This limitation is even more critical when MP immobilization is at the heart of high throughput screening for drug discovery. An innovative way of addressing both limitations has been to immobilize GPCRs through microarray printing assays[138,139]. A nice example of this technique has been developed at Corning. Membranes in vesicular solutions of DPPC and DMPC or egg-yolk PC were printed *via* a robotic-pin printer on γ-aminopropylsilane (GAPS)-derivatized gold surfaces[140]. The details of the methods used with GAPS are proprietary to Corning. GAPS-coated slides exhibited high mechanical stability during printing, with no dependence on lipid phases, thereby overcoming the desorption encountered when withdrawing the slides through air[141].

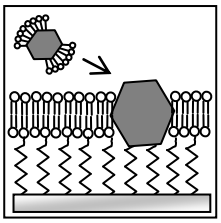
While covalently attaching membranes has been noted to impede lateral fluidity of membrane lipids, and consequently protein function, non-covalent printing techniques have proved to preserve GPCR and G protein functionality. Thus binding constants of known ligands were readily obtained, as well as compound selectivity between and within GPCR families. However, the assay in this format could not be used to differentiate between agonists and antagonists. Arrays of the β_1-adrenergic receptor, the neurotensin receptor (NTR1) and the dopamine (D1) receptor have been created. As an example, upon incubation with fluorescently labelled neurotensin, fluorescence was restricted to areas in which NTR1 had been immobilized, as expected. Binding constants consistent with literature were also determined for known small molecule ligands using the printed array[112,140]. A recent exciting advance in printing is the adaptation to porous substrates onto which the membranes are transferred, allowing access to both sides of the membrane[142].

Array printing is expensive due to the costs involved in acquiring such printers and also in time spent to optimise procedures which involve correct buffer compatibility, ligand specificities, affinities, and association and dissociation rates. However, although at present microarrays have

been analysed with fluorescent or radiolabeled[138] ligands, they can in principle be adapted for other applications such as SPR and electrochemistry, providing the surfaces are compatible. Information on cell-surface interactions and ligands for orphan receptors might also be derived from these microarrays, potentially enabling lead finding and validation, high throughput screening, and bioassays for compound screening.

3. Covalent interactions with linker/spacer to membrane

3.1 Via thiopeptide tethered membrane bilayers –MP: Cytochrome C oxidase



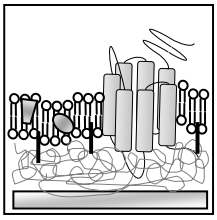
SPR applications require flat, metallic surfaces upon which proteins must be immobilized at very high densities and ideally, as close to the surface as possible. However, studies of immobilized MPs have revealed the need to create space between the protein and the surface to reduce steric hindrance of MP dynamics. Therefore molecules such as thiolipids[143] and thiopeptides have been developed as linkers, also called tethers, because they couple the membrane in close proximity to the surface but allow the membrane to remain fluid. Thiopeptides have been used previously[144,145] as a more hydrophilic alternative to thiolipids, for example, to immobilize cytochrome C oxidase (CcO)[146,147]. In this particular case, a thiopeptide-lipid monolayer was covalently linked to gold surfaces with subsequent incorporation of the MP within the lipid bilayer.

An oriented peptide monolayer was created by covalently binding an N-terminally thiolated, oligo-serine peptide to a gold surface[148]. To complete the tethered membrane mimic, vesicles obtained by dialyzing Lipoid Egg PC[149] were incubated with the monolayer surfaces to form the thiopeptide-lipid bilayer by adsorption. CcO, previously solubilized with Triton[146,150], was added to the cell containing the thiopeptide-lipid bilayer surface and spontaneously became incorporated into the bilayer with the micellar dilution technique. All steps of the process could be monitored by SPR, and final thicknesses of bilayers measured. Active proton transport driven by CcO had been established before, but it was measured here for the first time using impedance spectroscopy. This immobilization strategy proved to be compatible with the sensitive

measurements of proton transport through the lipid bilayer. Furthermore, kinetics and binding interactions of cyanide with CcO could be studied.

Due to the reported difficulty in preparing thiopeptide or thiolipid compounds in the bulk phase, this strategy allowed the simplified formation of a robust tethered bilayer in steps. Furthermore, the tethered bilayer allowed surface analytical techniques involving electric currents due to the metal surface used as a support, suggesting the technique may be adaptable to other applications with such requirements. Again, the challenge is to find the appropriate solubilization condition for the protein, but the space conferred between the surface and the bilayer environment of the protein by the tethered membrane bilayer can minimize steric hindrance of large, dynamic MPs and allow small molecule transport and diffusion.

3.2 Via polymer cushion- supported lipid bilayers –GPCR: Rhodopsin

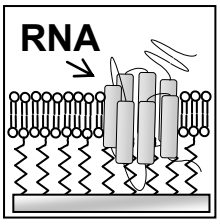


As discussed, previous studies revealed how immobilizing lipid bilayers containing MPs too close to the surface may hinder protein dynamics and membrane stability. An alternate solution to tethered membrane bilayers has been the introduction of a hydrophilic polymer cushion interspaced with hydrophobic anchors below the lipid bilayer The cushion, formed by a hydrophilic polymer interspaced with long alkyl chains, provides a lubricating surface which allows the lipid bilayer to remain mobile and provides a better mimic of the native environment[151,152].

The gold L1 SPR chip used for such polymer-supported lipid layers consisted of a covalently linked, carboxymethyl-modified, dextran polymer hydrogel where a large part of the sugar moieties were grafted with lipophilic alkyl side chains to which liposomes adhere[151,153]. Whereas the preparation of small unilamellar vesicles (SUVs) containing rhodopsin used to be necessary[153], here, fusion of crude rhodopsin-enriched membranes directly onto Biacore L1 chips was used instead of purification[151,153]. SPR, atomic force microscopy (AFM) and electrochemical impedance spectroscopy (EIS) were used to follow receptor immobilization. The receptors remained mobile in the plane of the lipid, suggesting that this strategy has the potential to be used to study receptor dimerization or interaction with other proteins[153]. Furthermore, the chips could

be used directly, without prior treatment, and repeatedly with different receptors after washes with detergents to remove earlier immobilized proteins. This may be generally applicable to other systems as the strategy allows on-surface enrichment for low yields of MPs.

3.3 In vitro synthesis in presence of a tethered lipid membrane – GPCR: Odorant receptor OR5

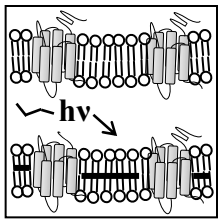


Biophysical analysis requires pure protein preparations functionally immobilized to a surface, and obtaining such conditions for GPCRs remains challenging due to loss of functionality and refolding problems typically encountered with GPCR solubilization and purification. To address this limitation, a new strategy that uses *in vitro* GPCR synthesis in the presence of a previously established[144,145], solid-supported, peptide tethered lipid membrane (tBLM) has been developed[113].

Using an approach similar to that described in Section 3.1, a hydrophilic peptide spacer was covalently attached to gold slides through amino-terminal thiol groups. The carboxyl group of the peptide was subsequently activated and amino-coupled to dimyristoyl-l-α-phosphatidylethanolamine (DMPE), forming a lipid monolayer. The monolayer was subsequently fused with lipid vesicles to create the final bilayer. The odorant receptor, OR5 from *Rattus norvegicus,* was expressed *in vitro* directly on the sensor surface[113]. This caused spontaneous integration of the GPCR into the tBLM upon biosynthesis. The procedure was monitored by surface plasmon enhanced fluorescence spectroscopy (SPFS) where fluorescent antibody binding to the GPCR created signals when the fluorophore was close to the surface. The OR5 receptor was successfully oriented on the surface in such a way that it mimics the orientation in the native endoplasmic reticulum, with the N-terminus facing the extracellular side. Ligand binding was monitored by surface-enhanced infrared reflection absorption spectroscopy (SERIAS) where absorbance differences were linked to effective binding of lilial, a small hydrophobic ligand for OR5.

Details were not provided concerning the stability of such slides, and although this application requires fluorescently labeled ligands, the success with GPCRs, which tend to be the most challenging MPs due to conformational heterogeneity, instability and low expression yields, suggests that the procedure has potential to be generally applied to other MPs and to analytical methods which do not necessitate labeling, such as SPR.

3.4 Via polymerized lipid monomers -GPCRs: Rhodopsin



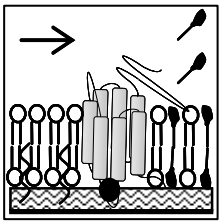
Rhodopsin has been used extensively to study efficient immobilization into lipid environments, but these lipids have shown to be unstable when used for immobilizing MPs on chips. This is because the hydrophobic interactions which keep the lipids adsorbed to the glass are not strong enough to resist eventual desorption after extensive washing. To address this issue, cross-linked synthetic lipids were developed to provide a more stable environment[154]. A planar supported lipid bilayer (PSLB) composed of 1,2-bis[10-2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphocholine (bis-SorbPC)[155] was used for these studies. The bis-SorbPC can be covalently cross-linked by exposure to UV light providing the necessary resistance to washing. Rhodopsin was solubilized in buffer containing OG for these experiments[156,157]. Reconstitution of rhodopsin into the PSLBs was carried out in a plasmon-waveguide resonance (PWR) cell. The PWR cell was set up in such a way that UV light could be directed to it from a Mercury lamp, with a band-pass filter to remove all visible light which would irreversibly photoactivate rhodopsin. More than 95 % of the bis-SorbPC was polymerised, as monitored by UV absorbance. PWR spectroscopy was used to characterise rhodopsin immobilized in the cross linked PSLBs. Within the PSLBs, the opsin isomerization and G protein activation could be monitored and rates were found to be similar to those obtained in a fluid dioleoylphosphatidylcholine (DOPC) bilayer.

MP immobilization via this procedure has the advantage of being completed in a few hours resulting in a system with intact and functional immobilized protein. Although no details were given on the reversibility of this polymerisation, it rendered the lipids resistant to Triton X-100 treatment. Although this method has high potential for on-surface purification and

immobilization of other GPCRs, preserving their functionality in such a cross-linked environment may not be as easy to achieve.

4. Covalent interactions with protein followed by lipid reconstitution

Via carbodiimide coupling of carboxymethyl-modified dextran with lipid reconstitution – GPCR: Rhodopsin



GPCR biosensor studies require high densities of pure and functional immobilized protein and this is the limiting factor with regards to most GPCRs. To address this limitation, a technique was developed in a flow cell to allow stepwise addition and removal of mixed detergent and lipid micelles and protein to create an on-surface lipid bilayer reconstituted around a solubilized GPCR[108]. In principle, large quantities of unfolded GPCR could be used as input for this approach.

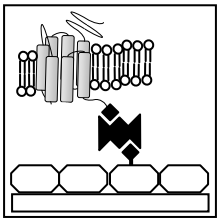
The gold L1 sensor chip was treated to create covalently linked carboxymethyl-modified dextran polymer with random glucose moieties substituted by alkyl groups[153]. The alkyl groups hydrophobically bound to mixed micelles of OG detergent and POPC lipid as they were injected over the surface to create the initial lipid layer. OG-solubilized rhodopsin[143] was injected and immobilized on the surface by both the amide chemistry and the strong hydrophobic interaction between the surface lipid layer and the OG micelles. The immobilized rhodopsin was immediately reconstituted in a POPC bilayer by injection of mixed OG and POPC micelles over the surface. The technique makes use of the high CMC of OG (25 mM). As buffer is injected over the flow cell, OG monomers detach much quicker than POPC and as they detach and wash away, the remaining POPC micelles spontaneously fuse into a continuous lipid bilayer over the sensor surface and the immobilized rhodopsin. The functionality of rhodopsin after immobilization was tested by surface plasmon resonance with light activation and measurement of transducin dissociation from the membrane as it consumed GTP[143]. Signalling was only detected in the sample flow cell containing rhodopsin-POPC. There was no signal in absence of lipids, which proved that their presence[158] and correct reconstitution[159] was necessary for

rhodopsin's functionality[108,160]. The ligand binding capacity of the receptor was therefore preserved through this immobilization procedure and could be repeatedly measured.

This technique resulted in reasonably high densities of up to 4 ng/mm^2 of immobilized protein while the use of a polymer hydrogel layer improved the stability of the immobilized bilayer. Although protein solubilization and lipid reconstitution were fast and straightforward via the commercially available Biacore systems, defining the correct lipid and detergent stoichiometries for new target proteins will require significant optimization[161]. The technique however can be adapted to different immobilization chemistries, including high-affinity interactions with antibodies to capture the protein in a defined orientation[162].

5.0 Specific immobilization of protein in native membrane via linkers

Via biotinylated protein bound to streptavidin or avidin – GPCR: Neurokinin-1 receptor



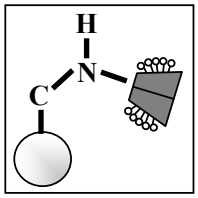
Many MPs have specific roles in binding extracellular ligands. Therefore, when immobilizing such proteins to study their biological activity, it is desirable to orient them in a controlled manner so as to maximise the accessibility of the ligand to its binding site. Specific interactions between the protein or membrane and the surface have been used in place of random ones for a better control of the protein's orientation. The most common strategies involve the biotin interaction with streptavidin or avidin[110,163,164,165]. In one case, a biotinylated Neurokinin-1 receptor was overexpressed in CHO cell lines and immobilized through a streptavidin linker to a biotin covered slide[165]. The procedure was very simple, consisting primarily of treating the quartz slides with biotinylated BSA, which results in a surface resistant to salt and detergent washes. The multivalent streptavidin was then used to bridge the biotinylated GPCR to the biotinylated BSA for immobilization. The cell lysates containing overexpressed C-terminally biotinylated Neurokinin-1 receptor were then injected into the chamber directly to yield a specifically oriented immobilization of the receptor. Total internal reflection fluorescence (TIRF) was used to detect binding of streptavidin to the BSA-biotin surface and to detect binding of the fluorescently

labelled substance P (SP), a Neurokinin-1 receptor agonist, to the oriented receptor. Binding studies revealed functionality of the receptor in accordance with previous studies[166] but used as little as 1 attomol of receptor due to the controlled orientation and high sensitivity of TIRF.

This simple immobilization was the first example of biotinylated MP on quartz surfaces directly from crude cell lysates without purification, thus allowing one to study e.g. mechanisms involved in GPCR and G protein interactions in the native system. This technique has the potential to be applied to a wide array of applications and if higher densities of proteins are needed for read-outs, on-surface purification and enrichment is possible due to the high affinity of the immobilization reagents. It was also suggested that artificial integration of biotinylated lipids into the plasma membrane of cells prior to homogenization could be an alternative and in some cases, chemically oxidized silicon surfaces may be used to avoid interactions with the lipids[164].

6.0 Specific immobilization of detergent-reconstituted protein via linkers

6.1 Via N-terminus of protein on sepharose resin –Potassium ion channel KcsA and membrane enzyme: DsbB (See Chapters 4 – 6).



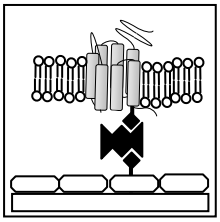
Fragment based drug discovery, an approach that consists of screening small molecules (< 300 Da) with weak interactions to a pharmaceutical target but with promising bioavailability properties[13], has gained considerable attention in the pharma industry. Sensitive techniques, such as crystallography and NMR, are required to detect the weak binding of fragments to the target. However, many biophysical techniques require large quantities of stable protein in a pure form, and that is generally not possible with many MPs. Although 60 % of all drugs target MPs[36], it has not proven possible to apply fragment methods to them. Target Immobilized NMR screening (TINS) has addressed some of these limitations[23] (Chapter 4) and shows promise as a fragment screening method on MPs. In TINS, 1D ^{1}H NMR spectra of the fragments are simultaneously acquired in the presence

of immobilized reference and target MPs in order to detect specific binders above the non-specific level of fragments interacting with detergents.

All proteins in this case were solubilized in dodecyl-phosphocholine (DPC) during the purification procedure, which involved simple metal affinity chromatography followed by gel filtration in the presence of the detergent. The immobilization procedure used Schiff's base chemistry between primary amines of the protein and aldehyde groups on the commercially available sepharose resin. The immobilization efficiencies were reported similar to previous studies on soluble proteins, with a final concentration equivalent of 100 μM of both the reference and target proteins on the resin. The functionality of immobilized DsbB was confirmed by an enzymatic assay which indicated that immobilized DsbB retained 90 % of its functionality. The functional immobilization of both KcsA and DsbB was further demonstrated by detecting binding of known ligands using TINS[23] (Chapters 4 & 5).

TINS addresses the protein demand by reusing a single sample of the target to screen the entire library, thereby requiring only 50 nmol of protein. Immobilization can be achieved via the N-terminus or a variety of other chemical strategies making it potentially broadly applicable to MPs. OmpA has been noted to retain conformation in a variety of surfactants[53,72,78,88,89] making it a useful reference. Although this was the first example of fragment based drug discovery applied to MPs, there remain several limitations. A full screen of 1000 fragments required a week to complete, and most MPs may not be stable in such conditions, although, in some cases, immobilization has proven to improve MP stability. Furthermore, the solubilization processes must still be tailored to each protein. However, alternative solubilization media such as Amphipols[167] and Nanodiscs[68,168], which appear to be compatible with TINS, offer the possibility to generalize the procedure (Chapter 6).

6.2 Via FLAG tag and biotinylated antibody linked to streptavidin or avidin on BSA-biotin surfaces -GPCR: β_2 Adrenergic receptor



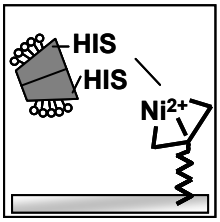
High-affinity interactions have been used successfully to immobilize GPCRs in their native membrane. However, many applications require pure protein in order to provide results with high signal to noise ratios. Here, the high affinity streptavidin-biotin interaction has been used to capture detergent solubilized GPCRs in a controlled and oriented manner and monitor activity by fluorescence microscopy[110].

To detect ligand binding, the detergent solubilized β_2 adrenergic receptor (β_2AR) was specifically labelled with fluorescein at Cys265, a conformationally sensitive site. The specific labelling of only Cys265 by the sulfhydryl-reactive fluorescent probe fluorescein maleimide was possible because the other cysteines of the protein were either inaccessibly located in the transmembrane domains, or non-reactive because of the presence of disulfide bonds[169]. For exploitation of the biotin-avidin interaction, two strategies were used in conjunction with an avidin or streptavidin linker. In the first, the protein was modified with an N-terminal FLAG epitope and indirectly linked to the avidin surface through a biotinylated anti-FLAG antibody. In the second strategy, a second cysteine residue was biotinylated and linked to the surface through avidin or streptavidin as in Section 5. Ligand-dependent activation of the β_2AR was detected with fluorescence microscopy by monitoring changes in fluorescence intensity upon ligand binding and receptor translocation. The β_2AR immobilized *via* the antibody displayed nearly identical responses to an agonist as the receptor in solution. The β_2AR directly immobilized by the biotin tag yielded less consistent responses that were significantly smaller than for the receptor coupled via the antibody. Whether this was an artefact of the immobilization itself or due biotinylation of the GPCR was not clear.

This method did not require protein reconstitution into a lipid bilayer and could immobilize protein with minimal loss due to the high affinity coupling. Although this detection method required the GPCR to be labelled with a fluorophore at a sensitive site, the immobilization strategy allowed the authors to study conformational changes of the protein and is compatible

with array technologies involving high throughput screening of MPs on chips for example, provided solubilization conditions have been found for a particular MP.

6.3 Via HIS tag on quartz surface – Serotonin-gated ion channel 5-HT$_3$R

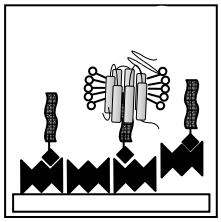


Many applications require pure protein immobilized at very high density. Immobilized metal affinity chromatography (IMAC) has been used to successfully immobilize the detergent solubilized serotonin-gated ion channel 5-HT$_3$R to quartz slides without the need for further lipid reconstitution[111]. This approach may be generally applicable to all MPs that can be solubilized and functionally expressed with a HIS tag. Prior to immobilization, the glass slides were first modified to contain thiol groups by gas-phase silanization. The thiol groups were then covalently linked to a lysine derivative of nitriloacetic acid (NTA) using a bifunctional maleimide-succinimide cross-linker. The newly created surface was fused to a Teflon spacer to create a flow-through cell and was subsequently charged with Ni^{2+}. The 5-HT$_3$R ion channel containing a HIS tag, was solubilized in nonaethyleneglycol monododecyl and immobilized via chelation to the Ni^{2+}-NTA.

The metal affinity based immobilization procedure was reversible, allowing quantification of immobilized receptors by elution with imidazole. Total internal reflection fluorescence spectroscopy was used to carry out competition binding experiments using the non-labelled competitor quipazine against GR-fluorescein ([1,2,3,9-tetrahydro-3-[(5-methyl-1H-imidazol-4-yl)methyl]-9-(3-amino-(N-fluoresceinthiocarbamoyl)propyl)-4H-carbazol-4-one]). The affinity of GR-fluorescein for the receptor in solution was identical to the surface-bound protein, demonstrating the feasibility of the process for identifying potential new drugs and quantifying their affinities with dose-response curves. This procedure was extremely simple and could potentially be applied to a variety of analytical techniques, given the usual constraints that the MP can be functionally solubilized and expressed with a HIS tag. The current application is limited to fluorescent ligands, but the sensitivity of the methods results in signal detection with as little as 1.6 attomol of immobilized MP, corresponding to the yield of a single mammalian cell! The detection is real-time, mass-independent and can be combined with micro-fluidic

applications to further explore high throughput analysis for drug discovery. Further, given the nature of the immobilization surface it seems likely that this procedure could be readily adapted to SPR applications.

6.4 Via biotinylated ligand – GPCR: Neurotensin receptor-1



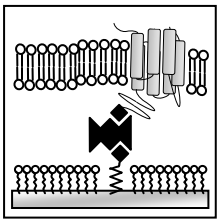
SPR analysis of GPCRs is often limited by the fact that low molecular weight ligands do not generate large signals. However, by immobilizing the ligand instead, the method allows one to study GPCR binding to biotinylated ligands immobilized to streptavidin-covered Biacore chips[170], without the need for lipid reconstitution.

For these studies the Neurotensin receptor-1 was expressed in *E. coli* as an N-terminal fusion product with the maltose binding protein, and a C-terminal His tag for stability and purification purposes. Receptor purification by IMAC and neurotensin affinity chromatography preceded solubilization in detergent micelles containing (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), DDM, and cholesteryl hemisuccinate (CHS). The peptide ligand Neurotensin (NT) was N-terminally biotinylated and immobilized onto the streptavidin-coated Biacore chips as the positive control. A ligand containing a scrambled version of the primary structure was also synthesized and immobilized on a second streptavidin-coated chip as a negative control. Binding was monitored by SPR by flushing the flow cells with the detergent-solubilized receptor, and highly specific interactions could be observed and confirmed. The chips could be regenerated by uncoupling bound receptors with high salt washes. The authors reported lower amplitude response than expected at higher levels of immobilized ligand, which they explained by potential ligand occlusion that could be prevented by the use of a longer linker between the surface and the ligand. Although no quantification of the binding affinities was provided, the technique has potential in array technology provided small molecular weight ligands are available and biotinylation does not affect their binding affinities to the target protein studied. Further, the technique could also be adapted to imaging mode SPR[110]. Although this technique was possible

with detergent-solubilized protein, the limitation of finding appropriate detergents for protein solubility remains a protein-specific issue.

7.0 Specific immobilization of lipid-reconstituted protein via linkers

7.1 Via biotinylated proteins in a mixed self-assembled monolayer -GPCR: Rhodopsin



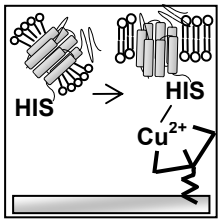
In order to study MPs which specifically bind ligands from the extracellular side of the membrane, methods have tended towards developing controlled, oriented immobilization in order to expose the appropriate side of the protein to the ligands. For such a controlled immobilization, a specific chemistry reacting to the appropriate terminus of the protein is necessary. Here, carbohydrate-specific chemistry[171,172] for N-terminal biotinylation of glycosylated proteins was combined with thiolipids to anchor rhodopsin to gold surfaces[66].

Gold sensor chip surfaces were covered with a homogeneous self assembled monolayer (SAMs) of ω-hydroxy-undecanethiol (HTA), interspaced with biotin attached to the surface through thiol groups, by micropatterned printing. The HTA layer was created to avoid protein immobilization in these areas. Subsequent addition of BSA blocked all non-specific binding sites and addition of streptavidin provided appropriate high affinity binding sites for the biotinylated receptor. The glycosylated receptor was specifically biotinylated on the carbohydrate chains near the N-terminus by oxidising the carbohydrate moieties with $NaIO_4$ prior to adding biotin-hydrazide[171]. Since only the extracellular facing portions of the receptor are glycosylated, rhodopsin was immobilized with the intracellular side facing away from the surface, allowing for maximal interaction with G proteins.

Surface plasmon resonance in the presence of GTP demonstrated the possibility of studying the receptor's constitutive activity. Interaction with the G protein was directly observed after a flash of light and the initial slope of the desorption signal was a good approximation of receptor density/unit area of surface. Relaxation of the activated G protein was also measured and binding of agonists such as 11-*cis*- or 9-*cis*-retinal was used to show that the immobilized rhodopsin was

functional. This immobilization strategy stabilized the GPCR for several hours and multiple cycles of ligand addition and removal, for which activation could easily be monitored. Furthermore, due to micropatterns with and without the receptor, non-specific binding was calculated as a localised reference, adding robustness to the data not otherwise available in SPR analyses. Although this particular biotinylation method may be limited to MPs with glycosylated regions near the targeted terminus, it is an attractive method for studying G protein mechanisms. However, when applying MPs in native environments, SPR often proves less sensitive than when used with the well behaved and highly overexpressed proteins such as rhodopsin.

7.2 Via lipid bilayer tethered through HIS-tagged protein -MP: Cytochrome c oxidase



Studying MPs by electrochemistry is often limited by the insulating properties of the lipids or detergents if they are applied directly onto the metallic surface. For detection of electron transfer, the protein therefore needs to be immobilized away from the surface. Here, a combination of lipid reconstitution and tethering the target protein through a HIS tag was used to immobilize Cytochrome c oxidase (CcO) to a silver surface[173,174]. The surfaces were roughened by electrochemical processes and functionalized with N-hydroxy succinimide ester (NHS) groups by addition of dithiobis-(N-succinimidyl propionate). An ion chelating layer of nitrilotriacetic acid groups (NTA) was finally created by binding the terminal amino groups of N-(5-amino-1-carboxypentyl) iminodiacetic acid to the existing NHS layer. Complexation of this new NTA monolayer layer with Cu^{2+} ions made it possible to reversibly immobilize HIS-tagged proteins. Immobilization was simply accomplished by flow-mediated addition of C-terminally HIS-tagged CcO in DDM. Finally, the lipid bilayer was established around the receptor by incubation with a buffer solution of DPGPC with subsequent removal of DDM. The heme groups of CcO remained intact and functional upon immobilization, as the ferric and ferrous states of hemes *a* and a_3 could be monitored repeatedly after electrode potential changes. The electron transfer rates of the protein were found to remain unchanged after immobilization, demonstrating an efficient electron exchange via the HIS tag.

An additional benefit of the IMAC methodology is that it can be used to purify and enrich the protein *in situ*[173]. This chemistry has been exploited by tagging C-termini of GPCRs[175,176,177,178] and is being used to produce high throughput screening platforms with flow cytometry[179]. Details were not supplied regarding stability of the surfaces or of the immobilized target. Although protein solubilization remains a challenge, a particularly exciting possibility is the potential to use crude cell lysates with this approach[173].

Table 1. Summary of direct (D) or indirect (I) immobilization methods with random (R) or oriented (O) protein on the surface, including type of membrane and protein involved, in order of appearance in the text.

Section	Ref	I/R	Method	Surface	Tethers	Membrane	Protein
1.0	107, 114	I	Lectin-gel bead adsorption	Sepharose 4B gel beads	-	Native membrane	Glucose transporter Glut1
2.1	116, 117, 118	I	Poly-Lysine, Silicon nitride interactions with native membrane	Poly-lysine coated glass or Silicon nitride surfaces	-	Native membrane	5-HT_3 receptor
2.2	119	R	Native vesicle budding from whole cells	Glass slides and/or functionalized glass	-	Native membrane	5-HT_3 receptor
2.3	124	R	Hydrophobic adsorption	Polyvinyltoluene fluorophor microbeads	-	Native membrane vesicles	Acetylcholine receptor
2.4	105, 106	I	Freeze-thawing	Superdex 200 gel beads	-	Native vesicles or proteoliposomes	Glucose transporter Glut1
2.5	67, 109	O	Freeze-thawing and hydrophobic glass adsorption	Glass slides	-	Reconstituted POPC/POPG	Fd coat protein
2.6	135, 136, 137	R	Hydrophobic, liposome adsorption onto monolayers	Platinum/glass and platinum/silicon slides	-	Synthetic bilayers formed by fusion of proteoliposomes to monolayers	Rhodopsin, acetylcholine-esterase, cytochrome oxidase, nicotinic acetylcholine receptor
2.7	112, 138	R	Hydrophobic adsorption by printing	γ-aminopropylsilane (GAPS)-derivatized gold slides	-	Vesicles of DPPC/DMPC egg-yolk PC	Adrenergic receptor, neurotensin receptor, dopamine receptor
3.1	146, 147	R	Hydrophobic tethers	Gold covered chromium layered glass	Thio-peptides	Triton X-114, Triton X-100, sodium cholate	Cytochrome C oxidase
3.2	151, 153	R	Hydrophobic adsorbance	Carboxymethyl-modified dextran polymer hydrogel	Polymer cushion	Native membrane vesicles	Rhodopsin
3.3	113	D	In vitro expression/ bilayer insertion	Gold	Peptides	Covalent peptide-DMPE fused with PC vesicles	OR5
3.4	154	R	Cross linking of bis-SorbPC by UV light	Cuvette	-	OG vesicles	Rhodopsin
4.0	108	D	Microprinting/affinity capture	Carboxymethyl-modified dextran	-	Reconstituted POPC lipid bilayer	Rhodopsin

Table 1. (Continued)

Section	Ref	I/R	Method	Surface	Tethers	Membrane	Protein
5.0	42, 110, 164, 165	O	Affinity capture	Glass slides Chemically oxidized silicon	Streptavidin-avidin + antibody	Native membrane vesicles	Biotinylated neurokinin-1 receptor, Gramicidin-A
6.1	23	O	Covalent coupling	Sepharose resin with reactive aldehyde groups	5 carbon chain-hydrophilic	DPC detergent	OmpA KcsA DsbB
6.2	111	O	Affinity capture	Glass or gold surfaces	Streptavidin-avidin + antibody + flag tag	DDM detergent	β2 adrenergic receptor with FLAG tag and fluorophore
6.3	170	O	Affinity capture	Glass modified to bind nickel (NTA)	Protein HIS tag	Nonaethylene/-glycol monododecylether ($C_{12}E_9$) micelles	5-HT_3R
6.4	66	O	Affinity capture	Streptavidin-coated chip	Streptavidin-biotin	CHAPS/DDM/ CHS detergent	Neurotensin receptor-1
7.1	173, 174	O	Microprinting /affinity capture	Gold sensor chips	Thiolipid and biotin	Self assembled monolayers (SAMs) of ω-hydroxy-undecanethiol (HTA)	Rhodopsin
7.2	111	O	Affinity capture	Silver modified to bind nickel (NTA)	Protein HIS tag	Reconstituted DPGPC	Cytochrome C oxidase

Table 2: Summary of protein immobilization solubilization requirements, shelf life, type of analytical results obtained, and type of analytical methods applicable, in order of appearance in the text.

Section	Ref	Protein	Surface covered	Solubilized	Shelf life	Studies allowed	Analysis
1.0	107, 114	Glucose transporter Glut1	44.6 μg/ml	No	One month	Binding, Interactions, Conditions	Frontal Chromatography
2.1	116, 117, 118	NK1 receptor and ion channel 5-HT3 receptor	-	No	-	Lipid and protein mobility in the native membrane, Binding Interactions	Fluorescence , Fluorescence after photobleaching (FRAP), Single molecule imaging
2.2	119	Ion channel 5-HT3 receptor	1750 receptor/ μm2	No	Several weeks	Binding interactions, interactions with other components in the cell membrane.	Fluorescence, Confocal microscopy, radioligand binding, fluorescence imaging
2.3	124	Acetylcholine receptor	12.5 -125 ng/mg	No	-	Binding studies, identification of orphan receptors	Radioligand binding
2.4	105, 106	Glucose transporter Glut1		No	Few months	Binding, Interactions, Conditions	Frontal Chromatography
2.5	67, 109	Fd coat protein	-	Cholate	-	Structural solid-state, ^{15}N labeled protein	Solid-state NMR
2.6	135, 136, 137	Rhodopsin, acetylcholinesterase, cytochrome oxidase, nicotinic acetylcholine receptor	50 ng/cm2	OG, DPPA, DPPE, DPPC and cholesterol	Few months	Binding studies	Radioligand binding, electrochemistry, fluorescence
2.7	112, 138	Adrenergic receptor, neurotensin receptor, dopamine receptor	-	No	-	Deorphanizing receptors, lead optimization and identification, bioassays, high throughput screening	Fluorescence based assays, radioligand binding assays*
3.1	146, 147	Cytochrome C oxidase	-	Triton X-114, Triton X-100, sodium cholate, and saccharose	-	Binding studies and kinetics	Impedance spectroscopy
3.2	151, 153	Rhodopsin	400 RU	No	-	Potential for hybridization and kinetics and binding	SPR, impedence spectroscopy, AFM
3.3	113	OR5	-	No	-	Binding studies, Protein orientation	SEIRAS

Table 2. (Continued)

Section	Ref	Protein	Surface covered	Solubilized	Shelf life	Studies allowed	Analysis
3.4	154	Rhodopsin	-	No	-	Isomerization and G-protein activation	Plasmon-waveguide resonance (PWR) spectroscopy
4.0	108	Rhodopsin	0.1 pmol/mm^2	Egg PC	-	Binding studies	SPR
5.0	42, 110, 164, 165	Biotinylated neurokinin-1 receptor	100-400 receptors/um2	No	-	Binding studies	Total internal reflection fluorescence
6.1	23	OmpA, KcsA, DsbB	100nmoles/ml	DPC	One month	Fragment based drug discovery	NMR
6.1	110	β2 adrenergic receptor with FLAG tag and fluorophore	-	DDM	-	Binding studies, activation under agonist	Fluorescence microscopy
6.2	111	Ion channel 5-HT3R receptor	-	Nonaethylene-glycol monododecyl-ether detergent (C12E9)	-	Binding	Total internal reflection fluorescence
6.3	170	Neurotensin receptor-1	250 RU	CHAPS/DDM/CHS	-	Binding	SPR
7.1	66	Rhodopsin	-	No	Several hours	Binding studies, activation with agonist	SPA
7.2	173, 174	Cytochrome C oxidase	-	DDM	-	Redox experiments	Electrochemistry

Perspectives:

Biophysical assays of protein function and/or ligand binding are playing an ever increasing role in both academic and industrial life science research. Applications in academic labs include determining and understanding protein interaction networks that lead to regulation of cell behavior (e.g. proteomics) while the primary use in industry has been for the discovery of ligands that modulate protein behavior. In order to scale these assays down and/or make efficient use of limited resources, the proteins are often immobilized on flat metallic or glass surfaces with little or no biocompatible characteristics. While the array of biophysical techniques that have been successfully applied to soluble proteins is impressive, until recently similar applications to membrane bound proteins were sparse. Here we have highlighted many of the success stories culled from the literature of the last few years. One common denominator of these success stories is the innovation and effort required to overcome the bio-incompatibility of the surfaces. A second recurring theme is that each solution must be tailored to the individual protein being studied. Together these two remaining issues represent a bottleneck to widespread, high throughput biophysical assays that could take advantage of, for example, printing techniques.

We see two developments that, when combined with methodologies that have been described, offer exciting opportunities for more generic application of biophysical techniques. Interestingly, these developments come from both sides of the problem, that is the protein itself and the media used to solubilize the protein, and therefore are potentially complementary. Approaching the problem from the protein point of view, the group of Christopher Tate at Cambridge University (UK) has developed a technique for selecting mutants that provide enhanced thermal and conformational stability while retaining desired ligand binding properties[70]. Importantly, these stabilized proteins are compatible with a much wider range of detergents than the wild type protein and thus should be more amenable to large scale immobilization studies. From the media point of view important advances have been made in developing alternatives to detergents for functional solubilization of MPs. Nanodiscs[68,73,74], bilayer lipid assemblies surrounded by a stabilizing protein, and amphipols[72,180,181], synthetic amphipathic polymers, have successfully

replaced detergents to solubilize a variety of MPs including GPCRs. Lipidic sponge phases represent an even more recent addition to this list, which, so far, shows promise for crystallization of MPs[182]. The crucial advantage of these new media is that they represent a more or less "one size fits all" solution that holds the promise of eliminating, or at least greatly minimizing, the requirement to precisely tailor each solution to the MP.

Ultimately applications which combine the high sensitivity of biophysical methods with the possibility of studying an MP in its native environment without the need for purification and reconstitution represent the Holy Grail for many research goals. While at present still out of reach, the growing body of information on production and immobilization strategies and the ever increasing sophistication of biophysical methods will undoubtedly conspire to enable this *in situ* approach. The combination of such biophysical studies with increasing success in the crystallization and NMR analysis of MPs should prove a powerful approach to both reveal molecular mechanisms of membrane protein function and enable rational elaboration of small molecule modulators of this function.

Acknowledgements

The authors would like to thank Prof. Karen Martinez for carefully reading the manuscript.

Glossary

Adsorption: The process of accumulating a substance onto a surface through hydrophobic interactions.

Bicelle: A micelle containing a bilayer of amphipathic molecules as opposed to just one layer.

Black membranes: An artificial planar membrane that forms over a hole in the partition between two aqueous compartments and is optically black when viewed in incident light; used to study the permeability of bilayer membranes and the mobility of bilayer components.

Critical Micellar Concentration (CMC): The concentration of amphiphilic molecules at which a micelle is formed.

Detergent Depletion Technique: Also called the micellar dilution technique. In a mixed detergent-lipid system, dilution with detergent and lipid free buffer will cause the detergents, which have a lower critical micellar concentration than lipids, to detach from the system and cause the remaining lipids to fuse together.

LB monolayers: A Langmuir–Blodgett monolayer: contains one layer of organic material, deposited from the surface of a liquid onto a solid by immersing (or emersing) the solid substrate into (or from) the liquid.

Liposomes: An artificial vesicle consisting of an aqueous core enclosed in one or more phospholipid layers.

Membrane vesicles: Closed, unilamellar shells formed when membranes are mechanically disrupted because the free ends of a lipid bilayer are highly unstable.

Micellar dilution technique: See Detergent Depletion Technique.

Micelle: A colloidal aggregate of a unique number (50→100) of amphipathic molecules. In polar media such as water, the hydrophobic “tails” of the amphiphilic molecules tend to locate away from the water while the hydrophobic “heads” are located towards the water.

Planar supported lipid bilayer (PSLB): Lipids arranged in micropatterns with high stability on a flat surface.

Proteoliposomes: A liposome with embedded membrane proteins.

Self assembled monolayers (**SAM**s) are surfaces consisting of a single layer of molecules covalently linked to a surface through functional groups.

Small unilamellar vesicles (SUVs): Small vesicles consisting of one layer of lipid molecules.

Solid supported membranes: Membranes immobilized to a surface through a linker. A hydrophilic polymer, often grafted with hydrophobic chains, is placed in between the membrane and the surface in order to provide a lubricating supportive, layer for the membrane.

Tethered bilayer lipid membrane (tBLM): A lipid membrane anchored to a surface through a long linker which does not impede membrane mobility

Chapter

3

Functional immobilization of histamine H_1 and adenosine A_1 receptors on sepharose beads:

A facile approach with broad applicability to membrane proteins

G protein-coupled receptors (GPCRs) are responsible for signal transduction across cell membranes and are involved in many pathologies. Emerging new biophysical techniques can potentially provide more detailed information on protein-ligand interactions at the atomic scale. However, many of these technologies require protein immobilization, which remains a challenge when applied to GPCRs due to the absence of a generally applicable procedure. Here we address this issue by developing a simple and widely applicable immobilization protocol and applying it to crude membrane preparations containing either the human histamine H_1 receptor (hH_1R) or the human adenosine A_1 receptor (hA_1R). Native G proteins involved in the signalling cascade are retained during this process due to immobilization of crude, non-solubilized membrane fractions. The immobilization is based on Schiff's base formation between aldehyde groups on the resin and primary amines present in membrane-spanning proteins. Radioligand binding assays and dot blots show that this methodology succeeds in consistently yielding between 1 to 2 pmol of functional receptor per ml of resin. Pharmacological characterisation indicates that both antagonists and inverse agonists have similar affinity for immobilized and non-immobilized receptors suggesting the approach should be sufficiently reliable to carry out analytical assays for ligand discovery and characterisation. Furthermore, receptor immobilization results in significant stabilization and therefore the ability to store them. Thus, the method is promising as a means to immobilize a wide range of membrane proteins, including GPCRs without prior modification, solubilization, or lipid reconstitution.

G protein-coupled receptors (GPCRs) are responsible for transducing sensory and chemical signals across the cell membrane, and as such, their involvement in a wide range of pathologies makes them important drug targets and study foci. It is reported that approximately 30 - 40 % of marketable drugs target this class of proteins[100]. The two model receptors used in the scope of this study are prime drug targets since the histamine H_1 receptor mediates a variety of allergic reactions[183] and the adenosine A_1 receptor is involved in neurotransmission and thus a variety of neurodegenerative diseases[84]. Most GPCRs are ligand activated, yet a substantial number remain "orphaned", where the native activating ligand is not known. Discovery of non-native, small molecule modulators of GPCR function is also an area of highly active research for which gaps remain in current technologies. These issues underline the need to improve our understanding of this group of proteins by developing tools that provide new information in an efficient and detailed manner.

High throughput ligand screening assays of GPCRs typically use membrane-based assays involving microprinting[66,112,139] or rely on cell-based assays[184,185,186], where as many as 150,000 compounds can be screened in 8 hours. These assays are good at finding "drug-like" (300-500 Da) modulators of GPCR function, along with "macroscopic" parameters such as IC_{50} values, as well as some biological functionality characterizing the ligand-GPCR interaction. For an alternative and more "microscopic" approach, however, new biophysical methods are being developed in which the atomic or molecular interactions between a ligand and a protein are emphasized. Biophysical methods present many advantages such as the ability to directly detect physical interactions and differentiate between reversible and non-reversible processes. Furthermore, known functionality of the target protein is not required. Due to their intrinsic sensitivity to weak intermolecular interactions, many biophysical methods can be used to screen small molecule libraries of so-called drug fragments (150 - 300 Da)[22] (that obey Lipinski's rules)[10]. As a result, subsequent stages of the drug discovery process should yield compounds that are more orally bioavailable and less toxic.

Although biophysical techniques for high throughput screening have been successfully applied to soluble proteins that have been immobilized via a multitude of chemical linkers[101,102,103], applications to immobilized GPCRs still pose a challenge. GPCR conformation, stability, and

functionality are all dictated by experimental conditions ranging from cell culture and storage to the composition of the necessary presence of the lipid membrane[148]. In addition to this sensitivity, it is difficult to immobilize GPCRs with sufficient density to allow reliable signal detection. Many immobilization strategies employ protein modification by biotinylation[66] or the adjunction of antibodies[162] to a surface in order to have a well defined target orientation. Functional GPCR immobilization is commonly achieved by adsorption[137,187] or anchorage[146] of lipids on flat glass or gold surfaces, and are typically applied to purified, solubilized preparations which undergo subsequent lipid reconstitution[108,162,173] (Chapter 2). Schiff's base chemistry has been used in liquid chromatography as an immobilization strategy, but it has been reported to result in high non-specific binding[188]. Solubilization and lipid reconstitution of GPCRs require specific protocols for each protein, and finding the correct mixture of detergents can be extremely time consuming[189] or even futile. Furthermore, solubilization and purification results in the removal of the native membrane and associated proteins, such as the appropriate G proteins, which are important players in the signalling cascades[85,190]. A generally applicable method to immobilize GPCRs within their native membrane would clearly be welcome.

The aim of this study, therefore, is to determine whether GPCRs, as exemplified by the hH_1R and the hA_1R receptors, remain functional when they are immobilized in their native membranes on sepharose beads without modification, purification, or lipid reconstitution (Figure 1). This would provide a ready alternative to gold or glass chip surfaces used in current research and would allow GPCRs to be studied by a variety of biophysical methods such as SPR[170] and Target Immobilized NMR[23,77].

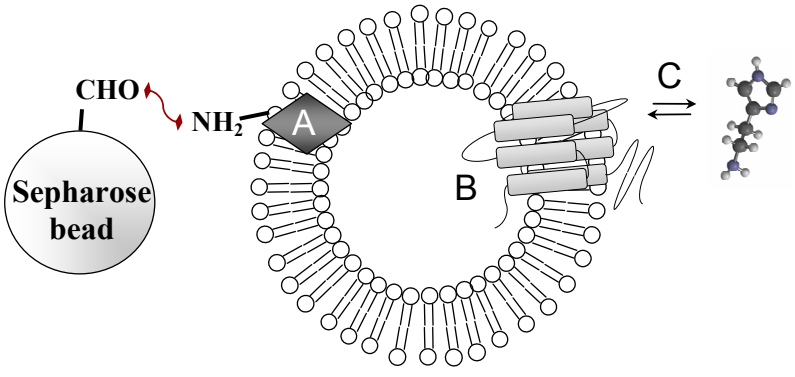


Figure 1. Immobilization of GPCRs on sepharose resin via native membrane vesicles. The immobilization is carried out *via* primary amines of the GPCRs (B) or of other integral or membrane associated proteins (A). The ligand binding properties (C) of such a system are essentially unchanged with respect to non-immobilized vesicles.

MATERIALS AND METHODS

Materials

The construction of the Chinese Hamster Ovary (CHO) stable cell line overexpressing the human H_1 receptor doubly tagged with heamaglutinin and 6-His (HA-hH_1R-HIS) will be reported elsewhere. The CHO stable cell line overexpressing the human A_1 receptor (hA_1R) was provided by Prof. Steve Hill at the University of Nottingham. HA-hH_1R-HIS and hA_1R had a maximum amount of binding sites (B_{max}) of 6.2 pmol/mg of total protein and 8.5 pmol/mg of total protein, respectively. All cell culture products such as Dulbecco's modified Eagle Medium, Penicillin, Streptomycin, Newborn bovine serum, G418, and Trypsin were purchased from standard suppliers. ALD Actigel coupling resin and coupling reagent $NaCNBH_3$ (sodium cyanoborohydride) were purchased from Sterogene (CA, USA). CH Sepharose 4B and NHS activated Sepharose 4 FF were purchased from GE Healthcare. [^{3}H]mepyramine (specific activity 32 Ci/mmol) was purchased from Amersham Biosciences (Roosendaal, NL) and [^{3}H]DPCPX (specific activity 127 Ci/mmol) was purchased from NEN (Du Pont Nemours, 's-Hertogenbosch, NL). Histamine and mianserin, as well as all chemicals used for buffer preparations, were obtained from Sigma-Aldrich (Zwijndrecht, NL). CPA, 8-CPT, and N0840 were purchased from RBI (Natick, MA, U.S.A) and ADA (adenosine deaminase) was acquired from Roche Biochemicals (Mannheim, Germany). The antibody recognising $G\alpha_{q/11}$ proteins (sc-392) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell culture

Cells were cultured weekly in 30 Petri dishes (15 cm) with 20 ml modified Eagle's medium containing 50 IU/ml penicillin, 50 µg/ml streptomycin, 10 % (v/v) newborn bovine serum, and 400 µg/ml G418, at 37°C in humidified 5 % CO_2.

Membrane isolation and preparation

Weekly, cells were harvested and prepared according to the method described previously[191], with minor modifications to create a finer suspension. Cells were rinsed with PBS, detached by scraping, and washed by centrifugation at 2700 rpm for 5 min, with resuspension in 30 ml cold membrane buffer (15 mM Tris, 1 mM EGTA, 0.3 mM EDTA, 2 mM $MgCl_2$, pH 7.4 at 4°C). Cells were then homogenized in a tight-fitting 30 ml Potter-Elvehjem tube with 10 slow up and down strokes at 700 rpm (pottering), and immediately centrifuged at 1000 rpm for 10 minutes to remove unwanted pelleted cell debris. The supernatant was collected, pottered, and precipitated *via* ultracentrifugation at 31000 rpm for 20 minutes. The pellet was resuspended in 20 ml cold membrane buffer, and the pottering and ultracentrifugation steps were repeated. The resulting pellet was resuspended in 4 ml cold phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2HPO_4; pH 7.4 at 4 °C). For hA_1R containing membranes, an additional 0.8 IU/ml ADA was added to the final preparation. Total protein concentration was determined using the bicinchoninic acid (BCA) assay[192] before storing membranes at -20 °C.

Membrane immobilization

The ALD Actigel resin was used as a 50 % slurry. When possible, all procedures were carried out at 4 °C. The resin was first washed with filtered water prior to being washed 3 x with cold phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2HPO_4; pH 7.6 at 4 °C) by centrifugation at 3000 rpm and resuspended in an equal volume of the same buffer. Membranes were thawed and pottered with 5 strokes before being added to the Actigel resin at a ratio of 1 mg total protein to 1 ml of resin. Coupling reagent (1 M $NaCNBH_3$) was added to a final concentration of 0.1 M. The tubes were placed at 4 °C and gently rotated for 18 h so as to avoid pellet formation. After immobilization, the supernatant was collected for quantification after a 5 minute centrifugation at 3000 rpm. The pelleted resin was resuspended with 1 ml Tris buffer (100 mM Tris; pH 7.6 at 4ºC) and 0.1 M coupling reagent for 2h at room temperature to block the remainder of the free aldehyde sites on the resin. Reducing agent was always removed prior to continuing with

experiments by washing the resin four times with Tris buffer (50 mM Tris; pH 7.4 at 4 °C). To determine the effect of linker length on GPCR immobilization, a similar procedure was followed using either activated CH Sepharose 4B which has an 8 atom linker or NHS activated Sepharose 4 FF which has a 14 atom linker. Membrane vesicle preparations were thawed and immobilized according to the manufacturer's suggestions for each of the three resins using 1 mg total protein per 1 ml of resin. Immediately before radioligand binding assays, the resin was washed 4 times with cold Tris buffer. The amount of functional receptors immobilized was quantitated by radioligand binding studies as described below.

Quantitation of total receptor immobilization efficiency

To obtain an approximate quantification of the total amount of HA-hH_1R-HIS or hA_1R immobilized, dot blots were used with anti-HIS tag or anti-A_1R antibodies. 10 µl of fresh membrane preparations overexpressing the receptors were used as reference, while equivalent preparations from the non-recombinant, parental cell line membrane preparations not overexpressing receptors ($CHOK_1$ for hH_1R and CHOkool for hA_1R) were blotted as negative controls. Corresponding volumes of supernatants before and after the immobilization procedure were also blotted. The pelleted resin containing immobilized receptors were also sampled for blotting, after a 1:1 dilution in denaturing buffer used in SDS-PAGE analyses, but lacking bromophenol blue (50 mM Tris/HCl pH 6.8, 1 % SDS, 15 % glycerol, 1 % β-mercaptoethanol at room temperature) and a 15 minute incubation at 95 °C for removal of protein from the resin. After a 3 minute centrifugation at 2500 rpm, the supernatant containing the membrane vesicles stripped off the resin, along with denatured receptors, was blotted. All samples went through the denaturing step in order to compare results, and each lane consists of a serial dilution by a factor of 0.5. The bands were quantified by volume density analysis. The background level from the negative control was subtracted from the final values which were extrapolated by comparing the standard curve obtained with the known B_{max} and density analysis from the positive control.

Detection of $G\alpha_{q/11}$ proteins present with immobilized receptors on resin

Western Blot analysis of whole cell lysates, isolated membranes, and immobilized receptors was carried out to identify the presence of native $G\alpha_{q/11}$ proteins in the immobilized receptor's environment. Whole cell lysates and isolated membranes were used as positive controls and prepared in equivalent volumes for direct comparison with immobilized receptors. To prepare whole cell lysates, densely cultured cells overexpressing the HA-hH_1R-HIS were washed 3 times with PBS and scraped into tubes on ice before being centrifuged at 4000 rpm for 5 min at 4 °C. Cells were then resuspended in RIPA buffer (150 mM NaCl, 1 % NP-40, 0.1 % SDS, 2 µg/ml aprotinin, 5 µM leupeptin, 50 mM Tris; pH 8.0) and incubated for an hour at 4 °C before being pottered. Membranes were isolated and immobilized as described previously, but with the presence of 2 µg/ml aprotinin and 5 µM leupeptin. The pelleted resin containing immobilized receptors was washed 3 x to remove non-immobilized material and incubated for 15 minutes at 95 °C to melt the agarose resin, leading to collection of protein which was bound to the resin in the supernatant. Samples were centrifuged at 3000 rpm for 3 minutes and the supernatant was diluted 1:1 in loading buffer. Whole cell lysates and isolated membranes were diluted 1:1 and 1:2 in loading buffer. All samples were loaded at 10 µl, separated by 12 % SDS-PAGE and blotted onto a polvinylidene difluoride membrane before being detected by an enhanced chemiluminescence assay, with the $G\alpha_{q/11}$ protein antibody as a primary antibody. The bands were quantified by volume density analysis with subtraction of the background levels from the negative control, extrapolated values from the standard curve obtained with the known B_{max} and density analysis from the positive control containing 1:1 cell lysates.

Pharmacological characterisation of non-immobilized and immobilized HA-hH_1R-HIS receptors and hA_1R receptors

Displacement studies were carried out by incubating HA-hH_1R-HIS membrane aliquots of 5 µg total protein for 30 min at 30 °C in 400 µl Tris buffer (50 mM Tris; pH 7.4 at 4 °C) containing final concentrations of 1 nM [^{3}H]mepyramine and increasing concentrations of displacer, either

mianserin (antagonist) or histamine (agonist). Saturation studies were carried out by incubating HA-hH_1R-HIS membrane aliquots of 5 μg total protein with increasing concentrations of [^{3}H]mepyramine ranging from 0.1 nM to 8 nM with non-specific binding determined by the presence of 1 μM mianserin. The incubations were stopped by rapid dilution with ice-cold Tris buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters (Whatman, Belgium) that had been treated with 0.3 % polyethylenimine as described previously[193]. Filters were washed four times with binding buffer, and radioactivity retained on the filters was measured by liquid scintillation counting. Binding studies for hA_1R were similar, however a 60 min incubation time at 25 °C was required with final concentrations of 1 nM [^{3}H]DPCPX with increasing concentrations of CPA (agonist), N0840 (antagonist), and 8-CPT (inverse agonist). Saturation curves were determined with increasing concentrations of [^{3}H]DPCPX ranging from 0.1 to 4 nM, using 10 μM CPA to determine non-specific binding. Filtration was done over Whatman GF/B filters without prior polyethylenimine treatment. Immobilized receptors were characterised in an identical manner to the respective non-immobilized receptors, by using an amount of resin which corresponded to 5 μg of total immobilized protein. All radioligand binding studies of membranes immobilized on resin required special attention because resin covered the filter surface and could easily be dispersed. Furthermore, the incubation steps were carried out without shaking to prevent loss of resin due to adherence on the edges of the tube.

In order to quantify the amount of functional receptor immobilization, samples were incubated with and without a displacer to determine saturation curves in the presence of the corresponding radioligand (1 nM). Appropriate controls were chosen to determine fmol of functional receptor present in the various steps. To determine the effect of the reducing agent on ligand binding, membranes were incubated with 0.1 M $NaCNBH_3$, which was subsequently removed by pelleting the membrane preparations and resuspending in Tris buffer. For stability studies, samples were kept for one week at 4 ºC and subjected to quantification as described above.

Data analysis

Receptor binding data were analysed using the non-linear regression curve fitting program Graph Pad Prism v. 4.01 (Graph Pad, San Diego, CA, U.S.A.). Statistical significance was evaluated with the student's T-test. Saturation experimental data (K_d and B_{max} values) were obtained by computer analysis of saturation curves. Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng & Prusoff equation $K_i=IC_{50}/(1+[C]/K_d)$, where [C] is the concentration of radioligand used in competition binding, and K_d its dissociation constant[194]. All values obtained are means of at least three independent experiments performed in duplicate. Values of functional receptor were derived by the following equation, assuming that each mole of radioligand binds to one mole of receptor: $R= [[C]/(K_d + [C])] \times [S/(2220 \times SP)]$ where R is the amount of functional receptors (moles) per 50 µl resin, [C] is the radioligand concentration, S is the radioligand specific binding (dpm), and SP is the radioligand specific activity (Ci/mmol). In all assays, care was taken to assure total binding never surpassed 10 % of total radioligand added. In order to determine how much total protein was immobilized, the amount of protein added to resin and the amount present in supernatants after immobilization were subject to BCA protein assays, after a wash with buffer and an ultracentrifugation step to remove $NaCNBH_3$ due to its negative effect on the assays. The volume density analysis of dot blots and western blots were carried out using Quantity One imaging software (BioRad, USA).

RESULTS

We sought an immobilization procedure that would both maintain the native environment and be widely applicable to an array of potential membrane protein targets, yet compatible with various biophysical assays. We therefore began by attempting to immobilize membrane preparations of cells stably expressing human GPCRs. We chose sepharose based resins that are well characterized for bio-compatibility, have low non-specific binding and are highly porous endowing large specific binding capacity. The Schiff's base chemistry used to immobilize proteins on commercially available sepharose resins is very mild, yet stable. Various membrane preparation methods were tried, but ultimately, that which resulted in a fine suspension by repeated pottering and centrifugation was used (see Methods). Using this method, a Heamaglutinin and 6-His tagged human H_1 receptor (HA-hH_1R-HIS) and untagged human A_1 receptor (hA_1R) were consistently immobilized on the Actigel ALD resin (Figure 2, panels **a** and **b** respectively).

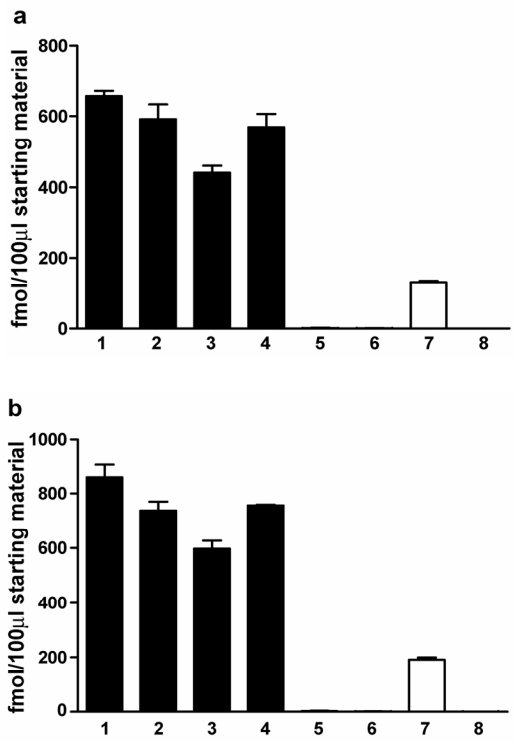


Figure 2. Immobilization efficiency of HA-hH_1R-HIS membranes (**a**) and hA_1R membranes (**b**) as measured by radioligand binding. The height of each bar represents the fmol of radioligand bound per 100 µl of starting material. Black bars represent controls of membrane preparations with the first being the starting material stored at -20 °C (1) and the second representing preparations that were maintained at 4 °C for the same duration as samples that were immobilized (2), the same as 2 but in the presence of reducing agent (3) and after removal of the reducing agent (4). Non-specific ligand binding to untreated resin was measured (5), along with the washing efficiency (6). The amount of functional, immobilized GPCR was determined (7) as well as that which remained in the supernatant after immobilization (8). The average of 3 experiments performed in duplicate is shown

The starting material consisted of membrane preparations that had been stored at -20 °C. We first titrated the amount of membrane preparation used for immobilization and observed a distinct optimum achieved when adding receptors at 1 mg total protein/ml concentration, at a maximum of 1 mg total protein per ml (settled bed volume) of resin (data not shown). Assuming 1:1 ligand binding stoicheometry, this amount corresponds to 620 fmol of active HA-hH_1R-HIS and 850 fmol of active hA_1R per 100 µl of starting material (Figure 2**a** and Figure 2**b**, column 1). Both receptors show consistency in the proportion of active receptors remaining at each step of this experiment. Simple storage of the membrane preparations at 4 ^{o}C for 18 h resulted in a 10 % loss of ligand binding capacity for both receptors (Figure 2**a** and Figure 2**b**, column 2). While the presence of the reducing agent sodium cyanoborohydride ($NaCNBH_3$, for reducing the Schiff's base to a primary amine) had a slightly negative impact on the radioligand binding assays, this was completely reversible (Figure 2**a** and Figure 2**b**, columns 3 & 4). There was little non-specific binding of the radioligand to the resin in the absence of receptor and the washing step was efficient in removing all receptors that were not irreversibly immobilized by $NaCNBH_3$ (Figure 2**a** and Figure 2**b**, columns 5 & 6). The mild procedure results in consistent functional immobilization of 20 - 25 % of both the hH_1R and hA_1R receptors. Essentially no detectable functional receptors remained in the supernatant fraction after immobilization for 18 h (Figure 2**a** and Figure 2**b**, columns 7 & 8). This equates to 1.3 pmol of functional HA-hH_1R-HIS and 2.1 pmol of functional hA_1R per ml of resin.

Since the radioligand binding assay could only detect functional receptors, we wished to know whether or not the coupling procedure was selecting for functional receptors leading to the apparent 20 - 25 % yield. Using a total protein quantification method, we found approximately 1 % of the input protein remaining in the supernatant after an 18 h immobilization (not shown). This data corresponds well to the amount of receptors in the supernatant after immobilization when determined by radioligand binding studies. We used a dot blot assay in order to specifically follow the fate of the HA-hH_1R-HIS during immobilization (Figure 3**a**).

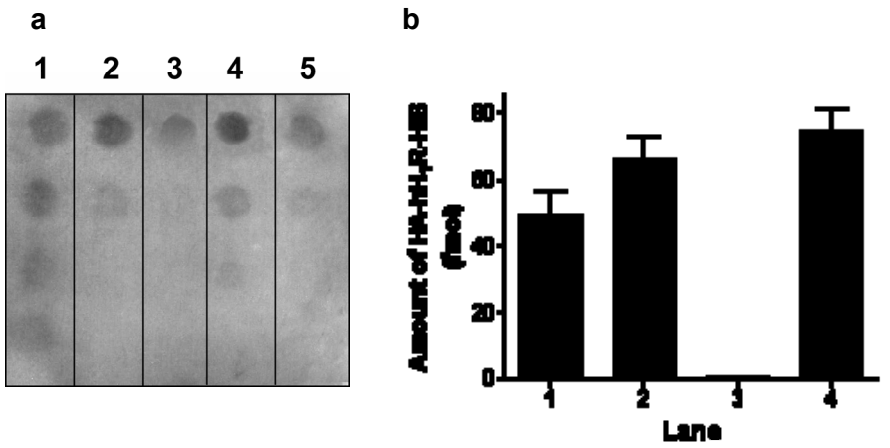


Figure 3. Immobilization efficiency of HA-hH_1R-HIS-containing vesicles. A dot blot assay was used to quantitate the amount of HA-hH_1R-HIS present in various fractions of the immobilization procedure using anti-HIS tag antibodies (**a**). A control vesicle preparation with approximately 6 fmol/µl of HA-hH_1R-HIS (lane 1) is compared to the supernatant of an immobilization reaction before (lane 2) and after immobilization (lane 3), from which it can be seen that the majority of receptor is removed from solution. Analysis of the supernatant after stripping the receptors off the beads (lane 4) indicates that the majority of receptors were tightly bound to the resin (lane 4). Vesicle preparations from the parental $CHOK_1$ cell line that does not express the receptor indicate the level of non-specific staining (lane 5). The dot volume densities were quantified by QuantityOne (BioRad) and the amount of HA-hH_1R-HIS in the first row of each lane is represented in (**b**) after subtraction of non-specific signal in lane 5.

The blots were quantified and the amount of receptor in the first row of each lane is represented in Figure 3**b** after subtraction of background signal from the negative control in lane 5. Fresh membranes stored at -20 ºC were blotted in (lane 1) and represent the positive control. Membrane suspensions before (lane 2) and after immobilization (lane 3) show that a significant fraction of the receptor has been removed from solution (compare lanes 2 and 3) and as expected, was bound to the resin (lane 4). The apparent increase in signal was due to concentration of the receptor sample upon immobilization which was difficult to precisely correct for due to the unknown efficiency with which the covalently bound protein could be removed from the resin by heating. The signal remaining in the post-immobilization supernatant was consistent with the amount observed with radioligand studies of the supernatant after immobilization, and is within the same level, within error, of the negative control membranes that do not express His-tagged receptors (lane 5). This data suggests that nearly all of the GPCRs have been successfully immobilized on the resin and that therefore, only 20 - 25 % of this population remained

functional as suggested by the data in Figure 1. Similar experiments were attempted for the hA_1R but dot blots were inconclusive as the anti-hA_1R antibodies apparently did not recognize denatured receptors.

The aim of immobilizing GPCRs via their native membrane was to keep as much of the native environment present as possible, specifically including all proteins necessary for signal transduction. Thus we sought to determine whether the appropriate G proteins for hH_1R, $G\alpha_{q/11}$, were co-immobilized on the sepharose beads. Western Blot analysis (Figure 4**a** and Figure 4**b**)

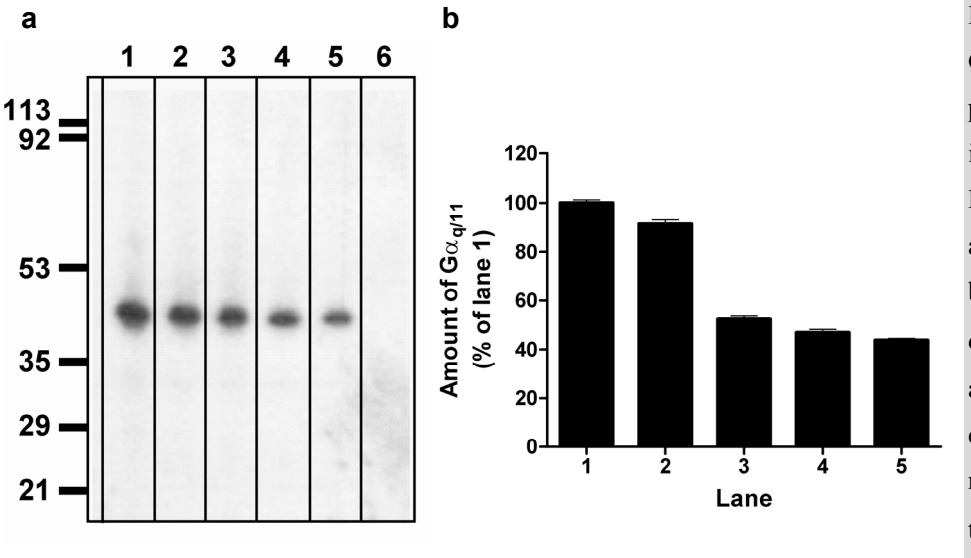


Figure 4. Detection of $G\alpha_{q/11}$ proteins (42 kDa) present on the resin with immobilized HA-hH_1R-HIS by Western Blot analysis. $G\alpha_{q/11}$ antibodies were used to detect $G\alpha_{q/11}$ proteins (**a**) and the corresponding quantities of $G\alpha_{q/11}$ are represented as percentages of lane 1 (**b**). Lanes 1 to 4 correspond to positive controls, where lanes 1 and 3 are lysates of whole CHO cells overexpressing the receptor, with no dilution and a dilution of ½ respectively, and lane 2 and 4 are the corresponding isolated membranes, with the same pattern of dilutions. Lane 5 corresponds to the supernatant containing membrane vesicles which have been denatured off the resin after immobilization, with no dilution. Lane 6 contains the standard proteins used as a ladder for the MW weights, and consists of a negative control due to the absence of $G\alpha_{q/11}$ proteins. The quantification reveals that 45 % of the proteins applied to the resin (lane 2) were collected from the resin after denaturation (lane 5).

show that the amount of $G\alpha_{q/11}$ proteins immobilized equates to 45 % of the amount present in HA-hH_1R-HIS whole cell lysates and isolated membranes. It was not our goal to quantify the exact amount of $G\alpha_{q/11}$ proteins which were co-immobilized, because the level of uncoupling from the resin achieved with our method is not fully quantifyable. Therefore, although it is

difficult to say whether there were more $G\alpha_{q/11}$ proteins immobilized but only 45 % were effectively stripped off the resin, this data suggests that the physiologically relevant $G\alpha_{q/11}$ proteins were co-immobilized on the resin and therefore enabled agonist binding pharmacology on the immobilized HA-hH_1R-HIS receptors.

We wanted to determine any possible influence of linker length on functional immobilization of receptors. The sepharose ALD has a relatively short 5 atom linker so we tested resins with hydrophilic linkers of 8 and 14 atoms for functional immobilization of hA_1R and HA-hH_1R-HIS receptors (Figure 5).

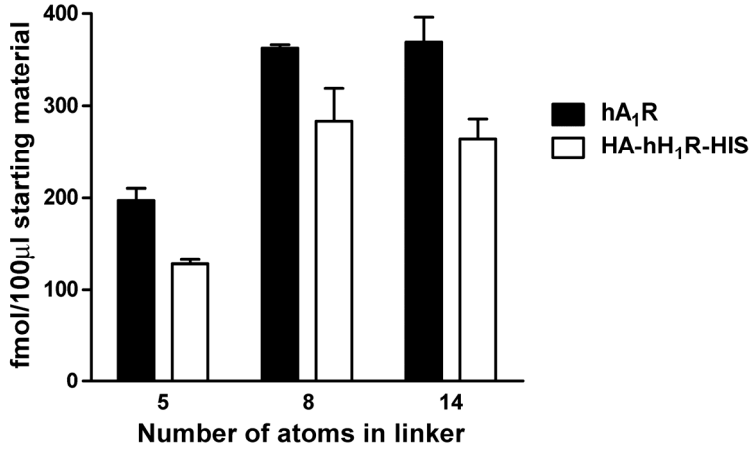


Figure 5. Determination of linker length on efficiency of functional immobilization of hA_1R and HA-hH_1R-HIS. The total amount of functional receptor bound was determined by saturation binding experiments. The average of 3 experiments performed in duplicate is shown.

Both resins resulted in a near doubling of the amount of functionally immobilized receptors. Since the immobilization chemistry of all three resins is very similar, it seems likely that there is a threshold of linker length required for maximal functionality. This data is consistent with the idea that the receptor is sterically hindered by shorter linkers[189].

To characterize the pharmacology of immobilized receptors, both saturation and competition binding studies were performed on non-immobilized and receptors immobilized on the ALD resin with a 5 atom linker. Values obtained from saturation studies (Figure 6) showed similar equilibrium dissociation constants (K_d) of [^{3}H]mepyramine for the non-immobilized and immobilized HA-hH_1R-HIS.

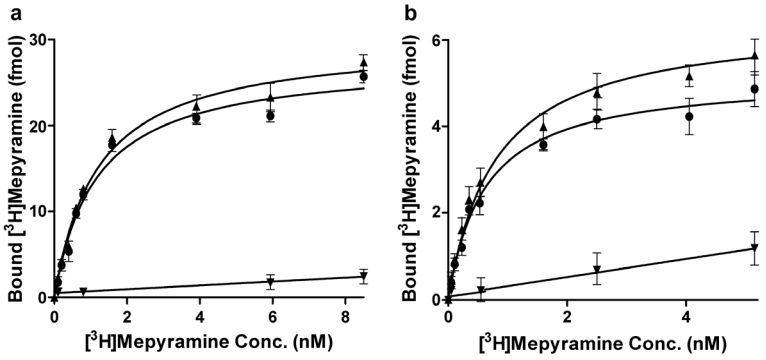


Figure 6. Saturation binding of [^{3}H]mepyramine to non-immobilized (**a**) and immobilized HA-hH1R-HIS (**b**). The amount of [^{3}H]mepyramine bound is indicated for the various fractions of total (▲), non-specific (▼),and specific binding (●). The average of 3 experiments performed in duplicate is shown.

As can be seen in Figure 2a, the immobilized receptor has a lower B_{max} (1.3 pmol/mg vs. 6.2 pmol/mg for non-immobilized, Table 1) while displaying a level of non-specific binding consistent to the levels found when the receptor was not coupled to the resin. Displacement studies were used to determine the binding constant of the agonist histamine and the antagonist mianserin on both non-immobilized and immobilized HA-hH_1R-HIS. In the case of histamine, both non-immobilized and immobilized receptor data were best fit by a 2-site model (Figure 7**a**, T-test; $p < 0.01$ and Figure 7**b**, T-test; $p < 0.01$, Table 1).

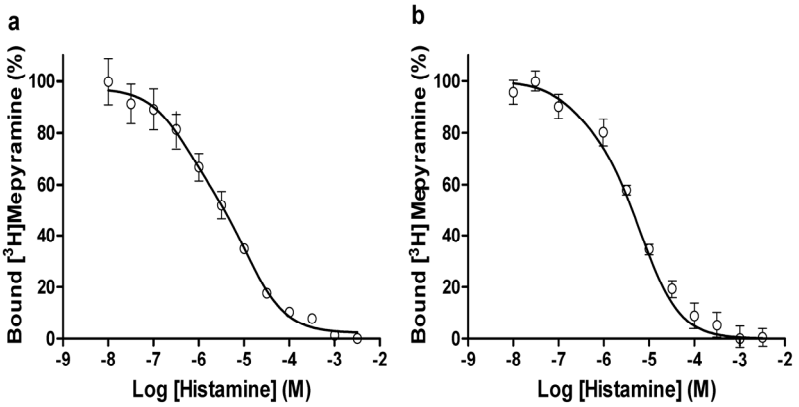


Figure 7. Displacement of specific [^{3}H]mepyramine binding to non-immobilized (**a**) and immobilized (**b**) HAhH_1R-HIS receptors by the agonist histamine. Both curves are best fit by a two-site model. The average of 3 experiments performed in duplicate is shown.

The affinity of the immobilized HA-hH$_1$R-HIS receptor for histamine was similar to the non immobilized receptor. Although there may be a slightly larger fraction of high affinity receptors in the immobilized sample (Table 1), it is not possible to distinguish a difference due to experimental uncertainty. hA$_1$R immobilized on the 5 atom linker ALD resin displayed the same pattern of ligand interaction as the HA-hH$_1$R-HIS receptor.

	N/I	Ligand	Binding state	Constant	B_{max}
A	N	[^{3}H]Mepyr-amine		K_d 1.1 ± 0.1 nM	6.2 ± 0.3 pmol/mg total protein
	I			K_d 0.7 ± 0.7 nM	1.3 ± 0.0 pmol/mg total protein
B	N	Histamine		K_L 20 ± 2 μM	
	N			K_H 0.4 ± 0.4 μM	
	N		2-site	R_H 55 ± 10 %	
	I			K_L 38 ± 6 μM	
	I			K_H 1 ± 2 μM	
	I			R_H 78 ± 24 %	
	N	Mianserin	1-site	K_i 0.3 ± 0.0 nM	
	I			K_i 0.6 ± 0.1 nM	

Table 1. Affinity and binding capacity estimates obtained from saturation binding curves (**A**) and competition binding curves (**B**) of non-immobilized (N) and immobilized (I) HA-hH$_1$R-HIS. Dissociation constants (K_d) and maximum amount of binding sites (B_{max}) were obtained from saturation curves, with 1 μM mianserin to determine non-specific binding. Competition binding constants of the agonist histamine and the antagonist mianserin provided low affinity constants (K_L), high affinity constants (K_H), and percentages of high affinity receptor populations (R_H) from 2-site binding curves and affinity constants (K_i) from 1-site binding curves.

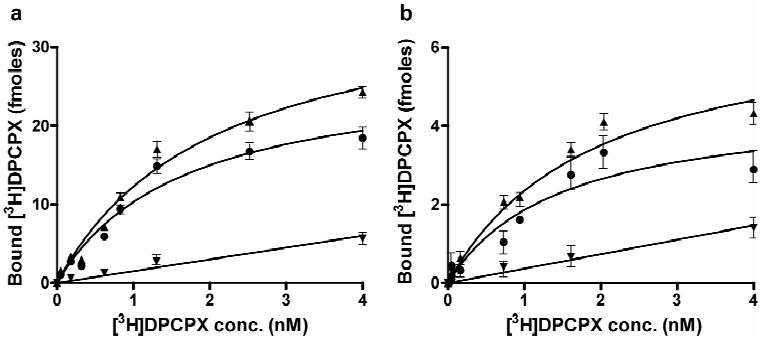


Figure 8. Saturation binding of [^{3}H]DPCPX to non-immobilized (**a**) and immobilized hA$_1$R (**b**) receptors. The amount of [^{3}H]DPCPX bound is indicated for the total (▲), non-specific (▼), and specific binding (●). The average of 3 experiments performed in duplicate is shown.

The saturation curve again indicated that approximately 25 % of the input receptor (relating to maximum functionality at 4 °C after 18 h incubation) had been functionally immobilized (Figure 8) and that non-specific binding, determined in the presence of 10 μM N^6-cyclopentyladenosine (CPA), was consistent with than for the non-immobilized preparation.

The equilibrium dissociation constant (K_d) of [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) for non-immobilized receptors was marginally larger than for immobilized ones, but still within experimental error (Table 2).

	N/I	Ligand	Binding state	Constant	Bmax
A	N	[3H]DPCPX		K_d 1.7 ± 0.7 nM	8.5 ± 0.8 pmol/mg total protein
	I			K_d 2.1 ± 0.4 nM	2.1 ± 0.2 pmol/mg total protein
B	N	CPA		K_L 250 ± 50 nM	
	N			K_H 3 ± 0 nM	
	N		2-site	R_H 55 ± 10 %	
	I			K_L 202 ± 100 nM	
	I			K_H 3 ± 2 nM	
	I			R_H 41 ± 7 %	
	N	8-CPT	1-site	K_i 28 ± 1 nM	
	I			K_i 39 ± 16 nM	
	N	N0840		K_i 440 ± 80 nM	
	I		1-site	K_i 540 ± 70 nM	

Table 2. Values obtained from saturation binding curves (**A**) and competition binding curves (**B**) of non-immobilized (N) and immobilized (I) hA_1R. Dissociation constants (K_d) and maximum amount of binding sites (Bmax) were obtained from saturation curves, with 10 μM CPA to determine non-specific binding. Competition binding constants of the agonist CPA, the inverse agonist 8-CPT, and the antagonist N0840 provided low affinity constants (K_L), high affinity constants (K_H), and percentages of high affinity receptor populations (R_H) from 2-site binding curves and affinity constants (K_i) from 1-site binding curves.

Agonist binding of CPA to non-immobilized and immobilized receptors was modelled on the basis of two different binding populations (Figure 9**a**, T-test, $p < 0.01$, Figure 9**b**, T-test, $p < 0.01$, Table 2). As for the HA-hH_1R-HIS receptor, the affinities of CPA for the receptors, whether immobilized or in solution, were very similar. In the case of the hA_1R receptor however, the

fraction of high affinity receptors is slightly less in the immobilized state than non-immobilized state and the difference is greater than the experimental error. The inverse agonist 8-cyclopentyl-1,3-dimethylxanthine (8-CPT) binds the immobilized receptor with slightly reduced affinity although again, within experimental error, while the antagonist N^6-cyclopentyl-9-methyladenine (N0840) displays lower affinity for the immobilized hA_1R than the non-immobilized receptor (Table 2).

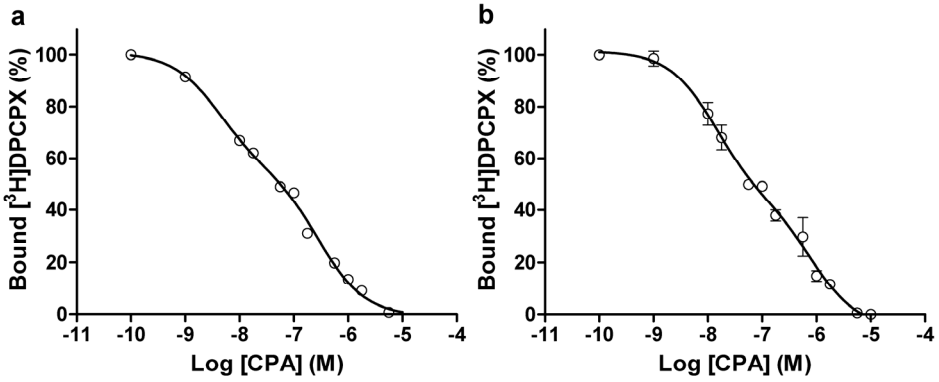


Figure 9. Displacement of specific [3H]DPCPX bin-ding to non-immobilized (**a**) and immobilized (**b**) hA1R receptors by the agonist CPA. Both curves are best fit by a two-site model. The average of 3 experiments performed in

Theory suggests that protein immobilization should result in stabilization due to decreased entropy of the unfolded state (from steric hindrance of the support). However, in the case of integral membrane proteins whose entropy in the unfolded state may be constrained by the membrane, it is not clear whether immobilization would have any effect. We investigated the effect of immobilization on GPCR stability by storing immobilized and non-immobilized preparation at 4 °C. After one week at 4 °C, both hA_1R and HA-hH_1R-HIS receptors maintained significantly higher activity when they were immobilized compared to when they were kept in solution (Figure 10).

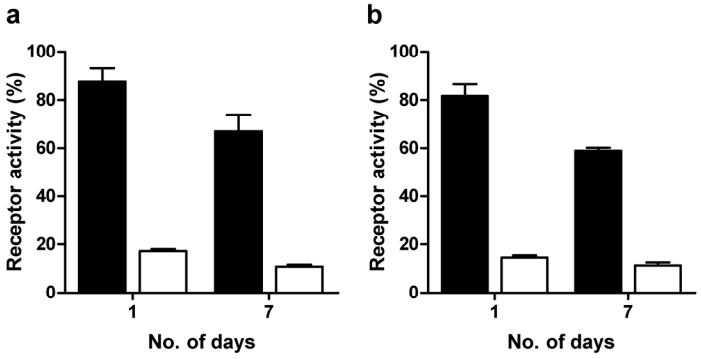


Figure 10. Stability of HA-hH_1R-HIS (**a**) and hA_1R (**b**) membranes as measured by radioligand binding and normalized to percentage on day 0. Membrane preparations, both immobilized and non-immobilized, were stored at 4 °C. Black bars represent immobilized membranes. White bars represent membrane preparations in solution.

DISCUSSION

Our results show that it is possible to immobilize functional GPCRs within their native membranes on sepharose beads without receptor solubilization, purification or lipid reconstitution. The method resulted in the co-immobilization of other membrane proteins, both integral and membrane associated with similar efficiency. Furthermore, receptors immobilized in this manner remained stable for up to a week at 4 ºC at least. The methodology applied was simple, repeatable, and consistently yielded up to 1 - 2 pmol of functional receptor per ml of resin with two different receptors. While we only used GPCRs in our work, it is reasonable to expect that a similar approach should also work for other integral and membrane associated proteins such as ion channels, chemokine receptors or cytochrome P450s.

A significant shortcoming of methods that rely on purification and solubilization is that upstream or downstream components of the signalling cascade may be lost upon immobilization. In the case of GPCRs, full functionality clearly requires the presence of other players within the cell membrane[195,196]. For example, $G\alpha_{q/11}$ proteins have been reported to increase constitutive activity of the histamine H_1 receptor[197] and have a role in dimerization[198]. Data in Figure 4 clearly indicate that $G\alpha_{q/11}$ proteins, which have been estimated to have a molecular weight of 42 kDa[199], were present in both non-immobilized and immobilized vesicles containing HA-hH_1R-HIS. We can not be certain about the proportion of immobilized $G\alpha_{q/11}$ proteins which were effectively stripped off the resin, because we may only be collecting proteins which were immobilized via other proteins in the membrane, and not those which were directly covalently linked to the resin. However, while not a complete inventory of all of the proteins required for GCPR signalling, the presence of the membrane associated $G\alpha_{q/11}$ proteins is strongly suggestive that other such proteins are likely co-immobilized as well. The presence of other members of the signalling cascade may play a role in maintaining a similar pharmacology for immobilized and non-immobilized receptors (see below) and likely contributes to the enhanced stability of receptors in immobilized vesicle preparations.

The efficiency of immobilization is an important aspect. We readily determined conditions under which the actual cross-linking process is quite efficient. Under these conditions the density

of receptor on the beads is then a simple function of the expression level. However, achieving a high level of functionality of the immobilized receptors required investigation of a number of parameters. Increasing the linker to 8 atoms from 5 yielded a near doubling of the amount of functional receptor immobilized. The reason for the sensitivity to linker length is not clear at this point, however, it is known that GPCRs require a fluid membrane environment[112] and space between the surface and the membrane for extracellular domain movement[143] for full functionality. It therefore seems possible that a short spacer leads to vesicle restriction and alters dynamics and mechanical properties.

When all of the optimisation steps were combined, it proved possible to functionally immobilize these two GPCRs with an efficiency of approximately 25 % in comparison to similarly treated but non-immobilized vesicle preparations. The yield of functional immobilized GPCRs was very similar to what we have observed for model bacterial membrane proteins that have been purified and solubilized in micelles (Chapters 4 - 6). The current yield of 25 % is likely not an upper limit as can be seen from improved functionality upon increasing linker lengths, and further investigation into various matrices and linking chemistries should prove useful for further improvement of the efficiency. Furthermore, although we use a pH that favours the reaction at the N-terminus, the current chemistry may still be affecting some lysines which may be important for activity, such as Lys5.39 in the hH_1 receptor ligand binding site, established from mutagenesis studies[200,201]. The N-terminus itself may also be too close to the binding site and steric hindrance may be limiting the activity of the immobilized GPCRs. However, the major limiting factor at this point appears to be the density at which the receptors were expressed. This issue could best be addressed by investigating other systems geared towards higher level expression. We have focussed on stably transfected mammalian cell culture as a source of material to develop the immobilization protocol. Clearly, one could produce similar vesicles from insect cells expressing the protein of interest at 10 - 100 fold higher levels. We are also investigating the possibility of on-bead enrichment strategies to improve the density of immobilized receptors to a level which would be sufficient for screening GPCRs with TINS.

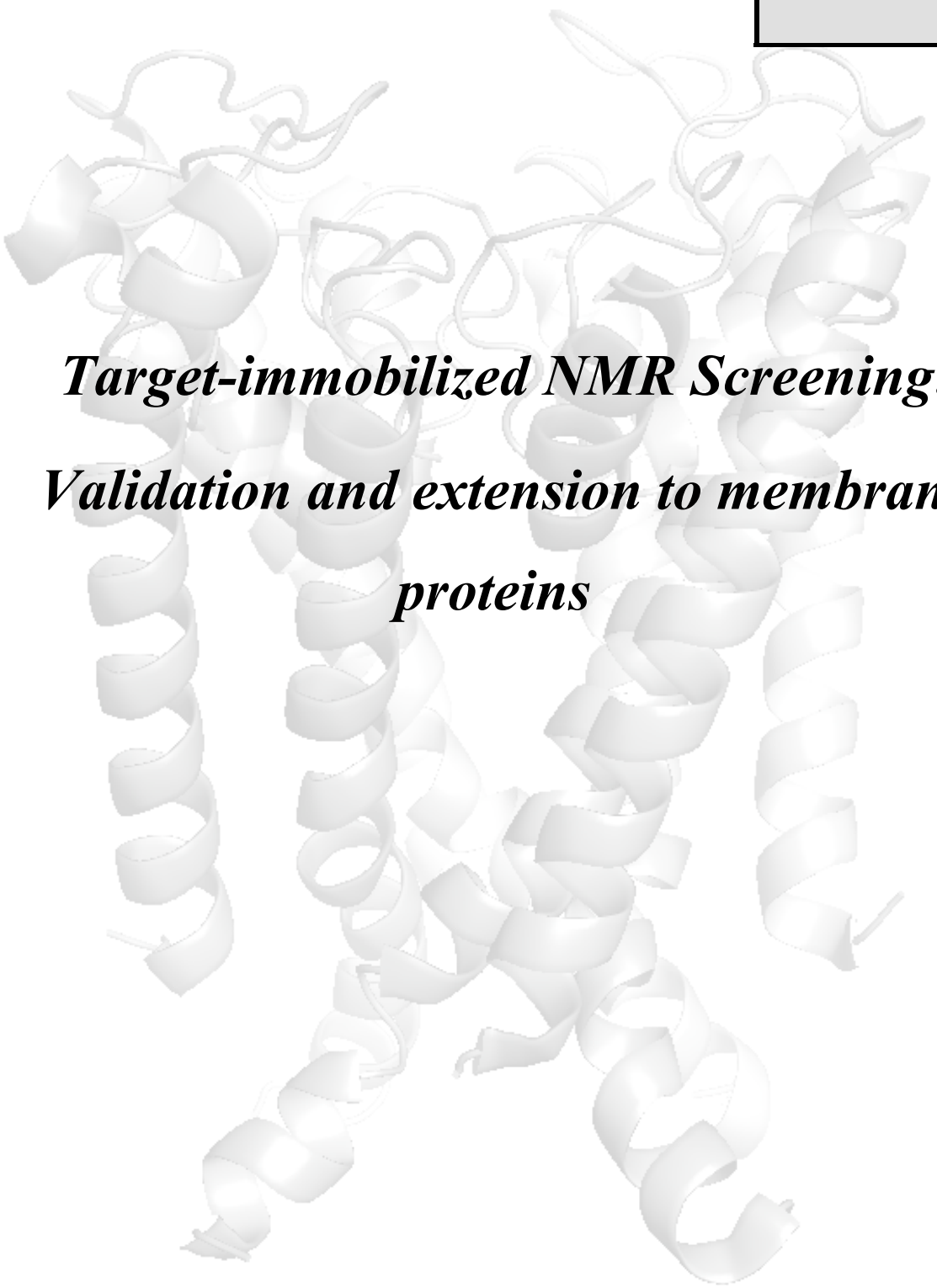
Immobilization of vesicle bound receptors had had only limited impact on their pharmacology. The immobilized HA-hH_1R-HIS and hA_1R populations bound antagonists and inverse agonists,

with affinity similar to non-immobilized receptors. Interestingly, the proportions of high and low affinity agonist populations typical of non-immobilized receptors[191] seemed to change slightly upon immobilization of the hA_1R while that of the HA-hH_1R-HIS is indistinguishable within the limits of experimental error. At present the basis for this difference is not known but may be related to the co-immobilization of the appropriate G proteins.

Screening of collections of compounds for target binding is often the first step in new drug discovery projects. Presently cell-based assays are the primary method of carrying out such programs for membrane bound targets. A generally applicable procedure for functional immobilization of membrane proteins could potentially be applied in any of the current bead based assays, for example our own NMR-based method[77] (Chapters 4 - 6) or Scintillation Proximity Assays. Use of an appropriate reference such as a parental cell line would enable these methods to be applied to membrane proteins. In some cases a secondary immobilization of the sepharose bead itself may also be necessary. Additional biophysical techniques to detect or characterise ligands for which the present immobilization method could be useful include frontal affinity chromatography-mass spectroscopy[202] and affinity capillary electrophoresis[203]. In addition to ligand screening, functional immobilization could be a powerful tool for deorphanization studies. The method has the potential of being generally applicable to all GPCRs without being G protein or secondary messenger system specific. Although challenging subjects for biophysical applications due to their fragile and exigent nature, GPCRs have, on the contrary, proven to be easy targets for this simple immobilization methodology. This fact is accentuated by the absence of a requirement for tags, biotinylation, or the use of antibodies as tools for immobilization. Furthermore, the increased stability of the receptors upon immobilization will allow longer high throughput screening experiments to be carried out. Our immobilization strategy therefore should enable the study of a broader range of membrane proteins, including GPCRs, in their native membranes using different analytical methods.

Chapter

4



Target-immobilized NMR Screening: Validation and extension to membrane proteins

Due to their involvement in a large number of pathologies and ease of access, membrane proteins are at the forefront of pharmacological interest. A number of high throughput screening technologies, including membrane and cell-based assays, have been used to develop lead compounds. Emerging biophysical techniques such as nuclear magnetic resonance (NMR) can potentially be used to find novel ligands, however to date they have only been applied to soluble proteins. We seek to apply our newly developed ligand screening technology, Target Immobilized NMR Screening (TINS), to membrane proteins. In TINS the target to be screened is immobilized on a porous support and flow-injection NMR spectroscopy is used to detect binders in mixes of compounds. Immobilization potentially allows TINS to be applied to membrane proteins. Here we address this issue by developing a simple and widely applicable immobilization protocol and applying it to model bacterial membrane proteins. Since TINS is a comparative method, we have developed a reference system to control for non-specific binding of hydrophobic compounds to lipids or detergents used to solubilize the membrane proteins. This control protein is of the same size as our target and refolded in the same lipid micelles. The proof of principle was tested with a limited screen small fragment (150 - 300 Da) library on a bacterial ion channel solubilized in dodecylphosphocholine micelles and a larger screen of approximately 1000 fragments was carried out on a membrane enzyme the Disulphide Bond Forming protein B. For both screens, 7 % of the fragments showed substantial changes in the NMR spectrum that were specific to the target and were considered binders. No loss of target binding capacity was detected after 1 month of storage of the samples. With the proof of principle validated, TINS is now ready to be applied to pharmaceutically[2] important, membrane bound drug targets.

[2] This chapter is a modified version of the published book chapter: Früh, V.; Heetebrij, R.; Siegal, G. Target Immobilized NMR Screening: Validation and Extension to Membrane Proteins. In *Fragment-Based Drug Discovery: A Practical Approach*; John Wiley & Sons: Chichester, 2008; Ch. 6.

Fragment based drug discovery (FBDD) methods have been widely embraced in the last few years. Nearly all of the major pharmaceutical firms have developed fragment screening and evolution programs and a number of biotech firms have sprung up that make exclusive use of the approach to develop small molecule therapeutics. Amongst the variety of fragment screening and evolution methods that have been implemented, there are two common themes. First, the collection of compounds to be screened consist of small (typically less than 300 Da), highly soluble molecules. As such, they typically interact with the target weakly, with binding constants in the range of 2 to 5,000 μM. Second, the low affinity hits discovered by screening such a collection must be developed into high affinity, high specificity ligands. This process is much more successful when 3D structures of target-compound complexes are available[21].

The promise of FBDD, that is compounds that through obeying Lipinski's rules[10] are more likely to make orally bioavailable, safe drugs, is starting to be put to the test as compounds begin to move into clinical trials. The number of such compounds is rising rapidly due to the successes of Plexxikon, Astex, Sunesis, SGX Pharma, and a host of other biotech companies that place FBDD at the core of their activities. However, a third common theme that applies to all FBDD to date is that is has been strictly applied to soluble targets. On the other hand, the attractiveness of membrane proteins as pharmaceutical targets has been well documented[204] with approximately 60% of all current targets being membrane proteins. Thus it would be a significant advantage to be able to apply FBDD to the class of targets that includes integral and membrane associated proteins.

We have developed a technology called Target Immobilized NMR Screening (TINS)[77,205] that in principle can be applied to screening of membrane proteins. In TINS, the target to be screened is immobilized on a commercially available chromatography resin in a simple and efficient process. The immobilized target, along with a second, reference sample, is placed in a flow-injection, dual cell sample holder in the magnet and the compounds to be screened are injected in mixes of about 5 compounds each[205]. Spatially selective spectroscopy[206] is then used to independently acquire a 1D ^{1}H spectrum of the compounds in the presence of the target or the reference. Comparison of the two spectra directly yields the identity of any compound that binds the target due to the simple reduction in peak amplitude of all resonances from the ligand. This

configuration yields a number of advantages for ligand screening. The combination of effective T_2 relaxation and chemical exchange endows the method with great sensitivity with specific binding as weak as 5 - 10 mM (K_d) being readily detected. On the other hand, the presence of a reference sample in routine use cancels the weak, non-specific interactions typically observed between many of the compounds to be screened and the target. Thus the presence of artefacts in TINS screens is greatly reduced as is the false positive rate. The sensitivity can also be used to reduce the concentration of immobilized target to as low as 5 μM solution equivalent, which combined with the fact that the entire compound collection is routinely screened with a single sample, means the screening can be carried out with as little as 5 nmols of the target.

TINS has been applied to a variety of soluble proteins and in this chapter we will present some of these results. In principle, immobilization should allow an extension of the range of targets to which TINS can be applied to include insoluble membrane proteins. This idea is not new and others have attempted to apply biophysical methods for detecting ligand binding to immobilized membrane proteins[151]. In particular, surface plasmon resonance (SPR) has been used for this application. Membrane proteins represent difficult targets for *in vitro* ligand screening studies however since they are insoluble, often require the presence of specific lipids for proper function, are highly challenging to purify, and rarely amenable to high resolution structural analysis. Furthermore, a general limitation that has always been encountered is the difficulty of functionally immobilizing membrane proteins in a form appropriate for the assay. SPR for instance requires a flat surface with an underlying metal layer (to provide the material with dielectric constant opposite that of water). While a few cases of successful immobilization of membrane proteins have been reported under these conditions, a widely applicable method is still lacking. Here we will report on our initial efforts in two areas, the ultimate goal of which is to allow routine *in vitro* fragment screening of a wide variety of membrane proteins.

General considerations for Fragment Screening

Fragments

We will focus on the principles and benefits of the TINS fragment library designed and tested as collaborative effort between ZoBio (www.zobio.com) and Pyxis Discovery (www.pyxis-discovery.com) of Delft, the Netherlands[207].

It is now a well accepted principle that the "rule of 3"[15] forms an approximate limit guiding the chemical nature of compounds that should be considered as a fragment for inclusion in a collection for ligand screening. At the other end of the spectrum, recent work from the Shoichet[208] lab suggests that including very simple fragments of less than approximately 150 Da could cause difficulties downstream during the lead evolution process. Clearly a number of *in silico* filters must also be employed to remove undesirable compounds such as known toxicophores or reactive groups. In our efforts we also placed great emphasis on water solubility of the compounds. In one of the first publications concerning fragment library design, only about 50 % of the selected fragments possessed sufficient solubility (1 mM) to be screened[18]. In more recent publications, better results for the water solubility of fragment libraries have been reported[31,209]. The prediction of water solubility however remains a challenge because one has to take into consideration both the crystal and solution state of the compound. Moreover, in our own analysis, we have not been able to find a simple correlation between the number of H-bond donors/acceptors and water solubility. Since computational methods for better prediction of water solubility are still under development, one must experimentally determine the solubility of a given fragment. However, by applying cut-off values based on experience, for properties that can be better predicted, such as cLogP and the number of hydrogen bond donors and acceptors, which have a profound influence on water solubility, the fraction of water soluble fragments can be increased considerably. In our own efforts, about 90 % of compounds that were selected were soluble as singletons at 500 µM in phosphate buffered saline and 5 % DMSO. Evotec has recently mentioned an in-house QSAR model to predict solubility which is claimed to be useful, but no data is presently available[210]. While originally our emphasis on water solubility stemmed

from practical aspects of making mixes of compounds at 500 μM each in aqueous buffer, this effort has been well served when screening membrane proteins since we feel that it is one of the important reasons that we have so far experienced a very low false positive rate.

Our library, which is intended to serve as a source of chemical diversity, is composed of compounds selected from four themes: (1) diversity using the scaffold-based classification approach (SCA)[61], (2) amino acid derivatives, (3) scaffolds found in natural products, and (4) shape diversity. All compounds were selected from a carefully prepared database representing 70,000 compounds that would make desirable starting points for drug discovery, including "rule of 3" compliance, and were commercially available from reliable suppliers. One of our explicit intentions in forming the library upon these design principles is to evaluate the performance of the various classes of compounds against different targets, both soluble and membrane bound. While it remains too early to make sensible conclusions from the roughly 10 targets that have been screened to date, in many cases there are up to two fold differences in hit rates between the different themes for a given target.

Immobilization and reference protein

The strength of TINS lies in the fact that it is a referential system. That is the signal acquired in the presence of the target protein is compared to the signal acquired in the presence of a reference sample consisting of a known protein immobilized at approximately the same density as the target. The requirement for a reference protein comes from the fact that TINS is highly sensitive to even very weak interactions between the compounds and the immobilized target. Therefore the choice of reference protein is important. Ideally one would like to have a reference protein which is convenient to produce in large quantities, can be readily immobilized, has the roughly "typical" amounts of exposed surface charge and hydrophobicity and has essentially no small molecule binding capacity. The PH domain of the cellular kinase AKT is a nearly ideal candidate which we use for screening of all soluble targets. Hajduk and colleagues showed that this protein was essentially refractory to small molecule binding using their well-known SAR by NMR assay[211].

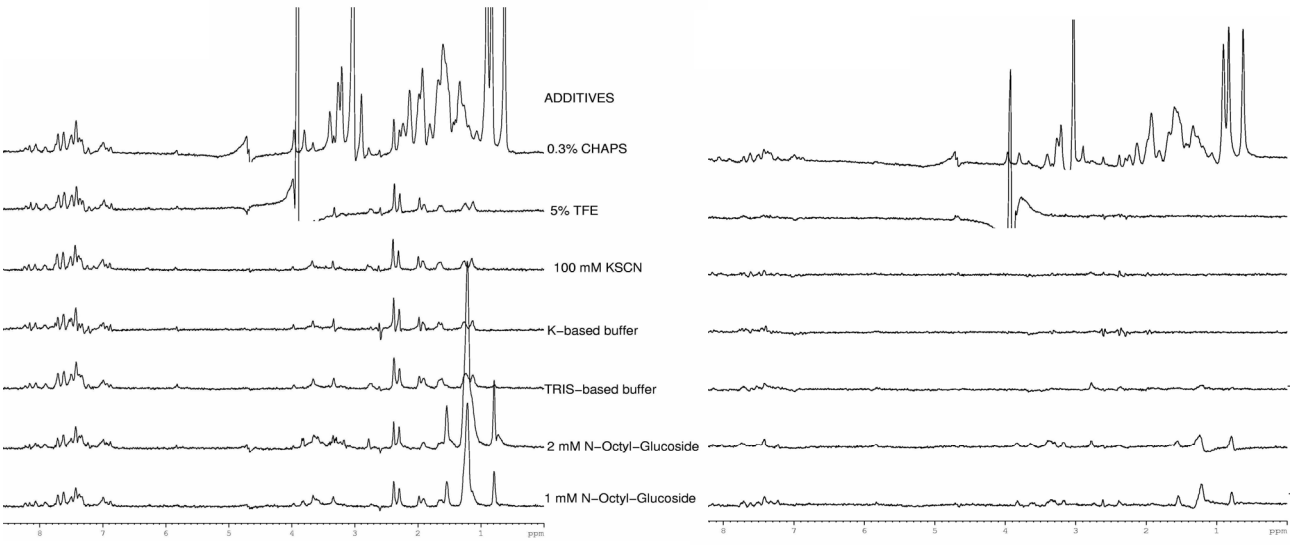


Figure 1. Cancellation of non-specific binding by the reference sample in TINS screening. The left hand panel shows difference ^{1}H NMR spectra of a mixture of non-binding compounds acquired in the presence of sepharose resin to which 6 mg/ml of an SH2 domain (111 amino acid residues) had been immobilized or just the resin itself. The indicated additive was included with each of the compound mixtures. The right hand panel shows the same difference spectra however the second spectra was acquired in the presence of a resin to which 6 mg/ml of FKBP had been immobilized. The improvement in cancellation when an immobilized protein is used as a reference is clear.

While we initially had concerns that this small protein would be unrepresentative of larger, potentially multi-domain targets, or that proper cancellation of non-specific binding would require accurate matching of total surface area, this turns out not to be the case as shown in Figures 1 and 2.

Immobilization is a constant source of questions with regards to TINS screening. In principle, one is free to choose any immobilization approach which is compatible with a) the biochemical function of the protein and b) the constraints of NMR. Specifically the major concern related to NMR is susceptibility mismatch between the solid support and the surrounding aqueous environment. The group of Meyer had originally demonstrated ligand binding to targets immobilized on glass beads[212]. However, the susceptibility mismatch was so severe in this case that magic angle spinning NMR was necessary to average out the inhomogeneity. Clearly this

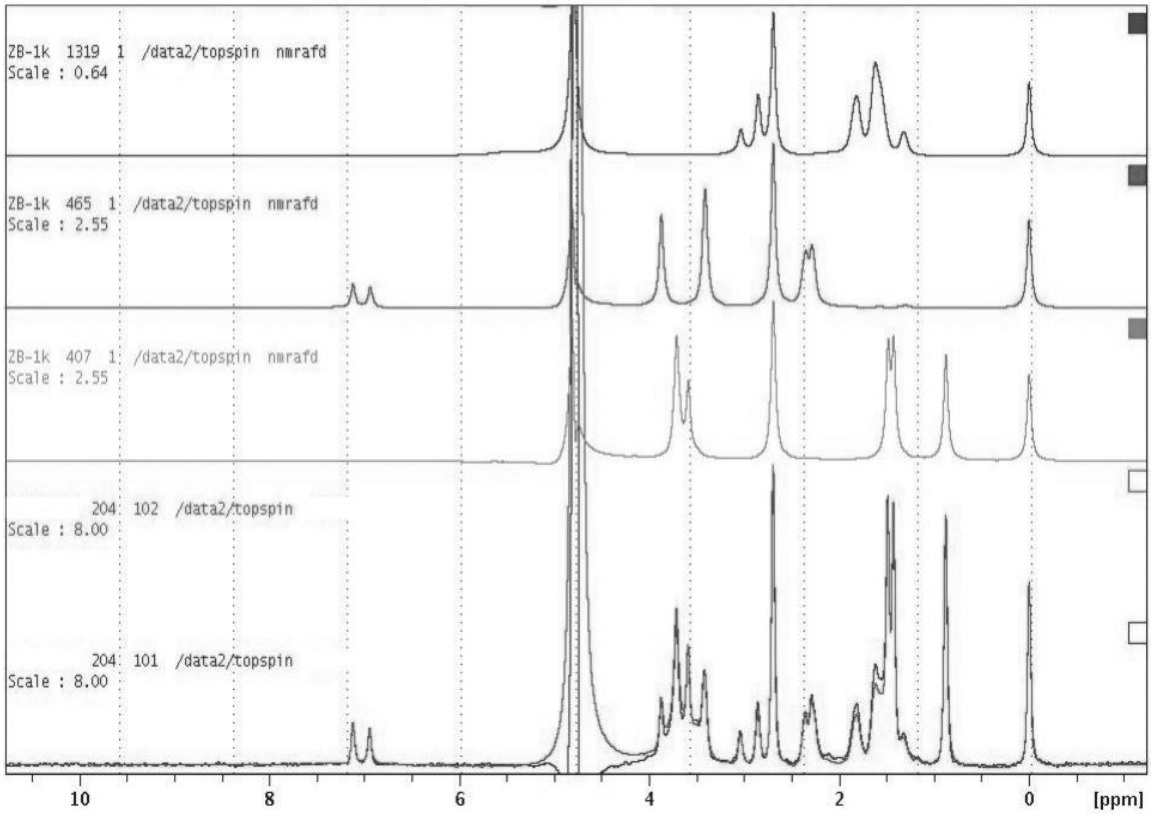


Figure 2. In this example taken from a screen of a soluble target, both the target and the reference protein (the PH domain of the kinase AKT) were immobilized on Actigel ALD (Sterogene, USA) at a solution equivalent of 100 μM. A mix consisting of three different compounds (upper three 1D 1H NMR spectra are of each compound in the mix separately) was applied simultaneously to the sample of immobilized target and reference protein in the dual cell sample holder. Spatially selective Hadamard spectroscopy was used to simultaneously acquire separate spectra of the compound mix in the presence of the immobilized target and reference. These spectra are overlaid at the bottom of the figure. The similarity of the two spectra indicates that none of the compounds specifically bind the target. The weak interactions with any immobilized protein that are observed for most compounds in the library are approximately the same for both the reference and target.

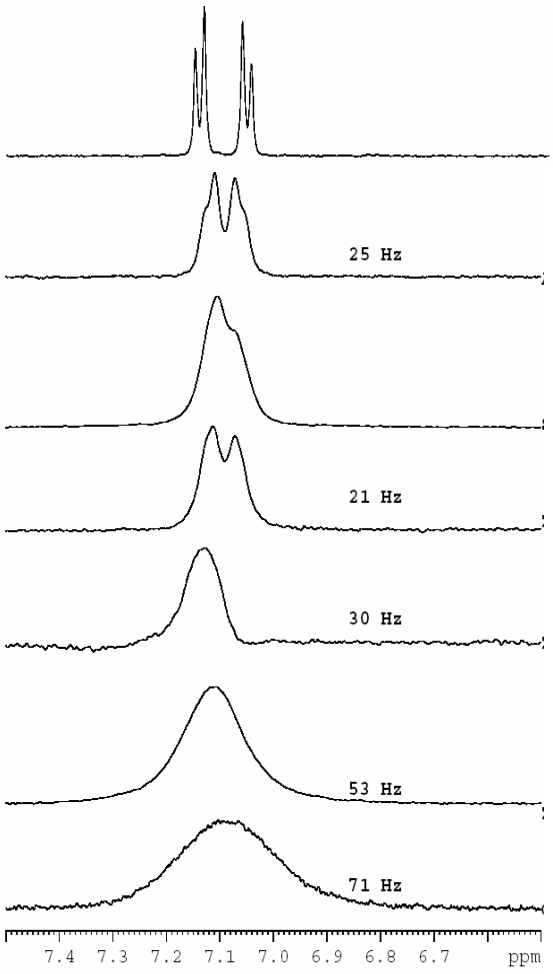


Figure 3. Effect of immobilization chemistry on the linewidth of compounds in solution. 1D 1H spectra of the aromatic protons of phosphotyrosine (pY) are shown with the fitted linewidth. From top to bottom, pY in solution, in the presence of Actigel ALD, streptavidin-sepharose, Zn-IDAA sepharose, Zn-NTA sepharose, Zn-NTA silica and controlled pore glass beads (for comparison).

arrangement would not be compatible with flow-injection NMR and so we sought a solid support which would not bind the compounds, would provide high capacity to immobilize proteins, and would minimize susceptibility differences. Sepharose based affinity resins turn out to be very useful in that they are very good matches for this list of requirements. In contrast to glass beads, sepharose beads can be more readily described as a three dimensional, bio-compatible mesh which is highly hydrated, yet sufficiently rigid to maintain good flow characteristics even after 300 applications of compound mixes. The susceptibility mismatch is minimal such that under our current screening setup, using the dual-cell sample holder made from KelF, we routinely obtain a linewidth of about 12 Hz. However, the nature of the immobilization chemistry of the sepharose bead also appears to play a role in the linewidth observed for the compounds, as can be seen in Figure 3.

A wide range of immobilization chemistries are commercially available in conjunction with sepharose beads. We have investigated a limited subset of these possibilities which include: direct, non-oriented immobilization via Schiff's base chemistry, oriented non-covalent immobilization *via* immobilized metal affinity chromatography

resins and oriented non-covalent immobilization via biotin-streptavidin binding. At present we favour direct, covalent attachment of proteins via primary amines since it is highly efficient (typically better than 85 % yield), minimizes leaching, and provides the best NMR results (Figure 3).

At the pH we typically carryout immobilization (pH 7.4), this reaction is rather specific for the amino terminus. In principle one could imagine that immobilization might interfere with the functionality of certain proteins, such as kinases that contain a lysine at an active site. Thus far we have not encountered this issue, but it is always possible to block access to this lysine by immobilizing in the presence of high levels of an ATP mimic such as AMPPNP. Kinases have been successfully immobilized for Biacore studies using related chemistry[213]. We have investigated the use of IMAC resins to immobilize proteins via a 6 his tag. While this method is convenient, it is not possible to use Ni^{2+} as the ion for chelating the tagged protein due to the potent paramagnetic relaxation. It is possible to immobilize his tagged protein using Zn^{2+} instead and leaching does not pose a problem. However, despite the fact that a sepharose resin is used in conjunction with a diamagnetic ion, there appears to be additional linebroadening effects (Figure 3). These may result from non-specific interactions with available NTA sites on the resin which turn out to be difficult to block. We have also used streptavidin sepharose to immobilize biotinylated ribonucleotides for ligand binding studies. This system is convenient and yields high quality NMR spectra (not shown). By blocking unoccupied binding sites with free biotin (and naturally using streptavidin sepharose as the reference sample) one should be able to limit small molecule binding to sites that are not on the target, however we have not carried out a full screen on such a system so it not possible to make a definitive statement at this time. Other affinity tags can also form the basis of successful, NMR compatible immobilization as well. For example, Haselhorst and colleagues have recently reported the use of Strep-tactin sepharose, a variant of streptavidin sepharose, to perform saturation transfer difference (STD) studies[214].

Ligand Screening

We decided to carry out our ligand screening studies using mixes of compounds at a very early stage in the process of developing TINS. This decision was made on the basis of throughput and robustness. Since our mixes consist of on average 5 compounds, obviously throughput is increased by a factor of 5 with respect to screening singletons. Also, since it is expected that only 1 (and occasionally 2) compounds per mix bind to the target, most peaks in the reference and target spectra should be of the same amplitude. If this is not the case it may be a sign that there is a problem with the screening sample. The use of mixes requires a strategy to design them properly. Given the constraint of increased linewidth generated by the heterogenous TINS system, the primary factor governing the selection of compounds for a mix is the number of well resolved peaks for each. We have therefore recorded a reference 1D ^{1}H spectrum of every compound in the ZoBio/Pyxis fragment collection at 500 μM in phosphate buffered saline (PBS) in the presence of a fixed amount of TSP. The reference spectra serve the dual role of quality control as well. The reference spectra are automatically peak picked and the peak positions stored in our database. We have developed an in-house algorithm to randomly select compounds from the collection and rapidly test them for TINS compatibility, that is at least 3 well resolved peaks for each compound (when available). This allows us to directly read out the ligand from the mix without further deconvolution (see below). The algorithm also places explicit limits on the number of aromatic compounds per mix and avoids mixing compounds with pKa extrema. Once designed, the mixes are then made at 500 μM for each compound in PBS. The mixes are stored at room temperature and subsequently visually inspected for signs of precipitation. About 1/3 of mixes are rejected at this point. Mixes that do not precipitate are subjected to ^{1}H NMR analysis where we expect to see that the NMR spectrum of the mix is a simple sum of the NMR spectra of the individual compounds using TSP as a reference. Changes to the NMR spectrum of the mix, which we rarely observe, are indicative of possible aggregation behaviour of the compounds.

In order to carry out a ligand screen, the resin bearing the target and reference proteins, which have been immobilized at a solution equivalent of about 100 μM, must be packed into the dual-cell sample holder. A homemade packing reservoir has been built to fit on top of the dual-cell

sample holder and double the volume of each cell. The resin (as a 50 % slurry) is pipetted in to each cell one at a time, allowed to settle by gravity and packed at a pressure of 0.5 bar. Once packed the cell can be connected to the sample delivery system via PEEK capillary tubes and inserted into the magnet using an aluminium arm. By attaching the cell to the aluminium arm we can readily orient it such that the plane that bisects each of the two cylindrical cells is parallel to one of the transverse gradients in our triple-gradient, flow injection probe[205]. In this way optimization of the NMR experiment for each screen is minimized. All that is necessary is to perform routine tuning and matching and shim, which we do using the FID of water. When known ligands are available, initial tests are performed to insure the integrity of the immobilized sample. This same experiment is repeated 4 - 5 times throughout and after the screen to detect possible target degradation (Figure 4). Once prepared, the mixes are placed in the Gilson autosampler in 96 positioned, deep-well plates and the Bruker HyStar software is programmed for each. We also use standard ICON NMR in Topspin to acquire the TINS data. A complete screen of about 1,500 unique compounds (including some replicates for quality assurance) requires about 7 days and runs without human intervention. Having evaluated a variety of different spatially selective NMR experiments, we have settled on the Hadamard sampling approach. The quality of the data using this experiment with carefully designed mixes is rather high, as can be seen in Figure 5.

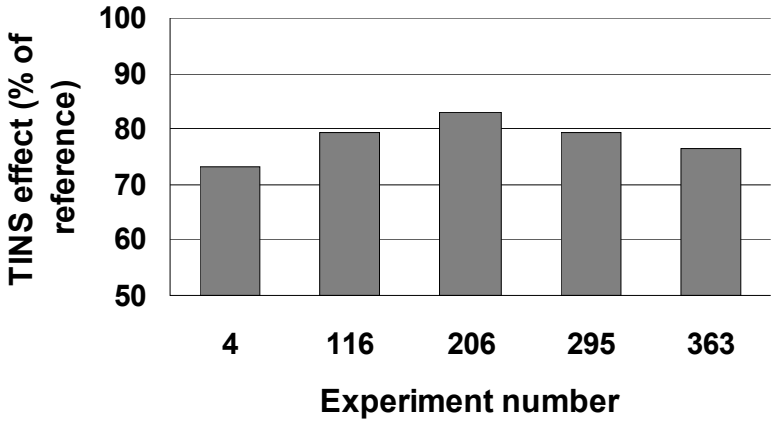


Figure 4. Determination of target integrity during a TINS ligand screen. A known ligand was applied to both the target and reference cells and the reduction in peak amplitude was measured ('TINS effect'). This experiment was carried out serially after the indicated number of mixes had been applied to the immobilized target.

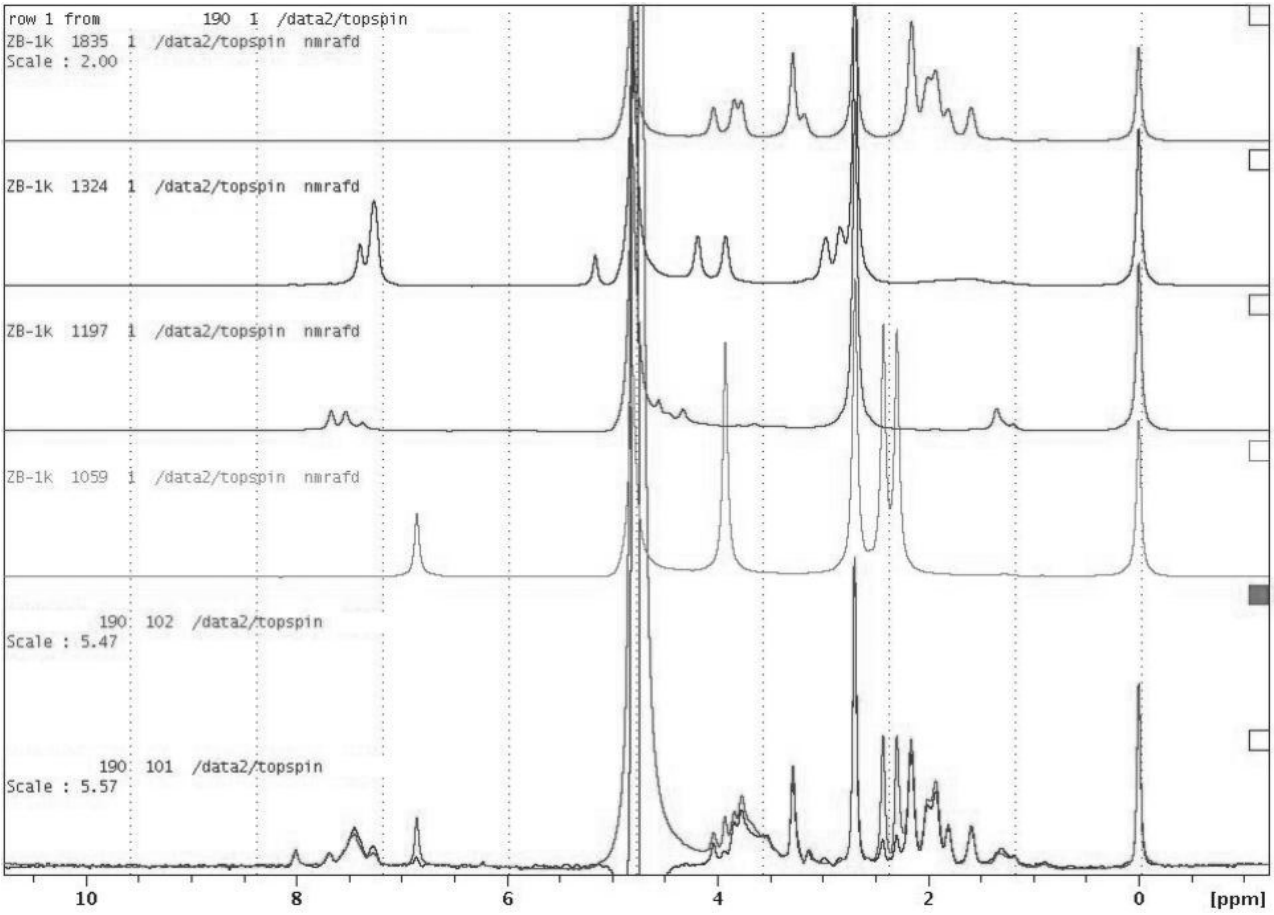


Figure 5. Direct determination of ligand identity using TINS. A mix of 5 compounds was applied to the dual sample holder containing immobilized target and the PH domain of AKT, both at 100 μM solution equivalent. The individual spectra of each cell, acquired with 30' of measuring time, are overlaid at the bottom of the figure. The ^{1}H spectra of four of the five compounds are shown above for reference. The identity of the ligand (fourth spectrum identifier 1059) is readily obtained by simple inspection.

We have now screened a number of different targets, both soluble and membrane bound, using TINS. The hit rate for targets has varied from a low of 3 % to a high of about 10 %, where we define a hit as having at least a 30 % difference in amplitude between the reference and target spectra for all well resolved peaks. This cut-off was chosen for practical reasons based on the fact that the difference was sufficiently large to overcome artefacts related to spectral noise, minor lineshape differences between the two samples and spectral crowding and therefore enabled

reliable detection of a hit. This latter fact is particularly important since we wish to automate the data analysis process. Since screening on these targets has only been carried out using TINS, it is not possible to directly compare the observed hit rates with other methods including High Concentration Screening (i.e. screens based on inhibiting and enzymatic activity). Where Hajduk and colleagues reported essentially a 0 % hit rate for the PH domain of AKT[211], we in fact do detect some compounds binding, but our "hit rate" is about 0.2 %, some 10 fold lower than the lowest rate obtained for a target that is expected to be "drugable". In their work Hajduk and colleagues reported hit rates of up to 1 % for SAR by NMR. Interestingly, the 3 % hit rate for TINS was found when screening a soluble "NTPase" in the NDP bound form. The hit rate for the apo protein was about 9 %. The low hit rate found when the nucleotide binding pocket is occupied is expected and suggests that the high hit rates that we observe are not due to artefacts, but rather to reliable sensitivity to binding events. This idea is further supported by follow-up biochemical studies that we have now performed for two targets with enzymatic activity. Considering a soluble enzymatic target for which we found a hit rate of 9.5 %, approximately 50 % of the TINS hits showed significant inhibitory activity at 500 μM, while we would expect this number to go up even further if tested at the 1 - 2 mM typically used in high concentration screening. A similar pattern has been observed for membrane proteins (see below).

Membrane protein considerations

Quantity limitations

Although TINS removes limitations such as size and solubility of the target protein to be applied, there still remain quantity limitations with regards to membrane proteins. At present the practical lower limit for screening is roughly 25 μM solution equivalent (*e.g.* nmol/ml settled bed volume). Since we typically prepare 500 μl of immobilized resin to fill one cell of the sample holder, we require about 15 nmol of target. For a 50 kDa protein this works out to slightly under 1 mg and therefore it is safe to use 1 mg as a lower limit. For soluble proteins in which structure

guided hit optimization is the primary means for evolving fragments, this limit does not generally present a problem. However, for many membrane proteins formidable efforts are required to produce even this quantity. Accordingly, efforts are underway in our laboratory to enhance the sensitivity of TINS towards an eventual goal of being able to screen recombinantly expressed proteins in their native membrane environment, that is, without purification. Below we present data demonstrating the feasibility of immobilizing such native membrane fragments. Since this approach is beyond the present sensitivity limits of our TINS ligand screening station however, current efforts utilize highly expressed, purified, and functionally solubilized membrane proteins.

Given the current requirement for about 1 mg of functional protein to carry out ligand screening, it is clear that an appropriate system must be available to produce large quantities. Due to the interest in pharmacology and structure of membrane proteins, tremendous efforts have been made in recent years in developing new means to express, purify and solubilize them. It is not our intention to catalogue these approaches here, merely to mention some which show promise with respect to producing sufficient quantities for ligand screening and subsequent structural studies. Conceptually the simplest method for membrane protein production is *via* cell-free expression. Recently 6 different GPCRs have been produced in mg quantities using an *E. coli* based expression system that included Brij78 as a solubilizing detergent[215]. Studies were performed to show that at least one of the *in vitro* expressed GPCRs was functional. Interestingly, all appeared to be dimeric. Bacterial expression of membrane proteins typically results in the protein being unfolded and located in inclusion bodies. While purification of proteins from inclusion bodies is easy, the requirement for refolding can represent a considerable hurdle. Nonetheless, companies such as M-fold have successfully produced isotope labelled GPCR using this approach and showed that the protein was amenable to NMR studies[216].

Beyond bacterial expression systems, a number of eukaryotic expression systems have also been developed. One simple method of producing functional membrane proteins is to generate recombinant transient or stable cell lines based on CHO or HeLa cells. Such cell lines have the benefit of providing appropriate posttranslational modifications such as glycosylation which are not available in prokaryotic expression systems[217]. Often these modifications are required for protein function as shown for rhodopsin where folding is inefficient when the glycosylation site

at its N-terminus is suppressed[218]. Unfortunately the yield of proteins from stable cell lines is more often than not insufficient for ligand screening studies. Transient expression of membrane proteins can increase the yield by as much as a factor of ten but results in other inconveniences such as repeatability issues. Alternatives that have seen increasing success include recombinant expression in insect SF9 cells[219], use of Semliki Forest virus infected cells[220], and expression in the yeast *Pichia pastoris*[58]. All of these systems are capable of yielding sufficient quantities of folded, functional membrane proteins for ligand screening and structural studies. Unfortunately none is perfectly general and the rate limiting step remains finding the best system for a particular target of interest.

The membrane environment

Membranes are structured as stable phospholipids bilayers which delimit the boundaries of the organelle or the cell. The membrane provides an environment where chemical signals can be emitted and detected, where energy can be converted into inter- and intra-cellular functions, and through which materials can be transported. For all these activities, there are complex networks of interactions between the membrane-associated proteins, such as receptors, ion channels, and enzymes, and the ligands which stimulate or inactivate them. The membrane itself plays more than a passive role in these processes. Current understanding suggests that interaction between the membrane and embedded proteins is at least required for and may regulate protein function. Therefore the ultimate goal of research in our group is to be able to perform NMR based ligand screening studies on membrane proteins in their native environment. However, in light of the discussion in the preceding section it is clear that this is not yet possible and therefore membrane proteins must be recombinantly expressed and purified. Given the intimate interaction between protein and membrane, functional solubilization represents a major hurdle.

In order to retain functionality of a membrane protein, it is imperative to refold it or reconstitute it into a synthetic lipid environment which mimics the properties of its natural membrane as closely as possible[61]. Integral membrane proteins must be solubilized before being purified, and this often calls for addition of detergents after the initial centrifugation steps. For

example, the potassium channel KcsA was extracted from the cell membrane by addition of DPC prior to purification using IMAC and gel filtration chromatography[221]. Transmembrane proteins have large hydrophobic domains which can cause aggregation during purification. This can be avoided by using high concentrations of urea to prevent random folding before reconstitution in lipids[222]. These solubilization and purification steps are important because lipid reconstitution success depends on the state of the protein at this point. Organic solvents are the simplest approach to mimicking a membranous environment, but have only been possible to use with proteins with stable native folds such as ATP synthase[223] or colicin E1 immunity proteins[224]. The simplest true mimic of a membrane occurs when ionic or non ionic surfactants in organic solvents or water create micellar vesicles[61]. Micelles, which are 10 - 100 kDa in size when there is low ionic concentration, are very convenient since they are readily formed and can be used to solubilize membrane proteins in a monomeric form amenable to high resolution structural studies. To date all TINS screening of has been applied to micelle solubilized membrane proteins. However, due to, at least in part the monolayer and the extreme curvature of micelles, they are only rarely compatible with native functioning of membrane proteins. Surfactants used for such preparations include, but are certainly not limited to, sodium dodecyl sulfate (SDS), cetyltrimethylammonium chloride and bromide (CTAC and CTAB), lysophosphatidylcholine (LPC), Triton X-100, and dodecylphosphocholine (DPC)[61]. For NMR studies, deuterated surfactants are at least convenient and many times may be required. At present only DPC and SDS are commercially available in this form, although the latter tends to denature some proteins[225]. Micelles are formed when the surfactant is in a higher concentration than its critical micellar concentration (CMC), which can vary from 0.01 mM for non-ionic ones to 10 nM for short chain ionic ones, such as SDS[61]. The equilibrium shifts from micellar to monomeric forms of the surfactant when diluted with buffers that do not contain the detergent and therefore buffers must always contain a concentration of surfactant above the CMC to prevent micelle disruption and loss of protein conformation. In our hands, there is rapid exchange of surfactant molecules from the micellar to the monodispersed form resulting in rapid breakdown of micelle bound proteins when the surfactant is not included (see below). Bicelles are micelles which are composed of phospholipids rather than detergents and are slightly more complex than micelles.

Usually bicelles are composed of long chain phospholipids such as dimiristoyl-phosphatidylcholine (DMPC) forming bilayers and one shorter chain phospholipid such as dihexanoylphosphatidylcholine (DHPC) which lines the hydrophobic edges of the bilayer[226]. Bicelles, being mostly planar, represent a better membrane mimic than micelles and should be more compatible with protein function. The utility of bicelles for functionally solubilizing membrane proteins has recently been demonstrated by their use in crystallization of the GPCR, β_2-adrenergic receptor[43]. However, we have not yet tested bicelles for compatibility with TINS. In addition, there are more complex stable bilayer or multilayer vesicles of synthetic phospholipids which can be used to immobilize and orient membrane proteins on glass slides in solid-state NMR[227], but there usefulness for membrane protein immobilization on supports that are compatible with static NMR studies is not yet known.

Immobilization

The TINS methodology, by definition, requires immobilized protein to allow flow-through screening of ligands. Clearly, the choice of the surface upon which the protein will be immobilized and the choice of the immobilization chemistry have to be made within the limitations of the TINS equipment. The general requirements for immobilization compatible with high resolution NMR have been discussed so we focus on issues specifically related to membrane proteins here. We have taken a pragmatic approach when attempting to apply the TINS methodology to membrane proteins by beginning with what has worked for soluble proteins. To date we have immobilized three purified, micelle solubilized membrane proteins KcsA, OmpA and DsbB, all of which are from bacterial sources. All three membrane proteins were solubilized in dodecylphosphocholine micelles (DPC). In all three cases we have simply utilized the same immobilization scheme that has been successfully applied to soluble proteins i.e. Schiff's base chemistry to primary amines. We have found that the yield of immobilized micelle solubilized protein is nearly identical to that of soluble proteins. Further, immobilization has not had any detectable effect on the functionality of the immobilized, micelle solubilized proteins. This has been checked in two ways. For KcsA a panel of known ligands was available and we simply

assayed for binding using TINS. Since DsbB has an enzymatic activity, we adapted a spectrophotometric assay[1] for use with beads containing immobilized protein.

Enzyme inhibition studies were carried out by adding a reduced partner enzyme, and ubiquinone, whose reduction can be monitored by measuring the absorption decrease at 275nm over time. In order to reduce non-specific interactions to the resin and thus to compare enzymatic activity of the target prior to and post immobilization, there was an equivalent presence of resin in both cases. Results showed an efficient enzymatic activity post immobilization. Considering the imprecision in determining the amount of immobilized enzyme, the rate of the reaction of immobilized enzyme (3 M Ubiquinone-5/M DsbB s^{-1}) was close to that of the enzyme in presence of, but not immobilized to, the resin (4 M Ubiquinone-5/M DsbB s^{-1}) (Figure 6).

Naturally more complex strategies can be envisioned and may prove necessary for membrane proteins that are less robust than those used so far. One interesting strategy immobilizes protein first, followed by subsequent reconstitution into a synthetic lipid environment[108]. As with soluble proteins, active site blockers may be necessary in cases where illicit immobilization of lysine side chains in close proximity to the binding site may occur and thereby inhibit protein function. Various native or synthetic lipid assemblies have been extended to encompass the use of high affinity immobilization reagents such as biotin and streptavidin[66,163,165,228], antibodies[229,230,231], or nickel affinity[173,174] in order to immobilize the protein in more oriented manners. Thus as with soluble proteins, these approaches should also be compatible with TINS.

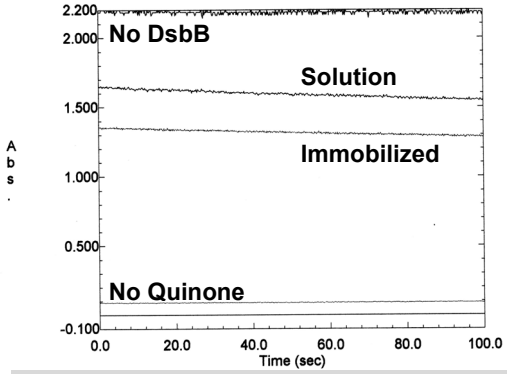


Figure 6. The target immobilized to the resin shows significantly similar enzymatic activity to the target in the presence of, but not immobilized to, the resin.

As a first step along the road to enabling TINS ligand screening for a truly broad range of membrane targets, we have begun to immobilize GPCRs in native membrane fragments (Chapter 3). In this experiment the idea was to use standard, stable animal cell expression systems such as CHO or HeLa cells as a source of material. In this way, all membrane proteins that can be

recombinantly expressed in these simple systems could potentially be used in fragment screening campaigns. Thus far we have succeeded in immobilizing membrane fragments produced by pottering (gentle disruption of animal cells) of post centrifugation membrane preparations. We have applied the procedure to both histamine receptors and adenosine receptors and in both cases, the pharmacology of immobilized receptors was similar to non-immobilized receptors. The efficiency of immobilization is quite reasonable with approximately 35 % of total receptors functionally immobilized and in comparison to non-immobilized ones; the immobilized receptors appear significantly more stable. At present the density of receptors is insufficient to perform NMR ligand screening but work is in progress to address this issue.

Screening

We have developed a diversity library for use in TINS and it is our intention to screen it against all targets. The design requirement for high solubility (to maximize oral bioavailability) pays dividends when used in membrane protein ligand screening since partitioning to the lipid phase is minimized. Nonetheless, as with soluble proteins, it remains important to use an appropriate reference system to cancel out non-specific binding events. We have used the *E. coli* protein OmpA as a successful reference protein in one partial screen of about 200 compounds and one complete screen of about 1,300 compounds. Its advantages include easy expression and purification, solubility in DPC and low small molecule binding. One potential way to avoid the use of a reference protein would be to screen using a known, competitive ligand. We are presently adapting the hardware of the TINS ligand screening station to enable competition ligand screening studies. In this arrangement the target is immobilized in both cells of the sample holder and the same mix applied to both cells while the competitor is added to only one of the cells. Competition ligand screening will eliminate the need for a separate reference protein but has the drawback that one can only find ligands to known binding pockets. When it becomes possible to screen proteins in native membrane vesicles, then a preparation of membrane vesicles of parental cell lines not expressing the target should serve as an ideal reference.

In order to further improve the robustness of TINS we include a reference compound in all mixtures that can be used to scale the two spectra post acquisition. With membrane proteins, even more than with soluble proteins, it is important to ascertain whether the reference compound interacts with the target or the surfactant used to solubilize it. The ideal reference compound has only one peak outside of the spectral range of all compounds and naturally, does not interact with the reference, target or surfactant. TSP fulfils most of these requirements but does bind to some targets. Alternatives we have used include glycine and tetramethylammonium chloride (TMA). A crude scaling factor for the two cells can be experimentally determined by integrating the water signal from each cell using a standard 1D imaging experiment with a single scan. Binding of potential reference compounds can readily be established by simply conducting TINS experiments on all, applying the scaling factor and analyzing the spectra for equal peak intensity in both cells. So far we have not encountered a case where more than one of the three potential reference compounds bound to the target.

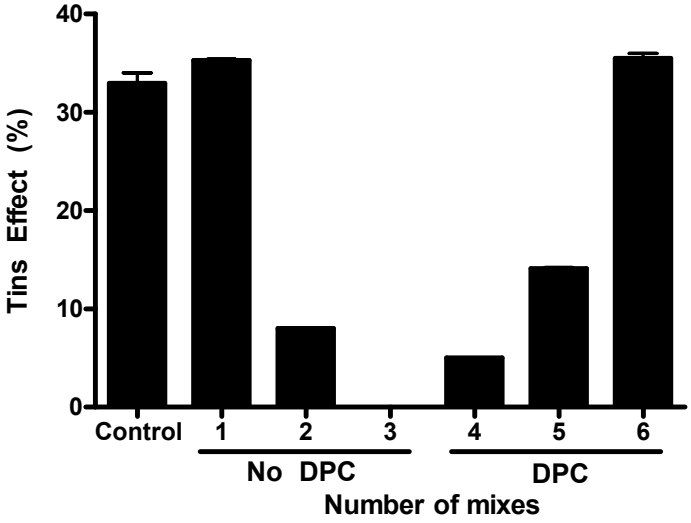


Figure 7. Requirement for the presence of detergent while screening micelle solubilized membrane proteins. In this series of experiments both the target (KcsA) and the reference (OmpA) were immobilized at a solution equivalent of 150 μM. The histogram represents the fractional difference in peak amplitude of a known ligand of KcsA in the presence of KcsA and OmpA. The bar labelled control represents the first application of the ligand. Subsequently 3 injections of the ligand were performed using buffers that contained no detergent. A further 3 injections were performed where the buffer used to wash the immobilized samples contained deuterated DPC.

As previously noted, individual detergent molecules rapidly exchange between the micellar and monomeric forms. Thus, washing of immobilized micelles in buffer without detergent leads

to rapid loss of protein functionality, as shown in Figure 7. At least for the case of KcsA, which consists of a single polypeptide, the loss of functionality (as measured by binding of a known ligand) appears to be perfectly reversible. Nonetheless, it is clear that DPC must be applied throughout the screening procedure. Since DPC is available in deuterated form its presence does not interfere with the acquisition of the NMR spectra of the compounds. For convenience we chose to include DPC only in the buffer used to wash the compounds out of the cells of the sample holder and not in the mixes themselves. Since this approach has lead to two successful screens of membrane proteins we are optimistic it will be general. In this way it may prove possible to acquire NMR spectra even in the presence of non-deuterated detergents since the concentration of the monomer is reduced by application of the compound mix in the absence of detergent. However, we have yet to test this hypothesis. Once the immobilized protein functionality has been verified, it is also important to create checkpoints at different time points of the screen with mixes containing a known binder as a positive control to check that protein functionality and thus conformation is maintained through the screen.

One final issue deserves special attention when considering carrying out ligand screening studies on a membrane protein, the kinetics of ligand binding. While low affinity ligands for soluble proteins nearly always exhibit rapid exchange kinetics on the NMR time-scale, this may not be the case for membrane proteins. For example, histamine binds the human H1 receptor with a K_d of 20 μM[232]. Such a small molecule (histamine fits well within the definition of a "fragment") binding with moderate affinity would normally imply a fast on rate. However, in this solid state NMR study, the on rate was found to be in the order of minutes! Likely mechanisms for such slow binding include access to the active site of the protein *via* the membrane or slow conformational exchange of the protein due to interaction with membrane (or membrane mimetic). Since the dynamic behaviour of detergents and phospholipids are strongly temperature dependent, it may be necessary to carryout screening at near physiological temperature where the long term stability of the target may be less than optimal. In such situations it may be necessary to prepare multiple samples in order to successfully carryout a screen of a complete fragment library.

Application of TINS to Ligand Discovery

Soluble Targets

To date TINS has been applied to five different soluble targets. We have immobilized the target at a range of concentrations for the various screens, from as high as 500 μM to as low as 100 μM solution equivalent. We now typically screen at 100 - 150 μM which represents an optimal balance between sensitivity, artefact suppression and protein consumption. In all cases we have used the PH domain of AKT as the reference. Typically we immobilize the target and reference on the activated sepharose, Actigel ALD (Sterogene, USA). The efficiency of immobilization is monitored by UV absorption of the supernatant and visual inspection to insure that no precipitation has occurred. If an enzymatic assay of the target is available, we use it at this stage to confirm that the immobilized protein remains functional. The derivatized supports are subsequently packed into the dual-cell sample holder under pressure (0.5 Bar/cell), connected to the solvent delivery lines from the sample handling system, and then placed into the magnet. In most cases a small number of known weak ligands (up to 6) are available to test whether the target has been functionally immobilized and to demonstrate that we can indeed detect ligand binding. One of the known ligands is then selected for use in monitoring the condition of the target during screening. We routinely monitor the condition of the immobilized target through repeated injection of the known ligand throughout the screen.

Once the immobilized target has been deemed functional we carry out the actual screen. The mixes are delivered in 1 ml volumes in deep 96 well plates to the Gilson autosampler. Sample handling is controlled by Bruker HyStar software which communicates with Bruker TopSpin to acquire the NMR data. Using the Hadamard sampling experiment described earlier we currently acquire data for 30 minutes with an additional 5 minutes for sample handling resulting in a cycle time of about 35 minutes. In a recent screen 324 experiments were run in total to assess binding of 1,393 compounds from our fragment collection. This number includes repeated assaying of the

positive control to assess target condition and some overlap of compounds (*e.g.* compounds appear in two different mixtures). This design allows us to assess the repeatability of the screening data. Such a screen was carried out without human intervention in under eight days. Finally, since the target and reference are immobilized, it is possible to change buffer conditions to closely match crystallography conditions without regard to protein stability. We routinely screen under solution conditions in which the reference protein would precipitate if not immobilized. Nonetheless it ligand binding characteristics vary only very moderately from one set of solution conditions to the next.

TINS proof of principle application to a bacterial membrane protein

TINS is a comparative method, where detection of ligand binding to the immobilized target is quantitated by comparison to an immobilized reference. With membrane proteins, partitioning of ligands can occur on the native or synthetic lipids surrounding the target present on the resin. An appropriate reference system had to be developed to control for non-specific binding of hydrophobic compounds to lipids or detergents used to solubilize the membrane proteins. An appropriate choice for such a reference protein would be one with few known binders, in order to minimize the chances of non-specific binding. The *E. coli* Outer membrane protein A (OmpA) was chosen for such qualities. This reference protein was of similar size as our intended target and also refolded in DPC micelles. To get an initial feel for whether we could detect specific binding to a membrane protein using TINS, we conducted a proof of principle study with a screen of a small subset (about 100 compounds) of our compound library using KcsA from Streptomyces as the target and OmpA as the reference.

Prior to screening it was necessary to establish an appropriate 1) level of DPC to include in the wash buffer to maintain the integrity of the immobilized, micelle solubilized target and 2) internal reference compound. If the DPC concentration in the environment of the target decreased to below its CMC, the micelles formed by DPC would start to slowly dissociate into monomers and be flushed away. Simple calculation suggested that it was necessary to use DPC at 5 mM in the wash buffer to in order to maintain the concentration above the CMC (1 mM) upon dilution with

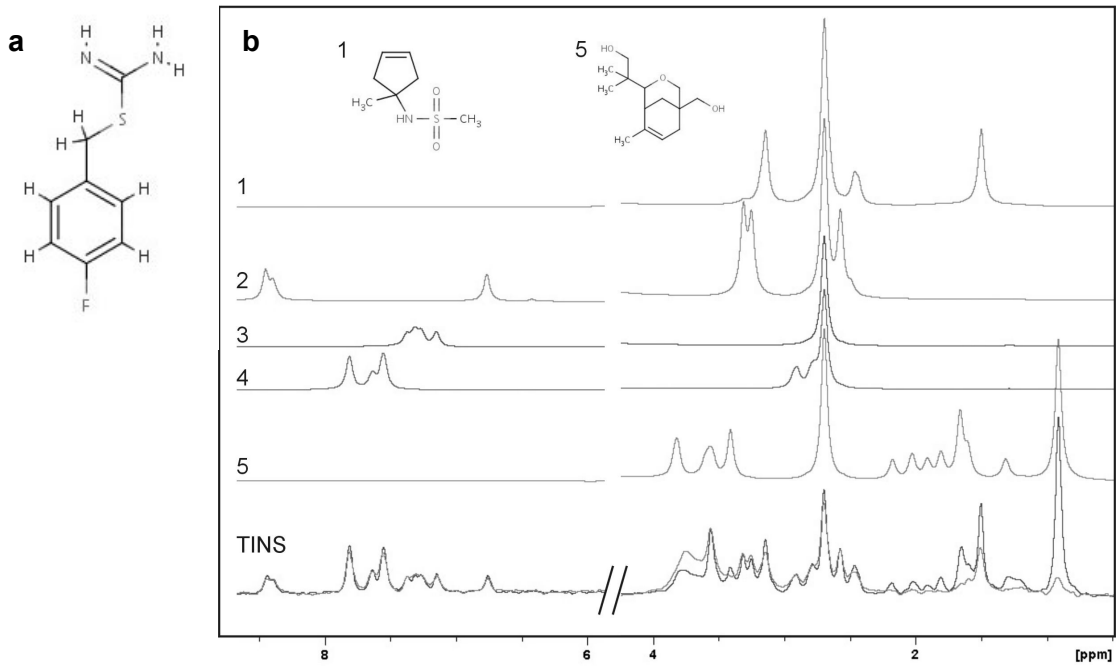


Figure 8. Proof of principle ligand screen against a bacterial membrane protein. **a**. Structure of the known ligand (4-fluorophenyl)methylsulfanylmethanimidamide used determine the integrity of the immobilized KcsA. **b**. Detection of ligand binding in one mix during the screen. A mix containing 5 different compounds was applied simultaneously to the cell containing immobilized KcsA and to the cell containing OmpA. The individual ^{1}H NMR spectra of each cell are overlaid (labeled TINS). The ^{1}H NMR spectrum of each individual compound, which has been intentionally linebroadened to approximately match the linewidth of the TINS spectra, is shown above (numbered). All peaks from compounds **1** & **5** were reduced in amplitude in the presence of the immobilized KcsA with respect to OmpA, indicating that these compounds bind to KcsA. The structures of compounds **1** & **5** are shown.

the compound mix absent DPC. We tested both TSP and TMA as possible internal standard by including both in a mixture with (4-fluorophenyl)methylsulfanylmethanimidamide (FPMSMA, Figure 8**a**), a known ligand for KcsA. These tests indicated that both TSP and the known ligand FPMSMA specifically bind KcsA and we therefore chose to use TMA as an internal standard.

Repeated application of TMA and FPMSMA, followed by washing with buffer plus 5 mM DPC demonstrated stability of the immobilized KcsA and so these conditions were used for the limited library screen. During the screen the immobilized target showed insignificant loss of binding capacity for the control compound and only 12 % loss after 3 months of storage. Out of

the 95 fragments that were screened, 7 % showed substantial changes in the NMR spectrum that were specific to the target and were considered binders after analysis of spectra intensities (Figure 8**b**). This is in line with target hit rates obtained for soluble proteins applied to TINS. Of the potential new hits, 2 structures had a similar scaffold to the known binder. The other hits had a variety of scaffolds with a variety of shapes and numbers of rings.

Development of a high affinity inhibitor of bacterial membrane protein DsbB using TINS

Very recently we have undertaken a program to develop high affinity inhibitors to the bacterial inner membrane protein DsbB in collaboration with Prof. John Bushweller's group at the University of Virginia (USA). DsbB is a redox enzyme involved in the production of toxin in gram negative bacteria[94] and as such is a potentially medically interesting target. The crystal structure of DsbB bound to its redox partner, DsbA has been solved[92] and the Bushweller group has solved the solution structure of a disulfide mutant of DPC solubilized DsbB[62]. For ligand screening we immobilized both the functional wild type DsbB (see above) and OmpA (as a reference) at a solution equivalent of 100 μM. We used the compound Ubiquinone-5 (Figure 9) which binds competitively with the native DsbB ligand. Similarly to KcsA, deuterated DPC was included only in the wash buffer.

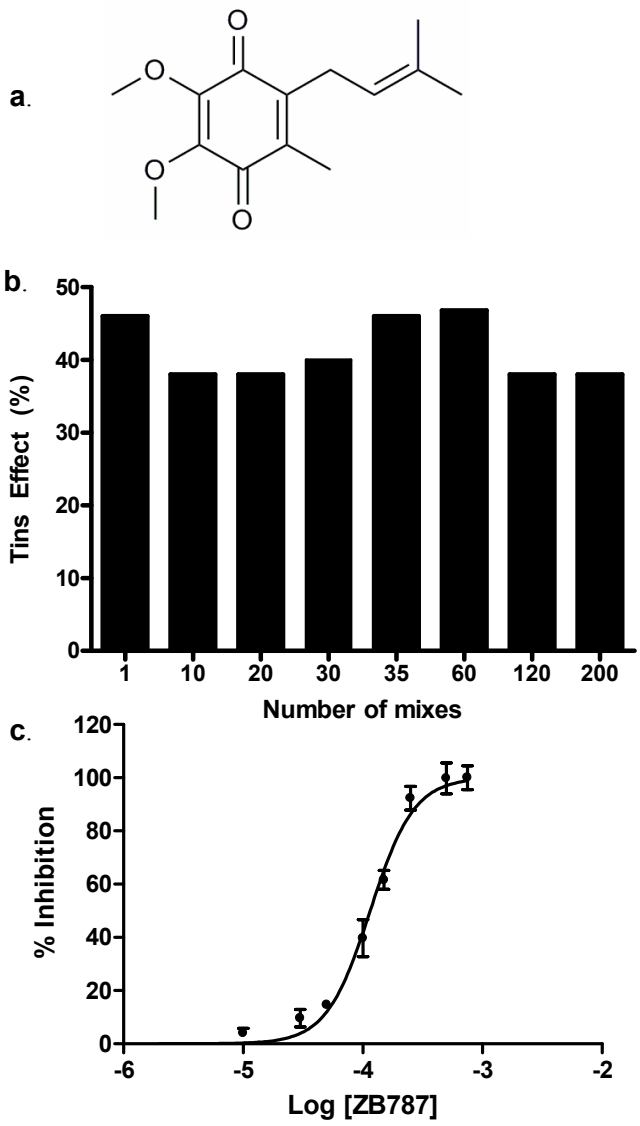


Figure 9. Ligand screening of a bacterial membrane protein. **a**. The structure of Ubiquinone-5 used to asses the integrity of immobilized DsbB during the screen. **b**. Ubiquinon-5 binding to immobilized DsbB during the screen. Binding is defined as in Figure 2. **c**. Enzyme inhibition curve of a hit from the screen.

Using this arrangement 1,270 fragments were screened in mixtures that averaged a little over five compounds each. Figure 9 demonstrates that the immobilized DsbB remains intact throughout the screen. In the screen we found 93 compounds that specifically bind DsbB for a hit rate of 7.3 %. The hits have been investigated for enzyme inhibition at 250 μM and the best 9 of these compounds had IC50s of 150 μM or better where a representative curve is shown in Figure 3. We have carried out both competition binding and competition enzyme inhibition analyses on a limited subset of the hits. Most of the hits are competitive with ubiquinone binding and this seems to represent the major small molecule binding pocket. However, one of the subsets of hits is not competitive with ubiquinone. Follow up biochemical and biophysical analyses are presented in Chapter 5 of this thesis.

Outlook

In the past decade an impressive repertoire of methods has been developed to enable drug development against soluble targets at the molecular level. In addition to fragment screening methods, structural biology has played a key role in this process. Although at present no drugs are marketed that are the exclusive result of the fragment approach the principles can clearly be seen in the remarkable specificity and potency of recently marketed kinase inhibitors such as Imatinib and Gefitinib and indeed, many fragment based drugs are in the late stages of clinical trials[21]. Membrane proteins represent a similar pharmacological challenge in that one would like to be able to specifically address individual targets from amongst large numbers of closely related members of a protein family. However, it is presently not possible to use the molecular methods developed for soluble proteins for drug discovery efforts on membrane proteins.

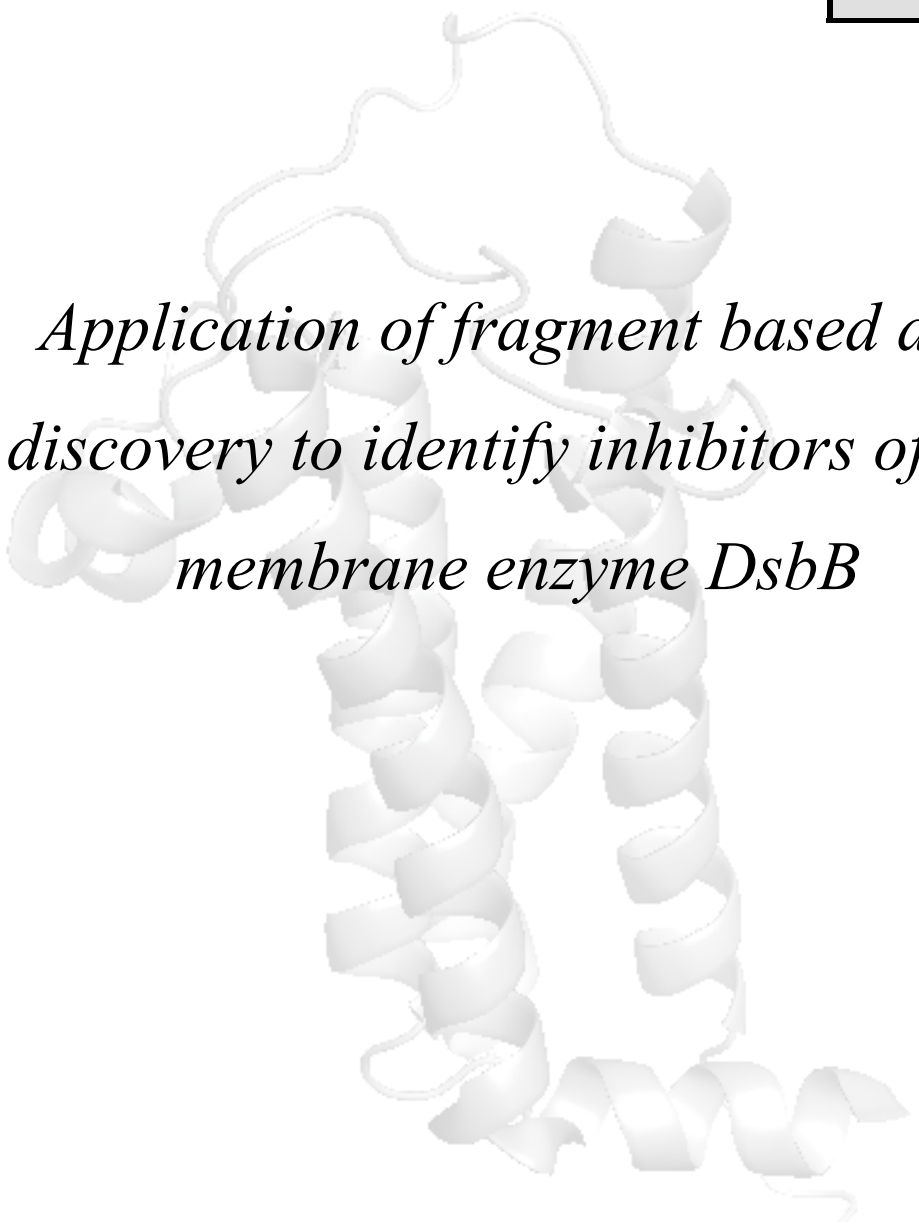
A major goal of the research in our laboratory is to adapt methods developed for soluble targets to membrane proteins or to develop alternative ones. While we are clearly only at the beginning stages of this process we have nonetheless made a promising start. We have been able to immobilize a variety of membrane proteins in functional form and have carried out ligand screening on two. Our current efforts are geared toward finding new ways to solubilize and immobilize membrane proteins that can be more widely applied. We are also looking towards a variety of methods to improve the sensitivity of TINS including experiments that are better optimized for the diffusion limited nature of the heterogeneous system we employ and possible implementation of a TINS cryoprobe.

Once one finds and validates hits, it is of course necessary to evolve these towards high affinity, high specificity ligands. The hit evolution process is greatly aided by the availability of three dimensional structural information of target-ligand complexes for soluble targets. Since crystallography of membrane proteins is not yet widely applicable it will be imperative to develop alternate approaches. We envision a number of such approaches that utilize the power of liquid or solid state NMR. In recent years both solid state NMR[233] and solution state NMR[234] have made significant progress in elucidating 3D structures of either the membrane protein itself or ligands bound to membrane proteins. While it is vital that these efforts continue, it is also

logical that NMR should be employed to take advantage of its unique ability to rapidly generate local, low-resolution structural information. For this we foresee new applications in chemical shift perturbation based modelling of protein-ligand complexes[235], sparse NOE based methods[236,237] and paramagnetic NMR[238]. With the foreseeable advancements in ligand screening and structural analysis, the era of molecular drug discovery on membrane protein targets should soon be upon us.

Chapter

5



Application of fragment based drug discovery to identify inhibitors of the membrane enzyme DsbB

Membrane proteins are an interesting class due to the variety of cellular functions and their importance as pharmaceutical targets, but they pose significant challenges for fragment based drug discovery approaches. Here we present the first successful use of biophysical methods to screen for fragment ligands to an integral membrane protein. Using the recently developed Target Immobilized NMR Screening approach, we screened 1,200 fragments for binding to the enzyme Disulphide bond forming protein B. Biochemical and biophysical validation of the 8 most potent hits revealed an IC_{50} range of 7 to 200 μM, which could be categorized as cofactor binding inhibitors or mixed model inhibitors of both cofactor and substrate protein interaction. Our results clearly establish the utility of fragment based methods in the development of inhibitors of membrane proteins, making a wide variety of important membrane bound pharmaceutical targets amenable to such an approach.

This chapter has been recently submitted as a manuscript: Virginie Früh, Yunpeng Zhou, Caroline Loch, Eiso AB, Herman Verheij, John H. Bushweller and Gregg Siegal. Application of Fragment Based Drug Discovery to Identify Inhibitors of the Integral Membrane Enzyme DsbB.
Nature Chemical Biology. Accepted for review 2009.

With 60 % of currently marketed drugs targeting membrane proteins[36], it is clear that finding small molecules to modulate the function of such proteins is essential. High throughput screening (HTS) methods have been successful in identifying such compounds, but because the methods of detection rely on functional assays, they are generally only sensitive to submicromolar interactions. Such relatively tight interactions are generally only observed for larger compounds (300 - 500 Da). However, it has proved challenging to simultaneously optimize potency and absorption, distribution, metabolism, and excretion (ADME) properties of these "lead-like" or "drug-like" compounds. Furthermore, such large compounds inefficiently explore the binding sites of proteins[17]. Fragment-based drug discovery (FBDD) has become a powerful complementary approach to HTS for generating novel chemical modulators of pharmaceutical targets. FBDD screens small libraries (1,000 - 20,000 compounds) of so-called drug "fragments" that are typically described by a "rule of threes"[15] (Ro3, M_r < 300 Da, cLogP < 3, H-bond donors < 3, H-bond acceptors < 3, number of rotatable bonds < 3 and TPSA (total polar surface area) < 60 Å^2) for binding to the target. Ro3 compliant compounds typically bind the target with K_d greater than 10 μM. In order to detect such weak binding, sensitive biophysical techniques are required. Commonly used techniques for detecting fragment binding include NMR, X-ray crystallography and surface plasmon resonance (SPR)[207]. Although these methods have been successfully applied to an array of soluble protein targets[239], they have failed in one way or another when applied to membrane proteins. There are two primary reasons for this failure: insufficient quantity of the target and non-specific binding of compounds to the solubilization media. Since many biophysical methods require tens or even hundreds of mg of protein and most membrane proteins are difficult to produce in even single mg quantities, many potential applications of FBDD to membrane proteins have been impractical. For those membrane proteins that can be produced in sufficient quantity, non-specific partitioning of fragments into the detergent micelle or lipid bilayer used to solubilize the protein has been a severe and limiting problem.

We have developed an NMR-based fragment screening approach which has proven, in principle, capable of overcoming the challenges posed by membrane proteins[23] (Chapter 4). The approach, called Target Immobilized NMR Screening (TINS)[77], involves immobilizing a target

and a reference protein in two compartments of a dual-cell sample holder[205] and simultaneously injecting mixtures of fragments in an automated process. For each mixture a 1D, spatially selective ^{1}H NMR spectrum, which only contains contributions from the fragments in solution, is recorded. Fragment binding to the target protein is readily detected by the decrease in peak amplitude resulting from the greatly enhanced transverse relaxation experienced in the bound state. The reference protein, which is selected for minimal specific small molecule binding, serves to cancel out non-specific binding of fragments to protein surfaces. Hits can therefore be easily detected by comparing spectra recorded in the presence of the target to those recorded in the presence of the reference. By repeatedly using the same sample to screen the entire fragment collection (~1,500 compounds) typically only ~25 - 50 nmol of protein is required. Furthermore, the reference system is expected to account for non-specific binding of fragments to the detergent micelles in which the membrane proteins are solubilized. We therefore sought to apply TINS to a *bona fide*, integral membrane pharmaceutical target.

The inner membrane protein of *E. coli* Disulphide bond forming protein B (DsbB), and its homologs in other gram-negative bacteria, is an oxidoreductase that is essential for protein disulfide bond formation in the periplasm. Periplasmic DsbA functions as the catalyst for protein disulfide bond formation and is reoxidized by DsbB with concomitant reduction of bound ubiquinone or menaquinone. Since many bacterial virulence factors are secreted proteins that require disulfide bonds for proper folding and function, the DsbA/DsbB system is a potential antimicrobial drug target[94,240,241]. DsbB is an ideal candidate to test the TINS methodology since it can be readily produced and solubilized in detergent micelles where it retains a robust enzymatic activity which is easily assayed. In addition, a wealth of biochemical data is available that describes the enzymatic activity of the wildtype as well as numerous relevant mutants[242,243,244,245]. Finally, the 3D structures of the wildtype DsbB bound to its redox partner DsbA[92] and of a mutant representing an enzymatic intermediate are available[62]. Selection of an appropriate reference protein is critical to insure the robust performance of TINS. Our previous experience using the *E. coli* Outer membrane protein A (OmpA) transmembrane domain, which has native structure under the same detergent micelle conditions as DsbB, in a limited screen of

about 200 compounds, suggested that it had minimal small molecule binding[23] (Chapter 4). Thus we have utilized OmpA as a reference protein.

Here we report the first complete screen of a fragment library against an integral membrane protein. Hits from the screen have been validated and characterized with respect to mode of action using an enzyme inhibition assay. Finally, the binding mode of two classes of inhibitors has been investigated by analysis of chemical shift perturbations induced upon fragment binding to isotope labelled, mutant DsbB.

Methods

Protein immobilization

DsbA, DsbB, and OmpA were expressed and purified as previously reported[1,79,99]. The Actigel ALD resin (Sterogene, Carlsbad, CA, USA), available commercially, was used as a 50 % slurry and all experiments were carried out at 4 ºC when possible. The resin was washed with cold phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2HPO_4, 100 mM NaCl) at pH 7.5. 200 nmol of DPC solubilized DsbB was added to 1 ml bed volume of resin. The reductant sodium cyanoborohydride ($NaCNBH_3$) was added to a final concentration of 0.1 M. After an overnight incubation at 4 °C, residual unreacted aldehydes were blocked by addition of 50 mM Tris buffer and $NaCNBH_3$ for another 2 hours. The same procedure was repeated for DPC solubilized OmpA. Quantification of immobilized protein was monitored by absorption of the supernatant at 280 nm before and after immobilization, and by SDS-page gel with a known standard curve and volume analysis. This data indicated that a final concentration of 100 μM of both immobilized DsbB and OmpA was achieved, equating to a 50 % yield.

DsbB activity assays

DsbB activity was quantified by measuring the capacity of the enzyme to reoxidize the protein DsbA or reduce its cofactor Ubiquinone-5, also called Coenzyme Q1 (UQ1) at pH 6.2. DsbA

was reduced with 10 mM DTT for 10 minutes on ice. DTT was subsequently removed by gel filtration on a PD-10 column pre-equilibrated with degassed distilled water containing 0.1 mM EDTA. EDTA was used to chelate metal ions which would otherwise give rise to spurious reoxidation of DsbA[244]. DsbA fluorescence (excitation at 295 nm and emission at 330 nm) was measured in the presence of DsbB and UQ1 in 50 mM sodium phosphate, 100 mM NaCl, 0.1 % detergent (DPC or DDM depending on which was used to solubilize DsbB) and 0.1mM EDTA) at 30 ºC. The activity of DsbB in terms of moles ubiquinone reduced/moles DsbB min^{-1} could be calculated by using the initial slope of fluorescence decrease upon DsbA oxidation, or by using the slope of absorption decrease at 275 nm upon reduction of UQ1[99].

To measure activity of immobilized DPC solubilized DsbB, resin was aliquoted and diluted with degassed activity assay buffer to a final protein concentration of approximately 20 nM. For an appropriate baseline, an equivalent amount of resin without protein (blank resin) was prepared in the same manner. Quinone reduction was monitored in both samples after addition of 20 μM coenzyme Q1 and 20 μM DsbA.

Target Immobilized NMR Screening

Immobilized, DPC solubilized DsbB and OmpA were each packed into a separate cell of a dual-cell sample holder[205]. The cell was attached to a Gilson 210 autosampler *via* capillary tubing and inserted into an 8 mm, ^{1}H selective, flow-injection probe in a 500 MHz magnet. Mixes of the 1,270 fragments were made by 200 fold dilution of a 100 mM stock of each compound in d_6-DMSO such that the final DMSO concentration was never greater than 5 %. Upon injection of each mix into the dual-cell sample holder, flow was stopped and spatially selective Hadamard spectroscopy[206] was used to acquire a 1D ^{1}H spectrum of each sample separately. A CPMG T2 filter of 80 ms was used to remove residual broad resonances from the sepharose resin. The cycle time was about 35 minutes, with 30 minutes required for the NMR experiment and 5 for sample handling, resulting in a total time of about 5 days to complete the screen. To maintain proper fold of each protein, 5 mM deuterated DPC was included in the buffer (20 mM phosphate buffer in D_2O, 100 mM NaCl, pH 7.6) used to wash the fragment mixes from

the sample holder. Scaling of peak intensities and positions between the two cells during data analysis was facilitated by adding glycine and TSP in all fragment mixes.

Biochemical hit validation

All fragments from the screen that were designated as positive for binding were assayed for DsbB inhibition at 250 μM. The amount of DMSO in all biochemical assay controls was adjusted to match the amount present when fragments were tested. Those compounds that showed more than 70 % inhibition at 250 μM were further characterized by titration from 0.0001 mM to 10 mM to generate IC_{50} curves. The mode of action for the 8 most potent fragments was determined from competition enzyme assays. For this analysis either DsbA or UQ1 was titrated in from 0.2 to 40 μM, while the other was kept constant at 40 μM. For each titration point, slopes were measured in the presence of 5, 10, and 75 μM of the fragment. DsbB activity data was analyzed using the non-linear regression curve fitting routines in Graph Pad Prism v. 5.01 (Graph Pad, San Diego, CA, U.S.A.). Statistical significance was evaluated with the student's T-test. Depending on the light absorbing properties of the fragments, they were used in either the fluorescence or UV-absorbance assay. Compounds which were not compatible with the assays due to high intrinsic fluorescence, high UV absorbance or irregular baselines were not included in the analysis.

Biophysical hit validation

Due to the poor quality of the NMR spectra of the wild-type DsbB, it was necessary to use a mutant that represents an intermediate in the disulfide oxidation pathway[62]. Accordingly, validated hits from the screen were titrated at 1, 5, and 10 mM into ^{15}N-labelled DsbB[CSSC] mutant (C44S, C104S). [^{15}N,^{1}H] HSQC experiments were acquired at 40 ºC in a Bruker DRX 600 MHz spectrometer equipped with a cryoprobe. A reference titration of DMSO and a non-binding fragment from the screen were used to subtract chemical shift perturbations not related to fragment binding.

RESULTS

Structure of a micelle solubilized DsbB disulfide intermediate

We have previously reported the solution structure of a mutated form of DsbB in which cysteines 44 and 104 have been mutated to serine, resulting in a stable disulfide bridge between Cys41 and Cys130 in DPC micelles (referred to as DsbB[CSSC], Figure 1)[62].

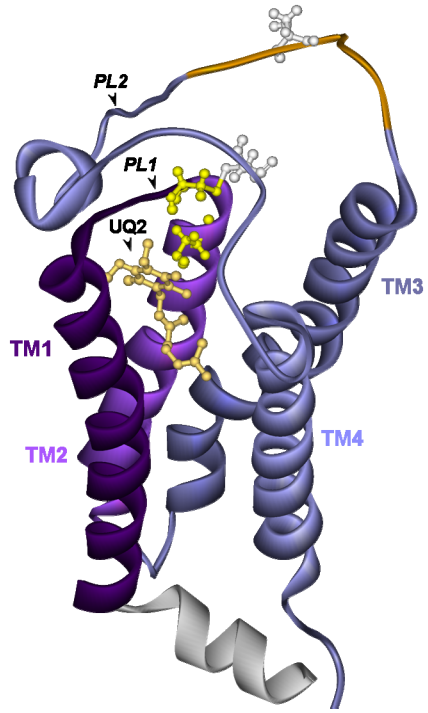
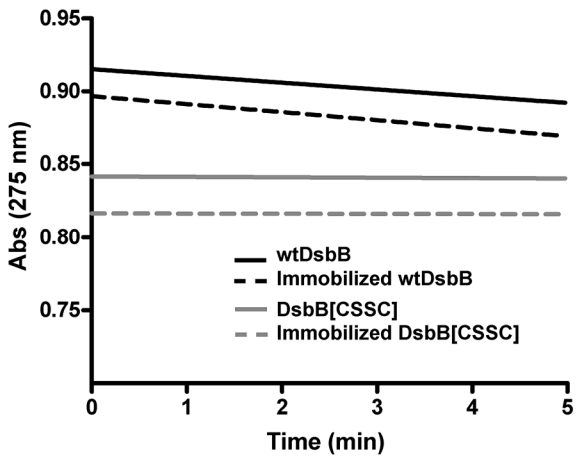


Figure 1. Solution NMR structure of DsbB[CSSC] (PDB 2K74), viewed approximately parallel to the membrane plane. The upper surface faces the periplasm while the lower surface faces the cytoplasm. DsbB has four transmembrane helices (TM 1 - 4) and an N-terminal cytoplasmic amphipathic helix (H1). The functionally important second periplasmic loop is divided into two parts (PL2 and PL2') by a short horizontal helix (H2). The four essential Cys residues (yellow) are located at the N-terminus of TM2 and in PL2 and PL2'. The binding site for the ubiquinone cofactor (black and red) lies on the periplasmic side of DsbB and is formed by TM1, TM2 and TM4. Two regions in PL2 and PL2' that form the DsbA binding site are colored red.

This form of the protein, with a disulfide bridge between periplasmic loops 1 and 2, is an intermediate in the redox reaction that results in transfer of electrons from DsbA to a ubiquinone buried in DsbB[246]. The N-terminus of micelle solubilized DsbB[CSSC] forms an amphipathic helix that runs parallel to the cytoplasmic side of the inner membrane. The ubiquinone binding site is near the periplasmic side of the inner membrane with the isoprenoid tail extending down the groove between TM1 and TM4. The interloop disulfide between Cys41 and Cys130 is approximately 7.5 Å from the benzoquinone ring.

a



b

Enzyme	K_{cat} (M UQ1/ M DsbB-min^{-1})
DsbB wt	267 ± 14
DsbB wt (immobilized)	238 ± 27
DsbB[CSSC]	13 ± 2
DsbB[CSSC] (immobilized)	10 ± 1
DsbB wt*	278

Figure 2: Enzymatic activity of wildtype DsbB (wt) and DsbB[CSSC] in solution and immobilized. Both forms of DsbB were assayed at 5 nM. The graph shows the reduction of UQ1 observed by a decrease in absorption at 275 nm (**a**). The calculated K_{cat} values for each form of DsbB are compared with literature values *[1] (**b**).

DsbB functional immobilization and enzymatic activity

Wildtype DsbB (containing endogenous quinone), solubilized in DPC micelles, was immobilized on a Sepharose resin containing a 6 atom hydrophilic linker terminating in an aldehyde *via* a Schiff's base intermediate. At the pH selected (7.4), this reaction is relatively specific for the free N-terminus. A final concentration of approximately 100 μM DsbB (nmol protein per ml settled bed volume) was readily achieved. The functionality of the immobilized enzyme was compared to non-immobilized, micelle solubilized enzyme. Figure 2 shows that immobilized wildtype DsbB retained 90 % activity in comparison to the non-immobilized protein and that the K_{cat} of both forms of the protein was close to values previously reported[1].

DsbB[CSSC] was assayed for enzymatic activity in both immobilized and non-immobilized forms and, as expected, had no activity (Figure 2)[1,62,245]. The ready immobilization with retention of enzymatic activity suggests that the N-terminus of DsbB is accessible in the micelle solubilized protein. Given the success of the immobilization strategy with DsbB, we used the same approach with OmpA which had also been solubilized in DPC micelles. We observed a similar yield of OmpA immobilization. Since OmpA has no enzymatic activity, we had to assume that its structure was not grossly perturbed by the immobilization process. Independent

experiments showed that immobilized samples of DsbB were stable for at least one month after storage at 4 ºC (data not shown).

Target immobilized NMR Screening (TINS)

In order to screen our fragment collection, DsbB and OmpA were immobilized at a solution equivalent of 100 μM and separately packed into cells of the dual-cell sample holder[205].

Preliminary studies clearly demonstrated that repeated cycles of compound application and washing in the absence of added detergent resulted in rapid degradation of DsbB activity, as would be expected of a membrane protein[23] (Chapter 4). Therefore deuterated DPC was included at a minimum concentration of 5 mM (5 x critical micellar concentration) in the buffer used to wash the compounds from the sample holder. To monitor the integrity of the DsbB sample during the screen, the binding of synthetic UQ1 was observed. The TINS effect, defined as the average ratio of the amplitude of peaks in the presence of DsbB to that in the presence of OmpA, remained constant for UQ1 throughout the screen[23]. This fact suggests that either the bound quinone was not cumulatively displaced or leached from the DsbB or that the injected UQ1 was sufficient to replace any lost quinone. A total of 1200 fragments, in 182 mixtures containing 3 - 9 fragments at 500 μM each, were assayed for binding to DsbB. A spatially selective Hadamard NMR experiment[206] was used to simultaneously acquire a 1D ^{1}H spectrum of compounds in the presence of DsbB or OmpA. The data resulting from the screen could be analysed directly without deconvolution because fragments could be directly identified by comparing peaks from TINS spectra with the fragment's individual reference spectra (Figure 3). The ready identification of binders enabled a totally automated analysis of the complete screening data,

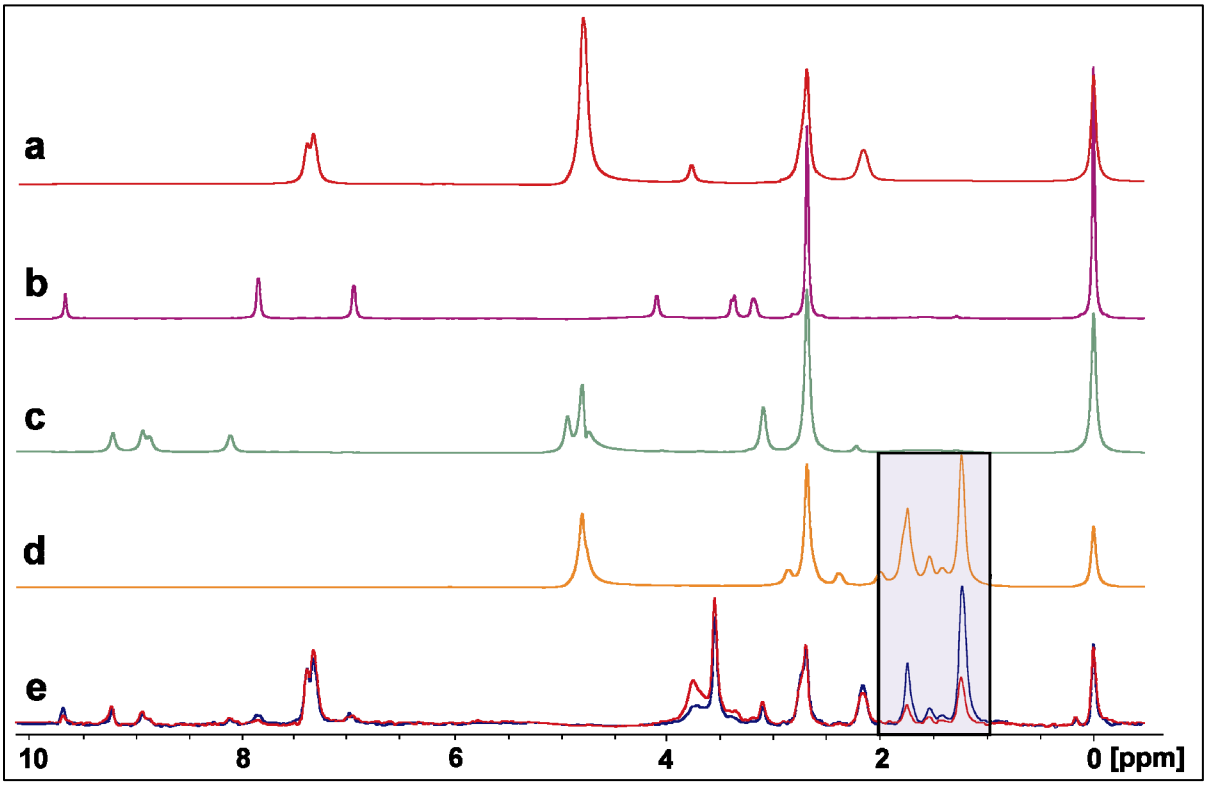


Figure 3. Detection of ligand binding to immobilized DsbB using TINS. The 1D ^{1}H NMR spectrum of 4 different fragments in solution (**a** - **d**) is shown for reference. The linewidths of the reference spectrum have been intentionally broadened to match those recorded in the presence of the sepharose support. The ^{1}H NMR spectrum of a mix of the 4 fragments in the presence of DsbB (**e**-red spectrum) or OmpA (**e**-blue spectrum) that have been immobilized on the sepharose support. The highlighted region shows the reduction in peak amplitude expected upon specific binding of a fragment to the immobilized target.

from Fourier transform and phasing to comparison of peak amplitudes and binder identification. The analysis was performed using in house written routines implemented in TopSpin, the spectrometer control and data analysis software. The screen resulted in 93 hits for DsbB, defined as fragments which had a TINS effect less than 0.3, as shown by an example of a mix containing a hit in Figure 3. This particular cut-off was chosen by virtue of a step-like relationship between the observed TINS effect and the number of "hits" whereby even slightly raising the cut-off gave a large increase (more than two fold) in the number of compounds that were selected as hits (not shown). The resulting hit rate for DsbB was 8.7 % which is well within the range we typically

observe with TINS (3 - 9.5 %). The higher hit rates of TINS with respect to other ligand based, NMR detected methods is readily explained by the sensitivity of TINS to binding as weak as 15 mM K_d, which is about 5 times lower than comparable screening assays. Application of the same criteria to OmpA binding identified 7 compounds as hits for a hit rate of 0.6 %, validating the earlier data suggesting that OmpA has minimal small molecule binding capacity and is therefore a good reference for membrane proteins.

Hit validation and characterization using enzymatic assays

Since the TINS assay simply identifies compounds that bind to DsbB, not necessarily hits which inhibit enzymatic activity, it is important to validate the hits in terms of biological activity. Enzyme inhibition studies using a single concentration (250 μM) were used to generate an initial ranking of the biological activity of the fragment hits (Figure 4). Each of the fragments identified as TINS hits was assayed for inhibition of DsbB-dependent reoxidation of DsbA. Compounds which interfered with the assay when run in either fluorescence or absorbance mode and were left out of the analysis. The remaining 74 hits exhibited a distribution of potencies against DsbB (Figure 4), including 60 % with better than 30 % enzymatic inhibition and 16 % with either less than 20 % inhibition or mild stimulation.

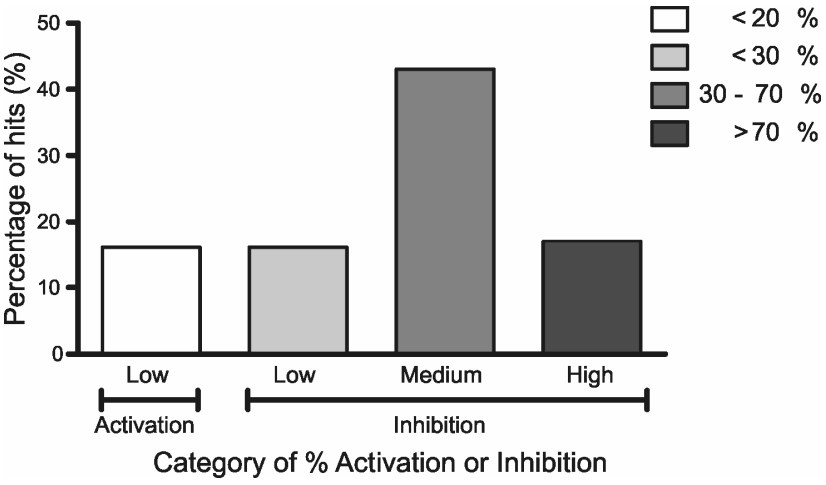
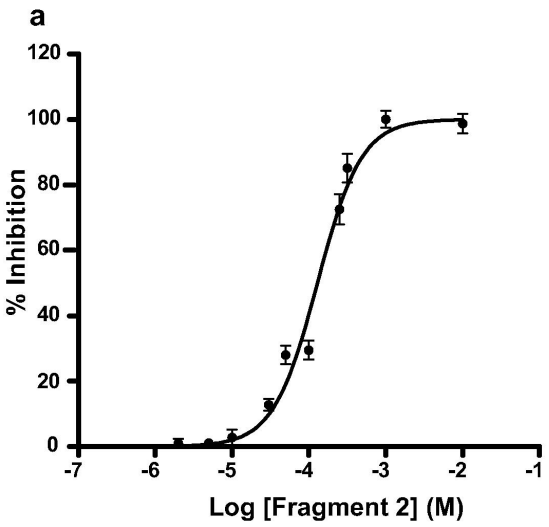


Figure 4. Distribution of biological activity of the hits found in the TINS fragment screen of DsbB. Each fragment was assayed singly at 250 μM.

a



b

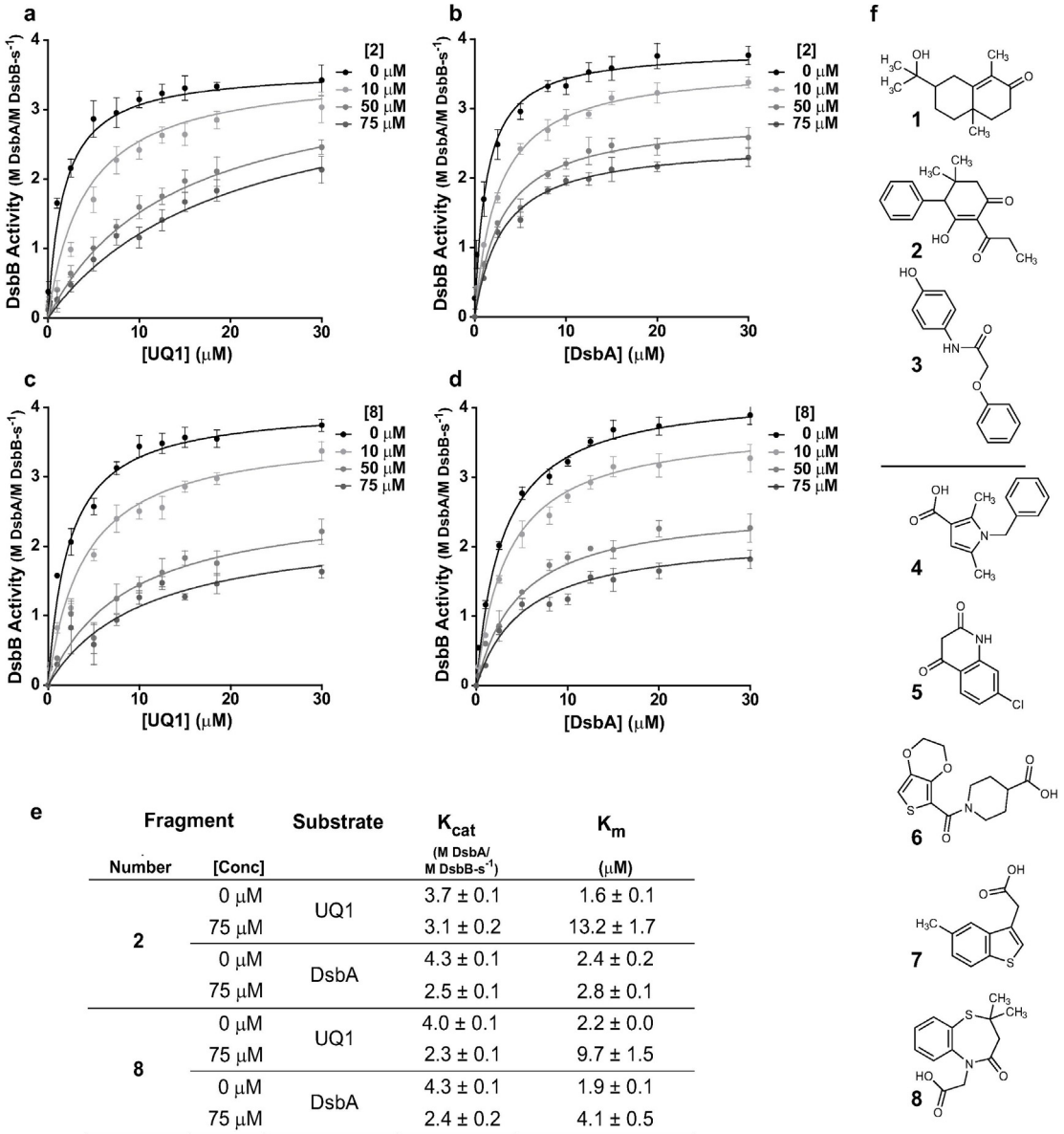
Fragment	IC50 (μM)	Hill coefficient
1	7 ± 1	0.80 ± 0.10
2	100 ± 10	1.40 ± 0.15
3	193 ± 11	1.20 ± 0.11
4	13 ± 1	0.80 ± 0.10
5	46 ± 12	0.80 ± 0.10
6	70 ± 10	1.00 ± 0.10
7	115 ± 11	1.15 ± 0.05
8	168 ± 10	1.40 ± 0.10

Figure 5. Potency determination for selected hits from the TINS screen. An example of an inhibition curve used to determine the IC_{50} (**a**). The curve represents the mean ± S.E.M. of three independent experiments performed in triplicate; (**b**) Inhibitory constants and Hill coefficients for the 8 most potent compounds.

The 13 fragments showing more than 70 % inhibition in the single concentration point assay were further analysed for potency (IC_{50}) by dose-response experiments (Figure 5). Dose response experiments were carried out with increasing fragment concentrations, from 0.0001 to 10 mM, while both DsbA and UQ1 were kept in excess. Three of the 13 fragments showed artefacts including signs of protein precipitation at higher compound concentration and/or steeper than expected Hill coefficients. The remaining 10 fragments titrated over 2 log orders and exhibited a Hill coefficient close to unity and are therefore well-behaved. The 8 most potent compounds had IC_{50} values between 7 and 200 μM and consisted of a variety of scaffolds (see Figure 6).

As an initial step towards delineating the molecular mechanism of DsbB inhibition, we carried out a more detailed kinetics analysis of the mode of action of the 8 most potent fragments. Substrate-velocity experiments were performed with either DsbA or UQ1 titrated in the presence of saturating amounts of the other. The titrations were then repeated in the presence of increasing amounts of the inhibitory fragment. There are several possible outcomes of such an experiment: a decrease in the apparent affinity for DsbA or for UQ1, in which case the fragment competes with

one of these for binding to DsbB, or mixed-model inhibition in which the apparent affinity for both decreases (Figure 6).



e

Fragment		Substrate	K_{cat} (M DsbA/ M DsbB-s^{-1})	K_m (μM)
Number	[Conc]			
2	0 μM	UQ1	3.7 ± 0.1	1.6 ± 0.1
	75 μM		3.1 ± 0.2	13.2 ± 1.7
	0 μM	DsbA	4.3 ± 0.1	2.4 ± 0.2
	75 μM		2.5 ± 0.1	2.8 ± 0.1
8	0 μM	UQ1	4.0 ± 0.1	2.2 ± 0.0
	75 μM		2.3 ± 0.1	9.7 ± 1.5
	0 μM	DsbA	4.3 ± 0.1	1.9 ± 0.1
	75 μM		2.4 ± 0.2	4.1 ± 0.5

Figure 6. Mode of action determination for the most potent DsbB inhibitors. Fragment **2** was assayed in competition with synthetic UQ1 (**a**), the electron acceptor, or DsbA (**b**) the electron source. Fragment **8** was assayed in the same manner (panels **c** and **d** respectively). The K_{cat} and K_m apparent determined from the data are shown in the table below (**e**) in the absence and presence of the indicated amount of each inhibitor. The structures of all 8 fragments are shown in **f**. The data indicates that fragments **1** – **3** are quinone competitive and fragments **4** – **8** are mixed mode inhibitors.

In this analysis, fragments **1 - 3** behaved similarly (Supplementary Figure 1). This group is exemplified by fragment **2** where increasing concentrations result in moderate perturbation of the maximum enzymatic turn over rate (K_{cat}) and apparent affinity of DsbA but a dramatic reduction (> 6 fold) in the apparent affinity of UQ1. This data suggests that fragments **1 - 3** compete for the same binding site as UQ1. On the other hand, addition of fragments **4 - 8** simultaneously decreased both the apparent affinity and the K_{cat} for Q1 and DsbA as best exemplified by fragment **8** (Figure 6 and Supplementary Figure 2). This data suggests a mixed model of inhibition of DsbB by these fragments. Mixed model inhibition could be explained if the fragments bound DsbB in such a way that limited access of both UQ1 and DsbA to their binding sites on DsbB. To investigate this possibility we sought 3D structural information on the binding site of the fragments.

Mapping of Fragment Binding on DsbB by NMR chemical shift perturbation

If the sequential assignment of a protein is available, analysis of chemical shift perturbation data affords rapid access to low resolution structural data to characterize ligand binding sites[28,236]. While the sequential assignment of wildtype DsbB is not available due to the poor quality of the NMR spectra, spectra of the DsbB[CSSC] double cysteine mutant are of high quality, resulting in a complete backbone resonance assignment for this form of the protein[62]. Accordingly, we titrated fragments **1 - 8** into ^{15}N labelled DsbB[CSSC] and acquired HSQC spectra at 1, 5 and 10 mM fragment concentrations. We titrated DMSO into ^{15}N DsbB[CSSC] to control for solvent induced chemical shift perturbations and subsequently a fragment was selected from the library that was scored as a non-binder in the TINS screen. As expected these titrations resulted in minimal shifts of peak positions, which were subsequently subtracted from the titrations of binding fragments. When purified from *E. coli,* DsbB[CSSC] contains the endogenous ubiquinone-8[99], thus compound specific for this site must compete with UQ-8 for binding. Synthetic UQ1 was titrated into a sample of ^{15}N DsbB[CSSC] to locate the resonances which were affected by *bona fide* binding to the ubiquinone site (Figure 8). We observed

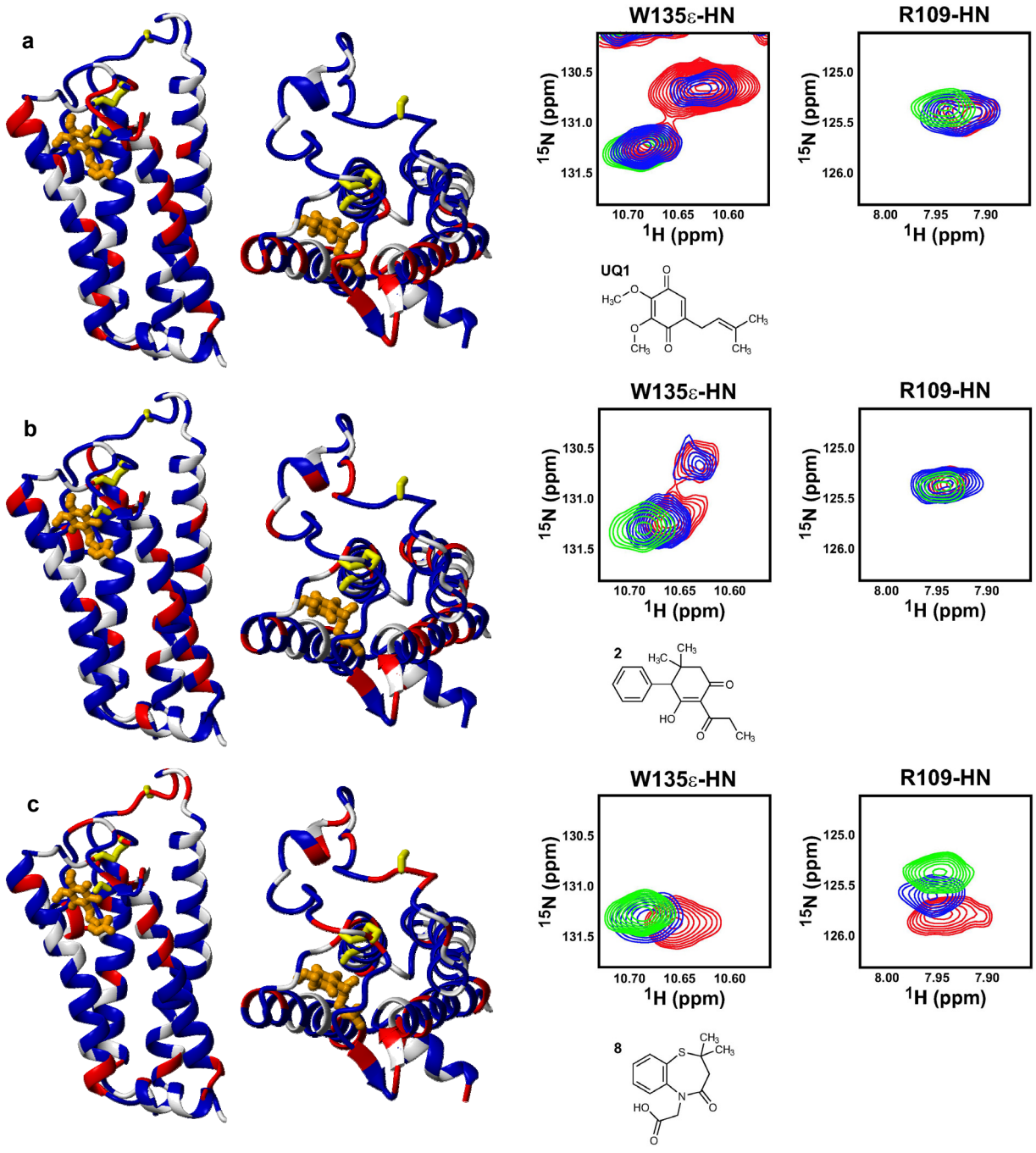


Figure 7: NMR analysis of fragment binding to DsbB. The 8 most potent fragments were titrated into ^{15}N DsbB[CSSC]. Data for the synthetic quinone UQ1 (**a**), competitive fragment **2** (**b**) and the mixed model fragment **8** (**c**) are shown.

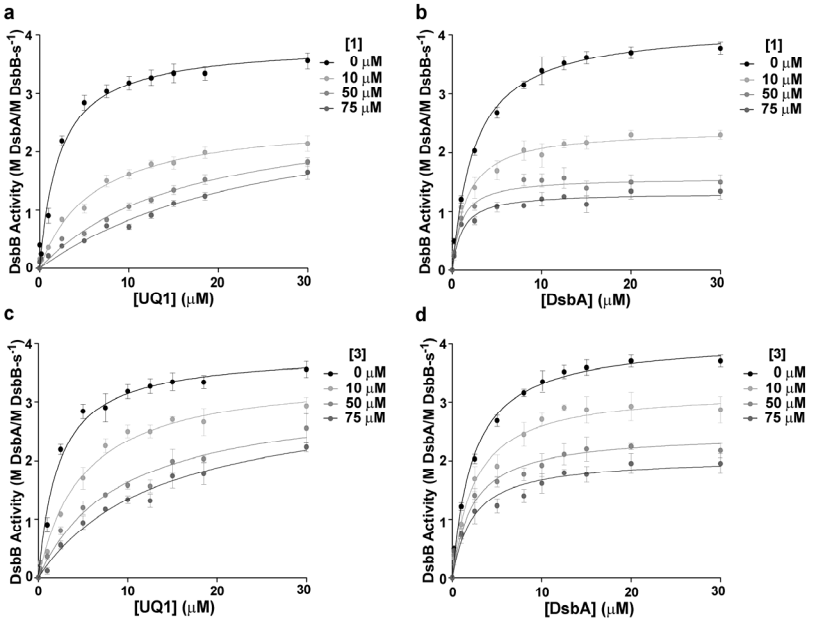
Figure 7 (cont.): For each of these three compounds, the pattern of peak perturbations mapped onto the backbone structure, characteristic peak perturbations and the structure of the compound is shown. Residues with largest perturbations upon titration of the are indicated in red on the DsbB[CSSC] backbone structure (modified pdb ID 2k74[62]) viewed from the same orientation as in Figure 1 (left) and from the periplasmic face (right). The side chains of C41, S44, C104, and S130 are indicated in yellow. The coenzyme UQ2 (orange) is shown to indicate its binding site and the 23 unassigned residues from this study and 10 prolines are colored white.

significant chemical shift perturbations for a number of residues including the backbone amides of A22, Q33 and D136 as well as the sidechain indole of W135, all of which are in the areacorresponding to the endogenous quinone binding site, as reported previously in both the crystal and solution structures of DsbB[62,92]. In all cases, titration of UQ1 resulted in the appearance of peaks at a new chemical shift and the simultaneous disappearance of peaks from the spectrum of DsbB[CSSC] bound to endogenous quinone. This pattern of peak changes is indicative of slow exchange on the NMR time scale (e.g. k_{off} < 30 Hz $\Delta\delta$ in Figure 7), a fact that is not surprising given the similarity of UQ1 to other known synthetic substrates of DsbB (e.g. UQ2). The appearance of the UQ1 HSQC spectrum and the lack of visible protein precipitation indicate that the displacement of endogenous quinone by UQ1 is a reversible phenomenon that maintains the overall fold of the protein.

Addition of all 8 fragments to ^{15}N labelled DsbB[CSSC] resulted in readily detectable changes in chemical shifts, suggesting that the fragments selected by TINS screening and biochemical assays on wild type protein also bind the cysteine mutated form. The presence of chemical shift perturbations in solvent exposed loops as well as portions of the protein buried within the micelle suggests that the fragments were specifically binding to the protein and not non-specifically partitioning into the micelle. Fragments **1 – 3,** which competitively inhibited ubiquinone binding, induced chemical shift perturbations located on the first periplasmic loop, in close proximity to the active site cysteine Cys41 and the mutated residue Ser44. Unfortunately the key residue Arg48, previously identified as being involved in ubiquinone binding[92] could not be clearly assigned in our experiments, along with a few other residues in close proximity to the ubiquinone binding site (Figure 7). The pattern of chemical shift perturbations induced by this group, as best

exemplified by fragment **2**, closely resembles those induced by UQ1. First, titration of **2** into ^{15}N DsbB[CSSC] resulted in chemical shift changes in the same residues as for UQ1 and further, these changes suggested slow exchange. Similarly, R109HN, which is minimally affected by UQ1, undergoes only minor chemical shift perturbations in the presence of **2**. Further, mapping all of the chemical shift perturbations induced by **2** onto the backbone structure of DsbB[CSSC] reveals a pattern that strongly resembles that induced by UQ1 (compare Figure 7**a** to 7**b**).

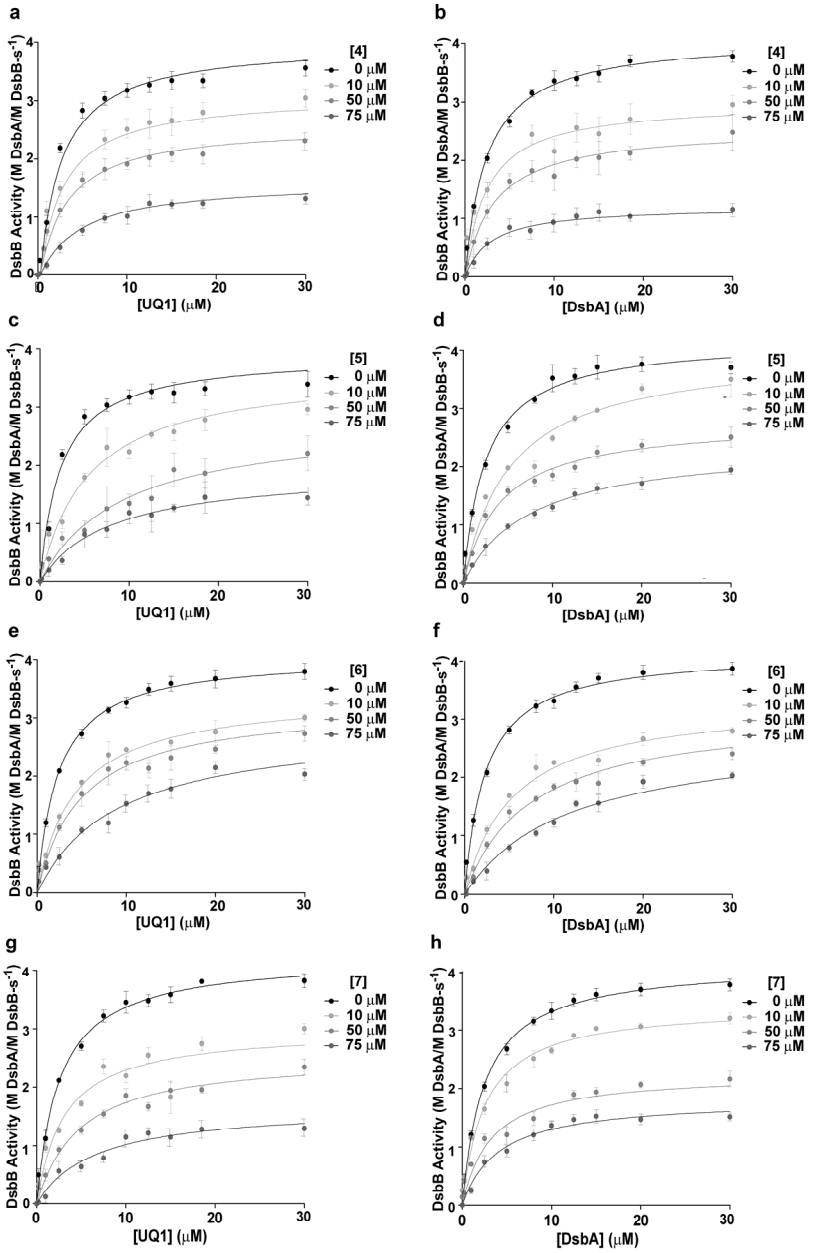
In contrast, the chemical shift changes induced by fragments **4** – **8** differ in both the overall pattern and the details (Figure 7**c**). Addition of **8**, for example, to ^{15}N DsbB[CSSC] resulted in chemical shift perturbations in TM1 and TM2 close to the quinone binding site as with **2**, but additional extensive perturbations in the second periplasmic loop, especially the segment containing Cys104, involved in transferring electrons from DsbA to DsbB and the segment containing Pro100-Phe106, involved in DsbA binding[92]. Interestingly, the section containing the hydrophobic residues Leu116 and Val120, which are suggested to be involved in associating with the membrane during charge transfer from DsbA to DsbB[92], were affected by titration of fragments **4** - **8**, but not by Q1 or fragments **1** - **3**. Moreover, the details of the chemical shift perturbations differed significantly between fragments **4** - **8** and UQ1 or **1** - **3**. There was no evidence for slow exchange for any of the fragments **4** - **8**, although **4** & **7** show signs of line broadening of the backbone resonance of Q33 that may indicate intermediate exchange (not shown). Addition of **8** resulted in gradual, concentration dependent changes to W135ε NH that are indicative of rapid exchange while the position of the bound peak is very different from that in the UQ1 and **2** titrations. In contrast, the backbone amide of R109, which is only mildly perturbed by UQ1 or **2,** is very dramatically perturbed by the presence of **8**. This data suggests that fragments **4** - **8**, which exhibit mixed mode DsbB inhibition, bind in either a different mode or different site to fragments **1** - **3** which are competitive with ubiquinone.



Supplementary Figure 1. Competition binding analysis of fragments showing UQ1-competitive inhibition of DsbB. The substrates UQ1 and DsbA were independently tested for competition by fragments **1** and **3**. In both cases, fragments showed competitive inhibition of UQ1 binding (**a**, **c**) and non-competitive inhibition of DsbA binding (**b**, **d**). The data is represented as the mean ± S.E.M. of three independent experiments performed in triplicates.

Fragment		Substrate	K_{cat} (M DsbA/ M DsbB-s^{-1})	K_m (μM)
No.	**[Conc]**			
1	0 μM	UQ1	3.8 ± 0.1	2.2 ± 0.2
	75 μM		3.7 ± 0.5	8.8 ± 0.4
	0 μM	DsbA	4.2 ± 0.2	2.5 ± 0.2
	75 μM		1.9 ± 0.5	2.3 ± 0,5
2	0 μM	UQ1	3.7 ± 0.1	1.6 ± 0.1
	75 μM		3.1 ± 0.2	13.2 ± 1.7
	0 μM	DsbA	4.3 ± 0.1	2.4 ± 0.2
	75 μM		2.5 ± 0.1	2.8 ± 0.1
3	0 uM	UQ1	3.8 ± 0,1	2,3 ± 0,2
	75 μM		2.9 ± 0.2	4.6 ± 0.2
	0 μM	DsbA	3.9 ± 0.1	2.3 ± 0.2
	75 μM		2.0 ± 0.5	3.2 ± 0.6

Supplementary Table 1. Summary of values for DsbB competition binding analysis graphically represented in Supplementary Figure 1. K_{cat} and K_m values for DsbB at 0 μM and 75 μM of fragments **1** to **3** are listed as the mean ± S.E.M. of three independent experiments performed in triplicates.



Supplementary Figure 2. Competition binding of fragments showing mixed model inhibition of DsbB. The substrates UQ1 and DsbA were independently tested for competition by fragments **4** to **7** and in all cases, fragments showed mixed model inhibition for UQ1 binding (**a**, **c**, **e**, **g**) and DsbA binding (**b**, **d**, **f**, **h**). The data is represented as the mean ± S.E.M. of three independent experiments performed in triplicates.

Fragment		Substrate	K_{cat} (M DsbA/ M DsbB-s^{-1})	K_m (μM)
No.	**[Conc]**			
4	0 μM	UQ1	3.9 ± 0.1	2.4 ± 0.2
	75 μM		1.6 ± 0.5	4.3 ± 0.6
	0 μM	DsbA	4.1 ± 0.2	2.2 ± 0.2
	75 μM		1.2 ± 0.1	3.0 ± 0.1
5	0 uM	UQ1	4.0 ± 0.1	2,4 ± 0,2
	75 μM		1.9 ± 0.1	6.3 ± 0,2
	0 μM	DsbA	4.2 ± 0.1	2.4 ± 0.2
	75 μM		2.3 ± 0.2	5.6 ± 0.1
6	0 μM	UQ1	4.0 ± 0.2	2.4 ± 0.4
	75 μM		3.6 ± 0.1	5.8 ± 0.3
	0 μM	DsbA	4.2 ± 0.4	2.3 ± 0.3
	75 μM		2.7 ± 0.5	7.8 ± 0.5
7	0 uM	UQ1	4.2 ± 0.0	2.5 ± 0.5
	75 μM		1.6 ± 0,1	5.9 ± 0.2
	0 μM	DsbA	4.1 ± 0.2	4.2 ± 0.5
	75 μM	DsbA	1.8 ± 0,3	5.9 ± 0.2
8	0 uM	UQ1	4.0 ± 0.1	2.2 ± 0.0
	75 μM		2.3 ± 0.1	9.7 ± 1.5
	0 μM	DsbA	4.3 ± 0.1	1.9 ± 0.1
	75 μM	DsbA	2.4 ± 0.2	4.1 ± 0.5

Supplementary Table 2. Summary of values for DsbB competition binding analysis graphically represented in Supplementary Figure 2. K_{cat} and K_m values for DsbB at 0 μM and 75 μM of fragments **4** to **8** are listed as the mean ± S.E.M. of three independent experiments performed in triplicates.

Discussion

The use of Ro3 compliant, so-called "drug fragments" as a starting point for drug discovery, in combination with 3D structural information of target-fragment complexes, has delivered a number of innovative compounds which are currently in clinical trials[21]. However, this success has so far been strictly limited to soluble, moderately sized protein targets. Membrane proteins have not made good targets for FBDD due to their challenging physicochemical properties. In particular, the difficulty of generating sufficient quantities of purified, functional protein and of detecting specific binding to the target, as opposed to non-specific partitioning into hydrophobic phases, have limited the applicability of biophysical ligand screening approaches. Here we have addressed these two issues by a) immobilizing the target and reusing a single sample to screen an entire fragment collection and b) using a reference sample to cancel out non-specific interaction of the fragments with the hydrophobic phase. An additional, likely important, factor contributing to the low false positive rate is that the fragments that make up the collection are highly soluble, with each having been tested at 500 μM in an aqueous buffer alone and in a mixture. Using our Target Immobilized NMR Screening approach we have screened a collection of nearly 1,100 fragments with a single sample of less than 2 mg of protein and found 93 ligands. A number of observations suggest that these fragments are directly binding to DsbB and not indirectly *via* the micelle. First, there is a range of potencies in the enzyme inhibition studies that includes a small number of non-inhibitors and activators. Second, and perhaps more critically, inhibition is saturable and occurs over 2 log orders, strongly suggesting a stoichiometric interaction. Third, titration of 8 different fragments into ^{15}N labelled DsbB resulted in chemical shift perturbations at well defined sites in both solvent exposed and micelle buried portions of the protein. In particular, the similarity of the chemical shift perturbations induced by the synthetic quinone UQ1 and fragment **2** indicate the compounds are binding to the same site.

The eight fragments with greatest potency in the single concentration enzyme inhibition assay were fully characterized for potency, mode of action, and binding site on DsbB. This analysis suggests that these fragments can be divided into two groups, one that competes only with

quinone for DsbB binding and a second that perturbs the apparent affinity of DsbB for both quinone and DsbA. The clearest examples of the different behaviour are fragment **2**, which is quinone competitive and fragment **8** which exhibits mixed mode inhibition. The difference is best exhibited by the differing effect on the apparent K_m for UQ1 or DsbA that these two compounds have. While addition of fragment **2** at 75 μM reduced K_m for UQ1 more than 8-fold, it had only a marginal effect on the K_m for DsbA (only 5 % greater than experimental error). In contrast, addition of **8** at 75 μM reduced K_m for UQ1 more than 4.4-fold and K_m for DsbA more than 2-fold.

Titration of fragments **2** and **8** into ^{15}N labelled DsbB[CSSC] further supports a different mechanism of action of these two inhibitors. The differences can be most clearly seen by concentrating on the resonances from the backbone amide of R109 and the sidechain indole of W135. Titration of UQ1 into ^{15}N labelled DsbB results in the simultaneous appearance of a new peak at a nearby position and the reduction of the peak from W135ε NḤ from the endogenous quinone bound form. Since the other indoles are largely unaffected, we have tentatively assigned the new peak to W135ε NH in the UQ1 bound form. Addition of **2** resulted in concentration dependent shifts in the position of W135ε NH and the simultaneous disappearance and appearance of a new peak, as with UQ1. This new peak has an almost identical chemical shift (^1H & ^{15}N) as the UQ1 bound form. This behavior is consistent with two processes occurring. The first is a competition between **2** and the quinone moiety of the bound UQ8, consistent with the competitive kinetics observed for this inhibitor. However, we have shown that the isoprenyl tail of UQ8 extends down the groove between TM1 and TM4, making extensive interactions with the protein. Therefore, displacement of the quinone moiety likely does not immediately result in dissociation of UQ8 from DsbB[CSSC]. Apparently, this occurs on a slower timescale, resulting in the observation of a new peak with a quite different chemical shift. Addition of **8** to ^{15}N labelled DsbB[CSSC] also causes chemical shift perturbation of the W135ε NḤbut these are exclusively concentration dependent and the bound state has a different resonance frequency than the bound state of UQ1 or **2**. Also, addition of **8** causes a large downfield shift in the resonance of R109N that is concentration dependent while UQ1 and **2** had no or only minor effects on this peak.

When the chemical shift perturbations are plotted on the structure of DsbB[CSSC], a distinct difference is observed between UQ1 and **2** on the one hand, and **8** on the other. UQ1 and **2** induce similar shift perturbations which are primarily located in TM 1, 2 and 4. Fragment **8** induces fewer and smaller shifts which are primarily in the periplasmic end of TM1 and 4, but significantly more and larger shifts in both PL2 and PL2'. We propose a simple structural model that is consistent with the results of the biochemical mode of action study and the chemical shift perturbation analysis. In this model, the quinone competitive fragments **1** - **3**, bind directly in the quinone binding pocket and displace the endogenous quinone. In contrast, the mixed mode fragments **4** - **8** bind on the periplasmic side of the ubiquinone binding site in the immediate neighbourhood of PL2' and do not displace endogenous ubiquinone. This binding site would both reduce electron transfer to the ubiquinone and perturb the conformation of the periplasmic loops that form the binding site for DsbA, thereby reducing the binding affinity of DsbA.

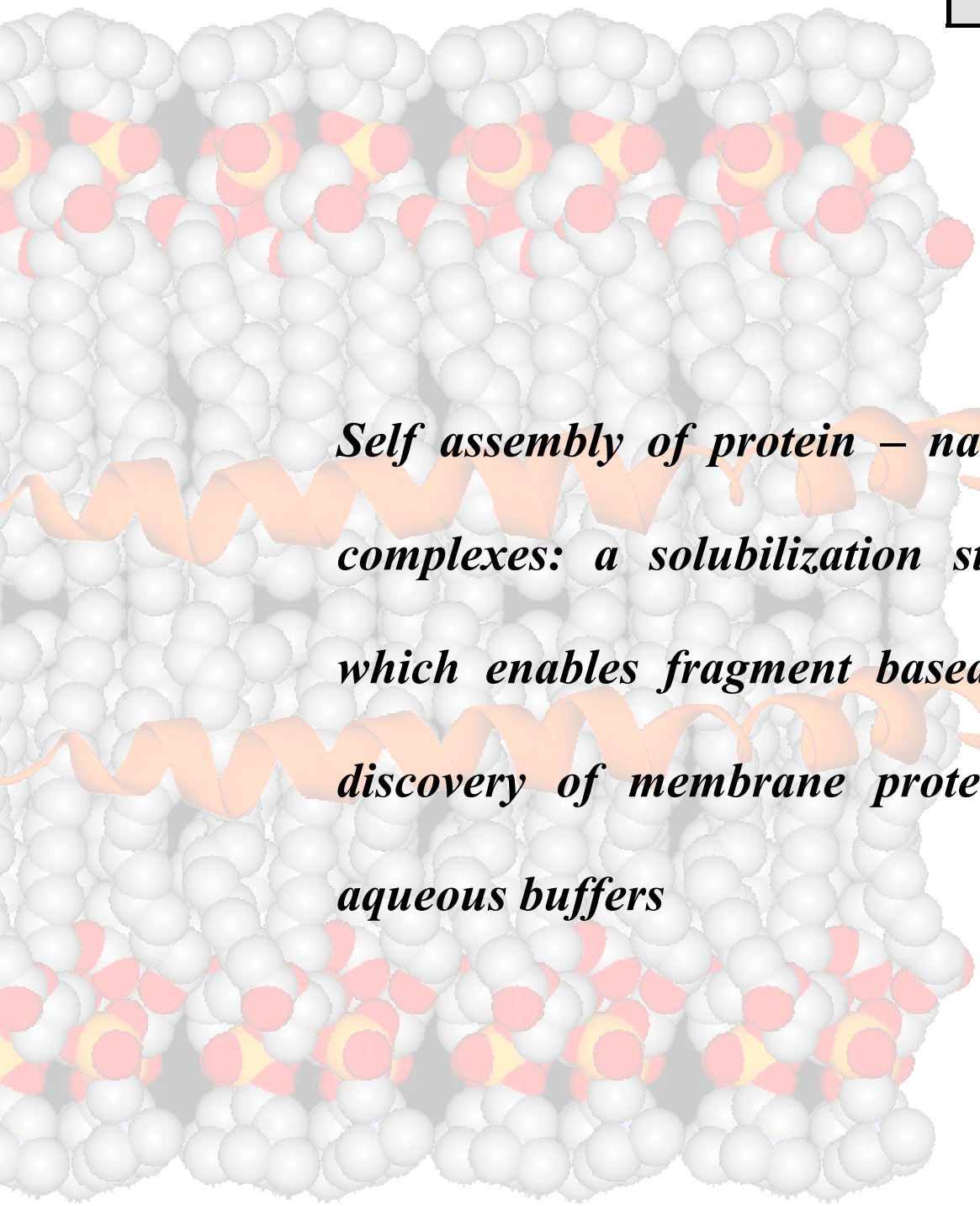
We note that the concentration of the fragments required to induce chemical shift perturbations in DsbB[CSSC] is significantly higher than the IC_{50} values measured for the wild type protein. In addition, UQ1 dependent chemical shift perturbations occurred in the same concentration range as the fragments. A likely explanation is that the conformation of the mutant differs slightly from the wild type protein, against which the fragments were selected. In addition, either the affinity for the quinone is higher for the DsbB[CSSC] mutant or more likely, the quinone binding site may be partially occluded. This latter possibility is clearly consistent with the reduced dynamic behaviour of DsbB[CSSC] with respect to the wild type protein, which results in the substantial improvement in the quality of the NMR spectra. This reduced dynamic behaviour of the disulfide mutant may be responsible for the slow exchange kinetics observed for UQ1 and **2** if release of the ligand from this binding site (endogenous ubiquinone, UQ1 or **2**) can only occur from a sparsely populated conformation.

Since TINS does not require any specific characteristics of DsbB, it should, in principle, be broadly applicable for fragment discovery with membrane proteins. Although the present study used deuterated detergent, this is not *a priori* a requirement. Many detergents that are compatible with membrane proteins have lower CMC values than DPC and therefore could be used at much lower concentration. Since the detergent would be at least 10 fold lower in concentration than the

compounds, its NMR signals would not interfere with the aliphatic signals of the compounds, while aromatic signals would occur in an entirely different portion of the spectrum. Furthermore, non-detergent media for solubilizing membrane proteins have been developed that are more compatible with protein function. These media, such as nanodiscs[68,73,74] and amphipols[72,180,181], are compatible with most membrane proteins and should prove highly complementary to the TINS technology for finding fragments that bind membrane proteins, as can be seen in Chapter 6.

Chapter

6



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.

This chapter is part of a manuscript: Früh, V.; Heetebrij, Grinkova,Y. N. , Sligar, S. G., Siegal, G. Self assembly of Protein – Nanodiscs Complexes: A solubilization strategy which enables fragment based drug discovery of membrane proteins in aqueous buffers. *Manuscript in preparation 2009.*

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.

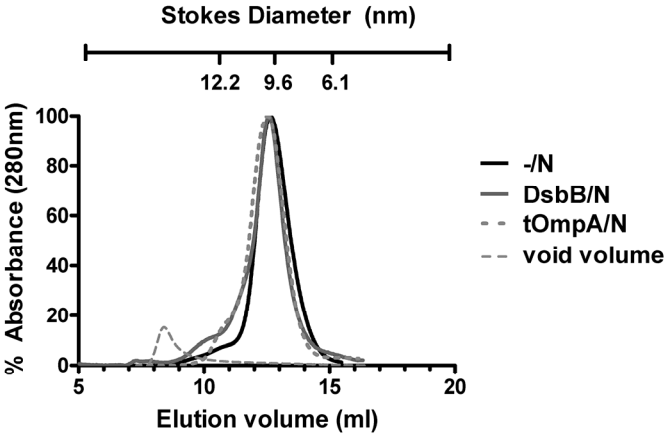


Figure 1. Characterization of empty NDs (-/N), DsbB/N and OmpA/N by gel filtration. The Stokes' Diameters were calibrated by using known proteins as standards.

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.

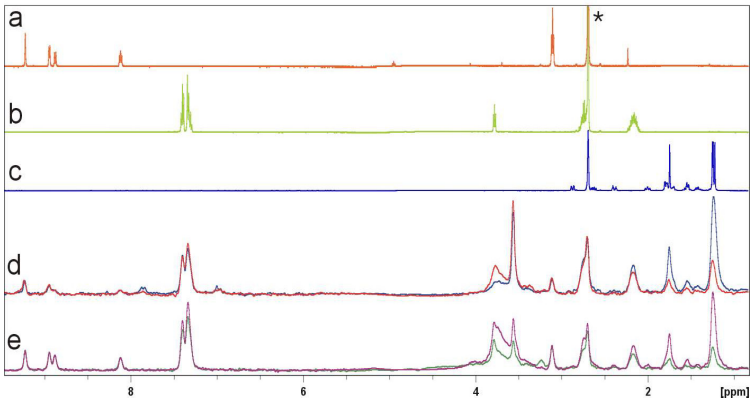


Figure 2. Effect of membrane protein solubilization system on NMR spectra of small molecule ligands. A reference NMR spectrum of each of three compounds (**a** - **c**). 1D ^{1}H NMR spectrum of a mix of the three compounds **a - c** in the presence of detergent solubilized OmpA (blue) or DsbB (red) (**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**).

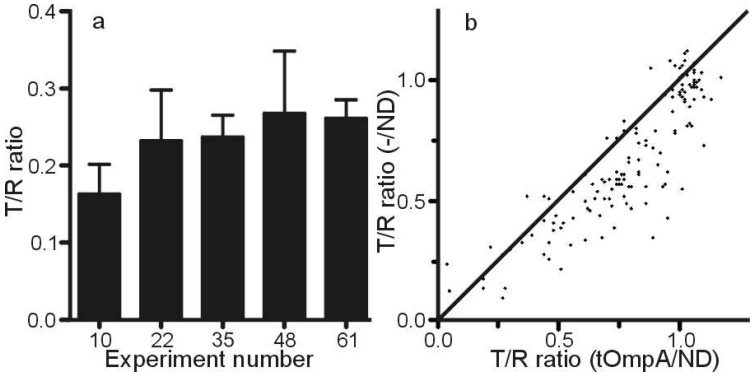


Figure 3. Stability of DsbB in nanodiscs (**a**, see text for details). Comparison of empty ND or OmpA in ND as a reference (**b**). The solid line indicates the position of a perfect correlation.

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.

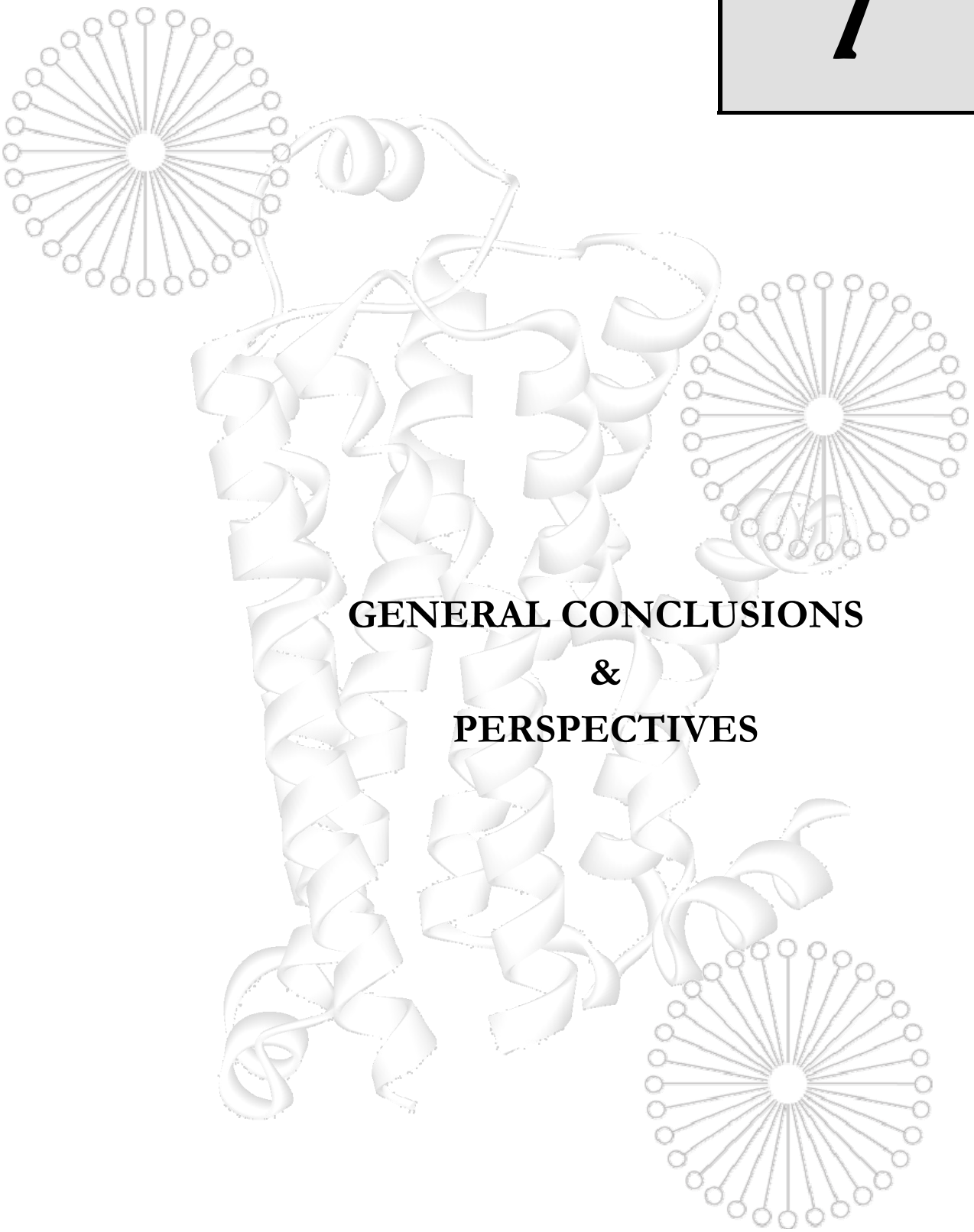
Once immobilized, the DsbB/ND and OmpA/ND were packed into two separate cells of a dual-cell sample holder[205] which enables capillary tubing to connect the cell to the autosampler Gilson 210. The dual-cell sample holder was then inserted into an 8 mm, ^{1}H selective, flow-injection probe in a 500 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

We are grateful to F. Zito for constructing the histidine-tagged variant of OmpA (Unité Mixte de Recherche 7099, Institut de Biologie Physico-Chimique, Paris).

Chapter

7



GENERAL CONCLUSIONS & PERSPECTIVES

General Conclusions

As described in the review of membrane immobilization strategies in **Chapter 2**, there exists a variety of intricate protocols to express, solubilize, and immobilize membrane proteins for a range of applications ranging from 'macroscopic' functional cell-based assays to biophysical studies at the molecular level. Most of these methods, however, have not enabled fragment based drug discovery in a high throughput manner on membrane proteins due to the challenging inherent properties of this class of proteins and the lack of general methods for their solubilization and immobilization. With the simple and mild Schiff's base chemistry used to immobilize membrane proteins, combined with Target Immobilized NMR Screening, the research presented in this thesis has shown that fragment based drug discovery now has the potential to be applied to membrane proteins in general. As described below, the Schiff's base chemistry made it possible to immobilize a wide class of membrane proteins including the histamine H_1 and adenosine A_1 GPCRs, the potassium ion channel KcsA, the membrane enzyme DsbB, and the reference membrane protein OmpA. Although the GPCRs were immobilized in a functional manner, the intrinsic expression levels were too low to allow us to apply these proteins to the TINS methodology. On the other hand, the high levels of bacterial expression of KcsA, DsbB, and OmpA enabled us to apply these proteins to TINS. With available protocols for DsbB biochemical and biophysical characterization, the fragment hits identified for this enzyme were validated as specific binders, proving that fragment based drug discovery is now applicable to membrane proteins with exciting new perspectives, as described further.

Simple and functional immobilization chemistry generally applicable to membrane proteins

The GPCRs used in **Chapter 3** were an example of immobilizing membrane proteins within their native environment. The immobilization efficiency was low, with only 20 % of the initial receptors functionally immobilized on the resin, with a maximum population of 2 pmoles of

receptor per ml of resin. This is approximately 3 log units less than what is required for the current parameters of a TINS screen but nonetheless, the pharmacology profile of these immobilized receptors was very similar to the receptors which had not undergone the immobilization process. Immobilized receptors not only presented native pharmacology with regards to antagonist binding, but they also presented agonist two site binding profiles which confirmed the presence of G proteins as indicated by western blots of resin samples. Immobilizing GPCRs also had a positive effect on the relative stability of immobilized GPCRs compared to those in native membrane vesicles. This physiologically relevant pharmacology of the receptors on the resin suggests that there is potential to apply this class of proteins to molecular based screening methods such as TINS. The limiting step in obtaining high amounts of immobilized GPCRs for TINS however, mostly has to do with the current low expression levels rather than the immobilization procedure itself. For example, the increase in functionality which we report to occur upon immobilization with longer linkers between the protein and the surface has been reported for other GPCRs and is explained by the larger space available for the protein's extracellular domain movement[112]. Other suggestions for obtaining higher functionally immobilized GPCRs are described in the perspectives section.

The Schiff's base chemistry also enabled the functional immobilization of detergent solubilized membrane proteins from bacterial sources, KcsA and DsbB (**Chapters 4 & 5)** and DsbB in the alternative solubilization agent, the nanodisc (**Chapter 6**). These bacterial proteins have been extensively studied by molecular methods in the past, such as crystallography[92,253] and NMR[62,91], and a variety of protocols exists for their purification and functional solubilization in detergent micelles. These conditions enabled us to produce sufficient amounts in *E.coli* to optimize the immobilization conditions. The functional immobilization could be monitored either by injecting a known binder at different time points of the screen, or by an existing functionality assay. The only protein for which we had a functionality assay available, however, was DsbB[93]. We had no means to detect whether OmpA was properly folded upon immobilization, but our sole requirement for this protein was minimal small ligand binding properties, a quality which **Chapters 4 – 5** demonstrated to be true. These three detergent solubilized proteins were immobilized with an efficiency of 50 %, with a yield of 100 μM

(volume equivalent) of immobilized protein, as required for TINS. Upon the solubilization of DsbB and OmpA in the nanodisc complexes (DsbB/ND, OmpA/ND), the immobilization yield increased to 75 %. This suggests that in detergent micelles, the N-terminus of both DsbB and OmpA may have been slightly buried into the DPC micelles and therefore less accessible to the immobilization reaction with the aldehyde groups on the resin. In contrast, the N-terminus of the nanodisc membrane scaffold proteins may have participated in the immobilization reaction, thereby increasing the final yield of functionally immobilized DsbB/ND and OmpA/ND. In contrast to the GPCRs in this study, the higher immobilization yield of DsbB (in detergents or nanodiscs) may be because the enzymes were immobilized in a more stable conformation due to the presence of the endogenous ligand, UQ8. Both DPC-solubilized DsbB (DsbB/DPC) and DsbB/ND were 90 % active on the resin compared to in solution and remained stable for a month. DsbB/ND however showed approximately 16 % increase in turnover rate compared to that in detergent micelles. This may be explained by the increased stability and functionality of membrane proteins in a better mimic of the native membrane, such as in the lipid bilayer of the nanodisc formation, as opposed to the less stable detergent micelle formation[248]. The lower level of activity of detergent-solubilized DsbB may also be explained by the partial solubilization and consequent lowering of the effective concentration of the cofactor into the micelles during the enzymatic activity assay.

Target Immobilized NMR Screening in DPC detergent

The functional immobilization of membrane proteins in detergent micelles enabled us to carry out a TINS screen in the presence of detergent to identify fragments binding to KcsA (**Chapter 4**) and DsbB (**Chapter 4 and 5**) with OmpA as a reference. The immobilization of DsbB and OmpA enabled an entire screen on the same protein samples for one week at room temperature, which resulted in a 7 % hit rate with a final set of 93 fragments which were listed for validation as DsbB inhibitors. By comparing the intensities of the fragments injected from the same mix in the presence of the immobilized target or reference, one could immediately identify a target binder within a cocktail of fragments without any deconvolution. This was facilitated by the

reference protein which accounted for non-specific levels of binding to proteins and to detergents, thereby providing information on specific binders to the target. This proved the principle that TINS can be applied to membrane proteins, provided the appropriate distribution and concentration of detergent is used in the system. We tested the amount of detergent required in the wash buffer to enable continuous and stable protein functionality during a screen, and established that a concentration 5 x above CMC was sufficient. With KcsA, which is a tetramer protein, we could see an immediate drop in capacity to bind a control known binder upon an initial wash with an aqueous buffer void of detergents. Interestingly, this drop in intensity was reversible with a fresh injection of buffer containing DPC, suggesting that the immobilization does indeed somewhat stabilize the protein. We established that keeping the detergent in the wash buffer rather than in the mixtures containing the fragments would limit the unwanted interactions between them while still enabling a full screen without loss of protein functionality. DPC was used as a deuterated detergent, thereby rendering its signal invisible in the NMR spectra of the fragments.

At this point, there was no method for us to establish whether the detergents were partially solubilizing the fragments, and, in doing so, were causing loss of fragment ^{1}H 1D signal intensity. Fortunately, the signal intensities remaining were high enough that fragments binding to the targets could be detected thanks to the reference system composed of immobilized OmpA in the same detergent micelles.

DsbB hit validation by biochemical and biophysical analyses

Of the 93 DsbB/DPC hits identified from TINS, a number of observations suggested that the fragments were indeed specifically binding to DsbB. First, a well-distributed population of inhibitors was found by adding 250 μM of fragments to the DsbB assay and calculating the subsequent percentage of enzyme inhibition induced by the fragments. This resulted in 16 % showing less than 20 % inhibition or mild stimulation, 60 % of the fragments showing better than 30 % inhibition of DsbB, and 17 % showing more than 70 % inhibition. These were only single concentration point experiments which did not provide information on the mode of action, nor

whether this inhibition was due to protein precipitation or other assay artefacts. Thus, secondly, the 13 fragments showing highest inhibition were chosen to be validated further by competition assays to quantify the potency (IC50) of the fragments as well as their mode of action (competition for the cofactor UQ1 or the DsbA binding site of DsbB). These assays revealed artefactual inhibition by 3 fragments, but stoichiometric inhibition by the remaining 10 fragments. The IC50 values ranged between 10 and 200 μM, and further competition analyses revealed two distinct binding modes. The most 8 potent fragments were thus grouped into two categories. 3 fragments caused the expected competition with the ubiquinone cofactor in the endogenous binding pocket of DsbB and the remaining 5 presented a mixed model inhibition, as defined by lowering the affinities of both DsbA and UQ1 for DsbB.

Finally, because the biochemical assays were not providing structural insight into the mechanisms of DsbB inhibition by the fragments, an orthogonal biophysical method was used and confirmed the mechanism of the fragment modes of action in a structurally relevant manner. Unfortunately, as is the limiting factor for many membrane proteins, DsbB in DPC micelles was dynamically unstable and although part of the crystal structure was obtained in complex with DsbA[92], there was no full structure nor sequential assignments available from NMR studies. We therefore used ^{15}N labelled DsbB[CSSC] for which the sequential assignments were available[62]. In this mutant, cysteines 44 and 104 have been mutated to serine, which had the consequence of creating a stable disulfide bridge between Cys41 and Cys130[62], resulting in a stabilized and inactive form of DsbB. Clearly, using the native protein would have been more ideal, but the mutant was a physiologically relevant intermediate of the disulfide oxidation pathway[62] which, nonetheless, turned out to be useful in providing us with information regarding the structural interactions between the fragments and the enzyme. HSQC experiments revealed detectable changes in the NMR spectra of DsbB[CSSC], which contained the endogenously bound UQ8 cofactor, upon titration of UQ1 (positive control) and all 8 fragments. This shows that the fragments identified by TINS and validated by biochemical assays also bound the double cysteine mutant, with a somewhat lower affinity as expected from this conformational variant of DsbB. The titrations revealed interesting information which confirmed the biochemical assay validation results. Titrations of the positive control UQ1 yielded patterns which suggested slow

exchange of UQ1 into the DsbB ubiquinone binding pocket (already mapped by structural studies by crystallography on wildtype DsbB in complex with DsbA[92]) but also some elements of fast exchange. The 3 fragments showed similar slow and fast exchange patterns on the same residues as affected upon UQ1 titration: these residues were located close to the first periplasmic loop of DsbB[CSSC] which contains the active cysteine pair in the wildtype DsbB, involved in reducing the ubiquinone in its native binding pocket. Although the second group of fragments showed some similar patterns of chemical shift perturbations as the UQ1 and competitive fragment titrations close to the ubiquinone binding site, they also presented a distinctive pattern in the area of the second periplasmic loop. The second periplasmic loop contains residues involved in DsbA binding (Phe100 - Phe106) and oxidation (Cys104) but also the hydrophobic residues (Leu116 and Val120) involved in associating DsbB to the membrane upon charge transfer from DsbA to DsbB[92]. The effects of mixed model fragment titrations also differed from the competitive fragment titrations in that they only presented patterns of fast exchange. These differences can explain the mechanism of DsbB inhibition by these fragments. Upon competitive inhibition, such as with competitive fragments or UQ1, the dissociation of the quinone within the binding pocket may have happened in two phases. The initial fast exchange displaced the quinone moiety which was bound towards the hydrophilic exposed part of the enzyme, but the dissociation of the isoprenyl tail, which extended deep into the hydrophobic groove, making extensive contacts with the hydrophobic residues between TM1 and TM4, happened at a slower rate. Upon mixed model inhibition however, the fragments did not displace the ubiquinone but rather caused a conformational change which affected electron transfer to the ubiquinone and binding of DsbA to DsbB, as predicted by biochemical assays. In conclusion, the fragments identified by TINS were validated as specific binders of DsbB and turned out to be either specific inhibitors of native ligand binding, or inhibitors of protein-protein interactions.

Target Immobilized NMR Screening in alternative solubilization medium: the nanodisc

An attractive alternative to detergents is the use of amphiphilic polymers or complexes which on the one hand satisfy hydrophobic needs of membrane proteins while on the other maintain surface polarity contacts with the surrounding aqueous buffer. In **Chapter 6** we have shown that the nanodisc system has been a useful alternative to detergent in a TINS screen. Although there were quite some conditions to test before obtaining a good population of well formed complexes, the procedure was simple and easy to characterize by gel filtration and SDS-PAGE gel band volume analysis. Not only was the immobilization yield more efficient in nanodiscs than in detergent due to the participation of the scaffold protein in the immobilization reaction, but the activity of DsbB was higher as well. As previously mentioned, this suggests that either the protein was more active in lipid bilayers, as can be expected, or suggests that the detergent may indeed have partially solubilized the cofactor UQ1 in the assays, thereby resulting in lower final effective turnover rates. This last possibility has been further supported by the better quality of NMR spectra in the presence of nanodiscs as opposed to DPC. The signal to noise ratio was double to that in detergents, which, by NMR standards, suggests that there may have been a loss of 30 – 40 % of the effective amount of fragments into detergent micelles. We had obtained particles of appropriate size upon gel filtration of nanodisc-embedded OmpA (OmpA/ND), but using these complexes as references for nanodisc-embedded DsbB (DsbB/ND) enabled us to identify only 5 known binders as opposed to 17 with the empty nanodiscs as a reference, and 18 with the DPC screen of DsbB with the reference protein OmpA. We used the same cut-off for all three screens, suggesting that the OmpA reference was now showing higher non-specific binding properties when embedded in a nanodisc complex. Due to the highly stable nature of OmpA previously solubilized in a variety of detergents, however, it is unlikely that it was unfolded upon nanodisc entrapment, but it may more likely be a matter of mismatch in heights of the protein transmembrane domain of 30 Å and the corresponding lipid bilayer of 50 Å in the nanodisc[254]. This mismatch may have exposed areas with high fragment binding properties, either from OmpA hydrophobic residues or from the lipids in the nanodisc. When using empty nanodiscs as

a reference, DsbB/ND proved to be stable throughout the screen without any addition of detergent. Naturally, in the event that a target protein can not be solubilized in nanodiscs, however, screening the protein in detergent micelles still remains an option due to the sufficient level of fragment ^{1}H 1D peak intensities remaining even after the fragments have been partially solubilized in the detergent micelles.

Perspectives

The possibility of using molecular methods such as NMR for fragment based drug discovery on membrane proteins opens an exciting new venture for drug discovery. Clearly, we are still limited from screening membrane proteins within their native membrane, and we could only obtain samples in the quantities and stabilities required for TINS by screening bacterial membrane proteins in detergent micelles. Work is underway to improve the conditions which would facilitate screening of membrane proteins in their native membrane. This includes enrichment of native membranes *in situ*, the use of more powerful NMR probes such as cryoprobes, improved NMR parameters, and downscaling of sample size by immobilizing proteins on the glass surfaces of microfluidic chambers compatible with the system. In the meantime however, in combination with new solubilization and stabilization strategies[70,71,255], there are a variety of perspectives for TINS on membrane proteins *in vitro*.

Membrane protein immobilization

In future studies, it would be interesting to see how we can maximize the population of functionally immobilized GPCRs and tailor the chemistry so as to immobilize these and other membrane proteins in a more oriented manner rather than the random Schiff's base chemistry. There exists a variety of immobilization chemistries which can be used with the TINS compatible aldehyde resin used in this thesis. In our case, although there were no structures available for the H_1 and A_1 receptors, mutagenesis studies on the adenosine receptor class has revealed the close homology between the recently solved A_{2A} receptor structure[45] and the A_1 receptor[256]. This type of information can be used to pinpoint structural elements of the receptors that can be involved in immobilization without affecting functionality, such as biotinylation of cysteines[110]. For instance, the C-terminus has been found to regulate G protein coupling to GPCRs[257], and should therefore be avoided in future immobilization reactions. GPCRs can also be embedded in biotinylated forms of nanodiscs, which can then be immobilized to avidin-covered surfaces with

the benefit of leaving the target GPCR unaffected by the immobilization reaction. In addition, GPCRs which are in the ligand bound state tend to maintain a higher conformational stability, as can be seen by successful crystallization of the β_2-adrenergic receptor in the presence of the partial inverse agonist carazolol[34]. This points to the potential of obtaining higher populations of functional GPCRs when they are immobilized in the presence of their ligands. The immobilization of membrane proteins will not only enable the elaboration of new drugs, but will also be interesting to use in studies looking at the physiologically relevant mechanisms. With regards to GPCRs for example, this includes allosteric modulation, dimerization, and coupling to G proteins and other GPCR-interacting proteins (GIPs) such as the receptor-activity-modifying-proteins (RAMPs)[257,258]. It may for example be useful to immobilize a GPCR in the presence of the ligand to establish whether it influences G protein coupling, as is the case for the C5a receptor, where C5a binding activates G protein coupling[259]. G protein coupling can therefore be potentially targeted in drug discovery by screening different mutants of a GPCR target with TINS to identify fragment scaffolds which interact with specific residues relevant for G protein coupling. The current immobilization procedure also has the potential to be used with other forms of solubilized membrane proteins, such as those in cubic lipid phases[260], and is simple and generally adaptable to use with other chemical immobilization strategies, and with other proteins, surfaces, and assays.

Target Immobilized NMR Screening

Perspectives for TINS on membrane proteins are numerous because the technique can be adapted to various solubilization and immobilization strategies for primary screening of fragment binding. Screening a focused library based on known important scaffolds for the targeted membrane proteins can minimize the time of a screen down to a few days rather than an entire week, limiting the problems faced by low protein stability or quantities. There may soon be improved NMR parameters with increased sensitivity which can enable one to downscale the amount of protein required per screen, thus enabling screens on proteins in their native membrane vesicles. In the meantime however, detergent or nanodisc solubilized membrane proteins may be

the only preparation available for current day fragment based drug discovery with TINS. This is possible with the new stabilization mutations which have found to enable one to produce, for example, high quantities of GPCRs in *E.coli*, stabilized in either the agonist or the antagonist state, as has been previously reported for the A_{2A} receptor[261] and the β_1-adrenergic receptor[70]. The advantage of this technique is that *E.coli* can be grown in fermentors to provide large quantities as opposed to the adherent mammalian cell cultures, and carrying out a TINS screen on a specific state of the receptor minimises the need to carry out future assays to determine whether ligands are agonists or antagonists. Furthermore, synergetic effects or allosteric modulation can be studied by designing appropriate libraries.

The application of TINS to membrane proteins in nanodisc formations also enabled us to carry out a screen in complete absence of detergents. The lower signal to noise ratio obtained in screens containing detergents was still high enough to identify potent fragment hits. This leads to the advantageous possibility that screening nanodisc-embedded proteins in the absence of detergent can be carried out with 30 – 40 % lower protein and fragment concentrations and still obtain detectable fragment ^{1}H 1D peak intensities. GPCRs and GPCR-like proteins have been previously successfully embedded in nanodiscs, both as monomers (b2-adrenergic receptor)[74] and as trimers (bacteriorhodopsin)[262], suggesting that there are exciting possibilities of screening nanodisc embedded Class A and Class C GPCRs, the latter of which are obligate dimers[263], with TINS. The use of empty nanodiscs as an appropriate and generalized reference, has now also been established for SPR technology[264], and we believe future TINS applications should, when possible, be applied to nanodisc embedded targets. Furthermore the use of the empty nanodisc as a reference would enable one to minimize the energy spent in finding an appropriate reference protein and developing a protocol for the appropriate self assembly into nanodiscs.

Research is currently replacing old ideas about physiological processes, as can be seen by the increase in reports on GPCR multimerization[50] and the multiprotein networks into what is now being coined 'receptosomes'[257]. Therefore, there are a variety of other topics aside from fragment based drug discovery to explore with TINS, in the event of obtaining better information for modulating a biological system in the body. Without much information available on orphan receptors, TINS can be used as a tool to compare hits between different classes of protein,

leading to deorphanizing studies. As previous studies have shown, classification of GPCRs can be predicted by which class of G proteins they bind[265,266], and therefore, deorphanizing studies in principle could also be carried out by immobilizing an orphan GPCR and injecting different G proteins instead of fragments. Furthermore, the pharmaceutical industry is finding new and exciting ways of targeting drugs, such as pharmacogenomic profiling[267,268] and the use of multitarget drugs[269,270]. Thus, why not screen several targets within a specific pathway, or screen several physiologically relevant target mutants and identify a common hit between them, as has been suggested previously for drugs targeting multiple mutated version of kinases as an efficient treatment for cancer[271].

Fragment hit elaboration

As we mentioned, structurally relevant information can be used to facilitate the elaboration of weak fragment hits into stronger, more potent and more specific ligands. For the hits which were found for KcsA and DsbB, clearly, obtaining structural key information of fragment binding to the wildtype protein would be an ideal next step. The intrinsic movement of the periplasmic loops upon DsbB enzymatic activity or upon KcsA dynamics may however hinder these kinds of experiments no matter how stabilizing the solubilization medium is. As with the stabilized GPCR mutants in literature[70,261], and the DsbB[CSSC] mutant we used in Chapter 5, combining mutagenesis and molecular methods has the potential to provide worthwhile information on ligand-protein interactions. However, some interactions are inherently weak, such as protein-protein interactions involved in kinase domains[272,273] and between G proteins and GPCRs[258]. Perhaps weak interactions in biological processes are currently undermined and targeting such interactions may enable to provide the medicinal chemistry realm with more specific inhibitors with lower amounts of side effects. The advantage of using fragment based drug discovery in such a context is that fragment hits may suffice as weak inhibitors of protein-protein interactions, for example. There are a variety of NMR based applications which can be applied to obtain valuable information on molecular interactions between ligands and proteins, as described in the outlook section of Chapter 4, such as modelling chemical shift perturbations[235], sparse NOE

based methods[236,237] and paramagnetic NMR[238], but it is important to integrate other applications of medicinal chemistry, biology, and computational methodologies in order to be fully effective[237]. The emerging era of new internet based software should be used to link data[274] across different databases, such as ones concerning diseases, targets, drugs, and clinical trials, in the global aim to make medicinal chemistry research more efficient by enabling the discovery of new connections between diverse data sets[275]. Currently, work is underway to better understand how we can modulate the NMR parameters in such a way that TINS can be used for fragment binding quantification and hence, the ranking of fragment hits, and also as a competition binding assay which can provide information on fragment kinetics in relation to a given target. TINS can be applied as a primary screening step, but once elaborated fragments have been made, it can also be worthwhile to use TINS as a secondary screening step.

In conclusion, TINS can now pave the way to applying fragment based drug discovery to membrane proteins in general because it enables one to identify weak fragment binding to membrane proteins, whether solubilized in detergents or nanodiscs, in smaller quantities than those required for biophysical methods in general. The interesting perspectives which follow such applications are numerous because the immobilized target and references can be modulated to include, for example, different states or mutants of the same protein, and libraries can be modulated to include studies on allosteric modulation, and synergetic or competitive effects on weakly interacting biomolecules.

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Summary

Sammenvatting

Résumé

Summary

This thesis describes how the Target Immobilized NMR Screening (TINS) method can be applied to identify small molecule hits on membrane proteins.

Screening small molecules, or fragments (< 300 Da), by fragment-based drug discovery (FBDD) has recently been shown to be an advantageous alternative to screening larger molecules, yet the concept is limited to soluble proteins. **Chapter 1** introduces the notion of FBDD and why it would be useful to find ways of applying this drug discovery approach to membrane proteins. In brief, FBDD has evolved with biophysical methods such as Nuclear Magnetic Resonance (NMR) because of the possibility of detecting weak binding of fragments on proteins. However, biophysical methods are demanding in terms of amount and purity of protein samples and, unfortunately, are currently limited to soluble proteins. 60 % of the drugs on the market however target membrane proteins which are involved in a variety of crucial cellular processes ranging from cell signaling to transport of ions and solutes in and out of the cell. As the name indicates, membrane proteins are present within the cellular membrane and require a hydrophobic environment to maintain their structure and functionality. **Chapter 2** reviews the extensive work carried out on immobilizing membrane proteins in a variety of membrane mimics, and shows that none of them are applicable to studying weak fragment binding on membrane proteins. It is precisely this hydrophobic nature which limits the study of such proteins by FBDD due to the low protein yield and stability, and the problem of non-specific binding of fragments to the membrane mimics used, such as detergents or lipids.

TINS is a reference system where the simultaneous screening of a reference protein (with minimal small molecule binding properties), solubilized in the same detergent as the target, can account for non-specific binding of fragments to the hydrophobic environment. Identification of fragments which bind specifically to the target can be done immediately without any deconvolution, by simple comparison of the 1D ^{1}H signal intensities of the fragments in the presence of the reference or target. This has the advantage over other NMR methods because no

structural information on the target is required, low amounts of protein are needed, and binders can be identified within cocktails of fragments, speeding up the process.

In **Chapter 3**, we showed that important pharmaceutical targets such as G protein coupled receptors (GPCRs) could in principle be studied by TINS due to their functional immobilization on a resin in a variety of biologically relevant agonist and antagonist conformations. This was possible because native membrane fragments of the cells overexpressing these proteins were immobilized, without further purification. This enabled the co-immobilization of other important players of the signaling cascade, such as G proteins. The low population of functionally immobilized proteins could be increased by increasing the linker length between the protein and the surface, yet the intrinsically low overexpression of these proteins was the limiting factor which prevented us from applying TINS to this class of proteins.

In **Chapter 4**, we proved the principle that membrane proteins in general could be applied to TINS. We used the bacterial potassium ion channel (KcsA) and the bacterial membrane enzyme Disulphide bond forming protein B (DsbB) as targets, which were immobilized and screened with the bacterial reference protein Outer membrane protein A (OmpA), all solubilized in dodecylphosphocholine detergent. The immobilization efficiency of all three membrane proteins was calculated by detecting the amount of protein in the supernatant before and after immobilization, with a constant yield of 50 % for all three proteins. The functionality of KcsA and DsbB was tested by using a known binder at various points of the screen. DsbB was further tested for functionality by a robust enzymatic assay which detects oxidation of the electron donor DsbA, a soluble partner protein, or the reduction of the electron acceptor, the synthetic cofactor ubiquinone-5, also named coenyzme Q1 (UQ1). The fragment mixtures were void of detergent, in order to limit hydrophobic interactions between fragments and detergent micelles. However, detergent was required at a concentration equivalent to 5 x the critical micelle concentration, below which the detergent micelles would dissociate into monomers and eventually cause loss of protein conformation and hence, functionality. These optimal conditions allowed us to carry out a small test screen of 100 fragments on KcsA and a full screen of 1000 fragments on DsbB, both leading to a 7 % hit rate.

TINS is an NMR method which enables to identify fragment hits on a target protein, but these hits should be further characterized for their potency and mode of action, as was carried out for DsbB in **Chapter 5**. Enzymatic assays were used to determine the potency range of the fragments, along with their mode of action. Bidimensional NMR experiments were used to identify which residues of the protein were affected upon titration of the fragments, such as those in proximity of DsbA or UQ1 binding sites on DsbB. Wildtype DsbB was too unstable to be studied by NMR, but a stabilized mutant from a physiologically relevant intermediate state of DsbB was used and resulted in the confirmation of the binding modes established by enzymatic assays. This suggested that the results found were not artefacts and that the fragments were binding specifically to DsbB. The final 8 fragments, with potencies ranging between 10 to 200 µM (IC_{50} values), were thus classified into two groups. The first group, containing 3 fragments, consisted of competitive inhibitors for the endogenous ubiquinone binding site of the enzyme. These fragments therefore inhibited UQ1 binding and subsequent electron transfer from DsbA to the respiratory chain. The second group, containing 5 fragments, represented mixed model inhibitors which inhibited both UQ1 and DsbA binding by binding in an alternative site to the first class of fragments. The existence of two binding modes provides exciting perspectives in chemically linking or elaborating these diverse fragment scaffolds from these two different groups into more potent and more selective DsbB inhibitors.

Chapter 6 demonstrates how an alternative solubilization technique enabled TINS in aqueous buffers, completely void of detergents. The proteins were encapsulated into a bilayer stabilized by an amphiphilic membrane scaffold protein in a complex called the nanodisc which was entirely soluble in aqueous buffers. We demonstrated here that nanodisc embedded DsbB, as opposed to detergent solubilized DsbB, was more active in enzymatic assays, was immobilized at a higher efficiency, and was stable throughout the test screen of 180 fragments. Furthermore, 18 of the 19 fragments which showed high DsbB inhibition in **Chapter 5** were also identified as hits in the nanodisc embedded DsbB screen, suggesting that TINS is a repeatable method. Empty nanodiscs were used as a reference system to account for the non-specific binding of fragments to the nanodisc environment. The advantages of using nanodiscs in TINS are numerous, as they

alleviate the need to produce, solubilize, and immobilize a reference protein, they are more stable, and make it easier to handle membrane proteins in the absence of detergents.

Finally, **Chapter 7** describes the conclusions and perspectives of this thesis. The results of this thesis show that TINS can be applied to a variety of membrane proteins, such as ion channels and membrane enzymes. The fragment hits identified for DsbB were characterized as inhibitors in the micromolar range and could be identified with different solubilized states of the enzyme. The use of nanodisc embedded targets enables one to screen a target in aqueous buffers with empty nanodiscs as a good standardized reference. These results, combined with the new methods for overexpression and thermostable mutagenesis should provide exciting new possibilities which may extend beyond the identification of fragments binding to membrane proteins. These include the identification of protein-protein or drug-drug interactions, depending on what one chooses to immobilize or inject in the place of proteins and fragments.

Samenvatting

Dit proefschrift beschrijft hoe de Target Immobilized NMR Screening (TINS) technologie kan worden gebruikt om kleine moleculen als binder aan membraaneiwitten te identificeren.

Recentelijk is aangetoond dat het screenen van kleine moleculen, of fragmenten (< 300 Da) door middel van fragment-based drug discovery (FBDD) een goed alternatief is voor screening van grotere moleculen, hoewel de techniek is beperkt tot oplosbare eiwitten. **Hoofdstuk 1** introduceert het begrip FBDD en waarom het nuttig zou zijn manieren te vinden om deze drug discovery benadering toe te passen op membraaneiwitten. In het kort, FBDD heeft zich met behulp van biofysische technieken als Kern Spin Resonantie (NMR) ontwikkeld vanwege de mogelijkheid om zwakke binding van fragmenten aan eiwitten te detecteren. Biofysische technieken zijn echter veeleisend met betrekking tot de hoeveelheid en zuiverheid van het benodigde eiwit en, helaas, tot nu toe alleen toepasbaar op oplosbare eiwitten. Van de geneesmiddelen op de markt richt 60 % zich echter op membraaneiwitten die te maken hebben met een reeks van cruciale cellulaire processen, van het signaleren van cellen tot transport van ionen en andere opgeloste stoffen de cel in en uit. Zoals de naam al aangeeft bevinden membraaneiwitten zich binnen in de celmembraan en hebben een hydrofobische omgeving nodig om structuur en functie te behouden. **Hoofdstuk 2** behandelt het uitgebreide werk dat is uitgevoerd op het gebied van immobilisatie van membraaneiwitten in verschillende systemen die membranen nabootsen en laat zien dat geen van alle toepasbaar zijn om zwakke bindingen van fragmenten aan membraaneiwitten te bestuderen. Het is juist de hydrofobe aard die het onderzoek aan deze eiwitten met FBDD limiteert door de lage eiwitopbrengst en stabiliteit en het niet-specifieke karakter van fragment binding aan de pseudomembranen, zoals oppervlakteactieve stoffen of lipiden.

TINS is een vergelijkende methode waarin de simultane screening van een referentie eiwit (met minimale binding eigenschappen aan kleine moleculen), opgelost in aanwezigheid van dezelfde oppervlakteactieve stof als het target eiwit, kan compenseren voor non-specifieke binding van fragmenten aan de hydrofobe omgeving. Identificatie van fragmenten die specifiek aan de target

binden kan direct worden uitgevoerd zonder enige deconvolutie, door eenvoudige vergelijking van de 1D ^{1}H NMR signaal intensiteiten van de fragmenten in aanwezigheid van het referentie en target eiwit. Dit heeft als voordelen boven andere NMR technieken dat geen structurele informatie van de target vereist is, kleine hoeveelheden eiwit nodig zijn en bindende moleculen kunnen worden opgespoord in mengsels van fragmenten, wat het proces versnelt.

In **Hoofdstuk 3**, lieten we zien dat belangrijke farmaceutische targets als G protein coupled receptors (GPCRs) in principe bestudeerd kunnen worden met behulp van TINS dankzij hun functionele immobilisatie op een resin in een serie van biologisch relevante agonist en antagonist conformaties. Dit was mogelijk omdat natieve membraan fragmenten van de cellen die deze eiwitten tot over-expressie brengen werden geimmobiliseerd, zonder verdere zuivering. Dit maakte de neven-immobilisatie mogelijk van andere targets die een belangrijke rol spelen in de signalerings cascade, zoals G proteinen. De lage populatie van functionele geimmobiliseerde eiwitten kon worden verhoogd door toename van de linker lengte tussen het eiwit en het oppervlak, maar de intrinsieke lage overexpressie van deze eiwitten was de beperkende factor die ons verhinderde om TINS toe te passen op deze klasse van eiwitten.

In **Hoofdstuk 4**, bewijzen we het principe dat TINS in het algemeen kan worden toegepast op membraaneiwitten. We gebruiken het bacteriele potassium ion channel (KcsA) en het bacteriele membraan enzym Disulphide bond forming protein B (DsbB) als targets, beiden geïmmobiliseerd en gescreened met het bacteriele referentie eiwit Outer membrane protein A (OmpA), en allen opgelost in dodecylphosphocholine detergent. De immobilisatie efficientie van alle drie de membraaneiwitten werd uitgerekend door het meten van de hoeveelheid eiwit in het supernatant voor en na de immobilizatie, met een stabiele opbrengst van 50 % voor alle drie de eiwitten. De functionaliteit van KcsA en DsbB werd getest door TINS metingen aan een bekende binder op verschillende tijdstippen tijdens de screen. Bovendien werd DsbB getest op functionaliteit met behulp van een robuust enzymatisch assay dat oxidatie waarneemt van de electron donor DsbA, een oplosbaar partner eiwit, of reductie van de electron acceptor, de synthetische cofactor ubiquinone-5, ook genaamd coenzym Q1 (UQ1). De fragmenten mixen bevatten geen detergent, om de hydrofobe interacties tussen fragmenten en detergent micellen te beperken. Het membraaneiwit, aan de andere kant, had detergent nodig bij een concentratie gelijk aan 5 x de

kritische micel concentratie. Bij lagere concentratie zouden de detergent micellen dissocieren tot monomeren en uiteindelijk verlies van eiwit conformatie, en dus functionaliteit, veroorzaken. Deze optimale condities maakten het mogelijk om een kleine test screen van 100 fragmenten uit te voeren op KcsA en een volledige screen van 1000 fragmenten op DsbB, beide resulterend in een hit rate van 7 %.

De NMR techniek TINS maakt het mogelijk om fragmenten hits van een target eiwit te identificeren, maar deze hits moeten verder nog gekarakteriseerd worden op hun potentie en manier van werken, zoals uitgevoerd voor DsbB in **Hoofdstuk 5**. Enzymatische essays werden gebruikt om het potentie gebied van de fragmenten, naast hun manier van werking, te bepalen. Tweedimensionale NMR experimenten werden gebruikt om aan te tonen welke eiwitresiduen, zoals die in de nabijheid van de DsbA of UQ1 binding sites bij DsbB, werden beïnvloed door titratie van de fragmenten. Wildtype DsbB was te instabiel om met NMR te bestuderen, maar een gestabiliseerde mutant van een fysiologisch relevante intermediaire toestand van DsbB werd gebruikt en resulteerde in de bevestiging van de binding karakteristieken die door enzymatische essays waren aangetoond. Dit leidde tot de veronderstelling dat de verkregen resultaten geen artefacten waren en dat de fragmenten specifiek aan DsbB binden. De uiteindelijke 8 fragmenten, met potenties variërend van 10 to 200 μM (IC_{50} waarden), werden nu geordend in twee groepen. De eerste groep, bestaande uit 3 fragmenten, waren competitieve inhibitors voor de endogeneuze ubiquinone binding site van het enzym. Deze fragmenten verhinderden op die manier UQ1 binding en achtereenvolgende electronen overdracht van DsbA naar de ademhalingsketen. De tweede groep bevatte 5 fragmenten en vertegenwoordigde gemengde model inhibitors die zowel UQ1 als DsbA binding verhinderden door binding in een andere site dan de eerste reeks fragmenten. Het bestaan van twee binding modes levert opwindende vooruitzichten op het gebied van chemisch linken of het nader onderzoeken van de fragmenten structuren van deze twee verschillende groepen tot meer potente en meer selectieve DsbB inhibitors.

Hoofdstuk 6 laat zien hoe het op een alternatieve manier in oplossing brengen van membraaneiwitten TINS mogelijk maakte in waterige buffers, totaal zonder toevoeging van detergent. De eiwitten werden ingekapseld in een bilaag gestabiliseerd door een amfifiele membraan structuur eiwit in een complex, nanodisc genoemd, dat volledig oplosbaar was in

waterige buffers. We toonden hier aan dat in nanodisc ingebedde DsbB, in tegenstelling tot in detergent opgelost DsbB, actiever was in enzymatische essays, met grotere efficiëntie werd geïmmobiliseerd, en stabiel was gedurende de test screen van 180 fragmenten. Bovendien, 18 van de 19 fragmenten die hoge DsbB inhibitie vertoonden in **Hoofdstuk 5** werden ook geïdentificeerd als hits in de in nanodisc ingebedde DsbB screen, suggererend dat TINS een reproduceerbare techniek is. Lege nanodiscs werden gebruikt als referentie systeem om te compenseren voor de non-specifieke binding van fragmenten aan de nanodisc omgeving. Er zijn veel voordelen aan het gebruik van nanodiscs in TINS, zoals het overbodig maken om een referentie eiwit te produceren, op te lossen en te immobiliseren. Ze zijn ook stabieler, en ze vereenvoudigen het werken met membraaneiwitten door de afwezigheid van detergent.

Ten slotte beschrijft **Hoofdstuk 7** de conclusies en vooruitzichten van dit proefschrift. De resultaten van dit proefschrift laten zien dat TINS kan worden toegepast op een verscheidenheid aan membraaneiwitten, zoals ion channels en membraan enzymen. De gevonden fragmenten hits voor DsbB werden gekarakteriseerd als inhibitors in het micromolaire gebied en konden worden geïdentificeerd met verschillende opgeloste toestanden van het enzym. Het gebruik van in nanodisc ingebedde targets maakt het mogelijk om een target te screenen in waterige buffers met lege nanodiscs als een goed gestandaardiseerd referentie eiwit. Deze resultaten, gecombineerd met de nieuwe methoden van overexpressie en thermostabiele mutagenesis zouden prikkelende nieuwe mogelijkheden moeten opleveren die verder strekken dan de identificatie van aan membraaneiwitten bindende fragmenten. Daarbij horen het opsporen van eiwit-eiwit of geneesmiddel-geneesmiddel interacties, afhankelijk van wat men kiest om te immobiliseren of NMR aan te meten in de plaats van achtereenvolgens eiwitten en fragmenten.

Résumé

Cette thèse décrit la méthode de « Target Immobilized NMR Screening (TINS) », qui peux etre appiquée aux protéines membranaires afin d'identifier de nouveaux ligands de petite taille.

La méthode de criblage de petites molécules, ou fragments (<300 Da), par « fragment-based drug discovery (FBDD) » a été récemment identifiée comme une bonne alternative au criblage de molécules plus larges, mais le concept est restreint aux protéines solubles. Le **1er Chapitre** présente la notion de FBDD ainsi que les raisons pour lesquelles il est intéressant de pouvoir appliquer ce genre de découverte médicinale aux protéines membranaires. En gros, le FBDD a évolué avec les méthodes biophysiques telles que la Résonance Magnétique Nucléaire (RMN) grâce à la possibilité de détecter les liaisons faibles entre fragments et protéines. De telles méthodes biophysiques sont exigeantes en ce qui concerne la quantité et le niveau de pureté d'une protéine. 60 % des drogues sur le marché pharmaceutique, cependant, ciblent les protéines membranaires qui sont beaucoup plus difficiles à produire et à purifier que les protéines solubles. Ces protéines sont à la base de multiples processus cellulaires vitaux au bon fonctionnement du corps, tels que la signalisation et le transport d'ions et de solutés intra et extra cellulaires. Comme le nom l'indique, ces protéines sont localisées dans la membrane cellulaire et requièrent un environnement hydrophobe pour maintenir leurs conformations et par conséquence, leur bon fonctionnement.

Le **2ème Chapitre** est une revue de différentes méthodes ingénieuses qui existent pour immobiliser les protéines membranaires dans une variété d'imitations de membranes cellulaires dont les propriétés ne sont pas évidentes à préserver. Ce chapitre démontre qu'aucune de ces méthodes d'immobilisation n'est applicable à l'étude des interactions faibles entre fragments et protéines membranaires. C'est précisément cette nature hydrophobe qui limite l'application de FBDD aux protéines membranaires, car les imitations synthétiques de membranes cellulaires, tels que les détergents ou les lipides, ont tendance à créer des liaisons non-spécifiques avec les fragments, ce qui engendre souvent de fausses lectures de résultats.

TINS est un système de référence qui permet le criblage simultané d'une protéine de référence (avec peu d'affinités pour les fragments), solubilisée dans le même détergent que la cible, pour permettre l'identification des liaisons non-spécifiques au niveau des protéines ainsi qu'au niveau de l'environnement hydrophobe présent autour des deux protéines. Il en découle la possibilité d'identifier les fragments qui se lient spécifiquement à la cible. Ceci peut être fait instantanément, sans déconvolution, en comparant simplement l'intensité des signaux 1D ^{1}H des fragments en présence de la cible et de la référence. Ceci à certains avantages par rapport aux autres méthodes par RMN car il n'y a *a priori* aucun besoin d'avoir résolu les structures protéiniques, de petites concentrations de protéine sont requises, et les fragments peuvent etre identifiés parmi des cocktails de plusieurs fragments à la fois, afin de rendre le processus plus rapide.

Dans le **3ème Chapitre**, nous avons démontré que les cibles pharmaceutiques importantes telles que les récepteurs couplés aux protéines G: les « G protein coupled receptors (GPCRs) », peuvent, en principe, être criblés par la méthode TINS. Leur immobilisation dans les conformations d'importance physiologiques, tels que sous formes agonistes ou antagonistes sur résine était possible. Ces différentes conformations pouvaient être présentes sur la résine car nous avons immobilisé des vésicules de membranes cellulaires natives, co-immobilisant d'autres protéines importantes à la cascade de signalisation et la fonctionnalité des GPCRs, tels que les protéines G. La quantité de GPCRs fonctionels sur résine pouvait être améliorée en augmentant la distance entre les vésicules et la résine, mais le faible niveau d'expression intrinsèque des cellules mères était le facteur qui nous a empêchés de pouvoir appliquer la méthode TINS à cette classe de protéines.

Dans le **4ème Chapitre**, nous avons établi que les protéines membranaires en général pouvaient être criblées par la méthode TINS. Nous avons utilisé le canal ionique de potassium bactérien (KcsA) ainsi que l'enzyme membranaire bactérien Disulphide bond forming protein B (DsbB) comme cibles, immobilisées et criblées en présence de la protéine Outer Membrane protein A bactérienne. Toutes trois protéines étaient solubilisées dans le même détergent dodecylphosphocholine (DPC). L'efficacité d'immobilisation des toutes les trois protéines était constamment de 50 %. Le fonctionnement de KcsA et DsbB pouvait etre testé par l'identification

de l'interaction avec un ligand connu par la méthode TINS. L'activité de DsbB pouvait etre d'avantage confirmée par un essai enzymatique qui permettait de suivre l'oxidation de la protéine partenaire DsbA, ainsi que la réduction du cofacteur de DsbB: le coenyzme Q1 synthétique (UQ1). Pour le criblage, afin de minimaliser les interactions entre fragments et détergents, les solutions de fragments ne contenaient aucun détergent. Cependant, le détergent était requis à une concentration équivalente à 5 x celle de la concentration de micelle critique dans le tampon de lavage, en dessous duquel les micelles de détergents se dissocieraient en monomères, éventuellement causant la perte de conformation protéinique et ainsi, leur bon fonctionnement. Ces conditions optimales nous ont permis de compléter un petit criblage de 100 fragments sur KcsA ainsi qu'un criblage complet de 1000 fragments sur DsbB.

TINS est une méthode par RMN qui permet d'identifier les fragments qui se lient par faible affinité aux cibles protéiniques immobilisées, sans donner d'informations quantitatives ou qualitatives en ce qui concerne leur constante d'inhibition enzymatique ni leur mode d'action (site de liaison). Nous avons donc poursuivi l'étude de ces caractéristiques pour les ligands identifiés par TINS sur DsbB dans le **5ème Chapitre.** Les fragments démontrant plus de 70 % d'inhibition dans les essais enzymatiques ont démontré une rangée de constante d'inhibition entre 10 et 200 μM (IC50s). Les essais compétitifs enzymatiques ont aussi démontrés que parmi ces 8 fragments finaux, il existait trois fragments compétitifs pour le site de liaison du cofacteur UQ1, ainsi que cinq fragments présentant le mode mixte, à savoir, un effet inhibiteur sur le cofacteur UQ1 ainsi que sur la liason de DsbA avec DsbB (inhibition d'interaction entre protéines). Ces résultats ont été confirmés par des expériences parallèles par RMN sur des échantillons de DsbB mutants. Le mutant est la seule forme de DsbB suffisamment stable pour ces expérimentations, et représente une conformation physiologiquement importante qui représente une conformation intermédiaire de cette protéine dans le processus d'échange d'électrons. Ces expériences nous ont permis ainsi d'identifier les zones d'acides aminés qui participaient dans les liaisons avec les fragments. L'existence des deux sites de liaison nous permettra dans l'avenir de lier chimiquement des fragments des deux groupes distincts afin d'obtenir des composés inhibiteurs de DsbB avec d'avantage de spécificité et d'efficacité.

Le **6ème Chapitre** nous démontre la possibilité d'utiliser une technique de solubilisation alternative qui permet de répéter le criblage TINS sur DsbB en l'absence de détergents. Les protéines DsbB et OmpA étaient encapsulées dans une couche lipidique stabilisées par une ceinture de protéine amphiphile (membrane scaffold protein MSP) dans un complexe nommé le nanodisque. Ce nanodisque est complètement soluble dans l'eau grace aux propriétés de l'MSP. Nous avons démontré que DsbB, une fois encapsulée dans le nanodisque (DsbB/ND) est plus active, peut être immobilisée avec d'avantage d'efficacité, et peut être criblée en l'absence totale de détergents en gardant sa fonctionnalité intacte. De plus, sur les 19 fragments à haute efficacité inhibitrice, 18 ont été identifiés dans le criblage en utilisant les nanodisques vides (sans DsbB mais avec une couche lipidique) démontrant que les résultats d'une telle méthode sont répétables et qu'il est possible d'éviter la nécessité de produire, purifier, et solubiliser une protéine de référence.

Le **7ème chapitre** décrit les conclusions et les perspectives de cette thèse. Les résultats de cette thèse prouvent que TINS est une méthode que l'on peut appliquer de façon générale aux protéines membranaires, telles que les canaux ioniques et les enzymes. Les fragments identifiés comme ligands pour DsbB ont été caractérisés comme des inhibiteurs dans la gamme micromolaire et pouvaient être identifiés par TINS malgré les différents états solubilisés de l'enzyme. L'utilisation des cibles solubilisées dans les nanodiscs permet d'examiner une cible dans un environnement aqueux avec les nanodiscs vides comme bonne référence standardisée. Ces résultats, combinés avec les nouvelles méthodes pour la surexpression et la mutagénèse thermostabilisante devraient fournir de nouvelles possibilités passionnantes qui peuvent se prolonger au delà de l'identification des fragments ligands aux protéines membranaires. Ces expériences incluent l'identification des interactions entre protéines ou entre drogues, selon ce qu'on choisit d'immobiliser ou d'injecter au lieu des protéines et des fragments.

Curriculum Vitae

List of publications

Acknowledgements

Curriculum vitae

Virginie's profile was drawn by her brother, Johan Früh

Virginie Früh was born in Lausanne, Switzerland, on the 22nd of April, 1978. At the age of 9 months she was brought to travel the world with her parents, where she attended international schools in Manila, Jakarta, and Sri Lanka. After obtaining her International Baccalaureate in Colombo, she studied Biology at the University of Lausanne and transferred to complete her Bachelor of Science in Coastal Marine Biology (Scarborough, United Kingdom) in 2001, with a First Class Honours degree. She carried out several voluntary positions in the tropics in the aim to protect coral reef and fishermen communities. She returned to Switzerland to work for a pharmaceutical company for a temporary contract of 5 months, and another of 8 months, where she was responsible of cell culture media development. She continued to apply for PhD positions when Dr. Gregg Siegal and Prof. Ad. IJzerman accepted her as a candidate with shared time between the LIC and LACDR of the University of Leiden. She started her PhD in September 2004 in the aim to adapt the Target Immobilized NMR Screening (TINS) methodology to the challenging membrane proteins such as GPCRs, ion channels, and enzymes. The project also involved applying TINS to membrane proteins with alternative solubilization media. Several collaborations with other academic research groups and industries were established during her PhD project, including the group of Prof. Rob Leurs (LACDR, Amsterdam, Netherlands), Prof. Lukas Tamm and Prof. John Bushweller (University of Virginia, USA), Prof. Jean-Luc Popot (IBPC, France), Dr. Gregg Siegal and Dr. Rob Heetebrij (internal collaboration with ZoBio, Leiden, Netherlands) and Herman Verheil (Pyxis Discoveries, Delft, Netherlands). Part of the work was presented at the Figon Medicine Days in October 2008, where she was awarded the first prize for the oral presentation. She now has returned to Switzerland where she aims to continue working in the field of biochemistry, and to continue work on her association *ekolibrium,* co-founded with Didier Thomas and Ayi Ardisastra, which aims at encouraging and gathering ecological research through the production of documentaries made by the Swiss youth for the Swiss public.

List of publications

1- Früh, V.; Heetebrij, R.; Siegal, G. **Fragment-Based Drug Discovery: A Practical Approach**; John Wiley & Sons: Chichester, 2008; Chapter 6, pp 135-158.

2- Früh, V.; IJzerman, A.P., Siegal, G. **How to catch a membrane protein in action: A review of functional membrane protein immobilization strategies and their applications**. *Accepted for review. Chemical Reviews (2009)*

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4- Früh, V.; Heetebrij, Grinkova,Y. N. , Sligar, S. G., Siegal, G. **Self assembly of Protein – Nanodisc Complexes: A solubilization strategy which enables fragment based drug discovery of membrane proteins in aqueous buffers.** *Manuscript in preparation (2009).*

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