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Ligand screening by saturation-transfer difference (STD) NMR spectroscopy.

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Abstract

NMR based methods to screen for high-affinity ligands have become an indispensable tool for designing rationalized drugs, as these offer a combination of good experimental design of the screening process and data interpretation methods, which together provide unprecedented information on the complex nature of protein-ligand interactions. These methods rely on measuring direct changes in the spectral parameters, that are often simpler than the complex experimental procedures used to study structure and dynamics of proteins. The goal of this review article is to provide the basic details of NMR based ligand-screening methods, with particular focus on the saturation transfer difference (STD) experiment. In addition, we provide an overview of other NMR experimental methods and a practical guide on how to go about designing and implementing them.

Key words

Nuclear magnetic resonance (NMR), ligand screening, saturation transfer difference (STD), protein, nuclear Overhauser effect (NOE) and relaxation.

I. Introduction

Nuclear magnetic resonance (NMR) spectroscopy provides a variety of powerful tools for identifying and characterizing protein-ligand interactions. Since the first report appeared in 1996 [1] describing the use of NMR spectroscopy to screen for potential drug molecules, the field of NMR-based screening has evolved rapidly. Over the last several years, a variety of novel approaches have been introduced and have found widespread application in both pharmaceutical and academic research settings. NMR screening has become an important component in an integrated arsenal of biophysical, biochemical, and computational methods designed to discover and optimize drug leads. The current state of the technology is also synergistic with combinatorial chemistry, high throughput screening, structure-based drug design, proteomics and metabolomics.

This review focuses on the theoretical and practical aspects of one of the most commonly used NMR-based screening approaches, the saturation transfer difference (STD) experiment. The emphasis is to introduce the theory of steady state nuclear Overhauser effect (NOE) and how it relates to STD spectroscopy in the presence of chemical change. For information on alternative NMR-based screening approaches and to a wide range of applications, the reader is directed to the many excellent reviews available on the subject [2-11].

The article is organized as follows: Section II presents the theoretical aspects of the chemical exchange phenomena that form at the foundation of STD-NMR screening experiments along with the equilibrium kinetics of ligand binding, and their importance in the measured NMR parameters. Section III elucidates the other commonly used ligand and receptor-based NMR approaches and their implementation. Section IV provides a

general experimental description of how to design and perform NMR-based ligand screening and section V concludes with the summary of the active areas of technology development for NMR based ligand screening.

II. Saturation Transfer Difference NMR Spectroscopy

Saturation transfer difference (STD) spectroscopy is a versatile technique for NMR-based screening. There are several advantages of STD experiments in comparison with other techniques (*vide infra*), such as reduced protein (or receptor) requirements, relative ease of implementation, and applicability to large molecular weight proteins [12]. As the name suggests, STD takes the difference of two experiments as shown schematically in Figure 1. In the on-resonance experiment, the receptor proton magnetization is selectively saturated via a train of frequency selective r.f. pulses. The r.f. train is applied to a frequency window that contains only the receptor resonances (e.g. 0.0 to -1.0 ppm where majority of the methyl resonances of folded proteins appear [13]). It is important that the choice of the on-resonance irradiation frequency does not overlap with any of the ligand resonances. The saturation propagates from the selected receptor protons to other receptor protons via the vast network of intramolecular ^{1}H-^{1}H cross-relaxation pathways. This process, also known as spin diffusion [14, 15] is quite efficient due to the typically large molecular weight of the receptor. As shown in Fig.1, the saturation is transferred to binding compounds via intermolecular ^{1}H-^{1}H cross-relaxation and chemical exchange mechanisms at the ligand-receptor interface. The small molecules then dissociate back into solution where the saturated state persists due to their small spin-lattice relaxation rate constants (R_{1L}). At the same time, more "fresh" unsaturated ligand exchanges on and off the receptor while saturation energy continues to enter the

system through the sustained application of r.f., increasing the population of saturated free ligands. A reference off-resonance experiment is then recorded that applies the identical r.f. train far from the on-resonance, such that no NMR resonances are perturbed (typically the carrier frequency is shifted by 25-50 ppm from the on-resonance experiment). The on and off resonance experiments are recorded in an interleaved fashion to avoid any experimental inconsistencies and minimize the effect of any r.f. induced sample temperature change. The resulting difference spectrum yields only those resonances that have experienced saturation, namely, the receptor and the binding compound resonances. Because they are present at minimal concentration, receptor resonances will usually not be visible and, if so, can be eliminated by R_2 relaxation filtering prior to detection. The result is a simple 1D ^{1}H spectrum that reveals only the binding compounds.

II.A. Theory

The performance of NMR-based ligand screening is based on three important and inter-linked concepts [16]; (a) The biochemistry of protein-ligand interactions governed by the equilibrium kinetics, (b) The phenomenon of chemical exchange with reference to NMR spectroscopy and (c) Relative difference between the time scales as measured by the NMR parameters. The theoretical basis for protein-ligand interaction follows that of the equilibrium enzyme kinetics [17]. A broader description of how the various kinetic parameters are influenced by different NMR experiments are given in detail by Peng and co-workers recently [2, 3] and this section focus on details relevant to STD experiments.

II.A. 1 Equilibrium kinetics of binding

Assuming that the protein receptor [E] has a single binding site for the ligand [L], in this case the STD indicator, the reversible binding process is then described by

$$[E] + [L] \rightleftarrows [EL]. \qquad [1].$$

The temperature dependent, equilibrium dissociation constant of the reaction is then given by

$$K_D = [E][L]/[EL] = k_{off} / k_{on}, \qquad [2]$$

where k_{off} , the unimolecular rate constant, is inversely related to the lifetime of the protein-ligand complex, while the bimolecular rate constant k_{on} is the probability of a productive interaction between the protein and the ligand. At any given time, the fraction of the bound ligand concentration L_B, is given by

$$L_B = [EL]/([E] + [L]). \qquad [3]$$

Combining equations (2) and (3) yields,

$$L_B = [L]/([L] + K_D). \qquad [4]$$

Equation (4) is one of the fundamental equations that elucidate the outcome of the NMR experiment. It shows that increasing the ligand concentration [L] will increase fractional occupation of the receptor-binding site L_B, in a hyperbolic function of [L]. In the limit $[L] << K_D$, the fractional population of the ligand is directly proportional to the concentration of the ligand. In the other extreme, $[L] >> K_D$, the protein receptor is completely saturated by ligand ($L_B = 1$). The interesting situation occurs when $[L] \approx K_D$, the receptor is half-saturated; that is, half of the receptor molecules exist in a one-to-one complex with the ligand. Therefore, the ligands with weaker affinity have larger K_D and require the addition of more ligand to saturate the receptor-binding site. Thus, by adjusting the receptor and ligand concentrations, it is possible to "select" the maximum K_D for an optimum observable NMR signal. This tuning of detection threshold is very useful in ligand based screening experiments and in particular the STD experiments, for

screening large compound libraries. At equilibrium, the transient presence of the ligand molecule at the protein site is responsible for chemical exchange, a parameter that is used exclusively by NMR methods in the ligand screening experiments.

II.A. 2 Transfer of magnetization between sites.

Protein-ligand biochemistry is responsible for bringing the receptors and ligands to close proximity and at equilibrium conditions, the ligand transiently adopts NMR parameters that are characteristic of the typically much larger receptor. Alternatively, from the receptor's perspective, the ligand transiently perturbs the binding site microenvironment(s), which may alter distribution of conformations sampled by the ensemble of receptor molecules. In either case, the exchange modulates the NMR parameters, such as relaxation rates, chemical shifts and NOE of both the ligand and receptor molecules [18, 19]. A ligand screening experiment optimizes the transfer of information through the magnetization difference between the receptor and ligand.

Due to inherent difference between the size of the receptor and the ligand, often spin relaxation based screening tend be the choice of the experimental scheme. Nuclear spin relaxation occur due to a range of mechanisms [20], of which the chemical exchange based mechanism is of critical importance in ligand screening experiments. It is important to design NMR experiments that can distinguish how relaxation and exchange phenomena affect the measured parameters. Exchange effects can be broadly classified into direct and indirect effects [21]. In the direct effect the relaxation of a spin is influenced by its own exchange mechanism, while in the indirect effect, the spin does not undergo exchange by itself, but exchange effects of another nearby spin is affects the relaxation of this spin. The direct effects are a result of physical transfer of nuclei between sites and result in magnetizations in all sites that are part of the network of exchanging spins. In

the following mathematical treatment, the transfer of magnetization between the protein and the ligand sites is initiated either by cross relaxation or chemical exchange or in some rare occasions both. In order to provide an insight to the experimental design, the expressions for STD NOE for a two-spin system is derived.

For a spin ½ nucleus exchanging between two sites, protein receptor (E) and the ligand (L), using the energy level diagram and the transition probabilities shown in Figure 2 we can derive the expression for STD NOE. The equation of motion for the line intensities is then obtained from the master equation for populations [21],

$$d\frac{P_i}{dt} = \sum_j W_{ij}\left[\left(P_j - P_j^0\right) - \left(P_i - P_i^0\right)\right] \qquad [5]$$

Where, P_i is the population of site 'i' (protein or ligand), W_{ij} represent the probability of a transition from state j to i and the superscript 'o' is the equilibrium value of the population. The intensity of the ligand and protein resonance, are then $P_{L\alpha} - P_{L\beta}$ and $P_{E\alpha} - P_{E\beta}$, respectively. Using the transition probabilities and populations from Figure 2 and from Eq.[5] the populations at protein and ligand site are written as [15],

$$d\frac{P_{E\alpha}}{dt} = -\left(W_{1E} + W_o\right)\left(P_{E\alpha} - P_{E\alpha}^o\right) + W_{1E}\left(P_{E\beta} - P_{E\beta}^o\right) + W_o\left(P_{L\beta} - P_{L\beta}^o\right) \qquad [6a]$$

$$d\frac{P_{E\beta}}{dt} = -\left(W_{1E} + W_o\right)\left(P_{E\beta} - P_{E\beta}^o\right) + W_{1E}\left(P_{E\alpha} - P_{E\alpha}^o\right) + W_o\left(P_{L\beta} - P_{L\beta}^o\right) \qquad [6b]$$

Taking the difference between equations 6a and 6b,

$$d\frac{\left(P_{E\alpha} - P_{E\beta}\right)}{dt} = -2\left(W_{1E} + W_o\right)\left[\left(P_{E\alpha} - P_{E\alpha}^o\right) - \left(P_{E\beta} - P_{E\beta}^o\right)\right] + W_o\left[\left(P_{L\alpha} - P_{L\alpha}^o\right) - \left(P_{L\beta} - P_{L\beta}^o\right)\right]$$

[7]

A similar equation can be written for the ligand ($P_{L\alpha} - P_{L\beta}$) as well. As the difference in the population is equal to the expectation values of the Z-magnetization, $\langle I_z \rangle$, Eq.7 for protein and ligand is written as

$$d\frac{\langle I_{zE}(t)\rangle}{dt} = -\rho_{1E}\left(\left\langle I_{zE} - I_{zE}^{o}\right\rangle\right) - \sigma_{EL}\left(\left\langle I_{zL} - I_{zL}^{o}\right\rangle\right) \qquad [8a]$$

$$d\frac{\langle I_{zL}(t)\rangle}{dt} = -\rho_{1L}\left(\left\langle I_{zL} - I_{zL}^{o}\right\rangle\right) - \sigma_{LE}\left(\left\langle I_{zE} - I_{zE}^{o}\right\rangle\right) \qquad [8b]$$

where $\rho_{1E} = 2W_{1E}+W_o$, $\rho_{1L} = 2W_{1L}+W_o$, $\sigma_{EL} = \sigma_{LE} = -W_o$, and in general $I_{zE}^{o} = I_{zL}^{o} = I_o$ the equilibrium population. Equation [8] is derived on the basis of Solomon's equation of relaxation coupled two-spin system [22]. ρ_{1E} and ρ_{1L} are also referred as the spin-lattice relaxation rates, R_{1E} and R_{1L} respectively. W_o can be defined on the basis of two mechanisms; intramolecular relaxation and chemical exchange. In the biomolecular limit ($\omega_o\tau_c >> 1$, ω_o is proton resonating frequency in radians and τ_c is the time the protein takes to rotate by one radian or rotational correlation time, in seconds). For ^{1}H-^{1}H cross relaxation, W_o is given by [20],

$$W_o = \sigma_{EL} = k_{DD}\,\tau_c/(r^6), \qquad [9]$$

where is k_{DD} is a constant ($\gamma^4\hbar^2/10$, γ: gyromagnetic ratio and $\hbar$: Plank constant), r is the internuclear distance between the spins.

For the exchange mechanism, W_o is defined as the probability per unit time of a nuclear spin transferring from the ligand to the protein and thus it can be defined as the first order rate constant for the exchange process as

$$W_o = \frac{Number\ of\ nuclei\ from\ Ligand\ transferring\ per\ unit\ time}{Total\ number\ of\ nuclei\ in\ the\ binding\ site\ of\ the\ protein} = \frac{k_{ex}[E]V}{[E]V} = k_{ex} \qquad [10]$$

where, [E] is the concentration of the nuclei in the binding sites of receptor (same as defined in Eq.[1]) and V is the volume of the system. The cross relaxation rate becomes

$$\sigma_{LE} = -W_o = k_{ex}, \qquad [11]$$

The mechanism for transfer of magnetization in this case is due to the biochemical nature protein ligand interaction as described in Eq.[1]. When the protein spin magnetization is saturated, STD experiment is similar to the conventional steady state NOE [21]. It is hence straightforward to solve the master equation under the steady state condition ($d\frac{\langle I_{zE}(t)\rangle}{dt} = 0$). Thus Eq.(8b) becomes,

$$\langle I_{zL}\rangle = I_{zL}^{o} + \sigma_{EL} I_{zE}^{o} / \rho_{1L} \qquad [12]$$

Using Eq.[11], in Eq.[12], with $I_{zE}^{o} = I_{zL}^{o} = I_o$, the fractional enhancement in the integrated intensity of the ligand resonance upon saturating the protein signal is given by

$$\eta_L(E) = 1 - \frac{k_{ex}}{2W_{1L} + k_{ex}} \qquad [13]$$

For $k_{ex} >> 2W_{1L}$, Eq.[13] yields $\eta_L(E)$ = -1, which physically means that the transfer of magnetization between ligand to the protein spins is much more rapid than the interconversion of α and β spins by the relaxation process operating at the ligand site. This regime is extremely useful for the STD experiments. However, two requirements for the exchange must be met in order for this to be useful.

(a) $k_{ex} < \Delta\omega$, where $\Delta\omega$ is the chemicals shift (in radians per second) between spectral lines corresponding to different exchange sites. If this condition is not satisfied, the lines will overlap and the NOE enhancements between them will not be possible.

(b) W_1 and k_{ex} must be similar magnitude in order that information on exchange rate can be derived from the STD NOE measurements. If the exchange is very slow ($k_{ex} << W_1$), then

$$\eta_L(E) = -\frac{k_{ex}}{2W_{1L} + k_{ex}} \approx \frac{-k_{ex}}{2W_{1L}} \approx 0 \qquad [14].$$

It is possible to estimate range of values of the ratio of $\frac{k_{ex}}{2W_{1L}}$ over which STD experiments will be beneficial. Assuming that the smallest measurable signal is 3% of Z-magnetization (NOE enhancement in Eq.[14]). Rearranging Eq.[14] leads to the limits of detectability as $-0.03 \geq \eta \geq -0.97$ and correspondingly $64.7 \geq \frac{k_{ex}}{2W_{1L}} \geq 0.06$. This rough estimate suggests the limits of the STD experimental methods in practice and it is however a range that is not readily available for many other experimental techniques for ligand screening.

II.A. 3 The fast exchange approximation

In the STD experiments, the ligand resonances are used to read out the protein-ligand complex formation. In the two-state equilibrium given by Eq.[1], ligand and receptor molecules will exist in either a free (L, E) or complexed (EL) state. In the free state, both receptor and ligand retain their intrinsic NMR parameters (e.g. chemical shifts, relaxation rates, translational diffusion coefficients). In each other's presence, the mutual binding affinity of ligand and receptor drives an exchange process that toggles both sets

of molecules between the free and complexed states. Here, the focus is on what is 'fast exchange' limit and how it is important for ligand screening experiments.

All the NMR based ligand-screening experiments assume that interaction is in the fast exchange limit; this is a necessary condition that only at this condition, the NMR based ligand screening is useful. These experiments are typically carried out with $L_T/E_T >$ 10 (ratio of total ligand concentration), and the binding compounds, have $K_D \sim 100$ μM. If k_{on} is well approximated by a diffusion-limited value (10^7-10^9 $M^{-1}s^{-1}$), then the slowest k_{ex} values lie in the range $1000 < kex < 100{,}000$ s^{-1}. Ligand-based NMR screening methods are primarily ^1H based; consequently, k_{ex} exceeds most differences in intrinsic ^1H relaxation rates and rotating frame precession frequencies, thus providing assurance that the fast exchange assumption is valid. In addition, at this limit the equations describing the kinetics are more attractive due to the algebraic simplicity.

In the fast exchange limit, the total effect of protein-ligand complex is simply the sum of the contributions of the protein and ligand. If 'Q' is the NMR parameter, then

$$\langle Q \rangle = P_B Q_B + P_F Q_F \qquad [15]$$

Here, $\langle Q \rangle$ is the exchange-averaged parameter between its free (F) and bound (B) states, with respective populations P_F and P_B. Observed differences between $\langle Q \rangle$ and Q_F provide a signature measure of receptor binding and often a indicator of a 'hit' in screening a large library of compounds. The ability to detect binding with adequate sensitivity depends critically on the bound state contribution ($P_B Q_B$) being significantly larger relative to the contributions from free state ($P_F Q_F$). However, typical screening conditions where $L_T >> E_T$, automatically make $P_B << P_F$ and subsequently $\langle Q \rangle$ is amplified in the bound state (i.e. $Q_B >> Q_F$).

Following the arguments of Peng at al [10], the population-weighted average of the NMR observable ⟨Q⟩ in Eq.[15] can be related to ligand binding affinity . Defining $\Delta Q = \langle Q \rangle - Q_F$, the differential response of a NMR parameter with respect to its free state, then

$$\Delta Q = (E_T/L_T)\,(Q_B - Q_F)[L]/([L] + K_D) \qquad [16]$$

Equation 16 is similar to Eq.[4] and essentially the Langmuir isotherm[23] rearranged and is a hyperbolic function of [L]; hence, increasing [L] increases ΔQ. To experimentally measured ΔQ, Q-related NMR signal intensities are measured in the presence of receptor and Q_F is determined either by performing a reference experiment for the compound in the absence of receptor or by designing an experimental protocol in which $Q_F = 0$. Equation 16, also demonstrates that ΔQ increases with ligand addition and then plateaus at $(Q_B - Q_F)$ when the binding site is saturated ($L_T >> K_D$). Provided the ligand solubility is not problematic, ligand titration data can be fitted to this simple relation to estimate binding affinity. Specifically, for a large ligand excess ($L_T/E_T >> 1$), one can approximate $[L] = L_T$, and a fit of ligand titration data to Eq.[16] to obtain estimates for K_D and $(Q_B - Q_F)$.

II.B Experimental Details of STD spectroscopy.

The pulse sequence for the STD experiment is shown in Figure 3, following the original design [12]. Figure 3a shows the pulse sequence when the spectra need to be recorded in D_2O and additional water-suppression schemes (Fig 3b or 3c) are inserted in the arrow when the samples are in H_2O. Typical saturation trains involve *N* repetitions of 50 ms frequency selective shaped pulses (e.g. Gaussian or Seduce) [24, 25] and longer saturation times (2-3 s) are necessary. Sometimes, a pulsed field gradient is applied after this saturation train to ensure that only *z*-magnetization remains, but not essential. A

90° (ϕ_1) pulse and subsequent spin-lock (ϕ_2) serves as a relaxation filter for residual receptor signals. Water suppression can either be achieved by WATERGATE block [26, 27] (Fig.3b) or DPFGSE (double pulsed field gradient spin echo) block (Fig 3c) [28] that simultaneously suppresses water while further relaxation filtering the residual receptor signal. On-resonance saturation yields a spectrum of intensity I_{SAT}, while off-resonance saturation yields the equilibrium value I_0. Appropriate phase cycling of the receiver subtracts the two intensities to yield the STD response $\eta_{STD} = \eta_L(E) = I_{STD}/I_o$ (Eq.[12]) as shown in the last row of Figure 1.

STD is ideally suited to receptors with large masses (>30,000 Da). Receptors with large molecular masses possess large rotational correlation time τ_c that enhance spin diffusion and, consequently, saturation transfer within the receptor and to the ligand. STD experiments require relatively low concentrations of receptor (~1 μM). STD spectrum contains signals only from the *bound* state of the ligand and corrections for free state contributions are not required. This is especially advantageous when an experimental design with high ligand-to-receptor ratios $L_T/E_T >> 1$ is employed.

Optimal setup, use and interpretation of the STD experiments require a familiarity with the exchange processes at work and have been the subjects of several methods development studies in the literature. To better quantitative the amplification of resonances in the STD experiment, Mayer and Meyer [25] introduced the "STD amplification factor" A_{STD}. Because the STD response arises directly from the receptor-ligand complex, it is proportional to [EL]. Hence, I_{STD} can be written as

$$I_{STD} = C.\ \alpha_{STD}.\ [EL] \qquad [17]$$

where C is a proportionality constant that makes the appropriate unit conversions and α_{STD} is a dimensionless scaling factor that represents the maximum STD amplification. I_{STD} corresponds to $\langle Q \rangle$, since there is no contribution from [L] (i.e. $Q_F = 0$), while the reference, or equilibrium, intensity I_o is just proportional to L_T ($I_o = CL_T$) and thus the steady state NOE, STD in this case (η_{STD}) becomes

$$\eta_{STD} = I_{STD}/I_o = \alpha_{STD} \cdot [EL]/L_T \qquad [18]$$

Since $[EL]/L_T$ is the bound fraction of the ligand [3], using the relation in Eq.[16] the STD amplification factor A_{STD} is given by

$$A_{STD} = \alpha_{STD} \cdot [L]/([L] + K_D) \qquad [19]$$

This equation is the hyperbolic dose-response curve and analogous to the well-known equation for the Henri-Michaelis-Menten enzymatic reaction rate v_0 [3, 29, 30]:

$$v_o = V_{max}[S]/([S] + K_M) \qquad [20]$$

Using an analogy to enzyme kinetics, A_{STD} will act in a manner similar to that of v_o: It will increase with increasing L_T until the maximum amplification α_{STD} is reached when the receptor-binding site is saturated ($L_T >> K_D$). After the point of receptor saturation, continued increase of L_T will monotonically decrease the fractional response of η_{STD}. In principle, if L_T well-approximates to [L], ligand titration data can be fitted to the form of Eq.[19] to estimate K_D and α_{STD}.

Following the enzyme argument, A_{STD} gives the average number of saturated ligands "turned over" per receptor. As such, it provides a convenient means for gauging the inherent sensitivity of the experiment. Mayer and Meyer provide an example in which A_{STD} =10, which implies that a receptor concentration of 50 μM yields an effective saturated ligand concentration of 500 μM [25]. The latter concentration is more than

sufficient for sensitive detection by current high-field magnets and probes. Additional motivation for using A_{STD} is that spectra from samples having different receptor concentrations may still be compared. This would be relevant for titration and competition experiments that may involve samples with variable amounts of receptor.

Assuming a diffusion-limited on rate, the K_D range of the STD method has been estimated to be $10^{-8} < K_D < 10^{-3}$ [12]. For weak binders having $K_D > L_T$, over half of the receptor molecules will have no ligand in the binding site. As K_D increases further, the population of the ligand-receptor complex [EL] decreases, which leads to a reduction and, ultimately, disappearance of the STD signal. For the case of strong binders, decreasing K_D increases the receptor -ligand lifetime ($1/k_{off}$) and thus decreases the exchange rate constant k_{ex}. At sufficiently small K_D, the free state residence times of the ligands can exceed their free state R_{1L} values. Under these conditions, exchange is so slow that the free ligand magnetization "forgets" its visit to the saturated receptor by relaxing back to equilibrium at a faster rate than the receptor is able to "turn over" newly saturated ligands. The initial population of saturated ligands decreases and ultimately disappears, thereby eliminating the STD signal.

Recent applications of STD experiments demonstrate their versatility beyond simple enzyme systems. For example, Benie et al.[31] have used STD to identify ligands targeting HRV2 (human rhinovirus serotype 2), a macromolecular complex of 8.5×10^6 Da. Meinecke and Meyer have characterized the interactions of peptide ligands binding to the extracellular region of an integral membrane protein (Integrin $\alpha_{IIb}\beta_3$) reconstituted in liposomes [32]. Other examples of alternative targets include small RNA fragments [33] and macromolecules immobilized on solid-support beads [34].

II.C Practical aspects of STD experiments:

The intrinsic sensitivity of the STD experiment is limited by the efficiency of the intended spin energy transfer pathway from receptor (source) to ligand (recipient). The main factor limiting magnetization transfer at the recipient (ligand) end is the ligand spin-lattice relaxation time (R_{1L}). Since the ligands are usually low-molecular-mass compounds (<1000 Da), the free state R_{1L} values are small and therefore any non-equilibrium magnetization state (like saturation) dissipates quite slowly. In contrast, when bound to the receptor, the ligand spin-lattice relaxation rate (R_{1L}) can be much larger. Hence, efficient spin energy transfer requires that the ligand dissociate from the receptor at a rate faster than the bound state relaxation time.

The source (receptor molecule) receives a constant influx of energy by the applied r.f saturation train. This constant energy input is what enhances the sensitivity of STD over other methods such as transferred-NOE spectroscopy. In practice, one often assumes 100% saturation of the receptor shortly after application of the r.f train due to intramolecular spin diffusion. However, the actual extent of receptor saturation depends critically on the competition between energy influxes and the various relaxation and/or "leakage" mechanisms [35-37].

The leakage mechanism can be very important for some systems. For example, Mayer and James have demonstrated the effects of exchange-mediated leakage from solvent molecules by comparing the STD responses of RNA-binding ligands in H_2O versus D_2O [33]. The overall STD response is significantly less in H_2O due to the additional dipole-dipole interactions between the RNA protons and hydration waters. Another factor that can compromise saturation is low receptor molecular mass. Although

low mass (<20 000 Da) is a rare concern for therapeutic targets, one occasionally encounters a target whose rapid tumbling leads to inefficient spin diffusion and, hence, poor saturation. In these cases, it is possible to increase the effective transfer by applying longer saturation trains or, alternatively, by addition of viscosity enhancing reagents (e.g. glycerol) and/or screens at lower temperatures that help to shorten the rotational correlation time of the receptor.

III. Overview of Other NMR Based Methods

In addition to the STD experiments, NMR based screening methods utilizes other spectral changes induced due to the interaction between the ligands and the protein. The change in the spectral features (secondary-chemical shifts, linewidths, relaxation rates) is monitored in a controlled manner to screen a array of ligands against macromolecules. NMR based screening of ligands can be broadly classified into two categories: protein based and ligand based [2, 3, 10]. In the protein-based methods the interaction is monitored by following the changes in the NMR parameters of the protein while in the ligand based approach spectral follows the spectral features of the ligand. Both these methods have distinct advantages as well as disadvantages with reference to each other and the choice of the method is to a large extent determined by the nature of the biological system of interest. Protein based experiments are limited to the changes in the chemical shifts, while in the ligand based approach a variety of mechanisms, such as relaxation, exchange and diffusion properties can be utilized. In particular, protein based experiments provide direct structural information that can help to rationalize design of inhibitors, while the ligand based methods are versatile for screening for ligands from a library of small molecules. From a practical point, protein based experiments need some form of isotopic

enrichment of the proteins (^{15}N and/or ^{13}C) to increased sensitivity, while ligand-based experiments do not have that requirement.

III. A. Protein based approaches

Fesik and co-workers first introduced the method referred to as SAR (structure–activity relationship) approach that uses ^{15}N-labeled proteins [1, 38]. It is perhaps the most utilized protein based experimental approach. In this method, the binding of the ligand is detected by comparing 2D ^{1}H–^{15}N HSQC (footnote [1]) [39, 40] spectra of the ^{15}N-enriched target protein in the absence and presence of ligand to elucidate ligand-induced changes in the chemical shift. The overall change in the chemical shift of a cross peak in the HSQC spectrum is defined by $\Delta\delta$, root-mean-square deviation of the chemical shift difference of ^{1}H and ^{15}N nuclei, with and without the ligand. Typically $\Delta\delta$ greater than 0.1 ppm for at least two peaks in the spectrum is considered significant. Binding constants are then determined by following the $\Delta\delta$ as a function of ligand concentration. This method further allows the option of adding the second ligand to the complex, thus allowing this method to progressively identify multiple ligands for the same protein.

The advantages of the SAR based screening method include, complete elimination of background signals from test compounds due to use of spectral editing using the ^{15}N nucleus and concomitant identification of ligand binding site location. Further this method has the unique advantage of developing simple binding assays that

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[1] HSQC: Heteronuclear single quantum correlation also known as a X-^{1}H-COSY (X=^{15}N or ^{13}C) show the single bond correlation between the X-^{1}H pair. In particular, ^{15}N-^{1}H HSQC is highly sensitive to small changes protein-ligand interactions.

do not require other functional assays. This method has been successfully used in several systems, including FKBP, Stromelysin, Human papillomaviruses, Src homology domains, Erm Methyls transferase, Urokinase, Adenosine kinase and Leokocyte function associated antegen-1 (LFA-1). The system that highlights the best of the technique is the design of high affinity ligands FKBP (FK506 binding protein) that blocks T-cell activation when complexed to the immunosuppressant FK506 [41, 42].

Chemical shift perturbation experiments have been the primary source of information enabling localization of ligand interactions with the receptor. Perturbations in ^{15}N chemical shifts, while indicating which amide protons are perturbed by ligand binding, do not provide precise information regarding binding geometry. McCoy and Wyss [43] have developed an alternative, higher resolution method for localizing ligand binding sites by using previously unexploited information contained in binding-induced protein chemical shift perturbations. The method recognizes the high prevalence of aromatic rings in drug-like molecules and that chemical shift perturbations of the protein upon binding must be due in part to ring current shifts induced by the ligand. By quantifying the spatial dependence of the ligand ring current field, and the consequent effects on the local magnetic fields of neighboring spins, the ligand binding site can in principle be more accurately characterized. Since localization proceeds from an analysis of shift perturbations alone, there is no need for a lengthy structure determination of the protein-ligand complex.

III.B Ligand based approaches

Ligand based experimental techniques use a diversely different mechanisms to screen and detect ligand in comparison with that of the protein directed method. The

major advantage of monitoring the small molecule resonances is that there is no need to isotopically enrich the target protein and no upper limit to the size of target that can be screened. The main disadvantages are that ligands with high affinity ligands will be missed and that no information regarding ligand-binding sites is directly available from the screening data as the binding interaction is manifested in the signals observed for free ligand. The ligand must be in fast exchange between the free and bound states for the binding interaction to be detected, thus ligands in slow exchange will appear as non-binders. The ligand based experiments, can be broadly classified into three categories, based on the particular mechanism that differentiate the ligand properties from that of the protein. These mechanisms are (a) rotational motion as sampled by relaxation parameters, (b) translational mobility by the self-diffusion coefficients and (c) inter and intra-molecular magnetization transfer as measured by chemical exchange and nuclear Overhauser effect (NOE), respectively

III.B. 1 Relaxation for the screening mechanism

In this approach the difference in the relaxation mechanisms between the protein and the ligand is exploited to identify the affinity of the ligands. The relaxation of the protein is strongly influenced by the lager number of protons through dipole-dipole relaxation in comparison to that of the relatively much smaller ligand. This allows to design experimental method that can distinguish from compounds that bind to the ones that do not. The experimental methods are generally designed to either use the difference in the longitudinal or transverse relaxation mechanisms. Relaxation rate constants (both R_1 and R_2) are weighted means of the contributions from the 'free' and 'bound' ligand in the solution and hence are sensitive to the relative populations and binding constant. As the rate constants depend on the rotational correlation time of the protein and ligand,

bound ligand assumes that correlation time of the protein and thus would display an enhanced rate constant. For example, the line width of a ligand binding to the protein will show an increased broadening and often could be measured directly from a one-dimensional NMR spectrum. Modulation of the relaxation rate constants due to ligand binding depends roughly on the relative ratio of the molecular weights of the receptor to ligand. The effect is hence more pronounced for large receptors due to longer overall rotational correlation time.

Transverse relaxation is more often used for screening than the longitudinal relaxation mechanisms. The potential difficulties associated with these methods are lack of distinction in the NMR spectra upon binding and effects due to intermediate exchange between the bound and free ligand in chemical shift time scales (see section II.A.2). Relaxation based experiments, though powerful, have not been used in NMR screening because of the problems of achieving selective excitation for a large library of chemically diverse compounds.

III.B. 2 Molecular Diffusion based screening

Similar to the use of rotational motion of the protein and ligand in the relaxation based ligand screening, the translational diffusion rate can also be used to detect complex formation [44-47]. Self-diffusion coefficient of a nearly spherical molecule is inversely related to the radius through the Stokes–Einstein equation ($D=kT/6\pi\eta r$, k: Bolzmann constant, T: temperature, and η: solvent viscosity). As the ligands are small they have diffusion constants much larger than the protein. When the ligand binds to protein transiently, it will acquire the diffusion behavior of the protein and in the fast exchange limit it will amount to the population weighted average of the diffusion coefficient of the ligand and protein.

Experimentally, any of the standard pulse sequences to measure self-diffusion coefficient in the solution NMR can be used. However, bi-polar gradient pulses with eddy-current delay and its variations serve as a optimum experiment for better performance [48, 49]. Due to inherent difficulties in quantifying the diffusion coefficients (for example, accounting for hydration effects), the results are often analyzed qualitatively to identify compounds that bind to a receptor from a mixture of non-binding molecules. Typically, first the diffusion coefficient of the mixture of ligands in measured in the absence of the protein. Then, the same spectra for the chemical mixture in the presence of the protein are recorded at low and high gradient strengths and subtracted to produce a spectrum that contains only the signals of the compounds not interacting with the protein. The resulting subtracted spectrum is then subtracted from the spectrum of the chemical mixture recorded in the absence of the protein to obtain a spectrum that contains only the signals of the molecule that binds to the receptor.

NMR screening with the diffusion-based approach are useful for screening ligands with weak dissociation. In this method a signal reduction of more than fourfold is observed compared to the acquisition of a normal 1D spectrum. A factor of two is lost in the diffusion part of the experiment and another factor of two is lost in the two spectral subtraction procedures. Further signal loss occurs due to longitudinal relaxation during the diffusion period. These problems are amplified, in particular when the difference in the diffusion coefficients between the protein and the ligand are not large.

III.B.3 Intramolecular and intermolecular magnetization transfer.

Protein structure determination in the solution state, strongly depends on nuclear Overhauser effect (NOE) and intermolecular exchange between water and protein protons. In the same spirit, several ligand-screening methods, put these mechanisms for

effective use. The basic principle of this method is similar to the earlier methods that the transient binding of the ligand to protein acquires a population-weighted average of the relevant NMR parameter.

III.B. 3.1 Transferred NOE (TrNOE):

TrNOESY (Transferred Nuclear Overhauser Effect SpectroscopY) experiments [50-54] are routinely used to detect ligand binding to a target protein under conditions of fast exchange (ligands that bind with μM to mM dissociation constants). The advantages of the trNOESY method are that it does not require large amounts of pure, labeled protein, it is not limited by the size of the protein, and it can provide information about the structure of bound form of the ligand. In the experiment, the intensity of each intra-ligand NOE cross-peak is governed by the population-weighted cross-relaxation rate [55]. Thus the binding event is relatively straightforward to detect and does require time-consuming chemical shift assignments. A strong positive NOE cross-peak is observed for binders, as opposed to weakly negative or zero NOE cross-peaks for the same mixture of compounds in the absence of the target receptor. Thus the sign flip of the NOE cross peak between the free versus bound states acts as a simple binary filter to distinguish binders from non-binders [2, 10, 56].

Transferred NOE (TrNOE) experiment is used extensively to determine weak to medium affinity ligands when bound to a large proteins. The important distinction of this method is that it uses cross-relaxation that responsible for NOE. Therefore the ligand NOEs are often opposite in sign to that of the proteins. Assuming that the magnitude of the rate is significantly larger for the bound state, at equilibrium conditions, the total NOE is predominantly determined by the bound form. A drawback of this method is the

requirement of large ligand concentrations and hence may not be suitable for identification of ligands with poor solubility. In addition, the presence of strong diagonal peaks in the two-dimensional experiment can hamper the observation of cross peaks between ligand resonances having similar chemical shifts.

STD experiments (section I) also fall in this categorization. Variations of the experiments to higher dimensions have also been proposed that combines a TOCSY (foot note [2]) or ^{13}C-HMQC (foot note [3]) at the end of the standard STD experiment. These experiments are useful in investigating the conformational changes of the ligand that are induced due to binding. It is important to note that the observed intensity of the ligand resonance is not a direct measure of the affinity of a particular ligand. For example, tight binders may produce a small changes because of their long residence time on the protein, while weak binding ligand could produce a larger difference. This experimental also allows to examine structural information of the ligand surface that is directly in contact with the protein.

III.B.3.2 Protein-water exchange (waterLOGSY):

It known that solvent water molecules play a critical role in protein-ligand interactions. High-resolution crystal structures of protein-ligand complexes show that a

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[2] TOCSY: Total correation spectroscopy is a homonuclaer through bond correlation experiment used to identify J-coupled networks in a molecule.

[3] HMQC: Heteronuclear multiple quantum correlation. This experiment is similar to a HSQC experiment and uses the multiple quantum coherence between the ^{13}C-^{1}H for correlation.

dense interaction of protein atoms with that of water. In the solution state, the residence times of water in protein cavities range between a few nano-seconds to several seconds. WaterLOGSY (Water–ligand observed via gradient spectroscopy) [57-63]experiment utilizes the large bulk water magnetization to transfer magnetization via the protein–ligand complex to the free ligand in a selective manner. There are two competing mechanisms that are responsible for the performance of these experiments; intermolecular NOE transfer between the water protons to protein and exchange of magnetization from labile protons with water. This combination allows the resonances of non-binding compounds appear with opposite sign and tend to be weaker than those of the interacting ligands and is a powerful tool for primary screening of compound mixtures by NMR. Several variations of the basic waterLOGSY experiments have been propose and of which the ePHOGSY sequence can be considered as a robust scheme [57]. Similar to STD based screenings titration binding experiments, waterLOGSY can also be performed in order to extract an approximate value of the binding constant of weak affinity ligands. However, the major drawback of the method is that very low-affinity ligands cannot be detected and may not impact the overall performance if ligands of high-affinity are of interest.

In implementing these experimental methods, except for waterLOGSY, STD and transferred NOE experiments adopt only the conventional setup, with slight modification such as improved water-suppression. The fundamental limitation of ligand based NMR screening techniques is that it is these can be used to detect strongly binding ligands with slow dissociation rates due to the fact that these experiments are generally performed at least 10 fold excess of ligand concentrations. As a rule of thumb, roughly diffusion-limited on-rate of 10^{-8} M^{-1} is the upper limit of detection using this method. Further,

ligands that have poor solubility that are potential ligands are difficult to detect since the methods require the observation of the ligand signals.

III.B.3.3: Note on non-specific binding:

Like any other screening methods, NMR based screenings methods also have a tendency to produce some false-positive results from nonspecific protein–ligand interactions. Ligand based screening methods generally tend to use a moderate excess of ligand relative to a target receptor used. Under these conditions, binding of the ligand to secondary binding sites with lower affinity or nonspecific interaction with protein surfaces may occur. This would also contribute to the observed change in the NMR parameter, complicating the interpretation due to an averaging from several conformations bound at different sites. Murali et al. showed the significance of this effect in TrNOE experiments [64]. In fast exchanging conditions, the measured NMR parameters are expected to be dominated from the bound ligand. In obtaining this condition, there is sufficient latitude in the choice of P_F/P_B (see equation 15 and discussion). Since the sensitivity as well as the ease of experimental measurement is optimized by choosing as large a value of P_F as this condition allows, ligand concentrations exceeding that of the receptor (sites) by a large factor (10-30). In particular, for charged ligands (such as the nucleotides), the likelihood of association due to electrostatic interactions to the protein surface is high and will lead to non-specific interactions. The extent of such weak nonspecific binding, the multiplicity of such sites, and the relative dissociation constants depend on the particular receptor-ligand complex and the sample preparation protocol. If non-specific interactions are suspected, control experiments with non-binding receptors may be performed to eliminate such possibilities. In addition, a buffer system containing

both high salt concentration (to prevent nonspecific ionic interactions) and an organic solvent (to prevent nonspecific hydrophobic interactions) may also be considered.

IV. General Experimental Considerations

Several groups, including our own, have been routinely using NMR screening methods to simultaneously and rapidly provide binding information. Here we present the methods in detail based on our experience [65, 66] and recent review articles [2, 10].

III. A Screening library of small molecules

Any screening method requires a collection small molecules and in particular such a library has to meet some stringent conditions for the NMR based screening. Typically some of the published NMR based screening methods has used libraries containing 10,000 compounds. However NMR based screening becomes efficient if a smaller collection of well-chosen compounds are considered. Compounds for NMR screening libraries are selected based on their drug-like character, which selects compounds that have similar characteristics as known drugs that have desirable pharmacokinetic characteristics or scaffolds that occur frequently in known drug. Some of the general approaches are:

(a) The molecular weight of the ligands does not exceed 350. This is an important criteria as most affinities range 100 M , low molecular weight molecules will have easy access for elaboration to build new scaffolds that have a higher affinity and selectivity.

(b) Water solubility of the ligands is an important requirement. It enhances the potential success of other studies such as calorimetry, enzymology, crystallization and NMR structural studies.

(c) It is necessary for the library of compounds to be suitable for chemical elaboration by traditional or combinatorial chemistry methods.

SHAPES [67-69] strategy of library design developed by Vertex pharmaceuticals provide a good example. This library is a small collection of diverse, low molecular weight, water-soluble compounds whose molecular shapes represent those most commonly found in known drug molecules. The basis of the library is an outcome of systematic analysis of the comprehensive medicinal chemistry (CMC) database. For example, SHAPES contains 32 different frameworks that describe close to 50% of all known drugs. These are commercially available, soluble, non-aggregated at 1 mM, chemically and isomerically pure, and non-reactive small molecules. The molecular weight range of the selected compounds is 68–341 Da. Compounds were also required to have at least two protons within 5 Å apart in order to be amenable to TrNOE-based screening.

IV. B Designing competition binding assays

NMR based ligand screening method can also be designed to perform competition binding and assay development and thus allowing high throughput screening. The strategy can be outlined as follows:

(a) First the identification of a weak affinity ligand its complete characterization. Typically this is done by screening of few hundreds of soluble, well-characterized molecules by ligand based screening (STD and/or WaterLOGSY experiments).

(b) The identified binders are subsequently studied with isothermal titrating calorimetry (ITC) in order to determine their binding constants. Based on the binding constant

and specificity NMR or X-ray structure of the complex is selected as the reference compound for the competition binding experiments.

(c) Additional NMR experiments are performed on this molecule in the presence of the protein to optimize the experimental conditions for screening and for quantifying, according to binding strength, and for the next round of screening.

(d) Chemical mixtures are then screened against the target macromolecule in the presence of the reference compound, preferably using the high throughput approaches.

Completion of a traditional high throughput screen are often triaged in some manner before consideration as lead chemical templates. For example, whether the hits actually bind to the target of interest. Simple yes/no answers from secondary NMR-assays can be an important aspect of the triage funnel. NMR-derived K_D values can also be used to rank order the screened ligands. The choice of the ligands for the first screening could be limited if other computational or biophysical methods such as mass spectrometry could be used for screening. For example, we have recently developed NMR competition assay based on transferred nuclear Overhauser spectroscopy (trNOESY) to help us to identify sets of ligands on the surface of TeNT fragment C (TetC) [65, 66]. Using this assay, we can more rationally optimize sets of ligands to synthetically link together for use as detection reagents, as well as estimate the relative binding constants for ligands competing for the same site.

IV. C Sample preparation

Key points of the NMR process for ligand screening are given below:

(a) Stock solutions of each compound are made in 1 M DMSO. If necessary, the pH of the 1 M stock solution is adjusted with acetic acid or ethanolamine so that no pH change is observed upon a 1/10 dilution into phosphate buffer. This is important since small changes in pH can lead to chemical shift changes that could complicate analysis of the spectra.

(b) Reference spectra of the target can also be collected at slightly different pH values in order to distinguish pH-induced chemical shift changes from ligand-induced chemical shift changes.

(c) Stock solutions are then prepared in DMSO that contains 8–10 compounds at a concentration of 100 mM each. For the screening, NMR samples are prepared by adding the DMSO stock solution to 0.4 mL buffered protein solution. Typically, the final concentration of protein is 0.3 mM and the final concentration of each organic compound is 1 mM.

Both protein and ligand based experiments are used at this stage of screening. In protein-based experiments, well-equipped laboratories could perform 100–120 ^{1}H–^{15}N HSQC spectra 24 h period, thus allowing about 1000 compounds to be screened per day. In order to be a truly high-throughput screening technique, it should be possible to screen more than 100,000 compounds in about a week. This could be achieved by screening 50 M protein solutions against mixtures of 100 compounds at 50 M each in about 10 min. Improved NMR probes, such as cryoprobe technology and new experimental methods could be are needed to achieve such turnover. Typically, HSQC spectra suitable for screening were obtained on a 300 M solution of a 100 kDa protein in 30 min using a cryoprobe on a 500 MHz spectrometer.

IV.D Other important considerations

Most biologists consider that NMR screening methods treat the target protein to harsh and unrealistic conditions. By performing several control experiments will ensure that the NMR based screening is a valuable method to transcribe the results to experiments in-vivo. Generally, small aliquots of concentrated ligand stocks in deuterated DMSO solution are added to achieve the required concentration. It is also important to examine the stability and activity of the target protein as a function of organic solvent concentration, pH (buffer type and concentration), and temperature as well as length of the screening process. For example, micro-drop optimization method developed by Vertex Pharmaceuticals [67-69] is a useful method to optimize sample conditions prior to actual NMR experiments. Several research group also point out the importance of critically maintaining the pH range of the mixture with and without the target protein. The choice of molecules in the mixture to screen also plays a critical role in the performance of the experiment. Larger number of ligands can be mixed in the protein based experiments, while in the ligand based method it is restricted between 4-8 well chosen library of molecules.

Considering the variability in the sample conditions and experimental method used in the NMR based screening, it is not easy to predict that one particular approach is the 'best'. Requirement of the protein, ligand as well the choice of the experimental method dictates the quality and in particular sensitivity of the method. Table 1 shows the comparison of the performance of the various experimental methods following the original description [10]. In protein based experiment, Table 1 assumes a protein of mass 20 kD, 10 compounds per mixture and a total of 500 μL NMR sample. For the ligand-based

estimates it is generated for a 40 kD protein with 4 compounds in the mixture for a same 500 μL sample volume.

V Summary and Future Directions

NMR is currently one of the methods of choice for fragment-based approaches. NMR screening has a growing track record of discovering submicromolar lead candidates even for difficult targets where conventional methods failed. In addition, NMR can identify novel scaffolds in cases where high-affinity ligands are known but have undesired properties. One way to practically evaluate the usefulness of the NMR based screening method is by asking, are there any drugs on the market or in clinical development that were identified or optimized or otherwise strongly influenced by NMR? The answer is yes: two compounds from NMR screening have reached or are about to reach clinical development [70]. Among those are compounds that target leukocyte function-associated antigen-1 [71] and matrix metalloproteinase (MMP-3) [72]. In addition, Merck (Germany) is developing the N-methylated c(RGDFV) cyclic peptide that was identified by spatial screening. Those compounds identified by NMR that are now in clinical trials are the most spectacular proof of the value of biomolecular NMR in drug discovery.

Though serial NMR analysis methodologies with moderately high-throughput capabilities are becoming routine, the analysis of chemical libraries dictate that alternative approaches need to be formulated. The development of different techniques for high speed and/or parallel NMR data acquisition has occurred only over the past few years, but this is clearly a rapidly evolving area of research. Currently, development is focused on the areas of pulse sequence techniques, data processing and probe hardware

design. In addition to the potential for cryoprobe technology [73], high-throughput can also be increased by simultaneous data collection on multiple samples [74-76] by four-coil, flow-through multiplex sample NMR probe [77]. Incorporation of capillary separation with a multicoil NMR probe and the associated capillary fluidics to deliver the samples to and from the coils is the next step in probe development [78]. With the development in probe technology, parallel sampling NMR techniques along with high-speed multidimensional data acquisition will play an important role in high-throughput chemical analysis and drug screening. A major thrust is to increase the number sample coils, although one ultimate limitation is imposed by the extent of the homogeneous region of the magnet.

NMR based ligand screening is expected to have continued advances in instrumentation and methodologies will lead to faster throughput and to the application of NMR screening to ever larger systems. Screening proteins in living cells will also provide novel information for drug discovery. As NMR is an extremely versatile biophysical technique, its applications can be tailored to answer a variety of different questions. The abilities to deal with weak interactions and to structurally characterize binding events are unique features of NMR, which have important applications in all phases of drug discovery. Recent developments in NMR screening technologies are contributing to its increasing importance in industrial drug research. NMR screening is now established as a field in its own right, and one that benefits from both novel applications of well-established methods and the adoption of new techniques emerging in general NMR research; both of which will provide future innovation in the field.

In this review, I have made an effort to provide a comprehensive overview of STD NMR based ligand screening. In addition to the theoretical description of the

method, it has also provided the technical details of how these experiments may be carried out and how these methods have been used in practice. In this rapidly growing area of NMR based ligand screening, this review by no means complete in providing all the information. As mentioned in the introduction, several excellent review articles are available in the literature from research groups that have pioneered these methods. The reader must follow these original work to obtain additional details. Though the focus here is mainly on STD experiment, the other methods can be of benefit as well. What approach is the most appropriate for a given target and library of possible ligands depends therefore on the size of the target, the availability of its structure and chemical shift assignments and the number of drug candidates to screen. It may be necessary to perform more than one experimental approach and decide upon the optimal experimental and sample conditions. The developments described in this review, together with the very promising advances in hardware that are being presented by manufacturers suggest that NMR will definitely have a role to play in future structure-based drug discovery research initiatives.

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Figure Captions

Figure 1: Schematic representations of the mechanism of saturation transfer difference (STD) experiment. In the off-resonance (**Off**)condition (r.f irradiation far from the spectral region) normal NMR spectra of the ligands is observed. Protein signals are not observed due to their large molecular weight and low concentration. When the protein resonances are irradiated (**On**) using frequency selective r.f (lightning bolt) causes selective saturation (shading) of the protein magnetization. Irradiation is applied for a sustained interval during which saturation spreads throughout the entire receptor via spin diffusion (^{1}H-^{1}H cross-relaxation). Saturation is transferred to binding compounds (triangles) during their residence time in the receptor binding site and thus showing a enhanced signal in the NMR spectrum. When a difference between the two experiments is taken (**Difference**), the fractional enhancement of the bound ligand reaonances is only observed.

Figure 2: The energy level diagram for a spin-1/2 nucleus exchanging between a receptor (E) and ligand (L) sites. W_{1E} and W_{1L} are the total probabilities (by all mechanisms) for spin transitions in the protein and ligand site, respectively. W_0 is the probability for transfer if the spin between protein and the ligand.

Figure 3: Pulse sequence for the one-dimensional saturation transfer difference (STD) experiment. (a) Pulse sequence when samples are dissolved in D_2O. Additional water suppressed detection of resonance is employed for spectra in H_2O; WATERGATE and DPFGSE blocks are shown in (b) and (c), respectively. The upper and lower staffs show proton r.f and field gradient pulses, respectively. Selective r.f saturation occurs for 1-3 s via a train of frequency selective r.f pulses. Generally, a 50 ms Gaussian or Seduce-

1 90° pulses (field strength of 35-70 Hz) separated by an inter-pulse delay of ~1 ms. Two experiments are performed, which apply saturation on and off resonance with receptor protons in an interleaved fashion. The on- and off-resonance frequency of the selective pulse is therefore switched between 30 and -0.4 ppm after every scan. The 1D signals are stored in separate locations, and their spectral differences are taken via inverting the receiver phase. d1 is an additional short relaxation delay and typically set to 100-200 ms. Phase cycling for (a): ϕ_1 = x, -x, -x, x, y, -y, -y, y, -x, x, x, -x, -y, y, y, -y; ϕ_2 = 2(y, -y), 2(-x, x); and ϕ_r = 2(x), 2(-x), 2(y), 2(-y), 2(-x), 2(x), 2(-y), 2(y). For the WATERGATE block, the pulsed-field gradients are of equal intensity and sign. Water suppression can be achieved here by a binomial 3-9-19 pulse sandwich or a WATERGATE-5 pulse train, resulting in inversion of all signals except the HDO signal at the carrier frequency. The delay between the 3-9-19 pulses is 0.2 ms each. Phase cycling is: ϕ_1 = 16x, 16(-x); ϕ_2 = y; ϕ_3= (x, y, -x,-y); ϕ_4 = 4x, 4y, 4(-x), 4(-y); and ϕ_r = 2(x, -x, x, -x, -x, x, -x, x), 2(-x, x, -x, x, x, -x, x, -x). The pulse width of the two selective 180° pulses in the DPFGSE sequence is about 2 ms, which can be adjusted to obtain the best water suppression. The phase cycle is ϕ_1 = x; ϕ_2 = y; ϕ_3 = 4(x),4(y),4(-x),4(-y); ϕ_4 = 4(x,y,-x,-y), 4(-y,x,y,-x), 4(-x,-y,x,y), 4(-y,x,y,-x); ϕ_r= 4(x), 4(y). The durations and the strengths of the gradients are ~ 2ms and at strengths 15G/cm (g_1) and 7G/cm (g_2).

Table 1: Comparison of the NMR based screening methods

Scheme	Range of binding affinity	Ligand:protein ratio	Minimum protein concentration (μM)	Amount of protein required per 100 ligands (mg)
Protein based methods				
SAR-HSQC	< nM-mM	1-2	300	30
Ligand based				
Relaxation	μM	< 2	100	50
Diffusion	μM – mM	< 2	100	50
TrNOE	μM – mM	5-50	20	10
STD	μM – mM	10,000	0.1	0.05
WaterLOGSY	μM – mM	5-50	1-5	10

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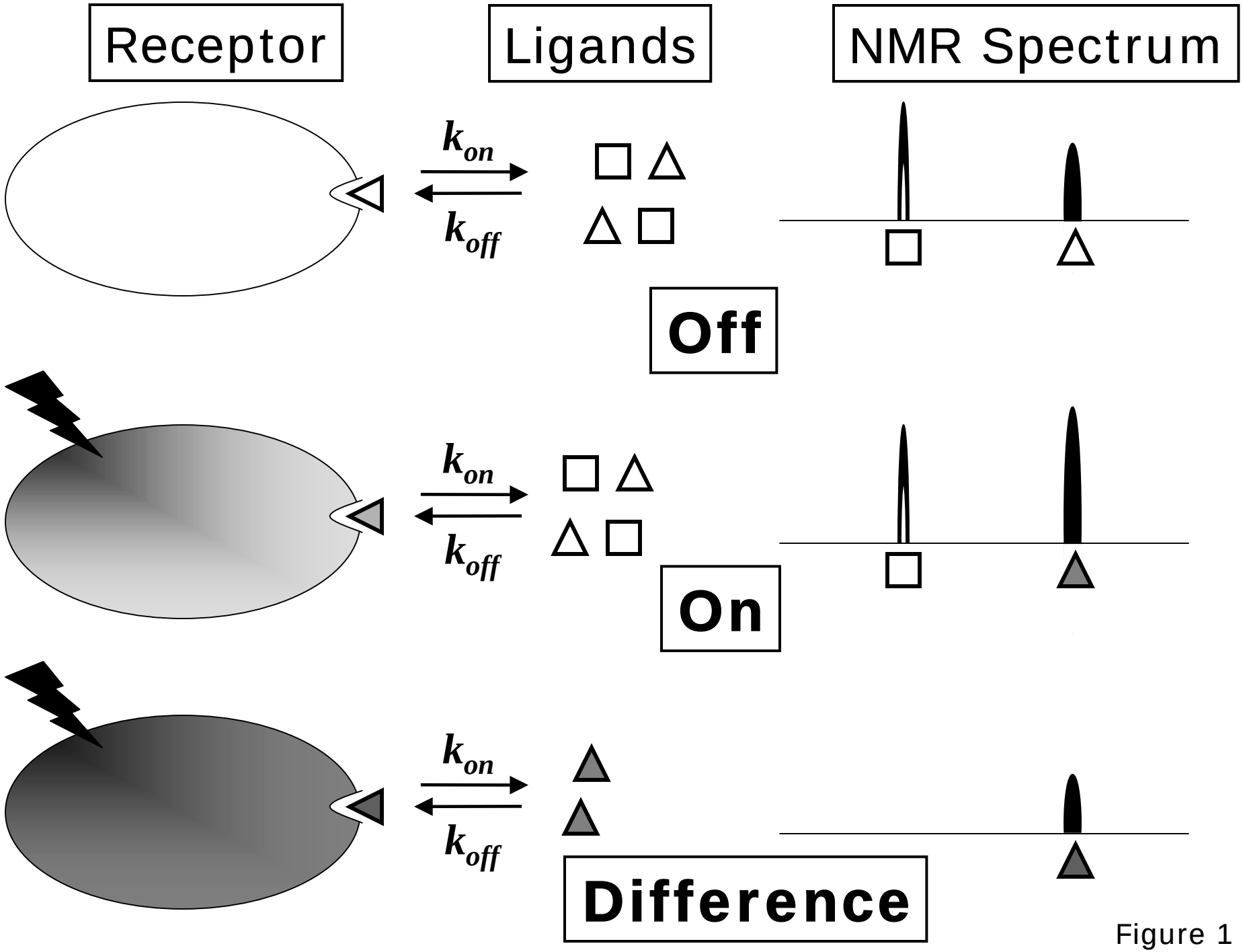
Receptor
Ligands
NMR Spectrum
k_{on}
k_{off}
Off
k_{on}
k_{off}
On
k_{on}
k_{off}
Difference

Figure 1

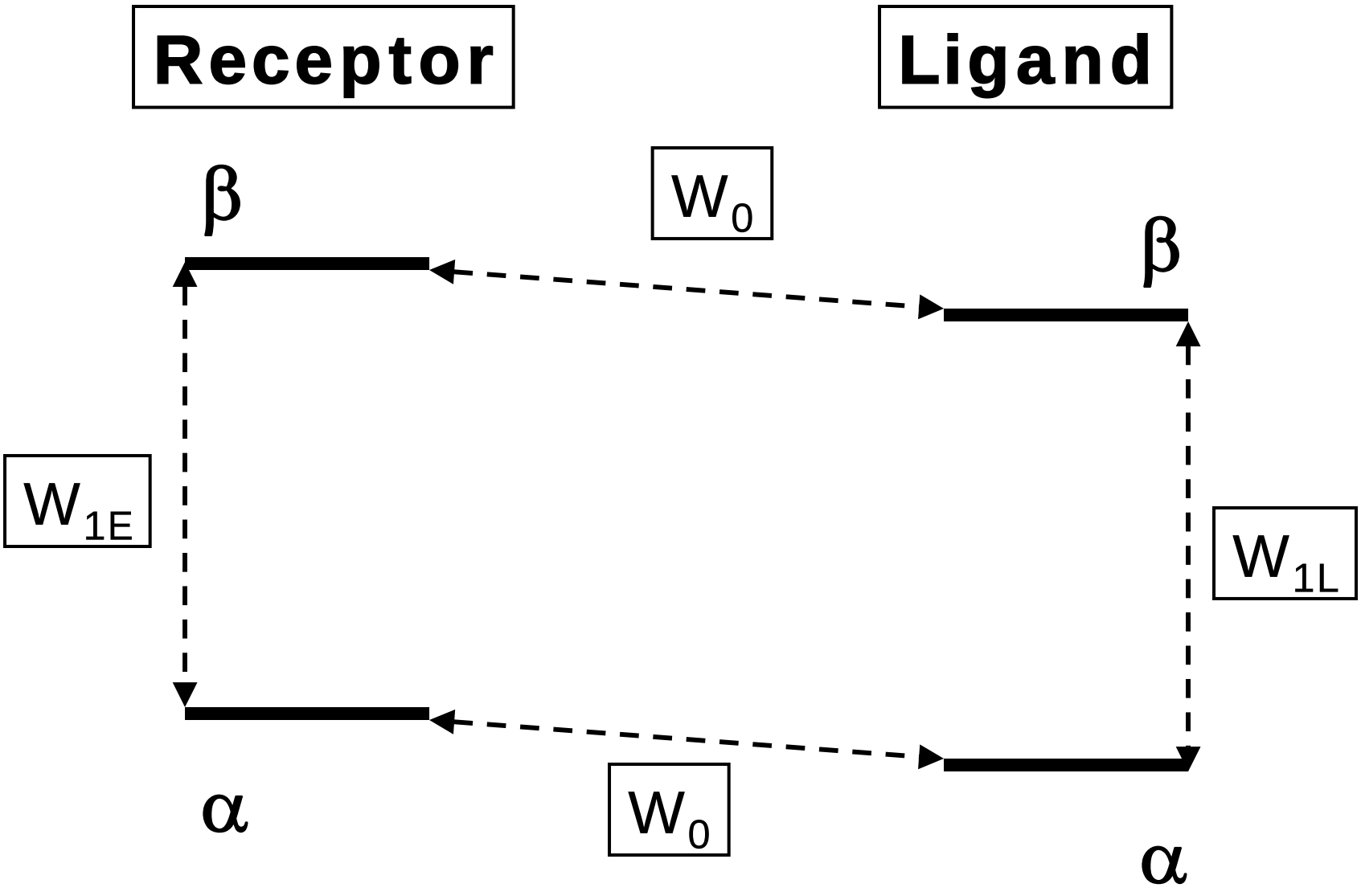

Figure 2

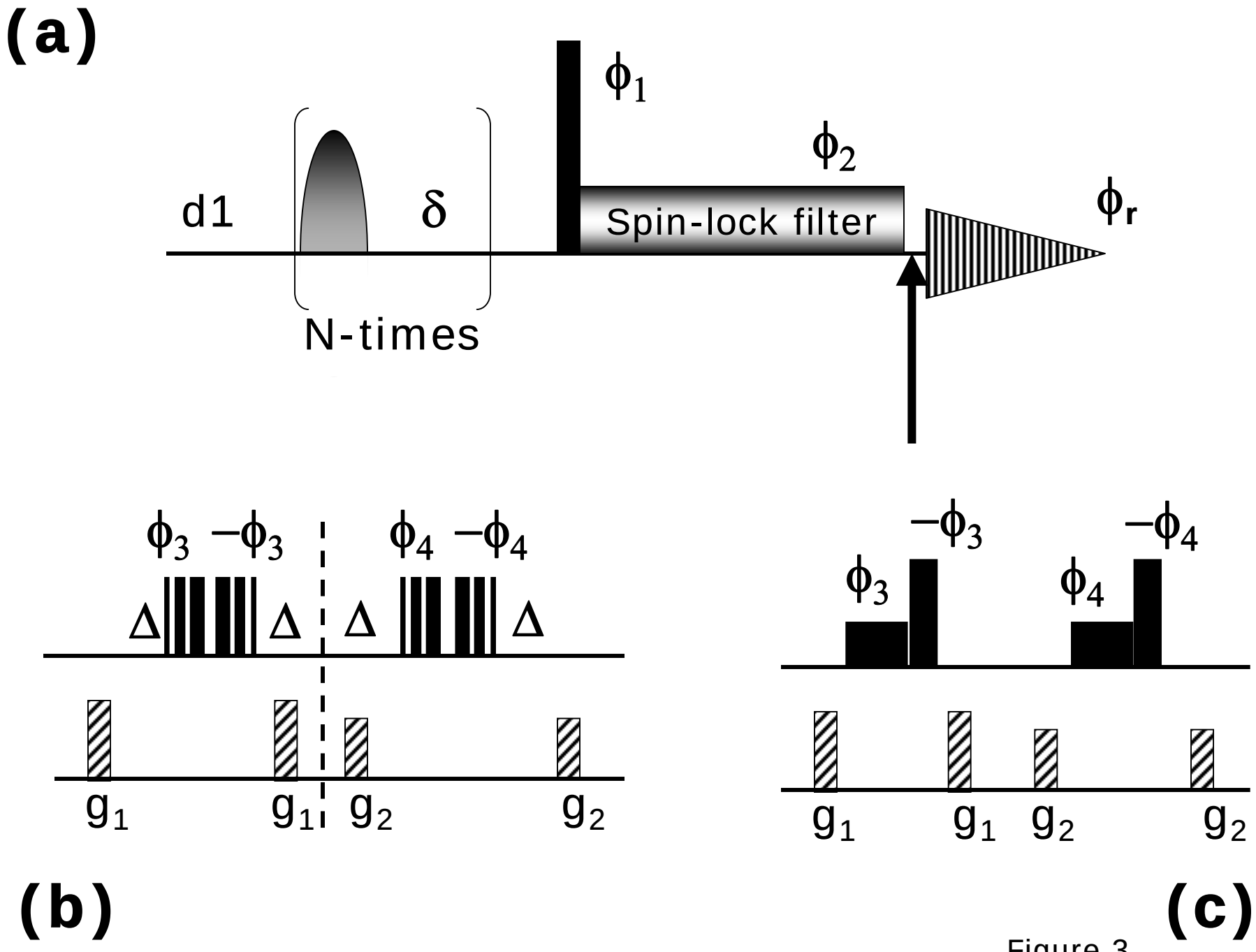
(a)
d1
δ
N-times
ϕ1
ϕ2
Spin-lock filter
ϕr
ϕ3 −ϕ3
ϕ4 −ϕ4
Δ
Δ
Δ
Δ
g1
g1
g2
g2
(b)
−ϕ3
−ϕ4
ϕ3
ϕ4
g1
g1
g2
g2
(c)

Figure 3