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Electron Spin Resonance (ESR) Spectroscopy

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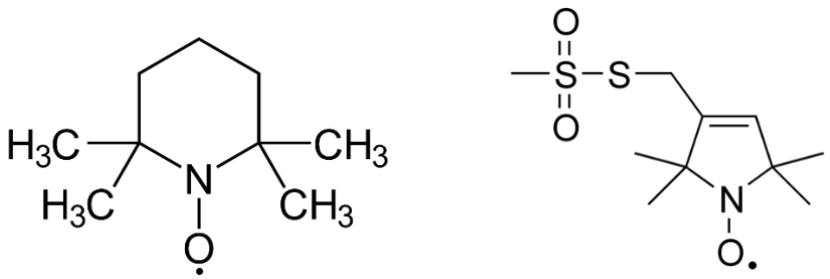
1. Experiments

1. Measure the CW ESR spectra of 200 µM TEMPO in different concentrations of glycerol (0%, 40%, 60%, 80, and 90%).
2. Measure the CW ESR spectra of 100 µM MTSL in water and MTSL labeled papain.
3. Simulate the above spectra using the program EasySpin and determine the rotational correlation time (τ_c).
4. Calculate the τ_c for experiment 1 from the linewidth and peak-to-peak amplitude using the equation (33). Plot the correlation time as a function of the viscosity and extract the apparent radius for TEMPO using the equation (34).
   Use the viscosity of 8.93, 40.46, 127.58, 666.46, 2081.30 (10^{-4} Ns/m^2) for glycerol concentrations of 0%, 40%, 60%, 80, and 90% respectively.
5. Calculate the τ_c for the spin labeled papain (experiment 2) using Stokes-Einstein equation, compare with the simulated value from step 3, and compare with MTSL in solution. Assume a viscosity (η_s) of 8.9274 (10^{-4} Ns/m^2) for the buffer and a radius (r_H) of 2.49 nm for the papain.



TEMPO MTSL

Fig. 1.1 Chemical structure of the nitroxide spin labels used for the experiments. (Left) TEMPO (2,2,6,6-tetramethylpiperidine 1-oxyl). (Right) MTSL (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl methanethiosulfonate).

2. Theoretical background

2.1. Brief overview of ESR spectroscopy

Electron Spin Resonance (ESR) or Electron Paramagnetic Resonance (EPR) spectroscopy is an important tool for studying local structures and electronic and dynamic properties of paramagnetic systems. The property of paramagnetism arises from the presence of unpaired electron(s) in the system, whose spin angular momenta generate a permanent magnetic moment, which in turn can interact with electromagnetic radiation under the influence of an external magnetic field (B). In simple terms, ESR spectroscopy allows the measurement of resonant microwave absorption of a sample in an external magnetic field.

A few examples of paramagnetic systems include organic radicals, transition metal complexes, radical intermediates produced by photolysis or redox processes, and atomic or molecular gases. Organic radicals include paramagnetic spin labels which can be stably attached to otherwise diamagnetic biomolecules. This renders proteins and nucleic acids accessible to ESR spectroscopy for probing their chemical environment and local or global conformational flexibility.

The ESR technique relies on the interaction of the magnetic field component of the electromagnetic radiation with magnetic moments within the sample. By applying an external magnetic field (B), these magnetic moments are aligned with the applied field. Resonance absorption occurs when the frequency (energy) of electromagnetic radiation matches the energy separation between the spin states and induces the spin transition. The electromagnetic radiation used in ESR spectroscopy is in the microwave range. Thus, ESR transitions are characterized by long wavelengths and small energy gaps between the spin states (Fig. 2.1)[1].

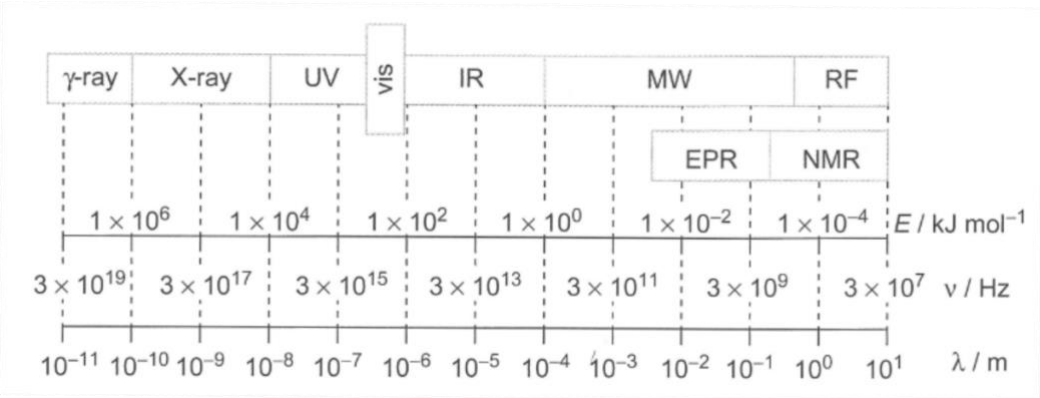


Fig. 2.1 ESR spectroscopy and the electromagnetic spectrum. The subdivision of the spectrum is based on the wavelength and the corresponding frequency and energy. ESR spectroscopy operates with smaller wavelengths than NMR spectroscopy[1].

Both continuous wave (CW) and pulsed approaches are commonly used in ESR spectroscopy. In CW ESR spectroscopy, microwaves of a fixed frequency are continuously irradiated to the sample while the applied magnetic field is swept. Pulsed techniques operate by applying short intense microwave pulses to the sample at a constant magnetic field. A CW ESR spectrum represents microwave absorption of the sample as a function of the applied external magnetic field strength. In CW ESR spectroscopy, the signal is detected as the first derivative of the absorption spectrum. A simple example of such a spectrum is shown in Fig. 2.2.

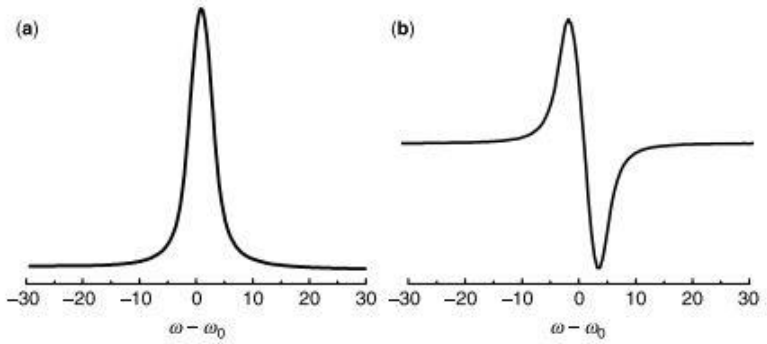


Fig. 2.2 An example of an ESR spectrum, represented as an absorption curve in (a) and as the first derivative in (b). The expression $\omega - \omega_0$ represents the deviation of the microwave frequency from the resonance frequency[1].

The CW ESR spectra of paramagnetic systems often differ from the one shown above because an electron is usually not solitary, but interacts with other electron(s) or nuclei nearby, which cause the ESR spectrum to be more complex.

2.2. Electron angular momentum and magnetic moment of an electron

An electron is a negatively charged particle with a net angular momentum composed of the orbital angular momentum (as it moves around the nucleus) and a spin angular momentum (as it spins about its own axis).

The spin angular momentum $\boldsymbol{S}$ characterizes an intrinsic property of the electron called spin. The magnitude of $\boldsymbol{S}$ is quantized and given by:

$$|\boldsymbol{S}| = \sqrt{S(S+1)}\hbar \tag{1}$$

Where S is the electron spin quantum number and $\hbar$ the reduced Planck's constant. Given an arbitrary direction z, which coincides with the direction of an applied external magnetic field, the z-component of the spin angular momentum S_z is given by:

$$S_z = m_S\hbar \tag{2}$$

Where m_S is the spin projection quantum number, ranging from $-S$ to S in steps of one:

$$m_S = -S, -S+1, \ldots, S-1, S \tag{3}$$

This quantization generates $2S+1$ values for m_S. The allowed values for S are non-negative integers for bosons (such as photons) or half-integers for fermions (such as electrons, protons or neutrons). In the case of an electron, S has the value of $1/2$. The allowable values for m_S are therefore $\pm 1/2$, characterizing two possible spin states denoted as 'spin-up' (for $m_S = +1/2$, labeled with $\uparrow$ and called the α-spin state) and 'spin-down' (for $m_S = -1/2$, labeled with $\downarrow$ and called the β-spin state). The length of the vector $\boldsymbol{S}$ is $\sqrt{3}\hbar/2$. The vector representation for the electron-case is shown in Fig. 2.3.

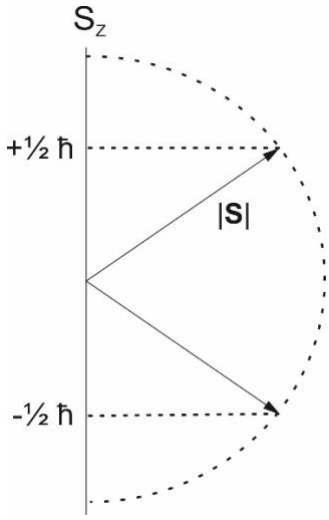


Fig. 2.3 Vector representation for the quantized spin angular momentum $\boldsymbol{S}$. S_z represents the space quantization axis and coincides with the direction of the applied magnetic field.

Without an external magnetic field, the two electron spin states have the same energy and the probability of an electron being in either of the states is equal, or the spin states are degenerate.

The most significant physical effect of the spin angular momentum of the electron is the associated magnetic moment $\boldsymbol{\mu_S}$. In classical terms, the magnetic moment is created by considering an electron as a particle of mass m_e and charge e rotating about an axis with spin angular momentum $\boldsymbol{S}$ producing a circulating current, which in turn generates a magnetic moment. The following equation describes the relation between the spin angular momentum $\boldsymbol{S}$ and the generated magnetic moment $\boldsymbol{\mu_S}$:

$$\boldsymbol{\mu_S} = \gamma \boldsymbol{S} = -g_e \mu_B \frac{\boldsymbol{S}}{\hbar} \tag{4}$$

With:

$$\mu_B = \frac{e\hbar}{2m_e} \tag{5}$$

In these equations, γ is the gyromagnetic ratio given as $\frac{-e}{2m_e}$, m_e is the electron mass, e is the electron charge, and g_e with an approximate value of 2.0023 is the free-electron g value, a dimensionless correction factor for the discrepancy between the magnetic moment of the quantum electron and the classical result[1]. The Bohr magneton μ_B describes the magnetic moment for one unit of quantum mechanical angular momentum. The negative sign arises because the magnetic moment of the electron is collinear but antiparallel to the spin itself. The z-component of the magnetic moment μ_z can be related to the z-component of the spin angular momentum value m_S :

$$\mu_z = -g_e \mu_B m_S \tag{6}$$

This results in two electron magnetic dipole moments corresponding to $m_S = +1/2$ for the α-spin state and $m_S = -1/2$ for the β-spin state, aligned anti-parallel (anticlockwise) or parallel (clockwise) to the applied external magnetic field (Fig. 2.4).

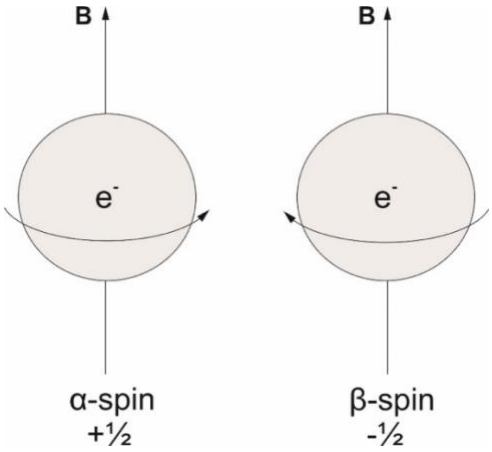


Fig 2.4 The two oppositely oriented electron magnetic dipole moments with respect to the magnetic field, which arise due to the two allowed values for m_S corresponding to the α-spin state (left) or the β-spin state (right).

2.3. The electron Zeeman effect

By applying an external magnetic field, the degeneracy of the two electron spin states is removed. The magnetic moment of the electron interacts with the applied magnetic field $\boldsymbol{B}$ and their interaction energy in classical terms is given by:

$$E = -\boldsymbol{\mu_S}.\boldsymbol{B} \tag{7}$$

The interaction of the electron magnetic moment with the applied magnetic field is called electron Zeeman interaction. If the magnetic field is defined along the z direction (the space quantization axis), the scalar product simplifies to:

$$E = -\mu_z B \tag{8}$$

Where B is the magnetic field strength (i.e. the magnitude of the vector $\boldsymbol{B}$). Substituting the z-component of the magnetic moment given in equation (6) results in the following term for the interaction energy:

$$E = g_e \mu_B m_S B \tag{9}$$

Since $m_S = \pm 1/2$, only two energy states are available, called the electron Zeeman levels, which are degenerate in the absence of a magnetic field. As the magnetic field strength increases, the degeneracy increases linearly as illustrated in Fig. 2.5. The splitting of the electron spin energy levels in a magnetic field is referred to as the electron Zeeman effect. The higher energy state, labeled E_2, corresponds to the electron magnetic moment aligned anti-parallel to the applied field (the α-spin state) and the low energy state E_1 arises due to the parallel alignment of the magnetic moment (the β-spin state).

As described previously, an ESR sample containing unpaired electrons is irradiated with microwave radiation under an external magnetic field. Absorption leading to ESR resonance occurs only, if the energy gap ΔE between the two electron Zeeman levels created by the applied magnetic field can be matched to the given quantum of the radiation ($h\nu$). To maintain angular momentum, the selection rule in ESR spectroscopy is $\Delta m_S = \pm 1$. This results in the following condition for resonance absorption:

$$\Delta E = E_2 - E_1 = h\nu = g_e \mu_B B \tag{10}$$

Where h is the Planck's constant and ν is the frequency of the applied radiation. Since there are typically more electrons in the lower state due to the Maxwell-Boltzmann distribution, there is net absorption of energy which is monitored and converted into a spectrum.

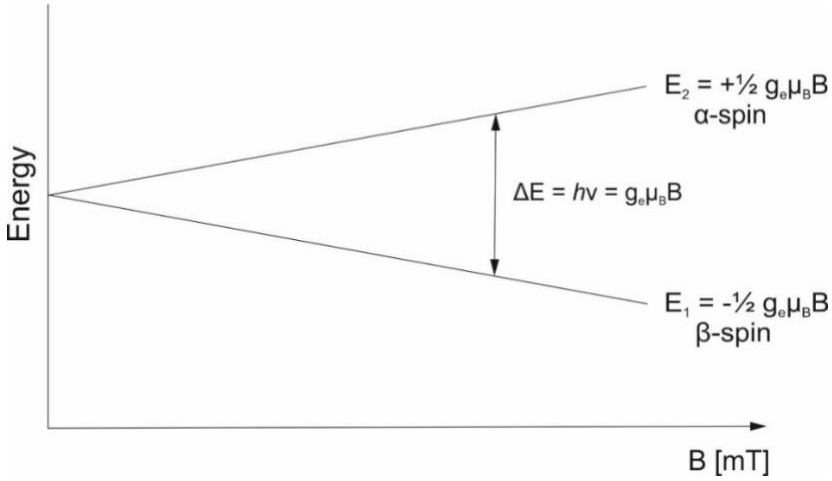


Fig. 2.5 Lifting of the degeneracy by increasing the magnetic field strength results in electron Zeeman splitting and two electron Zeeman levels separated by ΔE, denoted as E_1 and E_2. A spin transition from the lower energy level to the higher energy level is induced when an electromagnetic radiation with a matching frequency is irradiated to fulfill the resonance condition.

Typically, in a CW ESR experiment, the frequency is held constant while the applied magnetic field is swept. Equation (10) shows that the microwave frequency required for the spin transition is about 2.8 MHz per Gauss (or per 0.1 mT) of the applied field. Thus, the corresponding electromagnetic radiation belongs to the microwave region. In case of organic radicals, the applied magnetic field is in the region of 3400 Gauss and the corresponding microwave frequency is about 9 to 10 GHz, known as the X-band frequency.

The magnetic field that the electron experiences in a molecule differs from the applied magnetic field $\boldsymbol{B}$. The presence of local fields at the electron adds to the applied field so that the exact resonance frequency of the ESR signal reflects the chemical environment of the unpaired electron. The effective magnetic field $\boldsymbol{B_{effective}}$ experienced by the electron can be written as a sum of the applied external field $\boldsymbol{B}$ and the local field $\boldsymbol{B_{local}}$ generated at the location of the electron in the molecule:

$$\boldsymbol{B_{effective}} = \boldsymbol{B} + \boldsymbol{B_{local}} \tag{11}$$

Although $\boldsymbol{B_{effective}}$ should replace $\boldsymbol{B}$ when looking at the interaction energy of the electron magnetic moment with the applied magnetic field, it is more convenient to retain $\boldsymbol{B}$ because the applied field can be measured, but therefore replacing g_e with an effective g value[1].

$$\boldsymbol{B_{effective}} = \frac{g}{g_e}\boldsymbol{B} \tag{12}$$

The g value is the key parameter of interest because it determines the resonant field position in an ESR spectrum and is influenced by the chemical environment of the electron, as seen by substituting the effective magnetic field given in Equation (11).

$$g = g_e + \frac{\boldsymbol{B_{local}}}{\boldsymbol{B}} g_e \tag{13}$$

The orbital angular momentum $\boldsymbol{L}$ also may contribute to the local field experienced by the electron. The orbital and spin angular momenta can interact through the spin-orbit coupling. This will result in a local magnetic field which adds to the external magnetic field and in turn yields g values that differ from the free-electron g value g_e. Systems with small atomic masses (e.g., organic radicals) show effective g values close to g_e because of little spin-orbit coupling, while the effective g value in systems with large orbital angular momentum can significantly differ from g_e. In addition, the effective g value can become anisotropic when the local fields are not collinear with the applied magnetic field and must be replaced by a 3x3 matrix $\boldsymbol{g}$.

2.4. The nuclear Zeeman effect and the hyperfine interaction

The electron Zeeman effect is a result of the electron magnetic moment interacting with the applied magnetic field. Similarly, the interaction of the nuclear spins present in the observed system with the applied magnetic field results in nuclear Zeeman splitting. Further interaction between the electron and nuclear Zeeman levels referred to as the hyperfine interaction leads to a multi-line ESR spectrum.

The nuclear Zeeman splitting

The nuclear spin shows similar properties as the electron spin. The magnitude of the nuclear spin angular momentum $\boldsymbol{I}$ is given by:

$$|\boldsymbol{I}| = \sqrt{I(I+1)}\hbar \tag{14}$$

Where I is the nuclear spin quantum number. The analogue of the electron spin quantum number m_S for the nuclei is the nuclear spin quantum number m_I, ranging from $-I$ to I in steps of one:

$$m_I = -I, -I+1, \ldots, I-1, I \tag{15}$$

This generates $2I+1$ values for m_I. Since nuclei consist of nucleons (i.e. protons and neutrons), the nuclear spin angular momentum is formed by coupling their angular momenta. Without an external magnetic field, the nuclear spin states for nuclei with $I \geq 1/2$ have the same energy, meaning they are degenerate. The degeneracy of the $2I+1$ nuclear energy levels is removed by applying an external magnetic field. In analogy to the electron magnetic moment $\boldsymbol{\mu_S}$, nuclei that possess a non-zero nuclear spin angular momentum (when the number of protons and neutrons are uneven) will also have an associated nuclear magnetic moment $\boldsymbol{\mu_I}$:

$$\boldsymbol{\mu_I} = g_N \mu_N \boldsymbol{I} \tag{16}$$

With:

$$\mu_N = \frac{e\hbar}{2m_P} \tag{17}$$

In these equations, g_N is the effective nuclear g value (which can be positive or negative), μ_N is the nuclear magneton, and m_P is the mass of a proton. The z-component of the magnetic moment μ_z can be related to the spin z-projection characterized by the m_I value:

$$\mu_z = g_N \mu_N m_I \tag{18}$$

The interaction of the nuclear magnetic moment with the applied magnetic field is called nuclear Zeeman interaction and results in nuclear Zeeman splitting with an interaction energy described by:

$$E = g_N \mu_N m_I B \tag{19}$$

In analogy to the electron Zeeman splitting, for every m_I value only two energy states called the nuclear Zeeman levels are available in the presence of an external field, one referring to the parallel alignment of the nuclear spin with the applied field and the other one to the anti-parallel alignment. The $2I + 1$ nuclear Zeeman energy levels are equally spaced. Compared to the electron Zeeman splitting, the energy gaps between the nuclear Zeeman levels are considerably smaller, due to the much smaller μ_N[1].

The electron spin energy can so far be summarized as:

$$E = g\mu_B m_S B - g_N \mu_N m_I B \tag{20}$$

The first term describes the electron Zeeman interaction (see equation (9)) and the second term represents the nuclear Zeeman splitting (see equation (19)). The additional term arising from the nuclear Zeeman interaction is illustrated in Fig. 2.6 for a two-spin system with $S = I = 1/2$. Each of the electron Zeeman levels is split into $2I + 1 = 2$ nuclear Zeeman levels.

The hyperfine interaction

As mentioned at the beginning of this chapter, the interaction between the electron and nuclear magnetic moments with each other will also give rise to an additional term in equation (20), referred to as the hyperfine interaction with the following interaction energy:

$$E = a m_S m_I \tag{21}$$

Where a is the hyperfine splitting constant, representing the extent of the hyperfine interaction. The hyperfine interaction creates a shift in the nuclear Zeeman levels towards higher or lower energy.

The final electron spin energy can now be expressed as:

$$E = g\mu_B m_S B - g_N \mu_N m_I B + a m_S m_I \tag{22}$$

For the two-spin system with $S = I = 1/2$ and therefore $m_S = m_I = \pm 1/2$ and positive g_N and a, this results in four energy levels labeled E_1 to E_4 in Fig. 2.6:

$$E_1 = -\tfrac{1}{2} g_e \mu_B B - \tfrac{1}{2} g_N \mu_N B - \tfrac{1}{4} a \tag{23.1}$$

$$E_2 = -\tfrac{1}{2} g_e \mu_B B + \tfrac{1}{2} g_N \mu_N B + \tfrac{1}{4} a \tag{23.2}$$

$$E_3 = +\tfrac{1}{2} g_e \mu_B B - \tfrac{1}{2} g_N \mu_N B + \tfrac{1}{4} a \tag{23.3}$$

$$E_4 = +\tfrac{1}{2} g_e \mu_B B + \tfrac{1}{2} g_N \mu_N B - \tfrac{1}{4} a \tag{23.4}$$

In CW ESR spectroscopy, the selection rules for the spin transitions are $\Delta m_S = \pm 1$ and $\Delta m_I = 0$, meaning the electron spin state has to change following the spin transition and the nuclear spin state remains unchanged[1]. This results in two allowed ESR transitions (from E_1 to E_3 and from E_2 to E_4) for the considered two-spin-system, indicated in Fig. 2.6 as EPR 1 and EPR 2. The difference in their arrow lengths represents different energy quanta $h\nu$ required for resonance absorption to occur:

$$\Delta E_{1\to 3} = E_3 - E_1 = h\nu = g_e \mu_B B + \frac{1}{2}a \tag{24.1}$$

$$\Delta E_{2\to 4} = E_4 - E_2 = h\nu = g_e \mu_B B - \frac{1}{2}a \tag{24.2}$$

This results in two resonance lines in the CW ESR spectrum. The hyperfine splitting constant a, in field units, describes the separation between these two resonance lines and is therefore directly related to the distance between peaks in the spectrum. Like the g factor, it is an important parameter of the ESR spectrum:

$$a = |E_1 \to E_3| - |E_2 \to E_4| \tag{25}$$

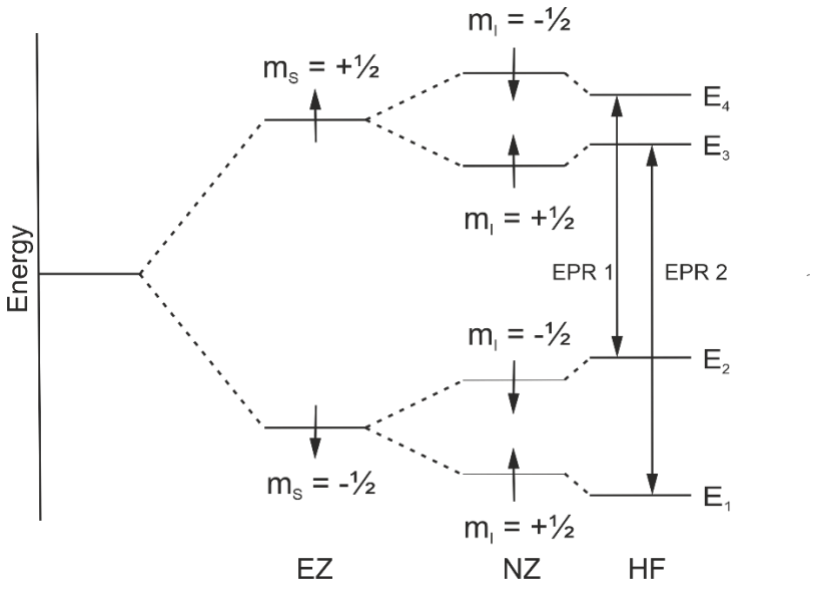


Fig. 2.6 Energy level diagram resulting from electron Zeeman interaction (abbreviated with EZ), nuclear Zeeman interaction (NZ), and hyperfine interaction (HF) for a $S = I = 1/2$ system. The CW EPR spectrum shows two transitions, which are labeled as EPR1 and EPR2.

There are two contributions to the hyperfine interaction that differ in their regions of space: the isotropic Fermi contact interaction that arises inside the nuclear volume and the anisotropic dipolar interaction as a result of interactions outside the nuclear volume. In general, the hyperfine splitting will be anisotropic, except for systems where the anisotropy is averaged out by fast molecular tumbling of the radical. Especially for radicals in the solid state, the anisotropy significantly effects the spectral shape and the hyperfine splitting constant a must be replaced by a 3x3 matrix $\boldsymbol{A}$ (further explained in the next chapter).

2.5. Isotropic and anisotropic CW ESR spectra

Isotropic ESR spectra of organic radicals in fluid solution

In an isotropic CW ESR spectrum, the g value presented as a scalar quantity determines the position of an ESR line along the magnetic field. Especially for organic radicals, the g value is usually similar to that of a free electron and therefore their spectra show ESR lines at roughly the same positions.

As mentioned earlier, the mechanism of isotropic hyperfine interaction between the electron and nuclear magnetic moments is explained by Fermi contact interaction, describing the interaction between an unpaired electron and a nucleus that are overlapping directly. From a quantum mechanical view, there is a non-zero probability for the electron to enter the nuclear volume. It arises exclusively for s-orbitals or orbitals with partial s-character such as sp^2 or sp^3 orbitals, which have some electron density at the nucleus. In these cases, the electron and nuclear magnetic moments can interact directly and the generated hyperfine field inside the nucleus is constant in all directions (i.e. it is isotropic). Sample orientation to the magnetic field does not affect the interaction. The isotropic hyperfine splitting constant a, in energy units, is given by:

$$a = \frac{2\mu_0}{3} g\mu_B g_N \mu_N |\psi(0)|^2 \tag{26}$$

Where μ_0 is the vacuum permeability and $|\psi(0)|^2$ is the probability density of the unpaired electron in the s-orbital or orbital with partial s-character. The absolute sign of the isotropic hyperfine splitting constant cannot be derived directly from the spectrum, but it still is very important, as it affects the relative positions of the energy levels E_1 to E_4 presented in Fig. 2.6 according to equation (22).

For these kinds of spectra, the splitting generated by the hyperfine interaction is the key parameter to assign an organic radical structure to an ESR spectrum. The energy of the hyperfine interaction is much smaller than the energy of the electron Zeeman interaction. This effect is referred to as the high field approximation ($|a| \ll g\mu_B B$) and thus the energy difference between the ESR transitions is very small (see Fig. 2.7 left)[1]. Hence the probabilities of these transitions are comparable and the CW ESR lines have roughly the same intensities (Fig. 2.7 right). In addition to that, the energy levels characterized by the m_I values are evenly spaced with respect to the m_S value and therefore the distances between the $2I + 1$ ESR lines determined by the a value are the same (in case of a system which shows more than two ESR lines). An example for such a system is a nitroxide radical with $S = 1/2$ and $I = 1$ as shown below.

Anisotropic ESR spectra

ESR spectra can also be taken for radicals in the solid state, in frozen or viscous solutions. In these cases, the paramagnetic centers within the sample are randomly oriented with respect to the applied magnetic field. The important spectral parameters g and a are no longer scalar quantities but have to be replaced by the anisotropic $\boldsymbol{g}$ and $\boldsymbol{A}$ tensors, which provide additional information about structure, electronic configuration and symmetry of the system. The spin-orbit coupling becomes orientation or angle dependent and different g values arise due to variations in the orientation of the external field $\boldsymbol{B}$ relative to the paramagnetic center. The isotropic scalar g value must be replaced by the anisotropic $\boldsymbol{g}$ tensor, which is a 3x3 matrix:

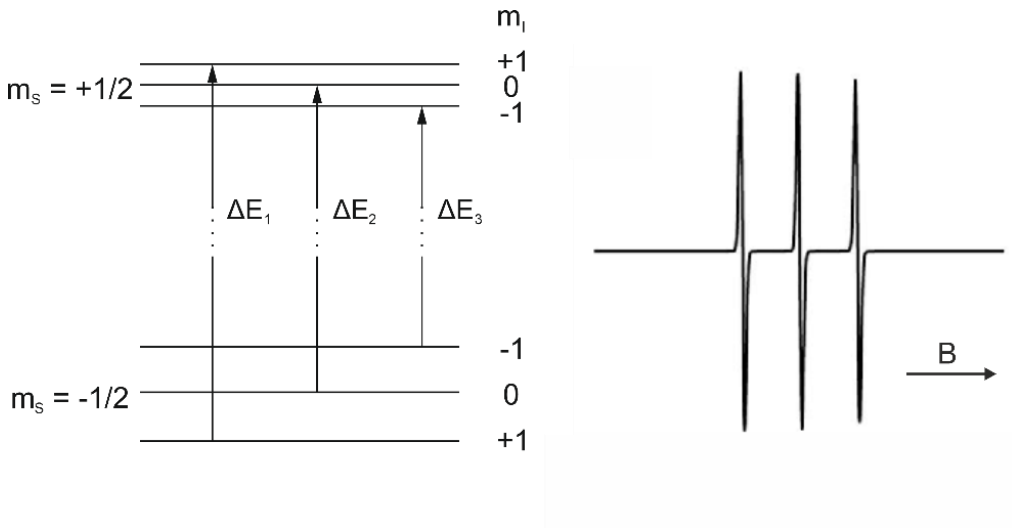


Fig. 2.7. Energy diagram (left) and resulting CW ESR spectrum (right) for a nitroxide radical with $S = 1/2$ and $I = 1$. In the high field approximation, the three lines show roughly the same intensities because the energy difference between the ESR transitions (e.g. $\Delta E_1 - \Delta E_2$) is small compared to the energy difference between the electron Zeeman levels denoted as $m_S = +1/2$ and $m_S = -1/2$.

$$\boldsymbol{g} = \begin{pmatrix} g_{xx} & g_{xy} & g_{xz} \\ g_{yx} & g_{yy} & g_{yz} \\ g_{zx} & g_{zy} & g_{zz} \end{pmatrix} \tag{27}$$

If the tensor is chosen so that its principal axes coincide with the coordinate axes (x, y, z), its off-diagonal elements equal zero and the resulting diagonal tensor is:

$$\boldsymbol{g} = \begin{pmatrix} g_{xx} & 0 & 0 \\ 0 & g_{yy} & 0 \\ 0 & 0 & g_{zz} \end{pmatrix} \tag{28}$$

As discussed in chapter 2.4, two effects contribute to the hyperfine interaction: the isotropic Fermi contact and the anisotropic dipolar hyperfine interaction. Both components will contribute to an anisotropic ESR spectrum. The dipolar hyperfine interaction arises from a classical dipole-dipole interaction between the electron and nuclear magnetic moments in the region outside the nuclear volume and is dependent on their relative orientation and distance, as illustrated by the term for their interaction energy:

$$E_{dipolar} = -\frac{\mu_0}{4\pi}\left(\frac{3cos^2(\theta)-1}{r^3}\right)\mu_e\mu_N \tag{29}$$

Where r is the distance between the magnetic dipole moments and θ is the angle between the magnetic field vector $\boldsymbol{B}$ and the vector that connects both dipoles. If the electron of the paramagnetic molecule occupies a s-orbital, which is spherically symmetric or when the molecule is undergoing rapid tumbling, the angle θ can take on all possible values and the averaged $< cos^2(\theta) >$ value becomes $1/3$ and therefore $E_{dipolar}$ becomes zero. In all other cases where the value for $E_{dipolar}$ is not averaging to zero, the magnitude of this interaction energy is anisotropic, meaning sample orientation to the magnetic field affects the interaction. The isotropic scalar a value must be replaced by the hyperfine $\boldsymbol{A}$ tensor, which is a 3x3 matrix, composed of the isotropic a value a_{iso} and the anisotropic dipolar part $\boldsymbol{T}$:

$$\boldsymbol{A} = \begin{pmatrix} A_{xx} & A_{xy} & A_{xz} \\ A_{yx} & A_{yy} & A_{yz} \\ A_{zx} & A_{zy} & A_{zz} \end{pmatrix} = a_{iso} + \boldsymbol{T} \tag{30}$$

With:

$$\boldsymbol{T} = \begin{pmatrix} T_{xx} & T_{xy} & T_{xz} \\ T_{yx} & T_{yy} & T_{yz} \\ T_{zx} & T_{zy} & T_{zz} \end{pmatrix} \tag{31}$$

The principal components of $\boldsymbol{A}$ can be expressed for a simple axial case as:

$$A = \begin{pmatrix} A_{xx} & 0 & 0 \\ 0 & A_{yy} & 0 \\ 0 & 0 & A_{zz} \end{pmatrix} = a_{iso} + \begin{pmatrix} a_{iso} - T & 0 & 0 \\ 0 & a_{iso} - T & 0 \\ 0 & 0 & a_{iso} + 2T \end{pmatrix} \tag{32}$$

The presence of anisotropy of the hyperfine interaction can result in non-equal values for distances between the lines within an ESR spectrum, corresponding to the angle between the applied magnetic field and the vector that connects the electron and the nuclear magnetic moments.

2.6. Linewidth of CW ESR spectra

ESR spectra do not consist of a discrete set of infinitely sharp lines. The lines are broadened by dynamic effects (molecular tumbling or rotational diffusion, spin relaxation, or chemical exchange) or static effects (orientational disorder, unresolved hyperfine splitting, or inhomogeneous nature of the sample). Linewidth is an important parameter in ESR spectroscopy, as it can add additional information about the structure and the dynamics of the spin system. Linewidth in absorption mode can be described by the full width of half maximum (FWHM), while in first derivative mode, in which an ESR spectrum is usually detected, the peak-to-peak linewidth is commonly used. Both types of linewidth are illustrated in Fig. 2.8.

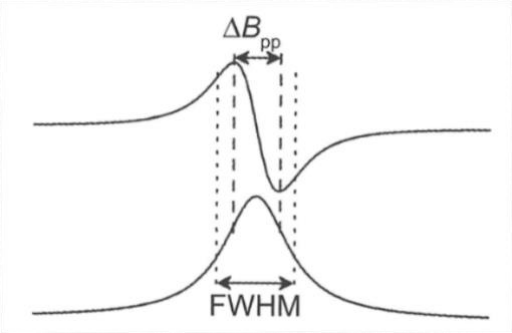


Fig. 2.8 Linewidth of a CW ESR spectrum shown as the full width of half maximum (FWHM) for the absorption spectrum and as the peak-to-peak linewidth ΔB_{PP} for the spectrum in first derivative mode[1].

Effect of molecular tumbling on the spectral shape

In an isotropic ESR spectrum of a sample in fluid solution, all anisotropies are averaged out due to fast molecular tumbling. The rotational correlation time τ_c characterizes the molecular tumbling and is, in simple terms, defined as the mean time required for a molecule to rotate one radian. To completely average out all the anisotropies, the tumbling frequency $(1/\tau_c)$ of a radical needs to be much faster than the timescale of the ESR measurement, or to be more precise much faster than the difference in resonance frequency for different molecular orientations. For nitroxides such as MTSL or TEMPO, the difference in resonance

frequencies is about $8 \times 10^8\ s$ and therefore the rotational correlation time must be shorter than $10^{-9}\ s$ (below $10^{-11}\ s$ in practice)[1]. Very small molecules in low-viscosity solutions can possess such short correlation times and thereby produce isotropic spectra.

The effect of molecular tumbling is divided into fast and slow motion. Fast motion describes the case, where $1/\tau_c$ is larger than the difference in resonance frequencies for different molecular orientations or where the correlation time is shorter than $10^{-9}\ s$. The ESR spectra of fast motion show ESR lines at the same field positions but become broader with increasing correlation times. Hence the peak amplitudes become smaller. The precise line shape depends on whether the molecular tumbling is isotropic or anisotropic, which in turn depends on the size and shape of the molecule, the viscosity, and the temperature at which the measurement is carried out. Small nitroxide molecules in solution often produce fast motion spectra and the value for the correlation time can be calculated from the relative peak heights[1].

$$\tau_c = 6 \times 10^{-9} \frac{s}{mT} \cdot \Delta B_{pp} \cdot \left(\sqrt{\frac{h_0}{h_{-1}}} + \sqrt{\frac{h_0}{h_1}} - 2 \right) \tag{33}$$

Where ΔB_{pp} describes the peak-to-peak linewidth of the central line and h_0, h_{-1}, and h_1 are the peak-to-peak amplitudes of the central, high and low field lines, respectively. Alternatively, the correlation time of an isotropic fast motion spectra can be obtained from simulations with the program EasySpin (www.easyspin.org) using the *garlic* function.

Slow motion describes the case, where $1/\tau_c$ is smaller than or similar to the difference in resonance frequencies or where the correlation time is $10^{-9}\ s$ or longer. The spectra are more complex and the line shape heavily depends on the type of rotational diffusion. Molecules in viscous solvents especially at low temperatures produce slow motion spectra. Slow motion often includes spin label molecules attached to macromolecules such as proteins. The tumbling of the spin label molecule is restricted by the attachment to the protein and by interactions with its surroundings as compared to the tumbling of the free molecule in solution. The shape of the spectrum and the obtained correlation time depends on the tumbling of the whole system and on the local motions of the spin label. In most cases, the attachment to a macromolecule introduces anisotropic effects, since the tumbling of the system is insufficiently fast to average them out. Extracting parameters accurately from such complex spectra is challenging. The *chili* function of the program EasySpin can be used for simulating a slow motion spectrum.

Experimentally, the correlation time of a molecule can be very roughly estimated using the Stokes-Einstein equation:

$$\tau_c = \frac{4\pi\eta_s {r_H}^3}{3k_B T} \tag{34}$$

Where η_s is the viscosity of the solvent, r_H is the effective hydrodynamic radius of a hypothetically spherical molecule, k_B is the Boltzmann constant, and T is the temperature.

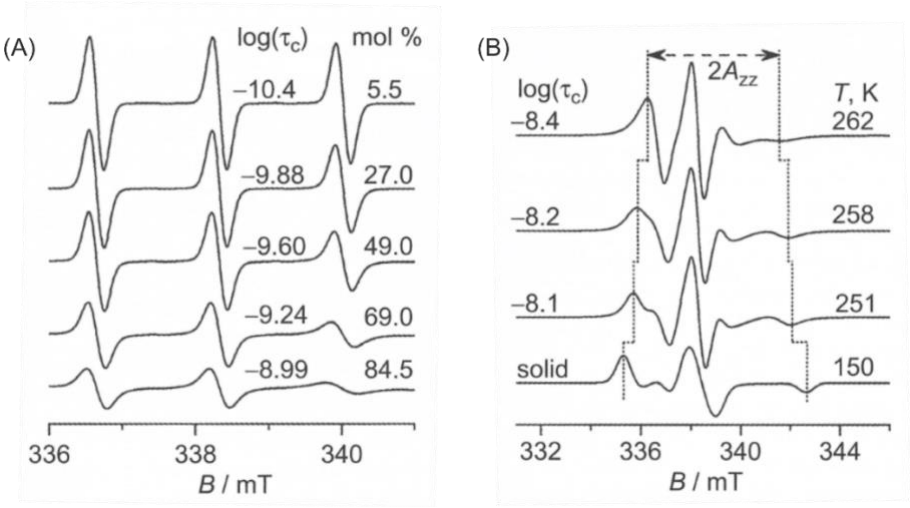


Fig. 2.9 Effect of (A) viscosity and (B) temperature on the line width of the ESR spectra.

3. Application in biological systems

ESR spectroscopy can be effectively used to provide valuable structural and dynamic information on a wide variety of biological systems. Conventional techniques like X-ray crystallography or NMR spectroscopy are often limited when one moves to large protein complexes or protein-lipid assemblies. ESR spectroscopy allows studying such complex systems without any size limitations. But this requires the presence of an unpaired electron spin in the system. Some biological systems contain intrinsic paramagnetic centers like organic-based radicals or ESR active transition metals. However, using site-directed spin labeling (SDSL) it is possible to introduce an artificial paramagnetic label (spin label) into any desired site in a protein. CW ESR spectroscopy of spin labeled proteins is an important method in structural biology, as it can provide information about structure, dynamics, and the surrounding environment. The study is of great importance, as proteins play a crucial role in almost all cellular functions (e.g. transport processes, enzyme activity, immune and drug response, biogenesis of the membrane itself) and can go through large changes in conformation during their function. Furthermore, inter-spin distances between two spin labels attached to a protein can be determined using pulsed ESR techniques such as double electron-electron resonance/pulsed electron-electron double resonance (DEER/PELDOR).

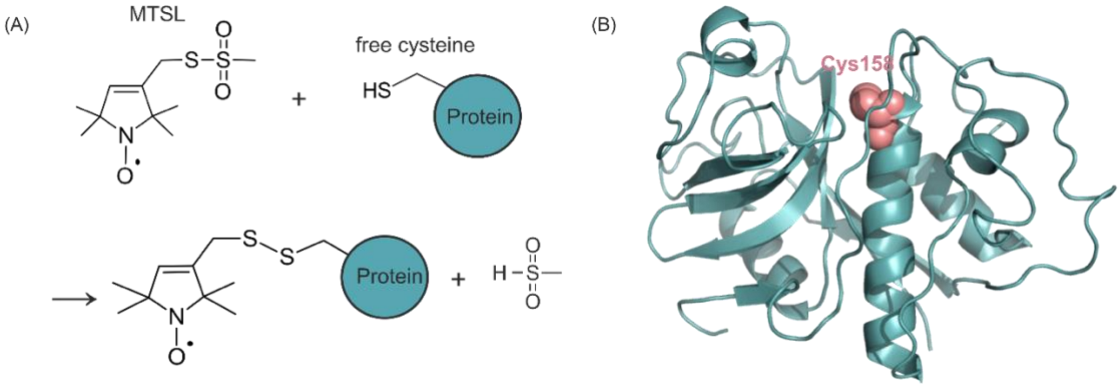


Fig. 3.1 (A) Reaction scheme of MTSL with a free cysteine residue in the protein. (B) Structure of a proteolytic enzyme, Papain (PDB ID: 1PPP). A single reactive cysteine (Cys158) that can react with MTSL creating a spin probe in the protein is highlighted in salmon colour.

In most cases of SDSL, the spin label is attached to a cysteine residue in the protein, which can be introduced into the protein using site-directed mutagenesis. The cysteine is subsequently reacted with a reagent containing a paramagnetic center to generate a stable spin labeled sidechain. Most widely used spin label is the nitroxide based radical called methanethiosulfonate spin label (MTSL). An example of a spin labeling reaction scheme of MTSL with the protein papain is shown in Fig. 3.1A. The ESR spectrum of the spin labeled protein can be used to observe the motion of the spin label. As mentioned earlier, a free MTSL label that moves rapidly in solution gives an isotropic spectrum with three narrow peaks and a small correlation time. When attached to a larger moiety like a protein, the motion of the spin label becomes restricted. As a result, anisotropic effects are introduced into the spectrum. The correlation time depends on the degrees of freedom for the dihedral angels connecting the spin label to the protein, the motion of the backbone, and the protein itself.

According to equation (34), the correlation time of a spin label can be calculated from its hydrodynamic radius, which can be approximated as 0.2 nm for the free radical TEMPO. For macromolecules such as proteins, the hydrodynamic radius can be roughly estimated from the molecular mass M_r, the specific volume of the protein $\bar{V}$ and the hydration layer r_W:

$$r_H = \left(\frac{3\bar{V}M_r}{4\pi N_A}\right)^{1/3} + r_W \tag{35}$$

In which N_A is the Avogadro constant. For the protein papain, the hydrodynamic radius is approximately 2.49 nm.

4. The CW ESR Spectrometer

4.1. Hardware components

As described previously, a CW ESR spectrum is obtained by applying continuous microwave radiation to a paramagnetic sample under an external magnetic field. The detected spectrum is plotted as a function of the applied magnetic field in the first derivative mode. A simplified construction of a CW ESR spectrometer is shown in Fig. 4.1.

The most important hardware components are the microwave resonator, or cavity, which contains the sample to be measured, the microwave bridge, and the magnet. The microwave bridge (indicated as the dashed box in fig. 4.1) produces the microwave radiation with the desired frequency and passes it on to a directional coupler which divides the microwave radiation into two pathways, one leading in the direction of the resonator and the other one operating as a reference path. A circulator passes on the incident microwave radiation to the sample in the resonator, which is designed to enhance the microwave magnetic field at the sample. The *iris screw* controls the amount of microwave energy entering and leaving the cavity. At a unique position, the full microwave power is contained inside the cavity with no reflection back to the circulator. At this point, the cavity is critically coupled. Placing a sample into the resonator leads to resonance absorption of microwave energy by the sample and results in a modification of the coupling profile. Thus, microwave power is reflected back to the circulator from where it is directed towards the detector. Components of the reference pathway are responsible for capturing the correct amplitude ratios and the phase-sensitive detection enhances the sensitivity, which also results in the detection of the signal in the first derivate mode (see the next section). The magnet system generates the homogenous external magnetic field with a field range determined by the frequency of the microwave radiation and sweeps it linearly across this range.

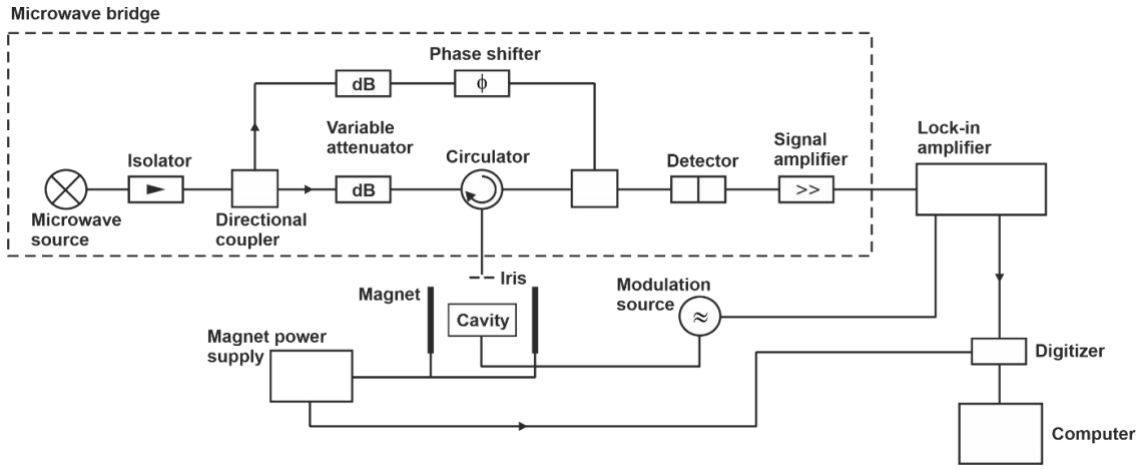


Fig. 4.1 Simplified construction representing a typical CW ESR spectrometer. The sample to be measured is placed in the resonator or cavity (Adapted from 1).

4.2. Experimental settings

The most important experimental parameters include the microwave frequency and the microwave power, the modulation frequency and amplitude, the receiver gain, as well as the conversion time and the time constant. The most commonly used microwave frequency for organic radicals is the X-band frequency (9-10 GHz). The microwave power must be optimized in order to detect the correct line shape and to achieve the best signal-to-noise ratio (S/N ratio). Modulation frequency and amplitude are two important parameters, characterizing a small additional oscillating magnetic field, which is applied to the external magnetic field $\boldsymbol{B}$, typically at a frequency of 100 kHz. When the additional oscillating magnetic field is present, the detector output also oscillates with the same frequency and with a peak-to-peak amplitude that corresponds to the gradient of the detected absorption curve of the sample. This results in a first derivative profile of the absorption spectrum (Fig. 4.2)[2]. Through phase-sensitive detection in the signal channel, only output signals with the same modulation (i.e., 100 kHz) are detected, thereby suppressing the low-frequency signals coming from noise. By adjusting the receiver gain, the amplitude of the signal can be further increased. The conversion time is the time spent at each point as the magnetic field is swept to detect the signal. The time constant filters out the noise by limiting the detection bandwidth and it is important to set a suitable ratio between conversion time and time constant to detect weak signals with small signal-to-noise ratios.

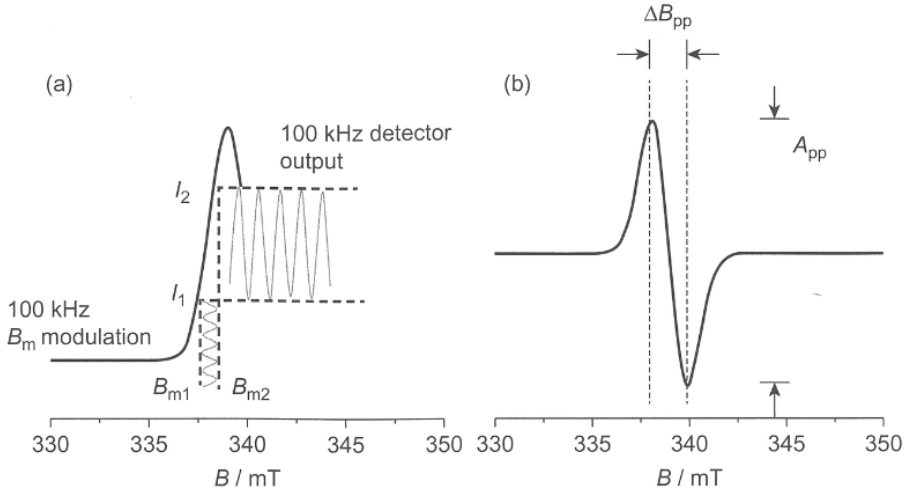


Fig. 4.2 Absorption spectrum (in a) and first derivative profile (in b) as a result of field modulation. The detector output oscillates with the frequency of the field modulation. The peak-to-peak linewidth ΔB_{PP} is the field difference between the points of inflection in the absorption spectrum[2].

5. Simulations of CW ESR spectra using EasySpin

EasySpin is an advanced computational software package for spectral simulation and analysis in ESR spectroscopy. This is a freely available tool running on the MATLAB platform.

There are several functions in EasySpin that simulate continuous-wave ESR spectra: *chili* (slow-motion), *garlic* (fast-motion and isotropic limit), or *pepper* (solid-state limit). For example, a free radical in solution is in fast-motion dynamics. Hence garlic function is used to simulate such a spectrum. However, when the spin label is attached to a protein, one needs to use chili function as the system is in a slow-motion regime. Depending on the dynamics of the label, simplifications in such computations are made. In the rigid and isotropic limits, rotational dynamics can be entirely ignored. In the fast-motion regime, the rotational diffusion can be treated as a small perturbation affecting only line widths. Only in the slow-motion regime a full quantum dynamical treatment is necessary[3].

The basic layout of the EasySpin in MATLAB window is shown in Fig 5.1. The Command Window is where you type in MATLAB commands and it gives the results instantly in the same window. The Editor window is used when you have a long collection of commands to run. Here, the command script can be saved and loaded for later use. The Workspace shows the list of variables that are currently defined. The simulated spectrum opens in a new figures window when you run the commands.

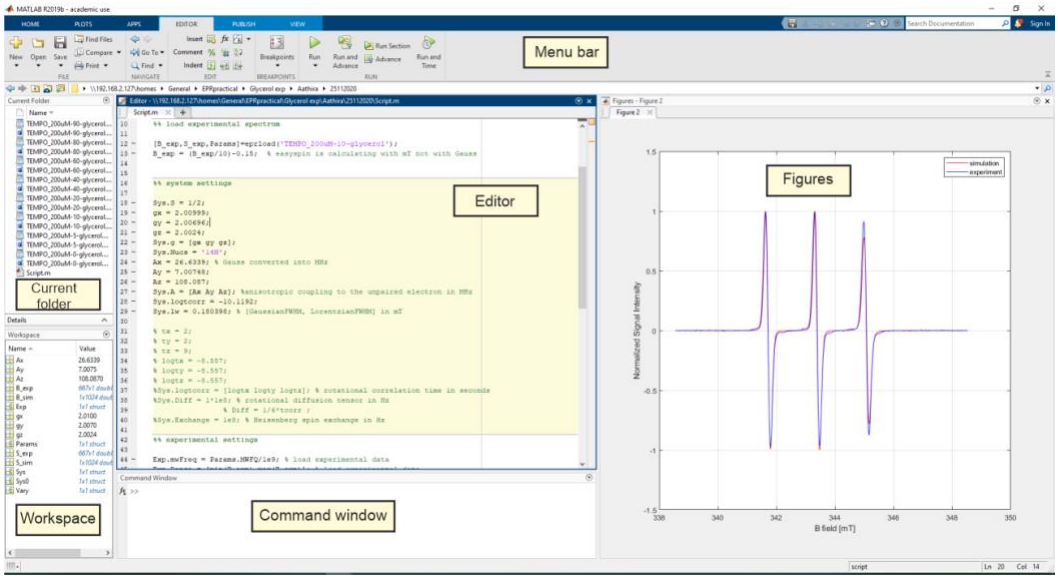


Fig 5.1 Layout of the Easyspin in the MATLAB window

In order to analyze and simulate the spectrum, the experimental data has to be imported. EasySpin uses the *eprload* function for loading experimental data. One needs to specify the spin system parameters and the experimental parameters used. The spin system should contain all information about the spin Hamiltonian specifying the spin quantum numbers, interaction parameters, matrices and tensors, relative orientation angles for these matrices and tensors as well as details about broadenings. The experimental parameters define spectrometer settings like microwave frequency, sweep width, modulation amplitude, etc. The function for simulating the data (*chili*/*garlic*/*pepper*) must be chosen carefully based on the dynamics of the spin label as mentioned earlier. Fitting the simulated data with the experimental data can be done manually or using the function *esfit*. During manual fitting, one must manually change the A value, g value, τ_c, and the line width. While using the *esfit*

function, these values are varied in a given range for the best fit using a least-squares fitting algorithm.

6. Experimental setup and basic instructions

6.1. Sample preparation

Required chemicals:

- Water
- Glycerol
- TEMPO stock solution (2 mM)
- MTSL stock solution (100 µM)
- MTSL labeled Papain

Procedure:

1. Thaw the stock solutions and MTSL labeled papain on ice.
2. Prepare TEMPO samples with a volume of 20 µl each, with the following concentrations:

Sample	**1**	**2**	**3**	**4**	**5**
c(TEMPO)[µM]	200	200	200	200	200
v% water	100	60	40	20	10
v% glycerol	0	40	60	80	90

3. Transfer the samples into an analytical micro pipette and seal the lower end.
4. Measure the CW ESR spectra of the five 200 µM TEMPO samples (see 8.2.).
5. Measure the CW ESR spectra of 100 µM MTSL and MTSL-labeled papain (see 8.2.).

6.2. CW ESR measurement

- Switch on the EMXnano spectrometer (Bruker). Open the Xenon software on the computer, which is connected with the spectrometer. A window as shown in figure 6.1 opens.
- Open tuning panel. A microwave bridge tuning window opens.
- The microwave bridge will be in Standby mode. Set it to Tune mode. A typical resonance dip is seen in the microwave bridge tuning panel.
- Insert the ESR capillary tube containing the sample into the resonator.
- Press the lock search mode on the microwave bridge tuning panel. Wait for the automatic tuning of the system. Once completed, the system goes into Operate mode.
- Change the attenuation to 20 dB.
- Press the On button at the AFC fine tuning area. When fine-tuning is completed, it automatically goes back to off.
- Close the microwave bridge tuning panel.
- Press the parameters panel at the bottom and set the necessary parameters for the experiment as given in Table 6.1. The number of scans is adjusted to achieve a satisfactory signal to noise ratio for the spectrum.
- Start the measurement by clicking on the start button at the bottom left corner.
- Save the data when the measurement is completed.

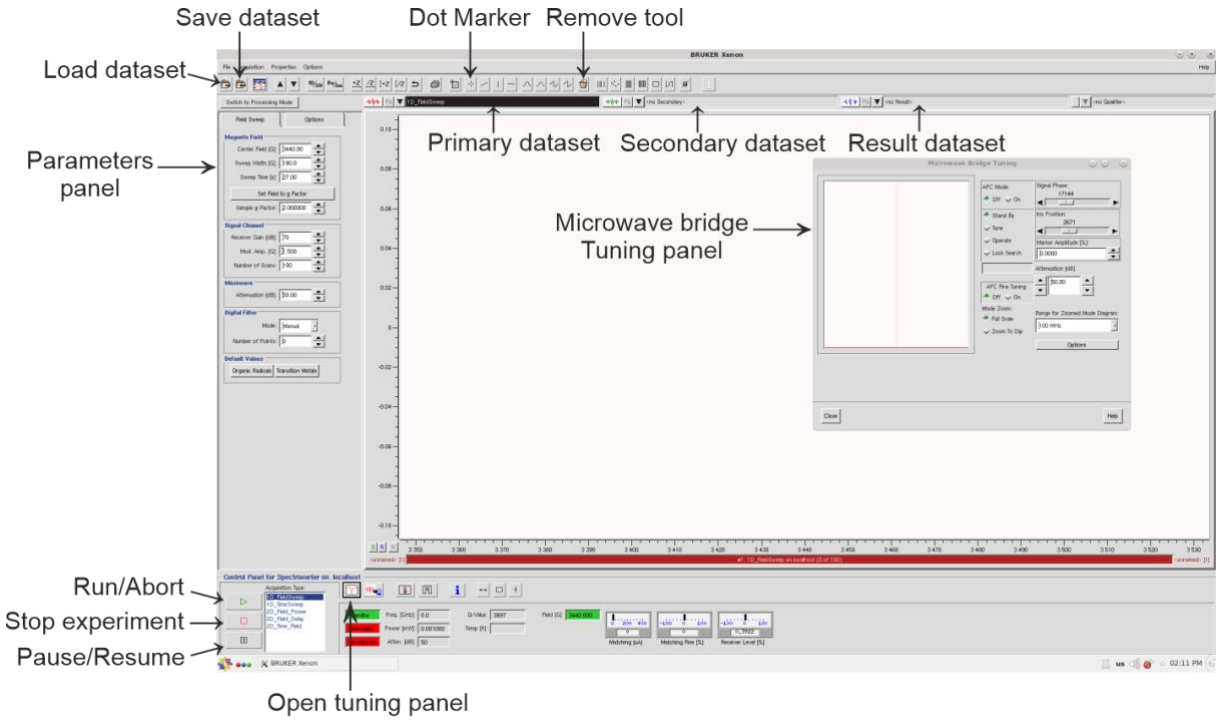


Fig 6.1 The Xenon software for controlling the EMXnano spectrometer.

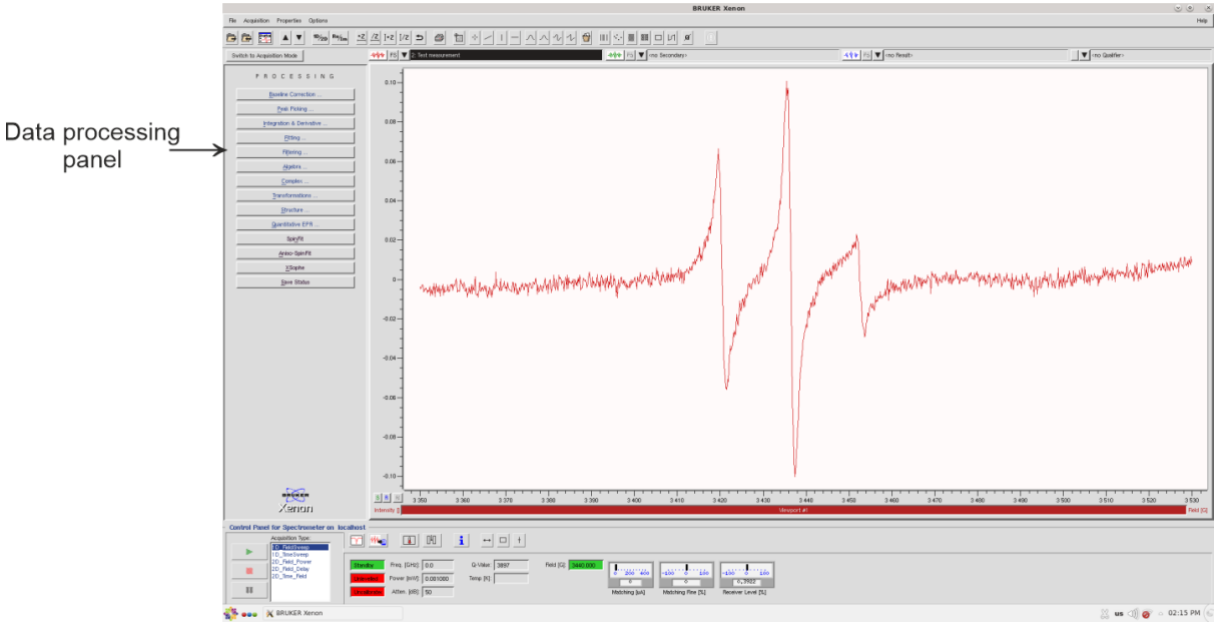


Fig 6.2 The data processing window **of the** Xenon software.

Field Sweep	
Magnetic Field	
Center Field [G]	3437
Sweep Width [G]	100
Sweep Time [s]	15
Sample g-Factor	2.000000
Signal Channel	
Receiver Gain [dB]	55
Mod. Amp. [G]	1.5
Number of Scans	10, 25, or 50
Microwave	
Attenuation [dB]	20
Digital Filter	
Mode	Manual
Number of Points	0
Options	
Magnetic Field	
Number of Points	667
Field Settling	Wait LED off
Settling Delay [s]	0.0
Sweep Direction	Up
Field Flyback	On
Signal Channel	
Modul. Freq. [kHz]	100
Modulation Phase	0.0
Conv. Time [ms]	22.49
Time Constant [ms]	5.12
Dual Detection	OFF
Pts / Mod. Amp.	
Scan	
Fine Tune Each Scan	Off
Auto Scaling	On
Replace Mode	Off
Auto Offset	On

Field Sweep	
Magnetic Field	
Center Field [G]	3437
Sweep Width [G]	100
Sweep Time [s]	15
Sample g-Factor	2.000000
Signal Channel	
Receiver Gain [dB]	70
Mod. Amp. [G]	1.5
Number of Scans	50
Microwave	
Attenuation [dB]	20
Digital Filter	
Mode	Manual
Number of Points	0
Options	
Magnetic Field	
Number of Points	667
Field Settling	Wait LED off
Settling Delay [s]	0.0
Sweep Direction	Up
Field Flyback	On
Signal Channel	
Modul. Freq. [kHz]	100
Modulation Phase	0.0
Conv. Time [ms]	22.49
Time Constant [ms]	5.12
Dual Detection	OFF
Pts / Mod. Amp.	
Scan	
Fine Tune Each Scan	Off
Auto Scaling	On
Replace Mode	Off
Auto Offset	On

Table 6.1 Parameter settings for measuring TEMPO and MTSL in water (on the left) or MTSL labeled papain (on the right).

7. References

1. Victor Chechik, Emma Carter, and D. M. *Electron Paramagnetic Resonance*. (Oxford University Press, 2016).
2. Hemminga, Marcus A., Berliner, L. *ESR Spectroscopy in Membrane Biophysics*. (Springer Science+Business Media LLC, 2007).
3. Stoll, S. & Schweiger, A. EasySpin: Simulating cw ESR spectra. *Biol. Magn. Reson.* 27, 299–321 (2007).

8. Questions

- How is ESR spectroscopy classified in the electromagnetic spectrum?
- Why does the ESR spectrum of a nitroxide radical ($S = 1/2$ and $I = 1$) give three lines? Explain it based on the splitting of the energy levels.
- Why the spectrum is distorted at higher microwave power or modulation amplitude?
- What are typical applications of ESR spectroscopy in biological systems?

Questions in German:

- Wie wird die ESR-Spektroskopie in das elektromagnetische Spektrum eingeordnet?
- Warum zeigt das ESR-Spektrum des Nitroxyl-Radikals ($S = 1/2$ und $I = 1$) drei Peaks? Erklären Sie dies anhand der Aufspaltung der Energieniveaus.
- Warum erscheint das Spektrum bei höherer Mikrowellenleistung oder Modulationsamplitude verzerrt?
- Was sind typische Anwendungen der ESR-Spektroskopie in biologischen Systemen?