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Affinity-based, biophysical methods to detect and analyze ligand binding to recombinant proteins: Matching high information content with high throughput

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ABSTRACT

Affinity-based technologies have become impactful tools to detect, monitor and characterize molecular interactions using recombinant target proteins. This can aid the understanding of biological function by revealing mechanistic details, and even more importantly, enables the identification of new improved ligands that can modulate the biological activity of those targets in a desired fashion. The selection of the appropriate technology is a key step in that process, as each one of the currently available technologies offers a characteristic type of biophysical information about the ligand-binding event. Alongside the indisputable advantages of each of those technologies they naturally display diverse restrictions that are quite frequently related to the target system to be studied but also to the affinity, solubility and molecular size of the ligands. This paper discusses some of the theoretical and experimental aspects of the most common affinity-based methods, what type of information can be gained from each one of those approaches, and what requirements as well as limitations are expected from working with recombinant proteins on those platforms and how those can be optimally addressed.

1. Introduction

Biophysical methods can have an impact in several valuable areas in early drug discovery. The available technologies have evolved in recent years such that the reliability, throughput, high-quality and orthogonality of approaches now compromise a tool-box of methods essential to modern drug discovery programs. Biophysics can allow rapid and reliable quality control checks on recombinant target proteins, and the assays in which they are used. This forms an important first step in establishing a suite of approaches focused on finding hits and leads.

While high-throughput screening of corporate compound collections has been the main approach used within the pharmaceutical industry to identify hits and leads, these methods have had limited success in identifying novel drug candidates. This fact, along with an increase in the number of biophysical approaches that can be applied, both to primary and secondary screening, as well as in lead optimization, has led the pharmaceutical industry to invest heavily in biophysical screening approaches in recent years. Some biophysical methods have the required throughput to compete directly with traditional biochemical screens such that they can be considered as truly primary hit finding assays. Yet more methods have sufficient throughput to allow focused screening for particular targets or for using selected compound libraries.

Although suitable for characterizing interactions of compounds covering a wide range of molecular weights, biophysical technologies are most often employed to focus on smaller libraries of low-molecular weight compounds. These so-called fragment-based lead generation approaches are being used increasingly, alongside or even instead of traditional high-throughput screening (see Albert et al. (2007) for a detailed review about the philosophy and strategy for fragment-based lead generation within AstraZeneca). The reasons for this are twofold: high throughput biochemical assays are already established as a route to screen larger compounds, but the probability of finding compounds showing optimal interactions is low; and perhaps more importantly, highly sensitive, high-quality biophysical assays are essential in order to detect the interactions of smaller compounds, due to their often weaker affinities. Given that the mean molecular weight of a drug molecule is around 335 Da, (median around 320 Da) and the mean molecular

Abbreviations: BACE, β-secretase; DBA, direct binding assay; DSC, differential scanning calorimetry; ED, equilibrium dialysis; EDC, N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide; ESI, electrospray ionization; FAC, frontal affinity chromatography; HTS, high-throughput screening; IMS, ion mobility spectrometry; ISA, inhibition in solution assay; ITC, isothermal titration calorimetry; LC, liquid chromatography; MS, mass spectrometry; MW, molecular weight; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; RU, resonance units; OWG, optical waveguide grating; SAR, structure activity relationship; SPR, surface plasmon resonance; TDC, target definition compound; TS, thermal shift.

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weight of a bioactive compound is 455 Da (median around 450 Da, see Tyrchan et al., 2009), biophysical methods capable of utilizing fragment start points with molecular weights in the range 150–250 Da may be an extremely useful primary screening approach. Identifying lower molecular weight start points may support several rounds of medicinal chemistry design-make-test cycles, during which the tendency is usually to increase molecular weight (Smith, 2009). This contrasts with using HTS approaches, which may successfully identify larger bioactive compounds, but the necessity will be to optimize compound properties with little or no change in molecular weight, in order to fit the profile for marketed drugs. Of course, this oversimplifies the situation somewhat, as there are many other considerations of compound properties that are important in drug discovery, although it does provide a convenient backdrop for the increased application of biophysical methods coupled to fragment screening.

Thus, biophysical methods are becoming increasingly established as complementary approaches to traditional hit finding routes, and are being actively exploited across the industry. The hope is that these biophysical methods will add an extra dimension to drug discovery by providing an opportunity to create hits and leads, rather than just finding them from within the corporate compound collection.

Thus, coupled to the incorporation of these new screening methods have been efforts to improve compound libraries for use with these technologies. These improvements include building fragment libraries which can be used as chemical start points, extended fragment libraries exploiting protein–ligand recognition principles, and target-specific focused libraries.

The result is that there are now a large number of method-library combinations which can be employed to monitor ligand binding in drug discovery.

In order to exploit these developments in technology and library design most effectively, it is necessary to consider the system and the information required before choosing which approach to use. Important considerations are the availability of the protein and well-characterized reference compounds, including the amenable concentration range, the functionality and the stability. The availability of tool compounds should also be investigated, as even if these are not essential, they may provide routes to additional valuable experiments for screening or evaluation. Different biophysical methods also offer a range of information content, so it is important to determine what information is critical to the stage of the project, and employ the most suitable method to extract that information from the collected data.

It may be necessary or desirable to combine approaches in order to identify and characterize compounds, to access the information required, in the most resource and time efficient manner. Consideration should be given to the most appropriate combinations of methods with the appropriate read-outs and level of confidence in order to achieve the desired goals. By combining techniques in this way, it should be feasible to provide medicinal chemists with data on the kinetics and thermodynamics of an interaction, which can then be interpreted alongside available structural information. This, almost full characterization of a binding event (lacking may still be the mean structures and in most cases the dynamic ensemble populations of one or both of the free interacting partners), should be invaluable in assigning some rules for guiding optimization of the appropriate parameters to meet the required candidate drug target profile.

So, the pharmaceutical industry is realizing that front-loading biophysical screening, or using it in conjunction with established HTS methods can be advantageous, as these methods can provide important information early in the drug discovery process about the required routes for lead generation for particular targets, and the potential success rates of HTS. This knowledge can be useful in helping to reduce the rate of attrition for valuable targets. It can also be useful in providing a more thorough description of protein–ligand interactions allowing attempts to optimize compounds towards profiles that appear to be favored in marketed drugs, for example larger negative enthalpies (Freire, 2008) and slower off-rates (Swinney, 2009).

This review highlights some of the available biophysical approaches that can be used to identify hits, provide data and information on the fundamental properties of the target protein–ligand interaction, and to give insights into how the thermodynamic and kinetic properties of that interaction may be modified in order to improve potency during the medicinal chemistry phase of a project.

2. Thermodynamic methods – ITC

Over the past decade ITC has been established as the gold standard method for directly measuring ligand binding affinity and thermodynamics (for a review see Freyer and Lewis, 2008). The technique often allows the affinity, enthalpy and stoichiometry of a binding interaction to be measured in a single experiment usually taking under one hour. Recent advances in sensitivity, reduction in cell volume, and automation have allowed the approach to evolve from a technique predominantly used for bespoke compound thermodynamic characterization, to one which can now begin to be applied in compound screening. The combination of thermodynamic and structural data has always been powerful in helping to guide molecular design, but the opportunity to characterize increased compound numbers relatively quickly, will see the use of ITC extended in medicinal chemistry design-make-test cycles.

The ITC experiment involves the monitoring of the heat produced (for an exothermic binding event) or absorbed (for an endothermic binding event) during the binding reaction (for a comprehensive protocol see Holdgate, 2010). As the name suggests, the experiment occurs at (almost) constant temperature with the ligand solution usually titrated from the injection syringe into the protein solution contained within the calorimeter cell. Modern calorimeters operate via power compensation, whereby the difference in the variable power, proportional to the binding heat, applied to the sample cell and the constant power applied to the thermal reference cell (in order to maintain a zero temperature difference between the two cells) is monitored by the instrument.

During the titration, in which small, typically 2–5 μL aliquots of the ligand solution are added, the first injections generate the largest heat change as the largest number of moles of protein–ligand complex are generated. As the titration progresses through subsequent injections, the protein becomes increasingly saturated with ligand, and the amount of newly generated complex falls (although the total amount of complex increases), resulting in a lower measurement of instrumental power. Finally, once all of the protein binding sites are occupied by ligand at the end of the titration, no further incremental complexation occurs and no further heat change is detected. Sometimes significant, non-zero heats following saturation are observed. These are often attributable to the heat associated with dilution of the ligand, as this is often larger than that associated with protein dilution (see Fig. 1).

Depending upon the binding affinity and the amounts of available reagents, it is often possible to arrange the experimental conditions so that a single experiment can provide precise estimates of the affinity (K_d), the enthalpy (ΔH) and the stoichiometry (n) of the binding interaction. This also allows calculation of the entropy (ΔS) from the Gibbs–Helmholtz equation:

$$\Delta G = \Delta H - T\Delta S = RT \ln K_d.$$

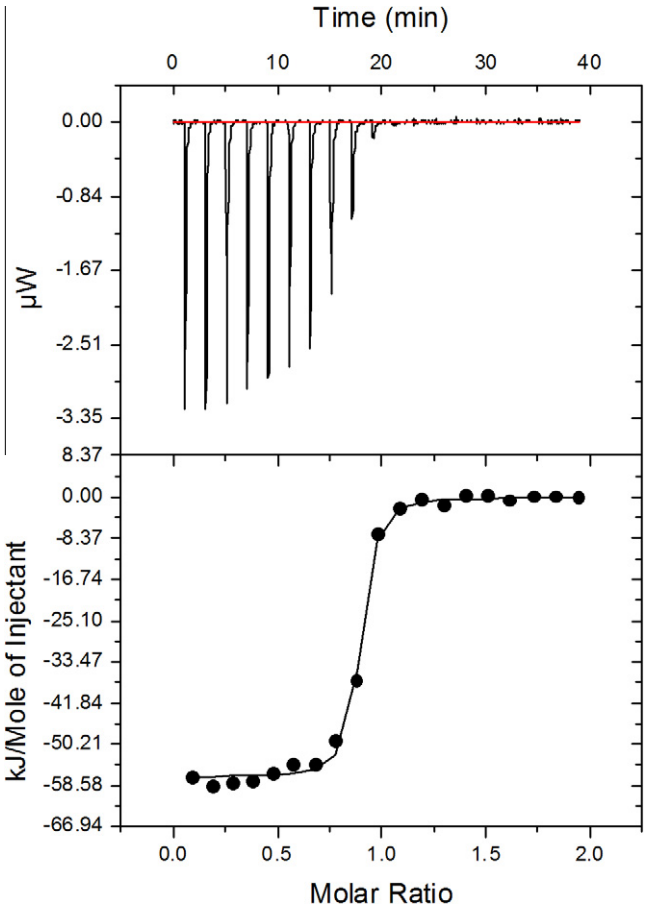

Fig. 1. Typical ITC data. Shown is a thermogram for a test compound binding to a 24 kDa fragment of the DNA gyrase B subunit. The top panel presents the raw data, whereas the bottom panel presents the integrated data with the binding isotherm.

Useful concentrations of protein are typically estimated by rearranging the relationship $5 \leqslant c \leqslant 100$, where the value of c is given by: $c = n \cdot [\text{protein}]/K_d$.

However, experiments with c values outside of this range can still yield valuable information. Low c values are often encountered for fragments, since they bind only weakly to the target protein. Under usual titration conditions this low c value would yield a flat, featureless binding isotherm, from which it would be difficult to extrapolate values for the binding parameters. However, affinity values can often be accessed for these low c value situations as long as the stoichiometry of the interaction is known and the final ligand concentration ensures almost full saturation of the protein (Turnbull and Daranas, 2003).

High c values, typical of tight-binding compounds, lead to thermograms taking the shape of step functions where information on the ΔH is not only amenable, but very precise, but little information about affinity can be determined. In this case, it may be possible to lower the protein concentration, within the confines of the magnitude of ΔH, in order to bring the c value back on scale. Alternatively, displacement experiments with lower affinity competing compounds may allow the affinity of the tight-binder to be measured.

ITC experiments within the range of c values above typically require protein concentrations of around 5–10 µM, which can translate into 0.1 mg of protein per titration with the newer low volume instruments, or up to 1 mg of protein per titration in the older instruments with large cell volumes, for a protein M_r of 50,000. These relatively high protein requirements have tended to limit the application of ITC to a few high interest compounds during a drug discovery project. However the prevailing difficulty in predicting ligand binding thermodynamics, along with an improving ability to generate large amounts of purified protein and recent advances in instrumentation provide the motivation to study larger libraries of related compounds, in order to build up more complete understanding of the structure–activity relationships. This will facilitate exploitation of thermodynamic signatures during lead generation and optimization.

ITC has a wide application in drug discovery focusing on protein–ligand interactions (Ward and Holdgate, 2001; Weber and Salemme, 2003), ranging from protein quality control applications (where it can allow an evaluation of protein functionality) through its use in crystallization protocols (allowing demonstration of complex formation with test compounds prior to set-up of crystallization trials), and of course including the use of the thermodynamic data gathered on compounds or series of interest to inform molecular design during lead optimization. ITC also can be applied to diverse applications including enzyme kinetics, examination of protonation effects, nucleic acid recognition and even in certain circumstances in the determination of association and dissociation rate constants for reversible binding interactions (Bjelic and Jelesarov, 2007).

We have viewed ITC for a number of years as part of a tool-box of techniques which can be employed to evaluate a range of properties associated with assessing the quality of protein preparations. We believe investigating the identity, purity, concentration, functionality and stability of proteins is an essential first step in any biophysical study of protein–ligand interactions. We first used ITC in this way to assess the quality and reproducibility of recombinant protein preparations for work carried out on the enoyl (acyl carrier protein) reductase (Ward et al., 1999). The ability to assess the functionality of the protein by observing substrate or tool compound binding with the expected affinity, as well as determining the functional protein concentration from the stoichiometry value allows batch to batch variation in different protein batches, obtained from different purification protocols, or produced at different times during the life-span of the project to be assessed. ITC can also be used to assess the suitability of different storage conditions, by monitoring changes in these parameter values. The identification and removal of non-functional protein, which may be present in some purified protein preparations, allows the use of fully functional protein which reduces the risk of detecting compounds with artefactual activity.

The high precision of ITC data means that it can be used to assess the validity of other assays, which is useful for drug discovery as the requirement for high throughput and low reagent consumption dictates that other, perhaps less rigorous, assay methods have to be used. This is of particular value in situations where model substrates are employed in the assay, either for convenience or for availability or cost purposes. For example, peptides are often used as alternatives to full length proteins because they are easier to produce and purify. ITC can test the reliability of peptides as models of the full length protein, by comparison of the binding parameters of the full length protein with that of the model substrate.

In a similar manner, recombinant DNA technology is often used to produce protein samples for structural studies and assays during drug discovery. Sometimes these purified protein constructs may lack the full-length wild-type functionality. ITC can be used to verify the validity of using these recombinant proteins, by evaluating the SAR of ligand binding. If the same SAR for the authentic and recombinant protein is observed, then the model protein can be used within the drug discovery project with increased confidence. We have used ITC in this way during several drug discovery projects, for example by investigating the binding thermodynamics of ATP and the antibiotic novobiocin to 24 and 43 kDa fragments of the

antibacterial target DNA gyrase. These fragments of the B subunit of DNA gyrase lack the topoisomerase-linked ATPase activity of the intact A_2B_2 heterotetramer, and so traditional biochemical assays following catalytic activity were impossible. The similar binding affinities observed for these and other ligands binding to these two recombinant proteins, compared to the wild-type protein allowed us to substitute for the full length gyrase tetramer during the structural studies (Holdgate et al., 1997).

Similarly, ITC has been used to characterize the binding of ligands to non-activated kinases. Non-activated kinases typically show little catalytic activity. Again, following test compound binding by steady state enzyme assays would result in assays having low signal:noise. Thus biophysical methods, such as ITC, allow ligand binding to be characterized without having to set-up complex enzyme assays, in order to search for and characterize compounds preventing kinase activation. It is possible to configure prevention of activation assays, which can identify binders to the non-activated kinase present in the assay. However, because the concentration response relationship for binding to the non-activating kinase is the same as that for test compound binding to the upstream activating kinase, ITC offers a simpler assay format for measuring affinities. Examples from our work include MEK protein kinase and p38α MAP kinase (VanScyoc et al., 2008; Sullivan et al., 2005).

Perhaps the most useful application of ITC is in the characterization of compounds produced by medicinal chemists during the lead optimization phase of drug discovery. Understanding the thermodynamics of a molecular interaction is key in drug discovery, as it allows modifications to be made to test compounds in more meaningful way. Thermodynamic measurements are fundamental in trying to understand molecular interaction, and in applying that learning in the pursuit of compounds, not only with higher affinity, but with the appropriate thermodynamic and kinetic profiles for their biological function (Holdgate, 2007). The binding affinity of a test compound is related to the free energy of the interaction, which is dependent upon the enthalpic and entropic components. The situation is complicated further by other factors such as influence of the solvent water on the binding thermodynamics and the change in dynamics and conformation of the ligand and the protein between the free and bound states, making these parameters incredibly difficult to predict. ITC allows the quantification of the enthalpic and entropic contributions to ligand binding. Because ITC is effectively a dual probe technique, allowing measurement of affinity and enthalpy in a single experiment, it can be useful in highlighting discontinuities in SAR which affinity only techniques may miss. It has been suggested that the change in enthalpy particularly can provide a valuable additional tool for the selection of compounds in lead identification and for helping to guide lead optimization. Examples of enthalpic optimization, where the enthalpy of interaction is increased from early drugs to later compounds with advantages in the clinic have been presented for the statins and the HIV protease inhibitors (Freire, 2008; Ladbury et al., 2010). The approach is theoretically simple: rather than establishing SAR on the binding affinity alone to improve the affinity of test compounds, more efficient optimization can be achieved if the contributions of enthalpy and entropy are considered and improved simultaneously (Freire, 2009). The thermodynamics of the AstraZeneca statin, Crestor, were evaluated in the context of both the structure and kinetics of the complex with the target protein, HMG-CoA Reductase (Holdgate et al., 2003). However, it should be remembered that although improving enthalpy is a useful strategy for medicinal chemists, sometimes interpreting changes, even relatively small changes in structure can be difficult. In a recent study of pyrazole and azaindoles binding to p38 MAP kinase, using van't Hoff analysis no discernible relationship between compound IC_{50} and enthalpy or entropy could be established (Papalia et al., 2008).

Only by the increased use of methods able to access thermodynamic parameters, such as ITC, and by exploring the relationship between thermodynamics and structure, will we really begin to be able to increase and exploit our knowledge of molecular interaction. In order to do this we will need to make use of databases providing structural and thermodynamic data. A recently described database, PDBcal (Li et al., 2008), has been created to provide a single source of structural and calorimetrically derived thermodynamic data. This database may be useful in developing our understanding of the relationship between structure and thermodynamics and may provide some impetus for the development of improved models to predict binding affinity from computational approaches.

3. Thermodynamic methods – thermal shift

The ligand-induced thermodynamic stabilization of proteins is the biophysical basis of the technique commonly known as thermofluor, introduced by 3DP, now part of Johnson & Johnson (Pantoliano et al., 2001; Cummings et al., 2006). The enhanced stability of a protein can be monitored in a variety of ways, utilizing either physical (temperature, pressure) or chemical denaturants (guanidine hydrochloride or urea). In thermofluor, the stabilization is manifest as an increase in the thermal stability conferred to the protein following the ligand-binding event. The assay has a homogeneous format, without the need for labeling protein or compound and can be applied in both 96- and 384-well plate formats. The approach has been demonstrated for a wide range of protein classes and has been applicable to both allosteric and active site binding compounds.

Further benefits of the method are its rapidity, as well as the lack of any requirement for custom assay development or the need for expensive instrumentation. This allows broad applicability both in the sense of amenable target proteins, but also in terms of non-biophysical laboratory settings (for a general introduction see Pantoliano et al., 2001). The protein thermal unfolding in the thermofluor approach is monitored indirectly utilizing extrinsic fluorescence, whereas other thermal unfolding approaches make use of direct read-outs, such as the change in heat capacity (DSC), optical rotation (CD) or the degree of light-scattering (Stargazer-384 from Harbinger Biotech).

Thermofluor uses a dye, such as 1,8-ANS, 2,6-TNS or Sypro Orange, which binds selectively to hydrophobic patches exposed on the protein during the unfolding process. The fluorescence of the dye is quenched in water, but shows strong fluorescence in the hydrophobic environment. The temperature of the protein–dye solution is incrementally raised with the fluorescence signal increasing proportionally with the degree of unfolding as the dye binds. This change in fluorescence intensity is easily measured in plate-based instruments, requiring relatively low amounts of material. Typical protein requirements are around 0.5 mg per 96-well plate. This process allows the mid-point of the unfolding transition or melting temperature, at which 50% of the protein is unfolded, to be determined (see Fig. 2). Comparison of the melting temperature in the absence of ligand, T_0, with that in the presence of ligand, T_m, allows the degree of stabilization to be determined. The extent of the T_m shift, ΔT_m, is dependent upon the ligand binding energetics, including the affinity and enthalpy, as well as the ligand concentration.

Using this approach there is usually a broad correlation between the ligand binding affinity and the measured ΔT_m, with tighter binding ligands giving larger shifts in T_m, when all other variables are the same. However, the relationship between ΔT_m and ligand concentration shows no saturation with ligand concentration, so there is not a simple saturation-based method for

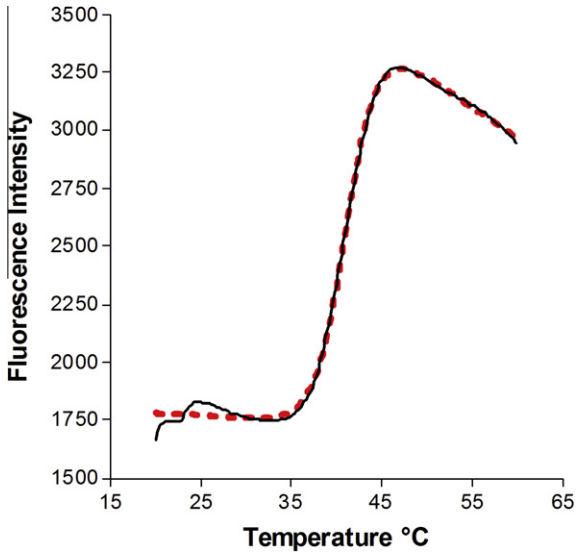

Fig. 2. Typical TS data. Shown is the thermal unfolding transition for BACE-1. Raw data is displayed by a solid line, with the fitted curve in red.

determining ligand binding affinities in the thermofluor approach (Matulis et al., 2005). Converting changes in T_m to a binding affinity at the relevant temperature requires knowledge of the ligand binding enthalpy as well as the protein unfolding enthalpy. The exact relationship between the ligand binding and the T_m shift is given by:

$$K_{L,N(T_{rel})} = K_{L,N(T_m)} \exp[(-\Delta H_{L(T_{rel})}/R)(1/T_{rel} - 1/T_m) + (\Delta C_{PL}/R) \times (\ln(T_{rel}/T_m) + 1 - (T_{rel}/T_m))]$$

where $K_{L,N}$ denotes the equilibrium association constant for ligand binding to the native protein at a relevant temperature (T_{rel}) or at the T_m; ΔH_L is the enthalpy of ligand binding; R is the gas constant; ΔC_{PL} is the change in heat capacity for ligand binding.

In principle the thermofluor approach appears to be a simple and generic approach, but there are instances when the method is less useful, because of issues such as protein precipitation, or reduced unfolding of the protein. Both of these situations may lead to a lack of dye binding and hence a reduced thermofluor read-out. There may also be difficulty in interpreting small shifts in T_m. Our experience with thermofluor shows that the standard deviation for control assays of target proteins used in drug discovery projects can often be around 0.4 °C, and that even in situations where the standard deviation is lower (in 96 controls for the kinase protein p38, the standard deviation was 0.12 °C, data not shown), the range of measured T_m values can still be reasonably large. In the p38 example the measured control T_m values covered a range differing by 0.7 °C. This would suggest that confidence in measuring a real difference in T_m may only be achieved if the ΔT_m is larger than around 1–1.5 °C, especially in cases where there may be relatively few control wells. This potentially limits the use of the approach for fragments, where the affinity may be low, relative to the solubility limit of the compound, and hence the expected T_m shift may not be in this range.

This problem may be compounded by the fact that at high ligand concentrations, non-specific effects may also occur more prevalently as well as increasing risk of possible artifacts to any auto-fluorescence of the compounds at these elevated concentrations. Also the method will tend to give positive shifts in T_m, not just for reversible, noncovalent binders, but also for covalent or irreversible compounds. Thus, by using the technique cautiously, as guide rather than an as an absolute determination of binding for T_m shifts which are below 1.5 °C, confidence in the interpretation of the data and classification of the compounds may be enhanced. As with other methods, orthogonal approaches to verify and characterize binding are recommended.

As well as identifying compounds that stabilize the protein, it is also feasible to identify compounds which destabilize proteins through non-specific aggregation or other types of promiscuous effects. It is possible for the protein in the presence of these compounds to exhibit a negative ΔT_m.

The ability of thermofluor and other thermal unfolding based methods to identify compounds binding to target proteins, as well as their ability to help to identify optimal buffer and additive conditions conferring stability for assay and storage has led to widespread use in primary (especially fragment) compound library screening, buffer screening for assays and optimization of conditions for crystallization and NMR studies.

An obvious use of the thermal unfolding approach is in the screening of compound libraries for binders and potential inhibitors of target proteins. Lo et al. (2004) report the use of the iCycler instrument (BioRad Laboratories) for thermal unfolding measurements for hit identification versus the pharmaceutical target BACE-1. Thirteen compounds from four chemical structural classes were evaluated in thermal unfolding and ITC assays. The results illustrate that true hits can be identified based on thermal shifts under appropriate conditions. The determined binding affinities were shown to be similar to those measured by ITC.

An extension to this screening approach for proteins of known function is to use thermal shift assays in order to search for ligands that bind proteins of unknown function. Binding information obtained in the thermofluor approach can then be combined with biochemical, sequence and structural evidence in order to assign putative functions to those orphan proteins. An example of this application of thermofluor has been published for an essential gene from *Streptococcus pneumoniae* (Carver et al., 2005). Here, the thermofluor method was used to screen 3000 compounds specifically selected to provide information about speculative biological functions.

The production and storage of large amounts of suitable quality protein is a challenge that must be overcome in order to allow the biophysical and biochemical studies that make up a drug discovery campaign to be completed. An approach that has been taken to do this is to engineer multiple constructs of the target protein in order to assess which may lead to optimal expression, purification and stability. Testing the stability in parallel of multiple constructs can easily be achieved using thermal unfolding. A recent review (Bommarius et al., 2006) highlights some examples where proteins have been engineered for increased stability.

A related approach is then to evaluate the most favorable conditions in which to produce and store the wild-type, or a chosen protein construct, which shows behavior indistinguishable in terms of ligand-binding characteristics from the wild-type. The thermofluor approach is well suited to scan buffer conditions, such as concentration and identity of buffer salts and pH for protein storage and assay, since it allows a variety of buffer conditions to be evaluated in a single run. The approach can also be used to monitor the effects of other additives and excipients such as detergents, cofactors, metal ions or in the case or crystallization trials, precipitants that may be included in the buffer solution. This application has been demonstrated for several proteins of interest to the pharmaceutical industry, and the systematic approach is illustrated nicely for Akt-3 and cFMS (Mezzasalma et al., 2007).

Thermofluor has also been used in identification and prioritization of ligands for cocrystallization trials. The premise is that those compounds showing large stability shifts are likely to be tighter binders and may be better start points for cocrystallization trials than those compounds displaying small or no thermal shift. Of

course, there will be systems where both strong and weak binders will tend to yield crystals, so again it is suggested that the approach should be used as a guide rather than as an absolute surrogate for success. The method has been applied to 25 *Escherichia* coli proteins, where the thermofluor results were used to identify stabilizing and destabilizing additives. The results showed a twofold improvement in the number of crystallization leads identified when the stabilizing additives were included (Ericsson et al., 2006).

In a recent extension to the thermofluor method the combination of thermal unfolding with the different fluorescent properties of flavin-containing proteins in the folded and unfolded state has been used to explore stability and crystallizability (Forneris et al., 2009). This method exploits the properties of the flavin prosthetic group present in 2–3% of proteins, and potentially highlights the applicability of other fluorescent cofactors and prosthetic groups for use in this way. In this technique, the flavin cofactor is used as an intrinsic probe to monitor protein folding and stability, taking advantage of the different fluorescent properties of flavin-containing proteins between the folded and denatured state.

Thermofluor can also be used to probe mechanism of action, by measuring multiple independent binding events. For example, monitoring the change in T_m occurring under a matrix of substrate and inhibitor concentrations may allow classification of the mechanism of inhibition. Uncompetitive inhibitors would be expected to show larger T_m shifts in the presence of substrate (as the effect would be dependent upon the additivity of the individual binding free energies, whereas competitive compounds would tend towards the showing the T_m shift of the ligand giving the greatest degree of saturation.

We have used a range of fluorescent plate readers with heating functions to access many of the applications described above. Many of the target proteins we have worked with show unfolding transitions in the range 40–50 °C. Obviously, for proteins having T_m values 10–15° lower than this, the thermal unfolding approach provides relatively little useful information without the comparative data on different conditions or different constructs, since proteins with melting temperatures only slightly above room temperature would be inherently thermodynamically unstable. Thus, we have tended to use the technique in a comparative manner mostly to probe pH and additive effects.

4. Optical biosensors – SPR

Optical biosensors typically generate a measureable change in some characteristic property of light that is coupled to the sensor surface by making use of the evanescent-wave phenomenon. SPR is by far the best-known optical biosensor that makes use of that phenomenon to enable the real-time measurement of protein–ligand interactions. During the last two decades SPR biosensor technology has seen a rapid evolution starting with the launch of the first commercial Biacore instrument by Pharmacia Biosensor in 1990 (Jonsson et al., 1991). This evolution relates not only to the emerging and newly established types of alternative instrument platforms (see Rich and Myszka, 2007 for available biosensor platforms) but even more to the expanding range of applications: to mention but two, the advances in working with membrane proteins (Navratilova et al., 2006; Karlsson and Lofas, 2002) and small-molecule work including fragment screening (Danielson, 2009).

SPR is a phenomenon that occurs when plane-polarized light hits a metal film under conditions of total internal reflection. The SPR angle, which is the angle of the incident light that results in the lowest intensity of reflected light at a constant wavelength, depends mainly on the properties of the metal film and the refractive index of the medium that is close to that film. By monitoring alterations in the refractive index, SPR is able to measure changes in the mass of dissolved material in the aqueous layer (biosensor surface) close to the metal film, which allows the interaction of proteins with other molecules or ligands to be monitored in real-time (for a detailed review of the basics of SPR see (Huber and Mueller, 2006). This enables the kinetic parameters and equilibrium constants for a given system, i.e. on-rate, off-rate and apparent dissociation constants, to be determined. If these systems can be studied within a large temperature interval it allows even for the determination of thermodynamic binding parameters in conjunction with a van't Hoff analysis and can provide a sound alternative to ITC methods (Papalia et al., 2008).

A critical success factor in conducting SPR experiments is the careful design and execution of the assay. Key to all SPR assays employing a DBA format is the successful tethering of the recombinant target protein to the biosensor surface by using different coupling strategies without compromising the activity/functionality of the protein or the access to the ligand binding pocket(s). The most common approach is random-orientation immobilization via accessible primary amines on the protein surface by activating the carboxymethylated dextran-matrix with a mixture of EDC and NHS in order to create NHS-esters that can react with amino-containing molecules. Besides other available chemistries (OShannessy et al., 1992) this approach works quite well for the majority of soluble recombinant target proteins and only a small fraction require a different coupling chemistry or in some cases even more sophisticated strategies. These strategies involve quite often the generation of suitable protein constructs that permit a more directed and orientation-controlled immobilization process via engineered tags (e.g. Streptavidin affinity tags for the use with Streptavidin-coated Biosensors (Li et al., 2006) or fusion proteins (e.g. hAGT fusion proteins Huber et al., 2004). For work with membrane proteins, and in particular G-protein coupled receptors, a labor-intensive strategy involving an antibody-capturing step with detergent-solubilized receptor can be required (Navratilova et al., 2006). To make use of a key advantage of SPR technology, namely the ability to utilize the same sensor surface during multiple cycles thus consuming very small amounts of recombinant protein, it is also essential to establish suitable conditions that allow for the reproducible regeneration of the sensor surface without losing binding activity and functionality of the immobilized protein after multiple ligand binding experiments. This becomes even more important in working with ligands or compounds that show an unspecific binding component (promiscuous or aggregation-based inhibitors) either to the sensor surface or the protein, which makes an accurate analysis of subsequent ligand binding experiments unfeasible.

As most of these central factors can be addressed in one way or the other by applying different strategies or approaches, there is one remaining but significant aspect that is related to the attainable sensitivity and thus the dynamic range of the measurements and is partly out of the control of the scientist as it is dictated by the system to be studied. The SPR signal intensity is dependent on several factors, namely the MW, the immobilized amount and remaining binding capacity (i.e. functionality) of the protein as well as the MW, the total concentration $[L_T]$ and dissociation binding constant K_D of the ligand. Simulations displaying the minimum required ratio $[L_T]/K_D$ for a reliable detection of the binding as a function of the MW of the ligand and the protein show that the dynamic range gets smaller with increasing MW of the protein and decreasing MW of the ligand (Dalvit, 2009). This obvious sensitivity limit presents a real challenge for working with fragments or fragment-like ligands but also with ligands that display low affinities combined with low solubility and will thus exclude this type of studies for larger protein systems.

The flexibility in the SPR assay format allows for the design of a tailored assay format and enables some of the key aspects

described earlier to be tackled. The DBA, where the protein is immobilized on the sensor surface and compounds are passed over the surface, is commonly used and has been published for a large number of different systems. Alternative assay formats such as the surface competition assay (SCA) or the ISA are viable alternatives to a DBA (Karlsson et al., 2000). In particular the ISA format helps to deal appropriately with the challenges for setting up and conducting a DBA as well as the limitations in the affinity range and analyte masses and thus makes systems amenable for small-molecule work that are usually deemed unfeasible for a DBA. We have thus employed a general strategy to assess ligand binding by making wide-ranging use of the ISA format.

The fundamental difference between the ISA and the DBA format is the tethering of a target definition compound (TDC) to the biosensor surface which serves as a probe for the binding site, instead of immobilizing the recombinant protein and monitoring the binding directly (see Fig. 3). The interaction with the TDC and the ligand to be investigated occurs simultaneously and due to this competition it is possible to derive the dissociation binding constant K_D for this particular ligand (Karlsson, 1994). If the analysis is performed on a biosensor surface with very high binding capacity and thus density of the TDC (typically 200–400 pg/mm^2) in conjunction with very low flow rates (<20 μl/min) it is feasible to generate conditions of mass transport limitations. Something that one usually would try to avoid in a DBA as it will obscure the kinetic analysis helps actually to simplify the analysis of the ISA data as the observed binding is solely determined by the mass transfer of the protein to the surface and not anymore by the interaction kinetics, and as a result the signal becomes concentration-dependent. This situation is typically characterized by a constant initial binding rate (RU/s) over a certain period of time which allows for the precise determination of the free protein concentration as the initial binding rate (RU/s) is directly proportional to that. Usually, the inhibition studies are carried out by pre-incubation of the recombinant protein with the ligands and subsequent injection over the TDC-modified sensor surface. The initial binding rate is used to determine the percentage of free protein in solution which will change by varying the concentration of the competing ligand. By plotting the free protein concentration against the logarithm of the ligand concentration one can apply sigmoidal dose–response curve-fit models available in standard software packages to determine the K_D-value. The assay can even be used for a more qualitative affinity ranking of different ligands without the need to determine their affinity, if experiments are conducted at similar ligand concentrations.

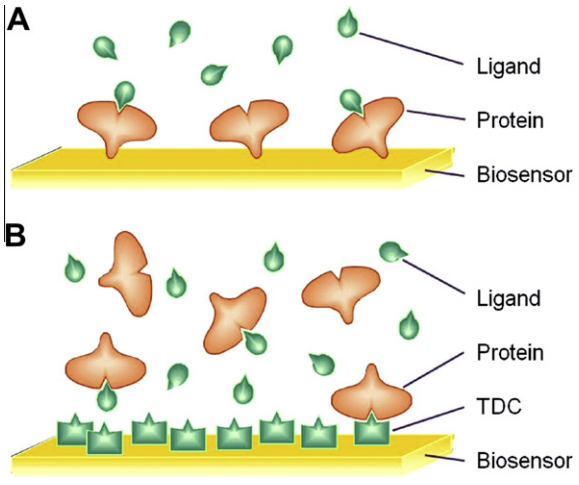

Fig. 3. Assay flexibility in SPR. Shown is the general principle of a direct binding assay (A) versus an inhibition in solution assay (B).

An important step in the development of an ISA is the identification of a suitable ligand to be used as a TDC. This ligand should ideally cover the entire binding site, should display a rapid association phase to enable binding studies under conditions of mass transport limitations and ideally a slow dissociation phase so that this becomes negligible during the initial association. A high affinity (K_D < 1 μM) of the TDC is desirable as the affinity dictates the concentration of the protein to be used in the assay (protein concentrations are typically in the range of 20–200 nM) in order to achieve an good binding signal (ideally RU/s > 1) and thus determines the overall protein consumption. A good choice of a ligand to be used as a TDC could be a substrate-analog or a commercially available compound. An absolute prerequisite is the possibility to immobilize the ligand preferably via primary amines without compromising the binding to the target protein, which presents a challenge if those functionalities are involved in direct contacts in the binding pocket. A successful strategy in our hands has been the chemical modification of appropriate ligands by attaching a carbohydrate- or ethylenglycol-linker that contain a free primary amine at their end. Great care has to be taken in choosing the correct length of the linker as it needs to sufficiently protrude out from the protein surface to enable efficient binding without facing steric hindrance, but should not be too long to avoid reduction of the free binding energy owing to the entropic penalty that arises upon binding of a molecule with increased flexibility and mobility.

There are obvious benefits of the ISA format compared to the more traditional DBA. First of all and most importantly one gains full control of the dynamic range of the assay via variation of the protein concentration as there are no MW-limitations for either the recombinant protein or the ligands to be studied. The interaction of the ligand and the protein occurs truly in solution and the assay set-up enables read-out of the free protein concentration without disturbing the equilibrium, thus enabling the determination of exact K_D-values. The procedures to establish protocols for the immobilization as well as the regeneration are much more predictable and usually straightforward, as quite harsh and powerful conditions for the immobilization of such small organic ligands as well as the regeneration of the modified sensor surface can be applied, which are usually not compatible with a DBA using immobilized protein. Clear shortcomings are of course that the described approach will not allow for the determination of the kinetic parameters of the protein–ligand interaction and that the consumption of recombinant protein will be significantly larger.

We have effectively applied the concept of the ISA as a follow-up for an NMR-based screening of a fragment library against BACE-1 (Geschwindner et al., 2007) but have recently also used a similar approach for direct screening of larger fragment libraries, which even involved the interrogation of fragment pools to increase the throughput. For the characterization and analysis of fragment binding to BACE, we designed an ISA using an P1 (S)-statine substituted substrate analog (sequence: KTEEISEVN(Sta)DAEF, where Sta is the transition state mimetic), which was reported to be a nanomolar inhibitor of BACE activity and binds specifically to the active site, as TDC. In order to show that the fragments that have been identified by the NMR-based screening might also act as broad aspartic protease warheads we also configured an ISA using the renin-inhibitor H-142 (sequence: PHPFHL^{R}VIHK, where R depicts the reduced isostere of the scissile peptide bond between residue Leu10 and Val11 in human angiotensinogen) as TDC and probed the fragment binding to the aspartic protease endothiapepsin in a similar fashion. Those substrate analogs have been particularly useful as TDCs, as they display very high affinities with their respective binding partners and could be readily immobilized using amine-coupling without any modifications but retained binding activity. For the ligand-binding studies we made use of BACE protein that has been produced as a C-terminal fusion protein with the Fc part of human

IgG1, which increases the MW of BACE to >100 kDa and consequently would not have been considered as a suitable protein construct for a DBA. The NMR screen helped to identify a low-molecular weight isocytosine fragment hit (MW = 153.19 Da) that displayed very weak affinity (K_D = 4.5 mM) but could be easily detected and validated in the ISA. By using analogs of the original hits at a single concentration an emerging SAR could be determined that correlated really well with the NMR results and quickly helped to identify molecules with higher affinity that could subsequently developed into inhibitors with nanomolar potency and cellular activity (Edwards et al., 2007).

The ISA has been a very efficient tool, not only for the rapid affinity ranking which guided the selection of suitable fragment hits, but most importantly for the determination of quantitative SAR of those very weak inhibitors during the 'analoging phase' of the fragment screening campaign. As such the ISA helped to effectively bridge the existing affinity gap until those fragment-derived inhibitors became potent enough to be picked up in a conventional enzymatic assay. By looking again at simulations displaying the minimum required ratio $[L_T]/K_D$ for a reliable detection of fragment binding (Dalvit, 2009) and using both the MW of the untagged BACE construct (approx. 45 kDa) and the MW of the original fragment hit we would have needed a concentration of around 3–4 mM to reliably detect this fragment in a DBA, a concentration that would never be considered for screening purposes. The increased dynamic range of the ISA tolerates performing those experiments at a much lower ligand concentration as it obviously allows for the detection of much weaker interactions compared to a DBA. Consequently the ISA can also be used as an attractive alternative for the reliable detection of weak fragment binding during a primary fragment screen.

5. Optical biosensors – OWG

As an emerging complement to SPR-technology (and probably also owing to the big impact of SPR in opening up the entire field of label-free analysis of protein–ligand interactions), alternative biosensor platforms have been developed that exploit related physical phenomena but offer different throughput and approaches to study those interactions. Evanescent field sensing provided by optical waveguides has recently found a wider acceptance within the affinity-based community as two technology providers, Corning and SRU Biosystems, have recently developed and successfully launched related systems (EPIC and BIND system, respectively) that allow for increased sample throughput by using a plate-based technology platform.

In optics, electromagnetic evanescent waves are formed when light waves that are traveling through a boundary between two media of different refractive indices undergo total internal reflection because they strike it at a critical angle of incidence. Evanescent wave coupling is usually accomplished by placing two or more electromagnetic elements such as optical waveguides close together, thus enabling propagation of a wave from one element to the next. In order to perform as a biosensor, some portion of those elements needs to be in contact with the liquid test sample. The change in the refractive index at the interface between the sensor surface and the liquid which occurs as a consequence of a ligand-binding event will modify the wave coupling and triggers a change in the reflected or transmitted output. This permits accurate determination of the alterations in mass at the sensor surface, thus allowing highly sensitive measurements of changes in binding or adherence in the proximity of the sensor surface (see Fig. 4 and for a review of waveguide-based biosensors and their principles please see Mukundan et al., 2009). Most of the related technologies operate with planar optical waveguides, with the EPIC system making use of resonant waveguide grating (Fang et al., 2006) while the BIND system applies nanostructured optical grating also known as 'photonic crystals' (Cunningham et al., 2004) which can be used to conduct either biochemical or even cell-based applications in various standard assay formats such as 96-, 384- and 1536-well microtitre plates.

The latter leads to some very interesting and novel approaches in working with GPCRs. In brief, ligand binding to GPCRs leads subsequently to trafficking of molecules and protein complexes, receptor internalization and translocation. This movement of proteins within a cell has been termed dynamic mass redistribution and can be followed by applying OWG, thus permitting studies in cell signaling as well as the screening of compounds against

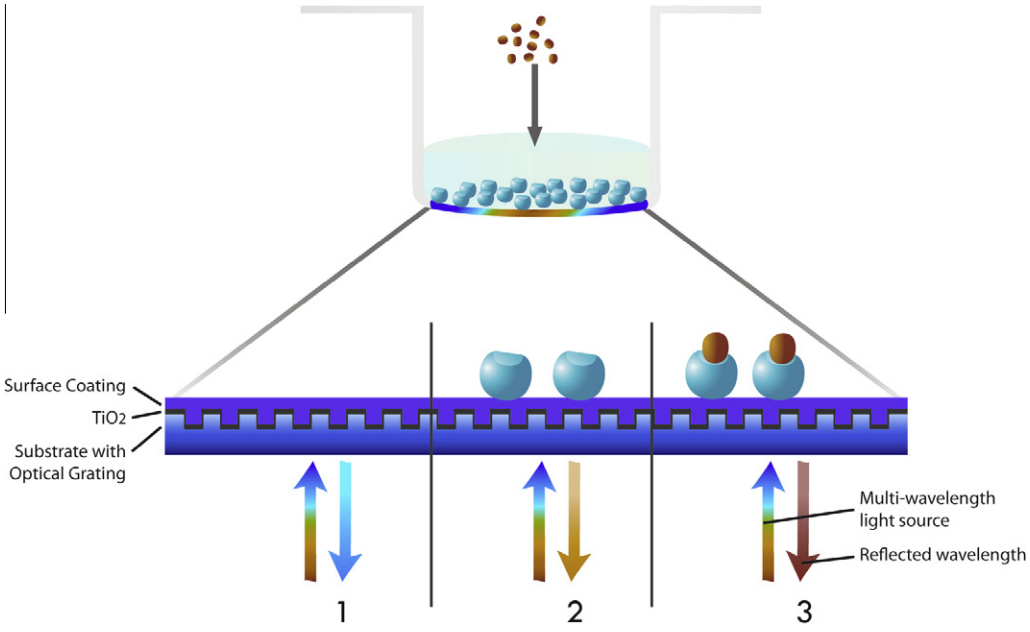

Fig. 4. The principles of OWG to measure ligand-binding events as exemplified for the BIND system. The increase in mass within the proximity of the biosensor surface causes an increase in the reflected wavelength both upon immobilization of a protein (2) and the specific binding of a ligand after protein immobilization (3).

endogenous receptors (Fang et al., 2007). Studies have shown that this can be successfully used both for primary screening as well as an orthogonal approach to enable rapid follow-up from other hit finding sources (Dodgson et al., 2009). This furnishes the interesting opportunity to find novel ligands that would not have been identified using a classical screening approach, thus enhancing success rates.

For biochemical assays, i.e. assays that use biosensor-coated recombinant proteins to study protein–ligand interactions, one naturally tends to compare OWG with SPR but one needs to be aware that there are fundamental differences from existing SPR-technology that can present particular opportunities as well as unique challenges for the application of OWG platforms in those studies. We have used both platforms in multiple projects and this puts us in a position to form an initial judgment about the possibilities as well as limitations of this approach. The most striking difference, which is due to the plate-based nature of the device, is the absence of any microfluidics and liquid flow. The consequent inability to measure accurate kinetics (while being able to determine accurate K_D-values) in a biochemical assay is somewhat compensated by the possibility to add several binding components (cofactors, substrates or substrate-analogs, competitive and noncompetitive inhibitors, etc.) either simultaneously or sequentially into the same well of the plate and study their interdependency. This possibility to sequentially add or take away different components is a unique advantage that can be optimally utilized by adopting a similar assay strategy as described for SPR, i.e. using an ISA format instead of a DBA format.

But why should one consider using an ISA format on OWG platforms? First of all, most of the restrictions described earlier for SPR are also applicable for OWG platforms, and in particular the MW-limitations for either the protein or the ligands to be studied present a severe sensitivity issue. Secondly, all the sensors should ideally be resistant to non-specific binding of the sample, as this will either mask the specific binding signal or even lead to wrong interpretations of ligand binding. All the in-house data that we have collected so far point towards a much larger degree of unspecific ligand binding in OWG platforms as compared to others which is reflected in a significantly increased hit rate in primary screening due to a larger fraction of false positive hits. In our opinion, this is for the most part related to the difference in the protein density on the sensor surface as compared to SPR. As opposed to Biacore technology, which uses a 3-dimensional hydrogel with a height of approximately 100 nm (Karlsson, 1994) for the covalent attachment of proteins, the sensor surfaces of the currently available OWG biosensors are rather 2-dimensional in nature and thus require a significantly higher immobilization density in order to achieve an attainable ligand binding signal. This high local protein concentration can be in the range of up to 100 mg/ml or more and can lead to non-productive binding of ligands to aggregated or poorly folded proteins. Using immobilized compounds instead and making use of the ISA format will enable to appropriately address those key challenges and preliminary in-house results in applying that concept in fragment screening are very encouraging. Another important aspect to consider is of course the increased consumption of recombinant proteins as compared to SPR, which can be several orders of magnitude higher for a DBA using OWG platforms. In addition there is currently only a very narrow range of immobilization surface chemistries available, which needs to be addressed as it limits the application of this technology for biochemical assays.

6. Spectroscopic methods – NMR

Since the introduction of the SAR by NMR method by Fesik and co-workers in 1996 (Shuker et al., 1996), NMR has evolved into an important tool for ligand screening of expressed proteins. In the pharmaceutical industry setting ligand screening is now a more important application of biomolecular NMR than protein–ligand complex structure determination, which is almost exclusively done by X-ray crystallography. NMR has been and remains a reliable work horse among affinity-based techniques in detecting, monitoring and characterizing molecular interactions using recombinant target proteins.

NMR binding studies can be conducted over a wide range of affinity regimes. However, the major strength of NMR is that binding of ligands with weak affinity, with K_D values in the μM to mM range, can be reliably detected. This defines one of the main niches for NMR, namely in fragment screening for which affinities indeed tend to be weak due to the low complexity of the compounds. Most target proteins can be subjected to ligand-binding studies by NMR provided that sufficient quantities of expressed protein can be obtained. NMR assays tend to be simple and robust, and generally generate few false negatives or false positives. NMR ligand-binding studies have two main applications: in fragment screening and in mode of action studies of compounds coming from, for instance, HTS or medicinal chemistry programs. Both protein-observe 2D NMR and ligand-observe 1D NMR techniques are routinely used. We typically deploy three NMR approaches to detect ligand binding: 2D NMR chemical shift mapping, 1D NMR direct binding and 1D NMR reference displacement (see Fig. 5).

Protein-observe 2D NMR chemical shift mapping experiments can be conducted either using ^{15}N–^{1}H or ^{13}C–^{1}H correlations in HSQC experiments (Shuker et al., 1996; Hajduk et al., 2000). This gives a fingerprint of the amide or methyl groups in the protein, respectively. Residue specific changes in the protein are then monitored upon addition of ligands. This means that specific binding site information can be observed directly in the protein. Residue peak assignments can be obtained through a series of additional 3D NMR experiments, although this is not a strict requirement. With such assignments more advanced analyses of binding mode can be performed. Even in the absence of such assignments residues in the binding site can generally be found, although not explicitly identified, as was done in the case of prostaglandin D synthase (PGDS) described below. With 2D NMR methods screening can therefore be performed without prior knowledge about binding sites.

In addition to being highly information-rich, 2D NMR binding assays tend to be very robust with low false positive rates. Non-specific binding is generally not an issue since such binders are either not detected at all or give rise to general non-specific line broadening. While high affinity (nM) binding is readily detected, quantitative K_D measurements are most reliably determined in the low affinity (μM to mM) and fast ligand exchange regime. 2D NMR offers some technical challenges on the expressed protein, since it requires uniform or amino acid specific 15N or 13C isotope labeling, or both if residue assignments are to be obtained (for a comprehensive review see Wagner, 2009). In practice this typically means that 2D NMR methods are limited to proteins that can be expressed in *E. coli*, although for instance cell-free expression systems have also been used successfully (Kigawa et al., 1995). This is indeed the main blocker to the deployment of 2D NMR for a given target. Another disadvantage of 2D NMR is the high demand for expressed protein, typically on the order of 0.5 mg per sample for a 30 kDa protein. The size of the protein is also limiting. For routine applications the size limit is on the order of 30–40 kDa. Uniform deuteration of the target protein, achieved by expressing the protein in D_2O instead of H_2O, reduces dipolar relaxation effects and enhance spectral quality. This can roughly double the routine applicable size range to 60–80 kDa. A rather unique feature of 2D NMR is that a single domain that has been labeled can be studied as part of a multi-domain complex.

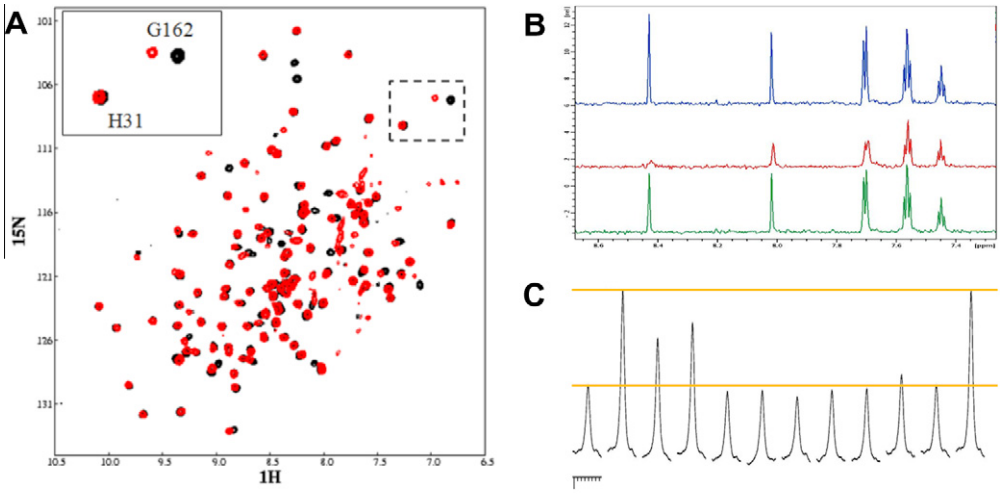

Fig. 5. NMR binding assays. (A) 2D NMR chemical shift mapping experiment showing spectrum of ligand bound form in red on top of spectrum of apo protein in black, with residue assignments shown in small rectangle. (B) 1D NMR direct binding experiment. Ligand signals are observed directly (top blue spectrum) with reduction of intensity upon binding to protein (middle red spectrum), followed by regained intensity upon displacement by high affinity ligand (bottom green spectrum). (C) 1D NMR reference displacement experiment. Different levels of displacement of signal from reference ligand with twelve different ligand additions. The blank (DMSO) addition, far left, and high affinity ligand addition, second from left, define minimum and maximum displacement, respectively.

The most common ligand-observe 1D NMR techniques are waterLOGSY, saturation transfer difference (STD), and T1ρ and T2 filtered experiments (Dalvit et al., 2000; Mayer and Meyer, 1999; Hajduk et al., 1997). Generally ^{1}H detection is used for 1D NMR but other nuclei such as ^{19}F may also be used (Dalvit et al., 2003). 1D NMR techniques are unique in that the ligand signals are observed directly, which results in a built-in quality control and solubility measure for the ligands under investigation. Another major advantage is that a robust ligand binding assay can be set up for most protein targets given that sufficient quantities have been expressed. Protein consumption varies with set-up but is on the order of 10–50 μg per sample for a typical 30 kDa protein. The protein material can be derived from bacteria, as well as more complex sources such as insect and mammalian cells. There is no limit to protein size. In fact, it is actually advantageous to use larger proteins due to the increased relaxation effects, which in turn result in magnified ligand binding effects. 1D NMR techniques are generally not adversely affected by the various tags and fusion proteins used to increase protein expression and solubilization, although non-specific ligand binding interactions may then become more pronounced. In the case of BACE described in the SPR-section, expression with the C-terminal fusion with the Fc part of human IgG1, actually meant that screening could be performed with very low protein concentrations (3 μM).

1D NMR screening can be run either in direct binding mode where putative binding of each individual ligand is observed directly, or by monitoring the modulation in binding of an established reference ligand, sometimes referred to as spy molecule or reporter, upon the addition of ligands of interest. Both approaches rely on competition with a ligand that is known to bind to a site of interest, in order to establish that the binding interaction is indeed specific and to a distinct site (Dalvit et al., 2002a,b; Jahnke et al., 2002; Siriwardena et al., 2002). The direct binding approach is quite unique among affinity-based methods in that significant binding effects are observed even at ligand concentrations much below the K_D. This in turn means the ligand solubility becomes less of an issue, which can be of major importance when analyzing the output from, for instance, an HTS. In direct binding experiments, the magnitude of binding effects is non-linear, which makes it difficult to extract K_D values from such experiments. Simply observing binding effects in a 1D NMR experiments without any sort of competition is not recommended as binding effects are highly dependent on the nature of the ligand, particularly in terms of lipophilicity. Such an assay set-up would run the risk of simply measuring non-specific interactions. Certain expressed proteins are particularly sticky and prone to such effects. In addition, the magnitude of the binding effects decrease when the binding approaches the high affinity (nM) and slow ligand exchange regime. This is a major limitation of the 1D NMR direct binding approach, and could potentially lead to false negatives. In contrast, reference displacement experiments are not subject to such affinity limitations and the accompanying potential for false negatives. K_D values for competing ligands can be determined readily, given that the K_D of the reference is known (Dalvit et al., 2002a; Wang, 1995). A drawback of both competition approaches is of course that some prior knowledge of ligands is required. However, even in the absence of known small molecule ligands these experiments can be informative. Competing ligands could comprise of, for instance, interacting protein domains. Cross-wise competition of novel identified ligands can also be used. First the binding of one ligand is observed, a second binding ligand is then added to see if binding of the first ligand is perturbed. The reversed experiment is then performed and if any competition between the two ligands is observed then the binding is most likely to a distinct site.

Advances such as cryoprobe technology and advanced robotics systems (Folmer and Fetzer, 2004) have increased sensitivity and throughput of NMR over the past decade. Thousands of compounds may be screened against a given target protein in a relatively short time period. Yet even with efficient robotics that prepare samples fresh and make measurements in a fully automated fashion the throughput for NMR screening is modest compared to HTS. Screening of mixtures is routinely done and offers way to increase throughput significantly. In 1D NMR direct binding experiments mixtures can be screened without subsequent deconvolution, provided that reference spectra for the ligands in the mixture have been recorded. Both 2D NMR screening and 1D NMR reference displacement screening requires deconvolution to identify putative hits in a mixture. This can be done in several ways, for instance by the subsequent addition of each component of the mixture until the hit has been identified. In general terms when using mixtures, hundreds of compounds can be screened per day, which means that a full NMR screen of 3000–4000 compounds generally takes anywhere from a few days to several weeks depending on screening

mode. Data analysis of either 1D or 2 NMR screening data is straight forward and can be done with standard NMR software. However, semi-automated solutions may be beneficial when dealing with large quantities of data. Screening libraries are generally divided into general sets and target specific sets based on prior knowledge about the protein structure and/or known ligands. A typical NMR screen will encompass 500–4000 fragments. In order to perform a 1D NMR screen typically at least 10 mg of expressed protein is required. Typical conditions are 1–10 μM protein and 30–1000 μM ligand depending on which experimental method is used, but ligand concentration may be even higher with the reference displacement method. A 2D NMR screen typically requires at least 50 mg of isotope labeled protein, sometimes significantly more. Typical conditions are 50 μM protein and 400–1000 μM ligand. In some cases it may be possible to reduce protein consumption by recycling the protein, provided that the protein is sufficiently stable over time. NMR screening can be conducted over wide range of buffer conditions. Although many buffer components, as well as DMSO, give rise to NMR signals, these are either not observed in the NMR spectrum, as is the case with 2D NMR, or can be suppressed during 1D NMR experiments. Many buffer components are also available in deuterated form, making them NMR-silent.

A rather unique niche for NMR screening is the ability to identify binders to a second adjacent site once binders to a primary site have been identified. A major challenge to such experiments is that the affinity for a second site tends to be very weak. Furthermore, it may be difficult to distinguish second site binding from binding to the primary site. There are NMR methods that cope with both of these challenges. Inter-ligand NOE measurements can be used to screen for binders adjacent to an identified binder (Li et al., 1999). Another approach is to chemically link a spin-label to an identified binder. Only ligands binding simultaneously to the spin-labeled compound are identified in regular 1D NMR screening experiments (Jahnke et al., 2001).

The recent example of prostaglandin D synthase (PGDS) illustrates how NMR screening can be used to drive a fragment-based lead generation campaign (Hohwy et al., 2008). The modest size of the protein (23 kDa) and the fact that it could be produced from *E. coli* routinely yielding up to 75 mg/l, made this target readily amenable to 2D NMR. This approach was also particularly suitable due to the lack of high affinity reference compounds at start of the campaign, and allowed for the exploration of alternative binding sites to the substrate binding pocket. Chemical sift assignments were not obtained but HSQC peaks corresponding to residues in or near the active site could readily be identified when reference compounds were added. Initially a set of 2500 fragments, 450 from a targeted set and 2000 general fragments, was screened. Compounds were screened in mixtures of 12 at 400 μM with 100 μM of 15 N-labeled PGDS, giving a K_D cut-off of roughly 500 μM. The primary screen was effectively completed in 9 days. This resulted to the identification of 24 primary hits (a 1% hit rate), which were subjected to K_D determination in the NMR assay, with affinities in the range of K_D 50–500 μM. X-ray structure determination of protein–ligand complexes with the hits enabled a hit optimization process whereby increasingly more potent inhibitors from our compound collection were identified. Two iterative cycles of analog screening were carried out, comprising NMR screening, molecular modeling, X-ray crystallography, and *in vitro* biochemical testing for PGDS glutathione-*S*-transferase activity. Nine high-resolution PGDS complex structures were determined and 300 hit analogs were tested in total, resulting in a detailed pharmacophore model. This rational drug design procedure culminated in the discovery of 24 compounds with an IC_{50} below 1 μM in the *in vitro* biochemical assay. The best inhibitor (IC_{50} = 21 nM) is one of the most potent inhibitors of PGDS described to date with an excellent ligand efficiency of 0.65 kcal mol^{-1} per heavy atom.

The example of BACE-1 discussed earlier (Geschwindner et al., 2007; Edwards et al., 2007) illustrates another effective application of NMR in fragment screening. A set of 2000 general fragments were screened using 1D NMR waterLOGSY competition experiments. Compounds were screened in mixtures of six at 300 μM each with 3–5 μM BACE, resulting in a hit rate of 0.5%. One of the primary hits, an isocytosine, that gave a K_D of 4.5 mM in the SPR assay was investigated further. Initial fragment SAR could be generated using related analogs available from our compound collection. The magnitude of the displacement effects observed in the waterLOGSY experiments correlated very well with the single-point SPR measurements. This example illustrates nicely how the two orthogonal affinity screening methods complemented each other and increased confidence in the results. Combining multiple affinity-based methods is indeed a common way to guide the evolution and design of fragments, until affinity has improved sufficiently such that activity can be measured in conventional biochemical and cell-based assays.

7. Spectrometric methods – MS

The application of mass spectrometry to ligand screening has developed in a number of directions over the past 20 years. Advances in both instrumentation and techniques have broadened the scope of MS such that protein–ligand binding affinities, binding site information, and even induced conformational shifts can be studied. The diversity of MS methods encompasses many widely varying approaches that cannot be satisfactorily addressed within the scope of this review. Our intention is to briefly outline the principles of MS techniques, focusing attention on the relative merits and disadvantages in the context of ligand screening and analysis, rather than MS instrumentation or experimental detail. We have made an attempt to bias the article towards what we subjectively perceive to be the more relevant MS strategies offering potential for screening, while acknowledging that this categorization is in itself an area open for discussion and debate.

MS methods for ligand study may be loosely grouped into four basic approaches: (1) direct detection/identification of the ligand, (2) detection of the protein:ligand complex, (3) analysis of the ligand binding site and in the case of an enzymatic assay, ligands may be identified indirectly by monitoring substrate or reaction product concentration (4).

The first of these four categories itself encompasses a number of discrete techniques that share a common principle, whereas the latter three approaches are more easily defined.

7.1. Direct detection of ligand

The first and most highly populated group of techniques is characterized by MS analysis of the ligand itself. MS analysis of small organic molecules is well-established, routine technology. One clear strength of MS data in ligand detection is that hits can be identified by mass (or by mass fragmentation signature with MS/MS) from a mixture of candidate compounds. The novel part of these techniques is derived from the initial separation of ligands from non-binding compounds prior to the MS analysis step, and has been tackled in different ways via size exclusion chromatography using either centrifugation or liquid chromatography, ultrafiltration, equilibrium dialysis, frontal affinity chromatography and ligand quantitation. The first four of these approaches are better suited to LC–ESI-MS analysis since LC or ionization parameters can be adjusted to promote protein denaturation thereby releasing bound ligand prior to MS detection. In the case of the latter two techniques, the analysis relies only on detection of free ligand in solution and is amenable to a wider range of ionization mechanisms.

For size exclusion based separations, target protein is incubated with one or a mixture of potential ligands before chromatography facilitated by either centrifugation or an LC pump (Annis et al., 2007; Siegel, 2007). In both cases the chromatographic step rapidly separates unbound small molecules from ligands bound to the target protein. The latter are analyzed and identified by LC–ESI-MS. Naturally these two subtly different approaches share a number of pre-requisites, advantages and drawbacks. A relatively pure source of target protein must be available, since the presence of significant quantities of contaminating proteins will lead to an unnecessarily high level of false positives. The size exclusion approach is amenable to higher throughput screening by virtue of its ability to deal with incubations with multiple compounds, and this is a parameter which heavily influences the consumption of target protein per compound tested. The centrifugation-based separation can be performed in a 96-column well plate format, the consumable hardware for which is commercially available (e.g. Pierce Zeba 96-well Spin Desalting Plates, Thermo Fisher Scientific, Rockford IL USA).

Owing to the simplicity of the read-out, assay development is minimal when compared to more conventional screening methods. Size exclusion chromatography has excellent compatibility with incubation buffer components. Ligand binding affinity estimations can be made via saturation titration experiments, though in practical terms this becomes difficult when the binding affinity becomes weaker ($K_d \geqslant 20\ \mu M$; Siegel, 2007). This binding affinity restriction probably also represents the practical limit for ligand detection, making size exclusion approaches unsuitable for fragment screening. However, it has been amply demonstrated that these size exclusion/MS approaches can be effective and are established methods for drug discovery screening (Siegel et al., 1998; Muckenschnabel et al., 2004; Blom et al., 1999; Annis et al., 2004).

Using ultrafiltration the target protein is incubated with potential ligands, typically subsets from combinatorial libraries. Post incubation the sample is concentrated in a centrifugal device fitted with a semi-permeable membrane. The sample is treated to two successive rounds of dilution and concentration, such that unbound compound able to pass through the membrane will become diluted to an insignificant concentration. Bound ligands are retained in the protein-containing sample, and are identified by LC–ESI-MS. The implementation of this screening technique has been demonstrated as part of a drug discovery process (Comess et al., 2006; Wendt et al., 2007), and shares similarities with size exclusion chromatography in its applicability and capabilities. Again, throughput and protein consumption depend largely on the number of compounds used per incubation. Ultrafiltration is also compatible with a wide range of incubation buffer components, and assay development is straightforward. The limit of ligand binding affinity detectable by this method is considered to be $\sim$10 μM, and K_d estimations are possible by analyzing filtrate samples in addition to the protein-containing sample (Cloutier and Comess, 2007).

Equilibrium dialysis (ED) entails an alternative use of the semi-permeable membrane, in which compound mixtures are allowed to fully equilibrate between two chambers separated by a membrane, one chamber of which contains target protein. The contents of each pair of chambers are analyzed quantitatively by LC–ESI-MS; bound ligands should be identifiable by a bias in concentration towards the protein-containing chamber (Wan and Rehngren, 2006). ED has the advantages that it has good compatibility with a wide range of buffer components and requires no modifications to the protein, simplifying assay development. However, protein consumption will be high and throughput relatively low unless large combinatorial compound libraries are used.

Frontal affinity chromatography is a fundamentally different approach in which the target protein is immobilized onto a liquid chromatography column bed. Compounds are injected and detected post column by ESI-MS; modulation in retention volume indicating a degree of binding to the column enables ligands to be identified (Chan et al., 2003). The introduction of known ligands into the system allows competition experiments for the estimation of binding affinity of a second ligand. This technique has been shown suitable to produce more refined, lower throughput data such as ligand ranking (Chan et al., 2007). FAC has been implemented as a form of high-throughput screening with the use of combinatorial compound libraries (Ng et al., 2005), but is not widely regarded as a universally applicable screening technique due to the unpredictable step of protein immobilization. This step can be time-consuming and lead to a relatively high requirement for target protein, and depending on the form of implementation, the chromatography step is not necessarily compatible with the full range of buffer components.

The final category of direct ligand quantitation covers a wide spectrum of potential assay formats in which the utilization of MS for the final quantitation seems relatively incidental. It is a broad topic that has been fully discussed in another publication (Höfner et al., 2007), from which this article cites a number of references and is recommended as a further source of information regarding all uses of MS in ligand study or screening. A central theme among the possible assay formats is to incubate compounds with target protein, separate the protein (e.g. by precipitation), and monitor the modulation in free (non-bound) compound concentration by quantitative MS. Alternatively, the separated protein fraction may be treated to release bound ligand, and this may be detected quantitatively by MS. Variations in experimental design include competition experiments using known ligands, which provides binding affinity data. The technique does not lend itself to the use of combinatorial compound libraries which has a negative effect on throughput, and a greater cost in the amount of target protein consumed.

7.2. Detection of protein–ligand complex

A relatively recent enhancement of electrospray ionization (and nanoelectrospray ionization) has been the development of robust systems that allow folded proteins (or multi-subunit assemblies) to be ionized without disrupting tertiary or quaternary structure (Ashcroft, 2005; Tito et al., 2000). There is evidence to suggest that alterations to protein structure due to desolvation under vacuum can be sufficiently subtle that the use of mass spectrometry to study intra- and intermolecular interactions is valid (Patriksson and Marklund, 2007). This technique (sometimes referred to as 'native mass spectrometry' (van den Heuvel and Heck, 2004) was found capable of identifying and studying noncovalent protein ligand complexes under near-physiological conditions (Daniel et al., 2002; Heck, 2008). The concept of using native MS as means of screening for ligands is well documented (Hofstadler and Sannes-Lowery, 2007; Vivat Hannah et al., 2010) and has been suggested as a viable approach for the more challenging task of screening fragment compounds (Erlanson et al., 2004).

Typically, the protein concentration required for native MS tends to be in the region of $\sim$10 μM. In addition, the practical limit in employing combinatorial libraries has been found to be limited, particularly if weaker ligands are to be identified. When individual incubation volumes are taken into account, all these factors mean that native MS is not easily employed in high-throughput screening due to the amount of highly purified target protein required (author's own findings, unpublished data). However, native MS's ability to identify relatively weak interactions (Swayze et al., 2002) together with the relatively small size of fragment compound libraries mean that native MS has potential for fragment compound screening.

A future direction for small molecule ligand study by native MS is in the use of ion mobility spectrometry coupled to mass spectrometry (IMS-MS). This configuration of instrument enables an ion's average cross-sectional area to be estimated along with its mass. This allows discrete conformations of a given protein to be differentiated (Smith et al., 2007), and where a ligand induces a specific conformational shift, it may be possible to characterize the ligand binding mode as a result of the conformational shift identified.

The tethering concept requires the target protein to have an accessible cysteine residue on its surface, and ideally, close to a known site of potential ligand binding (either a naturally occurring cysteine or an artificially introduced mutation). The technique utilizes compound libraries in which members all contain a thiol group (typically, relatively low-molecular weight compounds often referred to as fragments) and a low concentration of a reducing agent, such that binding of fragments via a disulfide bond is reversible. However, if the fragment has some affinity for binding to the protein in addition to the disulfide bond, this will stabilize the protein-fragment conjugate to allow detection and identification by MS. Fragments can subsequently be elaborated or combined with one another to yield higher affinity ligands (Erlanson et al., 2004). As a consequence of the specific modifications to the target protein that may be necessary, and also of the specialized compound library required, developing this form of assay requires significant effort. While not being amenable to high-throughput screening, tethering is ideally suited to fragment screening (Cancilla et al., 2008).

7.3. Analysis of the ligand binding site

When a protein is exposed to an environment of D_2O solvent, the backbone amide hydrogen atoms tend to exchange to deuterium at a rate that depends on their individual environments. The presence of a ligand bound to the protein can offer a level of protection from the solvent and therefore slow the rate of H/D exchange for the backbone amides at its binding site. This difference in H/D exchange (between ligand-bound and ligand-free samples of protein) is detectable by mass spectrometry, and the concept has been used to design different variations of experiment. One of the more prominent is the use of enzymatic digestion post deuterium exchange to fragment the target protein, and to identify the peptide(s) whose deuterium exchange mass shift is affected by pre-incubation with ligand (prior to H/D exchange and digestion). This latter technique allows the site of ligand binding to be mapped with considerable precision (Chalmers et al., 2006). Protein consumption is high and experiments tend to be designed with single ligands, making this technique much more suited to ligand characterization than screening. The sizeable topic of H/D exchange is discussed much more fully elsewhere (Zhu et al., 2007; Hamuro et al., 2007).

7.4. Enzymatic assay/MS

The principle behind this approach can be readily appreciated. Briefly, an enzymatic process in which a substrate is converted into a product can be monitored by measuring the concentrations of substrate, product, or both. When screening compounds in an enzymatic assay, these may modulate substrate/product concentrations and thereby identify potential inhibitory (or effector) ligands. In this approach the use of MS is merely the means of quantitatively monitoring those concentrations. It does, however, have the advantage that the use of fluorescent labels, radioactive isotopes, or other labeling systems are not required for assay development. This screening technology is established and commercially available (Jonas et al., 2009). The potential for high-throughput screening with this technology is clearly good, although the rate of consumption of target protein will depend on whether the screen incubates with individual compounds or combinatorial libraries. However, both approaches will use catalytic amounts of enzyme, which may be advantageous compared to the other biophysical methods exemplified in this review. Compatibility with buffer components may not be as flexible as other techniques, but ligand activity measurements can clearly be made using this approach.

Table 1 provides a superficial summary of each the MS approaches in the context of ligand screening/analysis, but is necessarily subjective and open to debate. In particular, combinatorial compound libraries that are commonly applied to these techniques greatly affect both the throughput potential and the consumption of protein. The column designating potential for K_D estimation could also be a contentious subject; some techniques require much more effort or consume much greater quantities of protein than others to achieve a similar quality of affinity estimation. However, in compiling the table, an attempt was made to reflect the manner in which the techniques have been implemented in published literature.

8. Summary and future perspectives

The biophysical methods and approaches described here have become a widely accepted and well-implemented tool-box that allow for a detailed interrogation of protein–ligand interaction. If selected carefully they can provide a set of highly complementary but unique data that enlights the protein–ligand interaction from multiple viewpoints. The value of applying orthogonal methods

Table 1
Summary of important parameters to be considered for ligand screening and analysis for the different MS approaches.

Technique	Throughput	Consumption of target protein	Covalent modifications tags immobilization	Compatibility with cofactors, salts, metals etc.	Ligand identification	K_D estimation	Fragment screening compatibility
SEC/centrifugation	Excellent	Intermediate	None	Excellent	Yes	Intermediate	Poor
SEC/LC driven	Good	Low	None	Excellent	Yes	Intermediate	Poor
Ultrafiltration	Good	Low	None	Excellent	Yes	Intermediate	Poor
Equilibrium dialysis	Poor	Intermediate to high	None	Excellent	Yes	Good	Poor
Frontal affinity chromatography	Poor	Intermediate to high	Immobilization	Intermediate	Yes	Good	Poor
Ligand quantitation	Good	High	None	Good	Yes	Good	Poor
Native MS	Intermediate	Low	None	Very poor	Partial	Good	Intermediate/good
Tethering	Intermediate	Intermediate	Introduction of surface cysteines	Good	Yes	No	Excellent
Deuterium exchange/MS	Very poor	High	None	Good	No	Poor	Poor
Enzymatic assay/MS readout	Excellent	Target and screening strategy dependent	Variable	Poor	No	Good	Poor

can been easily envisaged via the exclusion of model system inherent artifacts, as some of those technologies can provide a more integrative response including multiple effects like, e.g. ITC. The versatility of the available technology platforms is making a critical contribution to the full mechanistic understanding of ligand binding by making use of the unique strengths that each of those biophysical methods provide and will in some cases even serve as an enabling approach in the screening for novel ligands, e.g. in fragment-based lead generation.

Furthermore one can not underestimate the high value of some of the described biophysical methods for the quality control of recombinant proteins that are subsequently used for biophysical screening and/or in structural studies. A thorough investigation using preferably a combination of different biophysical methods will provide confidence in the reagents and will ensure that specific and relevant results are obtained. Very often the production of recombinant protein is primarily focusing on increasing the amount of soluble target protein and sometimes lacks the proof of functionality early in that optimization process. As an example, biophysical tools like ITC or Biacore ISA can be used to assess the functionality by simultaneously determining the ligand binding capability and capacity. A comparison of the stoichiometry obtained by ITC with the known number of binding sites allows estimation of the proportion of functional protein. If the protein concentration that is used in the Biacore ISA is higher than the K_D value of the test ligand (meaning it is above the tight binding limit), an estimate of functional protein concentration can be obtained as well. Additionally, some of these methods can be very effectively applied during the optimization of recombinant protein production and/or protein storage conditions. As an example, measuring the thermodynamic stability using thermal shift assays is very suitable for quickly optimizing reagent buffer conditions for long-term protein storage or for identifying an appropriate protein construct to be moved forward into large-scale production.

The recent technology development as well as method expansion, the significant reduction of reagent consumption as well as the availability of high throughput biophysical platforms have positioned them very well in the drug discovery process, where those biophysical parameters can and will be used to optimally drive the rational design of novel, improved lead compounds. An important aspect in that process is the understanding of the biological relevance of those parameters and how those link to improved efficacy and we still have some way to go in order to unleash the full potential of all the data for drug discovery. There is the associated risk that the identified binders will not display the desired activity, in particular if not great care has been taken in producing a biologically relevant protein construct or if the protein structure is compromised in any way during the binding process. The use of OWG in cell-applications can serve as a tool that helps bridging that gap between *in vitro* and *in vivo*, as many of the systems that are studied by affinity-based methods are intentionally artificial and simplistic in nature due to the applicability of many orthogonal methods and the clear linkage between actions and responses. Also other affinity-based technologies are having their play that have not been covered in this review, but it is imperative to make a careful technology choice based on the type of information that is required as well as the limitations that are set by the system to be studied. The use of some of those technologies can be severely constrained to a small number of high-value measurements so increasing the throughput and minimizing the protein consumption have become a continuous trend within the industry. For example recent efforts in thermodynamic approaches using enthalpy arrays are holding some promise to apply the concept of thermodynamic profiling onto a wider range of biomolecular interactions and systems (Recht et al., 2008). Also some of the key limitations of optical methods that relate to the requirements for immobilization as well as the sensitivity limit can be hopefully addressed in the near future by techniques that allow for the exact measurement of refractive index changes in free-solution irrespective of the size of the protein–ligand complex. Back-scattering interferometry can display such an opportunity as it is capable to provide a label-free, homogenous and mass-independent detection in free-solution with much lower requirements on sample quantity and purity (Bornhop et al., 2007). This could open up for biophysical studies on systems that are currently not amenable for small molecule ligand-binding studies using currently implemented affinity-based technologies. So the future looks bright for the application of affinity-based technologies in drug discovery as well as recombinant protein quality control and it is expected that the impact and scope of those methodologies will significantly grow over the next couple of years.

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