C6770 NMR Spectroscopy of Biomolecules

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Contents

Lecture 1a
NMR as a tool for structural biology

1.1 Aim of the course

The aim of our course (C6770 NMR Spectroscopy of Biomolecules) is to give students interested in structural biology an insight into methods of NMR spectroscopy applicable to biologically important molecules (mostly proteins and nucleic acids). Principles of methods are explained in details, but using classical physics instead of quantum mechanics. It should be emphasized that classical physics describes NMR correctly as long as we discuss macroscopic samples, consisting of large numbers of molecules. Graphical description and explanation in text is preferred to derivations based on series of plain equations. The theoretical background is limited, those interested in the theory of nuclear magnetic resonance (based on quantum mechanics) may choose the course C5320 Theoretical concepts of NMR.

Most topics of our course, except for NMR spectroscopy of nucleic acids, are described very well in Cavanagh et al., Protein NMR spectroscopy, 2nd. ed., Academic Press 2006. However, the book is based on the quantum mechanical approach and is not easy to read for a beginner without a solid physical background. General ideas and technical issues are explained in Keeler, Understanding NMR spectroscopy, 2nd. ed., Wiley 2010, and in Levitt: Spin dynamics, 2nd. ed., Wiley 2008. Both books are written for non-experts and present NMR in a very understandable manner, but they do not cover applications to proteins and nucleic acids. The mentioned limitations of the available literature where the major motivation to write this study text. The intention was to complement the lectures by writing a short review of NMR techniques applicable to biomacromolecules. The main purpose of the lectures is to explain the basic ideas in an interactive manner. The text should summarize the topics in a more fluent form and to provide a broader context.

1.2 Structure and structure determination

It should not surprise anybody that *the structure* is what a structural biologist is mostly interested in. Therefore, we have a good reason to start our discussion of nuclear magnetic resonance with the topic of *structure determination*. There are of course good reasons to start somewhere else (e.g., to show applications where no other method can compete with NMR), but I feel an obligation to acknowledge the importance of the structural information right at the beginning.

Before we start talking about structure determination, we should ask what the word *structure* means. The question may sound silly but it is really useful to define this term clearly if we wish to avoid possible confusions. When structural biologists talk about *structure* they usually refer to a model of little balls representing atoms and sticks representing covalent chemical bonds connecting the atoms. The way how the atoms are connected is described by the chemical term *configuration*. Limitations of the described model are obvious. The model is rigid and cannot describe molecular motions that are always present or chemical reactivity of the molecule.

Macromolecules of our interest, i.e., proteins and nucleic acids, are composed of a small number of basic building blocks, *residues*. Configuration is almost completely described by the *sequence* of the residues in the studied molecule. Configuration of the individual building blocks is well defined and depends very little on the actual position of the residue in the macromolecule. In other words, the sticks have a certain length and fit to holes drilled into the balls,

and there is very little that we can do about it. Even the way how to assemble the individual residues is given, so our task is only to find out how to join the residues together. Various *sequencing techniques* have been designed in order to determine the order of residues in a particular macromolecular chain. Once we build the chain of residues, and do some little adjustments like connecting correct disulfide bridges in proteins, the (chemical) configuration of our molecule is defined.

Knowing the configuration, we have enough information to draw a pretty scheme of our macromolecule, in the same way as the authors of the organic chemistry textbooks describe somewhat smaller molecules. Note that organic chemists call such pictures "structures", they use the term *structure* at a level equivalent to *configuration*. In the case of small molecules, the drawing is giving is a fairly good idea about the shape of the molecule (described by the ball-and-stick model). The situation is different if the molecule has a size of a protein. Why? It is possible to rotate more or less freely about most chemical bonds without changing the configuration. An astronomic number of possible ball-and-stick models can be generated by the rotation about various bonds. If we need to refer to one of them, we use the term *conformation*. Now it should be clear that the term *structure* is typically a synonym of *conformation* in structural biology, unlike in organic chemistry (where *structure* often refers to *configuration*).

Now, when we know that *structure determination* is nothing else than the process of finding correct conformation of the studied molecule, we can briefly discuss mathematical description of the structures. Position of every ball in our model can be described by three *coordinates*. Very often, the familiar Cartesian x, y, and z coordinates are used. Cartesian coordinates are very transparent but they also present some problems. If the molecule moves or rotates a little bit, the numbers describing its Cartesian coordinates completely change. It is difficult to say if two molecules are identical just by looking at their Cartesian coordinate lists. Another way of numerical description of the molecule is to list all bond length, angles between bonds, and torsions about the bonds (in terms of so-called *dihedral angles* or *torsion angles*.[1] The latter way represents *internal coordinates* of the molecule, independent of the position or orientation of the whole molecule. It should not surprise you that if six *external coordinates* (three defining position of the molecule in the chosen coordinate system and three describing overall orientation of the molecule with respect to the axes) are added to the internal coordinates, we obtain a list of numbers exactly as long as the list of Cartesian coordinates. The internal coordinates can be divided into those describing configuration of the molecule (i.e., bond length and bond angles) and those describing conformation (torsion angles). This is useful because configuration can be taken as granted and the whole structure can be described by torsion angles only.

1.3 Macromolecular structure from interatomic distances

Positions of individual atoms in biologically important macromolecules are most typically determined by techniques that somehow depend on diffraction of waves: X-ray crystallography, electron microscopy, neutron diffraction. The general requirement is that the wavelength must be similar to the distances between atoms (or shorter). Obviously, NMR spectroscopy does not meet this criterion. There are waves used in NMR spectroscopy, as we discuss later , but their wavelength is several decimeters. How can such waves provide information about atomic distances. The answer is: *NMR is an indirect method*. This is a big disadvantage because NMR structure determination is less straightforward and based on less complete data than when structures are solved using diffraction techniques. But the indirect nature of NMR structure determination is also a great advantage because limitations of diffraction techniques do not apply to NMR spectroscopy. Let us now compare the direct and indirect approaches to the structure determination. Instead of equations and technical argument, we use a story.

Imagine you are a sailor and you visit a beautiful Pacific island with friendly people. You wish to live there for ever. You burn your yacht, crash your smartphone, and build a shanty. After couple of days, you find out that the inhabitants do not have any map of their land. You decide to draw one for them.

There are several ways how to draw a map. For example, you can make a hot-air balloon, inflate it, sail over your island, and draw a map when watching the landscape from the balloon's basket. Or you can just walk from one site to another one, count your steps and measure distances between caps, bays, mountains, and other sites that should appear in the map.

The first approach requires long preparation but makes drawing the map rather easy. It resembles the diffraction techniques. The second approach does not need any preparation. You can start striding immediately. However, it

[1] Note that mathematical definitions of torsion and dihedral angles differ in sign.

takes a long time to walk between all sites and the distances measured in this way will not be too precise. It is also not so straightforward to construct a map from distances as to draw what you see from the sky. You have to draw circles of radii corresponding to the measured distances. Positions of the important points are given by intersections of the circles and every error in the measured distances makes the intersections less accurate. Accuracy can be improved if you use more circles for every point, but it would be time consuming to compete with the first approach.

The latter approach resembles NMR structure determination. The NMR methods discussed in our course are applied to molecules in solution.[2] You just need to dissolve your molecule in a buffer and add some heavy water as a sort of internal standard. However, the measurement itself requires a whole set of different experiments, and often takes several weeks. Like in the case of the sailor, the more experiment you run, the more accurate structural model you obtain. Analysis of the measured data is also more tedious than it was in the case of crystallography. Finally, it is very hard to determine structure of molecules larger than approximately 25 kDa. Not only the measured spectra are more complex, but it is difficult to get any signal at all. Currently, NMR is used as a structure determination method for small proteins, individual domains of large proteins, and structurally interesting fragments of nucleic acids. Approximately 15 % structures in the databases were obtained from NMR data.

In order to explain how NMR structure determination really works, we have to modify the sailor's story a little bit. We let our sailor use a more sophisticated means of measuring distances by giving him a set of walkie-talkies, tunable to different frequency channelsl (Figure 1.1). The sailor can give them to his friends living at various sites of the island. Each of these "radio-operators" can estimate how far her or his neighbors are by evaluating signal intensity. The procedure is simple. A guy sitting in a certain town tries to tune in all his fellows that he can hear, and writes down how strong the signal is (your operator locates the position of your mobile phone in a similar manner). Then the sailor collects the notes from every walkie-talkie and begins drawing the map.

The process of NMR structure determination is very similar to the walkie-talkie tale. We make the atomic nuclei transmit radio waves and then we measure how much of the signal has been passed to the nuclei in their vicinity. But such an experiment is not where we usually start. It certainly helped the sailor if he first made something like a telephone directory of his walkie-talkie operators: a list of their names and transmitting frequencies of the devices given to them. It is almost a rule that the NMR structure determination (actually, any NMR study of a biologically interesting macromolecule) starts with "writing a telephone directory". It is not so easy because nuclei do not have their frequencies written on their backs. We have a list of nuclei defined by the amino-acid (or nucleotide) sequence in one hand and a list of measured frequencies in the other hand. It is our task to find which frequency belongs to which nucleus. There is a whole suite of NMR experiments designed just for this purpose and analysis of their results is a routine well known to anybody who uses NMR as a structure determination tool. The process of writing "nuclear phone directory" has been given a self-explanatory name *assignment*. Only when the assignment is done, the structural data starts to make sense.[3] It can take longer to assign frequencies to (almost) all nuclei than to collect information about distances among nuclei. If you later feel that I spend too much time talking about the assignment experiments which tell us nothing about the structure,[4] please, be patient a remember what I just told you.

1.4 List of applications

We have described NMR as a method of structure determination, working reasonably well for smaller proteins and nucleic acid fragments. However, NMR is a much more flexible technique. Other applications are perhaps even more useful than building a structural model. In many cases, the assignment provides a basis for a variety of structural and functional studies. What looked just as an additional effort in the context of structure determination, opens doors to many interesting experiments. Let us now list at least some of them.

- *Is my protein folded?* We start our overview with a very simple but very useful application. It is an experiment telling us if the prepared protein has a well-defined structure, is only partially folded, or is present as a random

[2] NMR spectroscopy of biomacromolecules in solid state is a rapidly developing field and is already able to provide structure with atomic resolution.

[3] In theory, we do not need to assign frequencies to nuclei in advance. Structural model can be obtained just from a set of internuclear frequencies. However, protocols following this idea are currently less successful than the "orthodox approaches" described in the rest of this book.

[4] This is not quite fair, useful structural information can be obtained already from the assigned frequencies.

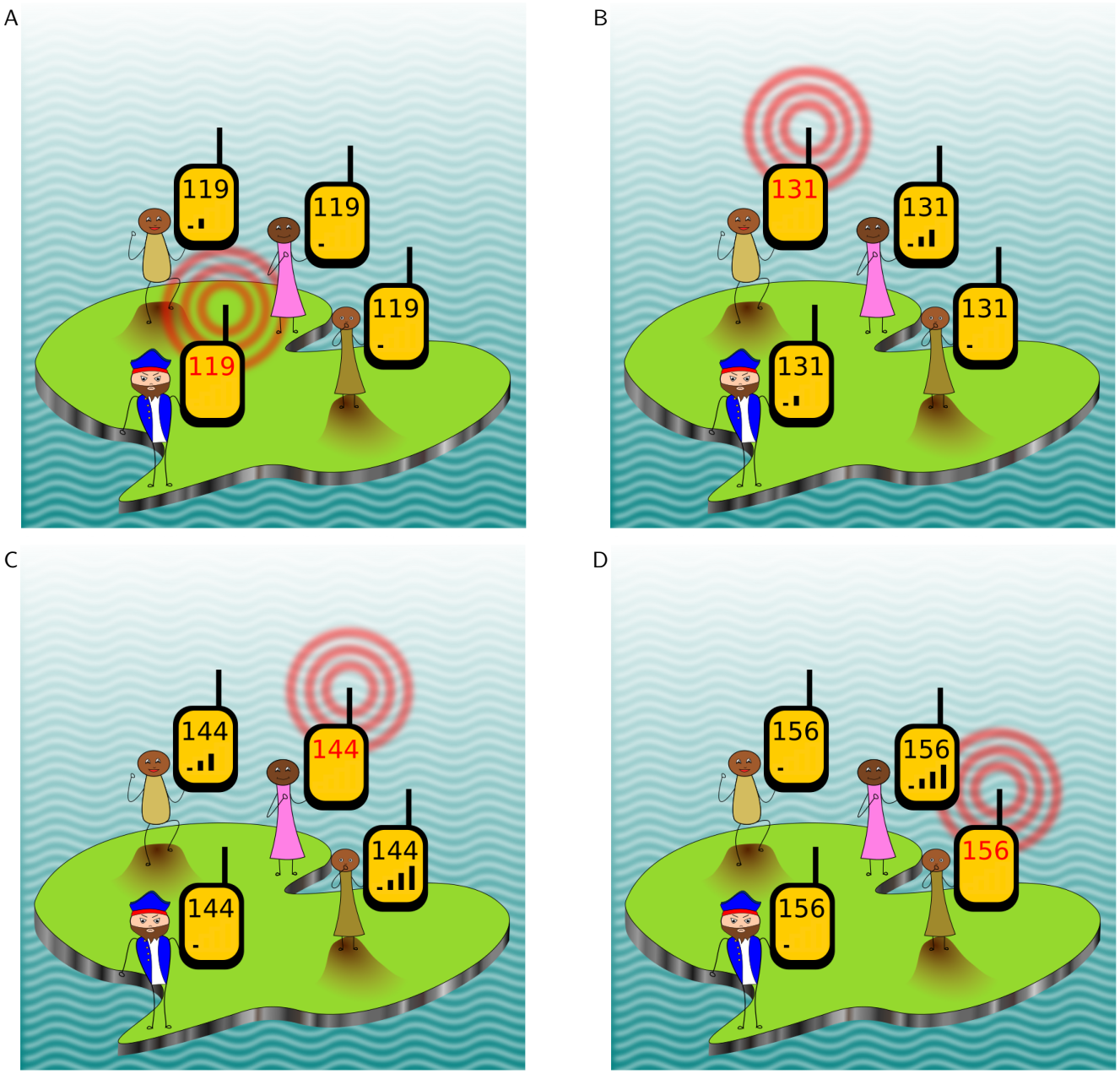


Figure 1.1: Walkie-talkie method of distance measurement. Technically, you decided to use PMR 446 (personal mobile radio operating at 446 MHz). Your walkie-talkies are tuned to four frequencies (four channels) that distinguish individual devices: 446.11875 MHz, 446.13125 MHz, 446.14375 MHz, and 446.15625 MHz. The transmitted and received frequencies are shown on the walkie-talkie displays in red and black, respectively (for better visibility, only three digits following 446 MHz are displayed). When switched to the receiver mode, the displays show also the strength of the received signal (the ▂▄▆▉ indicator).

coil. This information can be obtained in several minutes. The spectra provide atomic resolution (unlike the circular dichroism spectra, giving only percentage of secondary structure elements). The atomic resolution may seem useless when the assignment is not yet available, but it allows us to count signals corresponding to observed amino acids. If we count as many signals as we expect, and if their distribution is typical for a well folded protein, we know that the protein is likely to be prepared correctly. What I have said should already convince you that this experiment is a very useful test of the sample. It is always run prior to more complex NMR experiments. It is also a good idea to record a simple NMR spectrum before we try to crystallize a protein (if the protein is not too large).

- *3D structure determination* has been already described in Section 1.2.

- *Specific structural details.* We already know that NMR data are different from data provided by diffraction techniquess. Each NMR frequency can be attributed to an individual nucleus (and to the effects of its neighbors). On the other hand, diffraction of X-rays at each atom contributes to all reflections in a predictable way. This is a great advantage of X-ray crystallography. The diffraction pattern can be back-transformed into the electron density map of the molecule. However, the local nature of the NMR data can be an advantage under certain circumstances. There are cases when we are not interested so much in the whole structure but we would like to learn more about some detail (e.g., mutated active center of an enzyme, or binding site occupied by various ligands). We can save time and look just at the interesting site instead of solving the structure completely. Of course, the assignment must be known in advance, it is only the structural part of the study that can be simplified.

- *Intermolecular interactions.* Once the assignment is completed, interactions of a protein with another molecule (I call it "ligand" in the following text but it can be almost anything: metal ions, small molecules, other proteins, nucleic acids) can be monitored very quickly. We take the protein and titrate it with the ligand. After each addition of the ligand, a simple NMR experiment is run. Such experiments do not need to take more than an hour. A qualitative information at the atomic-level resolution is obtained immediately from the spectra. Nuclei that change frequency upon addition are likely to be close to the binding site. A real structural information requires distance measurements, like when determining the whole structure. But NMR makes our life easier again. It is possible to distinguish intramolecular and intermolecular distances using a clever combination of isotope labeling and design of the NMR experiment. This approach eliminates many ambiguities and produces highly reliable information about intermolecular contacts.

- *Molecular motions.* Crystal structures and EM images of proteins reflect protein flexibility to some degree. Less defined electron density may indicate internal motions. Missing loops are likely to be flexible. NMR methods tell us much more about the molecular dynamics. *Hydrodynamic behavior* of macromolecules may seem not too interesting from a biological point of view. Still, it is useful to know how a molecule moves in solution because the hydrodynamic properties depend on the size and shape of the molecule. This type of information is useful when we need to check if a molecule is monomer or dimer, if it is present in complex with another large molecule or not, etc. *Internal motions* are often essential for the biological function of biomolecules. Motions on various time scales can be distinguished and described quantitatively with atomic resolution. Motions on the time scale of seconds (and slower) can be studied in real time as you can expect. More interestingly, very fast motions (faster than 10^{-9} s) can be described relatively easily in terms of their amplitude and often frequency (at atomic resolution, of course). It is also an advantage of NMR that the signals affected by the fast motions are averaged but not missing. Various experiments have been designed to study slower, microsecond to millisecond, motions.

- *Kinetics and thermodynamics.* The fact that NMR can be applied to kinetic studies has been already mentioned in the previous paragraph. It is very easy to monitor molecular processes at various temperatures. Activation energies can be obtained at least in some cases. In principle, NMR spectroscopy can also provide thermodynamic data such as binding constants. However, the typical concentration range used in NMR experiments (higher than 100 μM) is rather high to study interactions with high affinities.

- *In vivo measurements.* Mild conditions of the NMR measurements offer the possibility to study molecules in living cells. Major limitation is relatively low sensitivity of the method and high complexity of the molecular

mixture inside the cell. Small molecules have been studied in applications like metabolic profile monitoring or intracellular pH measurements. It is currently also possible to measure *in vivo* NMR spectra of proteins and nucleic acids, although at higher than physiological concentrations. This allows us to estimate how are molecules influenced by the cellular environment and to check if spectra obtained *in vitro* are the same as those measured *in vivo*. Recently published *in vivo* studies already addressed important biological issues.

- *Spatial resolution.* So far, we looked at applications where NMR spectra were measured in the entire volume of the sample. It is also possible to monitor distribution of certain type of nucleus in space. *Magnetic resonance imaging*, used in medical diagnostics, is a typical example. Requirements for spectral and spatial resolutions can be combined, and *in situ* spectra (limited to a small volume) can be measured.

1.5 When can a structural biologist use NMR

I have tried to describe NMR as a wonderful method offering so much useful information and you may ask why it is not used more frequently by biochemists and biologists. I must admit that NMR has also its requirements and that some of them can be tough for a particular biological macromolecule. The common source of the limitations is an inherently low sensitivity. So, how should a biomolecular sample look like to be ready for an NMR study in general (and for structure determination, in particular)?

- *Solubility.* Sensitivity of the method requires relatively high concentrations. Samples of 1 mM macromolecules promise good results, higher concentrations are better. Working with concentrations below 0.1 mM is a challenge.

- *Stability.* Not only the molecule of interest should be well soluble, but it should also stay in solution for a long time. Ideally, the sample survives for month at room temperature. Molecules that are decomposed in less than a week are not suited for a long-term study such as structure determination. Measurements at temperatures close to 0 °C are possible but sensitivity is much worse (especially for large molecules).

- *Stable isotope labeling.* We do not see all nuclei in NMR. Almost all hydrogen and all phosphorus nuclei are active but only every hundredth nucleus of carbon is visible. The most abundant isotope ^{12}C is stable and silent in NMR experiments. Natural samples contain about 1 % of the isotope ^{13}C. It is also stable but can be observed in NMR experiments. Finally, trace amounts of a third isotope are present in Nature. The third isotope, ^{14}C, is created by cosmic rays in atmosphere, undergoes a radioactive decay, and is silent in NMR experiments. Unfortunately, the natural amounts of ^{13}C can be observed only in the most sensitive NMR experiments. The situation is even worse for nitrogen, and oxygen is completely useless for biomolecular application. Therefore, artificial molecules of proteins and nucleic acids are often prepared for NMR experiments by replacing the most abundant, NMR silent, isotope ^{12}C with the NMR active isotope ^{13}C. Also, ^{14}N is replaced by a more suitable ^{15}N. This effects NMR spectroscopy to proteins that are expressed in well behaving host organisms (often bacteria) and to the synthetically available molecules.

- *Scaling up production.* It follows from the previous paragraph, that proteins for NMR studies must be prepared in milligram amounts and with completely changed ratio of isotopes. Fortunately, *recombinant* methods of molecular biology nicely fit our needs. As most proteins are prepared in a recombinant form anyway, protein sample preparation does not present any extraordinary requirements. Samples of nucleic acids are not obtained so easily and labeling with NMR active isotopes is relatively expensive in this case.

- *Size.* Sensitivity of the NMR methods for large molecules is very poor. It is not molecular mass but rate of tumbling in solution that is critical. Large rigid molecules (or rigid molecules in more viscous solvents, e.g. in cold water) tumbles slowly and give weaker NMR signal. The sensitivity can be regained by using special tricks. Molecules of a relative mass close to one million have been successfully studied. On the other hand, the process of full structure determination is very difficult and has been applied to only few monomeric proteins larger that ∼30 kDa so far.

- *Separation from impurities.* Purity of the NMR sample is usually not a big problem. Obviously, signals of impurities are observed in the spectra, may obscure signals of our interest, and should be eliminated. Paramagnetic

compounds (typically various metal ions) should be avoided or removed from the sample. In theory, molecular oxygen dissolved in water may interfere as a paramagnetic molecule, but its presence causes no problems when working with large molecules. Bacterial contamination represents more serious danger than chemical impurities. Addition of an antimicrobial agent, such as sodium azide, is necessary to keep samples stable for a long time.

- *Salt content.* High ionic strength should be avoided for purely technical reasons. A decrease of sensitivity can be noticed with concentration of salt above 100 mM. Higher than 0.5 M salt concentrations should not be used at all. NMR spectroscopists just do not like salty stuff.

Lecture 1b
NMR as a physical phenomenon

1.6 Spin and magnetic moment

Nuclear magnetism is a phenomenon exact description of which requires an advanced physical theory of *quantum electrodynamics*. Yet, we can use very simple pictures to understand the basic ideas. Let us start with a really naïve model of the hydrogen atom shown in Figure 1.2A. Let us imagine an electron orbiting a proton like our Earth orbits Sun. The electron is charged and its circular motion is equivalent to the electric current (electric current in a wire is nothing else than traveling electrons). This circular current induces magnetic field like in an electric motor. But this is not all. The Earth is also spinning about its own axes. Let us assume for a moment that the electron is spinning in the same manner. The *spin* is also a circular motion and induces its own magnetic field. If we pull the electron out of the atom, the orbital motion (and the orbital magnetism) disappears, but the spin (and the spin magnetism) remains. If we put the electron inside a magnet, we can observe its spin magnetism (actually, this is how the spin of electrons was discovered).

Magnetism of the electrons is quantitatively described by the *magnetic moment*. It is a vector property. Magnitude of the magnetic moment describes strength of the magnetic field generated by the electron, and the direction of the magnetic moment defines orientation of this magnetic field. The magnetic moment is directly proportional to the *angular momentum*.[5] Behavior of the magnetic moment and of the angular momentum is therefore similar (except for the numeric value) and we will inspect both vectors together.

As any vector, magnetic moment or angular momentum can be decomposed into three perpendicular components oriented along the x, y, and z axes. Perhaps a more convenient way of description is the spherical representation, where the radius of the sphere defines the magnitude and two angles define direction in the same way as geographical latitude and longitude define the position on the Earth's surface.

How does a spinning electron move in a magnetic field? Well, in a similar way as a spinning top moves in the gravitation field of Earth. If you steal such a toy from children and set it spinning, there is a little chance that the axis of rotation is precisely perpendicular to the Earth's surface. More often, the axis is slightly askew. Gravitation pulls the spinning top down, but it does not fall to the ground immediately. Instead, the angular momentum rotates the spinning axis so that it undergoes a slow *precession* motion. You do a similar thing when you ride your bicycle. Your body does not stay straight up but it is swinging from left to right. Thanks to the angular momentum of your wheels you do not fall down, you are only forced to continually change direction. It should not surprise you that magnetic moments of free electrons are precessing in static homogeneous magnetic fields.

In order to proceed in our description of electrons in a magnetic field, we must borrow some results from quantum theory.[6] So far we have not noticed any difference between behavior of the electrons and our everyday experience. One might get a feeling that we do not need quantum theory at all. This feelings disappear as soon as we realize one big difference between electrons and macroscopic rotating bodies such as the Earth or the spinning top. Spinning of the electron cannot be stopped. It cannot be accelerated or slowed down. The angular momentum of the spinning top

[5] Angular momentum is a vector oriented along the axis of rotation and its absolute value is rmv, were r is radius of the circular orbit and m and v are mass and velocity of the electron, respectively.

[6] It is interesting that quantum mechanics itself does not explain why the spin exists. Only in combination with the special theory of relativity, the quantum theory predicts spin as a necessary property of particles like electrons.

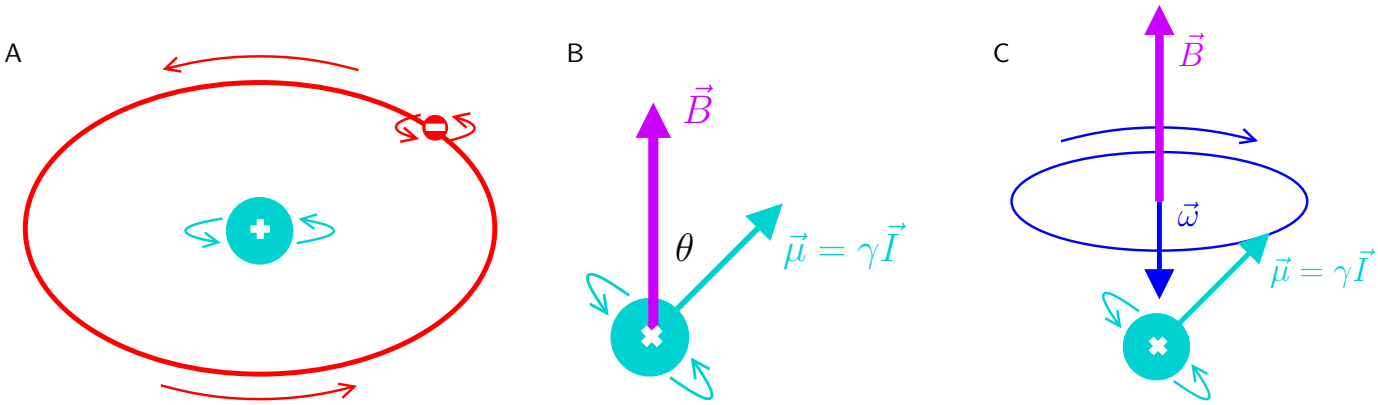


Figure 1.2: A, A naïve model of hydrogen atom. B, A magnetic particle in a homogeneous magnetic field $\vec{B}_0$. C, Precession of a magnetic dipolar moment in a homogeneous magnetic field $\vec{B}_0$.

is introduced by external forces of our fingers but the angular momentum of the electron is an inherent property of the particle. It is even misleading to take the spin as a kind of rotation motion. Experiments and theory show that electrons are not spinning, they simply *have spin*. I agree that the idea of an angular momentum[7] without physical rotation is very strange but we have to live with it.

Another consequence of the quantum theory that deserves our attention is the relation between the electron's spin and magnetic moment. In the classical physics, the proportionality constant is equal to one half of the charge-to-mass ratio. Quantum theory gives a value twice as big in the first approximation. Further corrections are needed to account for interactions of the electron with its own electromagnetism, but these effects are small and can be easily calculated. In the case of electron, the quantum theory can predict the magnetic moment with an astonishing precision of eleven digits.

Quantum theory of course describes electron and its spin in more details. For example, it tells us that only some physical quantities can be measured simultaneously. It also shows that two particular orientations of the magnetic moment in a magnetic fields are special. They are called stationary states and traditionally labeled α and β. We do not need such details for the description of NMR because we are not interested in isolated particles but in macroscopic samples. It is not only unnecessary, but confusing and physically incorrect (but unfortunately frequent in the literature[8]) to apply quantum theory of single particles to NMR samples.

1.7 Nuclear magnetism

In spite of our deep interest in atomic nuclei, we talked about electrons so far. The only reason was that electrons behave as simple particles, nicely described by quantum electrodynamics. Because the word "simple" may suggest various things, physicists call electron and other well-behaving particles *Dirac particles*.[9] On the other hand, nuclei are *composite particles* and their theoretical description is more complex. Even the nucleus of the hydrogen atom, a single proton, is a composite particle. Protons and neutrons consist Dirac particles, *quarks*. Each quark possesses its own spin and magnetic moment. Quarks are held together by very strong interactions, mediated by particles called *gluons*. Calculation of the magnetic moments of protons and neutrons, and consequently of the whole nuclei, is very difficult, as the resulting magnetic moment is a combination of spin and orbital moments of quarks and gluons. One of the following three general results can be obtained.

[7] The word "spin" is just a short name of the inherent angular momentum of a particle – do not confuse it with the angular momentum describing orbital motion of an electron around the nucleus.

[8] Quantum theory can be used to describe macroscopic samples but it must incorporate the statistical approach. The textbooks recommended in Section 1.1 use the quantum theory correctly.

[9] Paul Dirac laid the foundations for the development of quantum electrodynamics.

1. The magnetic moments are canceled completely and the nucleus does not show any magnetism. The most abundant isotope of carbon, ^{12}C, composed of six protons and six neutrons, is a typical example.

2. The magnetic moments are combined such that the magnetic behavior of the nucleus resembles magnetic behavior of a Dirac particle. The nucleus has the same spin as electrons.As uqntum theory describe this type of particles with the spin quantum numbers $\pm\frac{1}{2}$, a term *spin-1/2 nuclei* is used. It follows from the theory that these nuclei have spherically symmetric electric field, which is a great advantage for NMR measurements. Typical examples are isotopes ^{1}H, ^{13}C, ^{15}N, ^{31}P.

3. The third category includes all other combinations. Such nuclei have larger angular momenta (spins) and asymmetric electric fields. They are not suitable for biomolecular NMR investigations. The most abundant isotope of nitrogen (^{14}N) is an example.

It follows from the previous discussion that we can ignore the first and third type of nuclei[10] and treat nuclei of the second type as *Dirac-like particles*. Such an approach leads to correct qualitative conclusions but the numerical values must be taken as empirical constants.

Now it is a good time to turn our attention to quantitative issues. Fortunately, we do not need heavy math to understand biomolecular applications NMR spectroscopy.[11] It is good to remember three simple equations relating four physical quantities (i) spin or angular momentum $\vec{I}$, (ii) magnetic moment $\vec{\mu}$ and its (iii) energy E and (iv) angular precession frequency $\vec{\omega}$ in a magnetic field of *magnetic induction* $\vec{B}$ (Figure 1.2B,C):

$$\vec{\mu} = \gamma\vec{I}, \tag{1.1}$$

$$E = -\vec{B}\cdot\vec{\mu} = -\gamma\vec{B}\cdot\vec{I}, \tag{1.2}$$

$$\vec{\omega} = -\gamma\vec{B}, \tag{1.3}$$

where the symbol γ describes the proportionality constant between spin and magnetic moment, known as the *magnetogyric ratio*.

We have already discussed relation between $\vec{\mu}$ and $\vec{I}$ (Eq. 1.1) for electron and nuclei. The value of γ is provided by quantum mechanics with a great precision for Dirac particles and should be determined experimentally for Dirac-like particles, including nuclei.

Eq. 1.2 defines energy of the magnetic moment in a magnetic field. The classical physics shows that a magnet in a magnetic field has the lowest energy if its magnetic moment is oriented along the magnetic field (parallel with the vector of the magnetic induction $\vec{B}$. The expression $\vec{B}\cdot\vec{\mu}$ is a short version of the sum $B_x\mu_x + B_y\mu_y + B_z\mu_z$ (B_x is the x component of the vector $\vec{B}$ etc.). Traditionally, the z axis is defined by the direction of the magnetic field $\vec{B}_0$ inside the strong magnet of the NMR spectrometer. Therefore, the B_z component of $\vec{B}_0$ must be equal to the total length of the vector,[12] B_0, while B_x and B_y must be equal to zero. Eq. 1.2 can be simply written as

$$E = -B\mu_z = -\gamma B_0 I_z = -\gamma B_0 I\cos\theta, \tag{1.4}$$

where θ is the angle between the magnetic moment $\vec{\mu}$ and the vector of magnetic induction $\vec{B}$.

Finally, Eq. 1.3 shows that the angular precession frequency $\vec{\omega}$ is proportional to the external magnetic field and that the proportionality constant is again γ. Note that the angular precession frequency is presented as a *vector quantity* in Eq. 1.3. The magnitude $|\omega|$ is the speed of the precession (in the units of radian per second, precession frequency in the units of Hertz is $|\omega|/(2\pi)$), whereas the direction of $\vec{\omega}$ defines axis of the precession motion. The minus sign reminds us that the axis of precession is oriented opposite to the magnetic induction.

In summary, Eqs. 1.1–1.3 tell us that the spin and magnetic moment are intrinsic properties of the nucleus but the energy E and precession frequency $\vec{\omega}$ obviously depend on the external magnetic field.

[10] Only the nucleus of heavy hydrogen (deuterium) ^{2}H is somewhat exceptional. Its electric field is not spherically symmetric, but is relatively small and is observed in some biomolecular applications.

[11] Much more mathematics is needed if we ask *why* the NMR experiment works as they do. Another course at Masaryk University, C5320 Theoretical concepts of NMR, tries to ask such questions.

[12] Total length or magnitude, usually called absolute value or amplitude in physical literature, is labeled simply by the letter symbolizing the vector written without the arrow (B_0 in our case), or by the letter surrounded with the absolute value symbols ($|B_0|$).

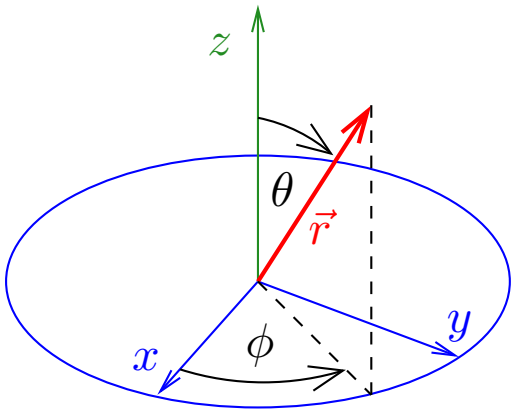


Figure 1.3: Orientation of a vector $\vec{r}$ (red) described by spherical coordinates ϕ, θ (black) and by the Cartesian coordinates x, y, z. The longitudinal direction (z) is shown in green, the transverse plane (xy) is shown in blue.

1.8 Nuclei in the test-tube

After discussing the nuclear magnetism as a phenomenon, we can look at it from a more practical point of view. We are now interested not in microscopic processes inside isolated atoms but in a measurable macroscopic quantity describing magnetism of the whole sample. Such quantity is called *total magnetization* and labeled with symbol $\vec{M}$. As we moved from microscopic particles to macroscopic samples, classical (non-quantum) physics should be sufficient to describe what we observe.

In physics, magnetization is defined as the *sum of magnetic moments per unit volume.* Magnetic moments of the same nucleus in different molecules have the same magnitude but different orientation. The orientation in a coordinate frame formed by axes x, y, z is described by two angles (Figure 1.3), (i) the angle θ between the magnetic moment and the z axis induction vector of the external magnetic field $\vec{B}$ (analogy of geographic latitude), and (ii) the angle ϕ describing the *azimuth*, i.e., the angle telling us how much is the component of the vector perpendicular to z rotated from the direction y in the xy plane (analogy of geographic longitude). Therefore, the result of addition of many little vectors of magnetic moments of individual nuclei depends on their angles θ and ϕ.

Real chemical and biological samples contain huge numbers of nuclei. The only practical approach to such a large collection of nuclei is a statistical description. Let us ask a typical statistical question: What are the most probable angles θ and ϕ? The answer depends on the magnetic field, describing forces acting on the magnetic moments. In the following paragraphs, we explore three types of magnetic environments.

1.9 No magnetic field

First, we can consider a sample of a biologically interesting molecule sitting on our laboratory bench (Figure 1.4A). If we ignore magnetism of the Earth and all other magnetic fields that surround us every day, the nuclear magnetic moments can be described as completely uncorrelated vectors, oriented randomly, pointing to all directions in space with the same probability (Figure 1.4B). No wonder that such sample has a zero total magnetization.

1.10 Static magnetic field

Second, we put the sample inside a static, homogeneous magnetic field defined by the magnetic induction vector $\vec{B}_0$ and ask again: What are the most probable angles θ and ϕ if the axis z is given by the direction of $\vec{B}_0$? The answer is simple for ϕ: all values of ϕ (all azimuths) are equally probable because the energy of a magnetic moment in $\vec{B}_0$ does not depend on ϕ (Eq. 1.4). As a consequence, components of magnetic vectors perpendicular to $\vec{B}_0$ (along x and y axes in our coordinate frame) are uniformly distributed and therefore they sum to zero. Only the components of

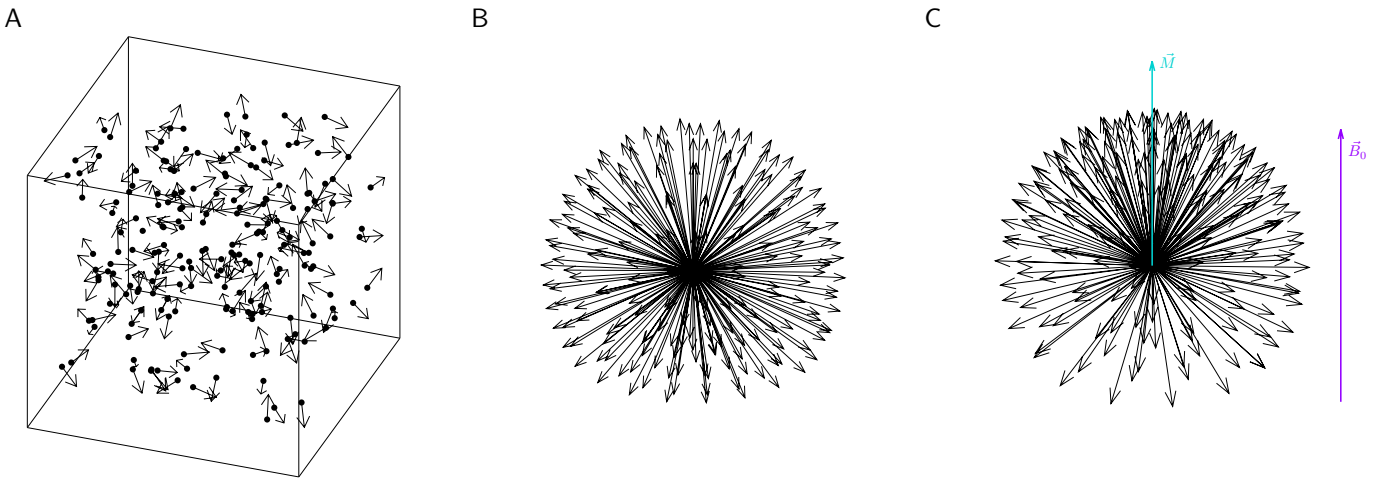


Figure 1.4: A, a schematic representation of an NMR sample. Dots represent molecules, arrows represent magnetic moments (only one magnetic moment per molecule is shown for the sake of simplicity, like e.g. in compressed $^{13}C^{16}O_2$). B, Distribution of magnetic moments in the absence of a magnetic field. The molecules are superimposed to make the distribution of magnetic moments visible. C, distribution of magnetic moments (black arrows) in a homogeneous magnetic field $\vec{B}_0$. The cyan arrow represents the macroscopic magnetization. Even if all black arrows precess with the same frequency about $\vec{B}_0$, the cyan arrow remains static.

magnetic moments parallel with $\vec{B}_0$ depend on $\vec{B}_0$. These components are described only by the angle θ. Therefore, we have to statistically analyze θ in order to evaluate the only non-zero component of the sum of magnetic moments, the component along the z axis of our coordinate frame.

On one hand, we have seen (Eq. 1.4) that energy of the magnetic moment in a magnetic field depends on the angle θ. Eq. 1.2 shows that the orientation of the magnetic moment parallel with $\vec{B}_0$, i.e. orientation with $\theta = 0$, has the lowest energy. One might expect that magnetic moments of nuclei should have this most favored orientation, at least in the classical physics. On the other hand, there is only one orientation with $\theta = 0$ (the magnetic moments pointing to the "northern pole"), but there are many possible orientations of magnetic moments for $\theta = 90\,^\circ$ (magnetic moment pointing to the "equator"). We can see that we obtained two contradictory answers: if we ignore statistics and consider only energy, the most probable θ is zero, if we ignore energy and consider only statistics, the most probable θ is $90\,^\circ$. The correct balance between energy and statistics is described by the Boltzmann law. Nuclei are not isolated from the rest of the world. They interact with the environment full of randomly moving molecules. This stochastic motion is manifested as the *temperature*, and represents a rich source of energy for the nuclear magnets. According to the Boltzmann law, fraction of magnetic moments in each orientation is indirectly proportional to the exponent of the ratio of the magnetic energy to the thermal energy. If we study biological molecules in liquid solutions, we work at temperatures high compared to the magnetic energies of nuclei in the strongest magnets. Therefore, the exponent in the Boltzmann law is a very small number and the exponential dependence is very close to a linear relation. Going through the somewhat tedious calculation[13] yields the average value of $\cos\theta$ at the thermal equilibrium:

$$\overline{\cos\theta^{\mathrm{eq}}} = \frac{|\mu||B_0|}{3k_{\mathrm{B}}T}, \tag{1.5}$$

where $k_{\mathrm{B}} = 1.38 \times 10^{-23}\,\mathrm{JK}^{-1}$ is the Boltzmann constant and T is the thermodynamic equilibrium. For the sake of simplicity, we assumed that the molecule contains only one type of nuclei with a magnetic moment, for example protons. As shown in Figure 1.4C, the distribution of magnetic moments is *polarized* in the direction of $\vec{B}_0$ (most favored orientation of a magnetic moment).

If we use the value of $|\mu|$ provided by quantum mechanics ($\sqrt{3}\gamma\hbar/2$, where $\hbar$ is the Plank constant divided by 2π,

[13]The following equation is derived in Course C5320 Theoretical concepts of NMR and the derivation is presented in the study material for the course.

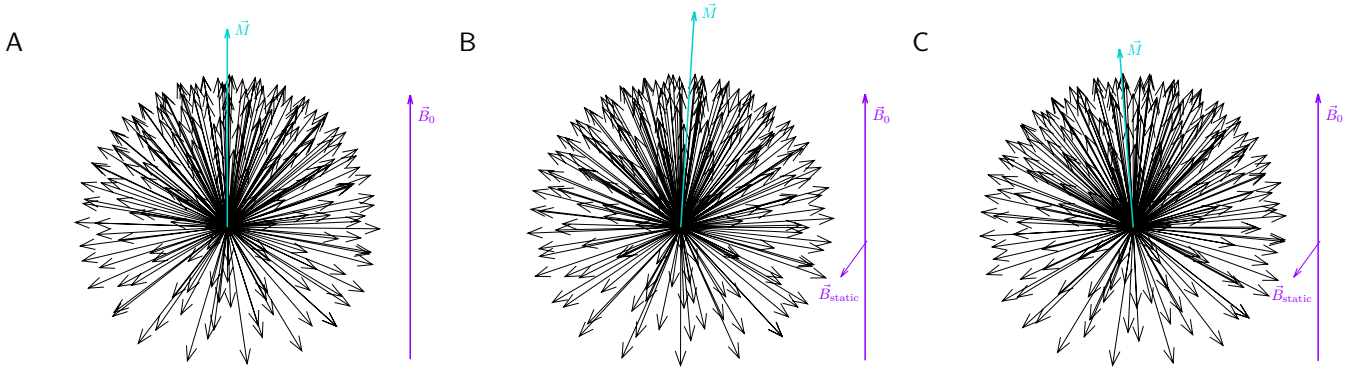


Figure 1.5: Distribution of magnetic moments in the presence of an external homogeneous magnetic field $\vec{B}_0$ (vertical violet arrow) is such that the macroscopic magnetization of nuclei (shown in cyan) is oriented along $\vec{B}_0$ (A). Application of an another static magnetic field $\vec{B}_1$ rotates magnetization away from the original vertical orientation down in a clockwise direction (B). However, the magnetization also precesses about $\vec{B}_0$. After a half-turn precession (C), the clockwise rotation by the additional magnetic field $\vec{B}_1$ returns the magnetization towards its original vertical direction. Therefore, a static field cannot be used to turn the magnetization from the vertical direction to a perpendicular orientation.

$\hbar = 1.054 \times 10^{-34}\,\mathrm{J\,s}$), we can express the z component of magnetization at the equilibrium as

$$M_z^{\mathrm{eq}} = \mathcal{N}|\mu|\overline{\cos\theta^{\mathrm{eq}}} = \mathcal{N}\frac{\gamma^2\hbar^2}{4k_{\mathrm{B}}T}, \tag{1.6}$$

where $\mathcal{N}$ is the number of magnetic nuclei per unit volume.

The x and y components of magnetization

$$M_x^{\mathrm{eq}} = \mathcal{N}|\mu|\overline{\sin\theta^{\mathrm{eq}}}\cdot\overline{\cos\phi^{\mathrm{eq}}}, \tag{1.7}$$
$$M_y^{\mathrm{eq}} = \mathcal{N}|\mu|\overline{\sin\theta^{\mathrm{eq}}}\cdot\overline{\sin\phi^{\mathrm{eq}}} \tag{1.8}$$

are equal to zero because $\cos\phi$ and $\sin\phi$ average to zero when all values of ϕ are equally probable. The magnetization vector pointing in the direction z tells us that the magnetic moments are polarized along the z axis (so-called *longitudinal polarization*).

Note that we added moments undergoing precession and obtained a macroscopic magnetization that does not move at all (Figure 1.4C). Although all summed magnetic moments precess at the same frequency and the total magnetization is not zero in this case, the rate of precession cannot be measured.

1.11 Creating a signal

We see that having a magnet is not enough to measure precession frequency. We also need something that would disturb the macroscopic magnetization from its equilibrium orientation along $\vec{B}_0$. Not surprisingly, this "something" is again a magnetic field. Let us try to orient the additional field, described by the induction $\vec{B}_1$, perpendicular to the original external field (now we need to distinguish two independent external fields, the original field $\vec{B}_0$ and the new one $\vec{B}_1$). The magnetic force of $\vec{B}_1$ would pull the magnetic moments from their equilibrium orientations. Unfortunately, static $\vec{B}_1$ would not work as we wish. It is shown in Figure 1.5. The magnetic moments precess around $\vec{B}_0$, so they would feel the force of $\vec{B}_1$ pulling them from their equilibrium orientation at the beginning but back to the equilibrium orientation a fraction of microsecond later. The static $\vec{B}_1$ would perhaps introduce fast fluctuations but not net change in the total magnetization.

Obviously, $\vec{B}_1$ must be synchronized with the precession caused by $\vec{B}_0$. For example, the magnetic field $\vec{B}_1$ could be a field that rotates at the frequency which *resonates* with the precession frequency of nuclear magnetic moments. This is why we talk about nuclear magnetic *resonance*. In such case, the precessing magnetic moment would feel a

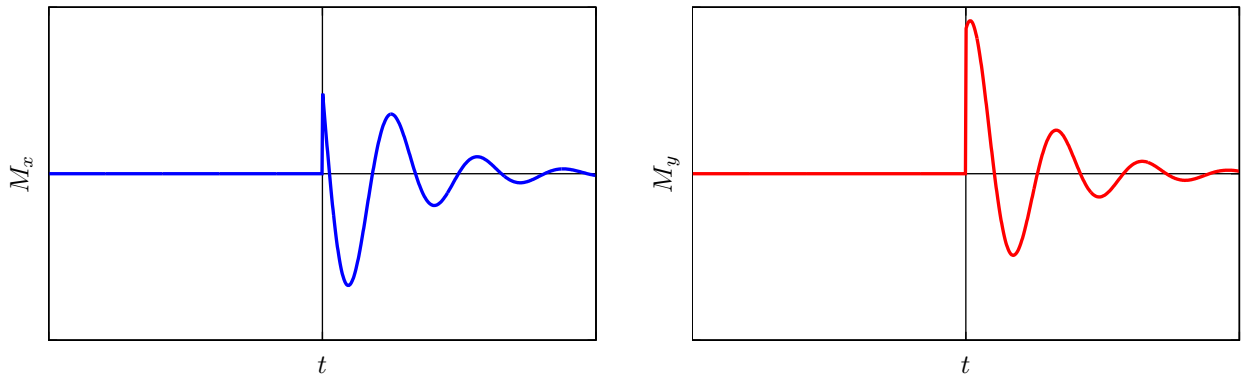


Figure 1.6: Values of M_x (blue) and M_y (red) components of a magnetization vector rotating with the initial phase of 60 °. The values decay due to a loss of coherence.

constant pull in one direction. This direction would precess together with the magnetic moment but would always point away from the equilibrium orientation. It is difficult to imagine the effect of the synchronized $\vec{B}_1$. Physicists like to make things simpler and so they introduced a coordinate system rotating together with the precessing magnetic moment. It is like jumping on a carousel that rotates with the precession frequency. Once we jump on it, the magnetic moments seem not to move any more and also $\vec{B}_1$ looks like a static field (Figure 1.7A). If $\vec{B}_1$ is static, it must have the same effect as $\vec{B}_0$, it must introduce a circular (precession) motion of the magnetic moments. The total magnetization is not parallel to $\vec{B}_1$ but perpendicular to it. So, it does not stay in its original position, defined by $\vec{B}_0$ but starts to rotate about a new axis defined by $\vec{B}_1$. The rotation of the magnetization is result of the additional precession introduced by $\vec{B}_1$, and has the same frequency as the new precession. Outside the rotating frame, we observe two superimposed rotations about two perpendicular axes. In our spherical representation of vectors, the tip of the arrow symbolizing the total magnetization draws spirals on a spherical surface. Such motion has been given a name *nutation* by astronomers.

I must admit that current technology does not allow us to rotate magnets at the precession frequency of nuclei. Fortunately, a magnetic field oscillating in one direction has a very similar effect if it is much weaker than $\vec{B}_0$ (Figure 1.7B). Radio waves represent a readily available source of a magnetic field oscillating in one direction with a frequency resonating with the precession frequency of the magnetic moments. Application of a radio wave for a well chosen period of time, known as the *radio-wave pulse*, thus rotates the magnetization vector from the z direction to the xy plane. After the pulse, the magnetic moments are not polarized vertically, but horizontally (Figure 1.7C). The direction of the horizontal, or *transverse* polarization then rotates with the precession frequency of the polarized magnetic moments. In other words, the M_x and M_y components of the magnetization vector oscillate as cosine and sine functions with the frequency equal to the precession frequency of individual nuclei. The cosine and sine functions are actually shifted in time by a phase factor depending on the phase of the radio wave, as shown in Figure 1.6.

We should stress that the *radio-wave pulse* did not create the transverse (horizontal) polarization from an unpolarized distribution. It only rotated the already existing longitudinal (vertical) polarization induced by the much stronger static field $\vec{B}_0$. Therefore, the magnitude of the magnetization rotating in the xy plane is proportional to $|B_0|$ according to the Boltzmann law (Eq. 1.6).

Bringing the magnetization to the xy plane is called *creation of coherence* in the NMR literature. The term *coherence* has its origin in statistical quantum mechanics, discussed in detail in Course C5320 Theoretical concepts of NMR. At this moment, we use the word "coherence" as a synonym for transverse polarization (we extend its meaning when we take into account mutual interactions between nuclei). The coherence is preserved as long as the magnetic moments move together, *coherently*. It does not take too long (usually seconds or less) to lose the coherence and, finally, to restore the equilibrium distribution of magnetic moments. The loss of coherence and return to the

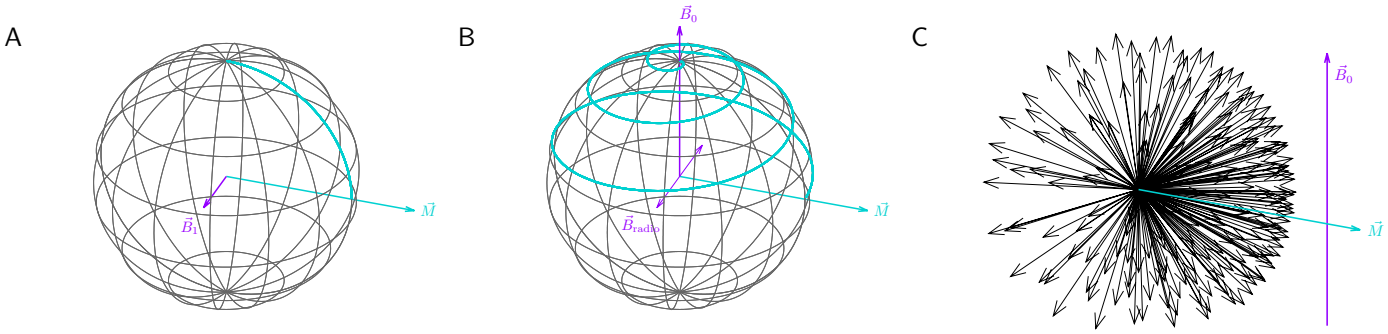


Figure 1.7: Effect of the radio waves on the bulk magnetization (A, in the rotating coordinate frame; B, in the laboratory coordinate frame) and distribution of magnetic moments after application of the radio-wave pulse (C). The thin purple line shows oscillation of the magnetic induction vector of the radio waves, the cyan trace shows evolution of the magnetization during irradiation. If the perpendicular magnetic field oscillates with a frequency equal to the precession frequency of magnetization, it rotates the magnetization clockwise then it is tilted to the right, but counter-clockwise when the magnetization is tilted to the left. Therefore, the magnetization is more and more tilted down from the original vertical direction. The total duration of the irradiation by the radio wave was chosen so that the magnetization is rotated to the plane perpendicular to $\vec{B}_0$ (cyan arrow). Note that the ratio $|\vec{B}_0|/|\vec{B}_{\mathrm{radio}}|$ is much higher in a real experiment.

equilibrium are known as *relaxation*. We discuss relaxation in more detail later in our course. Before doing so, we explain other issues important for NMR spectroscopy and often ignore the relaxation effects for the sake of simplicity. However, we should never forget that relaxation exists and influences all NMR experiments.

Lecture 2
NMR as a method reflecting chemistry

2.1 Nuclei are affected by their environment

We discussed a very simple situation in the previous lecture. Nuclei were placed into a homogeneous magnetic field of the magnet sitting in our laboratory. The result was a precession motion at a frequency given (1) by the internal structure of the nucleus and (2) by the strength of our magnet. The observable magnetization, tilted to the transverse plane by a pulse of a radio wave, rotated et the same frequency. Clearly, such frequency carried no information about the molecule built of atoms containing our nuclei. The chemical and biological applications are only possible because the molecular environment modifies the magnetic field felt by the nuclei. We will look at the most interesting effects of the molecular environment in this section.

2.2 Interactions with close nuclei

Magnetic dipolar moments of the nuclei experience not only the external magnetic fields. The nuclei themselves are also sources of little magnetic fields. The effective magnetic field perceived by a certain nucleus is modified by its nuclear neighbors. The effect of a close nucleus depends on mutual orientation of both nuclei, i.e., the observed nucleus (shown in cyan in Figure 2.1) and its neighbor (shown in green in Figure 2.1) modifying the field felt by the observed nucleus. Let us suppose that the neighbor's magnetic moment is pointing up (vertical green arrow in Figure 2.1). If the neighbor is placed in direction perpendicular to the induction of the external field (Figure 2.1A), the magnetic field generated by the neighbor at the site of the observed nucleus is oriented opposite to the external field, and the effective field is decreased. If the neighbor is positioned in the direction of the external field, its magnetic moment creates a field that is added to the external field (Figure 2.1C). Quantitatively, the field created by the neighbor nucleus is proportional to $3\cos^2\theta - 1$, where θ is the angle between a line connecting the nuclei and the direction of the external field. Graphically, the direction of the neighbor's field is described by the shape of the magnetic induction lines of the neighbor's magnetic dipole. The effects are exactly opposite if the neighbor's magnetic moment is pointing down. This type of interaction is called *dipole-dipole coupling* (or *dipolar coupling*) in the NMR literature.

The effects of the close nuclei can be observed directly as shifts of precession frequency if the molecules are ordered in space (in crystals, or at least partially in liquid crystals). If the molecule freely rotates and if the rate of the rotation is the same in all directions (*isotropic rotation*), the position of the neighbor varies in time and the effects of the neighbor's magnetic field average to zero (if we calculate the average value of $\cos^2\theta$ for all possible values of θ, we find that is equal to 1/3, and therefore the average of $3\cos^2\theta - 1$ is zero). Does it mean that no information about the molecular structure can be obtained? Not at all. The fluctuating electromagnetic fields resulting from the molecular tumbling can be viewed as electromagnetic waves (or photons) of certain frequency. The frequency of molecular rotation changes randomly as the molecule collides with other molecules, and once a while it matches the precession frequency of the neighbor nucleus. When this happens, the x and y components of the magnetic induction of the stochastic waves generated by the molecular rotation resemble the radio waves applied by us: frequency of oscillation in the xy plane resonates with the precession frequency of the close nucleus and rotates its magnetization as shown in Figure 1.7 until a next collision changes the frequency of the molecular rotation. Such exchange of energy between

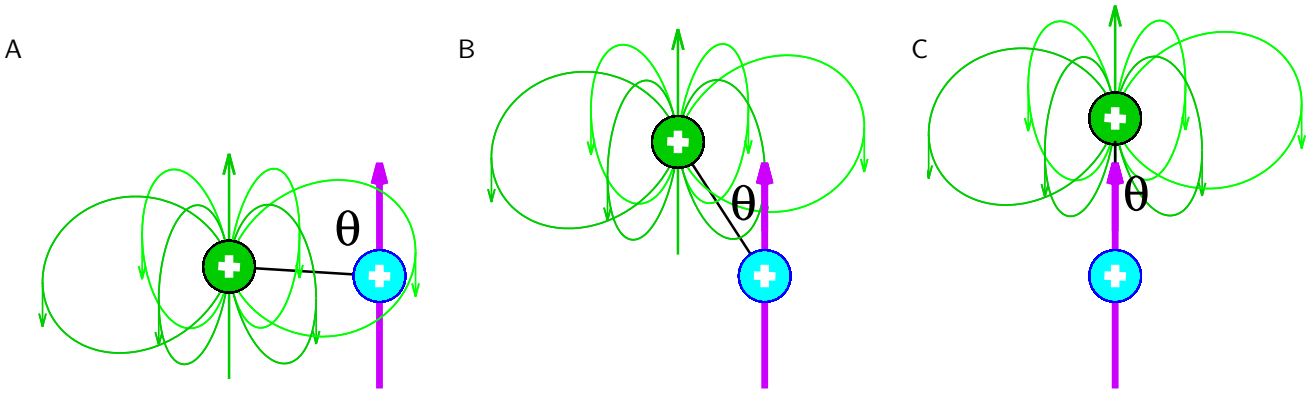


Figure 2.1: A, Classical description of interaction of a spin magnetic moment of the observed nucleus (shown in cyan) with a spin magnetic moment of another nucleus (shown in green). The thick purple arrow represents $\vec{B}_0$, the thin green induction lines represent the magnetic field $\vec{B}_2$ of the green nucleus (the small green arrows indicate its direction). The black line represents the internuclear vector $\vec{r}$. As the molecule rotates, the cyan nucleus moves from a position where the field of the spin magnetic moment of the green nucleus $\vec{B}_2$ has the opposite direction than $\vec{B}_0$ (A), through a position where $\vec{B}_2$ is perpendicular to $\vec{B}_0$ (B), to a position where $\vec{B}_2$ has the same direction as $\vec{B}_0$ (C).

nuclei is more probable if the nuclei are closer in space. Distances between nuclei can be estimated by measuring the efficiency of the described energy exchange, known as the *nuclear Overhauser effect* in the NMR literature. The interactions with magnetic moments of nuclear neighbors thus represent the physical nature of the allegory about the sailor and his walkie-talkie method (Section 1.3). Later we also show that dipole-dipole interactions varying due to the molecular motions cause *relaxation* (loss of coherence and return to equilibrium distribution of magnetic moments).

2.3 Interactions with electrons

Every nucleus in a molecule is surrounded by electrons. The magnetic fields of electrons significantly modify the field felt by the nucleus. Interactions with the spin magnetic moments of electrons are not so important because most electrons are paired and their spin magnetic moments canceled.[1] What is more interesting is the effect of the *orbital* magnetic moments. The orbital magnetism is induced by the external magnetic field and is always oriented against it. The external field is *shielded* by the paired electrons. Therefore, the precession frequency of a nucleus is decreased by orbital magnetic moments of electrons of the same atom (Figure 2.2A). Nuclei are also influenced by orbital magnetic moments of electrons of other atoms. The effect then depends on the orientation of the whole molecule in the magnetic field (Figure 2.2B,C). The precession frequency can be slowed down (shielding) but also speeded up (deshielding). The orientation of the molecule in the magnetic field influences the interaction with the orbital magnetic moments of electrons in a similar manner as described for the dipole-dipole interactions in Section 2.2, including relaxation. However, the isotropic molecular tumbling does not eliminate the shielding. As a result, the precession frequency is shifted even in perfectly spherical molecules. This phenomenon is known as the *chemical shift* (chemical because it does not reflect nuclear properties but molecular structure). Chemical shift is a sensitive probe of the structure of the molecule because it depends on the distribution of electrons in the molecule. Empirical values are routinely used as the key identifiers in the process of determining chemical configuration of small organic molecules. Unfortunately, interpretation of subtle chemical shift variations is very difficult and the use of chemical shifts in structure determination of biomacromolecules is limited.

[1] Unpaired electrons are mostly observed in proteins containing metal ions. They affect nuclei through the same mechanism as described in Section 2.2, but the interaction is much stronger.

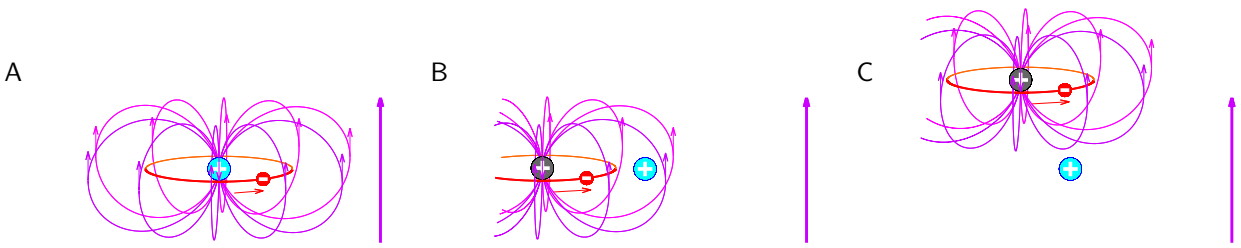


Figure 2.2: A, Classical description of interaction of an observed magnetic moment with the orbital magnetic moment of an electron of the same atom. The observed nucleus and the electron are shown in cyan and red, respectively. The thick purple arrow represents $\vec{B}_0$, the thin purple induction lines represent the magnetic field of the electron (the small purple arrows indicate its direction). The electron in $\vec{B}_0$ moves in a circle shown in red, direction of the motion is shown as the red arrow. The field of the orbital magnetic moment of the electron in the same atom decreases the total field in the place of the observed nucleus (the small purple arrow in the place of the cyan nucleus is pointing down). B, Interaction of an observed magnetic moment with the orbital magnetic moment of an electron of the another atom (its nucleus is shown in gray). In the shown orientation of the molecule, the field of the orbital magnetic moment of the electron in the other atom increases the total field in the place of the observed nucleus (the small purple arrow close the cyan nucleus is pointing up). C, As the molecule rotates, the cyan nucleus moves to a position where the field of the orbital magnetic moment of the electron in the other atom starts to decrease the total field (the induction lines reverse their direction in the place of the cyan nucleus).

2.4 Interactions between nuclei mediated by electrons

We described how magnetic moments modify the external magnetic field felt by their nuclear neighbors. The little magnetic moments of nuclei also interact with the magnetic moments of electrons forming bonds between atoms. Therefore, nuclei interact not only directly, as described in Section 2.2, but also through a cascade of interactions with magnetic moments of electrons forming chemical bonds between them. This type of interaction is known as the *J-coupling*.

The origin of the J-coupling is interesting. The dominant component of the J-coupling is the *Fermi contact interactions*, which can be explained as follows. It is not surprising that magnetic moments of nuclei interact with spin magnetic moments of electrons, but this is just a regular dipole-dipole interaction. We already learned in Section 2.2 that the shape of the magnetic field of a nuclear dipole is described by magnetic induction lines of the dipole, and that the numerical value is given by $3\cos^2\theta - 1$, which averages to zero if the nucleus freely rotates in the external magnetic field (Figure 2.3A–C). This is true, but with one exception, depicted in Figure 2.3D.

If the electron is present *exactly* at the nucleus, the vector of the electron spin magnetic moment $\vec{\mu}_e$ has the same direction as $\vec{B}_e$ and the magnetic energy is proportional to the scalar product of the vectors of the magnetic moments of the electron and the nucleus $(-\vec{\mu}_n \cdot \vec{\mu}_e)$. This energy is lowest when the magnetic moments are parallel, and obviously does not depend on the orientation of the molecules (the scalar product is scalar, not a vector). Therefore, this interaction is often called *scalar coupling*. The exact co-localization of electron and nucleus may look strange in our classical (non-quantum) discussion of NMR, but the interaction between the nucleus and electron *inside* the nucleus can be simulated by a hypothetical current loop giving the correct magnetic moment when treated classically. The direction of the magnetic induction lines *inside such loop* (*inside the nucleus*) is *parallel* with the magnetic moment of the electron.

When we discussed interactions between nuclear magnetic moments in Section 2.2, we did not pay any attention to the field inside the nucleus because no other nucleus can be found there, nuclei are always separated in molecules. But this restriction does not apply to electrons! The shape of the atomic s-orbital suggests that the electron can be found with non-zero probability exactly at the position of the nucleus (Figure 2.3E).

Let us now look at a pair of two nuclei connected by a single bond, interacting with two electrons with non-zero probability to be present exactly at the positions of the nuclei (Figure 2.3F,G). The electrons in pairs that form chemical bonds have the opposite spin (red arrows in Figure 2.3F,G). It has the following consequence. If the magnetic moment of the neighbor (shown in green in Figure 2.3F,G) is pointing up (Figure 2.3F), the electron co-localized with

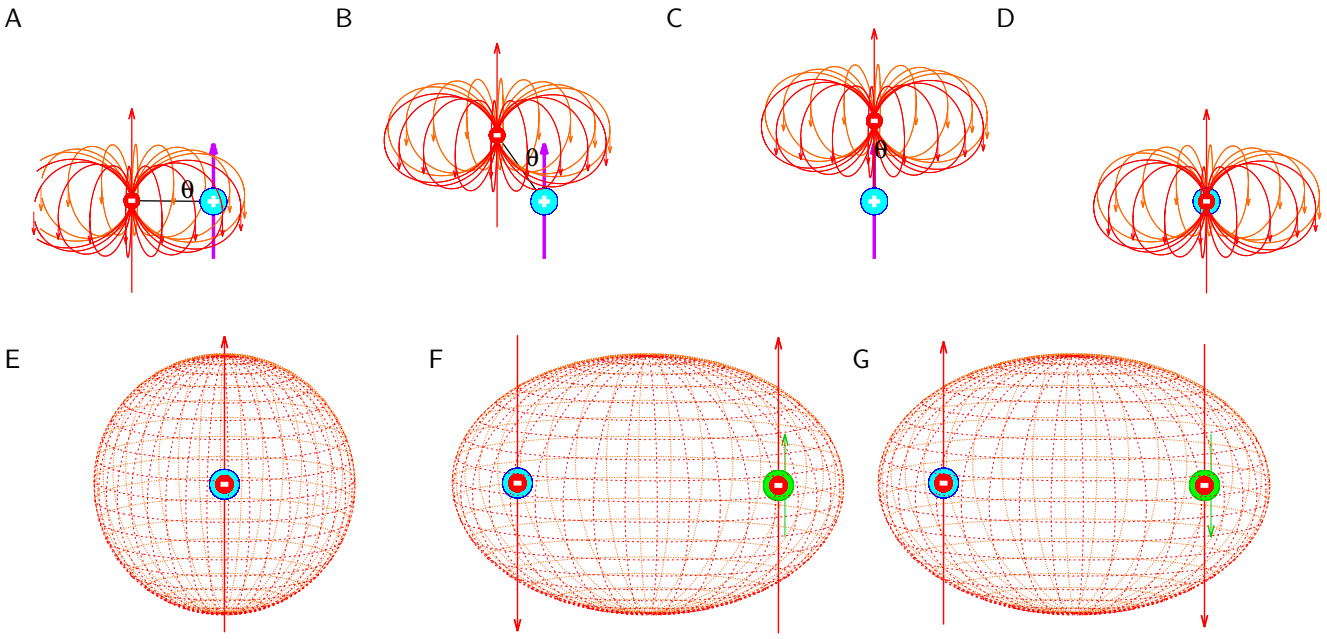


Figure 2.3: *J*-coupling. A–C, magnetic fields of an electron (red induction lines) outside the nucleus (cyan), influencing the nucleus as expected for regular dipole-dipole interactions. D, magnetic field of an electron localized at the position of the nucleus. E, probability of finding an electron in the hydrogen atom at particular coordinates is described by the probability density. The probability density described by the orbital 1s (depicted as a sphere) has a non-zero value at the position of the nucleus (shown in cyan). Therefore, there is a non-zero probability of finding electron (red circle) exactly at the site of the nucleus. The field produced at the site of the nucleus by the electron's magnetic moment (red arrow) does not depend on the orientation of the atom if the positions of the nucleus and electron coincide. Therefore, the interaction of the nucleus with the electron is not averaged to zero if the atom rotates isotropically. F and G, the probability density described by the sigma orbitals (depicted as an ellipsoid) in molecules has also non-zero values at the sites of nuclei. The spin states of the electrons in the bonding sigma orbital are opposite (indicated by the opposite direction of the red arrows) and perturbed by the magnetic moment of the nuclei. The parallel orientations of magnetic moments is energetically favorable for a nucleus and an electron sharing its position. As a consequence, precession frequency of the cyan nucleus is lower in Panel F and higher in Panel G.

the observed nucleus (shown in cyan in Figure 2.3F,G) lowers precession frequency of its magnetic moment (the field of the electron, corresponding to the left red arrow in Figure 2.3F, has the opposite direction than $\vec{B}_0$, pointing up). If the magnetic moment of the neighbor (shown in green in Figure 2.3F,G) is pointing down (Figure 2.3G), the electron co-localized with the observed nucleus (shown in cyan in Figure 2.3F,G) increases precession frequency of its magnetic moment (the field of the electron, corresponding to the left red arrow in Figure 2.3G, has the same direction than $\vec{B}_0$, pointing up).

We have analyzed only the vertical direction of nuclear magnetic moments when discussing Figure 2.3F,G. Other directions have similar effects. However, if the precession frequencies of the cyan and green nuclei differ, the horizontal polarization of the green nucleus rotates quickly in a coordinate frame of the cyan nucleus. Therefore, the effect of the horizontal polarization of the green nucleus on the precession frequency of the cyan nucleus fluctuates rapidly and is quickly averaged to zero.

The described electron-mediated interactions of course depend on the electron distribution in the molecule. The existence of an interaction so closely related to the chemical structure is obviously very useful. When we discuss the NMR methodology in details, we find out that the *J*-couplings are the corner stones of almost all modern NMR techniques.

2.5 NMR experiment

After discussing the physical phenomena important for understanding nuclear magnetic resonance, we proceed to more technical issues. The use of NMR spectroscopy in chemistry and structural biology relies on our ability to measure precession frequencies of nuclear magnetic moments in the studied molecules. We already know that this requires a strong static homogeneous magnetic field and a much weaker magnetic field oscillating with frequencies close to the measured precession frequencies. By accident, the required frequencies are in the same range as those used in frequency modulated broadcasting. We see that the instrument able to measure precession frequencies, the *NMR spectrometer*, must contain three components: magnet (as a source of the static magnetic field $\vec{B}_0$), radio transmitter (as a generator of the oscillating field $\vec{B}_1$), and radio receiver (as a detector).

Scenario of the basic NMR experiment is rather simple. We put our sample inside a strong homogeneous magnet and let it reach thermal equilibrium. Then we switch on our radio transmitter for a precisely defined time period. This event is called the *radio-frequency pulse*. Duration of the pulse should be such that the pulse rotates the total magnetization just by 90 ° in order to achieve the highest sensitivity.[2] Finally, we switch off the transmitter and turn on the radio receiver. Our sample is now a source of magnetic field oscillations. These oscillations have frequencies very close to the frequency of the applied radio pulse (only nuclei resonating with this frequency were excited from their equilibrium states), but not exactly identical. Slight variations in frequency have their origin mostly in the electron shielding (the chemical shift). Nuclei in different chemical moieties are shielded to different extent and feel slightly different magnetic field (see Section 2.1). Now we see why the external field $\vec{B}_0$ must be very homogeneous. Variations of $\vec{B}_0$ would cause the precession frequencies to vary as well. Even relatively small variability in the inherent frequencies of nuclei would completely mask the tiny shielding effects of electrons.

The megahertz radio frequency is *modulated* by the variable shielding, causing deviations ranging from zero up to several kilohertz. In the same manner, megahertz radio waves are modulated with the audio frequencies in the FM radio broadcasting. The megahertz frequency serves as a *carrier* for the kilohertz frequencies generated by the electron environment of nuclei in the case of NMR or by vibrations of musical instruments or human voice in the case of the radio. The NMR receiver does the same job as an ordinary radio receiver. It subtracts the carrier frequency from the signal (technical details are discussed in Section **??**). The remaining lower frequencies called *frequency offsets* Ω, are converted to a digital signal and recorded. The saved file has the same format as a typical audio record. If plotted, it shows a sum of sine (or cosine) curves. The amplitude of oscillations gradually decreases, as the nuclear magnetic moments lose their coherence, and as they return back to their equilibrium distribution. NMR spectroscopists use the term *free induction decay*, or simply *FID*, for the damped oscillating signal. When the system of nuclei arrives sufficiently close to the equilibrium, a new experiment can start. Waiting for the return to equilibrium is typically slightly longer than one second in the case of biological macromolecules.

The output of the NMR experiments is an audio record. We can therefore easily *listen* to the NMR data. Well, listening is not a typical way of analyzing chemical data. We would like to present our records in some graphical representation. Simple plotting does not make us happy. The damped sine curves interfere and several sinusoids already produce a mess. Fortunately, the signal, recorded as a function of time, can be easily converted to a *spectrum*, which is a function of frequency. We now examine the procedure leading to nice looking spectra in some detail.

2.6 From oscillations to spectrum: Fourier transformation

Certain people, including the author of this text, have hard time to remember their shoe size. Some shops take this handicap into account. They keep a special device, a board with the footprints of various sizes. Imagine somebody really silly, who starts with the smallest size and keeps comparing his foot with the footprints on the board to the largest size. He ranks each trial on a scale of comfort ranging from zero to 100 %. Clearly, the shoe size that fits his foot gets high comfort mark, perhaps two closest sizes get some low mark, and the other sizes are ranked as zero comfort. This is roughly the same procedure as used by computers converting FID to a spectrum.

The procedure described in the previous paragraph closely resembles *Fourier transformation*. Fourier transformation does not compare footprints but sine (or cosine) curves (Figure 2.4). Instead of the board with various footprints, Fourier transformation works with a set of testing sine (or cosine) functions of gradually increasing frequency. Each

[2] We learn soon that the first pulse is followed by a whole sequence of carefully timed pulses in all biomolecular experiments.

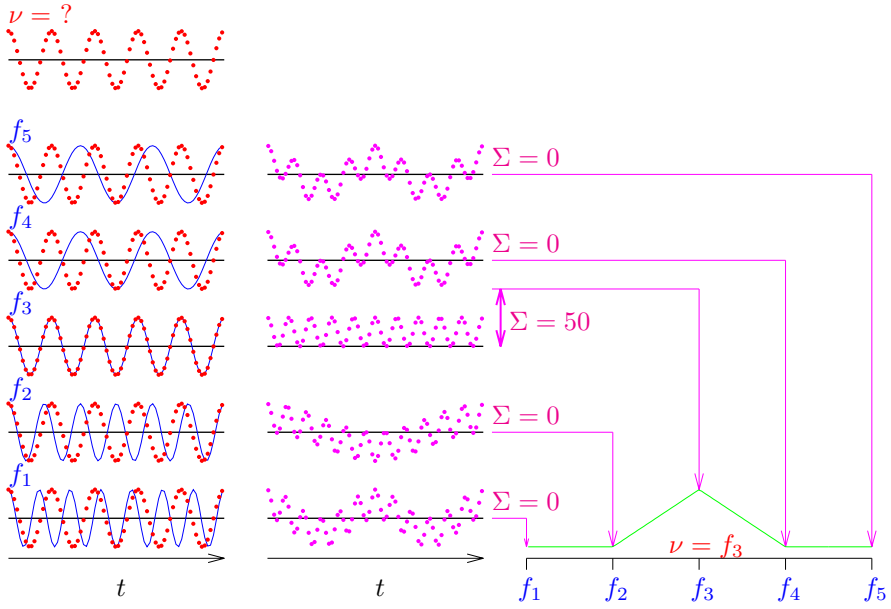


Figure 2.4: The basic principle of Fourier transformation presented for experimental data (red) and five testing functions (blue). Experimental data multiplied by the testing functions are displayed as magenta dots, sums (integrals) of the products as magenta lines. The sums are plotted in the right diagram and connected with the green lines.

trial represents calculating product of the tested curve (experimental data points shown in red in Figure 2.4) with one of the testing curve (blue curves in Figure 2.4). In case of different frequencies, the oscillations are uncorrelated and the products at individual points (shown in magenta in Figure 2.4) oscillate around zero. The total product (sum over all points) is equal or very close to zero. On the other hand, if the frequency of the tested curve matches the frequency of the testing curve, the oscillations coincide and we obtain some positive number as the product at each point (with the exception of points where both curves pass zero). The total product is a large number. Then we plot the total product as a function of frequency of the testing curve (green plot in Figure 2.4). The obtained plot has the familiar shape of a resonance curve with a peak at the frequency that matched the frequency of the testing curve. We obtained a *spectrum.*

The really attractive feature of Fourier transformation is its *additivity.* Two or more cosine curves oscillating at different frequencies just show two or more peaks in the spectrum. Figure 2.5 documents that the additivity allows us to convert a signal that is very difficult to interpret (three interfering cosine curves in the left plot) to a spectrum clearly showing presence of three frequencies and allowing us to read their values with a good accuracy (maxima of three peaks in the right plot). The width of each peak is given by the damping rate – the faster a cosine curve decays, the broader the resonance peak is.

Interactions described in Sections 2.2–2.4 affect the shape of the spectrum in a clear manner:

- *Dipole-dipole interactions* make the peak broader (contribute to relaxation) but do not shift the average frequency (peak maximum.
- *Chemical shift* shifts the average frequency (peak maximum) and its dependence on molecular orientation makes the peak broader (contributes to relaxation). The size of the shift is given by the electron distribution in the molecule and is linearly proportional to B_0 (because the effect is shielding B_0). Therefore it is presented in relative units (ppm). In principle, the chemical shift should be equal to $(\omega_0 + \gamma B_0)/(-\gamma B_0)$. In practice, it is reported relative to a reference signal from a standard compound $(\omega_0 - \omega_{0,\mathrm{ref}})/\omega_{0,\mathrm{ref}}$. The standards used in biochemistry are sodium 3-(trimethylsilyl)propane-1-sulfonate $(^{13}\mathrm{C}^1\mathrm{H}_3)_3\mathrm{SiCH}_2\mathrm{CH}_2\mathrm{CH}_2\mathrm{SO}_3\mathrm{Na}^+$, liquid ammonia $^{15}\mathrm{NH}_3$, and 85% phosphoric acid $\mathrm{H}_3{}^{31}\mathrm{PO}_4$. Chemical shift is the value reported in the literature as a property of the given

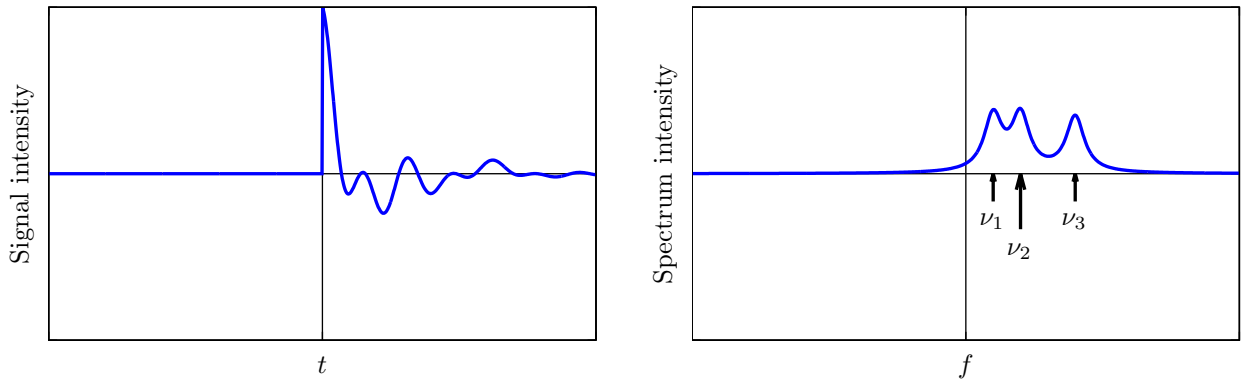


Figure 2.5: Signal (left) and frequency spectrum (right) with three precession frequencies.

compound. It is given by the molecular structure, is influenced by chemical and physical conditions (temperature, ionic strength, pH) but does not depend on the experimental setup (on B_0 or frequency of radio waves used in the experiment). As $-\gamma B_0$ is negative for nuclei with positive γ, peaks of the most shielded nuclei (with low values of the chemical shift and positive Ω) appear in the right-hand side of the spectrum, i.e. the Ω values increase from left to right, and the chemical shift values increase from right to left. As the spectrometers do not distinguish sign of the frequency, the chemical shift values *also increase from right to left* in spectra of nuclei with negative γ, and Ω values increase from right to left, which is, strictly speaking, physically incorrect.

- *J-coupling* splits the peak to a *multiplet* (several peaks of slightly different frequency[3]) but does not contribute to broadening (does not change as the molecule tumbles[4]). The frequency differences (splittings) are given by the electron distribution in the molecule and do not change with B_0 (J-coupling does not depend on the external field). Therefore they are presented in units of frequency (Hz).

2.7 Evolution of coherences

The coherence, introduced by the $\vec{B}_1$ field, evolves under the influence of magnetic fields acting on the observed nucleus, and the macroscopic magnetization of the sample varies. In order to track the changes, it is useful to decompose the overall magnetization into individual contributions rotating with different frequencies.

- First, we distinguish magnetizations of different nuclides (^{1}H, ^{13}C, ^{15}N, ^{31}P) because the magnetization vector rotates about $\vec{B}_0$ with the frequency $\vec{\omega}_0 = -\gamma\vec{B}_0$ (Eq. 1.3) and each nuclide is characterized by a unique value of γ.

- Second, we distinguish magnetizations of the same nuclides in different chemical groups because they are surrounded by different distributions of electrons resulting in different chemical shifts. As a consequence, precession frequencies ω_0 and frequency offsets Ω of magnetic moments of the same nuclide in different groups slightly vary.

- Third, we distinguish magnetizations of the same nuclides in different chemical groups coupled via covalent bonds to nuclei with different polarizations. As shown in Figure 2.3, polarization along $\vec{B}_0$ of a J-coupled neighbor

[3] In the case of nuclei with spin number of 1/2 and with sufficiently different precession frequencies, each nucleus coupled to the observed nucleus splits the observed frequency to a doublet. E.g., spectrum of three mutually coupled nuclei consists of three quartets (doublets of doublets), i.e., of twelve peaks. However, the central peaks may overlap if the interactions with both neighbors are of similar size, and the quartet may look like a triplet with doubled intensity of the middle peak.

[4] J-coupling does not contribute to peak broadening via molecular tumbling, but it may cause relaxation (broaden the peaks) if the structure of the molecule and/or distribution of magnetic moments changes during the experiment.

slows down the precession frequency (Figure 2.3F) whereas polarization against $\vec{B}_0$ of a J-coupled neighbor speed up the precession frequency (Figure 2.3F). In other words, evolution of transverse polarization (coherence) of the observed nucleus *correlates* with the longitudinal polarization of the neighbour. The difference between frequencies of the cyan nucleus in Figure 2.3F and Figure 2.3G is called *coupling constant* and usually labeled as J.

The correlation underlying the third distinction is not easy to grasp. An attempt to explain it is present in Table 2.1. In the second column of the table, four magnetic moment distributions of two nuclei are drawn. We assume that the nuclei are different nuclides with very different precession frequencies, e.g. the left nucleus is ^{1}H (proton) and the right nucleus is ^{13}C (or ^{15}N).

The first row (labeled "In-phase") shows distributions immediately after applying a 90° pulse a frequency very close to the ^{1}H precession frequency. The drawings are oriented so that the magnetic field is pointing to the right. Traditionally, the direction of $\vec{B}_1$ is used to define the x axis of the rotating coordinate frame introduced in Section 1.11. According to a convention described in Section **??** the axis of rotation is the same as direction of $\vec{B}_1$ for protons and other nuclei with positive γ. Therefore, the ^{1}H magnetic moments are polarized towards us (direction $-y$) after the pulse. In the second column, most polarized magnetic moments of ^{1}H are colored in cyan and magnetic moments of ^{13}C (not influenced by the radio wave and therefore keeping their equilibrium distribution) in the *same molecules* are colored in green. We see that there is no obvious correlation between orientations of magnetic moments shown in cyan and in green. Before we discuss diagrams in other columns, we look at the second row.

The second row (labeled "Anti-phase") shows distributions after time equal to $1/(2J)$. During this time, the transverse polarization of ^{1}H rotated due to the presence of $\vec{B}_0$ modulated by the chemical shift (*chemical shift evolution*). The apparent speed of rotation in the rotating coordinate frame depends on the exact choice of the radio wave frequency (which defines the frequency of the rotating coordinate frame). To simplify our analysis, let us assume that the radio wave has exactly the same frequency as the precession frequency of the observed proton. Therefore, the most polarized magnetic moments would not rotate in the rotating coordinate frame and stay in the $-y$ direction (pointing to us in the view used in Table 2.1) if there were no coupling between the nuclei.

However, the nuclei *are* coupled. Protons coupled to ^{13}C with magnetic moment pointing up precess slower (counterclockwise in the rotating coordinate frame) and protons coupled to ^{13}C with magnetic moment pointing down precess faster (clockwise in the rotating coordinate frame). As a result, the originally polarized magnetic moments of ^{1}H (cyan arrows in the second row) spread in a belt surrounding the "equator" in our drawings. At time equal to $1/(2J)$, the polarization of ^{1}H is lost completely, its magnetization is zero. However, the distribution is still coherent! It is just another type of coherence, present in samples with coupled nuclei. To distinguish the different types of coherences, the former one, associated with measurable proton magnetization, is called *in-phase*, and the latter one associated with zero proton magnetization, is called *anti-phase*. In the second row of Table 2.1, fractions of proton magnetic moment oriented most to the right (x direction in our coordinate system) and most to the left ($-x$ direction in our coordinate system) are colored in cyan. At the first glance, distribution of proton magnetic moments looks like for the in-phase coherence (the first row). However, coloring the distributions *separately* for the cyan arrows pointing to the right and pointing to the left reveals a striking difference (the third column in the second row of Table 2.1). The distributions of the magnetic moments of the nuclei are *correlated*, the right direction of the cyan arrows correlates with the up direction of the green arrows, the left direction of cyan arrows correlates with the down direction of the green arrows.

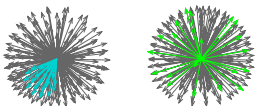
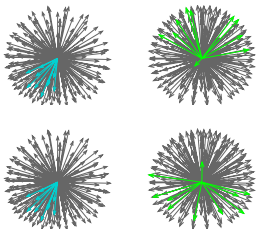
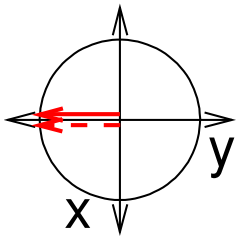
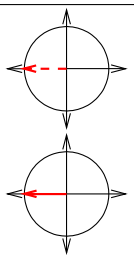
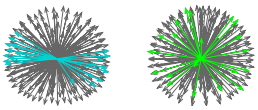
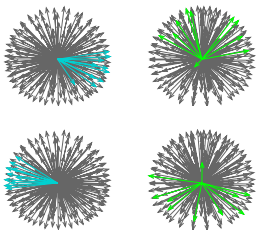
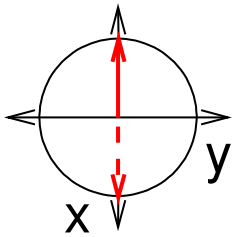
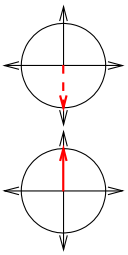
We can now return to the first row and divide the highlighted magnetic moments based on the direction of the green arrows (magnetic moments of ^{13}C). The result, shown in the third column confirms that no correlation exists between cyan and green arrows.

To avoid tedious drawing the distributions, we introduce simple arrows (the third column in the second row of Table 2.1) specifying directions of the magnetic moments of protons attached to ^{13}C with the magnetic moment pointing up (dashed arrow) and pointing down (solid arrow).

In the following sections, we use the solid and dashed arrows to analyze evolution of coherences in various modules of pulse sequences. Here, we only mention the effects of the chemical shift and J-coupling in the absence of radio-wave pulses:

- *Chemical shift* causes transverse polarization of nuclei in different chemical groups to rotate about $\vec{B}_0$ with different frequencies. The size of transverse magnetization is preserved (except for relaxation effects, neglected

Table 2.1: Examples of two graphical representations of in-phase and anti-phase coherences: as distributions and as arrows (used in Figure **??**). Cyan arrows represent magnetic moments resonating with the applied radio wave (magnetic moments of ^{1}H in our example) most aligned along the $-y$ direction. Green arrows represent magnetic moments that do not resonate with the radio frequency (magnetic moments of ^{13}C or ^{15}N in our example) in the same molecules. The solid and dashed arrows presented in the last two columns correspond to partial distributions of proton magnetic moments, shown in cyan in the third column. The direction of the arrow is given by the average direction of the cyan arrows, the type (dashed or solid) of the arrow is given by the average direction of the green arrows in the third column (up or down, respectively). In the second and third columns, the directions of the x and z axes in the are right and up, respectively, and the y axis is perpendicular to them, pointing away from us (behind the plane of the paper).

Coherence	depicted as distributions molecules with $\vec{\mu}$ of ^{1}H closest to $-y$ selected	decomposed distributions based on $\vec{\mu}$ of ^{13}C/^{15}N being closer to $+z$ vs. to $-z$ in the selected molecules	depicted as arrows	decomposed arrows
In-phase				
Anti-phase				

here), only the direction changes. Any direction in the xy plane can be expressed as a linear combination of x and y components of the magnetization. Both components oscillate as the magnetization rotates, and these oscillations are detected as the NMR signal. Accordingly, the in-phase coherence can be decomposed into x and y components. Chemical shift does not convert in-phase coherences to anti-phase coherences or vice versa.

- *J-coupling* makes transverse polarization and magnetization of nuclei in the same chemical group to disappear and reappear periodically. This can be described as an oscillation between "visible" in-phase coherences and "invisible" coherences. The consequence is an oscillation of the NMR signal given by the value of the interaction constant J.

Note that although the anti-phase coherence is "invisible", the polarization is hidden in it: after another time of $1/(2J)$, i.e., at time $t = 1/J$ after excitation, the magnetization is recovered, now in the direction pointing to us from the plane of the paper.