Exploring Ligand Affinities for Proteins by NMR of Long-Lived States

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"Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover."

Mark Twain

Abstract

The detection of molecules that can bind to active sites of protein targets and the measurement of their affinities is a promising application of NMR. Nowadays, the screening of drug candidates is routinely done by NMR in pharmaceutical industry.

We have proposed to use the relaxation of Long-Lived States (LLS) for drug screening by NMR. Long-lived states are nuclear spin states whose decay time constant T_{LLS} can be much longer than the longitudinal relaxation time T_1. LLS can be used to screen and determine the dissociation constant K_D of molecular fragments that bind weakly to protein targets. The use of LLS for fragment screening leads to a spectacular increase in contrast between free and bound ligands, and thus allows one to characterize binding of fragments with very weak affinities, with K_D in the millimolar range, which is difficult to achieve by other methods such as ITC. By exploiting the LLS behavior of a spy molecule, we experimentally demonstrate that it is possible to measure dissociation constants K_D as large as 12 mM, corresponding to very weak binding, where most other biophysical techniques fail, including other NMR methods based on the observation of ligands.

Furthermore, we have combined LLS for screening for improved contrast with ^{1}H dissolution-DNP to enhance the sensitivity. DNP-enhanced screening for measuring LLS signals of a weak ligand allows one to use very low concentrations of ligands and proteins. We observed dramatic differences between the spectra of the ligand in the presence or absence of a protein, or in the presence of the protein combined with a stronger ligand.

Moreover, we have explored LLS involving pairs of ^{19}F nuclei to study binding phenomena. Indeed, fluorine detection is quite interesting because it offers the possibility to perform screening experiments without any problems due to overlapping signals. In a custom-designed fluorinated ligand that binds trypsin, we have observed a promising ratio T_{LLS} / $T_1 > 4$. This fluorinated ligand has been used as spy molecule in competition experiments, which allowed us to rank the affinities and estimate dissociation constants of arbitrary ligands that do not contain any fluorine.

Keywords: drug discovery, fragment screening, ligand binding, competition experiments, Long-Lived States, dynamic nuclear polarization, trypsin, Hsp90, fluorine.

Sommario

La rilevazione di molecole che si legano al sito attivo di proteine e la misura della loro affinità è una promettente applicazione dell'NMR. Nell'industria farmaceutica, al giorno d'oggi lo screening di molecole con potenziale farmaceutico è abitualmente eseguito con tecniche NMR.

Abbiamo proposto l'uso del rilassamento degli stati a lunga vita (LLS) per lo screening di ligandi tramite NMR. Gli stati a lunga vita sono stati di spin nucleari la cui costante di rilassamento T_{LLS} può essere molto più lunga della costante di rilassamento longitudinale T_1. Gli LLS possono essere utilizzati per selezionare ligandi e determinare la costante di dissociazione K_D di frammenti molecolari che si legano debolmente a proteine. L'uso degli LLS per lo screening di frammenti molecolari permette uno spettacolare aumento del contrasto tra ligandi liberi e legati, permettendo quindi di caratterizzare il legame di frammenti con affinità molto deboli, con K_D nell'ordine del millimolare. Questo è un traguardo normalmente molto difficile da raggiungere con altri metodi, per esempio con l'ITC. Sfruttando il comportamento degli LLS in una molecola spia, abbiamo dimostrato sperimentalmente che è possibile misurare costanti K_D fino a 12 mM, corrispondenti a legami molto deboli, dove molte altre tecniche biofisiche falliscono, compresi molti metodi NMR basati sull'osservazione dei ligandi.

Inoltre, abbiamo combinato l'alto contrasto dello screening effettuato tramite LLS con la DNP per aumentare la sensibilità dell'esperimento NMR. L'uso della DNP per misurare i segnali LLS di un ligando debole permette l'uso di concentrazioni molto basse di ligando e proteina. Con questo approccio, abbiamo osservato enormi differenze tra gli spettri del ligando in presenza o in assenza della proteina, o in presenza della proteina e di un ligando più forte.

In aggiunta, abbiamo testato la possibilità di usare LLS su coppie di nuclei di fluoro per studiare questo tipo di fenomeni. Infatti, la rilevazione dei segnali del fluoro è piuttosto interessante perchè offre la possibilità di eseguire esperiementi di screening evitando problemi derivanti dalla sovrapposizione di segnali. In un ligando fluorurato che si lega alla tripsina, abbiamo misurato un promettente rapporto T_{LLS} / T_1 > 4. Questo ligando fluorurato è stato usato come molecola spia in esperimenti di competizione, permettendoci di confrontare le affinità e stimare le costanti di dissociazione di ligandi che non contengono atomi di fluoro.

Sommario

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1. Introduction

The overall cost of the development of a new drug is about $800 million to $1 billion.[1] These numbers may seem to be exaggerated, but two factors can explain them. The first one is the complexity of the research and development process: for every 5000-10000 compounds that enter the selection process, on average only one gets approved for the market. Hundreds of other molecules are dropped during the intermediate selection steps and the final cost must include the expense of these failures. The second reason is the length of the process: on average 10-15 years are needed to develop a new drug from the beginning of the discovery campaign to the final approval. These numbers are quite impressive. It means that a drug which enters into the market today is the result of a process that has started in the 2000. At that time, the twin towers were still standing and Wikipedia did not yet exist.

1.1 The drug discovery and development process

During the past 40 years there has been a huge acceleration in the understanding of molecular mechanisms that underlay disease processes. As a consequence, modern pharmaceutical research has become progressively based on target-focused discovery, where the aim is to modulate the biological activity of a particular molecular target and thus provide a cure for a disease. The 'post-genome era' has further increased the number of targets of therapeutic interest for which there are not yet known small-molecule modulators, stimulating many new studies and opening the way to fight diseases that were hitherto incurable.

The entire process consists in two parts. The first one is the phase of "**drug discovery**", during which a few molecules are identified, studied and optimized in order to be subsequently tested as potential drugs. The second one is called "**drug development**" and consists of an ensemble of clinical tests needed to get the final approval for marketing.
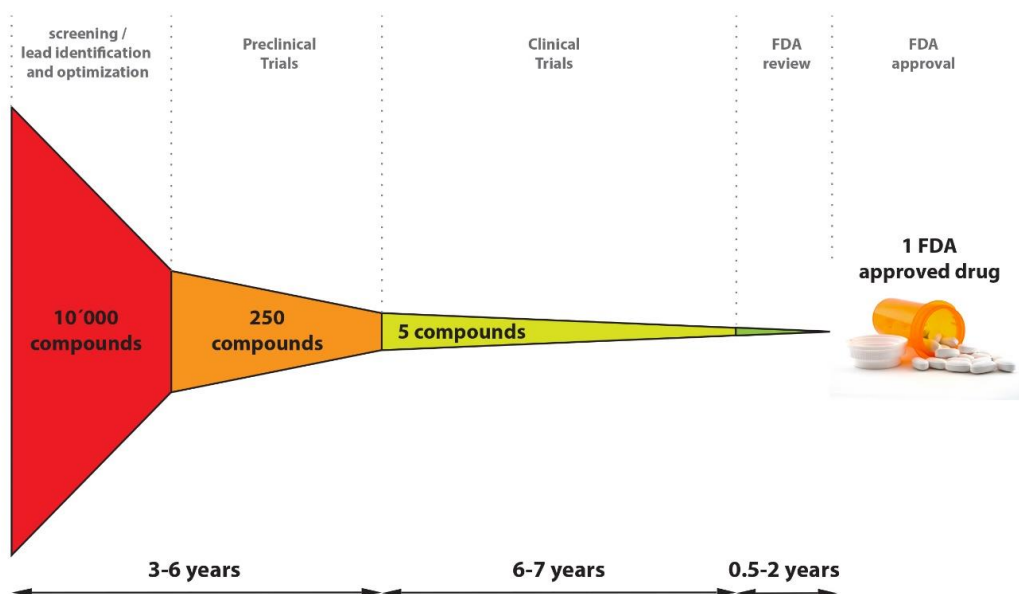
Figure 1 The drug discovery and development timetable. After the target identification, screening campaigns are performed in order to identify a few hit compounds, which have to be optimized in order to address requirements such as affinity, specificity, absorption, distribution, metabolism, excretion and toxicological properties (ADMET). Before accessing to the clinical trials, the compounds have to pass some preclinical tests, in order to verify their efficacy and safety. During the clinical trials, composed of three phases, potential drugs are tested on humans in order to test the safety and efficacy and to optimize the dose. At the end of these clinical phases, the FDA decides if the molecule can go to the market. The whole process can take 10-15 years.

1.1.1 The drug discovery phase

The first step of drug discovery is the understanding of a disease. Scientists try to understand how genes are altered, how proteins are overexpressed, etc., and how these abnormalities affect the health of the patients. In many cases, the major biopharmaceutical companies are not the only sources of knowledge of this step; many smaller companies, research centers, universities and other nonprofit institutions provide significant contributions to the basic knowledge of the disease etiology.

Once the knowledge of a disease allows it, pharmaceutical researchers select a target for a potential new drug. Already at this step there is the risk of failure: the chosen target has to be "druggable", *i.e.*, it should be possible to regulate its activity with high affinity and selectivity by a drug-like molecule, and its role in the disease has to be validated. In other words, researchers have to demonstrate that the chosen target is relevant to the disease being studied through experiments in both living cells and in animal models of the disease. A recent study estimated that among the 30000 genes in the human genome, only 3000 might code for druggable proteins.[2] Only about 400 of such targets have been studied so far.

Once the target is chosen, scientists look for a molecule, a "hit compound", which may act on it to alter the course of the disease. There are different approaches to search for a hit compound:

- Natural compounds: molecules present in nature can be starting points for developing a new drug;
- *De novo*: computer modeling can be used to design a molecule from structure base knowledge that may bind and modulate the target's activity;
- Screening: Few hundred thousand up to few millions compounds can be tested against the target to identify promising compounds;
- Biotechnology: Researchers can genetically engineer living systems to produce biological molecules that can fight a disease.

The next phase involves its optimization. The aim is to enhance properties such as specificity, efficiency and safety. Typically scientists synthetize hundreds of analogues of the initial hit and test them with the aim of improving the above-cited properties. For example, they can make the compound less likely to interact with other chemical pathways in the body, thus reducing potential side effects. The resulting molecule is called lead compound.

1.1.1 The drug discovery phase

Once a few lead compounds have been identified, they have to go through a series of tests to study their pharmacokinetics properties. In fact, a drug should be 1) absorbed into the bloodstream, 2) distributed to the proper site of action, 3) metabolized efficiently and effectively, 4) successfully excreted from the body and 5) demonstrated not to be toxic.

Before being allowed to test a candidate drug on humans, several preclinical tests need to be performed. Scientists have to understand how the drug works and what its safety profile looks like. Several *in vitro* and *in vivo* tests need to be carried out. Agencies like the FDA (Food and Drug Administration) require the molecules to go through severe tests before being applied to humans.

All the above steps can take from three to six years. After starting with 5000-10000 molecules, scientists may have identified a group reduced to one to five molecules which will be studied in clinical trials as candidate drugs.

1.1.2 The drug development phase

Before starting any clinical trials, an Investigational New Drug (IND) application has to be submitted to the FDA. This file includes the results of the preclinical work, the molecular structure and the hypothetical mechanism of action in the body, a list of any side effects and a detailed clinical plan for the next studies. FDA must be regularly updated on results of on-going tests and can stop the trials at any time if problems arise.

In Phase 1 trials, the candidate drug is tested on about twenty to one hundred healthy volunteers. These are the first tests on humans and they are mainly aimed at getting information on the safety profile and the definition of the safe dosing range.

In Phase 2 trials the potential drug is tested on about 100 to 500 patients who suffer from the disease. The aim of these tests is to evaluate the effectiveness of the drug, while keeping possible short-time side effects under observation. In this stage, scientists also optimize the dose strength and schedules for use of the drug.

In Phase 3 trials, the candidate drug is tested on a larger number (about 1000-5000) patients to get statistically significant data about safety, efficacy and overall benefit-risk relationship of the drug. This is the costliest, longest and most critical phase.

At the end of the third phase, all data are evaluated. If the results demonstrate that the potential drug is safe and effective, the company can file a New Drug Application (NDA) to the FDA requesting approval to market the drug. At this point, FDA reviews the application and can decide to 1) approve the medicine, 2) request more information or studies, or 3) deny approval.

It can take 7-9 years from the first tests of Phase 1 and the FDA approval. Research on a new drug continues even after approval since potential long-term side effects can occur. The company is asked to submit periodic reports to the FDA.

A famous proverb says "Rome wasn't built in a day". "Neither was a drug generated in a day", we could add.

1.2 Fragment-Based Drug Discovery

Nowadays, the search for hit compounds is usually achieved through screening campaigns. Large libraries (>10^5) of molecules are usually screened against the target of interest and their potential interactions are detected by biochemical or cell-based functional assays. The molecules identified through this procedure, called High-Throughput Screening (HTS), are then optimized via medicinal chemistry in order to improve their pharmacokinetic properties.

Progress in robotics and engineering allows one to accelerate the speed of the process. Nowadays, it is possible to monitor up to 100 million reactions in ten hours.[3] Nevertheless, the use of HTS has often proven to be inefficient for drug discovery resulting often in false positive and false negatives. About half of the HTS campaigns fail, mainly because the library does not contain any good small molecules as starting points.[4] The probability to fail is even higher for new classes of targets, such as protein-protein interactions (PPIs), for which there are not many historical precedents.[5, 6]

The hit molecules identified through this strategy may be complex and suffer from a substantial lipophilicity. These compounds therefore often have limitations with respect to the criteria of Absorption, Distribution, Metabolism, Excretion and Toxicological (ADMET) that cannot be easily overcome during the following optimization step.

FBDD	HTS
About 10^3 compounds of small size (<300 Da)	>10^5 compounds (>300 Da)
High coverage of chemical space	Poor coverage of chemical space
Low-affinity hits (100 µM < K_D < 10 mM)	High-affinity hits (K_D in the low µM or stronger)

Table 1 Main differences between Fragment-Based Drug Discovery (FBDD) and High-Throughput Screening (HTS)

In the past decade, an alternative approach called Fragment-Based Drug Discovery (FBDD) has emerged. FBDD involves the use of small libraries of fragments that are low molecular weight compounds. The idea is that scientists can look for small binding fragments and then either expand a fragment or combine two of them to achieve the affinity one expects from HTS.

The top part of Figure 2 shows an example of high-throughput screening: many compounds are screened against a target to identify a hit that binds. This will be then optimized through medicinal chemistry. The central part of Figure 2 represents the fragment-linking approach: the screening identifies two small molecules that bind to nearby sites. They can then be linked together and optimized via medicinal chemistry. This is the principle of a well-known strategy known as structure-activity relationship (SAR), implemented by NMR for the first time by Fesik and co-workers.[7]
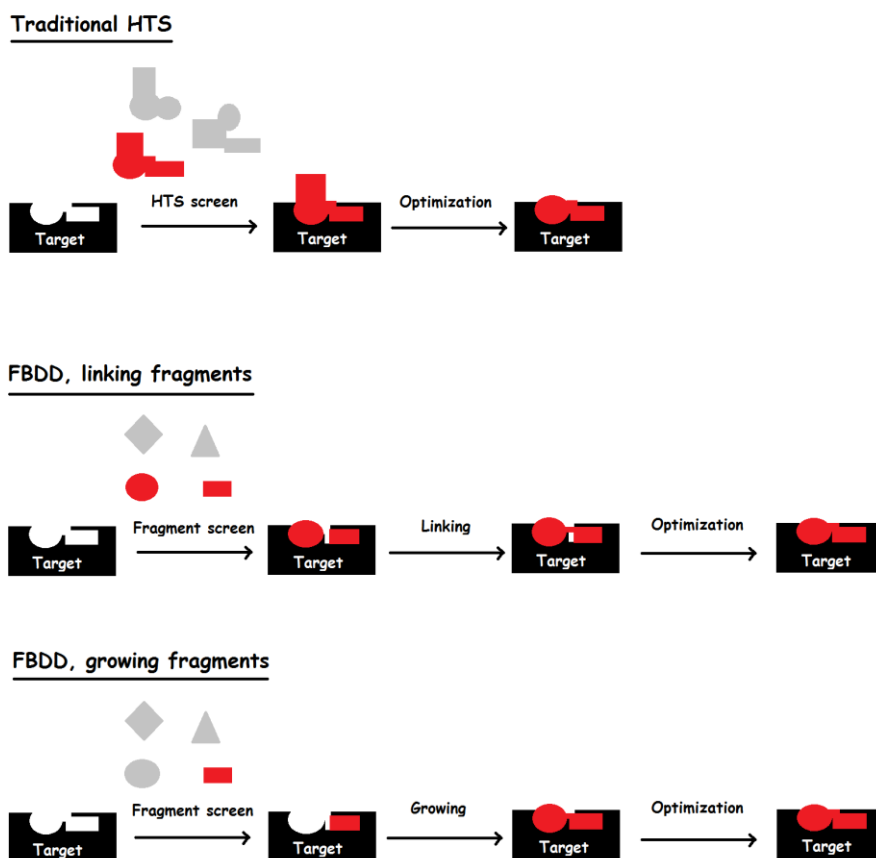
Figure 2 Graphical representation of the main approaches to develop a drug. (Top) In high throughput screening (HTS), libraries of relatively complex molecules are screened to identify hit compounds with high potency. (Middle) in FBDD, screening can identify fragments that can be successively merged in order to generate a ligand with higher potency. (Bottom) in FBDD, a fragment with low affinity can be identified and then optimized to improve its potency *via* medicinal chemistry.

1.2 Fragment-Based Drug Discovery

Although extremely elegant, many scientists have found that the linking is much more challenging than might be expected. The main difficulty is that much of the potency of the two fragments will be lost if they are not perfectly positioned, so that the affinity of the resulting molecule will not be as good as expected. Therefore, a frequently used alternative strategy is "fragment growing", shown in the bottom part of figure 2: a single fragment is expanded or "grown" by medicinal chemistry to increase the potency of the initial fragment.

The hit rates of screening campaigns of fragment libraries is usually higher than those of HTS. This is due to the fact that the larger the molecules, the more complex their structures. As consequence, each additional moiety has an increasing probability of interfering with binding. On the other hand, fragments give an opportunity to better sample the active site of the target, giving important information to medicinal chemists, who have to link different fragments or to optimize one of them.
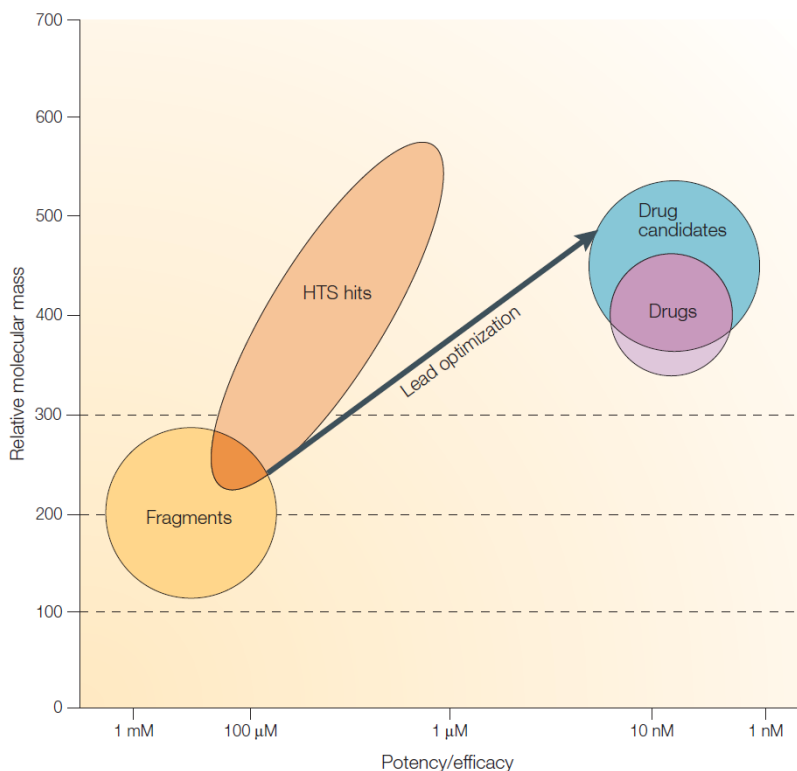
Figure 3 Correlation between the potency and the molecular mass of molecules considered in FBDD and HTS, and of approved drugs. Reproduced from [8]. FBDD starts with smaller and less potent molecules, giving medicinal chemists more opportunities to improve important properties needed to develop a successful drug.

1.2.1 What is a fragment?

The idea of FBDD to work with small fragments has been supported by some empirical evidence, summarized in Lipinski's famous rule of five (RO5).[9] Lipinski and co-workers observed that most orally administered drugs are relatively small and moderately lipophilic molecules. Note that the rule of five does not apply to certain classes of drugs, for example to antiviral drugs.

Based on these observations, the rule summarizes molecular properties which turn out to be important for the pharmacokinetics of a drug:

1) The molecular mass should be less than 500 Da. This allows one to work with molecules that can efficiently explore the binding pocket and represent the variety of chemical space;
2) An octanol-water partition coefficient log P (ClogP) not greater than 5. Solubility is a critical parameter for fragment libraries. Fragments require a certain level of hydrophilicity to be soluble up to 1-2 mM, but they should be sufficiently hydrophobic to interact properly with the target; indeed, many of the druggable protein targets have pockets with strong hydrophobic contributions to binding;
3) No more than 5 hydrogen bond donors;
4) No more than 10 hydrogen bond acceptors;

Lipinski's rules have been successively refined for the fragments. Results of the analysis of a diverse set of fragment hits show that such hits seem to obey a 'rule of three' (RO3).[10] The average molecular weight is less than 300 Da, the number of hydrogen bond donors is not greater than 3, the ClogP is less than 3, and the number of hydrogen bond acceptors is not greater than 3. In addition, the results suggest that the number of rotatable bonds (NROT) should not be greater than 3 and the polar surface area (PSA) should not be greater than 60 Å^2.

These are only guidelines, but nowadays many companies follow them while designing libraries of fragments.

1.2.2 Ligand efficiency

We could assume that a fragment behaves like an ant. If it invades a picnic, a guy can easily squash it. But if he watches the ant escape with a crumb, the answer is different: ants can carry at least ten times their own body weight.

It is the same for fragments. Due to their small size, fragments bind their target very weakly. Despite of this, they often bind tightly for their dimensions. In order to express the binding affinity of a fragment in the light of its molecular mass, the most widely used parameter is called ligand efficiency (LE).[11] LE can be defined as the ratio between the free energy of ligand binding and the number of heavy atoms in the ligand. The 'free energy of ligand binding', ΔG_{bind}, is equal to $-RT\ln K_D$, where R is the ideal gas constant, T the temperature, and K_D the dissociation constant. The number of heavy atoms refers to the number of non-hydrogen atoms in the ligand. Alternative parameters exist, as the binding efficiency index (BEI), which is defined simply as the ratio between the free energy of ligand binding and the molecular weight.
A drug with a K_D of 10 nM and a molecular weight of 500 Da (about 38 heavy atoms) would have LE = 0.3 Kcal/mol/heavy atom. Thus, the aim is to reach ligand efficiencies of 0.3 Kcal/mol/heavy atom or better. Ligand efficiency values can vary considerably based on the target: for many kinases, inhibitors can have LE above 0.5 Kcal/mol/heavy atom, while for more challenging targets (as most protein-protein interactions) LE can fall below 0.3 Kcal/mol/heavy atom.

1.2.3 FBDD compounds in clinical trials

Table 2 shows an updated list (January 2015) of drugs that entered clinical trials starting from fragments. Almost half of the targets are protein kinases, demonstrating that it is relatively straightforward to identify fragments with high LE that bind to the purine-binding site of this class of proteins.
August 17th, 2011, marked history of FBDD, when the FDA approved the first drug deriving from fragment-based screening. The drug is sold with the name Zelboraf (vemurafenib) and targets a mutant of kinase B-Raf. It can extend life of patients with metastatic melanoma, where it displayed an impressive activity.[2, 12] Vemurafenib was discovered at Plexxikon and developed in partnership with Roche. It is the result of a particularly rapid

drug discovery and development process: initiated in February 2005, it took just six years to reach approval. Figure 4 shows the initial fragment and its optimization into the final drug, reporting the potency and ligand efficiency of each step.

	Drug candidate	Company	Role
Approved	Vemurafenib	*Plexxikon*	B-Raf(V600E) inhibitor
Phase 2/3	ABT-199	*Abbott*	selective Bcl-2 inhibitor
	LEE011	*Novartis/Astex*	CDK4 inhibitor
	MK-8931	*Merck*	BACE1 inhibitor
Phase 2	AT13387	*Astex*	HSP90 inhibitor
	AT7519	*Astex*	CDK1,2,4,5 inhibitor
	AT9283	*Astex*	Aurora, Janus kinase 2 inhibitor
	AUY922	*Vernalis/Novartis*	HSP90 inhibitor
	Indeglitazar	*Plexxikon*	pan-PPAR agonist
	Linifanib (ABT 869)	*Abbott*	VEGF & PDGFR inhibitor
	LY2886721	*Lilly*	BACE1 inhibitor
	LY517717	*Lilly/Protherics*	FXa inhibitor
	Navitoclax (ABT 263)	*Abbott*	Bcl-2/Bcl-xL inhibitor
	AZD5363	*AstraZeneca/Astex/CR-UK*	AKT inhibitor
	AZD3293	*AstraZeneca/Astex/Lilly*	BACE1 inhibitor
	PLX3397	*Plexxikon*	FMS, KIT, and FLT-3-ITD inhibitor
Phase 1	ABT-518	*Abbott*	MMP-2 & 9 inhibitor
	ABT-737	*Abbott*	Bcl-2/Bcl-xL inhibitor
	AZD3839	*AstraZeneca*	BACE1 inhibitor
	DG-051	*deCODE*	LTA4H inhibitor
	IC-776	*Lilly/ICOS*	LFA-1 inhibitor
	JNJ-42756493	*J&J/Astex*	FGFr inhibitor
	AT13148	*Astex*	AKT, p70S6K inhibitor
	LP-261	*Locus*	Tubulin binder
	LY2811376	*Lilly*	BACE1 inhibitor
	PLX5568	*Plexxikon*	kinase inhibitor
	SGX-393	*SGX*	Bcr-Abl inhibitor
	SGX-523	*SGX*	Met inhibitor
	SNS-314	*Sunesis*	Aurora inhibitor
	AZD5099	*AstraZeneca*	Bacterial topoisomerase II inhibitor
	RG-7129	*Roche*	BACE1 inhibitor
	Undisclosed	*Vernalis/Servier*	Bcl-2 inhibitor

Table 2 Drug candidates currently under clinical evaluation (January 2015).[13]
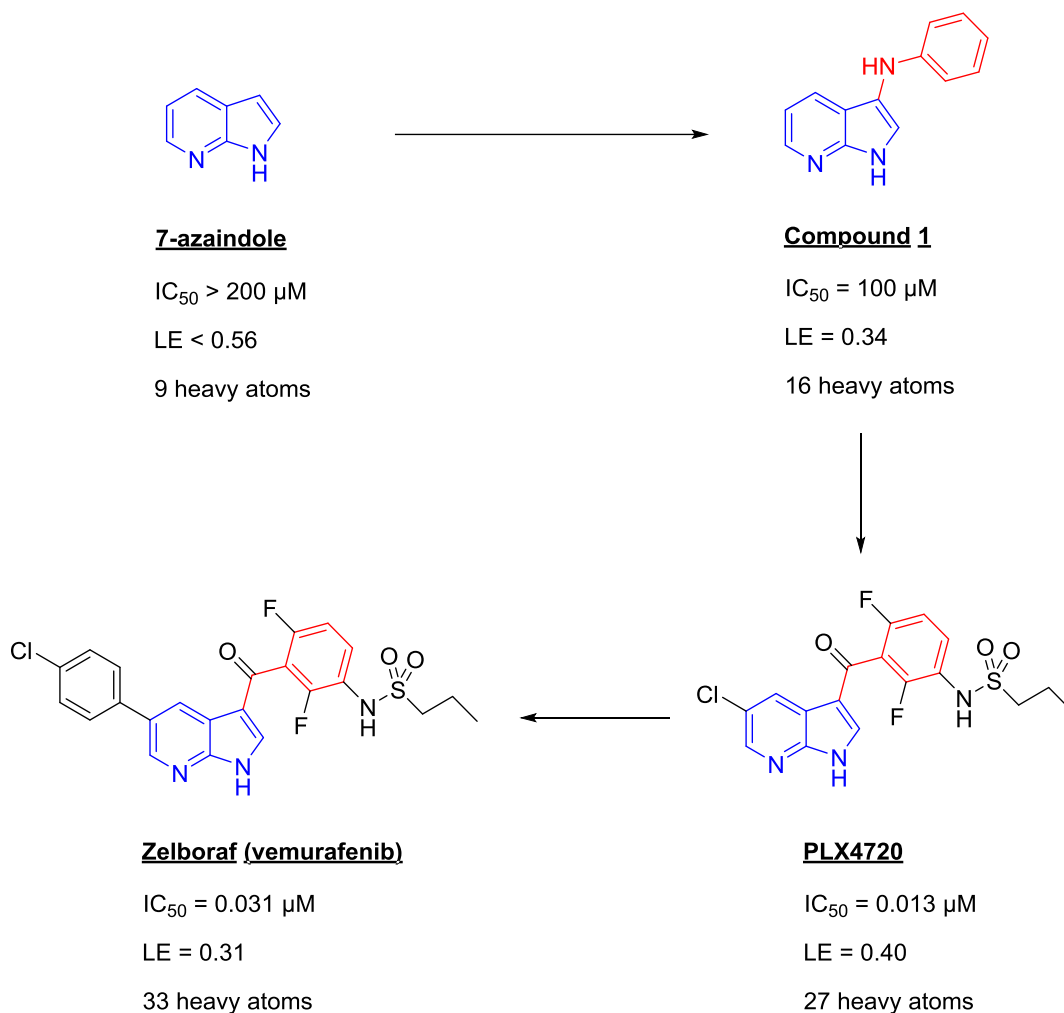
Figure 4 Pathway for the discovery of the first drug based on fragment-based screening. The initial hit compound (7-azaindole) was optimized, maintaining a good LE while enhancing its potency and selectivity for the desired target.

1.2.4 Screening of fragments libraries

HTS screening campaigns are typically performed at low ligand concentrations (typically 10 µM) and normally deliver potent hits.[14] However, some screens fail to identify a good starting point for lead optimization. This can be due to 1) assays that are not well configured, 2) problems of solubility or stability of the compounds in the screening libraries or 3) lack of good compounds in the libraries. To overcome the last two problems, screening collections are continuously updated with new compounds, based on

experience that has been gathered during other screening campaigns, in view of achieving a better coverage of chemical space.

Another approach is to screen libraries of fragments, which due to their size and low complexity are likely to interact much more weakly with the target. This therefore requires one to reconfigure screening assays for much higher concentrations. Issues of solubility of screening compounds are aggravated by the high concentrations used. Small quantities of organic solvents like DMSO can be used to improve the solubility and light scattering techniques can be employed to confirm the absence of aggregates.[12]

There are two ways to carry out fragment screening: biochemical screening (High Concentration Screening, HCS) or biophysical screening.

1.2.4.1 Biochemical assays at high concentration

This approach consists in performing classical biochemical assays, but working at higher concentrations (up to 1 mM). In this way, it is possible to use the same technology used for the classical high-throughput screening technology for detection, and one can identify some hits very quickly. In addition, the amounts of protein required are very small and many 'difficult' targets (like GPCR or ion channel targets) can be studied. Nevertheless, many problems can occur. For example, high ligand concentrations can interfere with the assay or be toxic for cells. Moreover, compound aggregations can give rise to false positives, or false negatives can result from a lack of solubility of the compounds.

1.2.4.2 Biophysical techniques

There are several biophysical techniques to screen fragments. Different biophysical techniques have different problems and provide complementary information about binding. For example, quantitative affinity data can be obtained from isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), or NMR spectroscopy, while X-ray crystallography can provide detailed atomic resolution of the binding mode.

Throughput and material requirements can differ dramatically between different techniques, determining the stage of the FBDD campaign for which they are best suited. Primary screening is carried out using techniques with high throughput that require small amounts of target protein, while secondary screening can be performed with techniques that have a lower throughput. Traditionally, X-ray crystallography and ITC are considered

as low throughput techniques, while SPR and thermal shift measurements are higher throughput methods. Depending on whether *ligand-* or *target-detected* techniques are used, NMR spectroscopy can offer a high or low throughput and potentially provide an atomic model of binding.

X-ray crystallography

X-ray crystallography provides direct information about the binding mode at atomic resolution.[15] Usually, protein crystals are soaked with a solution containing high concentrations (about 50 mM) of the fragment and binding is detected directly by observation of the fragment bound to the protein in the electron density. There is no risk of false positives, but false negatives can occur when binding sites are occluded by crystal contacts or when ligand binding requires a conformational change of the target protein that is inhibited by the crystal framework. The latter problem can be avoided by attempting co-crystallisation of the protein in the presence of the ligand. In general, X-ray crystallography provides high resolution data, and is able to detect fairly weak binders, up to $K_D \sim 5$ mM .

The low throughput of the technique does not allow its use for primary screening, but it is generally considered as the gold standard for final hit validation. Furthermore, the throughput of X-ray crystallography has recently been increased.[16] The use of automated, rapid data collection at powerful synchrotron beam sources that allow the collection of high-resolution data in minutes, employing sample-changing robots, and semi-automatic processing and structure solution has speeded up the process considerably. Molecular replacement strategies can reduce structure solution to manual inspection of ligand placements in different electron maps. Ligand orientations are obtained by ligand fitting routines, employing similar strategies as those used in molecular docking [17], with the advantage to optimize the fit of the ligand with the experimental electron density. If a cocktail of several ligands is used, it is important to use a diverse set of ligands to make them identifiable from the shape of their electron density, and to minimize the chances that two or more ligands compete for the same binding site.

The technique requires the protein of interest to crystallize in a reproducible way. Problems of solubility of the ligand can be addressed by using low concentrations of organic co-solvents, such as 1 to 10% DMSO. X-ray crystallography does not provide information about binding affinities, so X-ray observations must be combined with data

obtained by other methods. Ideally, X-ray crystallography should be used in conjunction with a method with higher throughput like SPR, or verified by ITC after crystallization.

NMR spectroscopy

NMR spectroscopy was the first technique used for experimental FBDD screening.[18] In the *SAR by NMR* methodology [7], the binding of a fragment to a protein is detected through the change in protein chemical shifts. These changes are due to the different chemical environments experienced by the nuclei of the protein upon binding. In its first application, two fragments identified in this way were merged, yielding a binder with improved potency.[16]

SAR by NMR is an example of *target-detected* method. In such methods, the chemical shifts of the target protein are observed in two-dimensional NMR experiments. As a consequence, these methods are limited to small proteins with molecular masses of about 30 to 40 kDa and require relatively large amounts of isotope-labelled proteins. On the other side, *target-detected* methods can provide information about the binding site, if the protein resonance assignments are available.

As an alternative, several *ligand-detected* methods have been developed, such as Saturation Transfer Difference (STD) [19], WaterLOGSY [20] or Target-Immobilized NMR Screening (TINS).[21] These methods require less protein, no isotope labelling, and are also applicable to larger proteins. On the other hand, they usually do not provide information about the binding site, but they are generally faster and simpler (usually, one-dimensional NMR experiments suffice). NMR methods for ligand screening will be the topic of chapter 2.

Surface Plasmon Resonance (SPR)

In this technique, the protein is immobilized on a metal-coated surface and ligands flow past.[22] The binding of a ligand to the protein determines changes in the refractivity/reflectivity properties of the metal. In fact, these properties are directly correlated with the mass of the protein and the mass of the ligand and can be detected by an optical device.

SPR is a high-throughput technique, well suited for primary screening. Screening campaigns are rapid and straightforward to set up. The immobilization of the protein on

the metal surface implies the need of very small amounts of protein. SPR allows one to obtain important data about affinity. Indeed, the recorded sensogramm is time-dependent and the approach represents a continuous-flow system, so ligands first saturate the protein and are then washed off, thus allowing one to determine kinetic information encoded in the k_{on} and k_{off} rates.

With SPR, several thousands of compounds can be screened in a few days, making it ideally suited for prioritizing subsequent X-ray experiments.[23]

Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry (ITC) measures the heat released upon ligand binding.[24, 25] ITC is the technique of choice for the precise determination of binding constants. It is the only widely used biophysical technique that is able to deconvolute the contributions of enthalpy and entropy to ligand binding. These properties are essential to understand the importance of polar and hydrophobic interactions and to guide medicinal chemists during the fragment expansion process. One drawback of ITC, however, is that it requires large amounts of protein and has a rather low throughput. As consequence, ITC is well suited for secondary screening and hit confirmation.

Thermal shift assay

In a thermal shift assay [26-28], the unfolding temperature of a target protein is determined in the presence and absence of a ligand by optical strategies. Indeed, folded and unfolded proteins have different fluorescence properties. In fact, the binding of small ligands to proteins stabilizes the protein's folded state by increasing its heat capacity. As a consequence, ligand binding leads to an increase of the unfolding temperature.

Thermal shift assay is a good technique for "yes/no" binding information and has a high throughput. It is mainly utilized as an enrichment process before applying a secondary screening technique. On the other hand, this strategy is also subject to false negative, since fragments sometimes do not display changes in the protein unfolding temperature.

Computational techniques

Nowadays, computational techniques can be used to identify molecules with a greater probability of binding a biological target. The process of screening virtual libraries is called Virtual Screening (VS). There are two main approaches to VS: docking and *de novo* design. Neither are widely used as stand-alone tools. The use of experimental knowledge to enrich data from virtual screening campaigns plays a key role to obtain useful results.

Docking

Molecular docking was used for the first time more than 20 years ago.[29] This computational tool combines a search algorithm to generate putative binding modes of a ligand and its receptor with a scoring function that ranks them.

The realization that the conformation of small molecules that form a complex with a macromolecular target does not imperatively correspond to a global minimum [30] and that proteins experience structural rearrangements upon binding [31] highlighted the necessity to include flexibility in docking algorithms. This means that docking algorithms should consider the fluctuations of bond distances and dihedral angles in addition to the rotational and translational degrees of freedom of the ligand. This is not feasible, due to the size and flexibility of macromolecular receptors and the time constraints that must be fulfilled for docking algorithms. At present, most docking algorithms consider the target as a rigid structure, and only the degrees of freedom corresponding to dihedral angles of the ligand are explored.

Despite of this simplification, most docking programs nowadays correctly predict the binding modes for 70-80% of all known protein-ligand pairs within a root mean square deviation (RMSD) of 2 Å. Recent studies have shown that VS results can improve when the flexibility of the receptor is included in the algorithm.[32] Nevertheless, flexible receptor docking is very prone to generating false positives.[33]

1.2.4.2 Biophysical techniques

De novo Design

It has been estimated that over a 10^6 million organic molecules could exist with molecular weights not greater than 500 Da.[34] It is evident that VS libraries (usually containing 10^5-10^7 molecules) cover only a small part of the chemical space.

De novo design methods [35] offer a way to explore the whole chemical space. They rely on the design of ligands from scratch, by merging fragments from pre-defined libraries and testing the complementarity to the receptor with the same or similar scoring functions as used for docking.

The molecules proposed by *de novo* methods are in most cases unknown. This can be a limitation, considering that the synthetic feasibility is generally ignored so that the chemical structures proposed can often not be easily synthesized. Recent improvements in this field provided programs with outputs prioritized according to their chemical accessibility.

De novo design methods are complementary to experimental fragment screening methods. Experimental approaches can identify small fragments that need to be extended to become lead molecules, while *de novo* design methods can benefit from limiting the search to chemical scaffolds that are known to bind an active site.

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2. Nuclear magnetic resonance for ligand screening

Until the mid 1990s, the role of NMR in the pharmaceutical industry was quite limited. Its main use was focused on the three-dimensional structure determination of peptides and proteins (as well as on the study of small molecules and metabolites). In this context, NMR was restricted mainly to a small subset of NMR-accessible targets (with molecular masses lower than 20 KDa and expressible in *E. Coli*). Moreover, the development of a drug candidate usually requires a considerable amount of structural information beyond the simple *apo* structure of the protein without ligand. Typically, a loop of structure determination, modeling and chemistry has to be repeated in order to achieve the potency, selectivity and ADMET properties required for a drug candidate. In this context, NMR is not able to generate structural data at the same resolution and with the same speed as X-ray crystallography.

Something changed after 1996. The milestone work of Fesik and co-workers [1] showed that NMR could give a huge contribution to drug discovery. SAR by NMR [1] proved the possibility to start a drug discovery project from compounds which would have been normally considered to bind too weakly to be relevant for classical medicinal chemistry.[2] Alternative techniques have been proposed to find such weakly binding fragments, for instance X-ray crystallography [3], mass spectrometry [4] and high-concentration enzyme assays [5], but NMR screening remains one of the most robust and reliable techniques for identifying ligands with dissociation constants between 10 µM and 10 mM or greater.[6] In fact, it is possible to study binding phenomena through the dramatic changes in several NMR parameters, which occur when a small and rapidly tumbling molecule binds to a slowly tumbling macromolecular target.

NMR screening has transformed magnetic resonance from a marginal tool to obtain structural information about proteins into a fundamental instrument for the discovery of lead compounds. Nowadays, there are many companies whose drug-discovery platforms are strongly dependent on NMR. Several pharmaceutical industries use NMR as their principal tool for both screening campaigns and ligand interaction studies.

2.1 The dissociation constant

E quilibrium processes involving non-covalent interactions are very common in chemical and biochemical systems. It is possible to define a *molecular complex* as "a non-covalently bound species of definite substrate-to-ligand stoichiometry that is formed in an equilibrium process in solution".[7]

The binding of a small molecule to a macromolecular target is in general considered as an equilibrium process and leads to the formation of a complex:

$$L + P \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL \tag{1}$$

where *L* is the small molecule (often called ligand or binder), *P* is the macromolecular target and *PL* is the resulting molecular complex. The dissociation rate constant k_{off} is inversely proportional to the lifetime τ_B of the ligand-target complex. The association rate constant k_{on} can be considered to be an estimate of the probability of a productive encounter between protein and ligand. k_{on} is often assumed to be diffusion-limited and consequently values varying between 10^7 and 10^9 $M^{-1}s^{-1}$ are assigned to it. Of course, this approximation does not take into account the potential complexity of intermolecular forces that may attract or repel protein and ligand.

The binding affinity can be described by the temperature-dependent equilibrium dissociation constant:

$$K_D = \frac{[L][P]}{[PL]} = \frac{k_{off}}{k_{on}} \tag{2}$$

where [*L*], [*P*] and [*PL*] are the concentrations at equilibrium of ligand, protein and ligand-protein complex, respectively.

Combining equation 2 with the definitions of some known experimental parameters, as the total ligand concentration $[L]_{tot} = [L] + [PL]$ and the total target concentration $[P]_{tot} = [P] + [PL]$, leads to:

$$[PL] = \frac{[P]_{tot} + [L]_{tot} + K_D - \sqrt{([P]_{tot} + [L]_{tot} + K_D)^2 - 4[P]_{tot}[L]_{tot}}}{2} \tag{3}$$

Figure 1 shows the trend of the bound target fraction $p_B^P = [PL]/[P]_{tot}$ as a function of the total ligand concentration $[L]_{tot}$, for different values of K_D. In general, increasing $[L]$ leads to an increase of p_B^P. When $[L] \ll K_D$, p_B^P is proportional to $[L]$; When $[L] = K_D$, 50% of the protein is saturated; When $[L] \gg K_D$, the protein tends to be completely saturated.
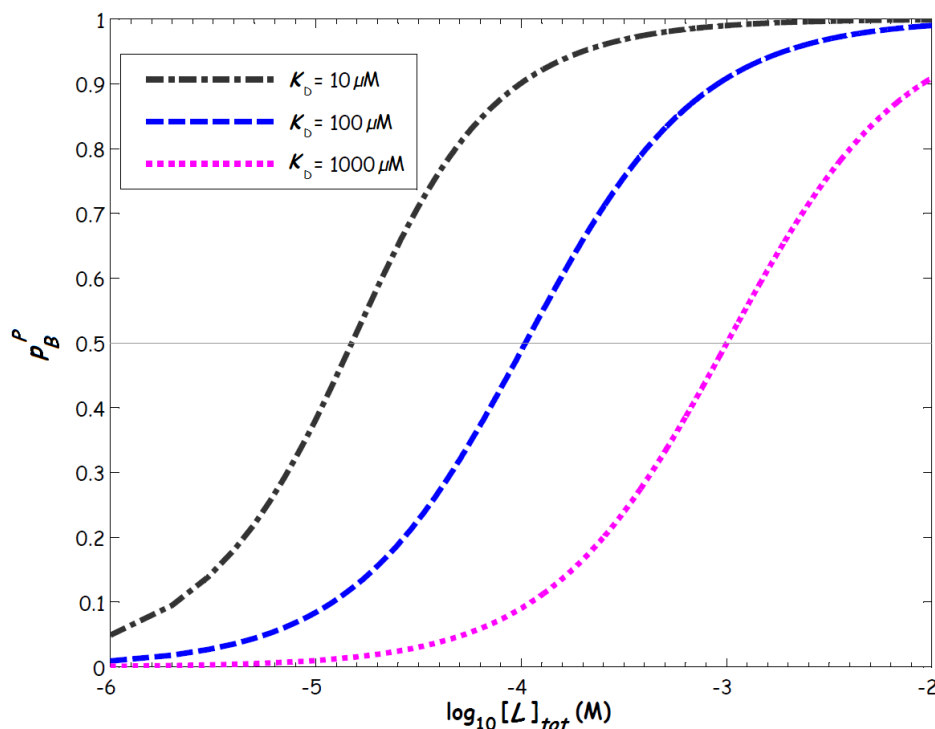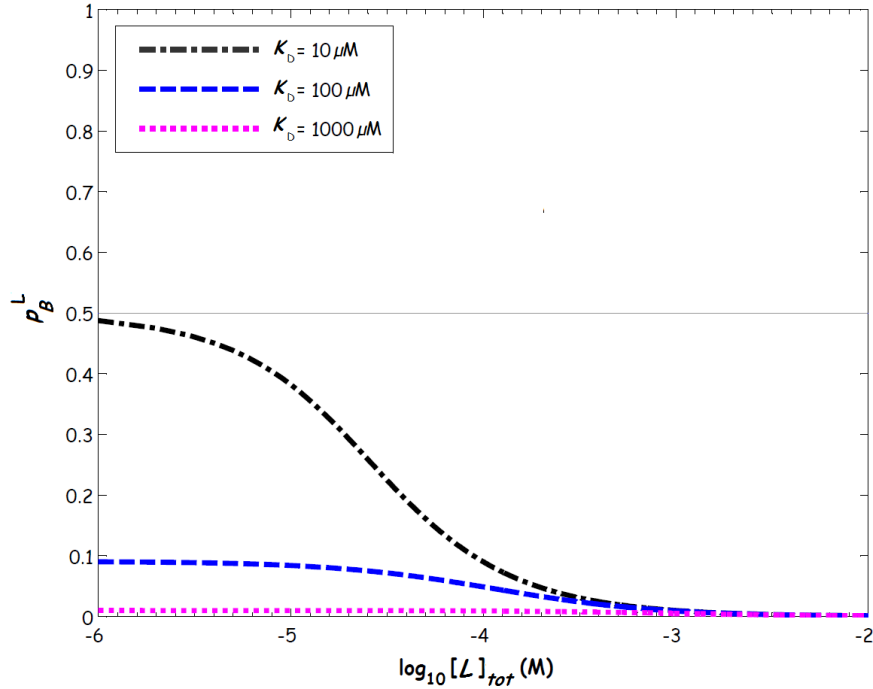

Figure 1 Bound protein fraction $[PL]/[P]_{tot}$ as a function of the ligand concentration $[L]_{tot}$. The protein concentration used in the calculation is $[P]_{tot} = 10\ \mu M$.

Ligands of weaker affinity have larger K_D and as consequence require more ligand to reach the same value of p_B^P. A value of K_D in the mM range gives rise to a 1:1'000 ratio of free to bound states in a equimolar mixture of P and L, while a K_D in the µM range implies a 1:1'000'000 ratio of these states, *i.e.*, a more stable ligand-protein complex with less free species present.

Figure 2 shows the trend of the bound ligand fraction $p_B^L = [PL]/[L]_{tot}$ as a function of the total ligand concentration $[L]_{tot}$. p_B^L can assume values in the range $0 \leq p_B^L \leq 1/\varepsilon$ [8],

where $\varepsilon = [L]_{tot}/[P]_{tot}$ is the ligand-to-protein ratio. The highest value is reached for low values of $[L]_{tot}$. The protein saturation condition occurs for high ligand-to-protein ratios, but in this case p_B^L tends to zero, because of the ligand is in excess compared to the target.

Figure 2 Bound ligand fraction $[PL]/[L]_{tot}$ as a function of the ligand concentration $[L]_{tot}$. The protein concentration used in the calculation is $[P]_{tot} = 10 \, \mu M$.

2.1.1 Dissociation constants in competitive binding equilibria

The situation described so far is the simplest case. Often, different species compete for the same binding site of the protein. In this case, the equilibria of the system can be described in the following terms:

$$PL \underset{k_{off}^L}{\overset{k_{on}^L}{\rightleftharpoons}} L + P + I \underset{k_{off}^I}{\overset{k_{on}^I}{\rightleftharpoons}} PI \tag{4}$$

where *L* and *I* are two competing ligands. Addition of *I* reduces $[PL]$; in fact, *I* competes with *L*, hampering the interactions of the latter with the protein.

Figure 3 shows the bound ligand fraction p_B^L as a function of the total competitor concentration $[I]_{tot}$ for different dissociation constants of the competitor. p_B^L decreases rapidly with increasing concentration and/or binding affinity of the competing ligand *I*. As consequence, p_B^L can be seen as a "marker" that indicates the presence of a second, competing molecule *I*. From now on, the ligand *L* will be called *spy molecule* or *reporter*, while ligand *I* will be dubbed *competitor*.

The K_D of the spy molecule determined in the presence of a competitor is called apparent dissociation constant, $K_{D,L}^{app}$ and contains information about the affinity of the competitor for the protein. The relationship between the two values is the following:

$$K_{D,I} = \frac{[I]_{tot} K_{D,L}}{K_{D,L}^{app} - K_{D,L}} \tag{5}$$

where $K_{D,L}$ is the true dissociation constant of the spy molecule and $K_{D,I}$ is the dissociation constant of the competitor. The denominator has to be greater than zero, hence $K_{D,L}^{app} > K_{D,L}$ and consequently the bound fraction of the spy molecule *L* in presence of the competitor *I* is lower than in its absence.
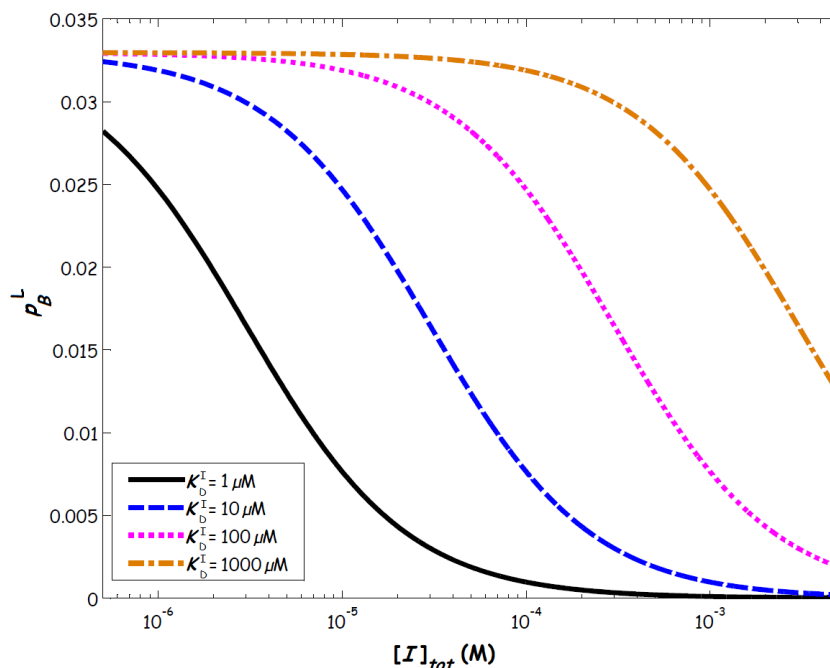
Figure 3 Bound ligand fraction $[PL]/[L]_{tot}$ as a function of the competitor concentration $[I]_{tot}$, for different values of the dissociation constant of the competitor. The concentrations of protein *P* and ligand *L* used in the calculation are 10 µM and 200 µM respectively.

2.2 Effect of binding on NMR parameters

I f a nucleus that "jumps" between two magnetically non-equivalent sites is observed, the appearance of the observed signal depends on the rate of the exchange process. For example, it is possible to consider the following complex formation equilibrium:

$$A \rightleftharpoons AB \tag{6}$$

where A and AB are two distinct environments or sites. Let's define ν_A and ν_{AB} as the Larmor frequencies of the nucleus in sites A and AB, respectively. If the system is characterized in a coordinate system that is rotating at a frequency ν_0, defined as the average of ν_A and ν_{AB},

$$\nu_0 = \frac{1}{2} \left(\nu_A + \nu_{AB} \right) \tag{7}$$

the nuclei in the two sites precess in opposite directions: nuclei in site A precess at frequency $(\nu_0 - \nu_A)$, while nuclei in site AB precess at frequency $(\nu_{AB} - \nu_0)$. Different cases are possible:

1) Slow exchange. This means that a nucleus in site A precesses many times before it leaves that site. The same happens for a nucleus in site AB. There is plenty of time for absorption of energy from the radiofrequency field B_1 and two distinct resonance peaks will appear at ν_A and ν_{AB} in the NMR spectrum;
2) Intermediate exchange. The resonance peaks tend to become broader. Indeed $(\delta E)(\delta t) \approx h$, where δE and δt are uncertainties associated with energy and time measurements and h is the Planck constant. Defining $\delta E = h\delta\nu$ and identifying δt with the state lifetimes τ, it results $\delta\nu \approx 1/(\tau)$: this means that the width of the peaks $\delta\nu$ increases as the state lifetimes decrease.
3) Fast exchange. A nucleus in site A does not have enough time to precess many times before it leaves that site. The same happens for a nucleus in site AB. From the point of view of the rotating frame, the nuclei are essentially stationary. As consequence, a single resonance peak will appear at $\nu_0 = \frac{1}{2} \left(\nu_A + \nu_{AB} \right)$.

A binding event such as described in equation 1 represents a two-state equilibrium for both protein P and ligand L. Both species, in fact, exist either in a free (P, L) or bound state (PL). The binding affinity drives the ligand and protein through an exchange process between their free and complexed forms. In this situation, the ligand transiently affects the NMR parameters characteristic of the protein and perturbs the chemical environment of the binding site. In other words, the exchange process promoted by the mutual binding affinity of ligand and protein modulates the NMR parameter of the two species.

A complete analysis of the influence of chemical exchange on the NMR parameters for arbitrary exchange timescales would require the use of Hahn's, Maxwell's and McConnell's equations.[9, 10] However, in most NMR screening experiments, the systems are in fast exchange and the situation is greatly simplified. Indeed, these kinds of experiments are typically performed with a large excess of ligand with respect to the protein ($[L]_{tot}/[P]_{tot} > 10$) and the ligand is weak, *i.e.*, $K_D \geq 100\ \mu M$. As discussed in paragraph 2.1, k_{on} is often approximated by the diffusion-limited rate (10^7-10^9 M^{-1}s^{-1}) [8], so the slowest reasonable values of the exchange rate $k_{ex} = (k_{on}[P] + k_{off})$ are in the range $10^3 < k_{ex} < 10^5\ s^{-1}$. These values exceed most differences in rotating frame precession frequencies, thus validating the fast exchange assumption.

Under the fast-exchange regime, the observed NMR parameters ε_{obs} can be defined as simple averages

$$\varepsilon_{obs} = p_B \varepsilon_B + p_F \varepsilon_F \tag{8}$$

$$\varepsilon_{obs} = p_B \varepsilon_B + p_F \varepsilon_F + \varepsilon_{ex} \tag{9}$$

where ε_B and ε_F are the values of the NMR parameter ε (e.g., a relaxation rate, a chemical shift, etc.) in the bound and free forms, respectively. Equation 9, which contains an additional term ε_{ex}, applies to parameters for which chemical shift modulations can give relevant contributions, for instance the transverse relaxation rate R_2.

Observation of differences between ε_{obs} and ε_F allows the detection of ligand binding. Equation 8 shows that the ability to detect binding depends on the magnitude of the term $p_B \varepsilon_B$ compared to $p_F \varepsilon_F$. Unfortunately, under typical conditions of screening experiments ($[L]_{tot} \gg [P]_{tot}$), $p_B \ll p_F$. For this reason it is most convenient to choose for NMR parameters which are amplified in the bound state, *i.e.*, with $\varepsilon_B \gg \varepsilon_F$.

Since $p_F = (1 - p_B)$ and $p_B = p_B^P/\epsilon$, it is possible to write

$$\epsilon(\varepsilon_{obs} - \varepsilon_F) = \frac{(\varepsilon_B - \varepsilon_F)[L]_{tot}}{[L]_{tot} + K_D} \tag{10}$$

Equation 10 shows that $\epsilon(\varepsilon_{obs} - \varepsilon_F)$ (where $\varepsilon = [L]_{tot}/[P]_{tot}$ is the ligand-to-protein ratio) increases with ligand addition and reaches a plateau at $(\varepsilon_B - \varepsilon_F)$, when $[L]_{tot} \gg K_D$, *i.e.*, when the binding site is saturated.

2.3 Ligand-Based and Protein-Based Screening

NMR offers a rich source of parameters that are sensitive to the changes in physical properties associated with binding and differ significantly between the free and bound states. As consequence, a great variety of NMR methods have been developed to perform screening experiments.

The NMR methods used to detect the binding of small molecules to macromolecular targets fall into two categories: the ones detecting changes in the parameters of the ligand are defined as *ligand-based* techniques, while the ones detecting changes in the properties of the protein are defined as *protein-based* techniques. Both approaches are routinely used and present advantages and limitations.

Protein-based NMR methods consist in the identification of perturbations of assigned protein resonances due to binding events. Therefore, this approach gives direct information about the binding site and allows the discrimination between specific and nonspecific binding. Moreover, it does not rely on fast exchange to retrieve information about the bound state, thus allowing the detection of ligands with K_D values from nM to mM. On the other hand, the direct observation of the protein usually requires the experiments to be performed with isotopically-enriched targets at rather high concentrations. Furthermore, problems of signal overlap and fast transverse relaxation rate are obviously correlated with the molecular mass of the target and impose severe limits on the molecular mass; generally, this approach is applied for proteins with molecular masses smaller than 40 KDa.[6]

Ligand-based NMR methods consist in observing a change of an NMR parameter of the ligand upon binding. They require only small amounts of protein and do not suffer from any limitations in molecular mass. Since the ligand concentration is usually high and the detection is based on the observation of nuclei with high gyromagnetic ratios (such as ^{1}H or ^{19}F), there is no need for isotopic labeling. On the other hand, observation of the ligand fails to give any information about the binding site; moreover, detection of binding is limited to weakly interacting molecules in the fast exchange regime, since the approach relies on

the exchange-mediated transfer of information from the bound state to the free state. Displacement of spy molecules by stronger competitors may allow one to circumvent the latter limitation and to discriminate between specific and nonspecific interactions.

The elaboration of different strategies to study ligand-protein interactions has followed progress in technology. At the beginning there were not many choices. In the mid-1970s, the only way to perform these kinds of studies was by means of R_1 or R_2 relaxation rates. With the 100 MHz spectrometers available at that time, there was little hope to resolve any protein signals. So the earliest NMR studies in this field were based on the observation of the ligands. With the increase of the available magnetic fields and the availability of pure and isotopically enriched proteins, direct protein-observed studies have started to be performed from the mid-1980s to present times. During the last two decades, the development of a series of new ligand-observed methods (mainly based on magnetization transfer effects) has lead to a renaissance of ligand-observed experiments. The limitations of protein-based approaches confine the number of targets to which the technique is applicable. Many new and interesting targets are too large, express too poorly or are too unstable to be suitable for this approach. As a consequence, ligand-based methods are nowadays more often used in pharmaceutical industry.

2.4 Protein-Based Methods

f the protein of interest is amenable to direct studies by NMR (*i.e.* if it is stable in solution and if it can be expressed in relatively large amount), protein-based methods can provide a unique set of information. In particular, if the structure of the protein has been studied and the assignment of its resonances is available, an atomic scale resolution of the ligand-binding site is obtained directly from screening experiments. So far, this approach has been mainly used for the detection of ligands that bind to proteins.

These experiments are based on the observation of chemical shift perturbations (CSPs) in ^{15}N-^{1}H and/or ^{13}C-^{1}H correlation spectra of the target protein in the presence of a ligand or a mixture of up to 50 ligands. In common experiments, a ^{15}N-labeled protein sample (at a concentration between 10 and 100 µM) is tested against a set of compounds by measuring ^{15}N-^{1}H TROSY-type or HSQC-type experiments. CSPs are considered significant if they are greater than 0.1 ppm for at least two peaks of the spectrum.[11] The chemical shift perturbations $\Delta\delta$ are defined as:

$$\Delta\delta = \sqrt{\left[\left(\delta\left(\ ^1H, ppm\right)_{free} - \delta\left(\ ^1H, ppm\right)_{obs}\right)^2 + 0.04\left(\delta\left(^{15}N, ppm\right)_{free} - \delta\left(^{15}N, ppm\right)_{obs}\right)^2\right]} \quad (11)$$
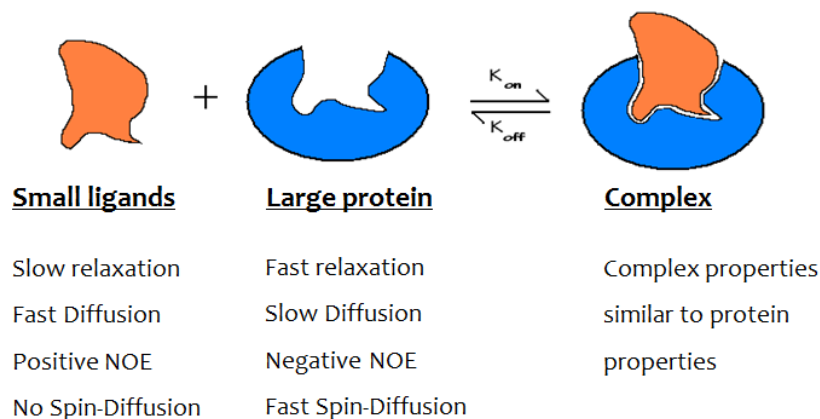
Once a binder is identified, CSPs can be monitored as a function of ligand concentration during a titration in order to determine the dissociation constant.[12]

This approach is usually limited to proteins with molecular masses lower than 30 kDa, but it can be extended to larger systems by performing ^{13}C-^1H HMQC with selectively labeled proteins. ^{13}C-labeling of methyl-containing amino acids such as methionine, isoleucine, leucine, alanine or valine is the most common strategy.[13]

The importance of providing information about the binding site has been firstly demonstrated in a landmark work by Fesik and co-workers.[1] Starting from the hypothesis that the binding energy of a ligand can be described as a sum of all interactions [14], it has been demonstrated that different fragments, identified as weak inhibitors in their own right, can be combined to drastically improve the potency of the resulting molecule. These structure-activity relationship studies are called "SAR by NMR". In the above-mentioned work, this approach was validated by the successful design of potent inhibitors of FKBP and stromelysin. The strategy consists in screening fragments in order to find molecules that bind the protein at two distinct sites that are close enough on the protein surface. Structural information obtained using intermolecular NOE data are then exploited in order to design a chemical linker that does not modify the binding mode of the two moieties. In this way it is possible to efficiently combine weak fragments with $K_D's$ in the millimolar range to get an inhibitor with a dissociation constant in the nanomolar range.

2.5 Ligand-Based Methods

Ligand-based methods are based on the dependence of different NMR parameters on the tumbling regime of the molecule studied. Molecules with $\tau_c\omega_0 \ll 1$, where τ_c is the correlation time and ω_0 the Larmor frequency, have relatively long relaxation times, large translational diffusion coefficients and positive NOEs. If such molecules bind to a slow tumbling molecule, so that $\tau_c\omega_0 \gg 1$, their behavior will change: they will assume the properties of the slow tumbling molecule, thus having shorter relaxation times, small translational diffusion coefficients and negative NOEs.

Small ligands	Large protein	Complex
Slow relaxation	Fast relaxation	Complex properties
Fast Diffusion	Slow Diffusion	similar to protein
Positive NOE	Negative NOE	properties
No Spin-Diffusion	Fast Spin-Diffusion	

Figure 4 Dependence of different NMR parameters on the molecular tumbling regime. Ligand-based methods are based on the fact that small, fast tumbling molecules assume the properties of large, slowly tumbling macromolecules when they bind to them.

As discussed in paragraph 1.2.1, compounds used in NMR screening have masses that are typically below 500 Da and thus $\tau_c \omega_0 \ll 1$, while targets have usually masses greater than 10 KDa so that $\tau_c \omega_0 \gg 1$. As consequence, small molecules which bind to a macromolecular target can be distinguished from those that do not by observing an NMR parameter that is sensitive to the correlation time τ_c. Unlike protein-based methods, high molecular masses of the protein are an advantage for the sensitivity of ligand-based methods.

Alternatively, one can exploit magnetization transfer pathways between the ligand and the protein, which obviously exist only in the bound state. These can be either intramolecular ligand effects, as in the case of transferred NOEs, or transfer between protein and ligand after the selective excitation of one species, as in the case of Saturation Transfer Difference (STD).

In a ligand-protein binding equilibrium, the properties mentioned above are dominated by the effects of the bound population, with only a small contribution from the free population. Therefore, fast exchange is needed in order to transfer information from the bound state to the free state.

A general consideration, valid for all relaxation parameters, needs to be made before we examine different experiments in more detail. The main relaxation mechanisms in ^{1}H NMR are ^{1}H-^{1}H dipole-dipole (DD) interactions between pairs of proton spins. The DD relaxation rate of a proton can be written as a double sum

$$R(V) = \sum_m a_m \sum_j \frac{1}{r_j^6} J_j(m\omega) \tag{12}$$

where the inner sum runs over all protons j that have dipolar interactions with the proton under investigation, while the outer sum represents a linear combination of the spectral density functions $J_j(m\omega)$ evaluated at different multiples m of the Larmor frequency ω. The coefficients a_m depend on the spin order V (e.g., I_z, I_x, multiple quantum coherences, etc.) and on the rate constant considered (e.g. R_1, R_2, etc.). Each spectral density $J_j(\omega)$ can be defined as

$$J(\omega) = \frac{2}{5}\frac{\tau_c}{1 + (\omega\tau_c)^2} \tag{13}$$

The spectral density function $J(\omega)$ at $\omega = 0$ is

$$J(0) = \frac{2}{5}\tau_c \tag{14}$$

Relaxation rates that strongly depend on $J(0)$ are highly sensitive probes for binding, since τ_c increases drastically upon binding, thus leading to an amplification of the corresponding relaxation rate.

The following paragraphs will explore different commonly used methods, starting from relaxation-filtered experiments and concluding with magnetization transfer-based experiments.

2.5.1 Transverse Relaxation Rates

The transverse relaxation rate $R_2 = 1/T_2$ is perhaps the best-established NMR assay. In fact, the strong dependence of R_2 on the overall molecular rotational correlation time τ_c causes the difference between its values in the free and the bound form to be particularly large. This is due to the strong dependence on $J(0)$. Indeed R_2 of a proton under ^{1}H-^{1}H DD relaxation can be defined as

$$R_2 = \frac{\hbar^2\gamma_H^4}{8}\sum_{j=1}^{N}\frac{1}{r_j^6}\left\{5J_j(0) + 9J_j(\omega_H) + 6J_j(2\omega_H)\right\} \tag{15}$$

As a result, when the ligand is bound to the protein, one has $R_{2,bound} \gg R_{2,free}$, since $\tau_{c,complex} \gg \tau_{c,free\ ligand}$. Fast exchange allows the transfer of information from the bound to the free state, so that the observed transverse relaxation rate $R_{2,obs}$ can be defined as in equation 9:

$$R_{2,obs} = p_B R_{2,bound} + p_F R_{2,free} + R_{ex} \tag{16}$$

where R_{ex} is

$$R_{ex} = (\Omega_F - \Omega_B)^2 \frac{p_F p_B}{k_{ex}} \tag{17}$$

In order to detect binding, $R_{2,obs}$ obviously needs to be different from $R_{2,free}$. This means that $p_B R_{2,bound}$ needs to be significant relative to $p_F R_{2,free}$. However, under conditions typical for ligand-based methods, the ligand is present in large excess ($[L]_{tot}/[P]_{tot} \gg 1$), so that p_B is much smaller than p_F. Nevertheless, thanks to its $J(0)$ dependence, $R_{2,bound} \gg R_{2,free}$. As consequence, $p_B R_{2,bound}$ can be significant despite the large excess of the ligand.

The rate R_{ex} expresses the line broadening due to the difference between the chemical shifts of the free and bound states. In some circumstances, R_{ex} may be very large, so that effects of binding on $R_{2,obs}$ can be observed even if $p_B R_{2,bound}$ is not significant. In particular, R_{ex} may be large if k_{ex} is small and/or if the difference $(\Omega_F - \Omega_B)$ between the chemical shifts of the free and bound forms is large. The latter case is common in ^{19}F NMR, due to the large range of chemical shifts of fluorine.

The linewidth of a resonance is proportional to R_2. If one neglects contributions due to field inhomogeneities, etc., the linewidth in Hz can be defined as $LW = R_2/\pi$. As consequence, binding events can in principle be detected by comparing the linewidths of a small molecule in the presence and absence of a protein. In practice, if the effect is small or if spectral crowding hampers direct comparison of the two spectra, direct observation of line broadening can be difficult.

Alternatively, it is possible to use R_2 experiments designed to observe differences in transverse relaxation behavior in the presence and absence of a protein.[15] Typically, R_2 relaxation can be monitored using Carr-Purcell-Meiboom-Gill (CPMG) pulse trains [16, 17] or continuous-wave irradiation as in $R_{1\rho}$ spin-lock methods.[18] In such experiments,

2.5.1 Transverse Relaxation Rates

the presence of a radiofrequency irradiation during the transverse relaxation period leads to different expressions for R_{ex}:

$$R_{ex}^{CPMG} = \frac{p_F p_B (\Omega_F - \Omega_B)^2}{k_{ex}} \left(1 - \frac{2\tanh(k_{ex}/t_{cp})}{k_{ex} t_{cp}} \right) \tag{18}$$

$$R_{ex}^{1\rho} = \frac{p_F p_B (\Omega_F - \Omega_B)^2 (\sin \Theta_{rf})^2}{k_{ex}} \left(\frac{k_{ex}}{(k_{ex})^2 + (\Omega_{SL})^2} \right) \tag{19}$$

where t_{cp} is the delay between two consecutive 180⁰ pulses and Ω_{SL} is the carrier frequency of the spin lock. In $R_{1\rho}$ experiments, on-resonance spin locking correspond to the spin magnetization locked by the RF field along the x- or y-axis, so that $R_{1\rho} \approx R_2$. In both cases, comparison of R_2-filtered experiments acquired with and without protein reveals binding through one or several resonances that have been attenuated because of an increase of R_2 upon binding.

2.5.2 Paramagnetic relaxation enhancement

A variation of relaxation filtering is the approach called SLAPSTIC (spin labels attached to protein side chains as a tool to identify interacting compounds).[19] This method involves the use of a spin label, such as 2,2,6,6-tetramethyl-1-piperidine-1-oxyl (TEMPO), which is covalently attached to selected protein side chains. Ligands that bind in the proximity of the spin label will relax more rapidly, because of the electron-proton DD interaction with the unpaired electron of the radical.
The observed transverse relaxation rate $R_{2,obs}$ can be written as follows:

$$R_{2,obs} = p_B R_{2,para} + p_B R_{2,0B} + p_F R_{2,0F} + R_{ex} \tag{20}$$

where $R_{2,para}$ is

$$R_{2,para} = \frac{\hbar^2 \gamma_e^2 \gamma_H^2}{8} \sum_{j=1}^{N} \frac{1}{r_j^6} \{ 4J_j(0) + 3J_j(\omega_H) \} \tag{21}$$

The j sum runs over all N spin labels in the proximity of the proton under consideration. The electron gyromagnetic ratio γ_e is about 660 times larger than γ_H. As consequence,

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electron-proton DD interactions can give a huge contribution to relaxation. The large $R_{2,para}$ in the bound state assures that the contribution of the bound form to the $R_{2,obs}$ is significant, since $p_B R_{2,B} \gg p_F R_{2,F}$ (where $R_{2,B}$ is the sum of the contributions of the paramagnetic relaxation rate $R_{2,para}$ and of $R_{2,0B}$ due to all other relaxation mechanisms). As consequence, small fractions p_B of bound ligands can be used. In some circumstances, the use of spin labels can lead to the reduction on the order of 50 times of the required protein concentration.[19]

For this approach to be successful, the target of interest should have amino acids that are amenable to spin labeling (for instance lysine, tyrosine, cysteine, histidine, and methionine) in the vicinity of the binding site. Moreover, target tailoring should not significantly modify the structure and the flexibility of the macromolecule, since the accidental occlusion of the site of interest would preclude binding. As consequence, prior knowledge of the 3D structure of the protein is required.

2.5.3 Longitudinal relaxation rates

The longitudinal relaxation rate $R_1 = 1/T_1$ is another parameter that is sensitive to the correlation time. When a small molecule is interacting with a macromolecular protein, the observed longitudinal relaxation rate $R_{1,obs}$ can be defined as

$$R_{1,obs} = p_B R_{1,bound} + p_F R_{1,free} \tag{22}$$

where $R_{1,bound}$ and $R_{1,free}$ are the longitudinal relaxation rates in the bound and free states, respectively.

It is necessary to distinguish between non-selective relaxation rates $R_{1,ns}$ and selective relaxation rates $R_{1,s}$; the former can be measured by inverting all resonances contained in a spectrum, while the latter is determined by selectively inverting only one resonance. The corresponding relaxation rates due to dipolar interactions between a proton *i* under investigation and neighboring protons *j* can be defined in terms of spectral densities [20]:

$$R_{1,ns} = \frac{\hbar^2 \gamma_H^4}{10} \sum_{j=1}^{N} \frac{1}{r_j^6} \left\{ \frac{3\tau_c}{1+\omega^2\tau_c^2} + \frac{12\tau_c}{1+4\omega^2\tau_c^2} \right\} \tag{23}$$

2.5.3 Longitudinal Relaxation Rates

$$R_{1,s} = \frac{\hbar^2 \gamma_H^4}{10} \sum_{j=1}^{N} \frac{1}{r_j^6} \left\{ \frac{3\tau_c}{1+\omega^2\tau_c^2} + \frac{6\tau_c}{1+4\omega^2\tau_c^2} + \tau_c \right\} \tag{24}$$

The last term of equation 24 indicates a direct dependance of $R_{1,s}$ on $J(0)$. Figure 5 shows the selective and non-selective longitudinal and transverse relaxation rates as a function of the correlation time τ_c. One can distinguish between slow tumbling regime ($\omega\tau_c < 1$), where $R_{1,ns}$ is smaller than $R_{1,s}$, and fast tumbing regime ($\omega\tau_c > 1$), where $R_{1,ns} > R_{1,s}$. Anyway, $R_{1,ns}$ does not show a direct dependence on τ_c [21]: when a small molecule binds to a large macromolecule, its $R_{1,s}$ increase rapidly, while $R_{1,ns}$ does not.
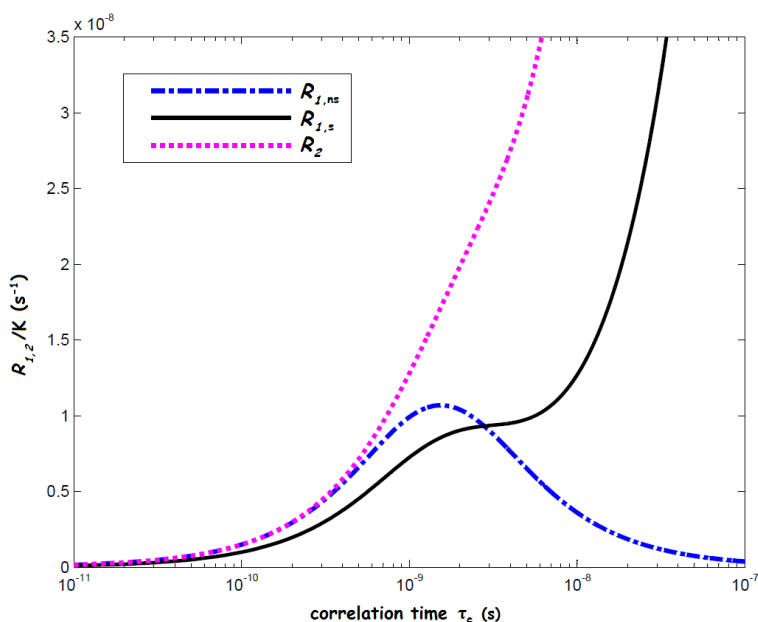
Figure 5 $R_{1,ns}$, $R_{1,s}$ and R_2 as a function of the correlation time. All rates were divided by the constant $K = \hbar^2\gamma_H^4/10 \sum_{j=1}^{N} 1/r_j^6$. These simulations were performed for a Larmor frequency $\omega = 400\,MHz$.

While testing mixtures of several compounds, it can be challenging to perturb several chosen spins selectively. Indeed, different selective inversion pulses may have to be applied for each compound. However, this difficulty can be circumvented by selectively inverting a single resonance of a suitable spy molecule in competition experiments.

2.5.4 Transverse ^{19}F relaxation

Most methods that are used for ligand-target interaction studies are based on proton detection. Nevertheless, a few methods exploit the favorable properties of fluorine NMR spectroscopy. Fluorine detection offers some unique advantages: (a) high sensitivity ($\gamma_{^{19}F}/\gamma_{^{1}H} = 0.94$) and 100% natural abundance of the ^{19}F isotope; (b) no interference of signals due to non-fluorinated solvents, buffers or detergents; (c) absence of overlap with signals of other non-fluorinated species in solution; (d) high sensitivity of the transverse relaxation rate R_2 to binding events.

Point (d) deserves to be discussed in more detail. There are two main reasons that explain the high sensitivity of fluorine R_2 to binding events: (i) the contribution of $J(0)$ for the DD interactions and for the chemical shift anisotropy (CSA) and (ii) the important contributions of exchange broadening.

Fluorine transverse relaxation R_2 is dominated by large contributions due to DD interactions with proton spins in the surrounding, and CSA. For fluorine, R_2 can be defined:

$$R_2 = R_2^{DD} + R_2^{CSA} + R_2^{others} \qquad (25)$$

where R_2^{DD} is the contribution of the DD interactions between the isolated ^{19}F nucleus and surrounding protons on the ligand (and on the protein in the complex), R_2^{CSA} is the contribution of CSA, and R_2^{others} stems from other sources. The rates R_2^{DD} and R_2^{CSA} can be defined as follows [20, 22]

$$R_2^{DD} = \frac{\gamma_F^2 \gamma_H^2 \hbar^2 \tau_c}{20} \sum_{H_i} \frac{1}{r_{FH_i}^6} \left\{ 4 + \frac{1}{1+(\omega_F-\omega_H)^2\tau_c^2} + \frac{3}{1+\omega_F^2\tau_c^2} + \frac{6}{1+\omega_H^2\tau_c^2} + \frac{6}{1+(\omega_F+\omega_H)^2\tau_c^2} \right\} \qquad (26)$$

$$R_2^{CSA} = \frac{2}{15} \Delta\sigma^2 \left(1 + \frac{\eta_{CSA}^2}{3}\right) \omega_F^2 \tau_c \left\{ \frac{2}{3} + \frac{1}{2(1+\omega_F^2\tau_c^2)} \right\} \qquad (27)$$

where $\Delta\sigma$ is the CSA of the ^{19}F nucleus which can be defined as $\Delta\sigma = \sigma_{zz} - (\sigma_{xx}+\sigma_{yy})/2$, where σ_{xx}, σ_{yy} and σ_{zz} are the principal components of the chemical shift tensor. The asymmetry parameter is defined as $\eta_{CSA} = (3/2)(\sigma_{xx}-\sigma_{yy})/\Delta\sigma$.

The trends of the dipolar and CSA contributions to R_2 as a function of the correlation time τ_c are shown in figures 6 and 7, respectively. Both of these contributions are monotonic
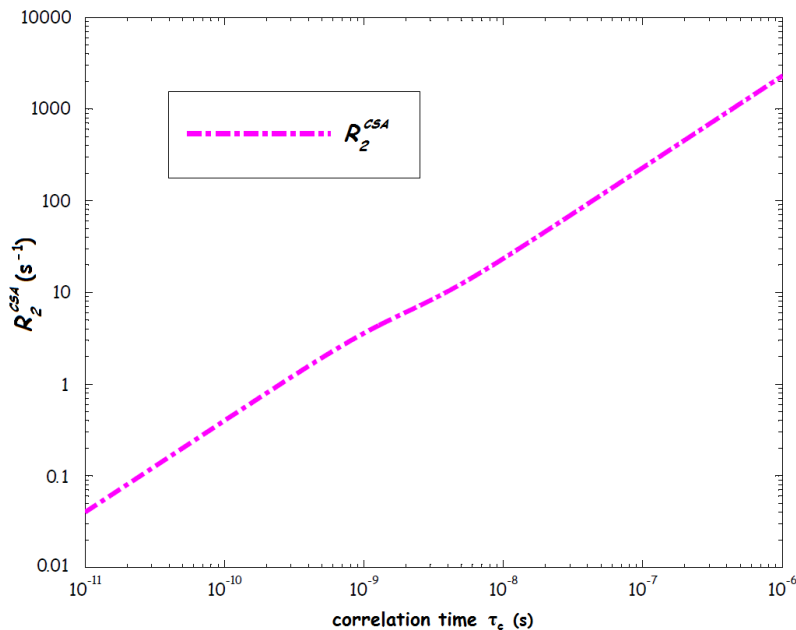
2.5.4 Transverse ^{19}F relaxation

functions of τ_c and can be used for screening small molecules that interact with a protein. The relaxation of the bound state is further boosted by additional intermolecular dipolar interactions between the ^{19}F under investigation and protons of the macromolecular target.

Proteins that have been selectively labeled with fluorinated amino acids have been studied by ^{19}F NMR since the 1970s. However, the initial results were disappointing: large CSA effects lead to extensive line broadening of the ^{19}F NMR spectra [23]. This fact is well described by figure 7; the slower the molecular tumbling, the larger the CSA contribution to the transverse relaxation. Despite the negative consequences for the quality of the ^{19}F spectrum of the protein, this effect turns out to be useful for detecting binding events [24, 25]. Because of the strong dependence of CSA contributions on the correlation time, large differences occur between the transverse relaxation rates of the free and bound states, allowing one to detect very small fractions of ligands in the bound state.
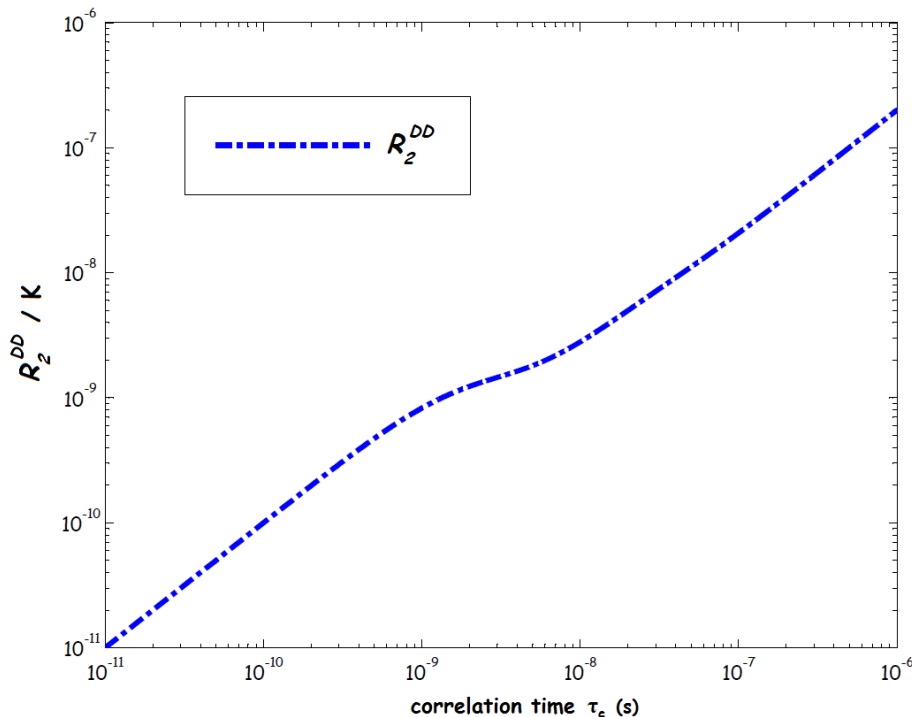
Figure 6 R_2^{DD} of ^{19}F as a function of the correlation time. The rates were divided by the constant $K = \hbar^2 \gamma_H^2 \gamma_F^2 / r_{FH_i}^6$. These simulations were performed for fluorine and proton Larmor frequencies 376 and 400 MHz, respectively.

Figure 7 R_2^{CSA} of ^{19}F as a function of the correlation time. The simulations were performed for a fluorine Larmor frequency 376 MHz. Typical CSA values of fluorobenzene were used ($\Delta\sigma = 71.5$ ppm and $\eta_{CSA} = -1.32$).

The transverse relaxation rates R_2 of ^{19}F are also extremely sensitive to chemical exchange. If the relaxation rate behavior is studied through a CPMG experiment, the observed transverse relaxation rate can be expressed as:

$$R_{2,obs} = p_B R_{2,bound} + p_F R_{2,free} + p_B p_F^2 \tau_{res} 4\pi^2 (\delta_f - \delta_b)^2 \left\{ 1 - \left[\frac{p_F \tanh(\frac{\tau}{p_F \tau_{res}})}{\frac{\tau}{\tau_{res}}} \right] \right\} \tag{28}$$

where δ_f and δ_b are the chemical shifts of the spin under investigation in the free and bound state, respectively, while $\tau_{res} = 1/k_{off}$ is the residence time of the ligand in the bound state and τ is the spin-echo delay in the CPMG scheme. Simulations show that the exchange term of equation 28 reaches its maximum value of $R_{ex} = p_B p_F^2 \tau_{res} 4\pi^2 (\delta_f - \delta_b)^2$ for $2\tau \gg \tau_{res}$, with the 80 % of the maximum value reached with $\tau \approx 5\,\tau_{res}$.[26] Indeed, if 2τ is smaller than the residence time, the dephasing resulting from the exchange can be neglected. Conversely, if 2τ is larger than τ_{res}, the ligand exchanges many times between free and bound state and this will lead to maximum dephasing. This effect is evident in figure 8. The four intensities represented in the picture are the result of four ^{19}F CPMG experiments with a constant total relaxation period of 80 ms, with two different τ

values (left and right). In absence of protein, different spin-echo delays do not lead to different intensities (bottom part); conversely, if exchange events occur, the exchange contribution described above leads to a significant difference of intensities between the two experiments.
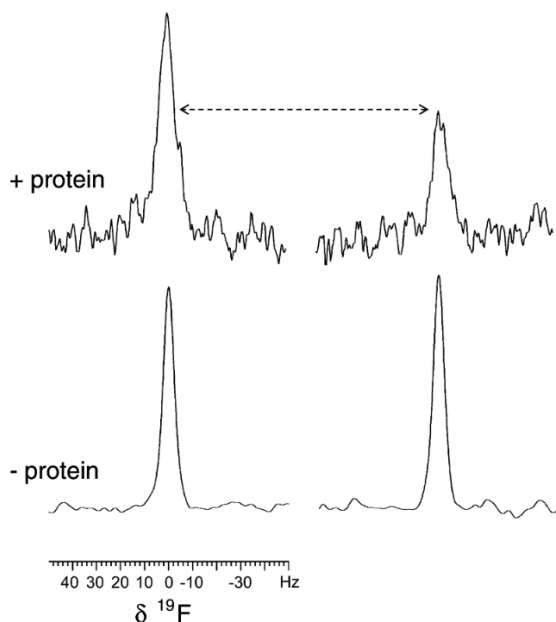
Figure 8 ^{19}F CPMG spectra of a CF$_3$ containing ligand in the presence (top) and absence (bottom) of a protein, recorded with two different τ values (200 μs on the left, 10 ms on the right). The difference in intensities between experiments performed with different τ values is noticeable only in presence of the protein, when the exchange term influences the observed relaxation rate. Reproduced from [26].

2.5.5 Translational diffusion

Diffusion experiments typically use a spin echo pulse sequence with a magnetic field gradient applied during the spin-echo delays. Before the refocusing pulse, the gradient spatially encodes the nuclear spins, while the one after the refocusing pulse decodes them. Only spins that do not move during the interval between the gradients will contribute to the signal. It is possible to measure translational diffusion coefficients by incrementing either the gradient strength or its duration.

The translational diffusion rate D of a sphere of radius r in a continuous medium of viscosity η can be defined by the Stokes-Einstein equation, [27] $D = (KT)/(6\pi\eta r)$, where K is the Boltzmann constant and T is the temperature. Of course, the translational diffusion

coefficient D is closely related to the molecular size. A large molecule will diffuse slower than a small one.

In order to be able to apply the fast exchange approximation, the ligand needs to be in fast exchange not only on the time scales of the chemical shifts but also on the timescale of the diffusion measurement (typically a few hundred milliseconds) [28]. If this is the case, the observed translational diffusion coefficient D_{obs} is:

$$D_{obs} = p_B D_{bound} + p_F D_{free} \tag{29}$$

where, as usual, D_{bound} and D_{free} are the translational diffusion constants of the bound and free state, respectively.

Diffusion-based filtering was one of the first proposed ligand-based methods.[15] Despite this, it has not proved to be as generally applicable as some other methods. This is mainly due to the limited dynamic range of D, which usually differs only about one order of magnitude between free and bound states. As consequence, low ligand-protein ratios are required to achieve substantial changes of D_{obs} upon binding. Under these conditions, rapid relaxation can lead to significant line broadening; as a result, this method is usually applied to small and intermediate size proteins, which are highly soluble and available in large amounts.

2.5.6 Transferred NOEs

There is a huge difference in correlation times between small molecules and macromolecular proteins, with τ_c values in the region of 10^{-12} and 10^{-8} s, respectively.[29] Slowly tumbling molecules show strong negative NOEs, while rapidly tumbling molecules have weak positive NOEs. In the presence of a binding event, the small ligand acquires transiently the correlation time of the protein, thus showing NOE cross-peaks that change sign in 2D-NOESY.[30] Transferred NOEs are easily distinguishable from NOEs due to the ligand in the free state, since the latter have opposite sign, and are slower to build in intensity.

The transferred Nuclear Overhauser Effect Spectroscopy (trNOESY) experiment relies on relatively short mixing times (100-500 ms) to probe for binding-induced changes in *intra*-ligand magnetization transfer. Indeed, the mixing time should be long enough to allow the trNOE to build up, but short enough to have negligible NOE intensities due to the free

population. This limits the sensitivity of the method: the same binding information can be obtained from a 1D STD experiment in 0.5 h as from a 2D trNOESY experiment in 4 h.[8] Consequently, this method has been progressively supplanted by other methods as STD or waterLOGSY.

Despite this, the change in sign of trNOEs makes this method useful when other screening strategies give ambiguous results. trNOEs have proven to be extremely useful in giving information about the bound conformation to assist modeling [31, 32], when a 3D structure of the ligand-protein complex cannot be obtained.

An important variant of the method is the inter-ligand trNOE (ILNOE) [33], where the trNOE is detected between two different ligands that bind to a protein in proximal sites. This strategy can be important to extend the SAR by NMR approach to systems which are difficult to crystallize, since it gives indications about the relative proximity of the ligands involved in the generation of the trNOE effect.

2.5.7 NOE pumping

The previously described trNOE effects are based on *intra*-ligand magnetization transfer. Alternatively, one can exploit *inter*-molecular NOE pathways between ligand and protein as a signature of binding events.

In NOE pumping experiments [34], the magnetization of a small molecule is selectively saturated by a diffusion filter, while the protein magnetization remains unchanged. A subsequent mixing period allows a magnetization transfer from the protein to the ligand. An additional CPMG filter can then be used in order to filter out the residual protein signals. The method can suffer from a lack of sensitivity due to the fact that the magnetization is stored on the macromolecular protein and its short transverse relaxation times T_2 can result in rapid losses of magnetization. A solution to this problem is provided by an inverse experiment, where the magnetization is transferred from the ligand to the protein. This experiment is known as reverse NOE pumping [35] and uses a CPMG filter to saturate the protein magnetization. The magnetization stored on the ligand is then transferred to the macromolecule during a mixing period. The result is a reduction in intensity of the ligand signals, which would not occur in the absence of a macromolecular protein. In order to discriminate signal losses due to NOE effects from losses due to ligand relaxation, the experiment is performed twice: in a reference experiment, a CPMG filter is inserted after the mixing period. In this latter experiment, the protein magnetization has not been

saturated and the ligand signals do not suffer from intensity losses due to magnetization transfer to the macromolecule.

2.5.8 Saturation Transfer Difference

The idea of saturation transfer was already developed in the early 1960s.[36] This approach has been applied in the late 1990s to identify binding events between small molecules and biological targets, and has become known as Saturation Transfer Difference (STD).[37, 38] The method exploits the magnetization transfer between an irradiated protein and a bound ligand. The identification of the binding molecule is obtained through the analysis of the difference between two experiments performed with or without saturation.

In the 'on-resonance' experiment, a train of shaped pulses (typically N repetitions of 50 ms frequency-selective pulses with Gaussian or Seduce-1 profiles) saturates some resonances of the protein. To selectively saturate only the protein, the irradiation is limited to a frequency range that contains only protein resonances; usually a region shifted upfield (between 0 and -1 ppm, containing resonances of methyl groups of the protein) or downfield (9-10 ppm). Only a small subset of the protons of the protein is saturated by the pulse train, but ^{1}H-^{1}H cross-relaxation pathways rapidly transfer this saturation across the protein. After saturation, which usually goes on for a few seconds, a $\pi/2$ pulse can be followed by a CPMG filter to suppress the protein signals and by a water suppression sequence to remove solvent resonances. If a small molecule binds to the protein, some magnetization transfer will also occur at the ligand-protein interface, *via* intermolecular DD interactions between protein and ligand. This will result in the partial saturation of the ligand resonances.

In the 'off-resonance' experiment, the same experiment described above is performed, but the pulse train is applied far from the protein resonances. In this case, none of the magnetization transfer pathways described before can occur. The signals of the on-resonance experiment are then subtracted from those of the off-resonance experiment, usually by interleaving scans and using an inversion of the receiver phase on alternate scans. In the resulting difference spectrum, only resonances which experienced saturation (by direct irradiation or *via* magnetization transfer) will appear, thus identifying the interacting ligand.

The STD amplification factor STD_{af} can be defined as following:

2.5.8 Saturation Transfer Difference

$$STD_{af} = \frac{(I_0 - I_{sat})}{I_0} * \frac{[L]_{tot}}{[P]_{tot}} \tag{30}$$

where I_0 and I_{sat} are the signal intensities in the off- and on-resonance experiments, respectively. Important structural information can be obtained from a detailed analysis of the amplification factors of individual signals of the ligand. It may be possible to discern the ligand surface that is in contact with the target (epitope mapping).[39]

Titration experiments based on the STD effect allow one to determine the dissociation constant K_D of the ligand. When STD_{af} is plotted against the total ligand concentration $[L]_{tot}$, the curve can be fitted to the following equation to extrapolate K_D:

$$STD_{af} = \frac{-STD_{af}^{max}}{1 + (\frac{[L]_{tot}}{K_D})} + STD_{af}^{max} \tag{31}$$

where STD_{af}^{max} is the maximum STD_{af} effect.

The STD method has some unique advantages. First of all, the technique works particularly well with high-molecular mass drug targets, since efficient spin diffusion within the protein is needed in order to transfer the magnetization to the bound ligand. Moreover, in systems where fast exchange prevails, slow relaxation of the ligand in its free state allows one to accumulate a high concentration of saturated ligand molecules over the duration of the saturating pulse train. This permits to saturate large amounts of ligand with low concentrations of protein. As consequence, STD experiments can be performed with a 50-500-fold excess of ligand.

The NOE cross-relaxation σ_{NOE} rate between two protons depends on the correlation time τ_c:

$$\sigma_{NOE} = \frac{\gamma^4 \hbar^2}{10 r_{ij}^6} \left\{ \frac{6\tau_c}{1 + 4\omega^2 \tau_c^2} - \tau_c \right\} \tag{32}$$

In the presence of small or medium-sized proteins with a relatively small τ_c, magnetization transfer *via* DD interactions may be inefficient, so that the complete saturation of the protein may fail. Moreover, the observed STD_{af} cannot be linked directly to the affinity of a ligand: a strong binder will show weak STD signals since a small k_{off} limits the turnover of ligand molecules in the binding site. Another problem can be saturation leakage [40]

via chemical exchange with the solvent; for this reason the STD experiment is preferentially performed in D_2O rather than H_2O. Nucleic acid proteins suffer from a low proton density which limits cross-relaxation within the protein, so that the STD technique is not very useful for this kind of systems.

The epitope mapping from the analysis of STD_{af} of ligand signals can only be performed for binders with weak affinities. Indeed, if the residence time is long compared to cross-relaxation of the ligand in the bound state, the magnetization spreads over all ligand protons and leads to a uniformly small STD effect.[21]

STD technique is useful for the study of ligands with $10^{-8} < K_D < 10^{-3}$ M.[37] For weaker ligands and if $[L]_{tot} < K_D$, less than half of the protein molecules will be bound to a ligand. This means that the saturation transfer from the protein to the ligand is inefficient. Conversely, for stronger ligands, the kinetic off-rate constant k_{off} is very small, thus diminishing the saturation of the magnetization on the ligand. In particular, if the residence time of the ligand in the bound state is long on the timescale of its R_1 in the bound state, the ligand cannot "remember" its visit to the binding site of the saturated protein and relaxes back to equilibrium.

2.5.9 WaterLOGSY

The NOE pumping and STD methods described in the previous two paragraphs rely on the selective perturbation of the magnetization of either ligand or protein and exploit the magnetization transfer between the two species to characterize the binding properties of the molecule under investigation. The so-called "water-ligand observed *via* gradient spectroscopy" (water-LOGSY) method [41] represents an elegant modification of these approaches.

WaterLOGSY does not need to create a pool of magnetization on either ligand or protein. It is based on indirect excitation of the magnetization of the ligand-protein complex and of the free ligand by selective perturbation of bulk water magnetization. The perturbation of bulk water can be achieved either by selective saturation or by selective inversion of the water signal. Nowadays, the most robust scheme to achieve efficient selective inversion of the water signal is ePHOGSY,[42, 43] which consists of a water-selective 180⁰ refocusing pulse sandwiched between two pulsed field gradients.

Different pathways can be involved in the transfer of magnetization from bulk water to either bound or free ligand molecules. Bound ligand molecules benefit from magnetization

2.5.9 WaterLOGSY

transfer from water molecules that reside in the binding site and in the hydration sphere around the ligand-protein complex. Moreover, chemical exchange between bulk water and exchangeable protons of the protein, *i.e.*, labile carboxyl, amino, hydroxyl, imidazole, guanidinium and amide protons [44, 45], pumps protein magnetization that can be transferred to the ligand. When the ligands are bound to the protein, the magnetization transfer within the ligand-protein complex occurs rapidly because of its long correlation time τ_c, generating large negative signals.

In the waterLOGSY experiment, magnetization is also transferred directly from the bulk water to the free ligand molecules. This transfer occurs *via* DD interactions between the hydration shell and the free ligand molecule, and possibly *via* chemical exchange through exchangeable protons of the ligand. In this case, the DD interactions fluctuate with short correlation times, leading to weak positive NOE's that generate small positive signals. The spectra are usually shown phased so that bound signals are positive and free signals negative. As consequence, the observed waterLOGSY signals I_{WL} result from the sum of negative signals due to free ligands that cannot bind and positive signals of ligand molecules that can bind to the protein [8]:

$$I_{WL} = C([PL]\sigma_{bound} + [L]\sigma_{free}) \tag{33}$$

Where C is a proportionality constant that accounts for the appropriate unit conversions, $[PL]$ is the concentration of the bound ligand, $[L]$ is the concentration of the free ligand, while σ_{bound} and σ_{free} are the cross-relaxation rate constants describing the transfer of magnetization from water to ligand protons in the bound and free states, respectively.

As the contribution of the free state becomes more significant, the resulting waterLOGSY spectrum of the ligand can pass through a null or be negative. For instance, if $[L]_{tot} \gg [P]_{tot}$ the negative contribution of the free state $[L]\sigma_{free}$ may overwhelm that of bound state, resulting in a false negative. For this reason, the experiment should not be carried out with large ligand/protein ratios. The reference spectrum $I_{WL,free}$ recorded in the absence of the protein can be subtracted from the spectrum I_{WL} obtained in the presence of the protein. The difference $I_{WL} - I_{WL,free}$ can be defined as [8]:

$$I_{WL} - I_{WL,free} = \frac{[P]_{tot}(\sigma_{bould} - \sigma_{free})[L]}{[L] + K_D} \tag{34}$$

Fitting the curve of $I_{WL} - I_{WL,free}$ during a titration to equation 34 and approximating $[L] \approx [L]_{tot}$ allows one to estimate K_D. The estimated lower limit of K_D that can be detected is $K_D \approx 0.1$ µM.[46] Ligands with a greater affinity have longer residence times so that the transferred magnetization is lost due to longitudinal relaxation before the ligand leaves the binding site.

WaterLOGSY can be used when STD is not applicable, as for nucleic acid proteins.[47] The bulk water can be seen as a pool of magnetization that compensates for the lack of proton DD cross-relaxation pathways in proteins with low proton density.
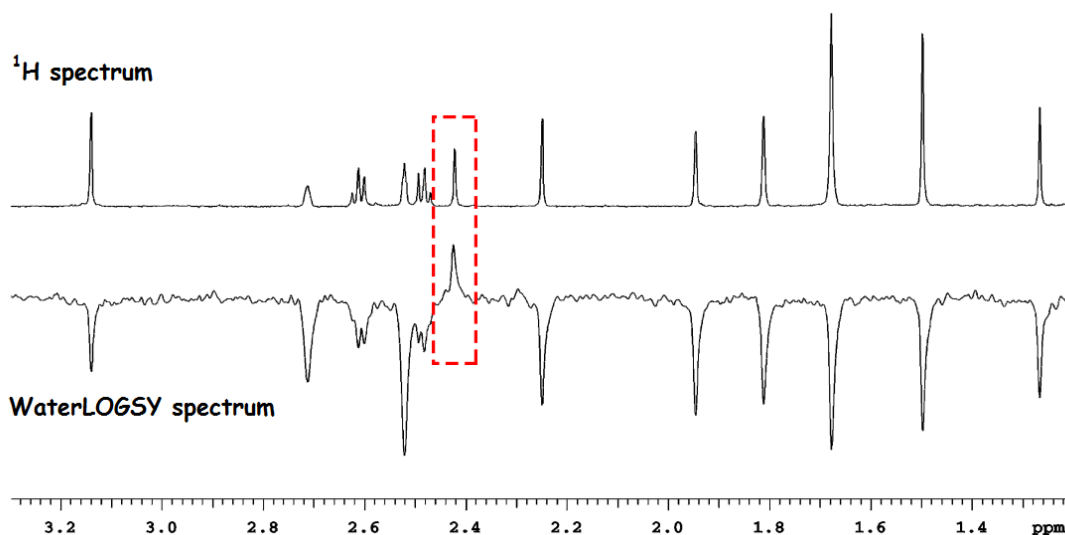
Figure 9 Identification of a ligand that binds to the protein cdk2 in a mixture of 10 compounds using waterLOGSY. Top: [1]H spectrum of the mixture in absence of protein. Bottom: waterLOGSY spectrum of the mixture in the presence of the protein. The positive waterLOGSY peak framed in a dashed red square indicates binding of the corresponding compound to the protein. Spectra reproduced from [21].

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3. Nuclear Long-Lived States

Two particles with spin $I = 1/2$ can couple to form a composite system with total spin $I = 0$ or 1. The spin $I = 0$ state can only have the magnetic quantum number $m = 0$, while the spin $I = 1$ configuration comprises three distinct energy levels ($m = -1$, 0, 1). These two categories of spin states are called respectively singlet state and triplet states.

When the two spin-1/2 particles are both electrons, there is usually a large energy difference between the singlet and triplet states, mainly due to the electron exchange interaction, which arises from the overlap of the electronic wavefunctions. In this situation, the electronic singlet and triplet states are nearly exact eigenfunctions of the Hamiltonian of the system. In many cases, an excited electronic singlet state is converted into lower-energy electronic triplet states through a process known as intersystem crossing (ISC).

If the two spin-1/2 particles are both nuclei, the situation is different. Nuclear exchange couplings are usually small, because the nuclear wavefunctions are strongly localized and there is no overlap. Since the nuclear exchange coupling usually vanishes, the singlet-triplet energy splitting is extremely small, and it is often dominated by weak symmetry-breaking interactions such as the chemical shift difference. As a consequence, the use of the language of singlet and triplet states is less widespread in NMR, compared with the fields of electronic spectroscopy or molecular quantum mechanics. However, the concepts of singlet and triplet states are very important to understand the relaxation properties of the nuclear spin systems.

The singlet and triplet states of a nuclear spin-1/2 pair can be described by the wavefunctions [1]:

$$
\begin{aligned}
|S_0\rangle &= {}^1\!/_{\sqrt{2}}\, (|\alpha\beta\rangle - |\beta\alpha\rangle) \\
|T_{-1}\rangle &= |\beta\beta\rangle \\
|T_0\rangle &= {}^1\!/_{\sqrt{2}}\, (|\alpha\beta\rangle + |\beta\alpha\rangle) \\
|T_{+1}\rangle &= |\alpha\alpha\rangle
\end{aligned}
\tag{1}
$$

where the symbols α and β refer to angular momentum components $\pm\hbar/2$ respectively, along a defined quantization axis (usually, the external magnetic field axis). If the system

is exposed to an external magnetic field, the configuration of the energy eigenstates of the spin-1/2 pair is different if the nuclear sites are magnetically equivalent or inequivalent. If the nuclear sites are magnetically equivalent, the nuclei experience identical local magnetic fields generated by the local electronic environment. In this situation, the singlet and triplet states are exact eigenstates of the Hamiltonian of the system. The three triplet states are split by the nuclear Zeeman resonance frequency, which is proportional to the applied magnetic field, as shown in figure 1.
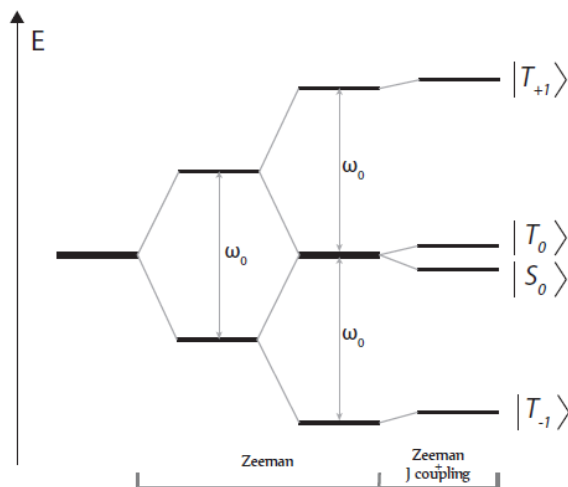
Figure 1 Energy levels for a system of two magnetically equivalent spins with $I = 1/2$.

If the nuclear sites are magnetically inequivalent, the nuclei experience local fields which can be significantly different, depending on the size of the chemical shift difference and the magnitude of the external magnetic field. If this difference is much larger than the scalar coupling between the two spins, the spin-pair is said to be weakly coupled. In this situation, the energy eigenstates of the system are close to the individual Zeeman states $|\alpha\alpha\rangle$, $|\alpha\beta\rangle$, $|\beta\alpha\rangle$ and $|\beta\beta\rangle$, as shown in figure 2.

Usually, the relaxation of nuclear spin systems is described by Redfield's equations, which involve transition probabilities between pairs of eigenstates. The classical Redfield theory of weakly-coupled spin pairs does not use the singlet and triplet states, since these are not eigenstates of a system of two inequivalent spins.

However, the use of singlet and triplet states in the Redfield formalism can show an important property: just as in electronic spectroscopy, where intersystem crossing between singlet and triplets is a slow process, nuclear singlet-triplet transitions break spin-

exchange symmetry and are therefore hindered. In addition, if the coherent singlet-triplet transitions are suppressed, the lifetime of the nuclear singlet order is often longer than the longitudinal relaxation time T_1. This fact provides the interesting possibility of storing nuclear spin order for times much longer than T_1.[2-4]
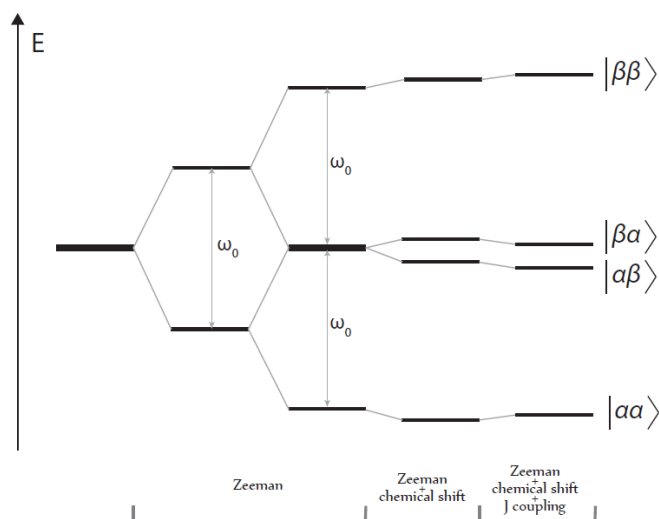
Figure 2 Energy levels for a magnetically inequivalent 2-spin-1/2 system.

However, in order to exploit these longer lifetimes, two problems must be overcome. In the case of two inequivalent spins, spin order cannot be deposited in a state that is not an eigenstate of the Hamiltonian of the system. Indeed, rapid singlet-triplet interconversion would lead to a rapid depletion of the non-equilibrium state. On the other hand, singlet states cannot be observed directly, since they have total spin zero and therefore they do not give rise to any NMR signal.

3.1 The principle of symmetry-switching

The solution to both problems outlined above is to switch the symmetry of the spin interactions at different points during the experimental procedure. When the symmetry prevails, coherent singlet-triplet transitions are quenched, so that the lifetime of the singlet state is extended. When the symmetry is broken, singlet-triplet

transitions are allowed, so that spin order to be deposited in the singlet state and be converted into detectable magnetization.

A typical singlet NMR experiment comprises three steps:

- In the excitation interval, the symmetry is broken, so that coherent singlet-triplet transitions are allowed. These coherent transitions are used to create an imbalance between the populations of the singlet and triplet states, abbreviated as TSI (triplet-singlet imbalance);

- In the storage interval, coherent singlet-triplet transitions are quenched by imposing spin-exchange symmetry. During this period, the TSI decays with a time constant T_{TSI} (also known as T_S), which may be longer than the longitudinal relaxation time T_1;

- In the detection interval, the symmetry is broken again. This permits the observation of singlet-derived NMR signals. In fact, nuclear singlet order is non-magnetic, but the broken symmetry restores coherent singlet-triplet transitions, which are used to obtain observable nuclear magnetization.

If a pair of magnetically equivalent spins is considered, symmetry is a starting feature of the system. The symmetry has to be broken both to populate the TSI and subsequently during the detection interval. Conversely, if an inequivalent two-spin system is considered, symmetry must be imposed during the storage interval.

All para-hydrogen-based methods belong to the first category and exploit the use of chemical reactions to break the symmetry of the two magnetically equivalent spins during the excitation and detection intervals.[5, 6] Dihydrogen gas is prepared in a metastable state of enriched nuclear singlet order by thermal equilibration at low temperatures (typically 40 K) in the presence of a metal catalyst, followed by separation from the catalyst and warming to ambient temperature. The symmetry of the hydrogen molecule is usually broken by a reaction in a metal complex. In this state of broken symmetry, coherent singlet-triplet transitions permit the conversion of hyperpolarized singlet order to hyperpolarized magnetization, thus leading to large NMR signals.

It has been recently demonstrated that a singlet-triplet imbalance can also be created directly in a system of magnetically equivalent spins by dynamic nuclear polarization (DNP); the resulting long-lived states have been nicknamed Hyperpolarized Equivalent Long-Lived States (HELLS).[7] The detection is performed after the symmetry has been broken, by means of enzymatic or chemical reactions. Alternatively, the TSI can be

transferred by cross-relaxation to observable, enhanced signals of protons and coupled ^{13}C spins.[8]

Alternatively, many traditional methods use a change of the magnetic field to which the nuclei are exposed to impose or remove the symmetry in a system of magnetically inequivalent spins. In field-cycling singlet NMR, a strong magnetic field is applied to break the exchange symmetry. Then, exchange symmetry is restored by removing this magnetic field and coherent singlet-triplet transitions are suppressed. To obtain detectable magnetization, radiofrequency pulse sequences can be applied at the nuclear resonance frequency.[1, 2, 9] In high-field NMR, the nuclear singlet order is maintained by using a resonant spin-locking field which suppresses effects of chemical shift differences and establishes an effective spin-exchange symmetry in the nuclear spin Hamiltonian.[1, 3, 10]

3.2 Applications

The extended lifetime of nuclear singlet states can suggest new applications of hyperpolarized NMR. In fact, they can provide a possibility to reduce losses of hyperpolarized spin order due to relaxation in the interval between the generation of hyperpolarized spin order and its use.

Dynamic nuclear polarization (DNP) consists in the doping of the sample with paramagnetic species and the application of a resonant microwave field slightly offset from the electron Larmor frequency at low temperatures. Under suitable conditions, a large polarization of nuclei builds up. It has been shown that the frozen sample can be dissolved using injection of a hot solvent and transferred to an NMR magnet, resulting in an enormous increase in signal strength.[11] Nevertheless, this dissolution method (D-DNP) has a serious limitation: hyperpolarized magnetization has a limited lifetime, which decays with the time constant T_1. Relaxation causes important losses of hyperpolarization during the warming process, the transport to the second magnet, or the transport of the hyperpolarized substance to the site of interest. The use of singlet states has enormous potential in combination with dissolution-DNP. Tayler and coworkers have shown that a TSI can be directly populated before the transfer by DNP for the two inequivalent ^{13}C spins in 1,2-$^{13}C_2$-pyruvic acid.[12] They demonstrated that a singlet-triplet population imbalance could be created directly by hyperpolarization, *i.e.,* without any extra manipulations of the

sample. However, in this experiment the magnitude of the TSI, which depends on the nuclear polarization reached, was very limited. As discussed before, alternative strategies involve the use of magnetically equivalent spin systems and particular strategies for the detection of the otherwise NMR-silent singlet order. [7, 8] However, most of the current experiments where D-DNP is combined with LLS rely on *rf* pulses sequences to prepare the LLS after the transfer of the hyperpolarized sample to the detection spectrometer.[13-15]

The extended lifetime of singlet states allows a greater interval for the transport to take place, while still maintaining the memory of the nuclear spin system. This allows the measurement of smaller diffusion coefficients or slower flow rates. Singlet states can also allow the study of slower exchange processes than is normally the case.[4, 16, 17]

The relaxation of nuclear singlet states is sensitive to the presence of nearby magnetic centers such as other magnetic nuclei and can therefore be used as a probe of molecular geometry. Its sensitivity to changes in molecular structure can be used to detect protein folding and unfolding.[18]

3.3 Long-Lived States: the principles

Nuclear magnetic resonance can give structural and dynamic information on molecules containing nuclei with a non-vanishing magnetic moment. Many studies, like the examination of slow translational diffusion, chemical exchange or folding of proteins, are limited by the longitudinal relaxation time T_1, which gives a measure of the time needed for the nuclear spins to return to thermal equilibrium. T_1 is usually regarded as the maximum lifetime of the memory of nuclear spins.

Nevertheless, Carravetta and Levitt have recently demonstrated that there are nuclear spin states whose decay time constant T_{LLS} is much longer than T_1.[1, 10, 19, 20] These nuclear states are called Long Lived States (LLS). LLS are based on peculiar properties of singlet states, *i.e.*, spin states that can be represented by an antisymmetric combination of Zeeman spin states of two nuclei. A singlet state is immune to the dipolar interaction between the two spins of the system, which is the main relaxation mechanism in solution-state NMR. If a population imbalance between singlet and triplet states can be created, it can be be stored for a long time.

Nuclear spin states are conveniently labeled as $|\alpha\rangle$ and $|\beta\rangle$ according to the projection of their angular moment onto the z-axis:

$$I_z|\alpha\rangle = \frac{1}{2}|\alpha\rangle \tag{2}$$

$$I_z|\beta\rangle = -\frac{1}{2}|\beta\rangle \tag{3}$$

Singlet and triplet states are defined as antisymmetric and symmetric superpositions, respectively, of the Zeeman spin states. As mentioned in equation 1, they can be written as follows:

$$|S_0\rangle = \frac{1}{\sqrt{2}}(|\alpha\beta\rangle - |\beta\alpha\rangle)$$
$$|T_{-1}\rangle = |\beta\beta\rangle$$
$$|T_0\rangle = \frac{1}{\sqrt{2}}(|\alpha\beta\rangle + |\beta\alpha\rangle)$$
$$|T_{+1}\rangle = |\alpha\alpha\rangle$$

with

$$I^2|S_0\rangle = I(I+1)|S_0\rangle = 0 \tag{4.1}$$

$$I^2|T_m\rangle = I(I+1)|T_m\rangle = 2|T_m\rangle \tag{4.2}$$

where $I^2 = I_x^2 + I_y^2 + I_z^2$ is the square of the spin angular momentum and $m = -1, 0, +1$ is the quantum number for the projection of I onto the quantization axis.[21]
These states can be classified according to their symmetry properties with respect to the spin exchange operator **P**:

$$\boldsymbol{P}|S_0\rangle = -|S_0\rangle \tag{5.1}$$

$$P|T_m\rangle = +|T_m\rangle \tag{5.2}$$

The singlet state is anti-symmetric while the triplet states are symmetric with respect to the exchange of the two spins. The singlet and triplet states are eigenstates of the spin Hamiltonian for a magnetically equivalent spin pair.

When the two spins are in magnetically equivalent environments, the nuclear spin Hamiltonian can be written as follows:

$$H = \omega_0 I_z + 2\pi J I_1 I_2 \tag{6}$$

where J is the spin-coupling constant, $\omega_0 = -\gamma B$ is the Larmor frequency and B is the applied static magnetic field. Its matrix representation in the singlet-triplet basis is:

$$H = \begin{array}{cccc} |S_0\rangle & |T_{+1}\rangle & |T_0\rangle & |T_{-1}\rangle \\ \begin{pmatrix} -\dfrac{3}{2}\pi J & 0 & 0 & 0 \\ 0 & \omega_0 + \dfrac{1}{2}\omega J & 0 & 0 \\ 0 & 0 & \dfrac{1}{2}\omega J & 0 \\ 0 & 0 & 0 & -\omega_0 + \dfrac{1}{2}\omega J \end{pmatrix} \end{array} \tag{7}$$

If the two spins are in magnetically equivalent environments, the three triplet states are spaced by energy dfferences γB and there is a field-independent energy difference of $2\pi J$ between the singlet state and the central triplet state.

The singlet state is non-magnetic and does not induce any NMR signal. In practice, with the inversion-recovery method, in a system of magnetically equivalent spin pairs, only the relaxation to equilibrium within the triplet manifold is measured. The time constant T_1 is thus an exclusive property of the triplet states.

However, the situation is different if the population of the singlet state is perturbed with respect to that of the triplet state. In this case, the re-establishment of thermal equilibrium

needs singlet-triplet transitions. Here is the heart of the phenomenon. These transitions can be far slower than T_1.

The reason why T_s is usually longer that T_1 can be explained by symmetry considerations. It has been demonstrated in equation 5 that the singlet state is antisymmetric under exchange, while the three triplet states are symmetric with respect to exchange. However, the conversion of an exchange-antisymmetric state into an exchange-symmetric state requires a mechanism that is itself exchange-antisymmetric. Since many of the strongest relaxation processes are exchange-symmetric (including the homonuclear dipole-dipole coupling between the nuclei, which is the strongest relaxation mechanism in a system of two coupled spin-1/2 in solution), they cannot contribute to T_s. In general, a relaxation mechanism can induce singlet-triplet transitions only if it does something 'different' to the two nuclear spins.

When the two spins are in magnetically inequivalent environments, the nuclear spin Hamiltonian can be written as follows:

$$H = \omega_0(1 + \delta_1)I_{1z} + \omega_0(1 + \delta_2)I_{2z} + 2\pi J I_1 I_2 \tag{8}$$

where δ_1 and δ_2 are the two chemical shifts. Its matrix representation in the singlet-triplet basis is the following:

$$H = \begin{array}{cccc} |S_0\rangle & |T_{+1}\rangle & |T_0\rangle & |T_{-1}\rangle \end{array}$$
$$\begin{pmatrix} -\dfrac{3}{2}\pi J & 0 & \dfrac{1}{2}\omega_0\Delta\,\delta & 0 \\ 0 & \omega_0(1 + \dfrac{1}{2}\Sigma\,\delta) + \dfrac{1}{2}\omega J & 0 & 0 \\ \dfrac{1}{2}\omega_0\Delta\,\delta & 0 & \dfrac{1}{2}\omega J & 0 \\ 0 & 0 & 0 & -\omega_0(1 + \dfrac{1}{2}\Sigma\,\delta) + \dfrac{1}{2}\omega J \end{pmatrix} \tag{9}$$

where the sum and the difference of the chemical shifts are:

$$\Sigma \delta = \delta_1 + \delta_2 \qquad\qquad (10.1)$$

$$\Delta \delta = \delta_1 - \delta_2 \qquad\qquad (10.2)$$

The relaxation theory for inequivalent spin-1/2 pairs does not predict any states that have a lifetime longer than T_1. The reason is apparent in equation 9: there are two terms $^1/_2\, \omega_0\, \Delta\, \delta$ that connect the singlet state to the central triplet state. These terms indicate that singlet-triplet transitions are induced by the chemical shift frequency difference $\omega_0\, \Delta\, \delta$. Therefore, the long-lived nature of the singlet state is masked by the chemical shift difference. In order to fully exploit the long lifetime of singlet states the chemical shift difference must be suppressed.

3.4 The singlet NMR experiment

In the case of magnetically equivalent spin pairs, singlet and triplet states are eigenstates of the spin system. However, the singlet state is non-magnetic and does not provide an NMR signal. In the case of magnetically inequivalent spin pairs, when the nuclear spin Hamiltonian is expressed in the singlet-triplet basis, the cross terms between singlet and triplet states demonstrate that coherent singlet-triplet transitions are allowed by chemical shift frequency difference.

Evidently, to exploit the long-lived nature of the singlet state (and hence to have $T_s = T_{LLS}$), a combination of the two previous situations has to be devised. Starting from a magnetically inequivalent spin pair (IS spin system), the trick is to switch the symmetry of the spin interactions at different points during the experimental procedure:

1. Singlet preparation: in a situation of broken spin-exchange symmetry; the two spins are magnetically inequivalent and the system Hamiltonian is the one given in equation 9. The goal is to obtain a magnetization which corresponds, in the next step, to the maximal difference between singlet and triplets populations;

2. TSI sustaining: in this step, by imposing spin-exchange symmetry, coherent singlet-triplet transitions are prohibited and the singlet population decays with a constant time $T_s = = \ T_{LLS}$ which can be much longer than T_1;
3. Detection: in the last step, spin-exchange symmetry is broken again and nuclear spin order is transformed into a detectable magnetization.

The two most used procedures for sustaining TSI are the followings:

a) Field cycling: the magnetic field is temporarily reduced (either by removing the sample from the region where the magnetic field is strong or by reducing the current in the main solenoid) and magnetic equivalence is thus established;
b) Radio-frequency spin-locking: the effect of the chemical shift difference is suppressed by applying a resonant radio-frequency field.

The mechanical procedure for the field cycling method is often slow and complicated. Where possible, it is more convenient to apply resonant radio-frequency irradiation to suppress the chemical shift difference. This is the solution that we adopted in the experiments shown in this thesis, and it will be described in more detail in the following paragraphs.

3.4.1 TSI preparation

In this step, a spin density operator corresponding to singlet nuclear spin order must be created. The normalized operator Q_{LLS}, which describes the population difference between singlet and triplet states, is given by:

$$Q_{LLS} = \frac{\sqrt{3}}{2}\left[|S_0\rangle\langle S_0| - \frac{1}{3}(|T_{+1}\rangle\langle T_{+1}| + |T_0\rangle\langle T_0| + |T_{-1}\rangle\langle T_{-1}|)\right] \qquad (11)$$

Since singlet and triplet states are not eigenstates in a magnetically inequivalent spin pair, it is more convenient to define two distinct basis sets:

- IS spin system: Product Basis set (PB) $\rightarrow \Phi_{PB} = \{|\alpha\alpha\rangle, |\alpha\beta\rangle, |\beta\alpha\rangle, |\beta\beta\rangle\}$;
- I₂ spin system: Singlet-Triplet Basis set (STB) $\rightarrow \Phi_{STB} = \{|S_0\rangle, |T_{+1}\rangle, |T_0\rangle, |T_{-1}\rangle\}$

It is thus possible to convert the density operator from the Liouville space expressed in the Φ_{STB} base to the Liouville space expressed in the Φ_{PB} base and vice-versa.[4] The operator Q_{LLS} expressed in the product basis is:

$$Q_{LLS} = -\frac{2}{\sqrt{3}}\left(I_x S_x + I_y S_y + I_z S_z\right) = \frac{1}{\sqrt{3}}(-2ZQ_x - 2I_z S_z) \tag{12}$$

where $ZQ_x = [|\alpha\beta\rangle\langle\beta\alpha| + |\beta\alpha\rangle\langle\alpha\beta|]/2$ and $2I_z S_z = [|\alpha\alpha\rangle\langle\alpha\alpha| - |\alpha\beta\rangle\langle\alpha\beta| - |\beta\alpha\rangle\langle\beta\alpha| + |\beta\beta\rangle\langle\beta\beta|]$.

In order to excite LLS, a zero-quantum coherence ZQ_x and/or a longitudinal two-spin order $2I_z S_z$ must be created.[4]

3.4.2 TSI storage

As mentioned above, the chemical shift difference between spins in magnetically inequivalent sites leads to coherent singlet-triplet transitions in the presence of the main field B. To reveal the long lifetime of the singlet state, this source of relaxation must be suppressed.
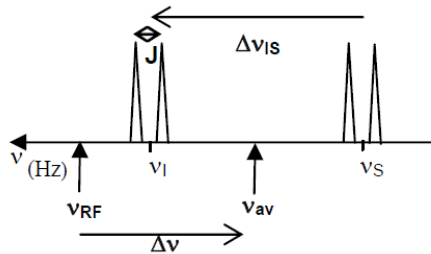
Figure 3 Sketch of an NMR spectrum of a weakly coupled two spin ½ system with some important parameters.

The Hamiltonian of a two-spin system irradiated by a continuous RF field is given by:

$$H = \omega_1(I_x + S_x) + \Delta\omega(I_x + S_x) + 2\pi J I S + \frac{1}{2}\Delta\Omega(I_x - S_x) \tag{13}$$

where ω_1 is the RF amplitude, $\Delta\omega = \omega_{RF} - (\Omega_I + \Omega_S)/2$ is the offset of the carrier ω_{RF} from the center of the spectrum $(\Omega_I + \Omega_S)/2$ and $\Delta\Omega = \Omega_I - \Omega_S$ is the chemical shift difference.[22] While the first three terms in the equation above are invariant with respect to a permutation of the two spins, the last term is anti-symmetric. This causes singlet-triplet transitions that lead to the leakage of singlet state population.

The application of a radio-frequency (RF) field allows the suppression of the chemical shift difference, making the effective Hamiltonian symmetric with respect to permutation. The ratio $\Delta\Omega/\omega_1$ between the magnitude of the chemical shift difference and the RF amplitude is an important parameter that affects the singlet lifetime. The ratio $\Delta\omega/\omega_1$ between the offset and the RF amplitude is another important parameter. When $\Delta\omega/\omega_1 = 0$ and $\Delta\Omega/\omega_1 \ll 1$, the term proportional to the chemical shift difference $\Delta\Omega$ is rendered ineffective. However, when the offset $|\Delta\omega|/\omega_1 > 0$, part of the term that is proportional to $\Delta\Omega$ remains secular with respect to the first dominant term. In other words, moving the carrier away from the center of the spectrum $(\Delta\omega \neq 0)$ re-introduces the chemical shift difference. To efficiently sustain the singlet state, it is necessary to know the value of the two chemical shifts.

A sufficiently strong RF field imposes a spin-exchange symmetry on the nuclear spin system and locks the anti-symmetric singlet state. However, to sustain the TSI, the RF field must be applied for a long interval. Therefore, the applied RF field should be as weak as possible in order to minimize sample heating.

Spin-locking can be achieved with an unmodulated RF field, often referred to as continuous-wave (CW) irradiation. Assuming that the RF carrier frequency corresponds to the chemical shift δ_{RF}, the resonance offset frequencies are given by

$$\Omega_1 = 2\pi\gamma B(\delta_1 - \delta_{RF}) \tag{14.1}$$

$$\Omega_2 = 2\pi\gamma B(\delta_2 - \delta_{RF}) \tag{14.2}$$

A good singlet spin-locking by a CW RF field requires that the nutation radiofrequency ω_{nut} is considerably larger than both resonance offset frequencies ($|\omega_{nut}| \gg |\Omega_1|, |\Omega_2|$). For example, in a 600 MHz spectrometer (14 T), two protons with a chemical shift difference of 1 ppm are spaced by 600 Hz. The resonant frequencies are +/- 300 Hz away from the spin-locking field and an RF nutation frequency of at least 1 kHz would be necessary for efficient singlet spin-locking. This amplitude can be applied to the sample for intervals as long as a few minutes without problems.

If the chemical shift difference is larger, the situation is more complicated. For example, a chemical shift difference of 50 ppm (*i.e.*, 7.5 kHz in 14 T) is common for ^{13}C. Under these conditions, it is impossible to lock the singlet state of such a spin pair without risking sample heating and damage to the NMR probe. Therefore, singlet spin-locking using CW RF fields requires that the two spins have similar chemical shifts and that the RF field is applied close to resonance for both spins.

Spin-locking can also be obtained by modulated spin-locking fields. Trains of broadband refocusing pulses can be used [4] as well as WALTZ-16 modulation of the RF field [10]. With this technique, a much broader bandwidth of the spin-locking field can be achieved with respect to the average chemical shift, but not with respect to the difference of the chemical shifts. This is a strong restriction on singlet NMR performed in high magnetic fields. In most cases, if the chemical shift difference is very large, the field-cycling method must be used instead of spin-locking

.

3.4.3 Detection

As described by equation 12, during the sustaining time of the singlet state, the nuclear spin order is comprised of zero-quantum coherence and longitudinal two-spin order. At the end of the sustaining time, the density operator must be converted into detectable magnetization. For this purpose, a procedure that resembles the time-reversal of the singlet preparation step can be used.[4]

3.4.4 LLS pulse sequence

As mentioned above, the LLS experiments presented in this thesis were performed using the optimized LLS pulse sequence described by Sarkar.[4] Hereunder the individual steps of the pulse sequence are described.
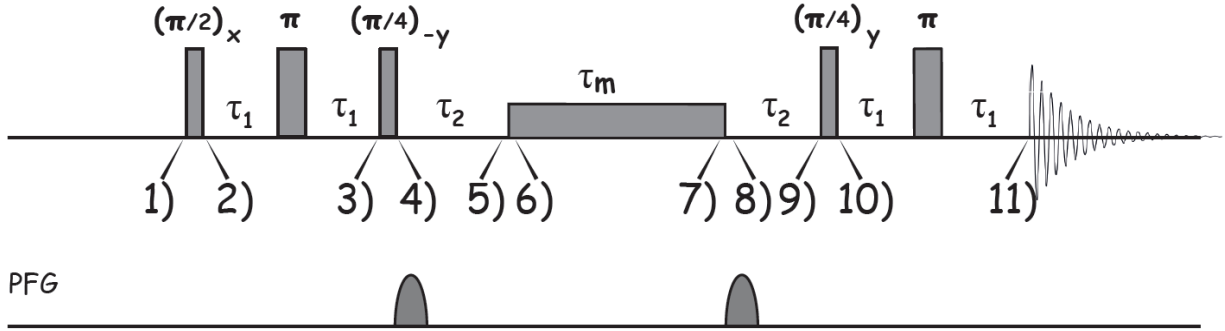
System: two non-equivalents spins $I = {}^1/_2$ and $S = {}^1/_2$ with a chemical shift difference $\Delta\nu_{IS} = (\Omega_I - \Omega_S)/(2\pi)$, and scalar coupling constant J_{IS}.

1. Boltzmann equilibrium, the magnetization is aligned with the z axis [23]:

$$\sigma(1) = a(I_z + S_z) \tag{15}$$

2. The first $\pi/2$ pulse with a phase parallel to the x axis flips the magnetization towards the transverse plane of the rotating frame:

$$\sigma(1) \xrightarrow{(\pi/2)_x} \sigma(2) = a(-I_y - S_y) \tag{16}$$

3. The $\tau_1 - \pi - \tau_1$ sequence convert the in-phase magnetization into anti-phase magnetization if $\tau_1 = 1/(4J_{IS})$:

$$\sigma(2) \xrightarrow{\tau_1-\pi-\tau_1} \sigma(3) = a(2I_xS_z + 2I_zS_x) \tag{17}$$

3.4.4 LLS pulse sequence

4. The $\pi/4$ pulse along the $-y$ axis generates a superposition of longitudinal two-spin order and two-spin coherence:

σ(3) = 2IxSz +2IzSx   σ(4) = 2IzSz -2IxSx
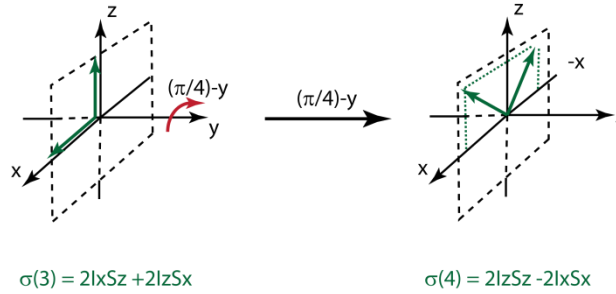
$$\sigma(3) \xrightarrow{(\pi/4)-y} \sigma(4) = b(2I_zS_z - 2I_xS_x) \tag{18}$$

Using the definitions of zero (ZQ_x) and double- (DQ_x) quantum coherences [24]:

$$\begin{cases} ZQ_x = \dfrac{1}{2}\left(2I_xS_x + 2I_yS_y\right) \\ DQ_x = \dfrac{1}{2}\left(2I_xS_x - 2I_yS_y\right) \end{cases} \tag{19}$$

we have $2I_xS_x = ZQ_x + DQ_x$ and hence:

$$\sigma(4) = b(2I_zS_z - ZQ_x - DQ_x) \tag{20}$$

The DQ_x term can be suppressed by a pulsed field gradient (PFG) which affects neither the $2I_zS_z$ nor the ZQ_x terms, so that one retains after this PFG:

$$\sigma(4) = b(2I_zS_z - ZQ_x) \tag{21}$$

5. To avoid that $2I_zS_z$ and $-ZQ_x$ cancel each other, a delay $\tau_2 = 1/(2\Delta\nu_{IS})$ is inserted to let $-ZQ_x$ evolve into $+ZQ_x$ under the chemical shift difference:

$$\sigma(4) \xrightarrow{\tau_2 = 1/(2\Delta\nu_{IS})} \sigma(5) = b(2I_zS_z + ZQ_x) \tag{22}$$

6. A continuous-wave (CW) radiofrequency (RF) field with an amplitude ν_1 larger than the chemical shift difference between the two spins ($\nu_1 > 5\Delta\nu_{IS}$) is usually applied to render the two spins I and S equivalent. During the 'sustaining delay' τ_m, the spin system is better best described in the singlet-triplet basis (STB) given by $\Phi_{STB} = \{|T_{+1}\rangle, |T_0\rangle, |S_0\rangle, |T_{-1}\rangle\}$. The conversion from the product basis to the singlet-triplet basis can be summed up as follows:

$$2I_zS_z \rightarrow \frac{1}{2}\begin{pmatrix} 1 & & & \\ & -1 & & \\ & & -1 & \\ & & & 1 \end{pmatrix}_{STB} \rightarrow \quad S_0 \;\; \begin{array}{c} \bullet \;\; T_{-1} \\ \circ \;\; T_0 \\ \bullet \;\; T_{+1} \end{array}$$

$$ZQ_x \rightarrow \frac{1}{2}\begin{pmatrix} 0 & & & \\ & 1 & & \\ & & -1 & \\ & & & 0 \end{pmatrix}_{STB} \rightarrow \quad S_0 \;\; \begin{array}{c} - \;\; T_{-1} \\ \bullet \;\; T_0 \\ - \;\; T_{+1} \end{array}$$

where filled circles indicate a population excess with respect to thermal equilibrium, while empty circles indicate a population deficiency. Hence one obtains:

$$\sigma(5) = b\left(2I_zS_z + ZQ_x\right)_{PB} \xrightarrow[\text{base-change}]{CW} \sigma(6) = b\frac{1}{2}\begin{pmatrix} 1 & & & \\ & 0 & & \\ & & -2 & \\ & & & 1 \end{pmatrix}_{STB} \rightarrow \quad S_0 \;\; \begin{array}{c} \bullet \;\; T_{-1} \\ - \;\; T_0 \\ \bullet \;\; T_{+1} \end{array}$$

$$\sigma(6) = b(|S_0\rangle\langle S_0| - \tfrac{1}{2}(|T_1\rangle\langle T_1| + |T_{-1}\rangle\langle T_{-1}|))_{STB} \tag{23}$$

7. During the sustaining delay τ_m, the population $|S_0\rangle\langle S_0|$ of the singlet state $|S_0\rangle$ is isolated from the three triplet states. The flow of populations between the singlet and triplet states is largely suppressed as long as the magnetic equivalence is maintained by the RF field. However, the populations of the three triplet states will equilibrate with a time constant T_1. If we multiply all populations by an arbitrary factor three for the sake of clarity, we have at the end of the sustaining delay τ_m:

3.4.4 LLS pulse sequence
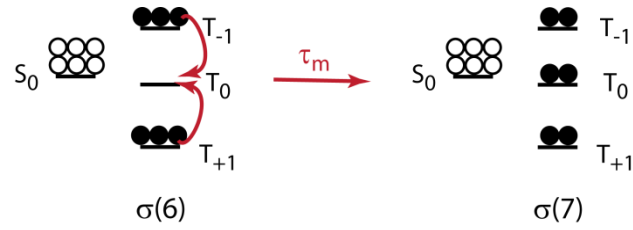
$$\sigma(7) = c(|S_0\rangle\langle S_0| - \frac{1}{3}(|T_1\rangle\langle T_1| + |T_0\rangle\langle T_0| + |T_{-1}\rangle\langle T_{-1}|))_{STB} \tag{24}$$
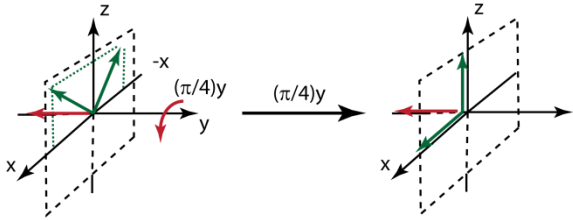
8. When the CW RF field is switched off, the two spins become inequivalent again, and are best described in the product basis (PB):

$$\sigma(7) = \frac{c}{2}\begin{pmatrix} 1 & & & \\ & 1 & & \\ & & -3 & \\ & & & 1 \end{pmatrix}_{STB} \xrightarrow{CW\ off} \sigma(8) = c(2I_zS_z + ZQ_x)_{PB} \tag{25}$$

9. Again, ZQ_x is converted to $-ZQ_x$ via the chemical shift difference during a delay $\tau_2 = 1/(2\Delta\nu_{IS})$. Remembering the definition of $-ZQ_x$ (see step 4) , we obtain:

$$\sigma(9) = c(2I_zS_z - ZQ_x) = c(2I_zS_z - 2I_xS_x - 2I_yS_y) \tag{26}$$

10. A last $(\pi/4)$ pulse along the y-axis partly converts the first two terms into observable anti-phase magnetization, while $-2I_yS_y$ remains unobservable:

$$\sigma(9) \xrightarrow{(\pi/4)_y} \sigma(10) = d(2I_xS_z + 2I_zS_x - 2I_yS_y) \tag{27}$$

11. An optional $\tau_1 - \pi - \tau_1$ sequence with $\tau_1 = 1/(4J_{IS})$ can be inserted to convert the antiphase magnetization into in-phase magnetization:

$$\sigma(10) \xrightarrow{\tau_1-\pi-\tau_1} \sigma(11) = d(I_y + S_y - 2I_yS_y) \tag{28}$$

The sensitivity of LLS experiments is limited by the requirements of magnetization transfer. Ignoring relaxation, the presence of two $\pi/4$ pulses leads to a signal which is about one-half of the signal derived from the initial polarization. In addition, the experiments suffer from transverse relaxation of the zero-quantum component $T_2(ZQ)$ and from longitudinal relaxation of the I_zS_z term $T_1(I_zS_z)$ during the two intervals $\tau_2 = 1/(2\Delta\nu_{IS})$.

To boost the sensitivity, one can use pulse schemes such as WASTE [25] for homonuclear decoupling of the two spins that participate in the LLS during signal acquisition. The collapse of the two doublets into two singlets would increase the observed signals intensities.

3.5 Relaxation of Long-Lived States

It has been previously mentioned that LLS are immune to relaxation by fluctuations of the dipolar coupling between the two nuclei of the spin system. As shown by equation 5, singlet states are antisymmetric with respect to the permutation of the two spins, while the dipole-dipole interaction is a mechanism which acts symmetrically on the spin pair and thus cannot mix symmetric triplet states with the antisymmetric singlet state.

In its simplest form, relaxation can be described by Bloch's equations, which provide a phenomenological picture of relaxation. Bloch's formulation does not provide any microscopic explanation of the origin of relaxation, nor does it allow to predict the magnitude of the rate constants. This limitation can be overcome by treating relaxation quantum mechanically.

In Liouville space, the relaxation rates of populations and coherences can be obtained as eigenvalues of the matrix representation of the relaxation superoperator in an appropriate operator basis. The set of basis operators is chosen as the one containing all eigenoperators Q_r obtained by commutation with the total spin angular momentum along the z-axis I_z:

$$I_z Q_r = p_r Q_r \tag{29}$$

where the integer value p_r is called the coherence order.

If a superoperator A commutes with the superoperator Iz, the matrix representation of A is block-diagonal in the chosen basis set b with a block for each possible value of the coherence order p [21]:

$$\begin{pmatrix} \blacksquare & \blacksquare & 0 & 0 & 0 & 0 \\ \blacksquare & \blacksquare & 0 & 0 & 0 & 0 \\ 0 & 0 & \blacksquare & \blacksquare & \blacksquare & 0 \\ 0 & 0 & \blacksquare & \blacksquare & \blacksquare & 0 \\ 0 & 0 & \blacksquare & \blacksquare & \blacksquare & 0 \\ 0 & 0 & 0 & 0 & 0 & \dots \end{pmatrix}$$

The matrix block containing operators with the same coherence order p will be denoted as $[A]_p^b$. In the context of singlet states relaxation, it is convenient to use an operator basis where the basis operators are built from an appropriate combination of all the 16 products of the type [10]:

$$|s\rangle\langle r| \tag{30}$$

where $|s\rangle \in \{|S_0\rangle, |T_{-1}\rangle, |T_0\rangle, |T_{+1}\rangle\}$
$\quad \langle r| \in \{\langle S_0|, \langle T_{-1}|, \langle T_0|, \langle T_{+1}|\}$

Furthermore, it is sufficient to consider only the zero-quantum block of the relaxation matrix to examine the relaxation properties of LLS because the singlet state belongs to this block and is not coupled to any blocks with different coherence orders.[21]
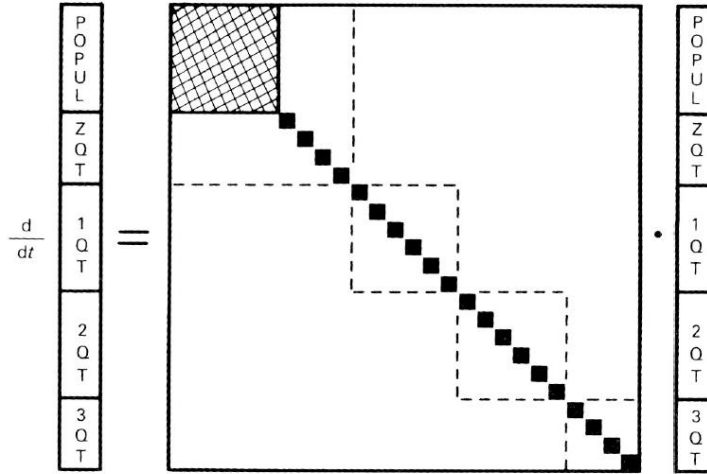
Figure 4 Redfield kite structure of the relaxation supermatrix [26].

The zero-quantum block ($p = 0$) of the operator basis is:

$$STB_0 = \left\{ \begin{array}{c} \frac{1}{2}(|S_0\rangle\langle S_0| + |T_{+1}\rangle\langle T_{+1}| + |T_0\rangle\langle T_0| + |T_{-1}\rangle\langle T_{-1}|) \\ \frac{1}{2\sqrt{3}}(3|S_0\rangle\langle S_0| - |T_{+1}\rangle\langle T_{+1}| - |T_0\rangle\langle T_0| - |T_{-1}\rangle\langle T_{-1}|) \\ \frac{1}{\sqrt{2}}(|T_{+1}\rangle\langle T_{+1}| - |T_{-1}\rangle\langle T_{-1}|) \\ \frac{1}{\sqrt{6}}(2|T_0\rangle\langle T_0| - |T_{+1}\rangle\langle T_{+1}| - |T_{-1}\rangle\langle T_{-1}|) \\ |S_0\rangle\langle T_0| \\ |T_0\rangle\langle S_0| \end{array} \right\} \tag{31}$$

The first operator in this zero-quantum block ($Q_1^{STB_0}$) is one-half of the unity operator and commutes with all other spin operators. The second basis operator ($Q_2^{STB_0}$) has an expectation value proportional to the deviation of the singlet population from a uniform population distribution. The third ($Q_3^{STB_0}$) and the fourth ($Q_4^{STB_0}$) operators represent perturbations of the triplet populations, the third one having an expectation value

proportional to the total longitudinal magnetization for the two nuclei. Finally, the last two operators $(Q_5^{STB_0})$ and $(Q_6^{STB_0})$ represent zero-quantum singlet-triplet coherences.

3.5.1 The homogeneous master equation

The dynamics of a spin system can be described by the homogeneous Liouville-von Neumann master equation:

$$\frac{d}{dt}\sigma(t) = -L(t)\sigma(t)$$

(32)

where $\sigma(t)$ is the density matrix representation and L the Liouvillian superoperator, which represents a superposition of both coherent and incoherent effects on the spin-system, described respectively by the commutation superoperators H_0 and Γ:

$$L(t) = -iH_0(t) + \Gamma$$

(33)

In a frame rotating at a frequency ω_{RF} (preferably corresponding to the average of the chemical shifts of the two spins), the coherent superoperator can be written as [27]

$$H_0 = \omega_I I_z + \omega_S I_z + 2\pi JIS + \omega_{nut}(I_x + S_x)$$

(34)

where ω_I and ω_S are the offsets of the two spins I and S, respectively, and ω_{nut} is the amplitude of the CW RF field with frequency ω_{RF} applied to suppress the chemical shift difference of the two spins, making them equivalent in the sense of average Hamiltonian theory.

The superoperator L contains a superposition of the contributions of all possible relaxation mechanisms:

$$\Gamma = \Gamma_{DD} + \Gamma_{CSA} + \Gamma_{SR} + \Gamma_{PS} + \sum_{j,k} \Gamma^{jk}$$

(35)

where Γ_{DD} is the contribution due to the dipole-dipole (DD) interaction between the two spins, Γ_{CSA} is the contribution of the chemical shift anisotropy, Γ_{SR} is the contribution of the spin rotation mechanism and Γ_{PS} is the contribution of interactions with the paramagnetic species, while $\sum_{j,k} \Gamma^{jk}$ is the sum of all cross-correlation terms, such as DD-CSA, etc..

From now on it is necessary to distinguish the types of mechanisms that cause relaxation. In the following paragraph, the attention will be focused on the dipolar mechanism between the spin pair, in order to demonstrate its inefficiency for the relaxation of LLS.

3.5.2 The dipolar relaxation mechanism

As mentioned, it is possible to demonstrate, by symmetry arguments, that the singlet state of two spin-1/2 nuclei is immune to the DD relaxation mechanism.

Singlet states are anti-symmetric with respect to spin exchange, while the dipole-dipole interaction acts symmetrically on the two coupled nuclei and thus cannot mix symmetric and anti-symmetric states. Therefore, singlet and triplet states are never interchanged.

A theoretical explanation requires examination of the zero-quantum block of the matrix representation of Γ_{IS}^{DD} in the STB_0 basis [21] :

$$[\Gamma_{IS}^{DD}]_0^{STB_0} = -\frac{b_{IS}^2 \tau_c}{10} \begin{pmatrix} 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 15 & 0 & 0 & 0 \\ 0 & 0 & 0 & 9 & 0 & 0 \\ 0 & 0 & 0 & 0 & 5 & 0 \\ 0 & 0 & 0 & 0 & 0 & 5 \end{pmatrix} \tag{36}$$

where $b_{IS} = -(\hbar \mu_0 \gamma_I \gamma_S)/(4\pi r_{IS}^3)$ is the dipolar coupling constant.

The zero-quantum block is diagonal and $Q_2^{STB_0}$ is, indeed, an eigenoperator of the superoperator Γ_{IS}^{DD}. Its eigenvalue corresponds therefore to the decay rate of the singlet population. At the same time, the longitudinal magnetization $-Q_2^{STB_0}$ has a non-vanishing decay rate. Hence in the extreme narrowing regime [21]:

$$R_S^{DD} = 0 \tag{37}$$

3.5.2 The dipolar relaxation mechanism

$$R_1^{DD} = \frac{3}{2} b_{IS}^2 \tau_c \tag{38}$$

The relaxation rate of the singlet state R_S^{DD} due to the dipolar interaction between the spin pair vanishes. The singlet state population is immune to this kind of interaction. On the other hand, the correspondent longitudinal magnetization decay rate R_1^{DD} does not vanish and is consistent with that derived by Redfield relaxation theory.

As a result, if a population imbalance between singlet and triplet states is excited, the main relaxation mechanism in solution is switched off. Relaxation of this long-lived state will occur due to secondary mechanisms, like dipolar couplings with "out-of-pair" nuclear spins or paramagnetic species, chemical shift anisotropy, spin rotation, and so on.

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4. The use of Long-Lived States for studying ligand-protein interactions

The first step of drug discovery is commonly referred to as *hit identification.* Screening techniques such as enzyme-linked immunosorbent assays (ELISA),[1] surface plasmon resonance (SPR, also known under the trade name Biacore),[2] isothermal titration calorimetry (ITC),[3] and an ever-expanding range of nuclear magnetic resonance (NMR) techniques [4-6] allow one to recognize ligands for a defined protein contained in extensive libraries of chemical compounds.

Because one can choose from a wide range of observable parameters, NMR spectroscopy offers several methods to study interactions between small ligand molecules and macromolecular targets. It is possible to extract dissociation constants [7] and to obtain structural information about the protein and its complex with the ligand.[8] Provided the exchange between the free and bound forms of the ligand is faster than the difference of their resonance frequencies,[8, 9] *i.e.*, when $k_{ex} \approx k_{off} \gg (\pi/\sqrt{2})\Delta v$, where Δv is the chemical shift difference (in Hz) of the signals in the bound and free states, any observable quantity ε_{obs}, be it a frequency or a relaxation rate, is determined by a weighted average of the free and bound forms [10]:

$$\varepsilon_{obs} = p_B \varepsilon_B + p_F \varepsilon_F \tag{1}$$

where p_B and p_F are the mole fractions of the bound and free ligands, respectively, while ε_B and ε_F are the values of the parameter ε in the bound and free forms, respectively. Observation of differences between ε_{obs} and ε_F allows the detection of ligand binding. Provided that $\varepsilon_B \neq \varepsilon_F$, differences between ε_{obs} and ε_F can be detected if there is a certain amount of ligand in the bound form, *i.e.*, $p_B > 0$.

The sensitivity of a parameter ε to binding events can be expressed by the experimental contrast C_ε, which is a function of the "observable" parameters ε_{obs} and ε_F:

$$C_\varepsilon = \left| \frac{\varepsilon_{obs} - \varepsilon_F}{\varepsilon_{obs}} \right| * 100 \tag{2}$$

The larger the contrast, the more sensitive the parameter ε to ligand–protein binding, *i.e.*, smaller the fraction of ligand in the bound form p_B that is required to detect binding.

Several NMR methods based on such a contrast are extensively used nowadays to determine dissociation constants of ligand–protein interactions. The quantity ε_{obs} can be determined by the chemical shifts of one or more selected nuclei of either target proteins [11] or ligands,[12] the translational or rotational diffusion constant of the ligand,[13] the relaxation rates T_1, T_2 or $T_{1\rho}$, the rate of magnetization transfer by cross-relaxation (Overhauser effect) between protons belonging to the ligand,[14] the saturation transfer from proteins to ligands determined by difference spectroscopy,[15] or "waterLOGSY" that exploits differences in the rate of transfer of magnetization from bulk water to free or bound ligands by cross-relaxation.[16] Several of these methods rely on differences in rotational correlation times between the free ligand and the protein–ligand complex.[17]

It has been recently demonstrated that so-called Long-Lived States (LLS), also known as singlet states (SS) in isolated two-spin systems, can be used very efficiently to investigate protein-ligand interactions.[18] In the following paragraphs, the use of LLS for this purpose will be described and experimental results will be discussed.

4.1 LLS contrast

As mentioned in the previous paragraph, detection of ligand-receptor binding is obtained through observation of a difference between ε_{obs} and ε_F. Equation 1 nicely shows that such a difference is possible only if $\varepsilon_B \neq \varepsilon_F$. In particular, the larger the difference between ε_B and ε_F, the smaller the fraction of ligand in the bound form p_B required to generate a sufficient difference between the parameters ε_{obs} and ε_F. In other words, for a fixed ligand-to-receptor ratio $[L]/[P]$, *i.e.*, a fixed p_B, large differences between ε_B and ε_F lead to a large experimental contrast C_ε, while small differences between ε_B and ε_F lead to a small value of C_ε. Combining equation 1 with the definitions $p_B = [PL]/[L]_{tot}$ and $p_F = ([L]_{tot} - [PL])/[L]_{tot}$, one gets:

$$\varepsilon_{obs} = \frac{[PL]}{[L]_{tot}}(\varepsilon_B - \varepsilon_F) + \varepsilon_F \tag{3}$$

Equation 3 can be used to fit the variation of an observed parameter during a titration in order to extrapolate the dissociation constant K_D, since the ratio $[PL]/[L]_{tot}$ is a function of K_D. It we rearrange equation 3, we obtain:

$$(\varepsilon_{obs} - \varepsilon_F) = \frac{[PL]}{[L]_{tot}}(\varepsilon_B - \varepsilon_F) \qquad (4)$$

Equation 4 correlates directly the experimental difference $(\varepsilon_{obs} - \varepsilon_F)$ with the difference $(\varepsilon_B - \varepsilon_F)$ between the values of the parameter ε in the bound and free forms, showing that the larger the difference $(\varepsilon_B - \varepsilon_F)$, the smaller the bound fraction $p_B = [PL]/[L]_{tot}$ required to obtain a detectable $(\varepsilon_{obs} - \varepsilon_F)$.

The LLS relaxation rate R_{LLS} is extremely sensitive to ligand-protein binding. Long-lived states have the unique property that their populations relax with time constants that can be much longer than longitudinal relaxation time constants $(T_{LLS} \gg T_1)$. The intensity I of the LLS signal decays mono-exponentially as a function of the sustaining delay τ_m:

$$I(\tau_m) = I_0 \exp(-R_{LLS}\tau_m) \qquad (5)$$

where I_0 is the LLS intensity at $\tau_m = 0$ and R_{LLS} is the LLS relaxation rate. The LLS relaxation rate R_{LLS} and thus the LLS lifetime $T_{LLS} = 1/R_{LLS}$ can be extracted by fitting the signal intensity observed as a function of the sustaining delay τ_m. For pairs of protons, T_{LLS}/T_1 ratios as large as 60 have been observed in R-CH=CH-R' systems.

Glycine residues in peptides contain two diastereotopic H$^\alpha$ protons, so it is straightforward to excite LLS in virtually any glycine-containing peptide.[19] It has been shown by phage display [20] using a peptide library and consensus sequence analysis that peptides that bind to Urokinase-type Plasminogen Activator (uPA) must contain at least one arginine residue.[21] We therefore considered the GGR tripeptide, which turns out to be a weak binder for uPA.

The two H$^\alpha$ protons of the central glycine residue are not magnetically equivalent, due to the vicinity of the chiral C$^\alpha$ carbon of the arginine residue. Hence, it is possible to excite long-lived states with the pulse sequence described in chapter 3.[22] The lifetime T_{LLS} of this pair of diastereotopic H$^\alpha$ protons was determined to be $T_{LLS} = (8.0 \pm 0.2)\,s$ at 8 °C and 9.4 T.[18] Under the same conditions, the longitudinal relaxation time T_1 turned out to be $T_1 = (0.40 \pm 0.08)\,s$, leading to a ratio T_{LLS}/T_1 as large as 20.
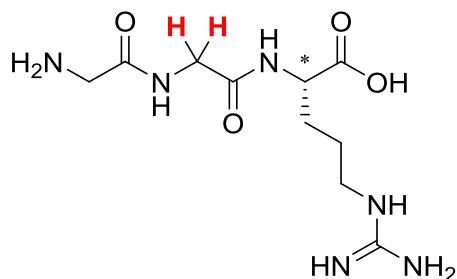
Figure 1 Chemical structure of the tripeptide GGR. The two Hα protons of the central glycine residue are highlighted in red. The chiral Cα carbon of the arginine residue is indicated by a star.

The situation changes drastically if there are interactions between ligands and proteins. We simulated the dipole-dipole contributions (the main relaxation pathways in solution-state NMR spectroscopy if chemical shift anisotropy can be neglected) to the R_1 and R_{LLS} relaxation rates of the free and bound tripeptide GGR. For the conventional longitudinal relaxation rates R_1, the dipole-dipole interactions between the protons of the ligand and those lining the binding site of uPA contribute roughly to 85% of the observed contrast. Moreover, the structures of free GGR and of the GGR-uPA complex in water were optimized using Gromacs 4.5.3 [23] using the GROMOS 53a6 force field.[24] The chemical shifts of the free and bound ligands were calculated by using these structures as input for Camshift 1.35.[25] The chemical shifts of the two Hα protons of the central glycine residue in GGR in the free and bound forms were estimated by modeling to change by ca. 0.5 ppm (200 Hz at 9.4 T in our experiments). Conventional longitudinal relaxation is not influenced by such a shift, but it has a dramatic effect on the lifetimes of the LLS.[26, 27] Indeed, during the LLS relaxation period, a radiofrequency (RF) field must be used to mask the chemical shift difference between the two Hα protons involved in the LLS. This field is most efficient when the carrier frequency coincides precisely with the center between the shifts of the two Hα protons, as we chose for the free ligand. When the ligand is bound, there is a frequency mismatch due to the change in chemical shifts upon binding, and the LLS decays rapidly, so that its lifetime is reduced. Indeed, figure 2 shows that ca. 75% of the decay rate of the LLS signal is due to the coherent effect of the frequency mismatch that occurs upon binding. This effect can be enhanced by using weaker RF fields or higher static fields.
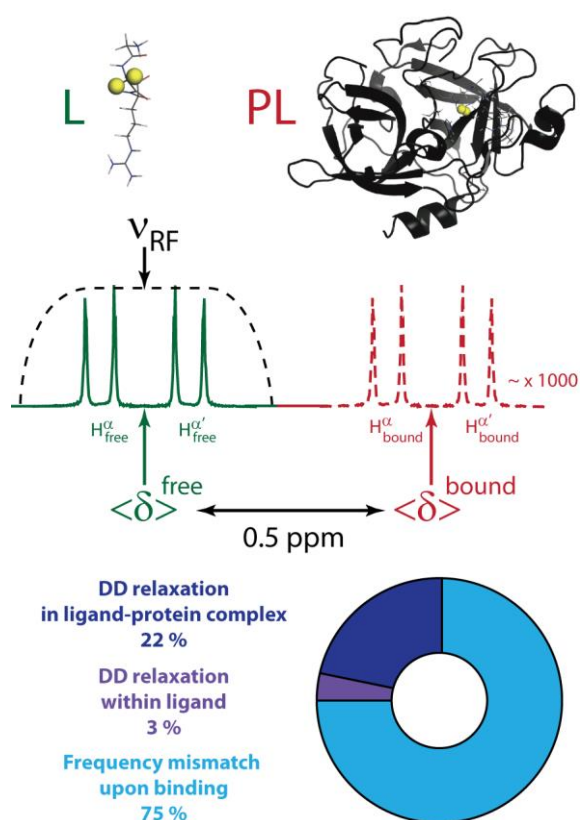
Figure 2 (Top) The tripeptide glycine-glycine-arginine (GGR) and its complex with Urokinase-type Plasminogen Activator (uPA). (Middle) Frequency jumps of the two H^α protons of the central glycine of GGR when it forms a complex with uPA. It is estimated that the two chemical shifts of the H^α protons jump by 0.5 ppm (200 Hz at 9.4 T) as the ligand goes from the free (green solid line) to the bound forms (red dashed line). The spectrum of the bound ligand cannot be observed directly because of fast exchange. The chemical shifts are weighted averages of the values in the free and bound forms. (Bottom) Relative contributions to the decay rate $R_{LLS} = 1/T_{LLS}$ of the long-lived state of the two H^α protons in the complex. The RF field used to sustain the LLS was monochromatic with an amplitude of $\nu_1 = 1\ kHz$, well above the minimum required to mask the chemical shift difference $\delta(H^\alpha) - \delta(H^{\alpha'}) = 100\ Hz$ in the free ligand.

The combination of a slow relaxation rate $R_{LLS}^{free} < R_1^{free}$ of the ligand in its free state with a rapid relaxation rate R_{LLS}^{bound} in its bound state makes the difference $(R_{LLS}^{bound} - R_{LLS}^{free})$ particularly large, thus making the relaxation of long-lived state an extremely sensitive NMR parameter to ligand-protein binding.

To demonstrate the enhancement of the contrast C_{LLS} with respect to the contrast C_1 and $C_{1\rho}$, binding experiments were carried out for a 1 mM solution of the tripeptide ligand GGR in the presence of its protein target trypsin in the range 0.5 < $[P]$ < 50 μM, using various methods (T_{LLS}, T_1 and $T_{1\rho}$). Figure 3 shows that the LLS method can work with a protein-ligand ratio that is ~25-fold lower that required for the well-known $T_{1\rho}$ method, whereas

the T_1 contrast remains below $C_1 < 10$ % even at the highest protein concentration $[P] = 50$ µM.
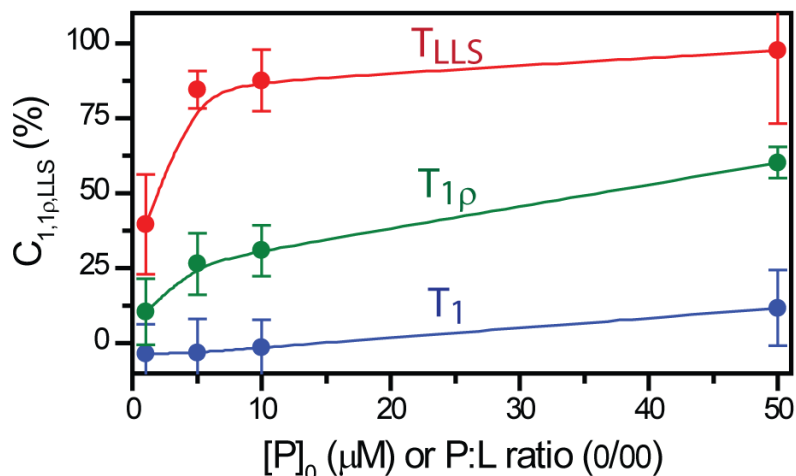
Figure 3 Experimental contrast for T_{LLS} (red), $T_{1\rho}$ (green), and T_1 (blue) methods for the diastereotopic pair of protons on the central glycine residue of the tripeptide GGR in a solution with a fixed concentration $[L] = 1$ mM and a variable trypsin concentration 0.5 µM $< [P]_0 < 50$ µM in D$_2$O at 8 °C and 11.7 T.

4.2 Competition experiments

Equation 3 can be used to fit the variation of the observed LLS relaxation rate R_{LLS}^{obs} during the titration of a ligand against a target:

$$R_{LLS}^{obs} = \frac{[PL]}{[L]_{tot}}\left(R_{LLS}^{bound} - R_{LLS}^{free}\right) + R_{LLS}^{free} \tag{6}$$

The fitting allows one to estimate the LLS relaxation rate of the bound form R_{LLS}^{bound} and the molar fraction of the ligand in the bound form $p_B = [PL]/[L]_{tot}$. The latter parameter is a function of the dissociation constant K_D [28]:

$$\frac{[PL]}{[L]_{tot}} = \frac{[P]_{tot}+[L]_{tot}+K_D-\sqrt{([P]_{tot}+[L]_{tot}+K_D)^2-4[P]_{tot}[L]_{tot}}}{2[L]_{tot}} \tag{7}$$

As consequence, it is possible to determine the dissociation constant by fitting the variation of the observed relaxation rate during a titration experiment. For example, the ligand GGR was titrated over a range 0.5 mM $< [L]_{tot} <$ 10 mM in the presence of $[P]_{tot} =$ 10 µM uPA at 8 ºC. The curve in figure 4 was fitted to equation 6, yielding K_D = 220 ± 10 µM and $T_{LLS}^{bound} = 1/R_{LLS}^{bound} = 30 ± 10$ ms. In contrast, as shown in figure 4, binding had virtually no effect on the longitudinal relaxation rates $R_1 = 1/T_1$ of the same Hα protons.
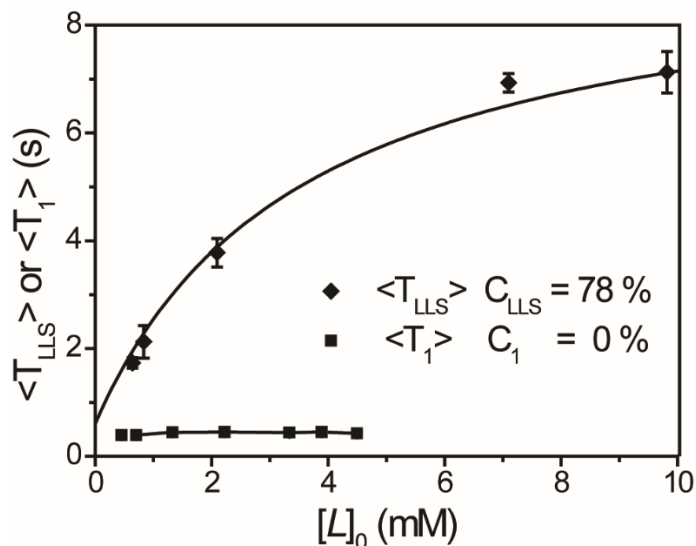
Figure 4 Lifetimes T_{LLS} of the long-lived state associated with the two Hα protons of the central glycine residue of the weak ligand L = GGR and their conventional longitudinal relaxation times T_1 in the presence of $[P]_{tot}$= 10 µM uPA as a function of $[L]_{tot}$ at 8 ºC and 400 MHz in D₂O. The curve shows a fit of the experimental data to equation 6.

Experiments based on the direct observation of ligands suffer from some limitations: non-specific binders may give similar effects as specific ones, ligands are difficult to detect if their solubility is low, and strong ligands in slow exchange are easily mistaken for non-binders. Indeed, when binding is too strong, the lifetime of the ligand-protein complex may be too long on the NMR time scale, so that the conditions for equation 1 are not fulfilled and the rates are not properly averaged.

To overcome these drawbacks, Dalvit and co-workers [29] introduced competition experiments for ligand screening. In this approach, a weak-affinity ligand is used as a spy molecule; a stronger binder partly displaces the spy molecule, and the latter's expulsion from the binding site of the macromolecular target leads to a decrease of relaxation rates of nuclei that belong to the displaced spy ligand. Of course, the contrast defined above should be sufficient. The concentration of the competitor that is required to displace the

spy molecule is inversely proportional to the former's affinity for the macromolecular target: the higher the affinity, the lower the concentration needed. Figure 5 shows the LLS signals of vanillic acid diethylamide, which is a weak ligand for the heat shock protein Hsp90, in three different solutions. The signals of vanillic acid diethylamide (which are easily observed in absence of protein, top spectrum in figure 5), disappear in the presence of Hsp90 (middle) because of the enhanced LLS relaxation caused by interactions with the protein. When Astex's clinical Hsp90 inhibitor AT13387 is added (bottom), the signal is almost completely restored, demonstrating that both vanillic acid diethylamide and the high-affinity inhibitor bind Hsp90 to the same ATP binding site, and that the latter is the strongest binder of the two.
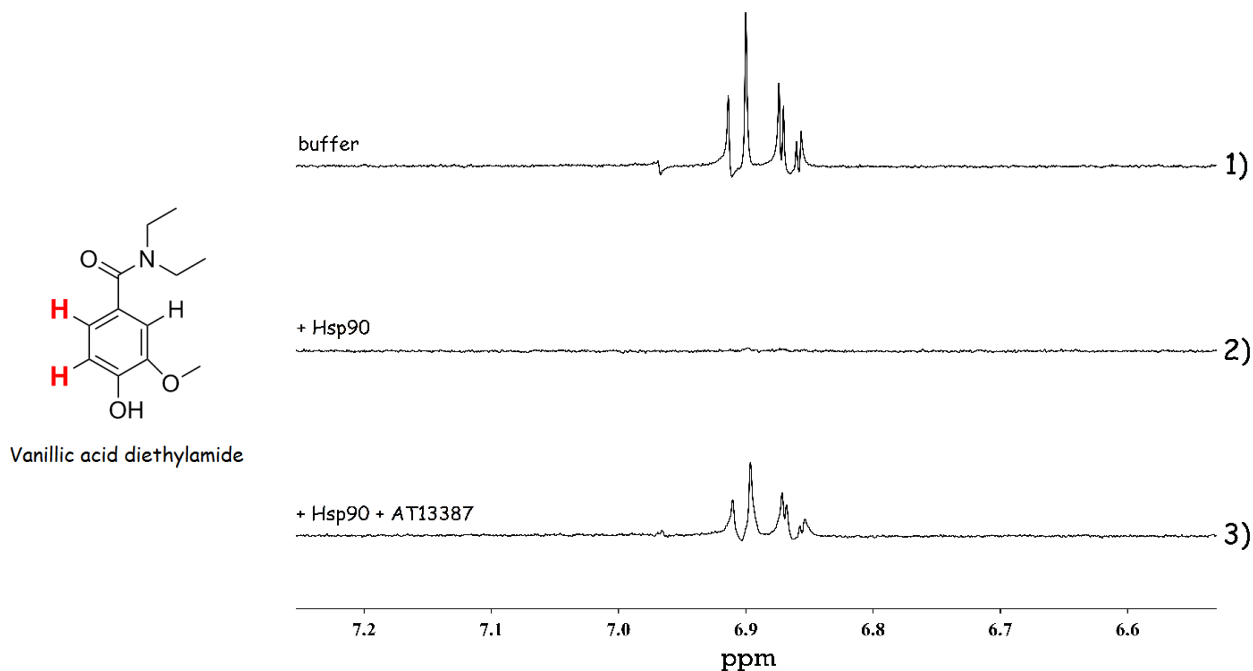
Figure 5 LLS spectra of vanillic acid diethylamide in three different solutions, sustaining the LLS during a delay τ = 2.5 s: (1) 500 μM vanillic acid diethylamide in the absence of Hsp90; (2) 500 μM vanillic acid diethylamide in the presence of 10 μM Hsp90; (3) 500 μM vanillic acid diethylamide in the presence of 10 μM Hsp90 and 10 μM AT13387. In the latter case, vanillic acid diethylamide is partly expelled from the ATP binding site of Hsp90 so that its LLS signal is partially restored.

The observed relaxation rate R_{LLS}^{obs} of the spy ligand gives information about the dissociation constant K_D^{comp} of the competitor. The dissociation constant determined by titration of the spy molecule in the presence of a competitor has come to be known as *apparent dissociation constant* $K_D^{spy,app}$ that allows one to determine the true dissociation

constant K_D^{comp} of the competitor. The relationship between these two constants is expressed by the following equation:

$$K_D^{comp} = \frac{[L_{comp}]_{tot}\, K_D^{spy}}{K_D^{spy,app} - K_D^{spy}} \tag{8}$$

where $[L_{comp}]_{tot}$ is the concentration of the competitor and K_D^{spy} is the true dissociation constant of the spy molecule.

Note that the competitor does not need to contain any spin pairs that can sustain an LLS. Moreover, by keeping the concentration of the spy ligand low, one can study competing ligands with limited solubility. Furthermore, as the changes in R_{LLS}^{obs} need only to be observed for the spy molecule, there are no requirements for the competitor to fulfill the fast-exchange condition. This implies that the dissociation constants K_D^{comp} of the competitor can lie anywhere in a wide range below 100 µM. Following this approach, GGR was used as spy molecule and titrated over a range 0.5 mM < $[L_{spy}]_{tot}$ < 10 mM in the presence of $[P]_{tot}$ = 10 µM uPA and $[L_{comp}]_{tot}$ = 10 µM of two different competitors at 8 ºC. In this fashion, K_D^{comp} = 89 ± 20 µM was determined for 4-aminobenzamidine, which has a better affinity than GGR but is not strong enough to inhibit uPA. Next, the bicyclic peptide ligand UK-18 has been investigated. This ligand has a high affinity and specificity for uPA [30], and indeed K_D^{comp} = 180 ± 20 nM was determined by the LLS method [18], in good agreement with previously published results obtained by Heinis and co-workers (K_D^{comp} = 157 ± 39 nM) [30] and Stubbs and co-workers (K_D^{comp} = 180 nM).[31]

4.3 Spin-pair labeling for ligand LLS experiments

A drawback of ligand screening by LLS is that the ligands (or only the spy ligand in the competition approach) must carry a pair of nonequivalent spins with *I* = 1/2. When such ligands are not immediately available in the compound libraries, synthetic labeling strategies can circumvent this issue. These approaches include two steps: 1) the identification of a spy ligand that binds weakly to the target protein, and 2) the functionalization of this ligand by attaching a *spin-pair label* that can carry LLS. By way of illustration, 3-bromothiophene-2-carboxylic acid (henceforth called "BT"), which is known to have long lifetimes T_{LLS} [32], has been covalently attached to the tripeptide GGR.

The resulting spin-pair-labeled tripeptide was called BT-GGR (figure 6b). It is a weak ligand for trypsin. [33]

Despite some steric effects and long-range dipolar relaxation mechanisms in the spin-pair-labeled tripeptide BT-GGR, the two aromatic protons of the bromothiophene group retain a remarkably long lifetime $T_{LLS}(BT) = 11.7 \pm 0.7$ s in the absence of protein. In this particular tripeptide, both diastereotopic pairs of H^α protons on the two glycine residues of BT-G_1G_2R can be used to excite LLS. They have lifetimes $T_{LLS}(G_1) = 10.4 \pm 0.5$ s and $T_{LLS}(G_2) = 9.3 \pm 0.5$ s again in the absence of protein.

The spin-pair-labeled ligand BT-GGR was added to a solution containing $[P]_{tot} = 25$ µM trypsin over a range 0.5 mM $< [L]_{tot} < 40$ mM. At each concentration, the observed relaxation times $T_{LLS}^{obs} = 1/R_{LLS}^{obs}$ of the three different pairs of protons were measured. Figure 6a shows how the titration curves can be fitted to equation 6. As expected, nearly the same dissociation constants were obtained for the three proton pairs that can sustain LLS in BT-GGR: $K_D(BT) = 0.18 \pm 0.03$ mM, $K_D(G_1) = 0.24 \pm 0.01$ mM, and $K_D(G_2) = 0.21 \pm 0.02$ mM.
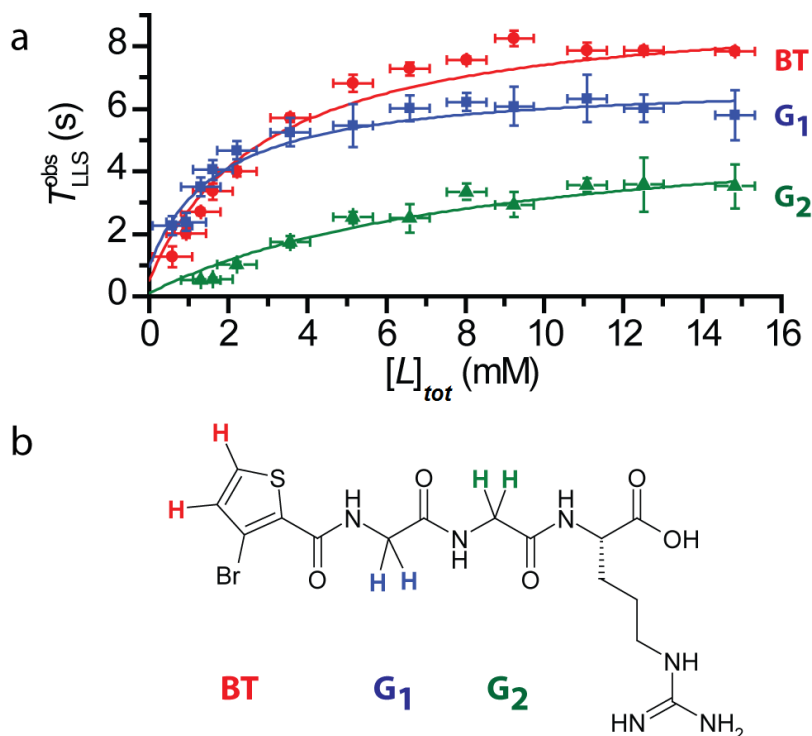
Figure 6 LLS titration experiments. a) Observed LLS lifetimes of the three proton pairs on the spin-pair-labeled tripeptide BT-GGR as a function of the ligand concentration $[L]_{tot}$, in the presence of 25 µM trypsin in D_2O at 25 °C and 11.7 T. b) Pairs of protons capable of sustaining LLS in BT-GGR: on bromothiophene BT (red), on the N-terminal glycine G_1 (blue) and on the central glycine G_2 (green).

The functionalized ligand can be used as spy molecule in competition experiments, once the dissociation constant K_D^{spy} of the spin-pair labeled spy ligand and its LLS lifetime in the bound form T_{LLS}^{bound} are known. It is possible to optimize the competitor $[L_{comp}]_{tot}$ and protein $[P]_{tot}$ concentrations to rank strong competitors according to their binding strengths. Figure 7 shows the calculated T_{LLS}^{obs} (BT) of the bromothiophene protons in BT-GGR if $[P]_{tot}$ = 25 µM and $[L_{comp}]_{tot}$ = 50 µM as a function of the dissociation constant K_D^{comp} of the competitor. Under these conditions, T_{LLS}^{obs} changes dramatically in the range between K_D^{comp} = 100 µM and K_D^{comp} = 1 µM.
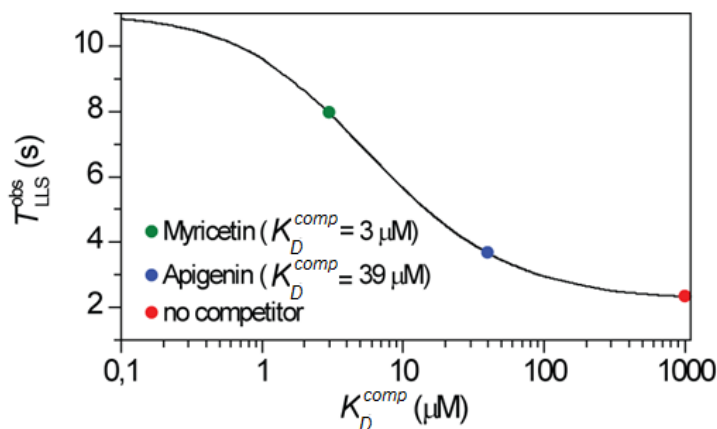
Figure 7 Effect of a competitor on the lifetime T_{LLS}^{obs} of a spy molecule. The lifetime of the pair of aromatic protons of bromothiophene (BT) depends on the dissociation constant of the competing ligand, calculated using equations 6, 7 and 8. The parameters of the spy molecule BT-GGR were obtained from the fit of the data in figure 6: K_D = 0.2 mM, T_{LLS}^{bound} = 0.1 s, T_{LLS}^{free} = 11 s, $[L_{spy}]_{tot}$ = 0.5 mM, $[P]_{tot}$ = 25 µM, and $[L_{comp}]_{tot}$ = 50 µM. The three points correspond to T_{LLS}^{obs} in the presence of myricetin (K_D^{comp} = 3 µM, green), apigenin (K_D^{comp} = 39 µM, blue) and in the absence of any competitor (red) calculated for these conditions.

A library of competing ligands can thus be ranked according to their affinities by observing the LLS signal of a spy ligand. Under the conditions shown in figure 7, one can easily rank competing ligands with great accuracy, provided 1 µM < K_D^{comp} < 100 µM. The ranking of ligands can be achieved by performing LLS experiments with a single sustaining delay τ_m. As the observed relaxation rate R_{LLS}^{obs} of the spy ligand is attenuated in the presence of a stronger competitor, the LLS signal intensity of the spy ligand after a suitably chosen delay τ_m will be enhanced. For instance, LLS spectra of 0.5 mM BT-GGR were recorded with τ_m = 3 s in the presence of $[P]_{tot}$ = 25 µM trypsin with four different competitors, all with $[L_{comp}]_{tot}$ = 50 µM: myricetin (K_D^{comp} = 3 µM), morin (K_D^{comp} = 30 µM), apigenin (K_D^{comp}

= 39 µM) [34] and benzamydine (K_D^{comp} = 39 µM).[31] Figure 8a shows three of the five LLS spectra, obtained either without competitor (red), with apigenin (blue), or with myricetin (green). Figure 8b shows the signal intensities of the spy ligand BT-GGR in the presence of one of the four competing ligands.

Note that the spin-lock duration τ_m has to be chosen carefully to tune the experiment to the expected range of affinities. Equation 8 can be used to estimate the apparent dissociation constant of the spy molecule $K_D^{spy,app}$, and equations 7 and 6 then allow one to estimate the relaxation rate R_{LLS} of the spy molecule in the presence of a competitor. Once this relaxation rate is known, the spin-lock duration can be set to maximize the difference between the signals of the spy molecule with and without competitor.
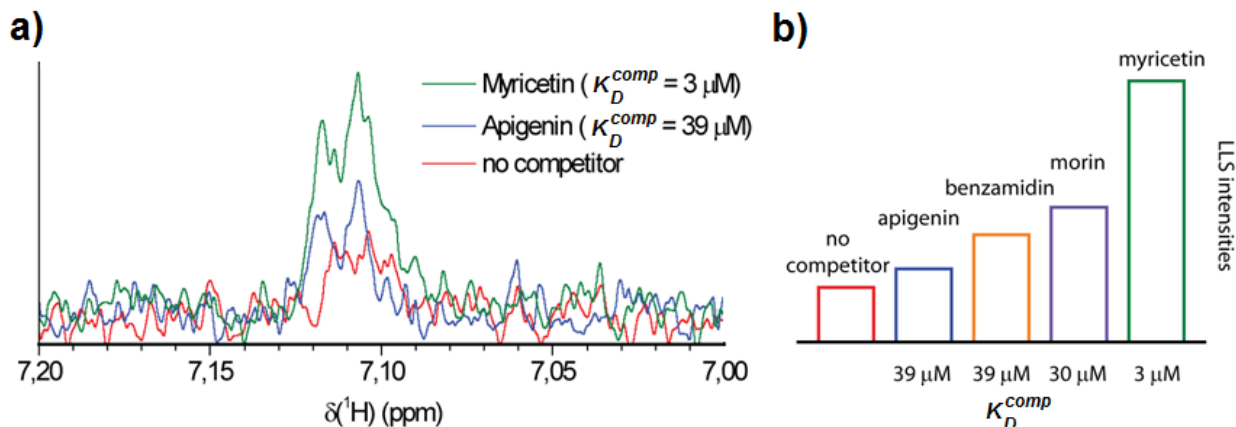
Figure 8 LLS competition binding experiments. a) Signals of one of the two aromatic protons of bromothiophene (BT) in the spy ligand BT-GGR at a concentration of 0.5 mM in the presence of 25 µM trypsin, sustaining the LLS for τ_m = 3 s in D$_2$O at 25 ºC and 11.7 T: 1) in the absence of any competitor (red), 2) in competition with 50 µM of the intermediate ligand apigenin (blue), and 3) in competition with 50 µM of the stronger ligand myricetin (green). b) Peak intensities of one of the aromatic protons of BT-GGR under the same conditions as in a), without competitor and in the presence of apigenin, benzamidine, morin, or myricetin. The better the binding, the smaller the dissociation constant K_D^{comp}, the more effective the displacement of the spin-pair-labeled spy ligand BT-GGR, and the more intense its LLS signal.

4.4 Hyperpolarized LLS ligand screening experiments

I t is obviously desirable to use low concentrations of both proteins and ligands, not only to save expensive materials, but also to avoid protein and ligand aggregation and to be able to study poorly soluble ligands. The ligand concentrations cannot be very low because of the intrinsic poor sensitivity of NMR. At ligand concentrations $[L]_{tot}$ < 100 µM, NMR spectra require extensive signal averaging to show sufficient signal-to-noise ratios (SNR).

Hyperpolarization of nuclear spins by dissolution dynamic nuclear polarization (D-DNP) [35] can overcome this problem. By microwave irradiation of samples containing radicals at temperatures close to T = 1.2 K, the polarization of electron spins can be transferred to protons or other nuclei, followed by rapid dissolution of the hyperpolarized samples and their transfer to a high-resolution NMR spectrometer for detection. Enhancements ε_{DNP} up to five orders of magnitude can be obtained for nuclei with low gyromagnetic ratios, while enhancements 100 < ε_{DNP} < 1000 can be achieved for ^{1}H or ^{19}F nuclei.[36] The technique has not been very popular for ^{1}H and ^{19}F nuclei so far, because rapid T_1 relaxation tends to cause losses of polarization during the transfer from the polarizer to the spectrometer.

Ligands with covalently attached spin-pair labels such as BT-GGR contain protons with long T_1 values and are therefore suitable for hyperpolarization by dissolution DNP. Indeed, provided $T_1(^1$H) > 1 s, a sufficient fraction of the hyperpolarized magnetization can be preserved during the transfer from the DNP polarizer to the NMR spectrometer.

In our laboratory, BT-GGR has been hyperpolarized by DNP and used as a spy ligand in LLS competition binding experiments.[33] We shall give a brief protocol for these experiments. In a glass-forming solvent mixture H_2O/D_2O/DMSO-d6 (*v*/*v*/*v* = 5:35:60), 10 mM BT-GGR are dissolved with 25 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL). Five frozen beads (5 x 10 µL) of this solution are loaded together with five frozen beads (5 x 10 µL) of 3 M ascorbate [37] into a home-built DNP polarizer [38, 39] operating at B_0 = 6.7 T and T = 1.2 K. The sample is irradiated with microwaves at a frequency f_{mW} = 188.3 GHz and power P_{mW} = 100 mW. At B_0 = 6.7 T, a proton polarization up to $P(^1$H) = 90 % can be obtained [39], while $P(^1$H) is only ~ 40 % in polarizers operating at B_0 = 3.35 T. After ~ 15 min of microwave irradiation, a steady-state proton $P(^1$H) can generally be reached.

The DNP sample can be dissolved rapidly in 0.7 s with 5 mL of hot D_2O (P = 1 MPa, T = 400 K) and transferred to a 11.7 T NMR spectrometer in 4.5 s through a "magnetic tunnel"

so that $B_0 > 0.8$ T during the transfer, [40] which is particularly important to preserve the polarization of ^{1}H and ^{19}F nuclei.[41] A fraction of 400 µL of the hyperpolarized solution is then injected in ~ 2 s into a 5 mm tube waiting in the NMR spectrometer containing 250 µL D$_2$O, 3.65 µM trypsin and 3.65 µM of a competitor such as myricetin. After injection and concomitant dilution, the sample has a concentration of 1.4 µM trypsin, 1.4 µM competitor, and 120 µM hyperpolarized spy ligand BT-GGR. After a 3 s interval to allow for proper mixing, a reference free induction decay is observed in 0.5 s after exciting transverse magnetization with a single 5⁰ pulse to control the quality of the hyperpolarized sample and to normalize the signal intensity of the spy ligand with respect to its known concentration. This is immediately followed by the LLS sequence described in chapter 3, using a fixed sustaining time τ_m = 3 s.
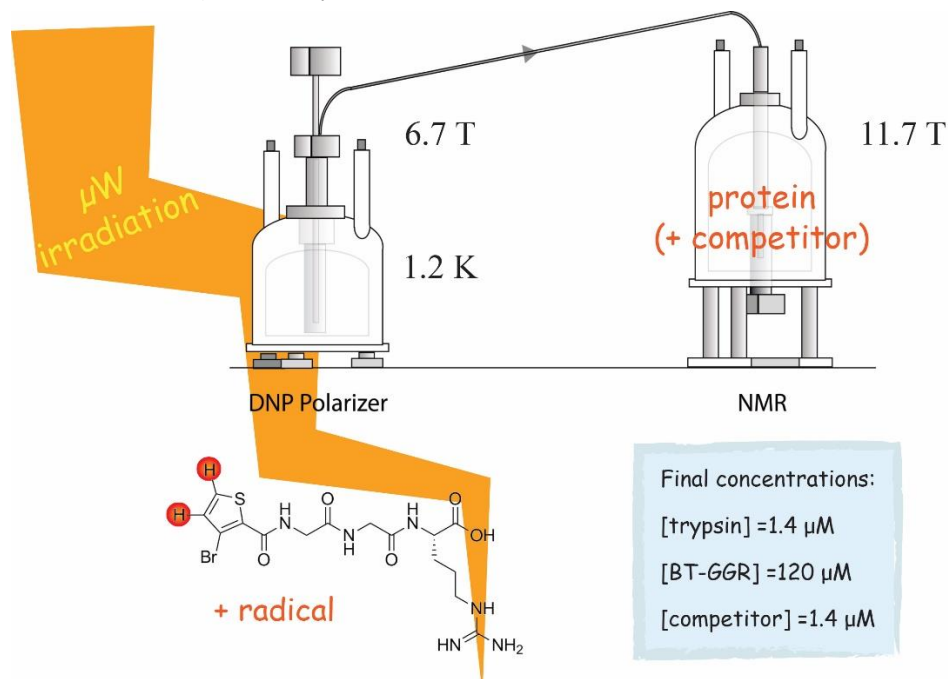
Figure 9 DNP setup to perform hyperpolarized LLS screening experiments. A sample containing the spy ligand BT-GGR and TEMPOL is initially cooled down to 4.2 or 1.2 K. By microwave irradiation, the electron spin polarization is transferred to the protons. The frozen sample is then rapidly dissolved with hot solvent (usually D$_2$O) and transferred to a conventional high-resolution spectrometer, where the LLS experiment can be recorded after a delay of a few seconds to allow proper mixing of the solution.

Figure 10 shows DNP-enhanced LLS spectra of (i) 120 µM of the spin-pair-labeled spy ligand BT-GGR in the absence of protein, (ii) the same with 1.4 µM trypsin, and (iii) the same with 1.4 µM trypsin and 1.4 µM myricetin as competitor. A dramatic decrease of the

LLS signal intensity stemming from BT-GGR is observed in the presence of trypsin. An equimolar amount of the competitor myricetin leads to a partial displacement of the spy ligand that can be readily detected through the revival of its LLS signal. With only 120 µM of BT-GGR, the DNP-enhanced LLS spectrum of figure 10 recorded in a single scan after τ_m = 3 s has a signal-to-noise ratio of 130. Under the same conditions, but without DNP, an accumulation of 225 transients for ~1 h was necessary to reach the same SNR.
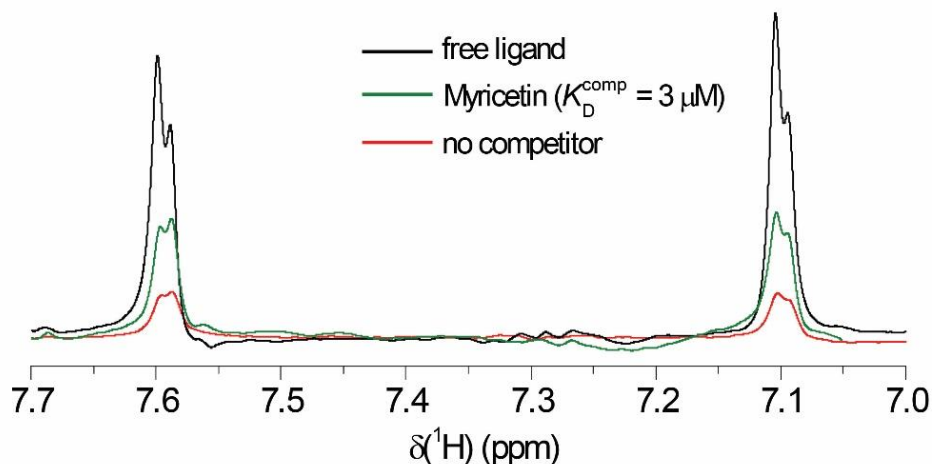
Figure 10 DNP-enhanced LLS competition binding experiments. DNP-enhanced LLS spectra of the two aromatic protons of bromothiophene in 120 µM BT-GGR after a sustaining time τ_m = 3 s, (i) without protein (black), (ii) in the presence of 1.4 µM trypsin (orange), (iii) with 1.4 µM trypsin and 1.4 µM myricetin as competitor (green). All spectra were acquired in a single scan in D₂O at 25 ºC and 11.7 T.

A DNP-enhanced LLS spectrum of BT-GGR with a concentration as low as 10 µM could be recorded with a SNR of 16. Clearly, DNP allows one to decrease the concentration of ligands, but the protein concentration should not be decreased further. Indeed, lower protein concentrations would diminish the observed contrast. Figure 11 shows the protein concentration $[P]_{tot}$ necessary to obtain a satisfactory contrast C_{LLS} = 50 %, depending on the ligand concentration, for different dissociation constants. If K_D = 200 µM, a protein with cannot be diluted to nanomolar concentrations without sacrificing contrast. It is clear that when $[L]_{tot} < K_D$, it is does not make sense to decrease the protein concentration further, since this leads to a loss of contrast C_{LLS}.

Without DNP, using a 50-fold increase in ligand concentration (figure 8), 256 transients had to be accumulated in 100 min to obtain a SNR of 8. The experimental conditions can be adapted depending on the primary objective: low concentrations of either protein or ligand, rapid throughput, high sensitivity for the displacement by a competitor, or high SNR. In figure 10, the conditions were optimized for high SNR and high contrast upon

addition of a competitor, albeit at the expense of a slightly higher ligand concentration and longer polarization build-up time. To attain faster throughput, one could polarize at a higher temperature T = 4.2 K and B_0 = 6.7 T, where proton polarization $P(^1H)$ = 25 % can be reached by DNP in ~ 2 min.[39] The price to pay would be an approximate three-fold lower SNR. Similarly, at T = 1.2 K and B_0 = 3.35 T, as in commercially available DNP polarizers, $P(^1H)$ = 40 % can be reached in ~ 6 min.[32]
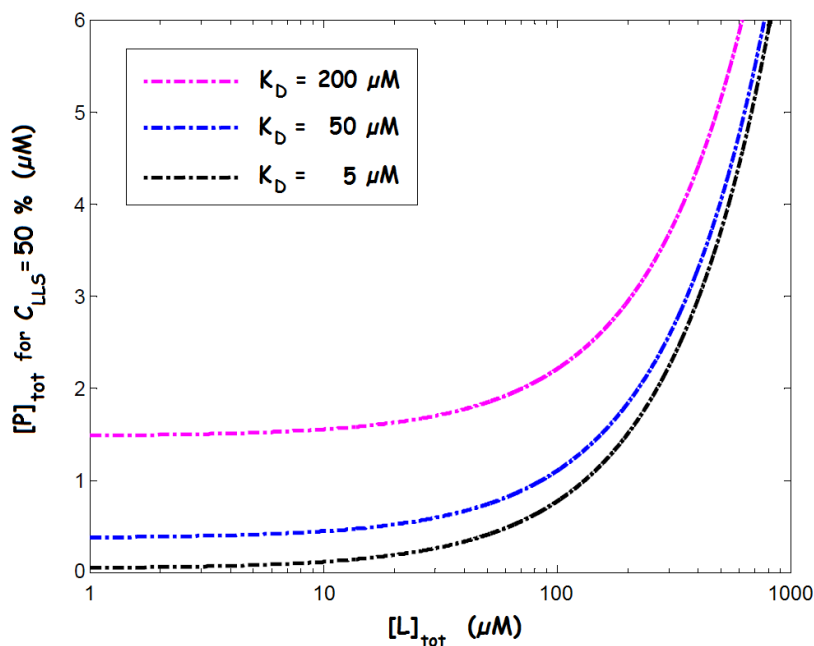
Figure 11 Protein concentrations required to obtain a 50 % LLS contrast for different ligand concentrations. When $[L]_{tot} < K_D$, the required protein concentration to obtain a sufficient contrast approaches a plateau. The decrease of the protein concentration below the plateau leads to a drop of contrast. These simulations are based on equations 6 and 7.

With the set-up used to perform these experiments, the time required for the transfer from the polarizer to the detection magnet is similar to $T_1(^1H)$ of the spy ligand BT-GGR. A significant fraction of the proton hyperpolarization is lost during the 10 s interval between dissolution and signal acquisition. Nevertheless, a faster sample injection device [41] could decrease this interval to 1.2 s. An acceleration of the transfer would enhance the remaining proton polarization and thus the SNR. Such improvements would allow either a further decrease in ligand concentration or an increase in sample throughput.

After dissolution, the sample temperature could not be monitored in our experiments. Nevertheless, one can assume that during the "voyage" through the magnetic tunnel, the hyperpolarized solution reaches room temperature. In any case, potential temperature

drifts of the final solution after dissolution should not affect the ranking of competitors, since the temperature should affect the restoration of the signals of the spy molecule in a uniform and reproducible manner.

4.5 Exploring weak ligand-protein interactions by LLS

I n fragment-based drug discovery (FBDD), fragment screening is performed using relatively small libraries of carefully chosen compounds with low molecular weights (120-250 Da). Useful fragments typically have dissociation constants K_D ranging from 0.1 to 10 mM or greater. Techniques that can detect ligand-protein complexes, such as X-ray crystallography, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and high-concentration assays can be used for fragment screening. The output of these target-based methods depends on the fraction of bound protein with respect to the total protein concentration.[42] If the binding affinities are weak, the equilibrium can only be shifted by increasing the concentration of the fragments, which must therefore be highly soluble, a requirement that is difficult to meet.

In ligand-based methods the output is given by the fraction $p_B = [PL]/[L]_{tot}$ of bound ligands with respect to the total ligand concentration.[42] So despite its low intrinsic sensitivity, the detection of ligands by NMR spectroscopy can be used over an extremely wide dynamic range of dissociation constants K_D while requiring only relatively low protein and ligand concentrations. In contrast to the above-mentioned biophysical techniques, NMR allows one to perform screening with ligand concentrations $[L]_{tot}$ that are orders of magnitude lower than the corresponding dissociation constants K_D.
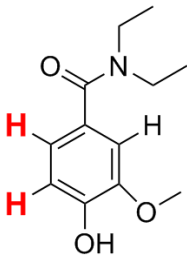
As has been shown in chapter 2, the fraction p_B of ligand in the bound form depends on the affinity of the ligand. In particular, for a given ligand $[L]_{tot}$ and protein concentration $[P]_{tot}$, the larger the dissociation constant, the lower the fraction of ligand in the bound form p_B (see figure 2, chapter 2). As consequence, the detection of weak interactions turns out to be challenging.

Equation 4 shows that, for a fixed value of p_B, the ability of detecting the binding event depends on the difference $(\varepsilon_B - \varepsilon_F)$ between the values of the parameter ε in the free and bound forms. In the case of long-lived states, the difference $\Delta R_{LLS} = (R_{LLS}^{bound} - R_{LLS}^{free})$ can be much larger than ΔR_1, ΔR_2, etc., so that it is possible to achieve a high contrast C_{LLS} even for high ligand/protein ratios, making LLS-based screening particularly attractive for a fragment-based approach that seeks to identify weakly binding ligands.

4.5 Exploring weak ligand-protein interactions by LLS

Figure 12 shows mole fractions p_B of bound ligands for different ligand/protein ratios and the corresponding contrast C_{LLS} during a titration of vanillic acid diethylamide against the N-terminal ATPase domain of heat shock protein 90 (Hsp90). Long-lived states were excited on the two aromatic protons of the ligand. Even for a large ligand-to-protein ratio $[L]_{tot}/[P]_{tot}$ = 272, one observes a dramatic 45 % contrast.

A contrast C_{LLS} = 23 %, corresponding to a ratio R^{obs}/R^{free} = 1.3, can be achieved with a ratio $[L]_{tot}/[P]_{tot}$ = 707, *i.e.*, under conditions where less than 0.2 % of the ligand is bound to the protein. Compared to other ^{1}H-detected NMR methods, which suffer from lower contrast, this allows ligand binding to be detected for low protein concentrations and/or low binding affinities. One can thus more easily customize the concentrations of proteins and ligands to study very weak affinities in screening assays. For example, to detect ligands with K_D ≤ 1 mM and [L] = 500 µM, one would require a protein concentration [P] = 3 µM; alternatively, if [P] = 20 µM one can detect binding even if K_D > 10 mM. Such weak affinities are typically encountered for fragments that bind to protein-protein interfaces. This offers considerable advantages over fragment screening by traditional ligand-based NMR methods.

$[L]_{tot}/[P]_{tot}$	X_b / mol %	Contrast C_{LLS} / %
56	0.74	72
125	0.49	63
202	0.36	54
272	0.28	45
366	0.22	41
548	0.16	29
707	0.13	23

Vanillic acid diethylamide

Figure 12 (Left) Structure of vanillic acid diethylamide. The pair of aromatic protons that is suitable for the excitation of LLS is indicated by bold red letters. (Right) Molar fractions of bound ligands for different ligand/protein ratios and the experimentally observed contrast C_{LLS} for a titration of vanillic acid diethylamide (K_D = 790 µM) in the presence of the protein Hsp90.

LLS screening is most effectively carried out in competition mode, as proposed by Dalvit et al. [29] for traditional R_1 and R_2 experiments: a strongly binding ligand partly displaces a weakly binding 'spy' ligand from the binding site, so that one observes a decrease of the relaxation rate R_{LLS} of the displaced spy ligand.

Obviously, the search for weak binders by a competition approach is a difficult task, since the spy ligand has to be displaced by a weaker competitor. Nevertheless, it has been demonstrated that LLS can efficiently reach this goal. Vanillic acid diethylamide (K_D = 790 µM) has been used as spy ligand in LLS competition screening experiments against Hsp90.[43] The performance of LLS screening in competition mode with a mixture containing known binders and known non-binders has been tested. Indeed, if a library of, say, 1000 compounds is to be screened against a protein target, it is most efficient to screen 'cocktails' containing typically 3-10 ligands, to reduce experimental time and protein consumption. In the absence of competing binders, the interaction between the spy ligand and the protein leads to rapid LLS relaxation and hence to the attenuation of the LLS signal (spectrum 1 in Figure 13); conversely, the presence of a competitor leads to a partial displacement of the spy ligand, hence to slower LLS relaxation and a partial restoration of the LLS signal of the spy (spectrum 2 in Figure 13). This change in LLS signal is due to a mere 13 % change in the amount of bound ligand, which itself is only 0.3 % of the total ligand concentration.

Once the presence of a binder in a mixture has been demonstrated, a deconvolution step is needed to identify the hit, as shown in Figure 13 (spectrum 3), which allowed the identification of 3-hydroxyindazole as a weak binder for Hsp90.

Note that to displace a weak spy ligand by fragments that are binding even more weakly, the latter must be present at similar concentrations. This is a considerable advantage over other NMR methods used in competition mode, which because of lower contrast require much higher concentrations to achieve effective displacements, as they require small ligand/protein ratios to detect weak ligands. Furthermore, if the mixtures comprise many components, the NMR resonances of the spy molecule may be obscured by overlapping signals [44], which may hamper all NMR methods when used in competition mode. Fortunately, the LLS sequence in effect eliminates signals that do not stem from long-lived states. As shown by spectrum 2 in Figure 13, resonances that arise from other compounds are considerably reduced, compared to the conventional ^{1}H spectrum of the same mixture (spectrum 4).
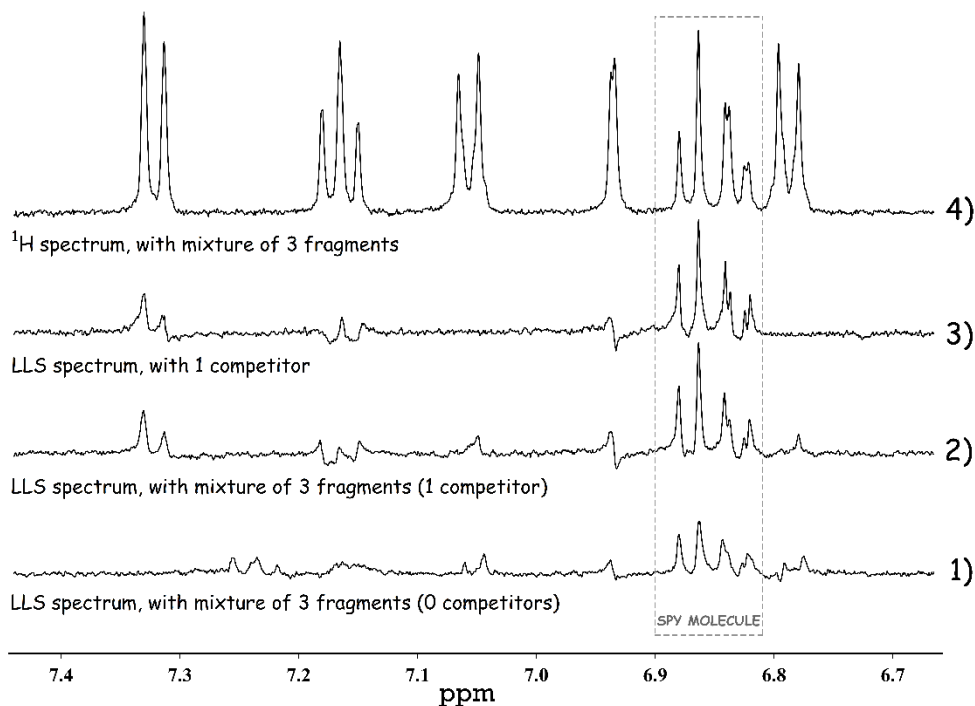
Figure 13 Identification of a weak binder in a mixture. (1) Weak LLS signals of the spy ligand after sustaining the LLS for τ_m = 2.5 s in the absence of any competing binder in mixture 1 ($[L_{spy}]_{tot}$= 500 µM with K_D = 790 µM, protein [Hsp90] = 2.5 µM and three non-binding ligands: 600 µM tyrosine, 600 µM 3,4-difluorobenzylamine and 600 µM 4-trifluoromethyl-benzamidine). (2) Enhanced LLS signals in the presence of a weak binder (mixture 2 contains 600 µM of the weakly binding ligand 3-bromo-5-methyl-pyridin-2-ylamine (K_D= 2.2 mM) instead of 600 µM of the non-binding ligand 3,4-difluorobenzylamine). (3) LLS signals observed in the presence of only the binding fragment (mixture 3 contains 500 µM spy ligand, 2.5 µM protein [Hsp90], and 600 µM of the weakly binding ligand 3-bromo-5-methyl-pyridin-2-ylamine). (4) Conventional ^{1}H spectrum of mixture 2.

Once weak binders have been identified, their dissociation constants K_D can be determined from $K_D^{spy,app}$ upon titration of the spy ligand in the presence of a constant amount of a weak binder or *vice versa*.[29] Titration of a spy ligand allows one to keep the same experimental set-up for different fragments. The highest concentrations of the competing ligands are limited only by their solubility. At each concentration, the rates R_{LLS} can be obtained from the ratio of the LLS signal intensities observed with two different sustaining delays τ_a and τ_b. The affinity of 2-amino-pyrimidine was determined with 10 µM Hsp90, using a fixed concentration [2-amino-pyrimidine] = 7 mM, and by titrating 500 µM < $[L_{spy}]$ < 5 mM. The measured dissociation constant K_D = 11 ± 2 mM of 2-amino-pyrimidine suggests very weak binding of this ligand to the protein. Binding must be specific to explain these observations. The fragment bound to Hsp90 was also observed by X-ray crystallography by Murray et al.[45]
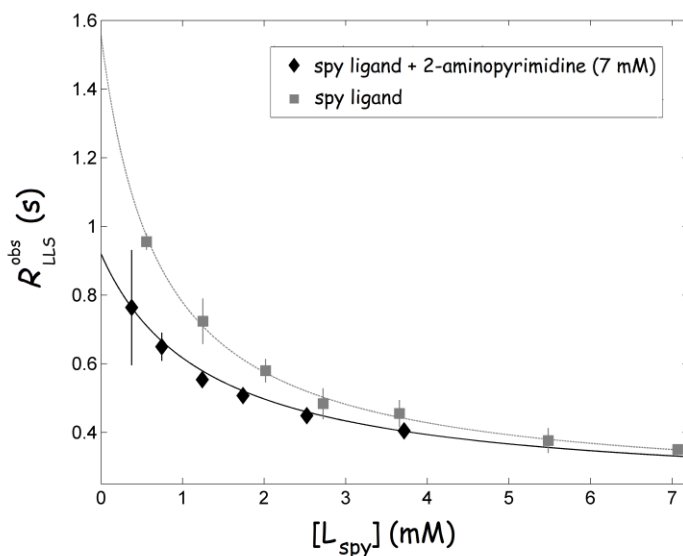
Figure 14 (Grey line) Direct titration of the spy ligand vanillic acid diethylamide in the presence of 10 µM Hsp90 protein. (Black line) Competition experiment with titration of vanillic acid diethylamide as a spy ligand in the presence of 10 µM Hsp90 protein and a constant concentration of 7 mM of 2-amino-pyrimidine.

Note that the choice of the spy molecule determines the experimental conditions of the LLS assay. With a spy molecule such as vanillic acid diethylamide (K_D^{spy} = 790 µM), a concentration of 7 mM of the competing 2-amino-pyrimidine (K_D = 12 mM) gives rise to 19% contrast (first point of black curve in Figure 14). This can be reduced to 3.3 mM to give rise to a 10% contrast, which is sufficient to show binding in screening experiments, as shown in Figure 13 for 3-hydroxyindazole (difference between spectra 1 and 2). If the expected affinities of fragments for a particular target are on the order of K_D = 5 mM or higher, it is most convenient to identify and use a weaker spy molecule that would ensure a 10% contrast while working at lower fragment concentrations. As a consequence, one can effectively screen and identify weak fragments with very low solubility.

The ability of measuring accurate binding constants in the mM range, where methods such ITC and high concentration assays may fail, in particular when the ligand solubility is limited, enables the investigation of structure-activity relationships and the guidance of initial steps of hit optimization chemistry.

4.6 Extending LLS ligand screening to ¹⁹F nuclei

In competition experiments, the concentration of the competitor that is required to displace the spy molecule is inversely proportional to the former's affinity for the macromolecular target: the higher the affinity, the lower the concentration needed. The study of weakly binding fragments turns out to be challenging since high concentrations and thus high solubility are required. Moreover, if mixtures of competitors are tested, the risk of signal overlap must be circumvented by a careful choice of the cocktail of molecules.

The excitation of LLS involving pairs of ¹⁹F nuclei belonging to spy ligands that have been designed to feature a favorable contrast C_{LLS} between free and bound forms allows one to study weak protein-ligand interactions while avoiding signal overlap. ¹⁹F detection offers several advantages [46] over ¹H detection: a) high sensitivity, since ¹⁹F has a high gyromagnetic ratio and 100% natural abundance; b) absence of overlap with protonated solvents, buffers or detergents; c) absence of overlap with other molecules if experiments are performed on chemical mixtures with many components; d) high sensitivity of transverse R_2 relaxation of ¹⁹F to binding, as seen in chapter 2.

Long-lived states have so far only been observed in systems comprising ¹H, ¹³C or ¹⁵N nuclei.[47] We have synthetized a molecule that contains a pair of diastereotopic aliphatic fluorine atoms: 1,1-difluoro-1-phenylacetyl-Gly-Arg, abbreviated as DFPA-GR (figure 15). The presence of an arginine residue assures a weak binding affinity for the active site of trypsin [21], thus allowing one to explore the behavior of LLS of pairs of ¹⁹F nuclei upon binding. Aliphatic fluorine atoms have been preferred to aromatic ones, in order to minimize LLS relaxation due to CSA contributions. In fact, amino acids with ¹⁹F-labels in aliphatic positions have shown smaller CSA values than in aromatic compounds with ¹⁹F substituents on the ring. [48, 49] [48, 49] [48, 49]
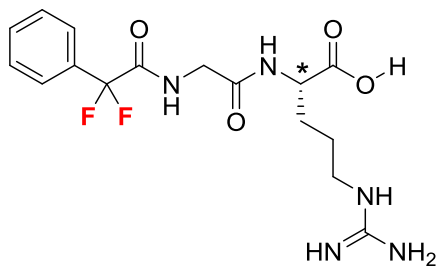
Figure 15 Structure of DFPA-GR that contains a pair of aliphatic diastereotopic fluorine atoms (red), which are magnetically inequivalent because of the vicinity of a chiral center (*).

No less than seven bonds separate the fluorine nuclei from the closest chiral center (C^α of the arginine residue, indicated by a star in figure 15). The chemical shift difference $\Delta\nu_{IS}$ between the two diastereotopic fluorine nuclei is only 0.8 ppm or $\Delta\nu_{IS}$ = 301 Hz at B_0= 9.40 T (400 and 376 MHz for ^{1}H and ^{19}F, respectively). As consequence, an RF field ν_1= 1.5 kHz is sufficient to sustain the LLS and to achieve T_{LLS}= 2.63 s. In general, $\Delta\nu_{IS}$ can be controlled by varying the distance between the fluorine nuclei and the chiral center. For instance, when the glycine residue is deleted, one observes an increase of the chemical shift difference between the two diastereotopic fluorine nuclei to $\Delta\nu_{IS}$ = 2.05 ppm, thus requiring a *ca.* 2.5-fold increase of the RF field amplitude ν_1 to sustain the LLS. The resulting T_{LLS} = 2.06 s is slightly shorter than the one in DFPA-GR. On the other hand, the insertion of an additional Gly residue would lead to a further reduction of the chemical shift difference $\Delta\nu_{IS}$.

The excitation of LLS for the pair of fluorine nuclei of DFPA-GR was achieved with the pulse sequence described in chapter 3, with the addition of continuous-wave ^{1}H decoupling during the acquisition period. In the absence of protein, the LLS relaxation rate of the ligand was found to be R_{LLS} = 0.38 s^{-1}, while the longitudinal relaxation rate is R_1 = 1.64 s^{-1}, leading to a favorable ratio R_1/R_{LLS} > 4. This shows that it is possible to achieve LLS with sufficiently long lifetimes for pairs of fluorine nuclei.

Of all parameters, the transverse relaxation rate $R_2(^{19}\text{F})$ = $1/T_2(^{19}\text{F})$ is one of the most sensitive to binding phenomena, because $R_2(^{19}\text{F})$ benefits from significant exchange broadening effects.[46] The rates $R_2(^{19}\text{F})$ of the ligand DFPA-GR have been determined in the presence or absence of trypsin. The resulting $R_2(^{19}\text{F})$ contrast lies in the range 32 < C_2 < 40 % for the two diastereotopic ^{19}F nuclei for 370 µM DFPA-GR with 2 µM trypsin (*i.e.*, a 185-fold excess). On the other hand, if we switch our attention to LLS, the contrast C_{LLS} is as large as 87% under the same conditions. This confirms that R_{LLS} is extremely sensitive to binding.

The affinity of DFPA-GR was determined with a fixed concentration of 2 µM trypsin, titrating 400 µM < [DFPA-GR] < 8 mM. The measured dissociation constant K_D(DFPA-GR) = 106 ± 26 µM indicates that the fast exchange condition is easily fulfilled, *i.e.*, k_{ex} = $(k_{on}[\text{P}] + k_{off}) \approx k_{off}$ >> $\Delta\omega$, where [P] is the concentration of the free protein and $\Delta\omega$ = $2\pi\Delta\nu$ is the difference in chemical shifts between the bound and free forms of the ligand. Indeed, the dissociation constant K_D must be equal to the ratio between the kinetic dissociation and association rate constants, K_D= k_{off} /k_{on}, and since the latter is usually assumed to be limited by diffusion ($10^7 < k_{on} < 10^9$ M^{-1}s^{-1}),[4] k_{off} must be in the range 10^3-10^5 s^{-1}.
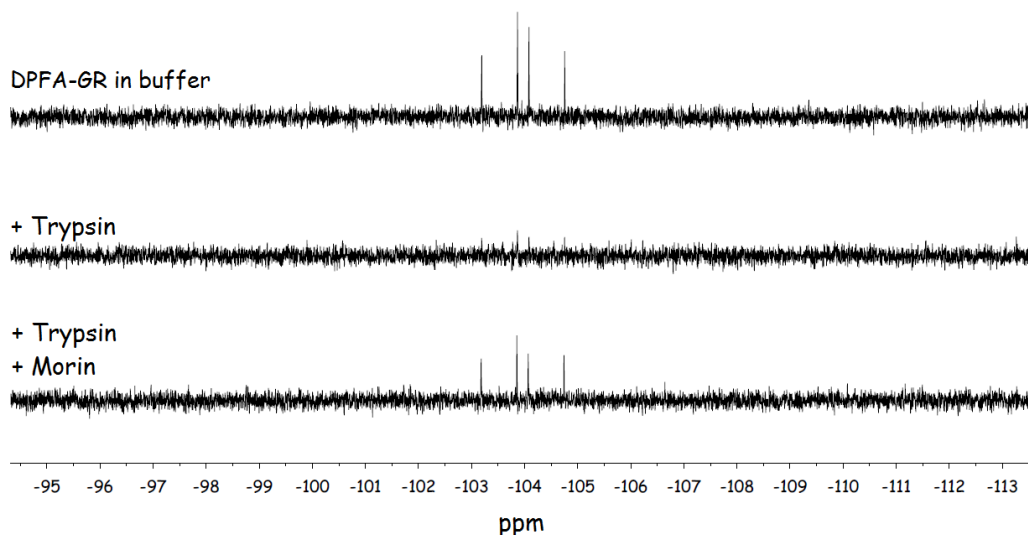
Figure 16 (Top) Signals derived from ¹⁹F-¹⁹F LLS of 500 µM DPFA-GR in the absence of trypsin after τ_m = 0.7 s. (Center) the same in the presence of 2 µM trypsin. (Bottom) in the presence of 2 µM trypsin and 485 µM morin as competitor, which partly displaces DPFA-GR from the binding site of the protein, leading to a partial restoration of its signals. A total of 128 scans were recorded for each spectrum, with acquisition and repetition times of 0.7 and 3 s, respectively.

Once a suitable spy ligand such as DFPA-GR that fulfills the fast exchange regime has been identified, libraries of potential binders can be screened by competition experiments [29] by observing changes in the LLS decay rates of the spy ligand. Figure 16 shows the LLS spectrum of 500 µM DFPA-GR in the absence of protein (top), in the presence of 2 µM trypsin (center) and with 2 µM trypsin plus 485 µM morin, a well-known trypsin inhibitor [50] (bottom). Since a competitor like morin partly displaces the spy molecule from the binding site of the protein, its presence leads to a partial restoration of the LLS signals of the spy ligand.

Once a competitor has been identified, its dissociation constant needs to be determined. To do so, the spy DFPA-GR can be titrated in the presence of a constant concentration of competitor or *vice versa* [29]. The curve of T_{LLS} vs. $[L_{spy}]$ can be fitted using equation 6 to extrapolate an apparent dissociation constant $K_D^{spy,app}$. The dissociation constant K_D^{comp} of the competitor can be calculated from the knowledge of $K_D^{spy,app}$ using equation 8.
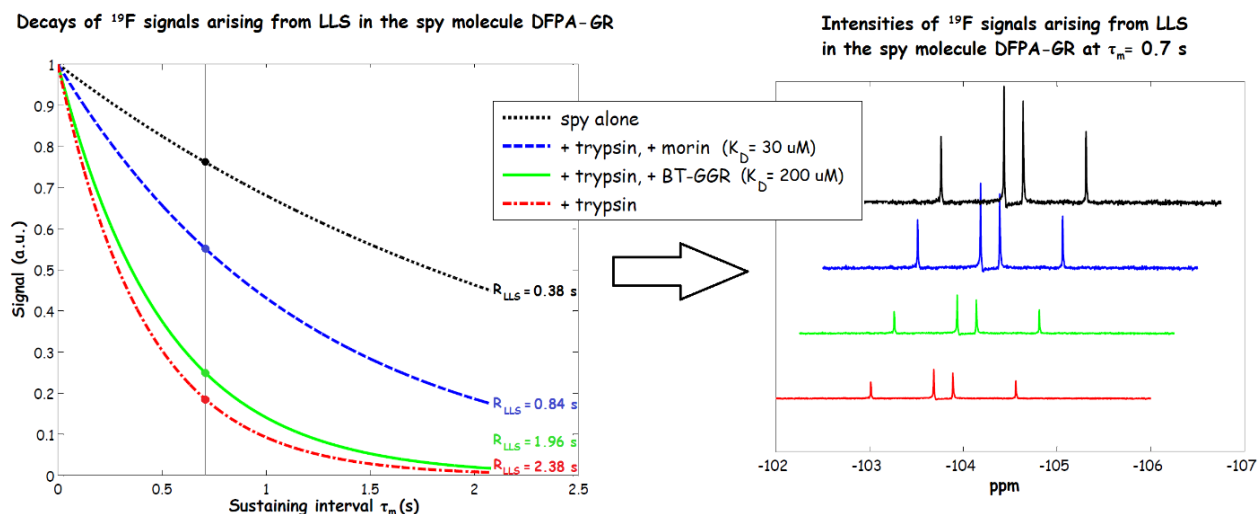
Figure 17 (left) LLS decays of 495 µM DFPA-GR (black) in the absence of protein and competitor, (blue) in the presence of 2 µM trypsin and 500 µM morin, (green) in the presence of 2 µM trypsin and 500 µM BT-GGR and (red) in the presence of 2 µM trypsin. The exponential decays in the figure are derived from experimental R_{LLS} values; (right) LLS intensities of the spy DFPA-GR in the four solutions described above, after sustaining for τ_m= 0.7 s.

A quick estimate of K_D^{comp} can be obtained from a single titration point: knowing R_{LLS}^{obs}, the mole fraction p_B of the spy molecule in its bound form can be estimated through equation 6. At this point, $K_D^{spy,app}$ can be calculated by rearranging equation 7. Following this approach, the K_D^{comp} values [33] have been estimated to be K_D^{comp} = 28 µM for morin and K_D^{comp} = 250 µM for BT-GGR, in reasonable agreement with values reported in the literature (30 and 200 µM).[33, 34] This shows that competition screening experiments and quick estimates of the affinities of competitors can be performed on the same sample. More accurate K_D^{comp} measurements require full titration experiments.
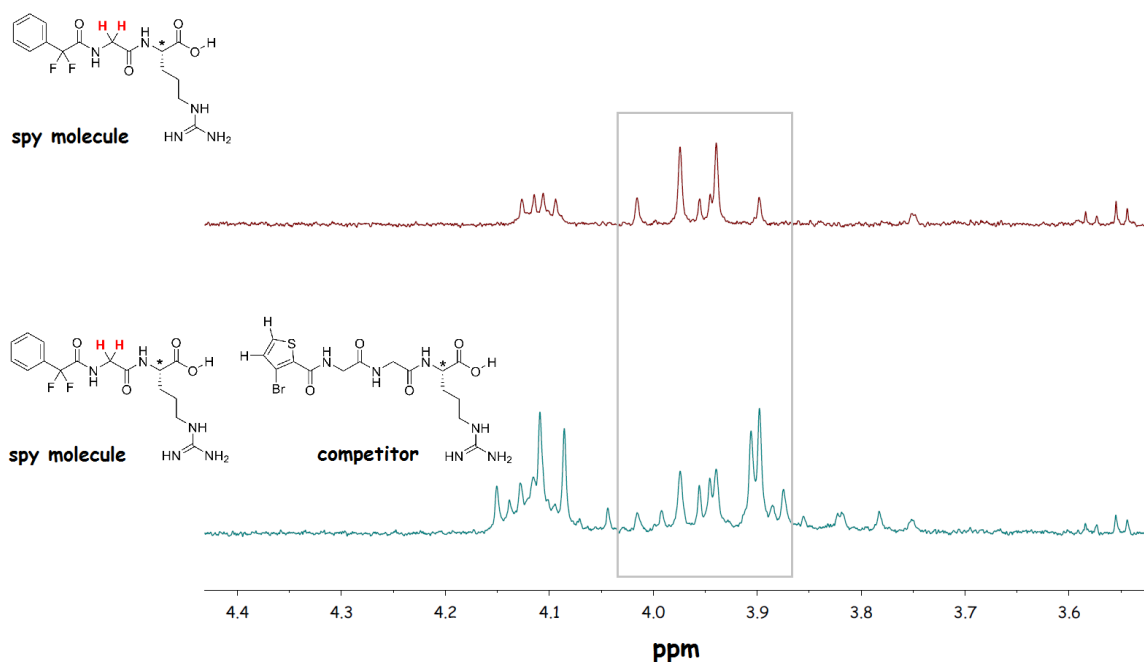
Figure 18 (top) Proton spectrum of 495 µM DFPA-GR (spy); (bottom) Proton spectrum of 495 µM DFPA-GR and 500 µM BT-GGR. The grey square shows the range where the signals of the α-protons of the glycine residues occur.

Often, screening is performed by testing mixtures (also known as "cocktails") of 3-10 putative competitors, in order to reduce experimental time and minimize protein consumption. If one observes proton signals, such experiments need a careful choice of the mixtures in order to avoid overlap of signals of the putative ligands with those of the spy molecule. For example, figure 18 shows a comparison between the ¹H spectra of the spy molecule DFPA-GR alone and mixed with BT-GGR, a competitor with a similar molecular structure. The overlap between the proton signals of the spy molecule and those of the competitor are too severe to allow one to perform any competition screening experiments. The problem can become even more severe when looking for weak binders. Indeed, the larger the dissociation constants, the higher the required concentrations of the competitors. In this context, ¹⁹F NMR has no rivals. It allows one to perform experiments with high sensitivity while avoiding problems of overlap with protonated buffers and signals of mixtures. The combination of the high sensitivity to binding phenomena offered by the LLS method and the lack of overlap in ¹⁹F NMR can be put to good use for fragment-based drug discovery.

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5. Experimental procedures

All the experiments presented in this thesis were performed at 298 K on 500 MHz (11.7 T) or 600 MHz (16.4 T) Avance Bruker spectrometers equipped with 5 mm CryoProbes. The following paragraphs give some details about ligand titrations, competition experiments, fitting of data and synthesis of ligands. Further details can be found in references [1-3].

5.1 Ligand titrations

Ligand titrations, as in figure 4 of Chapter 4, were performed by addition of small aliquots (from 1 to 2 µL) of a concentrated ligand solution (from 150 to 200 mM) to 400 µL of a buffered D_2O solution containing the protein (from 10 to 20 µM). Lifetimes T_{LLS} were obtained by mono-exponential fitting of signal intensities observed with the LLS pulse sequence described in Chapter 3, using 10 different spin-lock durations $0.5 \text{ s} < \tau_m < 5T_{LLS}$. A short-cut can sometimes be used. For example, the relaxation rates R_{LLS} reported in figure 14 were obtained from ratios of the signal intensities observed using only two different sustaining delays τ_a and τ_b, repeating each of them twice in order to compare four pairs of signal intensities $I_a(\tau_a)/I_b(\tau_b)$. In this case, the relaxation rates where obtained from the ratio $R_{LLS} = (\log(I_a/I_b))/(\tau_b - \tau_a)$. Typically, we used τ_a = 0.5 s, while τ_b was chosen in the vicinity of the estimated value of R_{LLS}.

Competition experiments were performed in the same manner. The only difference was the presence of a potential competitor in the protein solution.

Ligand concentrations were carefully measured using the PULCON technique.[4]

5.2 Fitting of titration curves

The experimental data obtained were fitted to equation 6 of Chapter 4 to determine the fraction of ligand in the bound form $[PL]/[L]_{tot}$. Equation 7 was used to determine the dissociation constant K_D.

For competition experiments, equation 7 of Chapter 4 gives the apparent dissociation constant of the spy molecule $K_D^{spy,app}$. Using this value, equation 8 of Chapter 4 gives the dissociation constant of the competitor.

5.3 Hyperpolarized LLS experiments

A detailed protocol for DNP-LLS experiments is described on page 95. Further details are described in reference [2].

5.4 Chemical synthesis of ligands

Two ligands mentioned in this thesis, BT-GGR and DFPA-GR, are not commercially available and were synthetized in our laboratory. Since the synthesis of BT-GGR is described in the supporting information of reference [2], we describe here the preparation of DFPA-GR.

The synthesis of 1,1-difluoro-1-phenylacetyl-Gly-Arg (DFPA-GR) was performed by solid-phase peptide synthesis (SPPS) using 2-chlorotritylchloride resin and Fmoc protected amino acids. The first step is a SN1 substitution of Fmoc-Arg(Pbf)-OH on the resin. All remaining reactive 2-chlorotrityl groups were then capped with MeOH. The molecule was obtained by coupling of Fmoc-protected Gly in the presence of HOBt and TBTU, followed by deprotection of the N-terminus of the dipeptide. Finally, difluoro-phenyl-acetic acid was attached at the N-terminus of the dipeptide. Cleavage from the resin, followed by deprotection of the arginine side chain, afforded DFPA-GR.

N-Fmoc-Arg(Pbf)-O-resin (2): After swelling with dry DCM (80 mL) for 5 min, the 2-chlorotrityl chloride resin (0.83 mmol.g^{-1}, 1 equiv, 1 mmol, 1.2 g) was treated with a solution of Fmoc-Arg(Pbf)-OH (**1**) (1.2 equiv, 1.2 mmol, 0.78 g) in dry DCM (10 mL) and DIPEA (2.5 equiv, 6.23 mmol, 0.81 g) shaken at 125 rpm at room temperature for 2 hrs. The reaction was performed in 4x10 mL filtration tubes with polyethylene fritters. MeOH (10 mL) was added to cap the free sites, and the reaction mixture was shaken for 1. The resin was washed with DCM (3 × 12 mL), DCM/MeOH 1:1 (3 × 12 mL), MeOH (3 × 12 mL), diethyl ether (3 x 12 mL) and dried for 12 hrs *in vacuo* to give the N-Fmoc-Arg(Pbf)-O-resin (**2**).

N-Fmoc-Gly-Arg(Pbf)-O-resin (3): N-Fmoc-Arg(Pbf)-O-resin (**2**) was suspended in a solution of 20% piperidine in DMF (10 mL) for 30 min and shaken to give the N-deprotected resin. The resin was washed with DMF (4 × 10 mL) and DCM (4 × 10 mL). Fmoc-Gly-OH (4 equiv, 4 mmol, 1.19 g), HOBt (4 equiv, 4 mmol, 0.54 g), TBTU (4 equiv,

4 mmol, 1.29 g) and DIPEA (4 equiv, 4 mmol, 0.52 g) were dissolved in DMF (10 mL). The solution was added to the N-deprotected resin. The reaction mixture was shaken at room temperature for 3 hrs to give Fmoc-Gly-Arg(Pbf)-O-resin (**3**) which was washed with DMF (4 × 10 mL).
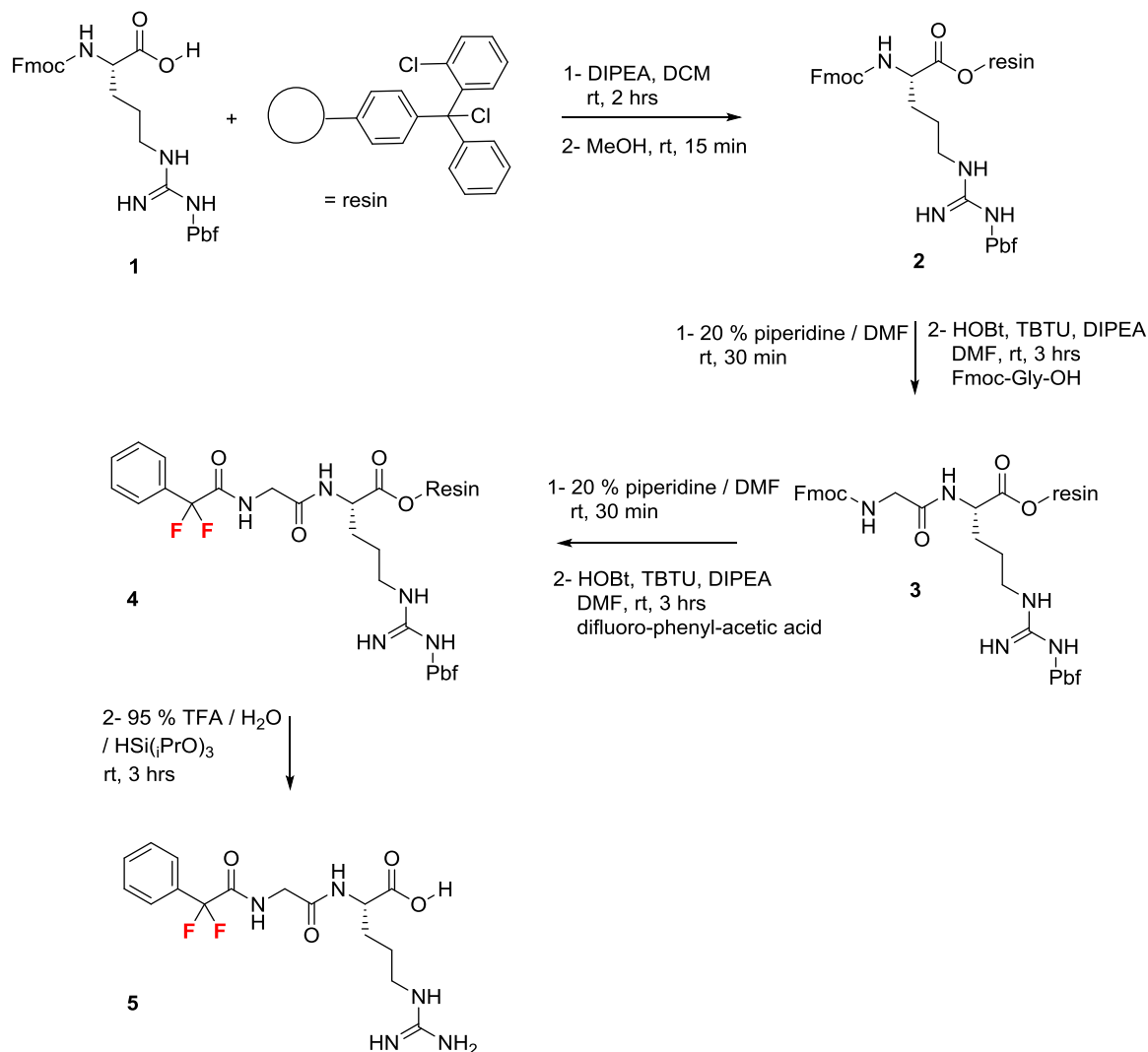
Scheme 1. Synthesis of the ^{19}F labelled ligand for trypsin **5**.

DFPA-Gly-Arg(Pbf)-O-resin (4): Fmoc-Gly-Arg(Pbf)-O-resin (**3**) was suspended in a solution of 20% piperidine in DMF (30 mL) for 30 min and shaken to give the N-deprotected resin. The resin was washed with DMF (4 × 10 mL) and DCM (4 × 10 mL).

5.4 Chemical synthesis of ligands

Difluoro-phenyl-acetic acid (4 equiv, 4 mmol, 0.69 g), HOBt (4 equiv, 4 mmol, 0.54 g), TBTU (4 equiv, 4 mmol, 1.29 g), and DIPEA (4 equiv, 4 mmol, 0.52 g) were dissolved in DMF (30 mL). The solution was added to the N-deprotected resin. The reaction mixture was shaken at room temperature for 3 hrs at 125 rpm to give DFPA-Gly-Arg(Pbf)-O-resin (**4**) which was washed with DMF (4 × 10 mL) and DCM (4 × 10 mL).

DFPA-Gly-Arg (5): Cleavage from the resin and deprotection of the side-chain was carried out with 10 mL TFA:H_2O:TIS (95:2.5:2.5) for 3 hrs. TFA was then removed by evaporation and the final product (**5**) was obtained after lyophilization.

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6. Conclusions

The drug discovery and development process is a very long and expensive pathway. It can take 12-15 years from the first, preliminary tests to the final approval for marketing.

In the first stage of drug discovery, screening campaigns are usually performed in order to identify a "hit compound", which may bind to a macromolecular target and alter the course of a disease. NMR offers a rich source of parameters that are sensitive to changes in physical properties associated with binding. As consequence, a great variety of NMR methods have been developed to perform screening experiments.

Long-Lived States (LLS) are nuclear spin states whose decay time constant T_{LLS} can be much longer than the longitudinal relaxation time T_1. The goal of the present work was to develop a new NMR strategy to study the interactions between ligands and proteins, by exploiting some peculiar properties of the LLS.

In the regime of fast exchange on the NMR time scale, i.e., when the exchange between the free and bound forms of a weak ligand is fast compared to the difference of their resonance frequencies, the observable relaxation rate of a long-lived state associated with a weak ligand results from a weighted average of the bound and free forms. As described in Chapter 4, the contrast between the averaged LLS relaxation rate and the LLS relaxation rate of the free ligand is proportional to the difference of the relaxation rates of the free and the bound forms. Because of the long lifetimes of LLS in the free form and the boosted LLS relaxation in the bound state, this difference is particularly large, making the relaxation of long-lived states one of the most sensitive NMR parameters to ligand-protein binding.

LLS have been successfully excited in glycine residues in short polypeptides in order to quantify their affinities for protein targets. The use of competition experiments allowed the study of binders which do not contain any spin system that can sustain a long-lived state. Whenever there is no ligand that can carry a long-lived state, the functionalization of a binder with a spin-pair label can provide a molecule that can be used as reporter in LLS competition experiments. This strategy was used to synthetize a ligand containing a bromo-thiophene group, which can carry an LLS on its two aromatic protons. The relatively long T_1 of these spins allowed us to perform a dissolution DNP experiment, where the functionalized ligand was hyperpolarized, transferred to a conventional NMR spectrometer and used as LLS reporter in competition experiments. This strategy allows

one to perform screening experiments with high contrast while working with low concentrations of ligand and proteins.

The superior sensitivity of the LLS method can be exploited in order to quantify the affinity of a weakly binding fragment to a protein target. By exploiting the LLS behaviour of a spy molecule, we experimentally demonstrated that it is possible to measure dissociation constants up to 12 mM, where most other biophysical techniques fail, including NMR methods based on the observation of ligands.

We also explored LLS involving pairs of ^{19}F nuclei to study binding phenomena. In a custom-designed fluorinated ligand that binds trypsin, we have observed *a* ratio T_{LLS} / T_1 > 4. We found a dramatic effect on the LLS lifetime T_{LLS} of the fluorinated ligand and great contrasts have been observed between signals derived from T_{LLS} with/without protein. This fluorinated ligand has been successively used as spy molecule in competition experiments, which allowed us to rank the affinities of arbitrary ligands that do not contain any fluorine. The extension of LLS to pairs of ^{19}F nuclei is an important achievement, since fluorine detection allows one to perform screening campaigns without suffering from problems due to overlapping signals. The combination of fluorine detection with DNP will be the next frontier of screening by LLS. It could provide biophysical scientists with a very sensitive and innovative tool to perform ligand-protein interaction studies.

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From now on, I want to spend a couple of words for all the people I met during this amazing experience, assigning a funny *nickname* at the end of the description. I would like to start with Dr. Simone Ulzega, who has been the first person to welcome me in Lausanne in the far 2011 during my Erasmus experience. He probably remembers those first days when I was almost not able to communicate in English and consequently he was my Italian lifebelt. I will never forget our scrupulous analysis of the Serie A and Champions league results and, above all, his innate skills in mimic the way of kicking the ball of famous players (not always with positive results). *Football mentor.*

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I shared my office for more than one year with Dr. Pavel Kaderavek. He is a very nice person and a good scientist. I thank him for having kept constantly active our office during this period (weekends included). I thank also Dr. AJ Perez-Linde for having contributed to very funny moments in the lab life. *Right?*

I would like to thank Dr. Sami Jannin, Jonas Milani and Basile Vuichoud for contributing to the nice atmosphere of the lab and for the fruitful collaboration in one of my projects. Coming back to the funny part, they are all members of the Swiss team, which wants to convince people that Switzerland is better than other many countries not only for cheese, chocolate and banks. They want to convince people that Switzerland is better also for wines and soccer skills…and, above all, they want to convince Italian people that pineapple pizza is good. *Reckless*.

I want to thank a lot also my current office-mates. Daniele Mammoli and Estel Canet are for me not only nice colleagues, but primarily great friends. We shared not only a space and some scientific projects, but also very nice experiences. Dinners. Parties. Bottles of wine. Venetian dialect songs. I will never forget our road trip in California, Nevada and Arizona. Daniele's obsession for optimization. Estel's liveliness. *Milestones*.

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I am sure you arrived to read this line skipping several parts of this wonderful manuscript. Anyway, I am glad that you read at least a small part of it. So, thank you!

Lausanne, September 2015

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2006 – 2009
<u>B.Sc. in Chemistry</u>
University of Padova, Italy
Bachelor thesis: "DJ1-DAQ conjugates in Parkinson disease (a topological study with molecular dynamic simulations)"

Awards

2013	Prize for the best poster, 49th International Conference on Medicinal Chemistry, Nice, France
2015	2015 SCNAT/SCS Chemistry Travel Award, offered by the Swiss Chemical Society and the Swiss Society for Foood and Enviromental Chemistry

Professional and research experience

EPFL
- Investigation of fundamental aspects of NMR spectroscopy in liquid states
- Development of NMR experimental methods
  - Detection of ligand-protein binding with new strategies, which allow to save materials and to work with cheaper proteins
  - Definition of chemical strategies to render screening methods always applicable
  - Combination of new screening methods with innovative techniques (dissolution-DNP) to increase the sensitivity of the experiments
  - Development of screening strategies based on ^{19}F detection, which allow to speed-up the screening processes
  - Investigation of relaxation properties of water in gas phase
  - Development of strategies to detect para-water in bulk
- Supervision of several student projects
- Organization of interdisciplinary projects for students, to be developed in different EPFL laboratories
- Teaching at all levels (B.Sc, M.Sc. and Ph.D)

Programming skills

- Experience with Matlab, Origin
- Experience with Bruker NMR software for implementation/modification of pulse sequences
- Basic experience with programming languages as Pascal, Java

Patent applications

130

EP20110793393, Method for the nmr based determination of the affinity of drugs for a target protein , EPFL

Hobbies and interests

Passionate of wines and agriculture, traveling, sports. I am currently the vice-president of Italaus, an association of Italian students and PhDs (recognized by EPFL) for the promotion of the Italian culture in Lausanne.

List of Publications

1. Bornet A., Milani J., Wang S., Mammoli D., Buratto R., Salvi N., Segawa T.F., Vitzthum V., Mieville P., Chintalapalli S., Perez-Linde A.J., Carnevale D., Jannin S., Caporini M., Ulzega S., Rey M., Bodenhausen G. *Dynamic Nuclear Polarization and Other Magnetic Ideas at EPFL*, Chimia **(2012)**, 66(10):734-740.

2. Salvi N., Buratto R., Bornet A., Ulzega S., Rentero Rebollo I., Angelini A., Heinis C., Bodenhausen G. *Boosting the Sensitivity of Ligand-Protein Screening by NMR of Long-Lived States*, J Am Chem Soc **(2012)**, 134(27):11076-11079.

3. Buratto, R., Bornet, A., Milani, J., Mammoli, D., Vuichoud, B., Salvi, N., Singh, M., Laguerre, A., Passemard, S., Gerber-Lemaire, S., Jannin, S. and Bodenhausen G. *Drug Screening Boosted by Hyperpolarized Long-Lived States in NMR*, ChemMedChem **(2014)**, 9: 2509-2515.

4. Buratto, R., Mammoli, D., Chiarparin, E., Williams, G. and Bodenhausen, G. *Exploring Weak Ligand-Protein Interactions by Long-Lived NMR States: Improved Contrast in Fragment-Based Drug Screening*, Angew Chem Int Ed **(2014)**, 53: 11376-11380.

5. Mammoli D., Salvi N., Milani J., Buratto R., Bornet A., Swhgal A. A., Canet E., Pelupessy P., Carnevale D., Jannin S., and Bodenhausen G. *On the path to para-water in bulk: overcoming proton exchange and other hurdles*, Phys Chem Chem Phys **(2015)**, in press.

Conferences

1. EUROMAR Conference, Dublin, Ireland, June 30th-July 5th, 2012.

Poster title: Boosting Sensitivity of Ligand-Protein Screening by NMR of Long-Lived States

2. ENC Conference, Asilomar, California, April 14-19th, 2013.
Poster title: Protein-Ligand Affinity using Long-Lived States and Long-Lived Coherences

3. RICT Conference, Nice, France, July 3-5th, 2013.
Poster title: Towards DNP-Enhanced LLS and LLC for Ligand Screening

4. SCS fall meeting, Lausanne, Switzerland, September 6th, 2013.
Poster title: Protein-Ligand Affinity using Long-Lived States and Long-Lived Coherences

5. ENC Conference, Boston, USA, March 23-28th, 2014.
Poster title: DNP-Enhanced Ligand-Protein Screening by NMR of Long-Lived States

6. RSC NMR DC, Spring Meeting 2014, Cambridge, UK, April 10-11th, 2014.
Oral presentation: Beyond the millimolar range: measuring ultra-weak ligand-protein affinities using NMR of Long-Lived States

7. EUROMAR Conference, Zurich, Switzerland, June 29th-July 3rd, 2014.
Poster title: Exploring weak ligand-protein interactions by Long-Lived States in NMR: improved contrast in Fragment-Based Drug Screening

8. FBLD Conference, Basel, Switzerland, September 21-24th, 2014.
Poster title: Exploring weak ligand-protein interactions by Long-Lived States in NMR: improved contrast in Fragment-Based Drug Screening

9. NMRS Conference, Amritsar, India, March 6-9th, 2015.
Invited oral presentation: Ligand-Protein Interaction Studies by NMR of Long-Lived States

10. ENC Conference, Asilomar, California, April 19-24th, 2015.
Oral presentation: Ligand-Protein Screening by Long-Lived States of Fluorine-19 Nuclei

11. EUROMAR Conference, Prague, Czech Republic, July 5-10th, 2015.
Oral presentation: Long-Lived States of pairs of Fluorine-19 Nuclei: a new tool for Ligand-Protein Screening