Abstract

Nuclear magnetic resonance (NMR) is an important analytical technique that has been around for several decades. In the late 1990s and early 2000s, the use of NMR in drug discovery greatly increased. The increase in NMR application was closely related to the rise of fragment-based drug discovery (FBDD). Despite the early enthusiasm about the use of NMR in drug discovery, some pharmaceutical companies have terminated their NMR research in later years. In this literature thesis, I will investigate the role of NMR in drug discovery. The main applications of this versatile technique will be explained and compared to competing technologies. Also, the present use and the future expectations of NMR in drug discovery are explored.

1 Introduction

Since the 1990s, pharmaceutical industry has invested heavily in high-throughput screening (HTS) and combinatorial chemistry to find new drug leads [1, 2]. However, the number of drugs approved by the Food and Drug Administration (FDA) has declined [3] and HTS technology has not been able to reverse this trend [1]. The high attrition rates can at least in part be assigned to the quality of chemical leads [4]. Fragment-based drug discovery (FBDD) is a new approach that has emerged as an alternative to HTS [4, 6, 7] and is used extensively in both industry and academia nowadays [8]. It is even often favoured over conventional HTS [9]. Nuclear magnetic resonance (NMR) has many applications in FBDD [10]. In this report, an overview of the different applications of NMR in pharmaceutical industry will be presented. NMR is mostly used in the early stages of drug discovery, that is, lead discovery, lead validation, and lead optimisation (Figure 1) [5, 10]. There is also a limited role of NMR in target discovery [3]. NMR can be used for screening moderately sized libraries with higher reliability and information content than most other screening techniques [11]. Also, it is an important alternative to X-ray crystallography to determine high-resolution protein structures [3]. Recent advancements in using NMR for the determination of membrane protein structures, which constitute a large group of drug targets, are described. NMR can also be applied in metabolomics to identify new targets or evaluate the effect of a drug lead on a cell line to alleviate the need for animal trials [12, 13]. The majority of the applications of NMR is in the field of fragment-based drug discovery. Therefore, a short overview of the principles of FBDD is presented first.

2 Fragment-based drug discovery

HTS is based on finding lead compounds from screening up to a few million drug-sized compounds [2, 4]. In FBDD, much smaller libraries (∼10^3–10^4 compounds) are screened, which consist of relatively small molecules (50-250 Da) – ‘fragments’ [2, 14]. Similar to the Lipinski rule-of-five [15], a rule-of-three has been established for fragments suitable to develop into drugs: a molecular weight of ≤300, a cLogP of ≤3, and a maximum of 3 hydrogen bond donors as well as a maximum of 3 hydrogen bond acceptors [16]. Although these are simply guidelines – especially the last two rules are under debate – they have proven valuable in the composition of fragment
more laborious, as the hits often show lower ligand optimisation [4]. For HTS hits, this process is much
10 nM inhibitor can be achieved through careful
only 20-100 molecules and a rule-of-five compliant
R represents the universal gas constant, and
ligand efficiency
≥ FBDD than in HTS [11]. When a fragment has a
the temperature. The LE is typically higher in
50% inhibited, HAC is the number of heavy atoms in
IC50, which is defined as follows [2]:

\[ LE = \frac{-\Delta G}{HAC} = \frac{-RT\ln(IC_{50})}{HAC} \]

where \( IC_{50} \) is the concentration at which the target is
50% inhibited, HAC is the number of heavy atoms in
the ligand, \( \Delta G \) is the Gibbs free energy difference,
R represents the universal gas constant, and T is
the temperature. The LE is typically higher in
FBDD than in HTS [11]. When a fragment has a
ligand efficiency \( \geq 0.3 \) kcal/mole per heavy atom, nM
leads can almost always be prepared by synthesizing
only 20-100 molecules and a rule-of-five compliant
10 nM inhibitor can be achieved through careful optimisation [4]. For HTS hits, this process is much
more laborious, as the hits often show lower ligand

efficiency (Figure 2). They are often evolved into
a high-molecular weight ligand that is not a suitable
drug candidate [2].

The number of possible compounds, or chemical
space, increases exponentially with the molecular
weight. There are about \( 10^9 \) fragment-sized drug-like
compounds, whereas for drug-sized compounds, the
estimates vary between \( 10^{20} \) and \( 10^{200} \) [4]. Therefore,
the smaller libraries in FBDD (\( 10^3-10^4 \)) actually
screen a larger portion of the chemical space com-
pared to HTS, which typically has a library size of \( \sim 10^9 \) [6]. Larger sampling of chemical space represents
an increased chance of finding a structural match
[6]. The larger compounds in HTS have increased
chances of mismatched groups as illustrated in Figure
3 [6, 17]. Other advantages of FBDD include easier
synthesis of fragments and circumventing patents
through the identification of hits in multiple chemical
classes [11].

In order to successfully employ FBDD, a number of
challenges have to be overcome. Firstly, the bioas-
says used in HTS are generally not suitable for de-
tecting weak binders [11]. Therefore, a key challenge
in FBDD is the development of reliable, specialised
methods with high sensitivity in the intermediate
binding affinity region used to perform fragment-
based screens [4, 7]. NMR has been a pioneering
technique in FBDD screening and has remained a
very popular choice for performing such screens [9–
11]. A second challenge is the optimisation of the hit
fragment to a lead molecule, which often starts with
determining the binding mode of the fragment. X-ray
crystallography is the preferred method to determine
the binding mode, while NMR is a less frequently
used back-up technique [11]. Finally, it should be
kept in mind that there is a fundamental difference
between a binding fragment and inhibition of the
target [6]. Therefore, additional experiments should
be performed to obtain inhibition data.

The range of targets FBDD can be applied to
is somewhat limited, because it heavily relies on
structural information in the lead optimisation process
[2, 7]. The small fragments allow for much creativity
in developing hits [6] but the number of possibilities
to expand the fragment is so large that structural
biology is virtually essential to prioritise the ideas [18].

At present, the results of fragment-based leads seem promising. One drug originating from FBDD, vemurafenib [19], which targets mutant BRAF kinase to treat metastatic melanoma, became the first FDA approved drug discovered by fragment-based screening in 2011 [6, 11]. Many more drugs are currently in several stages of clinical trials [6]. Full evaluation of the success of FBDD, however, is not yet possible as the technique only started to become popular in the early 2000s, whereas drugs often require ten to fifteen years to reach the market [1].
3 NMR screening

NMR is a popular tool in fragment-based screening. The versatility of NMR has given rise to many different methods over the years [10], of which an overview will be presented in this section. For every technique, a difference in one or another NMR parameter signifies binding. Methods can be divided into two groups: those that detect binding in the protein spectrum (target-based) and those that are based on changes in the ligand spectrum (ligand-based). Ligand-based methods can be further divided into techniques based on detecting differences in $^1$H spectra and those that measure the $^{19}$F spectrum to detect binding. These different classes of NMR screening methods will be discussed in succession.

3.1 Protein-based screening

One of the oldest and most widely used methods to detect binding by NMR is the use of chemical shift perturbations (CSPs), i.e. changes in chemical shift of certain resonances in the NMR spectrum [3]. The chemical shifts of atoms in the binding site will change when a ligand binds. Because of fast exchange between bound and unbound state, the observed chemical shift is a weighted average of the chemical shifts in the bound and unbound state [9]. CSPs are almost exclusively used for target-based screening [20], while other parameters are often monitored for ligand-based screens [21]. Typically, a 2D $^1$H-$^15$N-heteronuclear single quantum correlation (HSQC) spectrum, which requires labelled protein, is obtained in target-based screening [22]. Alternatively, a $^1$H-$^13$C-HSQC spectrum can be measured. However, $^1$H-$^15$N-HSQC spectra are preferred, because $^{13}$C-labelling is more expensive than $^{15}$N-labelling and $^1$H-$^13$C-HSQC protein spectra are more complex than their $^{15}$N counterparts [21]. Also, $^{15}$N-labelling allows for the identification of amides, which are often used for structure interpretation because of their strategic location in proteins. A limitation of using amide assignment is the lack of information on the amino acid sidechains, which often requires additional experiments [23]. Heteronuclear spectra are preferred over homonuclear 2D $^1$H$^1$H-experiments, such as total correlation spectroscopy (TOCSY), since the CSPs are often difficult to identify due to peak overlap and therefore, identification of amino acids involved in ligand binding is often impossible [21]. It should be noted that there are no ligand resonances visible in the heteronuclear spectra, because the ligand is not isotopically labelled [24]. Therefore, the ligand cannot be identified from the protein spectrum [21].

The requirement to obtain 2D NMR spectra is a major limiting factor for target-based experiments [11, 22]. There have been improvements in acquisition time [22] and >200 spectra can now be obtained in 24 hours if the spectra are well-resolved [11]. Additionally, a relatively high concentration of protein is required to obtain a reasonable signal to noise (S/N) ratio and, with the additional requirement of isotopic labelling, acquiring a sufficient amount of purified, labelled protein is relatively expensive and often technically difficult [5, 20]. Furthermore, there is a limitation on the protein size [24]. For large proteins, the number of signals increases, the signals themselves become broader due to reduced molecular tumbling, and larger molecules are subject to faster relaxation rates [5, 22, 24]. The spectral overlap increases with molecular weight and the structure becomes more difficult to solve. However, the size limit has increased steadily throughout the years as NMR becomes more sensitive by using higher magnetic fields, cryogenic probes, miniaturised samples and optimised acquisition protocols [25]. Currently, the size limit generally stands at 40-50 kDa [11, 26].

Specialised methods have been developed in order to deal with larger proteins, of which the two most important techniques are transverse relaxation optimised spectroscopy (TROSY) [23] and $^{13}$C-labelling of Val, Ile and Leu [27].

TROSY is a method that is not specifically designed for screening, but for extending the molecular weight limit of structure determination of proteins by NMR. Screening with TROSY still relies on the measurement of CSPs: the difference is that larger proteins can be screened when TROSY is used. TROSY is based on a pulse sequence that increases transverse relaxation time (Figure 4). Line broadening and low sensitivity are two main limiting factors for obtaining structural information at high molecular weights, but TROSY improves both by reducing the relaxation rate [23]. In order to acquire maximum decrease of relaxation rate, $^2$D-labelling is required since hydrogen atoms provide a major contribution to relaxation. Unfortunately, $^2$D-labelling decreases the signal to noise ratio, since $^1$H is the most sensitive of all nuclei in terms of NMR signal. Therefore, proteins are typically deuterated to a certain degree (~70 % of C-H moieties) to retain part of the signal. Backbone amides are not deuterated since they are in exchange with the solvent, H$_2$O [23].

Two or more different relaxation mechanisms must be present to use TROSY, because it makes use of the interference between the two. An important example is the $^{15}$N amide moiety, where protons couple either spin up or spin down to the $^{15}$N nucleus. Therefore, two signals are found in the spectrum. The different line widths of the signals show they have dissimilar relaxation rates, which is caused by interference between the two. In regular NMR methods, the two resonances are collapsed by decoupling, however, TROSY selects only the slowly resonating signal. Although only half of the potential signal is selected, the slower relaxation rates for large molecules ensure the final signal strength is still superior. A gain in signal is achieved for molecular weights above 15-20...
kDa when using a 700 MHz spectrometer [23]. The best TROSY spectra are obtained at high magnetic fields, since interference is optimal at about 1,000 MHz.

For homomultimeric proteins, signals of the different monomers usually overlap. However, TROSY is still very useful to increase signal strength and reducing line widths [23]. Using TROSY, the structure of a 900 kDa GroEL-GroES complex was solved [28], significantly above the regular molecular weight limit of about 40-50 kDa [11]. Structural assignments were possible because GroEL (800 kDa) is homotetradecameric and GroES (72 kDa) is homohexameric [28], which greatly reduced the peak overlap. Other large systems that become feasible for structure determination using TROSY are membrane proteins incorporated in micelles (discussed later in Section 6) [23].

Solvated exposed amides with TROSY (SEA-TROSY) is a method that is not suitable for full structure determination, but for fast partial backbone assignments [29]. It is based on the premise that only solvated-exposed amides are important for interacting with the ligand as opposed to the buried amides which are unlikely involved in ligand binding [29]. Magnetisation is transferred from water molecules to solvated exposed amides via proton exchange [21]. The appearance of the spectrum is the same as a regular TROSY spectrum, but only resonances from backbone amides are visible, thereby greatly reducing spectral complexity [29]. Binding is be detected with CSPs. This method can be easily combined with other techniques that obtain information concerning ligand binding, protein structure, etc. to simplify the use of NMR with large proteins [29].

Specific labelling also helps to reduce overlap for very large monomeric proteins. In contrast to TROSY, a specific $^{13}$C-labelling method is only suitable for screening ligand binding. In this experiment, spectral simplification for large proteins is achieved by labelling only Val, Leu, and Ile ($^{13}$C only) with $^{13}$C [27]. It relies on the presence of carbons near a ligand binding site, in contrast to most methods which rely on backbone amides. An analysis of 191 non-degenerate structure showed that in 92% of cases, a carbon is found within 6 Å of a ligand heavy atom. For amides, only 82% of ligands satisfied these conditions, showing the validity of the approach [27]. Detection of ligand binding is achieved by change in $^{13}$C chemical shifts. Usually, methyl signals are relatively narrow and are therefore often well separated in the spectrum [5]. However, for very large proteins, the sensitivity of the method greatly decreases due to significant overlap of the $^{13}$C Val-, Leu-, and Ile-signals. Also, deuteration is necessary for large proteins to reduce relaxation rates as in TROSY. A main advantage of $^{13}$C-labelling is that it works well at any magnetic field strength, whereas TROSY requires high magnetic fields ($\geq$800 MHz) for optimal sensitivity enhancement [11, 27]. $^{13}$C-based methods require more protein than TROSY experiments, but recycling the protein throughout the screen is possible with reasonable yields of 50-80% [11].

NMR has potential to be used in vivo for screening and structure determination. This was shown, for example, by Inomata and colleagues [30], who measured the in vivo spectrum of $^{15}$N-labelled ubiquitin with three residues mutated to Ala (Ub3A) in a HeLa human cell line. The protein was delivered into the cell by conjugating it to a cell penetrating peptide (CPP). In the cells, the CPP was cleaved off the Ub3A. The resulting in vivo spectra were of high enough quality to solve the NMR structure [30]. Not only Ub3A, but also wildtype Ub spectra were acquired. However, the spectral resolution of the wildtype Ub spectra was too poor to allow for resonance assignment. Presumably, the line broadening is caused by interactions with binding partners, which were avoided by introducing three alanines on key binding positions in Ub3A [30]. This shows that the in vivo characterisation of proteins interacting with other proteins is not yet possible by NMR. In a second experiment, they applied target-based screening in vivo with the model system of immunosuppressants FK506 and rapamycin binding to the target protein, peptidyl-prolyl cis–trans isomerase FKBP12. A CPP-Ub-FKBP12 fusion protein was able to penetrate the cells, and in cellular environment, FKBP12 was cleaved off, allowing for measurement of ligand binding. Similar CSPs were observed in vivo as in an in vitro control experiment. With in-cell NMR screening, the efficiency of drug delivery into cells can be determined directly and it can also be established whether drug leads make the same interactions in vivo or not. However, the technique is highly demanding and is therefore not suitable for high-throughput screening with the present methodology [30].
3.2 Ligand-based $^1$H methods

Small molecules have slow relaxation rates, negative Nuclear Overhauser Effect (NOE) cross-peaks and large diffusion coefficients, whereas large protein molecules exhibit fast relaxation rates, positive NOE cross-peaks and slow diffusion coefficients [20]. Ligand-based methods are often based on the ligand adapting such properties of the target upon binding (Figure 5). Measurement of CSPs is not suitable for ligand-based methods because a $^1$H spectrum is generally recorded, which shows much less chemical shift dispersion. Therefore, CSPs of ligands are often too small to measure reliably. Generally, ligand-based methods require the measurement of a 1D spectrum and are therefore faster than target-based methods [5]. Also, ligands can often be screened in mixtures of 10-20 ligands at a time, because identification of the binding compound is possible [9]. Mixtures should be carefully composed, since ‘bad actors’ (e.g. aggregators) can infest measurements of the entire mixture [11]. Also, the ligands in the mixture should not significantly overlap in resonances [5]. The division of the library into mixtures is therefore easier when the ligand spectra are known [11]. Scanning in mixtures saves time and, often even more importantly, reduces the protein requirement [5, 11, 20]. Unlike target-based methods, there is no need to isotopically label the protein and additionally, there are often no limits in molecular weight of the protein [5]. However, it is often difficult to measure very high affinities with ligand-based methods because the protein signal is usually suppressed. If a ligand binds the protein very tightly, the signal of that ligand will be suppressed along with the protein signal [5]. Some of the most interesting hits may therefore be missed. False-negative as well as false-positive rates are higher in ligand-based methods [11] and although some screens provide some crude information on the binding site, detailed structural information is not available [5].

The most widely used method in ligand-based screening is saturation transfer difference (STD) NMR [11, 31]. Although the principle for saturation transfer NMR was already known since 1979 [32], it was first applied to the screening of protein-ligand binding in 1999 by Mayer and Meyer [33]. It relies on the transfer of NOE information [11]. Two spectra are recorded: one where a $^1$H protein signal is saturated (‘on-resonance’) and one where saturation is applied at a frequency where no $^1$H resonances are found (‘off-resonance’) [32]. The saturation of the proton signal of the protein in the on-resonance experiment will spread by spin-diffusion throughout the protein and also to ligands bound to the protein. Saturation of a signal causes the populations of the energy levels to be equal, and not distributed according to the Boltzmann distribution as is normally the case. Therefore, saturated protons do not show any signal. The off-resonance spectrum is simply a reference where none of the signals are saturated. When the difference spectrum of the reference and the saturated spectrum is obtained, the bound ligands will show up because they lost their signal in the saturated spectrum. Non-binding ligands have roughly the same signal intensity in the reference and the saturated spectrum and therefore, they have approximately zero intensity in the STD spectrum [21]. The protein signal is removed from the difference spectrum by applying filters [21]. An example of ligand binding detection with STD-NMR is shown in Figure 6. It is important that the on-resonance spectrum is obtained at a frequency where no ligand resonance are recorded. A popular choice is around -1 ppm, where the broad methyl signals of the protein can be excited and ligand signals are hardly ever found [21]. If there are no ligands with aromatic groups, an alternative is exciting in the aromatic region around 7 ppm [32]. The off-resonance spectra can be recorded at any frequency where no $^1$H resonances are found (e.g. at 40 ppm) [21].

Saturation of the protein and ligand is very fast ($\sim$100 ms) compared to longitudinal ($T_1$) and transverse ($T_2$) relaxation times for small molecules ($\sim$1 s). If the ligand has a fast off rate, multiple ligands

![Figure 5: Fragments adapt properties relevant to NMR when they bind to a macromolecule. From [5].](image)

![Figure 6: Example of ligand binding detected by STD-NMR. (A) Reference spectrum of 120 kDa Ricinus communis agglutinin (RCA120) protein (50 μM binding site concentration). The broadness of signals is normal for such a large protein, the sharp peaks arise from small molecule impurities. (B) The STD NMR spectrum (i.e. reference – saturated spectrum). The on-resonance spectrum was recorded after excitation at $\delta \sim -2$ ppm. A fully saturated protein should not give any signal and indeed, the difference spectrum is quite similar to the reference. Also, the impurities are filtered out, since they give the same signal in the reference and saturated spectrum. (C) Spectrum recorded with a $T_{1\rho}$ filter which eliminates all protein signal. As expected, only the impurities are visible. (D) Reference spectrum of RCA120 (binding site concentration 40 μM) in presence of 1.2 mM β-GalOMe, a known inhibitor and (E) the corresponding STD NMR spectrum. (F) STD NMR spectrum as in (E) with $T_{1\rho}$ filter. In both (E) and (F), there are clear signals from β-GalOMe, indicating that it binds. From [21].](image)
can bind to the same target molecule to be saturated and the STD NMR signal increases [21]. A high concentration of ligands is used to make optimal use of this increase in sensitivity. Typically, the ligand concentration is about 100 times as high as the protein concentration, which makes low protein consumption possible [21, 22]. The protein requirement of STD NMR is one of the lowest among available NMR screening techniques [21]. A high affinity ligand often has a slower off rate and counter intuitively, a very strong binder therefore shows lower STD signal than a ligand with intermediate affinity [21]. Nevertheless, STD NMR is still sensitive for a relatively large range of affinities [22].

A major advantage of STD NMR is that it can be combined with other NMR techniques because of the nature of its pulse sequence [21]. Additionally, there is no upper size limit for the protein. Moreover, it is more sensitive for larger proteins, since increased molecular weight leads to reduced molecular tumbling and increased correlation time, which increases STD effects [5]. STD NMR can also be performed on immobilized proteins and is therefore particularly promising for applications with membrane proteins [21, 22]. There is a lower size limit for STD NMR of about 10 kDa, because small proteins are not saturated effectively by spin diffusion. Another limitation is a requirement for high ligand concentrations (∼50 μM), which may cause protein solubility difficulties [21].

The principles of STD NMR can also be used to screen complex biological samples. Claassen and colleagues [34] detected binding of the pentapeptide cyclo(RGDfV) to the surface glycoprotein integrin α1β3β3 of intact human blood platelets using saturation transfer double difference (STDD). Intact human blood platelets provide a complex sample where many small molecules and proteins interact with each other. Therefore, an STD spectrum will not only show binding of the pentapeptide, but also from many other small molecules interacting with proteins. In order to obtain a difference spectrum that contained only the binding of interest, two STD spectra were collected: one of the blood platelet sample and the second of that same sample, but with the added pentapeptide [34].

In this manner, when a double difference spectrum is calculated, all the background binding in the blood platelets is not observed in the final spectrum [34]. The complexity of the samples that can be analysed by NMR in this manner is greatly increased in comparison with other techniques, and may therefore have considerable impact in the future [5]. However, this method is not fast enough to measure large libraries of compounds, since the measurement of a single sample takes almost an hour [34].

Water-ligand observation by gradient spectroscopy (WaterLOGSY) is another much used technique similar to STD NMR. The difference is that it uses the transfer of magnetisation from bulk water to the protein instead of direct protein magnetisation [35, 36]. An off- and on-resonance spectrum are recorded in WaterLOGSY as in STD NMR, although selective inversion of the water signal is an alternative for on-resonance saturation in WaterLOGSY [21]. The water magnetisation is transferred to the protein via two pathways: (1) transfer from water molecules in protein cavities and (2) hydrogen exchange of labile protons on the protein [35]. Magnetisation is also transferred to binding ligands via water molecules bound in the protein cavity. Such water molecules are almost always present and have sufficient residence time in the cavity to transfer magnetisation, but do not stay long enough to change their chemical shifts, which would prevent them from being saturated with the bulk water [35]. Magnetisation is also transferred to ligands in solution. However, binding and non-binding compounds can still be distinguished, because the difference in molecular tumbling between the protein-ligand complex and the free ligand cause them to have opposite NOE signals. For small molecules, the NOE is positive, but it is negative for proteins and the ligands binding to the protein. Therefore, non-binding small molecules show negative peaks in the difference spectrum (i.e. off-resonance reference – on-resonance saturated water), whereas protein-bound ligands have a positive signal (Figure 7) [36].

WaterLOGSY screens are sensitive and can easily handle mixtures of ligands [35]. Limited amounts of protein are required, though compared to STD NMR the amount of protein required is quite high [21]. WaterLOGSY is especially useful when either the complex or the target is strongly hydrated, which, for example, is the case for many RNA targets [21]. WaterLOGSY is also a good alternative to STD NMR when the proton density of the target is not very high. In such cases, the STD effect is smaller because magnetisation transfer is more difficult [5]. Similar to STD NMR, WaterLOGSY has difficulties in detecting very strongly bound ligands, because it also relies on the transfer of magnetisation to multiple ligands [35, 36].

STD NMR and WaterLOGSY can also be executed in the presence of competitive binders or
reporter compounds [37]. Such an experiment is often performed in succession of the primary screen with a known ligand to find out if hits bind in the catalytic site or elsewhere [37]. Generally, STD NMR and WaterLOGSY are widely used because they are carried out relatively easily with standard NMR instrumentation [31]. The most important requirements are that the protein is of sufficient size (≥15-20 kDa) and soluble in the presence of high fragment concentrations [31].

STD NMR and WaterLOGSY both rely on the transfer of NOE signal. However, the transferred NOE (trNOE) signal itself can also be measured to determine whether a compound binds to the target [38]. This method relies on 2D NOESY spectra of the ligand. If a compound does not bind the target, it will show weak NOE peaks of small intensity opposite to the diagonal signal. However, a protein-binding compound will show high intensity signals with the same sign as the diagonal peak [38], because NOE information is transferred from the protein to the binding ligand. Though this technique is very reliable and sensitive, it is time consuming due to the need for 2D spectra [38]. Similar to STD NMR and WaterLOGSY, an excess of ligand is required to attain sufficient NOE transfer [21].

The final technique based on NOE transfer that is described here is NOE pumping [39]. In this experiment, ligand signals are suppressed and NOE signal is pumped via the protein to the ligand during a mixing time. This NOE signal can then be detected. Unfortunately, NOE pumping requires optimisation of mixing time and ligand excess [21] and ligand signal suppression is difficult [40]. Therefore, reverse NOE pumping [40] was also developed. Here, the protein signal is suppressed instead of the ligand signal. The loss of ligand signal due to NOE pumping to the protein is then detected by decreased ligand signal intensities [40].

A completely different ligand-based screening technique, target immobilised NMR screening (TINS) [41], allows for reduced protein consumption through immobilisation of the target. The protein is immobilised on a surface in random orientations, after which ligand binding is assessed based on 1D 1H-spectra. Ligands that bind to the surface are broadened beyond detection because of the lack of molecular tumbling. Therefore, the signal of a binding compound is not detected and binding is shown by comparing spectra in absence and presence of immobilised protein [41]. Binding ligands will not lose their entire signal, because part of the ligand molecules will not come near the target immobilised surface during the NMR experiment timescale (~80 ms). Nevertheless, weakly binding ligands can easily detected: weak binding of 1 mM still results in a signal decrease of about 35%. However, TINS cannot detect very weak binding (~10 mM) in contrast to, for example, STD NMR. The main advantage of TINS is the very limited protein requirement. Only 3-5 mg of protein is required to screen a moderately sized library, since the same batch of immobilised protein can be used throughout a TINS screen of 2,000 compounds without change in binding capacity [41]. Additionally, it has little requirements concerning the target: there are no solubility or aggregation concerns and it has great potential for screening membrane proteins, because membrane proteins can be immobilised in a natural environment-mimicking membrane [41, 42].

Techniques based on differences line width and diffusion rates have also been developed, but are only used in a very limited number of cases [21]. Broadening is observed for ligand signals when the ligand binds the protein, but measuring line broadening requires well separated signals. Therefore, use of large mixtures in screening is difficult [21]. Difference spectra with and without protein would resolve these problems, because only binding ligands would contribute, but a careful experimental set-up is required and artefacts in the difference spectra are difficult to remove [21].

Diffusion rates can be measured by NMR with gradient technology [21]. Ligands bound to the target adopt the target’s diffusion rate. If the target is too large, line widths of the ligand become so broad that accurate determination of the diffusion constant is difficult. The major limitation is the requirement that a large portion of ligand is bound, because the total diffusion rate is sum of the product of the fraction of free ligand and the diffusion rate of free ligand and the product of the fraction of the bound state with the diffusion rate of the bound state [21].

### 3.3 19F-based screening methods

A relatively new range of techniques in ligand-based screening are based on the detection of 19F chemical shift perturbations [43]. Clearly, fluorine spectra are much simpler than proton spectra since there are far less different signals. Therefore, larger mixtures of compounds can be screened simultaneously compared to 1H screening, which greatly reduces measurement time [43]. The 19F isotope has 100% natural abundance and 83% of the sensitivity of 1H [22]. To achieve optimal sensitivity, a specialised 19F probe is required [17]. In sharp contrast to 1H ligand-based screening methods, 19F-based experiments often measure CSPs [22]. CSPs are easy to measure, because the chemical shift range is large due to the large influence of nine electrons and Van der Waals and electrostatic forces [22]. In contrast to 1H spectra, background signals are not a problem for 19F spectra [43], since 19F does not occur in buffers, detergents, proteins, or even in entire cells [22, 43]. Their absence in cells offers possibilities to screen in vivo [44].

The main concern for 19F-based screening methods is the coverage of chemical space by fluorine-
makes use of the large chemical shift anisotropy of fluorine, which causes major changes in line widths of the fluorine peak of free and bound state [45]. FAXS is fast, requires limited amounts of protein and can provide an estimate of binding constants [45]. The affinity of the spy molecule determines the range of chemical shifts that can be measured. Strong main pocket binders are always detected with this method because they will certainly displace the spy molecule [22]. Binders at allosteric sites, however, may not be identified as they probably do not displace the spy molecule. An important benefit of FAXS and 3-FABS is that they do not require a fluorinated library [22].

Not only ligand-based experiments, but also target-based approaches exist for $^{19}$F-based methods. However, their application depends to a great degree on the target. Target-based $^{19}$F experiments require introduction of fluorine atoms in the protein. This can be done either via introduction of a biosynthetically fluorinated amino acid or by conjugation of activated cysteines in a chemical reaction [22]. Since the background signal of $^{19}$F is very low, these target-based approaches do not have spectral overlap difficulties [22]. Possible sites of $^{19}$F introduction vary from case to case. Ligand binding is mainly detected by CSPs in 1D $^{19}$F spectra, but other experiments can also be performed dependent on the site of fluorine introduction and general properties of the target [22]. Target-based $^{19}$F experiments are limited in use [22], because they do not provide detailed structural information as is the case for target-based $^1$H approaches and are relatively complicated because much depends on the site and manner of fluorine introduction [22].

### 3.4 Other screening techniques

Due to the great versatility of NMR, the experiments above are not a complete overview of all existing methods to determine whether a ligand binds to a target. However, the most widely used techniques have been dealt with. There are, however, some techniques that fall outside of each of the categories dealt with so far and these will be described briefly here. One method, labelled ligand displacement (LLD) [46], is a $^{13}$C ligand-based method. It is very similar to 3-FABS, only it is based on the displacement of a $^{13}$C-labelled competitive binder from the protein pocket instead of a CF3-containing spy molecule. The advantage over 3-FABS is that there is no need for a fluorine group, however, the sensitivity is lower for $^{13}$C.

A method quite different from the ones dealt with so far is the NMR-based antagonist induced dissociation assay (AIDA) [47], which measures the inhibition of complex formation. It requires a large protein fragment (>30 kDa) and a small protein fragment (<20 kDa) and measures the $^1$H-$^15$N-HSQC spectrum of the complex. When an inhibitor is added, the crosspeaks signifying the interactions between the...
two proteins will (partly) disappear and the binding event is established. This experiment is very sensitive, but of limited application due to its requirement of a small and a large protein fragment that bind each other [47].

Very recently, a new experiment was published where the mobile regions of immobilised $^{13}$C-labelled proteins could be detected using NMR [48]. Normally, detection of protein is not possible when it is immobilised, but it was now achieved by using high resolution-magic angle spinning (HR-MAS) technology and grafting the protein on affinity beads. The binding of ligands makes the loops less flexible, and their signal therefore decreases for stronger binding ligands. The handling of proteins becomes easier when they are immobilised on beads, however, assay responses are biased compared to solution assays [48].
4 Fragment to lead

4.1 Structure determination

Fragment optimisation in FBDD relies heavily on structural information [2, 7] because of the very large number of possibilities to extend the fragment molecule [18]. NMR can be used to obtain structural information, but X-ray crystallography is generally considered to be superior to NMR in obtaining macromolecular structures [11]. Compared to NMR, structure determination by X-ray crystallography is faster, easier, and of higher resolution [10, 11] and is used by pharmaceutical industry in almost all cases where crystal structures can be obtained [3, 10]. An additional advantage of X-ray crystallography over NMR is that it does not require isotopically labelled protein [3]. However, it was estimated that for 20-40 % of proteins, the structure can only be solved by NMR [3]. Also, it is sometimes difficult to obtain a protein-ligand complex in X-ray crystallography [11], since the crystal lattice may not relax sufficiently to allow ligand binding [17] or the water channels are too narrow for the ligand to diffuse easily [11]. A possible solution is co-crystallisation of the protein and the ligand, but this does not always work [11]. NMR may play an important role in the future for the structure determination of membrane proteins (as will be discussed in Section 6), which is notoriously difficult by X-ray crystallography [42], and NMR gives additional information on structural dynamics. A limitation of NMR in structure determination is the peak overlap for high molecular weight proteins [3]. This phenomenon was discussed before in Section 3.1, where it was shown that methods such as TROSY can help to extend the limit. Alternatively, structures of separate domains can be obtained, as most domains fall within the molecular weight limit for structure determination by NMR [3]. The overall process of obtaining protein structures of NMR is slow and difficult. Whereas determination of crystal structures is highly automated, full automation is not often incorporated in structure determination by NMR [49]. Automation is difficult, because the variability of the technique allows for many slightly different approaches that require slightly different software [50]. Also, the structural elucidation consists of several steps, increasing the error margin at each step. Therefore, NMR structure determination is often semi-automated, where software is developed to automate one of the steps and an expert checks the results after every step. In total, over a 100 programs or algorithms performing one part of the process or another have been published so far [49]. The first step in traditional structure determination by NMR is peak picking. Although this may seem trivial, complex spectra cause difficulties because of peak overlap, noise, artefacts, baseline distortions, and phase distortions. Therefore, checking spectral assignment by eye often produces better results than peak picking by even the most advanced methods [49]. Next, chemical shift assignments are usually carried out to match the peaks to the atoms. Cross peaks allow for sequential assignments, but there are many mistakes because of peak overlap and spectral imperfections. The many tools developed to automate this step usually output lists of varying completeness and precision [49]. In the next step, NOEs are assigned to obtain distance restraints between atoms. This process is iterative. A number of clear crosspeaks are assigned to obtain a preliminary structure and gradually, the number of unassigned NOEs is decreased. When all NOEs are assigned, the structure is updated to a final structure. Again, there are difficulties due to spectral imperfections in this process. Also, there are not always enough NOEs available to obtain a first preliminary structure. Ambiguous NOEs have to be used in such cases that represent several possibilities in NOE assignment. Clearly, earlier mistakes in peak picking and chemical shift assignment will decrease the accuracy of NOE assignment, yielding an overall lower quality structure [49]. Because of the additive nature of mistakes in NMR assignment, experts usually validate the outcome of the sequential steps, although the first pipeline that allows entirely automatic structure determination by NMR has been available for some years [49]. However, this pipeline simply consists of a combination of automated steps, so the quality of the resulting structures may not be optimal. Automatic structure determination using NMR is therefore at present only possible for relatively simple spectra [49]. The classical approach to structure determination is not the only manner in which a structure can be derived from the NMR spectra [49]. There are several alternative approaches, one of which will be highlighted here. This method uses NOE distance restraints directly to create a spatial proton distribution. The protein can be fitted to this distribution, similar to the fitting of proteins in electron density obtained by X-ray crystallography. Successful application requires high quality spectra and little peak overlap. This method is therefore not used routinely [49].

4.2 Fragment optimisation with known protein structure

There are three main strategies to increase the potency of a fragment hit: fragment growing, fragment merging and fragment linking [11]. Fragment growing is the most commonly applied strategy [18]. Functional groups are added to the fragment hit in order to try and grow it into a secondary site near the original binding site. It is applied with mixed success, as the ligand efficiency often drops. The possibilities for interactions with the protein structure surrounding the binding pocket are vital for successful application of fragment growing [11]. The second optimisation
A widespread uptake [17] of the principle of SAR by NMR, which may prevent a more widespread use of structure-activity relationships (SARs) that are linked together with a linker based on that structural information [24]. SAR by NMR has been employed successfully in many cases, though the use of the technique is somewhat declining [18]. Abbott has patented SAR by NMR, which may prevent a more widespread uptake [17].

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Several specialised methods have been developed to screen for a secondary site binder for fragment linking. The most straightforward technique is to saturate the first binding site with a known binder that is as small and as potent as possible and then perform a regular screening campaign to find hits in other binding pockets. Since the binding affinity of secondary site binders is expected to be very low, the screening method must be sensitive and the fragment concentrations must be high [11]. A second approach to identify secondary site binders makes use of paramagnetic labels [51]. Paramagnetic centres greatly increase the relaxation rates of the groups surrounding them, which is observed through distance-dependent line broadening. If the first site binder is paramagnetically labelled, compounds binding in proximity can thus be identified [11]. Finally, second site binders can be identified with interligand NOEs (ILOEs) if ligands bind in close proximity and transfer magnetisation to each other. However, this signal is often very weak because of insufficient saturation of the binding sites [11]. Improvements in ILOE signals can be attained when the first site binder is covalently linked to the protein near the binding site via a flexible linker that allows it to bind the protein in its native state. The success of such experiments varies greatly, because suitable linkers and conjugation sites are not always found [11].

Fragments in different binding sites can be identified simultaneously, which eliminates the need for a traditional first screen before identifying second site binders. The method that achieves this is named structure-activity relationships by interligand nuclear Overhauser effects (SAR by ILOE) [52]. It is based on NOE peaks between two ligands instead of NOE peaks between ligand and protein. When two fragments bind near each other, they will transfer magnetisation, which is established by an NOE peak in the NOESY spectrum [52]. Thus, two binding fragments are identified simultaneously [11]. Subsequent linking of the two fragments can give a very potent drug lead. A major error source in SAR by ILOE arises from ligands binding the same site competitively. These ligands can transfer magnetisation via the protein to the next binding ligands and therefore, NOE peaks will also exist between them, even though they do not bind in different pockets [11].

4.3 Fragment optimisation without protein structure

Determination of a full protein structure is not always necessary for fragment optimisation. Several methods are discussed here that derive relevant structural information without the need for complete structure determination. Such processes usually save much time [11]. If the crystal structure and back-bone NMR assignments are already known, identification of a few intermolecular NOEs can already provide

Figure 9: An outline of the SAR by NMR method in five steps. A ligand is identified and then optimised. Subsequently, a second ligand is identified in a proximal pocket and also optimised. Finally, the optimised ligands are linked together, creating a high affinity drug lead. From [5].
The principle of interligand NOEs. Two competitive ligands, A (in green) and B (in blue) bind to the same site on the target. First, A binds and transfers magnetisation to a proton on the target T. Then, this magnetisation is transferred back to the other ligand, B, when it binds. Hence, magnetisation is transferred between A and B, giving rise to an NOE peak. From [54].

A good protein-ligand binding model [11]. If no protein resonance assignments are available, some lower resolution information of the binding site can still be obtained by various methods.

STD NMR can be used for group epitope mapping (GEM), where a rough map of the binding epitope is obtained. The protons closest to the protein are the first to be saturated through spin diffusion from the protein and will therefore show the highest STD signal. However, this difference is only visible if the ligand off rate is high enough. If the ligand resides too long on the target, the saturation will transfer throughout the ligand and it can no longer be determined which protons are directly involved in binding [21, 32].

The orientation and solvent accessibility of a ligand can be determined by a technique based on WaterLOGSY: solvent accessibility, ligand binding, and mapping of ligand orientation by NMR spectroscopy (SALMON) [53]. Magnetisation transfer via the protein is prevented by deuterating the protein on all non-exchangeable positions. Only direct magnetisation transfer from water molecules is then possible to achieve a WaterLOGSY effect. Therefore, it can be determined which hydrogens in the ligand are solvent accessible. For example, this was used to determine the orientation of CB1954 in the binding pocket of quinone oxidoreductase 2. The crystal structure for this binding pocket allowed for two possible orientations of the ligand and using SALMON, the right orientation could be determined [53].

In a third method, the relative orientations of two ligands can be observed by competitive binding in a method named interligand NOEs for pharmacophore mapping (INPHARMA) [54]. Competitive binding in the same pocket with fast exchange is required to carry out this procedure. As one of the ligands binds, it transfers magnetisation to protons on the target. When the other ligand binds, the target partly transfers that magnetisation back to the other ligand, which causes an NOE peak between the two ligands (Figure 10). A number of interligand NOEs will be identified. The NOEs indicate which protons bind the same site in the pocket and the ensemble of NOEs will therefore describe the relative orientation of the ligands with respect to each other. This offers a fast and easy method to determine the binding epitope if the manner of binding of another ligand is already known [54].

A method called structural information using Overhauser effects and selective labelling with NMR (SOS-NMR) [55] is based on STD NMR spectra from proteins that have been deuterated for all amino acid types except one. The non-deuterated amino acids are often hydrophobic residues found at the bottom of a pocket. With SOS-NMR, it can be determined which ligand atoms are bound near, for example, leucine atoms [55]. This method thus requires extensive specific labelling and provides only information on one amino acid type at a time. The final method that obtains information on the binding epitope mentioned here is NOE matching. In NOE matching, several protein-ligand binding poses are simulated. Predicted NOE patterns of these binding poses are compared to the experimental NOE pattern to find the most likely manner of binding [11].
5 Comparison of NMR methods in screening and optimisation

Target-based screening is very robust [11]. It can distinguish non-specific from specific binding and detects protein aggregation and pH-effects [5]. Therefore, the false negative rate is low for target-based NMR screening [11]. The detection range is also larger for target-based methods compared to ligand-based screening, especially at the high-affinity end [5]. However, the most valuable advantage of target-based screening lies in the possibility to obtain detailed structural information [5]. The requirements and costs of target-based approaches are quite high: large amounts of isotopically labelled proteins and long acquisition times are needed. Also, no ligand mixtures can be measured [5]. Only proteins of limited size can be dealt with in target-based approaches, since an increased number of signals and line broadening due to reduced molecular tumbling cause much spectral overlap [5, 22]. With the use of labelling or adapted pulse schemes, larger proteins can also be screened, though such experiments are relatively challenging [23, 27].

Generally, ligand-based methods are faster than target-based methods for individual measurements and can screen mixtures of 10-20 compounds simultaneously [5, 9]. The total throughput is therefore much higher for ligand-based methods. Additionally, the use of mixtures saves much protein consumption [5, 11, 20]. There are generally no upper molecular weight limits and there is no need for isotopic labelling [5]. The main limitation of ligand-based methods is difficulty in measuring very high affinities [5]. Ligand-based methods are less accurate and less detailed than target-based methods [11]. In contrast to target-based methods, artefacts such as aggregation are often not detected [11]. Crude structural information can be obtained with ligand-based techniques such as SALMON, INPHARMA, NOE matching, GEM using STD, or SOS-NMR (Section 4.3), however, detailed structural information is not available [5].

The most used NMR methods are generally the ones that are the most widely applicable and the easiest to implement. Additionally, the amount of protein required and the speed of the method are important in screening. The use of methods with a large range of possible application is logical because pharmaceutical companies prefer to set up a screening pipeline that is simple and can be used for multiple screening campaigns. The most widely used methods for screening are STD NMR and WaterLOGSY, followed by 19F screening based on CSPs. STD NMR and WaterLOGSY are very easy to implement since they require only standard NMR equipment and can be applied to a wide range of targets [31]. 19F screening often requires a specialised probe [17], but a wide range of applications is possible in a fast and sensitive manner [43]. Use of TINS may be increase in the future, since it excels in protein requirement and sensitivity for strong binding. Especially, the possible application of all these methods for membrane proteins is very important to have widespread uptake in drug discovery. The use of target-based methods in screening is limited, but possible for small fragment-based libraries [20]. The requirement for 2D spectra make target-based strategies too slow to screen larger libraries [22] and the protein requirement is also too high [20].

In hit validation and optimisation, the number of samples decreases greatly. Therefore, there is a gradual increase in preference of high-quality target-based measurements over high-throughput ligand-based methods [5]. Additionally, the requirement of target-based methods for much labelled protein becomes a smaller concern [20]. However, ligand-based methods are still used because protein structures cannot always be solved by NMR.

The hits from a primary screen are often validated to remove false positives [5]. At this stage, target-based and ligand-based methods are both applied. Determination of $K_d$ is often desired at this stage, which is possible with STD NMR and WaterLOGSY and routine for target-based methods, but is not possible with all ligand-based methods [20].

In hit optimisation, target-based methods become more popular, mainly because they offer detail in the manner of ligand binding [5], which is crucial to determine novel interaction possibilities. Also, target-based methods offer far better detection of high-affinity ligand binding [5]. This is important as an increase in potency is one of the main goals of fragment optimisation. Determinations of the $K_d$ are more precise with target-based methods than with ligand-based methods, especially for ligands in the higher affinity range [20]. An exception to that rule is the use of ligand-based competitive binding experiments to determine the $K_d$. 19F-based competition experiments offer the additional advantage of low background signals [5]. The pioneering SAR by NMR method has been used extensively in hit optimisation [5], however, since fragment linking is not always viable, fragment growing and merging strategies based on CSPs in the 2D spectra are also exploited very often [8].
6 Membrane protein structures with NMR

Membrane-embedded proteins are an important class of targets for pharmaceutical industry, since they represent 30% of all encoded proteins and even 60% of all drug targets [22]. The most important protein family for drug targeting are the G-protein coupled receptors (GPCRs), to which approximately 50% of drugs are targeted. Moreover, only 10% of GPCR types have been targeted. The remaining 90% of GPCRs therefore have tremendous pharmaceutical potential [26]. If available, X-ray structures are preferred [26], but the number of crystal structures available for membrane proteins is still very low, since such structures are difficult to obtain [42]. This is in part due to difficulties in crystallisation, but obtaining a sufficient amount of well-purified membrane protein for the trial-and-error process of crystallisation is also difficult, with the exception of rhodopsin [26, 42]. For NMR purposes, enough purified protein can be obtained routinely [56]. Obtaining structures of membrane proteins is also difficult when using NMR, but obtaining lower resolution information is usually possible [42]. Also, information on structural dynamics can be obtained with NMR. Such information could be used to address questions about flexibility of G-protein and ligand binding loops in GPCRs that have not been answered so far [26]. So far, most applications of NMR on membrane proteins have been aimed at answering specific questions, not full structure determination. However, structure determination is possible [26].

Both solid state NMR and in solution NMR can help in solving membrane protein structures [56]. Peak overlap is a major difficulty for both. Therefore, $^{13}$C and $^{15}$N are the most popular nuclei, since they have large chemical shift ranges [26]. The relatively high molecular weight of most membrane proteins already causes many, relatively broad signals, but additionally, the chemical shifts of transmembrane helices are narrowly dispersed [56]. There is little difference between the chemical shifts of different amino acids, because the helices are generally very uniform in shape and also contain many similar (often hydrophobic) amino acids. In total, 50-70% of the residues in transmembrane helices are either leucine, valine, isoleucine or alanine. The overlap in their signals is significant, causing great difficulty in assigning chemical shifts. Whereas assigning the back-bone chemical shifts is difficult, the assignment of side-chain resonances is even more difficult, since the methyl signals are greater in number and more narrowly distributed in chemical shifts [56]. Sparse restraints between ‘rare residues’ in transmembrane helices, such as phenylalanine or tryptophan, are of great help in determining lower resolution structures [56].

To increase structural accuracy, extensive labelling is vital. For example, specific labelling of single amino acid types can be used to correctly identify the signals. An alternative is reverse labelling: then, all residues are labelled except for one or two amino acid types, which can be identified through their disappearing signals. However, even with such extensive labelling, unambiguous chemical shift assignment is difficult [56].

The main reason for difficulties in obtaining solution NMR data on membrane proteins is the high apparent molecular mass [22]. Due to their hydrophobic nature, membrane proteins are typically incorporated in micelles to prevent aggregation. Micelles increase the apparent molecular weight of the protein, thereby broadening its NMR signals. Especially when a membrane protein forms oligomers, the molecular weight is often too high to determine the structure [22]. The choice of detergent is essential to obtain spectra with minimal line broadening and has to be made on a case-to-case basis in a trial-and-error process [26, 56]. The retainment of the native protein structure when a membrane protein is incorporated in a micelle is a major concern in solution NMR of membrane proteins [56], but membrane incorporation is not possible for solution measurements. The molecular weight limit for membrane protein structure determination lies around 40 kDa [26]. With the use of TROSY and extensive labelling, the molecular weight limit can be increased. Using such techniques, the structure of several β-barrel membrane proteins has been solved [23], but no full-length GPCR proteins have been solved by in solution NMR. An alternative to incorporation of membrane proteins in micelles is measuring their structure in organic solvents, however, retainment of native conformation is even more questionable in such experiments [26].

Solid state NMR (SS-NMR) is more promising in membrane protein structure determination, because it does not have difficulties with slow molecular tumbling and can provide more native-like conditions [26]. There are two important techniques in SS-NMR: (1) the more traditional approach of using high resolution magic angle spinning (HR-MAS) NMR to gain structural restraints, and (2) oriented sample (OS) SS-NMR which measures uniformly oriented lipid bilayers that are aligned with the magnetic field to gain orientational restraints [56]. The latter is based on the angle dependence of the anisotropic spin interaction component parallel to the magnetic axis. The angle with the $z$-axis can thus be measured, which provides the angle relative to the membrane since the membrane was aligned with the $z$-axis [56]. $^{15}$N is preferred over $^{13}$C for such experiments because it has less homonuclear interactions, which simplifies the spectra. Orientational restraints are accurate, as a difference in chemical shift of 10 ppm causes an error of only 3° [56]. Preparing samples with uniform orientation used to be ‘more of an art than a science’ [56], but sample preparation is not a large limitation any more. Still, the technique is still quite laborious.
because it often requires extensive specific labelling to resolve individual residue assignments [56].

MAS spectroscopy is an approach with a goal similar to solution NMR: obtaining torsional and distance restraints [56]. It relies on mechanical spinning about the ‘magic angle’ of $54.7^\circ$, because this angle averages out the anisotropic spin interactions ($3\cos^2 \theta - 1 = 0$ when $\theta = 54.7^\circ$), thereby greatly reducing the line width [42, 56]. In MAS spectroscopy, proteins are often incorporated in liposomes, so their native structure should largely be retained. Assignments of spectra obtained by MAS spectroscopy gives restraints similar to solution NMR and the structure determination is therefore also similar. Unfortunately, few restraints can be obtained for protein side-chains, especially for residues facing the lipid bilayer. These side-chains are therefore often simply modelled with molecular dynamics [56].

A combination of OS NMR and MAS spectroscopy is the most powerful for solving structures with SS-NMR [56]. The most important achievement in this field so far is the structure determination of the full-length GPCR-family member CXCR1, a high affinity receptor for CXC chemokine interleukin-8 [57]. A new variant of OS SS-NMR based on orientation-dependent motional averaging of dipolar coupling powder patterns to obtain orientational restraints on CXCR1. Then, they were able to assign 97% of backbone resonances from uniformly $^{13}$C/$^{15}$N labelled CXCR1 with MAS spectroscopy [57].

Altogether, SS-NMR is the most promising NMR technique for structure determination of membrane proteins, especially when a combination of OS and MAS spectroscopy is applied [56]. However, the structure determination of CXCR1 is the only structural characterisation of full-length helical protein performed as of 2013 [56, 57]. Other proteins characterised by SS-NMR are often small helical proteins derived from full-length proteins [36]. Solution NMR has solved structures of several $\beta$-barrel domains and small helical proteins, but the native protein structure is often not retained [26]. Characterisation of membrane proteins by NMR is not yet widely applied in drug discovery, because the experiments are very challenging, very time-consuming and cannot be performed routinely [22, 26]. It is therefore still very difficult to find leads for membrane protein targets with FBDD, since it so heavily relies on protein structures [25]. The largest applications of NMR in drug discovery with a membrane-embedded target are therefore found in ligand-based screening techniques such as STD NMR or TINS, which can be applied to a membrane protein as well as to other proteins (Section 3.2).
7 NMR metabolomics

An entirely different use of NMR in drug discovery lies in the analysis of biological samples by NMR metabolomics. A typical sample in NMR metabolomics consists of a cell suspension or a bodily fluid, often urine or blood [12].

A main application of NMR metabolomics is identifying novel biomarkers [3]. Identification of new biomarkers is based on classification of healthy and diseased biological samples [13]. Often, the 1D $^1$H NMR spectrum is acquired in which metabolites are identified [13]. These spectra show much peak overlap and therefore, only the most abundant metabolites can be identified. Two-dimensional spectra are obviously much richer in information, but typically take several hours instead of a few minutes to acquire [12]. The intrinsic variability of biological samples requires that a significant number of samples is collected in order to reliably classify them [13]. Classification is typically performed using principal component analysis (PCA) or orthogonal partial least squares-discriminant analysis (OPLS-DA) [12]. OPLS-DA is a technique that, unlike PCA, maximises class distances and is increasingly popular. A common concern is overfitting of the data, since OPLS-DA is a supervised method, although this can be resolved by decent cross-validation [12].

An essential part of NMR metabolomics is data preprocessing. Noise exclusion, peak normalisation and intelligent bucketing methods to prevent peak splitting are required to obtain a good classification [12]. Finally, the results of the classification show which peaks are responsible for differences between healthy and diseased organisms. These peaks can be assigned to a certain metabolite using databases of metabolite spectra [13]. Such a metabolite varies in concentration between healthy and diseased individuals and is therefore a candidate biomarker.

The biological activity of a candidate drug molecule can be elucidated using NMR metabolomics. Then, the differences between samples treated with the drug and reference samples are analysed with PCA or OPLS-DA, similar as in biomarker identification [13]. The difference in the level of metabolites can be used to establish (1) the in vivo activity of the drug, (2) its selectivity to the target or (3) evaluate toxicity [13]. The in vivo activity can be identified by comparing the impact of the drug on the metabolome with the impact of other drugs. For example, Halouska and co-workers [58] were able to discriminate between several classes of antibiotics acting on the bacterium *Mycobacterium smegmatis*. The classification could then be used to predict the biological mechanisms of drugs for which this was unknown [58]. Selectivity to a target can be investigated with differential NMR metabolomics [13]. In this experiment, there are four groups of samples: cell lines where (1) the target was knocked out, (2) the drug was administered, (3) a combination of the knocked-out target and administered drug was applied, and (4) a reference group. If the drug selectively inhibits the target, the metabolome is similar between the cell line with the knocked-out target and the cells where drug was administered. Those groups then cluster together in the score plots [13]. Toxicity problems can often be identified by increased or decreased concentrations of certain metabolites. Early detection of toxicity can therefore prevent needless time-consuming and expensive animal trials [3].

An alternative technique to NMR for metabolomics is mass spectroscopy (MS). Both MS and NMR have their limitations and they are viewed as being complementary to each other [12]. The main limitation of NMR is lower sensitivity, since it can often only identify the most abundant metabolites in a 1D spectrum [3]. However, it often has redundant means of metabolite identification due to the multiple peaks arising from a single metabolite [12]. MS is more sensitive and can detect more diverse metabolites (not only $^1$H), but requires chromatography in advance to the spectrometer. Therefore, quantification is difficult and additionally, not all metabolites will be detected [12].
8 Use of NMR in drug discovery

8.1 Comparison NMR to other screening and hit optimisation methods

Many techniques other than NMR are used for screening in drug discovery. These experiments are, similar to the different NMR methods, of variable high-throughput and high-content [25]. Some of the most important techniques are summarised in Table 1. Important characteristics of screening techniques are broad applicability, sensitivity to a wide range of binding constants and low false positive and false negative rates [31]. Parameters such as speed, cost, and protein use are often dependent on the throughput required – slower methods give more detailed and more reliable information but require more time and material [25].

Several experiments are suitable to screen relatively large fragment-based libraries (10,000-50,000 compounds) [2]. Surface plasmon resonance (SPR) is based on a change in the refractive index of a target protein bound to a surface [59]. It uses little protein and compound compared to NMR and is suitable to screen larger libraries [59]. It can also be used to quantify the strength of the interaction, though it cannot offer any structural information and detecting very weak binding (>1mM) is difficult [31]. Binding can also be measured by a thermal shift, where stabilisation by a small molecule changes the melting temperature [59]. Another alternative is high concentration screening (HCS) which is a variant of the HTS bioassay, but with higher ligand concentration, weakly binding fragments can be identified for some targets [17]. The obvious advantage is easy incorporation of the method in the existing HTS frame in pharmaceutical industry [17], but its applicability is limited because the sensitivity is often too low to detect weak binding, or because the biological activity cannot be measured in vitro [31]. Mass spectrometry (MS) has also become an established screening technique, where binding of a ligand is measured by a change in molecular weight of the complex [25]. The main advantage of MS is that 400-2500 compounds can be screened simultaneously, since peak resolution is high and unbound molecules are not detected [59]. Using MS, stoichiometry, specificity (through competition) and relative binding affinities can easily be obtained, although affinities are likely somewhat different in the gas-phase [25]. An advantage of HCS and MS is that they do not necessarily need highly purified protein, in contrast to NMR, X-ray, SPR and thermal shift measurements [59].

The only technique used for screening that is higher in information content than NMR is X-ray crystallography. Unfortunately, the throughput of X-ray crystallography is limited at a maximum of about 1,000 compounds [18]. The most detailed structural information is obtained with X-ray crystallography, but a crystal structure can only be solved for 35% of proteins [2]. Additionally, false negatives in binding may arise from undetected ligand solubility problems [8] and failed ligand binding due to narrow water channels [11].

NMR sensitivity has increased in the past years by availability of higher magnetic fields, cryogenic probes, miniaturised samples and optimised acquisition protocols [25]. Despite these advances, the technique is still quite limited in throughput and relatively protein consuming [2]. The key advantage of NMR and X-ray crystallography over all other methods is their ability to provide structural information in the screening phase [5, 9]. However, the throughput of X-ray crystallography and target-based NMR is too limited to screen reasonable fragment libraries. NMR can detect a large range of fragment affinities and does not suffer the same artefacts as bioassays [11]. The structural information in NMR assays can also be used to identify novel allosteric ‘hot spots’ in drug targets [11, 37]. Such secondary binding pockets can help tremendously in increasing the specificity of a drug on major target classes such as protein kinases and proteases that often have similar main binding sites [37]. Additionally, NMR is suitable to design protein-protein interaction inhibitors to targets that are generally deemed ‘undruggable’ [11].

All screening techniques have their own benefits and limitations. A combination of several techniques is therefore desirable in FBDD [31, 60]. Execution of several screening steps is viable in FBDD due to the very small libraries compared to HTS campaigns. Generally, the first step employs a high-throughput technique and gradually, experiments yielding more detailed information are used [60]. A common approach is, for example, to do a ‘pre-screen’ using NMR, SPR, HCS or virtual screening and validate the hits with X-ray crystallography [18]. However, the correlation in the hits between different techniques is typically 30-40% [8]. Therefore, there is a risk of filtering out false negatives at every step.

8.2 Use of NMR

NMR methods are very near the high-information content and low-throughput end of the spectrum of screening techniques (Table 1). Only ligand-based screening methods are usable as a first-step screening method for a fragment-based library. Often, X-ray crystallography is used as the final step, because it offers the highest quality information [18]. Most companies combine several screening methods, decreasing in throughput and increasing in quality. Therefore, NMR is at risk of being skipped in the line of screening methods as the second highest quality technique.

NMR changed its traditional role as structure determination tool to become a pioneering method in FBDD screening [50]. Some suggest NMR
screening is the most widespread in use [9], others claim STD NMR, SPR, and HCS are used most often [31]. Apparently, industry still uses NMR quite extensively, but there are also concerns that the application of NMR in pharmaceutical industry is declining: some companies make only limited use of it and others do not use NMR at all [3, 10, 50]. Though declination of NMR use may in part be due to the development of other techniques [50], NMR itself also suffers from several obstacles that hinder its widespread application. First, FBDD has not been widely recognised as a suitable path to develop drug leads. The role of NMR in industry closely correlates with the role of FBDD, because NMR finds its major applications in FBDD [9, 11, 18]. In some companies that do not recognise the role of FBDD, NMR is seen as a more costly and redundant version of X-ray crystallography [2, 3]. The cost of NMR equipment is also high, which may hinder investments in the technology [2]. Another concern is the inability of NMR to deliver protein structures on a short timescale. Typically, industry requires a structure within 1-2 weeks to continue with new compound design and synthesis [50]. Determination of crude binding information by ligand-based methods, such as INPHARMA or SOS-NMR (Section 4.3), can match this timeline, but the most applied NMR methods determine a high-resolution protein structures, which requires considerable time because NOEs have to be assigned [50]. Another problem is that there is little synergy between NMR and other techniques. If a ligand cannot be co-crystallised, obtaining an NMR structure is the ideal alternative, but using the crystal structure to assign NMR spectra is difficult and lacks widely accepted methods [50]. Also, comparing the results of the NMR experiments to data from other analytical techniques is required for optimal use of NMR in drug discovery, which is an intrinsically interdisciplinary field that cannot rely on any stand-alone method [10].

Ironically, one of the great strengths of NMR, its versatility, forms an obstacle for widespread use of NMR [50]. The wide range of methods allows for tailor-made solutions to many problems, but simultaneously makes finding a consensus on the ideal NMR experiment virtually impossible [50]. This problem also shows in the lack of general acceptance and common use of the same software. Initiatives such as CCP4 in X-ray crystallography are not successful yet in NMR – even though there is a similar initiative called CCPN [50]. Therefore, many slightly different analyses and data processing methods are carried out. In contrast, X-ray crystallography is much more straightforward – there is only one way to obtain a structure from a protein crystal [50]. A lack of consensus in NMR therefore creates difficulties in the automation of NMR processes, which poses a major limitation on its use in pharmaceutical industry. Another aspect that can inhibit NMR use is that the versatility of NMR can confuse non-experts. The possibilities and limitations of NMR are often unclear to them [50]. There are many NMR experiments that yield different kinds of information – from simple screening to structure determination, dynamics characterisation and even in-cell spectroscopy, metabolomics and membrane structure determination with solid state NMR – and all methods have their own limits in applicability and accuracy. Again, this is in sharp contrast with other techniques such as X-ray crystallography, of which the only goal is obtaining structures [50]. Extensive communication is key in the successful application of NMR in a drug discovery company because the non-experts have to understand what has to be achieved with the NMR experiments. The NMR experts have the responsibility to communicate well and produce data that is usable by other scientists [50]. Lack of experience in how to use NMR in screening in many companies may also limit its application compared to easy to implement techniques such as HCS. Finally, there is a

<table>
<thead>
<tr>
<th>Approach</th>
<th>Typical throughput per screen (compounds)</th>
<th>Quality of information about ligand binding mode</th>
<th>Resource and instrumentation requirements</th>
<th>Protein structure required</th>
<th>Key technical considerations</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTS</td>
<td>100-1000K</td>
<td>None</td>
<td>Specialised infrastructure required</td>
<td>No</td>
<td>Not suitable for fragments</td>
</tr>
<tr>
<td>High concentration beaoyy</td>
<td>10-50K</td>
<td>None</td>
<td>Very straightforward method</td>
<td>No</td>
<td>High false-positive rates can often hinder interpretation of data</td>
</tr>
<tr>
<td>Surface plasmon resonance</td>
<td>10-50K</td>
<td>None</td>
<td>Straightforward method, but requires costly instrumentation</td>
<td>No</td>
<td>Protein or compounds must be immobilised, false positives possible</td>
</tr>
<tr>
<td>Affinity mass spectrometry</td>
<td>10-50K</td>
<td>None</td>
<td>Straightforward method, but requires costly instrumentation</td>
<td>No</td>
<td>Limited applications reported</td>
</tr>
<tr>
<td>Covalent disulphide and SS</td>
<td>10-50K</td>
<td>None</td>
<td>Specialised infrastructure required</td>
<td>No</td>
<td>Requires cysteine residue close to active site</td>
</tr>
<tr>
<td>Dynamic, combinatorial chemistry and LC/MS</td>
<td>1-10K</td>
<td>None</td>
<td>Straightforward method</td>
<td>No</td>
<td>Limited range of chemistry in variable</td>
</tr>
<tr>
<td>Ligand-detected NMR (182D)</td>
<td>1-10K</td>
<td>Can distinguish active site vs. non-active site binders</td>
<td>Straightforward methods using 1H and 15N; but requires costly instrumentation</td>
<td>No</td>
<td>Protein typically &gt;25kDa in size, moderate protein requirements</td>
</tr>
<tr>
<td>Protein-detected NMR (365)</td>
<td>1-10K</td>
<td>Information of principle interactions between ligand and protein</td>
<td>Requires &gt;500NMR resonance assignments for amide groups. Requires costly instrumentation</td>
<td>Usually</td>
<td>Protein typically &gt;100kDa in size, high protein requirements</td>
</tr>
<tr>
<td>X-ray crystallography</td>
<td>500-10000</td>
<td>Detailed binding mode elucidated</td>
<td>Specialised infrastructure required</td>
<td>Yes</td>
<td>Limited to ~35% drug targets where structures can be solved; moderate protein requirements</td>
</tr>
</tbody>
</table>

Table 1: A comparison of methods in fragment screening, ordered from high throughput to low throughput. Generally, low throughput methods are richer in information content and are also often more reliable. It should be emphasised that NMR techniques are more versatile than other techniques: the subtle but important differences between the many NMR methods cannot be shown in such a table. Adapted from [3].
lack of suitable NMR education in programs aimed at drug discovery compared to other techniques for lead generation and optimisation in the United States [10]. The natural tendency is to give a concise overview of all methods available, whereas few programs go deeply into the underlying theory of NMR [10].

Despite all difficulties, NMR is still a much applied technique in screening fragment-based libraries [9, 17, 31], though the discrepancy in NMR use between different companies is large [50]. The lack of application of FBDD in some companies is expected to correlate with little NMR use. The interest of industry in the application of NMR for drug discovery can also be expressed by the great number of publications cited here where one or more of the authors are from industry: [1, 2, 4, 7–11, 14, 16–19, 22, 24, 25, 27, 29, 31, 35–41, 43–46, 48, 50–56, 59]. The great majority of publications from industry on NMR in drug discovery concern screening and fragment optimisation techniques, which is in accordance with the use of NMR in industry. In contrast, advances in structure determination, such as increases in the molecular weight range and determination of membrane protein structures, are typically achieved in academics, presumably because industry will resort to different methods for such targets since development of such methods requires much work with little chance of a substantial profit. Although methods to increase molecular weight limit to study proteins by NMR have been adopted by industry, this is not the case for areas that are in their infancy such as membrane protein structure determination by NMR. For membrane protein targets, drug design still mostly relies on HTS and not on FBDD [25, 37]. NMR metabolomics has not been widely adopted by industry, but may play a more important role in the future if system biology techniques are adopted by industry. The effect of a drug on several cell lines could then be evaluated by NMR metabolomics [3].

Outside of FBDD, the use of NMR is much more limited [59]. Small HTS libraries can be screened by ligand-based NMR methods, which provides much more detailed and more reliable information than conventional HTS, but this is not often applied in practice [3, 22]. HTS is able to screen much larger libraries than NMR and it is unlikely that NMR ever achieves such speed and efficiency [9]. NMR does have great potential in the validation of HTS hits [10, 50] as a very robust technique with high information content [9, 11], although it is used mostly in the absence of available crystal structures. Important limitations for validating high affinity HTS hits are the limited detection in the high affinity range for ligand-based methods and protein size limitations for target-based methods [22].

A continued great impact of NMR is foreseen by many (e.g. [3, 11]). Additionally, NMR is thought to play important future roles in designing drugs against challenging targets such as protein-protein interaction inhibitors and intrinsically disordered proteins [11]. Its role as structure determination tool has been somewhat forgotten in recent years, but may return because the combination of screening and structural information is what make NMR such a valuable technique [50]. The information on structural dynamics obtained by NMR has been hardly used so far, but may provide an additional possibility in the future for NMR to distinguish itself [3, 50]. Finally, technological advances may enable NMR to screen larger libraries in less time with less material [11].

Altogether, NMR is used mainly in fragment-based screening and also frequently in hit optimisation efforts. NMR is the only technique that can get information on the binding mode and quantify the interactions simultaneously [50]. Ligand-based methods are more often applied in screening than target-based approaches because they are much faster and less costly due to lower protein requirements. Approaches such as STD NMR, WaterLOGSY and 19F-based methodologies are among the most often used experiments to screen fragment libraries. In subsequent hit validation and hit optimisation steps, target-based methods become increasingly popular because they provide more detailed information, although X-ray crystallography is often preferred when crystal structures are available. Applications of NMR in other areas such as metabolomics and the structure determination of membrane proteins have given some promising results, but have not yet been used extensively in industry so far. Outside FBDD strategies, the role of NMR in drug discovery limited. It is then mainly used as an alternative to X-ray crystallography when no crystal structure can be obtained. However, in FBDD, NMR is well able to compete with other methods through its high versatility and information content.

8.3 Personal opinion

The SAR by NMR method was one of the very first methods in the field of fragment-based screening and NMR has played a pioneering role ever since. At the time, NMR had been an established biophysical technique for several decades, and straightforward, reliable NMR experiments could be developed fast to screen weak-affinity ligand binding. In contrast, other methods lacked the possibility to easily measure weak binding affinities. Therefore, NMR was the most used technique in the late 1990s and early 2000s. However, other methods such as HCS and SPR have been developed or adapted in order to measure weak binding affinities. I expect it is mainly through these competing techniques that the use of NMR has declined over the years. Main limiting factors for widespread use of NMR in primary screening of fragment-based libraries are the high protein consumption, slow measurements and expensive equipment. Also, limited experience with NMR in a company and failure to comprehend
the possibilities of using NMR may prevent more widespread use.

Most techniques are less reliable than NMR, but it is common practice in industry to validate the results from a primary screen by several subsequent screening steps. Whenever possible, the final screening step is X-ray crystallography, because it offers the most detailed information. The use of X-ray crystallography as final step may limit the use of target-based NMR experiments, because detailed target-based experiments are often more difficult and time-consuming than solving crystal structures. Nevertheless, NMR is still the most valuable alternative to X-ray crystallography in obtaining high-resolution information. Therefore, I expect target-based methods will remain important for hit validation and hit optimisation.

In ligand-based screening, the use of NMR techniques is threatened by techniques where higher throughput and lower protein consumption is possible. I expect it will be difficult in the future for ligand-based NMR screening techniques to compete with more HTS-like fragment-based screening methods, because NMR methods are more expensive and limited in throughput. However, STD NMR, which is used extensively nowadays, may be an exception, mainly because it consumes very little protein compared to other NMR methods.

Altogether, the great versatility in useful NMR experiments ensures its continued use in the field of drug discovery. However, the competition with other techniques such as SPR, HCS and X-ray crystallography has lead to a decrease in use compared to a decade ago.
9 List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>One-Dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>Two-Dimensional</td>
</tr>
<tr>
<td>3-FABS</td>
<td>Three Fluorine Atoms for Biochemical Screening</td>
</tr>
<tr>
<td>AIDA</td>
<td>Antagonist Induced Dissociation Assay</td>
</tr>
<tr>
<td>CSP</td>
<td>Chemical Shift Perturbation</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell Penetrating Peptide</td>
</tr>
<tr>
<td>FAXS</td>
<td>Fluorine chemical shift Anisotropy and eXchange for Screening</td>
</tr>
<tr>
<td>FBDD</td>
<td>Fragment Based Drug Discovery</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FKBPI2</td>
<td>pepidyl-prolyl cis – trans isomerase</td>
</tr>
<tr>
<td>GEM</td>
<td>Group Epiteo Mapping</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>HCS</td>
<td>High Concentration Screening</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Correlation</td>
</tr>
<tr>
<td>HR-MAS</td>
<td>High Resolution Magic Angle Spinning</td>
</tr>
<tr>
<td>HTS</td>
<td>High Throughput Screening</td>
</tr>
<tr>
<td>ILOE</td>
<td>InterLigand nuclear Overhauser Effect</td>
</tr>
<tr>
<td>INPHARMA</td>
<td>Interligand NOEs for PHARmacophore MAPPING</td>
</tr>
<tr>
<td>LE</td>
<td>Ligand Efficiency</td>
</tr>
<tr>
<td>LLD</td>
<td>Laballed ligand Displacement</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectroscopy</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Effect SpectroscopY</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>Orthogonal Partial Least Squares-Discriminant Analysis</td>
</tr>
<tr>
<td>OS SS-NMR</td>
<td>Oriented Sample SS-NMR</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to Noise</td>
</tr>
<tr>
<td>SALMON</td>
<td>Solvent Accessibility, Ligand binding, and Mapping of ligand Orientation by NMR spectroscopy</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-Activity Relationship</td>
</tr>
<tr>
<td>SEA-TROSY</td>
<td>Solvent Exposed Amides with TROSY</td>
</tr>
<tr>
<td>SOS-NMR</td>
<td>Structural information using Overhauser effects and Selective labeling with NMR</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
</tr>
<tr>
<td>SS-NMR</td>
<td>Solid State NMR</td>
</tr>
<tr>
<td>STD</td>
<td>Saturation Transfer Difference</td>
</tr>
<tr>
<td>STD T2</td>
<td>Saturation Transfer Double Difference</td>
</tr>
<tr>
<td>T1</td>
<td>Longitudinal relaxation time</td>
</tr>
<tr>
<td>T2</td>
<td>Transverse relaxation time</td>
</tr>
<tr>
<td>TINS</td>
<td>Target Immobilised NMR Screening</td>
</tr>
<tr>
<td>TOCSY</td>
<td>T0tal Correlation SpectroscopY</td>
</tr>
<tr>
<td>trNOE</td>
<td>transferred NOE</td>
</tr>
<tr>
<td>TROSY</td>
<td>Transverse Relaxation Optimised SpectroscopY</td>
</tr>
<tr>
<td>Ub3A</td>
<td>Ubiquitin with Three residues mutated to Alanine</td>
</tr>
<tr>
<td>WaterLOGSY</td>
<td>Water-Ligand Observation with Gradient SpectroscopY</td>
</tr>
</tbody>
</table>

References


