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Applications of saturation transfer difference NMR in biological systems

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The method of saturation transfer difference (STD) nuclear magnetic resonance (NMR) is an indispensable NMR tool in drug discovery. It identifies binding epitope(s) at the atomic resolution of small molecule ligands (e.g. organic drugs, peptides and oligosaccharides), while interacting with their receptors, such as proteins and/or nucleic acids. The method is widely used to screen active drug molecules, simultaneously ranking them in a qualitative way. STD NMR is highly successful for a variety of high molecular weight systems, such as whole viruses, platelets, intact cells, lipopolysaccharide micelles, membrane proteins, recombinant proteins and dispersion pigments. Modifications of STD pulse programs using ^{13}C and ^{15}N nuclei are now used to overcome the signal overlapping that occurs with more complex structures.

The study of protein–ligand interactions is an important domain in the field of drug design by pharmaceutical companies. The starting point of developing novel drugs is often the high throughput screening of virtual-compound libraries. The process of validation of such drug-able candidates, known as leads, generally involves time-consuming tasks with several steps. There are various biochemical methods e.g., enzyme-linked immunosorbent assay (ELISA) and biophysical methods like surface plasmon resonance (SPR), fluorescence polarization assay or fluorescence resonance energy transfer (FRET) are used extensively in the pharmaceutical industry as screening methods. During the early and mid-1990s, nuclear magnetic resonance (NMR) spectroscopy was used for the first time as a tool to screen and validate lead compounds by directly identifying their sites of interactions [1]. In both pharmaceutical and academic research, NMR-based screening has since become a means of investigating several structural issues, including a way to discover optimized drug hits. The high-resolution X-ray crystal structure of a receptor, co-crystallized with a small molecule, provides important information about the compound, such as the chemical scaffolding. This clue serves as the foundation for computational chemists to use it to screen compound libraries, which are then further shortlisted by NMR screening strategies, enabling the development of precise knowledge of the conformations of the bound ligand on the interacted surface of a protein. Hence, the molecular interactions can be revealed at an atomic level.

Generally, there are two major NMR experimental approaches for screening a compound library that contains a large number of compounds. In the first approach, changes of chemical shifts of a protein are observed in the presence of ligands. The experimental result provides information about the ligand-binding domains of the protein. In addition, the second approach is ligand-based screening. There are several different experimental methods [e.g. chemical shift perturbation, line shape analysis, transferred nuclear overhauser effect spectroscopy (trNOESY), nuclear overhauser effect (NOE) pumping, water ligand observation with gradient spectroscopy (waterLOGSY), interligand NOEs for pharmacophore mapping (INPHARMA), target immobilized NMR screening (TINS) and saturation transfer difference (STD)] that can follow any of the two different approaches to enable the establishment of the screened compound library. These methods are complementary and the advantages and limitations of each have been reviewed extensively elsewhere [2–7].

In this article, we briefly describe recently developed NMR methods for receptor–small molecule interactions, such as

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waterLOGSY, TINS and INPHARMA, because they are not conventional NMR spectroscopic techniques. The waterLOGSY technique is based on the excitation of the receptor–ligand complex through a selective radio frequency (r.f.) pulse sequence. In this method, selective perturbation of the reservoir of bulk water magnetization is achieved as opposed to direct perturbation of receptor magnetization. The intended transfer of magnetization is in the sequence of water–receptor–ligand [8]. This method is effective for studying the interactions of nucleic acids with small molecules. Another effective method for drug screening is TINS, which reduces the amount of target required for fragment-based approaches toward drug discovery [9]. The strategy is that the protein is immobilized on a resin and whether the ligands, which were flowing across the resin, bind to the receptor can then be assessed; this is reflected by the reduction in NMR amplitudes, compared with a control experiment. This method is well suited for the screening of targets that are difficult to produce or insoluble in water, such as membrane proteins. Recently, Griesinger and co-workers have developed a technique, INPHARMA, for the characterization of ligand–ligand interactions through the target protein [10]. In this technique, first NOE transfer is achieved from a ligand (H_A) to the target protein (H_T). Next, the achieved NOE is retransferred from the host protein (H_T) to another ligand (H_B), effectuating receptor-mediated ligand-to-ligand NOE transfer. Thus, this method has become established as a way of mapping ligand effectiveness on the basis of interactions with the host receptor. Here, we focus primarily on the applications of STD NMR [11], which emerged as powerful NMR-based screening techniques just one decade ago. The technique has matured and become a robust approach in medicinal chemistry. STD is widely used to unravel receptor–small molecule interactions. Furthermore, the new generation of STD experiments with modifications in the pulse programs are now able to show how a small molecule binds to its receptor channelized by its internal dynamics. To achieve this, not only is highly resolved proton NMR used, but also various combinations of both ^{13}C- and ^{15}N-edited NMR are used with an ^{1}H probe to examine intermolecular interactions. However, there are few sensitivity issues surrounding the use of heteronuclear STD NMR, whereas working with isotope-labeled compounds is only applicable to a small subset of receptor–ligand systems. The large chemical shift dispersions of ^{13}C and ^{15}N nuclei are helpful in determining the STD effects arising from attached protons.

STD and its importance

Saturation transfer NMR was a pioneering technique developed in 1979 by Wüthrich's group to obtain resonance assignments of the heme group in oxidized cytochrome c-557 from *Crithidia oncopelli* [12]. Saturation transfer NMR spectroscopy characterizes the bound ligand–receptor complexes at the free and bound states of the ligand. If a ligand is revealed with two different signals because of the slow exchange between the bound and the free state, a transfer of saturation is possible within these two states by irradiating the signals of the free ligand; as a result, the signals of the bound ligand can be identified and vice versa. The simplicity of this method for gaining insights into receptor–ligand complexes is based on monitoring the ligand resonances in either their bound or free states. It is better to choose easily separated ligand resonances to avoid unnecessary resonance complexity [13–15].

In 1999, Mayer and Meyer introduced STD to elucidate ligand binding to the receptor [11]. In STD NMR experiments, a sample should contain both the ligand and the receptor; with the excess of ligand (usually an approximately 100-fold molar excess) so that both the quantification of receptor-bound and free ligand is possible (Box 1). However, because many other factors influence the STD NMR, quantification of the exact fraction of bound ligand with high accuracy is not possible. A simple 'on-resonance' one-dimensional ^{1}H NMR spectrum then recorded with irradiation on the protein signals at a frequency range where no ligand protons happen to resonate. Typically, in the case of protein–small molecule interactions, the on-resonance frequency might be fixed either at the up-field region (approximately −1 ppm) or at the down-field region (approximately 7.0 ppm) of the NMR spectra to avoid the 'direct' saturation of the ligand (Fig. 1; Box 1). In a similar fashion, a second spectrum (denoted as 'off-resonance' spectrum) is recorded using an irradiation frequency set to a value that is significantly different from the protein and the ligand frequency region (Fig. 1). Subtraction of the second spectrum from the former yields a difference spectrum, containing signals resulting from saturation transfer from the protein to the ligand (Fig. 1). The difference spectrum dictates the receptor and ligand interactions

BOX 1

Practical aspects of STD NMR

- On- and off-resonance frequencies are important parameters for STD NMR experiments. To avoid direct saturation of the ligand in STD NMR experiments, it is necessary to determine the 'safe' on-resonance frequency. For example, if the ligand shows no signal in the aromatic region (approximately 6–8 ppm) then it is a good choice for the saturation of the protein signal at this region. However, if the ligand contains aromatic groups, the saturation frequency must be shifted to a frequency of approximately –1 ppm. Similarly, determination of the off-resonance frequency is important to find out the 'remaining' saturation of the receptor so that it does not produce artifacts in the difference spectra.
- STD NMR experiments generally perform at a high molar excess of ligand over the protein. A protein:ligand ratio of 1:100 is normally appropriate.
- The STD NMR spectrum depends on the saturation time (t_{sat}) for the study of protein–ligand complex interactions. Generally, 1.5–2 s is suitable saturation time over which to perform initial STD NMR experiments. However, to avoid T1 bias and to determine group epitope mapping, it is recommended that STD NMR experiments are run over several saturation times, from 0.5 to 5 s. d1 is an additional short relaxation delay and typically set to 100–200 ms.
- It is advisable to apply a spin-lock filter after the STD pulse sequence to suppress 'unwanted' background protein signals. Normally, a spin-lock pulse of 10–20 ms with a strength of 5–10 kHz is sufficient to remove the protein signals.
- The STD NMR experiment must be performed in a solvent containing 100% D_2O to achieve sufficient magnetization transfer from protein to ligand. By contrast, with a solvent containing 95% H_2O and 5% D_2O, there is a chance of a waterLOGSY effect.
- Suppression of the residual water signal using either Watergate 3-9-19 or W5 is useful.

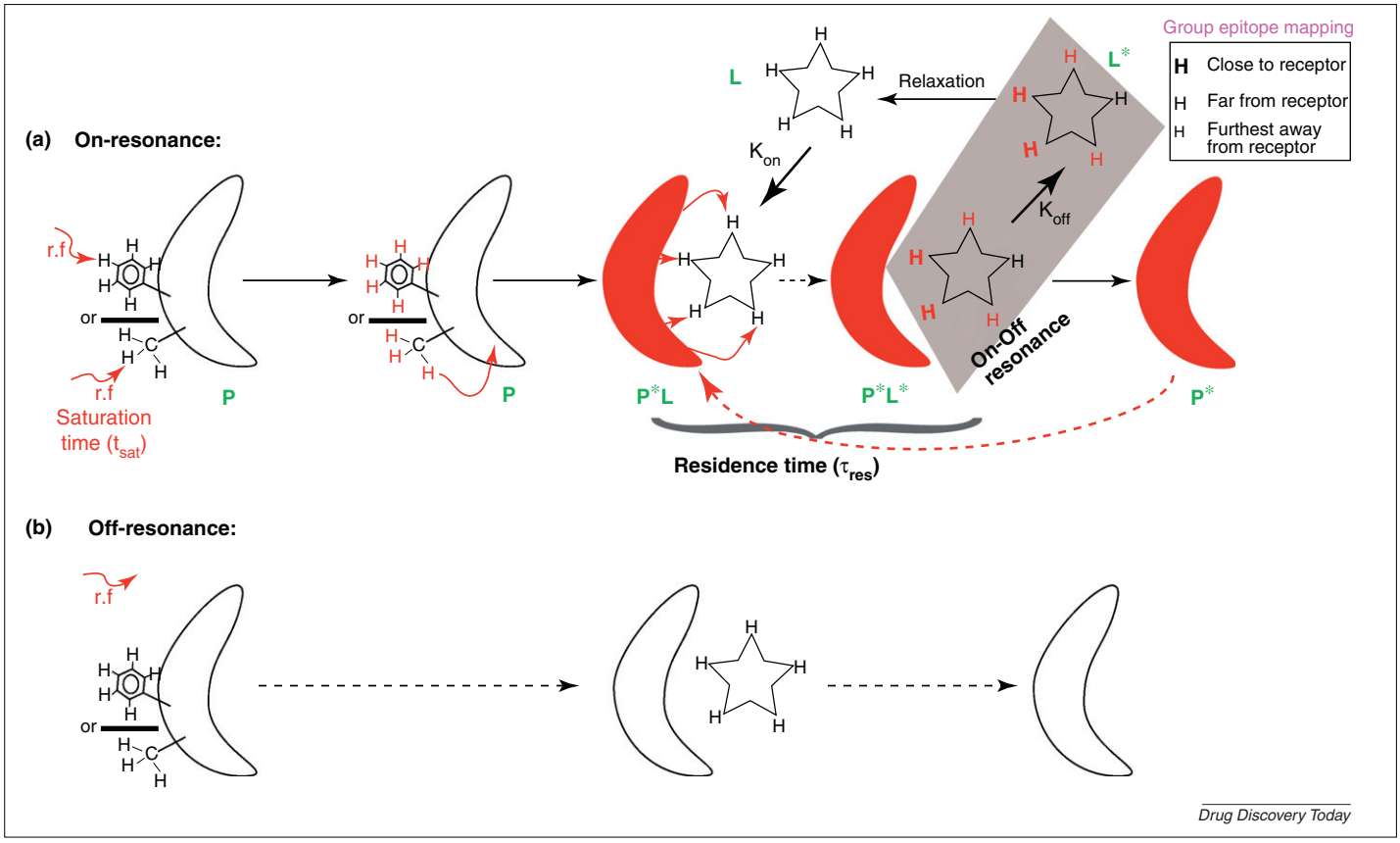

FIGURE 1

The mechanism of saturation transfer difference (STD) experiments. **(a)** In the 'on-resonance' spectrum, the protein resonances are saturated (t_{sat}) at a frequency (e.g. either methyl or aromatic protons of proteins) where no ligand resonates. The frequency selective radio frequency causes the saturation of the protein magnetization and it then spreads over all the protein using spin-diffusion (P*). If the ligand binds to the receptor protein, the saturation also transfers to the binding compounds (L*) during the residence time (τ_{res}) in the receptor-binding site, thus showing an enhanced signal in the STD nuclear magnetic resonance (NMR) spectrum. **(b)** In the 'off-resonance' condition, the selective radio frequency is set to a value where neither protein nor ligand resonates, resulting in the normal NMR spectra of the ligand being observed. Subtraction of the two spectrums (on-resonance minus off-resonance) leads to a 'difference spectrum' (shown here in a grey background) where the fractional enhancement of the bound ligand resonances is only observed (inset). The concept of group epitope mapping (GEM) is derived on the basis of proximity of ligand protons to the receptor core.

(Fig. 1). STD NMR was first developed to study carbohydrate–protein interactions and was used to screen a library of carbohydrate molecules with various receptor-binding affinities [16–21]. It has since become a popular way of screening a new library of various types of molecule, such as metabolites, drugs and binding receptors, such as proteins and nucleic acids.

Another important use of STD NMR is for the development of ideas for group epitope mapping (GEM), a technique that enables ligand–receptor binding interactions to be characterized at the atomic level (Fig. 1) [22]. In GEM, the intensity of a proton from the ligand that is situated at the nearest proximity to the receptor causes the highest STD effects and vice versa. Knowledge of the epitope of the ligand is fundamentally important when designing a new drug.

The main parameter in an STD NMR experiment is the saturation time (t_{sat}) in which intermolecular transfer of magnetization from the saturated protein to the ligand occurs. However, receptors that have inherently poor spatial proton density experience inefficient spin diffusion and thus generate poor sensitivity in STD NMR, whereas waterLOGSY pulse sequences enhance the sensitivity to a large extent because they use the magnetization transfer of the bulk water to ligand through the receptor. The degree of saturation largely depends on the so-called 'residence time' (τ_{res}) of the ligand in the binding site of protein or other macromolecule (Fig. 1; Box 1). The T1 or longitudinal relaxation time differs from one proton to another in the same ligand molecule in the free state. Therefore, the T1 value has an important role in the GEM of a molecule based on STD NMR experiments [23]. Very high or low values of T1 make an accurate interpretation of STD effects difficult (Box 2). Because the molar fraction of free ligand in STD NMR experiments is greater than that of the bound fraction, the corrections in T1 value are contributed largely by the activities of the free state of the ligand (Box 1). In 2004, Mayer and James introduced a new curve-fitting analysis where they could nullify the T1 bias and hence determine accurate STD analyses (Box 2) [24]. This fact is adequately explained with the example of the binding of acetropromazine in complex with RNA at two different saturation times, in addition to the STD effects calculated based on the build-up curves of saturation time [24]. The general trend is that protons with short T1 values increase their relative STD intensities, whereas saturation times of short-time domains are applied for the analysis.

BOX 2

Equations of relevance to STD NMR

Intensity of STD NMR spectrum : $I_{STD} = I_o - I_{sat}$ (I)

where I_o is the intensity of the off-resonance spectrum and I_{sat} is the intensity of the on-resonance spectrum.

STD effect : $\eta_{STD} = \frac{I_o - I_{sat}}{I_o} = \frac{I_{STD}}{I_o}$ (II)

STD amplification factor : $A_{STD} = \frac{I_o - I_{sat}}{I_o} \times \text{ligand excess}$ (III)

To determine the STD build-up curves:

STD amplification factor, $A_{STD} = STD_{max}(1 - e^{k_{st}t})$ (IV)

where STD_{max} is the maximal STD intensity, t is the saturation time and k_{st} is the saturation rate constant.
From Eq. (IV), the initial slope, which is known as the total STD value, can be obtained using Eq. (V):

$STD_{total} = STD_{max} \times k_{st}$. (V)

The initial slope of the STD isotherm can be plotted as a function of ligand concentration to obtain the dissociation constant, K_D:

$STD_{total} = \frac{B_{max} \times [\text{ligand}]}{K_D + [\text{ligand}]}$ (VI)

An STD pulse sequence is derived from the combination of several soft pulses with a duration of approximately 49 ms and the delay (δ_1) between the pulses is kept at approximately 1 ms (Box 1; Fig. 2). A soft pulse is introduced in the STD pulse sequence for delineating side-band problems that occur in cases of non-selective rectangular pulses. Generally, a train of Gaussian pulses is used to assist the saturation transfer of the protein. This cycle is repeated several times (usually $n = 40$) to achieve the saturation transfer of approximately 2 s from receptor to ligand (Fig. 2). The on- and off-resonances of the selective pulse is switched after every scan. A trail of other pulse sequences [e.g. STD-total correlation spectroscopy (TOCSY) or STD-HSQC (Heteronuclear Single-Quantum Coherence); Fig. 2] can be combined to provide better dispersion of the STD effects (Box 2). A spin-lock pulse (Fig. 2) can also be used after the saturation transfer cycle to suppress the interference of proteins or other large molecular receptors (Box 1).

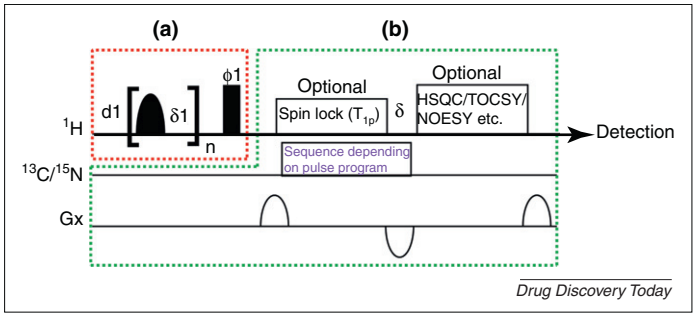

FIGURE 2

STD Pulse sequence. **(a)** A generalized pulse program scheme for saturation transfer difference nuclear magnetic resonance (STD NMR) spectroscopy (red-dashed line). Generally, 49-ms Gaussian soft pulses separated by an inter-pulse delay of 1 ms, are used in a loop ($n = 40$) for a total saturation of approximately 2 s. d1 is an additional short relaxation delay and typically set to 100–200 ms. The subtraction of on- and off-resonances is performed after every scan using phase cycling (δ_1). **(b)** denotes a variable NMR spectroscopy pulse sequence (green dashed line) that generates a whole suite of STD NMR experiments, such as STD Heteronuclear Single-Quantum Coherence (HSQC) spectroscopy or STD total correlation spectroscopy (TOCSY). The broad resonance signal of the protein can be eliminated with the help of a spin-lock filter ($T_{1\rho}$) for approximately 10–20 ms with a strength of 5 kHz.

Protein–carbohydrate interactions

In the first STD NMR experiments, carbohydrate as a ligand was introduced to interact with a protein, generating a substantial saturation transfer because of a low binding affinity (i.e. the dissociation rate constant, K_D, was high) toward the protein core [16–21]. The carbohydrate was liable to leave the interaction site and showed some capacity to be able to drift toward binding with the protein. To achieve a sufficient saturation transfer effect, the ligand should not be tightly bound so that the magnitude of the T2 relaxation rate of the bound ligand is high. The main point is whether the ligand spends more time in the core of the receptor, rather than being tightly attached to it, so that the ratio of the off-rate constant (k_{off}) and on-rate constant (k_{on}) is not high. K_D should have a value that is greater or equal to the magnitude of the T2 relaxation rate because the ligand has to leave the binding site before all saturation has been equally distributed among all the spins in the ligand as a result of spin diffusion.

The efficiency of the STD NMR experiment was first used in Bernd Meyer's laboratory [11] for the study of binding of *N*-acetylglucosamine (GlcNAc) to wheat germ agglutinin (WGA). In addition, the same group used the GEM method for the identification of binding of the surface of ligand, methyl β-D-galactoside (β-D-GalOMe) in presence of a 120-kDa protein, *Ricinus communis* agglutinin (RCA_{120}) [22]. It was possible to identify that only the H2, H3, H4 and H6 protons of galactose were responsible for binding to the protein, which is in agreement with previously published results [25]. Following this initial study, other groups began working on protein–carbohydrate interactions to facilitate either screening of compound libraries of carbohydrate molecules or epitope mapping for the designing of better inhibitors. Thus, we discuss below interesting observations of STD that helped answer many outstanding questions.

The carbohydrate molecule has a limited spectral width; therefore, there are severe problems associated with resonance overlapping. To obtain higher quality STD spectra, a second dimension becomes helpful by virtue of the combination of the STD pulse sequence with other pulse sequences, including TOCSY, COSY (correlation spectroscopy) and HSQC (Fig. 2), to resolve the spectral overlap for unambiguous quantification of STD effects. It was possible to screen a compound library comprising 20 dimethylated monosaccharides to identify the functional groups involved in competitive binding to a lectin, *Sambucus nigra* agglutinin (SNA) from elderberry using a simple and straightforward STD TOCSY experiment [26]. In addition, a new generation of STD NMR spectroscopy, called 1D STD TOCSY, was specifically used to work out how an oligosaccharide interacts with its receptor antibody [27]. Recent developments in STD NMR have produced edited-filtered strategies of gaining STD effects [28]. The convenience of using edited-filtered STD NMR is that it can be used to generate a compound library; a reference guest where isotopic labeling is used to monitor the differential binding affinities of different small molecular candidates toward the receptor. The resonance of the

reference guest is kept separate from that of the screening candidates so that it is easy to understand the proneness of the ligands to be attached to the receptor in a qualitative manner [28].

Rama Krishna and co-workers extended the application of complete relaxation and conformational exchange matrix analysis (CORCEMA) theory [29,30] in STD NMR experiments to determine theoretical STD effects (CORCEMA-STD), taking into account the binding kinetics, thermodynamics and protons involved in the binding site [31,32]. The good correlation between theoretical and experimental STD effects based on NOE R-factor analysis indicates that the STD effects obtained experimentally are devoid of T1 bias [31,32]. The theoretical STD values utilizing CORCEMA-STD were first implemented for the X-ray crystal structure of sialoadhesin, co-crystallized with its ligand 3′-sialyl lactose (1QFO.pdb) [33]. Excellent correlation between experimental and theoretical STD values yielded a reliable structural model for the complex of sialoadhesin with 3′-sialyl lactose in aqueous solution [21]. The successful development of STD NMR with an intensity-restrained CORCEMA optimization procedure for the refinement of the hybrid structure is elucidated based on the complete relaxation and conformational exchange matrix calculation and simulated annealing. This strategy is further used to refine bound conformations of a weakly binding ligand, positioned within the binding pocket of a target protein [34,35]. A recent study demonstrated that the group epitope mapping, with consideration of relaxation (CRL) of the ligand (GEM-CRL), is a more suited approach compared with only GEM. The GEM-CRL technique is useful for the CORCEMA-ST result because the transferred magnetization rate gives an additional parameter for the comparison between calculated and measure values [36]. Recently, Angulo and co-workers [37] presented a novel protocol based on STD NMR spectroscopy for the direct measurements of dissociation constants of protein–carbohydrate complexes using single ligand titration experiments (Box 2).

Earlier work by Meyer and co-workers [38] demonstrated how STD NMR can be effective in drug screening for scar proteins. In their experiment, several carbohydrate candidates tried to bind to the receptor protein WGA. Identification of the protein–carbohydrate complexes was executed by the use of high resolution magic angle spinning (HRMAS) NMR spectroscopy in combination with STD methods. Thus, STD NMR has become a pioneering technique that facilitates high-throughput screening for scar proteins.

Cell–peptide interactions

Although STD NMR is a useful tool for protein–ligand interaction studies, for large receptors, molecular assemblies or cells, it is not an effective technique to elucidate cell–ligand interactions at a submolecular level. For pharmaceutical industries where cell culture for drug discovery involves a huge amount of work, STD NMR is not a useful technique to find out which types of drug bind to infected cells. In addition, for membrane proteins, which in general do not dissolve easily in aqueous medium, drug–membrane protein interaction studies by STD NMR are unsuccessful. A new method, a second generation of STD NMR termed saturation transfer double difference (STDD) NMR spectroscopy [39,40], has become an effective tool in the epitope mapping of drug molecules when they are bound to cell and/or membrane proteins or any other complicated macromolecular assemblage. In STDD NMR [39,40], a STD spectrum of a cell plus ligand system is recorded. Then another spectrum containing only the cell is recorded at the same concentration as the previous spectrum. Next, a difference spectrum of these two systems is computed to perform the epitope mapping of the drug, bound to the cell. Meyer and co-workers realized that the 'double difference' spectroscopy is more advantageous compared with the STD NMR experiments with spin-lock, which is generally a popular scheme to suppress signals from large molecules [39]. With the aid of a spin-lock pulse, the signal-to-noise ratio of the STD spectra of small molecule decreases owing to the loss of saturation transfer from the ligand as a result of T1 and T2 relaxation processes.

Recently, the application of STDD NMR experiments has been implemented in several biological systems; for example, STDD NMR experiments were able to elucidate the mechanistic interactions of pesticides comprising anthropogenic chemicals with humic acid, which are generally used as a soil supplement in agriculture [41]. In other applications, STDD NMR experiments were used to confirm interactions between haplosamate A, a water-soluble cannabinoid agonist, and human cannabinoid G-protein-coupled receptors (GPCRs), CB1 and CB2 in a stable transformed Sf21 cell [42].

In our laboratory, we used STDD NMR probing interactions of live *Escherichia coli* cells with a designed antimicrobial peptide YW12D (NH_2-YVKLWRMIKFIR-amide) [43] (Fig. 3). Residual STD signal observed from *E. coli* cells (Fig. 3a,b) was subtracted, yielding a double difference spectrum containing STD signals only from the peptide (Fig. 3). Remarkably, a strong saturation transfer was observed for the aromatic protons of the residues Y1, W5 and F10 (Fig. 3d), indicating that these residues are in close contact with *E. coli* cells. STD signals were also observed for the side chains of residues V2, K3, L4, M7, R6, I11 and R12. The STD studies suggested that the YW12D interacts with the lipopolysaccharide (LPS) layer of the outer membrane of *E. coli* cells.

Potenza and co-workers [44] demonstrated that trNOESY with STD NMR can be an effective tool in measuring the binding affinities of integrin ligands with membrane bound proteins, directly suspended in the living cells without isolating the protein receptor.

Protein–peptide interactions

STD NMR has also been used to study various significant biological ligand–receptor systems. A small molecule study revealed that STD NMR could be a useful way to decipher the architecture of small molecule–protein complexes. STD NMR was used to monitor competitive binding to ubiquitin ligase protein, β-TrCP between the phosphorylated motifs of activating transcription factor-4 (ATF-4) and β-catenin [45]. This study was attempted to characterize the ATF-4 binding epitope [45]. Meyer and co-workers [46] have also shown using straightforward STD NMR spectroscopy that the cyclic peptide, cyclo(RGDfV), which is a specific inhibitor of integrin $\alpha_v\beta3$, can displace the open chain peptide RGD (arginine-glycine-aspartate) from the binding site. In addition, by performing STD NMR, one can also determine the binding epitope of the cyclo(RGDfV) to liposome-embedded integrins [46].

Isotope enrichment of carbon and nitrogen enhances the resolution of STD NMR spectroscopy to a great extent. The ^{13}C- and ^{15}N-enriched peptide RGDLXXL (XX indicates the two different amino acid residues) reduces the signal overlap to a major extent in

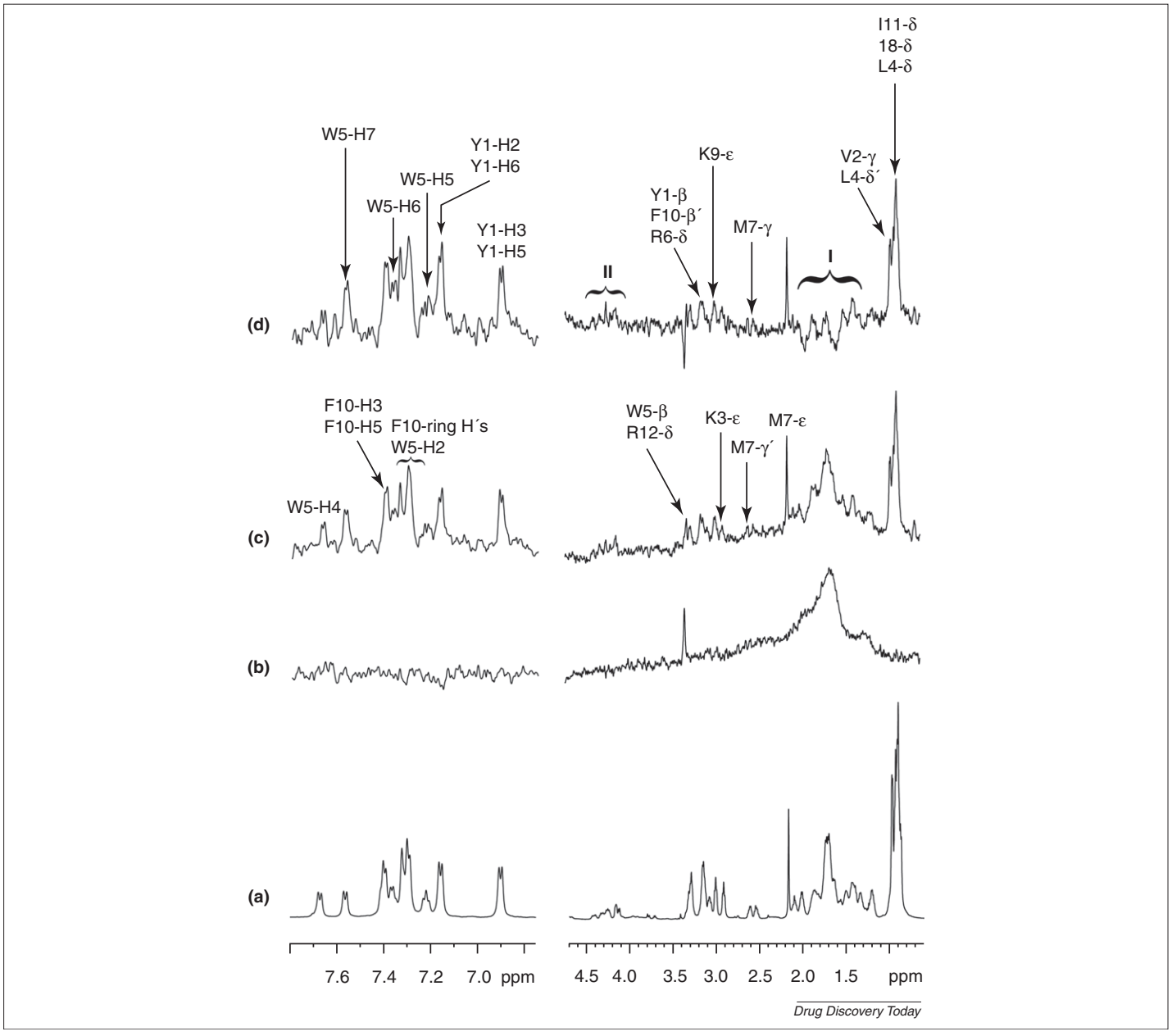

FIGURE 3

Saturation transfer double difference (STDD) technique. **(a)** The reference ^{1}H nuclear magnetic resonance (NMR) spectrum of YW12D in phosphate buffered saline (PBS), pH 4.8 at 298 K and 600 MHz. **(b)** The STD NMR spectrum of *Escherichia coli* BL21 cells in the absence of YW12D peptide. **(c)** The STD NMR spectrum of YW12D peptide in the presence of *E. coli* BL21 cells using the same condition as in (b). **(d)** The saturation transfer double difference (STDD) spectrum showing the filter effect of the double difference method in eliminating background signals from *E. coli* cells (a).

the 2D ^{13}C STD HSQC spectrum when it binds to integrin $\alpha_v\beta6$ [47]. The major advantage of STD NMR is that the STD pulse sequence can be combined with any pulse sequence of NMR spectroscopy to generate a well-suited, modified STD NMR spectroscopy. Interestingly, the ^{15}N-edited STD HSQC spectrum provided novel contact points between Val12 of the ligand and the $\alpha_v\beta6$ integrin, signifying the different orientations of the peptide in the presence of integrin [47].

Although it is well known that STD NMR is an appropriate tool for screening a compound library for binding to a receptor protein, Bernd Reif and co-workers [48] exploited the STD NMR technique to characterize the chemical exchange between soluble and aggregated states of amyloid-β peptides. Using the STD NMR experiment, it was possible to identify the chemical groups that were important for binding to mono- and oligomeric states of Aβ proteins. Surprisingly, the shift of equilibrium between mono- and oligomeric states can be stabilized by the formation of an intermediate structure, by changing the anionic strength of the buffer. Recently, Ongeri and co-workers [49] identified a sugar-based peptidomimetic that efficiently reduces the amount of typical amyloid fibrils. STD NMR experiments confirmed that the sugar-based peptidomimetic molecule interacts with the Aβ aggregated species through their hydrophobic amino acid residues. Bernd Reif's group also performed STD NMR to determine the

interactions between a yeast prion peptide, Sup35 and heat shock protein (Hsp)-104 under non-equilibrium conditions [50]. Strikingly, STD NMR in conjunction with a diffusion ordered spectroscopy (DOSY) experiment confirmed the strong interactions between Hsp104 and the tetra-/hexameric forms of Sup35. However, no interaction was detected between higher oligomeric states of Sup35 and Hsp104. This result suggests that the low-oligomeric species of Sup35 is important for prion propagation in yeast [50]. By contrast, STD NMR was used effectively to understand the inhibitory interactions of anti-prion peptide with prion protein during prion aggregation [51].

Lipid–peptide interactions

STD NMR is thought to be a robust technique in drug screening, although it was assumed that the cut-off size for the performance of STD NMR has a particular value. Contradictorily, it has been reported that STD NMR experiments can be suitably executed for micelle-forming lipopolysaccharide (LPS), although it has a molecular weight of approximately 100 kDa (the molecular weight of monomeric LPS is approximately 10 kDa) [52–57].

STD NMR is a useful method for identifying atoms of ligands that are significantly involved in binding to the receptor molecule. It has been shown that two atoms (geminal protons) have similar chemical shift values so that in one-dimensional proton NMR spectra, it is hard to separate them. In these specific cases, heteronuclear multidimensional STD NMR has been found to be a useful way of differentiating almost isochronous protons with respect to their binding affinities. Therefore, all atomic probes, namely ^{1}H, ^{13}C and ^{15}N, can be used to penetrate the structural insights of host–guest assemblies in different biological systems with a high atomic resolution [56]. The applications of STD NMR experiments were extended to map the binding surface of homodimeric antimicrobial peptides (AMPs) in the presence of LPS micelle. The synergistic effect of AMPs was also well proven by use of multidimensional STD NMR experiments [57]. Previously, STD NMR experiments had been used to identify the binding epitopes of encephalin [58] and bradykinin [59] peptides, which interact with ganglioside GM1.

Recently, the application of STD NMR experiments was extended to identify the binding localization of drugs to the small bicelles, which comprise 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dicaproyl-sn-glycero-3-phosphocholine (DHPC) [60].

Virus–carbohydrate interactions

STD NMR is a tool that resembles a sophisticated binocular with high resolution and that can separate the binding part of small molecules (ligands) and solve their functionalities in the presence of heterogeneous large molecular weight receptors, such as viruses [61–63] or virus-like particles (VLPs) [64–66]. A STD NMR experiment was used successfully to investigate the binding of the synthetic entry inhibitor, Repla 394 with the fully functional complete viruses [61]. It has also been reported that the state-of-the art STD technique could reveal the atomic details of recognition of histo-blood group antigens (HBGA) and fragments with rabbit hemorrhagic disease virus (RHDV)-like particles [64]. A recent article reported that STD NMR is a useful technique to show that citrate could compete with HBGA for norovirus binding [67].

Other applications

STD NMR is applied not only in regular biomolecular systems but also in colloidal matrices. A recent study using STD NMR to investigate the interactions of dispersion pigments in a colloidal system showed how useful it is in this situation [68]. A new STD NMR-based approach for nucleotide-dye binding studies showed that Sepharose CL-6B interacted less with 5′-mononucleotides than with beaded cellulose. This procedure was used to try to identify non-specific interactions between 5′-mononucleotides and matrices commonly used in affinity chromatography systems and also to clarify the contribution of a thiacarbocyanine dye immobilized onto cellulose beads in a biorecognition process [69].

Recently, Barbero and co-workers [70], using STD NMR in conjunction with CORCEMA-ST, calculated that the drugs, docetaxel and discodermolide, prefer a binding site that is different from the conventional binding site of microtubules at the lumen. This site is specifically the first binding site of the drug before its internalization by the microtubule lumen. STD NMR has also been used to study the binding interactions of two natural alkaloids, berberine and cinnamaldehyde, to FtsZ, a prokaryotic protein molecule [71,72]. Interestingly, STD NMR in combination with AutoDock confirmed that, although the molecules, berberine and cinnamaldehyde, bind two different parts of FtsZ, they can still halt cell division. Hence, they act as antibiotic molecules in nature.

Modified STD pulse sequences for better applications

The robustness of STD NMR is based on the understanding of interactions between isotopically unlabeled proteins and ligands. Because both the protein and ligand are unlabeled, their production costs are economical, especially for the screening of compound libraries. However, in some cases, the STD effect for a host–guest assembly pair is not achievable mainly because of severe signal overlaps. In this case, a novel modified STD experiment in which group-selective (GS) saturation of amide protons attached to ^{15}N labeled host can be used [73]. This kind of experiment comprises a train of Bird^{d} pulses, inverting only the protons, attached to ^{15}N, which results in the saturation transfer of amide protons while the magnetization of the background protons is much less affected. Partial saturation of the unlabeled protons by this method can be completely cancelled in the difference spectra, switching ^{15}N carrier frequency between on- and off-resonances. The author claimed that this ^{15}N GS STD NMR experiment can be applicable for all types of host–guest interaction, including screening of compounds from libraries [73]. In particular, host size in STD NMR experiments matters a lot. If the size of the host is small (<10 kDa), performing STD NMR will become problematic because of the failure of the strategy of using spin diffusion in STD NMR for producing the macromolecule–ligand interactions [4].

One interesting use of STD NMR is to map receptor–small molecule interactions at low concentrations. Clean STD NMR is the latest generation of STD experiments developed to overcome false positive results previously observed in the STD NMR spectrum because of the spillover of power in r.f. irradiation [74]. This new method achieves a high degree of saturation of resonance with the aid of digital editing of two STD NMR spectra to produce a difference spectrum in which sensitivity is enhanced by at least threefold.

Simultaneous action of STD and DOSY can reveal a strategy to generate knowledge of competitive interactions of small molecules with their receptor. DOSY-based STD NMR was introduced in a recent study where small molecules were screened simultaneously [75].

A new method of STD NMR has been developed based on the use of ^{13}C nuclei as the STD probe. By detecting the STD spectrum in the ^{13}C channel, it was possible to eliminate the signal of residual water in the STD NMR spectrum [76]. This is a simple method whereby an INEPT sequence was introduced in the pulse program so that magnetization transfer from the ^{1}H to the ^{13}C channel was possible. This work is the extension of work by Diercks and co-workers, who performed the same strategy using ^{19}F as a STD probe [77]. The advantage of using ^{13}C nuclei as the STD probe is the possibility of using natural ligands without any isotope labeling.

In a recent study, Tashiro and co-workers [78] developed a new STD pulse sequence that was more efficient and had greater applicability on the basis of ligand signals compared with that of the waterLOGSY experiments. A combination of presaturation (WET) with the STD sequence using repeated Z-filters provided effective water suppression, which led to the magnification of the resolution of the spectra provided by this modified STD pulse sequence.

Concluding remarks

STD NMR is a versatile technique that can be used to investigate a wide range of molecular recognition systems. It is a key method for the identification of active drug or drug-able molecules against the targets of complex biomolecular system. The usefulness of STD NMR experiments in mapping crucial chemical moieties at an atomic resolution is indispensible for the development of new therapeutics. Recently developed STD techniques, with higher robustness and sensitivity, are likely to have a significant impact on drug discovery pipelines in the future.

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