Journal of Magnetic Resonance 306 (2019) 102–108

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

Pulse EPR in biological systems – Beyond the expert's courtyard

Daniella Goldfarb

Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot, Israel

ARTICLE INFO

Article history:
Received 2 April 2019
Accepted 8 July 2019
Available online 9 July 2019

Keywords:
Pulse EPR
Distance measurements
PD-EPR
DEER
PELDOR

ABSTRACT

Application of EPR to biological systems includes many techniques and applications. In this short perspective, which dares to look into the future, I focus on pulse EPR, which is my field of expertise. Generally, pulse EPR techniques can be divided into two main groups: (1) hyperfine spectroscopy, which explores electron-nuclear interactions, and (2) pulse-dipolar (PD) EPR spectroscopy, which is based on electron-electron spin interactions. Here I focus on PD-EPR because it has a better chance of becoming a widely applied, easy-to-use table-top method to study the structural and dynamic aspects of biomolecules. I will briefly introduce this technique, its current state of the art, the challenges it is facing, and finally I will describe futuristic scenarios of low-cost PD-EPR approaches that can cross the diffusion barrier from the core of experts to the bulk of the scientific community.

1. Introduction

Application of EPR (Electron Paramagnetic Resonance) spectroscopy to biological systems has many faces and encompasses a large variety of methods [1]. Among these are the 'good old' CW-EPR (Continuous Wave EPR), which is still highly effective for deriving a wealth of information from EPR spectra, transient EPR, EPR imaging, pulse EPR, as well as the emerging fields of electrically and optically detected EPR in conjunction with single-molecule spectroscopy and sensing. Here, I focus on pulse EPR, which is closest to my heart. Pulse EPR developments and applications are currently practiced mainly in expert labs, with expensive, highly dedicated instrumentation. Additionally, effective use of the techniques commonly requires rather extensive training at the hardware/instrumental level as well as a good understanding of the fundamental theory of EPR and the associated spin physics. Therefore, the use of the rich pulse EPR toolbox remains within a rather small circle of experts and their collaborators. In a few cases, one can find centers that offer applications of pulse EPR as a service, leading to a broader range of users. When considering the future prospects of pulse EPR contributions to biological systems, providing structural (spatial and electronic) and dynamical information, the immediate question that comes to mind is whether pulse EPR methodology can be transformed into an easily accessed, benchtop, turnkey technology available to all interested. Can it become a user-friendly "black box", which is used routinely by non-EPR experts? Before trying to address these questions, I will briefly describe the state of the art of some aspects of pulse EPR and then proceed to speculate about a utopic future situation.

In general, pulse EPR techniques in the context of biological systems can be divided into two main categories: (i) Techniques that probe electron-nuclear interactions and are sensitive to length scales up to 1 nm, and are grouped under the name Hyperfine Spectroscopy. (ii) Techniques that focus on electron-electron spin interactions, probing length scales of 1–16 nm and are referred to as pulse dipolar EPR (PD-EPR) spectroscopy. Hyperfine spectroscopy targets primarily intrinsic paramagnetic centers in biological systems, e.g., transient and stable radicals and paramagnetic metal centers serving as catalytic active sites. Using hyperfine spectroscopic techniques, one can identify the various magnetic nuclei in the close environment of the electron spin, derive their hyperfine coupling, and consequently obtain information on the spatial and electronic structure of the paramagnetic center, which is a key to their function. The variability among the systems under study is enormous in terms of the spectroscopic properties of the center investigated (metal ions vs organic radicals, monomeric metal centers vs clusters and others). This means a great variation in terms of the choice of the most suitable technique and its experimental parameters, the frequency band to be used, and the temperature dictated by the relaxation times. It is highly plausible that in the future, the sensitivity will increase, measurement methods will become simpler, and the quantum chemical calculations necessary for deriving the structure of the paramagnetic center from spin Hamiltonian parameters will become highly automated, fast, and reliable. Nevertheless, the concept of "one size fits all" black box operation is hard to envision without the assistance of an expert because of the large spectroscopic variability. Therefore,

E-mail address: daniella.goldfarb@weizmann.ac.il

https://doi.org/10.1016/j.jmr.2019.07.038
1090-7807/

I do not envision Hyperfine spectroscopy EPR expanding much beyond the expert arena.

However, the situation is different for PD-EPR, which measures nanoscale distances. In the context of biological applications, PD-EPR relies heavily on spin labeling, which means introducing artificial paramagnetic centers to an otherwise diamagnetic biomacromolecule. Although there is a great variability in terms of the biological systems under study, the diversity in terms of spectroscopic properties depends on the choice of the spin label and currently the types of spin labels in use are relatively small. Moreover, the measured magnetic interaction, the electron-electron dipole interaction, is rather simple as it is mostly geometry related and the electronic structure of the paramagnetic center, which plays a considerable role in Hyperfine spectroscopy, can be ignored. Therefore, envisioning a black box's simple operation is not science fiction and this idea is developed next, after a brief discussion of the current state of the art of PD-EPR in biological systems.

2. PD-EPR

PD-EPR's application to biology usually reports on the distance between two spin labels, typically, but not necessarily, of the same type, conjugated to the biomolecule (protein or nucleic acid) at well-defined specific sites. This structural information is very important, in particular, when a series of spin label pairs can be independently engineered. For example, PD-EPR can be used for tracking conformational changes of a protein upon its interaction with small ligands, proteins, and nucleic acids, probing interactions between subunits in large complexes, and highlighting protein flexibility in solution [2–5].

PD-EPR measures the dipolar interaction between spin labels. In the simplest case of two spins, A and B, the dipolar interaction is given by

$$\omega_{dd} = \omega_{dd}^0\left(1 - 3cos^2\theta\right), \omega_{dd}^0 = \frac{\mu_0\mu_B^2 g_A g_B}{4\pi\hbar}\frac{1}{r_{AB}^3}, \tag{1}$$

where ω_{dd} is the dipolar splitting, μ_0 is the permeability of vacuum, $\hbar$ is the reduced Planck's constant, g_A and g_B are the g-factors of spins A and B, respectively, r_{AB} is the distance between spins A and B, and θ is the angle between the distance vector and the external magnetic field [6]. Currently, all PD-EPR sequences produce an echo, the intensity of which oscillates with the dipolar frequency, according to:

$$F(t) = 1 - \lambda\int_0^\infty P(r)\int_0^\pi \left[1 - \cos\left(\omega_{dd}t\right)\right]dr\sin\theta d\theta, \tag{2}$$

where$P(r)$is the distance distribution function and λ represents the percentage of spin pairs that contribute to the dipolar evolution in the experiment. Eq. (2) takes into account a powder average over all different possible orientations of the dipolar interaction relative to the magnetic field because measurements are usually carried out at low temperatures on frozen solutions. Eq. (2) holds under the weak coupling approximation. Because the electron-electron dipolar interaction is of a long range character, the inter-molecular dipolar interactions also contribute to the experimental signal and they are manifested as a background decay, given by B(t). The final expression for echo intensity, V(t) is given by:

$$V(t) = F(t)B(t) \tag{3}$$

Accordingly, extracting a distance distribution from V(t) requires evaluating the decay B(t) and its removal. For the most popular PD-EPR experiment, DEER (double electron-electron resonance, also called PELDOR), carried out on a frozen solution with a homogenous distribution of biomolecules, B(t) has an exponential decay form [7] $B(t) = e^{-Kc\lambda/t}$, where c is the spin concentration and K is a constant. It is possible to extract the distance distribution directly from the PD-EPR time domain trace using software packages kindly made available to the community by their developers [8]. Although data analysis is seemingly straightforward, it still has a few shortcomings and requires further development in terms of the analysis method used and evaluation of the error limits in the distance distribution. The background removal can introduce significant uncertainties in the distance distribution for long and broad distance distributions when the experimental evolution time is limited due to insufficiently long phase memory times [6]. In principle, this problem can be eliminated by reducing the sample concentration such that the intermolecular dipolar interactions become negligible. This, in turn, requires increased sensitivity. Another notorious problem is the presence of small peaks in the distance distribution, owing to SNR (signal-to-noise) limitations and background removal uncertainties. Such peaks are currently usually ignored and cannot be unambiguously attributed to minor conformations of the biomolecule. These pitfalls are particularly alarming for non-experts.

The first PD-EPR technique was actually the spin-echo decay [9], followed by the three-pulse DEER (double electron-electron resonance, or pulse electron-electron double resonance, PELDOR) experiment, which was introduced in the early 1980s by the Novosibirsk group [10,11]. Next appeared the single frequency "2 + 1" PD-EPR technique [12]. A major advance was introducing the four-pulse dead time-free DEER technique [13,14], which led to the great increase in DEER applications we have witnessed since then. An additional PD-EPR technique developed after DEER was the single-frequency DQC (double quantum coherence) technique [15]. The first application of DEER to proteins was reported in 2001 [16] and of DQC in 2002 [17]. Other PD-EPR methods such as the SIFTER [18] (single-frequency technique for refocusing dipolar couplings) and RIDME (relaxation-induced dipolar modulation enhancement) [19,20] followed. Further improvement of the DEER sequence involved implementing the 5-[21] and 7-pulse sequences, which require the use of shaped pulses [22]. This became possible with the recent introduction of arbitrary wave generators to EPR spectrometers, [23–25] which not only make new experiments [22] possible but also increase considerably the sensitivity of existing experiments [26–28].

In practice, the sensitivity of PD-EPR experiments is determined according to the minimal amount of sample needed to obtain results with a good SNR (signal-to-noise ratio) within a reasonable time. Currently, a typical measurement time for 10–100 μM protein solutions (amounts of protein in the nanomole range) are in the range of 1–12 h, depending on the sample concentration, the distance measured, and the spin label spectroscopic properties. Sensitivity improvement can be achieved by three measures: (i) Developing the instrumentation, for example, increasing the frequency, improving the resonator, increasing power, decreasing the noise characteristics of microwave components, and more. (ii) New measurement methodologies such as developing new pulse sequences and introducing shaped pulses. (iii) Development of new spin labels with optimal spectroscopic and spin dynamics properties. Among these, the spectral width and phase memory time play a major role. The rational design of spin labels with optimized properties is therefore of utmost importance and once done, the implementation is considerably cheaper than developing instrumentation. Protein deuteration is also an attractive option for increasing SNR and the accessible distance range [29]. In this context, introducing light-generated spin labels with non-Boltzmann polarization [30,31] is a direction with potential that is far from being fully realized.

2.1. Spin labels

The spin label employed in PD-EPR is of utmost importance and should be carefully chosen according to several criteria: (i) It does not disturb the structure and the function of the system. (ii) The conjugation chemistry to the biomolecule should be specific and effective, generating high labeling efficiency to minimize the number of singly labeled proteins in the sample. (iii) The conjugating linker should be short and potentially rigid to avoid significant contributions to the distance distribution. (iv) Unspecific interactions with the protein should be avoided to allow the spin label contributions to the distance distribution to be easily predicted. Additionally, the spin label should have (v) chemical stability that adheres to the system properties and the conjugation chemistry. (vi) It should exhibit optimized spectroscopic properties (spectral width, spin-lattice relaxation and phase memory times) for high-sensitivity measurements. (vii) It can be described by a simple spin Hamiltonian that allows a straightforward analysis of the experimental traces to generate the distance distribution.

Currently, the most commonly used spin labels are based on stable nitroxide radicals [32], which perform best at X- and Q-band frequencies. In recent years, measurements at Q-band have become the standard, owing to their increased sensitivity [33], thus outdating the formerly widespread X-band measurements. As high-field spectrometers (W-band and above) have become available and robust, other spin labels that perform well at high fields such as Gd(III) [34,35] and Mn(II) [36] chelates have been introduced during the past decade. The motivation for carrying out measurements at room temperature has led to the introduction of trityl radical spin labels [37], which deliver high sensitivity due to their narrow EPR spectrum. Cu(II)-based spin labels are also being considered [38], as well as optically generated triplet states [30,31]. Part of the development of new spin labels has been motivated by the addition of in-cell structural measurements [39–49] to the PD-EPR repertoire. In-cell PD-EPR has a great potential because of the minimal background signals and its insensitivity to the molecular size. These types of applications require redox stable spin labels.

The most common and straightforward method for labeling proteins is by conjugation to thiols of native or engineered cysteins [50], forming either S—S bonds or C—S bonds, depending on the linker chemistry. The dependence on cysteine poses difficulties when the protein has too many native cysteins or when cysteins are essential for the protein's activity and therefore they cannot be replaced or modified. This difficulty can be overcome by incorporating unnatural amino acids [51,52], such as *p*-acetyl-L-phenylalanine reacting with a hydroxylamine reagent to generate a nitroxide side chain. Other methods include the engineering of a small peptide loop that acts as a lanthanide binding site [53], or introducing a pair of histidines on a helix that can attach to a Cu(II) complex [38]. Several types of nitroxide-based [54,55] and a Gd(III)-based spin labels [49,56] specific for nucleic acids have been developed as well. The current availability of several types of spin labels and labeling chemistries allows for distance measurements in proteins between dissimilar spins-labels, such as Gd(III)-nitroxide [57,58], trityl-nitroxide [59] and Gd(III)-trityl [60] which feature some advantages over the homo-labeling-type schemes, depending on the system under study. Additionally, one can perform measurements between a native paramagnetic center and a spin label such as Cu(II)-nitroxide [61], Fe(III)-nitroxide [62], and Fe(III)-trityl [39]. For such spin pairs the RIDME experiment was found to be highly efficient [63]. The availability of different types of spin labels enables one to measure three distances per sample [64], as opposed to the standard one distance per sample. In this context, a two-dimensional PD-EPR experiment, called TRIER, aiming at correlating distances between three spin labels, has been recently developed [65].

In terms of applications, a new frontier is in-cell PD-EPR measurements, the potential of which has been demonstrated [66]; however, its development is still ongoing. Here, the main challenges are achieving sufficient sensitivity that can target physiological concentrations and the in-situ production of a stable spin-labeled biomolecule [45,67].

2.2. Current challenges

In the context of applications of PD-EPR spectroscopy in biology, I identified challenges in the following fronts: (i) Instrumentation and measurement methodology aimed at increasing the sensitivity needed mainly for the development of in-cell applications and for overcoming uncertainties in the distance distribution arising from the background decay. (ii) The labeling schemes and sample preparation, targeting in-situ labeling of biomolecules and optimized spin labels for high-sensitivity measurements. (iii) Data analysis, namely, deriving reliable distance distributions and associated uncertainties. (iv) Deriving atomic-level structural information in terms of conformational ensembles from a series of distance distribution. (v) Establishing room temperature measurements for tracking the conformational cycle of a protein during function and determined the associated kinetics.

There is active research in all of these fronts. The first three have reached a stage that allows many labs, which have access to pulse EPR spectrometers, to apply PD-EPR and obtain high-quality and significant results. Nonetheless, the issue of the best method for extracting the distance distribution from the time domain data as well as for correctly evaluating the error limits in the distance distribution [68–71] are still under debate. A different approach altogether for the data analysis, using machine learning methods, has been recently introduced [70] and will certainly be further developed. As for the fourth aspect, currently software for predicting distance distributions using known 3D structures is readily available [72–74]; however, refining structural models based on a series of distance distributions is possible mostly through collaboration with expert theoretical labs [75–77]. There have been efforts to make such calculations accessible to the community and an example is the very recent version of the MMM (Multiscale Modeling of Macromolecules) toolbox [78]. In its current version, MMM can build models based on the domain structure information from PDB files, different types of EPR restraints, selected types of NMR restraints, small-angle X-ray scattering (SAXS), and small-angle neutron scattering curves. I foresee that the above-mentioned (i–iv) challenges will probably be met within the next decade. Challenge (v) is more far-reaching and currently tracking conformational changes using a rapid freeze quench approach is still at the early infancy stage [79].

3. The future

The development of PD-EPR during the past two decades has been tremendous, from only 6 papers published in 2000, it increased steeply to 73 in 2016, according the ISI database. However, the increase in published PD-EPR papers seems to have leveled off in 2016–2018. It is interesting to compare these trends to single-molecule (sm) FRET (Förster resonance energy transfer), which is a method that in certain aspects is similar to PD-EPR, although the two methods should be considered complementary rather than competing techniques. Here the number of publications in 2000 was 8 and in 2018 it increased to 286. Interestingly, the average citation per paper for PD-EPR and sm FRET is the same,

26. These numbers and my acquaintance with the literature suggest that PD-EPR, in spite of its tremendous development and broad range of applications, has reached saturation in terms of applications. I interpret this leveling off as an indication of its restriction to EPR expert labs and their collaborators, namely, using magnetic resonance jargon, PD-EPR has not yet crossed the diffusion barrier over to the large community of Structural Biologists and Biophysicists. The question is why? Is it just because it has not yet caught the interest of non-expert labs? Or are there other reasons?

It has been realized that in order to obtain the structure and dynamics of large and complex biomolecular assemblies, applying the individual conventional biophysical techniques of X-ray crystallography, NMR spectroscopy, electron microscopy, and small-angle x-ray scattering (SAXS) is insufficient. Other techniques that provide structural and dynamic information of proteins and their assemblies should be included. This approach is referred to as "Integrative structural biology" and it is described in a recent perspective in Science magazine [80]. The other methods mentioned are "sequence comparisons of related proteins, co-purification, hydrogen-deuterium exchange mass spectrometry (HDXMS), single-molecule fluorescence, atomic force microscopy, analytical spectroscopy (both electron paramagnetic resonance and double electron-electron resonance), light scattering, chemical cross-linking, and mutagenesis." PD-EPR made it to the list (but not into the associated figure). The challenge is how to combine the very different types of information provided by these methods to generate the final structure and its conformational space. This perspective [80] suggests that PD-EPR begins to draw attention beyond the magnetic resonance community and that there are other reasons for the barrier. The two major obstacles that come to my mind are the limited availability of appropriate pulse EPR spectrometers, which is directly related to their high costs, and the EPR expertise required for both setting up the measurement and analyzing the data. The sample preparation is less of a problem because it employs fairly standard molecular biological and biochemical methods and because the most commonly used spin labels are commercially available. Another minor issue is that most of the experiments are not carried out under physiological conditions but instead at low temperatures, although this does not seem to affect the boom in cryo-EM use. It seems that in the future room-temperature PD-EPR will become more abundant [37,81,82], but its association with immobilization to prevent averaging of the dipolar interaction may not be more attractive in terms of mimicking physiological conditions than freezing would. Finally, dissemination of PD-EPR outside the magnetic resonance community may be lacking.

How can PD-EPR be transformed into an integral part of the tool box of a structural biologist? How can it become easily accessible to the community? One option is to have a central facility with state-of-the-art spectrometers and experts providing services to the scientific community. An example of such a center is ACERT at Cornell University. I envision a different scenario: The availability of a low-cost, table-top PD-EPR spectrometer (with a price comparable to an FPLC), operating at Q-band with a permanent magnet and controlled with an AWG unit, using a very high sensitivity micro-resonator and a cryo-free attachment that allows measurements in the range of 50–328 K (see Fig. 1). This spectrometer is dedicated and optimized for PD-EPR (probably DEER) on nitroxide spin labels such that the users do not have to adjust any experimental parameters and tuning is automatic. Just press start and wait for sufficient SNR, which can be automated as well. The spectrometer is equipped with an automatic sample changer, similar to standard high-throughput NMR spectrometers, affording measurements of a large series of samples around the clock. Here one can speculate that labeled proteins can be produced in the cell, skipping the purification and labeling steps. In this scenario each sample tube will contain cells with different overexpressed protein mutants. The data are then fed into a workstation that produces the distance distribution, with the option to generate a structure or an enable of structures for simple cases. In the more complicated cases, it can be fed into an integrative structural biology software for further analysis. This will generate structures of biomolecules in their native environment and will make the observation of proteins during their function inside the cell possible. This dream scenario does not seem too far reaching to me, since commercial table-top CW EPR spectrometers already exists.

A more far-reaching scenario would be an AFM (atomic force microscope) type machine having a cantilever with a sensing element based on nitrogen-vacancy (NV) centers in a diamond [83] that detects at the single-molecule level the dipolar interaction between two spin labels with sensitivity comparable to smFRET,

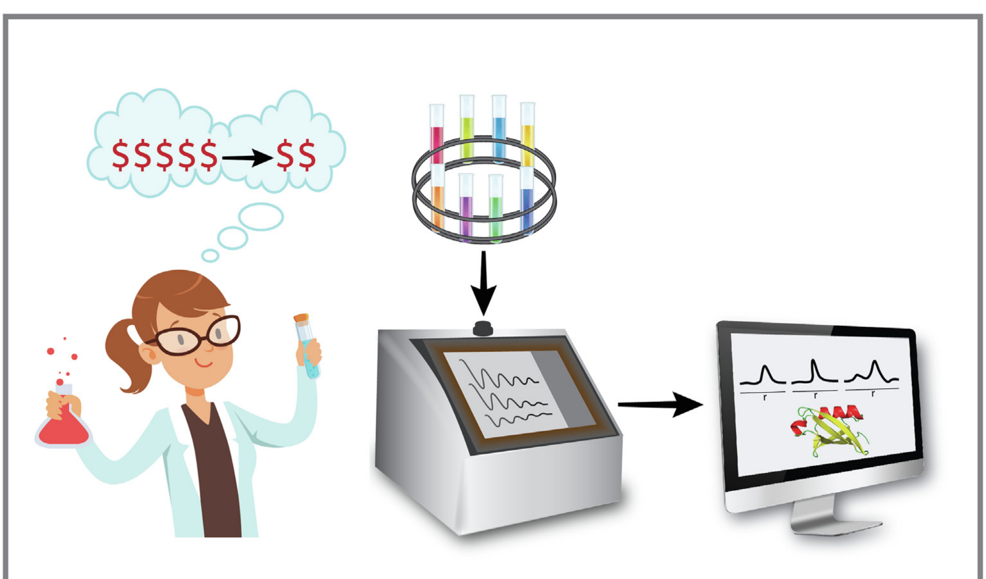

Fig. 1. A schematic illustration of a low-cost table-top PD-EPR spectrometer.

such that statistics can be collected in a short time to generate a histogram of accurate distances. This future scenario relies on recent exciting developments associated with the spin of a single nitrogen-vacancy (NV) center in diamond, which is a highly sensitive magnetic-field sensor. Briefly, optical excitation of the NV center generates a triplet state with a non-Boltzmann population distribution among its levels, having different decay rates back to the singlet ground state. A resonant microwave (MW) irradiation within the triplet levels changes their relative populations, thus affecting the fluorescence intensity just like normal ODMR (optical detection of magnetic resonance) [84]. Shi et al. [85] demonstrated the use of single NV center in bulk to measure the nitroxide spectrum of a labeled protein on the surface, in proximity to an NV center, under ambient conditions. DEER was applied at a fixed magnetic field with the MW echo generating pulses set to the NV frequency and the pump pulse frequency was swept in the range of the nitroxide spectrum. This generated a change in the NV fluorescence signal when the pump pulse was on resonance with the nitroxide and was used to record the spectrum of a single nitroxide label [86]. In a follow-up work the EPR spectrum of individual DNA duplexes labeled with a nitroxide spin label and tethered to a surface placed in aqueous buffer solutions was measured at ambient temperatures [85]. In these two works the DEER sequence was used to couple the NV detector to the paramagnetic center of interest. This can be further extended to measure dipolar coupling between two spin labels in a molecule by fixing the pump frequency to the frequency of one spin label, adding a second pump pulse with a third MW frequency applied to the second spin label, and sweeping the time of this pump pulse as is done in conventional DEER. This set-up has been recently applied to a polyproline peptide with two nitroxide spin labels separated by a few nanometers, adsorbed on a diamond surface [87]. Here only a few molecules were detected, owing to the high dilution of the sample.

With these pioneering works in mind and the necessary increase in SNR, I envision two approaches: one that has sufficient spatial resolution such that the position of the spin-bearing moiety in the molecule can be determined just from the spatial distance in an image; here, however, one will have to worry about the orientation of the molecules on the surface. The other option is to do this spectroscopically and measure the actual frequency shifts due to the dipolar coupling between the two as described above. In this case, one has to worry about the orientation of the magnetic field relative to the orientation of the molecules. In both cases, the issue of the orientation can be solved by analyzing many molecules with different orientations. The major obstacle in this approach is the need for orders of magnitude increase in sensitivity. The cost of the set-up should be low since the magnetic field is low, the optical excitation is standard and the MW frequencies are low, and therefore the components are cheap.

In the above attempt to examine PD-EPR in biology in the next decades I assumed that structural biology will still be of interest. However, as often happens in science, burning questions and subjects of today may be obsolete tomorrow and in this case there will be no driving force for the development of methods/instruments. However, looking back into a history of 75 years of magnetic resonance, I am optimistic that PD-EPR will prevail and tackle new exciting open questions.

Acknowledgments

This work was supported by Israel Science Foundation (ISF) – National Natural Science Foundation of China (NSFC) (grant number 118768). I thank Dr. Angeliki Giannoulis, Dr. Akiva Feintuch, Mr. Thorsten Bahrenberg, Prof. Sharon Ruthstein, and Prof. Arnold Raitsimring for critically reading the manuscript and Dr. Amit Finkler for helpful discussion on the future of NV centers in diamonds in magnetic resonance. This research was made possible in part by the historic generosity of the Harold Perlman Family. D.G. holds the Erich Klieger Professorial Chair in Chemical Physics.

References

[1] S. Stoll, D. Goldfarb, E.P.R. Spectroscopy, Fundamentals and Methods, John Wiley & Sons, 2018.
[2] D.P. Claxton, K. Kazmier, S. Mishra, H.S. McHaourab, Navigating nembrane protein structure, dynamics, and energy landscapes using spin labeling and EPR spectroscopy, Meth. Enzymol. 564 (2015) 349–387.
[3] C. Pliotas, Ion channel conformation and oligomerization assessment by site-directed spin labeling and pulsed-EPR, Meth. Enzymol. 594 (2017) 203–242.
[4] M.C. Puljung, H.A. DeBerg, W.N. Zagotta, S. Stoll, Double electron-electron resonance reveals cAMP-induced conformational change in HCN channels, Proc. Natl. Acad. Sci. U.S.A. 111 (2014) 9816–9821.
[5] G. Jeschke, DEER distance measurements on proteins, Annu. Rev. Phys. Chem. 63 (2012) 419–446.
[6] G. Jeschke, Y. Polyhach, Distance measurements on spin-labelled biomacromolecules by pulsed electron paramagnetic resonance, Phys. Chem. Chem. Phys. 9 (2007) 1895–1910.
[7] A.D. Milov, Y.D. Tsvetkov, Double electron-electron resonance in electron spin echo: conformations of spin-labeled poly-4-vinilpyridine in glassy solutions, Appl. Magn. Reson. 12 (1997) 495–504.
[8] G. Jeschke, V. Chechik, P. Ionita, A. Godt, H. Zimmermann, J. Banham, C.R. Timmel, D. Hilger, H. Jung, DeerAnalysis2006 - a comprehensive software package for analyzing pulsed ELDOR data, Appl. Magn. Reson. 30 (2006) 473–498.
[9] S.A. Dzyuba, A.M. Raitsimring, Y.D. Tsvetkov, Investigation of the paired radical-paramagnetic ion spatial distribution function by the electron spin echo method, Theor. Exp. Chem. 14 (1978) 150–156.
[10] A.D. Milov, K.M. Salikhov, M.D. Shirov, Application of the double resonance method to electron spin echo in a study of the spatial distribution of paramagnetic centers in solids, Fiz. Tverd. Tela (Leningrad) 23 (1981) 975–982.
[11] A.D. Milov, A.B. Ponomarev, Y.D. Tsvetkov, Electron-electron double resonance in electron spin echo: model biradical systems and the sensitized photolysis of decalin, Chem. Phys. Lett. 110 (1984) 67–72.
[12] V.V. Kurshev, A.M. Raitsimring, Y.D. Tsvetkov, Selection of dipolar interaction by the "2 + 1" pulse train ESE, J. Magn. Reson. 81 (1989) 441–454.
[13] M. Pannier, S. Veit, A. Godt, G. Jeschke, H.W. Spiess, Dead-time free measurement of dipole-dipole interactions between electron spins, J. Magn. Reson. 142 (2000) 331–340.
[14] R.E. Martin, M. Pannier, F. Diederich, V. Gramlich, M. Hubrich, H.W. Spiess, Determination of end-to-end distances in a series of TEMPO diradicals of up to 2.8 nm length with a new four-pulse double electron electron resonance experiment, Angew. Chem. Int. Ed. 37 (1998) 2833–2837.
[15] P.P. Borbat, J.H. Freed, Multiple-quantum ESR and distance measurements, Chem. Phys. Lett. 313 (1999) 145–154.
[16] M. Persson, J.R. Harbridge, P. Hammarström, R. Mitri, L.-G. Mårtensson, U. Carlsson, G.R. Eaton, S.S. Eaton, Comparison of electron paramagnetic resonance methods to determine distances between spin Labels on human carbonic anhydrase II, Biophys. J. 80 (2001) 2886–2897.
[17] P.P. Borbat, H.S. McHaourab, J.H. Freed, Protein structure determination using long-distance constraints from double-quantum coherence ESR: study of T4 lysozyme, J. Am. Chem. Soc. 124 (2002) 5304–5314.
[18] G. Jeschke, M. Pannier, A. Godt, H.W. Spiess, Dipolar spectroscopy and spin alignment in electron paramagnetic resonance, Chem. Phys. Lett. 331 (2000) 243–252.
[19] L.V. Kulik, S.A. Dzuba, I.A. Grigoryev, Y.D. Tsvetkov, Electron dipole-dipole interaction in ESEEM of nitroxide biradicals, Chem. Phys. Lett. 343 (2001) 315–324.
[20] S. Milikisyants, F. Scarpelli, M.G. Finiguerra, M. Ubbink, M. Huber, A pulsed EPR method to determine distances between paramagnetic centers with strong spectral anisotropy and radicals: the dead-time free RIDME sequence, J. Magn. Reson. 201 (2009) 48–56.
[21] P.P. Borbat, E.R. Georgieva, J.H. Freed, Improved sensitivity for long-distance measurements in biomolecules: five-pulse double electron-electron resonance, J. Phys. Chem. Lett. 4 (2013) 170–175.
[22] P.E. Spindler, I. Waclawska, B. Endeward, J. Plackmeyer, C. Ziegler, T.F. Prisner, Carr-Purcell pulsed electron double resonance with shaped inversion pulses, J. Phys. Chem. Lett. 6 (2015) 4331–4335.
[23] P.E. Spindler, S.J. Glaser, T.E. Skinner, T.F. Prisner, Broadband inversion PELDOR spectroscopy with partially adiabatic shaped pulses, Angew. Chem. Int. Ed. 52 (2013) 3425–3429.
[24] A. Doll, M. Qi, N. Wili, S. Pribitzer, A. Godt, G. Jeschke, Gd(III)-Gd(III) distance measurements with chirp pump pulses, J. Magn. Reson. 259 (2015) 153–162.
[25] C.E. Tait, S. Stoll, Coherent pump pulses in double electron electron resonance spectroscopy, Phys. Chem. Chem. Phys. 18 (2016) 18470–18485.
[26] A. Doll, M. Qi, S. Pribitzer, N. Wili, M. Yulikov, A. Godt, G. Jeschke, Sensitivity enhancement by population transfer in Gd(III) spin labels, Phys. Chem. Chem. Phys. 17 (2015) 7334–7344.
[27] A. Kuzhelev, D. Akhmetzyanov, V. Denysenkov, G. Shevelev, O. Krumkacheva, E. Bagryanskaya, T. Prisner, High-frequency pulsed electron-electron double

resonance spectroscopy on DNA duplexes using trityl tags and shaped microwave pulses, Phys. Chem. Chem. Phys. 20 (2018) 26140–26144.
[28] T. Bahrenberg, Y. Rosenski, R. Carmieli, K. Zibzener, M. Qi, V. Frydman, A. Godt, D. Goldfarb, A. Feintuch, Improved sensitivity for W-band Gd(III)-Gd(III) and nitroxide-nitroxide DEER measurements with shaped pulses, J. Magn. Reson. 283 (2017) 1–13.
[29] H. El Mkami, R. Ward, A. Bowman, T. Owen-Hughes, D.G. Norman, The spatial effect of protein deuteration on nitroxide spin-label relaxation: implications for EPR distance measurement, J. Magn. Reson. 248 (2014) 36–41.
[30] M. Di Valentin, M. Albertini, E. Zurlo, M. Gobbo, D. Carbonera, Porphyrin triplet state as a potential spin label for nanometer distance measurements by PELDOR spectroscopy, J. Am. Chem. Soc. 136 (2014) 6582–6585.
[31] C. Hintze, D. Bucker, S. Domingo Kohler, G. Jeschke, M. Drescher, Laser-induced magnetic dipole spectroscopy, J. Phys. Chem. Lett. 7 (2016) 2204–2209.
[32] M.M. Haugland, J.E. Lovett, E.A. Anderson, Advances in the synthesis of nitroxide radicals for use in biomolecule spin labelling, Chem. Soc. Rev. 47 (2018) 668–680.
[33] H. Ghimire, R.M. McCarrick, D.E. Budil, G.A. Lorigan, Significantly improved sensitivity of Q-band PELDOR/DEER experiments relative to X-band is observed in measuring the intercoil distance of a leucine zipper motif peptide (GCN4-LZ), Biochemistry 48 (2009) 5782–5784.
[34] R. Ward, A. Bowman, E. Sozudogru, H. El-Mkami, T. Owen-Hughes, D.G. Norman, EPR distance measurements in deuterated proteins, J. Magn. Reson. 207 (2010) 164–167.
[35] D. Goldfarb, Gd^{3+} spin labeling for distance measurements by pulse EPR spectroscopy, Phys. Chem. Chem. Phys. 16 (2014) 9685–9699.
[36] D. Banerjee, H. Yagi, T. Huber, G. Otting, D. Goldfarb, Nanometer-range distance measurement in a protein using Mn^{2+} tags, J. Phys. Chem. Lett. 3 (2012) 157–160.
[37] Z. Yang, Y. Liu, P. Borbat, J.L. Zweier, J.H. Freed, W.L. Hubbell, Pulsed ESR dipolar spectroscopy for distance measurements in immobilized spin labeled proteins in liquid solution, J. Am. Chem. Soc. 134 (2012) 9950–9952.
[38] T.F. Cunningham, M.R. Putterman, A. Desai, W.S. Horne, S. Saxena, The double-histidine Cu^{2+}-binding motif: a highly rigid, site-specific spin probe for electron spin resonance distance measurements, Angew. Chem. Int. Ed. 54 (2015) 6330–6334.
[39] J.J. Jassoy, A. Berndhauser, F. Duthie, S.P. Kuhn, G. Hagelueken, O. Schiemann, Versatile trityl spin labels for nanometer distance measurements on biomolecules in vitro and within cells, Angew. Chem. Int. Ed. 56 (2016) 177–181.
[40] A. Martorana, G. Bellapadrona, A. Feintuch, E. Di Gregorio, S. Aime, D. Goldfarb, Probing protein conformation in cells by EPR distance measurements using Gd3+ spin labelling, J. Am. Chem. Soc. 136 (2014) 13458–13465.
[41] M. Qi, A. Gross, G. Jeschke, A. Godt, M. Drescher, Gd(III)-PyMTA label is suitable for In-cell EPR, J. Am. Chem. Soc. 136 (2014) 15366–15378.
[42] R. Igarashi, T. Sakai, H. Hara, T. Tenno, T. Tanaka, H. Tochio, M. Shirakawa, Distance determination in proteins inside Xenopus laevis oocytes by double electron-electron resonance experiments, J. Am. Chem. Soc. 132 (2010) 8228–8229.
[43] I. Krstic, R. Haensel, O. Romainczyk, J.W. Engels, V. Doetsch, T.F. Prisner, Long-range distance measurements on nucleic acids in cells by pulsed EPR spectroscopy, Angew. Chem. Intl. Ed. 50 (2011) 5070–5074.
[44] M. Azarkh, V. Singh, O. Okle, D.R. Dietrich, J.S. Hartig, M. Drescher, Intracellular conformations of human telomeric quadruplexes studied by electron paramagnetic resonance spectroscopy, Chemphyschem 13 (2012) 1444–1447.
[45] F.C. Mascali, H.Y.V. Ching, R.M. Rasia, S. Un, L.C. Tabares, Using genetically encodable self-assembling Gd(III) spin labels to make in-cell nanometric distance measurements, Angew. Chem. Int. Ed. 55 (2016) 11041–11043.
[46] G. Karthikeyan, A. Bonucci, G. Casano, G. Gerbaud, S. Abel, V. Thome, L. Kodjabachian, A. Magalon, B. Guigliarelli, V. Belle, O. Ouari, E. Mileo, A bioresistant nitroxide spin label for in-cell EPR spectroscopy: In vitro and In oocytes protein structural dynamics studies, Angew. Chem. Int. Ed. 57 (2017) 1366–1370.
[47] Y. Yang, F. Yang, Y.J. Gong, J.L. Chen, D. Goldfarb, X.C. Su, A reactive, rigid Gd(III) labeling tag for in-cell EPR distance measurements in proteins, Angew. Chem. Int. Ed. 56 (2017) 2914–2918.
[48] Y. Yang, F. Yang, Y.-J. Gong, T. Bahrenberg, A. Feintuch, X.-C. Su, D. Goldfarb, High sensitivity in-cell EPR distance measurements on proteins using an optimized Gd(III) spin label, J. Phys. Chem. Lett. 9 (2018) 6119–6123.
[49] F. Wojciechowski, A. Groß, I.T. Holder, L. Knörr, M. Drescher, J.S. Hartig, Pulsed EPR spectroscopy distance measurements of DNA internally labelled with Gd^{3+}-DOTA, Chem. Commun. 51 (2015) 13850–13853.
[50] W.L. Hubbell, C. Altenbach, Investigation of structure and dynamics in membrane proteins using site-directed spin labeling, Curr. Opin. Struc. Biol. 4 (1994) 566–573.
[51] M.R. Fleissner, E.M. Brustad, T. Kalai, C. Altenbach, D. Cascio, F.B. Peters, K. Hideg, S. Peuker, P.G. Schultz, W.L. Hubbell, Site-directed spin labeling of a genetically encoded unnatural amino acid, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 21637–21642.
[52] E.H. Abdelkader, A. Feintuch, X. Yao, L.A. Adams, L. Aurelio, B. Graham, D. Goldfarb, G. Otting, Protein conformation by EPR spectroscopy using gadolinium tags clicked to genetically encoded p-azido-L-phenylalanine, Chem. Commun. 51 (2015) 15898–15901.
[53] D. Barthelmes, M. Granz, K. Barthelmes, K.N. Allen, B. Imperiali, T. Prisner, H. Schwalbe, Encoded loop-lanthanide-binding tags for long-range distance measurements in proteins by NMR and EPR spectroscopy, J. Biomol. NMR 63 (2015) 275–282.
[54] Y. Ding, P. Nguyen, N.S. Tangprasertchai, C.V. Reyes, X.J. Zhang, P.Z. Qin, Nucleic acid structure and dynamics: perspectives from site-directed spin labeling, in: B.C. Gilbert, V. Chechik, D.M. Murphy (Eds.) Electron Paramagnetic Resonance, vol. 24, 2015, pp. 122–147.
[55] N.S. Tangprasertchai, X.J. Zhang, Y. Ding, K. Tham, R. Rohs, I.S. Haworth, P.Z. Qin, An integrated spin-labeling/computational-modeling approach for mapping global structures of nucleic acids, Meth. Enzymol. 564 (2015) 427–453.
[56] Y. Song, T.J. Meade, A.V. Astashkin, E.L. Klein, J.H. Enemark, A. Raitsimring, Pulsed dipolar spectroscopy distance measurements in biomacromolecules labeled with Gd(III) markers, J. Magn. Reson. 210 (2011) 59–68.
[57] L. Garbuio, E. Bordignon, E.K. Brooks, W.L. Hubbell, G. Jeschke, M. Yulikov, Orthogonal spin Labeling and Gd(III)-nitroxide distance measurements on bacteriophage T4-lysozyme, J. Phys. Chem. B 117 (2013) 3145–3153.
[58] I. Kaminker, H. Yagi, T. Huber, A. Feintuch, G. Otting, D. Goldfarb, Spectroscopic selection of distance measurements in a protein dimer with mixed nitroxide and Gd^{3+} spin labels, Phys. Chem. Chem. Phys. 14 (2012) 4355–4358.
[59] B. Joseph, V.M. Tormyshev, O.Y. Rogozhnikova, D. Akhmetzyanov, E.G. Bagryanskaya, T.F. Prisner, Selective high-resolution detection of membrane protein-ligand interaction in native membranes using trityl-nitroxide PELDOR, Angew. Chem. Intl. Ed. 55 (2016) 11538–11542.
[60] A. Giannoulis, Y. Yang, Y.-J. Gong, X. Tan, A. Feintuch, R. Carmieli, T. Bahrenberg, Y. Liu, X.-C. Su, D. Goldfarb, DEER distance measurements on trityl/trityl and Gd(iii)/trityl labelled proteins, Phys. Chem. Chem. Phys. (2019).
[61] D. Abdullin, N. Florin, G. Hagelueken, O. Schiemann, EPR-based approach for the localization of paramagnetic metal Ions in biomolecules, Angew. Chem. Int. Ed. 54 (2015) 1827–1831.
[62] C.L. Motion, J.E. Lovett, S. Bell, S.L. Cassidy, P.A.S. Cruickshank, D.R. Bolton, R.I. Hunter, H. El Mkami, S. Van Doorslaer, G.M. Smith, DEER sensitivity between iron centers and nitroxides in heme-containing proteins improves dramatically using broadband, high-field EPR, J. Phys. Chem. Lett. 7 (2016) 1411–1415.
[63] A.V. Astashkin, Mapping the structure of metalloproteins with RIDME, Meth. Enzymol. 563 (2015) 251–284.
[64] Z. Wu, A. Feintuch, A. Collauto, L.A. Adams, L. Aurelio, B. Graham, G. Otting, D. Goldfarb, Selective distance measurements using triple spin labeling with Gd^{3+}, Mn^{2+} and a nitroxide, J. Phys. Chem. Lett. 8 (2017) 5277–5282.
[65] S. Pribitzer, M. Sajid, M. Hulsmann, A. Godt, G. Jeschke, Pulsed triple electron resonance (TRIER) for dipolar correlation spectroscopy, J. Magn. Reson. 282 (2017) 119–128.
[66] F.X. Theillet, A. Binolfi, B. Bekei, A. Martorana, H.M. Rose, M. Stuiver, S. Verzini, D. Lorenz, M. van Rossum, D. Goldfarb, P. Selenko, Structural disorder of monomeric alpha-synuclein persists in mammalian cells, Nature 530 (2016) 45–50.
[67] M.J. Schmidt, J. Borbas, M. Drescher, D. Summerer, A genetically encoded spin label for electron paramagnetic resonance distance measurements, J. Am. Chem. Soc. 136 (2014) 1238–1241.
[68] L. Fabregas Ibanez, G. Jeschke, General regularization framework for DEER spectroscopy, J. Magn. Reson. 300 (2019) 28–40.
[69] M. Srivastava, J.H. Freed, Singular value decomposition method to determine distance distributions in pulsed dipolar electron spin resonance: II. Estimating uncertainty, J. Phys. Chem. A 123 (2019) 359–370.
[70] S.G. Worswick, J.A. Spencer, G. Jeschke, I. Kuprov, Deep neural network processing of DEER data, Sci. Adv. 4 (2018) eaat5218.
[71] T.H. Edwards, S. Stoll, Optimal Tikhonov regularization for DEER spectroscopy, J. Magn. Reson. 288 (2018) 58–68.
[72] G. Hagelueken, R. Ward, J.H. Naismith, O. Schiemann, MtsslWizard: in silico spin-labeling and generation of distance distributions in PyMOL, Appl. Magn. Reson. 42 (2012) 377–391.
[73] G. Hagelueken, D. Abdullin, R. Ward, O. Schiemann, mtsslSuite: in silico spin labelling, trilateration and distance-constrained rigid body docking in PyMOL, Mol. Phys. 111 (2013) 2757–2766.
[74] Y. Polyhach, E. Bordignon, G. Jeschke, Rotamer libraries of spin labelled cysteines for protein studies, Phys. Chem. Chem. Phys. 13 (2011) 2356–2366.
[75] S.M. Islam, R.A. Stein, H.S. McHaourab, B. Roux, Structural refinement from restrained-ensemble simulations based on EPR/DEER data: application to T4 lysozyme, J. Phys. Chem. B 117 (2013) 4740–4754.
[76] C.M. Hammond, T. Owen-Hughes, D.G. Norman, Modelling multi-protein complexes using PELDOR distance measurements for rigid body minimisation experiments using XPLOR-NIH, Methods 70 (2014) 139–153.
[77] S.J. Hirst, N. Alexander, H.S. McHaourab, J. Meiler, RosettaEPR: an integrated tool for protein structure determination from sparse EPR data, J. Struct. Biol. 173 (2011) 506–514.
[78] G. Jeschke, MMM: a toolbox for integrative structure modeling, Protein Sci. 27 (2018) 76–85.
[79] A. Collauto, H.A. DeBerg, R. Kaufmann, W.N. Zagotta, S. Stoll, D. Goldfarb, Rates and equilibrium constants of the ligand-induced conformational transition of an HCN ion channel protein domain determined by DEER spectroscopy, Phys. Chem. Chem. Phys. 19 (2017) 15324–15334.
[80] A.B. Ward, A. Sali, I.A. Wilson, Integrative structural biology, Science 339 (2013) 913–915.
[81] M. Granz, N. Erlenbach, P. Spindler, D.B. Gophane, L.S. Stelzl, S.T. Sigurdsson, T. F. Prisner, Dynamics of nucleic acids at room temperature revealed by pulsed EPR spectroscopy, Angew. Chem. Int. Ed. 57 (2018) 10540–10543.

[82] V. Meyer, M.A. Swanson, L.J. Clouston, P.J. Boratynski, R.A. Stein, H.S. McHaourab, A. Rajca, S.S. Eaton, G.R. Eaton, Room-temperature distance measurements of immobilized spin-labeled protein by DEER/PELDOR, Biophys. J. 108 (2015) 1213–1219.
[83] M.S. Grinolds, M. Warner, K. De Greve, Y. Dovzhenko, L. Thiel, R.L. Walsworth, S. Hong, P. Maletinsky, A. Yacoby, Subnanometre resolution in three-dimensional magnetic resonance imaging of individual dark spins, Nat. Nanotech. 9 (2014) 279.
[84] J. Wrachtrup, A. Finkler, Single spin magnetic resonance, J. Magn. Reson. 269 (2016) 225–236.
[85] F.Z. Shi, F. Kong, P.J. Zhao, X.J. Zhang, M. Chen, S.Y. Chen, Q. Zhang, M.Q. Wang, X.Y. Ye, Z.C. Wang, Z.Y. Qin, X. Rong, J.H. Su, P.F. Wang, P.Z. Qin, J.F. Du, Single-DNA electron spin resonance spectroscopy in aqueous solutions, Nat. Meth. 15 (2018) 697–699.
[86] F. Shi, Q. Zhang, P. Wang, H. Sun, J. Wang, X. Rong, M. Chen, C. Ju, F. Reinhard, H. Chen, J. Wrachtrup, J. Wang, J. Du, Single-protein spin resonance spectroscopy under ambient conditions, Science 347 (2015) 1135.
[87] L. Schlipf, T. Oeckinghaus, K. Xu, D.B.R. Dasari, A. Zappe, F.F. de Oliveira, B. Kern, M. Azarkh, M. Drescher, M. Ternes, K. Kern, J. Wrachtrup, A. Finkler, A molecular quantum spin network controlled by a single qubit, Sci. Adv. 3 (2017) e1701116.