Recommended NMR Textbooks

*Modern NMR Techniques for Chemistry Research*, Pergamon Press

Wüthrich, K. (1986)
*NMR of Proteins and Nucleic Acids*, John Wiley and Sons

*NMR of Macromolecules: A Practical Approach*, Oxford Univ. Press

Cavanagh, J., et al. (1996)
*Protein NMR Spectroscopy, Principles and Practice*, Academic Press

Evans, J. N. S. (1999)
*Biomolecular NMR Spectroscopy*, Oxford Univ. Press
Useful websites

http://www.ch.ic.ac.uk/local/organic/nmr.html
NMR Spectroscopy. Principles and Application.
Six second year lectures given at Imperial College, U.K.

http://uic.unl.edu/nmr_theory.html
http://www.shu.ac.uk/schools/sci/chem/tutorials/molspec/nmr1.htm
Theoretical principles of NMR
Courtesy of Sheffield Hallam University, U.K.

http://www.nature.com/nsb/wilma/v4n10.875828203.html
links to various NMR and structural biology web sites
simulation and analysis software; NMR research groups, etc.
Origin of the NMR signal

Nuclear subatomic particles have spin

1. If the number of neutrons and protons are both even the nucleus has 0 spin
   i.e. \(^{12}\text{C}\) (6 neutrons + 6 protons = 12) has \(I = \varnothing\) spin

2. If the number of neutrons plus protons is odd the nucleus has a half-integer spin (1/2, 3/2, 5/2)
   i.e. \(^{13}\text{C}\) (7 neutrons + 6 protons = 13) has \(I = 1/2\) spin

3. If the number of neutrons and protons are both odd then the nucleus has an integer spin (1, 2, 3)
   i.e. \(^{14}\text{N}\) (7 neutrons + 7 protons = 14) has \(I = 1\) spin; spin 1 nuclei are quadrupolar (relax fast)

For high resolution applications, we use spin ½ nuclei (\(^{1}\text{H},^{13}\text{C},^{15}\text{N},^{31}\text{P}\) in biology);
Nuclear spins and the energy levels in a magnetic field

• A nucleus of spin I has $2I + 1$ possible orientations (a nucleus with spin 1/2 has 2 possible orientations)

• Each level is given a magnetic quantum number $m$

• For a spin $\frac{1}{2}$ nucleus like $^1\text{H}$, the two possible values of $m$ are $+1/2$ and $-1/2$

• In the absence of an external magnetic field, these orientations have equal energy; if a magnetic field is applied, the energy levels are split
Nuclear spins and the energy levels in a magnetic field

The energy of a particular level is given by:

\[ E = \gamma \ h \ m \ B_0 \]

where: \( \gamma \) is the the gyromagnetic ratio, a nuclear property (a measure of the polarizability of the nucleus)
\( h \) is Planck's constant divided by \( 2\pi \) (\( h = h/2\pi \))
\( B_0 \) is the strength of the magnetic field

The difference in energy between levels (the transition energy)

\[ \Delta E = (1/2 - (-1/2)) \ \gamma \ hB_0 = \gamma \ hB_0 = h\omega_0 \]

If the magnetic field is increased, so is \( \Delta E \) (as \( \Delta E \) increases, so does sensitivity)
Nuclear precession in a magnetic field: semi-classical description

The nucleus possesses a magnetic moment $M$ proportional to its spin $I$.

- In a magnetic field, the axis of rotation will *precess* about the magnetic field $B_0$: $\frac{dM}{dt} = -\gamma M \times B_0$.

- The frequency of precession ($\omega_0$, *Larmor frequency*) is identical to the transition frequency ($\omega_0 = -\gamma B_0$).

- The precession may be clockwise or anticlockwise depending on the sign of the gyromagnetic ratio (+$\gamma$ or -$\gamma$).
# NMR properties of nuclei of common use in biology

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Spin</th>
<th>Abundance</th>
<th>Magnetogyric ratio</th>
<th>NMR frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(I)</td>
<td></td>
<td>$\gamma/10^7 \text{ rad T}^{-1}\text{s}^{-1}$</td>
<td>MHz (2.3 T magnet)</td>
</tr>
</tbody>
</table>

Derivation for $^1\text{H}$: $(26.7519 \times 10^7 \text{ rad/T/S}) \times 2.3 \ T/(2\pi \text{ rad}) = 97.9 \ MHz$

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<tr>
<td>$^1\text{H}$</td>
<td>1/2</td>
<td>99.985 %</td>
<td>26.7519</td>
<td>100.000000</td>
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<tr>
<td>$^2\text{H}$</td>
<td>1</td>
<td>0.015</td>
<td>4.1066</td>
<td>15.351</td>
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<td>100</td>
<td>7.08013</td>
<td>26.466</td>
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<td>12.26</td>
<td>-5.9550</td>
<td>22.193173</td>
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Origin of a macroscopic (observable) NMR signal

Out of a large collection of moments, a surplus have their z component aligned with the applied field, so the sample becomes magnetized in the direction of the main field $B_o$.

- The parallel orientation is of lower energy than the antiparallel.

- At equilibrium, spins will be distributed according to Boltzmann distribution between the two energy states.

- A net magnetization parallel to the applied magnetic field arises because of the small population difference between states.
S/N in NMR is poor because energy levels are so close.

According to Boltzmann distribution:

\[
\frac{n_1}{n_2} = e^{-\frac{(E_1 - E_2)}{kT}}
\]

If the system is exposed to a frequency:

\[
\nu = \frac{E_2 - E_1}{h}
\]

then the energy absorbed is proportional to the difference

\[
\Delta E \gg kT
\]

If

(as is the case for optical spectroscopy), then essentially all the molecules will be in their ground state configuration.
S/N in NMR is poor because energy levels are so close

\[ \frac{n_1}{n_2} = e^{-\frac{(E_1 - E_2)}{kT}} \]

If instead \( \Delta E \approx kT \), then the net absorption of energy will be very small because the rate of upward transitions is equal to the rate of downward transitions.

For this reason, we use magnets of increasing strength to separate energy level more and increase the sensitivity of the experiment.
The frequency of absorption of the NMR signal depends on the external field as we have seen
\[ \nu_0 = \gamma B_0 \]

Let us now introduce a quantity that describes the fact that different nuclei in the sample experience slightly different magnetic fields because of chemical structure and conformation
\[ \nu = (1-\sigma) \gamma B_0 \]

Finally, let us introduce a scale that is field-independent, so that we can compare directly data recorded on different spectrometers:
\[ \delta = (\nu - \nu_0) / \nu_0 \times 10^6 \]

We use a standard sample (e.g. DSS) to reference all of our spectra, so that we can report the resonance frequency for our proton in a universal, field-independent manner
Amino acid chemical structure leads to distinct shift for each residue: random coil chemical shift values

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>αH</th>
<th>βH</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met</td>
<td>8.42</td>
<td>4.52</td>
<td>2.15,2.01</td>
<td>γCH2, εCH3 2.64, 2.64</td>
</tr>
<tr>
<td>Cys</td>
<td>8.31</td>
<td>4.69</td>
<td>3.28,2.96</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>8.09</td>
<td>4.70</td>
<td>3.32,3.19</td>
<td>2H 7.24, 4H 7.65, 5H 7.17, 6H 7.24, 7H 7.50, NH 10.22</td>
</tr>
<tr>
<td>Phe</td>
<td>8.23</td>
<td>4.66</td>
<td>3.22,2.99</td>
<td>2,6H 7.30, 3,5H 7.39, 4H 7.34</td>
</tr>
<tr>
<td>Tyr</td>
<td>8.18</td>
<td>4.60</td>
<td>3.13,2.92</td>
<td>2,6H 7.15, 3,5H 6.86</td>
</tr>
<tr>
<td>His</td>
<td>8.41</td>
<td>4.63</td>
<td>3.26,3.20</td>
<td>2H 8.12, 4H 7.14</td>
</tr>
</tbody>
</table>
1D NMR spectrum of a protein – the chemical shift scale

amides  NH$_2$  H$_\alpha$  Side chain CH$_2$

Side chain CH$_3$
How are experiments recorded: the Radiofrequency field (RF)

Modern NMR use radio-frequency pulses to generate observable signals and manipulate the spin state of the system under study: this is the basis of 2D/3D NMR but also of all FT NMR.

The alternating voltage applied across the ends of the coil in the NMR probe induces an alternating magnetic field in the sample.

The geometry of the coil is arranged so that this field is perpendicular to the applied field (in the xy plane).

This oscillating $B_1$ field is substantially smaller than the external field $B_0$ (thousands of times smaller), and close to the Larmor frequency.
FT-NMR spectroscopy relies on weak RF pulses to excite signal

1. Static (z-axis) magnetization $M_0$ is not observable as NMR signal

2. Turn RF on: $M_0$ driven around x axis

3. Turn RF off after a p/2 pulse (control pulse power or length)

4. $M_0$ precesses in the xy plane at its Larmor frequency

5. The receiver records the radio signal generated by the precessing magnetic moment $M_{x,y}$
The NMR Signal and Spectrum

• The emission signals are oscillatory and physically damped (damped harmonic oscillations)

• This signal is called the Free Induction Decay or FID

• The actual spectrum is recovered from the FID via Fourier transformation, which transforms the time interferogram into a frequency spectrum

• Without FT NMR, it would take the square of the time to obtain an equivalent signal/noise ratio
Example: FID and a 1D spectrum

FID

Fourier Transform

1D spectrum
The effect of relaxation

No relaxation: infinitely sharp lines

Relaxation has two components: inhomogeneity (line width, $T_2$) and return to thermal equilibrium ($T_1$)
1D spectra contain structural information, but is hard to extract: need multidimensional NMR

Dispersed amides: protein is folded

Hα: protein contains β-sheet

Downfield CH3: Protein is folded
1D vs 2D homonuclear NMR Spectroscopy

Basic structure of 1D experiment

Basic structure of 2D experiment (e.g. NOESY)

Jeener and Ernst, 1972
2D homonuclear NMR Spectroscopy

Two basic experiments: NOESY and COSY
“cross-peaks” in multidimensional NMR carry structural information

Diagonal is closely related to 1D spectrum

2D contour representation: peaks outside diagonal are called cross-peaks
The NOESY experiments detects interactions between spins that are close in space and dipolar coupled: the magnetization transfer mechanism is essentially the same as FRET in optical spectroscopy (fluorescence).
Nuclear Overhause Effect Spectroscopy (NOESY)

1. Excite with 90° pulse

2. During t1, spins are labeled with their Larmor frequency

3. The second 90° pulse exchanges rotates magnetization to -z

4. Magnetization is exchanged between spin I and S during the mixing time

5. The final 90° pulse makes the signal observable and signal is acquired in t2
Nuclear Overhause Effect Spectroscopy (NOESY)

When magnetization is exchanged, spin I signal contains information on spin S and vice versa (cross-peaks)

Cross-peaks appear between spins which are close in space (<5-6 Å) (assignments and structure)
Nuclear Overhause Effect Spectroscopy (NOESY)

The mixing coefficients, $a_{IS} = a_{SI}$ are proportional to the NOE between these two nuclei.

The NOE is related to the distance $r$ between the two spins and the correlation time $t_c$ (the time for reorientation of the IS vector in the molecule): structure and motion.

$$\text{NOE} \propto r_{IS}^{-6} f(t_c) \tau_m$$
A simple example: a small immunogenic peptide

1D spectra contain structural information, but is hard to extract: need multidimensional NMR.

Dispersed amides: protein is folded

Ha: protein contains β-sheet

Downfield CH3: protein is folded

A simple example: a small immunogenic peptide
Patterns of NOE interactions define protein secondary structure

<table>
<thead>
<tr>
<th>Distance</th>
<th>α-helix</th>
<th>3_10-helix</th>
<th>β</th>
<th>β_p</th>
<th>turn I^a</th>
<th>turn II^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>d_{αN}</td>
<td>3.5</td>
<td>3.4</td>
<td>2.2</td>
<td>2.2</td>
<td>3.4</td>
<td>2.2</td>
</tr>
<tr>
<td>d_{αN} (i,i+2)</td>
<td>4.4</td>
<td>3.8</td>
<td></td>
<td></td>
<td>3.6</td>
<td>3.3</td>
</tr>
<tr>
<td>d_{αN} (i,i+3)</td>
<td>3.4</td>
<td>3.3</td>
<td></td>
<td></td>
<td>3.1-4.2</td>
<td>3.8-4.7</td>
</tr>
<tr>
<td>d_{αN} (i,i+4)</td>
<td>4.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_{NN}</td>
<td>2.8</td>
<td>2.6</td>
<td>4.3</td>
<td>4.2</td>
<td>2.6</td>
<td>4.5</td>
</tr>
<tr>
<td>d_{NN} (i,i+2)</td>
<td>4.2</td>
<td>4.1</td>
<td></td>
<td></td>
<td>3.8</td>
<td>4.3</td>
</tr>
<tr>
<td>d_{βN}</td>
<td>2.5-4.1</td>
<td>2.9-4.4</td>
<td>3.2-4.5</td>
<td>3.7-4.7</td>
<td>2.9-4.4</td>
<td>3.6-4.6</td>
</tr>
<tr>
<td>d_{αβ} (i,i+3)</td>
<td>2.5-4.4</td>
<td>3.1-5.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Observable NOE interactions (<5 Å) in regular protein secondary structures
1D spectra contain structural information, but is hard to extract: need multidimensional NMR.

- **Dispersed amides:** protein is folded
  - **Ha:** protein contains b-sheet
- **Downfield CH:** protein is folded

**COherence transfer SpectroscopY (COSY)**

The COSY experiments detects interactions (correlation) between spins that are scalar coupled: beware, it can only be understood through quantum mechanics.
1. Excite with $90^\circ$ pulse

2. During $t_1$, spins are labeled with their Larmor frequency

3. During $t_1$, if spins are scalar coupled, the signal encodes this information as well

4. The second $90^\circ$ pulse exchanges magnetization between spins: spin I now has memory of spin S and viceversa

5. Signal is acquired in $t_2$
COherence transfer SpectroscopY (COSY)

When magnetization is exchanged, spin I signal contains information on spin S and vice versa (cross-peaks)

Cross-peaks appear between spins which are scalar coupled (assignments)

The cross-peak fine structure contains information on scalar coupling (structure)
Structural information: scalar couplings directly gives you the torsion angles that define protein or n.a. structure

\[ {^3J_{\text{HaN}}} = 5.9 \cos^2 \phi - 1.3 \cos \phi + 2.2 \]

\[ {^3J_{\alpha\beta}} = 9.5 \cos^2 \chi_1 - 1.6 \cos \chi_1 + 1.8 \]

(Karplus, 1958)

Cross-peaks in COSY experiments occur only between residues that are scalar coupled; in turns, these couplings can be measured in COSY experiments.
1D spectra contain structural information, but is hard to extract: need multidimensional NMR.

Dispersed amides: protein is folded.

Ha: protein contains β-sheet.

Downfield CH₃: protein is folded.

COSY and NOESY connectivities in the polypeptide unit:

COSY (broken lines) and NOESY (continuous lines)
Amino acid identification from scalar coupling patterns

Different pattern of scalar couplings allows amino acid type identification in correlated spectra (COSY, 2QF-COSY, TOCSY)

This is the first step towards complete spectral assignments of a protein spectrum (at least before heteronuclear NMR)
NOE interactions, scalar couplings (and chemical shifts) can be combined to define protein secondary structure.

<table>
<thead>
<tr>
<th>d_{\alpha\beta}(i,i+4)</th>
<th>d_{\alpha\beta}(i,i+3)</th>
<th>d_{\alpha\beta}(i,i+3)</th>
<th>d_{\alpha\gamma}(i,i+2)</th>
<th>d_{\alpha\gamma}(i,i+2)</th>
<th>d_{\alpha\gamma}</th>
<th>d_{\alpha\gamma}</th>
<th>d_{J_{\alpha\alpha}}(Hz)</th>
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<tbody>
<tr>
<td>α-Helix</td>
<td>3_{10}-Helix</td>
<td>Turn I</td>
<td>Turn II</td>
<td>Turn I’</td>
<td>Turn II’</td>
<td>Half-Turn</td>
<td></td>
</tr>
<tr>
<td>β,β'</td>
<td>α-Helix</td>
<td>3_{10}-Helix</td>
<td>Turn I</td>
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NOE interactions (<4.5 Å) and scalar coupling patterns in regular protein secondary structures.
Even 2D spectra can be (and indeed are) very crowded.

Realistic limit of homonuclear NMR: proteins of 100-120 amino acids; spectra of larger proteins are too crowded.
3D Heteronuclear NMR
Useful nuclei such as $^{15}$N, $^{13}$C are rare

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Spin (I)</th>
<th>Natural abundance</th>
<th>Magnetogyric ratio $\gamma/10^7$ rad T$^{-1}$s$^{-1}$</th>
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Requirements for Heteronuclear NMR: isotope labeling

• Isotopically labelled proteins can be prepared straightforwardly in *E. coli* by growing cells in minimal media (e.g. M9) supplemented with appropriate nutrients (¹⁵NH₄Cl, ¹³C-glucose)

• Metabolic pathways can be exploited and appropriate auxotrophic strains of *E. coli* can also be used for selective labelling: e.g. use acetate instead of glucose and obtain selective labeling of certain side chain CH₃

• Isotopic labelling of protein expressed in eukaryotic cells is expensive but can be done (post-translational modifications can be studied but you need $$$$)
Heteronuclear NMR exploits 1-bond scalar couplings

- 1-bond couplings are large (20-150 Hz) compared to HH couplings (1-10 Hz)

- They are independent of conformation: no structural insight but wonderful for assignments
The basic building block of Heteronuclear NMR (INEPT)

- Polarization of $^{13}\text{C}$ and $^{15}\text{N}$ is low: start with $^1\text{H}$ polarization
- Use 1-bond scalar couplings to transfer magnetization from $^1\text{H}$ to the nucleus of interest
- Delay $\Delta$ must be set to $1/4J$ for optimal transfer: in the absence of relaxation, magnetization transfer is 100% efficient
The basic building block of Heteronuclear NMR (INEPT-1D)

Consider a two spin system, I & S:

- **A**
  - Initial state: \( I_z \) and \( S_z \)
  - \((90\times I)\)

- **B**
  - \( \Delta = \frac{1}{4}J_{IS} \)
  - Free precession

- **C**
  - \( (180\times I) \) or \( (180\times S) \)
  - \( \frac{\Delta}{2} \) \([-I_y - 2I_xS_z]\)

- **D**
  - \( \Delta = \frac{1}{4}J_{IS} \)
  - Free precession

- **E**
  - \( (90\times y)I \) or \( (90\times S) \)
  - \(-2I_xS_z\) (Iy antiphase magnetization)

- **F**
  - Acquire on N or C channel

- **G**
  - \(-2I_zS_y\) (Sy antiphase magnetization)
The basic building block of 2D Heteronuclear NMR (HSQC)

- Polarization of $^{13}$C and $^{15}$N is low: start with $^1$H, transfer to $^1$H with INEPT (sensitivity increases by the ratios of $\gamma$, e.g. 10 for $^{15}$N)

- Label magnetization with $^{15}$N Larmor frequency in t1 and record $^{15}$N evolution in the first dimension

- Go back to $^1$H for detection with a reverse INEPT, i.e. from $^{15}$N to $^1$H and record $^1$H evolution in the direct dimension (high s/n)
HSQC is the building block and foundation for very many heteronuclear NMR experiments

- Measurements of relaxation properties (motion)
- Spectral assignments

- **3D versions of NOESY and COSY spectra (structure)**
The most important experiment for protein structure determination: 3D NOESY HSQC

- Measurements of $^1\text{H}-^1\text{H}$ distances (as in 2D NOESY)

- Resolution is spread in a third dimension (usually $^{15}\text{N}$ but also $^{13}\text{C}$; for nucleic acids mostly $^{13}\text{C}$; for protein/nucleic acid complexes you can observe only the protein, only the RNA or only the contact from one to the other)
Protein and nucleic acid 3D structure generation from NMR

Typical protocol for 3D structure generation from NMR data (with 3D data)