LABORATORY OF BIOPHYSICS FOR ADVANCED

Experimental exercises for III year of the First cycle studies

Field: “Applications of physics in biology and medicine”

Specialization: “Molecular Biophysics”

NMR signal assignment of the ubiquitin protein backbone using triple resonance experiments (PBdZ38)

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The objective

Nuclear magnetic resonance (NMR) spectroscopy is a research technique used to study physical and chemical properties of atoms and chemical molecules. It is widely employed for the elucidation of the molecular structure and dynamics of organic molecules in solution (solution-state NMR) and is the second, after crystallography, most commonly used method of determining the structure of biological macromolecules. The possibility of studying macromolecular conformation in solution enables NMR to be used to study molecules which do not form crystals, such as small proteins and peptides and also the so called intrinsically disordered proteins (IDPs), which are increasingly becoming a target of biophysical studies.

Whatever the final aim (structural elucidation, dynamics or binding studies) the first stage of NMR studies is usually signal assignment – that is the act of finding nuclei (or groups thereof) corresponding to the individual peaks visible on the spectra. Any errors in this assignment might lead to drawing erroneous conclusions from further investigations. As obtaining signal assignment for molecules as large as proteins is a complex task a rich methodology has been developed, including many specialized experiments. The aim of this laboratory project is to familiarize the student with this methodology by performing the signal assignment of a small, folded protein ubiquitin (76 amino acids, 8.6 kDa) using several „triple resonance" type experiments – multidimensional (3+) spectra acquired for isotopically labelled (13C/15N minimum) proteins. Such experiments lay at the foundation of modern protein spectroscopy.

Introduction

Basics

A theoretical description of the magnetic resonance phenomenon and of one-dimensional NMR spectroscopy was given in previous courses and can be review in the literature given at the end of this handout. Multidimensional NMR experiments are based upon precise control of the time evolution of the state of the spin system and their full description has to employ the methods of quantum mechanics of complex systems. In order to understand, what information can be gleamed from the analysis of multidimensional spectra, a simplified phenomenological description should however suffice. Here we will limit ourselves to impulse-based spectroscopy, in the liquid state, of nuclei of spin ½ and give only the information necessary to understand the experiments used in this lab project.

With every nuclei of spin ½ we can associate a property called its chemical shift δ (a real number). We should remember that each isotope (hydrogen-1, carbon-13) has its own chemical shift scale. Obtaining structural and/or dynamic information from NMR studies is conditional upon performing a resonance assignment, that is assigning chemical shifts to individual nuclei, observed on the NMR spectra.

In order to perform the resonance assignment we must first be able to determine the set of chemical shifts present in the molecule. As both the measurement precision of one-dimensional spectra and the linewidths of signals are limited for bigger molecules, especially proteins, we cannot distinguish individual signals on such spectra. In order to do so we must perform multidimensional experiments. Firstly the signals measured there are tuples of numbers (2 for two-dimensional spectra, 3 for three-dimensional…) - of chemical shifts - and signal separation should be much better. Secondly by properly designing the experiments we can limit the set of nuclei giving rise to signals on the spectra. For example on the ^{1}H-^{15}N HSQC spectrum (Fig. 1) only the signals from NH groups (chemical shift of amide protons in one dimension, chemical shift of directly bonded amide nitrogen in the other) are present, even thought on one-dimensional proton spectra signals from NH and aromatic protons (from side-chains) can overlap.

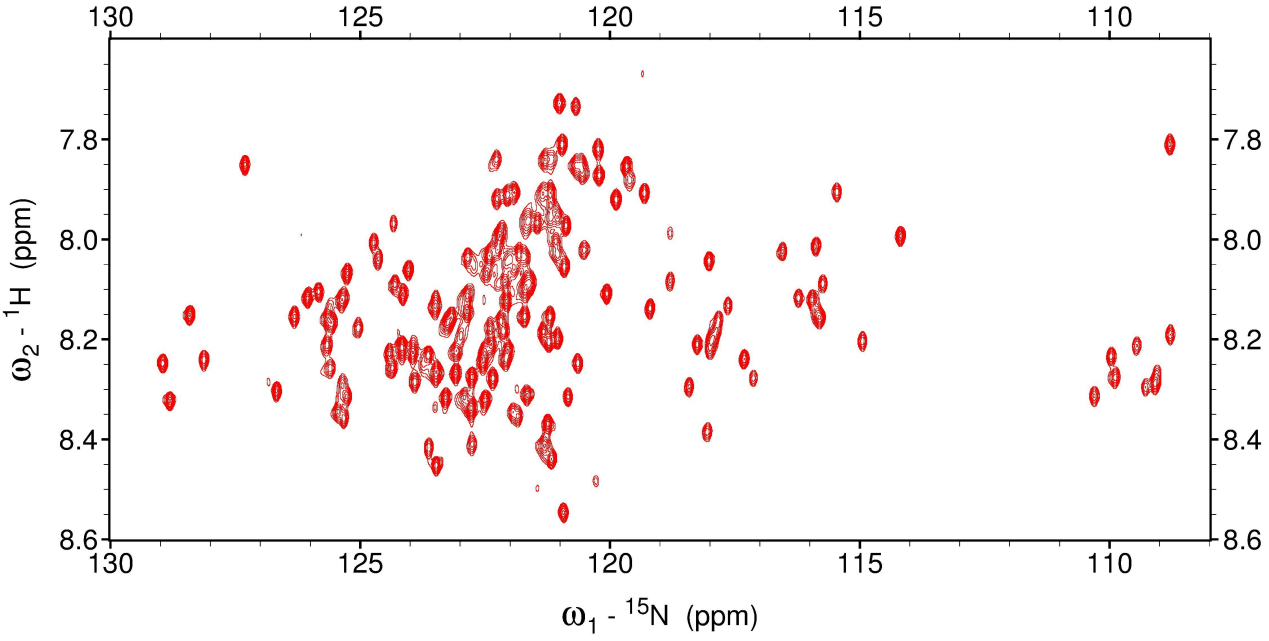

Fig. 1 Two dimensional ^{1}H, ^{15}N HSQC spectrum of the ERD14 protein (185 amino acid residues).

In addition to chemical shifts another important type of parameters are the J coupling constants for pairs of nuclei. Their values (measured in Hertz) are a measure of the strength of the interaction between nuclear magnetic moments, which is used to implement multi-dimensional experiments. As the values of the coupling constants are largely dependent upon the number of chemical bonds separating the nuclei the upper indices carry that information (^{1}J are for nuclei from directly bonded atoms, ^{2}J between the two protons of CH_2 group…). For proteins the values of ^{1}J and ^{2}J are similar for all amino acids and are mostly independent of their position in the protein sequence and structure. Their typical values in the protein backbone are show on Fig. 2.

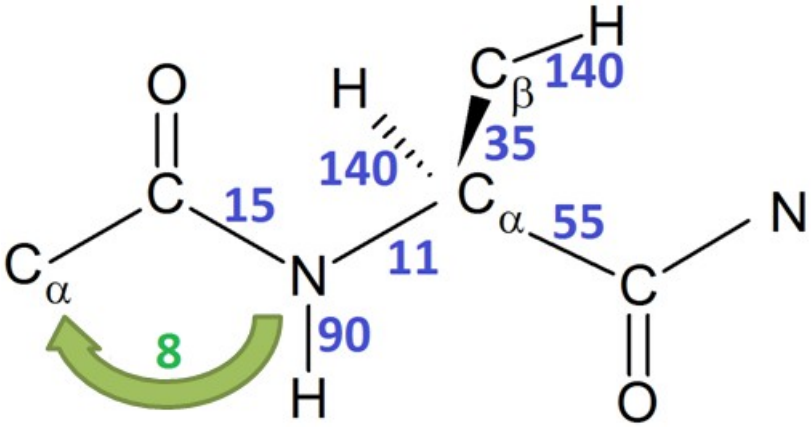

Fig. 2 Typical values of coupling constants (in Hertz) through single bonds and the two-bond $^{2}J_{N\text{-}CA}$ coupling constant in proteins. For other two-bond and higher order coupling constants their absolute value does not exceed 4 Hz.

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Multidimensional experiments

Simple multidimensional experiments follow a common scheme: the nuclei N_1 is excited and the excitation is transferred through the (spin ½) nuclei N_2 to N_n and is detected on the nuclei N_n. The consecutive nuclei through which the excitation is transferred is called the coherence transfer and the transfer of the excitation is called polarisation transfer. While designing NMR experiments we can choose, for which of the N_1 to N_{n-1} nuclei, the chemical shift will be measured (it is always measured for N_n) – that is we can determine the experiments dimensionality. Increasing the experiments dimensionality, as well as lengthening the coherence transfer, decreases the sensitivity of the experiment and lengthens the time needed to obtain a spectrum of adequate signal to noise ratio. Due to practical considerations the N_1 and N_n nuclei are usually protons, strongly coupled to the external magnetic field. The time needed to transfer the polarization is inversely proportional to the coupling constant between the nuclei and due to the competition with different (undesired) transfers we usually choose pairs of nuclei with J constant values of at least several Hertz. In practice this limits transfers to directly coupled nuclei, with the notable exception of the transfer between amide nitrogens and α carbons, where intra (^{1}J)- and inter-residue (^{2}J) pairs have similar coupling constants – around 11 and 8 Hz respectively. The use of this coupling for polarisation transfer leads to the splitting of signals on the spectrum and decreases its overall sensitivity.

Nomenclature of multidimensional experiments

The most straightforward method of constructing the names of experiments in multidimensional NMR spectroscopy is by writing, in sequence, the symbols of nuclei through which the polarisation is transferred. In case of proteins the main categories of nuclei have the following symbols: N (amide nitrogen), CA (α carbon), HA (α proton), CB (β carbon), HB (β proton), CO (carbonyl carbon) and HN (amide proton). The fragments HNN and NNH are usually shortened to HN and NH respectively. If for the given nuclei its chemical shift is not measured its symbol is written in parentheses. In the case of α and β protons (carbons) their chemical shifts are sometimes measured in a single dimension (as separate signals on the spectrum) and the sequence HBHA (or CBCA for carbons) corresponds to this dimension. If the coherence transfer is symmetric with respect to the middle nucleus (e.g. 3D (HN)CONH), the redundant fragment is omitted (here 3D HNCO).

Spin systems and resonance assignment

Groups of nuclei corresponding to separate signals on multidimensional spectra are called spin systems. The simplest strategy of resonance assignment is based on registering a pair of experiments in which the molecule is split into non-overlapping spin systems (as in the case of HNCO on Fig 3.). If the spin systems from the two experiments partially overlap it is possible, by comparing chemical shifts, to determine the sequence in which the spin systems occur in the protein. Groups of sequentially arranged spin systems are called chains. If the chains are long enough or we are able to obtain additional data about individual spin systems (like determining which of them are alanines) it might be possible to assign individual spin systems to specific positions in the protein backbone and thus assign their constituent chemical shifts to specific nuclei.

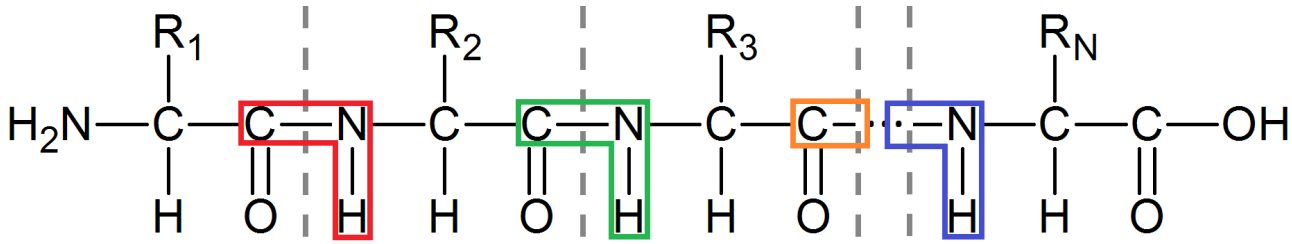

Fig. 3 Spin systems (different colours) corresponding to separate peaks visible on a 3D HNCO spectrum.

On overview of the experiments used in this project

Indices used in the figures illustrating coherence transfers indicate the relative positions of amino acids from which they came in the protein sequence.

- ^{1}H, ^{15}N HSQC

A simple example of a multidimensional experiment is the most common of protein NMR experiments – the two dimensional ^{1}H, ^{15}N HSQC (heteronuclear single quantum coherence). Here the amide protons are excited and polarisation is transferred to the directly bonded amide nitrogen. It is then transferred back to the amide protons and detected. Each amino acid residue (with the exception of prolines, which do not have amide protons, and the N-terminal amine group) gives rise to a single peak on this spectrum (plus there can be peaks from side-chain NH groups). Due to the above it is the basic spectrum used for to test the quality of a sample. By tracking the changes in peak position on this spectrum it is possible to ascertain the stability of a proteins conformation (e.g. with respect to temperature or buffering solution) or binding of other molecules.

- 3D HNCO

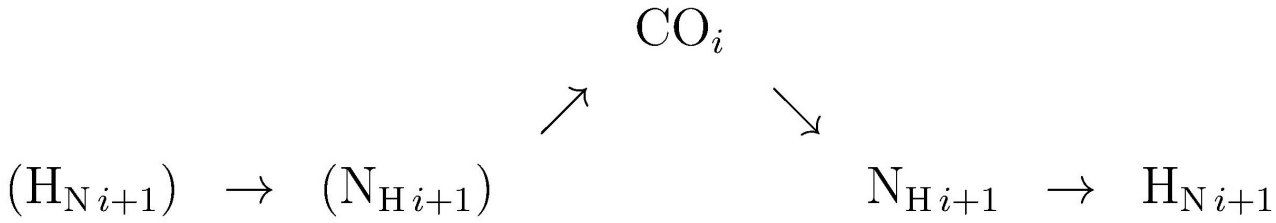

Fig. 4 Coherence transfer in the 3D HNCO experiment.

This is the most sensitive of all the „triple resonance" experiments (which utilize ^{1}H, ^{13}C and ^{15}N nuclei in a single experiment). The coherence transfer (Fig. 4) indicates that it splits the protein backbone into non-overlapping spin systems, with a single peak per amino acid residue - this will be a common theme of all the experiments presented here. As in the case of the ^{1}H, ^{15}N HSQC experiment due to the lack of amide protons proline residues (or rather the spin systems including their amide groups) do not give rise to peaks.

- 3D HN(CA)CO

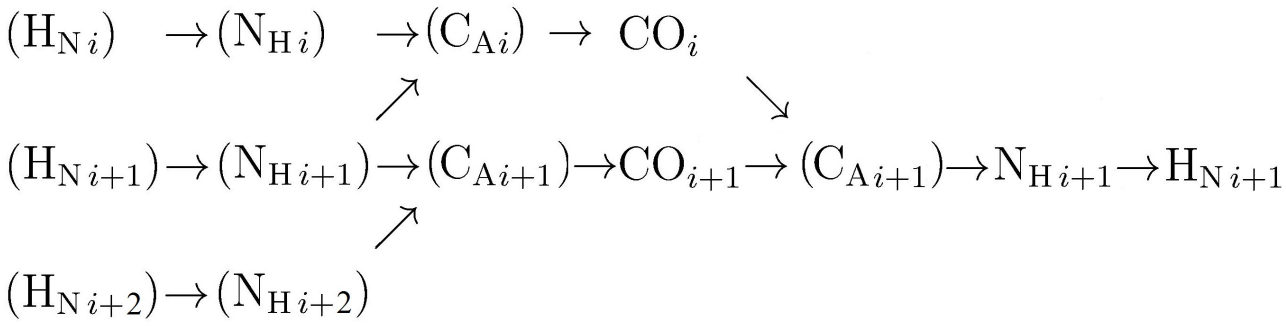

Fig. 5 Coherence transfer for the 3D HN(CA)CO experiment.

Due to its use of N-CA polarisation transfer (Fig. 5.) this spectrum contains two sets of signals – the first one is identical to the 3D HNCO spectrum (and can be easily identified by overlaying the spectra), the other one different. Together with 3D HNCO for every NH pair we obtain information about the chemical shifts of carbonyl carbons from both the same residue (intra signal) and the preceding one (inter signals, also present on HNCO). This information alone is usually not enough to sequentially arrange the spin systems but it can be used to augment and/or verify assignments performed using the experiments described below.

- 3D (HBHA)CBCA(CO)NH

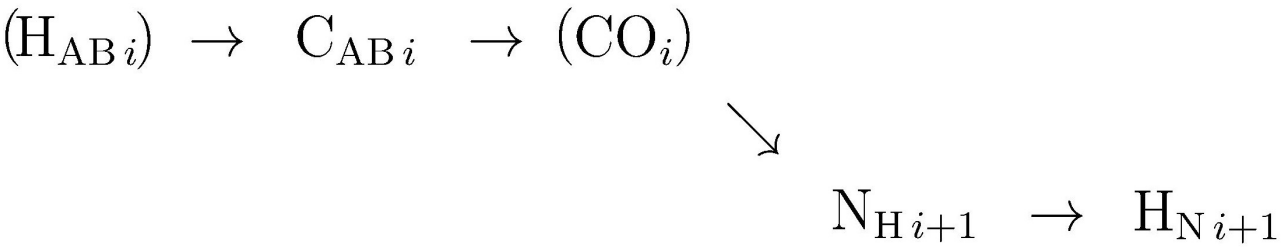

Fig. 6 3D (HBHA)CBCA(CO)NH coherence transfer.

Two sets of signals are present on this spectrum, corresponding to the triples $C_{A\,i}$-$H_{N\,i+1}$-$N_{H\,i+1}$ and $C_{B\,i}$-$H_{N\,i+1}$-$N_{H\,i+1}$. In the literature this experiment is most commonly referred to as 3D CBCA(CO)NH.

- 3D (HBHA)CBCANH

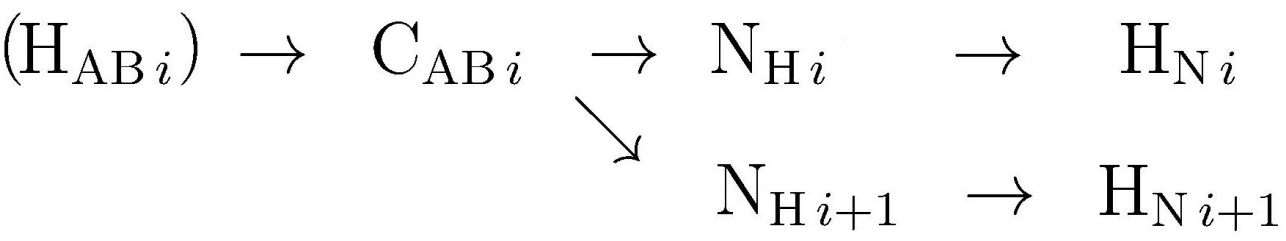

Fig. 7 Coherence transfer in the 3D (HBHA)CBCANH experiment.

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From the coherence transfer (Fig 7.) it follows that four sets of signals will be present on the 3D (HBHA)CBCANH spectra. Two of them will be the same as on the 3D (HBHA)CBCA(CO)NH (and can be identified be overlaying the spectra). The two new sets will correspond to the triples $C_{A\,i}$-$H_{N\,i}$-$N_{H\,i}$ and $C_{B\,i}$-$H_{N\,i}$-$N_{H\,i}$. Signals from CB and CA carbons have different signs, which can be useful in ambiguous cases. Together with the 3D (HBHA)CBCA(CO)NH spectrum this experiment furnishes us with both intra and inter type correlation of NH pairs with CB and CA chemical shifts. For small proteins this is usually enough to unambiguously arrange the spins systems into chains. The absence of β carbons for a particular spin system together with a characteristically lowered α carbon chemical shifts enables the identification of glycine residues. Alanines, serines and threonines can be identified by their β carbon shifts. Together with the sequentially arranged chains this type of data is usually enables the completion of the resonance assignment. This experiments is also known as 3D CBCANH.

Experimental procedure

Week one – Spectra acquisition and analysis program familiarization

Day 1.

After acquainting yourself with the spectrometer and operational procedures:

1. Place the sample in the spinner and after centering in the sample gauge inject into the spectrometer.
2. Tune the proton channel and check the tuning for the nitrogen and carbon channels.
3. Lock the sample and set the deuterium lock parameters.
4. Increase the magnetic field homogeneity by shimming the sample.
5. Calibrate the proton pulse by finding the duration of pulse rotating the water signal by 360 degrees.
6. Acquire the 2D ^{1}H, ^{15}N HSQC spectrum. Determine the phase correction factors and spectral widths for 3D experiments.
7. Acquire the ^{1}H-^{13}C plane of the 3D HNCO experiment. Determine the carbonyl spectral widths for 3D experiments.
8. Prepare the acquisition of the 3D HNCO spectrum so that it ends by the time the next day of the lab starts.

Day 2.

1. Acquire the ^{13}C-^{1}H plane of the 3D (HBHA)CBCA(CO)NH experiment. Determine spectral widths for the 3D experiments.
2. Prepare the acquisition of the 3D HN(CA)CO spectrum so that it ends by the time the next day of the lab starts.
3. Fourier transform the 3D HNCO data and convert to Sparky (UCSF) data format.
4. Familiarise yourself with the multidimensional spectrum analysis program Sparky.

Day 3.

1. Prepare the acquisition of the 3D (HBHA)CBCA(CO)NH and 3D (HBHA)CBCANH spectra so that it ends by the time the next week starts. While allocating time between these two experiments take into consideration the sensitivity and resolution requirements and available instrument time.
2. Fourier transform the 3D HN(CA)CO data and convert it to the UCSF format.
3. Familiarise yourself with the methods of working with multiple linked spectra in the Sparky program.
4. Lear how to prepare two-dimensional strips (strip plots) of higher dimensional data and their use in Winding sequential links between spin systems in the Sparky program.

Week two – Resonance assignment and writing the lab report.

Day 4.

1. Fourier transform all the acquired spectra and convert them to the UCSF data format.

Using the Sparky program:

2. Prepare a list of NH spin systems based on the 3D HNCO spectrum.
3. Using this list find peaks on the 3D HN(CA)CO, 3D (HBHA)CBCA(CO)NH and 3D (HBHA)CBCANH spectra. Inspect the spectra an check whether the list contains any errors or omissions.
4. Where possible determine the amino acid type for individual spin systems based on α and β carbon chemical shifts.

Day 5.
1. Arrange as many spin systems into sequential chains using the strip-plot method using the 3D (HBHA)CBCA(CO)NH and 3D (HBHA)CBCANH spectra pair. In ambiguous cases use the 3D HNCO and 3D HN(CA)CO pairs as well.
2. Perform resonance assignment based on spin system chains and identified amino acids.
3. Try to assign any leftover spin systems by combining the information on unassigned spin systems and missing resonance assignments.

Day 6.
1. Write/finish the laboratory report.

Laboratory report guidelines

It is expected that the final report will follow the conventional structure for experimental report, in particular it should contain:

- an abstract,
- a short theoretical introduction,
- descriptions of the equipment used, protein sample and experimental details (numbers of points, spectral widths) and data processing (weighting functions)
- data analysis which should include information on the assigned chemical shifts (types, number) and (if necessary) on unassigned shifts with the reasons they could not be assigned and how could this be rectified. The report should include a figure showing the ^{1}H, ^{15}N HSQC spectrum with the assigned peaks. The usefulness of the individual experiments in the process of resonance assignment should be commented upon - was there signal overlap, was the information obtained enough to perform the assignment, were all the experiments necessary to perform it. If the different spectra were congruent and are whether the assigned chemical shifts deviate from typical values for individual amino acid types.

Introductory test

Commencing with laboratory work is contingent upon passing the pre-laboratory test. The form of the test will be decided by the lab assistant. Participants should familiarize themselves with the literature given at the end of this handout. The exam will cover the following topics:

- Spin ½ isotopes (in the ground state) of biologically import ant elements and their natural

abundance.

- Spins in a (external) magnetic field. The magnetic resonance phenomenon, magnetogyric ratio.
- Nuclear shielding and the effective field. Definition of the δ chemical Shift and the reason for introducing this quantity
- Interactions between spins in a molecule. Coupling constant J. Fine structure and multiplet structure of NMR spectra.
- Qualitative description of the factors influencing the appearance of a NMR spectrum using a simple organic molecule (like ethanol) as an example.
- The Fourier transform and its applications in NMR spectroscopy.
- The motivation for acquiring multidimensional NMR spectra. Data furnished by correlation spectra (like COSY) and experiments using the nuclear Overhauser effect (NOESY).
- Chemical structure of proteins, general information on the structure of individual amino acids.

Literature

Basic:

- Sections 3.7 and 6.2 in
  David Sheehan *Physical Biochemistry. Principles and Applications. Second Edition*. Wiley-Blackwell, 2009

- Chapters J1 and J2 (details of the implementation of multidimensional experiments aren't required) in
  Igor N. Serdyuk, Nathan R. Zaccai and Joseph Zaccai, *Methods in Molecular Biophysics. Structure, Dynamics, Function*, Cambridge University Press, 2007

- Description of solution-state NMR in
  Peter Atkins *Physical Chemistry* OUP (any recent edition)

  Supplementary:

- Chapter J3 in
  Igor N. Serdyuk, Nathan R. Zaccai and Joseph Zaccai, *Methods in Molecular Biophysics*. Structure, Dynamics, Function, Cambridge University Press, 2007
  (A description of NMR spectroscopic investigation of protein and nu clear acid structure and Dynamics)

- Kazimierczuk, K., Misiak, M., Stanek, J., Zawadzka-Kazimierczuk, A. and Koźmiński, W. (2012). *Generalized Fourier transform for non-uniform sampled data*. W *Novel Sampling Approaches in Higher Dimensional NMR* (pages 79-124). Springer Berlin Heidelberg.
  (Mostly signal processing, that is the pathway from the measured signal to spectrum. It also contains a presentation of performing high dimensionality and/or resolution experiments using non-uniform sampling)

- James Keeler, *Understanding NMR Spectroscopy* Wiley, 2013
  (An accessible description of NMR theory on a basic level. Unfortunately there is some terminological confusion in the chapters with regard to quantum mechanics – especially the concepts of a mixed state, density matrix and reduced density matrix.)