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The World as Viewed by and with Unpaired Electrons

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Abstract

Recent advances in electron paramagnetic resonance (EPR) include capabilities for applications to areas as diverse as archeology, beer shelf life, biological structure, dosimetry, in vivo imaging, molecular magnets, and quantum computing. Enabling technologies include multifrequency continuous wave, pulsed, and rapid scan EPR. Interpretation is enhanced by increasingly powerful computational models.

Introduction

As noted by J. S. Hyde in the 1970s ‘there are spins everywhere’. Advances in our ability to probe those spins with new techniques, interpret observations, make predictions using evermore powerful computational models, and improved sensitivity to a diversity of characteristics of the samples, makes this an exciting time for electron paramagnetic resonance (EPR). Applications range from archaeology to zero-field splittings. In this article we seek to convey our perspectives on these opportunities. Although there is a tendency in modern science for researchers to become increasingly specialized, we seek to provide an overview of the wide range of opportunities for applications of EPR. For conciseness, most details are incorporated by citation to the literature. References are intended to be illustrative, not comprehensive, and we cite books and reviews rather than original papers for many areas. We do not claim to provide references to the “first” observation or interpretation of a phenomenon, because such action requires deep scholarship on every detail. Often we find that there is precedent in the literature for the articles that are commonly cited as the “first.”

1. Physical principles

Unpaired electrons occur in a wide variety of species. Many organic and biological molecules have oxidation states in which there are an odd number of electrons, commonly one electron that is not spin-paired. Some species with an even number of electrons can have two electrons unpaired, as in diradicals and triplet states. Many oxidation states of transition metals, lanthanoids, and actinoids have one or more unpaired electrons (e.g., 7 in the case of Gd^{3+}). The energy states of the unpaired electrons have separations that depend on the magnitude of an external magnetic field. Many physical techniques can measure the number of unpaired electrons, their interactions with their environment, including with other unpaired electrons or nuclear spins, and the kinetics of their changes. Bulk magnetic susceptibility is one measure. Nuclear spins measured by NMR techniques report interactions with electron spins. A method that is very specific to the nature and dynamics of

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electron spins is the resonant absorption of energy that is equal to the spacing between spin energy levels. This method is called electron paramagnetic resonance (EPR) or electron spin resonance (ESR). By analogy with NMR, the method is sometimes also called electron magnetic resonance (EMR). The match between energy separations and incident electromagnetic radiation, resulting in magnetic dipole transitions, can be achieved by scanning the radiofrequency (RF)/microwave frequency at constant magnetic field, or, more commonly, by scanning the magnetic field at constant RF/microwave frequency. Electron-nuclear spin interactions, and electron-electron spin interactions can result in many energy levels and many different allowed and forbidden transitions among them. The energy of interaction between electron spins and other electron spins can be of the same order as the energy of interaction between electron spins and nuclear spins. These interactions may lead to resolved splittings, but also commonly are within the experimental line width of the EPR transition, and require special methods for sorting out the interactions. Methods have been developed for studying these transitions, and many of the methods have acquired their own names and acronyms. For example, nuclear spin couplings to electron spins can be measured by irradiating the sample simultaneously with microwaves at the electron spin resonant frequency (e.g., ca. 9.5 GHz at 3400 G) and with RF at the nuclear spin resonant frequency (e.g., ca. 14 MHz at 3400 G). Usually, one sets the magnetic field and microwave frequency at the values for electron spin resonance, and scans the RF frequency observing changes in the EPR signal whenever a nuclear spin is in resonance. This method is called electron nuclear double resonance (ENDOR). This experiment can be performed with continuous wave (CW) microwave and RF, or with one or both pulsed. Historically, most EPR has been CW, but pulse methods are becoming much more common. The response to a single resonant microwave pulse results in a free induction decay (FID) as in pulsed NMR. However, many EPR spectra are so broad that only a portion of the spectrum can be excited with the pulse powers and pulse lengths available, so FID techniques are less common than spin echo techniques. Multiple microwave pulses can prepare unpaired electrons in defined states in the same way that multiple RF pulses are used in NMR. The challenge is that electron spin relaxation times are much shorter than nuclear spin relaxation times, so shorter pulse sequences are usually used in EPR than in NMR. One common technique is to observe a 2-pulse echo as a function of time between the pulses. In addition to relaxation decay of the echo, if the electron is in the vicinity of nuclear spins, the echo amplitude oscillates at the Larmor frequency of the nuclear spins plus quadrupole and other effects. This electron spin echo envelope modulation (ESEEM) reports the nuclear environment of the electron spin. More sophisticated analogs of ESEEM have been developed, one of which is the hyperfine sublevel correlation experiment (HYSCORE). Electron-electron interactions are strong enough to measure distances that are large on a molecular distance scale. This has become such a powerful tool in polymer chemistry and biochemistry that pulsed double electron-electron resonance (DEER) is reason by itself to have an EPR spectrometer.

In all of these techniques the physical principles are the same – interaction of the spin and orbital angular momentum vector with the microwave magnetic vector – just as in NMR. The primary differences in technology and methodology between NMR and EPR are caused by the much larger magnetic moment of the electron relative to nuclei. Electron interactions with the environment are larger, so concentrations have to be smaller to achieve high resolution, and relaxation times are shorter. EPR instrumentation consists predominantly of a very stable microwave source, a high-Q resonator in the gap of a variable-field electromagnet, a sensitive diode detector, and associated control and spectral analysis console. The most commonly-used frequency is X-band (9–10 GHz), which requires a magnetic field of ca. 0 to 1 T ($g = 2$ at ca. 0.34 T, 3400 G). For a long time, X-band was the frequency of choice. Now, however, frequencies from ca. 250 MHz (for in vivo imaging) to hundreds of GHz (for high-spin systems) characterize EPR.

2. Enabling Technologies

2.1. Operating Frequencies

EPR instrumentation has come a very long way from the routine X-band (9–10 GHz) continuous wave (CW) spectrometers that long dominated the field. The many applications of EPR depend on and stimulate developments in methodology and instrumentation. Recent developments in pulsed EPR methods have emphasized DEER [1–3] and double quantum coherence (DQC) spectroscopies [4]. Saturation recovery, long restricted to a few labs [5–7] is now available in the Bruker Elexsys spectrometers. As other applications of EPR create demand for spectrometers in new frequency ranges, or with other capabilities such as imaging, rapid scan, and other methods briefly mentioned in this article, one can anticipate that the range of commercially available instrumentation will increase accordingly.

State-of-the-art X-band CW spectrometers perform close to the signal-to-noise estimate calculated based on inherent losses between the resonator and the signal detector [8]. For particular applications such as very high power, high-Q (quality factor) resonators, or low modulation frequencies, improvements could be achieved with lower phase noise sources. It is anticipated that most future improvements at X-band will be via purpose-specific resonators. The power incident on the sample in CW spectrometers usually is less than about 200 mW. The power selected depends on spin relaxation times, and the information desired from the measurement. Usually, incident power is chosen such that the EPR transition is not saturated. Pulsed EPR spectrometers use much higher excitation power – often hundreds of watts incident on the resonator. Pulse lengths to achieve 90° turning angles commonly range from 10 to 100 ns. Dead times after pulses, and before measurements can be made, are of the order of 10s to 100s of ns.

Pulse and CW spectrometers are now available from Bruker BioSpin with operating frequencies ranging from 1 to 263 GHz. Instrumentation and experimental setup for pulsed EPR were described by Jeschke [9]. W-band (95 GHz) was daunting to many labs in the very recent past, when operating such an instrument meant ongoing high costs of replacing liquid helium, but now “cryogen-free” magnet systems open high-field EPR to more potential users.

Spectrometers operating at frequencies below 1 GHz and above 263 GHz are still purpose-made in individual labs. Extensive description of a pulsed 250 MHz spectrometer is in a series of papers from our lab [10–12]. The Freed lab 250 GHz spectrometer is based on a quasi-optical design to minimize power losses [13,14]. Nagy et al. described a spectrometer operating in the 75 – 225 GHz region [15]. A pulsed spectrometer operating from 110 to 336 GHz has been constructed [16]. A spectrometer operating up to 900 GHz was used for the study of Co(II) [17]. Initial operation of a free-electron laser-based EPR at 1.2–75 THz was illustrated with EPR spectra of DPPH at 56.1 T and a low-dimensional organic material, (C₆H₉N₂)CuCl₃ at 43.8 T [18].

2.2. Detection methods

Unlike NMR where the field is dominated by pulse methods, CW remains important in EPR because of wider spectral widths and faster relaxation times for electron spins than for nuclear spins. Historically magnetic field modulation, and phase-sensitive detection at the modulation frequency, has been used to improve sensitivity, yielding the characteristic first-derivative representation of EPR spectra. However, field modulation introduces other problems, especially a line shape vs. line amplitude compromise, loss of sensitivity for broad lines, such as when gradients are applied in imaging, microphonic noise caused by eddy currents, and difficulty of introducing modulation with some types of resonators [19].

Several methods to avoid magnetic field modulation in CW EPR have been explored [19]. One of these is multi-quantum EPR [20,21]. Another approach is frequency sweep [22,23], but application is limited because the EPR resonator bandwidth is small relative to the spectral width for most samples of interest. At W-band, a resonator with $Q \approx 100$ has a bandwidth of ca. 1 GHz, which corresponds to about 350 G between 3 dB points, so frequency sweep is more feasible at W-band than at lower frequencies [24,25]. Frequency sweep is increasingly common for measurement of zero-field splittings of species with spin $S = 1$, such as Fe^{2+} and Ni^{2+} [26–29], either at zero magnetic field or at a fixed magnetic field. Frequency domain magnetic resonance spectroscopy (FDMRS) is complementary to high field EPR (HFEPR) in which the frequency is held constant and the magnetic field is swept.

Diode detectors and double-balanced mixers are common detectors for CW EPR, and the absorption signal is measured. Usually, the microwave source is locked to the EPR resonator for frequency stability. Consequently, differently engineered systems are required to measure the dispersion signal. Pulse spectrometers usually use quadrature mixers so that post-acquisition analysis can use both the in-phase and quadrature signals.

Early in the development of NMR some transient effects, called “wiggles”, were observed after the magnetic field passed through resonance in a time that was short relative to T_1 and T_2 . This effect was used in CW NMR to guide shimming the magnetic field to maximum homogeneity, since inhomogeneity over the sample caused the oscillations to damp out more rapidly. Dadok, Gupta, and coworkers in the early 1970s [30,31] showed that since the oscillations following passage through an NMR line were characteristic of the spin system and the spectrometer, they could be accounted for experimentally or theoretically, and the slow-scan spectral features could be obtained by post-processing a rapid-scan NMR spectrum. Analogous oscillations in EPR signals were observed, starting with [32], and were developed into a third EPR regime, between CW and pulse, by the Denver EPR Center (Fig. 1) [24,33–36].

Direct-detected EPR, as in pulsed EPR, can be performed with magnetic field sweep or microwave frequency sweep, at rates ranging from very slow to very fast relative to relaxation times. Instrumentally, the difference is in scan drivers and detector bandwidth. When the scan is slow, one can record the final spectrum as it is detected. When the scan is fast, the relaxation-dependent spin response is deconvolved in post-acquisition processing to obtain the slow-scan spectrum [34,35]. In both cases, filters can be applied in post-acquisition processing. This is a new regime for EPR, and new aspects of methodology and new applications are being developed. The Hyde lab developed segmental acquisition of spectra, a few gauss at a time [37], while the Denver lab engineered faster and larger magnetic field sweeps to encompass spectra of most organic radicals [38], and to measure relaxation times [39]. The amplitude of the detected signal is larger for direct detection than for phase sensitive detection (Fig. 2). Rapid scan is analogous to pulsed EPR in the sense that the microwaves are resonant with the spins for only a brief period during the scan. Consequently, much higher power can be used relative to a normal CW scan [33,36], which provides improved signal-to-noise (S/N) (Fig. 1). Similarly, there is no magnetic field modulation broadening of the line. In terms of S/N per unit time, rapid scan is more beneficial the longer the spin relaxation time. For species such as N centers in diamond, spin relaxation times are so long that normal field-modulated CW EPR without power saturation or modulation broadening is extremely difficult, but rapid scan is straightforward to apply.

We propose that recent advances in arbitrary waveform generators (AWGs) and fast digitizers now make it possible to build an EPR spectrometer operating at frequencies up to 9.5 GHz with fully digital source and detection, which will provide greater flexibility in

experimental design and operating frequency than current generation spectrometers. The transition from analog to digital was crucial to improving NMR [40], microwave spectroscopy [41–43], and ion cyclotron resonance [44], and we propose that similar advantages await in EPR. The new approach replaces the largely analog microwave bridge of current EPR spectrometers with a digital source and digital detector. One other lab, in parallel with our efforts, has digitally generated the incident RF and directly digitized the EPR signal (at 300 MHz) [45,46]. Advances in AWGs and the development of cross-loop resonators (CLR) will make this feasible at X-band in the near future. Down-conversion and subsampling techniques (called time-locked subsampling, TLSS, in EPR applications), which are also extensively used in NMR [40] and MRI, have been used by the Hyde lab for recording multiharmonic X-band CW EPR [47–49] [25], by the Ohio State lab for L-band CW EPR [50], and by the NCI lab to digitize an FID [45]. An AWG was used to control pulse timing in a 26.5–40 GHz pulsed EPR spectrometer [51].

A major advantage of a digital spectrometer is its flexibility to be reconfigured, with little cost or effort, to do essentially all EPR experiments that now are performed by separate, stand alone spectrometers, and do them more efficiently, more accurately, with better signal-to-noise and at less total cost. While conventional spectrometers are constructed for one primary application over a limited frequency range, a digital spectrometer allows a multitude of applications over a wide band of frequencies. Each application requires only the appropriate resonator, and perhaps a microwave amplifier, plus software modules that can be linked together to provide the required functionalities. For example, the flexibility of an AWG as a source permitted implementation of the Frank sequence of phased pulses in EPR [52,53].

The “normal” CW EPR spectrometer acquires only the absorption or the dispersion EPR signal. A digital spectrometer can detect both the absorption and dispersion channels, and have precise orthogonality between the two channels. Thus, for any experiment one can combine the two channels to get a $\sqrt{2}$ improvement in signal-to-noise (S/N) (Fig. 3), which by itself is a major advantage [54]. Actualizing these advantages requires isolation from the source noise of an AWG, such as can be achieved with use of a CLR. In addition, digital detection of a field overmodulated signal permits calculation of the undistorted lineshape from multiple harmonics of the modulation, which can further improve S/N [55]. Pulsed EPR requires phase cycling to compensate for non-orthogonality, in addition to phase cycling to eliminate unwanted artifact signals. Less phase cycling is needed in a digital spectrometer because of the orthogonality of the two digitally-derived channels.

2.3 Differences between NMR and EPR

Magnetic resonance study of nuclear spins and electron spins differ dramatically, largely because of the difference in the nuclear and electron magnetogyric ratios, and the coupling of the electron spins to orbital angular momenta. To achieve magnetic dilution for the much larger electron magnetic moment requires low concentrations for most studies. Spin-spin and spin-orbit couplings cause electron spin relaxation rates to be many orders of magnitude faster than nuclear relaxation rates. To highlight differences between NMR and EPR, note that NMR spectra can be obtained on samples with molar concentrations, whereas samples for EPR typically have concentrations less than 1 mM. In fluid solution at room temperature, NMR relaxation times are msec to sec, but typical EPR relaxation times range from μ s for organic radicals to less than ns for transition metals. Whereas in fluid solution NMR line widths and resolution are usually determined by magnetic field inhomogeneity, EPR line widths are usually determined by unresolved nuclear hyperfine couplings and relaxation times. Another important difference is that in NMR often the spin Hamiltonian can be treated with 1st order perturbation theory because the nuclear Zeeman term dominates. In

EPR, the interactions can be of similar orders of magnitude making the interpretation and simulation more complicated, but simulation programs that perform calculations to the needed order are readily available (see section 3.)

Striving for signal-to-noise, EPR traditionally has used resonators with much higher quality factor, Q , than in NMR. This results in greater interaction between the resonator and the sample in EPR than in NMR. Automatic sample changers are rarely used in EPR. These differences are decreasing: NMR increasingly uses cryogenically cooled resonators (usually called cryoprobes in NMR) to increase resonator Q to improve S/N . EPR increasingly uses resonators with lower Q , sacrificing some inherent S/N to have wider bandwidths for pulsed and rapid scan measurements. For some EPR resonators, automatic tuning is available, increasing ease of use.

2.4 Imaging

The differences between nuclear and electron magnetic resonance are particularly evident in imaging experiments. Since MRI is better-known to many readers than EPR imaging, the comparison shown in Table 1 may assist understanding of special features of EPR.

Although increasingly NMR imaging and *in vivo* spectroscopy are focusing on low-concentration species, e.g., for the study of metabolism, the well-known applications of MRI use the high-concentration and long-relaxation-time features of ^1H in water and fat in the body. With a few possible exceptions, free radical concentrations *in vivo* are not high enough or persistent enough to measure them directly with EPR, so a probe is introduced (nitroxide or trityl in almost all cases), somewhat analogous to using a contrast agent or a hyperpolarized species in MRI. The resultant concentrations of electron spins are sub-millimolar, in contrast to the ca. $90\text{ M } ^1\text{H}$ spins in the body. Electron spin relaxation times are less than a μsec for nitroxides, and less than about $10\text{ }\mu\text{sec}$ for trityls. These short relaxation times, relative to ^1H and other nuclei, necessitate different approaches to EPR imaging than for NMR imaging. For example, pulsed field gradients of high linearity over animals even the size of mice, analogous to the way MRI is commonly conducted, are not feasible within the ca. hundreds of nanoseconds needed for projections in an EPR image. Instead, EPR imaging uses stepped constant gradients. Various CW, rapid scan, and pulsed FID and spin echo detected imaging modalities are being developed to get the best image in the shortest time for targeted *in vivo* information [59–61].

EPR imaging was developed largely independently in at least three labs [62], and has developed in many labs in many directions, including fundamentals of materials [63,64], basics of chemical and electrochemical reactions [65], and *in vivo* studies including physiology [61,66]. Synthesis of persistent triarylmethyl radicals (called trityl, or TAM in various papers) [67] stimulated new interest in *in vivo* imaging. Whereas nitroxide radicals are reduced *in vivo* at a rate high enough that it has to be considered in interpretation of images, trityl radicals are resistant to reduction. Further, the trityl radicals that are used for *in vivo* spectroscopy and imaging have long spin relaxation times and very narrow CW EPR lines, broadened only by small unresolved hyperfine coupling to distant nuclei in the molecule. Collisions of trityl radicals with O_2 in solution shorten the spin relaxation times and broaden the CW lines to a greater extent than for commonly available nitroxide radicals, which were used to initiate these studies before trityls became available [68]. However, so far, the greater synthetic versatility of nitroxides than of trityls has made nitroxides the probe of choice for many *in vivo* studies of oxidative stress [69] and other details of physiology (pH, redox, intracellular measurements, etc.).

In vivo O_2 concentration has been imaged [70]. An example of a spin echo image of O_2 in a mouse tumor obtained with a trityl radical is shown in Fig. 4. *In vivo* measurement of

oxygen (oximetry) [70,71] is crucial for the diagnosis and monitoring of tumors, wound healing, cardiac function and disease [72], and peripheral vascular problems [73–77]. The accuracy of oximetry by linewidth measurements was compared for nitroxides and fusinite [71]. Spectral-spatial imaging over short times is best accomplished using trityl radicals [78]. Point samples of implanted oxygen-responsive samples such as LiPc (lithium phthalocyanine) can be measured over long times [79]. The state of the art of *in vivo* oxygen imaging is about 1 Torr O₂ and 1 mm spatial resolution in about 10 minutes of acquisition [80]. Microscopic imaging is another exciting direction. Freed and Blank and coworkers achieved 3.5×7×11.4 μm resolution at X-band [81], sub-micron resolution at 17 GHz [82], and about 10 μm resolution at 15.4 GHz [83].

3. Enabling Computations

Simulations of EPR spectra have advanced from multiple locally-written programs for specific purposes to highly sophisticated general-purpose programs [84], of which those that are commonly used are EasySpin by Stefan Stoll (easyspin.org) [85,86] and Sophe and MolecularSophe by Graeme Hanson and others [87,88]. The appendices in [59] contain descriptions of simulation software by seven research groups. The availability of these high quality tools should greatly decrease the activation barrier to data analysis. The availability of the DEERanalysis software [89,90] (<http://www.epr.ethz.ch/software/index>) makes the technique more accessible to a wider range of users.

Molecular orbital calculations have improved to the point that useful calculations can be performed on open-shell molecules, which are much more difficult to calculate than are closed shell, diamagnetic, species [84,91–93]. ORCA (<http://www.thch.uni-bonn.de/tc/orca/>) and the commercial ADF (<http://www.scm.com/Products/ADF.html>) and Gaussian software (<http://www.gaussian.com/>) can calculate hyperfine couplings to all of the atoms in a free radical. Calculations of free radicals provide good values for both *g* values and hyperfine values [94–96]. Quantitative agreement with experiment requires further development of the calculational tools, especially the treatment of solvent interactions with the radicals [97–99]. However, these MO calculations are already useful for interpreting results and guiding experimental design. Even for transition metals [100] good agreement with experiment has been obtained. Some of the metals for which calculations have been reported include Cu(II) [100,101], Mo(V) [102], and vanadyl proteins and model complexes [103,104]. Early confirmation of molecular orbital predictions by measurements of hyperfine of free radicals was one of the triumphs of physical science. Now, enhancements in molecular orbital calculations are yielding estimates of hyperfine couplings of predictive utility.

4. Applications that Drive Innovation

Few fields are beyond the reach of EPR. There is not space to treat any in detail in this article, but a few are selected for brief description in order to illustrate the range of applications. For a much more extensive compendium of applications of EPR see the Annual Reports of the Royal Society of Chemistry, Physical Chemistry, and especially the reviews by Rhodes, 2010, and previous years.

4.1 Selection of operating frequency

Frequencies lower than the common X-band have been applied extensively to *in vivo* studies and many other applications [61]. For biological distance measurements pulsed Q-band (34 GHz) is the major tool, and DEER at Q-band provides substantial S/N improvement over X-band (Fig. 5) [105–107]. Frequencies above X-band have been useful for separating transitions with different *g* values, and for studies of molecular motion [108]. The best spectral resolution may occur at different frequencies for different *S* = 1/2 species, since

there is a tradeoff of g value differences, hyperfine, and g -strain and a -strain. However, for $S > \frac{1}{2}$ species, high-field, high-frequency EPR may be required to observe transitions for which the zero-field splitting (ZFS) is greater than the X-band energy. One such example is the $S = 2$ tetranuclear copper trifluoroacetate with quinoline [109]. Spectra obtained at 15 K and 345 GHz allowed assignment of $\Delta m_s = 1, 2, 3$, and 4 transitions. For a tetranuclear Ni(II) complex all 8 transitions within the $S = 4$ state can be observed at 172 GHz (Fig. 6) [110]. Some species which historically have been described as “EPR silent” can be observed with parallel instead of perpendicular B_1 [111] or with high-field EPR. For example, Telser et al. used a spectrometer [112] that could have a frequency in the range of 25 to 3000 GHz, and a continuous magnetic field sweep over 0 – 17 T, and thus choose a window for studying high-spin non-Kramers ions. A specific case studied was Cr(II) in a complex with zero-field splitting of ca. 2.2 cm^{-1} .

4.2 Relaxation times and electron-nuclear interactions

Understanding of relaxation times and development of EPR methodology and applications have been mutually supportive since the beginning of magnetic resonance. Early work on lasers and masers (then called quantum electronics) relied upon and stimulated understanding of relaxation at low temperature [113,114]. By the late 1960s, relaxation in the solid state at temperatures below about 20 K seemed to be well-described [115], and this is the picture that is in many subsequent books. However, much of chemistry and biochemistry is studied at higher temperatures in soft materials, requiring new models. Recent summaries extend understanding of relaxation [116–119]. Identifying actual spin relaxation mechanisms is a hot topic for current research.

Early efforts to determine relaxation times commonly used CW line widths and power saturation behavior to estimate the transverse, or spin-spin relaxation time T_2 , and the longitudinal or spin-lattice relaxation time, T_1 . If a detailed knowledge of hyperfine splittings is used to calculate the relaxation-determined line width, reasonable estimates of T_1 and T_2 can be made using CW methods [120]. However, pulse methods are much more powerful. Saturation recovery methods yield the best estimates of T_1 . In saturation recovery, a microwave pulse of high enough power to saturate the spin transition is applied for a time that is substantially longer than the longest relaxation process, and then the recovery of the saturated line to thermal equilibrium is monitored by a very low-power, i.e., non-saturating, CW observe power without magnetic field modulation. The signal recovers with a time constant, T_1 . Because of the need for low observe power and the lack of magnetic field modulation, the S/N of a saturation recovery signal is low. To measure T_2 directly, one uses a 2-pulse spin echo, measuring the amplitude of the echo as a function of the time between the two excitation pulses. If the spin concentration is low enough that instantaneous diffusion is not a contributor to the decay, the measured echo amplitude decay rate is T_2 . However, since T_2 is usually reserved to mean the electron-electron spin-spin interaction time in the Bloch equations, the experimental time is usually called T_m , the dephasing time of the xy magnetization following a pulse. A FID decay time reports magnetic field inhomogeneities and hyperfine-induced inhomogeneous broadening of the EPR line, so it is designated T_2^* , and the FID decay rate provides only a limiting measure of T_2 . One can use a strong pulse to invert the spins, and then monitor the recovery at increasing times after the inverting pulse, using either a single-pulse FID or a 2-pulse echo. In some spin systems this inversion recovery method yields an apparent T_1 that is shorter than the real T_1 because of spectral diffusion, the same problem that requires long saturating pulses in saturation recovery. The inversion recovery method can approximate the saturation recovery method if one uses a series of inverting pulses before monitoring the recovery. These methods are discussed in more detail in [116].

Nuclear and electron spins reciprocally report their interactions [121,122]. Relaxation enhancement of nuclear spins by paramagnetic species that may be native or added as probes or labels can complicate NMR spectra, but can be used to measure distances in substances studied. Similarly, nuclear spins couple to electron spins and via these couplings report the near environment of the electron spins which can be revealed by ENDOR [123,124] and by spin-echo methods such as ESEEM and HYSCORE. Coupling of the electron spins to the nuclear spins in the environment reciprocally affects the electron spin relaxation time, including in some cases sufficiently to affect the EPR linewidth, as is often demonstrated by changing solvents from normal isotopic abundance to deuterated solvents. Measurement of nuclei in the vicinity of a paramagnetic center has been a powerful tool in biochemistry, especially for revealing the coordination sphere of metals in enzymes and spin density distributions (Fig. 7) [125]. If the nuclear spin coupling to the electron spin produces energy levels that are excited by the pulses in a normal 2-pulse echo experiment, the nuclear contributions to the energy level splitting shows up as a modulation of the echo amplitude when the time between the pulses is increased. The Fourier transform of the echo decay yields the spectrum of interactions, which are characteristic of the identity and number of nuclei involved [126].

At low temperatures T_m is largely determined by nuclear spin diffusion, including interaction with nuclei rather distant from the electron spin [127]. It is rare for nuclear spin concentrations to be low enough not to strongly influence the electron spins. For example, even the low concentration of ^{29}Si in SiO_2 can dominate electron spin echo decay modulation in low magnetic fields [128]. Relaxation of P doped Si depends on the concentration both of P and of the ratio of ^{28}Si to ^{29}Si [129,130]. Dynamic nuclear polarization (DNP) enhancement of NMR sensitivity and EPR have been intertwined since the beginning. EPR linewidth and relaxation observations guide the selection of DNP agents, from Cr(V) to dinitroxide radicals and trityl radicals. Conversely, some “EPR imaging” exploits the Overhauser enhancement in the methods called OMRI [131].

4.3 Characterization of compounds

Most chemists know EPR as a method to characterize metal complexes and identify organic free radicals. Many elements in the periodic table can be studied by EPR under some set of conditions. Many transition metals have common paramagnetic states, and EPR is the method of choice for studying them, in fluid or frozen solution or in solids. Because they can be studied so easily, VO(IV), Cr(III), Mn(II), Fe(III), Cu(II) have been studied extensively, both in “inorganic” complexes and in metalloproteins [132]. Co(II) is also important, but is studied at cryogenic temperatures. Iron-sulfur proteins, commonly known now, were first identified via EPR [133]. Many defects in solids, whether caused by radiation, thermal changes, or purposeful introduction of atoms, have long relaxation times that make them amenable to detailed study by EPR. Many organic radicals are sufficiently persistent to be studied directly, or, if less persistent, can be trapped by another compound to produce a relatively persistent radical for study by EPR [134]. Some radicals, such as DPPH, BDPA, and nitroxides can be prepared as crystalline solids. Semiquinones are somewhat more transient, but have been studied extensively [135]. Nitroxides have so many interesting features, and can be synthesized with so many different ring structures and substituents, that they are as important as metal ions for exploring everything from small molecule motion in solution to protein structure. Several books provide background on organic radicals [134,136–138].

Recently, spin-spin interactions have reported distances between metals and spin labels, between pairs of spin labels, and between pairs of metals. High-field EPR helps identify orientation of the spin labels, yielding more specific information about the interspin distance than is available from lower frequency measurements [139–142]. EPR distance

measurement methods, including DEER, ESEEM, and HYSCORE are showing that the protein structure in frozen solution can in some cases be very different from the structure in crystals revealed by X-ray diffraction. Regions of proteins that are important in reactions often are disordered in crystals used for X-ray diffraction, but can be monitored by site-directed spin labeling. Among the applications of DEER is mapping folds in oligonucleotides [143]. Bird et al. [144] used DEER to obtain molecular conformations of bispeptides in order to develop new force-field parameters for modeling bispeptides. The primary limitation on the longest distance that can be measured in DEER studies is the general proton contribution that shortens T_m for the spin labels [145]. Deuteration of the solvent and of the protein considerably lengthen T_m [146], which permits determination of longer distances.

Much of the understanding of the detailed mechanism of photosynthesis has been developed via the insights of EPR, starting with the observation that there was a radical formed [147], then the important observation of the “special pair” [148], and continuing with the extensive and detailed experimental and calculational studies by the Berlin group [149–152]. Electron transfer aspects were reviewed by Kawamori [153].

4.4 Molecular Dynamics

Much of the detailed understanding of motion in molecular liquids has come from the studies by Freed and coworkers of the motion of nitroxides [154–156]. The hyperfine and g anisotropy contribute to CW line widths and spin echo phase memory times. 2D displays of T_m reveal subtle features that test alternative models of molecular motions.

Even in “rigid” solids and frozen solutions at low temperature, molecular motion can be measured by changes in phase memory time as a function of position in the spectrum [157–159]. The dependence of T_m on position in the spectrum reflects motion in the same way as was described for saturation transfer EPR in fluid solution [160]. Between the turning points in the spectrum, a small reorientation of the molecule causes a large change in the resonant magnetic field, due to the anisotropy. Spin labeling of biological molecules has provided unique insights on motion. Studies of nitroxides in membranes have been the major contributor to understanding of the dynamics of membranes [59] and of rafts in membranes [161].

Transient radicals occur in many systems. Identifying them, and monitoring their dynamics is crucial to explicating the chemistry. Methods of studying such transient radicals depend on the lifetime and on the concentration of the radicals. Rapid mixing permits measuring fast reactions of small molecules and of biochemical changes. Technology has been developed to facilitate stopped flow [162] and rapid freeze-quench [163,164] EPR. Spin trapping is a technique by which a transient radical reacts with another molecule producing a radical that is more persistent than the original radical. The most common spin trapping method involves reaction with a nitron to produce a nitroxide [134]. Commonly, the nitroxide produced is more reactive than the nitroxides used for spin probes and spin labels, because there is a H where the more persistent nitroxides have a CH_3 protective group. The spin traps called DMPO and 4-POBN have been used to detect $OH^\cdot$ and superoxide, including radicals generated by radiation in vivo [165–168]. Improvements in spin trapping have produced traps such as DEPMPO [169] and BMPO [170] that are more persistent or more selective for particular common transient radicals, such as $OH^\cdot$, $O_2^{\cdot-}$, $R^\cdot$, etc., to yield nitroxides that persist for many minutes. Other types of spin traps have been developed for NO [171] that cannot be detected by EPR in fluid solution because it is a diatomic molecule.

4.5 Defect centers

A vast array of defects occur in natural and synthetic diamonds. One of the early demonstrations of EPR imaging used an N defect in natural diamond [172]. The long relaxation times of the N defect in diamond is being explored for multiple uses, including quantum computing [173]. Davies ENDOR (Fig. 8) permitted characterization of the proton hyperfine coupling in the E_2' defect in quartz [174].

Radiation causes characteristic defects in many materials. In many cases the defects last for very long times, which is the basis for archeological dating of teeth and quartz grains [175–177], dosimetry [178], and accident dosimetry [179]. Defects in irradiated crystalline alanine can be used to monitor radiation doses with high accuracy [180]. There is an ASTM procedure (ISO/ASTM 51607-04) and fully developed monitoring systems are available from Bruker BioSpin. Seeking improved radiation dosimetric materials, and characterizing them, is a major subfield of EPR. EPR signals occur in cut fingernails [181,182]. Teeth appear to be the best monitor of human radiation exposure [183,184], because defect centers are exceptionally long-lived (millennia).

4.6 Stability of foodstuffs

Many aspects of food chemistry involve free radicals. EPR is used routinely to monitor food preservation by irradiation, shelf life stability of beer, and stability of oils in foods. A method for analyzing the stability of beer by EPR has entered into widespread commercial use, both for monitoring product and ascertaining improved production methods [185,186]. EPR signals are used to monitor whether food has been irradiated to preserve it. Defects persist for longer times the drier and more rigid the location in which the defect formed. Thus, cellulosic and boney materials retain EPR signals longer than do fleshy materials.

4.7 Other applications

To provide a glimpse into the fascinating breadth of applications of EPR, we list additional areas with a leading reference, usually a book or other review: atmospheric pollution [187,188], carbon chars [189–191], catalysis [192,193], electrochemistry [194–196], even spin systems [112], free radical reactions [197–199], fullerenes [200], gas phase radicals [201–203], gemstones [204–206], magnetic field effects on reactions [207], molecular magnets [208], magnetic resonance force microscopy [209], multiphoton transitions [210], optically detected magnetic resonance (ODMR) [211–213], polymer chemistry [136,214], quantum dots [215,216], quantum computing [217], radiation damage and color centers [218] [219], sculpture limestone [220,221], hydrogenated silicon [222], spin exchange [208,223], spintronics [224], and supercritical fluids [225].

5. EPR in the formal student curriculum

With so many important contributions to understanding our world, it is surprising that EPR is not part of the standard undergraduate science curriculum. There is brief mention of EPR in some physical [226] and inorganic chemistry textbooks [227,228], but it seems to have disappeared from mainstream analytical chemistry textbooks. Morse is using EPR to introduce the scientific method to pre-college students [229]. Several small EPR instruments are being marketed (Bruker, Jeol, Magnetech, Adani, Phywe, Active Spectrum, Oxford Instruments, Resonance Instruments, Keycom), but at prices well above the cost of NMR instrumentation of comparable performance relative to research spectrometers. It is hoped that readers of this article will be inspired to include EPR in undergraduate lecture and lab courses to stimulate the next generation of scientists to use this powerful way of looking at the world.

6. The Future

After 68 years of vigorous development, EPR is undergoing a renaissance. Important refinements of current tools will make possible studies that at present are beyond reach. We foresee three major thrusts in the near future. (i) In vivo EPR, and especially in vivo imaging, will be a major contributor to the general trend in science toward understanding biochemistry and medicine at the organism level, not just isolated biomolecules. This will require substantial development of low-frequency EPR, in the range of hundreds of MHz to achieve adequate depth penetration of the RF. We hope for commercial spectrometers to broaden participation in this field. (ii) High frequency, high-field EPR will make major contributions to understanding high-spin species, and many spin-concentrated species, such as molecular magnets. Commercial spectrometers have recently become available up to 263 GHz. For some experiments, magnet costs will probably continue to restrict high frequency EPR to National Labs and Centers. However, the reduction in costs of superconducting magnets for NMR opens the possibility of EPR spectrometers at frequencies ca. 680 times the routinely available NMR frequencies. The major impediments will be designing the magnet to minimize distance between source/detector and the resonator, and obtaining a sufficiently wide magnetic field scan without boiling off liquid helium. Spectrometers may be restricted to certain applications. (iii) The third major change will be a reinvention of the EPR spectrometer to be fully digital – source, excitation patterns, and detection – performing CW, rapid scan, and pulse. We venture so far as to predict that direct-detection (i.e., no field modulation) magnetic field or microwave frequency scan methods will replace normal CW EPR and some of pulsed EPR spectral acquisition as the routine EPR of the future. We also predict increased focus on utility of spin relaxation times, and hence on the power of pulsed EPR to establish special spin conditions and separate contributions to relaxation.

7. Further Reading

Beyond the selected specific references cited, several recent textbooks and monographs will reward the reader. The standard EPR textbook, written from a physical chemistry perspective, is Weil and Bolton [230], the third edition of the important text that started as Wertz and Bolton. A short introduction to analysis of CW EPR is given by Rieger [231]. An introductory chapter, written for analytical chemists, is in Ewing's Analytical Instrumentation Handbook, 3rd edition [232]. Eaton et al. [8] teaches in detail how to perform quantitative EPR measurements. Measurements at multiple frequencies commonly are needed to understand EPR spectra. The extensive scope of multifrequency EPR is covered by the recent monograph edited by Misra [233]. Those interested in the history of the field will profit from Foundations of Modern EPR [234]. EPR at Work, a special thematic issue of Concepts in Magnetic Resonance, written by James Hyde and us, brings into an easily accessible volume, with updates, many of the informally published documents that stimulated the early development of EPR [235]. Many of the volumes in the Biological Magnetic Resonance series provide chapters that extend and enhance the content of the present article: in vivo [61], distance measurements [236], instrumentation and calculational methods [237,238], high-frequency [239], biomedical [60,66], membranes [59], metalloproteins [240] EPR.

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Highlights

- Applications of EPR range from quantum dots to in vivo imaging.
- EPR instrumentation is expanding to both higher and lower frequencies.
- Digital sources and detection will make instrumentation more flexible.
- Calculations of EPR parameters are beginning to guide experiments.

Fig. 1.

Comparison of rapid scan (1.8 MG/s) and conventional CW EPR spectra of the low-field nitrogen hyperfine line of ^{15}N -mHCTPO. (A) As-recorded sinusoidal rapid scan signal obtained with a scan rate of 1.8 MG/s and microwave power about 80 mW ($B_1 = 0.14$ G). 12,288 averages were recorded in about 0.9 sec. (B) Slow-scan absorption spectrum obtained by deconvolution of signal in A. (C) First derivative spectrum obtained by pseudomodulation of the signal in B. (D) Single scan of a conventional field-modulated first-derivative CW EPR spectrum of the same sample, obtained in 0.9 sec using 5 mW power, 10 kHz modulation frequency and 0.13 G modulation amplitude. Modulation amplitude, power, and filter were chosen to maximize signal amplitude with less than 2% broadening.

Fig. 2. Comparison of the signal amplitudes obtained by conventional field-modulated phase-sensitive detection and by direct detection. (A) In phase sensitive detection the amplitude is a fraction of the total signal, which is determined by the magnitude of the modulation. (B) In direct detection the full signal amplitude is detected.

Fig. 3.

The percent improvement in signal to noise, κ , that is achieved by combining the absorption and dispersion channels from a quadrature detector, is a function of the phase difference between the two channels. When the two channels are exactly 90° out of phase the improvement is $\sqrt{2}$. The calculations assume that the noise floor is defined prior to the detector.

Fig. 4.

Slice of a 250 MHz pulsed EPR image of pO_2 in a tumor-bearing mouse leg. The pO_2 was obtained from 5 electron spin echo images of the trityl radical OX063 injected into the mouse intravenously. Images were obtained at 5 different echo times from 0.7 to 2.4 μs to obtain relaxation rates in each voxel. pO_2 is proportional to the relaxation rate $1/T_{2e}$. The tumor contour is obtained from a co-registered T_2 -weighted MRI. (H. J. Halpern, unpublished results)

Fig. 5.

Comparison of X- and Q-band DEER measurements. Positions of the pump (solid arrows) and observe (dashed arrows) pulses are shown on the field-swept echo-detected spectra for an enzymatically reduced, spin-labeled protein (top). Contributions from MTSL [241] (short dashes) and FAD SQ^{•-} [242] (long dashes) to the field-swept echo-detected spectra were simulated using a locally written program [243] and are shown, with arbitrary y-axis scales. Time domain DEER dipolar evolution signals for enzymatically reduced, spin-labeled protein (95 μ M MTSL, $\sim$ 45 μ M FAD SQ^{•-}) were recorded at 80 K, averaging 23 scans overnight, followed by a 2nd degree polynomial background subtraction (bottom). X- and Q-band data are shown in grey and black, respectively. Signal-to-noise at Q-band is enhanced by approximately 10 times relative to X-band. The sample within the active volume of the resonators was approximately 40 μ L for X-band and 5 μ L for Q-band [107].

Fig. 6.

Easy axis EPR data for a single crystal of a tetrameric nickel complex at 172 GHz as a function of temperature. All 8 transitions within the $S = 4$ state are observed. Peaks that are highlighted with vertical dashed lines correspond to transitions between states in higher lying ($S < 4$) multiplets [110].

Fig. 7. Hyscore spectra at X-band (9.77 GHz) obtained for *Anabaena* flavodoxin neutral semiquinone. Spectra with different tau values are shown in different colors: red, $\tau = 96$ ns; blue, $\tau = 128$ ns; green, $\tau = 168$ ns; purple, $\tau = 208$ ns. The field position is 348 mT. Some level curves have been eliminated to make the picture clearer. The four regions identified as containing specific spectral features are marked with dashed contour lines. A, B, C, and D regions correspond to H(N5), other protons, N10, and N1–N3 features, respectively. Details are given in the original text [125].

Fig. 8. Davies ENDOR spectrum of the E_2' center measured at 60 K. The pair of peaks centered at 14.7 MHz unambiguously characterizes the coupling to a proton. Reproduced with permission from [174]

Table 1Qualitative guide to comparison of MRI and in vivo EPR imaging^a

	NMR imaging	EPR imaging
T ₁	hundreds of ms to seconds	1 to 20 μ s
T ₂	tens of ms to seconds	0.1 to 10 μ s
echo time, TE	tens of ms	a few μ s
encoding	use gradient encoding	no gradient encoding because of short T ₁
gradient rise time	ca. 100 μ s rise time	constant, stepped between projections, or rotated
magnet and resonator orientation	usually solenoid magnet; resonator axis magnet axis	resonator axis $\perp$ magnet axis to facilitate animal access
RF pulse power	5–23 kW peak power	< 4 kW
magnet homogeneity	< 5 ppm	$\pm$ 40 ppm
magnetic susceptibility	effects are important; $\pm$ need to shim for each image	negligible; no shimming needed
resolution	Human: 1 to 5 mm ³ for imaging; 1 to 8 cm ³ for spectroscopy; usually larger in the 3 rd dimension Small animals: 50 μ m resolution	1 mm ³ for spectral-spatial imaging
readout bandwidth	<500 KHz	< 1 Hz for CW, up to 5–10 MHz for rapid scan and pulse

^aThis table does not include the specialized fields of NMR microscopy [56] or EPR microscopy [57,58].