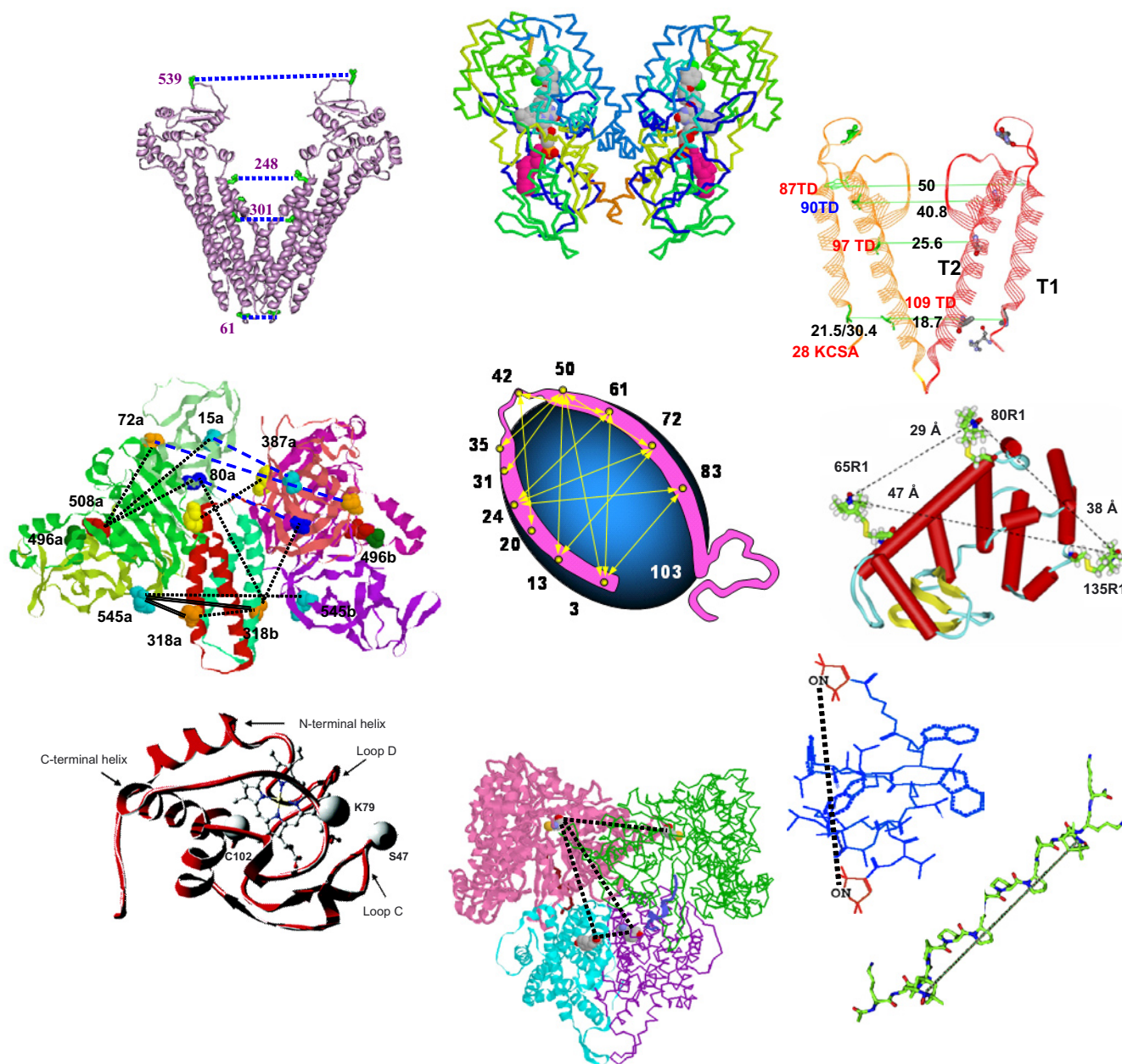


PROS AND CONS OF PULSE DIPOLAR ESR: DQC & DEER

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Front Cover. A collection of proteins and peptides studied at ACERT. The collection illustrates various points of PDS associated with each system studied.

Upper row. Left: Multi-drug ABC-transporter, MsbA; dimeric; reconstituted in membranes and detergent micelles; several functional states studied. **Center:** Monoamine oxidase, MAO-A (and MAO-B's from different organisms); oligomerization state in native membranes; reconstitution in detergent micelles and native membranes; spin-labeling with spin-labeled inhibitors and radical cofactors. **Right:** KcsA (Also MscL, and KvAP); tetramer membrane channel; tandem dimers and tetramers; liposomes; multiple distances in oligomeric state. (unpublished data).

Middle row. Left: Histidine kinase, CheA complex with CheW; triangulation; protein complex; oligomeric; heterodimers and heterodimeric complexes. **Center:** α -Synuclein: polymorphic (unstructured and highly structured); soluble and surface-bound to micelles and liposomes; MTSSL labeling; rigid-body modeling. **Right:** T4-lysozyme: multiple distances; deuterated solvents; triangulation; MTSSL labeling.

Bottom row. Left: iso-cytochrome c: folding; MTSSL labeling; wide distance ranges. **Center:** Ribonucleotide reductase (yeast), RNR: using radical cofactors and substrate; unlike spin labels. **Right:** Gramicidin doubly-labeled at C,N-termini: membrane-associated peptide; iod-acetamide labeling; equilibrium of different forms; aggregation aspects. **Far right:** Linear polyproline peptide Ac-OO-TOAC-PPPPPP-TOAC-OO-Amide. The peptide model was built with Insight II and edited using Pymol. The measured distance 27.86 Å is between nitrogen atoms of NO moieties of two TOAC residues. DQC provided 26.8 Å distance, 1.5 Å FWHM distribution. (Borbat & Kallenbach, unpublished data).

1. Introduction

Continuous-wave (cw) and pulsed ESR have been extensively applied to biological problems in the context of molecular dynamics [1–4] and are now increasingly applied to study biomolecular structure and function. cw ESR has been used to measure distances in the range of 6–20 Å between pairs of nitroxide spin labels [5–14]. Distance measurements using pulse ESR methods, a major advance in this area, are currently able to deliver long-distance constraints in the range of 10–80 Å [15–37]. The distance constraints from pulse ESR can for example be used to establish protein folding or orient and dock proteins and their subunits, yielding useful insights into the structure of a protein or a protein complex. They can also aid in refinement of NMR data. We refer to this emerging methodology as ‘pulsed dipolar (ESR) spectroscopy’ or PDS for short.

The PDS method¹ of double electron-electron resonance (DEER, also known as PELDOR) [38–41], was introduced more than two decades ago to circumvent problems in isolating weak electron-electron dipolar couplings from electron-spin-echo decays, which are usually dominated by relaxation and nuclear modulation effects [42, 43]. But there were few applications until the development of site-directed spin labeling (SDSL) as a useful tool of structural biology [44–46], as well as a modified version of DEER enabling its commercial implementation, and last but not least major efforts of dissemination. During that time, other pulsed methods of distance measurements were introduced [47–52], with the most useful being double-quantum coherence, (DQC) ESR [47, 48] (or DQC for short). Applications of DEER and DQC, to structural problems in biology have rapidly grown in number and scope in the last few years [3, 16, 18, 22, 23, 25, 28, 32, 33, 36, 37, 49, 53–56], with several reviews outlining the distance measurements [56–63].

We illustrate in this commentary PDS applications and methodology (both DQC and DEER) through examples from our laboratory, which cover many aspects of its applications to biomolecular structure and function. We only present here a short synopsis.

2. Distance Measurements

As ESR spectroscopists know well, the dipole-dipole part of the spin-Hamiltonian, H_{dd} between electron spins 1 and 2, (as relevant within this context) is given by

$$\frac{H_{dd}}{\hbar} = \frac{\gamma_e^2 \hbar}{r^3} (3 \cos^2 \theta - 1) \times \left[S_{1z} S_{2z} - \frac{1}{4} (S_1^+ S_2^- + S_1^- S_2^+) \right] \quad (1)$$

in high magnetic fields, where the non-secular terms (not shown) are unimportant [64]. One usually uses the point dipole approximation in employing Eq. (1), i.e. the electron spins are far enough apart that their distributions (in e.g. nitroxide p - π orbitals) are unimportant, (i.e. $r > 5$ Å for nitroxides). In Eq. (1), θ is the angle between the direction of the static magnetic field B_0 and $\mathbf{r} =$

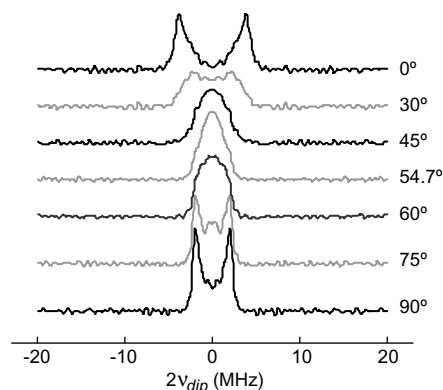


Fig. 1. An experimental dipolar spectrum of spin-labeled gramicidin A [24] obtained by 4-pulse DEER at several orientations in a macroscopically aligned lipid membrane bilayer of DMPC.

$(\mathbf{r}, \theta)$. The term in $S_{1z} S_{2z}$ in Eq. (1) is known as the secular term, and that in $S_1^+ S_2^-$ the pseudosecular term. The dipolar coupling in frequency units may be written as

$$A(r, \theta) = \omega_d (1 - 3 \cos^2 \theta) \quad (2)$$

with

$$\omega_d = \gamma_e^2 \hbar / r^3. \quad (3)$$

It leads to a splitting of the resonant line of each spin into a doublet². For the case of unlike spins, i.e. $\omega_d \ll |\omega_1 - \omega_2|$, (where ω_1 and ω_2 are the resonant frequencies of the two electron spins in the absence of dipolar coupling) the splitting is by $|A|$; the precise value of A depends on the angle θ , yielding a range of values of A from $-2\omega_d$ to $+\omega_d$. The PDS dipolar spectrum provides this splitting, which is shown in Fig. 1 as a function of the angle θ , obtained from a macroscopically aligned frozen sample. In the usual case of an isotropic frozen sample, one observes an average over θ , which yields a distinct dipolar spectrum, known as a Pake doublet [65], (cf. Fig. 2a). It shows a prominent splitting of ω_d , corresponding to $\theta = 90^\circ$, and another splitting of $2\omega_d$, corresponding to $\theta = 0^\circ$. The distance r is immediately and accurately obtained from a measurement of ω_d . This more familiar case of unlike spins corresponds to considering only the secular term in Eq. (1) and ignoring the pseudosecular term. In the case of like spins, i.e. $\omega_d \gg |\omega_1 - \omega_2|$, then the pseudosecular terms become important (a fact less appreciated) and Eq. (3) becomes

$$\omega_d = 3\gamma_e^2 \hbar / 2r^3.$$

¹ Both acronyms, PELDOR and DEER do not indicate the fact that they are solely concerned with dipolar couplings rather than dynamics. Also DQC obscures its application to dipolar couplings. Thus we prefer to use PDS to make more explicit the function of the methods.

² We leave out a discussion of electronic exchange, which for nitroxides is not significant above about 10 Å.

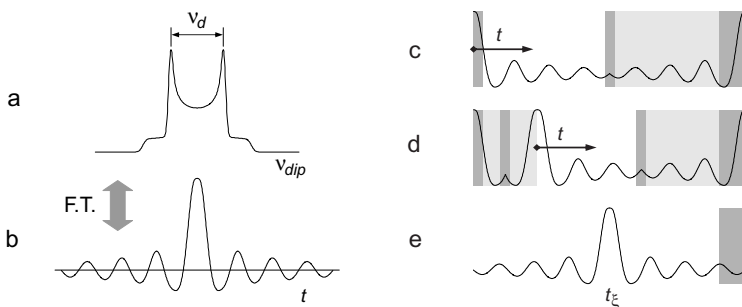


Fig. 2. a, b A dipolar spectrum in isotropic media (Pake doublet) from cosine Fourier transformation of the simulated time-domain signal. c, d and e Forms of dipolar signals for 3-pulse DEER (PELDOR), 4-pulse DEER, and DQC respectively. Shaded areas indicate repeats of the signals, which are not sampled. Wide dark bars depict dead-time zones, i.e. representing true dead-time when no signal can be detected due to overload conditions. Narrow bars correspond to conditions when the signal may be corrupted due to overlapping of pulses in the amplifier or due to bichromatic irradiation and step-wise signal phase shifts when pulses change their order. It is clear that 4-pulse DEER and DQC provide dead-time-free signals, with no off-resonance excitation effects in the latter. Usually, only one half of the signal is recorded in all three methods.

Otherwise the results (cf. Fig. 2) are equivalent. The intermediate case of $\omega_d \approx |\omega_1 - \omega_2|$ is more complex, and is handled by careful simulation using Eq. (1) including both secular and pseudosecular terms, (and using the full spin-Hamiltonian). In the case of nitroxide spin labels, the two nitroxide spins in a given molecule usually have their ω_1 and ω_2 substantially different. This arises from their different orientations with respect to the B_0 field, so their effective hf and g values (arising from their hf and g tensors) are different. At typical ESR frequencies this means that the unlike spin limit is valid generally only for ≥ 20 Å (9–17 GHz ESR).

If ω_d is sufficiently large, it can be determined from the broadening of the nitroxide cw ESR spectrum [7] but this is likely to fall into the regime where pseudo-secular terms are significant. Smaller couplings, ω_d require using pulse ESR methods. In all cases, accurate values of distances are produced from the measured dipolar couplings.

3. How do ESR and PDS Compare to Other Methods?

a) ESR vs. X-Rays and NMR

The primary sources of structure at atomic resolution are, of course, X-ray crystallography and NMR. Many biomolecules, however, are not amenable to study by NMR or crystallography for reasons such as insufficient quantities, inability to grow diffraction quality crystals, large molecular weight, poor solubility, or lack of stability, etc. Currently, determining the structure of a relatively small membrane protein is a challenge for both NMR and crystallography. The notable virtues of ESR-based limited structural

methods compared to X-ray and NMR methods are that the former require only tiny amounts (nano- to picomole [66] of proteins or other biomolecules), and they can be studied in a variety of environments, e.g. dilute solutions, micelles, lipid vesicles, native membranes, supported lipid bilayers, and more. There is no need to grow crystals or be concerned with long-term protein stability at high concentrations. Large biomolecules or complexes that are beyond the range of NMR or X-ray methods are not a major limitation; even unstable or transient biomolecules can be captured and studied. It is worth mentioning that PDS often relies on the availability of partial structural information provided by X-rays or NMR; and it may be employed synergistically, as was the case in recent applications [18, 32, 36].

b) ESR vs. FRET

FRET also provides distances over a range comparable to ESR. Its very high sensitivity, access to longer distances, and ability to operate at biological temperatures makes it a potent tool, but PDS has its distinct virtues. It has now become routine to express, purify, and spin-label dozens of mutants for nitroxide scan [67–69] or to produce and label a set of cysteine double-mutants for distance measurements. The distance between nitroxides as well as distance distributions is more accurately determined than between chromophores, since it is directly obtained from a simple frequency measurement, and there are no uncertainties in κ^2 as in FRET. There is usually a single type of reporter group, which is often a methanethiosulfonate spin label (MTSSL), and in most cases it introduces only a small perturbation

to the protein structure and function. Since the nitroxide side-chains are smaller in size than most fluorescent labels, the uncertainty of their positions relative to the backbone is less. A drawback of PDS, as well as of FRET, is that a limited number of constraints, which are themselves the distances between the reporter groups rather than the backbone C_α carbons may only provide limited insights into the structure. However, the detailed 3D structure is not always required, e.g. to elucidate the functional mechanism. But the fact that the distances are measured between the reporter groups does lead to a challenge in translating them into distances between the C_α carbons at the labeled sites. Modeling efforts, to overcome this, are in early stages of development [70].

c) CW and pulse ESR

CW ESR has been most often applied to nitroxides, whose powder spectra are dominated by the inhomogeneous broadenings from nitrogen hyperfine (hf) and g -tensors, and unresolved proton hf couplings. One has to extract what usually is a small broadening effect introduced by the dipole-dipole interactions between the spin labels to the nitroxide powder spectra. This is usually accomplished by spectral deconvolution [12] or by a rigorous spectral simulation with a multiple-parameter fit [7]. This often requires the spectra from singly-labeled species as a reference for the background broadening, which is a complication and not always an option. Incomplete spin labeling makes the task more complex [71]. For distances less than 15 Å, the dipolar coupling approaches other inhomogeneous spectral broadenings and then can be more easily inferred from cw ESR spectra. CW ESR is thus practical for short distances up to a maximum of ca. 15–20 Å, with the values for distances under 15 Å being more reliable [71].

Pulsed ESR is based on detecting a spin-echo, wherein the inhomogeneous spectral broadening cancels. Spin echo temporal evolution is governed by the weaker effects of spin relaxation and not refocused electron-electron dipolar and exchange couplings, and electron-nuclear super-hyperfine and nuclear quadrupole couplings. The dipolar and exchange coupling can be isolated from the rest by means of a suitable pulse sequence. This also helps to alleviate the problem caused by the presence of single labeled molecules. The direct signal from them is filtered out in some forms of PDS, but they do contribute to the background intermolecular dipolar signal, ▶

which is best suppressed by working at low concentrations. PDS is routinely used for distances longer than 15–20 Å [15, 18, 19, 22, 59, 32], and it works well all the way down to 10 Å [25], thus significantly overlapping with the cw ESR range, but it is much less affected by inefficient labeling and can readily yield distance distributions. The sensitivity of PDS is rather high as we show.

4. PDS at Work

The development of PDS has involved two stages. In the first, the fundamental aspects and details of the methods had to be developed, so they could be applied in the context of biomolecular structure and related applications. The second stage has been its practical use, wherein subtle details are of lesser concern, with the main goal being to solve structures by distance constraints. Substantial progress has been made, rendering such applications routine [18, 22, 32, 33, 60, 62, 63], although there is much room for further developments.

To illustrate the current stage of development of PDS at ACERT, we have assembled a small zoo, populated with selected species of proteins and peptides that we have studied, which are portrayed on the Front Cover. Although we cannot show here most of the signals and distance distributions, we note that they are of a very good or excellent quality, well in line with ACERT standards. The collage on the front cover represents cases that illustrate the following aspects: i) Protein environment (soluble, reconstituted in detergent micelles or liposomes, in natural membrane environments); ii) Oligomerization status (establishing the state of oligomerization, circumventing problems of multiple spins, heterodimers, tandem dimers and tetramers); iii) The state of folding (unfolding/refolding equilibrium in denaturants and freeze-trapped); iv) Spin-labeling aspects (naturally-occurring radicals, spin-labeled

substrates and inhibitors, MTSSL labeling, other nitroxide labels, termini labeling); v) PDS modes (single distance, multiple distances, triangulation); vi) PDS methods used (DQC, DEER); vii) Peptides (water soluble, organic solvents, lipid vesicles, macroscopically-aligned lipids; dimers, conformers, aggregates, spin-counting, equilibrium, affinity, membrane composition); viii) Functional studies (capturing functional states with substrate mimetics, pH, ligands); ix) Data processing aspects (background removal, distance distribution and refinement, distance embedding, rigid-body modeling); x) Oligonucleotides (long-distance constraints to aid NMR); xi) Protein complexes (binding and docking, tertiary and ternary structure, large supramolecular complexes, mobile subunits and domains).

a) Single-distance measurement

When a rough structure or the oligomeric state of a protein complex is of interest, a few distances may suffice [15]. This mode of PDS has been used most often to produce a critical distance or its change, providing insights into a key structural aspect, such as the location of a binding interface or the extent of conformational change.

b) Multiple-distance measurements

Obtaining more detailed structural information is usually more involved, since it requires obtaining several distances in order to select among possible conformations of a protein or a protein complex, by checking that all experimental distances are consistent with the model [35, 53]. The sites should be accessible for the spin-labeling reagent, and they should not alter protein structure or function; this may limit their selection.

c) Triangulation

The 'triangulation' approach to protein mapping [20, 32], is based on obtaining a network of distance constraints from a set of

spin labeled sites such that they uniquely define the coordinates of all (or most) of the sites. A sufficiently large rigid distance network (scaffold) based on tetrahedra [20, 32] strongly restrains positions of spin labels and thereby the possible conformations of the protein (cf. Fig. 3). Such constraints can be used to solve the protein structure at a low resolution of ± 5 Å. This task can be accomplished by making a sufficient number of double mutations and then measuring the distances between the respective pairs of spin labels in a 'one-at-a-time' manner. It is not feasible, in general, to obtain distances simultaneously amongst several spin labels due to the flexibility of the side-chains and the structural heterogeneity of proteins, which usually yield fairly broad distributions in each distance. However, there can be favorable cases [16, 32, 72] (cf. Fig. 4).

d) Oligomeric proteins

Many proteins are oligomeric and they require additional care to obtain the required set of constraints. Even the simplest case of a dimeric protein of CheA [32] required one to select mutation sites such that the measured distance could be isolated from other distances possible between more than two labels. Doubly-labeled homodimeric protein carries four spin labels, therefore six distances are possible in general. They can be resolved in cases when all (or most of them) are strongly immobilized. Otherwise the distance of interest should be well-isolated from the rest. This approach was successfully implemented in triangulation study of CheW binding to CheA [32, 62]. In the case of tetrameric membrane channels (KcsA), tandem dimers were also used to provide better resolved dipolar spectra. Tandem tetramers can also be expressed and folded for KcsA or KvAP, and they can be applied to set up triangulation. Another aspect of work with oligomeric proteins is to establish their oligomeric state in native environment which was accomplished for monoamine oxidase (MAO) in the outer mitochondrial membrane [37].

e) Protein complex

The potential problems are structural heterogeneity of the complex, and low affinity leading to weak dipolar signals compared to that from single-labeled proteins. This task is better suited for DQC, conducted at low concentrations and at a high frequency, possibly in Ka- or W-band, which may enable detection of just a few percent of dimers in a pool of single-labeled protomers.

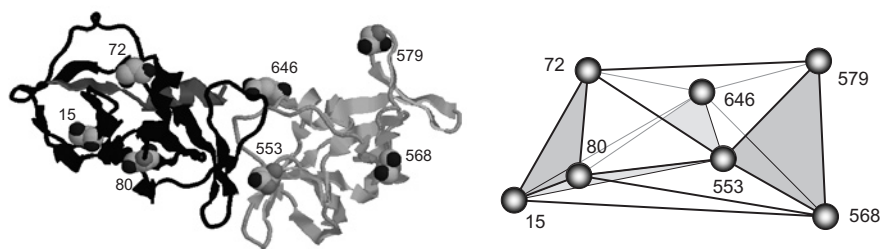


Fig. 3. Structure of P4/P5/CheW complex (P4 is not shown) determined by PDS and confirmed by X-ray. [32]. Residues mutated to nitroxides for PDS are shown in a space-fill representation; (right) also shows the rigid triangulation grid based on tetrahedra.

f) Embedding PDS constraints and rigid-body modeling

In the case of tertiary or ternary structure, which includes aspects such as the relative position of protein subunits or protein docking, knowing all possible distances amongst several labeling sites makes it possible to transform them into molecular coordinates of the sites by means of a generic method of embedding based on metric matrix distance geometry. This task was accomplished for the CheA/CheW complex; the results are shown in Fig. 3. When some constraints are missing, the problem of embedding requires a more advanced method, e.g. based on CNS software [73], which is up to the task. This approach was successfully applied to dock CheW to its binding site in CheA and also to determine the tertiary structure of α -Synuclein [63]. A recent proposal suggests using spin-label rotamer libraries [74].

g) Difficult labeling cases

Not all proteins can be successfully labeled with nitroxide using the SDSL approach. Some cysteines can be functional (RNR, MAO), or the protein may be destabilized, or its function significantly altered. In this case there are currently few approaches. It is possible to measure distances between radical cofactors to establish oligomerization state, quantify major structural change, or the pathway for electron transfer in some cases. Radical cofactors and spin-labeled inhibitors were used in the case of MAO [37], depending on protein environments, with only a spin-labeled inhibitor being suitable for labeling in native membranes. Optimistically, one could rely on future developments such as unnatural amino-acid mutagenesis that may permit spin-labeling with high specificity, spin label incorporation via protein splicing technology, etc. The low amounts required for PDS may be well in-line with these trends.

h) Structural and conformational heterogeneity, protein folding

Iso-cytochrome C unfolded by varying concentration of denaturants was explored at the outset of the L-curve Tikhonov and MEM method development with the goal of exploring the utility of distance distributions from PDS to study kinetically trapped folding intermediates [72, 91]. α -Synuclein, (α S), is unstructured in solution, but it assumes helical structures on micelle or membrane surfaces. The tertiary structure of α S was based on the multiple-distance approach and rigid-body modeling [63], and they have enabled us to

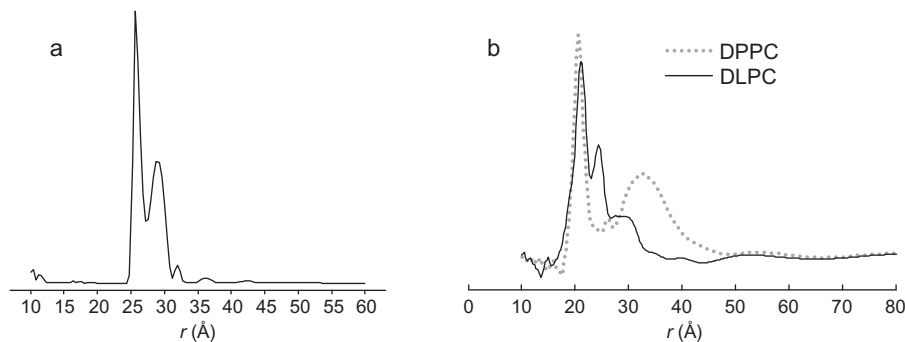


Fig. 4. a MEM reconstruction of distances between two symmetry-related sites in the dimerization interface of histidine kinase, CheA from *T. maritima* spin-labeled at site 318. The data were obtained using DQC at 17.4 GHz. Two peaks in $P(r)$ separated by 2.5 Å probably indicate the presence of two distinct conformations in this part of the protein. b Distance distribution reconstructed by the L-curve Tikhonov regularization method applied to 17.4 GHz DEER data from a doubly-labeled gramicidin A diluted with unlabeled gramicidin A in DLPC (solid line) and DPPC (dashed line). Gramicidin A is a monomer in DLPC with the distance between labels of about 20 Å, but also forms some double helical dimers in DPPC (solid line) visible as a broad distribution at 34 Å.

establish the arrangement of the two helical subunits.

All the above examples and applications have been addressed mainly by using just two PDS methods, which have worked the best, namely DQC and DEER. Taken together they cover most practical aspects that can arise in structure determination by ESR. Both have their strengths and weaknesses, which tend not to overlap.

5. PDS Toolbox

a) 3-pulse DEER

DEER in its original 3-pulse form [40] is based on the two-pulse primary spin-echo $\pi/2$ - τ - π - τ -echo sequence to which a 3rd pumping π -pulse is added. The primary echo from the $\pi/2$ - and π -pulses, separated by time interval τ , is applied to spins resonating at the frequency ω_A , to form an echo at the time 2τ after the $\pi/2$ -pulse. These spins are commonly referred to as A spins. The third (pumping) pulse is applied at the resonant frequency ω_A (at a variable time t) sufficiently different from ω_B that it does not have any direct effect on the A spins but instead inverts the spins resonating at ω_B , i.e. the B spins. The B spins, at a distance r from the A spins yield the electron dipolar coupling A (cf. Eq. (2)), which splits the resonant line at ω_A into a doublet. Thus flipping a B spin inverts the sign of the coupling sensed by the A spin. This results in the instant shift of the Larmor precession frequency of spins A; it was shown in [40] that the effect manifests itself as a modulation of the spin-echo amplitude, $V(t)$, which for like spins is:

$$V(t) = V_0[1 - p(1 - \cos A(r, \theta)t)] \quad (4)$$

for $0 < t < \tau$.

Here V_0 is the echo amplitude in the absence of the pumping pulse and p is the probability of flipping spin B. Powder averaging of $V(t)$ over an isotropic distribution of orientations of r , under the simplifying assumption of random orientation of the magnetic tensors of the A and B spins relative to r produces a decaying oscillatory signal (cf. Fig. 2c):

$$V(t) = V_0[1 - p(1 - u(\omega_d t))], \quad (5)$$

where

$$u(\omega_d t) = \int_0^{\pi/2} \cos[\omega_d(1 - 3\cos^2 \theta)t] d(\cos \theta) \quad (6)$$

is the desired ('dipolar') signal, oscillating with the frequency of $\nu_d = \omega_d/2\pi$, from which r is calculated as $r[\text{\AA}] = 10(52.04/\nu_d[\text{MHz}])^{1/3}$. Cosine Fourier transformation of $u(\omega_d t)$ vs. $2t$ (that is the full dipolar evolution time) yields the dipolar spectrum with the shape of a Pake doublet (cf. Fig. 2a). The remaining (and the larger) part of $V(t)$ amounts to background, which makes it difficult and sometimes impossible to separate weak $u(\omega_d t)$ from the effects modifying and destabilizing the background, which constitutes the major source of errors.

Equations (4) and (5) thus should be considered as a reasonable approximation for DEER, which is suitable for the majority of cases encountered in biological applications of PDS. In reality, a number of factors affect the signal, and their effects usually cannot be written in closed form or are unwieldy [48, 75–77]. What is significant is that DEER achieves a good separation of the dipolar coupling from relaxation effects in most practical cases, because the time between the $\pi/2$ and π spin-echo pulses at ω_A is constant, (i.e. τ in Fig. 5 is constant in the experiment; this is referred to as a constant time pulse se-

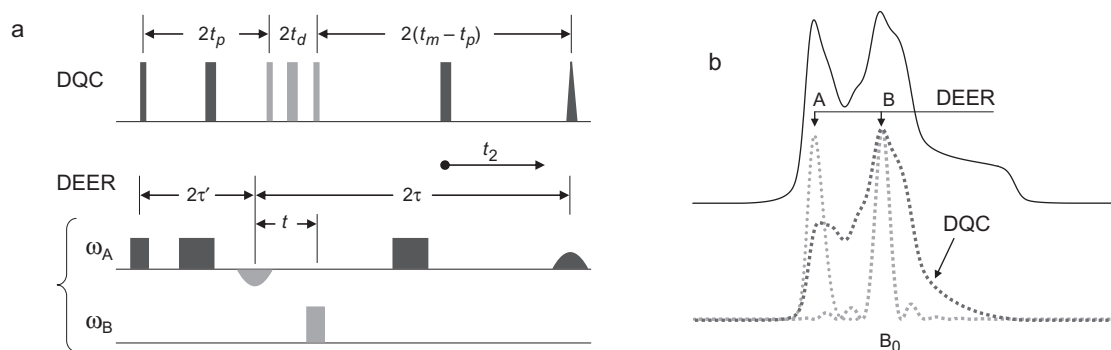


Fig. 5. **a** 6-pulse DQC (top) and 4-pulse DEER (bottom) sequences: The DQC 6-pulse sequence [47, 48] is based on intense pulses in order to probe the dipolar coupling between (nearly) all intramolecular pairs of nitroxide spins. The reference point $t_{\xi} = 0$ is well-defined due to the very short pulses used in DQC. The 4-pulse form of DEER is based on softer selective pulses, with detection of the refocused primary echo formed at ω_A of A-spins. **b** Excitation of the nitroxide spectrum at 17.3 GHz for DQC and DEER. The ^{14}N nitroxide ESR spectrum is plotted as a solid line and the spectral excitation profiles are plotted as dashed lines. The detection frequency in DEER is set at the low field edge of the spectrum (A) and the pump pulse frequency corresponds to positioning it at the center (B). The pumping pulse is 4 G (45 ns π -pulse) in DEER; The wide DQC excitation profile corresponds to a 48 G (3.7 ns) π -pulse.

quence), and relaxation effects introduced by the pumping pulse can normally be ignored. Nuclear ESEEM is also considerably suppressed, but still could be an issue.

b) The newer and better methods

i. 4-pulse DEER

The more recent methods of 4-pulse DEER [78] and 6-pulse DQC [1, 4, 20, 47, 48] are illustrated in Fig. 5. The 4-pulse DEER sequence is an improvement over 3-pulse DEER. It is based on the 3-pulse spin-echo sequence $\pi/2-\tau'-\pi-(\tau+\tau')-\pi-\tau$ -echo, which refocuses the primary echo formed by the first two pulses. The additional pumping pulse at ω_B is varied in time, t between the π -pulses at ω_A (cf. Fig. 5). Both τ and τ' are fixed, thus relaxation does not modify the signal envelope recorded vs. position of the pumping pulse. The signal is described by Eqs. (4) and (5) at the same level of approximation as 3-pulse DEER (also cf. Fig. 2d). This pulse sequence substantially simplifies its technical implementation, since the starting point ($t = 0$) is shifted away from the second pulse by τ' . This has enabled convenient commercial implementation.

ii. 6-pulse DQC

The 6-pulse DQC pulse sequence $\pi/2-t_p-\pi-t_p-\pi/2-t_d-\pi-t_d-\pi/2-(t_m-t_p)-\pi-(t_m-t_p)$ -echo (cf. Fig. 5) is based on a different principle. All pulses are applied at the same frequency ω_A , and it is important that they all be intense in order to excite the whole spectral distribution of spins, i.e. all the spins are regarded as A spins. The first interval, $2t_p$ is used to let the normal single-quantum coherence with spin character $S_{1y} + S_{2y}$ evolve into what is known as anti-phase single-quantum coherence between the coupled spins with spin character

$S_{1x}S_{2z} + S_{2x}S_{1z}$. Then the $\pi/2-t_d-\pi-t_d-\pi/2$ -pulse 'sandwich' (hatched bars in Fig. 5) converts this coherence into double-quantum coherence with spin character $S_{1x}S_{2y} + S_{1y}S_{2x}$ (by means of the first $\pi/2$ -pulse), then refocuses it by means of the π -pulse, only to convert it back to (unobservable) anti-phase coherence (by means of the last $\pi/2$ -pulse), which evolves back into the observable coherence $S_{1y} + S_{2y}$, giving rise to the echo. Both spins participate equally in the process. The first and the last π -pulses of the 6-pulse sequence are used to refocus in-phase and anti-phase coherences, thereby respectively enhancing the effectiveness of the double-quantum filtering (DQF) 'sandwich', and producing the echo at time $2t_m + 2t_d$. The signal in the ideal limiting case of intense and non-selective pulses can be written as [47, 48]

$$V = -V_0 \sin A(r, \theta) t_p \sin[A(r, \theta)(t_m - t_p)] \\ = \frac{V_0}{2} [\cos A(r, \theta) t_m - \cos A(r, \theta) t_{\xi}] \quad (7)$$

The signal is recorded vs. $t_{\xi} = t_m - 2t_p$, with t_m kept constant in order to keep relaxation effects, (which decay exponentially in time) constant. (Also t_d is kept short and constant.) Powder averaging gives

$$V = \frac{V_0}{2} [u(\omega_d, t_m) - u(\omega_d, t_{\xi})] \quad (8)$$

with $u(\omega_d, t_{\xi})$ is given by Eq. (6). For large $\omega_d t_m$ the first term in Eq. (7), which is constant in t_{ξ} , is close to zero, leaving just the desired 'dipolar' signal. The important feature of the double quantum coherence sandwich is that it very effectively filters out the single quantum signals arising from the individual spins, and only passes the signal from the interacting part of the two spins, which just

contain the dipolar oscillations. The only background that can develop is from the double quantum coherence signal that originates from the bath of surrounding spins, i.e. from intermolecular electron-electron dipolar interactions with other doubly-labeled molecules, (and singly-labeled molecules when they are present). The signal envelope $V(t_{\xi})$ is symmetric with respect to $t_{\xi} = 0$. This is referred to as being dead-time free, since the dipolar oscillations are a maximum at $t_{\xi} = 0$ (cf. cosine term in Eq. (7)).

Relaxation effects that decay exponentially but non-linearly in time in the exponent [20], or substantial differences in T_2 's from the two spins, can modify the signal as the positions of refocusing pulses and DQF are not fixed. The 6-pulse sequence generates a number of echoes, but with the proper phase cycling only the dipolar modulation of the double-quantum filtered echo is detected. The details can be found in [48].

The DQC experiment maintains phase coherence between the two coupled spins and treats them equally, whereas in DEER, phase coherence between the two coupled spins is of no importance. The independence of tuning of the pulse conditions at both frequencies, as well as its applicability to widely separated spectra, makes the DEER sequence quite flexible. Nevertheless, it can be shown that the dipolar signal recorded in DEER is based on the same type of evolution of in-phase and anti-phase coherences as in DQC. This is also the case with other related pulse sequences [48]. Although it may look complex, the DQC experiment, once it is set up using adequate equipment, is rather simple to use. The similarity in DQC and DEER means that the maximum useful time of

the experiment (i.e. $2t_m$) in DQC and 2τ in DEER will be comparable, except for respective differences in signal-to-noise (SNR) as discussed below.

DQC and DEER, even though both are not perfect, have proven to be the most useful methods, and together they address a wide range of applications. Additional methods of occasional use were introduced elsewhere [49–52, 79].

6. Relaxation and Distance Range

The amplitude of the primary echo V_0 decays with pulse separation due to phase relaxation. Therefore the maximum dipolar evolution time interval, $t_{\max}$ available for recording $V(t)$ is ultimately limited by the phase memory time, T_m . In the simplest case, $V(t) = V_0 \exp(-2t/T_m)$. This limits the maximum distance, $r_{\max}$ that one can measure, over a reasonable period of signal averaging. Depending on the signal strength, $t_{\max}$ is ca. $1\text{--}3 T_m$ and cannot be extended much further. Here $t_{\max}$ is essentially $2t_m$ in DQC and $2(\tau' + \tau)$ in DEER (cf. Fig. 5). The largest measurable distance, $r_{\max}$ is proportional to $t_{\max}^{1/3}$ in order to recover the dipolar oscillation [48]. Thus only a minor increase in $r_{\max}$ can be made by increasing $t_{\max}$, and this would necessarily require a large increase in signal averaging. For nitroxide-labeled proteins, T_m is largely determined by the dynamics of the nearby protons [80–82], especially those from methyl groups, leading to the simple exponential decay expressed above with T_m in the range of $1\text{--}2 \mu\text{s}$ for buried or partially buried labels. Such relaxation times are typical for hydrophobic environments that are encountered in lipid membranes and the protein interior [81]. This permits an $r_{\max}$ of typically $50 \text{ \AA}$ [134]. For water-exposed labels, relaxation at longer τ is dominated by $\exp[-(2\tau/T_m)^\kappa]$ with $\kappa \sim 1.5\text{--}2.5$ and $T_m \sim 3\text{--}4 \mu\text{s}$ [81]. A quadratic term in the exponent is governed by the nuclear spin diffusion mechanism [83, 84]. This permits an $r_{\max}$ of typically $\sim 55\text{--}60 \text{ \AA}$ (or $\sim 70\text{--}75 \text{ \AA}$ with low accuracy). Such types of relaxation could be partially suppressed by multiple refocusing and/or using deuterated solvent [19, 48, 85, 86]. This could extend $t_{\max}$ to ca. $6\text{--}8 \mu\text{s}$ in favorable cases [80], i.e. much less than in D₂O/glycerol-d₈, as there still is a bath of protons of the protein itself [80]. Using 6-pulse DQC helps to extend $t_{\max}$ when T_m is dominated by nuclear spin diffusion [19, 48]. This permits a more accurate estimate of $r_{\max}$ to ca. $70 \text{ \AA}$. Further improvement would

require much greater effort such as partial or complete protein deuteration, and this might extend $r_{\max}$ to $100\text{--}130 \text{ \AA}$ and make distances up to $80 \text{ \AA}$ much more accurate.

The longitudinal relaxation time, T_1 , determines how frequently the pulse sequence can be repeated, (usually no more frequently than $1.5/T_1$), and consequently the rate at which the data can be averaged. Both T_1 and T_2 are temperature dependent, as is the signal amplitude, which depends on the Boltzmann factor for spins in the dc magnetic field. The combined effect of all these aspects is such that for proteins in water solution or in membranes the optimal temperature as a rule is in the range of $50\text{--}70 \text{ K}$ for both DQC and DEER.

We summarize next the limiting distance ranges and what is optimum.

a) Long distances

As noted above, the ability to measure very long distances is limited by the phase memory time, T_m and for proteins $65\text{--}75 \text{ \AA}$ is about the upper limit with current technology. Also, distances measured in this range are typically not very accurate. This situation could be radically improved by protein deuteration. Alternatively, with a good spin labeling strategy, such long distances may be avoided.

b) Short distances

The π -pulse excites a spectral extent (in Gauss) of about B_1 . It is necessary to excite both components of the Pake doublet in DEER, which normally uses π -pulses longer than 20 ns (B_1 of $\sim 9 \text{ G}$). This provides a lower limit to DEER of ca. $15\text{--}20 \text{ \AA}$ (cf. Fig.

6). However, π -pulses of $30\text{--}60 \text{ ns}$ width are typical, since they provide a cleaner implementation of the method, which requires that the pump pulse and observing pulses do not overlap in spectral extent. This tends to limit DEER to ca. $20 \text{ \AA}$. The sensitivity to shorter distances decreases significantly because the coupling increases and both components of the Pake doublet can no longer be adequately excited [87]. Also, account must be taken of strong dipolar coupling during these long pulses [75]. (We also note that longer pulses render ESEEM effects negligible because of sufficient spectral separation.)

DQC uses intense pulses with B_1 of 30 G or greater, hence it can access distances as short as ca. $10 \text{ \AA}$ [25] (cf. Figs. 6 and 7). In this case the pseudosecular part of the dipolar term in the spin-Hamiltonian (cf. Eq. (1)) cannot be neglected, but this can be accounted for in rigorous numerical simulations [48]. The short distance range is more appropriate however for organic biradicals, buried spin labels or radical cofactors, TOAC, and similar cases, when radicals are substantially immobilized and their geometry is known or can be deduced. This range is less desirable for typical nitroxide labels with long tethers, with uncertain geometry.

c) Optimal range of distances

In our experience an optimal range of distances for the purposes of PDS is within $20\text{--}50 \text{ \AA}$ ($45 \text{ \AA}$ for membrane proteins, whose T_m 's are $0.7\text{--}1 \mu\text{s}$), even though larger distances can be measured with a longer period of signal averaging, but usually with reduced accuracy. Distances shorter than $20 \text{ \AA}$ introduce a relatively large uncertainty

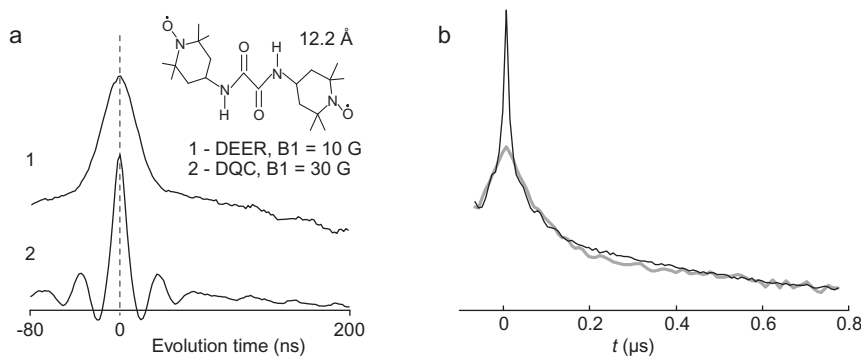


Fig. 6. a The challenges of short distances. DQC and DEER were applied to a rigid $12.2 \text{ \AA}$ nitroxide biradical. Detection pulses in DEER were $16/32/32 \text{ ns}$, the pumping pulse was 18 ns ($B_1 \sim 10 \text{ G}$). This is found to be insufficient to properly excite the dipolar spectrum. DQC using 6.2 ns π -pulse ($B_1 \sim 30 \text{ G}$) develops the $\sim 30 \text{ MHz}$ oscillations very cleanly. The longer pulses of DEER lead to a spread in the refocusing point of different spin packets, and the weaker B_1 , both smear out the high-frequency dipolar oscillations. (The biradical courtesy of R. G. Griffin). b The superimposed DQC (solid line) and DEER (dotted line) signals obtained for the CheA $\Delta 289$ heterodimer doubly-labeled in one of two protomers at two close sites [32]. DQC is able to detect a broader range of distances.

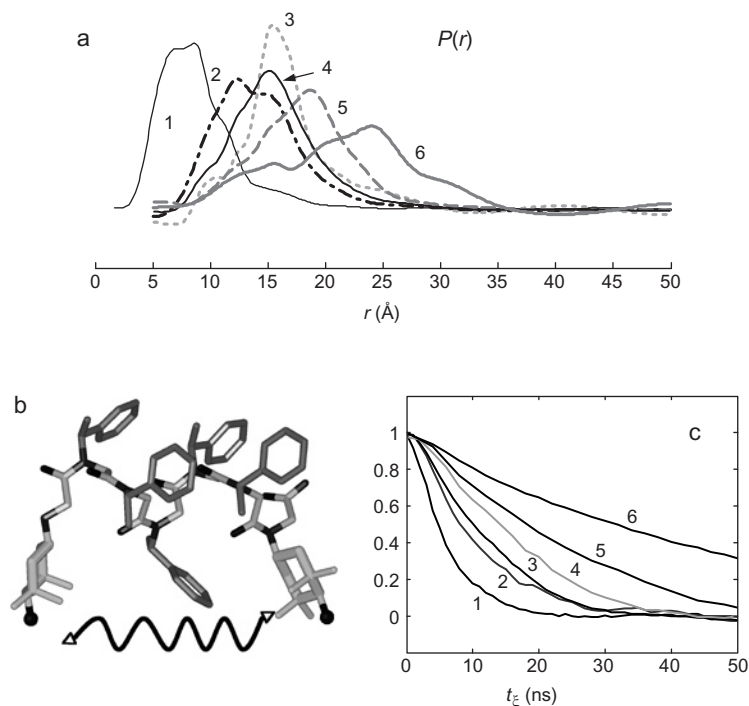


Fig. 7. a Distance distributions for a set of doubly-labeled peptides (b) with a range of end-to-end distances [25]. c Shows time-domain signals, which for the shortest distance, decay is less than 10 ns.

in estimating the C_α - C_α distances. Measurement of distances in the optimal range is fast and accurate in most cases. The labeling sites and distance network should thus be chosen such that they provide optimal conditions for PDS, by increasing the relative number of optimal distances, as needed. Optimal conditions are not readily available for oligomeric proteins due to multiple labels, and their typically large size. For an unknown structure, a preliminary scanning by several trial measurements may be very helpful.

7. Sensitivity of PDS

The sensitivity of PDS techniques, specifically DQC and DEER, has been discussed in [48], where the main criterion for sensitivity was based on the ability to perform a successful experiment, (i.e. of reliably measuring a distance) in a reasonable period of time. It was chosen to correspond to an acceptable SNR, nominally taken as a S_{acc} of 10, which has to be attained in an acceptable time of experiment nominally taken as 8 hrs of signal averaging. Such a SNR would make it possible to obtain a desired distance, given a sufficient length of, t_{max} . However, a S_{acc} of 10 is a bare minimum, and we usually require a SNR of at least 50 [72].

Even though it is possible to estimate sensitivity rather accurately from first principles [88], we prefer to use an experimental calibra-

tion in the spirit of [48] based on measurement of the spin echo amplitude using a two-pulse primary echo (PE). Such an experiment provides the SNR for a single-shot, S_1 (PE).

The calibration of DQC and DEER has been conducted for our pulse ESR spectrometer [32, 89] at the working frequency of 17.35 GHz on a nitroxide sample of 4-hydroxy TEMPO in a vitrified solution of 50% (w/v) glycerol in H_2O with a 20 μM spin concentration in a 10 μL sample volume at 70 K, where most PDS measurements are performed. The DEER calibration used a primary echo [90] generated by $\pi/2$ - π pulses (π -pulse of 32 ns) separated by 80 ns, with the pulses applied at the low-field edge of the nitroxide spectrum. A similar DQC calibration was based on $\pi/2$ - π pulses with a 6 ns π -pulse, and the same separation as in DEER, but pulses were applied in the middle of the spectrum. For the two measurements, the ratio of the echo amplitudes relevant for (DQC vs. DEER) was ca. 6.5 and the ratio of SNR's of the single-shot signals at the condition of optimal signal reception (i.e. given by the integration of the spin echo in the time window defined by the time points corresponding to 0.7 of the echo amplitude) was ca. 3.0, i.e. $S_1 \approx 0.42 \mu M^{-1}$ (DEER) and $S_1 \approx 1.25 \mu M^{-1}$ (DQC).

Based on these numbers, the estimates of the dipolar signals for the two methods according to the analyses given in [47, 48] are

summarized as follows. For 4-pulse DEER with 16/32/32 ns pulses in the detection mode and a 32 ns pump pulse, S_1 is 0.084 μM^{-1} , and for DQC based on a 3/6/3/6/3/6 ns pulse sequence, S_1 is 0.3 μM^{-1} , i.e. it is greater for DQC by a factor of 3.6. This ratio is supported by our experimental observations, (e.g. Fig. 8). Using the sensitivity analysis of [48] we estimate the SNR of the raw data of the full PDS experiment as

$$SNR = 2S_1 x^2 C \eta_c K(f, T_1) (f t_{exp} / n)^{1/2} \times \exp\left(-\frac{2t_{max}}{T_m} - 2kxCGt_{max}\right). \quad (9)$$

Here, t_{exp} is the duration of the experimental data acquisition; f is the pulse sequence repetition frequency; n is the number of data points in the record³; C is the doubly-labeled protein concentration (μM); η_c is the ratio of the sample volume ($\leq 15 \mu L$) to that used in the calibration (i.e. 10 μL). The terms in the exponent are consistent with those given in [48], namely the first accounts for the phase relaxation (where we use⁴ $\kappa = 1$ in Eq. (9)) and the second for instantaneous diffusion, where $k \approx 1 \mu s^{-1} mM^{-1}$ for nitroxides. G is method-specific [48] (and defined below in the next section), and for the pulse sequences defined above it is ca. 0.14 in DEER and ca. 0.52 in DQC. We also include the spin-labeling efficiency, x , which modifies the fraction of both spins that need to be flipped in PDS, showing its strong effect on the outcome of an experiment. Below we assume complete labeling for convenience in the discussion ($x = 1$). $K(f, T_1) = 1 - \exp(-1/fT_1)$ gives the ef-

³ Note that the factor of $n^{1/2}$ in Eq. (9) accounts for the effective averaging of each data point. But the raw signal can be processed in several ways in order to determine distances and the distributions in distances, when possible. In [48] the number of points was not included in the expression for the SNR, because their sensitivity analysis was conducted within the context of the maximum measurable distances. In that case, based on consideration of spectral analysis (i.e. by FT), there should be at least $n_{min} = 4t_{max}/T_{dip}$ sampling points in order to satisfy the Nyquist criterion for the highest dipolar frequency of the Pake doublet, $2\omega_d$ (and just 2 for $t_{max} = T_{dip}/2$). It is this n_{min} that should be used as N in Eq. (9) to estimate the SNR for the dipolar spectrum in the frequency domain. Oversampling does not degrade the SNR, which is determined by the total number of signal samples ($f t_{exp}$) and n_{min} , but it helps to reduce aliasing in the spectrum and may have other positive effects. For reliable recovery of distributions in distances by Tikhonov analysis, 50–100 data points are desirable with the SNR in the data record of at least 30 [72, 91] Eq. (9) thus gives a conservative estimate.

⁴ When $\kappa > 1$, e.g. for relaxation effects from nuclear spin diffusion, its partial refocusing in the DQC experiment provides an improved SNR [19].

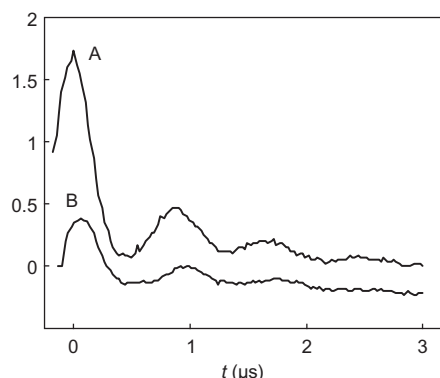


Fig. 8. Comparison of the DQC signal vs. t_z (A) and 4-pulse DEER vs. t (B) operating at 17.4 GHz for spin-label at position 340 in the cytoplasmic domain of band 3 protein [35]. The same resonator and sample was used in both cases, data collection time was 25 min, T was 70 K. In DQC 9 ns π -pulses, (i.e. 20 Gauss B_1) were used; 16/32/32 ns observing pulses and 28 ns pumping pulse were used in DEER. SNR of DQC is 142, in DEER it is 43. The DQC SNR may be improved by using shorter π -pulses. An additional advantage of DQC was due to its partial cancellation of nuclear spin diffusion. (Current operating performance for these conditions yields SNR's that are greater by a factor of 2.5.) (Unpublished data, the protein courtesy of Zheng Zhou.)

fect of incomplete spin-lattice relaxation for a given relaxation time, T_1 and repetition rate, f (K is 0.72 for the optimal repetition rate, when $fT_1 = 0.79$ and is unity when $fT_1 < 1$.) As an illustration of the capability of PDS in various regimes, we consider the following examples: fully supported by experiment.

a) Short distances, low concentrations

For a short distance of 20 Å ($T_{\text{dip}} \equiv \nu_d^{-1} = 154$ ns), we set $t_{\text{max}} = 0.48 \mu\text{s} \approx 3T_{\text{dip}}$ in order to provide very good resolution of distance; T_m is taken as 1.0 μs , i.e. the shortest within its typical range; 8 ns steps in t yielding 60 data points are taken as producing the signal record; a pulse repetition frequency f of 1 kHz should be optimal for a spin-labeled protein at 70 K. One finds from Eq. (9) that just $t_{\text{exp}} \approx 4$ min of signal averaging of the DQC signal provides a SNR of 10 for a C of 1 μM . DEER will require nearly one hour (50 min) to achieve this result. Note that this concentration corresponds to just 10 picomoles of protein. A high SNR of 100 for DQC could be attained in 6.5 hours for the same amount of protein.

b) Long distances

We assume $t_{\text{max}} = 4 \mu\text{s}$, a typical T_m of ca. 2 μs , and the steps in t are taken to be 50 ns. Then a SNR of 10 will be reached in 8 h for a C of 2.1 μM for DQC (while for DEER it would be 104 h). By using one period of T_{dip}

we find $R_{\text{max}} = 59$ Å; for half of the period, R_{max} is 75 Å. (Longer distances cannot be estimated reliably with this SNR). An accurate analysis of the distance distribution requires a higher concentration of at least 10 μM in order to provide a SNR of at least 50 [72, 91], under otherwise similar conditions.

c) Distances in the optimal PDS range

We consider 50 Å as an upper limit for the 'optimal' PDS distance range. T_{dip} is then 2.4 μs , therefore a t_{max} of 2.4 μs suffices to provide the distance sufficiently accurate for a structure constraint. We assume the rather challenging case of $T_m = 1.5 \mu\text{s}$; steps in t are taken to be of 32 ns; f is 1 kHz, C is taken as 25 μM ; but now we require a good SNR of 50. Such a SNR will be achieved in 16 min by DQC. DEER will require nearly 3.5 hours to achieve the same result, or else the concentration must be increased (by a factor 2–4). Shorter distances of 20–45 Å are measured faster, or else yield a better SNR or resolution.

Absolute spin sensitivity is closely related to the concentration sensitivity; however it does increase rapidly with an increase of the working frequency due to the smaller volume of a resonator used at a higher frequency, e.g. at Ku-band 25–250 picomoles of protein are routinely used in the optimal distance range. The smaller amounts are better suited for DQC. These amounts can be reduced by about an order of magnitude using smaller resonators than we currently employ, but by an even greater factor at a higher working frequency.

We remind the reader that the above estimates relate to our 17.3 GHz spectrometer; lower estimates of sensitivity, in particular absolute sensitivity, would apply to the typical pulse spectrometers that operate at 9 GHz, (cf. below).

8. Further Aspects of Sensitivity of PDS: Higher Frequencies

To complete our discussion of sensitivity, we address the single-shot SNR of the dipolar signal, S_1 in Eq. (9) in the absence of relaxation and the other factors considered above, with a view to estimate its frequency dependence. It is clear that this sensitivity is determined by the SNR of the relevant echo signal, which depends on the fraction of participating A spins giving rise to the echo, and is then modified with the factor (≤ 1) depending on the fraction of the B spins flipped by the pump pulse; (in DQC B spins are also A spins, but the approach below works similarly). The SNR

is highest when all (or nearly all) spins are excited, resonator Q matches the bandwidth of the echo (and that of excitation pulses), and the signal reception is optimized, e.g. by matched filtering. The single-shot SNR of the part of the echo modified by dipolar coupling, S_1 can be estimated as (cf. [90]):

$$S_1 = \beta_0 \omega C V_s G H (Q \omega / V_c F_N \Delta f)^{1/2} \quad (10)$$

wherein β_0 is a constant, $\omega = 2\pi f$, where f is the working frequency; C is the spin concentration in the sample; V_c is the resonator effective volume; $V_s = V_c \eta$ is the sample volume, with η being the filling factor of the resonator; G and H are the spectral excitations of A and B spins, respectively; Q is the loaded Q -value of the resonator; F_N is the system noise figure; Δf is the receiver bandwidth.

Often, Δf is set to match the signal bandwidth e.g. the spin-echo signal is integrated (usually between the points located at ca. 2/3 of the echo height). The spectral extent of the echo is proportional to γB_1 , thus for optimal signal reception $\Delta f \propto \gamma B_1$. Also, Q is set to accommodate short pulses in DQC and the frequency separation in DEER. We assume $Q \propto K_1 \omega / \gamma_c B_1$ with $K_1 \approx 0.1$ –0.2 for DEER and $K_1 \approx 1$ for DQC. For a modern solid-state receiver, F_N only slowly degrades with frequency increase; therefore it will be considered a constant.

Both, G and H are determined mainly by the spectral coverage of the π -pulses. Thus the better spectral coverage of DQC is included in these factors. In DQC $G \approx H$, and we assume for simplicity the same for DEER, which is usually the case. For DEER, typically, the pump pulse should be in the 20–40 ns range to avoid signal distortions, yet provide adequate excitation. In DQC π -pulses can be as short as 3–4 ns. For $B_1 \ll B_s$ we can let for both DQC and DEER $G \approx H \propto B_1/B_s$, where B_s is the spectral extent in Gauss; so $GH = K(B_1/B_s)^2$, with K dependent on pulse method [48, 76]. In the opposite case of large $B_1 \geq B_s$, appropriate for DQC, G and H levels off approaching unity. Given practical considerations, one chooses in DQC a value of $B_1/B_s = K_2 < 1$ (e.g. $K_2 \sim 0.7$ for DQC as compared to 0.1–0.2 for DEER).

With all these considerations, the achievable SNR for the integrated dipolar signal becomes⁵

$$S_1 \propto \omega^2 C V_c^{1/2} \eta B_s^{-1} K K_2 K_1^{1/2}. \quad (11) \quad \blacktriangleright$$

⁵ A larger number of pulses in DQC and the factor of 2 in the denominator (cf. Eq. (8)), makes the difference between the two methods less dramatic as follows from more accurate analysis.

For the same type of resonator⁶ $V_c = \alpha\omega^{-3}$, with α being determined by the resonator design. Then the SNR depends on concentration as

$$S_1(C) \propto C\alpha^{1/2}\omega^{1/2}\eta B_s^{-1}KK_2K_1^{1/2} \quad (12)$$

whereas the absolute sensitivity in terms of number of spins, N is:

$$S_1(N) \propto N\omega^{7/2}\alpha^{-1/2}B_s^{-1}KK_2K_1^{1/2}. \quad (13)$$

For very high frequencies $B_s^{-1} \propto \omega$, so

$$S_1(C) \propto C\alpha^{1/2}\omega^{-1/2}\eta KK_2K_1^{1/2}, \quad (14)$$

$$S_1(N) \propto N\omega^{5/2}\alpha^{-1/2}KK_2K_1^{1/2}. \quad (15)$$

We assumed above there is enough power available at the higher frequencies to maintain optimal SNR, so $S_1(C) \propto \omega^{-1/2}$ and $S_1(N) \propto \omega^{5/2}$. Thus it would appear that concentration sensitivity is not benefited by going to higher frequencies (e.g. in the mm-range) but absolute sensitivity should improve. On the other hand even at W-band the spectral width growth is not as dramatic, and there are opportunities to design resonators with a larger value of α . Given the use of open Fabry-Perot resonators that have relatively large V_c , circular polarization, and other factors, such as different spectral shapes, these matters require more detailed consideration.

10. Distance Distributions

Several approaches to determine distance distributions of paramagnetic centers in solids were utilized in the early applications of DEER and related methods [40, 42, 92]. Such methods have been improved [72, 91, 93–95] and the Tikhonov regularization method [96] became a routine for extracting distance distributions from the raw or preprocessed data from both DEER and DQC.

The time-domain dipolar signal for uniform spin distributions in the sample may generally be viewed as $V_{\text{intra}}A_{\text{inter}} + B_{\text{inter}}$ (B_{inter} originates from singly-labeled molecules and free label or pairs where one of the spins does not participate). The A and B terms are removed to the extent possible; and then, what is taken to be a reasonably accurate representation of V_{intra} is subject to inverse reconstruction by Tikhonov regularization or related methods. The ideal-case problem can

be represented by a Fredholm integral equation of the first kind

$$V_{\text{intra}}(t) = V_0 \int_0^\infty P(r)K(r,t)dr \quad (16)$$

with the kernel $K(r,t)$ for an isotropic sample (cf. Eqs. (2) and (3)) given by

$$K(r,t) = \int_0^1 \cos[\omega_d t(1-3x^2)]dx. \quad (17)$$

The inversion of the signal V_{intra} given by Eq. (16) to obtain $P(r)$, the distance distribution, is in principle achievable by standard numerical methods, such as by singular value decomposition (SVD), but it is an ill-posed problem which requires regularization methods in order to arrive at a stable solution for $P(r)$. In the practical implementation, the data are discrete and available over a limited time interval, and the actual form of the kernel $K(r,t)$ may differ from the ideal form given by Eq. (17).

Tikhonov regularization [72, 91, 95] recovers the full distribution in distance, $P(r)$. It is based on seeking an optimum $P(r)$, which tries to minimize the residual norm of the fit to the data while also trying to maximize the stability of $P(r)$ (i.e. to reduce its oscillations). The relative importance of both is determined by the regularization parameter, λ . The L-curve method [97] for optimizing λ is computationally very efficient and the most reliable to date, [91]. In the Tikhonov method the regularization removes the contributions of the small singular values, σ_i , in the SVD that are corrupted by the noise by introducing the filter function,

$$f_i \equiv \frac{\sigma_i^2}{\sigma_i^2 + \lambda^2} \quad (18)$$

which filters out those contributions for which $\sigma_i^2 \ll \lambda^2$. Further refinement of the $P(r)$ can be performed by means of the maximum entropy method (MEM) [72, 98], although it is computationally more time consuming. The latest versions of MEM and Tikhonov regularization permit one to simultaneously fit and remove the effects of A_{inter} and/or B_{inter} while optimizing the $P(r)$ from raw experimental data [72]. It was shown [70, 72, 91, 95] that distance distributions are recovered faithfully, from test data simulated using the ideal kernel of Eq. (17) even in the presence of significant noise (SNR of 10). However, real data departs from this ideal picture for several reasons, thus increasing uncertainty and requiring significantly higher SNR.

11. Some Technical Aspects of DEER and DQC

A preferred setup for 3-pulse DEER is based on using two independent power amplifiers (sources) for the two frequencies [40]. (And we find it beneficial in all cases.) A bimodal cavity resonator was used with this scheme in order to optimize sensitivity and reduce overlap between excitation profiles of pulses in pumping and detection modes. The gain in concentration sensitivity due to a higher Q -value and relatively large sample volume is offset by a low filling-factor. The modern approach, which is preferred for PDS, is based on using loop-gap (LGR) or dielectric resonators, which can be easily installed in commercial cryostats, providing days of continuous stable operation. Also, the sensitivity is higher and sample size can be much smaller resulting in small amounts, when this is needed.

3-pulse DEER can be successfully conducted with a single amplifier as we demonstrate (cf. Fig. 9a), but this usually necessitates using a TWTA in its linear regime, which is some 10–12 dB below the preferred saturation mode of operation. For this reason there may not be enough power at X-band to provide short pulses, but it was not a problem at Ku-band. Simultaneous application of bichromatic irradiation may also contribute a problem. On the other hand, the pulses in 4-pulse DEER do not need to be close, thereby avoiding some small but significant dead times effects in 3-pulse DEER. 4-pulse DEER thus can be readily set up with a single amplifier, and stronger pulses can be produced, leading to greater sensitivity. Pulse interaction is not entirely removed, but becomes less of a problem if the distance between the first two pulses is not too short.

Figure 9 compares 3-, 4-pulse DEER and DQC carried out in the same setup on the same sample with a single TWTA mode of operation. A better SNR in 3-pulse DEER compared to 4-pulse DEER is mostly due to the short relaxation times, T_1 and T_2 at the temperature of 200 K used. Note, that in both forms of DEER, the apparent dead time (time resolution) is limited by the pulse widths (one can see this point in Fig. 6), and thus is considerably longer than in DQC, which uses pulses as short as a few nanoseconds. DEER can be used, in principle, without phase cycling or even with incoherent pulses, (with performance degradation). However, DEER requires high instrument

⁶ Note that at a lower frequency V_c may be limited by available power, thus α needs to be smaller.

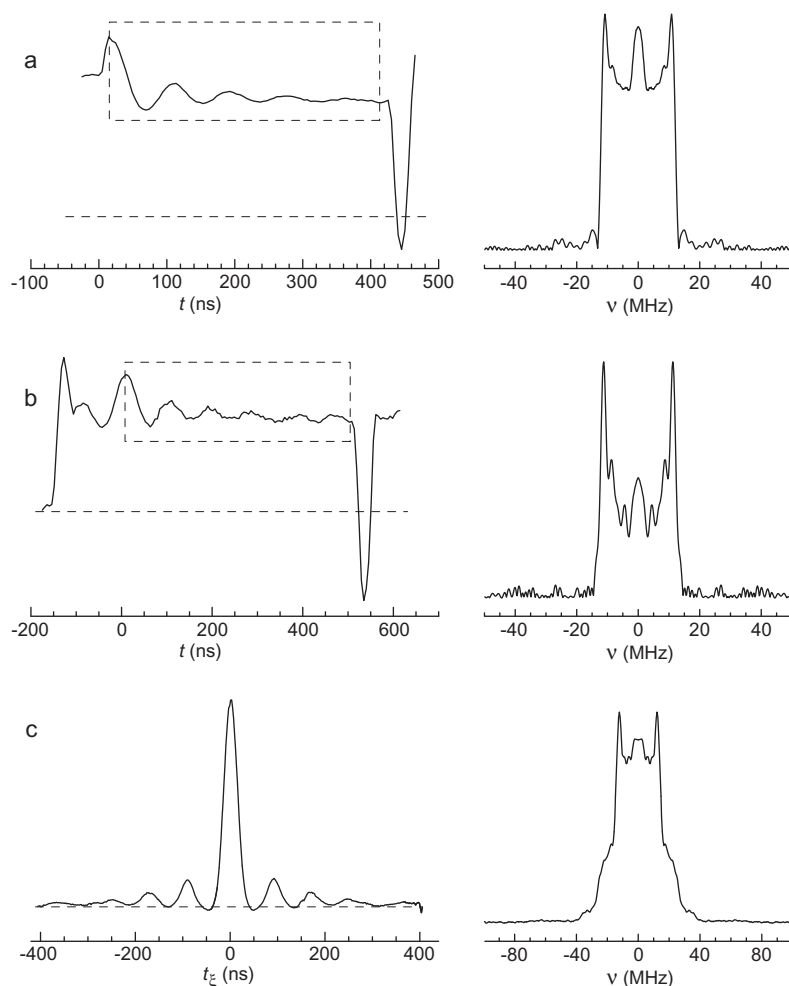


Fig. 9. 3- (a) and 4-pulse (b) DEER, and DQC (c) are compared for a 16.3 Å rigid biradical in LC phase V, rapidly frozen from the isotropic phase; at -80°C and 17.4 GHz. DEER was set up with a single power amplifier working in the linear regime at 10 dB below saturated output level. A low- Q dielectric resonator was used to accommodate the pulses at both DEER frequencies separated by ~ 100 MHz. $\pi/2$ - and π -pulses were 10 and 20 ns in DEER and 3.2 and 6.2 ns in DQC. The pumping pulse was positioned at the low-field portion of the nitroxide spectrum. The informative parts of the signal traces in DEER are enclosed in a rectangle. In 4-pulse DEER the maximum of the signal is shifted in time as in DQC, so both 4-pulse DEER and DQC are zero dead-time pulse sequences. The outer turn-over points of the Pake doublet are missing in the dipolar spectrum from the DEER signals. The DQC signal is considerably stronger and cleaner but decays somewhat faster due to spectral broadening caused by the pseudosecular term of the dipolar coupling. [48, 62].

stability in order to maintain gain, phase, field, etc. as all small drifts directly affect the echo amplitude, leading to low-frequency noise that could limit SNR. This requires state-of-the-art pulse generation and signal detection paths with low noise and drifts, thus very high overall stability, which may be difficult to achieve in a home-built instrument, unless it is designed and built with the care given by commercial equipment vendors.

A key virtue of DQC is the suppression of the large background signal (baseline) by means of its extensive phase-cycling, in particular its use of the double-quantum filter. Unwanted modulation of the signal due to low frequency noise and drifts in phase or

gain becomes less important, thereby simplifying implementation and use. This also helps to reduce nuclear ESEEM effects, which are mostly due to modulation of the large background from the single order coherence signals. The basic requirement is to provide reasonably accurate quadrature phase-cycling and sufficient B_1 , which requires a more powerful and thus more expensive TWTA. Once these requirements are met, DQC is easy to set up and use. Since a higher-power TWTA could be a less attractive option for a typical user, a sound alternative is to employ minute dielectric or loop-gap resonators, which were demonstrated up to 95 GHz [99, 100], yet a cavity resonator is still a viable alternative at 35 GHz and above [100, 101].

3-pulse DEER was introduced among other things to minimize nuclear ESEEM effects, since excitation and detection regions of the ESR spectrum are well separated. For a typical 4-pulse DEER experiment with a single power amplifier at X-band, ESEEM cannot be discounted. In both DQC and DEER, standard suppression techniques are very successful, [20, 85, 102]. Also, increasing the frequency from 9 GHz to 17 GHz virtually eradicates the proton ESEEM, but deuterium ESEEM, as we find, can remain a factor in DQC.

Finally we mention orientation selection in DEER [38, 76] due to the anisotropy of the nitroxide magnetic tensors and their orientations relative to the inter-spin vector. This is an issue for DEER due to its use of selective pulses. DQC with its hard pulses is much less sensitive to orientational selectivity, but when desired orientational correlations can be revealed in considerable detail in a 2D mode [48]. The reader is referred to analyses of orientational selectivity, and its potential for distorting the dipolar spectrum in DEER by [76, 103]. However, the flexibility of side-chain spin labels, such as MTSSL, considerably decreases correlation effects. On the contrary at high fields, where orientation selection could be objectionable in standard use, it can be exploited to obtain some additional information on orientation of nitroxide side-chains, and endogenous radical centers [23, 104].

12. Summary and Perspective

In most PDS studies conducted thus far, just a few distances were typically obtained, often with the goal of detecting an important structural change or establishing the oligomerization state [15, 37, 105]. On the other hand, cw ESR routinely employs extensive protein scans [67–69] to elucidate aspects of secondary and tertiary structures. PDS is certainly capable of extensive protein mapping as we have demonstrated [18, 32]. In all, at least 70 distances (including those using WT proteins) have been obtained in our work on the CheA/CheW complex of *T. maritima*. Our approach is based on implementing triangulation to determine the ternary structure. A similar mapping effort has focused on the helix topology of α -Synuclein [18].

At present, protein structure can be reasonably accurately evaluated just using self-consistent nitroxide side-chain modeling (as noted above) and by structure refinement by CNS for a sufficiently large set of ESR dis-

tance constraints, [63]. One could anticipate that future developments will enable ESR distance restraints combined with homology modeling, nitroxide side-chain geometry simulation, and structure prediction to be applied to generate detailed 3D structures of large proteins and their complexes.

Further technical improvements are expected in PDS, in particular in DQC, which is not yet at its optimum performance. Shorter pulses with higher B_1 's, better phase cycling, and resonators optimized for concentration and absolute sensitivity are expected in the near future. Both DEER and DQC will be developed at a higher frequency than Ku-band. The automation of sample processing is also planned at ACERT.

In this commentary we emphasized that PDS, as it applies to protein structure, is a rather straightforward technique in its principles and implementation, and is not overburdened with complexities. We have tried to convey our enthusiasm that PDS (both DEER and DQC) will become a standard technique for structure determination, given that it does have key virtues, which should lead to its wider acceptance.

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