

SPIN

EPR spectroscopy 80 years after its discovery

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Electron paramagnetic resonance (EPR) spectroscopy has evolved into a powerful tool for studying matter containing unpaired electrons. By probing electron spin interactions with their environment and with other spins, EPR provides insight into electronic structure, local chemical environments, molecular motion, and intermolecular interactions across broad spatial and temporal scales. This review outlines the principles and methodology of continuous-wave and modern pulse EPR, emphasizing the framework for quantitative spectral interpretation. Applications range from metalloproteins and reactive radicals to functional materials and catalytic systems. Recent advances, including high-field EPR, pulse dipolar EPR for nanometer-scale distance measurements, hyperfine techniques for resolving weak nuclear interactions, and integrated computational modeling, have expanded the role of EPR in structural biology, chemistry, and materials science. Improvements in instrumentation, spin labeling, and sensitivity enhancement continue to broaden its applicability to increasingly complex and dilute systems, while ongoing developments in automation and data analysis are making EPR more accessible across the physical and life sciences.

INTRODUCTION

Electron paramagnetic resonance (EPR) spectroscopy is a sensitive technique (actually a family of techniques) for studying paramagnetic materials, namely, those containing unpaired electrons. Such materials are ubiquitous across physical, biological, medical, and materials sciences. The requirement for nonzero electron spin is sometimes quoted as a limitation, but this specificity is in fact its great strength. When present, unpaired electrons are responsible for the important physical and chemical properties of paramagnets, and EPR is sensitive to the environment and dynamics of that site. Hence, information can be gained on, for example, electronic and geometric structures, reaction mechanisms and kinetics, dopants or defects in solids, charge transfer, redox, conduction, and molecular and spin dynamics. The electron spins can be intrinsic to the system or induced, for example, by spin labeling or photoexcitation. The samples can be solid, liquid, or gas phase. We start by giving a brief history in the context of the centenary of the discovery of electron spin.

In 1922, Gerlach and Stern (1) demonstrated the quantization of angular momentum in space: When a beam of silver atoms passed through an inhomogeneous magnetic field, it split into two components. This was direct evidence of a half-integer angular momentum, which we now know to be an intrinsic property of the electron. In 1925, Uhlenbeck and Goudsmit attributed this to “the spinning electron” (2, 3), also explaining the anomalous Zeeman effect, and Dirac soon after explained it based on relativistic quantum mechanics. Proton spin was proposed in 1927 and demonstrated in molecular-beam

experiments in 1933. Gorter reported failed attempts to detect magnetic resonance with perpendicular oscillating and static magnetic fields in 1936. Rabi reported successful nuclear magnetic resonance (NMR)-like experiments in molecular beams in 1938. In 1944, Zavoisky (4) (studying paramagnetic relaxation) detected the first magnetic resonance signal from condensed-phase materials, using sensitive electrical detection in contrast to Gorter’s calorimetric methods. He reported “paramagnetic absorption” of solutions of Mn^{2+} salts at ~10 MHz and showed dependence of absorption maximum with applied field: He noted that the results could be interpreted “on the basis of... spin-magnetic resonance.” Shortly after, further reports appeared from Cummerow and Halliday (5) on solid Mn^{2+} salts at 3 GHz, and from Bagguley and Griffiths on a single crystal of chrome alum at frequencies including 9 GHz, i.e., X-band (6). The group led by Bleaney at Oxford then undertook extensive studies of d- and f-block ions, where much of the foundational theory of EPR was developed (7). This work by physicists inspired important developments in coordination chemistry and biochemistry, including the development of ligand field theory. Further applications came with free radicals in solution, aided by higher-resolution spectrometers and solution-phase continuous-wave (CW) electron nuclear double resonance (ENDOR) (8), also pushing development of molecular orbital theory (9). Feher had previously introduced CW ENDOR in the solid state (10), and pulse EPR techniques followed the observation of the electron spin echo (11), including electron spin echo envelope modulation (ESEEM) (12) and pulsed ENDOR (13, 14). These opened many new applications, giving access to much weaker interactions than can be resolved in the CW-EPR spectrum, in addition to spin dynamics. An excellent account of the first 50 years of EPR is given in the book edited by Eaton *et al.* (15), and the same authors have written a recent short celebration of 80 years of EPR (16).

In the conventional EPR experiment, an applied magnetic field breaks the degeneracy of the $2s + 1$ sublevels (m_s) of the electron spin s ; a microwave (MW) field, perpendicular to the external magnetic field, can then induce transitions with a selection rule of $\Delta m_s = \pm 1$. EPR methods then provide structural information (spatial and electronic) through interactions between electron spins and their environments. The interactions are parameterized in the spin Hamiltonian

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(SH), a concept introduced by Abragam and Pryce (17), which is the cornerstone of spectrum analysis in magnetic resonance. Because it provides the language for the experimental observables in EPR, we start with a description of the typical SH.

In general, a static SH for a system of electron spins (indices i, j) and nuclear spins (index k) can be written (in energy units) as

$$\hat{H} = \sum_i \left(\mu_B \mathbf{B}^T \cdot \hat{\mathbf{g}}_i \cdot \hat{\mathbf{s}}_i + \hat{\mathbf{s}}_i^T \cdot \mathbf{D}_i \cdot \hat{\mathbf{s}}_i \right) + \sum_k \left(-\mu_n g_{n,k} \mathbf{B}^T \cdot \hat{\mathbf{I}}_k + \hat{\mathbf{I}}_k^T \cdot \mathbf{P}_k \cdot \hat{\mathbf{I}}_k \right) + \sum_i \sum_{j>i} \left(\hat{\mathbf{s}}_i^T \cdot \mathbf{J}_{ij} \cdot \hat{\mathbf{s}}_j + \hat{\mathbf{s}}_i^T \cdot \mathbf{D}_{ij} \cdot \hat{\mathbf{s}}_j \right) + \sum_i \sum_k \left(\hat{\mathbf{s}}_i^T \cdot \mathbf{A}_{ik} \cdot \hat{\mathbf{I}}_k \right) \quad (1)$$

where T indicates the transpose of a vector or vector operator. The terms, in the order in which they are written, parameterize the following:

1) The electron Zeeman interaction (described by the $\mathbf{g}$ tensor) describes the interaction with an applied magnetic field $\mathbf{B}$. μ_B is the electronic Bohr magneton. The g values differ from that of the free electron ($g_e = 2.002319\dots$) and are in general anisotropic, due to spin-orbit coupling (SOC)-driven mixing with excited states. Hence, $\mathbf{g}$ gives information on the orbital ground and excited states.

2) The zero-field splitting (ZFS) interaction (described by the traceless $\mathbf{D}$ tensor) is relevant for high spin, $s > 1/2$. As written, this includes only second-order spin operators, and can be extended as

$$\hat{\mathbf{s}}^T \cdot \mathbf{D} \cdot \hat{\mathbf{s}} = D \left[\hat{s}_z^2 - s(s+1)/3 \right] + E \left(\hat{s}_x^2 - \hat{s}_y^2 \right) \quad (2)$$

where D and E are the axial and rhombic ZFS parameters, respectively. In principle, operators are allowed up to order $2s$; extended Stevens operators are commonly used in this case. SOC and electron-electron dipolar interactions contribute to $\mathbf{D}$.

3) The nuclear Zeeman interaction, where μ_n and g_n are the nuclear magneton and g value, respectively.

4) The nuclear quadrupole interaction (described by the traceless $\mathbf{P}$ tensor) is relevant for $I > 1/2$. This can be written in terms of axial (K) and rhombic (η) parameters in a manner similar to the ZFS

$\mathbf{P}$ arises from electric field gradients at the nucleus.

$$\hat{\mathbf{I}}^T \cdot \mathbf{P} \cdot \hat{\mathbf{I}} = K \left\{ \left[3\hat{I}_z^2 - I(I+1) \right] + \eta \left(\hat{I}_x^2 - \hat{I}_y^2 \right) \right\} \quad (3)$$

5) Electron-electron exchange coupling, described by the $\mathbf{J}$ matrix, is relevant when there is more than one electron spin center. $\mathbf{J}$ can be broken down into isotropic, anisotropic, and antisymmetric terms

$$\hat{\mathbf{s}}_i^T \cdot \mathbf{J} \cdot \hat{\mathbf{s}}_j = J_{\text{iso}} \hat{\mathbf{s}}_i^T \cdot \hat{\mathbf{s}}_j + \hat{\mathbf{s}}_i^T \cdot \mathbf{J}_{\text{aniso}} \cdot \hat{\mathbf{s}}_j + \mathbf{d}_{\text{anti}} \cdot \hat{\mathbf{s}}_i \times \hat{\mathbf{s}}_j \quad (4)$$

The isotropic part (J_{iso} , a scalar), often dominant, arises from the overlap of the magnetic orbitals (this is formulated by some authors as $-2J_{\text{iso}} \hat{\mathbf{s}}_i^T \cdot \hat{\mathbf{s}}_j$ where the definition of J_{iso} differs by a factor of 2, and in sign, from that in Eq. 4). The anisotropic ($\mathbf{J}_{\text{aniso}}$, a symmetric, traceless matrix) and antisymmetric ($\mathbf{d}_{\text{anti}}$, a vector) parts arise from SOC-driven mixing of the ground state of one spin with the excited state of the other.

6) The dipolar interaction between electron spins, which varies as the inverse cube of the separation and can be formulated as

$$\hat{\mathbf{s}}_i^T \cdot \mathbf{D}_{ij} \cdot \hat{\mathbf{s}}_j = \frac{\mu_0}{4\pi} \frac{\mu_B^2}{r^3} \hat{\mathbf{s}}_i^T \cdot \left[\mathbf{g}_i \mathbf{g}_j - 3(\mathbf{g}_i \mathbf{r})(\mathbf{r}^T \mathbf{g}_j) \right] \cdot \hat{\mathbf{s}}_j \quad (5)$$

where μ_0 is the vacuum permeability, $\mathbf{r}$ is the interspin unit vector, and r is the separation. If the electron spins are delocalized, then this must be integrated over the spatial distributions.

7) The hyperfine interaction (described by the $\mathbf{A}$ matrix) between the electron and nuclear spins. This is often written in terms of an isotropic (A_{iso} , a scalar) and an anisotropic, dipolar part ($\mathbf{T}$)

$$\hat{\mathbf{s}}^T \cdot \mathbf{A} \cdot \hat{\mathbf{I}} = A_{\text{iso}} \hat{\mathbf{s}}^T \cdot \hat{\mathbf{I}} + \hat{\mathbf{s}}^T \cdot \mathbf{T} \cdot \hat{\mathbf{I}} \quad (6)$$

The isotropic part is due to electron spin density at the nucleus ($|\psi(0)|^2$), hence due to spin density in s-orbitals, and is given by

$$A_{\text{iso}} = \frac{2\mu_0}{3} g_e \mu_B g_n \mu_n |\psi(0)|^2 \quad (7)$$

In the point dipole limit, the dipolar part (neglecting orbital contributions) can be formulated as

$$\hat{\mathbf{s}}^T \cdot \mathbf{T} \cdot \hat{\mathbf{I}} = \frac{\mu_0}{4\pi} \frac{g_e \mu_B g_n \mu_n}{r^3} \hat{\mathbf{s}}^T \cdot \left[3(\mathbf{r} \mathbf{r}^T - \mathbf{1}) \right] \cdot \hat{\mathbf{I}} \quad (8)$$

where $\mathbf{r}$ is the interspin unit vector, r is the separation, and $\mathbf{1}$ is the unit matrix. If a point dipole model is invalid, then the matrix $\mathbf{T}$ involves integration over the spin density distribution. Therefore, $\mathbf{A}$ contains information on spin density distribution and on electron-nuclear distances.

The terms in Eq. 1 are the most commonly encountered in EPR spectroscopies. We have neglected chemical shift effects and nuclear spin-nuclear spin coupling as these are seldom resolved in EPR spectra (being on the hertz scale), although the former have been observed in high-field hyperfine EPR experiments, and the latter have been invoked in relaxation studies. The phenomenological SH parameters are derived from analysis and simulation of experimental spectra. They are related to the actual structure (geometric and electronic) via quantum chemistry and provide a rigorous test of such methods (Fig. 1) (18).

Because the SH parameters are, in general, anisotropic, EPR also gives important information on molecular dynamics (MD) via partial averaging of the relevant interaction matrices ($\mathbf{g}$, $\mathbf{A}$, etc.) on the timescale of the motion (quantified by the rotational correlation time, τ_c). When $1/\tau_c \gg \Delta\omega$ (the spectral anisotropy in frequency units), all anisotropic quantities are averaged, and the isotropic limit is reached. The rigid limit is reached when $1/\tau_c \ll \Delta\omega$, and a powder pattern spectrum is observed. In between these two regimes, τ_c can be determined, and anisotropy in rotational dynamics can also be assessed (19). This has been most exploited with nitroxides; for example, CW X-band EPR (where the anisotropy is dominated by the ^{14}N hyperfine) gives sensitivity to τ_c from $\sim 10^{-7}$ to 10^{-10} s. Motions on a longer timescale (to $\sim 10^{-3}$ s) can be accessible by saturation transfer or pulse EPR methods (20). The sensitivity of EPR to motion has been widely exploited in structural biology via site-directed spin labeling to probe internal motions in proteins (21).

For the reasons above, EPR spectroscopy represents a vast field, and exhaustive coverage is impossible in this overview. Instead, specific methods and applications in some areas are given in the following sections. We do not cover some important areas, such as EPR imaging and nitrogen-vacancy centers in diamond, and others are only mentioned in passing. We have aimed to reference extensive reviews and primary literature when we believe these are most helpful to the reader, but we apologize for the inevitable omission of many key publications.

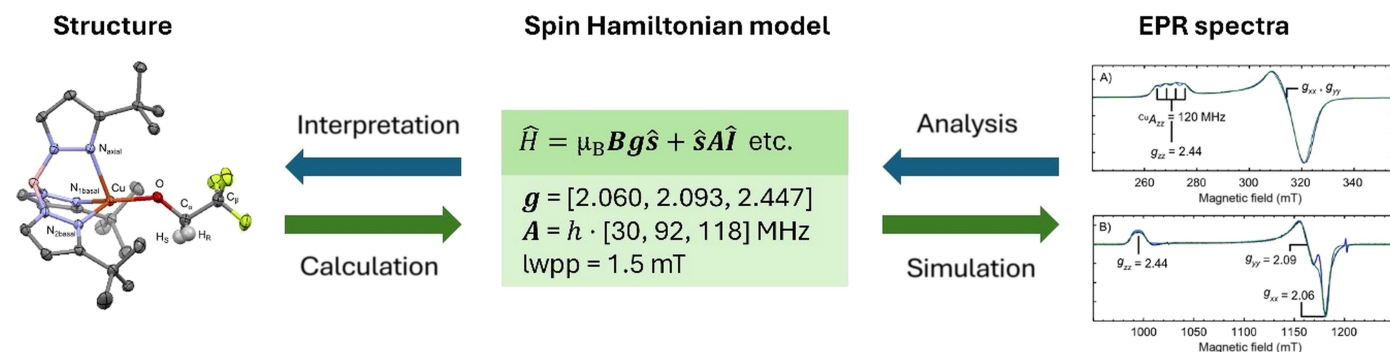


Fig. 1. Connection between electronic and nuclear structure, the spin Hamiltonian model, and the EPR spectrum.

CW EPR

In CW EPR, the MW frequency is kept constant while the static magnetic field is slowly swept to bring the spins through resonance and reveal MW energy absorption. A small additional modulation of the static field (typically at kilohertz frequencies) enables phase-sensitive lock-in detection and improves sensitivity, producing spectra that are recorded as the first derivative of the absorption line (“derivative-like” line shapes). For analytical applications, CW EPR remains the most commonly used method (22). The reason is that in liquid solutions, the relaxation times of most radicals and transition metal complexes are too short (in the nanosecond to picosecond range) to be detected by pulse EPR methods (different from the field of NMR spectroscopy). In addition, the spectral width of paramagnetic molecules is often too large to be entirely excited by MW pulses. Spectral widths for many radicals are larger than 100 MHz, whereas the frequency coverage of typically used MW pulses (with a pulse length of about 10 ns) is only about 30 MHz. The most commonly used MW frequency of such spectrometers is in the 9- to 10-GHz range (X-band), and they allow operation at variable temperatures and have access to optical excitation of the sample inside a resonator tuned to the MW excitation frequency. A number of commercial tabletop EPR spectrometers also exist and operate in this frequency band. The recorded spectra allow quantitative determination of radical concentrations (down to micromolar for small organic radicals in solution) by double integration of the typically recorded derivative CW-EPR signal. CW EPR can be used to investigate rotational correlation times of molecules with anisotropic interactions as described above. An example is shown for nitroxide radicals (see the “Chemistry” section) in Fig. 2A, where the sensitivity of the X-band CW-EPR spectrum of a nitroxide radical to the rotational correlation time is simulated. CW EPR is also an important analytical tool for detecting paramagnetic states in catalytic reactions. For this purpose, CW-EPR setups have been combined with electrochemical methods and other spectroscopic or structural methods, such as infrared spectroscopy (23, 24). The high sensitivity of CW EPR allows for operando detection of paramagnetic species in catalysis (25) and analytical applications (26).

Rapid-scan EPR

In recent years, rapid-scan EPR, where the magnetic field is rapidly swept through the EPR spectrum, has been developed (27). In this case, the field-modulation lock-in detection used for CW EPR is removed, and the time-resolved MW signal is directly recorded and lastly analyzed to obtain the EPR spectrum. Compared to CW EPR,

this method offers higher sensitivity and a higher temporal resolution for observing transient radical species, e.g., in chemical reactions (28), but requires additional instrumentation (ultrafast scan coils, high bandwidth detection, and corrections for resonator response distortions).

High-field EPR

For many high-spin systems with large ZFS, high MW excitation frequencies (above 100 GHz) are favorable, or even mandatory, to observe the full EPR spectrum (with resonance transitions at magnetic fields in the Tesla range). When the external magnetic field exceeds the ZFS, the quantization of the electron spin stays almost along the external field axis, allowing a direct extraction of the EPR SH parameters from the EPR spectra (Fig. 3). For integer spin systems, e.g., octahedral Ni^{2+} with $s = 1$ or Mn^{3+} with $s = 2$, it might be impossible to detect an EPR signal at X-band frequencies with typically applied magnetic fields below 1 T (EPR silent under such conditions), but at high MW frequencies, such spin systems will also become visible (29).

For half-integer spins larger than $1/2$, e.g., Mn^{2+} ions with $s = 5/2$, the spectral position of the central transition ($m_s = -1/2$ to $m_s = +1/2$) is, to first order, not affected by the ZFS term in the SH (Eq. 2) and therefore is much easier to observe. The inhomogeneous line-width of this transition becomes narrower at larger magnetic field strength B_0 , making the detection more sensitive and a quantitative analysis of the SH parameters is much easier (30). This is advantageous when using ENDOR to investigate in detail the nuclear spins surrounding the Mn^{2+} metal ion (31). High-field EPR is also an important analytical tool to distinguish different organic radicals, e.g., amino acid radicals in biological systems. This is achieved by resolving the anisotropic g tensor of such radicals (Fig. 2B), which strongly depends on the radical character and even its chemical surroundings (32). The determination of the chemical species and its chemical environment, e.g., noncovalent bonding to proteins, is typically done in combination with quantum-chemical density functional theory (DFT) calculations, which predict the anisotropic g tensor (33, 34).

Transient EPR

Transient EPR has been a technique of choice for investigating photo-generated transient paramagnetic states, including radical pairs and photo-excited triplet states produced by intersystem crossing (35, 36). In this method, continuous low-power MW is applied, and the resulting transient EPR signal is detected as a function of time. The

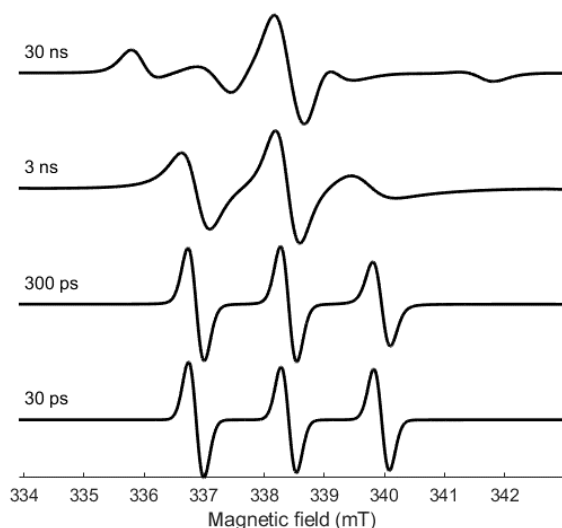
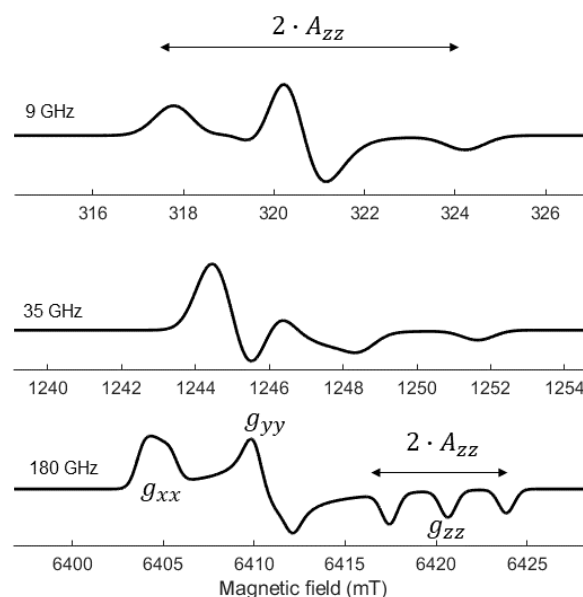
A X-band nitroxide cw-EPR as a function of τ_c **B** Solid nitroxide cw-EPR as a function of ν_{mw} 

Fig. 2. CW-EPR line shapes encode dynamic information. (A) X-band nitroxide CW-EPR spectra as a function of the rotational correlation time τ_c . The line intensities and widths depend on the rotational correlation time (from slow rotation top to fast rotation at the bottom). (B) Nitroxide CW-EPR spectra at different microwave frequencies (9 GHz/X-band, 35 GHz/Q-band, and 180 GHz/G-band, from top to bottom). At low MW frequencies (X-band), the spectral width is dominated by the ^{14}N nitrogen hyperfine tensor **A**, whereas at high MW frequencies (G-band), the anisotropic **g** tensor is fully resolved and determines the spectral width. All simulations are performed with EasySpin (154).

disadvantage of this method compared to pulsed MW excitation results from the low MW power applied, exciting only small portions of the EPR spectra. This disadvantage is compensated by the high spin polarization typically encountered in photoexcited paramagnetic states, leading to selective populations of the electron spin eigenstates far away from Boltzmann equilibrium (37).

PULSE EPR

As mentioned above, for most samples and under most conditions, CW EPR is the most sensitive experiment for measuring an EPR spectrum. It provides information on the largest interactions of the electron spin that give rise to resolved features, but a wealth of information is hidden due to the lack of resolution, particularly for solid samples. However, the interaction of interest can be separated from all other interactions using dedicated pulse sequences (38). Resolution is then limited only by the lifetime of electron spin coherence. This is the same as in NMR spectroscopy, and pulse EPR shares many concepts with NMR, but the number of shared pulse sequences is indeed small. This is because the much larger magnetic moment of the electron spin, the faster relaxation, and the larger g shifts compared to chemical shifts render most NMR experiments unfeasible (39). CW EPR can also provide some information on spin relaxation by measuring progressive power saturation, but longitudinal and transverse relaxation rates cannot be separated in this way. This can be achieved by pulse EPR, where the inversion-recovery and Hahn-echo decay measurements separate these two rates.

Decoherence and dynamical decoupling

Because electron spin decoherence is a principal limiting factor, its mechanisms and methods to counter it are outlined below. Under

conditions of fast tumbling in a liquid solution of sufficiently low concentration, decoherence is dominated by the transverse relaxation time T_2 . The same approaches as in NMR can be used to predict T_2 in this regime. However, most pulse EPR experiments are performed on solids at low temperatures. For many systems, the decoherence rate attains an asymptotic limit at sufficiently low temperatures. This regime is entered at about 100 K for organic radicals without methyl groups in glassy solution and at about 15 K for transition metal ions with $s = 1/2$ in single crystals, microcrystalline powders, and frozen solutions.

At moderate (about 1 mM for organic radicals and about 10 mM for transition metal complexes) to high concentration, the decoherence rate is dominated by the dipole-dipole coupling between electron spins. These couplings cause flip-flop transitions during free evolution and a stochastic change of the local field at the observer spin during a refocusing π pulse (40). The latter effect is termed instantaneous diffusion (41).

For sufficiently low concentration, tens of micromolar for organic radicals (42), and higher when only a small fraction of the EPR spectrum is excited by the pulses, decoherence is dominated by the nuclear spin bath (43). Typically, in this regime, protons contribute the strongest to electron spin decoherence, with methyl protons exhibiting a larger effect than other protons (44). The decay imposed by the nuclear spin bath is typically not monoexponential. Empirically, it can be described by a stretched exponential decay function (44) or a sum thereof (42). Such a stretched exponential decay is akin to echo decay from diffusion processes, which can be partially refocused by the Carr-Purcell (CP) sequence. Analogous decoherence suppression by repeated refocusing was developed in the quantum information processing (QIP) community and termed dynamical decoupling (DD) (45). In the context of pulse EPR, DD was first used for improving the resolution in measurements of small dipole-dipole

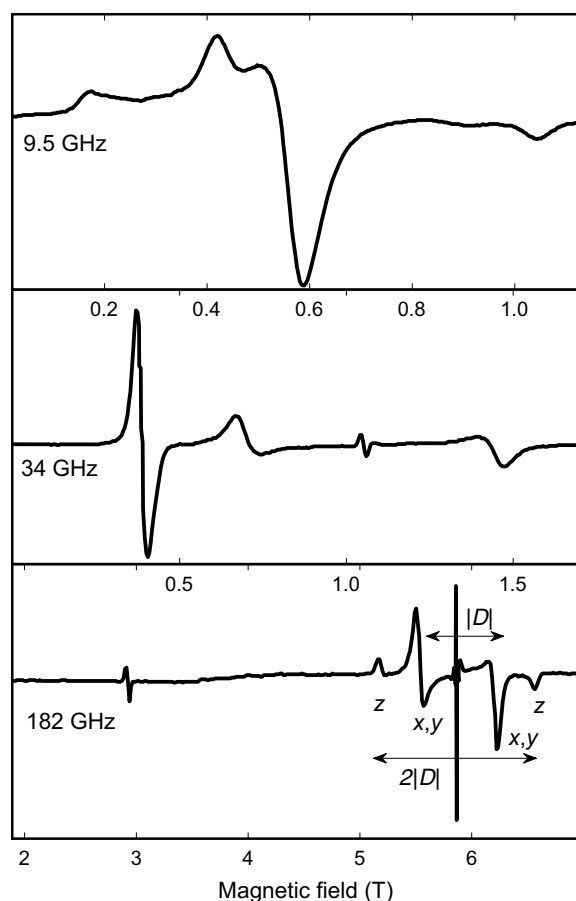


Fig. 3. The effect of the frequency on the EPR spectrum. CW-EPR spectra of $[\text{Ni}(\text{5-methylpyrazole})_6](\text{ClO}_4)_2$ as a powder at 100 K and at frequencies of 9.5, 34, and 182 GHz (top to bottom, respectively). The six-coordinate Ni^{2+} ion has $s = 1$; the large ZFS ($D = +0.72 \text{ cm}^{-1}$) requires high-field EPR to yield recognizable triplet spectra. Adapted from (274).

couplings for inference of distance distributions. To this end, the refocused-echo observer subsequence of the double electron-electron resonance (DEER) experiment was set up with delays corresponding to the CP sequence (46). By using shaped pulses, CP DD for this application could be extended to three π pulses (47). Decoherence suppression by CP and Uhrig DD sequences extends to at least five π pulses for water-glycerol matrices. It is effective even after exchanging protons by deuterons to slow down decoherence (48). For Gd^{3+} spin labels with spin quantum number $s = 7/2$, however, DD is not effective in suppressing an additional decoherence contribution that arises from ZFS. Decoherence due to the nuclear spin bath is also suppressed during a spin lock, i.e., in a strong MW field (49). This effect is largely due to hyperfine decoupling, which shifts the matching with nuclear dipole-dipole coupling to shorter distances r . As the number of nuclei around the electron spin scales with r^2 , this reduces decoherence.

Pulsed dipolar EPR spectroscopy

A set of pulse EPR methods that has gained considerable interest in the EPR and structural biology communities is pulsed dipolar EPR spectroscopy (PDS) (50). This toolbox of pulse sequences has been

shown to measure distances in the range of 15 to 160 Å (51). PDS methods select the dipolar coupling ω_{AB} (Eq. 9) between unpaired electrons from all other spectral contributions.

$$\omega_{AB} = \frac{\mu_B^2 g_A g_B}{\hbar} \frac{1}{r^3} (3 \cos^2 \theta - 1) \quad (9)$$

These unpaired electrons can either be intrinsic spin centers (e.g., Mn^{2+} , iron-sulfur clusters, semiquinone, and amino acid radicals) or spin labels, or combinations of these. The interest in PDS stems from ω_{AB} being directly related to the distance r_{AB} between the spin centers, thus providing access to the structure of a biomolecule (Fig. 4). Because PDS experiments do not distinguish between intramolecular and intermolecular distances, the intramolecular contribution to the time trace is convolved with the intermolecular contribution, which adds a decay function to the time trace. To avoid dynamic averaging of ω_{AB} , the molecules must be immobilized, usually by freezing the sample. Therefore, the PDS measurements record distance distributions associated with the conformational ensemble of the biomolecule present at the freezing point of the buffer (52, 53). Alternatively, PDS measurements can be performed at room temperature if the molecules are immobilized by binding to a surface (54). In any case, PDS measurements give access to conformational distributions because the distances are not averaged over the dynamics of the biomolecule. This is highly valuable for the study of intrinsically disordered systems and for gaining access to lowly populated, energetically higher-lying conformers, which are often responsible for the function of biomolecules.

Which PDS method to use depends on the EPR properties of the spin centers between which the distances are to be measured (see below). PDS methods have been applied to large biomolecular complexes such as ribonucleoproteins (55, 56) and membrane proteins (57, 58) in vitro and have recently been extended to in cellulose (59). PDS experiments have been combined with fast freeze-quench setups to follow conformational changes with time resolution, with times as short as 80 μs (60, 61). Last, all PDS experiments discussed below can and have been combined with shaped pulses, e.g., chirp pulses, reducing artifacts, expanding the distance range, and increasing the signal-to-noise ratio (SNR) (see the “Instrumentation” section).

Double-frequency experiments

A set of double-frequency PDS experiments is shown in Fig. 5. Out of these, pulse electron-electron double resonance (PELDOR, also known as DEER) is the most well-known and commonly used in its four-pulse version to measure distances between nitroxide spin labels (62, 63). Here, the detection sequence $\pi/2 - \tau - \pi - T - \pi$ is applied at an MW frequency ν_A on electron spin A, yielding a refocused echo, while a pulse at MW frequency ν_B flips electron spin B. Stepping this inversion pulse through the time interval T and plotting the intensity of the refocused echo against the time position of the inversion pulse yield a time trace that is modulated by the frequencies of the dipolar coupling ω_{AB} . In PELDOR experiments, the intermolecular decay function can usually be removed by fitting an exponential decay function to the unmodulated section at the end of the time trace. Fourier transform (FT) of the remaining intramolecular time trace (the form factor) yields the so-called Pake pattern. This experiment requires that the excitation profiles of the detection and inversion pulses do not strongly overlap. This is fulfilled for nitroxides at X- and Q-band when separating the frequencies of the inversion and detection pulses by 60 to 80 MHz. Recently, the program “autoDeer” has been released, which enables an automated setup of the PELDOR experiment (64).

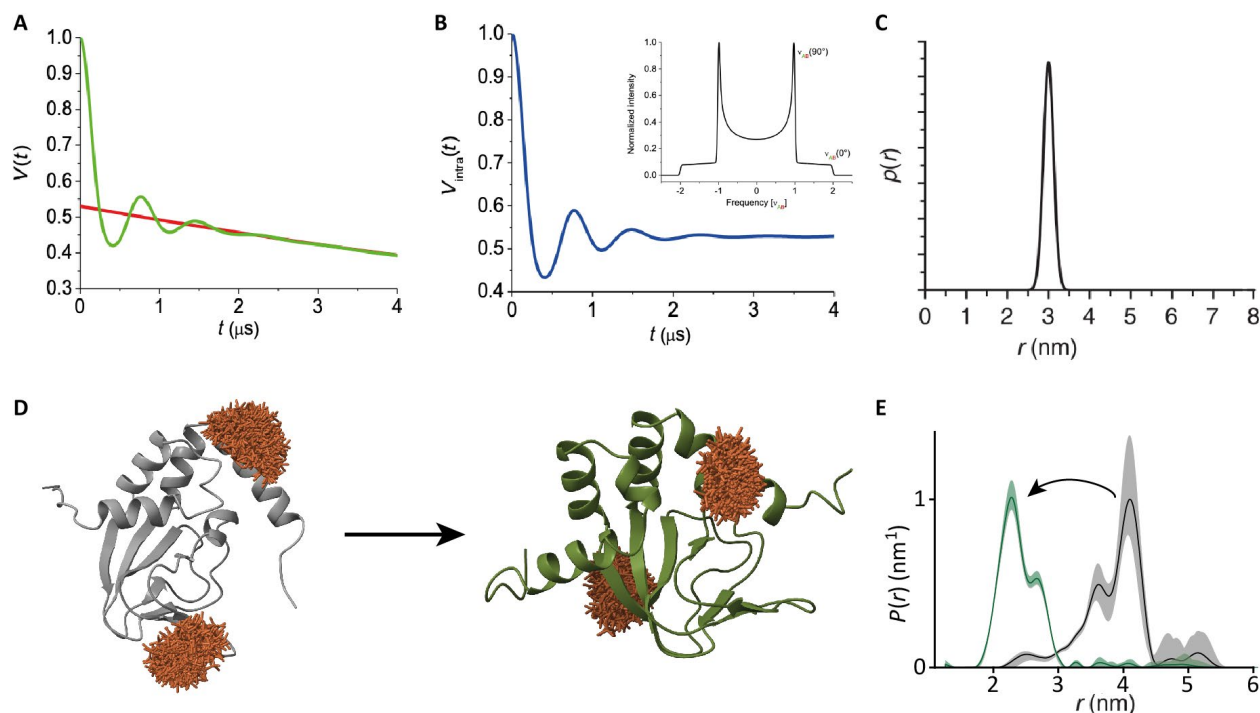


Fig. 4. PDS time trace and its transformation to distance distribution. (A) A PELDOR time trace (green) overlaid with the fitted background function (red). (B) The background-corrected intramolecular contribution to the time trace (blue) and its Fourier transform yielding the Pake pattern (black). (C) The corresponding distance distribution generated with DeerAnalysis (see below). (D) Structure of a cyclic nucleotide binding domain (CNBD) of the cyclic-nucleotide-gated (CNG) potassium channel of *Mesorhizobium loti* in the apo form (gray, 2kxl) and after (green, 2k0g) binding of cyclic adenosine monophosphate (cAMP). The orange sticks represent the conformer distribution of the spin label MTSL (generated with mtslWizard, see below). (E) The conformational change associated with the transition from apo to holo is observed in PELDOR in the form of a large change in the distance distribution (60).

A precursor of this experiment is the single-frequency “2 + 1” experiment performed at X-band using just one MW source, which had the disadvantage of large ESEEM contributions, and, because the “+1-pulse” (the inversion pulse) needs to be applied with a time delay after the $\pi/2$ pulse, the experiment could not recover the broad components of the Pake pattern (65). Using the three-pulse PELDOR version, in which two different, non-phase-correlated MW sources are used for the detection pulses and the inversion pulse, reduces ESEEM contributions substantially (66). In the deadtime-free four-pulse version, ESEEM is suppressed, and the full Pake pattern can be recovered (62, 63). Performing the four-pulse PELDOR experiment at Q-band frequencies and with high-power amplifiers for the inversion pulse yields high SNRs (67); samples with nanomolar spin concentrations have been measured (68). A white paper on how to record and document four-pulse PELDOR experiments has been published along with a multilaboratory test (69). If the orientation of the two spin centers is fixed with respect to each other, orientation selection can occur because the excitation bandwidth of the pulses is usually narrower than the spectral width. This orientation selection can be used to gather not only distance but also angular information, or it can be removed by adding time traces recorded with different frequency offsets (70). Last but not least, the modulation depth of the time trace provides information about the number of coupled spins, which means that it can be used, e.g., to quantify the number of monomers in a complex (71). Using shaped pulses, longer time windows and thus longer distances become accessible as shown for sech/tanh inversion pulses in the seven-pulse CP PELDOR (47).

Single-frequency experiments

A set of single-frequency PDS experiments is depicted in Fig. 6. If one of the spin centers is a slow relaxer and the other relaxes fast, e.g., a nitroxide spin label and a metal ion, respectively, then the relaxation-induced dipolar modulation enhancement (RIDME) experiment is very useful (72). In its deadtime-free five-pulse version (73), the pulse sequence is applied to the EPR spectrum of the slowly relaxing nitroxide, and the time interval T is chosen such that it matches the T_1 relaxation time of the fast-relaxing metal ion. Thus, the inversion pulse of the double-frequency experiments is replaced by a stochastic spin flip of the fast-relaxing B spin. This is especially beneficial if the EPR spectrum of the metal center is very broad and, if the experiment is performed at higher MW frequencies, e.g., Q-band, where ESEEM artifacts can be suppressed (74). In Q-band RIDME, the gain in SNR as compared to the PELDOR experiment can be roughly one order of magnitude (75). Combining Cu^{2+} with a trityl spin label enabled RIDME measurements at a concentration as low as 10 nM (76). In addition, RIDME has also been applied to cases of two metal centers with very good SNR. In the case of high-spin ions with small ZFS, double- and triple-quantum transitions may complicate the analysis (77). The background function, for which no analytical expression is known, and the artifacts in the time traces are still a challenge for RIDME experiments (78). Some of the artifacts can be reduced by using a six-pulse RIDME sequence (79), while the background can be removed by reference measurements.

If both spin centers are slowly relaxing and have narrow and overlapping EPR spectra, e.g., two trityl spin labels, the double quantum

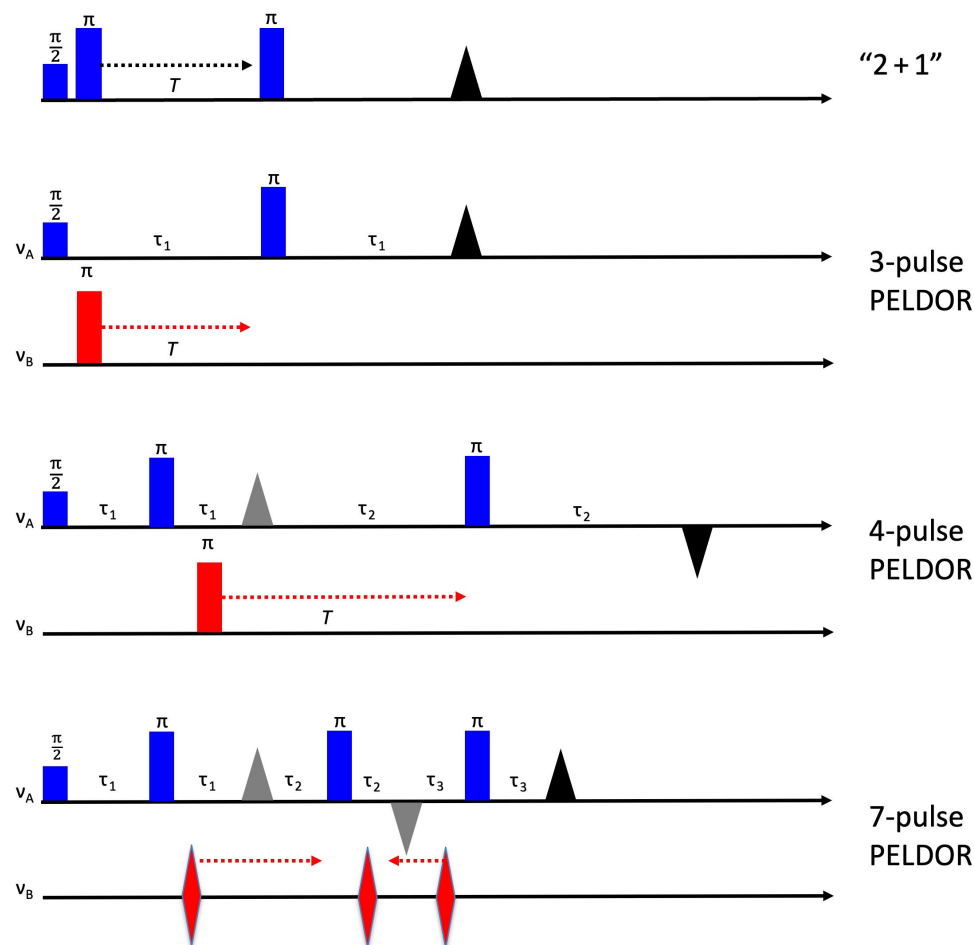


Fig. 5. Pulse sequences for double-frequency PDS experiments. MW pulses are shown as blue or red rectangles, where the two different colors indicate the two different frequencies. The "2 + 1" experiment is a single-frequency experiment but shown here in this figure for historic reasons. The shaped pulses in the seven-pulse PELDOR are depicted as rhombuses. Recorded echoes are colored black. Nonrecorded echoes are shown in some cases to visualize timings and are colored gray. Pulses/timings that are incremented in the sequences are indicated by dotted arrows.

coherence (DQC) experiment is advantageous (80, 81). Nonetheless, DQC has also been successfully applied to systems with two Cu^{2+} ions or two nitroxides, but with lower SNR (82, 83). DQC is a single-MW frequency experiment, and the subsequence $\pi/2-\pi-\pi/2$, together with a 64-step phase cycle, isolates the DQC from the other coherence pathways, yielding a time trace that is modulated by the dipolar coupling frequency. The background function plays a minor role in this experiment, because the modulation depth is 90% or more. DQC experiments in combination with two trityl spin labels yield very good SNRs, enabling measurements at 45 nM (81).

Another less-used experiment is the single-frequency technique for refocusing dipolar couplings (SIFTER), which is based on the solid echo (84). Artifacts and a more pronounced and nonexponential background function hampered its application. Nevertheless, a broad-band pulse SIFTER has been shown to be very effective (85).

Light-induced experiments

Light-induced PDS (Fig. 7) is a subset of PDS methods for systems with photo-excited triplet chromophores ($s = 1$) (86). In the case of one transient triplet spin center and one permanent radical ($s = 1/2$), pulse sequences with one laser pulse, like light-induced DEER or laser-induced magnetic dipole spectroscopy/refocused laser-induced

magnetic dipole spectroscopy, can be used, whereas light-induced triplet-triplet electron resonance spectroscopy, using two laser pulses of different wavelengths, has recently been published for two triplet chromophores (87). These methods can become particularly important if light-excitable cofactors and their involvement in the structure, dynamics, and function of biomolecules are to be studied.

Methods for distance distribution analysis

The conversion of the time traces into distance distributions can be done based on Eq. 9 with several freely available programs, such as DeerAnalysis, DeerNet, Comparative DeerAnalyzer, DeerLab, Long-Distances, DD, and PDSFit. A comparative summary of the first six programs has been published by Russell *et al.* (88). DeerAnalysis uses Tikhonov regularization to generate the distance distribution and is historically the most widely used program. It provides the user with the option to set variables or to run it in automation mode. DeerNet is based on a neural network, and the user has no variables to set. DeerLab performs background fitting and regularization in a single step and has additional features. Both approaches, a standardized DeerLab workflow and DeerNet, are combined in Comparative DeerAnalyzer, which provides the average of the results from DeerLab and DeerNet. All four programs provide confidence intervals

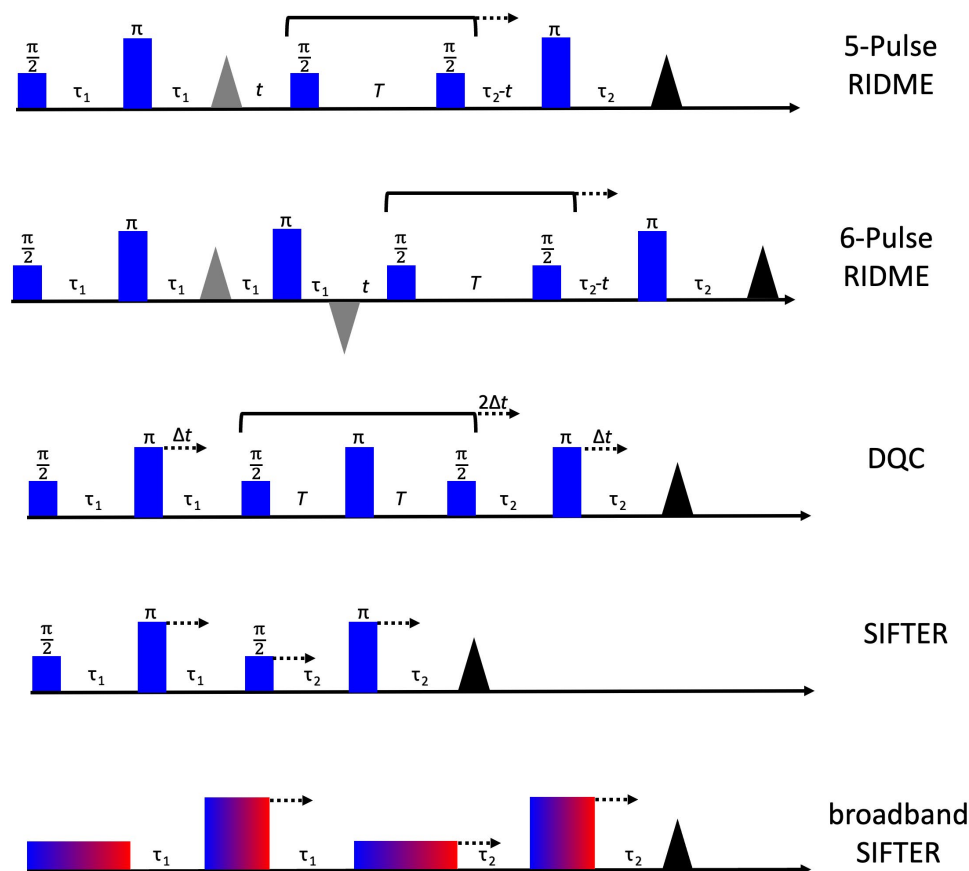


Fig. 6. Pulse sequences for single-frequency PDS experiments. Microwave pulses are shown as blue rectangles. Recorded echoes are colored black. Nonrecorded echoes are shown in some cases to visualize timings and are colored gray. Pulses/timings that are incremented in the sequences are indicated by dotted arrows. The chirped pulses in the broadband SIFTER are indicated by a color gradient from blue to red.

and a scale for the reliability of distances. All these programs are model-free.

In the program LongDistances, background fitting and distance distribution generation (model-free via Tikhonov regularization or model-based with a sum of Gaussian distributions) are done in two steps. DD uses a multi-Gaussian model. Both programs provide uncertainty analyses. PDSFit (89) is based on the input of a geometric model and is useful if spin centers with g values different from g_e , large g tensor anisotropies, orientation selection, or exchange coupling are present. The program can fit PELDOR and RIDME time traces and provides errors for distances and angles.

Which program to use cannot be answered in a generalized way because a comparative test of the programs is still pending. However, while results are very similar for high-quality data, the community white paper recommends a one-step analysis, i.e., fitting background and intramolecular contribution (the form factor) simultaneously, and cautions about confirmation bias with approaches that give too many options to the user (69).

Computational methods for deriving structural information from distance distributions

Modeling structure from distance distributions between spin labels requires predictions of the conformation distribution of the spin-label side chain in a given structural context. The most popular software packages for this purpose, MMM (90), mtsslWizard (91), and

chiLife (92), all provide similar prediction quality. These packages allow simulating label-to-label distance distributions for a given structure or ensemble of structures by generating spin-label side-chain rotamer ensembles using fast approaches such as accessible-volume exploration or precomputed rotamer libraries. Thus, these packages can be used directly for assessing whether crystal structures, cryo-electron microscopy structures, or predictions by AlphaFold and similar packages are consistent with experimental distance distributions.

Whereas the recent advances in cryo-electron microscopy and AlphaFold have revolutionized structure determination for intrinsically folded regions of proteins, characterization of the ubiquitous flexible and dynamic regions very much profits from EPR techniques (93). Compared to the mean values of geometric parameters that many other techniques in structural biology yield, distance distributions directly inform on the width of a conformation ensemble (Fig. 8). Because intrinsic disorder is ubiquitous in proteomes (94), this may be a necessity for generating realistic models of proteins and their complexes in solution. In cases where a protein can adopt several distinct conformation states, distance distributions reveal how the distribution between these states shifts in the presence of ligands or other stimuli (95). Models for such shifts can be derived with elastic network models (95), by random walk in backbone conformation space (96), by biasing AlphaFold predictions with distance distributions (97), or by modeling the structure from scratch (55). Distance

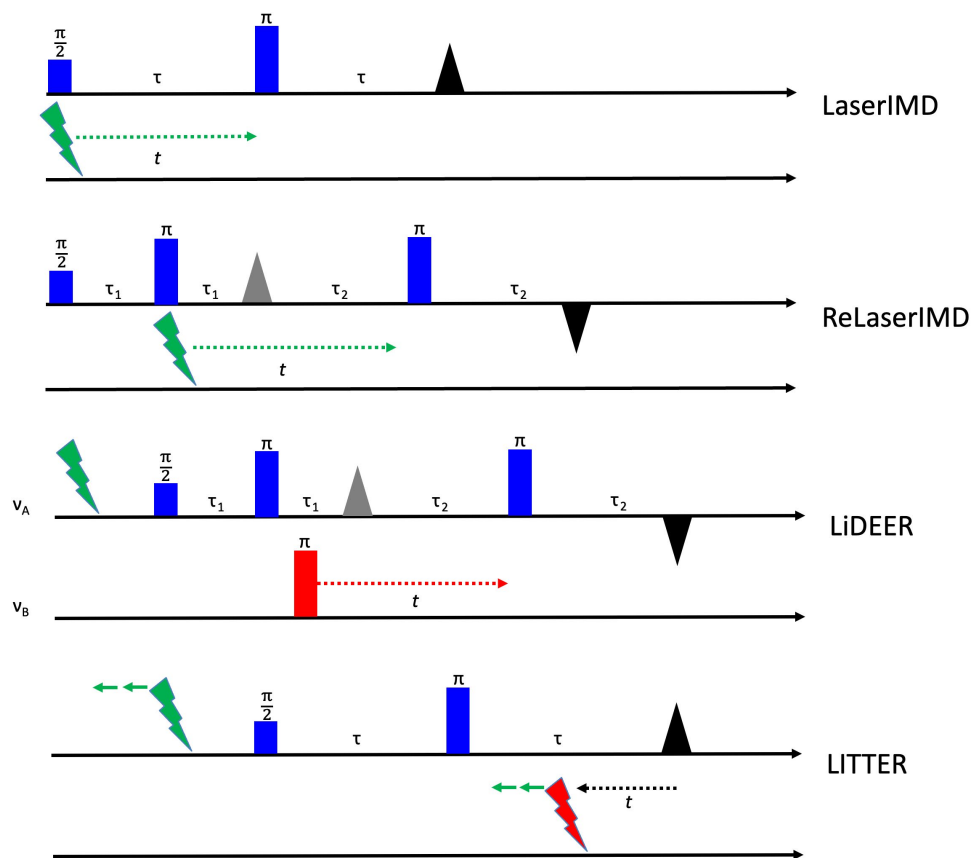


Fig. 7. Pulse sequences for light-induced PDS. Microwave pulses are depicted as blue rectangles. Laser pulses are depicted as green flashes. If microwave or laser pulses of different frequencies are used, the second frequency is colored red. Recorded echoes are colored black. Nonrecorded echoes are shown in some cases to visualize timings and are colored gray. Pulses/timings that are incremented in the sequences are indicated by dotted arrows.

distributions can also be used for validating or restraining MD simulations of proteins (98, 99).

HYPERFINE SPECTROSCOPY

Hyperfine interactions between electron and nuclear spins are a rich source of electronic and structural information in paramagnetic systems. They underpin studies ranging from metalloproteins and heterogeneous catalysts to materials and QIP. Strong hyperfine couplings, typical of nuclei with large electron spin density, are often directly resolved even in CW EPR spectra of powders or frozen solutions and can be quantified through spectral simulation. Examples include paramagnetic metal ions with nonzero nuclear spin (Cu^{2+} , Mn^{2+} , and VO^{2+}) and nitroxide radicals. In contrast, more distant nuclei experience weak hyperfine couplings that are rarely resolved in the CW EPR spectrum; these couplings cause broadening that is easily masked by other inhomogeneous effects. Yet, such weak couplings are ubiquitous and critically informative. These can be determined by hyperfine spectroscopy.

Hyperfine spectroscopic methods access the nuclear transition (NMR) frequencies of nuclei coupled to the electron spin by monitoring their influence on the EPR signal. From these frequencies, one identifies the nucleus via its Larmor frequency (e.g., ^1H and ^{13}C) and extracts hyperfine coupling constants and, for $I > 1/2$, also nuclear quadrupole parameters. The hyperfine interaction has an isotropic

part, which reports the electron spin density at the nucleus (related to the electronic structure), and an anisotropic part that encodes the geometry (see the “Introduction” section for the explicit SH term). For nonconjugated systems with electron-nucleus separations $r \gtrsim 0.3$ nm, the anisotropic interaction is well described by the point-dipole model ($\propto r^{-3}$). The static SH, along with expressions for nuclear transition frequencies, was established in the 1970s (7); comprehensive treatments can be found in (100, 101).

Over the past eight decades, EPR has progressed from CW approaches to pulse methodology, using sophisticated pulse sequences, including shaped and frequency-modulated MW pulses, and has expanded into high magnetic fields. The availability of commercial pulsed spectrometers considerably broadened access and applications. These developments substantially affected hyperfine spectroscopic methods, which fall into three main families, each detecting nuclear frequencies via different physical mechanisms.

Frequency- and time-domain ENDOR

ENDOR transfers polarization between electron and nuclear transitions using combined MW and radiofrequency (RF) irradiation [reviewed in (102)]. Accordingly, the resonator must admit both MW and RF with minimal compromise.

In CW ENDOR the magnetic field is set at an EPR line and saturating continuous MW is applied (103); a swept RF drives nuclear transitions that share levels with the selected EPR transition, partially

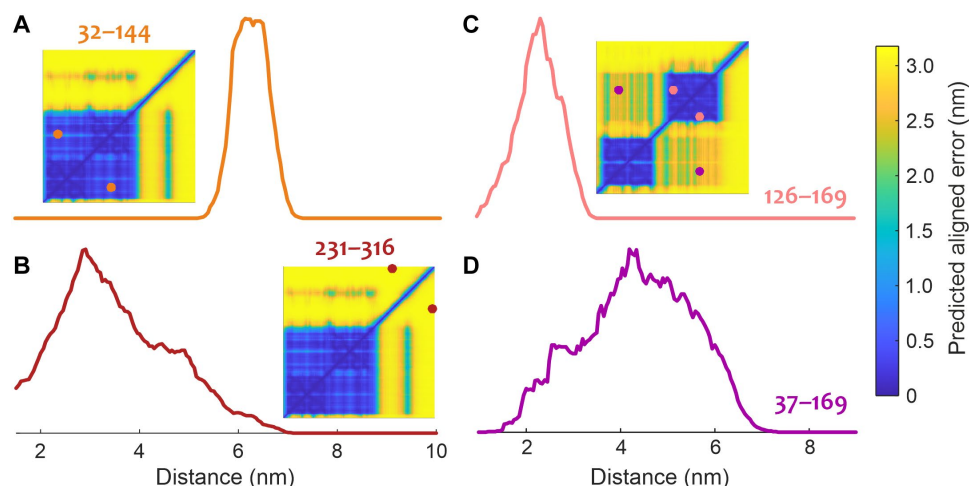


Fig. 8. Distance distributions between spin labels inform on the extent of disorder in proteins. The distributions were back-computed from ensemble models for the RNA-binding proteins hnRNP A1 {Protein ensemble database PED00213 [(A) and (B)]} and SRSF1 {PED00497 [(C) and (D)]}. (A) Label pair in the RNA recognition motif (RRM) of hnRNP A1. (B) Label pair in the C-terminal intrinsically disordered region of hnRNP A1. (C) Label pair in the RRM2 of SRSF1. (D) Label pair that characterizes arrangement disorder between RRM1 and RRM2 of SRSF1. The insets are AlphaFold-predicted aligned error matrices that denote the uncertainty of the position of residue y when the structures are superimposed at residue x . Dots in the insets denote the label pairs.

desaturating it and increasing the EPR signal. CW ENDOR operates in both solids and liquids and was pivotal for mapping spin densities in organic radicals and, thereby, in refining and confirming Hückel molecular orbital theory (104). The CW-ENDOR intensity critically depends on electron and nuclear relaxation rates.

Pulse ENDOR followed CW ENDOR with the Mims (13) and Davies (14) sequences (Fig. 9, A and B). Here, an electron spin echo, generated by MW pulses after manipulating the energy-level populations at a fixed field, is monitored while an RF pulse induces NMR transitions, reducing the echo intensity. In these experiments, the echo is monitored as a function of the RF and, therefore, they are referred to as frequency-domain (FD) ENDOR. Because the experiment relies on long electron spin lattice relaxation times, measurements are typically performed at cryogenic temperatures and are usually unsuitable for liquids. Pulsed ENDOR generally offers higher sensitivity and resolution, cleaner baselines, and fewer artifacts than CW ENDOR.

Davies and Mims ENDOR are complementary. Davies ENDOR excels for large couplings (roughly above 2 MHz); Mims ENDOR is most sensitive to weak couplings and longer distances. A practical limitation is sensitivity (ENDOR signals are often only a few percent of the echo) where, in general, Mims ENDOR offers better sensitivity than Davies ENDOR as the MW pulses do not have to be selective with respect to the hyperfine splitting. A number of FD-ENDOR refinements are as follows: variations of time intervals and repetition rates to determine the sign of the hyperfine couplings (105); echo-train (CP) detection to boost averaging efficiency (106); randomized RF acquisition to suppress RF-heating baselines and nuclear saturation (107); modified Davies ENDOR placing an RF pulse after echo detection to avoid NMR saturation during fast averaging (108); and cross-polarization ENDOR, which enhances transitions and reveals relative signs of couplings while reducing sensitivity to nuclear saturation (109). Pulse shaping further improved performance. Band-selective inversion pulses in Davies ENDOR enable full inversion of spin packets and raise sensitivity (110). For broad

ENDOR spectra where RF selectivity is not limiting, chirped RF excitation yields large sensitivity (111). Recently, chirp pulses were applied to the MW pulses in the Mims ENDOR sequences, allowing for high-resolution correlation between the ENDOR frequencies and the EPR spectrum, namely, providing orientation selective spectra in one ENDOR spectrum (112). This approach was also applied to Davies ENDOR, termed chirp echo EPR spectroscopy (CHEESY) ENDOR (113). Multidimensional ENDOR adds a second axis to disentangle overlap and correlate interactions. In two-dimensional (2D) Mims ENDOR, the $\cos(A\tau/\hbar)$ modulation of the ENDOR intensity is sampled as a function of the evolution time τ and RF frequency; Fourier transformation along τ correlates nuclear frequencies with hyperfine couplings (114). 2D TRIPLE (triple ENDOR) sweeps two RF frequencies to correlate transitions from the same center and m_s manifold; cross-peak shapes reflect the hyperfine tensor principal values and relative orientations (115). The trade-off is a longer acquisition.

Time-domain ENDOR (TD-ENDOR) directly records nuclear coherences. Replacing the conventional RF π pulse with a $\pi/2$ - t_1 - $\pi/2$ block creates a nuclear free induction decay (FID) that is reconverted to an electron echo (Fig. 9C) (116). FT of the FID yields the ENDOR spectrum, typically with higher resolution than FD-ENDOR. Short, broadband RF excitation is technically demanding, but chirped RF pulses (117) or micro-resonators with integrated micro-NMR coils (118) can provide the needed broadband coverage. A dead-time-free (DTF)-TD-ENDOR (Fig. 9D) generates a nuclear echo to eliminate deadtime that distorts the FT spectrum and enables direct observation of nuclear coherences relevant to quantum information (119, 120).

Historically, ENDOR mapped near-neighbor shells ($\lesssim 0.5$ nm) around metal centers and radicals. Recently, ^{19}F Mims ENDOR has been used to measure 0.7- to 2.0-nm distances, complementing PELDOR (121). Introducing a ^{19}F nucleus near a spin label leverages high gyromagnetic ratio (sensitivity) and the paucity of native fluorine (spectral selectivity). For such weak couplings (spectral widths often < 1 MHz), DTF-TD-ENDOR can deliver order-of-magnitude SNR gains at similar resolution and acquisition times

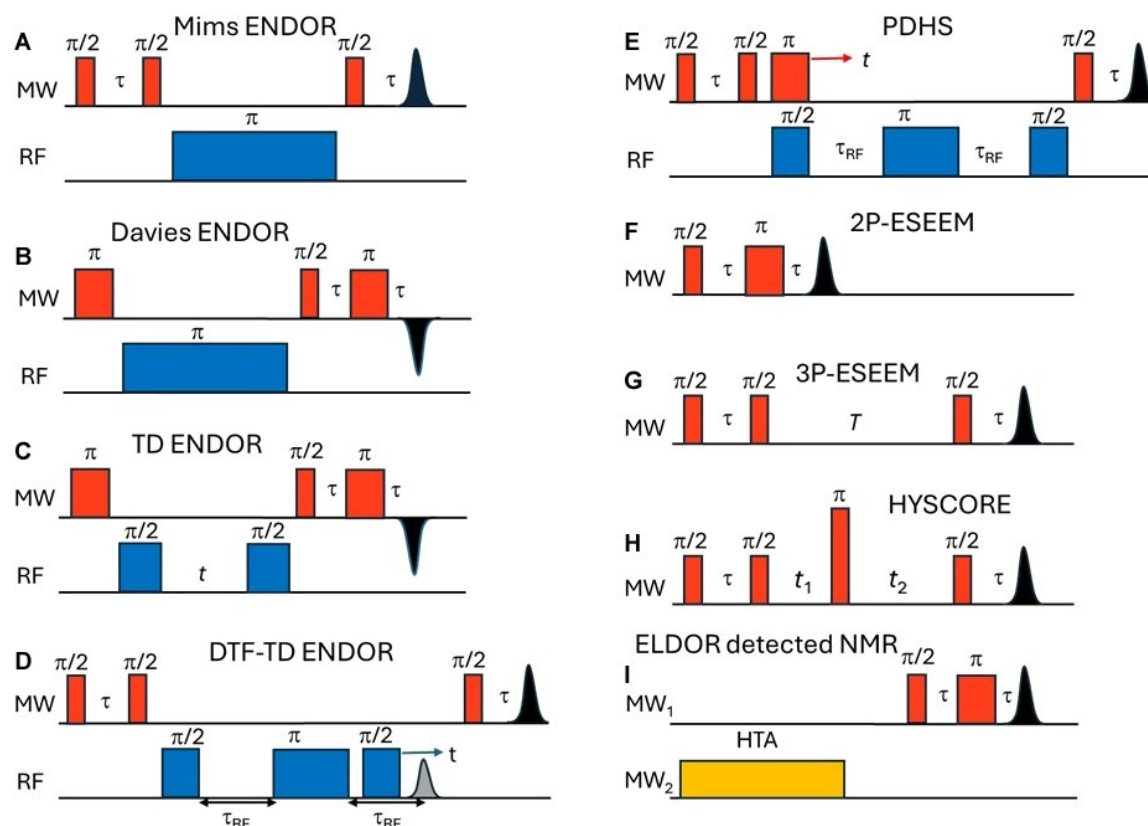


Fig. 9. Hyperfine spectroscopy pulse sequences. (A and B) Mims and Davies ENDOR sequences, respectively. The echo intensity is measured as a function of the RF. (C) TD-ENDOR, echo intensity is measured as a function of t . (D) DTF-TD-ENDOR, echo intensity is measured as a function of t . (E) Pulsed dipolar hyperfine spectroscopy (PDHS), echo intensity is measured as a function of t . (F) 2P-ESEEM, echo intensity is measured as a function of τ . (G) 3P-ESEEM, echo is measured as a function of T . (H) HYSCORE, echo intensity is measured as a function of t_1 and t_2 . (I) ELDOR detected NMR, echo intensity is measured as a function of the frequency difference, $MW_2 - MW_1$, where MW_2 is swept and MW_1 is fixed.

(122). Generating and refocusing nuclear coherences also enables line-narrowing strategies. A key resolution limit in ^{19}F ENDOR is dipolar coupling to surrounding protons. Pulsed dipolar hyperfine spectroscopy (PDHS) (Fig. 9E) uses a ^{19}F nuclear echo to refocus nuclear dipolar interactions and reintroduces the electron-nuclear dipolar evolution with an added MW π pulse, substantially enhancing resolution (Fig. 10A) (120).

High-field ENDOR (Q-, W-, and higher bands) offers major benefits: larger separation of nuclear Larmor frequencies (e.g., distinguishing ^1H and ^{19}F , or strongly coupled ^{14}N from weakly coupled ^1H), simpler spectra via reduced second-order hyperfine effects, and clearer assignments (122–124). Commercial 263-GHz EPR now makes such experiments accessible (125).

Electron-spin echo envelope modulation

Electron-spin echo envelope modulation (ESEEM) detects nuclear frequencies from modulations in the electron spin echo decay (126). The effect was first observed in two-pulse (2P) echo decays (127) and later extended to three-pulse (3P)-ESEEM (128). In 2P-ESEEM, the echo intensity is recorded versus the interpulse delay τ , and the echo decay is determined by the phase memory time, T_m ; in 3P-ESEEM, it is measured versus the evolution time T , during which the spin lattice relaxation dominates (Fig. 9, F and G). Accordingly, the latter provides higher resolution. FT yields nuclear frequencies

and their combinations, depending on the pulse sequence and the number of nuclei present. The modulations arise from pseudo-secular Hamiltonian terms that enhance “forbidden” transitions with simultaneous Δm_s and Δm_I changes; the modulation depth, and thus spectral intensity, is maximal when the nuclear Larmor frequency approaches the hyperfine coupling (13). Accordingly, modulation depth can be optimized by multifrequency operation (129). Because most hyperfine couplings that are unresolved in the EPR spectrum are small, in the range of a few megahertz, ESEEM is typically run at X-band (or even lower, C-band if available) where nuclear Larmor frequencies are close to this range. Nevertheless, measurements at W-band are valuable when couplings are large, i.e., tens of megahertz (130). ESEEM needs only one MW frequency and is easier to implement than ENDOR, but line shapes are often more complex, and analysis is less straightforward.

A breakthrough was the introduction of 2D HYperfine Sublevel CORrelation (HYSCORE) (Fig. 9H) (131). Adding a second spectral dimension separates overlapping features, correlates nuclear frequencies from different m_s manifolds as cross-peaks, and helps assign different nuclei (^1H , ^{14}N , ^{15}N , ^{13}C , etc.; Fig. 10B). Cross-peak shapes and orientations report anisotropic hyperfine tensors even in disordered systems; nuclei with $I > 1/2$ also reveal quadrupole interactions. HYSCORE has largely superseded 1D ESEEM.

The sensitivity and robustness of HYSCORE have been improved via matched-pulse variants (matched HYSCORE, single matched

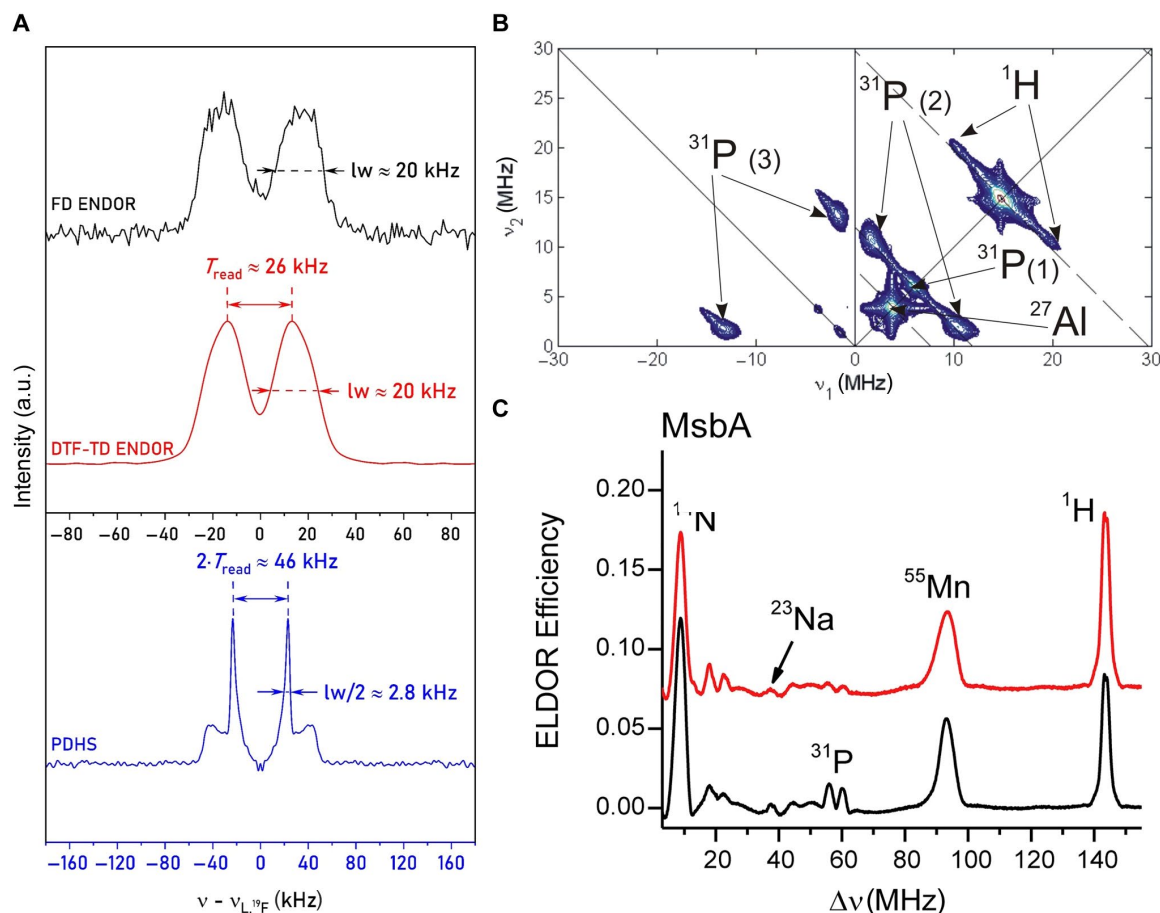


Fig. 10. Example of spectra acquired with various hyperfine spectroscopy techniques. (A) Sum spectra of three field positions acquired with FD ENDOR, FT of DTF-TD-ENDOR, and PDHS summed time traces. The sample is a model compound with a nitroxide- ^{19}F distance of 1.48 nm. Reproduced from (120) with permission. (B) HYSOCORE spectra of an oxidized VTiAlPO-5 sample. The spectrum contains signals stemming from the hyperfine interaction between the unpaired electron hosted in the V orbitals and nearby ^{31}P , ^{27}Al , and ^1H nuclei. In particular, two sets of cross-peaks [^{31}P (1) and ^{31}P (2)] appear in the (+, +) quadrant approximately centered at the ^{31}P nuclear Larmor frequency [$\nu(^{31}\text{P}) = 5.993$ MHz], while a third set of cross-peaks [^{31}P (3)] appears in the (−, +) quadrant separated by $2\nu(^{31}\text{P})$ and positioned at approximately (−13.8, +1.8) (−1.8, +13.8) MHz. Reproduced from (275) with permission. (C) ELDOR-detected NMR spectrum of the ABC (ATP binding cassette) transporter MsbA, without and with Mn^{2+} /AMPPNP [adenosine 5'-(β , γ -imido)triphosphate]. The peaks are assigned to nuclei identified on the panel. Reproduced from (276) with permission.

resonance transfer–HYSOCORE) that use high-turning-angle pulses (132, 133), five- and six-pulse sequences that alleviate suppression of weakly modulating nuclei in the presence of strong ones (134), and double nuclear coherence transfer–HYSOCORE, which correlates nuclear frequencies within the same m_s manifold to aid assignment (135). Frequency-swept (chirped) ultra-wideband (UWB) pulses extend ESEEM/HYSOCORE into regimes of larger hyperfine couplings (136) and enable 2D EPR/ESEEM and even 3D EPR/HYSOCORE correlations analogous to multidimensional NMR, where the full EPR spectrum comprises one of the dimensions. UWB operation requires broader resonator bandwidths (lower Q), which leads to lower sensitivity that can be offset by increased filling factors.

ELDOR-detected NMR

Electron-electron double resonance (ELDOR)-detected NMR (EDNMR) (137, 138) uses two MW frequencies and high-turning-angle (HTA) pulses to excite formally forbidden electron-nuclear transitions. The electron spin echo is recorded as the frequency of the HTA pulse is swept (Fig. 9I); the spectrum presents the nuclear transition

frequencies (Fig. 10C). Implementation is straightforward but requires a resonator with a wide bandwidth to scan broadly. EDNMR often offers high SNR, albeit with lower spectral resolution than ENDOR or ESEEM. Powder-pattern line shapes are less directly observed because signals arise from forbidden transitions, though they can be interpreted. A 2D EDNMR correlates nuclear frequencies from different m_s manifolds (139).

EDNMR particularly benefits from high fields, where nuclear Larmor frequencies are well separated; numerous W-band applications exist, and the method performs well at Q-band as well. The complementarity of EDNMR with ENDOR/ESEEM has spurred hybrid, multidimensional experiments: Hyperfine sublevel correlation ENDOR (HYEND) correlates ENDOR frequencies with hyperfine couplings (140), and triple resonance hyperfine sublevel correlation (THYCOS) experiment for assignment of electron-nuclear double resonance lines merges ENDOR and EDNMR concepts akin to 2D TRIPLE (141).

At low temperatures, where most of the experiments described in this section are conducted, anisotropic hyperfine interactions

dominate. These interactions not only carry structural information of interest but also add substantial complexity to the spectra. Under these conditions, the bandwidth of the applied MW pulses is much narrower than the width of the EPR powder pattern. As a result, only a limited subset of molecular orientations relative to the magnetic field contributes to the signal, a phenomenon known as orientation selection. To fully characterize the anisotropic interaction, measurements must be performed at several magnetic field positions across the EPR spectrum. Analysis of these orientation-selected spectra reveals the orientation of the hyperfine tensor relative to the g tensor (or another reference interaction) of the paramagnetic center, which defines the spectral anisotropy. When the orientation of the g tensor in the molecule is known, this information provides valuable structural insight (142, 143). Rigorous extraction of full hyperfine tensors typically requires numerical simulations.

SPECIAL METHODS

Electrical detection

While conventional EPR measures MW absorption or emission, electrically detected magnetic resonance (EDMR) instead monitors spin-dependent changes in a sample's conductivity and thus resistivity. This technique exploits the fact that spin polarization can influence charge transport and recombination processes in semiconductors, molecular conductors, or other materials containing paramagnetic centers (144).

In EDMR, the sample is placed in a resonant MW cavity or near a microstrip resonator within a static magnetic field. When the spins are driven into resonance, the EPR transitions alter the spin states, and if the spins participate in recombination, transport, or trapping/detrapping, this modifies the device's electrical response. The resulting variation in current or voltage is recorded as the EPR signal. EDMR spectra are determined not only by the SH (e.g., spin-coupling parameters) and the dynamics (e.g., spin-relaxation times) of the observed paramagnetic centers, like in EPR, but also by the dynamics of the electronic transitions. This large number of parameters complicates the interpretation of EDMR signals.

A major advantage of EDMR is its extraordinary sensitivity, often several orders of magnitude higher than conventional EPR, making it invaluable for studying thin films, interfaces, and nanoscale devices. EDMR was first performed in CW mode (1965) (145) and later on also for W-band (146). Pulse EDMR, introduced in 2002 (147), gave access to a broad series of quantitative parameters and timescales that define the nature of spin-dependent electronic transitions. Many pulse sequences known from pulse EPR have analogs in pulse EDMR, such as rotary echoes and Hahn echo sequences, echo decay measurements, inversion recovery, ELDOR, ENDOR, ESEEM, and the use of adiabatic pulses. Recently, EDMR-on-a-Chip has been reported, and this new generation of small, versatile spectrometers enables in situ and operando EDMR experiments (148).

Ultrahigh-sensitivity EPR at millikelvin temperatures

Most EPR experiments are performed under conditions where the high-temperature approximation is valid, electron spin polarization is thus much smaller than unity, and where the MW field can be described classically. In the past decade, EPR experiments were established in the quantum limit in the millikelvin temperature range. The first approach combined a Josephson parametric MW amplifier with a high-quality factor superconducting microresonator (149)

and improved the sensitivity of inductive detection to about 10^3 spins. This enabled observation of cavity-enhanced relaxation by the Purcell effect and of the influence of quantum fluctuations on spin dynamics (150). By extending such experiments to pulse EPR, the longitudinal relaxation time in this Purcell regime was found to depend on Rabi frequency, resonator detuning, and echo amplitude (151).

Detection by an MW photon counter provided another step change in sensitivity (152). This experiment enabled the detection of single spins of Er^{3+} ions in a Scheelite (CaWO_4) crystal. Such fluorescence detection of electron spins also relies on the Purcell effect that enhances the otherwise very low rate of spontaneous emission by excited electron spins.

SIMULATION

The CW-EPR spectrum of a paramagnetic spin center directly depends on the chemical structure of the center. Oxidation state, spin and charge delocalization, nuclear geometry, exchange interactions, and SOC all codetermine the positions and intensities of the spectral features (153). Therefore, an EPR spectrum reveals rich information about the electronic and nuclear structure of a paramagnetic center. To extract this information, one proceeds in two steps (Fig. 1). First, a simple model based on an SH is used as an intermediate. The SH (Eq. 1) is an effective Hamiltonian that is chosen such that its parameters, plus some line broadening information, are the minimal information needed to reproduce the EPR spectrum. The actual parameter values are obtained by least-squares fitting a simulated spectrum to the experimental one. This analysis can range anywhere from trivial (when parameters can be directly read off from the positions of spectral features) to challenging (when it is not clear which SH model is applicable, due to either a lack or an overabundance of features or nonexistent literature precedent). The fitted SH parameters represent a compact quantitative summary of the experimental spectrum. In a second step, the SH parameters are interpreted in terms of the underlying electronic and nuclear structure of the center.

The methodology for the simulation and fitting of EPR spectra is well developed (154). Algorithms depend on the dynamical regime of the sample (Fig. 2). If the paramagnetic center is tumbling on a timescale that is fast relative to the anisotropies of the spectral line positions, the anisotropies are averaged out. In this case, a simple SH model with isotropic interactions is applicable and can often be solved analytically or using perturbation theory. In the rigid limit, where the rotational tumbling rate is zero or at least much slower than any interaction anisotropy, these anisotropies need to be fully considered. In this case, approaches based on the diagonalization of the SH matrix (155) or anisotropic perturbation theory are used (156). In the regime between the isotropic and the rigid limit, rotational tumbling complicates the situation, and a Liouville-space approach using a combined spin-rotation basis is required (157). In this slow-motion regime, the rotational dynamics are generally modeled as Brownian, unless there are specific structural reasons to expect other processes such as multisite jumps. Over the years, many software packages have been developed and used for individual regimes or specific paramagnetic centers. In the last two decades, the software package EasySpin (154) has emerged as a tool that covers all regimes and a wide range of spin systems, including spin-polarized systems (158).

The rigorous simulation and analysis of pulse EPR spectra generally requires explicit modeling of quantum dynamics, i.e., a computational approach where the density matrix describing an initial state is

explicitly propagated through the pulse sequence, and appropriate averages over inhomogeneities (orientation, MW field, etc.) are taken. For sequences with many pulses and variable evolution periods, as well as for shaped pulses, this can be very resource intensive. Explicit propagation engines are implemented in EasySpin (154, 159) and in Spinach (160). The latter software package is a very general tool aimed at simulating pulse experiments across all subdisciplines of magnetic resonance. Recent non-EPR packages for quantum dynamics, such as QuTiP (161), provide general tools for quantum dynamics of coupled qubits.

Fitted SH parameters are often useful by themselves, as they allow comparison with literature. Also, they can serve as fingerprint indicators of structural or electronic change around a center. However, learning about the underlying electronic and nuclear structure is often the goal. The approach for this depends on the nature of the paramagnetic center. For transition metals, particularly d^1 and d^9 systems, simple analytical expressions connect g and A values to ligand field parameters (SOC constants and orbital splittings). For more complicated cases such as orbitally degenerate ground states, a calculation in the basis of the valence microstates can be successful at predicting SH parameters from electronic structure parameters. For delocalized systems such as organic radicals, and for centers with complicated energy level diagrams, an all-electron electronic structure calculation is required. This can be done with the quantum chemistry software ORCA, which is specifically geared toward spectroscopy (162). The calculation of g tensors, hyperfine tensors, and ZFSs can be performed at various levels of electronic-structure theory (SCF, CASSCF/NEV-PT2, and MP2). The accuracy of these methods is variable and strongly depends on the nature of the paramagnetic center. While $s = 1/2$ systems can be modeled reasonably well, methods can be highly inaccurate for high-spin systems. For modeling rotational dynamics in the slow-motion regime, MD has been used and can be directly combined with spectral simulation (163).

In the last decades, rapid progress has been made in the first-principles computational prediction of spin relaxation. Methods have been particularly successful for predicting decoherence dynamics of $s = 1/2$ systems in the low-temperature regime, where all motion and longitudinal spin relaxation are frozen out and dynamics is purely confined to spin space. In this regime, decoherence dynamics is determined by electron and nuclear spins (the “spin bath”) surrounding the paramagnetic center. A correct description requires the explicit time evolution of the large system of coupled spins, which is intractable. Instead, cluster correlation expansion (CCE) is used to approximate these dynamics. CCE is an approximate perturbative many-body approach that simulates the quantum dynamics of a specific spin (the central spin) under the influence of surrounding spins (164). In the absence of methyl groups, such simulations are in near-quantitative agreement with experiment (165, 166). Mechanistically, electron spin relaxation can be understood as an ESEEM effect between the electron spin and pairs of matrix nuclei (164). For protons, the main contributions arise from pairs at 0.5 to 1 nm from the electron spin (166, 167). The ESEEM results in an echo decay rather than an oscillation due to the presence of a large number of proton pairs with a broad distribution of interactions. The effect of methyl protons can be understood by CCE computations as well. It is a consequence of proton tunneling, with predictions being in reasonable agreement with experiment for both low and high tunnel barriers (168–170). The CCE approach works well as long as the intrabath spin-spin couplings are weak and the timescale of interest is

short. For strongly coupled spin networks, no efficient algorithms are currently available.

There has also been recent progress in predicting longitudinal relaxation dynamics (171–173). Electron spins relax due to coupling to local vibrations and to phonon modes in the surroundings. Density in the phonon spectrum at the spin resonance frequency drives electron spin relaxation.

Overall, EPR simulation methodology is mature in many respects. Current work focuses on modeling relaxation dynamics and on improving electronic structure methods for SH parameter prediction. Large spin systems continue to pose substantial challenges for software and hardware due to the multiplicative scaling of the spin state space with the number of spins.

INSTRUMENTATION

General EPR spectrometer design

An EPR spectrometer can be divided mainly into three parts: an MW transmitter, an MW resonator containing the sample and situated inside a magnet, and an MW receiver (Fig. 11). The MW is generated in the transmitter and coupled to the resonator by waveguides or coaxial cables. Typically, the experiments are performed in reflection mode, meaning that the MW signal reflected from the resonator travels back over the same transmission line to the receiver. The separation between excitation and detection is performed by the MW circulator, placed between these three subunits (or, at high frequencies, by a Faraday rotator and a linear polarizer extracting the linear polarized component of the circular polarized reflected signal, orthogonal to the linear polarized excitation wave). The MW resonator is central to the performance of EPR spectrometers. It defines the usable sample size (filling factor), the homogeneity of the MW field strength over the sample volume, and the quality factor Q (related to signal intensity and excitation bandwidth). Especially for pulse EPR applications, where a large MW field strength and short pulse lengths are required, there is a trade-off related to the quality factor Q : The MW field strength increases with the square root of Q , whereas short pulses require a large resonator bandwidth that increases proportional to the inverse of Q . In addition, resonators might be designed to allow additional sample manipulations in situ (as photoexcitation,

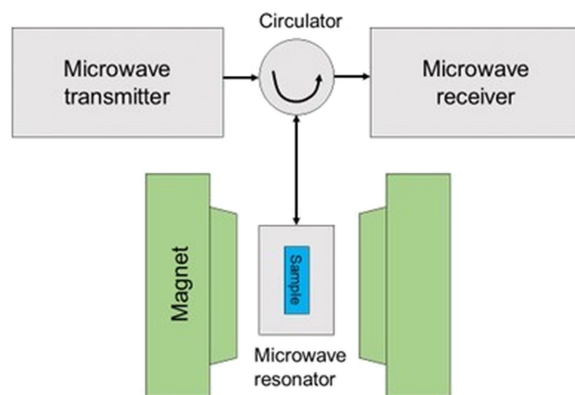


Fig. 11. Block diagram of an EPR spectrometer. It consists of an MW generating (and pulse forming for pulse EPR) transmitter, a resonator, and an MW receiver for signal detection. The sample is placed inside the resonator, mounted in the gap of a magnet. A circulator routes the MW from the transmitter to the resonator and from the resonator to the receiver.

electrochemical manipulation, and RF excitation for ENDOR or double MW excitation for PELDOR, and ultra-broadband resonators for chirp pulses). Some examples will be given in the “MW resonators” section.

Differences between CW EPR and pulse EPR setups

In CW-EPR experiments, the observed signal is based on the absorption of MW by the paramagnetic sample if the magnetic field matches the energy level splitting of the spin eigenstates as described by the SH in Eq. 1. The MW absorption leads to a mismatching of the resonator, tuned to the excitation frequency and to a very sensitive detection of the absorption signal (174). In contrast, in pulse EPR, the transient MW signal emitted by the sample after strong and short (in the nanosecond range) MW pulses is detected (38). To achieve strong enough MW fields that generate short and efficient excitation pulses, the MW output of the transmitter is further amplified by a high-power amplifier (up to kilowatts). As described above, the resonator further amplifies the MW excitation field strength at the sample. For pulse EPR applications, the MW resonator must have a larger bandwidth (lower quality factor Q), allowing the short pulses and the transient signal to be transmitted with minimal deadtime. In the receiver part of the pulse spectrometer, a protection switch is typically used to protect the sensitive low-noise detection amplifier from the reflected strong MW excitation power. Thus, signals can only be observed after a so-called “deadtime” (of typically 50 to 100 ns) after the MW pulses.

Historical pulse EPR applications

Pulse EPR spectrometers appeared shortly after the first pulse NMR experiments were performed (11). After that, several laboratories in the United States, Russia, The Netherlands, and Germany built their own pulse EPR setups, mostly in physics and physical chemistry departments. Most applications at that time were on developing pulse sequences for the determination of hyperfine couplings: ESEEM (127, 175), pulsed Davis (14) and Mims (13) ENDOR, and 2D HYSCORE (139), the measurement of transversal and longitudinal relaxation times of paramagnetic samples, and the determination of spin dynamics of transient paramagnetic states created after photoexcitation. Most of this work was done on solid or powder samples and at low temperatures. From the end of the 1980s, a commercial pulse EPR setup at X-band frequencies became available, which boosted the field of applications and enabled fast signal averaging and pulse setting changes (176). Only at this point did the sensitivity of pulse EPR start to reach the sensitivity of CW-EPR experiments. Unlike the field of liquid-state NMR, pulse EPR did not surpass the sensitivity of the CW-EPR method. The reason is that the MW power is not large enough to excite the full EPR spectrum. Therefore, the multiplexing advantage observed in pulse NMR is not obtainable (177, 178).

An important early research field using X-band pulse EPR became the study of (transient) paramagnetic states in photosynthesis (36). Here, the investigation of the spin dynamics of the coupled correlated radical pair after photoexcitation by the dipolar EPR methods discussed above became a prominent target for several research groups worldwide. In the beginning, most pulse EPR setups worked at X-band frequencies. More recently, the most prominent pulse EPR frequency became 35 GHz (Q-band), owing to the increased availability and performance of MW components at this frequency, and the increased sensitivity at higher MW frequencies, owing to the Boltzmann population and the induction signal detection (179).

Shaped MW pulses

As already mentioned in the “CW EPR” section, monochromatic MW pulses with a length of 10 ns only cover a spectral width of about 30 MHz. Most radicals have larger spectral widths even at low MW frequencies (X-band). Therefore, monochromatic MW pulses cannot excite the whole EPR spectra. Frequency-swept chirp pulses can overcome this limitation if the transverse relaxation time of the radical is long enough (in the microsecond range). With the availability of fast arbitrary waveform generators (AWGs), it has become possible to routinely shape MW pulses in phase and amplitude on a sub-nanosecond timescale. Whereas the first principles of this were already realized by a home-built pulse shaper at the ETH Zürich in 1986 (180), the first application with a commercially available and more generally usable AWG was performed in 2012 at X-band frequencies using optimum control theory-generated pulses for FT-EPR (181). Soon, the possibility of broadband chirp MW pulses (182) for EPR experiments was explored, and several research groups developed broadband MW pulse experiments for the characterization of hyperfine and dipolar couplings in EPR (183). The implementation of an AWG into commercial X- and Q-band spectrometers allowed more researchers to use such pulses. The development of shaped pulses in EPR follows, with a time lag of about 20 years, the developments in NMR, a time delay similar to the commercial development of pulse EPR. In the first case, this relates to the availability of AWGs with sub-nanosecond clock rate, and in the second case, this relates to the availability of fast, versatile pulse programmers and transient recorders with the possibility of fast data averaging and transfer. This time lag is roughly related to the difference in Larmor frequencies between nuclear and electron spins (roughly a factor of 1000) and Moore’s law, which predicts a factor-of-2 improvement in semiconductor technology every 2 years.

Miniaturized EPR setups

Related to the same law, which also holds for the miniaturization of semiconductor technology, an integration of EPR excitation (transmitter) and detection (receiver) onto semiconductor chips started (184, 185). Miniaturized EPR spectrometers [including also dynamic nuclear polarization (DNP) and NMR applications] have been realized on a single semiconductor chip with one or several coupled electrical circuits in parallel. In some cases, the sample and the resonant structure are integrated into the oscillator circuit, enabling a new and highly sensitive detection in CW-EPR and pulse-EPR mode. Such devices might in the future allow the use of EPR for routine applications in health or industrial settings, with EPR spectroscopy integrated into a consumer device or for highly sensitive detection of very small sample sizes (as single biological cells).

MW resonators

As explained above, the MW resonator is a crucial part for the performance of an EPR spectrometer. Therefore, different MW resonators have been developed for specific EPR setups and applications. For pulse EPR applications, where high MW field strengths are needed, loop-gap resonators (186) and dielectric resonators (187) have been implemented. For high-field EPR, a Fabry-Pérot resonator (188) or a bucket sample holder (189) has been introduced. In both cases, oversized structures much larger than the MW wavelength allow for extending the sample size compared to downscaled fundamental-mode resonators used at lower MW frequencies. For high-field DNP on liquid samples at room temperature, double-resonant structures have

been developed to avoid sample heating by the MW. They consist of a semi-confocal Fabry-Pérot resonator for the MW (EPR), where the flat mirror is at the same time a stripline resonance structure for the nuclear spin (NMR) (190). Also, an oversized photonic bandgap resonator for the MW has been used for such purposes (191). Resonant microscale loop-gap resonators have been used for EPR spectroscopy (192) on small and flat samples and for microscale imaging applications (193). An example of a self-resonant helical structure at X-band frequencies for nanoliter samples or small single crystals is shown in Fig. 12 (194). An overview of resonant structures for EPR on nanoscale samples, such as microfluidic devices, is given in (195). Another possibility is to couple the sample directly to the MW oscillator on a semiconductor chip (196).

EPR setups at high MW frequencies

EPR spectrometers working in the sub-terahertz frequency range (100 GHz up to 1 THz) were also developed. For such spectrometers, the design changes drastically; not only by using superconducting magnets with appropriate sweep capability, but also by using the rather different MW components and their transmission in the sub-terahertz frequency (far infrared) range. This work started with CW-EPR setups (197–200). Soon, pulse EPR setups (201) and pulse EPR/ENDOR setups (123, 202, 203) at 95 GHz (W-band) were implemented. This led to the development of a commercial pulse EPR/ENDOR setup at W-band frequencies (204). The main advantage of high-field EPR spectrometers is typically a higher absolute sensitivity but not a higher concentration sensitivity (because the sample size is scaled down at higher frequencies, proportional to smaller MW wavelengths). In addition, the increased g tensor resolution at higher magnetic fields allows distinguishing different radical species, as described in the “CW EPR” section. It also offers better separation of different types of nuclei in ENDOR.

The development of high-field EPR was further advanced by the implementation of quasi-optical MW devices and propagation

(205). High-field EPR spectrometers were extended to frequencies up to 260 GHz (J-band) commercially and to even higher frequencies at high magnetic field research support centers (206, 207). Also, methods based on broadband frequency sweeps were developed (208). This was possible because the performance (availability, reliability, and maximum output power) of solid-state MW amplifiers at such high frequencies increased considerably within the last 20 years. Still, there is a lack of high MW power at such high frequencies, especially important for pulse EPR applications. FT detection with high power synchrotron radiation was developed at BESSY in Berlin (209) and with a free electron laser in Santa Barbara (210).

In recent years, the strong interest in DNP performed at high magnetic fields (211) increased the interaction between the EPR and NMR communities and led to the development of combined high-field EPR and DNP spectrometers in several laboratories (212–217). Not only are the technical requirements of high-power MW tube devices (such as gyrotrons, gyro-amplifiers, klystrons, and backward-wave oscillators) for DNP and the quasi-optical transmission of the MW to the sample of common interest to scientists of both research fields, but the EPR properties of the used DNP agents at high magnetic fields (EPR lineshape and relaxation times at different sample temperatures) are also important for the quantitative understanding and further optimization of the performance of high-field DNP (218, 219).

Specific EPR setups

The special EPR methods mentioned in the “Special Methods” section also have specific instrumentation requirements. This holds for electrical detection on surfaces as well as the ultrasensitive detection at very low temperatures using parametric Josephson-junction quantum amplifiers. The references given in the “Special Methods” section also relate to the specific instrumentation needed for these experiments.

Other methodology developments in the direction of ultrahigh-sensitive detection of EPR (down to a single spin) are coupled to noninductive detection schemes, for example, optical detection of photoactive EPR probes (as in NV centers) (220), magnetic force detection (221), tunnel current detection (222, 223), or electrical detection of EPR of two coupled quantum dots (224). As already mentioned in the “Introduction” section, single-spin detection in EPR has already developed rather independently from the field of classical EPR spectroscopy and is not covered in this review article. The same is true for the field of EPR imaging for medical applications.

CHEMISTRY

Spin labels

The advances in EPR methodology are also connected with the development of new spin labels and labeling strategies (225, 226). A selection of labels for proteins is depicted in Fig. 13A [for the original references, see (226)]. For in vitro protein studies, the most used spin label is MTSSL 1. Site selectivity is achieved by mutating unwanted cysteines for other amino acids and placing cysteines into the amino acid sequence where the label should bind. MTSSL binds covalently to the protein by forming a disulfide bridge with the cysteine. The process of site-directed mutagenesis and labeling is called site-directed spin labeling (SDSL). Labeling protocols for MTSSL are well established. Other spin labels that form a thioether linker to cysteines are nitroxide labels with iodoacetamide 2 or maleimide groups 3. In these latter cases, the reaction conditions have to be carefully chosen to circumvent unwanted labeling of, e.g., lysines.

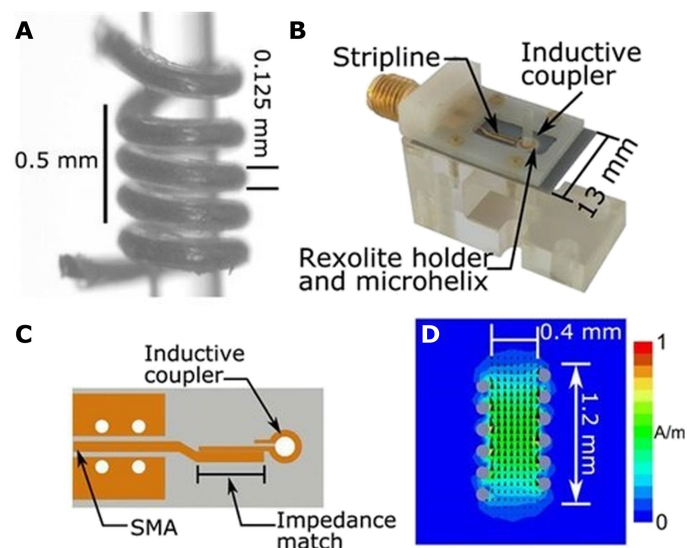


Fig. 12. A resonator for nanoliter samples. A self-resonant five-turn microhelix (A) placed in a coupling assembly (B) consisting of a stripline impedance matched to an inductive coupling loop (C). (D) Finite-element simulation of the MW magnetic field distribution at 9.5 GHz, demonstrating the very good conversion of MW power to magnetic field strength of this structure. Reproduced from (194).

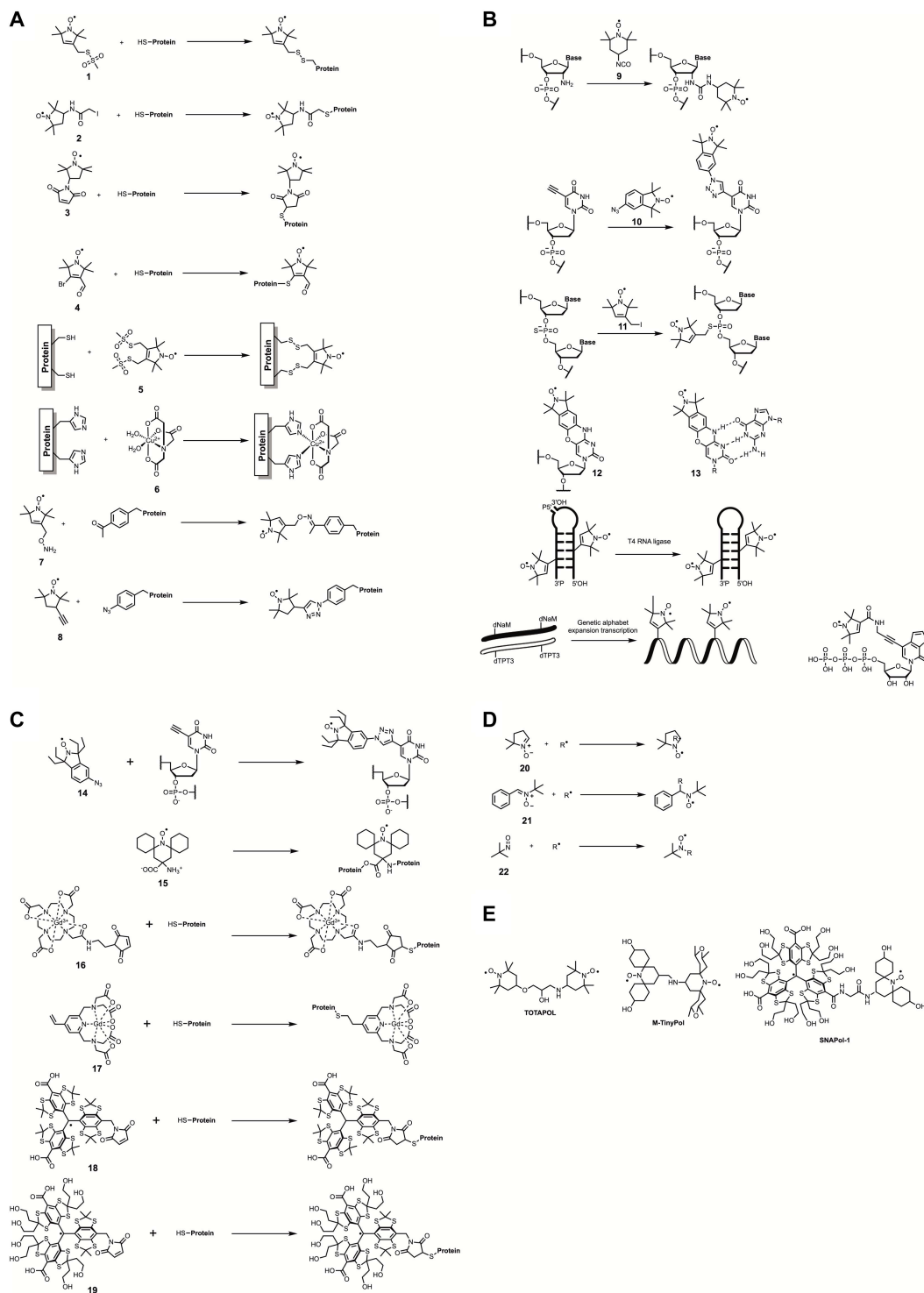


Fig. 13. Examples for spin labels, spin traps, and polarizers. (A) Spin labels for proteins, **(B)** spin labels for nucleic acids, **(C)** spin labels for in cellulose, **(D)** spin traps, and **(E)** polarizers.

All of these labels are bound to the protein via a single bond, and the linker group between cysteine and the nitroxide group contains several rotatable bonds. This leads to a conformational ensemble of the label, which is superimposed onto the protein's conformational ensemble. Differentiating between the two contributions can be challenging (227). In such cases, more rigid labels are useful, such as bisMTSL **4**, which connects via two disulfide bridges to the protein, or BASL **5**, for which the bromine atom restricts rotational freedom. Recently, an interesting alternative was introduced in which Cu²⁺NTA **6** binds site-selectively to a double-histidine motif (228). This approach is highly selective if the ratio of double histidine to Cu²⁺NTA is not more than 1:0.9, and this label yields very narrow distance distributions. It also opens up the possibility for orthogonal labeling, because, e.g., MTSSL could be linked to a cysteine and Cu²⁺ to a double-histidine motif. It is also an attractive solution if cysteines cannot be mutated because they are essential for the function of the protein. Alternatively, unnatural amino acids can be introduced, and then react with complementary spin labels **7**, **8**, but the protein yields are usually low (229).

For nucleic acids, a variety of spin labels are also available (Fig. 13B) (230). These connect to the sugar **9**, base **10**, or phosphate **11** groups. Commonly, labeling is performed on oligonucleotide strands that were synthesized with the respective modified nucleotide at the position where the label should be. While all former labels are connected via a single bond to the oligonucleotide, the ζ label **12** carries a nitroxide group fused to the oligonucleotide via two bonds. This label has been used frequently for the study of oligonucleotide dynamics (231). In addition, a noncovalent version of ζ **13** is also available, which binds to basic sites. Longer oligonucleotides that evade automated oligonucleotide synthesis can be labeled by labeling two shorter constructs and ligating these. Even longer oligonucleotides can be site-selectively labeled using a posttranscriptional method that makes use of nonnatural base pairs. With the latter method, a 377-nucleotide-long ribozyme was successfully labeled and studied by PDS (232).

For in cellulo studies, the labels must withstand the reducing conditions within the cell (Fig. 13C) (59). This means that the connecting group cannot be a disulfide, because it will be cleaved in cellulo, which emphasizes the thioether linker-forming maleimido or iodoacetamide groups. In addition, tertramethyl-nitroxides are reduced in cellulo within a couple of minutes. To overcome this challenge, tetraethyl **14** or even bulkier groups like bis-spirocyclohexyl **15** can be used. However, for studies in cellulo, mainly Gd³⁺ complexes **16**, **17** combined with a suitable linker group have been used. Recently, the toolbox for in cellulo labels has been expanded by trityl labels **18**, **19**. All these studies relied on expressing, purifying, labeling, and then transfecting the protein back into the cell. However, a recent study used Cu²⁺NTA (233) **6** to label a protein with a double-histidine motif in cellulo, without the need to label it in vitro and then transfect it back into the cell. How broadly this is applicable will be exciting to see.

Spin trapping

In many reactions, transient radicals form and quickly react further, such that their steady-state concentration is too low to be detected by EPR. In such cases, so-called spin traps (Fig. 13D) can be used, which are predominantly nitrones (DMPO **20**, PBN **21**) or nitroso compounds (MNP **22**) (234, 235). The transient radical reacts with the trap to form a stable nitroxide, which can then be detected by

CW X-band EPR. The *g* value and the ¹⁴N- and the ¹H-hyperfine coupling constants from the β -position (as in **20** or **21**) are then used to identify the radical trapped. However, one should keep in mind that different radicals present in the reaction may have different reactivities with the trap, meaning that a transient radical may be overlooked. In addition, if the type of transient radical is to be identified, the number of possibilities should not be too large because the parameter space of the formed nitroxide is small, and assignment may be difficult or ill-posed.

DNP polarizing agents

DNP enhances NMR sensitivity by transferring polarization from electron spins to nuclear spins. While early agents, such as trityl and nitroxides, laid the groundwork, EPR spectroscopy enabled the development of more efficient polarizers, as understanding how molecular structure and spin dynamics affect DNP efficiency relies critically on EPR characterization. DNP exists in several forms: (i) Dissolution DNP generates polarization at low temperatures and moderate fields (~3.4 T, W-band), followed by rapid dissolution and transfer to high-field NMR or MRI; (ii) liquid-state DNP aims for in situ hyperpolarization but remains technically challenging; and (iii) solid-state magic-angle spinning (ssMAS) DNP combines polarization and data acquisition at low temperatures and is now widely used, driving the development of advanced biradical polarizers (236, 237).

Under ssMAS DNP standard conditions (~9.4 T and 100 K), tailored nitroxide biradicals, like TOTAPOL (Fig. 13E), were identified as effective polarizing agents (238). Subsequent studies revealed that DNP efficiency requires the combination of a large enhancement factor and a short hyperpolarization time, *T_B* (239). To maximize this efficiency, the biradicals must have optimal electron spin relaxation times, orientation of electron *g* tensors, and electron-electron couplings to favor both large hyperpolarization and efficient polarization transport (237, 240). DNP and EPR experiments have established correlations between DNP enhancement and the product of electron spin-lattice (*T_{1e}*) and phase memory (*T_m*) times, as well as correlations between *T_B*/*T_{1e}* and *T_B* with the electron-electron couplings (240, 241).

At fields above 9.4 T, nitroxide biradicals with both dipolar and exchange interactions remain efficient, for example, AsymPol-POK (242) or M-TinyPol (243) (Fig. 13E), while hetero-biradicals combining a narrow-line radical (e.g., BDPA or trityl), with a broad-line nitroxide radical [e.g., the TEMTriPols family (244) and SNAPol-1 (245), Fig. 13E] have emerged as attractive alternatives. All these newer biradicals attempt to overcome issues with proton-rich media or for very high-field ssMAS DNP (243, 244). Comparative analyses of various polarizer families at different magnetic fields have been reported (237). ssMAS-DNP simulations, together with EPR, DFT, and MD calculations, help rationalize performance by providing *g*, *D*, and *J* parameters as well as conformational dynamics (242, 246, 247). Multi-frequency EPR line shape analyses have linked bis-nitroxide structures to DNP performance under various conditions (244, 247, 248).

In principle, studies of electron spin dynamics should be conducted under the same conditions as the DNP measurements. However, because of limited instrumentation, relaxation data have mostly been collected at lower fields. Combined 3.4-T DNP/NMR/EPR setups have demonstrated the value of integrated systems, enabling static-condition studies (214), where ELDOR experiments proved highly informative (249). Similar work was carried out later at 7 T (250). However, these fields remain below those of modern ssNMR. A 14-T (~400 GHz)

EPR/DNP system is under development (251). Recent work has begun to probe EPR properties under MAS conditions at 7 T (217).

d/f-block

The early years of EPR were dominated by studies of metal ions, starting with Zavoisky's first measurements on Mn^{2+} salts (4) and the work of the Oxford group on 3d and 4f ions (7). This is perhaps expected, because the vast majority of stable paramagnetic species arise from incomplete d^n or f^n configurations. This, and the nature of the materials studied—often doped (magnetically dilute) single crystals of high symmetry lattices—enabled the development of much of the early theory of EPR, including the SH concept (17). This work was also crucial in the development of the ligand field theory model of transition ion chemistry (252). Landmarks here include the observation of fine (ZFS) and hyperfine structure from metal ions (Cr^{3+} and Cu^{2+} , respectively), hyperfine coupling to ligand nuclei (in $[\text{IrCl}_6]^{2-}$; direct evidence of covalency in the metal-ligand bond), and electron-electron exchange coupling in $[\text{Cu}_2(\text{O}_2\text{CMe})_4(\text{H}_2\text{O})_2]$ (7).

The study of metal ions remains important to this day because EPR can give information on the metal ion identity, coordinated ligands, oxidation state (metal and ligand), spin state, orbital ground and excited states, coordination geometry, metal-ligand bonding, and chemical reactivity. The variety of species that can be studied is illustrated by considering even just the 3d metals in octahedral geometry: paramagnetic states are found for d^{1-9} configurations, with d^{4-7} having the possibility of high- and low-spin configurations, giving rise to all possible spin states between $s = 1/2$ and $5/2$, complicated by possible orbital singlet, doublet, and triplet states. The prevalence of $s > 1/2$ states, often with ZFS substantially larger than X-band MW photon energies, was one of the drivers for the development of high-field/frequency EPR (253).

Many important biological processes involve metal ions, and this has been a major area of EPR since the first study on hemoglobins (254). That work reported axial g values of 6 and 2, which are the effective g values from the $|m_s| = 1/2$ doublet of the $s = 5/2$ ferric ion with large $|D|$ and axial symmetry. The metalloproteins most studied by EPR are those involving V, Mn, Fe, Co, Ni, Cu, Mo, and W, all of which can exist in paramagnetic oxidation states; in some cases, an intrinsic diamagnetic ion can be substituted by a paramagnetic one, e.g., Zn^{2+} for Co^{2+} , or Mg^{2+} for Mn^{2+} . The metal ions are exploited for several roles, including catalysis, transport, and electron transfer. Multimetallic centers are common, and those studied intensively by EPR include, for example, the iron-sulfur clusters (first identified by EPR), which have been observed in redox states ranging from $s = 1/2$ to $9/2$ (255). Other examples are hydrogenases (256) and nitrogenases (257), and the oxygen evolving complex of photosystem II, where the active site is a Mn_4Ca cluster that cycles through five oxidation states (258). Metalloenzymes have inspired much small-molecule biomimetic chemistry, where EPR is also important. Biological EPR, which extends beyond metalloproteins, is a vast field and a comprehensive set of reviews up to approximately 2010 can be found in (259, 260). EPR can provide identification (and quantification) of metal sites, including local and macromolecular structure, and functional information, e.g., substrate binding, redox cycling, and transient intermediates (261). Hence, the full range of EPR techniques is important, including some such as ^{55}Mn and ^{57}Fe ENDOR (262, 263) that have been little exploited outside of biomolecular EPR.

Multimetallic active sites in biology inspired much interest in cluster chemistry, including magnetic properties. This led to the discovery

of the “single molecule magnets,” the first of which was $[\text{Mn}^{\text{III}}_8\text{Mn}^{\text{IV}}_4\text{O}_{12}(\text{O}_2\text{CMe})_{16}(\text{H}_2\text{O})_4]$ (“ Mn_{12} ”). The exchange coupling in Mn_{12} gives an $s = 10$ ground state with a large negative ZFS, a combination that leads to such slow relaxation of magnetization [at $\text{He}(\text{I})$ temperatures] that magnetic hysteresis is observed—a molecular magnetic memory effect. This led to a renewed interest in molecular magnetism more widely, and molecular clusters have allowed the study of phenomena like magnetization quantum tunneling, spin frustration, anti-symmetric exchange, and magnetocaloric effects in finite systems. EPR (including high-field and frequency-swept methods) is a crucial part of this (264), giving a direct probe of the spin levels in contrast to magnetometry. Such systems, and proposals to exploit molecules in quantum information science (265), also triggered renewed interest in magnetic relaxation mechanisms.

Many catalytic processes (including biological) involve paramagnetic ions (and intermediates) in their mechanisms. Hence, EPR methods are useful in their characterization, in both homogeneous and heterogeneous (solid/gas, solid/liquid) systems. Model or real systems can be studied under controlled conditions, e.g., to isolate a reactive species, or under operando conditions. Operando studies (e.g., high temperature/pressure, electro- or photolytic activation) tend to be restricted to CW EPR because of the temperatures involved, but can be coupled to complementary analytical techniques (23) and developments such as modulation excitation detection (pulsing in substrate gas enabling phase-sensitive detection) can give enhanced SNR (important for broad spectra) and time resolution (266). Of course, hyperfine methods can be applied to quenched or model systems. The metal ions may be present as, for example, a coordination compound (EPR has long been used in organometallic chemistry), in a metal oxide, or in a supported metal catalyst (including recent developments of single-atom catalysis), for example, in microporous solids (zeolites, metal-organic frameworks, etc.). To take one well-studied example, photoactivation of TiO_2 generates “trapped electrons” in the form of Ti^{3+} , and “trapped holes” in the form of O^- , both of which can be involved in further reactions and are EPR active; further insight can be gained by, e.g., incorporating ^{17}O ($I = 5/2$) and applying hyperfine methods (267).

Metal oxides, including metal-doped oxides, are also studied for their material properties, for example, optical, electronic, transport, and magnetic. EPR is a key technique in characterizing dopant sites, vacancies, defects, etc. that affect the physical properties (268). To take one old but important example, the masing and lasing properties of ruby arise from the $s = 3/2$ Cr^{3+} ions in the Al_2O_3 lattice. EPR (including hyperfine methods) can probe size effects, e.g., in nanoparticles and quantum dots. Dilute magnetic semiconductor metal oxides (e.g., metal-doped TiO_2 , ZnO , and SnO_2) are of interest in spintronics, displaying ferromagnetic and semiconductor properties. EPR can characterize dopant structure in the paramagnetic state, while ferromagnetic resonance can characterize the ferromagnetic state. Other important classes of materials studied intensively by EPR include perovskites (269) and electrode materials.

Lanthanide-doped lattices were some of the first materials to be studied by EPR (7): The first ESEEM and pulsed ENDOR measurements were on Ce(III)-doped CaWO_4 (12, 13). Such materials are still important because of their optoelectronic properties in, e.g., phosphors, upconversion, lasers, and quantum communication. Most of the EPR literature is on Kramers Ln^{3+} ions, where the spectra arise from the ground Kramers doublet of the $^{2S+1}L_J$ Russell Saunders term split by the crystal field. Pulsed studies are rarer (270), with the notable

exception of the orbital singlet $s = 7/2$ Gd^{3+} ion, but are of interest with a view to relaxation (important in NMR contrast and shift agents, EPR distance measurements, and potential quantum applications). Ln complexes have become of interest in molecular magnetism (much greater magnetic anisotropies than d-block), and advances in chemistry have made Ln^{2+} complexes, with unusual electronic configurations (271), available. EPR studies of actinides are much rarer (272) and are dominated by the most easily handleable U and Th ions. There is renewed interest in this area due to debates as to the extent of covalency in f-element ligand bonding, a problem suited to hyperfine methods (273).

OUTLOOK

Eighty years after its discovery, EPR spectroscopy continues to evolve in both capability and scope. Advances in MW technology, magnet design, pulse shaping, labeling, and detection schemes have improved resolution and sensitivity and extended the range of systems for which the structure, dynamics, and reactivity can be studied. The introduction of cryo-free cooling systems has lowered the cost of the essential low-temperature measurements. At the same time, progress in computation, ranging from quantum chemistry and MD to machine learning-assisted data analysis, has made it easier to conclude from EPR observables the underlying molecular structure.

Looking forward, several developments are poised to further expand the impact of EPR. Integration and automation of experiments, together with standardized acquisition and analysis workflows, will lower barriers to entry and improve reproducibility across laboratories. Continued miniaturization of resonators and electronics, including benchtop and chip-based instruments, promises reduced instrumentation cost and thus wider adoption in chemistry, biology, and materials research, as well as in operando and in cellulo studies. In parallel, ultrasensitive detection schemes and high-field implementations will enable access to systems at lower concentrations and improve spectral resolution. Last, further development of computational power will allow access to simulations of large spin systems.

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Acknowledgments

Funding: We acknowledge that we received no funding in support of this research. **Author contributions:** Conceptualization, writing, review, and editing: all authors. **Competing interests:** The authors declare that they have no competing interests. **Data, code, and materials availability:** All data and code needed to evaluate and reproduce the results in the paper are present in the paper and/or the Supplementary Materials.

Submitted 31 January 2026

Accepted 14 July 2026

Published 16 September 2026

10.1126/sciadv.aeg0169

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Sci. Adv. **12** (38), eaeg0169. DOI: 10.1126/sciadv.aeg0169

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