5.12: Electron Paramagnetic Resonance (EPR) of Membranes

Electron paramagnetic resonance (EPR) is a technique with applications in multiple branches of science, including physics, biology, and chemistry. The basic concepts of EPR are known to be analogous with Solid-state NMR, except it is electron spins that are excited as opposed to spins of atomic nuclei. EPR is often considered as a continuation of the renowned experiment conducted in 1922 by German physicists Otto Stern and Walter Gerlach, which demonstrated that an electron magnetic moment in an atom can assume only discrete orientations within a magnetic field, despite the spherical nature of the atom [1]. In 1945, Zavoisky recorded the first observation of an EPR peak when he detected a radiofrequency absorption line from a $CuCl_2 \cdot 2H_2O$ sample [1]. Ever since its inception, EPR has seen widespread use as a spectroscopic method for determining the dynamics, structure, and spatial distribution of paramagnetic species [2].

Fundamentals of EPR

Origin of an EPR signal

Since there is already a page dedicated to Electron Paramagnetic Resonance, this page will not go into the specific theories behind this branch of magnetic resonance spectroscopy. Instead, a brief overview of the origin of an EPR signal will be summarized. The magnetic moment of a molecule is mainly contributed from an unpaired electron. If the external magnetic field is increased, the gap between two energy states expands until it matches the energy of the microwaves. This phenomena is represented by the double arrow in the following diagram [3].

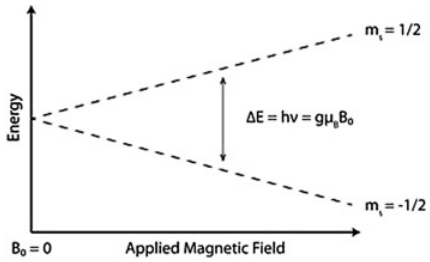

Figure 5.12.1. Energy levels for an electron spin ($M_s = \pm 1/2$) in an applied magnetic field B_0 [3]

As long as the resonance condition, $\Delta E = h\nu$ is followed, an unpaired electron can move between the two energy levels by either absorbing or emitting a photon of energy $h\nu$. The difference between the two energy states can be written as $\Delta E = h\nu = g\mu_B B_0$, where g is the electron's g-factor with a value of 2.002 319 304 386 [4] and μ_B is the Bohr magneton. As the intensity of the applied magnetic field increases, the difference in energy between the energy levels increases until it corresponds with the microwave radiation, thus resulting in absorption of photons. This is the basic principle behind EPR spectroscopy.

Hyperfine Coupling

It is reasonable to assume that all EPR spectra for one electron spin should consist of a single line since the source of an EPR spectrum is a change in an electron's spin state. On the contrary, the interaction of an unpaired electron via its magnetic moment with nearby nuclear spins causes the formation of additional allowed energy states, which would subsequently produce a multilined spectra. In these instances, the spacing between the EPR spectral lines demonstrates the degree of interaction between the unpaired electron and the perturbing nuclei. The hyperfine coupling constant of a nucleus is associated with the spectral line spacing [3]. Coupling is moderated by two processes: dipolar (through space) and isotropic (through bond) [5].

Electrons and nuclei can interact through two standard methods: Fermi contact interaction and by dipolar interaction. Fermi contact interactions apply mainly to the case of isotropic interactions, which are independent of sample orientation in a magnetic field. Conversely, dipolar interactions apply to anisotropic interactions, which are spectra dependent on sample orientation in a magnetic field. Spin polarization is a third method for interactions between an unpaired electron and a nuclear spin and are especially significant for π-electron organic radicals [1].

Spin-Labeling Method

Since the majority of chemical and biological samples of interest for EPR spectroscopy do not have an inherent stable unpaired electron, most EPR mechanisms rely on the use of spin-labeling reagents. Therefore, a radical must be introduced, which is usually referred to as a spin label or spin probe. The most frequently used radical is a nitroxide radical, which displays a three-line hyperfine structure whose peak shape and splitting are dependent on the radical's environment. This nitroxide label monitors

motion. The shape of the EPR signal also depends on the orientation of the magnetic field relative to the axis of the radical. Therefore, the spin probe method can be used to study the environment of the radical, which is also the structure of the polymer at a molecular level [1].

Generally, derivatives of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) are used since they offer adequate stability of the unpaired electron and remarkable EPR sensitivity mixed with adjustable moieties for binding to the sample through chemical reactions itself. Figure 5.12.2 shows the EPR spectra of TEMPO solution in water (0.02 wt%). The spectra contains three symmetric peaks since the NO* radical of TEMPO (or with 5-, 12-, and 16-doxylstearic acid derivatives) is free to move around the solution. All three peaks are nearly equal in height and symmetry [6].

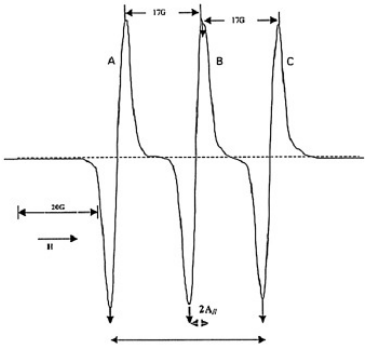

Figure 5.12.2: Electron paramagnetic resonance spectra of TEMPO solution in water (0.02 wt%) [6]

Block Diagram of EPR Spectrometer

Figure 5.12.3 depicts a block diagram for a standard EPR spectrometer. Klystron is commonly used as a radiation source. Klystrons are vacuum tubes known to be stable, high-power microwave sources, which have low-noise characteristics and thus give high sensitivity. Most EPR spectrometers operate at around 9.5 GHz, which is approximately 32 mm [1]. The radiation can be incident on the sample continuously or pulsed. Most EPR applications utilize continuous wave methods as the recording and interpretation of pulse EPR spectra requires sophisticated technical equipment and a more advanced theoretical background. The sample is placed in a resonant cavity between two electromagnets, which admits microwaves through an iris. Several different types of solid-state diodes are sensitive to microwave energy, thus allowing absorption lines to be detected when the separation of the energy levels are equal or very close to the frequency of the incident microwave photons. In practice, most of the external components are enclosed within a microwave bridge control [7].

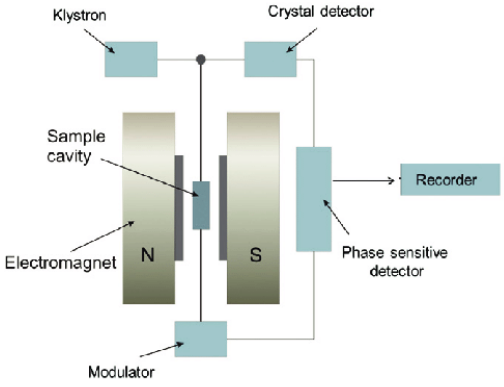

Figure 5.12.3. Block diagram for a typical electron paramagnetic resonance spectrometer [7]

Examples of EPR Applications on Membranes

Membrane Functions of Erythrocytes

In 2000, Tsuda et al. conducted research using EPR spectroscopy and spin-labeling to investigate the role of nitric oxide (NO) in the regulation of membrane functions of erythrocytes in patients with essential hypertension. The NO donor S-nitroso-N-acetylpenicillamine (SNAP) decreased the order parameter (S) for 5-nitroxide stearate (5-NS) and the peak height ratio (h_0/h_{-1}) for 16-NS obtained from EPR spectra of erythrocyte membranes in a dose-dependent manner [8]. Figure 5.12.4 depicts the EPR spectra of erythrocytes for the fatty acid spin-label agents obtained by the authors. The EPR spectra were used to distinguish any

changes in the freedom of motion in biological membranes and to contribute an indication of membrane fluidity. In the EPR spectra for 5-NS, the authors evaluated the values of outer and inner hyperfine splitting to calculate the order parameter (S). In the EPR spectra for 16-NS, they used the peak height ratio (h_o/h_{-1}) value to ascertain an index of the membrane fluidity [8].

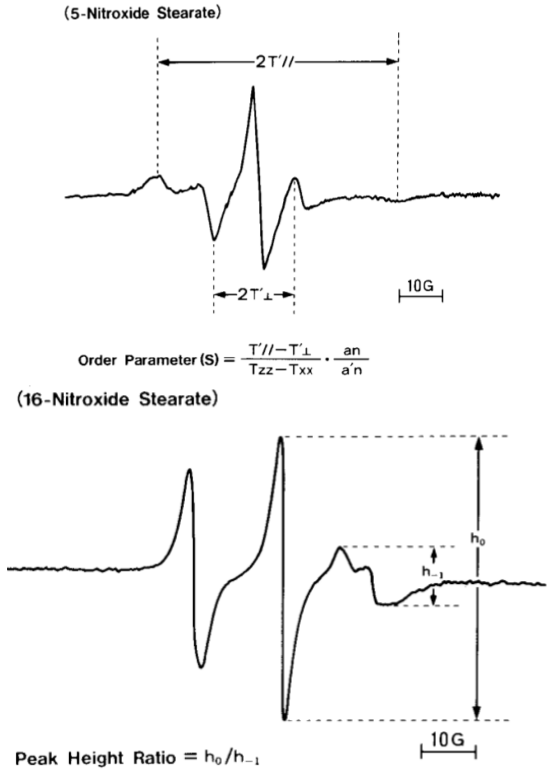

Figure 5.12.4: Standard EPR spectra of erythrocytes for the fatty acid spin-label agents (5-NS and 16-NS). Order parameter (S), outer hyperfine splitting (T'), inner hyperfine splitting ($T'_{\perp}$), hyperfine constants (T_{zz} and T_{xx}), peak height ratio (h_o/h_{-1}). The higher the values of the order parameter and the peak height ratio, the lower the membrane fluidity of erythrocytes [8].

Oxygen Transport in Thylakoid Membranes

In 1998, Ligeza et al. used EPR spectroscopy to study oxygen transport in thylakoid membranes of spinach chloroplasts by observing the collisions of molecular oxygen with spin labels [9]. Linewidths of the EPR spectra measured in the presence and absence of molecular oxygen were used to estimate the collision rates. Additionally, the authors estimated the oxygen permeability coefficient for the thylakoid membrane from the profile of the oxygen diffusion-concentration product across the membrane. This profile was developed by examining the oxygen broadening of the EPR spectrum of lipid-soluble spin labels located at multiple distances from the membrane surface, which was determined by the product of the local oxygen concentration and the local oxygen diffusion coefficient. Using these results, they were able to calculate the oxygen concentration difference that could be produced across the thylakoid membrane during chloroplast illumination. They concluded that under steady state conditions, the oxygen concentration difference across the thylakoid membrane should be inconsequential [9].

Furthermore, Ligeza et al. demonstrated that every collision of oxygen with a nitroxide radical in water catalyzes an observable line broadening of the EPR spectrum. These results allowed them to connect the Smoluchowski equation for colliding molecules [10],

$$\omega = 4\pi pRD(x)C(x), \tag{5.12.1}$$

with oxygen-induced line broadening of the EPR spin label spectrum:

$$\omega = \sqrt{3}/2\gamma\Delta Hpp(x). \tag{5.12.2}$$

where $C(x)$ is the local oxygen concentration at 1 atm partial pressure of oxygen, R is the interaction distance between oxygen and nitroxide radical, p is the probability that an observable event is recorded when a collision takes place, $D(x)$ is the diffusion constant, $\Delta H_{pp}(x)$ is the oxygen-induced peak-to-peak line broadening, and γ is the magnetogyric ratio of the electron [10].

The linewidth of the central component of the EPR spin-label spectrum was ultimately used as the most sensitive parameter to evaluate the broadening effect of oxygen dissolved in the lipid bilayer [9].

Oxidation of Lipid Membranes

EPR spectroscopy has also been used in the study of the aging process and in development of age-associated diseases. Gabbita et al. used EPR spectroscopy in conjunction with a site-specific spin label to investigate several hypotheses related to whether or not succinate stimulation of mitochondria results in oxidative modification of membrane lipids. The primary hypothesis that the authors tested was that caloric restriction protects brain mitochondria and its biomolecular components and decreases metabolic generation of oxygen radicals, thus modulating regulating lipid membrane damage [11]. Similar to Tsuda et al.'s previously analyzed research, Gabbita et al. obtained an EPR spectrum for 5-NS. After a 3 hour incubation at 22°C, EPR measurement of the amplitude (B_0) of the central resonance line of the 5-NS signal and the fluidity parameters ($T_{\parallel}'$ and $T_{\perp}'$) were performed, which is displayed in Figure 5.12.5

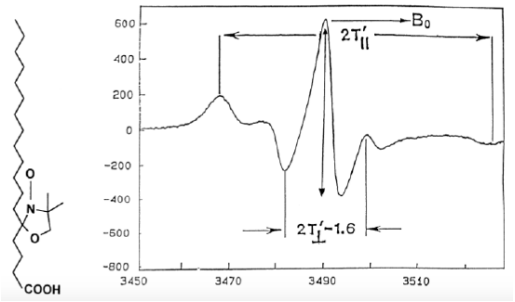

Figure 5.12.5. Structure and representative EPR spectrum of 5-NS [11]

The 5-NS EPR signal reflected an average of all the labeled membranes present in the mixed population of synaptosomes and mitochondria and was influenced by membrane lipid composition. From these results, the authors concluded that the overall succinate concentration effects observed with respect to loss in 5-NS signal amplitude and a decrease in order of synaptosomal and mitochondrial lipid membranes indicate that an induced metabolic stress to mitochondria may cause an increased production of oxygen radicals through the complex II-ubiquinone-cytochrome b region of the electron transport chain. There is a dose-dependent decrease in 5-NS signal amplitude consistent with an increase in generation of oxygen radicals upon mitochondrial respiratory stimulation with succinate.

Advantages	Disadvantages
Can be performed very quickly (15-20 minutes)	Can only be used to identify free radicals
Better selectivity than NMR spectroscopy	Process must be performed at low temperatures
Unlike X-Ray crystallography, EPR does not require a protein crystal	Requires multiple site-directed mutations
Results are easy to comprehend and understand	Can provide only meager distance restraints
Proteins are labeled before fibrillization, thus not necessary to pierce fibril core	

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