

5.10: Nuclear Magnetic Resonance (NMR) Theory and Solution NMR

Nuclear magnetic resonance (NMR) occurs when nuclei in an unmoving magnetic field is disturbed by an oscillating magnetic field; the nuclei generate an electromagnetic signal, whose frequency depends on the magnetic field applied. This happens near resonance, where the frequency of oscillation aligns with the frequency of the nuclei. The magnetic field strength, chemical environment, and isotope affect resonance. NMR spectroscopy is used to elucidate the structure of organic molecules, study crystals and non-crystals, and can be applied to medical diagnostic imaging. NMR has three basic steps: nuclear spins are aligned in a magnetic field, the nuclear spins are disturbed by a radio-frequency (RF) pulse, the NMR signal is detected during or after the RF pulse. NMR was described by Isidor Rabi in 1938 and he won the Nobel Prize in Physics in 1944. Felix Bloch and Edward Mills Purcell used NMR on lipids and solids and won the Nobel Prize in Physics in 1952. Yevgeny Zavoisky observed MNR in 1941, prior to Bloch and Purcell; however, he discarded the results as unreproducible. Alongside x-ray crystallography and cryogenic-electron microscopy, NMR is one of the three techniques that is used to elucidate the structure of proteins.

Nuclear Spin

Atomic nuclei of protons and neutrons have spin, an intrinsic quantum property; it is an angular momentum similar to the angular momentum of a revolving orb. The overall spin of nuclei is dictated by the spin quantum number, S . If the numbers of protons and neutrons in the nucleus are even, $S=0$, which means no overall spin. Electrons couple in non-degenerate atomic orbitals; even numbers of protons or neutrons, which are spin $1/2$ fermions, also give no overall spin.¹

Protons and neutrons have lower energy when spins are aligned; the parallel spin alignment of particles does not violate the Pauli exclusion principle. The quark structure of protons and neutrons is responsible for the lower energy; the spin ground state for the nucleus of deuterium, which has one proton and one neutron, is one, not zero. Tritium, due to the Pauli exclusion principle, has two anti-parallel spin neutrons, and a proton spin of $1/2$; the nuclear spin value for tritium is $1/2$, comparable to ^1H . The nuclear magnetic resonance (NMR) absorption frequency for tritium is akin to ^1H . For non-radioactive nuclei, overall spin is non-zero; as an example, ^{27}Al nuclei have an overall spin of $5/2$.¹

A non-zero spin is related to a non-zero magnetic dipole moment, μ : $\mu = \gamma S$; γ is the gyromagnetic ratio. This relates to the relation of the angular momentum and magnetic dipole moment of a rotating orb. They are vectors parallel to the revolution horizontal; the length increases directly with the rotating frequency. The magnetic moments' interaction with magnetic fields permits the study of NMR values; these are associated with alterations between nuclear spin levels during RF incitement or due to Larmor precession of the magnetic moment after stimulation. Nuclei with even numbers of protons and neutrons have zero nuclear magnetic dipole moment and lack an NMR signal. ^{13}C , ^{31}P , ^{35}Cl , and ^{37}Cl generate an NMR signal, whereas ^{18}O does not.⁴

In contrast to NMR, electron spin resonance (ESR) detects transitions in electronic spin, not nuclear spin.¹⁷ A smaller number of molecules with unpaired electron spins show ESR (or electron paramagnetic resonance (EPR)) absorption than NMR; however, ESR has a higher signal per spin than NMR.¹⁷ While NMR can be applied to proteins, EPR/ESR is mainly used to study metal complexes and organic radicals.¹⁷

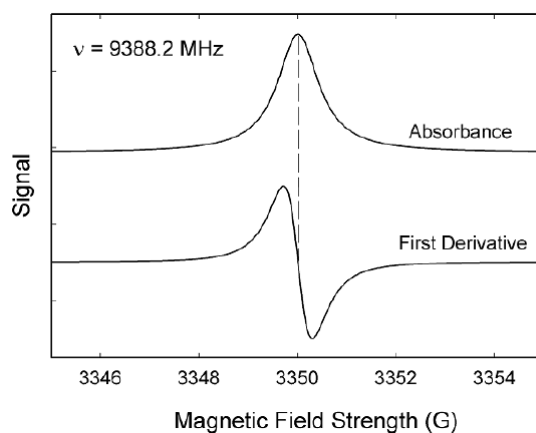


Figure 5.10.1. Magnetic field strength vs. signal spectra for EPR/ESR. The absorption signal is measured and the first derivative is calculated. dX/dB is the signal; it is the derivative of the magnetic susceptibility. When the spectrum passes zero, that is the absorption peak of the spectrum. This image is from: upload.wikimedia.org/wikipedia/commons/4/4a/EPR_lines.png.

Magic Angle Spinning (MAS)

In NMR, magic-angle spinning (MAS) is an approach used in solid-state NMR and ^1H NMR.² The sample is spun at a frequency of 1 to 130 kiloHertz (kHz) at an angle of $\theta_m = 54.7356^\circ$, in regards to the magnetic field.² Wide lines become more narrow, which increases the resolution of the spectra.² Nuclear spin experiences three interactions: dipolar, chemical shift anisotropy (CSA), and quadrupolar; the spectral lines generated are wide and difficult to analyze.² However, these interactions are dependent on position and MAS is used to average them.² Dipole interactions between nuclei are approximately zero at 54.7356° .⁶ Nucleus-electron interactions are known as CSA, and are non-zero.⁶ MAS can partially average the quadrupolar interactions.⁶ In solution, these interactions are averaged out due to molecular movement; in solids, MAS causes narrow spectral lines, which can be used to determine CSA.⁶

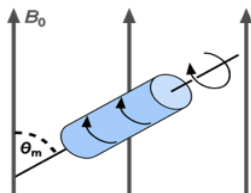


Figure 5.10.2. MAS NMR. The blue sample is rotating at 70 kHz in a magnetic field, B_0 . This image is from: [upload.wikimedia.org/wikipedia...leSpinning.svg](https://upload.wikimedia.org/wikipedia/commons/4/4e/leSpinning.svg); it was created by Dtrix.

Spin Angular Momentum

Nuclear spin is a quantized intrinsic angular momentum. The values of S are limited and the x, y, and z-components are quantized; they are (half)-integer multiples of \hbar , the reduced Planck constant. The (half)-integer quantum number related to spin alongside the z-axis or the magnetic field is the magnetic quantum number, m , which is $+S$ to $-S$, in integer values. For all nuclei, there are a total of $2S+1$ angular momentum states. The z-component of the magnetic moment is⁴

$$\mu_z = \gamma S_z = \gamma m \hbar. \quad (5.10.1)$$

Energy levels for a nucleus with spin quantum number 1/2

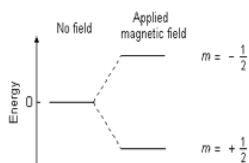


Figure 5.10.3. Nuclei spin energy splitting in an applied magnetic field. This image is from: <https://teaching.shu.ac.uk/hwb/chemi...spec/nmr1.htm#>.

Spin Energy in a Magnetic Field

Nuclei with a spin of $1/2$, like ^1H , ^{13}C , or ^{19}F have two independent linear spin states, where $m=1/2$ or $-1/2$ for the z-portion; if a magnetic field is absent, the states possess the same energy. At thermal equilibrium, the number of nuclei per state is the same. When placed in a magnetic field, the energy varies due to interactions of the nuclear magnetic dipole moment and the magnetic field applied. The energy of the magnetic dipole moment, μ , in a magnetic field, B_0 , is

$$E = -\mu B_0 = -\mu_x B_{0x} - \mu_y B_{0y} - \mu_z B_{0z}. \quad (5.10.2)$$

The z-axis lies with B_0 , and the new equation is

$$E = -\mu_z B_0 = -\gamma m \hbar B_0. \quad (5.10.3)$$

Therefore, in a magnetic field, energies vary. A spin state of $1/2$ means the spin is aligned with the magnetic field, and is the lower energy state; conversely, a spin state of $-1/2$ means the spin is not aligned with the magnetic field, and is the higher energy state. A positive gamma indicates that $m=1/2$ is the lower energy state. The difference between the high and low states is

$$\Delta E = \gamma \hbar B_0. \quad (5.10.4)$$

The lower energy state is preferred at thermal equilibrium. If more spins are up than down, net spin magnetization aside B_0 occurs.¹

Larmor Precession & Radio-Frequency Pulses

The spin magnetization is proportional to the sum of spin vectors of atomic nuclei in equal magnetic sites and moves in a cone around the field, B . At non-equilibrium, precession of magnetization in B_0 arises with the Larmor frequency

$$\omega_L = 2\pi\nu_L = -\gamma B_0. \quad (5.10.5)$$

The energy of the population does not change, as it is constant. The transverse magnetization formed by an oscillating field is detected in NMR when an RF field or pulse is applied.⁴

Chemical Shielding (Shift)

Not all nuclei of the same element resonate at the same frequency. Electrons are charged and rotate and produce a magnetic field that is the reverse of the applied magnetic field. The electronic shielding reduces the magnetic field at the nucleus; the frequency needed to obtain resonance is decreased. This is known as chemical shift, and provides an explanation for NMR's ability to ascertain chemical structures, which rely on electron density. Nuclei with higher degrees of shielding due to a higher electron density have their NMR frequency shifted up, which corresponds to a low chemical shift; in contrast, less shielding shifts NMR frequency down, which gives a high chemical shift. In solid state NMR, magic angle spinning averages these states to get frequency values at average shifts. In regular, NMR, molecular tumbling averages the CSA.¹

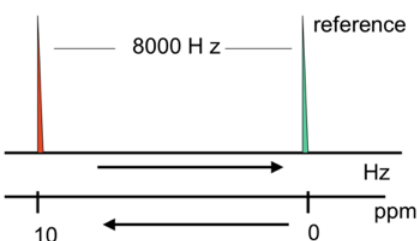


Figure 5.10.4. Example of chemical shift axis. The green peak is the reference compound for reference; the red peak is the sample compound. In this example, the distance between the two signals are 8,000 Hz; the frequency of the spectrophotometer is 800 MHz. If the reference peak is at 0, the distance between the two peaks is: $8,000(106)/800(106)=10$ ppm. This would be an example of a proton attached to an electronegative element, like oxygen. This image is from: <https://users.cs.duke.edu/~brd/Teach.../flemming.pdf>.

Relaxation

Relaxation occurs when nuclear spins revert to thermodynamic equilibrium; this is known as T_1 relaxation and indicates the average time it takes for nuclei to return to thermal equilibrium. Precessing nuclei eventually stop generating signals when they do not align; this is called T_2 relaxation. T_1 relaxation is normally longer than T_2 relaxation, because of small dipole-dipole interactions.¹

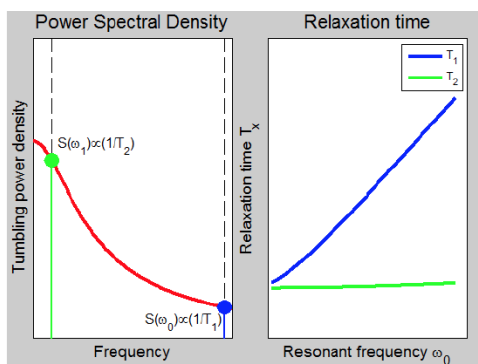


Figure 5.10.5. Animation showing the relation between Larmor frequency and T_1 and T_2 . T_2 is hardly affected. This image is from: commons.wikimedia.org/wiki/File:NmrrelaxM2.gif; it was created by Ponderthis.

Solution NMR

Less than 2% of the 120,000 structures of solved proteins are of membrane proteins; very few of those structures were solved via NMR.⁷ High quantities of protein must be used and they must be extracted from native environments using chemicals that maintain structural and functional integrity.⁸ If NMR is used, crystallization is not needed, but, new problems arise.⁸ Solution NMR can be used to determine the structures of macromolecules that go through fast rotational diffusion on timescales of less than 100

nanoseconds.¹⁵ As a result, proteins must be placed in lipid micelles, small bicelles, and nanodiscs.¹³ Proteins with a size of approximately 40 kiloDaltons or less can be obtained with solution NMR; this is due to dynamics and resonance line widths.¹⁴

Heteronuclear NMR methods used for soluble proteins can be applied to membrane proteins.¹⁸ For structural determination, proteins are ¹⁵N- and ¹³C-labeled by expression in minimal media with ¹⁵N-ammonium sulfate and ¹³C-glucose as nitrogen and carbon springs.⁵ Protein side-chains are deuterated via ²H,¹³C-glucose to obtain better spectral resolution.⁷ This lowers ¹³C relaxation, which causes lower resonance; however, less protons are available to measure ¹H-NOE's, which give distant restraints.¹¹ ²H₂O is used for culture growth and amide protons are replaced with the solvent during purification and preparation; if deuterium is used in the solvent, the NMR spectroscopy will reflect this.⁷ The rates of amide proton exchange provide information on exposed versus buried moieties.⁹

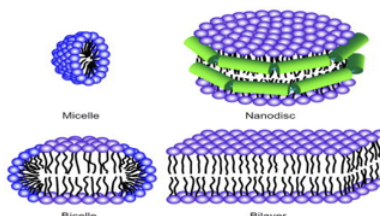


Figure 5.10.6. Solution and solid-state NMR sample preparation methods. Lipid micelles are used for membrane protein preparation for solution NMR. Dodecylphosphocholine (DPC) has a phosphocholine head and mild detergent activity makes it common in solution NMR. Short phospholipids, such as dihexanoyl-phosphatidylcholine (DHPC) or diheptanoylphosphatidylcholine can be used. Other detergents are lyso-phosphatidylglycerol, lauryl-dimethylamine oxide (LDAO), n-dodecyl- β -maltoside (DDM), and octyl- β -glucoside (β -OG). Bicelles are mixes of bilayer-making lipids; common ones are dimyristoyl-phosphatidylcholine (DMPC), and non-bilayer making lipids, such as DHPC. The ratio of long to short lipids is the q-value. The size of the bicelle depends on lipids used. In solution NMR, small bicelles with more non-bilayer making lipids works; the q-value ranges from 0.25-0.5. Small bicelles perform rotational diffusion, similar to micelles, which is detected by solution NMR. Nanodiscs are lipid bilayers enclosed by amphipathic helical proteins that stabilize the structure. Nanodiscs are made with two MSPD1's (a lipoprotein) at the edge. An empty nanodisc is around 150 kiloDaltons, which is too big for solution NMR. Short MSPD1 proteins have been made, lowering the size to 60-120 kiloDaltons, which is good for solution NMR. This image is from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5444674/>. The paper citation is: Liang, Binyong, and Lukas K. Tamm. "NMR as a Tool to Investigate Membrane Protein Structure, Dynamics and Function." *Nature structural & molecular biology* 23.6 (2016): 468.

Correlation-Time Problem

The rotational correlation time of proteins is important for NMR. It determines extraction conditions, instrumental analysis, and data processing methods. It is hard to obtain high-resolution spectra of large or aggregated proteins with NMR due to slow reorientation. The correlation-time problem can be dealt with in two ways when it comes to membrane proteins. In solid state NMR, membrane proteins in lipid bilayers or sizable bicelles are paralyzed by environmental conditions; radio-frequency irradiation, magic angle sample spinning, or sample alignment are replacements for molecular motions for the line-narrowing mechanism. For small membrane proteins, micelles or small bicelles can be used to prepare the samples; this leads to fast reorientation, which is detectable by solution NMR.⁴

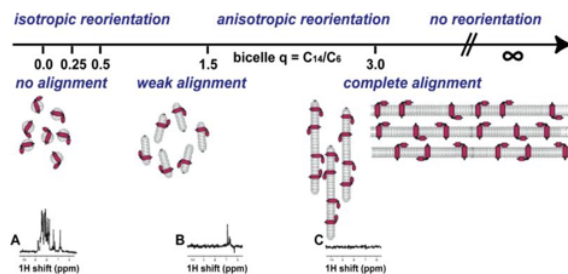


Figure 5.10.7. Linking of correlation times and alignments of proteins. Samples are labeled by q ratio for bicelles, which goes from 0 for isotropic micelles to 3 for large bicelles to infinity for bilayers. Spectra A-C are one-dimensional ¹H NMR of a membrane protein in various samples: (A) q=0 (isotropic micelle); (B) q=0.5 (medium sized bicelle); (C) q=3 (large bicelle). The correlation time problem is visible here. Large, weak signals from a protein in medium (B) and large (C) bicelles are different from a protein in a micelle (A). This image is from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3270942/>. The paper citation is: Opella, Stanley J., and Francesca M. Marassi. "Structure determination of membrane proteins by NMR spectroscopy." *Chemical reviews* 104.8 (2004): 3587-3606.

Protein NMR

NMR of proteins is unlike magnetic resonance imaging (MRI), which obtains an image directly; protein NMR uses algorithms to create three-dimensional models of the sample of interest.³ Protein NMR is conducted on thoroughly purified samples, which have

a volume of approximately 500 μL and a concentration of approximately 0.2 mM.¹⁶ Normally, proteins are obtained via recombinant DNA, which is performed by genetic engineering; this method permits isotopic labeling to track particular atoms.¹³ Once the proteins is dissolved in a buffer, it is placed in a thin NMR tube for spectroscopy.¹³

Large molecules, like proteins, have thousands of resonances, which will show overlaps of one-dimensional spectra.¹⁰ As a result, multidimensional experiments are conducted; this reduces overlaps and focuses on certain nuclei in one section of the sample.¹⁰ RF pulses permit the transfer of magnetization from chemical bonds and space (structure is irrelevant); this allows for the determination of chemical shifts and distance restraints, respectively.¹⁰ Experiments of higher dimensions require more time than lower dimension experiments.¹⁰

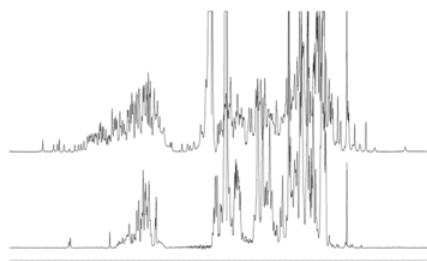


Figure 5.10.8. Folded (top) and unfolded (bottom) protein NMR spectra. This image is from: <https://users.cs.duke.edu/~brd/Teach...s/flemming.pdf>.

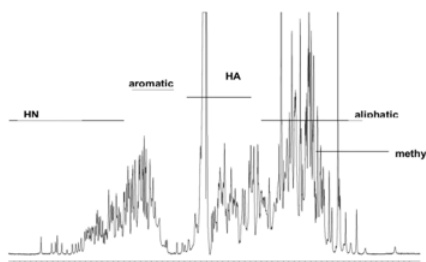


Figure 5.10.9. Proton NMR spectra of egg white lysozyme (hen). The -NH, aromatic, alpha-carbon, aliphatic, and methyl proton shift segments are labeled. This image is from: <https://users.cs.duke.edu/~brd/Teach...s/flemming.pdf>.

Homonuclear NMR

If the proteins of interest are unlabeled, correlation spectroscopy (COSY) is performed; two types of COSY are total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY).¹² These two-dimensional NMR's provide two-dimensional spectra.¹² Both axes are chemical shifts, in term of units.¹² These experiments build spin systems, a list of resonances of the chemical shift of protein's protons.¹² To link the spin systems in the right pathway, NOESY must be used, which uses spin-lattice relaxation.¹² Magnetization is transferred via space in NOESY, which can be used to calculate distance relations.¹² NOESY can also determine chemical and conformational changes.¹² Peak overlap is an issue with homonuclear NMR; as a result, it is limited to small proteins.¹²

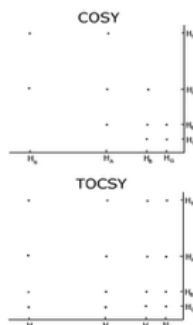


Figure 5.10.10. Comparison of two-dimensional COSY and two-dimensional TOCSY spectra for an amino acid (e.g. glutamate or methionine). TOCSY displays diagonal cross-peaks between all protons. COSY only displays cross-peaks between neighbors. This image is from: <upload.wikimedia.org/wikiped.../Tocsycosy.jpg>; it was created by Kjaergaard using GIMP.

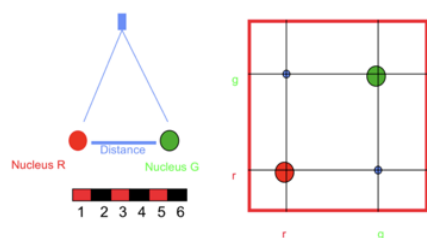


Figure 5.10.11. Two-dimensional NMR displaying the Nuclear Overhauser effect between two nuclei, G and R. The NOE is measured by the blue peak intensity at (r,g) and (g,r). This image is from: <https://users.cs.duke.edu/~brd/Teach...s/flemming.pdf>.

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