PELDOR Spectroscopy

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Relative Orientation of Rigid Nitroxides by PELDOR: Beyond Distance Measurements in Nucleic Acids**

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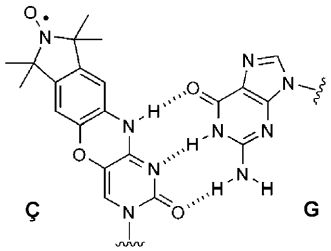
The central paradigm for the mechanistic understanding of biomolecular function is the idea that the structure and dynamics of a biomolecule determine its function. Of the methods capable of revealing structural data, X-ray diffraction has procured detailed information of large biomacromolecular complexes at atomic resolution.[1] However, it is not always possible to crystallize such large systems, and the structure in the crystal may not represent the functional state in solution.[2] High-resolution liquid-state NMR spectroscopy can be applied in solution and yields information about dynamics[3] but is currently restricted to systems smaller than approximately 50 kDa. Thus, for structural studies on large biomolecules in solution, other biophysical methods are required, for example fluorescence and electron paramagnetic resonance (EPR) spectroscopy.[4,5] Both methods enable the measurement of distances on the nanometer scale between fluorophores or paramagnetic centers, respectively, giving access to domain arrangements and movements. In EPR spectroscopy, techniques based on pulsed electron–electron double resonance (PELDOR or DEER)[6] or double quantum coherence (DQC)[7] have been used to determine distances in the range of 1.5 to 8 nm with high accuracy. Especially PELDOR is commonly applied for nanometer distance measurements between nitroxides, cofactors, and metal centers and to count the number of monomers in aggregates.[8,9] In both cases, the pulse sequences measure the distance-dependent dipolar coupling ν_d between the spin centers according to Equation (1):

$$\nu_d = \frac{52.16}{r^3}(3\cos^2\theta - 1) \quad (1)$$

where r is the spin–spin distance vector, θ is the angle between r and the external magnetic field B_0, and 52.16 [MHz nm^3] is the dipolar splitting constant for nitroxides.

In addition to distances, the angular dependence $(3\cos^2\theta-1)$ of the dipole–dipole interaction provides the possibility to unravel the relative orientation of spin labels, thus yielding considerably more structural information. For example, continuous wave (cw) EPR spectroscopy has been utilized to obtain relative orientations of spin-labeled cofactors,[10] and more recently, PELDOR experiments at high frequencies have disentangled the orientation between protein-bound radical cofactors[11] or radical-containing amino acids.[12] These studies benefited from the immobilization of the free radical in the rigid protein scaffold, whereas orientation studies using spin labels are hampered by the flexibility of most labels.[13] This limitation can be overcome by using rigid spin labels such as the recently described spin label **Ç**, in which a nitroxide group was incorporated into a polycyclic fused ring system of a cytidine analogue that forms a base pair with guanine (Scheme 1).[14] Spin label **Ç** enables grafting a rigid nitroxide onto chosen sites in nucleic acids as well as the preparation of samples with specific, fixed orientations of spin labels relative to the framework of the nucleic acid. We demonstrate herein that this new generation of spin labels allows determination of both distance and orientation of two labels in DNA through PELDOR measurements at common X-band frequencies.



Scheme 1. Spin label **Ç** base-paired to G.

Two **Ç**-spin-labeled DNA duplexes, **I** and **II**, were designed for a proof-of-principle experiment (Figure 1 a,b). Molecular modeling shows that **Ç** is projected into the major groove of the DNA helix,[14] and thermal denaturation studies have demonstrated that **Ç** does not appreciably affect the stability of DNA duplexes.[15] The distance and relative orientation of the spin labels, as inferred from molecular

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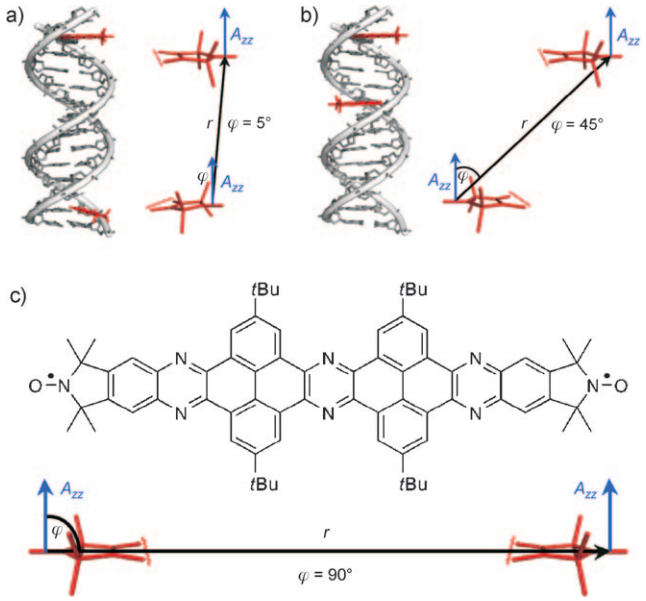


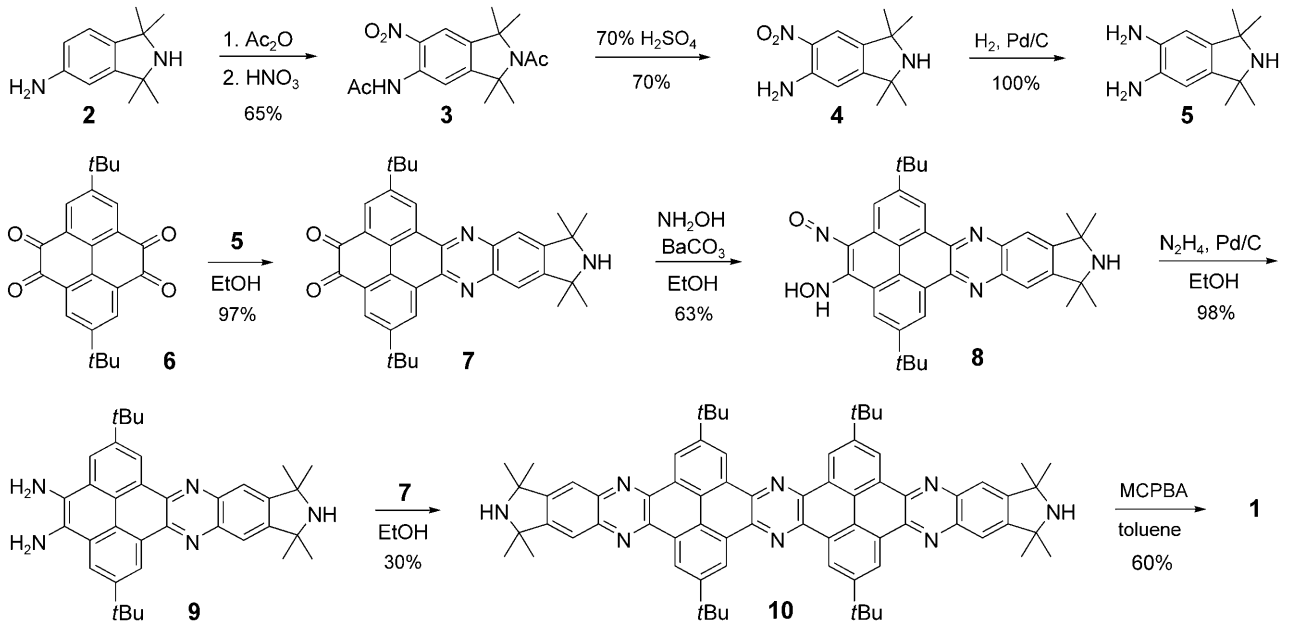
Figure 1. a) DNA **I** and b) DNA **II**, with the spin label **Ç** highlighted in red. c) The structure of **1**. Simplified schematic structures indicating the orientation of A_{zz} with respect to r are also shown.

modeling, differs in the two DNA molecules: $r = 3.7$ and 2.1 nm and $\varphi = 5$ and 45° for DNA **I** and DNA **II**, respectively. In this case, φ is defined as the angle between the z component of the ^{14}N hyperfine coupling tensor (A_{zz}) and r. Owing to geometry constraints, a DNA sample in which $\varphi = 90°$ could not be prepared. To circumvent this constraint, we synthesized biradical **1** (Figure 1c), in which the distance between the spin centers is 2.7 nm and φ equals 90°.

The synthesis of **Ç** and its incorporation into DNA was performed as described earlier.[14,15] Biradical **1** was synthesized according to Scheme 2. Amine **2**[16] was acylated, nitrated, and hydrolyzed to give nitroamine **4**, which was reduced to yield amine **5**. The diketone **7** was prepared by condensation of **5** with an excess of tetraketone **6**.[17] Conversion of the diketone to dioxime **8** and subsequent reduction with hydrazine yielded amine **9**. Pentadecacycle **10** was prepared by condensation of **7** and **9** and subsequently oxidized with MCPBA to give biradical **1**.

PELDOR experiments were performed on all three molecules with the usual four-pulse sequence.[18] The 12 ns pump pulse at microwave frequency ν_B was placed on the maximum of the nitroxide spectrum, thus exciting all orientations (Figure 2b). The detection pulses are applied at microwave frequency ν_A with a frequency offset $\Delta\nu = \nu_A - \nu_B$ of 40 to 90 MHz (Figure 2a). The detection sequence with pulses of length 32 ns has an excitation bandwidth of 26 MHz and therefore excites only a fraction of the nitroxide spectrum. For comparison, the nitroxide spectrum, which at X-band frequencies is dominated by the anisotropic ^{14}N-hyperfine coupling tensor $A(^{14}N)[A_{zz}=34$ G, $A_{yy}=5$ G, $A_{xx}=6.5$ G], has a width of about 210 MHz. Thus, varying the position of the detection pulse causes a selection of different components of $A(^{14}N)$. For $\Delta\nu = 90$ MHz, the detection pulses are placed on the low-field edge of the EPR spectrum, selecting mainly A_{zz} (Figure 2c). Decreasing $\Delta\nu$ in 10 MHz steps down to 40 MHz leads to a deselection of A_{zz} and an increasing contribution of A_{xx}, A_{yy}, and off-diagonal components (Figure 2d).

Importantly, the orientation of the nitrogen hyperfine tensor is fixed with respect to r, as the nitroxides in DNA **I**, DNA **II**, and **1** are geometrically rigid. Thus, selecting specific components of A selects at the same time specific components of the dipolar distance tensor, which depends on the molecular geometry. In the case of **1**, the selection of A_{zz} for $\Delta\nu = 90$ MHz leads to a selection of those molecules with the distance vector r perpendicular to B_0 ($\theta = 90°$), since A_{zz} is fixed perpendicular to r ($\varphi = 90°$). Decreasing $\Delta\nu$ in 10 MHz steps excites more of A_{xx} and A_{yy} and less of A_{zz} (A_{yy} is also



Scheme 2. Synthesis of biradical **1**. Ac_2O = acetic anhydride, MCPBA = *meta*-chloroperoxybenzoic acid.

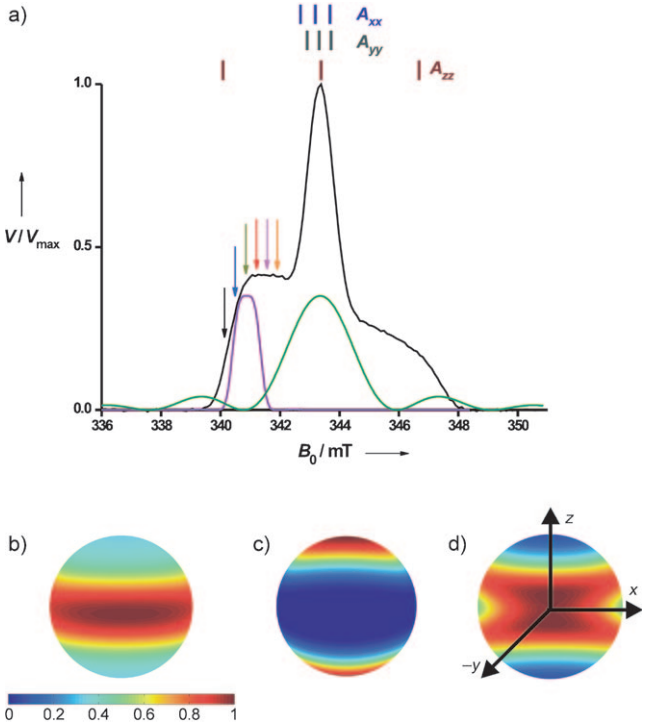


Figure 2. a) Field-swept EPR spectrum of DNA **I** at 40 K with the excitation profiles of the observer pulses (purple) and pump pulse (olive) and the corresponding ^{14}N stick spectrum. Arrows indicate observer pulse positions varying from $\Delta\nu = 40$ (orange) to 90 MHz (black). b) Excited orientations for the pump pulse and c) the detection sequence for $\Delta\nu = 90$ and d) 40 MHz.

perpendicular, but A_{xx} is parallel to *r)*. Therefore, at smaller frequency offsets, the parallel component ($\theta = 0°$) should contribute more to the PELDOR spectrum. This is exactly what is observed in the PELDOR time traces and the Fourier transformed spectra for **1** (Figure 3a, c).

In the case of DNA **I**, the geometry as obtained from molecular modeling is such that A_{zz} is nearly parallel ($\varphi = 5°$) and A_{xx} perpendicular to *r*. Therefore, the opposite trend compared to **1**, should be expected. For $\Delta\nu = 90$ MHz, the parallel component of the dipolar distance tensor ($\theta = 0°$) should dominate, and the perpendicular component should increase with decreasing $\Delta\nu$. This expectation is in full accordance with the experiment (Figure 3d,f). For the intermediate case of DNA **II**, where $\varphi = 45°$, neither of the Pake pattern singularities at $\theta = 0°$ or $\theta = 90°$ can be selected without large contributions from the other orientations (Figure 3g,i).

A qualitative estimation of φ can thus be easily performed on the basis of the experimental data. If φ is close to 0°, as in DNA **I**, the parallel component of the Pake pattern ($\theta = 0°$) increases by varying $\Delta\nu$ from 40 to 90 MHz, whereas it decreases if φ is about 90°, as in **1**. In cases of $\varphi \approx 45°$, as in DNA **II**, the shape of the Pake pattern remains constant with $\Delta\nu$ variation (Figure 3c,f,i). This qualitative analysis of orientations requires, however, two coplanar spin labels. With respect to distances, a variation of $\Delta\nu$ allows detection of both singularities of the Pake pattern and thus easy calculation of the distance by using the frequency corresponding to

Table 1: Experimental and modeled distances and φ values.

Molecule	PELDOR r[a] [nm]	Modeled r [nm]	φ [°]
1	2.7(0.1)	2.6	90(15)
DNA **I**	3.7(0.1)	3.8	5(20)
DNA **II**	2.1(0.1)	2.1	45(20)

[a]The numbers in brackets are the errors (see the Supporting Information).

$\theta = 90°$. This frequency amounts to 2.7, 1.0, and 6.0 MHz for **1**, DNA **I**, and DNA **II**, respectively, yielding distances of 2.7, 3.7, and 2.1 nm. All distances are in very good agreement with those gathered from molecular modeling (Table 1).

A more quantitative analysis was performed for all three molecules by simulations (Figure 3b,e,h) based on a program described earlier.[19] Parameters entering these simulations are the distance r and the angle φ, which are encoded in the frequency of the oscillation and the changes of the time trace with the frequency of the detection pulses, respectively. The line width and the *g*- and ^{14}N-hyperfine tensors were taken from simulations of cw EPR spectra, and both tensors were assumed to be collinear. The orientation of the ^{14}N hyperfine and the *g*-tensor of nitroxide A with respect to those of nitroxide B was fixed by the modeled geometry. The simulations were run with only one geometric conformer, thus excluding geometric distributions caused by dynamics. Experimental spectrometer settings such as detection pulse length and timings were taken as used in the experiment, and the pulse shape was calculated as described.[20] The simulations fit the experiment with respect to frequency and modulation depth variation with $\Delta\nu$ using the angles given in Table 1 and demonstrate at the same time that the observed effects are caused by orientation selection and are not due to experimental artifacts. The experimentally observed faster damping of the oscillation, especially in the case of the two DNA molecules, is attributed to conformational flexibility. This argument is supported by the far better agreement between the simulations and the experiment for the much more rigid molecule **1**.

In summary, we have shown that the parameter-free extraction of distances and relative orientations of spin centers in nucleic acids can be achieved by orientation-selective PELDOR experiments at common X-band frequencies. These studies lay the groundwork for the analysis of large biomacromolecular complexes, for example, domain arrangements, without prior structural models.

Experimental Section

Detailed experimental descriptions and information about the simulation program can be found in the Supporting Information.

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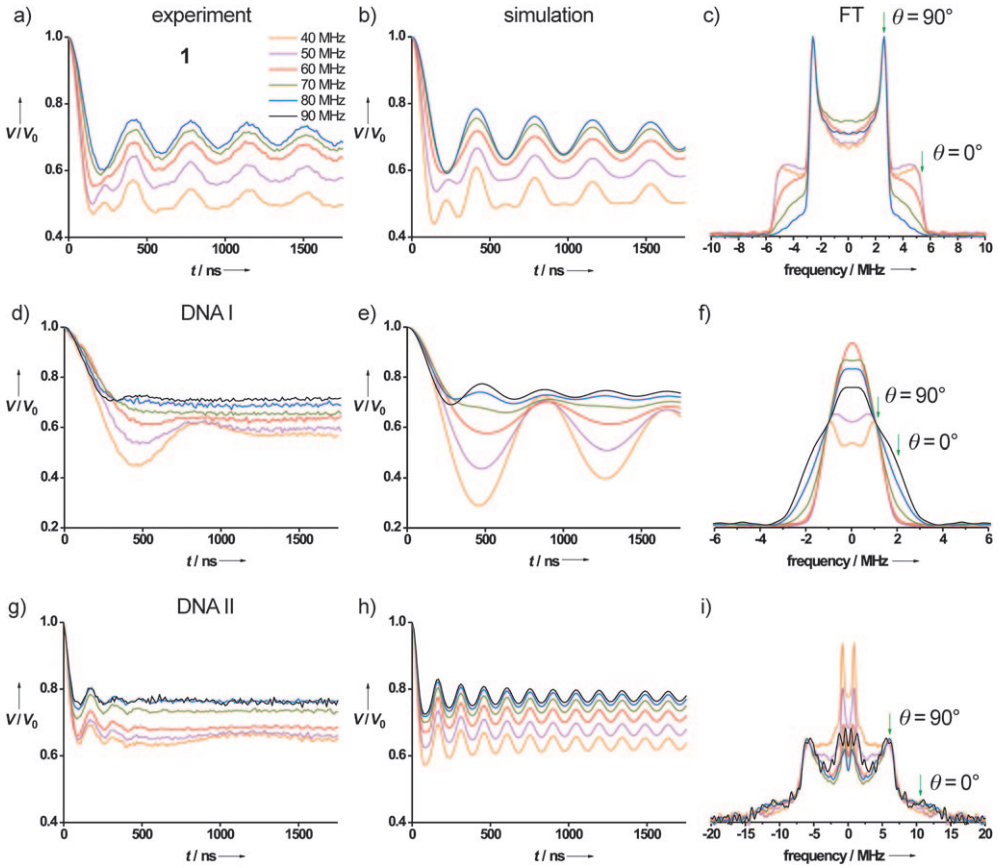

Figure 3. PELDOR spectroscopy data. a), d), g) PELDOR time traces recorded with $\Delta\nu$ values ranging from 40 to 80 MHz for **1** (a) and 40 to 90 MHz for DNA **I** (d) and DNA **II** (g). b), e), h) Simulated PELDOR time traces for **1** (b), DNA **I** (e), and DNA **II** (h). c), f), i) Fourier transformations of the experimental PELDOR time traces. The peak at about 1 MHz in (i) is attributed to end-to-end stacking of two DNA molecules.

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