ChemPubSoc Europe
DOI: 10.1002/chem.201403726
CHEMISTRY A European Journal
Full Paper

Spin Labels

Conformationally Restricted Isoindoline-Derived Spin Labels in Duplex DNA: Distances and Rotational Flexibility by Pulsed Electron–Electron Double Resonance Spectroscopy

Dnyaneshwar B. Gophane,[a] Burkhard Endeward,[b] Thomas F. Prisner,*[b] and Snorri Th. Sigurdsson*[a]

Abstract: Three structurally related isoindoline-derived spin labels that have different mobilities were incorporated into duplex DNA to systematically study the effect of motion on orientation-dependent pulsed electron–electron double resonance (PELDOR) measurements. To that end, a new nitroxide spin label, ^{Exlm}**U**, was synthesized and incorporated into DNA oligonucleotides. ^{Exlm}**U** is the first example of a conformationally unambiguous spin label for nucleic acids, in which the nitroxide N–O bond lies on the same axis as the three single bonds used to attach the otherwise rigid isoindoline-based spin label to a uridine base. Continuous-wave (CW) EPR measurements of ^{Exlm}**U** confirm a very high rotational mobility of the spin label in duplex DNA relative to the structurally related spin label ^{Im}**U**, which has restricted mobility due to an intramolecular hydrogen bond. The X-band CW-EPR spectra of ^{Exlm}**U** can be used to identify mismatches in duplex DNA. PELDOR distance measurements between pairs of the spin labels ^{Im}**U**, ^{Ox}**U**, and ^{Exlm}**U** in duplex DNA showed a strong angular dependence for ^{Im}**U**, a medium dependence for ^{Ox}**U**, and no orientation effect for ^{Exlm}**U**. Thus, precise distances can be extracted from ^{Exlm}**U** without having to take orientational effects into account.

Introduction

Electron paramagnetic resonance (EPR) spectroscopy has emerged as a powerful tool for investigating the structure and dynamics of biopolymers, such as DNA and RNA, under biological conditions.[1] Continuous-wave (CW) EPR spectroscopy is useful to extract information about the dynamics of specific sites through line-shape analyses of EPR spectra.[1b,e,2] In CW-EPR spectroscopy, the dipolar coupling between spin centers results in line broadening and can be used to extract information about distances in the range of 5–20 Å.[3] Pulsed EPR methods, such as pulsed electron–electron double resonance (PELDOR), also called double electron–electron resonance (DEER), and double quantum coherence (DQC) have been used to measure long-range distances from 20 to 80 Å.[4] PELDOR is also useful for the determination of the relative orientation of spin labels, in particular at high field.[5]

[a] Dr. D. B. Gophane, Prof. S. T. Sigurdsson
Department of Chemistry, Science Institute
University of Iceland, Dunhaga 3, 107 Reykjavik (Iceland)
Fax: (+354)5528911
E-mail: snorrisi@hi.is

[b] Dr. B. Endeward, Prof. T. F. Prisner
Institute of Physical and Theoretical Chemistry
J. W. Goethe University
Max-von-Laue-Str. 7, 60438 Frankfurt (Germany)
Fax: (+49)06979829404
E-mail: prisner@chemie.uni-frankfurt.de

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201403726.

Application of EPR spectroscopy to study nucleic acids requires incorporation of unpaired electron(s), since nucleic acids are inherently diamagnetic. The most commonly used spin-labeling method is incorporation of aminoxyl (nitroxide) spin labels by covalent attachment to a nucleic acid.[6] Most of the spin labels that have been described are attached with a tether that has some degree of flexibility. Due to this flexibility, such labels can move independently of the biopolymer and are, therefore, not optimal probes for distance measurements. The recently developed rigid spin labels **Ç** for DNA[7] and **Çm** for RNA[7b] (Figure 1 c) are able to give accurate distances between two spin labels and provide information about their orientation in nucleic acids.[5b,8] Native tyrosyl radicals have also been utilized for the same purpose in proteins.[5a]

Recently, we described isoindoline-derived probes ^{Im}**U** and ^{Ox}**U**[9] (Figure 1 a) and their incorporation into DNA. These probes are linked to the nucleobase by a single bond that lies on the axis of the nitroxide N–O bond and should be good probes for distance measurement in DNA duplexes, because rotation around the single bond does not cause displacement of the nitroxide relative to the DNA. The ^{Im}**U** spin label was shown by CW-EPR spectroscopy to be less mobile than the ^{Ox}**U** spin label due to intramolecular hydrogen bonding between the imidazole N–H and O4 of **U** (Figure 1 b).[9] Indeed, ^{Im}**U** displayed similar mobility to that of the rigid spin label **Ç** at low temperatures.[9] Inspired by these results, we decided to determine if ^{Im}**U** is useful for orientation-dependent distance measurements, which requires rigid labels.

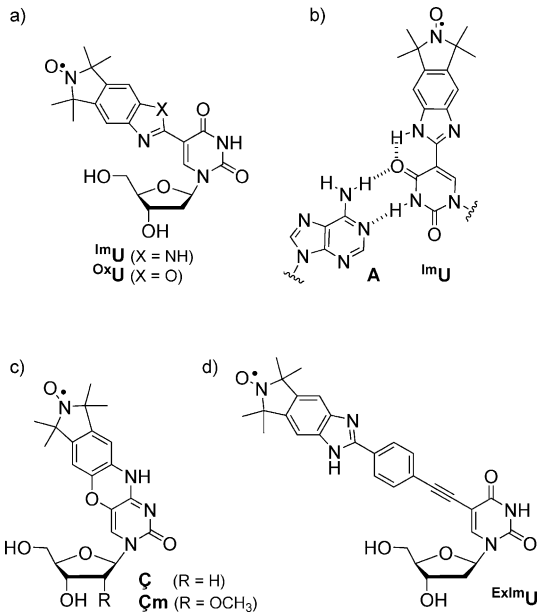


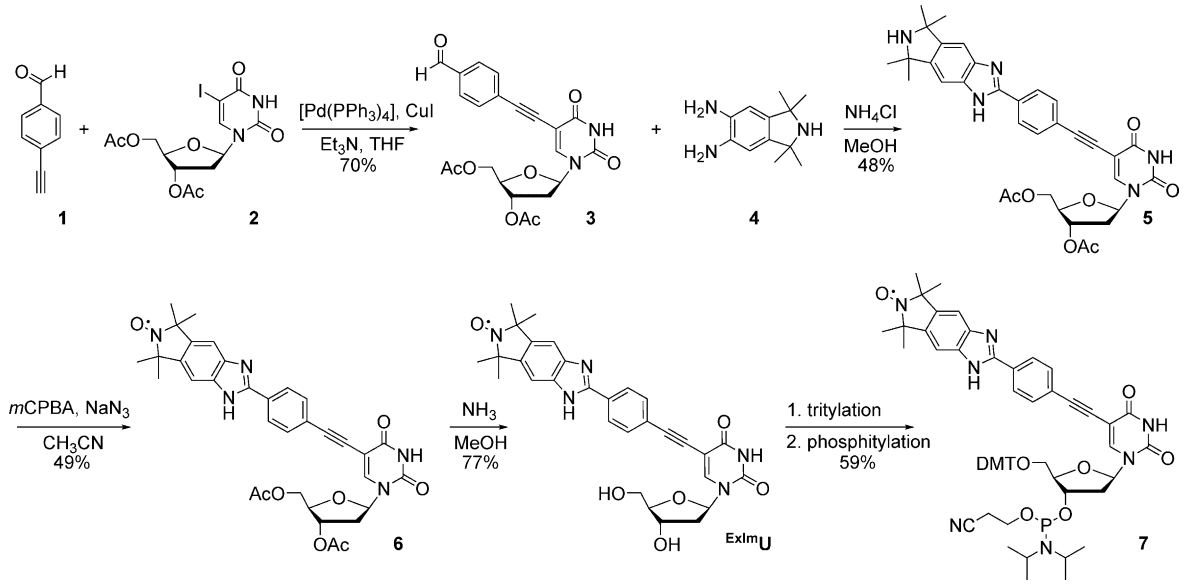
Figure 1. Spin-labeled nucleosides. a) Isoindoline-derived spin labels ^{Im}**U** and ^{Ox}**U**. b) Base pairing of ^{Im}**U** with **A**, showing the intramolecular hydrogen bond that restricts rotation around the bond connecting the nitroxide to the base. c) Rigid spin labels **Ç** and **Çm**. d) Extended benzimidazole spin label ^{Exlm}**U**.

Herein, we report that the ^{Im}**U** spin label shows a strong orientation dependence by PELDOR, similar to that of the rigid spin labels **Ç**[5b] and **Çm**.[8b] Interestingly, ^{Ox}**U**, which does not have the possibility to restrict movement of the spin label around the single bond through hydrogen bonding like ^{Im}**U**, still shows orientational effects in the PELDOR time traces. A structurally related spin label ^{Exlm}**U** (Figure 1 d) was also synthesized and incorporated into DNA for comparison with ^{Im}**U** and ^{Ox}**U**. In ^{Exlm}**U**, rotation is possible around the two single bonds flanking the acetylene bond that lie on the same axis as the N–O bond. CW-EPR measurements of ^{Exlm}**U** confirmed the high rotational mobility of ^{Exlm}**U** in duplex DNA. As expected, ^{Exlm}**U** only showed a very minor orientation dependence in the PELDOR measurements. To our knowledge, ^{Exlm}**U** is the first example of the incorporation of a conformationally unambiguous spin label[10] into nucleic acids for distance measurements. Accurate distances can be measured by a single PELDOR experiment with the new spin label ^{Exlm}**U**, unlike rigid labels that require summing of measurements at several probe frequencies to disentangle distance and orientation effects.[5b,8a,11]

Results and Discussion

Syntheses of spin-labeled phosphoramidites

The ^{Im}**U** and ^{Ox}**U** spin-labeled phosphoramidites were prepared by a previously reported procedure.[9] Synthesis of spin label ^{Exlm}**U** began with a Sonogashira coupling[12] of acetyl-protected 5-iodo-2′-deoxyuridine (**2**)[13] with 4-ethynylbenzaldehyde (**1**) to give compound **3** (Scheme 1). Treatment of compound **3** with 5,6-diamino-1,1,3,3- tetramethylisoindoline (**4**)[9] in the presence of NH_4Cl gave the extended benzimidazole derivative of 2′-deoxyuridine (**5**).[14] Oxidation of **5** proved somewhat challenging, similar to the previously reported spin-labeled nucleosides ^{Ox}**U** and ^{Im}**U**,[9] but sodium azide-facilitated *meta*-chloroperbenzoic acid (*m*CPBA) oxidation of **5** gave spin-labeled derivative **6** in good yields. Deprotection of the acetyl groups with methanolic NH_3 afforded the nucleoside ^{Exlm}**U**. The 5′-hydroxyl group was protected as a 4,4′-dimethoxytrityl ether and phosphitylation yielded the ^{Exlm}**U** phosphoramidite **7**, which was used for incorporation of ^{Exlm}**U** into DNA oligonucleotides.



Scheme 1. Synthesis of extended benzimidazole nucleoside ^{Exlm}**U** and its corresponding phosphoramidite.

Syntheses and characterization of spin-labeled oligonucleotides

Spin-labeled oligonucleotides were prepared by solid-phase synthesis and purified by denaturing polyacrylamide gel electrophoresis. Spin labels ^{Ox}**U** and ^{Im}**U** have previously been shown not to have any effect on the B conformation of duplex DNA and only a minor effect on DNA duplex stability.[9] ^{ExIm}**U** was incorporated into a 14-mer DNA (5′-d(GACCTCG^{ExIm}**U**ATCG TG)), verified by MALDI-TOF analysis (Table S1 in the Supporting Information). Circular dichroism (CD) spectra of both the unmodified and spin-labeled 14-mer duplex possessed negative and positive molar ellipticities at approximately 250 and 280 nm, respectively, characteristic of a right-handed B-DNA (Figure S1 in the Supporting Information). The ^{ExIm}**U** spin label slightly destabilized the DNA duplex by 6.5 °C (Table S2 in the Supporting Information), similar to that reported by Korshun and co-workers for a structurally related compound.[15]

CW-EPR analysis

To study the motion of the spin-labeled nucleoside ^{ExIm}**U** within a nucleic acid, the CW-EPR spectra of the nucleoside ^{ExIm}**U**, as well as the ^{ExIm}**U**-labeled DNA single strand and duplex, were compared (Figure 2, left column). Due to the rapid isotropic tumbling of the nucleoside in solution, its EPR spectrum shows three narrow lines. After incorporation into the 14-mer DNA, the tumbling of the nitroxide slows down and the resulting EPR spectrum is broader. However, there was not much difference between the spectral width of the single strand and the ^{ExIm}**U**-labeled duplex, thus indicating substantial mobility of the spin label independent of the nucleic acid. In contrast, the conformationally restricted ^{Im}**U** (Figure 2, right column) shows much lower mobility in duplex DNA, compared with single-stranded DNA (Figure 2; see Figures S2 and S3 in the Supporting Information for simulated spectra and Figure S3 for measurements at other temperatures).

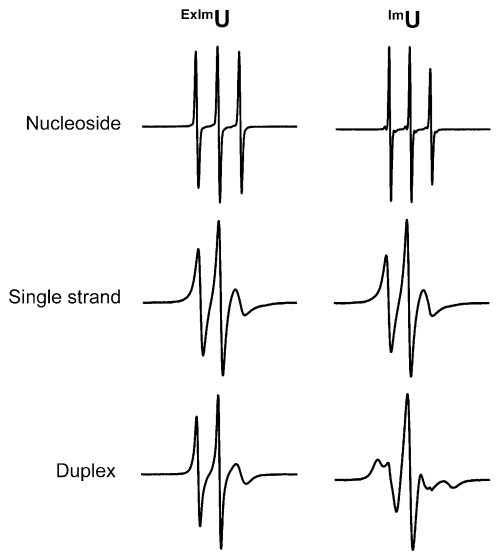


Figure 2. CW-EPR spectra of nucleosides ^{ExIm}**U** and ^{Im}**U** (top) and after their incorporation into single-stranded DNA (middle) and duplex DNA (bottom) at 20 °C (10 mM phosphate buffer, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0).

Spin labels have been used to study local structural perturbations in nucleic acids.[2c,16] To investigate if the ^{ExIm}**U** spin label could be used to probe base pairing in duplex DNA, four 14-mer duplexes containing either A, T, G, or C paired with ^{ExIm}**U** were prepared. The EPR spectrum of ^{ExIm}**U·A** is the least mobile and markedly different from the others (Figure S4 in the Supporting Information); ^{ExIm}**U** can, therefore, clearly distinguish between a "native"-like base pair and a mismatch.

Distance and orientation measurements by PELDOR

Two doubly labeled duplex constructs were used for spin-labeling, which contained either seven base pairs (DNA(1,9)) or ten base pairs (DNA(1,12)) between the labels. The distance between the ^{Im}**U** or the ^{Ox}**U** spin-label pairs were similar for both DNA constructs, but their relative orientations were different (Figure 3, Table 1). The distances for the ^{ExIm}**U** duplexes were different from those for ^{Im}**U** or ^{Ox}**U**. Molecular models of ^{Ox}**U** and ^{ExIm}**U** in both DNA constructs are shown in Figure S5 in the Supporting Information.

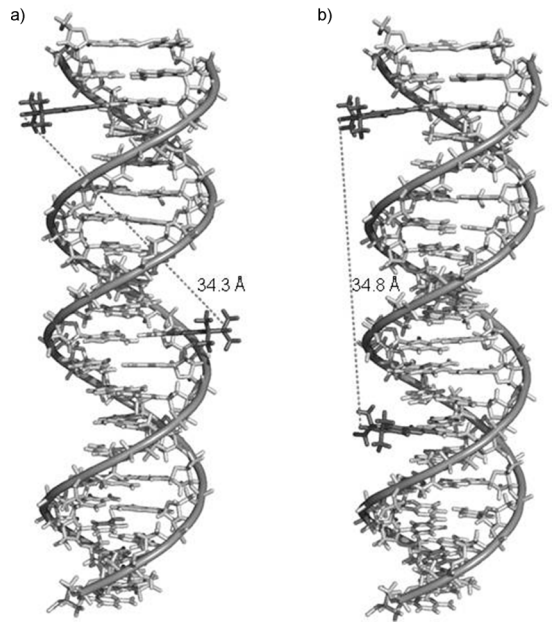


Figure 3. Molecular models of a) ^{Im}**U**-DNA(1,9) and b) ^{Im}**U**-DNA(1,12).

The dead-time-free four-pulse sequence was used for all PELDOR experiments.[17] The pump pulse was placed on the maximum of the nitroxide spectrum (Figure S6 in the Supporting Information), thus exciting all orientations. The detection pulses were applied at a microwave frequency with a frequency offset of 40 to 90 MHz from the pump pulse (Figure S6 in the Supporting Information). Due to the narrow excitation band-

Table 1. Distance measurements in duplex DNA by PELDOR.

Descriptor	Duplex	r [Å][a]
^{Im}**U**-DNA(1,9)	5′-d(GC^{Im}**U** AGT GCG C AC GCG CGA TC) 3′-d(CG A TCA CGC G ^{Im}**U**G CGC GCT AG)	34.3/34.3
^{Im}**U**-DNA(1,12)	5′-d(GC^{Im}**U** AGT CGC GCG C AC GCA TC) 3′-d(CG A TCA GCG CGC G ^{Im}**U**G CGT AG)	34.1/34.8
^{Ox}**U**-DNA(1,9)	5′-d(GC^{Ox}**U** AGT GCG C AC GCG CGA TC) 3′-d(CG A TCA CGC G ^{Ox}**U**G CGC GCT AG)	32.3/34.3
^{Ox}**U**-DNA(1,12)	5′-d(GC^{Ox}**U** AGT CGC GCG C AC GCA TC) 3′-d(CG A TCA GCG CGC G^{Ox}**U**G CGT AG)	34.1/34.8
^{Exlm}**U**-DNA(1,9)	5′-d(GC ^{Exlm}**U** AGT GCG C AC GCG CGA TC) 3′-d(CG A TCA CGC G ^{Exlm}**U**G CGC GCT AG)	40.1/38.4
^{Exlm}**U**-DNA(1,12)	5′-d(GC^{Exlm}**U** AGT CGC GCG C AC GCA TC) 3′-d(CG A TCA GCG CGC G^{Exlm}**U**G CGT AG)	35.8/35.3

[a] Measured/modeled distances between spin labels.

width of the detection pulses (31 MHz) only a fraction of the nitroxide spectrum is excited. Thus, varying the frequency of the detection pulses causes a selection of different components of the hyperfine tensor **A**, originating from coupling with the ^{14}N of the nitroxide. If the orientation of the nitrogen hyperfine tensor is fixed with respect to the spin–spin distance vector **r**, as is the case for rigid spin labels,[5b] selection of specific components of A also selects specific molecular orientations with respect to the external magnetic field. The dipolar interaction depends not only on the distance r, but also on the orientation of this vector with respect to the external magnetic field, so different detection frequencies will result in different PELDOR time traces. Thus, PELDOR is ideally suited to determine the rigidity of spin labels, since the dependence on the frequency offset is strongly reduced if the two spin labels have some conformational freedom with respect to each other and vanishes for very mobile labels.

The PELDOR time traces for the three spin labels in the two DNA constructs are shown in Figure 4. The PELDOR data clearly show orientation dependence for both ^{Im}**U** and ^{Ox}**U**, as judged by the degree of variation between the time traces recorded at different offsets. The ^{Im}**U** label has the strongest orientation dependence of the three, followed by ^{Ox}**U** and ^{Exlm}**U**. This is in agreement with CW-EPR data, which showed that ^{Im}**U** is less mobile than ^{Ox}**U**, presumably because an intramolecular hydrogen bond between the imidazole hydrogen and O4 of the nucleobase (Figure 1 b) limits the rotation around the single bond

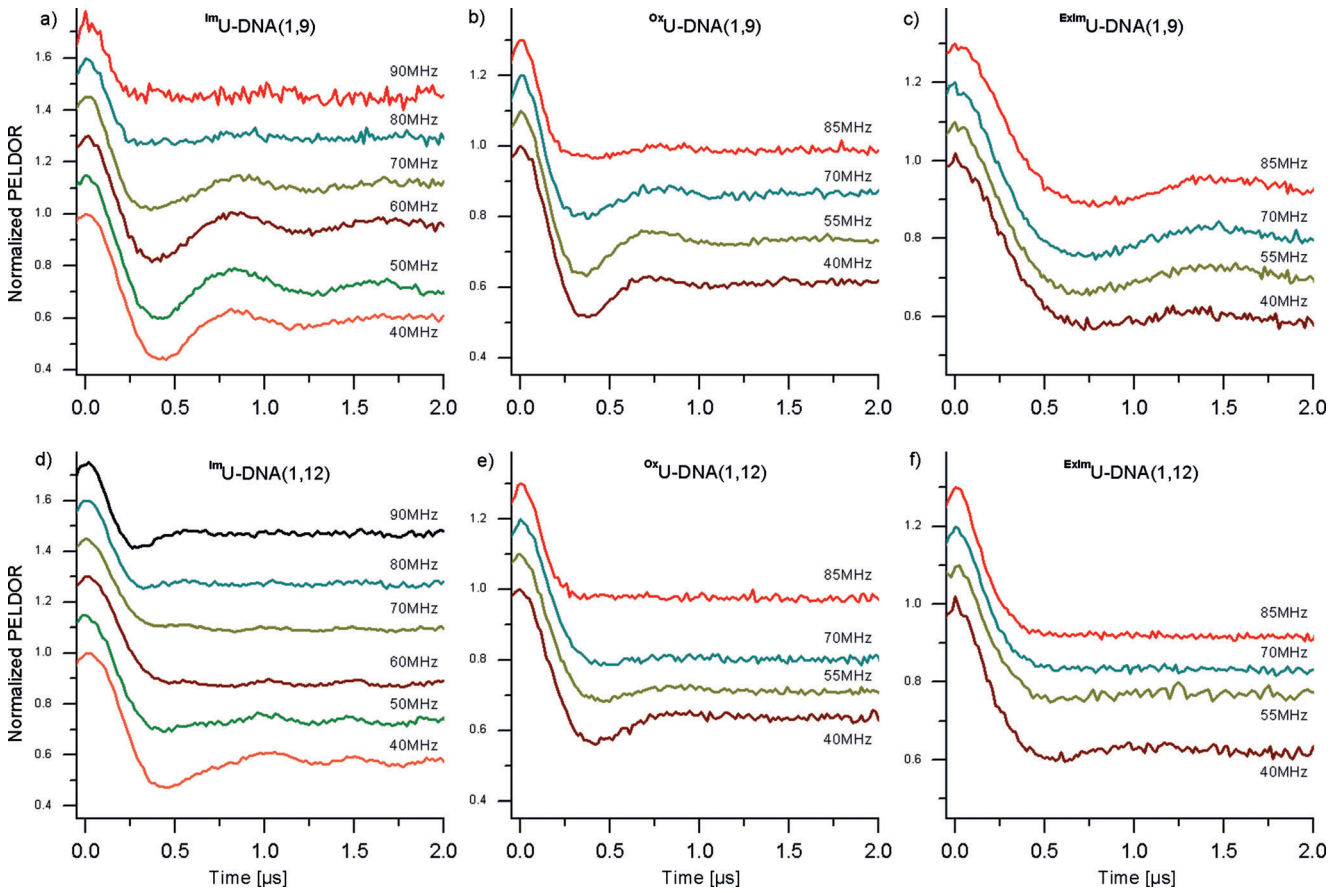


Figure 4. PELDOR time traces at different frequency offsets (see Figure S6 in the Supporting Information) for a) ^{Im}**U**-DNA(1,9), b) ^{Ox}**U**-DNA(1,9), c) ^{Exlm}**U**-DNA(1,9), d) ^{Im}**U**-DNA(1,12), e) ^{Ox}**U**-DNA(1,12), and f) ^{Exlm}**U**-DNA(1,12). The offset between fixed pump and different detection frequencies is indicated at the PELDOR time traces.

connecting the nitroxide to the base.[9] As expected, negligible orientation dependence was observed for ^{ExIm}**U**, which has free rotation around the two bonds flanking the acetylene linker.

For evaluation of the distance between two rigid spin labels that display orientation dependence, such as **Ç**,[5b] the time traces from different frequency offsets need to be summed and the intermolecular decay function has to be removed before performing a Tikhonov regularization. This was demonstrated for ^{Im}**U**, for which the 70 MHz offset (normally used for single-measurement distance determination) gave a distance distribution indicating two distances, whereas the summed traces gave a relatively sharp single distance distribution below 4 nm (Figure 5 a). In contrast, the spin label ^{ExIm}**U**, which showed a very small orientation dependency, allows extraction of distances directly from a single measurement at a fixed frequency offset (Figure 5 b).

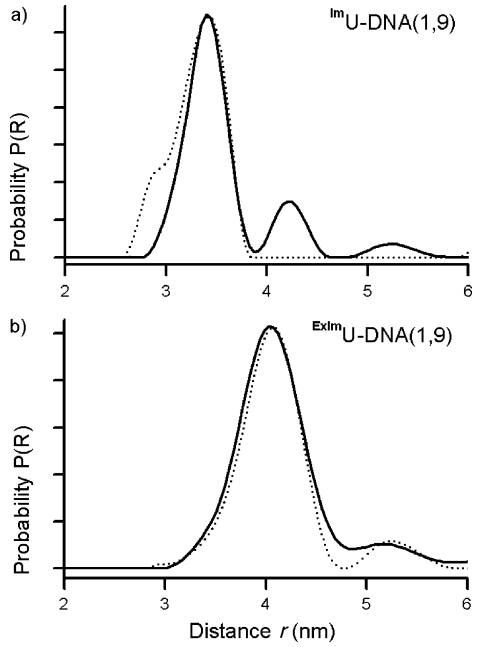


Figure 5. Evaluation of interspin distances in doubly spin-labeled DNA duplexes from PELDOR data for a) ^{Im}**U**-DNA(1,9) and b) ^{ExIm}**U**-DNA(1,9). Distance probabilities are derived from the experimental data by Tikhonov regularization from one PELDOR time trace (70 MHz offset, dotted black line) and all PELDOR time traces (sum of all offsets, solid black line).

Conclusion

The new spin label ^{ExIm}**U** was prepared and incorporated into DNA oligonucleotides for distance measurements by pulsed EPR spectroscopy. CW-EPR spectra of ^{ExIm}**U** in duplex DNA confirm its high mobility relative to the previously reported structurally related spin label ^{Im}**U**, which has restricted rotational mobility due to an intramolecular hydrogen bond. The ^{ExIm}**U** spin label can distinguish between pairing with its Watson–Crick partner A and any of the other mismatches by CW-EPR spectroscopy. PELDOR distance measurements using the spin labels ^{Im}**U**, ^{Ox}**U**, and ^{ExIm}**U** show that ^{ExIm}**U** exhibits only negligible orientation dependence, according to its design, unlike ^{Im}**U** and ^{Ox}**U**. Thus, PELDOR measurements using ^{ExIm}**U** in nucleic acids allow distance determination from a single measurement with a fixed detection frequency.

Experimental Section

General

All chemicals, except 2′-deoxyuridine and thymidine, were purchased from Sigma–Aldrich, Acros, or Fluka and used without further purification. 2′-Deoxyuridine and thymidine were purchased from Rasayan Inc., USA. Thin layer chromatography (TLC) was carried out using glass plates precoated with silica gel (0.25 mm, F-254) from Silicycle. Compounds were visualized by UV light and staining with *p*-anisaldehyde. Flash column chromatography was performed using ultrapure flash silica gel (Silicycle, 230–400 mesh size, 60 Å). Water was purified on EASYpure RoDi Water Purification Systems. CH_2Cl_2 and pyridine were freshly distilled over calcium hydride prior to use. Anhydrous Et_3N, *n*-hexane, and EtOAc were used directly as received. All moisture- and air-sensitive reactions were carried out in oven-dried glassware under an inert atmosphere of argon. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer. NMR chemical shifts are reported in parts per million (ppm) relative to the residual proton signal of solvents $CDCl_3$ ($\delta = 7.26$ ppm) and [D_6]DMSO ($\delta = 2.50$ ppm) for ^{1}H NMR spectroscopy, and $CDCl_3$ ($\delta = 77.0$ ppm) and [D_6]DMSO ($\delta = 39.43$ ppm) for ^{13}C NMR spectroscopy. ^{31}P NMR chemical shifts are reported relative to 85% H_3PO_4 as an external standard. Commercial grade $CDCl_3$ was passed over basic alumina shortly before use with tritylated compounds. Mass spectrometric analyses of all organic compounds were performed on an HRMS-ESI spectrometer (Bruker, MicroTof-Q) in positive-ion mode.

DNA oligonucleotide synthesis, purification, and characterization

The DNA oligonucleotides were synthesized by phosphoramidite chemistry on an automated ASM800 DNA/RNA synthesizer (Biosset, Novosibirsk, Russia) by using a trityl-off protocol and phosphoramidites with standard protecting groups on 1.0 μmol scale, 1000 Å CPG columns. All commercial phosphoramidites, CPG columns, and solutions were purchased from ChemGenes Corporation (Wilmington, MA). The spin-labeled DNA was prepared by using previously reported protocols;[18] the spin-labeled phosphoramidite was incorporated manually into the oligonucleotides by pausing the synthesizer program after completion of the prior cycle, removing the column from the synthesizer, and running 200 μL of standard activator solution and 200 μL of a 0.05 M solution of spin-labeled phosphoramidite in 1,2-dichloroethane back and forth through the column for 10–12 min. After manual coupling, the column was remounted on the synthesizer and the synthesis cycle completed. Treatment with 33% aq. NH_3 at 55 °C for 8 h deprotected the oligonucleotides, which were subsequently purified by 20% denaturing polyacrylamide gel electrophoresis. The DNA oligonucleotide bands were visualized under UV light, excised from the gel, crushed, and eluted from the gel with Tris buffer (2×10 mL; Tris (10 mM, pH 7.5), NaCl (250 mM), Na_2EDTA (1 mM)). The DNA elution solutions were filtered through a 0.45 mM cellulose acetate membrane (Whatman) and desalted using a Sep-Pak cartridge (Waters Corporation). The dried oligonucleotides were dissolved in sterile water (400 μL) and their final concentrations were calculated according to Beer's law based on UV absorbance of oligonucleotides

at 260 nm. Extinction coefficients were determined by using the UV WinLab oligonucleotide calculator (V2.85.04; PerkinElmer). Molecular weights of oligonucleotides were determined by MALDI-TOF analysis (Bruker, Autoflex III) after calibration with an external standard. UV/VIS spectra were recorded on a PerkinElmer Lambda 25 UV/Vis spectrometer. CD spectra were recorded on a JASCO J-810 spectropolarimeter at 20 °C with path length of 1 mm (Hellma), 10 scans, scanned from 500 to 200 nm with response of 1 s, data pitch of 0.1 nm, and bandwidth of 1.0 nm.

CW-EPR measurements and sample preparation

CW-EPR spectra were recorded on a MiniScope MS200 spectrometer using 100 kHz modulation frequency, 1.0 G modulation amplitude, and 2.0 mW microwave power. Each spectrum was scanned 100–120 times. The temperature was regulated by a Magnettech temperature controller M01 with an error of ±0.5 °C. The sample was prepared by dissolving spin-labeled, single-stranded DNA (2.0 nmol) and its complementary strand (2.4 nmol) in phosphate buffer (10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0; 10 μL, oligonucleotide final concn 200 μM). The resulting mixture was annealed by using the following protocol: 90 °C for 2 min, 60 °C for 5 min, 50 °C for 5 min, 40 °C for 5 min, 22 °C for 15 min. Samples (10 μL) were placed in a quartz capillary prior to EPR measurements.

PELDOR sample preparation

The DNA samples for PELDOR measurement were prepared by annealing 10 nmol of each strand with 10 nmol of its complementary strand in phosphate buffer (100 μL, 10 mM, pH 7.0), NaCl (100 mM), and EDTA (0.1 mM), followed by evaporation of the water. The annealed dried samples were dissolved in 20% ethylene glycol/H_2O (100 μL) before the PELDOR measurements.

PELDOR data collection

The dead-time-free four-pulse PELDOR sequence was used for all experiments.[17] A Bruker Elexsys E580 X-band spectrometer equipped with Flexline MS-3 probe in an Oxford CF935 cryostat and a PELDOR frequency unit was used. Microwave pulses were amplified by a 1 kW traveling-wave-tube amplifier (ASE 117x). Typical pulse lengths were 32 ns (π/2 and π) for the probe pulses and 12 ns (π) for the pump pulse. The delay between the first and second probe pulses was varied between 132 and 196 ns in 8 ns steps to reduce contributions from proton modulations. The pulse separation between the second and third probe pulses was between 2.5 and 3.0 μs, depending on the sample preparation. The frequency of the pump pulse was fixed to the central peak of the nitroxide powder spectrum to obtain maximum pumping efficiency. The probe frequency was chosen 40–90 MHz above this frequency (Figure S6 in the Supporting Information). This range corresponds to the smallest frequency offset that avoids strong pump–probe frequency overlap, and therefore large proton modulation artifacts. The 90 MHz offset is the frequency offset that excites the edge of the nitroxide spectrum. All experiments were carried out at 50 K.

Compound 3

CuI (5 mg, 0.026 mmol) and Et_3N (0.5 mL) were added to a solution of 3′,5′-di-*O*-acetyl-5-iodo-2′-deoxyuridine (100 mg, 0.23 mmol) and 4-ethynylbenzaldehyde (60 mg, 0.46 mmol) in THF (2 mL). The suspension was degassed by bubbling argon gas through the solution for 5 min, after which $Pd(PPh_3)_4$ (27 mg, 0.023 mmol) was added. After stirring the reaction mixture for 3 h at 22 °C, the solvent was removed in vacuo and the crude product purified by flash silica gel column chromatography using gradient elution (CH_2Cl_2/MeOH, 100:00 to 98:02) to give compound **3** as a yellow solid (70 mg, 70% yield). R_f = 0.45 (5% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz,$CDCl_3$): δ = 10.02 (s, 1 H), 8.48 (s, 1 H), 7.94 (s, 1 H), 7.85 (d, *J* = 8.2 Hz, 2 H), 7.72–7.60 (m, 2 H), 6.32 (dd, *J* = 7.8, 5.9 Hz, 1 H), 5.30–5.21 (m, 1 H), 4.61–4.11 (m, 3 H), 2.68–2.52 (m, 1 H), 2.36–2.19 (m, 1 H), 2.17 (s, 3 H), 2.13 ppm (s, 3 H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 191.28, 170.32, 170.01, 160.55, 148.93, 141.97, 135.72, 132.13, 132.05, 131.87, 129.54, 128.51, 128.48, 128.39, 100.25, 92.96, 85.75, 83.95, 82.73, 73.86, 63.71, 38.37, 20.84, 20.79 ppm; HRMS (ESI): *m/z* calcd for $C_{22}H_{20}N_2O_8Na$: 463.1112 [*M*+Na]$^+$; found: 463.1121.

Compound 5

Compound **3** (749 mg, 1.7 mmol) and 1,1,3,3-tetramethylisoindoline-5,6-diamine (**4**; 349 mg, 1.7 mmol) were dissolved MeOH (15 mL). NH_4Cl (364 mg, 6.8 mmol) was added and the resulting solution stirred at 60 °C for 2 h and then at 22 °C for 14 h. After removing the solvent in vacuo, the crude product was purified by flash silica gel column chromatography using gradient elution (CH_2Cl_2/MeOH, 98:02 to 85:15) to give compound **5** as a dark yellow solid (577 mg, 48% yield). R_f = 0.25 (25% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 13.41 (s, 1 H), 8.25 (d, *J* = 8.3 Hz, 2 H), 8.14 (s, 1 H), 7.77–7.57 (m, 3 H), 7.47 (s, 1 H), 6.18 (t, *J* = 7.0 Hz, 1 H), 5.22 (dd, *J* = 6.3, 3.0 Hz, 1 H), 4.34–4.17 (m, 3 H), 2.56 (dt, *J* = 14.5, 7.3 Hz, 1 H), 2.44–2.31 (m, 1 H), 2.11 (s, 3 H), 2.07 (s, 3 H), 1.77 ppm (s, 12 H); ^{13}C NMR (100.6 MHz, [D_6]DMSO): δ = 169.99, 169.94, 161.11, 151.80, 149.25, 143.69, 131.51, 129.70, 126.79, 123.54, 111.73, 104.21, 98.50, 91.73, 84.99, 84.07, 81.49, 73.63, 66.56, 66.45, 63.45, 40.05, 39.84, 39.64, 39.43, 39.22, 39.01, 38.80, 36.18, 29.00, 20.68, 20.52 ppm; HRMS (ESI): *m/z* calcd for $C_{34}H_{36}N_5O_7$: 626.2596 [*M*+H]$^+$; found: 626.2596.

Compound 6

NaN_3 (21 mg, 0.32 mmol) was added to a suspension of **5** (50 mg, 0.08 mmol) in CH_3CN and MeOH (5 + 1 mL) and the suspension was stirred at 22 °C. After 30 min, *m*CPBA (28 mg, 0.16 mmol) was added. After 3 h, the reaction mixture was concentrated in vacuo and the residue purified by silica gel column chromatography using gradient elution (CH_2Cl_2/MeOH, 100:0 to 95:5) to give compound **6** as a yellow solid (25 mg, 49% yield). R_f = 0.70 (10% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 13.43 (brs), 11.84 (brs), 8.13 (brs), 7.90 (brs), 7.70 (brs), 7.54 (brs), 6.19 (brs), 5.23 (brs), 4.31 (brs), 2.07 ppm (brs); ^{13}C NMR (100.6 MHz, [D_6]DMSO): δ = 169.75, 169.71, 165.72, 160.95, 149.02, 143.62, 133.05, 132.49, 130.73, 130.44, 128.55, 127.66, 127.31, 98.14, 90.91, 84.79, 81.33, 73.45, 63.34, 59.86, 36.09, 20.66, 20.48, 13.84 ppm; HRMS (ESI): *m/z* calcd for $C_{34}H_{35}N_5O_8$: 641.2480 [*M*+H]$^+$; found: 641.2465.

^{ExIm}U

A solution of **6** (150 mg, 0.23 mmol) in methanolic NH_3 (3 mL) was stirred at 22 °C for 14 h, after which the solvent was removed in vacuo. The crude product was purified by flash column chromatography (CH_2Cl_2/MeOH, 98:2 to 90:10) to give ^{ExIm}**U** as yellowish solid (100 mg, 77% yield). R_f = 0.30 (10% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 13.40 (brs), 11.79 (brs), 8.44 (brs), 8.12 (brs), 7.71 (brs), 6.15 (brs), 5.29 (brs), 5.21 (brs), 4.29 (brs), 3.84 (brs), 3.67 (brs), 2.19 ppm (brs); ^{13}C NMR (100.6 MHz, [D_6]DMSO): δ = 161.13, 149.07, 144.05, 130.76, 127.24, 124.14, 97.48, 90.64,

87.30, 84.62, 84.50, 69.57, 60.55 ppm; HRMS (ESI): *m/z* calcd for $C_{30}H_{31}N_5O_6$: 557.2269 $[M+H]^+$; found: 557.2285.

5′-Dimethoxytritylated spin-labeled nucleoside (DMT-^{ExIm}U)

Spin-labeled nucleoside ^{ExIm}**U** (50 mg, 0.09 mmol), DMTrCl (61 mg, 0.18 mmol), and *N*,*N*-dimethylaminopyridine (1.0 mg, 0.008 mmol) were weighed into a round-bottomed flask and kept in vacuo for 16 h. Pyridine (2 mL) was added and the solution was stirred at 22 °C for 2 h. MeOH (100 μL) was added and the solution stirred for 10 min, after which the solvent was removed in vacuo to give a crude orange solid. The solid was purified by column chromatography using gradient elution (CH_2Cl_2/MeOH, 100:0 to 93.5:6 + 0.5% Et_3N), with a column that was prepared in 99.5% CH_2Cl_2 + 0.5% Et_3N. **DMT-^{ExIm}U** was obtained as a yellow solid (58 mg, 75% yield). R_f = 0.65 (15% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.14 (brs), 7.70 (brs), 7.45 (brs), 7.31 (brs), 6.87 (brs), 6.17 (brs), 6.16 (brs), 5.74 (brs), 5.42 (brs), 4.33 (brs), 3.98 (brs), 3.66 (brs), 3.05 (brs), 2.10 (brs), 1.90 (brs), 1.19 ppm (brs); ^{13}C NMR (100.6 MHz, [D$_6$]DMSO): δ = 161.33, 157.96, 149.19, 144.57, 143.21, 135.43, 135.30, 129.56, 127.84, 127.54, 113.16, 85.99, 85.84, 85.16, 70.31, 63.45, 54.92, 45.32, 40.05, 8.42 ppm; HRMS (ESI): *m/z* calcd for $C_{51}H_{49}N_5O_8$: 859.3576 $[M+H]^+$; found: 859.3606.

^{ExIm}U phosphoramidite (7)

DMT-^{ExIm}U (25 mg, 0.03 mmol) and diisopropyl ammonium tetrazolide (8 mg, 0.05 mmol) were dissolved in pyridine (1 mL) and the pyridine removed in vacuo. The residue was kept in vacuo for 19 h, followed by dissolution in CH_2Cl_2 (2 mL) and CH_3CN (1 mL), then addition of 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphoramidite (27 mg, 0.09 mmol). The reaction mixture was stirred at 22 °C for 3 h, diluted with CH_2Cl_2 (10 mL), and washed successively with saturated aq. $NaHCO_3$ (3 × 10 mL) and saturated aq. NaCl (2 × 10 mL). The organic solution was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude solid was purified by precipitation by dissolution in CH_2Cl_2 (0.5 mL), followed by addition of *n*-hexane (50 mL). The liquid was decanted and the operation repeated thrice to furnish phosphoramidite **7** as a yellow solid (24 mg, 78% yield). R_f = 0.50 (100% EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.42 (brs), 7.48 (brs), 7.39 (brs), 6.81 (brs), 6.41 (brs), 4.71 (brs), 4.28 (brs), 4.23 (brs), 3.68 (brs), 3.34 (brs), 2.64 (brs), 2.45 (brs), 1.29 (brs), 1.19 (brs), 1.09 (brs), 0.91 ppm (brs); ^{13}P NMR (162 MHz, $CDCl_3$): δ = 149.16, 148.81 ppm; HRMS (ESI): *m/z* calcd for $C_{60}H_{66}N_7O_9P$: 1059.4654 $[M+H]^+$; found: 1059.4601.

Acknowledgements

S.Th.S. acknowledges financial support from the Icelandic Research Fund (080041023 and 120001021). T.F.P. acknowledges financial support from the German Research Foundation (CRC902: Molecular Principles of RNA-Based Regulation). We thank H. Gustmann and P. Wenk for help in collecting the PELDOR data, Dr. G. Reginsson for simulations of CW-EPR spectra, and Dr. S. Jónsdóttir for assistance in collecting analytical data for structural characterization of the compounds that were prepared.

Keywords: EPR spectroscopy · nucleic acids · PELDOR · DEER · aminoxyl radical

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Received: May 28, 2014
Revised: July 10, 2014
Published online on October 8, 2014