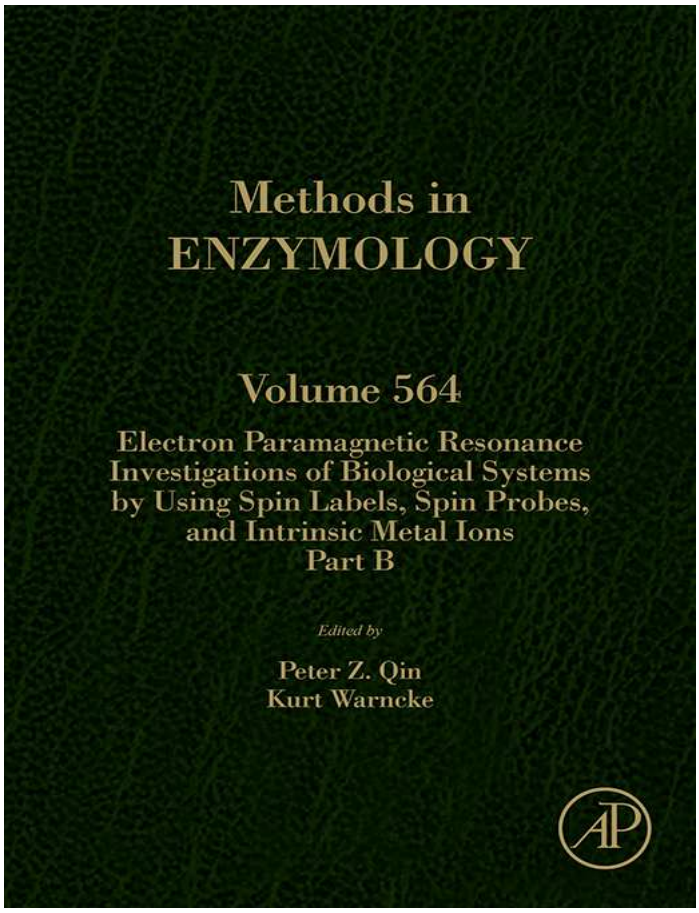
Provided for non-commercial research and educational use only.
Not for reproduction, distribution or commercial use.

This chapter was originally published in the book *Methods in Enzymology, Vol. 564* published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues who know you, and providing a copy to your institution's administrator.



All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at:

http://www.elsevier.com/locate/permissionusematerial

From B. Endeward, A. Marko, V.P. Denysenkov, S.Th. Sigurdsson and T.F. Prisner, Advanced EPR Methods for Studying Conformational Dynamics of Nucleic Acids. In: Peter Z. Qin and Kurt Warncke, editors, *Methods in Enzymology, Vol. 564*, Burlington: Academic Press, 2015, pp. 403-425.
ISBN: 978-0-12-802835-3
© Copyright 2015 Elsevier Inc.
Academic Press

Author's personal copy

CHAPTER FOURTEEN

Advanced EPR Methods for Studying Conformational Dynamics of Nucleic Acids

B. Endeward*, A. Marko*, V.P. Denysenkov*, S.Th. Sigurdsson†, T.F. Prisner*,[1]

*Institute of Physical and Theoretical Chemistry and Center of Biomolecular Magnetic Resonance, Goethe University Frankfurt am Main, Frankfurt am Main, Germany
†Department of Chemistry, Science Institute, University of Iceland, Reykjavık, Iceland
[1]Corresponding author: e-mail address: prisner@chemie.uni-frankfurt.de

Contents

1. Introduction 404
2. Spin Labels 407
3. Theory for Orientation-Selective PELDOR 408
4. Experimental Procedure for Multifrequency/Multifield PELDOR 410
   4.1 X-Band PELDOR (Low Magnetic Field) 410
   4.2 Q-Band PELDOR (Medium Magnetic Field) 412
   4.3 G-Band PELDOR (High Magnetic Field) 412
5. Analysis of Multifrequency/Multifield PELDOR Data 415
6. Summary and Outlook 418
Acknowledgments 421
References 421

Abstract

Pulsed electron paramagnetic resonance (EPR) spectroscopy has become an important tool for structural characterization of biomolecules allowing measurement of the distances between two paramagnetic spin labels attached to a biomolecule in the 2–8 nm range. In this chapter, we will focus on applications of this approach to investigate tertiary structure elements as well as conformational dynamics of nucleic acid molecules. Both aspects take advantage of using specific spin labels that are rigidly attached to the nucleobases, as they allow obtaining not only the distance but also the relative orientation between both nitroxide moieties with high accuracy. Thus, not only the distance but additionally the three Euler angles between both the nitroxide axis systems and the two polar angles of the interconnecting vector with respect to the nitroxide axis systems can be extracted from a single pair of spin labels. To extract all these parameters independently and unambiguously, a set of multifrequency/multifield

Methods in Enzymology, Volume 564
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2015.06.007

© 2015 Elsevier Inc.
All rights reserved.

Author's personal copy

pulsed EPR experiments have to be performed. We will describe the experimental procedure as well as newly developed spin labels, which are helpful to disentangle all these parameters, and tools which we have developed to analyze such data sets. The procedures and analyses will be illustrated by examples from our laboratory.

1. INTRODUCTION

Understanding the function of biomolecules relies on the knowledge of their structure and dynamics on an atomistic level. X-ray crystallography is the most successful technique to obtain structures of biological macromolecules, but requires crystals, a nonnatural environment for biomolecules. Also, due to this ordered crystallized state, limited information about conformational flexibility or dynamics of the molecules is available. Spectroscopic methods, like nuclear magnetic resonance or FRET (Förster resonance energy transfer), on the other hand, enable to gain information on structure and dynamics of the molecules in solution. PELDOR (pulsed electron–electron double resonance) (Milov, Ponomarev, & Tsvetkov, 1984; Milov, Salikov, & Shirov, 1981), also called DEER (double electron–electron resonance) (Larsen, Halkides, & Singel, 1993), is a method which can determine distances in the 2–8 nm range, similar to FRET.

PELDOR relies on the magnetic dipole interaction between two paramagnetic spin labels covalently attached to the macromolecule (Hubbell, Cafiso, & Altenbach, 2000). Pulsed EPR (electron paramagnetic resonance) techniques allow measurement of these weak interactions between the two spin labels, even if the strength of their interaction is much weaker than the interaction of the unpaired spin with the external magnetic field and surrounding nuclear spins. For a distance of 2 nm, the interaction strength in frequency units accounts to 7 MHz. The PELDOR time trace signal is modulated by this frequency and can be detected easily by Fourier transform of the signal or by other mathematical transformations (Chiang, Borbat, & Freed, 2005; Jeschke et al., 2006; Jeschke, Koch, Jonas, & Godt, 2002). For nitroxide spin labels, the time traces can usually be recorded for up to 10 μs at a temperature of 50 K. This results in a frequency resolution of about 100 kHz, corresponding to a very high distance accuracy of about 20 pm. Unfortunately, the internal flexibility of the covalently attached spin label itself blurs this high resolution. For the most commonly used nitroxide spin label MTSSL ((1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate) (Altenbach, Marti, Khorana, & Hubbell, 1990),

Author's personal copy

which can be covalently attached to cysteine amino acids residues, the distance distribution resulting from the rotameric freedom of the spin label itself limits the accuracy of the distance to about 300 pm (Fajer, Li, Yang, & Fajer, 2007; Jeschke, 2012; Sale, Song, Liu, Perozo, & Fajer, 2005).

Intrinsic paramagnetic cofactors, as amino acid radicals, metal ions, and iron sulfur centers exhibit a much higher accuracy in the distance determination, because they are usually rigidly incorporated into the protein environment (Becker & Saxena, 2005; Bennati et al., 2003; Biglino, Schmidt, Reijerse, & Lubitz, 2006; Denysenkov, Prisner, Stubbe, & Bennati, 2006; Kay, Elsässer, Bittl, Farrell, & Thorpe, 2006; Roessler et al., 2010; Van Amsterdam, Ubbink, Canters, & Huber, 2003). However, additional effort has to be undertaken to extract the distance information for such centers. This is caused by the broad anisotropic spectra, which inhibit an equal excitation of all random orientations of the interconnecting vector R by the microwave pulses for metal centers and clusters, and because of delocalized spin densities in coordinated metal ions and spin projection factors in FeS clusters (Bode, Plackmeyer, Bolte, Prisner, & Schiemann, 2009; Elsaesser, Brecht, & Bittl, 2005; Riplinger et al., 2009). Here, the orientation of the anisotropic hyperfine and g-tensors between both paramagnetic centers and with respect to the distance vector R also effects the dipolar oscillations of the PELDOR time traces and have, therefore, to be taken into account. On the other hand, if the orientation of these tensors within the molecular frame is known, this offers valuable additional angular information. Thus, instead of only determining spheres with a given distance between the two paramagnetic centers, all relevant angles between the two molecules can be determined, yielding complete structural information if the location of the paramagnetic cofactor in the protein environment is known. Several independent PELDOR experiments with distinct pump and detection frequencies have to be performed to extract this information unambiguously. Because the orientation between both paramagnetic centers cannot be assumed to be randomly distributed, Tikhonov regularization (Chiang et al., 2005; Jeschke et al., 2006, 2002) cannot be used to obtain the distance distribution function directly from the PELDOR time trace. Instead, more elaborate simulation methods have to be used to disentangle distances and angular information (Denysenkov et al., 2006; Margraf, Bode, Marko, Schiemann, & Prisner, 2007; Marko et al., 2010, 2009; Marko & Prisner, 2013; Polyhach, Godt, Bauer, & Jeschke, 2007; Schiemann, Cekan, Margraf, Prisner, & Sigurdsson, 2009).

Author's personal copy

The benefit of this additional angular information for structural biology was first demonstrated on a ribonucleotide reductase dimer. PELDOR measurements at X-band frequencies allowed determination of the distance between the two tyrosyl radicals of the dimer (Bennati et al., 2005, 2003). At high magnetic fields (6.4 T), the anisotropy of the tyrosyl *g*-tensor is fully resolved, which allowed the measurement of the dipolar interaction for all different orientations of the tyrosyl radicals with respect to the external magnetic field (Denysenkov, Biglino, Lubitz, Prisner, & Bennati, 2008; Denysenkov et al., 2006). From this set of measurements, the orientation of the two tyrosyl radicals with respect to the interconnecting *R* vector could be extracted, enabling full determination of the dimer structure just from a single pair of tyrosyl radicals.

As explained above, this orientation information is usually lost for covalently attached MTSSL spin labels, due to the large rotameric mobility of the spin label. Only in rare cases does interaction of the nitroxide spin labels with amino acid side groups or bound lipid molecules restrict the geometry of the labels (Abé et al., 2012; Endeward, Butterwick, MacKinnon, & Prisner, 2009; Sezer, Freed, & Roux, 2009). PELDOR experiments performed at different detection frequencies can easily detect such cases and indicate that more elaborate analysis of the PELDOR time traces is required to quantitatively interpret the data. Some spin labels, like the TOAC spin label (Karim, Kirby, Zhang, Nesmelov, & Thomas, 2004; McNulty & Millhauser, 2000) or newly developed spin labels for proteins (Hubbell, López, Altenbach, & Yang, 2013; Sajid, Jeschke, Wiebcke, & Godt, 2009), are more rigid and have the potential to allow determination of more precise distances and angular information in proteins.

Rigid spin labels already exist for nucleic acids, either intercalated into abasic sites of duplex DNA (Shelke & Sigurdsson, 2010) or as paramagnetic variants of the nucleobases cytosine or uracil. For the rigid spin labels, the orientation of the nitroxide moiety is locked to the structure of double-stranded DNA or RNA helices. This allows one to relate the obtained angular information directly to the tertiary structure of the oligonucleotide molecule. In this chapter, we describe spin labels for nucleic acids molecules with different degree of rotameric freedom and explain how the multi-frequency/multifield experiments, necessary to disentangle the distance and orientation information, has to be performed. In addition, we show how the data can be quantitatively analyzed to yield information about tertiary structure and conformational flexibility of the DNA and RNA molecules.

Author's personal copy

2. SPIN LABELS

Approximately 25 years ago, Spaltenstein, Robinson, and Hopkins (1989) developed a site-directed spin-labeling strategy for nucleic acids, where the spin label 2,2,5,5-tetramethylpyrroline-1-yloxy-3-acetylene (TPA), linked to the 5-position of uracil, was incorporated into DNA by solid-phase synthesis. This spin label was less flexible than other reported labels (Edwards & Sigurdsson, 2007; Piton et al., 2005; Qin et al., 2007; Qin, Hideg, Feigon, & Hubbell, 2003; Ramos & Varani, 1998; Schiemann et al., 2004, 2007; Sigurdsson & Eckstein, 1996; Strube, Schiemann, MacMillan, Prisner, & Engels, 2001), but still had a relatively large conformational freedom through rotation of the single bonds flanking the acetylene. Since this time, several other spin-labeling approaches have been reported (Shelke & Sigurdsson, 2012) some of which have less conformational flexibility than TPA. In particular, the rigid label Ç (c-spin) for DNA (Barhate, Cekan, Massey, & Sigurdsson, 2007) and the corresponding derivative for RNA, Çm (Höbartner, Sicoli, Wachowius, Gophane, & Sigurdsson, 2012; Fig. 1B) have an isoindoline nitroxide fused to a nucleobase, thus eliminating dynamics of the spin label independent of the nucleobase. These rigid labels were shown to be nonperturbing to DNA (Edwards et al., 2011) and RNA duplexes (Höbartner et al., 2012).

Due to the synthetic effort required to prepare Ç and Çm, the benzimidazole and benzoxazole derivatives ^{Im}U or ^{Ox}U were prepared (Fig. 1C; Gophane & Sigurdsson, 2013). Although rotation is possible around the single bond connecting the spin label to the base, making these labels less rigid than Ç, PELDOR experiments showed that due to an intramolecular hydrogen bond in ^{Im}U, the rotation around the single bond is strongly

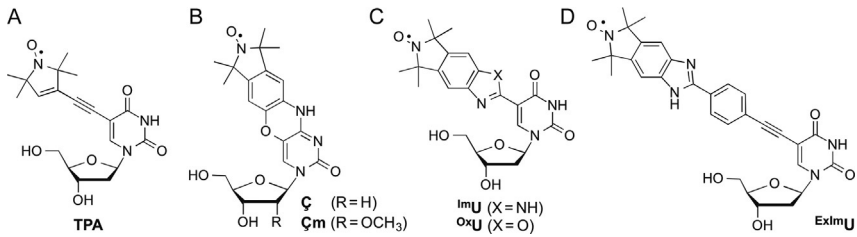


Figure 1 Semi-rigid and rigid spin labels. (A) The TPA spin label. (B) The rigid spin labels Ç and Çm. (C) The isoindoline-based spin labels ^{Im}U and ^{Ox}U. (D) The extended imidazole-based ^{ExIm}U spin label, which freely rotates around N–O axis (*x*-axis) of the nitroxide.

Author's personal copy

reduced (Gophane, Endeward, Prisner, & Sigurdsson, 2014). When the benzimidazole spin label was projected away from the nucleic acid with a longer and flexible linker, the orientation selection that was possible with ^{Im}U vanished. However, in this so-called conformationally unambiguous spin label, a rotation around the N–O axis (*x*-axis) of the spin label did not compromise the fixed position of the spin label relative to the nucleic acid. Therefore, the distance distribution that can be collected by one PELDOR time trace allows estimation of the distance as well as the flexibility of the nucleic acid. In this chapter, we will only concentrate on EPR applications of nucleic acid structures that contain rigid spin labels.

3. THEORY FOR ORIENTATION-SELECTIVE PELDOR

PELDOR is a two-frequency experiment which is performed on an ensemble of spin pairs in frozen disordered solutions (Milov et al., 1984, 1981). The dead-time-free four-pulse DEER sequence is commonly used (Fig. 2; Martin et al., 1998; Pannier, Veit, Godt, Jeschke, & Spiess, 2000). In order to observe the dipolar interaction between the two spins, the Larmor frequency of the first spin (A-spin) has to coincide with the detection frequency ν_A, and the Larmor frequency of the second spin (B-spin) has to be equal to the pump pulse frequency ν_B. For nitroxide spin labels, this condition is only fulfilled for a small part of the ensemble of spin-labeled molecules that have a special orientation with respect to the static magnetic field B_0, as explained below.

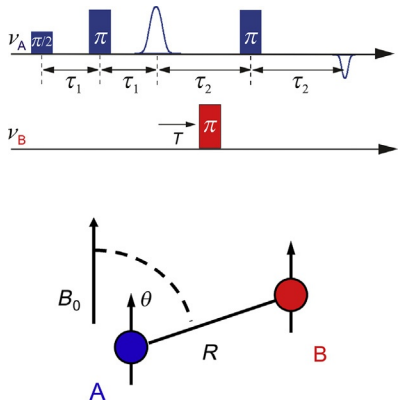


Figure 2 The dead-time-free four-pulse DEER sequence used for the experiments and a graphical representation of the parameters describing the magnetic dipolar coupling between both paramagnetic centers.

Author's personal copy

The A-spins which are excited by the three detection pulses, separated by the times τ_1 and τ_2, give rise to the refocused echo at $2(\tau_1 + \tau_2)$. The pump pulse inverts the B-spins that are resonant with the pump frequency. If a spin pair contains one A- and one B-spin, then the inversion of the B-spin changes the magnetic field at the position of the A-spin. This leads to a change of the A-spin Larmor frequency by the value ω_d determined by the strength of the magnetic dipolar interaction between the spin pair. The dipolar frequency

$$\omega_d(R,\theta) = \frac{D}{R^3}\left(1 - 3\cos^2\theta\right)$$

depends on the length of the distance vector R and its orientation θ with respect to the magnetic field B_0 (Fig. 2). $D = \mu_0\mu_B^2 g_A g_B/(4\pi\hbar)$ is the dipolar interaction constant, wherein μ_0 is the magnetic vacuum permeability, μ_B is the Bohr magneton, and $\hbar$ is the reduced Planck constant. For nitroxide spin labels, the g-values g_A and g_B correspond to 2.006, resulting in a value of the dipolar constant $D = 2\pi$ 52.04 MHz nm^3. The application of the pump pulse leads to a phase shift and a modulation of the refocused echo intensity. This phase shift, and therefore modulation of the intensity of the echo, depends on the time T between the A-spin Hahn echo and the pump pulse (Marko, Denysenkov, & Prisner, 2013). In nitroxide spin ensembles, only a fraction of the B-spin is flipped in each spin pair. Therefore, the refocused echo magnitude consists of a nonmodulated part $1-\lambda$ and a modulated part $\lambda\cos(\omega_d T)$, where λ is the probability to flip B-spins by the pump pulse.

Nitroxide spin labels in frozen solution have an inhomogeneous linewidth, which is dominated by the anisotropy of the nitrogen hyperfine interaction A (at X-band frequencies) or by the anisotropy of the g-tensor (at G-band frequencies). This inhomogeneous linewidth is larger than the spectral width of the mw-pulses, which allows excitation of different subensembles of the spin system by the pump and detection pulses (Fig. 3). Because the nitrogen hyperfine tensor A and the g-tensor are known (Möbius et al., 2005) in the molecular axis system, the specific orientations excited by pump and detection pulses can easily be calculated if the microwave field strength B_1 and the microwave frequency are known.

If the relative orientation of the nitroxides in the doubly labeled nucleic acid molecules of the ensemble is fixed, then the efficiency to excite simultaneously one spin by the detection pulse sequence and invert one spin by

Author's personal copy

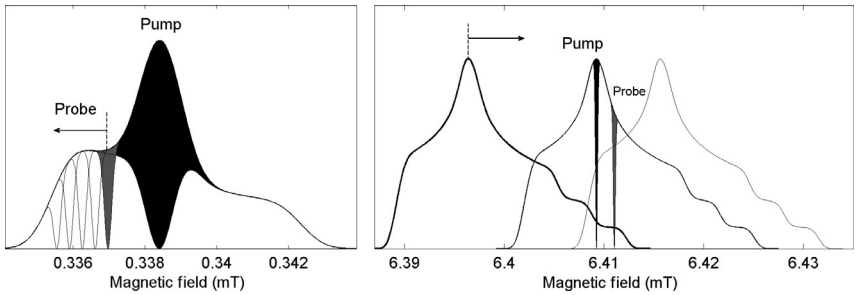


Figure 3 EPR absorption line shape for nitroxides at X-band frequencies (left) and at G-band frequencies (right). Typical pump (black) and detection (gray) pulse excitation profiles and positions are shown in the figures. Whereas at X-band frequencies the frequency offset between pump and detection pulses is varied, the magnetic field position is changed at G-band frequencies to obtain two-dimensional data sets.

the pump pulse strongly depends on the orientation of the nucleic acid molecule with respect to the external magnetic field B_0. The total normalized signal from an ensemble of such identical rigid biradicals in a powder sample is described by

$$S(T) = \mathrm{e}^{-\gamma T}\left(1 - \int_0^{\pi/2} \lambda(\theta)\left(\cos\left(\omega_\mathrm{d}(r,\theta)\,T\right) - 1\right)\sin\theta\,\mathrm{d}\,\theta\right)$$

Here, $\lambda(\theta)$ is the orientation-dependent B-spin flip probability density (Larsen & Singel, 1993; Milov, Maryasov, & Tsvetkov, 1998; Milov & Tsvetkov, 1997). The shape of this function is determined by the detection and pump pulses frequencies, relative orientation of nitroxides in double-labeled molecules, and the values of g- and hyperfine interaction tensors. The exponential factor corresponds to the intermolecular magnetic spin interaction.

4. EXPERIMENTAL PROCEDURE FOR MULTIFREQUENCY/ MULTIFIELD PELDOR

4.1 X-Band PELDOR (Low Magnetic Field)

At X-band frequency, the edges of the EPR spectra of nitroxides are governed by the large nitrogen hyperfine coupling along the molecular z-axis (out of plane axis). The contribution of the g-tensor anisotropy, as well as the hyperfine components along the x- (N–O bond direction) and y-axis, build up the intensity in the center of the spectrum (Margraf

Author's personal copy

et al., 2007). Therefore, in the center of the nitroxide EPR spectrum almost all orientation of the nitroxide with respect to the external magnetic field contribute, whereas the low- and high-field edges of the spectrum mainly relate to nitroxides, which have an alignment of the nitroxide z-axis parallel to the magnetic field. Thus, differently oriented subensembles of nuclear acid molecules can be selected by choosing different pump and detection frequencies. Taking advantage of these properties in the PELDOR experiment is called orientation selection (Margraf et al., 2007; Polyhach et al., 2007).

Typically, PELDOR experiments at 0.3 T magnetic field are performed by setting the pump pulse frequency resonant with spins in the center of the nitroxide spectra to reach a maximum inversion efficiency λ. The detection pulse sequence is set resonant with spins at the low-field edge of the nitroxide spectra to minimize spectral overlap between the pump and detection pulses. This simple picture already indicates some restrictions for performing orientation-selective measurements, where the detection frequency is varied (Fig. 3). A short pump pulse is chosen (typically 12 ns with an excitation bandwidth of 80 MHz) to excite a broad frequency range in the center of the nitroxide spectra resulting in a modulation depth parameter λ of approximately 0.43. To reach optimum B_1 field strengths, the frequency ν_B is adjusted to the microwave resonator frequency. The orientation selectivity is achieved by longer and more selective detection frequency pulses (typically 32 ns with a bandwidth of 30 MHz). Such pulses can be easily achieved off-resonant from the microwave cavity dip but have to be adjusted for each different offset. Experiments are performed with detection frequency offsets with respect to the pump pulse frequency, ranging from 40 to 100 MHz (up to the edge of the nitroxide spectrum). For the lowest offsets, a strong overlap between pump and detection pulse excitation occurs, which reduces the observable echo signal intensity and makes a quantitative prediction of the modulation depth parameter λ more difficult. Nevertheless, for quantitative analysis of the orientation selection, it is also important to acquire data sets where orientations of the magnetic field close to the x- and y-axis of the nitroxides are detected. Of course, such multi-frequency PELDOR experiments require more measurement time to achieve a good signal-to-noise ratio for all different detection frequencies. On the other hand, if only distance information is of interest, they can all be summed up, giving almost a similar signal-to-noise ratio as a classical 1D-PELDOR measurement. To average out the orientation selection, a data set with detection frequencies ranging from 30 to 90 MHz is required

Author's personal copy

(Prisner, Marko, & Sigurdsson, 2015). Further reducing the microwave power of pump and detection pulses increases the selectivity, which can be specifically beneficial for long distances. Of course, this also reduces the modulation depth further and, therefore, the signal-to-noise ratio of the dipolar oscillations. Thus, a compromise depending on the parameters of the specific system investigated has to be found. Gaussian or adiabatic pulses with better excitation profiles could improve the experiments for small pump–detection offsets.

4.2 Q-Band PELDOR (Medium Magnetic Field)

Q-band experiments offer a strongly improved signal-to-noise ratio even without a high power microwave amplifier (Gromov et al., 2001; Polyhach et al., 2012). For orientation-selective experiments, the situation is somewhat more complicated compared to X-band frequencies (where the hyperfine anisotropy is dominant) or G-band frequencies (where the *g*-tensor anisotropy defines the width of the spectrum). At Q-band frequencies, the contribution of the *g*-tensor and *A*-tensor anisotropies has similar sizes, making the orientation selection less intuitive and the analysis more complicated. Nevertheless, orientation selection can be observed at Q-band frequencies, as demonstrated on a double-labeled double-stranded DNA molecule in Fig. 4. Whereas the semi-rigid spin label ^{Im}U (Fig. 1; DNA spin labeled at two positions 8 nucleobases apart from each other) shows different PELDOR time traces for different offsets between pump and detection frequencies, such an effect is not visible for the spin label ^{ExIm}U, which can rotate around its N–O bond. Direct Tikhonov regularization leads to artifacts in the distance distribution in the first case, but such effects are not visible for the free rotatable spin label ^{ExIm}U (Fig. 4). Thus, a combination of both spin labels on the same nucleic acid molecule further simplifies the analysis of the data and the separation of orientation and distance information.

4.3 G-Band PELDOR (High Magnetic Field)

At high magnetic fields, PELDOR experiments of nitroxides appear at a first glance more challenging compared to standard frequencies, like X- or Q-band. This is due to a much broader EPR spectrum (about 250 G for nitroxides at 180 GHz, Fig. 3), and the fact that available excitation bandwidth of the pulses is much smaller (3 G at 180 GHz) due to limitations of available microwave power. Both effects decrease the sensitivity of the

Author's personal copy

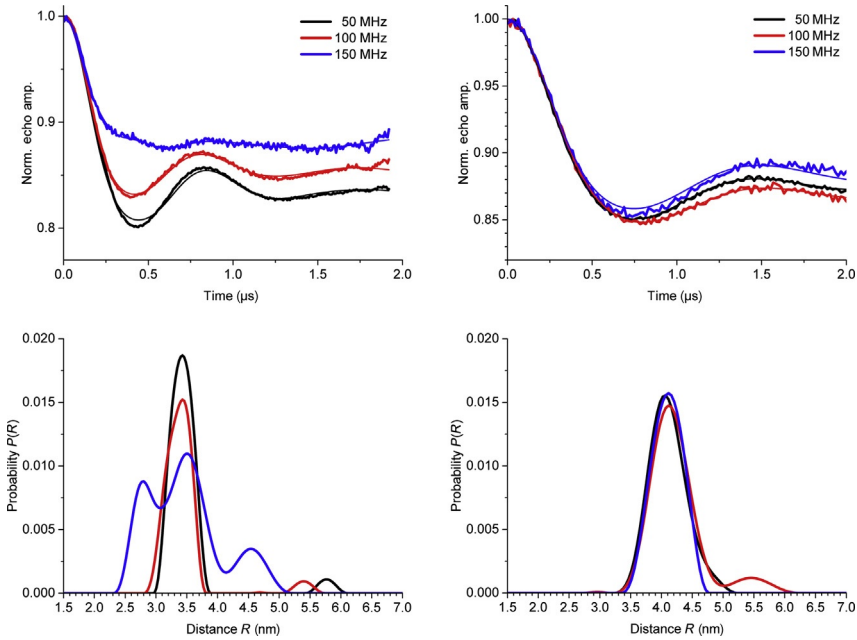


Figure 4 Orientation-selective PELDOR on a double-stranded DNA molecule measured at Q-band frequencies: upper row background corrected PELDOR time traces; lower row distance distribution estimated by Tikhonov regularization of PELDOR time trace. (A) ^{Im}U-spin-labeled DNA, eight residues apart. Different pump-detect frequency offsets at Q-band frequencies, due to the orientation selection is the distance analysis with standard Tikhonov regularization by using DeerAnalysis (Jeschke et al., 2006) total misleading. (B) ^{ExIm}U-spin-labeled but same DNA sequence and Q-band frequencies, all three distance distributions are equal, small differences are due to the direct interaction/overlap between the pump and detection pulses.

experiment because only very small fractions of the spins can be pumped and detected. On the other hand, only at high magnetic fields, all three molecular orientations can be distinguished by the *g*-tensor anisotropy, whereas at low fields the in-plane directions cannot be distinguished. Additionally, very high orientation selectivity can be achieved, overlap of pump and detection pulses is negligible and modulations by hyperfine interactions, which might lead to artifacts at low fields, are strongly suppressed at high fields.

Our available microwave power of 60 mW at 180 GHz, coupled to a cylindrical TE_{011} cavity with a $Q_{load} \sim 1000$, translates to an optimal $\pi/2$-pulse lengths of 30 ns at the detection frequency, the center of the cavity dip is tuned to that frequency. Due to the limited bandwidth of the resonator, the procedure to obtain 2D-PELDOR data sets differs from the variable detection frequency method that is used at X- and Q-band frequencies.

Author's personal copy

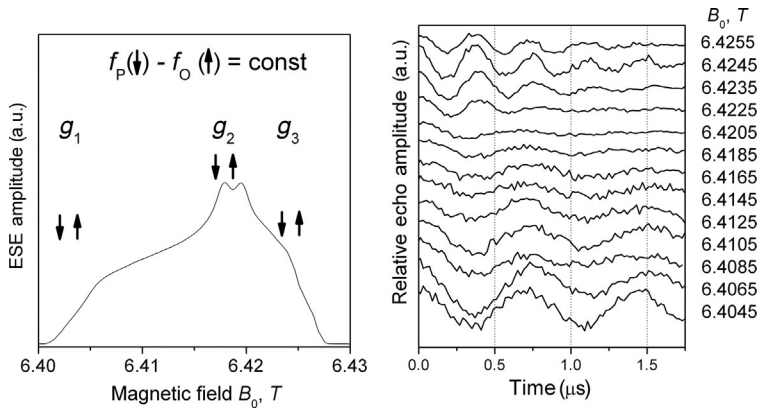


Figure 5 (Left) Field swept G-band spectrum of tyrosyl radical with pump–detection frequencies shown on the three main *g*-values. (Right) G-band PELDOR time traces recorded at different magnetic field values.

Here, the pump frequency f_P is set at a constant offset of 60 MHz from the detection frequency f_O, and PELDOR time traces are recorded for different external magnetic field values (Fig. 5). In this way, orientation-dependent dipolar modulation traces can be recorded throughout the whole EPR spectrum. This allows extraction of the mutual orientation of the two *g*-tensors of nitroxide spin labels that are rigidly attached to the nucleic acid molecule.

This procedure works nicely for flexible spin labels and in the case of collinear aligned spin labels. However, in a not collinear alignment of the two spins, offsets between pump and detection frequency in the full range of the spectral width need to be accessible. This is unfortunately not possible with a single-mode resonator. A possible solution to that problem is the use of a tunable double-mode resonator (Tkach, Sicoli, Höbartner, & Bennati, 2011) or by using a nonresonant sample holder (Cruickshank et al., 2009).

At our 180 GHz PELDOR setup, the time traces have only a few percent of modulation depth for nitroxide spin labels. One way to enlarge the modulation depth would be by using chirp pump pulses instead of fixed frequency rectangular pulses. A frequency chirp over 80 MHz of the pump pulse would already double the PELDOR modulation depth. The modulation depth and overall sensitivity of high-field PELDOR can be improved more significantly with a nonresonant sample holder and high transmitter output microwave power as demonstrated at the University of St. Andrews at W-band frequencies of 94 GHz (Cruickshank et al., 2009).

The first example demonstrating the power of orientation-selective PELDOR at high magnetic fields was the determination of the dimer

Author's personal copy

structure of ribonucleotide reductase. Class I ribonucleotide reductase is an ideal enzyme for this type of studies, since radical states are involved in each of the three principal mechanistic steps of the enzymatic turnover, i.e., the generation of the tyrosyl radical in the subunit R2, the radical initiation process between R2 and R1, and the chemistry of nucleotide reduction in R1. One major advantage in performing distance measurements between endogenous protein radicals by PELDOR is that they are located at very precise distances and orientations in the protein frame as determined by their role in the protein function (Bennati et al., 2003). This leads to orientation-selective dipolar modulation traces at high magnetic fields where the *g*-tensor anisotropy of these radicals is resolved.

Precise determination of the mutual orientation between both tyrosyl radicals could be achieved by analyzing the 2D-PELDOR data set at G-band frequencies (Fig. 5) acquired as previously described (Denysenkov et al., 2006). The principal values of the *g*- and proton *A*-tensors have been independently measured by EPR and ENDOR experiments before (Bender et al., 1989; Gerfen et al., 1993; Hoganson, Sahlin, Sjöberg, & Babcock, 1996) and were kept fixed within the simulation, which included the distance R between the tyrosyl radicals, determined by X-band PELDOR (Bennati et al., 2003). Assuming C_2 symmetry of the dimer structure a single set of Euler angles (α,β,γ) describing the rotation matrix between the two tyrosyl axis systems had to be optimized to fit the field-dependent PELDOR data. The fit was performed by varying the Euler angles in steps of $0.1°$. The effective field strength of the pump pulse was set to $\omega_{1B} = 4.0 \times 10^7$ rad/s, the field strength at the detection frequency was $\omega_{1A} = 5.2 \times 10^7$ rad/s corresponding to a π-pulse of 60 ns length.

By this procedure in one case the unknown dimer structure was determined, because the location of the tyrosyl radical within the monomer unit was known from X-ray crystallography (Denysenkov et al., 2008). In another case, where the dimer structure was already known from X-ray crystallography, a fine-tuning of the tyrosyl molecule in its radical state in the protein environment was possible, and the results were in perfect agreement with ENDOR experiments performed on single crystals (Kolberg et al., 2005).

5. ANALYSIS OF MULTIFREQUENCY/MULTIFIELD PELDOR DATA

Methods to analyze PELDOR time traces are well developed in the case where no orientational effects occur. No orientational effect means that

Author's personal copy

every orientation of the distance vector R of the spin pair with respect to the external magnetic field B_0 is detected with the same probability, leading to the well-known Pake-pattern of the dipolar coupling distribution (Borbat & Freed, 2007; Chiang et al., 2005; Martin et al., 1998; Pannier et al., 2000). Analyzing PELDOR time traces starts with the elimination of the background signal originating from the intermolecular dipole–dipole interaction between spin labels belonging to different proteins. The background corrected time domain signal is then converted into the distance distribution $P(R)$. This can be done by fitting the data with a model distance distribution function (e.g., one or more Gaussian distributions) or solving the integral equation employing the Tikhonov regularization of the ill-posed problem. The qualitative analysis of the time domain signal can already provide some information about the character of the distance distributions. If low-frequency oscillations are observable, then long distances can be expected between the spin labels. In such cases, observation time windows long enough to observe a full oscillation of the intramolecular interaction is necessary to quantitatively separate them from the intermolecular background signal.

In the case, where orientation selection exists, as explained above, the analysis procedure of the multifrequency/multifield PELDOR time traces has to include also the relative orientation between both spin labels (Marko et al., 2010; Schiemann et al., 2009). We have developed several approaches to analyze such data sets quantitatively. Simple geometrical modeling (Margraf et al., 2007; Schiemann et al., 2009) or comparison with MD simulations (Marko et al., 2009) can be used, if some prior knowledge about the molecule already exists. Analytical formula can be derived for specific cases and symmetries (Marko et al., 2010). A more general approach which does not require prior knowledge about the investigated molecule is to fit the PELDOR data with different pump/detection frequency offsets simultaneously to a calculated database containing each possible orientation and distance. This concept of a PELDOR signal database has proven to be very helpful for extracting the relative orientation between the spin labels from the experimental data set (Abé et al., 2012; Marko & Prisner, 2013). The signal database was generated for all possible relative orientations of the two radicals and stored. This is possible because all necessary parameters to fully describe the PELDOR signal of a nitroxide radical pair with a specific geometry are known. If the studied molecule is very rigid, then it is indeed possible to find unambiguously the corresponding signals from the database that fits best the experimental data set (Marko & Prisner, 2013). If the molecule can adopt several conformers, the program fits the

Author's personal copy

experimental data by an ensemble of structures from the database until a quantitative agreement is found. Although in all cases reported solutions fitting nicely to the experimental traces can be found, it is more difficult to prove the uniqueness of these solutions. The uniqueness of the solution depends very much on the quality and completeness of the multifrequency/multifield PELDOR data set and on the degree of flexibility of the molecule under study. Experiments taken at both low fields and high fields are mandatory to be able to determine the conformational flexibility of the biomolecule. Of course, several different spin labels and different spin label positions give further independent information, which can be used to exclude wrong fits or prove the correctness of the fit. An extraction of the true distance distribution function $P(R)$ can be achieved by averaging of the PELDOR time signals measured for different detection frequencies at X-band as explained above. A second approach is the use of two spin labels that distinguish themselves with their rotational freedom around the nitroxide N–O axis, as the ^{Im}U and ^{ExIm}U spin label. In such cases, the orientation information can be chemically switched on and off, allowing more easy separation of these parameters.

An example of such an analysis was the investigation of the conformational dynamics of double-stranded DNA molecules. Different models for the twist-stretch-bend motion of double-stranded DNA molecules existed and were debated in the literature. Where single-molecule fluorescence measurements of long DNA molecules attached to a magnetic bead were interpreted by a twist-stretch motion where mainly the radius of the double helix was changed within this motion (Gore et al., 2006), small angle X-ray scattering data on short double-stranded DNA molecules, where gold particles were attached to the ends, were interpreted by a correlated motion with a change in the pitch height (Mathew-Fenn, Das, & Harbury, 2008). Modeling studies on the other side predicted a substantial bending of the double-stranded DNA molecules (Becker & Everaers, 2007, 2009) as shown schematically in Fig. 6.

We could show with multifrequency/multifield PELDOR experiments (performed at X-band and G-band frequencies) that only the model with variable radius is consistent with our experimental data set. For this analysis, a 15-base pair long double-stranded DNA molecule was single spin labeled in both complimentary strands with the rigid spin label Ç (at 10 different positions). The PELDOR data sets were simulated with three different models and could be fitted only with Model B. The best simulation for the model (Model B) with a flexible radius and one pair of spin labels is

Author's personal copy

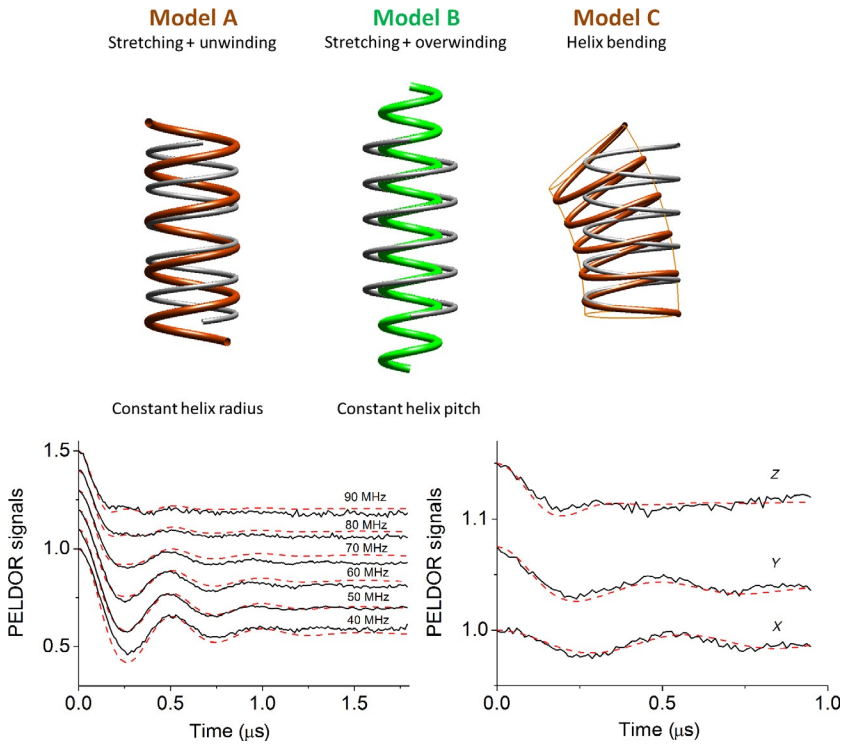


Figure 6 (Top) Three proposed conformation dynamics models of short double-stranded DNA helices. Model A describes double helix stretching with a constant radius and flexible pitch height. In Model B, the double helix shortens its radius and overwinds when stretched, keeping the pitch height constant. Model C allows the double helix to bend. (Bottom) Experimental data (solid) and best simulations (dashed) performed for the PELDOR signals of one single spin pair based on Model B showing good agreements for both X-band (left) and G-band measurements.

shown in Fig. 6. Analysis of the width of the distance distribution function $P(R)$ as a function of the number of base pairs between both spin labels additionally proved the correlation of the twist-stretch motion of Model B (Marko et al., 2011).

6. SUMMARY AND OUTLOOK

Acquiring a multifrequency/multifield data set is necessary for a comprehensive analysis of PELDOR time traces using rigid spin labels. Unfortunately, this makes the experiments more time consuming. However, this disadvantage is more than compensated by the much more precise distance

Author's personal copy

restraints and the additional gained angular restraints. Such an effort is especially beneficial if the conformational flexibility of the biomolecule is of interest. By means of rigid spin labels, such information can be extracted from multidimensional data sets. In such cases, Tikhonov regularization cannot be used anymore, because not only the distance but additionally the orientation between both spin labels determines the PELDOR time traces.

We have shown that a procedure we developed for rigid labels at X-band frequencies, where detection frequency offsets ranging from 30 to 90 MHz (in steps of 10 MHz) are taken and summed up, leads to an almost perfect averaging out of the orientation information for any given geometry (Prisner et al., 2015). The measurement time to acquire such a multifrequency data set is not much longer compared to a standard 1D-PELDOR experiment with a flexible spin label. As explained above, the distance accuracy is much higher making such spin labels attractive even if only distance information is of interest. Additionally, orientation information is available and can be extracted from the data set by fitting procedures (Marko & Prisner, 2013). Fortunately, all of the parameters beside the distance R, the Euler angels (α,β,γ), and the polar angles between the dipolar vector R and the nitroxide molecular axis system (θ, φ) are well known or can be calibrated from independent measurements. Something like a κ factor or a Förster radius R_0 in FRET spectroscopy does not exist! The only small uncertainties in the spin Hamiltonian are the exact values of the small in-plane hyperfine coupling values A_{xx} and A_{yy}, and the residual inhomogeneous linewidth arising from unresolved hyperfine couplings to other nuclear spins. This gives some uncertainty in modeling the exited detection and pump spins and therefore some inaccuracies in the modeling of the PELDOR time traces—especially at low field (X- and Q-band). The uncertainty becomes much less important at higher magnetic field values, where the orientation selection is strongly enhanced.

Another somewhat more serious problem arises from the pulse excitation profiles in the frequency domain, which are influenced by the resonator bandwidth and leads to rise time artifacts as well as B_1 inhomogeneities over the sample volume. The problem is, again, most pronounced at lower magnetic fields, where more overlap of pump and detection pulse excitation profiles can occur. At higher magnetic fields other experimental problems occur, only a small number of spins are detected and, more importantly, pumped, leading to very small modulation depths. Compensating this by better signal-to-noise requires a very high amplitude and phase stability of the detected echo signal. Nevertheless, with the very small modulation

Author's personal copy

depth (only few percent typically), the PELDOR time traces are very sensitive to experimental artifacts arising from the response of the detection channel to the excitation pulses.

Both problems described above may be overcome by hyperbolical pulses introduced recently as broadband pump pulses in PELDOR spectroscopy at X-band frequencies demonstrated improved performance (Spindler, Glaser, Skinner, & Prisner, 2013). The broader excitation profile of these pulses allows one to excite more spins, which could improve the modulation depth at high magnetic fields. Additionally, they also have a rectangular excitation profile in the frequency domain, which gives a well-defined selection of excited spins and can be used to avoid spectral overlap between pump and detection pulses. Recently, we carried out SIFTER dipolar experiment (Jeschke, Pannier, Godt, & Spiess, 2000; Schöps, Spindler, Marko, & Prisner, 2015) to show that such pulses have sufficient bandwidth to excite the full nitroxide spectrum at X-band frequencies without distortions. This offers a new and very promising perspective: to obtain the spectral dimension directly from the Fourier transformation of the echo signal. This would allow one to obtain all the orientation information without increased measurement time from the direct time domain Fourier transformation! Unfortunately, this dream is not reached yet; whereas a refocused and Hahn echo sequence can be created by such pulses using the Bohlen–Bodenhausen scheme (Bohlen, Rey, & Bodenhausen, 1989; Doll & Jeschke, 2014; Schöps et al., 2015), this does not hold true for the solid echo, which is used for the SIFTER experiment. The development of a phase coherent detection sequences with broadband pulses for SIFTER and DQC-EPR (Borbat, Mchaourab, & Freed, 2002) is in the focus of our current research efforts.

PELDOR experiments performed with rigid spin labels provide very detailed structural information on biomolecules. If these details are important and of particular interest, the extended effort in synthesis and data analysis is well spent. We believe that rigid spin labels will become more common for protein research as well. Because the spin label is rigidly linked to the much larger macromolecule, the overall tumbling rate is shifted into the slow-tumbling regime. This might allow to extend PELDOR experiments with such spin labels to room temperatures without the need of additional immobilization of the biomolecule (Babaylova et al., 2014; Meyer et al., 2015; Yang et al., 2012). Rigid spin label could be very attractive not only for distance measurements at physiological temperatures (avoiding freezing effects) but also to observe dynamics in the nanosecond to microsecond time range directly in the time domain PELDOR signal.

Author's personal copy

ACKNOWLEDGMENTS

Funding from the German Research Society (DFG) within the Collaborative Research Center CRC902 Molecular Principles of RNA-based Regulation is gratefully acknowledged as well as support from the Center of Biomolecular Magnetic Resonance (BMRZ) and the Center of Excellence Frankfurt Macromolecular Complexes (DFG).

REFERENCES

Abé, C., Klose, D., Dietrich, F., Ziegler, W. H., Polyhach, Y., Jeschke, G., et al. (2012). Orientation selective DEER measurements on vinculin tail at X-band frequencies reveal spin label orientations. *Journal of Magnetic Resonance*, *216*, 53–61.

Altenbach, C., Marti, T., Khorana, H. G., & Hubbell, W. L. (1990). Transmembrane protein structure: Spin labeling of bacteriorhodopsin mutants. *Science*, *248*(4959), 1088–1092.

Babaylova, E. S., Ivanov, A. V., Malygin, A. A., Vorobjeva, M. A., Venyaminova, A. G., Polienko, Y. F., et al. (2014). A versatile approach for site-directed spin labeling and structural EPR studies of RNAs. *Organic & Biomolecular Chemistry*, *12*(19), 3129.

Barhate, N., Cekan, P., Massey, A. P., & Sigurdsson, S. T. (2007). A nucleoside that contains a rigid nitroxide spin label: A fluorophore in disguise. *Angewandte Chemie, International Edition*, *46*(15), 2655–2658.

Becker, N. B., & Everaers, R. (2007). From rigid base pairs to semiflexible polymers: Coarse-graining DNA. *Physical Review E*, *76*(2), 021923.

Becker, N. B., & Everaers, R. (2009). Comment on 'remeasuring the double helix'. *Science*, *325*(5940), 538.

Becker, J. S., & Saxena, S. (2005). Double quantum coherence electron spin resonance on coupled Cu(II)–Cu(II) electron spins. *Chemical Physics Letters*, *414*(1–3), 248–252.

Bender, C. J., Sahlin, M., Babcock, G. T., Barry, B. A., Chandrashekar, T. K., Salowe, S. P., et al. (1989). An ENDOR study of the tyrosyl free radical in ribonucleotide reductase from Escherichia coli. *Journal of the American Chemical Society*, *111*(21), 8076–8083.

Bennati, M., Robblee, J. H., Mugnaini, V., Stubbe, J., Freed, J. H., & Borbat, P. (2005). EPR distance measurements support a model for long-range radical initiation in *E. Coli* ribonucleotide reductase. *Journal of the American Chemical Society*, *127*(43), 15014–15015.

Bennati, M., Weber, A., Antonic, J., Perlstein, D. L., Robblee, J., & Stubbe, J. (2003). Pulsed ELDOR spectroscopy measures the distance between the two tyrosyl radicals in the R2 subunit of the *E. Coli* ribonucleotide reductase. *Journal of the American Chemical Society*, *125*(49), 14988–14989.

Biglino, D., Schmidt, P. P., Reijerse, E. J., & Lubitz, W. (2006). PELDOR study on the tyrosyl radicals in the R2 protein of mouse ribonucleotide reductase. *Physical Chemistry Chemical Physics*, *8*(1), 58–62.

Bode, B. E., Plackmeyer, J., Bolte, M., Prisner, T. F., & Schiemann, O. (2009). PELDOR on an exchange coupled nitroxide copper(II) spin pair. *Journal of Organometallic Chemistry*, *694*(7–8), 1172–1179, Organo-Transition Metal Complexes Dedicated to Prof. Dr. Ch. Elschenbroich.

Bohlen, J.-M., Rey, M., & Bodenhausen, G. (1989). Refocusing with chirped pulses for broadband excitation without phase dispersion. *Journal of Magnetic Resonance (1969)*, *84*(1), 191–197.

Borbat, P. P., & Freed, J. H. (2007). Measuring distances by pulsed dipolar ESR spectroscopy: Spin-labeled histidine kinases. In A. C. Melvin I. Simon (Ed.), *Methods in enzymology: Vol. 423* (pp. 52–116). San Diego: Academic Press.

Borbat, P. P., Mchaourab, H. S., & Freed, J. H. (2002). Protein structure determination using long-distance constraints from double-quantum coherence ESR: Study of T4 lysozyme. *Journal of the American Chemical Society*, *124*, 5304–5314.

Author's personal copy

Chiang, Y.-W., Borbat, P. P., & Freed, J. H. (2005). The determination of pair distance distributions by pulsed ESR using Tikhonov regularization. *Journal of Magnetic Resonance*, *172*(2), 279–295.

Cruickshank, P. A. S., Bolton, D. R., Robertson, D. A., Hunter, R. I., Wylde, R. J., & Smith, G. M. (2009). A kilowatt pulsed 94 GHz electron paramagnetic resonance spectrometer with high concentration sensitivity, high instantaneous bandwidth, and low dead time. *Review of Scientific Instruments*, *80*(10), 103102.

Denysenkov, V. P., Biglino, D., Lubitz, W., Prisner, T. F., & Bennati, M. (2008). Structure of the tyrosyl biradical in mouse R2 ribonucleotide reductase from high-field PELDOR. *Angewandte Chemie, International Edition*, *47*(7), 1224–1227.

Denysenkov, V. P., Prisner, T. F., Stubbe, J., & Bennati, M. (2006). High-field pulsed electron–electron double resonance spectroscopy to determine the orientation of the tyrosyl radicals in ribonucleotide reductase. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(36), 13386–13390.

Doll, A., & Jeschke, G. (2014). Fourier-transform electron spin resonance with bandwidth-compensated chirp pulses. *Journal of Magnetic Resonance*, *246*, 18–26.

Edwards, T. E., Cekan, P., Reginsson, G. W., Shelke, S. A., Ferré-D'Amaré, A. R., Schiemann, O., et al. (2011). Crystal structure of a DNA containing the planar, phenoxazine-derived bi-functional spectroscopic probe Ç. *Nucleic Acids Research*, *39*(10), 4419–4426.

Edwards, T. E., & Sigurdsson, S. T. (2007). Site-specific incorporation of nitroxide spin-labels into 2′-positions of nucleic acids. *Nature Protocols*, *2*(8), 1954–1962.

Elsaesser, C., Brecht, M., & Bittl, R. (2005). Treatment of spin-coupled metal-centres in pulsed electron–electron double-resonance experiments. *Biochemical Society Transactions*, *33*, 15–19.

Endeward, B., Butterwick, J. A., MacKinnon, R., & Prisner, T. F. (2009). Pulsed electron–electron double-resonance determination of spin-label distances and orientations on the tetrameric potassium ion channel KcsA. *Journal of the American Chemical Society*, *131*(42), 15246–15250.

Fajer, M. I., Li, H., Yang, W., & Fajer, P. G. (2007). Mapping electron paramagnetic resonance spin label conformations by the simulated scaling method. *Journal of the American Chemical Society*, *129*(45), 13840–13846.

Gerfen, G. J., Bellew, B. F., Un, S., Bollinger, J. M., Jr., Stubbe, J., Griffin, R. G., et al. (1993). High-frequency (139.5 GHz) EPR spectroscopy of the tyrosyl radical in Escherichia coli ribonucleotide reductase. *Journal of the American Chemical Society*, *115*(14), 6420–6421.

Gophane, D. B., Endeward, B., Prisner, T. F., & Sigurdsson, S. T. (2014). Conformationally restricted isoindoline-derived spin labels in duplex DNA: Distances and rotational flexibility by pulsed electron–electron double resonance spectroscopy. *Chemistry: A European Journal*, *20*(48), 15913–15919.

Gophane, D. B., & Sigurdsson, S. T. (2013). Hydrogen-bonding controlled rigidity of an isoindoline-derived nitroxide spin label for nucleic acids. *Chemical Communications*, *49*(10), 999–1001.

Gore, J., Bryant, Z., Nöllmann, M., Le, M. U., Cozzarelli, N. R., & Bustamante, C. (2006). DNA overwinds when stretched. *Nature*, *442*(7104), 836–839.

Gromov, I., Shane, J., Forrer, J., Rakhmatoullin, R., Rozentzwaig, Y., & Schweiger, A. (2001). A Q-band pulse EPR/ENDOR spectrometer and the implementation of advanced one- and two-dimensional pulse EPR methodology. *Journal of Magnetic Resonance*, *149*(2), 196–203.

Höbartner, C., Sicoli, G., Wachowius, F., Gophane, D. B., & Sigurdsson, S. T. (2012). Synthesis and characterization of RNA containing a rigid and nonperturbing cytidine-derived spin label. *The Journal of Organic Chemistry*, *77*(17), 7749–7754.

Author's personal copy

Hoganson, C. W., Sahlin, M., Sjöberg, B.-M., & Babcock, G. T. (1996). Electron magnetic resonance of the tyrosyl radical in ribonucleotide reductase from Escherichia coli. *Journal of the American Chemical Society*, *118*(19), 4672–4679.

Hubbell, W. L., Cafiso, D. S., & Altenbach, C. (2000). Identifying conformational changes with site-directed spin labeling. *Nature Structural Biology*, *7*(9), 735–739.

Hubbell, W. L., López, C. J., Altenbach, C., & Yang, Z. (2013). Technological advances in site-directed spin labeling of proteins. *Current Opinion in Structural Biology*, *23*(5), 725–733, Protein-carbohydrate interactions/Biophysical methods.

Jeschke, G. (2012). DEER distance measurements on proteins. *Annual Review of Physical Chemistry*, *63*(1), 419–446.

Jeschke, G., Chechik, V., Ionita, P., Godt, A., Zimmermann, H., Banham, J., et al. (2006). DeerAnalysis2006—A comprehensive software package for analyzing pulsed ELDOR data. *Applied Magnetic Resonance*, *30*, 473–498.

Jeschke, G., Koch, A., Jonas, U., & Godt, A. (2002). Direct conversion of EPR dipolar time evolution data to distance distributions. *Journal of Magnetic Resonance*, *155*(1), 72–82.

Jeschke, G., Pannier, M., Godt, A., & Spiess, H. W. (2000). Dipolar spectroscopy and spin alignment in electron paramagnetic resonance. *Chemical Physics Letters*, *331*(2–4), 243–252.

Karim, C. B., Kirby, T. L., Zhang, Z., Nesmelov, Y., & Thomas, D. D. (2004). Phospholamban structural dynamics in lipid bilayers probed by a spin label rigidly coupled to the peptide backbone. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(40), 14437–14442.

Kay, C. W. M., Elsässer, C., Bittl, R., Farrell, S. R., & Thorpe, C. (2006). Determination of the distance between the two neutral flavin radicals in augmenter of liver regeneration by pulsed ELDOR. *Journal of the American Chemical Society*, *128*(1), 76–77.

Kolberg, M., Logan, D. T., Bleifuss, G., Potsch, S., Sjoberg, B. M., Graslund, A., et al. (2005). A new tyrosyl radical on Phe208 as ligand to the diiron center in Escherichia coli ribonucleotide reductase, mutant R2-Y122H: Combined X-ray diffraction and EPR/ENDOR studies. *Journal of Biological Chemistry*, *280*(12), 11233–11246.

Larsen, R. G., Halkides, C. J., & Singel, D. J. (1993). A geometric representation of nuclear modulation effects: The effects of high electron spin multiplicity on the electron spin echo envelope modulation spectra of Mn^{2+} complexes of N-*ras* p21. *The Journal of Chemical Physics*, *98*(9), 6704–6721.

Larsen, R. G., & Singel, D. J. (1993). Double electron–electron resonance spin-echo modulation: Spectroscopic measurement of electron spin pair separations in orientationally disordered solids. *Journal of Chemical Physics*, *98*, 5134–5146.

Margraf, D., Bode, B. E., Marko, A., Schiemann, O., & Prisner, T. F. (2007). Conformational flexibility of nitroxide biradicals determined by X-band PELDOR experiments. *Molecular Physics*, *105*(15–16), 2153–2160.

Marko, A., Denysenkov, V., Margraf, D., Cekan, P., Schiemann, O., Sigurdsson, S. T., et al. (2011). Conformational flexibility of DNA. *Journal of the American Chemical Society*, *133*(34), 13375–13379.

Marko, A., Denysenkov, V., & Prisner, T. F. (2013). Out-of-phase PELDOR. *Molecular Physics*, *111*(18–19), 2834–2844.

Marko, A., Margraf, D., Cekan, P., Sigurdsson, S. T., Schiemann, O., & Prisner, T. F. (2010). Analytical method to determine the orientation of rigid spin labels in DNA. *Physical Review E*, *81*(2), 021911.

Marko, A., Margraf, D., Yu, H., Mu, Y., Stock, G., & Prisner, T. (2009). Molecular orientation studies by pulsed electron–electron double resonance experiments. *The Journal of Chemical Physics*, *130*(6), 064102–064109.

Marko, A., & Prisner, T. F. (2013). An algorithm to analyze PELDOR data of rigid spin label pairs. *Physical Chemistry Chemical Physics*, *15*(2), 619–627.

Author's personal copy

Martin, R. E., Pannier, M., Diederich, F., Gramlich, V., Hubrich, M., & Spiess, H. W. (1998). 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. *Angewandte Chemie, International Edition*, *37*(20), 2833–2837.

Mathew-Fenn, R. S., Das, R., & Harbury, P. A. B. (2008). Remeasuring the double helix. *Science*, *322*(5900), 446–449.

McNulty, J. C., & Millhauser, G. L. (2000). TOAC—The rigid nitroxide side chain. In L. Berliner, G. R. Eaton, & S. S. Eaton (Eds.), *Biological magnetic resonance: Distance measurements in biological systems by EPR: Vol. 19* (pp. 277–307). New York: Kluwer Academic/Plenum Publishers.

Meyer, V., Swanson, M. A., Clouston, L. J., Boratyński, P. J., Stein, R. A., Mchaourab, H. S., et al. (2015). Room-temperature distance measurements of immobilized spin-labeled protein by DEER/PELDOR. *Biophysical Journal*, *108*(5), 1213–1219.

Milov, A. D., Maryasov, A. G., & Tsvetkov, Y. D. (1998). Pulsed electron double resonance (PELDOR) and its applications in free-radicals research. *Applied Magnetic Resonance*, *15*, 107–143.

Milov, A. D., Ponomarev, A. B., & Tsvetkov, Y. (1984). Electron–electron double resonance in electron spin echo: Model biradical systems and the sensitized photolysis of decalin. *Chemical Physics Letters*, *110*(1), 67–72.

Milov, A. D., Salikov, K. M., & Shirov, M. D. (1981). Application of the double resonance method to electron spin echo in a study of the spatial distribution of paramagnetic centers in solids. *Soviet Physics—Solid State*, *23*, 565–569.

Milov, A. D., & Tsvetkov, Y. D. (1997). Double electron–electron resonance in electron spin echo: Conformations of spin-labeled poly-4-vinilpyridine in glassy solutions. *Applied Magnetic Resonance*, *12*(4), 495–504.

Möbius, K., Savitsky, A., Wegener, C., Plato, M., Fuchs, M., Schnegg, A., et al. (2005). Combining high-field EPR with site-directed spin labeling reveals unique information on proteins in action. *Magnetic Resonance in Chemistry*, *43*(S1), S4–S19.

Pannier, M., Veit, S., Godt, A., Jeschke, G., & Spiess, H. W. (2000). Dead-time free measurement of dipole–dipole interactions between electron spins. *Journal of Magnetic Resonance*, *142*, 331–340.

Piton, N., Schiemann, O., Mu, Y., Stock, G., Prisner, T., & Engels, J. W. (2005). Synthesis of spin-labeled RNAs for long range distance measurements by PELDOR. *Nucleosides, Nucleotides & Nucleic Acids*, *24*(5–7), 771–775.

Polyhach, Y., Bordignon, E., Tschaggelar, R., Gandra, S., Godt, A., & Jeschke, G. (2012). High sensitivity and versatility of the DEER experiment on nitroxide radical pairs at Q-band frequencies. *Physical Chemistry Chemical Physics*, *14*(30), 10762.

Polyhach, Y., Godt, A., Bauer, C., & Jeschke, G. (2007). Spin pair geometry revealed by high-field DEER in the presence of conformational distributions. *Journal of Magnetic Resonance*, *185*(1), 118–129.

Prisner, T. F., Marko, A., & Sigurdsson, S. T. (2015). Conformational dynamics of nucleic acid molecules studied by PELDOR spectroscopy with rigid spin labels. *Journal of Magnetic Resonance*, *252*, 187–198.

Qin, P. Z., Haworth, I. S., Cai, Q., Kusnetzow, A. K., Grant, G. P. G., Price, E. A., et al. (2007). Measuring nanometer distances in nucleic acids using a sequence-independent nitroxide probe. *Nature Protocols*, *2*(10), 2354–2365.

Qin, P. Z., Hideg, K., Feigon, J., & Hubbell, W. L. (2003). Monitoring RNA base structure and dynamics using site-directed spin labeling. *Biochemistry*, *42*(22), 6772–6783.

Ramos, A., & Varani, G. (1998). A new method to detect long-range protein–RNA contacts: NMR detection of electron–proton relaxation induced by nitroxide spin-labeled RNA. *Journal of the American Chemical Society*, *120*(42), 10992–10993.

Riplinger, C., Kao, J. P. Y., Rosen, G. M., Kathirvelu, V., Eaton, G. R., Eaton, S. S., et al. (2009). Interaction of radical pairs through-bond and through-space: Scope and

Author's personal copy

limitations of the point–dipole approximation in electron paramagnetic resonance spectroscopy. *Journal of the American Chemical Society*, *131*(29), 10092–10106.

Roessler, M. M., King, M. S., Robinson, A. J., Armstrong, F. A., Harmer, J., & Hirst, J. (2010). Direct assignment of EPR spectra to structurally defined iron-sulfur clusters in complex I by double electron–electron resonance. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 1930–1935.

Sajid, M., Jeschke, G., Wiebcke, M., & Godt, A. (2009). Conformationally unambiguous spin labeling for distance measurements. *Chemistry: A European Journal*, *15*(47), 12960–12962.

Sale, K., Song, L., Liu, Y.-S., Perozo, E., & Fajer, P. (2005). Explicit treatment of spin labels in modeling of distance constraints from dipolar EPR and DEER. *Journal of the American Chemical Society*, *127*(26), 9334–9335.

Schiemann, O., Cekan, P., Margraf, D., Prisner, T. F., & Sigurdsson, S. T. (2009). Relative orientation of rigid nitroxides by PELDOR: Beyond distance measurements in nucleic acids. *Angewandte Chemie, International Edition*, *48*(18), 3292–3295.

Schiemann, O., Piton, N., Mu, Y., Stock, G., Engels, J. W., & Prisner, T. F. (2004). A PELDOR-based nanometer distance ruler for oligonucleotides. *Journal of the American Chemical Society*, *126*(18), 5722–5729.

Schiemann, O., Piton, N., Plackmeyer, J., Bode, B. E., Prisner, T. F., & Engels, J. W. (2007). Spin labeling of oligonucleotides with the nitroxide TPA and use of PELDOR, a pulse EPR method, to measure intramolecular distances. *Nature Protocols*, *2*(4), 904–923.

Schöps, P., Spindler, P. E., Marko, A., & Prisner, T. F. (2015). Broadband spin echoes and broadband SIFTER in EPR. *Journal of Magnetic Resonance*, *250*, 55–62.

Sezer, D., Freed, J. H., & Roux, B. (2009). Multifrequency electron spin resonance spectra of a spin-labeled protein calculated from molecular dynamics simulations. *Journal of the American Chemical Society*, *131*(7), 2597–2605.

Shelke, S. A., & Sigurdsson, S. T. (2010). Noncovalent and site-directed spin labeling of nucleic acids. *Angewandte Chemie, International Edition*, *49*(43), 7984–7986.

Shelke, S. A., & Sigurdsson, S. T. (2012). Site-directed spin labelling of nucleic acids. *European Journal of Organic Chemistry*, *2012*(12), 2291–2301.

Sigurdsson, S. T., & Eckstein, F. (1996). Site specific labelling of sugar residues in oligoribonucleotides: Reactions of aliphatic isocyanates with 2′ amino groups. *Nucleic Acids Research*, *24*(16), 3129–3133.

Spaltenstein, A., Robinson, B. H., & Hopkins, P. B. (1989). Sequence-and structure-dependent DNA base dynamics: Synthesis, structure, and dynamics of site, and sequence specifically spin-labeled DNA. *Biochemistry*, *28*(24), 9484–9495.

Spindler, P. E., Glaser, S. J., Skinner, T. E., & Prisner, T. F. (2013). Broadband inversion PELDOR spectroscopy with partially adiabatic shaped pulses. *Angewandte Chemie, International Edition*, *52*(12), 3425–3429.

Strube, T., Schiemann, O., MacMillan, F., Prisner, T., & Engels, J. W. (2001). A new facile method for spin-labeling of oligonucleotides. *Nucleosides, Nucleotides and Nucleic Acids*, *20*(4–7), 1271–1274.

Tkach, I., Sicoli, G., Höbartner, C., & Bennati, M. (2011). A dual-mode microwave resonator for double electron–electron spin resonance spectroscopy at W-band microwave frequencies. *Journal of Magnetic Resonance*, *209*(2), 341–346.

Van Amsterdam, I., Ubbink, M., Canters, G. W., & Huber, M. (2003). Measurement of a Cu-Cu distance of 26 Å by a pulsed EPR method. *Angewandte Chemie, International Edition*, *42*(1), 62–64.

Yang, Z., Liu, Y., Borbat, P., Zweier, J. L., Freed, J. H., & Hubbell, W. L. (2012). Pulsed ESR dipolar spectroscopy for distance measurements in immobilized spin labeled proteins in liquid solution. *Journal of the American Chemical Society*, *134*(24), 9950–9952.