Supporting material to

Additional text in black, text also given in the printed work in blue

ITC and NMR

Isothermal titration calorimetry (ITC)

ITC has gained much attention in drug discovery in recent years, because it has become apparent that enthalpy-driven optimization of hits and leads is favorable for obtaining compounds with balanced potency and physicochemical profile[1-3]. Hence, apart from applying van't Hoff analysis (temperature-dependent assessment of binding constants), ITC is at present the gold standard for decomposing binding free energies (ΔG) into enthalpy (ΔH) and entropy (ΔS) contributions. It gives direct access (Figure X1) in the measurement to ΔH, the binding constant K_d, and the stoichiometry n, while ΔG and ΔS are then derived by calculation from the primarily measured properties[4]. Further favorable features of ITC are that it is a label-free technique and it does not require immobilization of ligand or protein. During the experiment one component of the ligand-receptor complex is titrated into the other component and the incremental heat changes in µcal for each step of the titration are measured. This raw data is converted to a binding isotherm that needs to be fitted to a suitable binding model by non-linear least squares fit in order to retrieve the desired thermodynamic parameters[4,5]. The shape of this binding isotherm is represented by the ratio of the receptor concentration divided by the dissociation constant, also called the c value. It is generally accepted that c values in a certain range (5 to 500[5], better: 10 to 100[4,6]) provide the best sigmoidal shape for obtaining reliable K_d values. However, ligand and protein solubility can strictly limit the achievable c value, particularly for weak binders[7]. It has been demonstrated that for low affinity systems, even at $c < 10$, ITC can yield reasonable K_d values, given that the binding stoichiometry is known, protein and ligand concentrations are accurately measured, the signal-to-noise level is appropriate and the titration is pursued almost up to saturation levels[7]. However, enthalpies derived under such "low c" conditions should be interpreted only with great caution. In contrast, at "high c" conditions, the binding isotherm approaches a rectangular shape, from which no K_d value can be derived anymore. Still the enthalpy is well defined in this case. Based on these requirements, typical K_d values that can usually be determined by ITC, can range from low nM to high µM affinities. Measurement of high affinity ligands is limited predominantly by

the sensitivity of the machine to detect very small heats produced by the low concentrations necessary for an acceptable c value. In contrast, measurement of low affinity ligands is typically limited by the solubility of protein and compound required for obtaining a high enough c value. In some cases, inverse titrations or competition-based design of the experiment can help to overcome these limitations.[8]

Without a doubt, proper design of an ITC experiment is important for the quality of the resulting data. Still, a large variety of issues and systematic errors need to be avoided to produce reliable data. The accuracy of both the ligand and protein concentrations is certainly an important factor. Thus, they should be verified by additional analytical procedures. Because all processes that produce or require heat (e.g. mixing/dilution, protonation/deprotonation, aggregation/precipitation, alternative reactions, etc.) can interfere with the experiment quite substantially, ligand and protein buffers must be matched exactly and proper control experiments should always be performed. When the ligand is the titrant, such controls can include titration of buffer into buffer, ligand into buffer, and buffer into protein to highlight mismatches and dilution effects. If the same injection volumes are applied in the experiment and the controls, the experimental data may be corrected for dilution effects of the ligand and/or the protein by subtracting the controls[6]. A further source for introducing noise and artifacts into the titration experiment is the presence or generation of bubbles in the cell, which will lead to spurious heat signals that can compromise the integration and produce errors in the curve fitting to the binding isotherm[6]. To avoid this issue, degassing and cell loading protocols and equipment is typically supplied and recommended by the manufacturers. In consequence, sample preparation and setting up the experiment both requires time and attention to details. Automatization can certainly standardize and particularly speed up the process.

Various studies investigated the statistical errors in ITC curve fitting[9]. One parameter that can influence the quality of the curve fit is the number of injections and their volume. With an increasing number of injections more data points of the binding isotherm become available for the fitting. However, the volume error and heat error for injecting rather small volumes of titrant will limit the number of data points[6]. Thus, in some cases, fewer injections with significantly larger integrated heats can be favorable. It should be noted that the number of injections also strongly affects the duration of the titration, because after each injection the baseline needs to be stably reestablished[5]. In order to increase experimental throughput and, consequently, also foster the repetition of experiments, reduced injection and single injection methods have been proposed[5]. With the single injection method (SIM) a speedup of about 5-fold is plausible compared to a regular approach using 20 injections.

Another important step toward higher throughput is miniaturization. When reducing the cell volume by a factor of 7 to 200µl and the syringe volume to 40µl, MicroCal's iTC_{200} shows improved response times, reequilibrates faster, has a higher absolute sensitivity (by a factor of 2.5), and fairly reduced protein consumption (down to lower µg quantities for a 20kDa protein)[5].

In addition to precision, accuracy and repeatability, reproducibility is an important factor in ITC measurements, particularly, for the reported absolute values of ΔG, ΔH, and ΔS. Because of the multitude of possible pitfalls when planning, conducting, and interpreting an ITC experiment, it has been suggested that as a common standard, some validation reactions (e.g. Titrations of tris base in nitric acid, silver nitrate with sodium iodide/bromide, or bovine carbonic anhydrase II with CBS) should be performed and reported together with newly determined data[10].

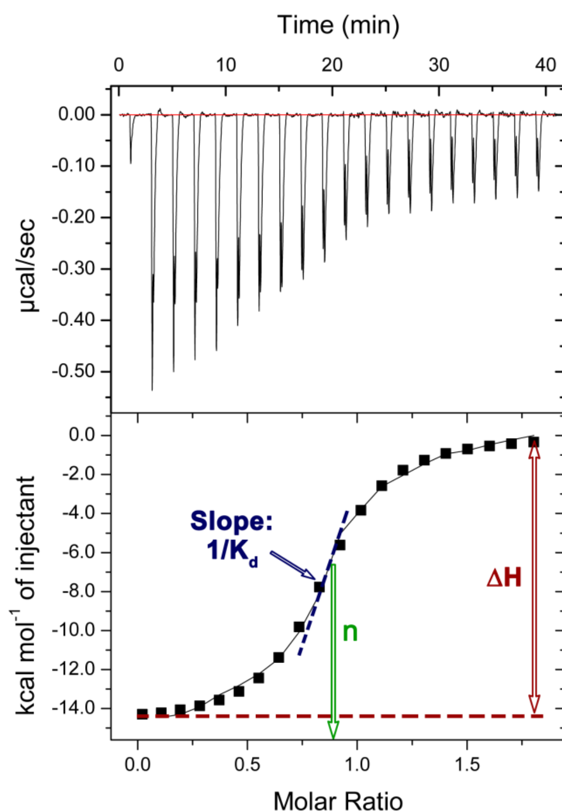

Figure X1: Raw data (upper panel) generated by an ITC experiment representing the heat released (or absorbed) during the duration of the titration (µcal/sec). Ideally, this heat should directly reflect the amount of binding, however, all reactions that can produce or consume heat can, of course, influence the measurement. This raw data is converted into the binding isotherm (below) by integration of each injection

peak giving the thermal energy (ΔH) of each titration step. Upon saturation of the protein in the cell with added ligand, the signal is reduced until only the background heat of dilution remains. Corrections for such heats (e.g. using control experiments) are essential. From the binding isotherm (heat in kcal/mol plotted against the molar ratio of ligand/protein), the change in enthalpy ΔH, the stoichiometry n, and the dissociation constant K_d can be derived after fitting the data to an appropriate binding model. The change in enthalpy is represented by the distance between the two asymptotic lines (red arrow) corresponding to the minimal and maximal heat formation. n is molar ratio at the inflection point (green arrow) of the sigmoidal curve. The slope at the inflection point (blue line) reflects the association constant ($K_a = 1/K_d$). ΔG and ΔS can be calculated from K_d, ΔH, and T. The figure was adapted from [4,11].

Nuclear Magnetic Resonance

NMR is of widespread use in rational drug design campaigns. Besides it classical applications for elucidating the constitution and structure of small organic molecules and, as an alternative to X-ray, the determination of the 3D structure of bio-macromolecules, it provides sensible probes for screening ligand binding to bio-molecular targets like proteins and nucleic acids [12-20]. Many successful applications have been reported from pharmaceutical and biotechnology companies in which NMR methods have been employed for hit identification, validation, and/or elaboration [12]. Many detailed reviews are available in the literature describing the large variety of NMR screening techniques [12-20] and we will only give a short summary here. Specific properties of the most important methods are summarized in Table 1. Depending on the signals observed in the experiment, the methods can be assigned to two categories: target or receptor-based and ligand-based. The first measures the change of chemical shifts of the macromolecule on ligand binding using 2D heteronuclear correlation spectra. The latter rely on the fact that many NMR observables differ in the complexed and uncomplexed state. A ligand in complex with its target takes over the dynamic properties of the latter. Therefore, it will experience much slower diffusion rates, slower tumbling leading to faster transversal relaxation and, in this way, to broadening of the signals, and negative intra-NOE signals. For a fast exchanging ligand, corresponding to a large k_{off} rate, these properties of the bound state are transferred to the free ligand in equilibrium and modulate the corresponding spectra. Since only the signals of the ligands are monitored here, specific labelling of the target is not needed and 1D ^{1}H-NMR spectra are often sufficient strongly reducing the acquisition time. Alternatively, methods relying on ^{19}F

resonances have started to be applied. Even if most applications use these methods to identify binders out of a large collection of molecules, identification of the binding epitopes of the ligand or the target as well as binding affinity determinations are possible directly or by replacement experiments.

	SAR-by-NMR	**STD**	**waterLOGSY**	**Spin labeling**	**Diffusion Editing**
protein > 30 kDa	Limited	Yes	Yes	Yes	Yes
Protein > 10 kDa	Yes	No	No	Yes	Yes
Labelling	Yes	No	No	No	No
Protein Binding Epitope	Yes	No	No	No	No
Ligand Binding Epitope	No	Yes	Yes	No	No
Amount of Protein at 500 MHz	25 nmol	0.1 nmol	25 nmol	~1 nmol	~100 nmol
K_D Tight Binding	No limit[(a)]	100 pM	100 pM	100 pM	~100 nM
K_D Weak Binding	~1 mM	~10 mM	~10 mM	~10mM	~1 mM
Identification of Ligand	No	Yes	Yes	Yes	Yes

Table 1: Comparison of methods for target- and ligand-based NMR screening in drug discovery (adapted from Ref. 17)
(a) Quantitative analysis can be compromised by line broadening in the intermediate-exchange regime

Target-based techniques all rely on chemical shift perturbation (CSP) on ligand binding, which are caused by changes in the electronic environment of atoms in the target due to the interactions with the ligands. These shifting signals are followed mainly in 2D ^{1}H-^{15}N correlation spectra, due to the need of only relative inexpensive ^{15}N labeling of the protein, but ^{1}H-^{13}C HSQC spectra of partially labelled proteins (e.g. methyl groups of Val, Leu, and Ile) can give more reliable results. One representative is the patented SAR-by-NMR approach [21,22]. To identify hits e.g. in a fragment library, mixtures of ligands can be screened. Those mixtures inducing CSPs in the target have then to be de-convoluted, i.e. each compound has to be measured individually, to identify the active ligands. After identification of a fragment for a specific binding

pocket, the screening are redone with a high concentration of this first fragment in the mixture. Fragments binding to other pockets of the binding site induce additional CSPs and high affinity ligands can be generated by linking the two fragments together. The two main advantages are the possibility to accurately determine binding affinities by non-linear fitting the size of the CSPs as a function of added ligand [17] and the identification of the binding epitope of the target (chemical shift mapping), if an assignment of the HSQC signals is available. For the latter it has to be kept in mind that changes in the conformation or the dynamics of the target can also lead to large CSPs even in regions further away from the ligand [23,24]. Disadvantages are the specific labelling and the high amount of protein needed as well as the long acquisition time for the 2D spectra. When applying this method for quantification of binding affinities in drug discovery projects (Figure X2)[25,26], there are several practical aspects that should be taken into account: (1) Trivial or uncharacteristic shifts should be discarded. Chemical shifts should be considered significant, if the average weighted $^{1}H/^{15}N$ chemical shift difference $\Delta\delta(^{1}H/^{15}N) = [(\Delta\delta(^{1}H))^{2} + (\Delta\delta(^{15}N)/5)^{2}]^{0.5}$ is greater than 0.04 ppm. This should help to avoid overinterpretation of meaningless peak deviations. Uncharacteristic shifts can only be identified by thoroughly studying the protein under modified buffer conditions. Some peaks might shift only because of small changes in the pH or ion concentration of the buffer system. Thus, it cannot be ruled out that ligands can manipulate the buffer conditions and therefore cause some chemical shift changes without actually binding to the target. (2) The number of concentrations should be sufficient for curve fitting. It is desirable to measure at least HSQC spectra for five different concentrations of the ligand. When fitting a quadratic saturation binding equation to the chemical shift difference data, the concentrations used should provide a decent definition of all relevant parts of this curve. It appears to be preferable to determine first the K_d value for each relevant chemical shift difference by making separate curve fits and then to calculate the mean K_d value and the standard deviations, instead of normalizing all relevant shift data and fitting the K_d to these normalized shifts as a whole. The statistic of K_d values obtained from multiple separate curve fits can provide additional insights about strange, possibly erroneous chemical shift changes. (3) Whenever peaks overlap or a shifting peak makes a transition though some other unchanged peaks, the shape of the peak can be distorted and it can become difficult to unambiguously identify the center of the peak and, thus, the correct chemical shift difference. In such a case the respective curve fit should be scrutinized and only used with caution. (4) Solubility of weak binders can be a strict limitation for obtaining reliable data. It needs to be taken into consideration that saturation with respect to solubility of the compound can mimic saturation of the binding site. Hence, chemical shifts might cease to increase upon use of higher concentrations only because the maximal soluble concentration of the

tested ligand had been reached. As a consequence, it should be ensured that before and during the measurement no precipitation has occurred.

Ligand-based methods can additionally be divided into two groups [12]. The amplifying methods include transferred NOE (trNOE) [27,28], saturation transfer difference (STD) [17,29], waterLOGSY [30,31], inter-Overhauser effect (ILOE) [32,33], inter-ligand NOE for pharmacophore mapping (INPHARMA) [34-36], as well as NOE [37] and inverse NOE [38] pumping are all based on nuclear Overhauser enhancement. Besides the possibility to identify binding, additional information on the bioactive conformation of the ligand can be obtained. TrNOE provides intra-ligand distances, which can be used to constrain the conformation e.g. in docking experiments [27,28]. Similarly, distances between ligands or fragments binding to the target at the same time are seen in ILOE experiments. In STD, magnetization is transferred from the target to the ligand, which is more effective at smaller distances. Therefore, it can quantify the relative closeness to the protein surface of parts of the ligand and, in this way, identify the binding epitope of the ligand [17]. In a similar way, INPHARMA uses the protein-mediated magnetization transfer between two competitive binding ligands, from which the relative orientation of the ligands in the binding site can be determined [34-36]. Finally, in waterLOGSY [30,31] the magnetization is coming from the solvent, which is then transferred to the free or bound ligand through the macromolecule. Since the signals of the just mentioned methods are proportional to the concentration of the bound ligand and accumulate in the course of several binding/unbinding events, they are superior to the second class, the non-amplifying techniques. In these latter, the signals, utilizing the enhanced relaxation or reduced diffusion rates of bound ligand, respectively, are proportional to the bound/unbound fraction of the ligand and, thus, low ligand concentrations should be used limiting the sensitivity. Therefore, possibilities to enhance the effects are desired. By immobilizing the target on a solid support as done in the TINS method (target immobilized NMR screening) [39], the molecular weight and, in this way, the relaxation are extremely increased. The relaxation can also be enhanced by introducing paramagnetic labels in the protein [40,41] or in reporter ligands or cofactors for second site screenings [42]. Compared to target-based methods, advantages of ligand-based screening are (1) the smaller amounts of the target needed in unlabeled form, (2) direct identification of the active compounds without the need to de-convolute the mixtures, and (3) that there is no upper limit for the target size [12]. However, they can result in false positives from unspecific binding to the target or to aggregation of other compounds in the sample. Using titration experiments of single ligands, binding affinities measurements were performed with most of the ligand-based methods (see e.g. [43-45]). Since fast exchange (large k_{off} rates) are needed especially for NOE-based experiments, the identification

of high affinity ligands is not possible directly. However, competition experiments can overcome this problem by using a reference ligand in the appropriate affinity range. This so-called spy, reporter, or probe molecule will be replaced by stronger binding ligands and the recovering of the signals of the free spy is recorded. Spy molecules are also expanding the use of ^{19}F NMR methods [46-48]. In contrast to proton-detecting experiments, there is no noise in fluorine spectra from the background from the target, solvent, or other solution components. Additionally, the signals are very narrow and have a very large spectral width, which allows for the analysis of mixtures with a large number of different ligands without spectral overlap. Unfortunately, the sizes of available fluorinated ligand libraries are limited and might not offer the diversity needed for specific screening studies. Still, one single hit of a weak binder from these libraries is already sufficient for competition experiments, in which the displacement of the fluorinated spy ligand by ligands from a large library is detected [49,50].

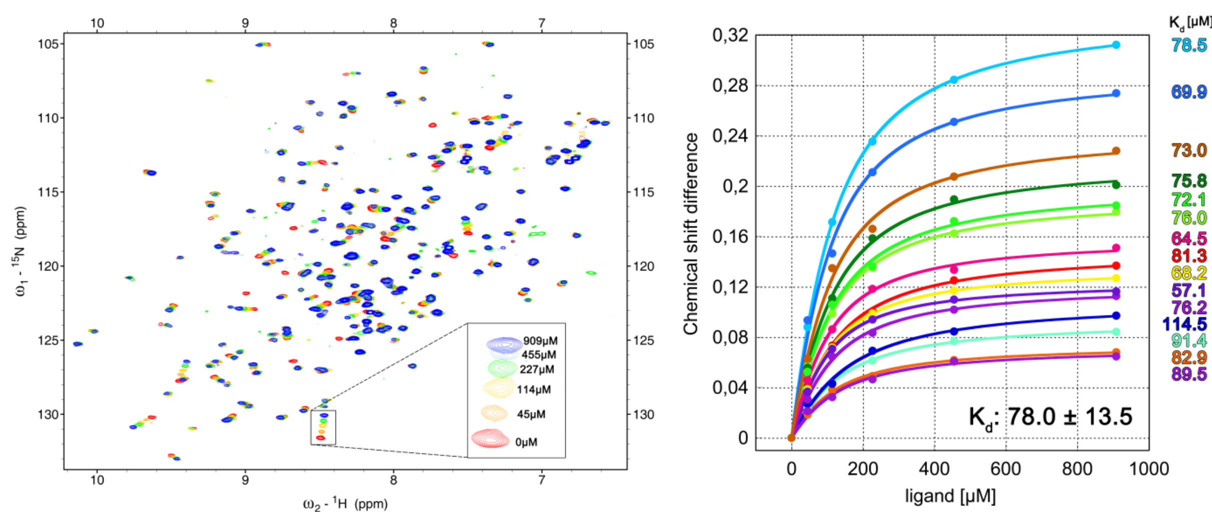

Figure X2: Overlay of ^{1}H-^{15}N HSQC spectra of protein without ligand (red) and increasing concentrations of a ligand (45µM, orange; 114 µM, yellow; 227 µM, green; 455 µM, cyan; 909 µM, dark blue). One of several significant chemical shift changes is shown as a close-up. Curve fits for all 15 significant chemical shift differences are shown at the right. The K_d value obtained from each individual curve fit is presented in color code next to the respective curve. The mean value can be calculated as 78.0µM, the standard deviation is 13.5µM.

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