NMR SPECTROSCOPY FOR STUDYING INTERACTIONS OF BIOACTIVE MOLECULES

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5.1. INTRODUCTION

One of the main prerequisites for the successful design of bioactive molecules and drugs is the elucidation of the three-dimensional structure of small molecular ligands, receptors and their complexes. Key steps in this process involve the identification of structural elements and groups responsible for bioactivity. On the other hand, our ability to design novel drug candidates simply from high-resolution structures of biomolecules is still limited. A deeper understanding of molecular mechanisms and dynamics involved in the interaction of ligands with macromolecules is of crucial importance. There are many techniques for probing ligand-receptor interactions, such as equilibrium dialysis, fluorescence spectroscopy, capillary electrophoresis, ultrafiltration, etc. Generally, these techniques require time-consuming separation steps which might influence binding equilibria or analyte derivatisation steps which may change ligand activity. NMR spectroscopy is a very useful method for the characterisation of ligand-receptor interactions since it can provide a wealth of information without destroying the sample. In this review, a description of the main NMR techniques to study the interactions of bioactive molecules with their biological receptors is given and the principal advantages and disadvantages are discussed.

Today, NMR spectroscopy is one of the most powerful and valuable methods in modern drug discovery and development. There is a plethora of one- and multi-dimensional techniques developed in recent years to study the structures of biologically important molecules such as proteins and nucleic acids and their interactions with other macromolecules or small molecules, for example. Many sequences have been proposed to screen for small molecular binders in the early stages of the drug discovery process. Drug discovery usually includes several critical stages, *i.e.* hit and lead generation, lead optimisation and preclinical development. Although knowledge of the 3D structures of ligand-receptor complexes might accelerate to a certain extent the search for molecules with higher activity, optimisation of physicochemical properties which dictate *in vivo* potency and efficacy play an important role as well. These properties include solubility, absorption, distribution, metabolism, excretion and toxicity, known as ADMET.

Initially, the main contribution of NMR spectroscopy to drug design included the determination of the three-dimensional structure of biological targets, mostly

proteins, and many efforts have been made by biological NMR groups to develop different two- and multi-dimensional techniques for that purpose. The first protein structures determined by NMR were published more than 25 years ago [1,2]. Although knowledge of protein structure is certainly an important factor for the drug discovery process, the predictions at that time regarding the impact of NMR were overly optimistic, primarily due to the fact that the size and solubility of proteins are still the biggest limiting factor for successful NMR analysis. Today, protein structure determination by NMR spectroscopy usually includes isotopic labelling of the protein over-expressed in genetically modified bacteria [3]. Hence, the availability of ^{13}C , ^{15}N and ^2H isotopically enriched proteins combined with numerous multidimensional NMR experiments enable sequential resonance assignment and solution state structure determination of proteins or protein-ligand complexes up to 82 kDa in size [4]. Recently developed sequences which reduce the transverse relaxation rates such as transverse relaxation optimized spectroscopy (TROSY) [5-7] have enabled the recording of high-resolution NMR spectra of macromolecules with masses up to 100 kDa and beyond. However, even for smaller proteins with good resonance dispersion and narrow line-widths, the process of structure determination can be very time consuming. Recent developments in membrane protein NMR studies have revived the role of structural NMR in pharmaceutical research [8]. More recently, new solid-state NMR techniques have emerged as a powerful tool to study proteins that were not accessible to solution state measurements. This has made possible studies of integral membrane proteins, protein aggregates and fibrous proteins, regardless of the solid state characteristics of the studied system, which is one of the main prerequisites for X-ray diffraction analysis where crystalline samples are needed. More recent information on protein structure analysis can be found in the related literature [9-13]. In modern drug discovery, NMR provides much more than macromolecular 3D structure determination since it has found widespread use in hit and lead generation, metabonomics and complex mixture analysis. Excellent discussions of the principles and applications of NMR techniques in metabonomics and mixture analysis can be found in a number of books and articles [14-21].

Apart from the structural information on the macromolecular target in the process of drug design, it is crucial to understand the principles of how a particular biological receptor interacts with a drug-like molecule. This is one of the prerequisites for the discovery of high affinity ligands for biologically important protein targets. Ligand-receptor interactions by using NMR might also include the elucidation of free and bound ligand conformations, the mobility of the ligand, receptor and their complex, determination of binding constants, determination of the reactive groups responsible for binding and also provide information on the orientation or immersion depth of the ligand in its target.

The binding of a ligand molecule to its receptor in its simplest form follows the bimolecular association reaction with second-order kinetics according to Equation 5.1:

where k_{on} and k_{off} are the on (association) and off (dissociation) rate constants.

The dissociation constant K_D can then be expressed by Equation 5.2:

$$K_d = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[R][L]}{[RL]} \quad (5.2)$$

As already mentioned, there have been many NMR sequences developed in the last two decades that can provide useful information on the interactions between biological targets and small molecules which can be used to screen for potential binders [22-28]. These can be divided into two main groups: ligand-based and receptor-based techniques. Theoretically, all NMR parameters are sensitive to binding processes and can be used to study ligand-receptor interactions. Most frequently used are those that are easily obtained and with highest sensitivity and include chemical shifts, translational diffusion coefficients, relaxation times and the nuclear Overhauser effect (NOE). Some of these parameters are simply used to indicate if binding occurs, while others provide information on the location of ligand binding sites and surface mapping features and determine binding constants and changes in the mobility of ligand-receptor complexes. NMR screening for biologically active molecules includes the identification of small molecule ligands for macromolecular targets by the observation of a change in an NMR parameter upon interaction. The parameters that are usually used to study interactions between ligand and their biological receptors are given in Figure 5.1.

Figure 5.1. NMR parameters used for studying ligand-receptor interactions

Both approaches have advantages and disadvantages. The receptor-based methods do not rely on fast exchange and therefore allow for the characterisation of both high and low affinity binders. They can also easily distinguish between specific and non-specific binding. The main prerequisite for this approach is *a priori* knowledge of the 3D structure of the protein obtained either from NMR or X-ray diffraction analysis. Monitoring changes in NMR parameters (usually chemical shifts) identifies not only binding of molecules, but also localises their binding sites. Some major drawbacks of this approach include the size of the target protein which prevents complete resonance assignment and structure elucidation as well as isotopic enrichments (^{13}C , ^{15}N , ^2H etc.) necessary for structural analysis. One of the main disadvantages of receptor-based techniques is the over-expression and purification of milligram quantities of a soluble protein, since in drug discovery, targets are selected on the basis of their therapeutic potential and not their compliance with NMR. For ligand-based methods, the target size is irrelevant and there is no need for isotopic labelling. The receptor concentration is much smaller, usually in the micromolar range. This approach mostly utilises 1D techniques, which are faster than 2D or higher dimensional techniques needed to solve protein structures. There are also some drawbacks connected to these techniques, such as the inability to *a priori* localise the binders on the receptor and the requirement for fast exchange between free and bound states. Also, the large excess of ligand molecules used in such experiments might increase the risk of non-specific binding. However, the ligand-based approach has broader applicability today and is frequently used for primary and secondary screening.

An excellent receptor-based method called structure-activity relationship (SAR) by NMR was developed by researchers at Abbott [29-31]. The method is based on perturbations in protein hetero-nuclear single quantum coherence NMR spectra (^1H - ^{15}N or ^1H - ^{13}C -HSQC) upon the addition of a compound mixture. It requires ^{15}N or ^{13}C isotopic enrichment of proteins. In the first step, a library of small molecules is screened to identify those that bind to the target protein. Binding is detected by comparing two-dimensional ^1H - ^{15}N or ^1H - ^{13}C -HSQC spectra of the protein prior to and after the addition of a ligand or ligand mixture to detect changes in chemical shifts upon binding. The ligands that bind to one or more pockets in spatial proximity are identified, optimised and linked together to produce high-affinity ligands. Since the first application of this method on the FKBP protein, it has been used to discover many high-affinity ligands for other therapeutically relevant proteins.

For experiments that rely on the observation of ligand molecules, the choice of NMR parameters is more diverse (Figure 5.1). The high sensitivity of ligand parameters to binding have enabled a number of valuable techniques to be developed to better understand the binding process at a molecular level and to identify new bioactive compounds [22-28]. Upon binding of a small molecule ligand to its target macromolecule, the chemical shifts of both the ligand and the target resonances are perturbed. Hence, for the detection of whether a ligand binds or not, in the case of well-separated signals it is sufficient to perform simple titration expe-

riments and observe changes in proton chemical shifts and line-widths. This is shown in Figure 5.2 for binding of the antibiotic erythromycin to the ribosome.

The dissociation constant may also be estimated by nonlinear curve fitting of the chemical shifts vs. concentration. Other methods to identify and characterise ligand-receptor interactions can generally be divided into NOE-based, relaxation-based and diffusion-based methods.

Figure 5.2. Proton spectrum of erythromycin (a) and proton spectra of erythromycin after the incremental addition of *E. coli* ribosome (lines b, c and d) recorded at 700 MHz

A number of NMR techniques based on the NOE effect have been developed recently. It is well known that NOEs are invaluable tools for determining 3D structures of small molecules and biomolecules. Upon binding of the ligand molecule to its biological receptor, the NOEs undergo significant changes leading to the observation of transferred NOEs or transferred nuclear Overhauser enhancements (trNOEs) which stem from different correlation times, τ_c , of free and bound molecules. Small molecular ligands have short correlation times and usually give rise to positive NOEs or no NOEs. On the other hand, large macromolecules exhibit strong negative NOEs. This is depicted in Figure 5.3. If a small molecule binds, it behaves like a macromolecule and shows strong negative trNOEs, providing a fast exchange regime between the bound and free states.

This is demonstrated in Figure 5.4 for binding of the macrolide antibiotic azithromycin to ribosomes isolated from *E. coli*. It can be seen that the 2D NOESY spectrum of azithromycin in buffered solution displays only a few positive NOE

Figure 5.3. Properties of the ligand, receptor and their complex

cross-peaks (all others vanish since $\tau_c \omega_0 \approx 1$, $MW = 774$). However, after the addition of 70S *E. coli* ribosomes, the observed trNOEs are all negative with higher intensities, indicating interactions between azithromycin and ribosomes and the formation of a complex. In practise, these experiments are performed with high excess of the ligand (from ten to hundred-fold excess) so that signals of the receptor molecules are not visible in the spectrum and no filtering techniques are necessary. Furthermore, the analysis of trNOEs provides information on the bound conformation. In addition, one can also observe intermolecular trNOEs between the ligand and receptor protein which allows for the determination of the orientation of bound ligands in protein binding pockets [32,33].

Another very popular and fast method that relies on the NOE effect is saturation transfer difference NMR spectroscopy (STD-NMR), proposed by B. Mayer and M. Meyer [34,35]. The method has become one of the most frequently used NMR methods to characterise interactions between ligands and receptors. It is based on the transfer of saturation from the receptor to the bound ligand which in turn is moved into solution by exchange and detected. As depicted in Figure 5.5, STD takes the difference of the two experiments. In the first experiment (on-resonance), only receptor resonances are selectively irradiated or saturated using selective pulses. The saturation propagates to all receptor protons *via* the network of an efficient intra-molecular proton-proton cross-relaxation process known as spin diffusion. As seen in Figure 5.5, compounds that bind to the receptor become saturated as well via inter-molecular proton-proton cross-relaxation. The saturation is then carried into solution by the exchange process and detected. Subsequently, a reference spectrum is recorded with the irradiation frequency set far from any signal giving rise to an off-resonance spectrum. The subtraction of the on-resonance spectrum from the off-resonance spectrum gives rise to a difference spectrum, showing only resonances that have experienced saturation.

Figure 5.4. (a) NOESY spectrum of azithromycin in TRIS buffer and (b) TrNOESY spectrum of azithromycin in TRIS buffer after the addition of 70S *E. coli* ribosomes

The degree of ligand saturation depends on the residence time of the ligand in the receptor binding pocket. When it dissociates from the receptor, the saturation is transferred to the solution where the free ligand gives rise to signals with narrow line widths. There are several advantages of the STD method.

First, STD has no limitation with respect to the size of the receptor and is ideal for large receptor proteins and even whole ribosomes (see Section 5.6). Second, it

Figure 5.5. Schematic representation of an STD experiment: (a) the off-resonance or reference experiment involves rf-irradiation off-resonance from ligand and receptor protons giving intensity I_0 , (b) the on-resonance experiment applies rf-irradiation for selective saturation of receptor resonances producing attenuated signal intensity I_{SAT} and (c) STD spectrum which gives only resonances of the binding compounds (receptor resonances are usually invisible due to either low concentrations or relaxation filtering)

requires low amounts of the receptor (usually in the micromolar range) and it benefits from the fact that only bound ligands show signals in the spectrum and therefore is very suitable for screening purposes. A typical one-dimensional STD pulse sequence is shown in Figure 5.6.

Figure 5.6. STD pulse sequence with a $T_{1\rho}$ relaxation filter to suppress unwanted receptor resonances

Furthermore, STD may be combined with other pulse sequences, thus generating experiments such as STD - total correlation spectroscopy (STD-TOCSY) or STD-HSQC, for example [36]. STD-NMR spectroscopy can be successfully used to identify groups of atoms in direct contact with the receptor since these experience the highest degree of saturation; this allows for epitope mapping or group epitope mapping (GEM) [35]. Protons closest to the target molecule are saturated to a higher degree, giving stronger STD enhancements. This information can be used to identify structural moieties that are important for binding. The binding constants can also be assessed from STD-NMR spectra by performing competition titration experiments. A variation of STD-NMR spectroscopy is the water ligand-observed gradient spectroscopy (waterLOGSY) method which uses the fact that intermolecular water-ligand NOE will be negative only when the ligand is bound to the protein [37,38]. The transfer of magnetisation is from water to receptor to binding ligand. The method utilises either the steady-state NOE experiment with on-resonance saturation applied to the water signal or selective inversion of the water resonance signal in the enhanced protein hydration observed through gradient spectroscopy nuclear Overhauser effect (e-PHOGSY NOE) experiment [39]. Protein-ligand interactions can also be studied by monitoring inter-molecular NOEs using NOE pumping or reverse NOE pumping (RNP) techniques [40]. RNP uses a relaxation filter followed by the NOE mixing element where polarisation is transferred from a binding ligand to the protein, resulting in a spectrum containing only the signals of the binding ligands. Recently, a method called INPHARMA (Interligand Noes for PHARmacophore Mapping) methodology was introduced [41]. It is based on the observation of spin-diffusion mediated, trNOE data between two ligands (L_1 and L_2) that bind weakly and competitively to a macromolecular receptor (R). As L_1 binds to the receptor, the magnetisation is transferred from the ligand to the

receptor protons during the mixing time of the NOESY experiment. During the same mixing time, L_1 dissociates from the receptor and L_2 binds, which allows the transfer of magnetisation from the receptor to L_2 protons.

The chemical exchange during INPHARMA experiment can be explained by two different models:

- a) A three-step model, which includes the state where both ligands and the receptor are found also in free form. The three-step model can be described by:

- b) A two-step model, where the receptor is never in free form, due to the presence of the two ligands in large excess. This model can be described by:

In contrast to STD and waterLOGSY experiments, the information obtained in the INPHARMA spectrum is highly site-specific. INPHARMA provides rapid determination of the relative orientation of different chemical structures in the macromolecule binding pocket. INPHARMA methodology was successfully applied to a mixture of baccatin III (L_1) and epothilone A (L_2) in the presence of tubulin (R). The results showed that the intermolecular NOEs between epothilone A and baccatin III were mediated by the protons in tubulin.

Binding of the ligand to the target will have a dramatic effect to its relaxation properties. Free small molecule ligands are characterised by small longitudinal and transverse relaxation rates, ($R_1=1/T_1$ and $R_2=1/T_2$). When bound, ligands share relaxation properties of the receptors with large relaxation rates. Therefore, binding will cause significant line-broadening of ligand resonances. A comparison of transverse relaxation times in the presence and absence of the receptor is very sensitive probe of binding due to its almost direct dependence on the overall molecular correlation time. T_2 can be determined by using a simple Carr–Purcell–Meiboom–Gill (CPMG) experiment [42,43] or, alternatively, one can use $T_{1\rho}$ determined from rotating frame Overhauser effect spectroscopy (ROESY)-type experiments to detect binding. A variation of the relaxation-based approach is the method called SLAPSTIC (Spin Labels Attached to Protein Side chains as a Tool to identify Interacting Compounds) [44,45] which allows for the screening of primary binding sites by covalently attaching spin labels to protein targets. The use of paramagnetic relaxation enhancement (PRE) experiments could provide further structural information on the orientation or immersion depth of the ligand in its target. Upon the addition of an inert paramagnetic agent to the solvent, relaxation enhancements of ligand nuclei depend on the insertion depth in the target. If the ligand is in fast exchange between the free and bound state,

solvent PREs are partly transferred to the free ligand where they can be observed with high resolution and without any size limitation of the receptor [46-49]. Information regarding the orientation of the ligand with respect to the receptor can be determined and further used for guiding docking calculations, provided that the three-dimensional atomic resolution structure of the receptor is available. Identifying parts of a drug which are buried deepest in the receptor can help with directing chemical modifications of the ligand to design better inhibitors.

Experiments based on translational diffusion can also be used to probe interactions of ligands with a target. The diffusion-editing techniques are based on the fact that, upon binding, the hydrodynamic radius r_H of the ligand increases substantially, leading to a decrease in the diffusion rate. The translational diffusion coefficients can be obtained according to Stokes-Einstein equation:

$$D = kT/6\eta\pi r_H \quad (5.5)$$

where k represents the Boltzmann constant, T is the temperature, η is the solvent viscosity and r_H is hydrodynamic radius. From Equation 5.5, it is clear that a small ligand molecule with a small hydrodynamic radius will have larger diffusion coefficient with respect to larger molecules such as proteins. Hence, if a ligand binds to the receptor, its diffusion coefficient will decrease. A variety of NMR pulse sequences have been developed to investigate diffusion coefficients in solution [50]. Diffusion-editing has been successfully used for the characterisation of interactions between small and intermediate sizes proteins. With the development of reliable gradient technology, many NMR pulse schemes using gradients have been proposed to determine diffusion coefficients such as bipolar pulsed field gradient longitudinal eddy-current delay (bpPFGLED), depicted in Figure 5.7.

Figure 5.7. The bpPFGLED pulse sequence.

The signal intensity during the diffusion experiment will decay exponentially according to Equation 5.6:

$$I = I_0 \exp[-D(\gamma\delta G)^2(\Delta - \delta/3)] \quad (5.6)$$

where I_0 is the signal intensity in the absence of field gradients, D is the diffusion coefficient, γ is the gyromagnetic ratio, δ is gradient length, G is gradient strength and Δ is the diffusion time. Diffusion coefficients can be determined from the slope of the linear dependence of the natural logarithm of both sides of the Equation 5.6. The experimental parameters can be optimised to suppress the signals of non-binding compounds and to detect the resonances of bound ligands.

Figure 5.8 shows the results from an experiment performed on a mixture of three compounds in the presence of porcine pancreatic elastase. It can be seen that only the signals of the compound that binds, *e.g.* phenylphosphonic acid, appears in the spectrum of the mixture, while signals of the other non-binding compounds are not present.

Figure 5.8. The bpPFGLED spectra of a mixture of phenylphosphonic acid and two small organic compounds at (a) low gradient strength, (b) high gradient strength and (c) after the addition of porcine pancreatic elastase at high gradient strength.

Diffusion-based methods can further be used to provide knowledge on which parts of the ligand are involved in the binding surface and to determine the binding epitopes. A key advantage of diffusion epitope mapping over the aforementioned GEM approach is that it bypasses problems of longitudinal relaxation, which in some cases might lead to erroneous STD enhancements in GEM experiments. The main disadvantage of diffusion experiments is their lower sensitivity compared to relaxation-based or NOE-based approaches and some limitations exist regarding the size of the receptor.

Although very powerful and efficient, ligand-based NMR methods also have some limitations. Most of these methods report cases of weak to medium affinity binding. Usually, these experiments are performed with a large excess of ligands which may lead to non-specific binding. Other limitations include molecules with low water solubility, those that bind with high affinity to the receptor and molecules that have small off-rate constants and hence are not detected. In such cases, competition experiments are very useful to monitor ligand-receptor

interactions, thus avoiding many drawbacks connected with the direct ligand-based methodologies [51-54]. Competition ligand-based methods are robust and reliable tools for providing valuable information on specific binding and could serve as quantitative measures of the ligand affinity for the receptor. A competition method that combines waterLOGSY and transverse relaxation has recently been proposed as a sensitive method for fragment screening [55].

The NMR methods discussed above will continue to play significant roles in the process of modern drug discovery, including hit generation, lead generation and optimisation phases, thus increasing the chances of success to produce drug candidates with high potency, reduced toxic effects and desired physicochemical properties.

In the next section, recent applications of the NMR techniques discussed above to study ligand-receptor interactions are reviewed.

5.2. LIGAND-PROTEIN INTERACTIONS

Ligand-protein interactions can be monitored by various experimental methods. As mentioned above, ligand-based NMR techniques are useful tools for investigations of such interactions.

STD-NMR spectroscopy was used to investigate the binding properties of the caged nucleotides guanosine 5'-*O*-(3-thiotriphosphate), *P* 3(*S*)-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl) ester (DMNPE-GTP- γ -S) and adenosine 5'-diphosphate, *P*²-(1-(2-nitrophenyl)ethyl) ester (NPE-ADP) to rabbit muscle creatine kinase (RMCK) and human annexin A6 (hAnxA6) [56]. The obtained results indicated the strong binding of caged nucleotides to the investigated proteins. Similarly, Blume *et al.* [57] used the same technique to study the interaction of the epimerase site of the bifunctional enzyme UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase with its natural substrate, UDP-*N*-acetylglucosamine (UDP-GlcNAc) and derivatives. STD-NMR allowed for ligand epitope mapping and the determination of ligand binding affinity. The binding epitopes of the uridine moieties of UMP, UDP, UDP-GalNAc and UDP-GlcNAc were similar, suggesting that the binding mode of the UDP moiety was the same in all cases. However, small differences in the binding epitopes of hexopyranose units of UDP-GlcNAc and UDP-GalNAc reflected the inability of the enzyme to process UDP-GalNAc. The results of STD titrations showed that UDP had the greatest binding affinity to the epimerase site of the enzyme.

Furthermore, a combination of STD-NMR and ¹H-¹⁵N HSQC experiments was used to investigate the binding of 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) and guanosine monophosphate (GMP) to histidine triad nucleotide binding protein (HINT1) [58]. The presence of ligand binding was checked using STD-NMR spectroscopy. The ligand binding site was established from ¹H and ¹⁵N chemical shift perturbations. The binding of AICAR to HINT1 revealed

considerable chemical shift perturbation similarity to GMP, indicating that the two ligands probably bind in the same basic location.

Ji *et al.* [59] applied STD-NMR spectroscopy to map a group epitopes and to measure the dissociation constant of specific interactions between L-tryptophan (Trp) and human serum albumin (HSA). STD spectra recorded in ligand-protein solutions with and without the inhibitor naproxen pointed towards the interaction between Trp and HSA. The STD spectrum in the absence of the inhibitor reflected specific as well as non-specific binding of Trp. However, in the presence of naproxen, the specific sites were occupied by the inhibitor. Hence, the resulting STD spectrum reflected only non-specific contributions to the interaction between Trp and HSA.

Several groups have studied competitive ligand binding using STD-NMR spectroscopy. Sadeghi-Khomami *et al.* [60] studied the binding of pNP- β -D-glucose to hydrolytically inactive nucleophile mutants of the family-3 retaining β -glucosidase DesR in the presence of erythromycin A. The results of competitive STD-NMR experiments indicated that erythromycin A binds to the active site and acts as a competitive glycosidase inhibitor. Similarly, STD-NMR spectroscopy was used to study the interaction between bifunctional galactofuranosyltransferase GlfT2 and two trisaccharide acceptor substrates [61]. The bifunctionality of the enzyme was explored by competition STD experiments and saturation transfer double difference experiments (STDD). The results showed that both acceptor substrates bind competitively at the same site.

STD-NMR spectroscopy provided insight into enzyme-ligand interactions in yeast hexokinase isoform PII under physiological conditions [62]. Competitive STD titration experiments allowed for ranking the ligands according to their binding affinities.

Goncalves *et al.* [63] investigated the binding of a synthetic Toll-like receptor (TLR4) antagonist to cluster differentiation antigen CD14 by STD-NMR. The results indicated that the binding was principally mediated through the lipid chains of the synthetic compound. These findings strongly suggest that TLR4 activation is inhibited by competitively occupying CD14.

Furthermore, the competition STD method was successfully applied to detect the presence of a competing high-affinity ligand, diazepam, in a compound mixture by the reduction of the STD signals of a low-affinity ligand, β -site amyloid precursor protein cleaving enzyme 1 [64].

Plesniak *et al.* [65] characterised the conformation of novobiocin in the pocket of histidine protein kinase EnvZ using STD and trNOE experiments. Thus, STD-NMR spectroscopy identified the novobiocin moieties that interacted with EnvZ. The authors suggested that novobiocin binds to EnvZ in a conformation and orientation similar to its binding to DNA gyrase B.

Similarly, Lee *et al.* [66] characterised binding inhibitors of *Escherichia coli* β -ketoacyl-acyl carrier protein synthase III (ecKAS III) using STD-NMR

spectroscopy. The results suggested that the antimicrobial compound, 4-cyclohexyliminomethyl-benzene-1,3-diol (YKAs3003), was a potent inhibitor of pathogenic KAS III. It was shown that the binding models of YKAs3003 and eKAS III determined from *in silico* screening correlated well with the STD results.

According to Houliston *et al.* [67] STD experiments enable the investigation of antibody-ligand interaction in target ganglio-oligosaccharides added directly to patient sera, without antibody fractionation. Very strong saturation transfer was observed in one-third of glycan resonances upon the addition of ganglio-oligosaccharides to patient sera; this delineated the region of contact with serum antibodies.

Murata *et al.* [68] have shown how STD-NMR spectroscopy can be applied to identify binding epitopes and to study the binding of gibberelin A₃ to the monoclonal antibody 4-B8(8)/E9. STD experiments provided useful information on the hapten-antibody interaction in the solution state. The results obtained by STD-NMR were in excellent agreement with the results of crystallographic analysis.

Assadi-Porter *et al.* [69] demonstrated the use of STD and STDD-NMR experiments for monitoring specific ligand binding to the human taste type 1 receptors 2 and 3 (T1R2 + T1R3). Subtraction of STD spectra recorded in the presence and the absence of sweet receptors yielded two-dimensional ¹H-¹⁵N HSQC STDD spectra containing signals only from specific binding. The results indicated that the mutant receptor (T1R2 + T1R3(D535Q)) binds the ligand brazzein much less tightly than does the wild-type receptor (T1R2+T1R3).

Streiff *et al.* [70] proposed the application of STD-NMR spectroscopy for anaesthetic binding proteins. The measurements were conducted on a series of protein/anaesthetic solutions. STD spectra provided evidence for Ca²⁺-dependent halothane binding to calmodulin. Those findings demonstrated the specificity of anaesthetics for a particular protein conformation.

Macnaughtan *et al.* [71] combined STD-NMR and trNOE experiments to study the conformation of UDP-GlcNAc and β-D-GlcpNAc-(1→2)-α-D-Manp(1→6)-β-D-Glcp-OOctyl, as well as their interaction with *N*-acetylglucosaminyltransferase V. In addition, the relative orientation of the two bound ligands was determined in a paramagnetic relaxation enhancement experiment using a spin-labelled ligand analogue, 5'-diphospho-4-*O*-2,2,6,6-tetramethylpiperidine-1-oxyl (UDP-TEMPO).

Furthermore, Wen *et al.* [72] used STD-NMR and molecular modelling protocols to probe the binding modes of the glycosidase inhibitors kifunensine and salacinol to *Drosophila melanogaster* Golgi α-mannosidase 11. A comparison of the experimental and theoretical effects of ligand protons in these complexes allowed for the selection of likely ligand binding modes. The results indicated that reliable binding modes of a ligand to a protein in aqueous solution can be obtained with the combined use of STD-NMR spectroscopy and a computational approach.

Becker *et al.* [73] used ligand-detected ¹H NMR experiments, T_{1ρ}-NOESY, diffusion and STD-NMR to distinguish the binding epitopes of propranolol enantiomers

with alpha(1)-acid glycoprotein. STD epitope mapping provided insights into different orientations of the enantiomers with respect to the alpha(1)-acid glycoprotein binding pocket. It was concluded that ligand-protein interactions can be elucidated by using the combination of several NMR epitope mapping experiments.

Several research groups have proposed the implementation of STD-NMR spectroscopy in chromatographic optimisation studies [74,75]. STD-NMR in combination with molecular dynamics simulations can be used to screen chiral stationary phases for evidence of molecular interactions as a predictor of chiral column selection and to study the mechanism of chemically selective displacement chromatography.

Recently, several improvements have been made in STD-NMR [76,77]. Novel competition STD experiments based on the combination of filtering and isotope labelling schemes have been demonstrated using a mixture containing either unlabelled or ^{13}C labelled D-maltose in the presence of malectin. The signals of the unlabelled ligand are present only in ^{13}C -filtered STD spectrum, while the edited experiment shows no signals. However, when using ^{13}C labelled maltose, only the ^{13}C -edited experiment produces STD signals. The combination of ^{13}C -filtered and ^{13}C -edited experiments allows for the separation of the STD signals of the labelled reference ligand from the signals corresponding to the unlabelled hit compounds. Similarly, a novel STD experiment was proposed for group selective saturation of amide protons in ^{15}N labelled hosts. The pulse sequence of the experiment is based on a train of bilinear rotation decoupling (BIRD) pulses that inverts only protons attached to ^{15}N and results in saturation of the amide protons. The ^{15}N GS STD experiment produces clean and artifact-free STD spectra. The feasibility study indicated that ^{15}N GS STD experiments can be used as the basis for studying intermolecular interactions where the standard STD experiment is difficult to optimise.

TrNOE and molecular dynamics simulations (MD) were used to study the interaction between *Serratia marcescens* chitinase B (*SmChiB*) and the dipeptide *N*-acetyl-Arg- $\{N^{\omega}$ -(*N*-methylcarbamoyl)-*N*-methyl-Phe [78]. The dipeptide was derived from the naturally produced cyclopentapeptide chitinase inhibitor argifin. Molecular dynamics simulations and trNOE experiments indicated that both argifin and the dipeptide bind to (*SmChiB*) with similar affinities. The less favourable van der Waals and non-polar solvation energies of the dipeptide compared with argifin were compensated for by the improved total electrostatic component.

Several authors have demonstrated the application of NMR methodology based on relaxation rate analysis. Spin-lattice relaxation rate enhancements of ligand protons were used in order to investigate the affinity of epinephrine and isoproterenol for two different systems: fibrinogen and platelets [79]. The affinity index for epinephrine-fibrinogen was three times greater than the isoproterenol-fibrinogen affinity index. On the contrary, the affinity indexes for epinephrine-

platelet and isoproterenol-platelet interactions were similar, indicating that the ligands interact with the receptor with the same strength.

Furthermore, Lerche *et al.* [80] applied hyperpolarised ^{13}C NMR screening as a competitive assay to characterise the binding of salicylate and ascorbate to human serum albumin. Signal enhancements were achieved using dynamic nuclear polarisation (DNP) ^{13}C NMR spectroscopy. Dynamic nuclear polarisation creates a non-equilibrium state and the enhanced spin polarisation returns to its equilibrium with the relaxation time T_1 . As enhanced polarisation is much faster in macromolecule-bound ligands than in free ligands, relaxation time T_1 can be used to distinguish free and bound states.

TROSY was applied to investigate ternary systems composed of liver bile acid binding protein, bile acids and membrane mimetic systems, such as anionic liposomes [81]. The absence of the folded protein resonances in the TROSY spectrum of the liposome-protein complex indicated the formation of partially unfolded membrane-bound species. NMR titration of the liposome-protein complex was performed with increasing amounts of bile acids. The signals of the folded protein became more intense at higher bile acid/protein ratios. Hence, the presence of bile acid produced a shift in equilibrium towards the ligand-bound folded protein.

Relaxation rate analysis can be combined with NMR techniques based on monitoring chemical shift perturbations. Shi *et al.* [82] combined the aforementioned method to investigate the human vinexin SH3 domain. Site-specific backbone amide (^{15}N) ^1H and side chain ^{19}F chemical shifts as well as relaxation analysis of SH3 domains in the absence or presence of a peptide ligand indicated different internal motions due to ligand binding at the three different sites. Similarly, Fenwick *et al.* [83] performed solution NMR experiments to gain insight into the structure and dynamics of the bilobed ligand-binding core of glutamate receptor 2 in complexes with a set of willardine partial agonists. The regions of the receptor experiencing structural and/or dynamic changes due to interactions with the ligand were identified by main chain amide $^1\text{H}/^{15}\text{N}$ chemical shift deviations. Backbone dynamics on the chemical shift timescale were detected using measurements of ^{15}N relaxation exchange rates (R_{ex}). The NMR results showed that conformational exchange dynamics vary largely with efficacy and the extent of desensitisation.

Bai *et al.* [84] used global ^1H - ^{15}N HSQC chemical shift perturbations to elucidate the structure of P2-ibuprofen complexes. Two-dimensional ^1H - ^{15}N HSQC spectra showed that the chemical shift information was communicated from one β -strand to another, in a “molecular wave” pattern. It was proposed that the ligand induced adjustments in hydrogen bonds stabilising the β -sheet structure to overcome unfavourable entropic contributions to binding. The information transfer across β -sheets could be explained by the correlation between the amide nitrogen temperature coefficient and the nitrogen chemical shift perturbation. The correlation between *R*- and *S*- ibuprofen temperature coefficients indicated

that there is little enantiomeric selection for these compounds. The same method was used for the structure determination of 19 distinct ligand-protein complexes in high-throughput ligand affinity screens [85]. The chemical shift perturbation data was used to guide and filter AutoDock calculations using the AutoDockFilter program. The results obtained using chemical shift perturbation experiments were in accordance with the original X-ray structures. Martín-Pastor *et al.* [86] applied chemical shift perturbation methods to study the interaction of the GH secretagogue receptor (GHS-R1a) with ghrelin and its des-acyl analogue in living cells. The results showed that ghrelin possesses higher binding affinity than des-acylghrelin and that the *n*-octanoic group is involved in the interaction with the (GHS-R1a) receptor. Furthermore, the differences between proton chemical shifts of bound and free ligand were used to elucidate the interaction of the isotopically labelled form of the fully active and chemically defined form of endotoxin, Kdo₂-Lipid A, with serum glycoprotein sCD14 [87].

Ligand-protein interactions have been recently investigated by solid-state NMR spectroscopy. Krabben *et al.* [88] determined the contact area of neurotoxin II from Asian cobra *Naja naja oxiana* when interacting with membrane-bound nicotinic acetylcholine receptors from *Torpedo californica*. The comparison of the chemical shift values for the free and receptor-bound toxin indicated that more than 75% of the assigned ¹³C resonances did not change within ±0.5 ppm. Hence, it was concluded that the observed chemical shift changes were related to large conformational modifications. The strongest chemical shift changes were associated with the residues at the ligand binding interface. Furthermore, Xu *et al.* [89] assigned uniformly microcrystalline ¹³C and ¹⁵N enriched yeast triosephosphate isomerase (TIM) using high-resolution solid-state NMR spectroscopy and compared the results with solution-state NMR assignments. An overall agreement between solid-state and solution-state NMR spectra was observed. Some of the observed chemical shift perturbations, as compared to the solution NMR assignment, were related to crystal packing interactions. Furthermore, the binding of a substrate analogue D-glycerol-3-phosphate (D-G3P) to TIM was studied by solid-state NMR for the first time. Site-specific perturbations of the enzyme's chemical shifts upon ligand binding indicated protein conformational changes. Similarly, solid-state ²H NMR spectroscopy was used to investigate the orientation and conformation of retinal within the rhodopsin binding pocket [90]. This approach elucidated the mechanism of rhodopsin activation as a guide for ligand-based drug design.

There are several articles concerning the combined use of NMR spectroscopy and isothermal titration calorimetry. This approach provides a complete thermodynamic characterisation of the investigated system, as was demonstrated with the aminoglycoside antibiotic paromomycin [91]. Furthermore, it was shown that isothermal titration calorimetry in combination with NMR methods was able to elucidate the structure of different enzyme-inhibitor complexes and the dynamics of their interaction [92-94].

NMR experiments based on the paramagnetic effects of lanthanide ions can provide unique insight to ligand-protein complex structure. This technique has been used for the structural characterisation of the ternary 30 kDa complex between the lanthanide-labelled N-terminal domain of the epsilon exonuclease subunit from *E. coli* DNA polymerase III, the theta subunit and thymidine [95].

Although modern ligand-based NMR methods are most widely used to monitor ligand-protein interactions, standard one- and two- dimensional NMR methods are able to structurally characterise the investigated ligand-protein complexes [96-98]. Alcaraz *et al.* [99] used ^{13}C NOESY-HSQC spectra to characterise the adducts of the catalytic domain of matrix metalloprotease-3 (MMP3) with three different non-peptide inhibitors. Similarly, Murail *et al.* [100] determined the structure of the mouse translocator protein (mTSPO) and the stability of its tertiary folding regarding the presence of (2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinoline carboxamide (PK 11195) ligand using two-dimensional NMR methods. The proton chemical shifts and ^{15}N - ^1H HSQC correlations indicated that PK 11195 binding enhanced the stability of the mTSPO tertiary fold.

As mentioned in the Introduction, ligand-protein interactions can be studied using protein-detected techniques, such as SAR by NMR. Tsantrizos *et al.* [101] used SAR by NMR to optimise small-molecule hepatitis C virus NS3 protease inhibitors. The observed results have shown that the binding affinity and potency were highly dependent on the ring size, the stereochemistry of each chiral centre and the electrostatic potential of the aromatic substituents.

5.3. LIGAND-DNA, LIGAND-RNA AND LIGAND MEMBRANE INTERACTIONS

NMR spectroscopy has also been used to study various interactions of small molecules (ligands) with DNA, RNA and membrane receptors.

Di Micco *et al.* [102] demonstrated an STD approach based on two parallel sets of STD experiments, in which saturation was elicited by irradiating at a suitable frequency chosen from specific DNA resonances for the characterisation of different DNA ligands. The signal-to-noise ratios of all the protons showing the STD effects were compared with the reference STD spectrum. The results suggested that the ligands making proximate contacts with aromatic base protons received more saturation upon the irradiation of DNA aromatic protons, whereas the external ligands were more affected by irradiation in the deoxyribose region.

Furthermore, ligand-DNA complexes have been studied by diffusion methods. Ramalho *et al.* [103] utilised a combination of, pulsed field gradient NMR (PFG NMR) spectroscopy, spin-lattice relaxation rate analysis and docking methods to investigate the interaction of 5-nitroimidazole radiosensitisers with duplex DNA. The intensity of radiosensitiser-macromolecule interactions was successfully evaluated using PFG NMR and docking data. Furthermore, the measurements of

spin-lattice selective relaxation rates represented a powerful tool to investigate the binding affinity of the ligand towards the receptor.

Moreover, novel methods have been proposed for studying ligand-RNA binding.

Kreutz *et al.* [104] applied ^{19}F NMR spectroscopy to identify site-specific ligand-RNA binding. The approach relied on site-specific labelling of RNA with 2'-deoxy-2'-fluoro(2'-F) nucleosides, thereby replacing the 2'-hydroxy group with a fluorine atom. The downfield shift of fluorine resonance upon ligand binding and a clear decrease of linewidth indicated that RNA has a more rigid structure in the bound state. Furthermore, Foloppe *et al.* combined NMR spectroscopy and a fluorescence resonance energy transfer (FRET) binding assay to identify aminoacyl-tRNA acceptor site (A-site) ligands [105]. The compounds that showed affinity for the A-site in a FRET-based binding assay were further investigated using NMR spectroscopy. Intermolecular NOEs confirmed the binding of these compounds to RNA.

The study of ligand-membrane interactions using a combination of NMR spectroscopy and other experimental or computational techniques have recently gained particular attention in order to understand metabolic pathways and develop drugs with the desired properties.

Melo *et al.* [106] studied the interaction between the dengue virus (DV) fusion peptide and different model membranes by fluorescence and NMR spectroscopy. The interaction was strongest in dodecylphosphocholine and anionic phosphatidylcholine/phosphatidylglycerol vesicles, which were the only vesicles fused by the DV fusion peptide. The three-dimensional structure of the DV fusion peptide bound to dodecylphosphocholine micelles and anionic phosphatidylcholine/phosphatidylglycerol vesicles was solved by transferred NOESY (trNOESY) and solution homonuclear NMR spectroscopy. The majority of the trNOE cross-peaks were from the hydrophobic triad, corroborating the dodecylphosphocholine-bound structure. Follet *et al.* [107] used a combination of electron spin resonance (ESR) and NMR spectroscopy in solution as well as solid-state NMR spectroscopy to investigate the physicochemical properties and membrane interactions of anti-apoptotic 2-(4-fluorophenyl)-3-(pyridine-4-yl)imidazo [1,2-*a*] pyridine derivatives with different side chain lengths and conformations. The results pointed towards the fact that improved solubility in biological media could be achieved by choosing a six-atom side chain length and that enhancement of membrane interactions/penetration could be achieved by hydroxyl substitutions on the C6 lateral chain. Cabeça *et al.* [108] applied molecular dynamics (MD) simulations and NMR spectroscopy to examine the interaction of the neutral and protonated species of the local anaesthetic prilocaine (PLC) with phosphatidylcholine (PC) bilayers. The diffusion-ordered 2D NMR spectroscopy (DOSY) experiments showed that neutral PLC has a higher affinity for the liposome environment. Furthermore, STD results revealed that the interaction of prilocaine molecules with the bilayer is pH-dependent. The findings were in accord with MD simulations.

The progress in NMR spectroscopy for studying interactions of bioactive molecules has been demonstrated in several reviews.

Fielding [109] recently reviewed NMR methods for the determination of protein-ligand dissociation constants. A protein and a ligand in thermodynamic equilibrium are characterised by the dissociation constant, K_D . In the simplest case of a protein with a single binding site, K_D is defined as:

$$K_D = \frac{[P][L]}{[PL]} \quad (5.7)$$

where [P], [L] and [PL] are the equilibrium concentrations of the protein, ligand and complexed state, respectively. The object of the NMR observation might be the ligand or the protein.

Hence, when viewing the ligand, the NMR experiment has to be able to distinguish between the ligand in the free and bound states, so that [L] and [PL] can be quantified. For a system in fast exchange, the observed NMR response (M_{obs}) of a ligand is a molar fraction weighted average of the NMR parameters of the free and bound states:

$$M_{\text{obs}} = X_{L(\text{free})}M_{L(\text{free})} + X_{L(\text{bound})}M_{L(\text{bound})} \quad (5.8)$$

where $X_{L(\text{free})}$ and $X_{L(\text{bound})}$ are the molar fractions of the free and bound ligand, and $M_{L(\text{free})}$ and $M_{L(\text{bound})}$ are the NMR parameters of the ligand in its free and bound states, respectively.

Alternatively, when observing the protein, the NMR experiment has to distinguish between and quantify [P] and [PL]. For the observation of a protein, the observed NMR response is defined as:

$$M_{\text{obs}} = X_{P(\text{free})}M_{P(\text{free})} + X_{P(\text{bound})}M_{P(\text{bound})} \quad (5.9)$$

where X_P and M_P are now the molar fraction and the NMR characteristic of the non-bound and occupied protein, respectively.

Kleckner and Foster [110] provided an overview of eight distinct NMR-based methods for studying protein dynamics. The methods are arranged by increasing experimental complexity and the observable timescale of the dynamic exchange τ_{ex} :

- a) real-time (RT) NMR ($\tau_{\text{ex}} > 1 \text{ s}$; $k_{\text{ex}} < 1 \text{ s}^{-1}$),
- b) exchange spectroscopy (EXSY) ($\tau_{\text{ex}} \approx 10\text{-}5000 \text{ ms}$; $k_{\text{ex}} \approx 0.2\text{-}100 \text{ s}^{-1}$; slow exchange; $k_{\text{ex}} \ll \Delta\nu$),
- c) lineshape analysis ($\tau_{\text{ex}} \approx 10\text{-}100 \text{ ms}$; $k_{\text{ex}} \approx 10\text{-}100 \text{ s}^{-1}$; slow-intermediate exchange; $k_{\text{ex}} \leq \Delta\nu$),
- d) Carr-Purcell Meiboom-Gill relaxation dispersion (CPMG RD) ($\tau_{\text{ex}} \approx 0.3\text{-}10 \text{ ms}$; $k_{\text{ex}} \approx 100\text{-}3000 \text{ s}^{-1}$; intermediate-fast exchange $k_{\text{ex}} \approx \Delta\nu$),
- e) Rotating frame relaxation dispersion (RFRD) ($\tau_{\text{ex}} \approx 20\text{-}100 \mu\text{s}$; $k_{\text{ex}} \approx 10000\text{-}50000 \text{ s}^{-1}$; intermediate-fast exchange $k_{\text{ex}} \geq \Delta\nu$),
- f) Nuclear spin relaxation (NSR) (ps-ns dynamics indirectly probed),
- g) Residual dipolar coupling (RDC) (ps-ms dynamics indirectly probed),

- h) Paramagnetic relaxation enhancement (PRE) ($\tau_{\text{ex}} \approx 10 \mu\text{s}$; $k_{\text{ex}} \approx 100000 \text{ s}^{-1}$; fast exchange $k_{\text{ex}} \gg \Delta I$).

Madl *et al.* [111] discussed the utility and computational approaches of combining solution-state NMR with small-angle X-ray and neutron scattering (SAXS/SANS) experiments for the structural analysis of large protein complexes. The combination of NMR and SAXS/SANS in particular is a valuable approach that complements crystallographic studies for structure determination and the investigation of dynamic properties of multi-domain proteins and their complexes in solution.

Reckel *et al.* [112] discussed the aim of in-cell NMR spectroscopy. The non-invasive character of in-cell NMR spectroscopy makes it the only biophysical method that enables the direct in-cell observation of conformational changes, post-translational modifications and binding events with high structural resolution in living cells. This unique ability is based on the sensitivity of the chemical shift of an NMR-active nucleus to changes in its chemical environment.

Grzesiek and Sass [113] have reviewed recent advances and applications of solution NMR methods for the characterisation of macromolecular structure and dynamics as well as molecular interactions. The combination of enhanced computational approaches and the in-cell detection of molecules enable a detailed characterisation of folding transitions and intracellular interaction studies.

Blobel *et al.* [114] have discussed different approaches to monitoring protein interactions by NMR at concentrations close to physiological values in unlabelled species. This review emphasises the role of small molecules and NMR active ^{129}Xe nuclei as reporters in the detection of protein-protein interactions.

Von Itzstein [115] reviewed the latest developments in structure-based inhibitor design for microbial-assisted and mammalian-assisted carbohydrate-recognising proteins. The inhibitors were studied using STD-NMR, crystallography and computational techniques.

Krishna and Jayalakshmi have provided a comprehensive description of complete relaxation and conformational exchange theory adapted for saturation transfer measurements (CORCEMA-ST) [116]. The CORCEMA-ST protocol can be applied to obtain quantitative data from STD-NMR spectra.

CORCEMA-ST is based on the two-state model depicted in Scheme 1. The two-state model involves the ligand and the receptor in their free (L_f , R_f) and bound (R_b , L_b) states. The exchange rate is determined by on- and off-rate constants ($k_{L,\text{on}}$, $k_{R,\text{on}}$ and $k_{L,\text{off}}$, $k_{R,\text{off}}$, respectively).

According to the two-state model, the expression of the observable magnetisation experiment is given by:

$$\mathbf{I}(t) = \mathbf{I}_0 + [1 - e^{-(\mathbf{R} + \mathbf{K})t}](\mathbf{R} + \mathbf{K})^{-1} \mathbf{Q} \quad (5.10)$$

Scheme 1.

where t is the time period in which the protons remain unsaturated. $\mathbf{I}(t)$ is the intensity matrix composed of column matrices $\mathbf{I}_f$ and $\mathbf{I}_b$ representing free and bound protons, respectively. $\mathbf{I}_0$ is the thermal equilibrium matrix, whereas the term $\mathbf{R} + \mathbf{K}$ is the dynamic matrix composed of relaxation rate matrix $\mathbf{R}$ and kinetic matrix $\mathbf{K}$. The matrix $\mathbf{Q}$ is composed of $\mathbf{Q}_f$ and $\mathbf{Q}_b$ submatrices corresponding to the free and bound states, respectively.

A feasibility study was performed utilising a known ligand-protein complex that demonstrated the ability of an STD-NMR intensity-restrained CORCEMA optimisation procedure (SICO) to identify the global minimum conformation for the bound ligand. Furthermore, it was shown that CORCEMA-ST can be successfully utilised in obtaining quantitative structural information on the conformation of ligands within the binding pockets of large proteins.

Goncalves *et al.* [117] reviewed a recent development in NMR methods for the structural characterisation of G protein-coupled receptors. This review outlines three key NMR interactions that yield structural information on G protein-coupled receptors:

- chemical shifts that provide information on the local environment, conformational changes and hydrogen bonding,
- dipolar couplings used to measure internuclear distances and orientations,
- quadrupolar couplings that are sensitive to molecular motion.

The investigations of structural changes occurring upon the activation of rhodopsin by a molecular switch mechanism demonstrated the ability of NMR spectroscopy to correlate the structure and function of G protein-coupled receptors.

Law *et al.* [118] discussed the role of computational chemistry, NMR spectroscopy and X-ray crystallography in fragment-based drug design (FBDD). The ability of FBDD to optimise ligand fragment properties is illustrated by several ligand-receptor complexes. It was outlined that computational chemistry, in combination with experimental techniques, could have a major impact on the success of FBDD programmes in the future and that it is ready to respond to the challenges that exist in the FBDD field.

Schanda [119] has provided an excellent review focused on the existing methods for fast multidimensional NMR spectroscopy used for biomolecular studies. The long data acquisition times required for the recording of conventional nD Fourier transform (FT) NMR spectra of biomolecules is very restricting for several reasons: limited stability of biological samples, instrumental costs and the inability to monitor many biologically relevant reactions in real time. These issues can be resolved using different fast multidimensional NMR techniques based on two approaches. The first replaces the regular $(n - 1)$ -dimensional sampling grid by some other time domain sampling that requires fewer sampling points. Several methods, such as sparse sampling, spectral aliasing and projection NMR, represent this approach. In the second approach, time-domain sampling is replaced by frequency-domain sampling (Hadamard spectroscopy) or a spatial encoding of evolution frequencies (ultrafast NMR spectroscopy).

Fast acquisition methods make NMR ready for use as a powerful tool in the analysis of the structure and dynamics of biological molecules. Hence, fast multidimensional NMR spectroscopy is capable of elucidating phenomena that are out of reach of many other techniques.

Donald and Martin [120] presented an overview of novel algorithms for protein structure determination, automated assignment, characterisation of membrane proteins and protein complexes as well as fold recognition using only unassigned NMR data. The methods presented in this review demonstrate how sparse dipolar couplings can be exploited to resolve the assignment problem, which is a key bottleneck in NMR structural biology.

Schwalbe *et al.* [25] emphasised the importance of NMR spectroscopy in understanding the structure and dynamics of biomacromolecules in drug discovery. The implementation of novel techniques improved the speed and accuracy of solution- and solid-state NMR spectroscopy. Hence, it was recognised that NMR spectroscopy was capable of determining the structure of medium-sized proteins in native and non-native states as well as characterising ligand-receptor interactions.

5.4. STRUCTURAL ANALYSIS OF LIGANDS

The structure of ligands is crucial for their function and interactions with the receptor. Some applications of NMR techniques for ligand structure elucidation are demonstrated by several examples in the following paragraph.

Modarresi-Alam *et al.* [121] used variable temperature 1H NMR spectroscopy to study restricted S–N rotation in aryl-*N*-(arylsulphonyl)-*N*-(triphenylphosphoranylidene) imidocarbamates. The doublets of the aryl substituents were employed to determine the barriers to rotation at the coalescence temperature. It was shown that a change in the substituents on the oxygen, sulphonyl and nitrogen of the imidoyl moiety had no appreciable influence on the observed barrier to rotation.

Seneci *et al.* [122] synthesised and characterised proapoptotic second mitochondria-derived activator of caspases (Smac) mimics and inhibitor of apoptosis protein (IAP) inhibitors. The SAR of this class of inhibitors was elucidated by computational methods and structural studies (NMR spectroscopy, X-ray diffraction). One lead molecule for further *in vitro* and *in vivo* evaluation was identified.

Fuchs *et al.* [123] prepared several heterocyclic analogues of thioflavones based on a Sonogashira-type coupling of *o*-halo(hetero)aryl chlorides with phenyl-acetylene. NMR spectroscopic studies provided the structure determination of the prepared compounds.

Zhuang *et al.* [124] used paramagnetism-based NMR constraints, including field-induced RDCs and pseudo-contact shifts (PCSs) for the structure determination of a galectin-3-carbohydrate. The paramagnetic Dy³⁺ ion was complexed to the peptide, making it possible to observe RDCs as well as PCSs for the protein and the ligand. The determined structure was in agreement with the crystal structure of a galectin-3-*N*-acetyllactosamine complex.

Tiziani *et al.* [125] applied DOSY to assess possible interactions between daidzein and daidzein soy protein and carotenoids. The results showed that the affinity between daidzein and the soy protein isolate was higher compared to the daidzein dissolved in the same model system. The shifting of the particular proton NMR signals pointed towards two main contributions to the interactions between the phytochemicals and the protein: hydrogen bonding from hydroxyl groups and hydrophobic interactions.

5.5. STRUCTURAL ANALYSIS OF PEPTIDES AND PROTEINS

A large number of proteins and peptides serve as ligand receptors. Hence, the elucidation of macromolecular structure and dynamics is crucial for the characterisation of ligand-protein complexes. The recent applications of NMR methods for peptide and protein structure determination are presented in the following section.

Arroyo and Mayo [126] elucidated the structure of angiostatic peptide anginex using NMR structural studies in dodecylphosphocholine micellar environment. Conformational modelling was performed using NOESY data. The results showed that anginex forms a three-stranded anti-parallel β -sheet conformation in a dodecylphosphocholine micellar solution.

Daly *et al.* [127] characterised the structure of several cyclotides (*cyclo peptides*), containing a head-to-tail cyclised peptide backbone and a knotted arrangement of three conserved disulphide bonds. Excellent signal dispersion in NMR spectra makes NMR spectroscopy the most commonly used technique for studying cyclotide structures. The NOESY spectrum of the cyclotide kalata B1 revealed a continuous cycle of sequential cross-peaks, which spanned all 29 amino acids in the sequence and provided evidence for its circular backbone. Furthermore, the

NMR analysis of kalata B1 and kalata B7 in the presence of dodecylphosphocholine micelles illustrated their binding to the membrane in different orientations.

Furthermore, NMR screening methods were applied to study the structural aspects of allosteric regulation by deuterated functionally active thermostable dimeric Lac repressor proteins [128]. The optimisation of conditions most suitable for lengthy NMR recordings was performed using a high-throughput thermofluor method based on the measurement of the fluorescence intensity of a protein solution at different pH values, temperatures and salt concentrations.

Yao *et al.* [129] applied PFG NMR for evaluating the estimates of protein rotational correlation times. The rotational correlation time, τ_c , can be expressed as a function of the translation diffusion coefficient, D_t :

$$\tau_c = \frac{2}{9} \frac{(R_h^{\text{ref}} D_t^{\text{ref}})^2}{D_t^3} \quad (5.11)$$

where R_h^{ref} and D_t^{ref} are the hydrodynamic radius and diffusion coefficient of the reference molecule, respectively.

The estimates of protein rotational correlation times can be useful if:

- a) isotopically labelled material is not available,
- b) relaxation rates are difficult to interpret,
- c) a full relaxation analysis is difficult because of limited sensitivity.

Kodama *et al.* [130] used time-sharing NMR measurements as a rapid, effective and unbiased approach for the identification of a protein-protein interface without resonance assignments. Only a single protein ^{15}N - and ^{13}C -labelled sample was required for the analysis. The measurements were performed using a new pulse sequence that allowed simultaneous aromatic ^1H - ^{13}C and ω_1 -TROSY-type backbone ^1H - ^{15}N correlations, together with single quantum methyl ^1H - ^{13}C correlations.

Sakurai *et al.* [131] investigated the structure, dynamics and folding of β -lactoglobulin as well as its interaction with ligands by NMR spectroscopy. The results indicated that β -lactoglobulin undergoes a conformational change during pH-dependent Tanford transition and ligand binding. The folding mechanism and perturbations due to the introduction of a mutation were explained by a schematic representation using folding funnels.

Buchko *et al.* [132] studied leucine-rich amelogenin protein (LRAP), a splice-variant isoform of amelogenin using NMR spectroscopy. The main difference in the ^1H - ^{15}N NMR spectrum of LRAP compared to the full-length amelogenin protein was an additional set of amide resonances for seven non-proline residues, which indicated the presence of two different conformations. The specific changes in molecular dynamics of LRAP upon NaCl addition, manifested

by the reduction in intensity and disappearance of ^1H - ^{15}N HSQC cross-peaks, pointed towards the formation of protein nanospheres.

Moriya *et al.* [133] determined the protein-protein interface and solvent accessibility upon complex formation between ubiquitin and a C90S mutant of yeast ubiquitin hydrolase 1 (YUH 1) using ^{13}C chemical shift perturbations induced by freely diffusing 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPOL).

5.6. MACROLIDE-RIBOSOME INTERACTIONS

Macrolide antibiotics, such as erythromycin, azithromycin and telithromycin (Figure 5.9) are effective therapeutic agents for treating infectious diseases [134,135]. They are still the centre of interest of many research groups and pharmaceutical companies and much effort is directed toward the discovery of new macrolide antibiotics by chemical modification of the existing classes of natural derivatives. The goal is to obtain novel therapeutic agents having an improved overall biological profile with a special emphasis on resistant bacterial strains. The continuing emergence of multidrug-resistant bacterial strains imposes a serious threat to the health-care community and intensifies the search for new and more effective agents in order to overcome this problem. Of particular interest are azalides, semisynthetic derivatives of erythromycin A [134,136], which have been found to display a wide antimicrobial spectrum and the ability to concentrate within host cells with high accumulation ratios.

There are two major mechanisms of resistance to macrolides, *i.e.* target site modifications by methylation that prevent the binding of the antibiotic to the ribosome (encoded by the *erm* gene) and efflux mechanisms, mediated by the *mef* (*streptococci*) and *msr* (*staphylococci*) genes. The methylation of ribosomal rRNA bases leads to cross-resistance to macrolides (M), lincosamides (L) and streptogramin B (S_B), the so-called MLS_B phenotype [137].

Figure 5.9. Chemical structures of (a) erythromycin, (b) azithromycin and (c) telithromycin

Macrolides exert their activity by interacting with the bacterial 50S ribosomal subunit at, or close to, the peptidyl-transferase centre and thus inhibit the growth

of the nascent peptide chain. As mentioned above, resistance to antibiotics has become a global problem and much effort is now directed toward new and more potent classes of drugs. An effective approach to overcoming this problem is to understand the principles of how these drugs interact with the ribosome [138]. Recently, crystal structures of some ribosome-macrolide complexes [139-141] have shed new light on the binding mechanisms of macrolides to ribosomes and hence provide a good basis for the rational design of new ligands and inhibitors. However, when analysing solid state structures of ribosome-macrolide complexes, one should keep in mind some discrepancies between the structures obtained for the halophilic archeon *H. Marismortui* [139,141,142] and *D. radiodurans* [140,143]. The proposed models differ even though ribosomal 50S subunits of the two bacteria have drug binding sites whose sequences are highly conserved. Furthermore, the crystal structure data obtained so far on complexes of macrolides with ribosomes isolated from clinically non-relevant bacteria do not explain all the effects of macrolides on different pathogenic strains. We believe that steps taken in the process of drug design should also include the elucidation of the solution-state structures of free and bound ligand molecules since the structural features of the complex may not be exactly the same in solution as in the solid state [142,145].

Novak *et al.* [146-150] have shown that a systematic approach combining NMR and molecular modelling calculations could be applicable to conformational studies of free and bound macrolides and their interactions with ribosomes. Their results [146-150], and those of other groups [151-155], have shown that macrolides adopt two major conformational families, folded-out and folded-in, referring to the outward and inward folding of the ring fragment 3C-5C (Figure 5.10).

Figure 5.10. Superposition of the two major conformations of azithromycin: folded-in (black) and folded-out (grey)

The authors concluded that the vicinal coupling constants $^3J_{H2H3}$ and NOE proton-proton contacts such as H3-H11 and H4-H11 are good indicators of aglycone fold-

ing. Furthermore, $^3J_{CH}$ coupling constants over the glycosidic bonds might provide information about the position and mobility of sugar units, with respect to the lactone ring. Longitudinal relaxation of methyl protons could also be useful to probe motions of methyl groups which reflect aglycone ring folding. Additionally, by applying trNOESY and STD-NMR experiments, it was possible to characterise the interactions of macrolides and ribosomes [146,148].

STD experiments have indicated three common regions closest to the ribosome surface: desosamine sugar, cladinose sugar and the methyl group at position 13 (Figure 5.11).

Figure 5.11. STD binding epitopes of a macrolide derivative.

These were also found by X-ray diffraction to be the reactive groups responsible for binding to the ribosome. The bound conformations of the studied macrolides were found to be very similar to those observed in the free state, which were also in agreement with the results obtained by crystallography [142,144]. Furthermore, it was found that the absence of cladinose sugar was the main cause of the inability of decladinosyl macrolides to bind to the ribosome. Recently, more detailed studies using a combination of STD-NMR and *in vitro* protein inhibition on a series of macrolide compounds have shown that hydrophobic interactions involving cladinose, methyl groups of the lactone ring and the 13-alkyl moiety are a prerequisite for optimal positioning of the desosamine sugar unit. A synergy between the desosamine 3'-dimethylamino and 2'-hydroxyl interactions with the ribosome has been proposed as crucial for successful protein synthesis inhibition. Recently, NMR self-diffusion and solvent paramagnetic relaxation enhancements have been used to determine the interaction strength and localization of a series of macrolide antibiotics with membrane-mimetic micelles. It has been proven that protonated nitrogen atoms play a crucial role in binding to membrane-mimetics [156].

STD-NMR spectroscopy has also been employed to characterise the epitopes of azithromycin, oleandomycin and telithromycin macrolide antibiotic binding to

bovine serum albumin (BSA) [157]. The structural parts of azithromycin and oleandomycin in intimate contact with bovine serum albumin were found to be similar, while those of telithromycin showed similarities but also some differences. The authors explained these findings by different structural elements of antibiotics that interacted with the protein, especially the alkyl-heteroaryl side chain in telithromycin and cladinose and desosamine sugars in azithromycin and oleandomycin.

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