Nuclear Magnetic Resonance

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Abstract. This article reviews the principles and methods of nuclear magnetic resonance spectroscopy, and gives examples of applications carried out at our Facility, which illustrate the capabilities of the technique.

1. Introduction

Nuclear Magnetic Resonance (NMR) is a useful tool for the determination of chemical compound structures. It is a powerful technique for dealing both with static and dynamic problems, and with molecular interactions. This ability makes it an essential tool in many scientific areas such as chemistry, biology, biochemistry, metabolomics and genomics and it has been of crucial importance for their research acceleration and dynamization, especially in the pharmaceutical industry.

NMR is a technique based on a physical property of certain nuclei that get oriented in the presence of an external magnetic field and its principles where established in 1938. The first measurements were done by Felix Bloch and Edward M. Purcell en 1945[1] and the most studied nucleus at these early times was ^{1}H. In 1950 the concept of chemical shift was introduced connecting it with chemical environment of protons on the molecule, and in mid 50's, the splitting of signals in the spectrum was connected with interactions between different nuclei. These early concepts, together with information coming from decoupling techniques and the Nuclear Overhauser Effect (NOE), are nowadays crucial for the determination of chemical structures. Since 1957, the development of ^{13}C NMR has opened the technique to the study of other nuclei such as ^{15}N, ^{31}P, ^{19}F or ^{113}Cd, some of them are essential today in the study of high-biological interest compounds.

In 1966 Richard R. Ernst[2] and Weston A. Anderson took a step forward in increasing the intrinsic low sensitivity of the technique by developing Fourier Transform pulsed NMR which allows simultaneous observation of the same species of nuclei on a wide range of frequencies, making feasible the co-addition of spectra. In 1970 Jean Jeneer conceived the idea of bidimensional experiments later developed by R. R. Ernst. The widespread use of 2D and 3D experiments has contributed to extend the technique to increasingly complex molecules, being essential today in the study of proteins[3].

Although the initial development of NMR focused on liquid state, overcoming of problems derived from strong interactions present in solid samples, allowed its extension to the latter samples (1960-1970). This application has increasing significance, not only for the study of materials, but also in the field of Biochemistry, as it allows the study of non-soluble great-sized proteins. Solid state NMR provides tools to better understand protein aggregation processes which happen to occur in the vast majority of neurodegenerative diseases.

The development of superconducting magnets has pushed forward stronger, more stable and more homogeneous magnets which has end up with improvements on sensitivity and spectral resolution. Latest advances on magnets are focused on decreasing their maintenance by lowering their cryogenic liquids demand and on ultrashield magnets which decrease magnetic field interferences in surrounding areas. More information on basic NMR can be found elsewhere[4,5].

Since 1982, the NMR Facility of the University of Barcelona, nowadays assigned to the Scientific and Technological Centers (CCiTUB), provides access to NMR instrumentation and scientific and technical support on NMR. Its activity is opened to researchers coming from both private and public institutions. The large number of instruments (12 magnets with "fields" ranging from 800 to 250 MHz) together with experienced staff converts this facility in a reference point in our state.

2. Applications and Methodology

The relationship between chemical shift and nuclei electronic environment is a key tool for structure determination. NMR spectrometers are able to detect subtle differences of chemical shifts providing identification of neighbouring chemical functionalities, changes on substitution, etc. It affords, too, a quick tool to differentiate among similar compounds, as illustrated in the detection of oversulfated Condriotin Sulfate (OSCS) as a contamination of Heparin; in this particular case the analysis is based on the differentiation between OSCS N-Ac methyl and heparin N-Ac methyl. In spite of the proximity of signals and of the great difference in intensity, it is possible to detect even

less than 1% of OSCS (Example 3.1). In fact, working with a 500 MHz NMR spectrometer, a detection limit of 0.01% of contamination has been established on an active pharmaceutical ingredient (API) analysis.

NMR technique affords also quantitative information (qNMR), as it is possible to correlate between the NMR signal area and the number of protons involved. With signal integration and a U.S. Pharmacopeia (USP) protocol, it has been possible to determine poloxamer composition (Example 3.2). In case no USP protocol or similar is available, a validation of protocols used in qNMR is necessary because the reliability of results is determined by sample characteristics and experimental conditions.

Stereochemical information can be inferred from scalar coupling constants (J_{HH}) on proton spectra and from dipolar coupling constants in NOE experiments. Scalar coupling data can afford direct information about, for instance, a cis/trans arrangement in a double bond or it also can be used both, directly or indirectly, in multiple bidimensional experiments. A simple example is the COSY experiment, where the correlation signals originate from magnetization transfer between scalar-coupled protons. The 2D experiments are very useful to study complex molecules since in these cases the mono-dimensional spectrum is pretty crowded and severe overlapping of the signals interferes with data interpretation. Some examples of 2D experiments are ^{1}H-^{1}H homocorrelation (COSY or TOCSY) where different fragments of structure can be identified, or ^{1}H-^{13}C heterocorrelation (HSQC) where directly bonded proton and carbon can be connected, taking advantage of the great carbon spectral width which reduces significantly overlapping. Some long range ^{1}H-^{13}C experiments (HMBC) are possible too. An appropriate combination of bidimensional experiments allows structure determination of natural products or synthetic compounds (Example 3.3).

The study of complex molecules such as proteins, DNA or RNA requires complex 2D and 3D experiments including some heteronuclei as ^{13}C and ^{15}N for proteins or ^{13}C and ^{31}P for nucleic acids. Professor K. Wutrich was the first to develop pulse sequences and assignment strategies for the study of proteins[3]. The low sensitivity of the technique and the complexity of this kind of samples requires ^{15}N labelling and often ^{13}C labelling too. For proteins larger than 250 residues, the use of ^{2}H labeling and TROSY[6] methodology in very-high-field instruments are necessary. An example of the largest macromolecular complex studied by NMR with this methodology is the GroEL-GroES complex, as big as 900 KD, carried out by Professor Wutrich[7]. In our NMR facility, protein studies are carried out on 600 and 800 MHz instruments where a wide variety of TROSY 2D and 3D sequences have been implemented (Example 3.8).

Detecting active sites and ligand-binding interactions is of great importance in the study of potential drug molecules. NMR can deal with it from two different approximations:

- Study of small chemical shift changes caused to nuclei close to the active site during binding. ^{1}H-^{15}N HSQC can be useful to detect these changes and has been used in our facility to study interactions of P53 tetramer and arginine enriched peptides[8].
- Study of changes caused to ligand when bound to a large molecule as it adopts characteristics of the latter as far as correlation time or relaxation concerns. “Information” from the bound state is transferred to the free ligand during the equilibrium between both species. In our facility, these kind of studies are run on 500 MHz instruments with autosampler. STD, Waterlogsy, CPMGT2, or NOE Transfer[9] experiments are commonly used (Example-3.5). If higher sensitivity is required 600 MHz instruments are used instead.

NMR is useful not only for ^{1}H, ^{13}C and ^{15}N nuclei, as there is a wide range of NMR active nuclei. ^{31}P and ^{19}F are widely used for the study of organometalic and fluorinated compounds, respectively. Experiments which combine both nuclei can provide simplified spectra (Example 3.6). Currently, ^{19}F NMR is used in our laboratory to determine association with proteins. Our facility can tackle with these nuclei and other less common such as ^{113}Cd, ^{195}Pt, ^{11}B, ^{29}Si, ^{27}Al, etc.

All instruments present in our facility are prepared to work with solution samples. In addition to that, our High Resolution Magic Angle Spinning (HRMAS) probe allows working with semi-solid samples such as tissues, resins chromatographic supports, gels, etc. Metabolite determinations on

tissues, stability of almond pulp or solid phase synthesis control are some examples of work done with this probe in our lab (Example 3.7).

Magnetic field strength determines the sensitivity and spectral dispersion of NMR spectra. Sensitivity increases in a relation of 7/4 with magnetic field B_0. The increase in spectral dispersion makes evident an smaller overlapping of signals (Figure 1). Occasionally, a simplification of coupling systems can also be obtained.

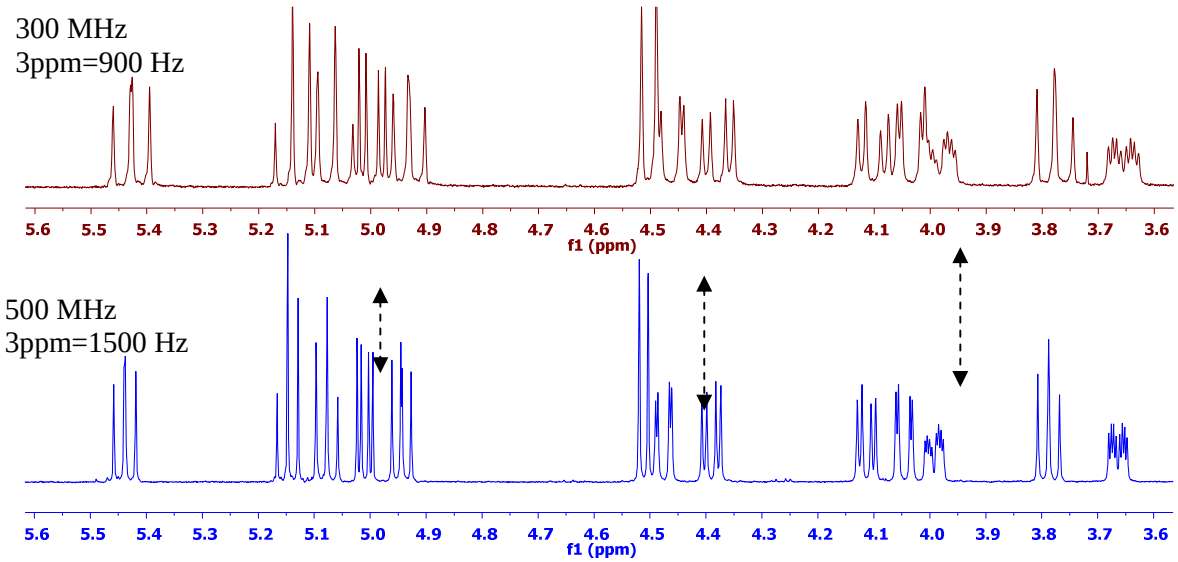

Figure 1 Expansion of a ^{1}H spectrum acquired in a 300 and 500 MHz spectrometer

The choice of spectrometer is determined by the complexity and availability of the sample under study. Generally speaking instruments with observation proton frequencies ranging from 300 to 500 MHz are suitable for the study of small and medium-sized molecules. In our facility the 800 MHz instrument is mainly used for the study of dynamic problems, biomolecular interaction or structure determination of high-molecular weight compounds such as proteins and nucleic acids. The 600 MHz instrument is used both for medium-sized molecules and initial studies with high molecular weight molecules. An important point to have into account in the choice of the best spectrometer is its particular characteristics, sensitivity (Table 1) and available probes. Detailed information on available spectrometers and probes can be found in our web site[10].

Table 1 S/N ratio on our main instruments (sample test 0.1% Etilbenzene in $CDCl_3$)

Nucleus	B800	B600-II	B600-I	V500S	V500	M400
^{1}H	>7000:1	>7000:1	700:1	730:1	720:1	220:1
^{13}C	> 700:1	> 750:1	335:1	240:1	83:1	158:1
^{31}P	---	---	200:1	135:1	24:1	183:1
^{19}F	---	---	450:1	450:1	---	200:1

3. Examples of applications performed in the NMR Facility

3.1. Heparin Analysis

Heparins are a complex mixture of highly sulfated glycosaminoglycans (CAGs) isolated mainly from the intestinal mucosa of pigs or bovine lungs and are widely used as anticoagulant and antithrombotic agents. The heparin consist of a mixture of sulfated disaccharide units, the major component is a 2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated glucosamine IdoA(2S)-GlcNS(6S).

The differences in the production processes of heparin lead to changes in the content of minor compounds. Some of these native impurities such as Dermatan sulfate (DS) are accepted with

restrictions but others such as Oversulfate Chondroitin sulfate (OSCS)[11], a non-native contaminant, are not allowed.

In 2008, there were an increase of incidents and deaths associated with allergic reactions caused by adulterated heparin. The Food and Drug Association (FDA) and several research groups identified OSCS as a heparin contaminant associated with adverse reactions. International organizations such as EP (European Pharmacopoeia) and USP included the use of NMR technique in heparin analysis[12] procedure to assess the presence of OSCS in the samples (Figure 2).

The proton spectrum of heparin must show, among others, characteristic signals at δ 2.04, 3.27 ppm (doublet), 4.34 ppm, 5.22 ppm, and 5.42 ppm (all with less than ± 0.03 ppm deviation). The chemical shift for OSCS N–Ac group in heparin sodium is reported at 2.16 ± 0.03 ppm and in any case it should not be observed. Subsequent revisions of the monographs include additional requirements on the profile of intensities of some signals of heparin in the proton spectrum. The latest methods are:

- USP monograph appeared in USP32; Pharmacopeia Forum 35(5) Oct 2009.
- European Pharmacopoeia 7.0 (8/2010:0333)

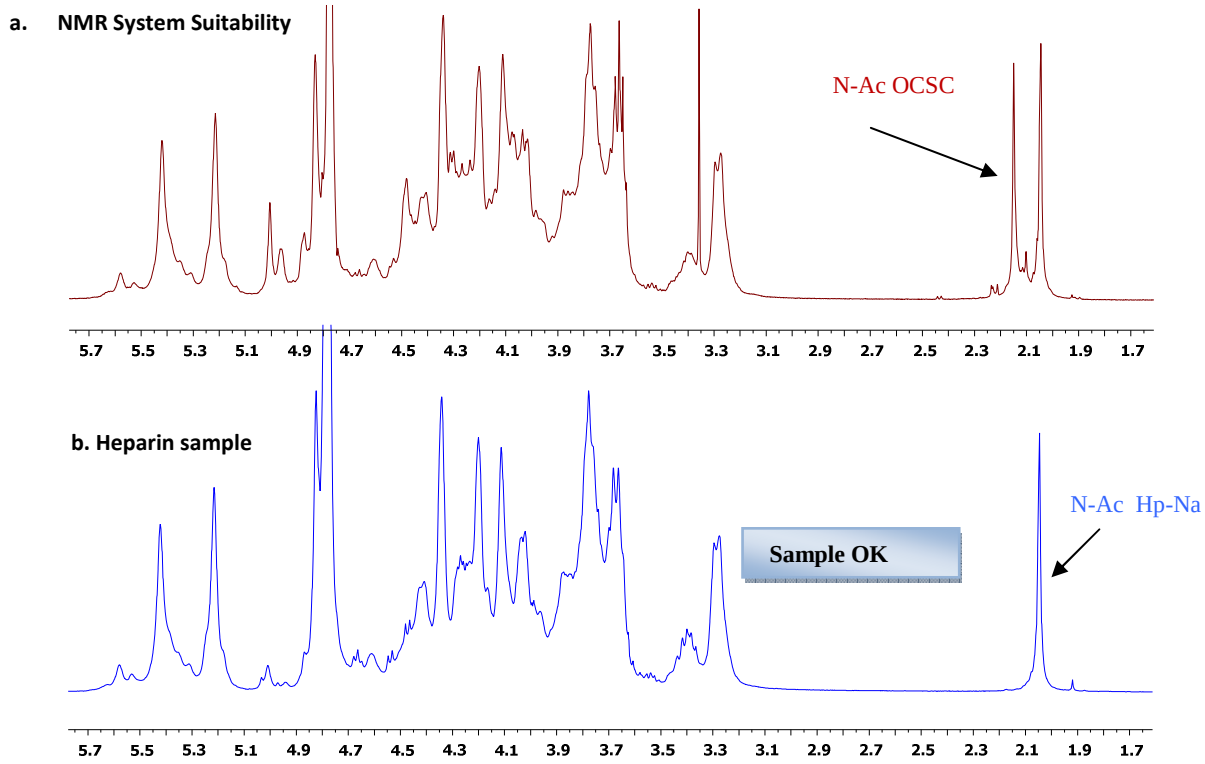

Figure 2 Upper spectrum: heparin System suitability sample spiked with OSCS. Lower spectrum shows heparin sample (suitable for marketing)

In our NMR Facility, heparin analyses are performed on NMR 500 MHz spectrometers, since it is essential to obtain a good separation and definition of the broad signals of the N-acetyl. It is also necessary that the signal to noise ratio in the area to examine must be higher than 1000:1. The ability to observe the ^{13}C satellites in the N-acetyl of the major components is an indication that the detection limit is higher than 0.5%.

Depending on the user's needs, a specific Standard Operating Procedure (SOP) can be elaborated. With NMR systems ISO9001 and GMP compliant

If the content of OSCS is small, the N-Ac peak of the impurity can be confused with the ^{13}C satellites of N-acetyl of the major components. In this case, acqusition of the proton spectrum with ^{13}C decoupling is necessary, as shown in Figure 3c.

When experiments are run in a 600 MHz spectrometer, ^{13}C decoupling is recommended.

3.2. Polaxamer Analysis.

A poloxamer is a synthetic triblock copolymer of ethylene oxide (EO) and propylene oxide (PO), with a general formula $HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_a OH$. The ability of poloxamers to form micelles makes them interesting for the pharmaceutical industry as drug-delivery vehicles. It is also used in pharmaceutical formulations because of its ability to form thermo-reversible gel, which at low temperatures, is a low viscosity liquid and at high temperatures a gel.

Figure 4 shows a proton poloxamer spectrum of the sample dissolved in $CDCl_3$. The oxypropylene units are characterized by a series of doublets centered at 1.08 ppm due to CH3 groups (region **A**). The signals from 3.2 to 3.8 ppm (region **B**) are due to the CH_2O groups of oxyethylene units, and also the CH_2O/CHO groups of the oxypropylene units.

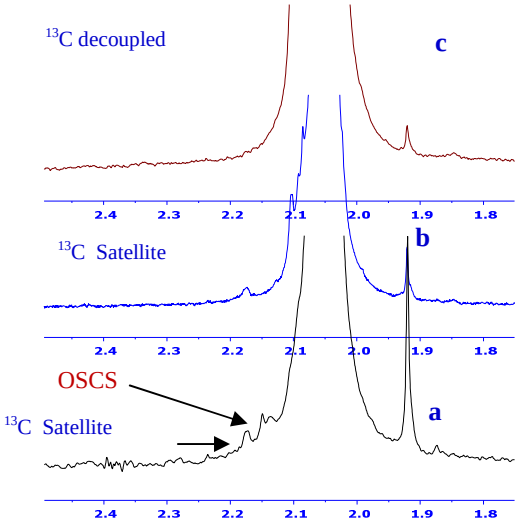

Figure 3: **a)**. ^{1}H-RMN of System suitability sample with the corresponding N-Ac of OSCS and ^{13}C satellite peaks. **b).** A typical spectrum of heparin sample (not containing OSCS) with the signal at δ 2.17 ppm attributed to the ^{13}C satellite. **c).** The proton spectrum of the previous sample performed with ^{13}C decoupling. Notice that the signal at δ 2.17 ppm disappears.

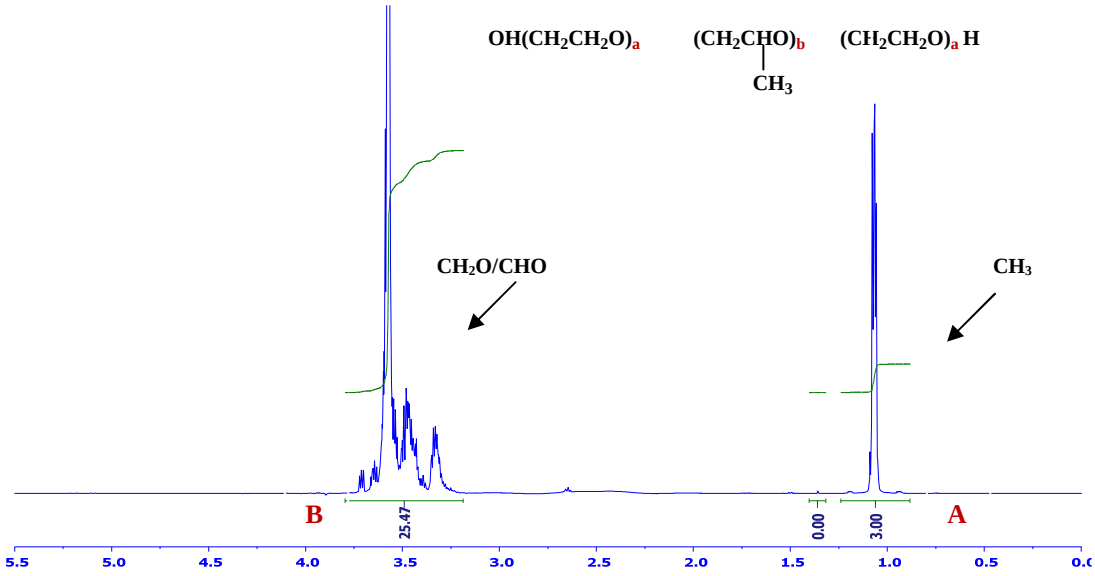

Figure 4. Proton spectrum of a poloxamer sample

In monograph USP32-NF27, a procedure to determine the ratio of oxyethylene/oxypropylene, using a Relative Method of Quantization <761> is described. The percentage of oxyethylene, by weight, in the Poloxamer is calculated by the following formula[13]

$$\text{Weight \% oxyethylene} = 3300\alpha / (33\alpha + 58) \quad \text{being } \alpha = (B / A) - 1.$$

Applying this formula to the spectrum of Figure 4**,** a value of 80.9% EO is obtained.

At the NMR Facility, poloxamer analysis are performed using 500 MHz spectrometers that fulfill ISO 9001-2008 regulations and GMP quality system.

3.3. Structural Elucidation by NMR Spectroscopy (small and medium size Organic Compounds)
An essential need of synthetic or chemical isolation processes is to unequivocally identify the obtained compounds.

Routine spectrometers in our facility are prepared with a series of tools which provide a friendly access to a wide variety of NMR experiments even for non-expert NMR users. Research users of Chemical and Pharmaceutical industry can make use too of these possibilities, depending on their particular demands, with no need to invest in high cost instrumentation.

NMR staff contribution to studies coming from industry can include data interpretation and fully elaborated documentation

The most frequently used strategy for structure determination of small and medium-sized molecules begins with a detailed study of proton 1D spectrum. Further information is obtained in a next step by ^{1}H-^{13}C heterocorrelation (either by HSQC or HMQC), which, if edited, affords differentiation between carbons according to the number of protons directly attached (**Figure 5**). Spin systems can be identified by ^{1}H-^{1}H homocorrelation experiments (COSY, DQF-COSY, TOCSY, etc). Assigned fragments obtained can be interconnected by long-range ^{1}H-^{13}C heterocorrelation (HMBC). Finally, a study of coupling constants and NOE data can define compound stereochemistry.

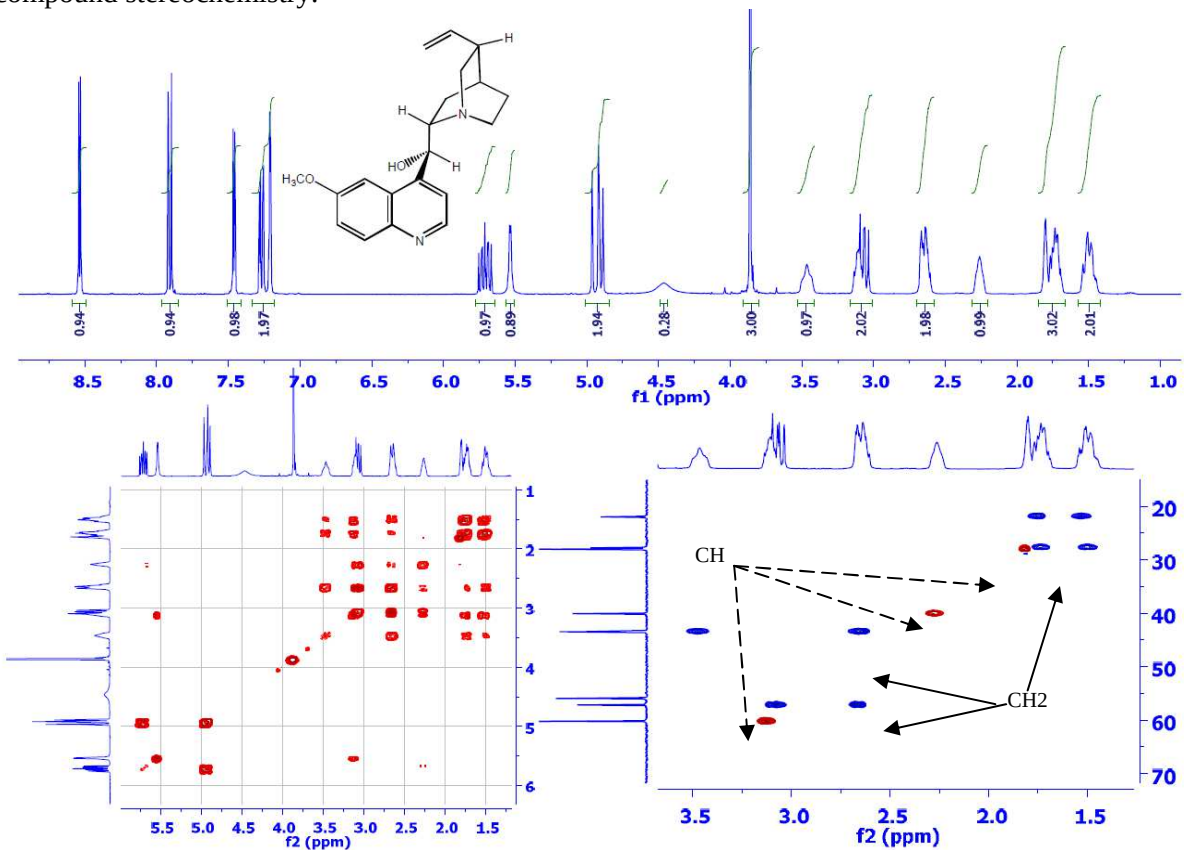

Figure 5. Basic experiments used for assignment 1D^{1}H, 2D COSY and 2D HSQC of Quinine in $CDCl_3$

A detailed example of a combined use of multiple 2D experiments for the study of Quinine is available on our web site as a tool for beginners on NMR in order to help them to choose proper experiments.

3.4. Fast 2D-NMR ligand screening using Hadamard spectroscopy[14]
The following is an example of methodological development carried out in the NMR facility in collaboration with the research group of Prof. Miquel Pons.

Chemical shift perturbation NMR experiments are widely used for structure-based drug discovery. The main advantage of NMR methods based on protein observation is the possibility to map the ligand interaction site, due to the single residue selectivity offered by 2D HSQC experiments.

There is an increasing interest in new NMR methods that can reduce the time needed to record 2D correlation spectra (SOFAST, Hadamard). Recent applications of Hadamard encoded spectroscopy allow a very fast acquisition of the spectrum , while maintaining a high-spectroscopic resolution in the 2D spectra. In ligand-screening experiments, the signals involved usually experience only small frequency changes (no more than 50-100 Hz). The knowledge of initial free protein spectrum can be used to speed up spectral acquisition using Hadamard encoding of the frequencies of interest. In Hadamard spectroscopy, the evolution time in the indirect dimension is replaced by phase modulated multisite selective excitation. In this way, only selected regions are being measured and the sensitivity per unit time can be increased by more than one order of magnitude.

Hadamard-encoded HSQC spectra only contain cross-peaks with selected heteronuclear frequencies (e.g. ^{15}N). The disappearance of a correlation signal of an amide proton (^{1}H-^{15}N) indicates that is involved in ligand-binding process. When the ligand-induced ^{15}N shifts exceed the excitation bandwidth, it results in the disappearance of the cross-peak. While this may be enough for screening applications, no information on the magnitude and direction of the shift is obtained. This information can be recovered by generating three frequencies for each signal to be studied. The excitation frequencies are those of the unperturbed signal (center) or have an offset of plus or minus 10 Hz. As shown in Figure 6 (residues 1 and 3), a signal intensity decrease in the Hadamard spectrum with the excitation frequencies of the free protein identifies the observed residues that are affected by ligand binding. Signals in Hadamard spectra with excitation frequency offsets provide information on the sign of the induced shifts.

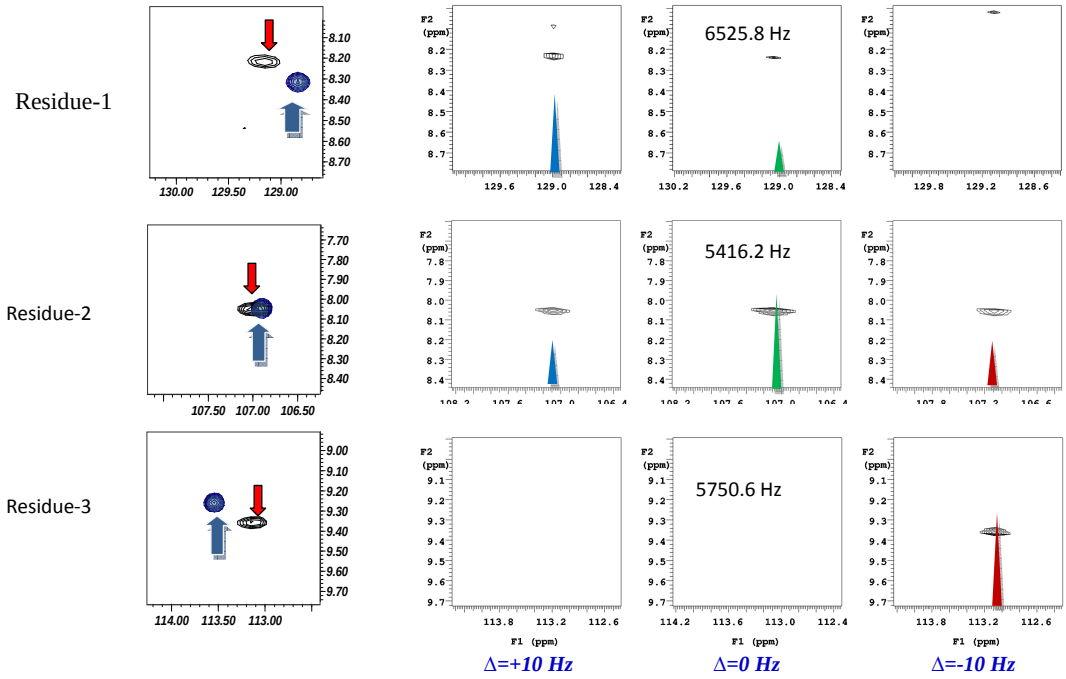

Figure 6. The top and bottom rows are from ligand shifted residues. The centre row is from an unperturbed residue. The leftmost column is a superposition of HSQC cross-peaks of a ^{15}N-labeled WW domain in the absence (↑) and in the presence (↓) of ligand. The other three columns to the right are expansions of Hadamard spectra in the presence of ligand at excitation frequencies corresponding to offsets of +10Hz, 0 Hz, and -10Hz, respectively, with respect to the frequencies in the free protein.

3.5. Saturation Transfer Difference a Method for HTS

The Saturation Transfer Difference (STD) was developed by M. Mayer and B. Meyer[15]. Using this method two experiments are acquired: in the first the selective saturation is applied on the target resonances (protein methyl's ~ 0 ppm); the second experiment is acquired with the irradiation off resonance (~ -35 ppm). Both spectra are subtracted and only the signals affected by the saturation are present. The saturation is transferred to all protein residues by diffusion mechanism, and also to the ligand bounded to protein, but the other ligands remain unaffected (Figure-7).

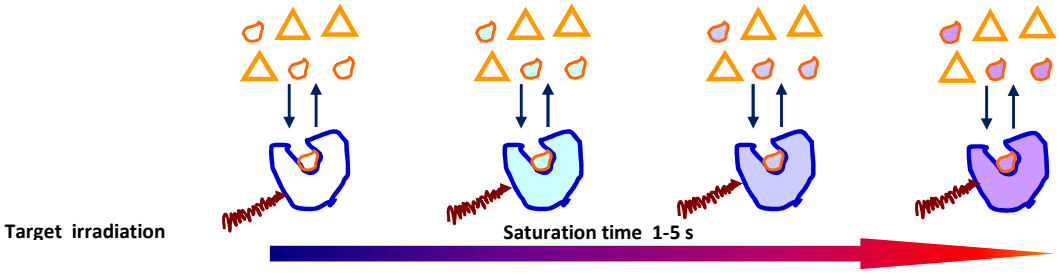

Figure 7: Schematic representation of the evolution of the STD effect

The degree of saturation in ligand resonance signals depend on the distance of the protons involved in the binding. Protons close to the target molecule are saturated in more extension and have a stronger STD effect. This information can be used to find the part of the ligand more involved in the association (*binding epitope on ligand*). The STD experiment is useful in the case of low and medium binding interaction. It can be applied to medium and large size proteins. No detailed protein information is required, and the concentration of the protein may be relatively low.

At In the NMR Facility this method, is regularly used by several public and private research groups. An example of this application is the collaboration with the research group of Dr. Ignasi Fita[16]. The STD experiment has enabled to check and complete the results of crystallographic studies on the binding sites on protein catalase-peroxidase KatG with INH and NAD^+.

The STD experiments of INH-KatG mixtures confirmed that the protons H2/H6 and H3/H5 of INH make interaction with the protein (Figure 8 (left)), consistent with the X-ray structures. The STD experiment also allows a mapping of binding epitope. In KatG/NAD^+ the H8 adenine ring and H1' of the adenosine ribose exhibit stronger STD signals than the others protons of NAD^+ , as it can be observed in the STD spectrum (Figure 8 (right))

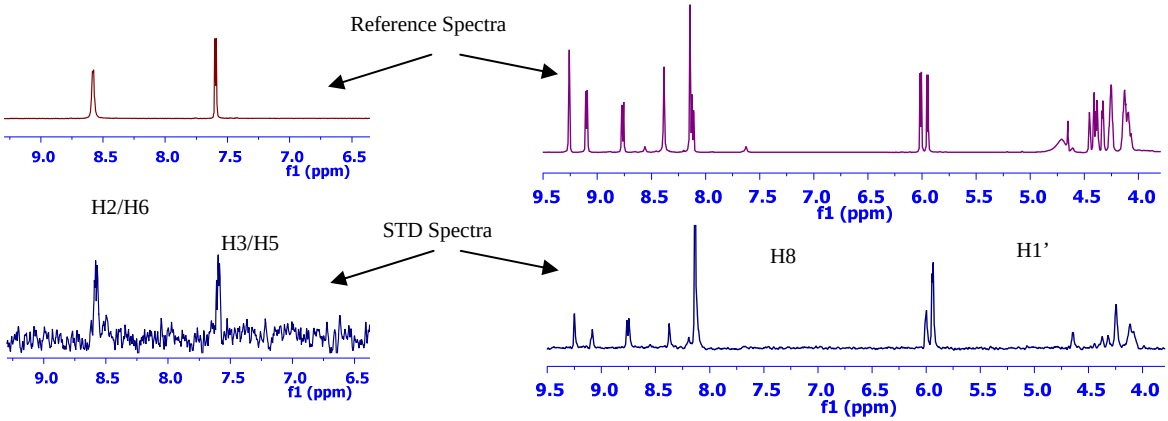

Figure 8. STD experiment of INH-KatG (left); STD experiment KatG/NAD^+ (right)

The protocol used in our NMR Facility for the study of chemical libraries includes an experiment CPMGT2 to confirm the results of the STD. The method allows the study of various ligands at the same NMR sample. The time required for the study of each sample varies between 30 and 100 minutes depending on intensity threshold set in the STD experiment.

3.6. ^1H-^{19}F Double resonance experiments

The high sensitivity of ^{19}F (83% of the ^{1}H sensitivity), makes ^{19}F NMR a powerful tool for analyzing different kind of compounds. However, often the spectra of ^{1}H and ^{19}F of these compounds are complex. This is so because ^{1}H and ^{19}F couple to each other ($^{2}J_{HF}$=50-45Hz; $^{3}J_{HF}$=27-7 Hz) , creating complex splitting patterns. The spectra can be simplified by decoupling techniques resulting in clearer assignments, more visible impurities and more reliable confirmations.

The application of $^{19}F\{^{1}H\}$ or $^{1}H\{^{19}F\}$ NMR methods is not always a routine. They need specialized hardware requirements:

- A probehead that can simultaneously be tuned to ^{1}H and ^{19}F.
- A console able to generate two high-band frequencies (^{1}H and ^{19}F) and often additional amplifiers.

These requirements are becoming more frequent in the latest generation of spectrometers, and are not as common in older instruments. Several spectrometers in our NMR Facility allow simultaneous work with ^{1}H and ^{19}F. Acquisition of ^{19}F spectra with ^{1}H decoupling in broadband mode is possible in our Varian Inova-300 spectrometer (Figure 9).

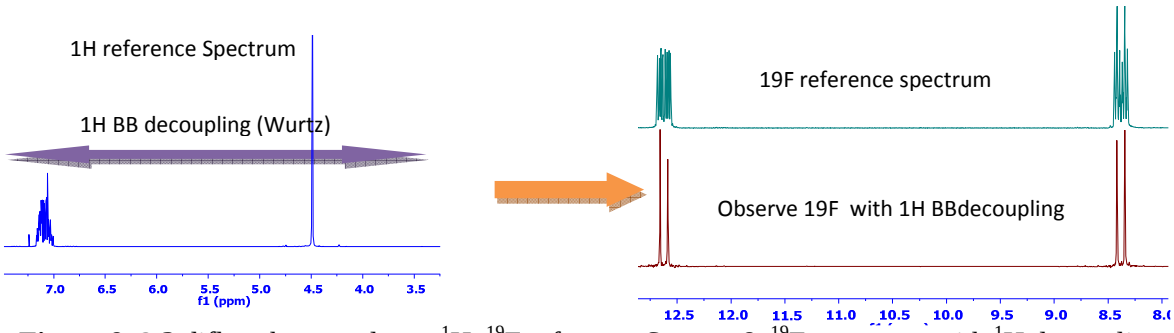

Figure 9. 2,3 difluro bromotoluene ^{1}H, ^{19}F reference Spectra & ^{19}F spectrum, with ^{1}H decoupling

The selective decoupling experiments (^{1}H or ^{19}F) may be useful to identify and measure J_{19F1H} . In the BrukerAvance 600 with a probehead (BBO BB/19F-1H/D), it is possible to carry out all 1D experiments $^{19}F\{^{1}H\}$ or $^{1}H\{^{19}F\}$ in broadband or selective mode, and also 2D NOESY ^{1}H-^{19}F.

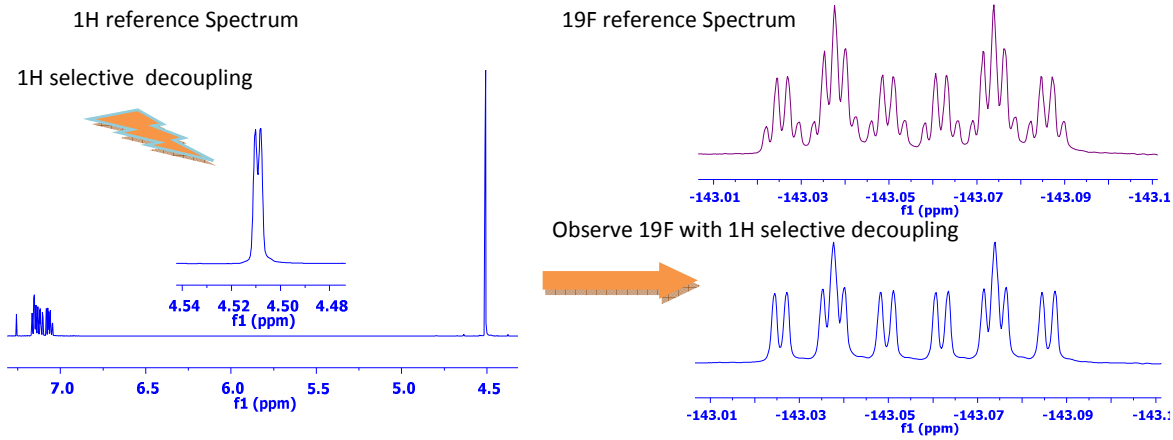

Figure 10 ^{19}F spectrum of 2,3 difluro bromotoluene with ^{1}H selective decoupling

Figure 10 shows the spectrum of ^{19}F with selective ^{1}H decoupling of the CH_2Br group, which allows checking the assignment of the signal at -143 ppm as the ^{19}F at position 2.

3.7. HRMAS (High Resolution Magic Angle Spinning)

There is a wide variety of samples unsuitable to be studied with common liquid-state NMR probes. The nature of these samples can be as different as polymer gels, lipids, biological tissues, swollen resins, food, etc. What they have in common is heterogeneity and restricted or anisotropic motion which results in broad NMR signals.

Figure 11. Small rotor being inserted into the magnet

High Resolution Magic Angle Spinning (HRMAS) NMR technique allows extending NMR studies to these heterogeneous samples. A special probe designed to perform solution type experiments while spinning the sample up to 16 kHz at the magic angle (θ=54.74°) is used. Under such conditions, broadening of NMR signals coming from differences in magnetic susceptibility is reduced, and leads to NMR spectra that display resolution approaching that of liquid samples. The quality of spectra depends only on the degree of mobility of the sample. All usual NMR experiments available on liquid probes (both 1D and 2D) are able to be run on a HRMAS probe.

ChT.4

Samples under study for this probe are prepared with some amount of solvent in specially designed zirconium oxide rotors with a final sample volume ranging from 12 or 80 µL (see on Fig. 11 a small white rotor being inserted into the magnet). Solvent added helps in increasing mobility.

Different nature of samples has been studied in our facility with this probe, and a pair of examples is shown in next figures. Figure 12 corresponds to an aminomethylated resin swollen in $CDCl_3$ and subsequent inclusion of a handle, characterized by an aldehyd function, easily detected on the spectrum by the characteristic signal at low field.

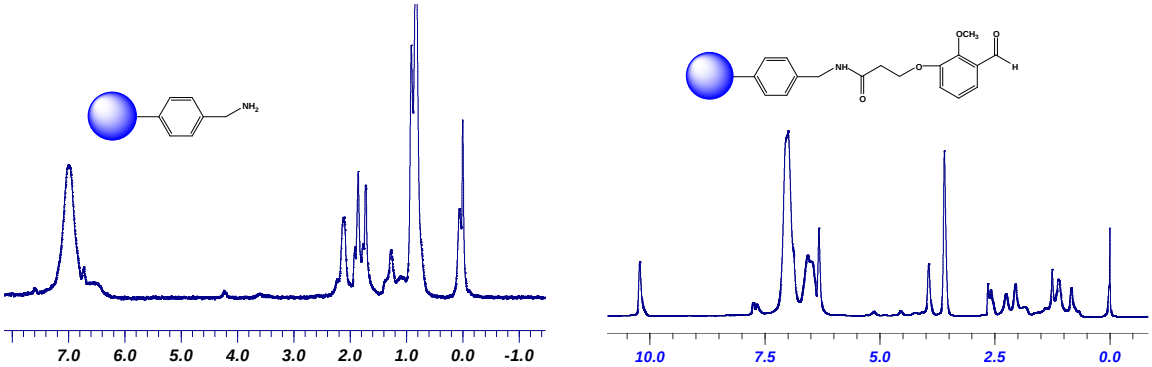

Figure 12 Anchoring of a handle in a resin followed by HRMAS

In our lab, in collaboration with Prof. C. Arús research group (UAB)[17] HRMAS has been used in a study to validate brain tumour biopsy classification by comparing meningiomas (MM), a benign tumour, with glioblastoma multiform (GBM), an aggressive tumour. Initial studies of this

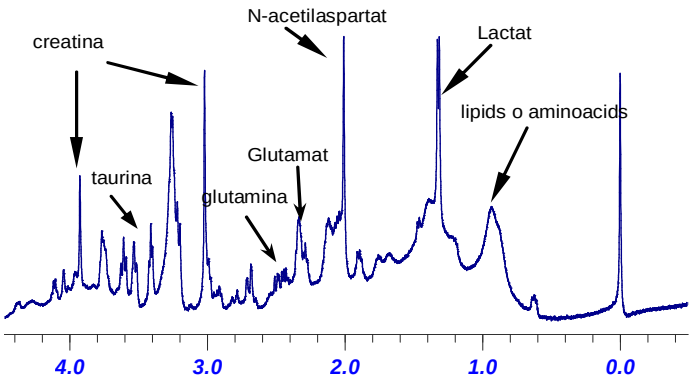

Figure 13. HRMAS spectrum of mouse brain sample

kind of samples with the aim to identify different metabolites on brain were run on mouse brain samples (Figure 13).

3.8. Structural and dynamic studies of proteins in solution by NMR Spectroscopy

NMR spectroscopy is a powerful tool for protein 3D structure determination at atomic resolution, as well as for the study of protein recognition processes and dynamic features of these macromolecular structures.

Strategies for structural studies by NMR largely depend on protein size. Peptides and small proteins (up to 10-12 kDa) can be studied with ^{1}H NMR spectroscopy using 2D TOCSY and DQF-COSY experiments to identify amino acid spin systems and 2D NOESY experiments to carry out the sequential resonance assignment[3], as the first step of the process. Secondly, structural restraints needed for structure determination, such as inter-proton distances and torsion angles, are calculated from NOE intensities in NOESY spectra and coupling constants in DQF-COSY, respectively. Finally, a computing algorithm is used to calculate a set of structures compatible with those NMR-derived constraints. Experiments above are regularly used in the high-field instruments in our NMR Facility and we have applied the described methodology to calculate the structure of several small proteins and peptides (see Figure 14 as an example), in collaboration with Prof. Ernest Giralt[18,19]

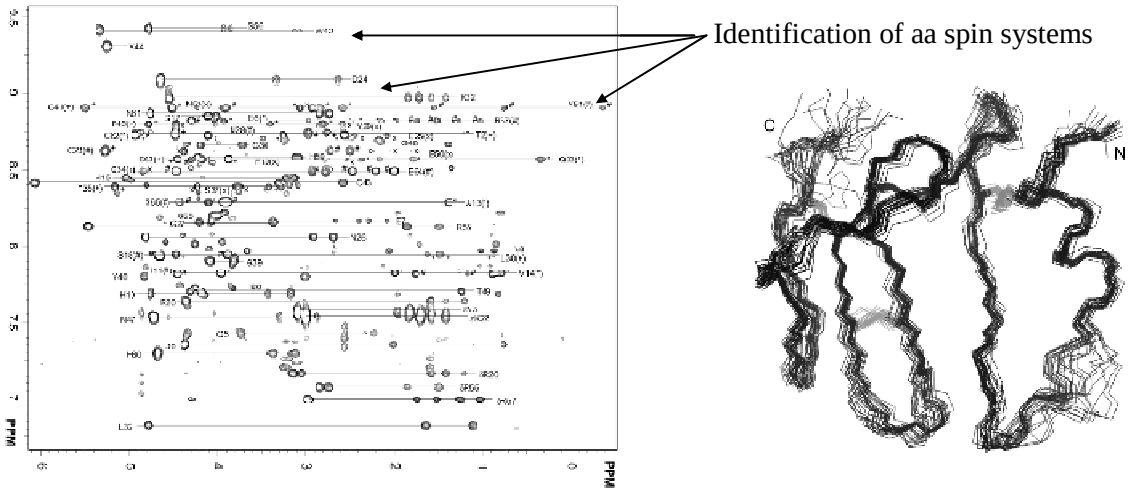

Figure 14. Region of a 2D TOCSY spectrum of the P41icf protein[16] (1mM, 15% D2O/ H2O, pH 5.7) recorded at 800 MHz and 288 K (left). Superposition of the 30 calculated structures of P41icf obtained by ^{1}H NMR spectroscopy (right).

The study of proteins up to 25-30 kDa requires the incorporation of isotopic labels (^{15}N, ^{13}C) to the protein to solve signal overlap problems in ^{1}H spectra. In this case, protein assignment[20] is based on 3D ^{15}N-edited TOCSY/ NOESY experiments and/or 3D triple resonance experiments correlating ^{1}H, ^{15}N and ^{13}C frequencies. A large set of 3D experiments is fully implemented in our 600 and 800 MHz spectrometers for the purpose of resonance assignment. Protein perdeuteration and/or the use of Transverse Relaxation Optimized Spectroscopy (TROSY)[5] are necessary to study proteins larger than 25 kDa in order to decrease transverse relaxation rates and to obtain satisfactory signal line widths. TROSY methodology applied to ^{1}H-^{15}N HSQC spectra combined with ^{15}N-selective labeling prove to be very useful in the study of binding events of large proteins, where the whole resonance assignment is not necessary, as has been demonstrated with POP, a serine protease of 80 kDa, one of the largest proteins studied in our Facility in collaboration with Prof. Giralt[21]

In addition to structural information, dynamics and stability of globular proteins can also be studied by NMR spectroscopy through hydrogen/deuterium (H/D) exchange experiments, by measuring the protection of labile hydrogen's against exchange with solvent deuterons. A very interesting example involves the H/D exchange study of amyloid fibrils formed from the SH3 domain of the α-subunit of bovine phosphatidylinositol-3'-kinase (PI(3)K-SH3) at pH 1.6, carried

out in our Facility in collaboration with Dra. Natàlia Carulla[22]. The site-specific hydrogen exchange behavior of this protein within amyloid fibrils was analyzed by 2D ^{1}H-^{15}N HSQC spectroscopy. Resonance assignment of the protein was previously done by standard 3D ^{15}N-edited TOCSY and NOESY experiments and 3D triple resonance experiments, CBCA(CO)NH and CBCANH. The degree of exchange was calculated for each hydrogen amide in the protein backbone from peak volumes in ^{1}H-^{15}N HSQC spectra (Figure 15, left) recorded after H/D exchange (V_{D2O}) relative to those before exchange (V_{H2O}), where V_{D2O}/ V_{H2O} =1 means no exchange (Figure 15, right). H/D exchange results indicated that SH3 molecules are highly resistant to exchange when incorporated into fibrils and that the majority of residues exchange to a similar degree. Complementary information derived from electrospray ionization mass spectrometry highlights that exchange is dominated by a mechanism of molecular recycling within the fibril population revealing the dynamic nature of amyloid fibrils.

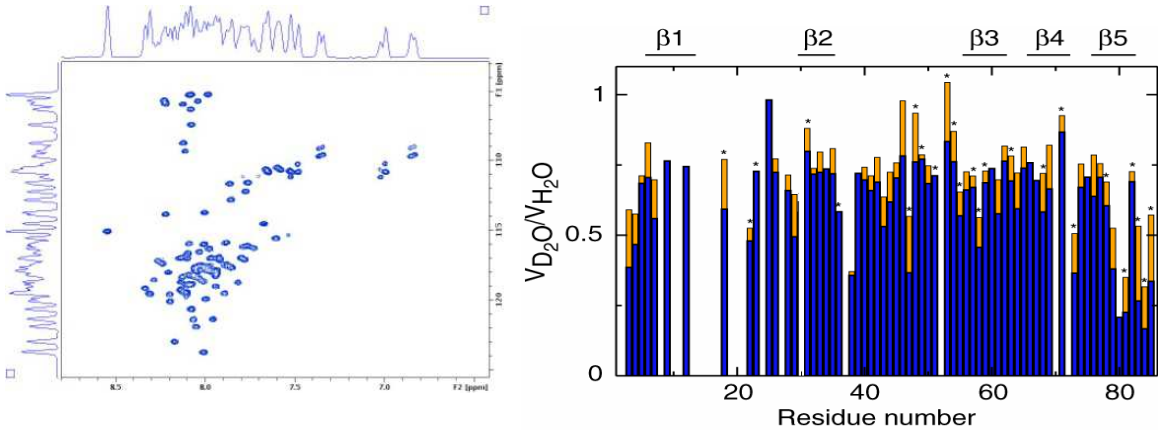

Figure 15. ^{1}H-^{15}N HSQC spectra of PI(3)K-SH3 at 800 MHz (left). H/D exchange of PI(3)K-SH3 amyloid fibrils monitored by NMR spectroscopy (right).

Acknowledgments

We thank Dr. Miquel Pons, Dr. Ernest Giralt, Dr. Natalia Carulla, Dr. Carles Arús, Dr. Xavier Salvatella and Dr. Ernesto Nicolás for their collaboration and permission to use part of their data in this document. We also wish to thank everyone who in one way or another has contributed to the completion and revision of this document, especially to all the technical staff of the NMR facility for their interest and dedication. We thank the Spanish Ministerio de Ciencia y Innovación (MICINN) for funding most of the high-field instrumentation and the European Regional Development Fund (ERDF) for funding the shielded Bruker 600 instrument.

References

[1] 1952 Nobel Prize: Felix Bloch & Edward Mills Purcell "for their development of new methods for nuclear magnetic precision measurements and discoveries in connection therewith" http://nobelprize.org/nobel_prizes/physics/laureates/1952.

[2] Nobel Prize1991: Richard R. Ernst "for his contributions to the development of the methodology of high resolution nuclear magnetic resonance (NMR) spectroscopy" http://nobelprize.org/nobel_prizes/chemistry/laureates/1991.

[3] K. Wuthrich; NMR of Proteins and Nucleic Acids (1986), Wiley, New York
Nobel Prize 2002: Kurt Wuthrich- "for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution" http://nobelprize.org/nobel_prizes/chemistry/laureates/2002.

[4] High-Resolution NMR Techniques in Organic Chemistry, Volume 27, Second Edition (Tetrahedron Organic Chemistry) [Paperback] T.D.W. Claridge

[5] A Complete Introduction to Modern NMR Spectroscopy, Wiley-Interscience (November 26, 2008), Roger S. Macomber

[6] K. Pervushin, R. Riek, G. Wider, K. Wüthrich; "Attenuated T2 relaxation by mutual cancellation of dipole–dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution"; Proc. Natl. Acad. Sci. USA (1997) 94, 12366–12371

[7] Jocelyne Fiaux, Eric B. Bertelsen, Arthur L. Horwich and Kurt Wüthrich; *"NMR analysis of a 900K GroEL–GroES complex"; Nature* 418, 207-211 (11 July 2002)

[8] M. Martinell, X. Salvatella, J. Fernández-Carneado, S. Gordo, M. Feliz, M. Menéndez, and Ernest Giralt. *"Synthetic Ligands Able to Interact with the P53 Tetramerization Domain. Towards Understanding a Protein Surface Recognition Event*" ChemBioChem7, 1005 (2006)

[9] Brian J. Stockman, and Claudio Dalvit, "*NMR screening techniques in drug discovery and drug design*" Progress in NMR Spectroscopy 41, 187 (2002)

[10] http://www.rmn.ub.es/cat/servicios/equipos/equipos.php

[11]http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/UCM112597

[12] http://www.usp.org/hottopics/heparin.html and http://www.edqm.eu/en/Search-519.html.

[13] USPC Official 12/01/09-4/30/10 NF Monographs Poloxamer; Pharmacopoeial Forum: Volume No. 33(4) Page 714; *European Pharmacopoeia* 01/2008 1664 E

[14] Miguel Feliz, Jesús García, Eric Aragón, and Miquel Pons* "*Fast 2D NMR Ligand Screening Using Hadamard Spectroscopy*"; *J. Am Chem Soc.* Vol 128,No 22, 7146 (2006)

[15] Mayer, M. and B. Meyer, *"Characterization of ligand binding by saturation transfer difference NMR Spectroscopy"*. Angewandte Chemie-International Edition 38 1784 (1999)

[16] Ben Wiseman, Xavi Carpena, Miguel Feliz, Lynda J. Donald, Miquel Pons, Ignacio Fita and Peter C. Loewen. *"Isonicotinic Acid Hydrazide Conversion to Isonicotinyl-NAD by Catalase-peroxidades ". Journal of Biological Chemistry* , 85, 26662 (2010)

[17] Daniel Valverde-Saubí, Ana Paula Candiota, Maria Antònia Molins, Miguel Feliz, Óscar Godino, Myriam Dávila, Juan José Acebes, Carles Arús; *"Short-term temperature effect on the HRMAS spectra of human brain tumor biopsies and their pattern recognition analysis"*; Magn Reson Mater Phy (2010) 23:203–215

[18] C. Chiva, P. Barthe, A. Codina, M. Gairí, F. Molina, C. Granier, M. Pugnière, T. Inul, H. Nishi, Y. Nishiuchi, T. Kimura, S. Sakakibara, F. Albericio, E. Giralt; "Synthesis and NMR structure of P41icf, a potent inhibitor of human Cathepsin L"; J. Am. Chem. Soc. (2003), 124, 1508-1517

[19] M. Gairí, P. Saiz, S. Madurga, X. Roig, J. Erchegyi, S. C. Koerber, J. C. Reubi, J. E. Rivier, E. Giralt;"Conformational analysis of a potent SSTR3-selective somatostatin analogue by NMR in water solution"; J. Pept. Sci. (2006) 12, 82-91

[20] M. Sattler, J. Schleucher, C. Griesinger; "Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients"; Progress in NMR Spectroscopy (1999) 34, 93-158

[21] T. Tarragó, B. Claasen, N. Kichik, R. Rodríguez-Mias, M. Gairí, E. Giralt; "A cost-effective labeling strategy for the NMR study of large proteins: selective 15N-labeling of the Trp side chains of Prolyl Oligopeptidase"; ChemBioChem (2009) 10, 2736-2739

[22] N. Carulla, G. L. Caddy, D. R. Hall, J. Zurdo, M. Gairí, M. Feliz, E. Giralt, C. V. Robinson, C. M. Dobson; "Molecular recycling within amyloid fibrils"; Nature (2005) 436, 554-558. DOI: 10.1038/nature03986