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Physiological-Temperature Distance Measurement in Nucleic Acid using Triarylmethyl-Based Spin Labels and Pulsed Dipolar EPR Spectroscopy

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Supporting Information

ABSTRACT: Resolving the nanometer-scale structure of biomolecules in natural conditions still remains a challenging task. We report the first distance measurement in nucleic acid at physiological temperature using electron paramagnetic resonance (EPR). The model 10-mer DNA duplex has been labeled with reactive forms of triarylmethyl radicals and then immobilized on a sorbent in water solution and investigated by double quantum coherence EPR. We succeeded in development of optimal triarylmethyl-based labels, approach for site-directed spin labeling and efficient immobilization procedure that, working together, allowed us to measure as long distances as ∼4.6 nm with high accuracy at 310 K (37 °C).

Site-directed spin-labeling (SDSL) with following measurement of interspin distances by pulsed dipolar electron paramagnetic resonance (EPR) spectroscopy is extensively used in studies of structure and functions of biomolecules.[1,2] For this purpose, two spin labels are introduced at selected sites of biological system and the value of dipolar interaction between them is measured by double electron−electron resonance (DEER or PELDOR)[3−5] or double quantum coherence (DQC)[6,7] techniques. Since the value of dipolar interaction strongly depends on the interspin distance, these methods provide high-accuracy data in the range of ∼1.5−10 nm.[8,9]

Most often, SDSL uses stable nitroxide radicals attached to proteins,[10,11] DNA and RNA.[12−17] As a rule, PELDOR/DEER experiments with nitroxide spin labels are carried out in frozen solutions at low temperatures ∼50−80 K.[9] The freezing allows the two following requirements of pulsed dipolar EPR to be simultaneously fulfilled. First, immobilization of biomolecule in frozen solution prevents averaging of anisotropic dipolar interaction between spins by rotational diffusion. Second, electron relaxation of spin label (phase memory time, T_m) must be long enough to provide sufficient time for recording at least one period of dipolar oscillation, and this is achieved only at cryogenic temperatures for nitroxides.[18] However, the drawbacks of low-temperature measurements on biomolecules are general uncertainty in coincidence of natural and frozen-state structures and frequently met necessity of using cryoprotectants that may affect distance distributions and conformational equilibria.[19,20] In order to overcome these drawbacks and to reach conditions close to the physiological ones, an intensive search for new spin labels with optimized relaxation properties has been carried out during last several years.[21−23]

Triarylmethyl (TAM) radicals represent a relatively new class of spin labels having long T_m on the order of microseconds in liquids at room temperature.[24] This profoundly long relaxation makes TAMs a promising alternative for nitroxide spin labels. In particular, TAM radicals were successfully applied recently for distance measurements at cryogenic temperature.[25,26] To date, only one pioneering work has been published where the ∼2 nm distance was measured using DQC in trityl-labeled protein attached to a solid support at 4 °C (i.e., nearly room temperature).[23] In particular, this work highlighted a necessity of further improvement of TAM-based labeling methodology in order to achieve longer T_m and allow for distance measurements beyond 2.5 nm. We emphasize also that, to the best of our knowledge, no room-temperature pulse EPR distance measurements on nucleic acids have been reported up to date. Perhaps this is due to the limiting short T_m of spin labels and consequent complications in creating the model systems with spin−spin distances of ∼2 nm. Here we demonstrate first distance measurement of ∼4.6 nm at physiological temperature 310 K (37 °C) in model DNA duplex.

The choice of the optimum TAM and an approach for its introduction into a nucleic acid are of particular significance for this task. The mobility of spin label relative to a biomolecule should generally be restricted, as this allows for narrow distance distributions and more precise distance measurements.[15,27] Therefore, the use of short semirigid linkers should be pursued. Recently we reported an efficient and practical protocol for the large-scale synthesis of tris(8-carboxy-2,2,6,6-tetramethylbenzo-[1,2-*d*;4,5-*d*′]bis[1,3]dithiol-4-yl)methyl, also known as Finland trityl.[28,29] In addition to its availability, the inherent long relaxation times in liquid solutions and a particularly narrow singlet EPR line favor its numerous applications in spectroscopy, materials science, and imaging. Moreover, Finland TAM

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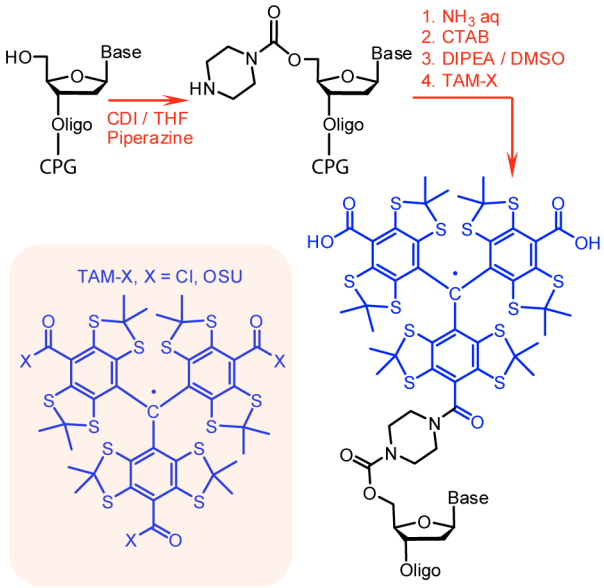
is known to be extremely stable against a majority of biological oxidants and reductants.[30,31] Therefore, Finland TAM has been chosen as a proper precursor for synthesis of two derivatives, i.e., TAM-Cl and TAM-OSU (Scheme 1), used alternatively as spin labeling reagents of oligonucleotides (details of synthesis are given in Supporting Information (SI)).

Scheme 1. Synthesis of TAM-Labeled Oligonucleotide



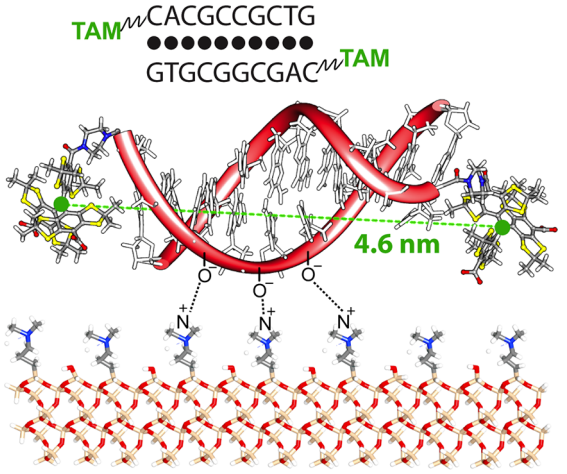
To prepare a model complementary DNA duplex for pulsed dipolar EPR distance measurements, we have synthesized two 10-mer complementary oligonucleotides D1 (5′-CACGCCGCTG-3′) and D2 (5′-CAGCGGCGTG-3′). Each of oligonucleotides was obtained by the phosphoramidite chemistry on controlled pore glass (CPG) support. The CPG-attached 5′-detritylated oligonucleotides were treated by *N*,*N*′-carbonyldiimidazole (CDI) in tetrahydrofuran (THF) (see Scheme 1). After washing the support was treated with 1,4-piperazine solution in anhydrous THF. Derivatives tethered 5′-piperazine residue (Pip-D*n*, *n* = 1 or 2) were fully deprotected in concentrated aqueous ammonia and purified by HPLC. Purified oligonucleotide was transferred to a water-insoluble cetyltrimethylammonium (CTAB) salt and dried. CTAB salt of oligonucleotide derivative was dissolved in anhydrous dimethyl sulfoxide/*N*,*N*-diisopropylethylamine (DIPEA) mixture (DMSO/DIPEA, 20/1, v/v) and treated with 10-fold excess of TAM-Cl. The latter operation afforded the required derivative of TAM to be attached to the only oligonucleotide molecule. Two remaining acyl chloride functions were efficiently hydrolyzed in the course of reaction and further workup, so they did not participate any notably in producing the bi- and trisubstituted TAMs. The details of reaction procedure, labeling efficiency, and spectroscopy data compiled for the title product are given in SI. Contrary to expectation, the alternative TAM-OSU showed negligibly low reactivity toward secondary aminogroup within 5′-piperazine-residue derivative of oligonucleotide, so this reagent was excluded from our further studies.

Remarkably, in course of the SDSL described above, each 5′-end of oligonucleotide has been modified by piperazine unit. This terminal piperazine block plays a role of both rigid linker and a reactive scaffold for attachment of TAM label. We believe that the use of (i) rigid piperazine block and (ii) efficient spin-labeling agent TAM-Cl has been crucial for the success of the synthesis and distance measurements described below. Note that this SDSL approach generally allows labeling of 5′ and 3′ ends of oligonucleotide or both of them simultaneously.

Circular dichroism (CD) study has shown that an attachment of TAM label does not severely perturb the B-form conformation of DNA duplex (SI). The absence of induced CD-signal from TAM-residue indicates that the interaction of label with chiral DNA duplex moiety is negligibly small. Melting temperature analysis additionally confirmed this observation: only insignificant increase of thermal stability (ΔT_{melt} = +1.5 °C) of the duplex arose from introduction of terminal TAM labels. The result of spin labeling of the oligonucleotides was independently confirmed by EPR data (see SI): The covalent attachment of TAM radical to functionalized oligonulceotides leads to partially resolved triplet splitting of EPR line induced by hyperfine interaction with piperazine nitrogen ($a_N \approx 0.022$ mT).

The synthesized TAM-labeled oligonucleotides TAM-Pip-D1 and TAM-Pip-D2 readily formed double stranded spin-labeled DNA duplex TAM-Pip-D1/TAM-Pip-D2 (see Scheme 2). The

Scheme 2. Structure of DNA Duplex TAM-Pip-D1/TAM-Pip-D2 (top)[a]



[a]Immobilization of doubly-labeled DNA on NucleosilDMA particles (bottom). Tertiary structure of this DNA duplex was obtained by MD simulation and visualized using USF Chimera.[32]

latter was then immobilized electrostatically on common ion-exchange sorbent NucleosilDMA by mixing 20 μL water solution of TAM-Pip-D1/TAM-Pip-D2 duplex (0.1 mM) with ∼5 mg of dried NucleosilDMA particles. At room temperature, the NucleosilDMA particles with immobilized duplexes settle down on the bottom of EPR sample tube, and this sorbent-rich part of the sample is introduced into the resonator. Note that the solution above sorbent contains negligible amount of radicals (no EPR signal), meaning that virtually all DNA duplexes are adsorbed. Their continuous wave EPR spectrum is similar to that in frozen water-glycerol solution at T = 200 K, confirming that the desired immobilization has been achieved (see SI).

All studied samples were prepared in D_2O to ensure maximum possible phase memory time (in frozen/liquid solutions)[24] and degassed by repetitive "freeze–pump–thaw" procedures to prevent the relaxation enhancement by oxygen.

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EPR experiments were carried out at X-band (9 GHz) using commercial EPR spectrometer Bruker Elexsys E580.

The phase memory time T_m was measured by two-pulse electron spin echo sequence (for details see SI). It has been found that TAM-Pip-D1/TAM-Pip-D2 duplex electrostatically immobilized on NucleosilDMA has the relaxation time T_m = 1.4 μs at 310 K, which is ~2 times larger than previously published value for immobilized TAM-labeled protein at 277 K (4 °C) in a degassed solution.[23] This increased relaxation time was one of the major factors that allowed us to extend the range of available distances to ~4.6 nm in aqueous solution. We assume that the reason for improved relaxation properties might be more rigid immobilization of the TAM label proposed in our work. In particular, weak electrostatic interaction of TAM with the sorbent might be responsible for this behavior. At the same time, as will be shown below, interaction of spin label with the sorbent has no influence on the distances obtained. Thus, we emphasize the finding of simple and efficient immobilization procedure allowing for significant increase of available distances.

Although two-frequency PELDOR/DEER technique is most often employed for distance measurements in nitroxide-labeled biomolecules, in the case of TAM-based labels the EPR spectrum consists of a single narrow line (inset to Figure 1a), and thus one-frequency DQC method is more suitable.[6] Figure 1a (top) shows the background corrected DQC trace obtained for the studied immobilized duplex at physiological temperature T = 310 K (see SI for details of DQC experiments). Corresponding distance distribution obtained by DeerAnalysis[33] is shown in Figure 1b.

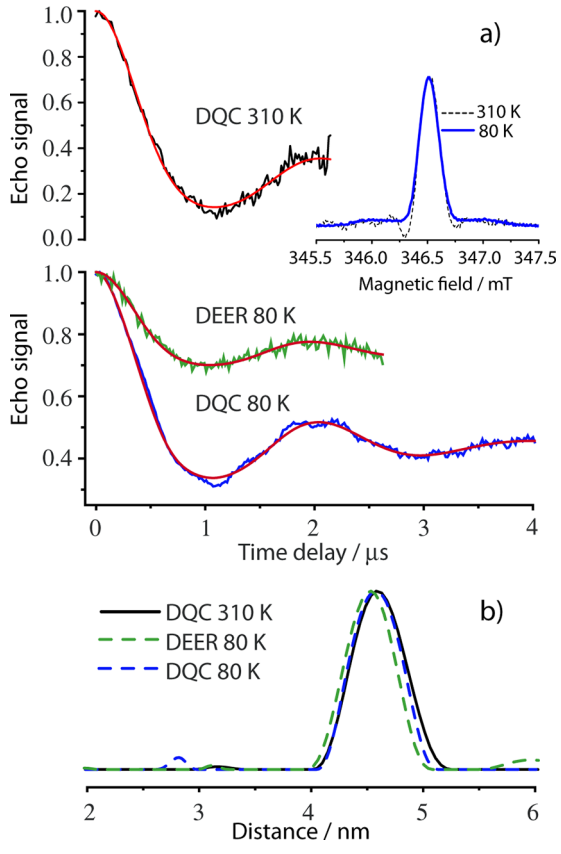

Figure 1. Distance measurements in TAM-Pip-D1/TAM-Pip-D2 duplex obtained by X-band EPR. (a) DQC trace of duplex attached to NucleosilDMA at 310 K in D_2O (black line); DQC (blue line) and DEER (green line) traces of "free" duplex at 80 K in D_2O-glycerol-d_8 (1:1) mixture. All traces were background corrected and normalized. Red lines show best fits obtained using DeerAnalysis2013.[33] Inset shows corresponding echo detected EPR spectra. (b) Obtained distance distributions for traces shown in (a) (normalized).

In addition, the data obtained at 310 K have been compared with the results of DQC and DEER experiments at 80 K in a frozen D_2O-glycerol-d_8 mixture (1:1) (see SI for details of experiments). The background-corrected DQC and DEER traces measured at 80 K are shown in Figure 1a (bottom). Distance distributions corresponding to these data agree well with the distribution obtained from DQC measurement at 310 K (Figure 1b). Tikhonov regularization parameter of 100 (the same for all data) has been found using L-curve approach. The following values of mean distance between spin labels $\langle r \rangle$ and standard deviation parameter σ have been obtained: $\langle r_{DQC} \rangle$ = 4.61 nm, σ = 0.21 nm for DQC at 310 K; $\langle r_{DQC} \rangle$ = 4.58 nm, σ = 0.19 nm for DQC at 80 K; $\langle r_{DEER} \rangle$ = 4.52 nm, σ = 0.19 nm for DEER at 80 K. Evidently, all obtained parameters are very similar, and the accuracy of measurement of mean distance at physiological temperature of 310 K is close to that in frozen solutions (see SI for details).

The coincidence of distance distributions obtained for TAM-Pip-D1/TAM-Pip-D2 duplex immobilized on NucleosilDMA at 310 K and for "free" DNA duplex in frozen solution is an important finding. It proves that weak electrostatic interaction between TAM-Pip-D1/TAM-Pip-D2 duplex and sorbent does not influence the structure of duplex, including orientations of spin labels relative to DNA helix. Thus, in general, the proposed immobilization approach is innocent in terms of structural perturbations, simple for use, and provides sufficient restriction of labels mobility to yield an increased relaxation time (T_m).

An accurate translation of the experimentally obtained distances into structural information has been done by molecular dynamics (MD) simulation of TAM-Pip-D1/TAM-Pip-D2 duplex. MD experiments were performed in explicit solvent at 300 K in NPT ensemble for 210 ns using the AMBER12 software.[34] The introduction of TAM labels does not change DNA duplex structure: root-mean-square deviation (RMSD) from the native structure is 0.6 Å. The spin−spin distance averaged over the trajectory was 4.64 nm with RMSD 0.21 nm (Scheme 2, for details see SI). This value is in excellent agreement with the one measured experimentally by DQC. One would expect that account of spin density delocalization in TAM[35] may lead to slight corrections of the obtained spin−spin distances, however the results of refs 23 and 26 suppose that such deviations of $\langle \Delta r \rangle$ ~ 0.03 nm can be neglected.

The obtained interspin distances (4.52−4.61 nm) exceed the distance between the label attachment sites at the nucleotide residues (~3.4 nm). The overestimation of the measured distances originates from finite size of the labels and their orientations relative to the DNA helix. However, such overestimation is comparable to those observed with standard nitroxide MTSSL label at low temperatures (up to 1.0 nm).[6,9,36] Note that a rigid piperazine linker in the developed SDSL is advantageous for obtaining high-accuracy distances with relatively narrow distributions.

In conclusion, we described the first physiological-temperature EPR distance measurement in nucleic acid using a 10-mer DNA duplex as an example. The essential milestones of this success have been (i) the use of triarylmethyl-based spin labels

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with long relaxation time, (ii) the development of SDSL strategy for attaching TAM label to nucleotide residue via a rigid piperazine linker, and (iii) the elaboration of the efficient and delicate immobilization procedure using NucleosilDMA sorbent. Simultaneously, these advances allowed us to measure as long distances as ~4.6 nm with high accuracy and in conditions simulating natural ones (water solution at 310 K = 37 °C). The developed approaches are rather flexible and universal. They can be adapted and applied in a broad range of biological studies involving nucleic acids, stimulating further progress in determination of nanometer-scale structure and dynamics of biological systems in natural conditions.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis of TAM-Cl and TAM-OSU. Synthesis of native and 5′-piperazine tethered derivatives of oligonucleotides. Synthesis of TAM-labeled oligonucleotide. UV spectra of spin-labeled oligonucleotides. Thermal stability data of D1/D2 and TAM-Pip-D1/TAM-Pip-D2 duplexes. CD data. Procedures of attachment of spin-labeled oligonucleotide to NucleosilDMA particles. Details of MD simulations. General EPR settings. CW EPR spectra. Phase memory time (T_m) measurements. Raw DQC and PELDOR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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