Distance and orientation measurements on nucleic acids using PELDOR and non-covalent spin-labeling

Gunnar Widtfeldt Reginsson
Distance and orientation measurements on nucleic acids using PELDOR and non-covalent spin-labeling

Gunnar Widtfeldt Reginsson

90 ECTS thesis submitted in partial fulfillment of a Magister Scientiarum degree in Chemistry

Advisor
Prof. Snorri Þór Sigurðsson

Faculty Representative
Dr. Olav Schiemann

Faculty of Physical Sciences
School of Engineering and Natural Sciences
University of Iceland
Reykjavik, April 2010
Distance and orientation measurements on nucleic acids using PELDOR and non-covalent spin-labeling
Distance measurements on DNA by pulsed EPR
90 ECTS thesis submitted in partial fulfillment of a *Magister Scientiarum* degree in Chemistry

Copyright © 2010 Gunnar Widtfeldt Reginsson
All rights reserved

Faculty of Physical Sciences
School of Engineering and Natural Sciences
University of Iceland
VRII, Hjardarhagi 2-6
107, Reykjavik
Iceland

Telephone: +354 525 4700

Bibliographic information:
Gunnar Widtfeldt Reginsson, 2010, Distance and orientation measurements on nucleic acids using PELDOR and non-covalent spin-labeling, Master’s thesis, Faculty of Physical Sciences, University of Iceland, pp. 1-68.

Printing: Háskólaprent
Reykjavik, Iceland, April 2010
Abstract

Numerous biological processes involve the interactions between proteins and nucleic acids. Determining the distance and orientation between DNA domains is therefore an imperative step towards the knowledge of the dynamics and function of such complexes. Labeling specific sites on DNA with spin labels and using pulsed EPR, distance and orientation can be measured between DNA domains. Until now, all site-directed spin-labeling (SDSL) techniques for nucleic acids have involved the incorporation of spin labels to oligonucleotides using chemical synthesis. By using the rigid spin label ç, which binds non-covalently to an abasic site opposite a guanine in duplex DNA, site-directed spin-labeling of dsDNA can be done without any synthetic chemistry. Using pulsed electron paramagnetic resonance (PELDOR) at 9.7 GHz, we have determined both distances and orientation between two ç labels bound non-covalently to abasic sites in dsDNA. In addition to measurements of distances beyond 7 nm, we have demonstrated the suitability of the rigid spin label ç for site-directed spin-labeling of nucleic acid–protein complexes, by following the bending of a synthetic Lac operator upon binding to a Lac repressor.
Fjöldi liffræðilegra ferla byggja á sambandi milli kjarnsýra og próteina. Ákvöðun á fjarlægð og innbyrðis afstöðu milli kjarnsýruþattra er þess vegna mikilvægt fyrir aukna þekkingu og skilning á virkni og hreyfingu slikra sambanda. Með staðbundinni spunamerkingu á DNA og púlsaðri EPR spektróskópiu má ákvæða fjarlægðir og afstöðu milli kjarnsýruþattra. Hingað til hefur staðbundin spunamerking kjarnsýra verið fólgin í því að tengja spunamerki við kjarnsýrfjölliður með efnasmiðum. Með notkun á stjarfa spunamerkinu č, sem bindst við guanósín gagnstætt abasískri stöðu í tvíþátta DNA (e. dsDNA) með ósamgildum tengjum, er staðbundin spunamerking dsDNA orðin mun einfaldari í framkvæmd. Með notkun PELDOR (pulsed electron double resonance) á 9,7 GHz örbylgjutíði voru fjarlægðir og afstaka milli tvéggja č spunamerkja, tengdum ósamgildum tengjum við abasískar stöður, á tvíþátta DNA ákvarðaðar. Ásamt því að mæla fjarlægðir lengri enn 7 nm milli tvéggja spunamerkja á DNA, var synt fram á hentuggleika č sem spunamerki fyrir staðbundna spunamerkingu á kjarnsýru–prótein sameindum með því að mæla bognun DNA af völdum LacI próteinsins (e. Lac repressor).
# Table of contents

Abstract ............................................................................................................................ iii

Útdráttur ........................................................................................................................... v

Table of contents .............................................................................................................. vii

List of figures ................................................................................................................... ix

List of tables ..................................................................................................................... xi

Abbreviations ................................................................................................................ xii

Acknowledgements ....................................................................................................... xiii

1 Introduction ................................................................................................................ 1
   1.1 Structure and dynamics of nucleic acids ............................................................. 1
   1.2 Spin-labeling ....................................................................................................... 1
   1.3 EPR spectroscopy ............................................................................................... 3
       1.3.1 CW-EPR ...................................................................................................... 3
   1.4 PELDOR .............................................................................................................. 7
       1.4.1 Pulse EPR ................................................................................................. 7
       1.4.2 Four-pulse PELDOR ............................................................................... 9
   1.5 Non-covalent binding of rigid spin label χ, to abasic nucleic acids ..................... 12
   1.6 Overview of thesis ............................................................................................ 14

2 Distance and orientation measurements on non-covalently spin-labeled DNA...... 15
   2.1 Abasic 20-mer dsDNA non-covalently spin-labeled ......................................... 15
       2.1.1 PELDOR measurements ........................................................................... 15
       2.1.2 Orientation averaging of PELDOR data for DNA 1_9 .............................. 19
       2.1.3 PELDOR simulation of DNA 1_9 .............................................................. 20
   2.2 Optimizing the sample preparation for PELDOR measurements ....................... 23
       2.2.1 Freezing the sample at different rates ....................................................... 23
       2.2.2 Optimizing the concentration of spin label .............................................. 24
       2.2.3 Increasing the relaxation time by using deuterated solvent ..................... 25
   2.3 PELDOR measurements on spin label χ ........................................................... 28

   2.4 Distance measurements on a TATA-box DNA sequence .................................. 29
       2.4.1 Designing the abasic TATA-box DNA ..................................................... 29
       2.4.2 PELDOR measurements on non-covalently spin-labeled 22-mer TATA- box dsDNA ................................................................. 30
       2.4.3 Orientation averaging of PELDOR data for 22-mer TATA-box dsDNA ...... 31
2.4.4 PELDOR simulation of 22-mer TATA-box DNA ........................................... 32
2.4.5 PELDOR measurements of abasic 22-mer TATA-box dsDNA with TBP ... 34
2.4.6 Binding study of TBP with abasic 22-mer TATA-box dsDNA .................. 35
2.5 Bending of DNA by Lac repressor ................................................................. 36
  2.5.1 Designing and spin-labeling the Lac operator ........................................... 36
  2.5.2 PELDOR measurements on non-covalently spin-labeled 29-mer dsDNA ... 38
  2.5.3 PELDOR simulation of non-covalently spin-labeled 29-mer dsDNA ....... 39
  2.5.4 Binding study of Lac repressor with abasic 29-mer dsDNA .................. 40
  2.5.5 PELDOR measurements of non-covalently spin-labeled 29-mer dsDNA bound to Lac repressor ................................................................. 41
  2.5.6 PELDOR simulation of abasic 29-mer dsDNA bound to Lac repressor .... 43
  2.5.7 Orientation averaging of PELDOR data for 29-mer dsDNA .................. 44

3 PELDOR simulation program ........................................................................... 46
  3.1 Simulation of PELDOR measurements on a nitroxide biradical at X- and W-band ........................................................................................................... 47
  3.2 Estimation of uncertainty in simulation parameters ......................................... 50

4 Conclusions ........................................................................................................... 52

Experimental .......................................................................................................... 53
  General procedures ............................................................................................... 53
  Hybridization of oligonucleotides ....................................................................... 53
  Synthesis and purification of DNA oligomers ....................................................... 53
  Preparation of DNA and protein samples ........................................................... 54
  Preparation of 20-mer abasic DNA oligomers for EPR measurements .............. 54
  Preparation of γ for EPR measurements ............................................................. 54
  Preparation of 22-mer abasic TATA-box DNA for EPR measurements ............ 54
  Preparation of 22-mer abasic TATA-box DNA for EMSA ................................ 55
  Preparation of TBP ............................................................................................... 55
  Preparation of 22-mer abasic TATA-box DNA with TBP ................................... 55
  Preparation of 29-mer abasic Lac operator DNA duplexes ................................ 55
  Preparation of 22-mer abasic Lac operator DNA for EMSA ............................. 55
  Preparation of Lac repressor .............................................................................. 56
  Preparation of 29-mer abasic Lac operator DNA with Lac repressor ............... 56
  CW-EPR measurements ....................................................................................... 56
  Pulsed EPR measurements .................................................................................. 57
  Data analysis and modeling ............................................................................... 57

References .............................................................................................................. 59

Appendix ............................................................................................................... 65
  CW-EPR measurements ...................................................................................... 65
List of figures

Figure 1. Rigid spin label Ç base paired with G. 2
Figure 2. Tetrahydrofuran abasic site and the rigid spin label ç. 3
Figure 3. Energy-level diagram for a free electron spin. 4
Figure 4. Rigid spin label ç. 4
Figure 5. Simulation of nitroxide EPR spectra. 5
Figure 6. Precession of the spin magnetic moment, µ around the magnetic field. 7
Figure 7. Microwave pulse excitation profiles. 9
Figure 8. Microwave pulse sequence for four-pulse PELDOR. 10
Figure 9. Simulated PELDOR time trace. 10
Figure 10. A simulated nitroxide EPR spectrum. 11
Figure 11. CW-EPR spectra of non-covalently spin-labeled dsDNA. 12
Figure 12. CW-EPR spectrum of covalently spin-labeled DNA. 13
Figure 13. CW-EPR spectra of spin label ç with unmodified 14-mer DNA duplex. 12
Figure 14. PELDOR data of spin-labeled DNA 1_9. 16
Figure 15. PELDOR data of spin-labeled DNA 1_9. 17
Figure 16. PELDOR data obtained from spin-labeled 20-mer dsDNA. 19
Figure 17. Orientation averaged PELDOR data of DNA 1_9. 20
Figure 18. Positions of spin labels relative to the DNA structure. 22
Figure 19. PELDOR data of DNA 1_9 shock frozen. 24
Figure 20. PELDOR time traces of DNA 1_9. 25
Figure 21. A two-pulse ESEEM time trace for DNA 1_9 in deuterated solvent. 25
Figure 22. A two-pulse ESEEM time trace for DNA 1_9 in deuterated solvent. 26
Figure 23. Background subtracted PELDOR time traces of free spin label ç. 27
Figure 24. PELDOR time traces of DNA 1_9 in protonated and deuterated solvent. 27
Figure 25. PELDOR data of spin label ç at 40, 50, 60, 70 and 80 MHz offset. 28
Figure 26. 16-mer TATA-box containing DNA duplex bound to TBP.

Figure 27. Double spin-labeled TATA-box DNA.

Figure 28. PELDOR data of spin-labeled 22-mer TATA-box DNA.

Figure 29. Orientation averaged PELDOR data of 22-mer TATA-box DNA.

Figure 30. Positions of "spin labels relative to the TATA-box DNA structure.

Figure 31. PELDOR data of spin-labeled 22-mer TATA-box DNA with TBP.

Figure 32. Electrophoretic mobility shift assay of 22-mer TATA box DNA and TBP.

Figure 33. Tetrameric (dimer of dimers) Lac repressor bound to DNA.

Figure 34. Spin-labeled DNA unbound and bound to Lac repressor.

Figure 35. PELDOR data of spin-labeled 29-mer dsDNA.

Figure 36. Positions of "spin labels relative to the 29-mer dsDNA structure.

Figure 37. Electrophoretic mobility shift assay of 29-mer Lac operator and LacI.

Figure 38. PELDOR data of spin-labeled abasic 29-mer dsDNA with LacI.

Figure 39. PELDOR data of spin-labeled Lac operator with and without LacI.

Figure 40. Orientations of spin labels relative to the 29-mer dsDNA bound to LacI.

Figure 41. Orientation averaged PELDOR data of 29-mer dsDNA.

Figure 42. A simulated W-band powder nitroxide EPR spectrum.

Figure 43. Conformational flexibility of a nitroxide biradical.

Figure 44. a) X- and W-band PELDOR time traces of a nitroxide biradical.

Figure 45. Estimation of the uncertainty of PELDOR simulation variables.
List of tables

Table 1. Parameters for simulation of PELDOR data for spin-labeled DNA 1_9. 21
Table 2. Distance between the two spin labels on DNA 1_9. 23
Table 3. PELDOR simulation parameters for spin-labeled TATA-box dsDNA. 33
Table 4. Distance between the two spin labels on TATA-box dsDNA. 33
Table 5. PELDOR simulation parameters for spin-labeled 29-mer dsDNA. 39
Table 6. PELDOR simulation parameters for spin-labeled DNA bound to LacI. 44
Table 7. Distance between the two spin labels on Lac DNA. 45
Table 8. PELDOR simulation parameters for nitroxide biradical at X- and W-band. 49
Abbreviations

A  adenine
C  cytosine
G  guanine/Gauss
T  thymine
U  uracil
Ap  apurinic and apyrimidinic
CW-EPR  continuous wave electron paramagnetic resonance
DEER  double electron electron resonance
dna  deoxyribonucleic acid
EDTA  ethylenediaminetetraacetic acid
EMSA  electrophoretic mobility shift assay
EPR  electron paramagnetic resonance
FFT  fast Fourier transform
FRET  fluorescence resonance energy transfer
K  kelvin
NMR  nuclear magnetic resonance
MES  2-(N-morpholino)ethanesulfonic acid
MPD  2-Methyl-2,4-Pentanediol
PAGE  polyacrylamide gel electrophoresis
PDB  protein data bank
PEG  polyethylene glycol
PELDOR  pulsed electron double resonance
PNE  10 mM Na$_2$HPO$_4$, 100 mM NaCl, 0.1 mM Na$_2$EDTA, pH 7.0
RNA  ribonucleic acid
SDSL  site-directed spin-labeling
TWT  travelling wave tube
µs  micro seconds
ns  nano seconds
Acknowledgements

I would first of all like to thank Professor Snorri Þór Sigurðsson for his guidance and confidence in my work. His enthusiasm for science has been a true inspiration for me. I would like to give special thanks Dr. Olav Schiemann for his guidance with my research project. His scientific knowledge and expertise has been inspiring and of great value to me.

I wish to thank the following people for their help and collaboration: Dr. Graham Smith, Dr. Hassane El Mkami, Dr. Paul Cruickshank, Dr. Robert Hunter, Prof. Malcolm White, Dr. Christophe Rouillon, Biljana Petrovic-Stojanovska. Group members of the Sigurdsson and Schiemann groups receive my thanks for their helpful discussions.

Last but not least I would like to give my deepest gratitude to my wife, Lilja and our children, María and Sara for their love and support.
1 Introduction

1.1 Structure and dynamics of nucleic acids

Nucleic acids are biopolymers with a diverse and important role in biological systems and in recent years their use in the fields of nanotechnology and materials science has increased because of programmable properties and the ability to self-assemble. Deoxyribonucleic acid (DNA), is a nucleic acid with the pivotal role of storing the genetic code in living organisms. Other forms of nucleic acid structures include, ribonucleic acid (RNA), DNA or RNA aptamers, ribozymes and quadruplex structures. Recognition of specific DNA base sequences and base lesions e.g. abasic sites, strand breaks and single-base mismatches by transcription factors and repair proteins respectively, are examples of the importance of nucleic acid–protein complexes. Among the factors that control the recognition of these sites by proteins are the inherent flexibility and dynamics of nucleic acids. Knowing the global structure and dynamics of nucleic acids and their interactions with proteins or ligands is therefore a key step towards knowledge of the function of these systems in biological environments. Among the most successful methods of structure determinations of biomolecules are X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, fluorescence resonance energy transfer (FRET) and electron paramagnetic resonance (EPR) spectroscopy. All of these methods have their strengths and weaknesses but most importantly, they complement each other. This thesis will focus on the method of pulsed electron electron double resonance for distance and orientation determinations in nucleic acids.

1.2 Spin-labeling

For structural determinations by EPR methods, nucleic acids have to contain paramagnetic centers. Ribozymes can contain intrinsic binding sites for divalent metals. With the exchange of the divalent metals for paramagnetic metal centers EPR methods can be used to study the structure of the binding site, but in most cases unpaired electrons have to be incorporated into nucleic acids. Site-specific incorporation of unpaired electrons to biomolecules has been extensively used, both for proteins and nucleic acids and is known as site-directed spin-labeling (SDSL). The most commonly used spin labels are nitroxides which are stable organic free radicals. In addition to being applicable to distance measurements with continuous-wave- (CW) or pulsed EPR techniques, the nitrooxide radical has a CW-EPR spectrum that can report on the inherent dynamics of the spin label, polarity and pH of the solvent. Nitroxides are therefore versatile spin labels. Considerable work has been done on nucleic acid spin-labeling. Through the attachment of nitroxides, by ways of flexible or semi-flexible tethers, to the phosphate backbone, sugar moieties or nucleobases of RNA and DNA, distances between spin labels and dynamics of...
individual nucleotides have been measured with EPR techniques.\textsuperscript{24, 25, 28-31} The accuracy and analysis of distances and especially dynamics deduced from EPR are greatly diminished and complicated by the inherent flexibility of the spin label and its linker.\textsuperscript{30, 32} Recently the synthesis and application of \( \mathcal{C} \), a rigid nitroxide spin label for nucleic acids, has been reported by Sigurdsson et al. (Figure 1).\textsuperscript{33-35} By means of automated oligonucleotide synthesis the phosphoramidite of \( \mathcal{C} \) is incorporated into DNA oligomers. When the spin-labeled oligomer is annealed to its complementary strand the spin label has the potential to base-pair with guanine through Watson-Crick base pairing (Figure 1).

![Figure 1. Rigid spin label \( \mathcal{C} \) base paired with G.](image)

This yields a rigid spin-labeled dsDNA, with negligible perturbation to the duplex.\textsuperscript{34} Not only do rigid spin labels report on the distance between spin-labeled sites with greater precision but they also give the opportunity to unravel the orientation of the spin label relative to the biomolecule in a disordered sample using advanced EPR techniques.\textsuperscript{21, 36-38}

Site-specifically spin-labeling nucleic acids, especially with rigid spin labels, involves laborious organic synthesis, not easily accessible to all scientific disciplines. Given the fact that \( \mathcal{C} \) has the potential to interact with nucleobases through base-pairing and base-stacking, the two main contributing factors of DNA duplex stability,\textsuperscript{4} motivated the development of a new method of site-specifically spin-labeling nucleic acids. The spin label \( \mathcal{C} \) (which is essentially the base moiety of the nucleoside \( \mathcal{C} \)) was synthesized and non-covalently incorporated into an abasic site of a DNA duplex (Figure 2), for structural studies on nucleic acids.\textsuperscript{59} Since oligonucleotides with abasic sites are commercially available and relatively easy to synthesize, SDSL of nucleic acids becomes a straightforward method. This method in conjunction with pulse EPR spectroscopy was used to determine distances in DNA and is described in this thesis.
1.3 EPR spectroscopy

In this section we will give a brief introduction to the theory of electron paramagnetic resonance (EPR) and discuss how the inherent properties of nitroxide radicals can be measured with EPR to study structure and dynamics of biomolecular systems.

1.3.1 CW-EPR

Electron paramagnetic resonance spectroscopy is a technique for studying chemical species with an unpaired electron. An electron has a spin magnetic moment, arising primarily from its spin angular momentum. The interaction of the spin magnetic moment with a static magnetic field (Zeeman interaction) lifts the degeneracy of the spin magnetic dipole energy levels. For a free unpaired electron with a spin quantum number of 1/2 (S=1/2), two levels arise (M_s = ± 1/2), with an energy difference that increases with the magnitude of the static magnetic field (Figure 3).
Figure 3. Energy-level diagram for a free electron in a magnetic field. \( \Delta E \) is the energy required for the transition between the two spin states. For a description of the various symbols see text.

The addition of an applied oscillating magnetic field \( (B_1) \) perpendicular to the static field \( (B_0) \), with the use of polarized microwaves, induces transitions between the two magnetic dipole energy levels. In continuous-wave EPR spectroscopy (CW-EPR), the absorption of a low power continuous microwave radiation by a paramagnetic sample is monitored as the static magnetic field \( (B_0) \) is varied.

The condition for transitions between magnetic dipole energy levels, i.e. the resonance condition, is given by the equation \( \hbar \nu = g_e \beta_e B \) where \( \beta_e \) is the Bohr magneton, \( g_e \) is the free-electron g-factor and \( B \) is the effective magnetic field. For most paramagnetic systems the g-factor is anisotropic and deviates from \( g_e \) because of spin-orbit interaction. Anisotropic g-factors are represented by 3x3 matrices and are referred to as g-tensors. Dipolar interaction between the electron-spin magnetic moment and magnetic dipoles from nuclei splits the electronic resonances into further \( 2I+1 \) lines, where \( I \) is the nuclear spin quantum number. This interaction is called nuclear hyperfine interaction and has both an isotropic and anisotropic part. Anisotropic hyperfine coupling is likewise represented by a 3x3 matrix, or A-tensor. 39, 40

The principal components of the nitroxide radical g-tensor are collinear to the nitroxide molecular frame (Figure 4). The EPR spectrum of a nitroxide depends on the orientation of the g-tensor relative to the static magnetic field (Figure 5 a). The anisotropic hyperfine coupling A (considered to be collinear to the g-tensor) between the unpaired electron spin \( (S=1/2) \) and the \(^{14}\text{N} \) nuclear spin \( I(^{14}\text{N})=1 \) splits each of the electronic transitions into three lines (Figure 5 a). 39

Figure 4. Rigid spin label \( \xi \) and the orientations of the g-tensor principal components relative to the molecular frame.
Simulations of EPR spectra for a nitroxide radical illustrate how the orientation of the nitroxide g-tensor, relative to the direction of the applied magnetic field, and dynamics affect the EPR line positions and splitting. A nitroxide single crystal, oriented with the three principal components of the g-tensor, \( g_{xx} \), \( g_{yy} \) and \( g_{zz} \) parallel to the static magnetic field shows the anisotropy of the g-factor and hyperfine coupling (Figure 5a). A nitroxide powder or glassy frozen sample will be composed of randomly oriented spin centers, the EPR spectrum will therefore be a sum of spectra from all orientations and reveal the full anisotropy (Figure 5a, top). Conversely, in a solution the nitroxide molecules rapidly and randomly reorient on the timescale of the EPR experiment. The recorded EPR spectrum therefore represents a time-averaged spectrum where the anisotropic g- and A-tensors are averaged out and only the isotropic g-factor and hyperfine coupling constant are visible (Figure 5b). By increasing the viscosity of the solution or decreasing the temperature the tumbling of the molecules is slowed down. As the molecular dynamics becomes slower the anisotropic interactions will not average out completely and the isotropic EPR spectrum becomes broader and asymmetric. Due to the anisotropy of the g- and hyperfine tensors, the EPR line shape broadens inhomogeneously, that is different EPR lines will have different line-widths. As the sample becomes a solid e.g. frozen glass, the nitroxide molecules are immobilized and the EPR spectrum will be a superposition of spectra of all orientations (Figure 5b, bottom).

![Figure 5. Simulation of nitroxide EPR spectra.](image)

Since the shape of a nitroxide EPR spectrum in solution reflects the inherent mobility of the spin center, dynamics of a nitroxide spin label and its local environment can be deduced from the shape of an CW-EPR spectrum. In addition, the nitroxide g- and hyperfine-tensors are influenced by the polarity of the solvent. The EPR spectrum can, therefore, also report on the spin label solvent and accessibility.

In the same way as an unpaired electron’s spin magnetic moment couples to nuclear magnetic moments, and causes hyperfine splittings, it interacts with the spin magnetic moment of other unpaired electrons which gives rise to the electron-electron exchange coupling and the anisotropic magnetic dipole-dipole coupling tensor. The dipole-dipole coupling between a pair of unpaired electrons, A and B is described by the Hamiltonian (eq. 1):
\begin{equation}
\hat{H}_{AB} = \frac{\mu_0}{4\pi\hbar} g_A g_B \beta_e^2 \left[ \hat{S}_A^T \cdot \hat{S}_B - \frac{3(\hat{S}_A^T \cdot \mathbf{r})(\hat{S}_B^T \cdot \mathbf{r})}{r^3} \right] \tag{1}
\end{equation}

where \( \hat{S}_A \) and \( \hat{S}_B \) are the spin operators and \( g_A \) and \( g_B \) the g-values for spin A and B respectively. The distance between the spin centers is given by \( r \), \( \mu_0 \) is the permeability of vacuum and \( \hbar \) is the reduced Planck constant. Expanding eq.1 and expressing the orientation of the interspin tensor in spherical coordinates, the Hamiltonian for the dipole-dipole interaction between two unpaired electrons becomes (eq.2),

\begin{equation}
\hat{H}_{AB} = \frac{\mu_0}{4\pi\hbar} g_A g_B \beta_e^2 (A + B + C + D + E + F) \tag{2}
\end{equation}

where

\[
A = S_z^A S_z^B \left( 1 - 3 \cos^2 \theta \right)
\]

\[
B = -\frac{1}{4} \left( S_+^A S_-^B + S_-^A S_+^B \right) \left( 1 - 3 \cos^2 \theta \right)
\]

\[
C = -\frac{3}{2} \left( S_+^A S_z^B + S_z^A S_+^B \right) \sin \theta \cdot \cos \theta \cdot \cos \varphi
\]

\[
D = -\frac{3}{2} \left( S_-^A S_z^B + S_z^A S_-^B \right) \sin \theta \cdot \cos \theta \cdot \sin \varphi
\]

\[
E = -\frac{3}{4} S_+^A S_z^B \sin^2 \theta \cdot e^{-2i\varphi}
\]

\[
F = -\frac{3}{4} S_+^A S_z^B \sin^2 \theta \cdot e^{2i\varphi}
\]

If the dipole-dipole coupling is small compared to the resonance difference of spins A and B, which at X-band frequencies is valid for distances exceeding 1.5 nm, and the paramagnetic centers have a weak g-anisotropy, only the first term in eq.2 needs to be considered. The energy of the dipole-dipole interaction is then described, in frequency, by eq.3,

\begin{equation}
\omega_{dip} = \frac{\mu_0 g_A g_B \beta_e^2}{4\pi\hbar r^3} \left( 1 - 3 \cos^2 \theta \right) \tag{3}
\end{equation}

where \( \theta \) is the angle between the dipolar distance vector and the static magnetic field. Furthermore, it is assumed that the dipole-dipole tensor can be described by the point-dipole approximation. For a nitroxide radical, this approximation assumes that the electron spins are localized to the center of the N-O bond. 32, 44-47

From eq.3 it can be seen that the dipole-dipole interaction depends on the distance between the paramagnetic centers. This attribute makes distance measurements between paramagnetic centers e.g. spin labels, on complex bio-systems possible. The through-space dipolar coupling between unpaired electrons causes splitting and/or broadening of a CW-EPR spectrum. If the two unpaired electrons are in the range of 2 – 2.5 nm apart, the distance can be deduced from the CW-EPR spectrum. 21, 48, 49 For longer distances the dipolar splitting can no longer be resolved from the inherent line broadening by CW-EPR methods and more elaborate and advanced EPR techniques have to be used, such as pulsed paramagnetic resonance (PELDOR), described in the next section.
1.4 PELDOR

In this chapter a short introduction to the theory of pulsed electron double resonance (PELDOR), also known as double electron electron resonance (DEER), EPR technique will be presented. The extraction of interspin distances, as well as the relative orientations of a pair of spin labels from PELDOR measurements will be discussed.

1.4.1 Pulse EPR

An unpaired electron with spin quantum number 1/2 (S=1/2) has two spin quantum states (M_S=±1/2) as mentioned in the previous chapter. In classical mechanics, the electron spin magnetic moment precesses about the direction of an applied magnetic field (conventionally assigned to the laboratory Z-axis) with a frequency known as the Larmor frequency (ν=gβ_eB/h). The z component of the spin magnetic moment is quantized accordingly and is given by μ_z = -g_eβ_eM_S (Figure 6).

![Figure 6](image)

Figure 6. A diagram that shows the precession of the spin magnetic moment, μ around the magnetic field Z-axis with a constant angle. The z component of the spin magnetic moment is depicted for the, M_S= -1/2 state.

The electronic Zeeman energy of the two spin states is given by eq.4, \(^{39}\)

\[
U = \mu_z B = \pm \frac{1}{2} g_e \beta_e B
\]

where the spin state M_S = -1/2 corresponds to the lower energy state U=gβ_eB/2.

Microwave radiation in resonance with the electron Larmor frequency induces transitions of the magnetic moment between the two spin states. The population difference of the two energy states in a sample of a paramagnetic species at thermodynamic equilibrium is given by the Maxwell-Boltzmann equation (eq.5), where ΔU is the energy difference between the two levels, k_b is the Boltzmann constant and T is the temperature of the sample.
\[
\frac{N_{upper}}{N_{lower}} = \exp \left( -\frac{\Delta U}{k_B T} \right)
\]  \hspace{1cm} (5)

Even at room temperature the population of the lower energy state is slightly higher which is enough to ensure a net absorption of microwave energy. The techniques of pulsed EPR utilizes high power microwave pulses with a duration of only a few nanoseconds in order to excite a broad distribution of spin species and flip the electron spin magnetic moment, most commonly $\pi/2$ or $\pi$ radians about an axis perpendicular to the applied magnetic field.\textsuperscript{50, 51} The excitation intensity of a microwave pulse with a flip angle of $\pi$ radians can be approximated by,

\[
I = \left( \frac{\omega_1}{\omega_{\text{eff}}} \right)^2 \sin^2(\omega_{\text{eff}} t/2)
\]  \hspace{1cm} (6)

and the following relations;

\[
\omega_1 = \frac{\pi}{t} \hspace{1cm} (7)
\]

\[
\omega = B_1 - \nu \hspace{1cm} (8)
\]

\[
\omega_{\text{eff}} = \sqrt{\omega_1^2 + 4\pi^2 \nu^2} \hspace{1cm} (9)
\]

where $t$ is the duration of the microwave pulse, $B_1$ is the magnitude of the microwave magnetic field and $\nu$ is the central frequency of the microwave pulse.\textsuperscript{46} Using eq. 6 the excitation profile of a $\pi$ pulse can be calculated as a function of pulse length. The excitation width of a microwave pulse broadens with shorter pulse duration, a consequence of the Heisenberg uncertainty principle\textsuperscript{52} (Figure 7).
Figure 7. Excitation profiles of a microwave pulse with a flip angle of π radians. The duration of the pulse is varied from 12 to 36 ns. The width of the excitation at half height is 67, 45, 33, 27 and 23 MHz for the 12, 18, 24, 30 and 36 ns pulses respectively.

1.4.2 Four-pulse PELDOR

Pulsed electron double resonance (PELDOR), also known as double electron electron resonance (DEER), experiments can be used to recover the through-space dipolar interaction between two unpaired electrons from the inhomogeneous line broadening. In the absence of exchange interaction between two spin centers the frequency of the dipolar coupling can be expressed by eq. 3.40

For two nitroxides, eq. 3 evaluates to eq. 11.

\[ \nu_{dip} = 52.16 \frac{1}{r^3} (1 - 3 \cos^2 \theta) \text{ MHz nm}^3 \]  \hspace{1cm} (11)

The four-pulse PELDOR experiment involves the setup of microwave pulses of two different frequencies. Pulses applied at frequency \( \nu A \) are denoted as detection pulses and the pulse applied at frequency \( \nu B \) is the inversion pulse (Figure 8). The first detection pulse (flip angle = \( \pi/2 \)) at time \( t = 0 \) flips a resonant spin packet (A spins) to the \( x-y \) plane where the different spin species magnetic moments start to precess around the \( z \)-axis in the \( x-y \) plane with different angular frequencies. The second detection pulse at time \( t = \tau 1 \) (flip angle = \( \pi \)) reverses the direction of all the magnetic moments so that the different spin magnetic moments will refocus and again fan out with their inherent frequency. These two first pulses create a Hahn echo at time \( t = 2 \tau 1 \). The fourth pulse (flip angle = \( \pi \)) at time \( t = 2\tau 1 + \tau 2 \) again refocuses the A spins to create a refocused echo at time \( t = 2\tau 1 + 2\tau 2 \). The third pulse, or the inversion pulse, (flip angle = \( \pi \)) at time \( t = T \) inverts the spin magnetic moments of a different resonant spin packet (B spins). The A spins that are coupled via dipolar interaction with the B spins will upon inversion of the B spins precess with the opposite direction in the \( x-y \) plane. The inversion of the coupled A spins during the time evolution of the A spins will affect the intensity of the refocused echo. As the time position of the inversion pulse is varied the refocused echo acquires a periodic modulation (eq. 12).53
\[ I(T) = \cos(\omega_{dip}(\tau_1 - T)) \]  \hspace{1cm} (12)

**Figure 8.** Microwave pulse sequence for four-pulse PELDOR.

When the resulting echo integral is recorded as a function of \( T \), a time trace is acquired that oscillates with the angular frequency, \( \omega_{dip}(r, \theta) \). The modulation amplitude, when all oscillation is damped, is given by \( 1 - \Delta \), where \( \Delta \) is the modulation depth (Figure 9a).\textsuperscript{46, 54, 55} When Fourier transformed, the time trace yields a Pake pattern with peaks at \( \pm \omega_{dip} \) and edges at \( \pm 2\omega_{dip} \), which correspond to an angle (\( \theta \)) of 90° and 0° respectively between the dipolar distance vector and the applied magnetic field (Figure 9b).\textsuperscript{32, 54}

**Figure 9.** a) A simulated PELDOR time trace, background subtracted. b) Fourier transformation of the simulated PELDOR time trace.

In a disordered paramagnetic sample, the dipolar time evolution data (PELDOR signal) contains contribution from both inter- and intramolecular dipolar interactions, \( V(T) = V(T)_{\text{intra}}V(T)_{\text{inter}} \). The intramolecular contribution to the signal can be expressed by

\[ V(T) = V_0 \left\langle \prod_{B=1}^{n} (1 - \lambda_B (1 - \cos(\omega_{dip}T))) \right\rangle \]  \hspace{1cm} (13)

where \( V_0 \) is the PELDOR signal at \( T=0 \) and \( \langle ... \rangle \) indicates averaging over all dipolar configurations. The intermolecular contribution is given by,
\[ V(T) = \exp \left( -\frac{2\pi \mu_0 \gamma^2 \hbar}{9\sqrt{3}} C \lambda_B T \right) \]  

(14)

where \( \gamma \) is the electron gyromagnetic ratio, \( C \) is the spin concentration in mol m\(^{-3} \) and \( \lambda_B \) is the fraction of B spins excited by the inversion pulse. \(^{32, 46, 55-57} \)

At X-band (9.7 GHz), the width of the nitroxide EPR spectrum is dominated by the \( z \) component of the anisotropic \(^{14}\)N hyperfine coupling \( A_{zz} \), while the \( A_{xx}, A_{yy} \) components and the anisotropic \( g \)-tensor remain unresolved (Figure 10). In a PELDOR experiment the applied magnetic field is held constant and the center frequencies of the detection and inversion microwave pulses are set to resonance with specific spin species. In order to know what frequencies to set the detection and inversion pulses to, a field swept CW-EPR spectrum is recorded (Figure 10) and the frequency positions for specific components of the spectrum calculated from field positions. For nitroxides at X-band, the optimum position for the inversion pulse is at the maximum of the field swept spectrum (i.e. at transitions for all orientations of the nitroxides) while the detection pulses are set to the low-field part of the spectrum i.e. at the transition for \( A_{zz} \) and off-diagonal components of the \( A \)-tensor (Figure 10). The detection and inversion pulses therefore excite different orientations of the nitroxides in the disordered frozen sample.

![Figure 10. A simulated echo detected field sweep EPR spectrum of a nitroxide at X-band. The microwave excitation profiles are overlaid in transparent colours. The blue excitation profile indicates the position of the inversion pulse. The green and red excitation profiles indicate the position of the detection pulses at either 40 or 80 MHz offsets respectively. The stick spectrum at the top of the figure shows the approximate positions of the \(^{14}\)N hyperfine coupling components for the different \( g \) components. Blue, green and red sticks represent the \( g_{xx}, g_{yy} \) and \( g_{zz} \) components respectively.](image)

With the inversion pulse applied to the maximum of the nitroxide spectrum it predominantly excites the \( A_{xx} \) and \( A_{yy} \) tensor components in addition to the \( A_{zz} \) (\( M_I = 0 \)). With the detection pulses set to the left wing of the nitroxide field sweep spectrum they mostly excite the \( A_{zz} \) (\( M_I = -1 \)) component. As the position of the detection pulses is moved closer to the maximum of the spectrum more of the off-diagonal components \( A_{xx}, A_{yx}, A_{zy} \) will be excited with the detection frequency (Figure 10). The frequency and modulation depth of the PELDOR time trace is dependent on the orientation of the dipolar distance vector relative to the applied magnetic field.\(^{55} \) If the orientation of the dipolar vector is correlated to the orientation of the spin labels, acquiring PELDOR time traces
with the detection pulse sequence at different positions of the low field part of the X-band nitroxide spectrum, specific orientations of the spin labels can be assigned to different PELDOR time traces.\cite{36}

For molecules with a fast rotation compared to the inverse dipolar frequency, the dipole-dipole interaction (eq.3) gets averaged to zero.\cite{32} For this reason, samples that are in solution have to be frozen into glass for PELDOR measurement. In addition, performing PELDOR measurements at low temperatures, (below 78 K), slows down the rate of spin transverse relaxation, which makes measurements of long distances possible.\cite{54, 58}

1.5 Non-covalent binding of rigid spin label $\zeta$, to abasic nucleic acids

As previously mentioned, distance measurements on dsDNA oligomers in the nanometer range, using EPR methods and site directed spin-labeling, is an established technique. Using non-covalently bound spin labels for distance and orientation measurements on dsDNA has to our knowledge not been tried before.

For successful spin-labeling of a dsDNA oligomer using a spin label that is to be bound non-covalently, the DNA has to contain a specific binding site that has high affinity for the spin label. In addition, it is imperative that the spin label has low affinity for unspecific binding to the DNA duplex. If indeed the spin label is able to non-covalently bind to the dsDNA abasic sites as proposed, the mobility of the spin label in solution with abasic dsDNA should be considerably impeded.

CW-EPR spectra from previous measurements on the abasic 14-mer dsDNA,

\[
\begin{align*}
5' - & \text{GAC - CTC - GA}_{\text{Ap}} - \text{TCG - TG} - 3' \\
3' - & \text{CTG - GAG - CGT - AGC - AC} - 5'
\end{align*}
\]

(Ap=abasic site) in aqueous 20% (v/v) ethylene glycol solution with one equivalent of spin label $\zeta$ per abasic site, show that with decreased temperature, the EPR spectrum becomes a superposition of nitroxide spectra from a mobile species and a species with decreased mobility. At 0 °C two peaks at low and high field are visible and increase in intensity with decreased temperature (Figure 11, blue arrows). At -20 °C the transitions from fast moving spin labels have decreased considerably (Figure 11, red arrows)\cite{59}.

![Figure 11. CW-EPR spectra of non-covalently spin-labeled dsDNA. Measured at 0 to -30 °C from left to right in steps of 10 °C. Blue and red arrows point to EPR transitions from immobilized and fast moving spin labels, respectively.](image)
Comparing these results with results from CW-EPR measurements that were carried out on the 14-mer DNA duplex,

\[
\begin{align*}
5' & \text{– GAC - CTC - GÇA - TCG - TG – 3'} \\
3' & \text{– CTG - GAG - CGT - AGC - AC – 5'}
\end{align*}
\]

covalently spin-labeled with the rigid spin label Ç (Figure 12)\(^59\), shows that the spectra for the non-covalently bound spin label ç (Figure 11) have a larger contribution of fast moving spin labels at all temperatures, except at -30 °C. It is, therefore, apparent that at temperatures above -30 °C there is less non-covalent binding of spin label.

![Figure 12. CW-EPR spectrum of a 14-mer DNA duplex, covalently spin-labeled with the rigid spin label Ç. Measured at 0 to -30 °C, from left to right in steps of 10 °C. Blue arrows point to EPR transitions from immobilized spin labels.](image)

To examine any unspecific binding of the spin label, CW-EPR spectra of spin label ç with the unmodified 14-mer DNA duplex,

\[
\begin{align*}
5' & \text{– GAC - CTC - GCA - TCG - TG – 3'} \\
3' & \text{– CTG - GAG - CGT - AGC - AC – 5'}
\end{align*}
\]

have been recorded (Figure 13)\(^59\). As the temperature reaches -30° C, a small fraction of spin labels show a restriction of mobility (Figure 13 blue arrows). Compared to Figure 11 the extent of immobilization is far less and is most probably due to groove binding and/or stacking of the spin labels at the end of the duplex. Most importantly, this shows that the binding of ç is to a small extent, unspecific.

![Figure 13. CW-EPR spectra of spin label ç with unmodified 14-mer DNA duplex, i.e. DNA not containing an abasic site. Measured at 0, -10, -20 and -30 °C (from left to right). Blue arrows point to EPR transitions from immobilized spin labels.](image)
1.6 Overview of thesis

In the first chapter of this thesis a brief introduction was given to nucleic acids and their importance in biological systems emphasized. It was discussed how site-directed spin-labeling can be used to obtain information on structure and dynamics of nucleic acids and the initiative to non-covalently spin label dsDNA, using $\mathcal{C}$. In addition, an introduction to the theory and techniques of EPR and pulsed EPR was given. The final chapter in the introduction contained an overview of previous CW-EPR studies performed on dsDNA containing one abasic site and non-covalently bound spin label $\mathcal{C}$. In chapter two the results from PELDOR measurements on doubly non-covalently spin-labeled dsDNA will be presented and a discussion on the information that can be obtained from the PELDOR time traces. Chapter three will contain detailed explanations of the PELDOR simulation program and determination of the uncertainty in simulation parameters. The fourth and last chapter contains a summary of results and conclusions. In the appendix, CW-EPR measurements, that were performed to verify the binding of spin labels to DNA abasic sites, will be presented.
2 Distance and orientation measurements on non-covalently spin-labeled DNA

In this chapter the results of PELDOR measurements on non-covalently spin-labeled synthetic dsDNA and the determination of distances and relative orientation of spin labels will be presented.

2.1 Abasic 20-mer dsDNA non-covalently spin-labeled

To assess the ability of spin label $\chi$ to function as a spin label for orientation selective distance measurements on dsDNA, 4-pulse PELDOR experiments were carried out on the 20-mer DNA duplex,

\[ 5' - GT_{Ap} - AGT - GCG - CGC - GCG - CGA - TC - 3' \]
\[ 3' - CAG - TCA - CGC - G_{Ap}G - CGC - GCT - AG - 5' \]

where Ap is an abasic site. For future reference this DNA duplex will be called DNA 1_9. To be applied to conformational and structural analysis of dsDNA the spin label has to bind tightly and specifically to the abasic sites to yield a high concentration of doubly labeled dsDNA with a well defined distance and orientation.

2.1.1 PELDOR measurements

4-pulse PELDOR experiments at X-band frequencies were carried out on samples of non-covalently spin-labeled DNA 1_9 at 50 K. As previously shown, CW-EPR measurements indicate that the amount of $\chi$, bound to the DNA duplex increases with decreased temperature and reaches a maximum at ca. -30 °C (Figure 11).

A sample of DNA 1_9 with two equivalents of spin label $\chi$ in aqueous 20% (v/v) ethylene glycol was thus cooled from 0 to -30 °C in steps of 10 °C over a period of about 15 minutes. After cooling, the sample was shock frozen. The first step of a PELDOR experiment is to set up a two pulse Hahn echo and acquire a field swept spectrum (Figure 14 a) to set the centerfield and guide the setup of frequencies for detection and inversion pulses (Figure 10). The next step is to set up a two-pulse electron spin echo envelope modulation (ESEEM) experiment (Figure 14 b). An ESEEM time trace is modulated by the hyperfine interaction between an unpaired electron and weakly coupled nuclei$^{60}$ and gives a quantitative estimation of the electron transverse relaxation time $T_2$. $^{61}$
exponential decay function was fitted to the ESEEM time trace of DNA 1_9 and revealed a $T_2$ of $4050 \pm 30$ ns for the nitroxide spins. The position of the fourth PELDOR pulse should be limited to $T_2$ if a PELDOR signal, with a good signal to noise (S/N), is to be obtained.

![Figure 14. a) An echo detected field sweep of non-covalently spin-labeled DNA 1_9. b) Two-pulse ESEEM time trace for non-covalently spin-labeled DNA 1_9.](image)

To establish orientation selectivity, PELDOR measurements were performed on non-covalently spin-labeled DNA 1_9 with the detections pulse sequence at 80 to 40 MHz higher frequency than the inversion pulse (80 to 40 MHz offset), in steps of 10 MHz (Figure 15). At 80 MHz offset the detection pulse sequence predominantly excites the $A_{zz}$ component while at 40 MHz offset the $A_{xx}$, $A_{yy}$, and off-diagonal terms are mainly excited.

A PELDOR time trace with the detection pulses at 80 MHz offset, i.e. detection of the $A_{zz}$ transition, showed a moderate modulation. Upon moving the detection pulses to 40 MHz offset, in steps of 10 MHz, the modulation amplitude increased dramatically (Figure 15 a, c). The variation of the modulation amplitude with different frequency position of the detection pulses indicates that the orientation of the dipolar distance vector is correlated to the nitroxides $^{14}$N hyperfine- and g-tensor orientations. Comparing the Fourier transformed time traces (Pake patterns) at different offsets (Figure 15 e) it can be seen that at 80 and 70 MHz offsets, where the $A_{zz}$ component is mainly excited, the singularities at the edges of the Pake pattern (approx. $\pm 5$ MHz) are more pronounced (Figure 15 e, black arrows). The edges of the Pake pattern represent the dipolar frequency for the parallel component of the dipolar interaction ($\theta = 0^\circ$), this shows that the $g_{zz}$ and $A_{zz}$ components are more parallel than perpendicular to the dipolar distance tensor. Reading of the frequency of the perpendicular singularity at 40 MHz offset, which is approximately 2.34 MHz (Figure 15 e, red arrows) and using eq. 11, 2.81 nm is obtained for the distance between the spin centers. Modeling the DNA 1_9 as a B-DNA duplex and superimposing $\epsilon$ spin labels on the cytosines, a distance of 3.12 nm is calculated between the nitroxides nitrogen atoms. The fast modulation in the PELDOR time traces (Figure 15 a, c, g), shown as the peaks at approximately $\pm 14.8$ MHz in the dipolar spectrum (Figure 15 e), is from the hyperfine coupling between spin label's unpaired electrons and nearby protons. Because the excitation profiles of the detection and inversion pulses partly overlap, especially at small offsets, (Figure 10) an unwanted ESEEM modulation signal is observed from weakly coupled nuclei, that can distort and interfere with the four-pulse PELDOR time trace.
Figure 15. PELDOR data of spin-labeled DNA 1_9. Non-covalently spin-labeled DNA 1_9 is on the left column, covalently spin-labeled DNA 1_9 is on the right column. a, b) Normalized PELDOR time traces. c, d) Normalized background subtracted time traces. e, f) Fourier transformed time traces. Red and black arrows point to the perpendicular and parallel components respectively. g, h) Normalized PELDOR time traces (solid lines) with simulated time traces overlaid (dotted lines) (see chapter 2.1.3). Time traces have been displaced on the y-axis for clarity. †Data was recorded by Dominik Margraf, Lab of Prof. T. F. Prisner, Center of Biomolecular Magnetic Resonance, Goethe-University, Frankfurt, Germany.
To get an assessment of the quality of the PELDOR data obtained from DNA 1_9, non-covalently spin-labeled with a \( \chi \), comparison was made with PELDOR data obtained from DNA 1_9, covalently spin-labeled with the rigid spin label \( \zeta \) (Figure 15).\(^{36,38}\) Firstly it is observed that the time traces for the covalently spin-labeled DNA 1_9 do not contain ESEEM proton modulation to the same extent as the non-covalently spin-labeled DNA 1_9. The reason for the appearance of ESEEM modulation in the PELDOR time traces for the non-covalently spin-labeled DNA 1_9, despite using proton-ESEEM averaging, is not fully understood. One likely explanation could be specific coordination between water molecules and spin labels that causes weak hyperfine couplings.

From the background subtracted time traces it is observed that the non-covalently spin-labeled DNA have less modulation depth. For the time traces at 40 MHz offset the modulation depth is 0.14 and 0.42 for DNA 1_9 spin-labeled with \( \chi \) and \( \zeta \) respectively, or about 33% less for the non-covalently spin-labeled DNA 1_9. The modulation depth of a PELDOR time trace is a function of the fraction of coupled spins and the mutual orientation between rigid spin centers.\(^{55,62}\) Assuming that the mutual orientation of the spin labels is approximately same for both non-covalently and covalently spin-labeled DNA 1_9, the fraction of coupled spins in non-covalently spin-labeled DNA 1_9 can be estimated to be about 33%. This does imply that the abasic sites in DNA 1_9 do not have high enough affinity for the rigid spin label \( \chi \) to populate all the binding sites.

Nonetheless, the quality of the data obtained from the non-covalently spin-labeled DNA 1_9 is sufficient to clearly observe a modulation and allow the extraction of a distance and information on the mutual orientation of spin labels as will be discussed in section 2.1.3. In addition, the time traces and dipolar spectra, for the non-covalently spin-labeled DNA, show approximately the same trends with different frequency offsets as the data for the covalently spin-labeled DNA. The modulation depth of the time traces decreases and the parallel components in the dipolar spectra appear with increased frequency offset. This indicates that the orientation and flexibility of the two spin labels, \( \chi \) and \( \zeta \), relatively to the DNA, is comparable.

Since the PELDOR data of covalently spin-labeled DNA 1_9 was measured using a different spectrometer (spectrometer B), instrumental setup and parameters settings than for the non-covalently spin-labeled DNA 1_9, the 20-mer dsDNA sequence,

\[
\begin{align*}
5' &- GT\zeta - AGT - CGC - GCG - CGC - GCA - TC - 3' \\
3' &- CAG - TCA - GCG - CGC - G\zeta G - CGT - AG - 5'
\end{align*}
\]

covalently spin-labeled with \( \zeta \), that had been previously measured using spectrometer B, was measured at 40 MHz offset using the same spectrometer (spectrometer A) as for the non-covalently spin-labeled DNA 1_9 to rule out any differences due to different instruments and settings. Comparing the PELDOR data at 40 MHz offset from the two spectrometers it can be seen that there is negligible difference between the time traces and dipolar spectra (Figure 16), although the position of the second detection pulse and the length of the inversion pulse were slightly different for the two measurements. Differences between the PELDOR data obtained from the non-covalently and covalently spin-labeled DNA 1_9 (Figure 15) can therefore be solely attributed to sample differences.
2.1.2 Orientation averaging of PELDOR data for DNA 1_9

To get an estimation of the average distance and distance distribution from the PELDOR data on non-covalently spin-labeled DNA 1_9 orientation averaging was used, as described by Godt et.al.\textsuperscript{63} The original time traces at 40 to 80 MHz offsets were normalized and then added together. The resulting time trace was then analysed with Tikhonov regularization, using DeerAnalysis 2009.\textsuperscript{64} For comparison, original PELDOR time traces of DNA 1_9 spin-labeled with Ç, at 40 to 90 MHz offset, were also orientation averaged (Figure 17). The distance distributions calculated from the orientation averaged time traces have the largest probability at 2.70 and 2.86 nm for non-covalently and covalently spin-labeled DNA 1_9, respectively (Figure 17 d). It is observed that the distance distributions are asymmetric towards longer distances. This could indicate that the dsDNA has more tendency to elongate than become shorter (Figure 17 d).
Figure 17. Orientation averaged PELDOR data of DNA 1_9. Red and black traces represent non-covalently and covalently spin-labeled DNA 1_9, respectively. a) Normalized time traces. b) Background subtracted time traces. Simulation from distance distribution is in red dotted line. c) Normalized dipolar spectra. d) Normalized distance distributions.

2.1.3 PELDOR simulation of DNA 1_9

In order to obtain information on the distance, mutual orientation of spin labels and dynamics, the background subtracted PELDOR time traces, obtained from non-covalently and covalently spin-labeled DNA 1_9, were simulated with a home-written Mathematica® program.

The angles between the $y$ and $x$ components of the spin label's $g$-tensors and dipolar distance vector ($\alpha$ and $\beta$ respectively (Figure 18)) were defined by a normal distribution, where the mean value and standard deviations were given explicitly. The angle between the $g_{xx}$ components (viewed towards the dipolar distance vector) was given a fixed value, $\gamma$ (Figure 18 e). The mean distance and standard deviation between spin labels was defined by a normal distribution. The goodness of fit between the experimental PELDOR time traces and the simulation was evaluated visually. A change in simulation parameter that resulted in a time trace with a reasonable fit to the experimental time trace (considering all frequency offsets) was estimated to be the uncertainty of the set parameter. The simulation
parameters used for the simulation of all frequency offsets are displayed in Table 1. The mutual orientation of the spin labels relative to the non-covalently and covalently spin-labeled 20-mer dsDNA is shown in Figure 18 a, b. For the non-covalently and covalently spin-labeled DNA 1_9 the angles $\alpha$ and $\beta$ were given identical values for both spins, A and B (Figure 18 c, d). The simulation program calculates the modulation depth of the PELDOR time trace from the fraction of coupled spins. In order for the simulated and experimentally observed modulation depths to fit for the non-covalently spin-labeled DNA the simulated modulation depth parameter had to be multiplied by 0.32. This is in agreement with the observed difference in modulation depth between the covalently and non-covalently spin-labeled DNA 1_9 (Figure 15).

Table 1. Parameters for simulation of PELDOR data for spin-labeled DNA 1_9. A and B refer to spin labels. Numbers within brackets are the uncertainty. Angles and distances are given as a mean value ± standard deviation.

<table>
<thead>
<tr>
<th>Spin-labeling</th>
<th>$\alpha_{A,B}$ ['']</th>
<th>$\beta_{A,B}$ ['']</th>
<th>$\gamma$ ['']</th>
<th>$r$ [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-covalent</td>
<td>59 (10) ± 15 (10)</td>
<td>71 (10) ± 15 (10)</td>
<td>40 (20)</td>
<td>2.70 (0.02) ± 0.15 (0.05)</td>
</tr>
<tr>
<td>Covalent</td>
<td>59 (10) ± 15 (10)</td>
<td>68 (10) ± 15 (10)</td>
<td>40 (20)</td>
<td>2.86 (0.02) ± 0.12 (0.05)</td>
</tr>
</tbody>
</table>
Figure 18. Positions of spin labels relative to the DNA structure. a) $\gamma$ inserted into DNA abasic sites. b) $\gamma$ covalently bound to the DNA sugar-phosphate backbone. The spin labels were exchanged for cytosines. Distance and orientation of spin labels was obtained from simulation parameters (Table 1). Distance between spin labels is given in Å. b) Orientation of spin labels relative to the dipolar distance vector, showing the relevant angles.
From the PELDOR simulations it is observed that the mean value for the interspin distance is slightly shorter for the non-covalently spin-labeled DNA 1_9. The mutual spin label orientation for the non-covalently and covalently spin-labeled DNA, is on the other hand identical, within the uncertainty of the simulation parameters (Table 1).

To summarize, the distance and mutual orientation between two rigid spin labels, non-covalently bound to a 20-mer abasic dsDNA, was estimated using PELDOR. Three different methods were used to obtain the mean distance between the spin labels. The first method used the positive frequency of the perpendicular ($\theta = 0$) component of the dipolar spectra and eq. 11 to obtain the distance between the spin labels. This method is only expected to give a reliable distance estimation if the distance distribution is negligible. In the second method the PELDOR time traces were added together and the resulting averaged time trace analyzed using Tikhonov regularization, which assumes that the PELDOR time trace is not affected by orientation selectivity. Analyzing a time trace that is an average of 5 time traces is, therefore, not expected to give a reliable distance distribution. The third method of obtaining the distance between the spin labels from the PELDOR data was by simulation of all time traces, that take into account the orientation selectivity of the PELDOR experiments. All time traces were simulated, using the same set of parameters obtained from a model representing the two spin labels and the distance vector between them. The interspin distances obtained from different methods and modeling are recapitulated in Table 2.

Table 2. The distance between the two spin labels on non-covalently spin-labeled DNA 1_9. Obtained by three different methods from PELDOR data. †The distance is given as a mean distance ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.81</td>
<td>2.70</td>
<td>2.70 ± 0.15</td>
<td>3.12</td>
</tr>
</tbody>
</table>

2.2 Optimizing the sample preparation for PELDOR measurements

2.2.1 Freezing the sample at different rates

The preferred way of preparing a sample for PELDOR measurement is to rapidly freeze the cryoprotected sample in freezing mixture (1:4 methylcyclohexane:iso-pentane at -165 °C) in order to obtain a homogeneous sample. Since the DNA 1_9 sample is dissolved in aqueous 20% (v/v) ethylene glycol solution, the sample will not become frozen immediately but will quickly reach a temperature of approximately -20 °C and then freeze into a glassy homogeneous sample. Therefore, we wanted to see if freezing the non-covalently spin-labeled DNA quickly in freezing mixture would result in less efficient spin-labeling as compared to slowly cooling the sample to -30 °C and then shock freezing it by quickly transferring it to freezing mixture. PELDOR measurement at 40 MHz offset of non-covalently spin-labeled DNA 1_9, rapidly frozen from room temperature in freezing mixture, reveals that there is not a detectable reduction in spin-labeling since the PELDOR time traces are practically identical for both freezing methods (Figure 19). The
most plausible explanation for this negligible difference in spin-labeling is that the dynamics of freezing is slower than the equilibration between bound and unbound spin labels.

**Figure 19.** PELDOR data of DNA 1_9 shock frozen from either -30º C or room temperature. Measurements were done at 40 MHz offset. a) Normalized PELDOR time traces. b) Normalized background subtracted time traces. c) Fourier transformed time traces.

### 2.2.2 Optimizing the concentration of spin label

Spin-labeling nucleic acids using the non-covalently bound spin label σ, that is dependent upon its affinity for DNA abasic sites, will result in an equilibrium between single labeled, doubly labeled and unlabeled DNA duplexes. For PELDOR measurement it is imperative that the concentration of doubly labeled molecules be as high as possible. Free spin labels and mono labeled dsDNA’s will only contribute to background decay of the PELDOR signal, which must be kept to a minimum especially if long distances are to be analysed. To determine if the concentration ratio of spin label to abasic site is optimised at approximately 1 equivalent, a series of three DNA 1_9 samples were prepared with 0.5, 1.0 and 1.5 equivalents of spin label per abasic site. PELDOR measurements at 40 MHz offset carried out on these samples show that varying the spin label concentration affects the PELDOR time traces. Increasing the spin label concentration to 1.5 equivalent per abasic site increases the modulation depth and amplitude moderately (Figure 20). Conversely, spin label concentration of 0.5 equivalent per abasic site considerably decreases the modulation depth and amplitude of the PELDOR time trace (Figure 20 b). From these experiments it is seen that moderately increasing the concentration ratio of spin label to abasic sites has the effect of increasing the concentration of doubly labelled dsDNA.
2.2.3 Increasing the relaxation time by using deuterated solvent

Using deuterated solvents significantly increases the electron spin transverse relaxation time and gives the opportunity to measure longer distances with PELDOR. To see what effects a deuterated solvent would have on the relaxation rate of the spin label $\mathcal{C}$, a sample of DNA 1_9, non-covalently spin labeled with $\mathcal{C}$, was prepared as above in D$_2$O with 20% (v/v) deuterated ethylene glycol. A two pulse ESEEM experiment shows a slowly relaxing nitrooxide spin with a $T_2$ of 12000 ±1000 ns (Figure 21).

Figure 20. PELDOR time traces of DNA 1_9 with 0.5, 1.0 and 1.5 equivalents of spin label per abasic site. The detection pulses were set to 40 MHz higher frequency than the inversion pulse. a) Original PELDOR time traces. b) Background subtracted time traces.

Figure 21. A two-pulse ESEEM time trace for DNA 1_9 in deuterated solvent.

At 10 $\mu$s there is still a detectable modulation from the two pulse ESEEM. In systems with a narrow distance distribution a PELDOR time trace containing only one period of modulation is sufficient for a reliable mean distance estimation. Using eq. 11 it can be seen that in principle it should be possible to run a PELDOR experiment with a time window of 10 $\mu$s and measure a distance up to about 8 nm for spin label $\mathcal{C}$ in deuterated solvent.
PELDOR measurements at 40 to 80 MHz offset of non-covalently spin-labeled DNA 1_9 in deuterated solvent show the same trend in modulation amplitude and dipolar spectra with different frequency offset as non-covalently spin-labeled DNA 1_9 in protonated solvent (Figure 22). With a deuterated solvent there is negligible proton ESEEM modulation visible in the PELDOR time traces.

![Figure 22](image)

**Figure 22.** PELDOR data of DNA 1_9, non-covalently spin-labeled with ç in deuterated solvent at 40 to 80 MHz offset. a) PELDOR time traces. b) Background subtracted time traces. The time traces have been displaced on the y-axis for clarity. c) Fourier transformed PELDOR time traces.

Taking a closer look at the time trace at 40 MHz offset (Figure 22 b) it can be seen that the modulation has a period of approximately 0.48 µs or a frequency of ~ 2 MHz, which is equal to the ESEEM frequency of deuterium. To assess the contribution of deuterium ESEEM modulation to the PELDOR time trace, a sample of free spin label ç in aqueous 20% (v/v) deuterated ethylene glycol solution was measured with PELDOR at 40 MHz offset. The time trace shows a strong deuterium ESEEM modulation with the approximately the same frequency as the PELDOR time trace of DNA 1_9 in deuterated solvent (Figure 23). Comparing the time traces for DNA 1_9 in deuterated and protonated solvent (Figure 24) shows that for DNA 1_9, deuterated solvent results in a PELDOR time trace that is distorted with deuterium ESEEM modulation at small frequency offsets. Even though the deuterium ESEEM modulation was suppressed in the PELDOR measurements, by positioning the second observer pulse at a deuterium ESEEM blind spot, it is still enough to impose a contribution to the four-pulse PELDOR signal.
Figure 23. Background subtracted PELDOR time traces of free spin label ç in aqueous 20% (v/v) deuterated ethylene glycol solution (red trace). DNA 1_9 in deuterated solvent (black trace).

Figure 24. PELDOR time traces of DNA 1_9 in protonated solvent (dotted lines) and deuterated solvent (solid lines). The time traces have been displaced on the y-axis for clarity.

Summarizing the observations from these optimization studies on DNA 1_9 it can be suggested that, i) rapidly freezing the DNA samples does not decrease site-specific spin-labeling, ii) increasing the concentration of spin label beyond one equivalent per abasic site results in moderate improvements of the PELDOR signal and iii) for measurements of long distances it would be necessary to use a deuterated solvent, but the contribution of deuterium ESEEM modulation to the PELDOR time trace would have to be assessed. For non-covalently spin-labeled DNA 1_9, the PELDOR modulation has approximately the same frequency as the unwanted ESEEM modulation. PELDOR measurements of non-covalently spin-labeled DNA 1_9 in deuterated solvent is therefore not advisable.
2.3 PELDOR measurements on spin label ç

As a control study, a sample of spin label ç in aqueous 20% (v/v) ethylene glycol solution was prepared. PELDOR measurements at 80 to 40 MHz offset, in steps of 10 MHz were performed to determine if dipolar interactions between free spin labels, at concentration used for DNA 1_9, could be contributing to the PELDOR modulation of non-covalently spin-labeled DNA 1_9 (Figure 25).

![PELDOR data of spin label ç at 40, 50, 60, 70 and 80 MHz offset. a) Normalized time traces. b) Normalized background subtracted time traces. The time traces have been displaced on the y-axis for clarity. c) Fourier transformed time traces.](image)

The PELDOR time traces for the free spin label only show the fast proton ESEEM modulation (Figure 25 a, b). These observations together with previous CW-EPR results indicate that there is no stacking of spin labels in solution that could give rise to visible PELDOR modulation.
2.4 Distance measurements on a TATA-box DNA sequence

To assess the applicability of the rigid spin label $\xi$, to problems of structural changes in DNA upon interaction with proteins, a proof of principle experiment was carried out. For this experiment we chose the transcription factor TBP (TATA-box binding protein), a subunit of the TFIID transcription factor that facilitates transcription of DNA. TBP binds specifically to the eight base pair sequence, T-A-T-A-@-A-@-N, where $@$ is either A or T and N is any base, through contact with the minor groove and bends the DNA, compressing the major groove.

2.4.1 Designing the abasic TATA-box DNA

A 22-mer DNA duplex with two abasic sites and a TATA-box sequence was designed. In designing the spin-labeled DNA duplex, three important structural restrictions had to be considered. First, since the TBP relies on contact with at least eight base pairs the two abasic sites have to be positioned so the local structural perturbation resulting from the abasic site do not interfere with the binding of the protein. Second, the spin labels have to be flanked by at least one nucleotide to either side and third, both distances have to be measurable and the change in distance upon binding of the TBP has to be greater than the inherent distance distribution.

As a guide to proper positioning of the spin labels the crystal structure of the 16-mer DNA duplex,

$$5' - CTG - CTA - TAA - AAG - GCT - G - 3'$$
$$3' - GAC - GAT - ATT - TTC - CGA - C - 5'$$

bound to human TBP was used (Figure 26).

![Figure 26. 16-mer TATA-box DNA duplex bound to TBP. The protein is coloured green and in cartoon representation. The DNA duplex is coloured blue and in stick representation. PDB ID: 1CDW.](image)

Based on the Sulfolobus rRNA promoter sequence (5' - GTC-TCC-CAT-ATA-AAT-CTA-ACC) we designed the 22-mer DNA sequence,
The position of the abasic sites was chosen as to minimize the distance between the non-covalently bound spin labels in the bent form, without placing them too close to the TATA-box. This resulted in placing the abasic sites 14 base pairs apart. The designed 22-mer DNA duplex, with $\varphi$ inserted into the two abasic sites, was modeled in its unbound form and compared to the crystal structure of the 16-mer bound DNA duplex (Figure 26) with the cytosine on the 5’ and 3’ ends replaced with $\varphi$ (Figure 27).

\[
\begin{align*}
5’ & - \text{CGT - ApGT - CCA - TAT - AAA - TCT - GAC - C - 3’} \\
3’ & - \text{GCA - GCA - GGT - ATA - TTT - AGA - ApTG - G - 5’}
\end{align*}
\]

The modeled distance between the spin label's nitrogen atoms in the unbound 22-mer DNA duplex is approximately 5.6 nm compared to 4.8 nm for the 16-mer DNA duplex bound to TBP. Given that the distance distribution for non-covalently spin-labeled DNA 1_9 was determined to be within 0.2 nm, a difference of 0.8 nm between the unbound and bound 22-mer TATA-box DNA should be sufficient to give unambiguously different PELDOR time traces.

2.4.2 PELDOR measurements on non-covalently spin-labeled 22-mer TATA-box dsDNA

A sample of the 22-mer abasic TATA-box DNA duplex was non-covalently spin-labeled with 1.0 equivalent of $\varphi$ per abasic site and measured with 4-pulse PELDOR. The detection pulses were set at 40 to 80 MHz higher frequency than the inversion pulse, in steps of 10 MHz. The time traces and dipolar spectra show that data acquired at different frequency offsets resulted in different modulation depth and dipolar spectra (Figure 28). Furthermore, the time trace at 80 MHz clearly shows a modulation from the parallel component of the dipolar tensor (a period of $\sim$1.3 $\mu$s). This indicates that the spin labels are rigid and their orientation is correlated to the dipolar distance vector with the z components.
of the g-tensors more parallel than perpendicular to the dipolar distance vector. The position of the perpendicular singularity at 40 MHz offset (Figure 28 c, red arrow), is approximately 0.311 MHz, which translates to a distance of 5.51 nm.

**Figure 28.** PELDOR data of non-covalently spin-labeled 22-mer TATA-box dsDNA at 40 to 80 MHz offset. **a)** Normalized PELDOR time traces. **b)** Normalized background subtracted time traces (solid lines) with simulated time traces overlaid (dotted lines). Time traces have been displaced on the y-axis for clarity. **c)** Fourier transformed time traces.

### 2.4.3 Orientation averaging of PELDOR data for 22-mer TATA-box dsDNA

An estimation of the average distance from the PELDOR data from non-covalently spin-labeled TATA-box DNA was obtained from orientation averaging. The original time traces at 40 to 80 MHz offsets were normalized and then added together. The resulting time trace was then analysed with Tikhonov regularization, using DeerAnalysis 2009. From the distance distribution it is seen that the most probable distance is at 4.97 nm. Since the PELDOR time traces only contain a little more than one modulation period, no conclusions or suggestions about the DNA structure or spin label conformation should be drawn from the shape of the distance distribution.
2.4.4 PELDOR simulation of 22-mer TATA-box DNA

The background subtracted PELDOR time traces of the non-covalently spin-labeled 22-mer TATA-box DNA were simulated to get an estimation on the interspin distance, mutual orientation of the spin labels and dynamics. The parameters used for a reasonable simulation of all frequency offset (Figure 28 c) are displayed in Table 3. The mutual orientation of the spin labels relative to the dsDNA and the interspin vector are shown in Figure 30. Both spin labels, A and B, were given identical values for the angles, α and β. The simulated modulation depth parameter had to be multiplied by 0.45 in order to fit the experimental time traces. This suggests that the TATA-box DNA has a slightly higher affinity for spin labels compared to DNA 1_9, since the simulated modulation parameter for DNA 1_9 had to be multiplied by 0.32. Detailed description of the simulation program can be found in chapter 3.

Figure 29. Orientation averaged PELDOR data of 22-mer TATA-box dsDNA. Black and red traces represent experiment and simulation, respectively. a) Normalized time trace. b) Background subtracted time trace. Simulated time trace calculated from distance distribution. c) Dipolar spectrum. d) Distance distribution.
Table 3. Parameters for simulation of PELDOR data for non-covalently spin-labeled TATA-box dsDNA. A and B refer to spin labels. Numbers within brackets are the uncertainties. Angles and distance are given as a mean value ± standard deviation.

<table>
<thead>
<tr>
<th>$\alpha_{A,B}$ [°]</th>
<th>$\beta_{A,B}$ [°]</th>
<th>$\gamma$ [°]</th>
<th>$r$ [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>93 (20) ± 22 (10)</td>
<td>63 (20) ± 22 (10)</td>
<td>180 (50)</td>
<td>4.95 (0.05) ± 0.30 (0.05)</td>
</tr>
</tbody>
</table>

Figure 30. Positions of $\varsigma$ spin labels relative to the TATA-box dsDNA structure. a) $\varsigma$ inserted into abasic sites with approximate distance and orientation according to simulation parameters from Table 3. Distance between spin labels is given in Å. b) Orientation of spin labels relative to the dipolar distance vector, showing the relevant angles.

To summarize, the distance and mutual orientation between two rigid spin labels, non-covalently bound to a 22-mer abasic TATA-box DNA, were estimated. Three different methods were used to obtain the mean distance between the spin labels. The obtained distances and distributions are recapitulated in Table 4.

Table 4. The distance between the two spin labels on TATA-box dsDNA. Obtained by three different methods from PELDOR data. The distance is given as a mean distance ± standard deviation

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5.51</td>
<td>4.97</td>
<td>4.95 ± 0.30</td>
<td>5.48</td>
</tr>
</tbody>
</table>

$^\dagger$
2.4.5 PELDOR measurements of abasic 22-mer TATA-box dsDNA with TBP

A sample of non-covalently spin-labeled 22-mer abasic TATA-box DNA in aqueous 20% (v/v) ethylene glycol solution with 1.3 equivalents of TBP was measured with PELDOR at 40 and 80 MHz offsets (Figure 31).

Figure 31. PELDOR data of non-covalently spin-labeled 22-mer TATA-box dsDNA with TBP (solid lines) and without TBP (dotted lines) at 40 to 80 MHz offset. a) Normalized PELDOR time traces. b) Normalized background subtracted time traces. Time traces have been displaced on the y-axis for clarity. c) Fourier transformed time traces.

It is observed when comparing the PELDOR data obtained from the 22-mer TATA-box DNA with and without TBP at 40 and 80 MHz offsets, that the time traces and dipolar spectra at both 40 and 80 MHz offsets are practically identical (Figure 31).

Since no change was observed in the PELDOR spectra upon introduction of TBP, it must be concluded that either the TBP does not have high enough affinity for the abasic DNA or that the bending did not result in the anticipated change in distance between the spin-labeled sites. Therefore, binding studies were performed to determine the affinity of TBP to the 22-mer TATA-box DNA.
2.4.6 Binding study of TBP with abasic 22-mer TATA-box dsDNA

To verify the binding of TBP to the abasic 22-mer TATA-box dsDNA, electrophoretic mobility shift assay (EMSA) was carried out on the TATA-box dsDNA with TBP from *S. Acidocaldarius* by Christophe Rouillon. The band shift assay indicates that about half of the DNA is bound at 50 µM of TBP (*Figure 32*). At 75 µM concentration of TBP there is a sudden decrease in binding, probably caused by TBP precipitation, and again increased binding at 100 µM concentration of TBP. From these observations it can be seen that the protein was unstable and/or had solubility problems. No decisive conclusions can therefore be drawn on why binding of TBP to the TATA-box was not observed from PELDOR measurements.

*Figure 32*. EMSA gel with $^{32}$P-labelled 22-mer TATA box DNA and TBP from Sulfolobus Acidocaldarius. The dark spots in the middle of the gel shows bound DNA, the spots on the bottom shows unbound DNA. The wedge at the top of the picture indicates increased TBP concentration.
2.5 Bending of DNA by Lac repressor

Because we were not able to demonstrate the applicability of spin label to problems of DNA-protein complexes, using a TATA-box DNA and TBP, we decided to try the homotetrameric Lac repressor protein, LacI, which has a high affinity and specificity for its consensus DNA sequence. A 29-mer dsDNA containing a 19-mer lactose operator and two abasic sites was non-covalently spin-labeled. The distance between the two labels as well as their orientation was monitored with PELDOR before and after addition of Lac repressor from Escherichia coli.

2.5.1 Designing and spin-labeling the Lac operator

The metabolism of lactose in Escherichia coli is regulated by the specific binding of the lactose repressor protein to the operators of the Lac operon. The 24 base pair primary operator (O₁) of the Lac operon binds specifically to the Lac repressor with a dissociation constant in the pico-molar range (Kₐ = 10⁻¹³ M). It has been determined that the 19-mer consensus sequence,

$$5' - TTG\text{-}TGA\text{-}GCG\text{-}GAT\text{-}AAC\text{-}AAT\text{-}T\text{-}3'$$
$$3' - AAC\text{-}ACT\text{-}CGC\text{-}CTA\text{-}TTG\text{-}TTA\text{-}A\text{-}5'$$

binds as tightly to the Lac operator as the longer 24-mer sequence. The Lac repressor binds to the major groove of the operator and distorts the DNA from its B-form by bending the center of the operator sequence through an angle of ~45°. In solution, the Lac repressor preferentially forms a dimer of dimers creating two operator binding sites (Figure 34).

![Figure 33. Tetrameric (dimer of dimers) Lac repressor bound to a 21-mer symmetric Lac operator. The Lac repressor is represented in surface mode. The Lac operator DNA is represented in cartoon mode. PDB ID. 1LBG.](image-url)
In designing the synthetic 29-mer DNA sequence containing a 19-mer Lac operator, the same restrictions had to be considered as for the design of the TATA-box DNA. First, since the Lac repressor relies on contact with the nucleobases, the two abasic sites have to be positioned so the structural perturbation resulting from the abasic sites do not interfere with the binding of the protein and concurrently the protein does not interfere with the binding of spin labels to the abasic sites. Second, the spin labels have to be flanked by at least one nucleotide to either side. Third, both distances have to be measurable and the change in distance upon binding of protein has to be greater than the inherent distance distribution. As a guide to proper positioning of the abasic sites, the NMR derived solution structure of the 23-mer DNA duplex,

\[
\begin{align*}
5' & \text{–} \text{GAA - TTG - TGA - GCG - GAT - AAC - AAT - TT} \text{–} 3' \\
3' & \text{–} \text{CTT - AAC - ACT - CGC - CTA - TTG - TTA - AA} \text{–} 5'
\end{align*}
\]

bound to Lac repressor was used (PDB ID. 1L1M). We designed the 29-mer DNA sequence, where Ap is an abasic site) using the 19-mer sequence above as a template and extended both 5’ ends with Ap and a cap of GCG and CGC. The positions of the abasic sites were chosen as to minimize the interspin distance in the dsDNA, bound to Lac repressor, without placing them to close to the 19-mer Lac operator. This resulted in placing the abasic sites 22 base pairs apart.

From the simulated PELDOR time traces of DNA 1_9 a distance of 2.7 nm was obtained. DNA 1_9 has the two spin labels, or abasic sites, separated by 8 base pairs. As an approximation, that gives a distance of 0.34 nm per base pair. Applying this base-pair distance to the 29-mer synthetic dsDNA gives an estimated interspin distance of 7.48 nm. Placing the abasic sites one base pair further away from the 19-mer operator would give an estimated interspin distance of 8.16 nm, which was believed to be too long and difficult to measure.

To get a rough estimate of the two interspin distances, the designed 29-mer duplex was modeled in its unbound state and compared to the solution structure of the 23-mer dsDNA, bound to Lac repressor (Figure 34). For the 23-mer dsDNA, 3 spin labels were inserted in place of the second adenine (A) on the 5’ – GAA… oligomer and on the end of the 3’ – CTT… oligomer. The 29-mer dsDNA was modeled and 3 spin labels inserted in place of cytosines (C). The modeled distance between the nitroxide nitrogen atoms in the unbound 29-mer dsDNA was approximately 7.4 nm and for the 23-mer dsDNA, bound to Lac repressor, the distance was approximately 6.3 nm (Figure 34). A distance change of ~1 nm should be clearly observed with PELDOR measurements.
Figure 34. Left) 23-mer dsDNA bound to Lac repressor (Lac repressor removed for clarity) with spin labels positioned two base pairs away from the 19-mer Lac operator. Right) 29-mer dsDNA with spin labels positioned two base pairs away from the 19-mer Lac operator. Distances between the nitrooxide nitrogens are given in Å.

2.5.2 PELDOR measurements on non-covalently spin-labeled 29-mer dsDNA

A sample of abasic 29-mer dsDNA was non-covalently spin-labeled with 1.0 equivalent of spin label σ per abasic site and measured with PELDOR at 40, 60 and 80 MHz offsets. Since a long distance has to be measured, the sample was dissolved in deuterated 20% (v/v) ethylene glycol/D$_2$O. In order to record at least one full period of the dipolar function, the time between the second and third detection pulses were set to 7.8 µs. At 40 MHz offset a full modulation period of the time trace is observed with an approximate modulation depth of 0.20 and a reasonable signal to noise. However, at 60 and 80 MHz offsets, due to decreased spin excitation at larger offsets, the timing between the second and third detection pulses had to be shortened to 7 µs in order to obtain a signal to noise ratio comparable to that obtained at 40 MHz offset. The time traces at 60 and 80 MHz offsets were recorded with a 30 ns time increment of the dipolar evolution time (T) (Figure 8) while the time trace at 40 MHz was recorded with T set to 20 ns. From Figure 35 it is observed that the modulation depth and amplitude of the PELDOR time traces has decreased upon increasing the excitation of the A$_{zz}$ ($M_I = -1$) component. This indicates that the spin labels are rigid and oriented in such a way that their $g_{zz}$ components are more parallel than perpendicular to the dipolar distance tensor. The perpendicular singularity at 40 MHz offset (Figure 35 c, red arrow), is approximately at 0.114 MHz, which translates to a distance of ~ 7.71 nm.
**Figure 35.** PELDOR data of non-covalently spin-labeled 29-mer dsDNA at 40, 60 and 80 MHz offset. a) Normalized PELDOR time traces. b) Normalized background subtracted time traces with simulated time traces overlaid in dotted lines. Time traces have been displaced on the y-axis for clarity. c) Fourier transformed time traces.

### 2.5.3 PELDOR simulation of non-covalently spin-labeled 29-mer dsDNA

The background subtracted PELDOR time traces of the non-covalently spin-labeled 29-mer dsDNA were simulated to estimate the interspin distance, orientation and dynamics of the spin labels. The parameters used for a reasonable simulation of all frequency offsets (Figure 35) are displayed in Table 5. The mutual orientation of the spin labels relative to the dsDNA are shown in Figure 36. The angles, α and β were set to identical values for both spins, A and B. The simulated modulation depth was multiplied by 0.32 in order to fit the experimental time traces. Detailed description of the simulation program can be found in chapter 3.

**Table 5.** Parameters for simulation of PELDOR data for non-covalently spin-labeled 29-mer dsDNA. A and B refer to spin labels. Numbers within brackets are the uncertainties. Angles and distance are given as a mean value ± standard deviation.

<table>
<thead>
<tr>
<th>α_{A,B} [°]</th>
<th>β_{A,B} [°]</th>
<th>γ [°]</th>
<th>r [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 (60) ± 22 (8)</td>
<td>66 (60) ± 22 (8)</td>
<td>80 (40)</td>
<td>7.1 (0.2) ± 0.30 (0.20)</td>
</tr>
</tbody>
</table>
Figure 36. Positions of \( \varsigma \) spin labels relative to the 29-mer dsDNA structure. a) \( \varsigma \) inserted into abasic sites with approximate distance and orientation according to simulation parameters from Table 5. Distance between spin labels is given in Å. b) Orientation of spin labels relative to the dipolar distance vector, showing the relevant angles.

2.5.4 Binding study of Lac repressor with abasic 29-mer dsDNA

Before preparing samples of the 29-mer DNA and Lac repressor for PELDOR measurements, the binding of LacI from E. coli to the synthetic 29-mer dsDNA was verified with EMSA. \(^{32}\)P labelled 29-mer dsDNA was incubated with LacI and loaded onto gel. The concentration of DNA was held constant at 50 nM while the concentration of LacI was increased from 1.25 to 25 \( \mu \)M. The band shift assay, carried out by Christophe Rouillon, shows an increased concentration of bound (slower moving) DNA's with increased concentration of Lac repressor (Figure 37). The second well (from left) of the EMSA gel has a LacI concentration of 1.25 \( \mu \)M, and shows about half of the DNA bound to protein. The concentration of Lac repressor refers to the concentration of monomers. Since the Lac repressor needs to form a dimer to bend a Lac operator (Figure 33) the effective concentration of LacI in the second well is therefore 625 nM, or 12.5 equivalents of LacI per dsDNA. This binding study shows that the Lac repressor is able to bind effectively to the 29-mer dsDNA.
Figure 37. Electrophoretic mobility shift assay of 29-mer Lac operator and Lac repressor, carried out by Christophe Rouillon. LacI was titrated at 1.25, 2.5, 5, 12.5 and 25 µM to 50 nM dsDNA (wells 2 to 6 from the left respectively). dsDNA without LacI is represented by lane 1.

2.5.5 PELDOR measurements of non-covalently spin-labeled 29-mer dsDNA bound to Lac repressor

A sample of abasic 29-mer DNA with 6.3 equivalents of Lac repressor monomers per dsDNA was non-covalently spin-labeled with 1.0 equivalent of spin label 3 per abasic site and measured with PELDOR at 40, 60 and 80 MHz offsets. The solvent contained 20% (v/v) deuterated ethylene glycol and ~60% D2O. Since the Lac repressor could not be further concentrated and the optimum concentration of spin label, for a good PELDOR signal, is 100 µM, the concentration of Lac repressor dimers became limited to 3.15 equivalents per dsDNA. The refocused echo for this sample had considerably less intensity than for the sample of abasic 29-mer DNA without LacI. Since the expected distance for the bound Lac operator is shorter than for the unbound, the time between the second and third detection pulses could be set to 6 µs in order to obtain a better signal to noise ratio and still obtain a full modulation period. Figure 38 shows the PELDOR data obtained from the 29-mer Lac operator with LacI. The PELDOR time trace at 40 MHz offset is clearly modulated with a shorter period than for the 29-mer dsDNA at the same microwave frequency offset. For the 60 and 80 MHz offsets, the modulation is less clearly observed, which indicates that the spin labels 3zz components are more parallel than perpendicular to the interspin vector. It is observed that the modulation depth for the 40 MHz offsets is about 0.10. The modulation depth for the covalently spin-labeled DNA 1_9, which is fully spin-labeled had a modulation depth of 0.42. This would indicate ~20% of the spin labels are coupled. This low concentration of doubly labeled DNAs could be a consequence from blocking of the abasic sites by LacI. The perpendicular singularity at 60 MHz offset (Figure 38 d, red arrow) is approximately at 0.148 MHz, which translates to a distance of 7.06 nm between the spin labels for the DNA bound to Lac repressor.
Comparing the PELDOR data obtained from the 29-mer dsDNA with LacI and data obtained from unbound 29-mer dsDNA (Figure 39), shows that the modulation period is shorter for the DNA with LacI, which translates to a shorter distance between spin labels, and hence abasic sites. It can therefore be concluded that a bending of the synthetic 29-mer dsDNA upon addition of LacI is observed. From Figure 33 it can be seen that LacI has two DNA binding sites bringing one end of each DNA duplex in close proximity. From modeling, the shortest inter-DNA distance within one LacI-DNA complex is estimated to be within 5 nm. That is shorter than the measured interspin distance for the 29-mer DNA bound to LacI. It can therefore be concluded that the observed distance from PELDOR measurements on DNA bound to LacI, is not between two DNA duplexes bound to the same tetrameric Lac repressor.
Figure 39. PELDOR data of non-covalently spin-labeled 29-mer Lac operator with and without LacI at 40, 60 and 80 MHz offsets. a) Normalized PELDOR time traces. Red traces represent the unbound dsDNA while the blue traces represent the dsDNA bound to LacI. b) Normalized background subtracted time traces. Red traces represent the unbound dsDNA while blue traces represent the dsDNA bound to LacI. Time traces have been displaced on the y-axis for clarity. c) Fourier transformed time traces.

2.5.6 PELDOR simulation of abasic 29-mer dsDNA bound to Lac repressor

The background subtracted PELDOR time traces of the non-covalently spin-labeled 29-mer dsDNA bound to LacI were simulated to estimate the interspin distance, orientation and dynamics of the spin labels, as described in chapter 3. The parameters used for a reasonable simulation of all frequency offsets (Figure 38) are displayed in Table 6. The mutual orientation of the spin labels relative to the interspin vector are shown in Figure 40. In order to fit the experimental time traces, the simulated modulation depth was multiplied by 0.17.
Figure 40. Orientations of \( \varsigma \) spin labels relative to the interspin vector for the 29-mer dsDNA bound to Lac repressor. 

(a) Distance between the spin labels nitrogen atoms is in Å.
(b) The angle between the \( g_{xx} \) components, viewed along the dipolar distance vectors. The distance and orientation of spin labels was obtained from simulation parameters in Table 6.

Table 6. Parameters for simulation of PELDOR data for non-covalently spin-labeled 29-mer dsDNA bound to LacI. A and B refer to spin labels. Numbers within brackets are the uncertainties. Angles and distance are given as a mean value ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>( \alpha[^\circ] )</th>
<th>( \beta[^\circ] )</th>
<th>( \gamma[^\circ] )</th>
<th>( r ) [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 70 (60) ± 20 (20)</td>
<td>A: 70 (60) ± 20 (20)</td>
<td>0 (40)</td>
<td>6.5 (0.2) ± 0.30 (0.20)</td>
<td></td>
</tr>
<tr>
<td>B: 50 (60) ± 20 (20)</td>
<td>B: 45 (60) ± 20 (20)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5.7 Orientation averaging of PELDOR data for 29-mer dsDNA

To get an estimation of the average interspin distance using Tikhonov regularization, PELDOR time traces obtained from the 29-mer dsDNA, with and without LacI, were normalized and added together. From the background subtracted time traces, a difference in modulation period is clearly observed upon addition of LacI to the dsDNA (Figure 41 b). The calculated distance distributions show the most probable interspin distances to be 6.44 nm and 6.88 nm for the 29-mer dsDNA with and without Lac repressor, respectively (Figure 41 c). It is observed from Figure 41 that the DNA bound to LacI seems to have a slightly larger distance distribution. This observation seems to be in disagreement with the expected decrease in dynamics of the DNA upon binding to a protein. Since the Tikhonov regularization is applied to a time trace that only shows one full modulation period, and is composed of only three time traces with different frequency offsets, the distance distribution can only represent a rough estimation of the true distribution.
**Figure 41.** Orientation averaged PELDOR data of 29-mer dsDNA. *a, c, d*) Red and black traces represent 29-mer dsDNA bound and unbound to LacI, respectively. *b*) Background subtracted time traces. Simulation from distance distribution is represented by a red dotted line. *c*) Normalized dipolar spectra. *d*) Normalized distance distributions.

Assuming the bending of the Lac operator is symmetric around the central base pair, using the obtained distances and simple trigonometry the bending angle of the 29-mer DNA is estimated to be ~47°. This is in agreement with a previously reported bending angle of ~ 45° observed from crystal structure.70

To summarize, the distance and mutual orientation between two rigid spin labels non-covalently bound to a 29-mer abasic dsDNA, containing a 19-mer Lac operator sequence have been estimated. Upon addition of Lac repressor the distance between spin labels becomes shorter. We have used three different methods to obtain the mean distance between the spin labels. The obtained distances and distributions are recapitulated in Table 7.

**Table 7.** The distance between the two spin labels on 29-mer dsDNA. Obtained by three different methods from PELDOR data. †The distance is given as a mean distance ± standard deviation. ‡The distance was measured from the solution structure of a 23-mer DNA bound to LacI.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>7.71</td>
<td>6.88</td>
<td>7.1 ± 0.30</td>
<td>7.4</td>
</tr>
<tr>
<td>DNA + LacI</td>
<td>7.06</td>
<td>6.44</td>
<td>6.5 ± 0.30</td>
<td>6.3‡</td>
</tr>
</tbody>
</table>
3 PELDOR simulation program

In order to extract the interspin distance and relative orientation of the two spin labels, the PELDOR time traces were simulated with a home-written Mathematica® program. In this chapter a detailed description of the program and method of simulation will be given.

The background subtracted PELDOR time traces were simulated by defining the orientation of the spin labels' g-tensor with respect to the interspin distance vector and generating either 20,000 or 30,000 conformers and molecular orientations. Mobility of the spin labels relative to the interspin vector was given explicitly. To represent the orientation of two spin labels (A and B) relative to the vector connecting their spin centers, three coordinate systems and 7 angles were defined. With the interspin vector \( r \) parallel to the Z-axis of the molecular coordinate system (X,Y,Z), the orientation of \( r \) with respect to the external magnetic field \( B_0 \) can be described by the polar \( \Theta \) and azimuth angle \( \Phi \). The orientation of the two spin labels are represented in the coordinate systems \((x_1,y_1,z_1)\) and \((x_2,y_2,z_2)\) and their orientation relative to the molecular coordinate system is represented by the angles, \((\alpha_A,\beta_A)\), \((\alpha_B,\beta_B)\) and \(\gamma\). These angles and the distance between the spin centers were explicitly supplied in the program as a mean value with a standard deviation. The standard deviation of the angles then represents the mobility of the spin labels.

The excitation profiles of the detection pulse sequence and inversion pulse was calculated from the experimental microwave frequencies and pulse lengths. For experimental inversion pulse lengths of 12 and 14 ns, an inversion pulse length of 18 and 16 ns, respectively, was used for the simulation in order to fit the experimentally observed modulation depth. A plausible explanation for this discrepancy is that the calculated excitation profile does not resemble the experimental excitation profile in the case of very short (< 16 ns) pulse lengths.

g-values, hyperfine couplings and EPR linewidths were obtained from parameters used to simulate experimental field-swept spectra. The experimental field position was given explicitly to calculate the spin labels resonance frequencies.

With the orientation of the spin labels relative to the interspin vector defined by a normal distribution, the program then generates a set amount of \( \sin(\Theta) \) weighted orientations of the interspin vector and calculates for each orientation the relative orientations of the spin labels g-tensors, using the given parameters (mean values and distributions). If e.g. 30,000 orientations are calculated for the interspin vector, then 30,000 different spin-pairs are generated. For each orientation of the g-tensors, the resonance frequency is calculated using known values for the g-tensor components, hyperfine couplings and linewidths. The resonance frequency for every generated orientation of the g-tensors is compared to the excitation profiles of the detection pulse sequence and inversion pulse. For each orientation of spin A or B, that is only excited by the detection pulse sequence, the program asks if the coupled spin, B or A, respectively, is only excited by the inversion pulse. If the answer to that question is yes, that specific spin couple contributes to the observed PELDOR modulation. The fraction of coupled spin pairs, excited by the microwave pulses, defines the modulation depth parameter.

The dipolar evolution function is then calculated from eq. 13 with the mean value of the interspin distance and its standard deviation given explicitly.
3.1 Simulation of PELDOR measurements on a nitroxide biradical at X- and W-band.

In order to evaluate the simulation program for simulation of PELDOR data at both X- and W-band frequencies, PELDOR measurements on a semi-rigid biradical (Figure 43 b), at X-band and W-band (94 GHz), were simulated. The biradical compound was dissolved in deuterated o-terphenyl and measured at 40 to 80 MHz offset (in steps of 10 MHz) at X-band. At W-band, three PELDOR time traces were recorded of the biradical, setting the observer and inversion pulses to different combinations of nitroxide orientations.

At X-band frequencies and small offsets (40 - 60 MHz) the PELDOR time traces contained deuterium ESEEM modulation with a period of ~ 0.5 µs. This modulation was visible as a distortion to the PELDOR modulation.

At W-band frequencies, the anisotropic g-tensor is fully resolved (Figure 42). It therefore becomes possible to perform a combination of orientation selective PELDOR measurements and obtain a detailed picture of the nitroxide orientation. No deuterium ESEEM modulation was observed in the PELDOR time traces at W-band.

![Figure 42. A simulated W-band powder nitroxide EPR spectrum. The spectrum is in frequency domain. g_{xx}, g_{yy} and g_{zz} represent the nitroxide g-tensor diagonal components. 1, 2 and 3 represent the three peaks of the A_{zz} hyperfine tensor component.](image)

For the simulation of all time traces, at both X- and W-band frequencies, it was assumed that the nitrooxides have a free rotation around the ester-linkages and a conformational flexibility centered around the carbon-carbon bond connecting the two phenyl groups (Figure 43).
Figure 43. a) Probability distribution of the nitroxide $g_{xx}$ component on a sphere. The $g_{xx}$ vector is represented by yellow dots. The coordinate system of the nitroxide $g$-tensors is represented by $x$, $y$ and $z$. b) The nitroxide biradical compound. The small cone represents the rotation cone of the nitroxide. The larger cone represents the conformational flexibility of the nitroxides. c) Equilibrium geometry structure of the nitroxide biradical.

Generating 20- and 30,000 rotations and conformers for the X-band and W-band data respectively, allowing the nitroxides to rotate on a cone of 22° and to have a flexibility of 62° about the molecular backbone, resulted in the best fit to all experimental time traces (Figure 44). The flexibility of the nitroxides was given by a normal distribution, centered around the edge of the nitroxide rotation cone (Figure 43). The distance between spin labels obtained from the PELDOR simulation was 1.95 ± 0.03 nm. The interspin distance, calculated between the centers of the N-O bonds, of the equilibrium geometry structure was 1.98 nm Figure 43 c). The distance and flexibility obtained from simulation of PELDOR time traces and molecular modeling are summarized in Table 8.
Figure 44. a) X-band PELDOR time traces at 40 to 80 MHz offsets. Simulated time traces are overlaid in dotted lines. The time traces have been normalized and displaced on the y-axis for clarity. b) W-band PELDOR time traces with simulated time traces overlaid in dotted lines. XX (observer pulses on g_{xx}, inversion pulse on g_{zz}). YZ2 (observer pulses on the second peak of the A_{zz} component, inversion pulse on the g_{yy} component). Z1Z3 (observer pulses on pulses on the third peak of the A_{zz} component, inversion pulse on the first peak of the A_{zz} component).

Table 8. Parameters used for the simulation of PELDOR time traces, both at X- and W-band. The flexibility is given as the standard deviation of the normal distribution describing the flexibility of the nitroxide about the molecular backbone. The interspin distance is given as a mean value ± standard deviation. The number within brackets is the uncertainty.

<table>
<thead>
<tr>
<th>Cone Angle [°]</th>
<th>Flexibility [°]</th>
<th>r [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 (12)</td>
<td>20 (5)</td>
<td>1.95 (0.01) ± 0.03 (0.01)</td>
</tr>
</tbody>
</table>

By measuring a semi-rigid biradical (Figure 43 b) with PELDOR at both X- and W-band and simulating the PELDOR time traces, the distance between the spin centers and the flexibility of the nitroxides have been estimated. Using a molecular model where the nitroxides have a free rotation around their linkers and relatively large conformational flexibility, the PELDOR data, at both X- and W-band, could be simulated with a reasonable fit, using the same set of parameters. The PELDOR experiments show that despite the large flexibility and random orientation of the nitroxides, as estimated from simulations, the PELDOR modulation frequency depends on the position of the probe pulse sequence even at X-band frequencies. Estimating the interspin distance distribution on semi-rigid biradicals, assuming random orientation of the nitroxides, could therefore result in erroneous results.
3.2 Estimation of uncertainty in simulation parameters

The uncertainty in the simulation parameters was estimated by running several simulations, varying each parameter individually from its optimum value, until the fit between the simulated and experimental time traces could be differentiated from the fit using the optimum parameter values. The evaluation of the fit between simulated and experimental time traces was done visually. Simulated and experimental time traces of covalently spin-labeled DNA 1_9 at 40 to 90 MHz offset, where each simulation parameter is individually varied between its optimum and estimated uncertainty, are displayed in Figure 45.
Figure 45. Simulated and experimental PELDOR time traces of covalently spin-labeled DNA 1_9 at 40 to 90 MHz offsets. The experimental time traces are represented by black solid lines. The simulated time traces with optimum parameter values are represented by blue dotted lines, time traces with one of the simulation parameters set to its uncertainty limit are represented by red dotted lines. a, b, c, d, e, f) Simulated time traces with \( \alpha, \beta, \sigma(\alpha, \beta), \gamma, r \) and \( \sigma(r) \), respectively, set to its optimum value and uncertainty limit. \( \sigma \) is the standard deviation.
4 Conclusions

By using non-covalent spin-labeling of nucleic acids with ç, we have determined the distance and orientation between two sites on synthetic dsDNA using PELDOR at X-band frequencies. PELDOR measurements on a non-covalently spin-labeled 20-mer dsDNA with two abasic sites, revealed a mean interspin distance of 2.70 nm, slightly shorter than the 2.86 nm, measured for an identical DNA sequence spin-labeled with the rigid spin label Ç. The shorter interspin distance for the 20-mer abasic dsDNA, spin-labeled with ç, could suggest that the energetically favourable position of the spin labels inside the abasic site is slightly shifted relative to the position occupied by Ç, that is covalently attached to the nucleotide sugar moiety. Since abasic sites are known to cause sequence dependent, local perturbations to double stranded DNA duplexes,73, 74 the observed difference in the interspin distance, between the non-covalently and covalently spin-labeled 20-mer dsDNA could also be due to the DNA abasic sites. This new method of site-directed spin-labeling was also used to measure the bending of DNA upon interaction with a protein that binds to DNA and bends the double helix. A DNA containing a TATA-box was non-covalently spin-labeled on two sites and measured with PELDOR with and without TATA binding protein (TBP). The distance between the spin labels for the unbound DNA was 4.95 nm, as determined from simulation of the PELDOR time traces. Since the binding study of TATA-box DNA and TBP indicated that the TBP was unstable and/or has solubility problems another protein was chosen for this proof of principle experiment. A 29-mer DNA containing a Lac operator was non-covalently spin-labeled on two sites and the distance between the spin labels measured with PELDOR before and after binding to Lac repressor. The distance for the unbound DNA was 7.1 nm and 6.5 nm for the bound DNA. This change in distance results in a bending angle of ~ 47˚ about the center of the Lac consensus sequence, which is in agreement with previous measurements on crystal structures of DNA bound to Lac repressor.

Results presented in this thesis have shown that using a non-covalently bound spin label for site-directed spin-labeling of nucleic acids is indeed a viable method. Although using a spin label that is not covalently attached to specific sites on the DNA, which does involve some degree of insufficient labeling, the affinity and specificity of ç for its complementary nucleobase opposite an abasic site is sufficient to yield modulated PELDOR time traces with a reasonable modulation depth at X-band frequencies. In addition it has been shown that the rigid spin label, although non-covalently bound, is capable of showing orientation selective PELDOR time traces, that not only report on the distance between the paramagnetic beacons but also indirectly report on the mutual orientation of the nucleobases opposite the abasic sites. Whilst site-directed spin-labeling of nucleic acids using ç is a promising and facile method, the prerequisite for abasic sites does impose some restrictions on the spin label's versatility.

The work done so far, using this novel way of spin-labeling nucleic acids is informative and gives a basis for further studies on the usability of ç as a spin label for nucleic acids. The suitability of ç to site-directed spin-labeling of abasic RNA and assessment on the effects of solvent and flanking bases on the affinity of ç to abasic sites, are examples of further studies. The binding of ç to DNA abasic sites could possibly be improved by extending the spin label with functional groups that would increase non-covalent interactions with the abasic site sugar and adjacent nucleobases.75, 76
Experimental

General procedures

NaCl, Polyethylene glycol 400 (PEG 400) and MES was purchased from Fluka. Na₂HPO₄, • 6 H₂O, Ethanol and EDTA was purchased from Fischer Scientific. Ethylene glycol was purchased from Aldrich. Trizma Base, KCl and Sodium Cacodylate trihydrate was purchased from Sigma. Spermine • 4HCl was purchased from Acros Organics. MPD was purchased from Merck Schuchardt OHG) Deuterated ethylene glycol was purchased from Cambridge Isotope Laboratories.

Spin label ç was synthesized by Sandip Shelke, Sigurdsson group, Science Institute, University of Iceland, Iceland. ç (1.1 mg) was dissolved in Ethanol (5 mL). The Nitrooxide biradical was dissolved in deuterated o-terphenyl and rapidly frozen in liquid nitrogen before PELDOR measurements. Concentration of DNA oligomers was calculated from Beer’s law based on measurements of absorbance at 260 nm, using a 50 Bio UV-VIS spectrometer from Varian, equipped with a 100 µL cell (optical path length = 1 cm). Extinction coefficients of DNA oligomers were determined, using the UV WinLab oligonucleotide calculator (V2.85.04, Perkin Elmer). ssDNA oligomers solutions (1 µL) were dissolved in sterilized water (99 µL) and transferred to spectrometer cell for measurements. Sterilized water was used as a reference sample. Evaporation of solvents under vacuum was carried out on a SPD 111V speed-vac from Savant equipped with a vapour trap and vacuum inversion. Preparation of all DNA samples for EPR measurements were done in sterile Biopur Eppendorf tubes (2 mL) with cap.

Hybridization of oligonucleotides

Hybridization of all DNA oligomers was performed with a PCH-2 heating block from Grant-bio. Complementary DNA strands were annealed according to the following program: 90°C for 2 minutes, 60°C for 5 minutes, 50°C for 5 minutes, 40°C for 5 minutes, 22°C for 15 minutes, 4°C until stopped.

Synthesis and purification of DNA oligomers

20-mer, 14-mer and 22-mer TATA-box DNA oligomers were synthesized by Sandip Shelke, Sigurdsson group, Science Institute, University of Iceland, Iceland. Unmodified and abasic DNA oligomers were synthesized by a trityl-off synthesis on a 1.0 µmol scale (1000 Å CPG columns) using an automated ASM 800 Biosset DNA synthesizer and phosphoramidites with standard protecting groups. 1,2-dideoxy D-ribose CED phosphoramidite were used as a building block for abasic oligomer synthesis. The DNA oligomers were deprotected in concentrated ammonia solution at 55 °C for 8 h and purified by 20% denaturing polyacrylamide gel electrophoreses (DPAGE). The oligonucleotides were visualized by UV shadowing and the bands excised from the gel were crushed and soaked in TEN buffer (10 mM Tris pH 7.5, 250 mM NaCl, 1 mM Na₂EDTA). The DNA elution solutions were filtered through a 0.45 µm polyethersulfone membrane (disposable
filter device from Whatman) and desalted using Sep-Pak cartridge (Waters Corporation) according to manufacturer’s instructions. After removing the solvent under vacuum, the oligonucleotides were dissolved in de-ionized and sterilized water (200 µL). All commercial phosphoramidites, CPG columns and solutions for DNA synthesis were purchased from ChemGenes Corporation.

29-mer Lac operator DNA oligomers were purchased from Eurogentec. Purification was done with DPAGE and quality control by MALDI-TOF Mass spectrometry.

**Preparation of DNA and protein samples**

**Preparation of 20-mer abasic DNA oligomers for EPR measurements**

Synthesized and purified DNA oligomers were reconstituted with sterile water in Biopur Eppendorf tubes (2 mL). Spin-labeled DNA duplexes were made by mixing appropriate single-stranded DNA oligomers (5 nmol) and spin label ç, dissolved in ethanol (10 nmol). The water/ethanol solution was evaporated in vacuum at 33°C and the dry sample dissolved in PNE buffer (Na₂HPO₄ 10 mM, NaCl 100 mM, Na₂EDTA 0.1 mM, pH 7.00) (100 µL). After annealing the DNA oligomers the solvent was removed under vacuum at −25°C. The dry sample was dissolved in sterile H₂O or D₂O with 20% (v/v) ethylene glycol (100 µL). All samples were transferred to a quartz EPR tube (inner diameter < 4 mm.), rapidly frozen in freezing mixture and stored in liquid nitrogen.

**Preparation of ç for EPR measurements**

Samples of ç spin label were prepared by transferring an appropriate amount of ç, dissolved in ethanol, to sterile Eppendorf tube and the ethanol removed under vacuum at 33°C. The samples were dissolved in PNE buffer (Na₂HPO₄ 10 mM, NaCl 100 mM, Na₂EDTA 0.1 mM, pH 7.00) (100 µL) and heated according to the annealing program for DNA samples. After heating the water was evaporated under vacuum at −25°C. The spin label samples were then finally dissolved in sterile H₂O or D₂O with 20% (v/v) ethylene glycol (100 µL). All samples were transferred to a quartz EPR tube (inner diameter < 4 mm.), rapidly frozen in freezing mixture and stored in liquid nitrogen.

**Preparation of 22-mer abasic TATA-box DNA for EPR measurements**

22-mer DNA oligomers (5 nmol) were mixed with 2 equivalents of ç (10 nmol) and the solvent removed under vacuum at 33°C. The dry sample was dissolved and annealed in Tris buffer (Trizma Base 20 mM, NaCl 100 mM, EDTA 0.1 mM, pH 7.02) (100 µL). The buffer was then evaporated under vacuum at −25°C. The dry sample was dissolved in TBP buffer (20mM Trizma Base, 500 mM NaCl, glycerol 10% (v/v) 1 mM EDTA, pH 8.0) (20.83 µL) and the solvent removed under vacuum at −25°C. The dry sample was then dissolved in sterile water with 20% (v/v) ethylene glycol (100 µL). The sample was transferred to an EPR tube, rapidly frozen in freezing mixture and stored in liquid nitrogen.
Preparation of 22-mer abasic TATA-box DNA for EMSA

22-mer DNA oligomers (0.47 nmol) were mixed in a Biopur Eppendorf tube and the solvent removed under vacuum. The dry sample was reconstituted in PNE buffer (Na₂HPO₄ 10 mM, NaCl 100 mM, Na₂EDTA 0.1 mM, pH 7.00) (100 µL) and annealed.

Preparation of TBP

Aliquots of TBP from *Sulfolobus Acidocaldarius* (332 µM) in Tris buffer (Trizma Base 20 mM, NaCl 200 mM, EDTA 1mM, pH 7.75) were obtained from our collaborator, Malcolm White, University of St Andrews, St Andrews. Electrophoretic mobility shift assay (EMSA) of the abasic 22-mer TATA-box DNA with TBP from *S. Acidocaldarius*, titrated at 2.5 to 200 µM, was carried out by Christophe Rouillon (Malcolm’s White group, University of St Andrews, St Andrews). ³²P-labelled 22-mer abasic TATA-box dsDNA (40 nM) was incubated at room temperature for 20 min. with TBP from *S. Acidocaldarius* in Hepes buffer (20 mM Hepes pH 7.05, 2 mM DTT, 50 mM NaCl, 0.002% Triton X100, 0.1 mg/mL BSA) before loaded onto 12% native acrylamide gel (90 mM Tris-Borate, 2 mM EDTA). Gel were run at 130V for 4 hours, exposed to phosphorimaging screen and visualized using a Fuji FLA5000 imager.

Preparation of 22-mer abasic TATA-box DNA with TBP

22-mer DNA oligomers (5 nmol) were mixed with 2 equivalents of ç (10 nmol) and annealed in PNE buffer (Na₂HPO₄ 10 mM, NaCl 100 mM, Na₂EDTA 0.1 mM, pH 7.00) (100 µL). The solvent was removed under vacuum at ~25°C and the dry sample dissolved in EMSA binding buffer (HEPES 20 mM pH 7.05, DTT 2mM, NaCl 50 mM, BSA 0.1 mg/mL) (8 µL of 5x concentrated EMSA buffer reconstituted with 32 µL sterile water). Tata-box binding protein (TBP) in Tris buffer (40 µL) and ethylene glycol (20 µL) were added to the sample. Final sample volume was 100 µL. After approximately 30 min the sample was transferred to an EPR tube, rapidly frozen in freezing mixture and stored in liquid nitrogen.

Preparation of 29-mer abasic Lac operator DNA duplexes

29-mer DNA oligomers (4 nmol) were mixed with 2 equivalents of ç (8 nmol) and annealed in PNE buffer (Na₂HPO₄ 10 mM, NaCl 100 mM, Na₂EDTA 0.1 mM, pH 7.00) (100 µL). The solvent was removed under vacuum at ~25°C and the dry sample dissolved in MES buffer (MES 20 mM pH 6.0, NaCl 300 mM, in ²H₂O) (80 µL), deuterated ethylene glycol (20 µL) and DMSO (5 µL). Final sample volume (105 µL) was transferred to an EPR tube, rapidly frozen in freezing mixture and stored in liquid nitrogen.

Preparation of 22-mer abasic Lac operator DNA for EMSA

29-mer DNA oligomers (0.5 nmol) were mixed in a Biopur Eppendorf tube and the solvent evaporated under vacuum. The dry sample was reconstituted in PNE buffer (Na₂HPO₄ 10 mM, NaCl 100 mM, Na₂EDTA 0.1 mM, pH 7.00) (100 µL) and annealed.
Preparation of Lac repressor

Aliquots of LacI from *Escherichia coli* (315 μM) in MES buffer (MES 20 mM pH 6.0, NaCl 300 mM, 50% in ²H₂O) were obtained from our collaborator, Malcolm White, University of St Andrews, St Andrews. Electrophoretic mobility shift assay (EMSA) of the abasic 29-mer Lac operator DNA with LacI, titrated at 1.25, 2.5, 5, 12.5 and 25 μM, was carried out by Christophe Rouillon (Malcolm’s White group, University of St Andrews, St Andrews). ³²P-labelled 29-mer abasic Lac operator dsDNA (50 nM) was incubated for 20 min. at room temperature with Lac repressor in MES buffer (MES 20 mM pH 6.0, NaCl 300 mM, 100% in ²H₂O) before loaded onto 12% native acrylamide gel (90 mM Tris-Borate, 2 mM EDTA). Gel were run at 130V for 4 hours, exposed to phophorimaging screen and visualized using a Fuji FLA5000 imager.

Preparation of 29-mer abasic Lac operator DNA with Lac repressor

29-mer DNA oligomers (4 nmol) were mixed with 2 equivalents of ç (8 nmol) and annealed in PNE buffer (Na₂HPO₄ 10 mM, NaCl 100 mM, Na²EDTA 0.1 mM, pH 7.00) (100 μL). The solvent was removed under vacuum at ∼25°C and the dry sample dissolved in MES buffer containing Lac repressor (315 μM) (80 μL) and deuterated ethylene glycol (20 μL). The sample was transferred to an EPR tube and rapidly frozen in freezing mixture approximately 10 min. after addition of LacI. The sample was stored in liquid nitrogen.

CW-EPR measurements

X-band CW-EPR measurements were done using a Bruker EMX spectrometer with an ER 4122SP 9705 cavity. DNA 1_9 was measured in a Pasteur pipette at temperatures above 100 K. The microwave power was set to 2.00 mW. Modulation frequency was 100 KHz and modulation amplitude was 1.00 G. The time constant and conversion time were set to 81.92 msec. Microwave frequency was about 9.5 GHz. The magnetic field was centered at 3390 G and swept through 150 G with a resolution in X-axis of 1024. ç was measured in a Pasteur pipette and with the same parameters settings as for DNA 1_9. At 100 Kelvin the spin label sample was measured in an EPR quartz tube with outer diameter less than 4 mm. The microwave power was set to 0.2 mW. Modulation frequency was 100 KHz and modulation amplitude was 1.00 G. The time constant and conversion time were set to 81.92 msec. Microwave frequency was about 9.5 GHz. The magnetic field was centered at 3385 G and swept through 150 G with a resolution in X-axis of 2048. The receiver gain was set to 5.02 x 10⁵.
Pulsed EPR measurements

Pulsed EPR measurements were done using a Bruker ELEXSYS E580 pulsed X-band EPR spectrometer with a standard flex line probe head, housing a dielectric ring resonator (MD4 or MD5). For measurements at cryogenic temperatures, a continuous flow helium cryostat (CF935) and a temperature control system (ITC 502), from Oxford instruments were used. All pulsed experiments were performed at 50 K. For PELDOR measurements a double microwave frequency setup available from Bruker was used. Microwave pulses were amplified with an (TWT) amplifier (117X) from Applied Systems Engineering. The cavity was over-coupled to a quality factor of about 50. Field sweep measurements were done using the two pulse sequence; $\pi/2-\tau_1-\pi-\tau_1$ -echo. The length of the $\pi/2$ pulse was set to 16 ns and the length of the $\pi$ pulse to 32 ns. The frequency was set to the resonance frequency of the resonator. The magnetic field was swept through 120 Gauss. Video bandwidth was set to 20 MHz and the video gain was maximized without clipping of data. The height of the echo was optimized through appropriate setting of parameters. Two pulse ESEEM experiments were performed using the same sequence as mentioned above but with $\tau_1$ incremented by 12 ns and a static magnetic field. PELDOR experiments were done using the 4 pulse sequence, $\pi/2(\nu_A)-\tau_1-\pi(\nu_A)- (\tau_1+t)-\pi(\nu_B)-(\tau_2-t)-\pi(\nu_A)-\tau_2$ -echo. To eliminate receiver offsets, the $\pi/2(\nu_A)$ pulse was phase-cycled by applying the microwave pulse consecutively through the $+<x>$ and $-<x>$ channels and subtracting the signals. The length of the detection pulses ($\nu_A$) were set to 16 ns ($\pi/2$) and 32 ns ($\pi$). The frequency of the inversion pulse ($\nu_B$) was set at the maximum of the nitroxide spectrum and the length was set to 16 ns in all of the PELDOR experiments where it maximized the inversion of the Hahn-echo. Amplitude and phase of the pulses was set to optimize the refocused echo. The frequency of the detection pulses ($\nu_A$) was set at 40 to 80 MHz higher than the inversion pulse ($\nu_B$). All PELDOR spectra were recorded with a shot repetition time of 4000 to 5000 $\mu$s, video amplifier bandwidth of 20 MHz and amplifier gain of 51 to 57. $\tau_1$ was set to 192 and 200 ns for samples in protonated matrix and to 380 ns for samples in deuterated matrix. To suppress any unwanted ESEEM modulation from the PELDOR signal, nuclear averaging is performed. To suppress proton modulation the position of the second detection pulse is set close to the proton ESEEM blind spot which is about 200 ns. In addition, the delay between the first and second detection pulses is increased by 8 ns eight times, whereby any residual proton ESEEM modulation is averaged out. On the other hand, if a sample contains deuterium the position of the second detection pulse is set to 380 ns, which is a blind spot for deuterium ESEEM modulation and averaging is performed over any proton ESEEM modulation as before.

Proton and deuterium modulation was suppressed by incrementing $\tau_1$ by 8 ns 8 times and adding the consecutive spectra. A time increment of 12 ns for $t$ was used for all PELDOR spectra. An average of 100 scans were accumulated with an approximate measurement time of 6 hours.

Data analysis and modeling

The background subtracted PELDOR time traces, Fourier transformed spectra and distance distributions were generated from experimental time traces with DeerAnalysis2009. The zero time was adjusted if necessary and a three dimensional
homogeneous background model was fitted to the experimental PELDOR data. The starting time for the background fit was adjusted to minimize any singularity in the dipolar spectrum at zero frequency. Distance distributions were calculated with Tikhonov regularization. The Tikhonov regularization parameter, $\alpha$ corresponding as close as possible to the corner of the L curve was chosen in every case. $T_2$ of two pulse ESEEM experiments were extracted by fitting an exponential function to the real data with Bruker's XEPR program. All graphs and mathematical manipulations were done in Origin 8 from OriginLab and Matlab from The MathWorks. Publication quality figures and distance measurements on DNA models were done in PyMOL from DeLano Scientific LLC.

Molecular modeling of $\eta$ spin label and nitroxide biradical was done using Spartan from Wavefunction. The equilibrium geometry of the spin label $\eta$ was calculated using the Møller Plesset perturbation theory to second order (MP2) and the 6-31 $G^*$ base. The equilibrium geometry of the nitroxide biradical was calculated using the Hartree-Fock (HF) method and the 3-21 G base. B-DNA structures were modeled using Spartan and make-na server from http://casegroup.rutgers.edu/Biomer/index.html. The spin labels were inserted into the DNA duplexes by superimposing them on the cytosines. Abasic sites were introduced into the DNA duplexes by deleting the corresponding cytosine and replacing the glycosidic bond with hydrogen. The model of DNA 1_9 covalently spin-labeled with $\eta$ was energy minimized with all atoms frozen, except those belonging to the nucleotide sugar and phosphate backbone connected to the spin labels.
References


Appendix

**CW-EPR measurements**

To probe the behaviour of spin label $\mathfrak{q}$ in solution with double abasic DNA, X-band (9.7 GHz) continuous wave (CW) EPR measurements were recorded of DNA 1_9 with 1.0 equivalents of $\mathfrak{q}$ per abasic site, in aqueous 20% (v/v) ethylene glycol solution. Since the binding of the spin label to hydrophobic abasic sites involves non-covalent interactions, the EPR measurements are recorded at 0 °C and below in order to make the binding of spin label to the abasic sites more favourable. In order not to freeze the sample, ethylene glycol is added to the sample as a cryoprotector. To get an estimation of the sample's freezing point, the temperature was decreased slowly and the state of the sample examined by quickly lifting the sample tube out of the spectrometer cavity. The sample was found to become partially frozen at approximately -17 °C.

The resulting spectra show a superposition of nitroxide spectra from a mobile species and a species with decreased mobility (Figure A 1). At 0 °C (273 K) two peaks at low and high field can been seen to appear and increase in intensity with decreased temperature. At -17 °C the transitions from fast moving spin labels have decreased considerably in intensity (Figure A 1).

![Figure A 1. CW-EPR spectra of non-covalently spin-labeled 20-mer DNA duplex (5'-GTAp-AGT-GCG-CGC-GCG-TC-3'/3'-CAG-TCA-CGC-GApG-CGC-GCT-AG-5', Ap=abasic site) in aqueous 20% ethylene glycol solution. The black arrows at 0 °C point to the slow moving transition. The red arrows at -17 °C point to the fast moving transition.](image-url)
Overlaying the EPR spectra of DNA 1_9 at 0, -10, -20 and -30 °C and zooming in on the slow moving transitions on the low field side, it can be seen that the concentration of immobilized spin labels increases with decreased temperature (Figure A 2).

Figure A 2. EPR spectra of DNA 1_9 at 0, -10, -20 and -30 °C overlaid. Only the low field transition from slow moving spin labels is shown.

The apparent mixture of spin labels with different mobility in the non-covalently spin-labeled DNA 1_9 sample can only be understood by proposing that a portion of spin labels are getting immobilized by the DNA duplex. There are four possible ways the spin labels can bind to the DNA duplex. 1) Binding to the complementary nucleobase opposite the abasic site, through hydrogen bonding. 2) Intercalation between any two nucleotides in the DNA duplex. 3) Stacking at the end of the DNA duplex. 4) Binding to the grooves of the DNA duplex.

As a control study, a sample of free spin label ç in aqueous 20% (v/v) ethylene glycol solution was measured with CW-EPR at 0 to -30 °C (Figure A 3). At 0 °C (273 K) the spin labels are rapidly tumbling and the EPR spectrum represents an average of the different g values and hyperfine coupling components. As the temperature is decreased, i.e. the dynamics of the spin labels are slowed down, the EPR spectrum is inhomogeneously broadened by the unresolved g- and hyperfine-tensors. At -173 °C (100 K) the spin labels have reached the rigid limit and are represented by a powder spectrum (a sum of all possible orientations of the g- and hyperfine coupling tensors, relative to the applied magnetic field) (Figure A 3). In addition, the spectrum does not show any broadening from exchange and dipolar coupling, indicating no stacking of spin labels in solution.
Figure A 3. CW-EPR spectra of free spin label χ in aqueous 20% (v/v) ethylene glycol solution.