Long Range Distance Information on Orthogonal Spin Pairs by EPR

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<th>Description</th>
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<tbody>
<tr>
<td>$A_k$</td>
<td>hyperfine tensor</td>
</tr>
<tr>
<td>$\vec{B}_0$</td>
<td>static magnetic field vector</td>
</tr>
<tr>
<td>$\overline{C}$</td>
<td>averaged relaxivity</td>
</tr>
<tr>
<td>$C(\tau)$</td>
<td>correlation function of a random process</td>
</tr>
<tr>
<td>$\Delta k$</td>
<td>relaxation enhancement</td>
</tr>
<tr>
<td>$\Delta k$</td>
<td>orientation averaged relaxation enhancement</td>
</tr>
<tr>
<td>$\Delta\kappa(O_2, Ln^{3+})$</td>
<td>relaxation enhancement induced by oxygen</td>
</tr>
<tr>
<td>Dy$^{3+}$</td>
<td>dysprosium ion</td>
</tr>
<tr>
<td>$g$</td>
<td>g tensor</td>
</tr>
<tr>
<td>Gd$^{3+}$</td>
<td>gadolinium ion</td>
</tr>
<tr>
<td>$g_e$</td>
<td>g value of the free electron [2.0023193043622]</td>
</tr>
<tr>
<td>$\hat{H}$</td>
<td>Hamiltonian</td>
</tr>
<tr>
<td>$h$</td>
<td>Planck’s quantum of action</td>
</tr>
<tr>
<td>$\hat{H}_0$</td>
<td>static spin Hamiltonian</td>
</tr>
<tr>
<td>$\hat{H}_{\text{DD}}$</td>
<td>dipole-dipole interaction Hamiltonian</td>
</tr>
<tr>
<td>$\hat{H}_{\text{EZ}}$</td>
<td>electron Zeeman Hamiltonian</td>
</tr>
<tr>
<td>$hf$</td>
<td>hyperfine</td>
</tr>
<tr>
<td>$\hat{H}_{\text{HF}}$</td>
<td>hyperfine interaction Hamiltonian</td>
</tr>
<tr>
<td>$\hat{H}_{\text{NZ}}$</td>
<td>nuclear Zeeman Hamiltonian</td>
</tr>
<tr>
<td>$\hat{H}_{\text{ZFS}}$</td>
<td>zero-field splitting Hamiltonian</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>inversion efficiency</td>
</tr>
<tr>
<td>$\hat{I}_k$</td>
<td>nuclear spin operator</td>
</tr>
<tr>
<td>$J(\omega)$</td>
<td>spectral density function</td>
</tr>
<tr>
<td>La$^{3+}$</td>
<td>lanthanum ion</td>
</tr>
<tr>
<td>Ln$^{3+}$</td>
<td>lanthanide ion</td>
</tr>
<tr>
<td>$\mu_0$</td>
<td>permeability of vacuum</td>
</tr>
<tr>
<td>$\mu_B$</td>
<td>Bohr magneton</td>
</tr>
<tr>
<td>$\mu_n$</td>
<td>nuclear magneton</td>
</tr>
<tr>
<td>O$_2$</td>
<td>oxygen</td>
</tr>
<tr>
<td>$r$</td>
<td>inter-spin distance</td>
</tr>
</tbody>
</table>
\( \hat{S} \) electron spin vector operator
\( S(r) \) suppression function
\( T_1 \) longitudinal relaxation time
\( T_2 \) transverse relaxation time
\( \tau_C \) correlation time
\( \omega_0 \) Larmor frequency
\( w_{DD} \) dipolar frequency

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BPP</td>
<td>Bloembergen-Purcell-Pound theory</td>
</tr>
<tr>
<td>CW</td>
<td>continuous wave</td>
</tr>
<tr>
<td>DD</td>
<td>dipole-dipole interaction</td>
</tr>
<tr>
<td>DEER</td>
<td>double electron-electron resonance</td>
</tr>
<tr>
<td>DFT</td>
<td>density functional theory</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane N,N',N'',N'''-tetraacetic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminopentaacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>2,2',2''-(Ethane-1,2-diyl)tetraacetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia [Latin: for example]</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ESE</td>
<td>electron spin echo</td>
</tr>
<tr>
<td>ESEEM</td>
<td>electron spin echo envelope modulation</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>EZI</td>
<td>electron Zeeman interaction</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transformation</td>
</tr>
<tr>
<td>M.D.</td>
<td>modulation depth</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>MTSSL</td>
<td>S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methane-sulfonothioate</td>
</tr>
<tr>
<td>m.w.</td>
<td>microwave</td>
</tr>
<tr>
<td>N(_2)</td>
<td>nitrogen</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>Au-NPs</td>
<td>gold nanoparticles</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance image</td>
</tr>
<tr>
<td>NZI</td>
<td>nuclear Zeeman interaction</td>
</tr>
<tr>
<td>PELDOR</td>
<td>pulsed electron double resonance</td>
</tr>
<tr>
<td>RE</td>
<td>relaxation enhancement</td>
</tr>
<tr>
<td>r.f.</td>
<td>radio frequency</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SDSL</td>
<td>site-directed spin labelling</td>
</tr>
<tr>
<td>SEDOR</td>
<td>spin-echo double resonance</td>
</tr>
<tr>
<td>S/N</td>
<td>signal-to-noise ratio</td>
</tr>
<tr>
<td>srt</td>
<td>shot repetition time</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-tetramethyl-1-piperidinyloxy terpyridine derivative</td>
</tr>
<tr>
<td></td>
<td>2.2,5-dihexyl-1-</td>
</tr>
<tr>
<td>TFE</td>
<td>trifluoroethanol</td>
</tr>
<tr>
<td>ZFS</td>
<td>zero-field splitting</td>
</tr>
</tbody>
</table>
Abstract

The determination of distances between labels, featuring unpaired electrons, by electron paramagnetic resonance (EPR) measurements can provide information about the structure or structural changes of bio-macromolecules and bio-macromolecular complexes on systems which are difficult to be crystallized and are too big to be evaluated by nuclear magnetic resonance (NMR) techniques. The currently available EPR techniques, capable to provide distance information in the nanometre range, are based on the site-directed spin labelling (SDSL) approach, where two identical spin probes are attached to the system of interest. The most commonly used spin pair consists of two nitroxide radicals, however since a few years distance determination between paramagnetic metal ion complexes can also be measured (e.g. Gd$^{3+}$-Gd$^{3+}$ spin pairs). The combination of different spin probes in orthogonal spin pairs, shown in this work, can build a bridge between several techniques and opens up the possibility for more complex experimental schemes with potentially higher information content.

The strength of the dipole-dipole interaction between two spatially separated spins encodes the distance between them and can be measured directly by double electron-electron resonance (DEER) and indirectly by relaxation enhancement ($\Delta k$). The two complementary techniques were applied to orthogonal spin pairs consisting of lanthanide ions (Ln$^{3+}$) and nitroxide spin labels. It was found that the independent addressability of gadolinium ions (Gd$^{3+}$) and nitroxide radicals is advantageous for the direct detection of the dipolar frequency ($w_{DD}$) by DEER. Dysprosium ions (Dy$^{3+}$) induce an enhancement of the longitudinal relaxation time ($T_1$) of nitroxide spin labels in the close neighbourhood, which can be measured and evaluated by the relaxation enhancement ($\Delta k$) approach. To examine the performance of both techniques, three model systems were studied that have different chelating agents, with different zero-field splitting (ZFS) parameters of the paramagnetic Ln$^{3+}$ ions. The developed and optimized techniques are able to provide distance information from 1.5 nm to at least 6 nm and can be applied for narrow as well as for broad distributions of distances. DEER measurements on Gd$^{3+}$-nitroxide spin pairs could be performed with a similar or even slightly better (at Q band) sensitivity compared to commonly used nitroxide-nitroxide spin pairs in the same concentration range ($\approx 50 \mu M$). At low frequencies (S band) long distances between Dy$^{3+}$ and nitroxide spin labels are best extracted from $\Delta k$ measurements. The first report on the reduction of the refocused echo intensity due to the presence of the pump pulse suggests an inter-play between the observed effect and
the characteristic ZFS parameters of the investigated Gd$^{3+}$ complexes. However, satellite features ('artefacts') in the distance distribution were detected, in the case of narrow distance distribution and small ZFS parameters for Gd$^{3+}$. From temperature dependent $\Delta k$ data not only the inter-spin distance ($r$) could be extracted, but also additional information on the relaxation time of the fast relaxing species ($T_{1f}$) could be obtained. This provides an interesting possibility to study the relaxation behaviour of paramagnetic species, which are not directly detectable by other pulse EPR techniques. The extraction of short distances ($r \leq 2$ nm) from $\Delta k(T)$ data was found to be problematic because very short distances are suppressed. Therefore the magnitude of the relaxation enhancement effect is disturbed, which can lead to a wrong mapping of distances.

The experimental results presented reveal that both techniques can be used in a broad inter-spin distance range and are capable to provide structural information for systems relevant in biological as well as materials science context.
Zusammenfassung


Die Stärke der dipolaren Wechselwirkung zwischen zwei räumlich getrennten Spins korreliert mit ihrem Abstand und kann entweder direkt über das Doppel-Elektron-Electron-Resonanz (DEER)-Experiment oder indirekt über die Bestimmung der Relaxationsbeschleunigung gemessen werden. Diese zwei komplementären Methoden wurden an orthogonalen Spinpaaren aus je einem Lanthanidion (Ln$^{3+}$) und einer Nitroxdidspinprobe durchgeführt. Hierbei ist die unabhängige Detektion der Gadoliniumionen (Gd$^{3+}$) und der Nitroxdidradikale vorteilhaft für die direkte Messung der dipolaren Frequenz $\omega_{DD}$, wohingegen die Anwesenheit von Dysprosiumionen (Dy$^{3+}$) eine Beschleunigung der longitudinalen Relaxationszeit ($T_1$) der Nitroxdidspinprobe bei der indirekten Detektion hervorruft. Um die Leistungsfähigkeit beider Methoden zu untersuchen, wurden drei Molekülsysteme mit definierten Eigenschaften der paramagnetischen Metallionen untersucht, bei denen unterschiedliche Komplexbildner eine Variation der Nullfeldaufspaltungsparameter (ZFS) hervorrufen.

Die entwickelten und verfeinerten Methoden sind in der Lage, Abstandsinformationen im Bereich von 1,5 nm bis mindestens 6 nm zu liefern und können sowohl auf schmale, als auch auf breite Abstandsverteilungen angewendet werden. DEER-Messungen an Gd$^{3+}$-Nitroxdid Spinpaaren können im selben Konzentrationsbereich ($\approx 50 \mu\text{M}$) mit ähnlicher und vor allem bei Q-Band-Frequenzen sogar mit leicht besserer Empfindlichkeit als DEER-Messungen an den routinemässig verwendeten Nitroxdid-Nitroxdid-Spinpaaren durchgeführt werden. Bei tieferen S-Band-Frequenzen können hingegen lange Abstände zwischen Dy$^{3+}$ und
Nitroxidspinsonden am besten mit Relaxationsbeschleunigungsmessungen ($\Delta k$) bestimmt werden.
Bereits die ersten Untersuchungen einer unerwünschten Echointensitätsniedrigung durch die Anwesenheit eines Pumppulses deuten auf ein Wechselspiel dieses beobachteten Effektes mit den ZFS-Parametern der untersuchten Gadoliniumkomplexe hin. Die Anwesenheit störender, wenn auch kleiner Satellitenmerkmale in der Abstandsverteilung wurde nur bei einer schmalen Abstandsverteilung in Kombination mit kleinen ZFS-Parametern von Gd$^{3+}$ beobachtet.
Aus temperaturabhängigen $\Delta k$-Daten kann nicht nur der Abstand bestimmt werden, sondern es können darüber hinaus zusätzliche Informationen über die Relaxationszeit der schnell relaxierenden Spezies ($T_1$) gewonnen werden. Dies stellt eine interessante Möglichkeit dar, das Relaxationsverhalten der mit anderen Puls-ESR-Methoden nicht direkt detektierbaren paramagnetischen Spezies zu untersuchen.
Die Bestimmung kurzer Abstände ($r \leq 2\text{ nm}$) aus $\Delta k(T)$-Daten muss mit Vorsicht durchgeführt werden, da durch die Unterdrückung sehr kurzer Abstände ($< 1.5\text{ nm}$) die Stärke des Relaxationsbeschleunigungseffektes gestört ist, was zu einer fehlerhaften Abstandsbestimmung führen kann.
Die experimentellen Resultate zeigen eindrücklich, dass beide Methoden in einem grossen Abstandsbereich Strukturinformationen über liefern können, die für biologische und materialwissenschaftliche Fragestellungen relevant sind.
1. General Introduction

This thesis is devoted to distance measurements in the nanometre range by electron paramagnetic resonance on orthogonal spin pairs. Distance information from 1 nm to 6 nm can be of crucial importance to get an insight into the structure or structural changes of bio-macromolecules and bio-macromolecular complexes.

In this chapter I first want to introduce the reader to the general idea of the applied spectroscopic method. Furthermore, a brief summary of the current methods used to determine the structure of macromolecular systems is given. The state of the art for distance determination by electron paramagnetic resonance will be presented and the underlying concept of orthogonal spin labelling is introduced.

1.1 Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR), is a spectroscopic method to study systems with at least one unpaired electron (paramagnetic species). Most compounds carrying unpaired electrons are chemically reactive, such as organic and inorganic free radicals or transition metal complexes used for catalytic reactions. On the other hand stable radicals like nitroxide radicals can be used as spin probes or labels.

In general EPR relies on the same basic concept as nuclear magnetic resonance (NMR), where the splitting of energy levels of a single spin is introduced by the presence of an external magnetic field. Therefore EPR and NMR belong to the same spectroscopic family of magnetic resonance techniques. The transitions between the split electron spin states can be induced by on-resonant microwave (m.w.) radiation. The measurement of electron spin transition frequencies and the evaluation of structural information from them is the main purpose of EPR [1].

Two main categories of EPR experiments can be distinguished: continuous wave (CW) and pulse EPR measurements. The first EPR experiment was performed with a continuous irradiation of low-power microwaves by Zavoisky [2]. Since then, CW EPR techniques have been successfully developed and established. The first pulse EPR experiment by Blume [3] observing a single pulse free induction decay (FID) paved the way to the state of the art experimental pulse schemes, where m.w. radiation is applied in the form of very short and
high-power pulses. Nowadays most pulse EPR experiments are based on electron spin echos (ESEs) [4, 5]. The extensive development of pulse EPR techniques blossoms into a number of spectroscopic tools, which provide the possibility to get an insight to the nature of the paramagnetic species and their surrounding. As pointed out for instance by Schweiger and Jeschke [1] the combination of different EPR techniques can provide unique information on the electronic structure since the magnetic parameters are related to the electronic wave function and the configuration of the surrounding nuclei with non-zero electron spin. The $g$ values and, for species with several unpaired electrons, the zero-field splitting (ZFS) parameters often provide fingerprint information on the type of paramagnetic centres. Hyperfine couplings characterize the bonding situation in more detail, and the exchange coupling between electron spins is related to the delocalization and overlap of the singly occupied molecular orbitals of a pair of electron spins. In many cases, electronic structure can also be interpreted in terms of geometric structure. Hyperfine couplings give access to distances between the nuclei and the unpaired electron up to about 0.5 nm, while couplings between electron spins provide geometric information up to distances of at least 5 nm [6–8]. The nuclear quadrupole interactions provide information on the bonding of nuclei. CW and pulse EPR spectroscopy are thus able to characterize the structure of systems lacking long-range order on length scales which are not easily accessible by other techniques. Moreover, with a frequency range up to several hundred gigahertz (S band: 4GHz; X band: 9.75GHz; Q band: 35GHz; W band: 95GHz) EPR spectroscopy can also access molecular and chemical dynamics down to the picosecond time-scale.

1.2 Structure Determination

The overall global structure of a protein of interest can not yet be predicted precisely from the amino acid sequence, also called the primary structure of the protein. Nevertheless, particular combinations of amino acids are prone to result in certain secondary structure elements like $\alpha$-helices or $\beta$-sheets with regular local sub-structures. These conformations are based on the properties of the single amino acids, such as polarity of the residue, and on formation of hydrogen (H) bonds through space. The tertiary structure of the protein determines the three-dimensional structure of a single molecule and is the construction of a compact global fold of secondary structure elements. The assembly of several proteins or peptides is called the quaternary structure and dimers, oligomers or polymers are formed in a homogeneous or heterogeneous way. Many proteins do not have a quaternary structure and the tertiary fold of the monomer is the functional form of the protein. The globular fold of the protein is strongly correlated to its function. The understanding of the function of bio-macromolecules is of great importance to get an insight into the ongoing processes in cells. NMR spectroscopy and X-ray crystallography are currently the only techniques
1.2. Structure Determination

capable of determining the structures of biological macromolecules like proteins and nucleic acids at atomic resolution, with only few exceptional structures solved by electron microscopy techniques. In addition, it is possible to study time dependent phenomena with NMR, such as intra-molecular dynamics in macromolecules, reaction kinetics, molecular recognition or protein folding.

X-ray crystallography was initially used to determine the structure of inorganic and organic crystals since the early years of the last century. The technique was first used for the elucidation of salt crystal structure, which for example gave Linus Pauling the basis for studying atomic distances. From the knowledge obtained from salt crystals Pauling proposed the α-helical and β-strand secondary structures [9], both of which have been confirmed by X-ray crystallographic analysis using crystals of myoglobin and hemoglobin in the early 1960ies by Kendrew and co-workers [10].

X-ray structures can exhibit high resolution by enabling the distinction of two points in space as close as 2 Å. Yet they depict a static structure, the result of a technique which requires large, stable protein crystals, where each protein unit is lined up in a regular lattice. One can recognize that these static structures do not fully explain function because the structures are mostly the average of millions of identical units. 'Loose' structural parts like surface loops often fail to be resolved and this leaves some protein structures incomplete. The development of nuclear magnetic resonance techniques could be used to overcome this problem. In contrast to protein crystals needed for X-ray diffraction, NMR makes use of protein solutions allowing for the determination of structures on very short time scales. Consequently, information on dynamics of flexible loops and on domain motion is, in principle, accessible.

The growth of protein crystals up to 1 mm in size from a highly purified protein source is required for X-ray crystallography. Crystal growth is an experimental technique and there exist no rules about the optimal conditions for a protein solution to result in a good protein crystal. The protocol has to be established for every new type of protein. Water soluble proteins are easier to crystallize than membrane proteins. Membrane proteins tend to precipitate from the solution due to unfavourable protein-protein and protein-solute interactions. To be kept soluble in aqueous solution, membrane proteins need the addition of detergents. The presence of detergents, however, often interferes with regular arrangement of the protein complexes in the crystal resulting in a diffuse diffraction pattern. If membrane proteins contain large out-of-membrane domains, these water soluble domains can be cleaved off from the membrane buried domain and crystallized individually.

Nuclear magnetic resonance is able to obtain the same high resolution using a very different strategy. NMR measures the distances between atomic nuclei, rather than the electron density in a molecule. The basic phenomenon of NMR was discovered in 1945: The energy levels of atomic nuclei are split up by a magnetic field, similar to the effect observed for electrons in EPR. Transitions between these energy levels are induced by exciting the sample with radio frequency (r.f.) radiation equivalent to the energy difference between the two levels. Since 1960 the field of NMR has seen an explosive growth which
started with the development of pulsed Fourier-transform (FT) NMR [11] and multidimensional NMR spectroscopy and still continues today. Besides the structure of the system of interest NMR spectroscopy can also extract further important information like molecular dynamics and chemical kinetics. NMR can be used to determine the atomic level structure of proteins in solution and is often providing structures as accurate as those determined by X-ray diffraction. Although structure determination by NMR is mostly limited to proteins with molecular weights less than 40-60kDa, NMR techniques provide an alternative method for structure determination if a protein cannot be crystallized, or if there is concern that crystal packing has distorted the true structure in solution. [12]

For the structure determination of proteins by NMR four principal elements have to be combined: (i) the nuclear Overhauser effect (NOE) as an experimentally accessible NMR parameter in proteins that can yield the information needed for the global fold determination of a polymer chain; (ii) sequence-specific assignment of the many hundred to several thousand NMR peaks from a protein; (iii) computational tools for the structural interpretation of the NMR data and the evaluation of the resulting molecular structures; and (iv) multidimensional NMR techniques for efficient data collection [13].

The structure determination by NMR is based on analysis of numerous spin-spin interactions between nuclei. This approach is efficient when the structure of a small or intermediate size protein has to be solved, but for bigger macromolecules it becomes challenging. In this situation long distance constraints are of crucial importance to support the modelling of the target structure from many available short distances. Recent development of the double electron-electron resonance (DEER) technique has established the possibility to determine distances in the range of 1.5 to 6 nm [8]. This approach can not only be used as a supplementary method for NMR but it also provides a stand-alone tool to study structures at a coarse grained level and conformational changes of macromolecules. The currently established, 'classical' modus operandi for DEER requires double labelling with nitroxide radicals.

EPR is only sensitive to paramagnetic species whereas most proteins are diamagnetic. Therefore normally proteins have to be mutated with an EPR active group, named spin label. If no native paramagnetic centre is present in the sample of interest site-directed spin labelling (SDSL) is commonly used in EPR to introduce paramagnetic nitroxide radicals spin labels [14, 15]. In this case cysteines can be specifically labelled and from doubly labelled proteins the distance between the two nitroxide spin labels can be measured by EPR. The diamagnetic core of the protein is EPR silent and does not disturb the detected EPR signal, therefore bio-macromolecules of essentially arbitrary size can be studied by this technique. For the same reason also the reconstitution of membrane proteins into a lipid bilayer does not pose a problem for the distance determination. The information content of a single sample is limited but very specific, and crowded signals due to solvent or matrix can be avoided. The disadvantage of SDSL is that the protein of interest has to contain only cysteine residues at desired sites and additional cysteines eventually present in the
protein must be eliminated.

In this thesis an alternative way of labelling a macromolecule with one nitroxide spin label and one lanthanide chelate complex as spin probes is introduced. Distance information by DEER of gadolinium ion (Gd$^{3+}$)-nitroxide radical spin pairs and by the enhancement of nitroxide radical relaxation induced by a fast relaxing lanthanide ion (Ln$^{3+}$) are obtained.

1.3 Distance Measurements by EPR

In this thesis I will focus on the determination of long range distance information by EPR. Distance information obtained by magnetic resonance is mainly extracted from dipole-dipole interaction of two spatially separated spins. The distance can be measured 1) directly from the dipolar interaction frequency or 2) indirectly via the change of the relaxation times of the interacting spins. In the course of my thesis I will investigate both possibilities applied to orthogonal spin pairs consisting of a lanthanide ion (Ln$^{3+}$) and a nitroxide radical.

Structure and dynamics studies of bio-macromolecules and bio-macromolecular complexes is of broad interest for many scientists. Especially structural changes in the system of interest can be monitored nicely by EPR with a limited amount of spin labelled mutants. The site-directed spin labelling (SDSL) approach is usually applied to introduce nitroxide radicals as spin probe into the system. The spin label attachment to the protein macromolecules has to be treated with care because it may in principle disturb the native structure of the investigated molecule. Pulse EPR usually is performed at low temperatures and the freezing of protein solutions may result in some distortion, too. However, bio-macromolecules and bio-macromolecular complexes are known to be sufficiently rigid compounds and a large number of proteins after freezing and thawing retain their activity. Even though the mutation of the protein in terms of exchange of amino acids or introduction of the a spin label can be performed routinely some combinations of interacting residues are crucial for the design of the protein and a disturbance in the interaction area can lead to a distortion of the molecular structure. Using the method of spin labels, as well as all methods of chemical modifications of biological systems thus implies the necessity to control the invariability of macromolecule structure and functional activity of the investigated samples.

Typically, nitroxide-nitroxide spin pairs are used to directly detect the dipolar interaction frequency by the so called double electron-electron resonance (DEER) experiment. Nowadays such pulsed EPR techniques are established to measure distance constraints in the range of 1.5 to 6 nm [16–19] and are applied in structure and dynamics studies of bio-macromolecules and bio-macromolecular complexes [8, 20–23]. The upper detectable distance limit depends strongly on the type of system studied and the transverse relaxation time of the spin label. For model systems and often for soluble proteins it can be as long as
8 nm if the sample is prepared in deuterated solvent [24]. This limit can reach beyond 11 nm if the macromolecule under study is completely deuterated [25]. Nevertheless, in many cases, especially for membrane-incorporated proteins, the transverse relaxation times of nitroxide labels are in the range 2-4 $\mu$s, rarely 5 $\mu$s [26], which restricts the detectable distance limit to not more than 5 nm even if deuterated solvents are used. The DEER experiment is also rather expensive in terms of the measurement time, with typical times for a single distance measurement of 12-24 hours at protein concentrations of approximately 50-200 $\mu$M. The sensitivity of DEER increases significantly if the measurement is performed at Q-band frequency ($\approx$ 34 GHz) instead of commonly used X-band ($\approx$ 9.5 GHz) [27–29]. The recent development of a high power W-band spectrometer [30] has yet more significantly increased the sensitivity of DEER measurements on nitroxide radicals and added flexibility with respect to optimization of measurement conditions. Still the sensitivity improvement comes on the expense of stronger orientation selection [31]. This may force one to measure series of DEER traces in a range of different magnetic fields [24] and to sum the obtained traces in order to get a reliable distance distribution. This increases the measurement time and thus reduces the sensitivity increase at higher bands. Therefore, despite the apparent success of nitroxide-label based techniques, the search for new types of spin labels and improved experimental conditions is currently an active field of research. In particular, the availability of separately addressable labels would be of interest for studies of protein complexes, where different subunits of a complex under study could then be marked with different types of labels and observed independently. To introduce such an approach is the aim of this thesis.

Apart from the traditional nitroxide-nitroxide spin pairs addressed by DEER also the combination of nitroxide radicals with transition metals for distance determination was already investigated in model systems [32] as well as in a biologically relevant Cu(II) loaded porphyrin [33]. Also the combination of two metal ions serving as spin probes was suggested by Raitsimring et al. [34] and Gd$^{3+}$ centres were reported as potential spin probes for high-frequency (Kα-band and W-band) DEER [34–36]. In frozen solutions, such spin probes are expected to have much less pronounced orientation selection due to a stochastic distribution of the eigenframe orientations and magnitudes for the ZFS [37]. As I will show combination of the two paramagnetic centres (Gd$^{3+}$ and nitroxide radical) opens up the possibility to achieve independent addressability of the spin labels [38] and selective distance measurements between nitroxide-nitroxide, Gd$^{3+}$-Gd$^{3+}$ and Gd$^{3+}$-nitroxide should become possible. The spin pair arrangement of a nitroxide with chelating agent capable of binding lanthanide ions can also be used to combine DEER techniques with relaxation-enhancement-based distance measurements [39].

The indirect detection of the dipolar interaction between two spins via relaxation-based experiments is widely used in NMR (e.g. NOESY) and provides important information for protein structure determination. In EPR, relaxation based measurements are mainly used by CW techniques for qualitative analysis of the accessibility. Kulikov and Likhtenstein [40] describe the influence of the
enhanced relaxation of nitroxide radicals in the presence of iron and obtained
distance information based on the reformulated Bloembergen-Purcell-Pound the-
ory (BPP).
The use of pulsed EPR techniques for taking advantage of relaxation informa-
tion in distance determination is rather rare. In nitroxide-nitroxide spin pairs
relaxation enhancement is typically out of the Redfield regime [41, 42] and thus
the second order perturbation description can not be applied. However few
interesting works are published on distance determination in iron containing
proteins and reviewed by Eaton and Eaton [6] although it was generally not
checked whether a Redfield regime approach is indeed applicable for iron(III). In
these cases the fast relaxing agent was intrinsically present in the sample and the
nitroxide label is introduced via SDSL. Jäger et al. [39] could show that in an
artificial model system with rather well defined distance the longitudinal relax-
tation time ($T_1$) of nitroxide radicals is influenced by the presence of fast relaxing
Dy$^{3+}$ ion. The obtained distance information from relaxation enhancement for
such a spin pair is in good agreement with molecular dynamics (MD) prediction
for an artificial model system. For this reason, a first systematic study of the
relaxation enhancement effect on orthogonal spin pairs, consisting of a nitroxide
radical and Ln$^{3+}$ as fast relaxing agent, is performed in the course of this thesis.

1.4 Orthogonal Spin Labelling

In case only one type of spin label is present in the system the available EPR
techniques are not able to distinguish between different spin pairs. The com-
bination of spin labels with different properties can provide the possibility to
selectively address one or the other spin probe. Hence, the separation of different
contributions can be achieved. The potential of orthogonal spin labelling was
already shown for most commonly used nitroxide spin probes, where an isotope
labelling with $^{14}$N ($I = 1$) and $^{15}$N ($I = 1/2$) allows for a better interpretation
of spin-spin interactions [43, 44].
The traditional site-directed spin labelling (SDSL) approach using cysteine
residues and thiophilic nitroxide spin labels was improved by Fleissner et al.
[45]. The developed orthogonal spin labelling strategy is based on the aid of
an unnatural amino acid in combination with a hydroxylamine reagent carrying
the desired spin label. This approach allows for the simultaneous introduction
different spin labels where one spin label can be specifically attached onto the
unnatural amino acid and the other one onto a cysteine. This provides the
opportunity to design spin probes with different reactive moieties and label
properties and enables to perform more advanced experimental schemes.
The design of orthogonal spin labelling methods was out of the main scope of
this thesis, but is an important requirement for the routine applicability of the
developed and refined techniques introduced in the following. It sound promis-
ing to combine the SDSL approach of Fleissner et al. with available chelating
agent labels in order to obtain orthogonal spin pairs consisting of lanthanide ions and nitroxide radicals as used in this thesis.
2. Theory

The broad and extensive theoretical background of electron paramagnetic resonance (EPR) is well established and conclusively illustrated in comprehensive textbooks [1, 46, 47].

In this chapter the theoretical basis needed to describe the properties of the investigated system consisting of nitroxide radicals and lanthanide ions (Ln$^{3+}$) is discussed. Therefore the quantum mechanical description is reduced to the essentials relevant for the understanding of the analysed system. This includes the representation of the interactions with the appropriate Hamilton operator and a brief overview of relaxation theory.

The last part of this chapter introduces the experimental basis of this thesis and deals with the technical aspects of double electron-electron resonance (DEER) and relaxation measurements.

2.1 Spin Hamiltonian

The quantum mechanical description of EPR spectra is based on the representation of different interactions by an appropriate Hamilton operator also called Hamiltonian ($\hat{H}$). The concept of using the spin Hamiltonian was derived by Abragam [46]. The energy levels of a paramagnetic system with an effective electron spin $S$ and $n$ nuclei with a nuclear spin $I_k$ are described by the static spin Hamiltonian ($\hat{H}_0$):

$$\hat{H}_0 = \hat{H}_{EZ} + \hat{H}_{NZ} + \hat{H}_{HF} + \hat{H}_{ZFS} + \hat{H}_{DD}$$  \hspace{1cm} (2.1)

where $\hat{H}_{EZ}$ is the electron Zeeman Hamiltonian, $\hat{H}_{NZ}$ is representing the nuclear Zeeman interaction, $\hat{H}_{HF}$ is the hyperfine (hf) interaction Hamiltonian, taking the interaction between the electron spin and the nuclear spin into account, $\hat{H}_{ZFS}$ is the zero-field splitting Hamiltonian and $\hat{H}_{DD}$ introduces the dipole-dipole interaction between two electrons.

The Zeeman interaction for electrons as well as for nuclei is field dependent, which opens up the possibility to perform EPR experiments at different fields to influence the contribution of these terms. Therefore the separation and manipulation of different interactions becomes feasible.

In the following the discussion will be limited of the terms relevant for this thesis. A more detailed description of all terms influencing the analysis of EPR
spectra is given in textbooks [1, 46, 47] and PC IV Magnetic Resonance lecture at ETH Zurich [48]. In many cases, the **electron Zeeman interaction (EZI)** is dominating the contributions of other terms of the spin Hamiltonian. It is given by

$$\hat{H}_{\text{EZ}} = \frac{\mu_B}{\hbar} \vec{B}_0^T \vec{g} \hat{S}$$  \hspace{1cm} (2.2)

where $\mu_B$ is the Bohr magneton, $\hbar$ Planck’s quantum of action, $\vec{B}_0^T$ the transpose of the static magnetic field vector, $\vec{g}$ the g tensor and $\hat{S}$ the electron spin vector operator. The electron Zeeman interaction leads to the splitting of the energy levels due to the presence of an external magnetic field. The g tensor includes the orientation dependence of the electron Zeeman splitting. The strength of the static magnetic field vector ($\vec{B}_0$) is proportional to the electron Zeeman interaction as shown in figure 2.1 for an $S = 1/2$ system with the g-value close to the one of the free electron ($g_e$). The energy of the different spin states is given by the eigenvalues of electron Zeeman Hamiltonian ($\hat{H}_{\text{EZ}}$) and calculates to

$$E_\pm = \pm \frac{1}{2} g \mu_B B_0$$  \hspace{1cm} (2.3)

with an effective g-value. From the field dependence of the electron Zeeman splitting illustrated in figure 2.1 it can be seen that the Boltzmann population difference at the same temperature will be higher at Q-band frequencies as compared to S-band frequencies. This leads to a general increase of sensitivity at higher fields.

**Figure 2.1:** Energy levels of a $S = 1/2$ spin system in a magnetic field due to electron Zeeman interaction. The common frequency range for EPR experiments performed in this thesis is indicated. The transition between two Boltzmann populated energy levels can be induced by microwave (m.w.) radiation.

As for the electron spin, also the nuclear spin $I_k$ is quantized in an external magnetic field resulting in **nuclear Zeeman splitting**, which scales with the
2.1. Spin Hamiltonian

The nuclear Zeeman interaction (NZI) can be expressed by

\[ \hat{H}_{\text{NZ}} = -g_n \frac{\mu_n}{\hbar} B_0 \hat{I}_k \]  

with the \( g_n \)-value for the nucleus, \( \mu_n \) the nuclear magneton and the nuclear spin operator \( \hat{I}_k \). The NZI can often be neglected for computation of EPR spectra since this interaction is much smaller than the EZI, due to the fact that the magnetic moment of an electron spin exceeds the one of protons by a factor of 660. This results also in the higher sensitivity of EPR spectroscopy as compared to NMR at a given spin concentration.

In case the electron spin \( S \) is coupled to \( n \) nuclear spins \( I_k \) in the close neighbourhood an additional term for the spin Hamiltonian has to be taken into account. The so called hyperfine interaction is defined as

\[ \hat{H}_{\text{HF}} = \sum_{k=1}^{n} \hat{S} \cdot T_A \hat{I}_k \]  

with \( A_k \) being the hyperfine tensor. The hyperfine interaction contains two contributions an isotropic part originating from the Fermi contact interaction and an anisotropic part due to dipolar interaction. Fermi contact (FC) interaction occurs due to non-zero electron density at the position of the nucleus. This is only possible for s-orbitals carrying the unpaired electron and leads to a purely isotropic coupling (\( a_{iso} \)).

\[ \hat{H}_{\text{HF-FC}} = a_{iso} \hat{S} \cdot \hat{I}_k. \]  

The dipolar contribution of the hyperfine interaction arises from incomplete averaging of the dipole-dipole coupling (DD) between the electron and nuclear spin. In this case a purely anisotropic coupling is observed and the consideration of the point dipole approximation gives

\[ \hat{H}_{\text{HF-DD}} = \frac{\mu_0}{4\pi \hbar} g_e \mu_B g_n \mu_n \left( \frac{(3\hat{S} \cdot \hat{r})(\hat{r} \cdot \hat{I}_k)}{r^5} - \frac{\hat{S} \cdot \hat{I}_k}{3r^3} \right) = \hat{S} \cdot T_{DD} \hat{I}_k \]  

with \( r \) representing the distance between the electron and the nucleus, \( \mu_0 \) the permeability of vacuum and \( T_{DD} \) the dipolar hyperfine tensor. It is important to mention that the hyperfine interaction is not field dependent and therefore the separation from Zeeman interaction can be simplified by frequency dependent measurements.

For the case of nitroxide radicals the situation can be illustrated as shown in figure 2.2. The single unpaired electron is partially located at the p-orbital of the nitrogen and has an electron spin 1/2 (\( m_s = \pm 1/2 \)). There are two stable isotopes of nitrogen: \( ^{14}\text{N} \) and \( ^{15}\text{N} \). By far the most common is \( ^{14}\text{N} \) with a natural abundance of 99.634% and a nuclear spin \( I_k = 1 \). The hf interaction between the unpaired electron and the nitrogen nucleus leads to a splitting of the energy levels and three transitions can be observed in a nitroxide radical spectrum. In figure 2.2A the energy level splitting is shown.
for the averaged case in liquid state or a particular orientation in a single crystal. The orientation dependence arises from the fact that the electron is not located in an s-orbital, but in a p-orbital. This leads to the orientation dependent dipolar contribution of the hyperfine interaction. The molecular frame of the 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) derivative can be aligned with the p-orbital carrying the unpaired electron. The hf splitting in z-direction is the biggest and the splitting in x- and y-direction is mostly hidden in the line-width at lower frequencies. At X-band frequencies the width of the nitroxide spectrum is determined by $A_{ZZ}$ and at higher frequencies (e.g. W band-95 GHz) the spectral shape is dominated by the small g-anisotropy of the nitroxide radical.

**Figure 2.2:** Theoretical description of a nitroxide radical mainly resulting from electron Zeeman interaction (EZI) and hyperfine (hf) interaction. A) Energy level diagram for an unpaired electron coupled to a $^{14}$N nucleus (liquid state/single orientation). B) Orientation dependence of the hf interaction with the biggest splitting in z-direction of the molecular frame of a TEMPO spin label. Figure adapted from [48].

**Zero field splitting (ZFS)** occurs if $m > 1$ electrons are distributed in degenerate orbitals of the same atom. Therefore, they are strongly coupled to each other and cannot be excited separately. That is why this coupled spins can be described as a single group spin $S = m/2$. The dipole-dipole coupling between the electron spins removes the $(2S + 1)$-fold degeneracy of the ground state with a further contribution mediated by spin-orbit coupling being dominant for transition metal and lanthanide ions. This interaction is field independent as the hyperfine interaction. The zero-field splitting (ZFS) contribution to the Hamiltonian is formally analogous to the nuclear quadrupole interaction and is written as

$$\hat{H}_\text{ZFS} = \hat{S}^T \hat{D} \hat{S}$$

(2.8)

with $\hat{D}$ being the traceless zero-field splitting tensor. In its eigenframe (principal axis system (PAS)), the D-tensor is diagonal and the zero-field splitting
Hamiltonian is

\[ \hat{\mathcal{H}}_{\text{ZFS}} = D_x \hat{S}_x^2 + D_y \hat{S}_y^2 + D_z \hat{S}_z^2 \]

\[ = D \left( \hat{S}_z^2 - \frac{1}{3} S(S + 1) \right) + E (\hat{S}_x^2 - \hat{S}_y^2) \quad (2.9) \]

with \( D = \frac{3}{2} D_z \) and \( E = \frac{D_x - D_y}{2} \). By convention the principal axes are assigned such that \( |D_z| > |D_y| > |D_x| \) and \( E \) cannot exceed \( D/3 \).

In case the ZFS is larger than the m.w. quantum and the electron Zeeman interaction, only part of the transitions are observable by EPR. For half-integer group spins at least one pair of low lying energy levels is degenerate in the absence of an external magnetic field and will split in its presence. Such a level pair is called a Kramer’s doublet and can be described with an effective spin \( S = 1/2 \). The effective \( g \) value of a Kramer’s doublet depends on the zero-field splitting parameters \( D \) and \( E \). Electron spin transitions can usually only be excited within Kramer’s doublets, but not between levels belonging to different doublets.

**Figure 2.3:** Dy\(^{3+}\) spectrum. X-band continuous wave (CW) EPR spectrum of a 1 mM frozen solution of a free dysprosium ion (Dy\(^{3+}\))-DOTA complex in water/glycerol mixture 1/1 at 5K. The \( g \) values (\( g_{\perp} = 14, g_{\parallel} = 4.2 \)) can be assigned. Microwave power: 21dB, modulation amplitude: 1mT.

In case of Dy\(^{3+}\) the zero-field splitting is very strong and lifts the ground state degeneracy. Due to that the levels of only the lowest Kramer’s doublet are significantly populated, so that Dy\(^{3+}\) can be treated as an effective spin \( S = 1/2 \) [49]. The CW EPR spectrum of Dy\(^{3+}\) is shown in figure 2.3 and the spread of the spectrum is correlated to the anisotropy of the g-tensor. The spectrum is obtained from a free Dy\(^{3+}\)-DOTA\(^1\) complex in a 1/1 mixture of water and glycerol as a cryoprotectant at 5K at X-band frequencies. Since the spectral shape has to be interpreted as an effective \( g \) value, it can be determined with \( g_{\perp} = 14 \) and \( g_{\parallel} = 4.2 \). This are the same values as literature suggest

\(^{1}\)1,4,7,10-tetraazacyclododecane N,N',N\(^{\prime}\),N\(^{\prime\prime}\)-tetraacetic acid
for a related Dy$^{3+}$ complex with 2,2',2''',2'''-(Ethane-1,2-diyldinitrilo)tetraacetic acid (EDTA) as chelating agent. The broad spectrum reveals a strong dependence on the orientation of the paramagnetic centre. This has to be taken into account by analysing the effect on the relaxation times of a nitroxide radical in the close neighbourhood.

For gadolinium ions (Gd$^{3+}$) the situation is different, because the ZFS is comparatively small and the high spin system with $S = 7/2$ can not be simplified. All transition have to be taken into account. Raitsimring, Astashkin, and Caravan [37] have shown that the spectrum of various Gd$^{3+}$ complexes can not be simulated with a single set of strong ZFS parameters but a rather broad distribution of D and E values have to be considered. This leads to a broadening of the spectral shape and a pronounced central transition corresponding to the $|-1/2\rangle \leftrightarrow |1/2\rangle$ transition is present. This central transition is broadened by the ZFS interaction only to the second order. The broad distribution of D and E values introduces a homogeneous overlap of different species with particular ZFS parameter [50]. The resulting broadening of the spectrum covers an eventual anisotropy of the g-tensor and an isotropic $g \approx 1.99$ fits the spectrum of the Gd$^{3+}$ centres (figure 2.4).

![Figure 2.4: Echo detected field sweep spectrum of Gd$^{3+}$ loaded DTPA (black). The Gd$^{3+}$ spectrum is simulated (green) for a broad distribution of D and E values. A) A D/E distribution is used for values form 0 to 1/3 with $< D > = 1500$ MHz and $\sigma(D) = D/10$ (inset). The experimental spectra a shown in black and were measured B) at X-band frequencies with the fit shown in light green and C) at Q-band frequencies with the fit shown in dark green.](image)

The dipole-dipole interaction (DD) between two electrons ($S_1$, $S_2$) has a classical analogy, the interaction between two magnetic moments $\mu_1$ and $\mu_2$, and is analogous to the one between two nuclear spins. For transition metal and rare earth ions $g$-anisotropy or strong ZFS can lead to a tilt of the magnetic moments with respect to the external magnetic field direction. The interaction energy is therefore given by

$$ E = -\frac{\mu_0}{4\pi} \mu_1 \mu_2 \frac{1}{r^3} (2 \cos \theta_1 \cos \theta_2 - \sin \theta_1 \sin \theta_2 \cos \Phi) $$

where $\theta_1$ and $\theta_2$ are the angles of the magnetic moments with respect to the inter-spin vector $\vec{r}$ and $\Phi$ the orientation of $\vec{r}$ with respect to the magnetic field.
2.1. Spin Hamiltonian

The dipole-dipole interaction contribution to the Hamiltonian has the form

\[ \hat{H}_{DD} = \hat{S}_1^T \frac{g_1 g_2 \mu_B^2}{4\pi \hbar} \left( \hat{S}_1 \hat{S}_2 - \frac{3}{r^2} (\hat{S}_1 \vec{r}) (\hat{S}_2 \vec{r}) \right) \]

(2.11)

where \( D_{DD} \) is the dipole-dipole coupling tensor. The dipolar interaction can be rewritten in the so-called dipolar alphabet:

\[ \hat{H}_{DD} = \frac{\mu_0 g_1 g_2 \mu_B^2}{4\pi \hbar} [ \hat{A} + \hat{B} + \hat{C} + \hat{D} + \hat{E} + \hat{F} ] \]

(2.12)

with

\[
\begin{align*}
\hat{A} &= \hat{S}_{1z} \hat{S}_{2z} (1 - 3 \cos^2 \theta) \\
\hat{B} &= -\frac{1}{4} [\hat{S}_1^+ \hat{S}_2^- + \hat{S}_1^- \hat{S}_2^+] (1 - 3 \cos^2 \theta) \\
\hat{C} &= \frac{3}{2} [\hat{S}_1 \hat{S}_2^+ + \hat{S}_1^+ \hat{S}_2] \cos \theta \sin \theta e^{-i\phi} \\
\hat{D} &= \frac{3}{2} [\hat{S}_1 \hat{S}_2^- + \hat{S}_1^- \hat{S}_2] \cos \theta \sin \theta e^{+i\phi} \\
\hat{E} &= -\frac{3}{4} \hat{S}_1^+ \hat{S}_2^+ \sin^2 \theta e^{-2i\phi} \\
\hat{F} &= -\frac{3}{4} \hat{S}_1^- \hat{S}_2^- \sin^2 \theta e^{-2i\phi}.
\end{align*}
\]

(2.13)

The \( \hat{A} \) term commutes with the electron Zeeman Hamiltonian, which implies that only the energy of the electron state is influenced but no mixing of different levels occurs. The \( \hat{B} \) term represents a zero quantum transition (flip-flop process) and is also pseudo secular if \( S_1 \) and \( S_2 \) are like spins. \( \hat{C} \) and \( \hat{D} \) contain a raising or lowering operator for each of the spins, therefore they characterize single quantum transition (spin flip) with \( \Delta m = \pm 1 \). The \( \hat{E} \) and \( \hat{F} \) terms involve a rising and lowering operator for both spins, representing double quantum transitions (flip-flip process) with \( \Delta m = \pm 2 \) \((\hat{E})\) or \(-2\) \((\hat{F})\).

The four-level diagram for a \( S_1 = 1/2, S_2 = 1/2 \) system is illustrated in figure 2.5, with the electron states denoted as \( \alpha \) and \( \beta \) and the transitions driven by the several dipolar alphabet terms being marked accordingly.

In case of strong Zeeman interaction the time dependent terms \( \hat{S}^+ \) and \( \hat{S}^- \) can be neglected as non-secular terms \((\hat{C} - \hat{F})\). As the through-bond exchange couplings in typical spin-labelled EPR probes of bio-macromolecules for distances larger than 1.5nm are by at least one order of magnitude smaller than the dipolar coupling, the exchange coupling can also be neglected. Therefore, the complete interaction between two electron spins can be described by the dipole-dipole interaction Hamiltonian, which simplifies to:

\[ \hat{H}_{DD} \approx \frac{\mu_0 g_1 g_2 \mu_B^2}{4\pi \hbar} \hat{A} \approx \omega_{12} \hat{S}_{1z} \hat{S}_{2z} \]

(2.14)
Figure 2.5: Four-level energy diagram of a two-spin system ($S_1 = 1/2, S_2 = 1/2$) with zero-quantum ($\hat{B}$), single-quantum ($\hat{C}, \hat{D}$) and double-quantum ($\hat{E}, \hat{F}$) transitions introduced by the terms of the dipolar alphabet.

with the dipolar evolution frequency

$$\omega_{12} = \frac{\mu_0}{4\pi\hbar} \frac{g_1 g_2 \mu_B^2}{r^3} (1 - 3 \cos^2 \theta)$$  \hspace{1cm} (2.15)$$

The $\hat{B}$ term was dropped since the resonance frequencies of lanthanide ions and nitroxide ions differ by much more than the dipole-dipole coupling for an overwhelming majority of spin pairs. The $g$ value of the unpaired electron for organic radicals, like nitroxide spin labels, can be approximated with the $g$ value of the free electron $g_1 = g_2 = g_e$. The dipolar coupling thus has a value of

$$\omega_{DD} = \frac{\omega_{12}}{1 - 3 \cos^2 \theta} = 2\pi \nu_{DD} = \frac{2\pi}{r^3/\text{nm}^{-3}} \frac{52.04 \text{ MHz}}{r^3/\text{nm}^{-3}}$$  \hspace{1cm} (2.16)$$

in frequency units. A more detailed discussion about the limitations for the direct detection of the dipolar interaction using this relationship will given in section 2.3.2. The indirect detection of the dipolar coupling via relaxation based measurements requires further exploration of the dipolar alphabet and will be discussed in the following chapter.

### 2.2 Relaxation

Relaxation is the sum of numerous processes which lead to the rebuild of the equilibrium magnetization after preparation of a non-equilibrium state. The relaxation rate describes how fast the spins are oriented back to the direction of the external magnetic field (convention: z-direction). According to the high-field approximation, in equilibrium the whole magnetization is aligned with the external magnetic field (longitudinal) and no magnetization is present in the
x-y-plane (transverse magnetization). Due to strong g-anisotropy, the equilibrium magnetization is tilted with respect to the z-axis for the effective spin $1/2$ of the low lying Kramers doublet of Dy$^{3+}$. This complication will be neglected here to keep explanations simple.

Relaxation occurs due to the interaction of the spin with its environment. The theoretical description of relaxation was already developed in the early history of magnetic resonance and can be explained by the Bloch equations [51] in the classical vector model. The ad hoc introduction of the phenomenological relaxation times $T_1$ and $T_2$ describes the decay of the longitudinal and transverse magnetization. The further development the classical transition-rate theory [41, 52] takes the transition between different states into account and the description was later extended to include transverse relaxation.

This thesis is based on a semi-classical description of relaxation processes, where the interaction of the observed spin system with the environment is expressed via a fast and randomly changing local field [46, 53–56]. A special example, discussed in the course of this thesis, is the induced relaxation on one type of spins (nitroxide radical) due to the interaction with a much faster relaxing paramagnetic species (lanthanide ions (Ln$^{3+}$)) in the close neighbourhood. The correspondent Bloembergen-Purcell-Pound theory (BPP) developed mainly for nuclear spins was reformulated by Kulikov and Likhtenstein [40] for electron spins.

The theory describing several approaches explaining relaxation processes is summarized in the following chapter. A more detailed description can be found e.g. in the textbook of Slichter [47] or the Advanced Magnetic Resonance lecture script at ETH Zurich [57].

### 2.2.1 Bloch Equations

For a magnetic moment $\vec{\mu}$ caused by a rotating charge with the angular momentum $\vec{L}$ an external magnetic field $\vec{B}$ is induces a torque $\vec{T}$, which equals a change of the direction of the angular momentum vector ($\frac{d}{dt} \vec{L} = \vec{T} = \vec{\mu} \times \vec{B}$). The motion of the magnetization vector ($\vec{M} = V^{-1} \sum \vec{\mu}$) therefore can be expressed by

$$\frac{d}{dt} \vec{M} = \gamma \vec{M} \times \vec{B}. \quad (2.17)$$
The magnetic field vector is directed by convention along the z-axis and the coupled system of differential equation can be solved to:

\[
\begin{align*}
\frac{d}{dt}M_x(t) &= \gamma M_y(t) \cdot B_0 \quad \Rightarrow \quad M_x(t) = M_x(0) \cos(\omega_0 t) - M_y(0) \sin(\omega_0 t) \\
\frac{d}{dt}M_y(t) &= -\gamma M_x(t) \cdot B_0 \quad \Rightarrow \quad M_y(t) = M_x(0) \sin(\omega_0 t) + M_y(0) \cos(\omega_0 t) \\
\frac{d}{dt}M_z(t) &= 0 \quad \Rightarrow \quad M_z(t) = M_z(0)
\end{align*}
\]

(2.18)

where the Larmor frequency \( (\omega_0) \) describes the precession of the magnetization. Experimentally it can be shown that the magnetization is turning back to align parallel to a static, external field \( \vec{B}_0 \), because this is the energetically most favourable situation. Hence, the precession of the magnetization has to undergo a damping process to arrive to this aligned direction.

The magnetization component along the z-axis is called longitudinal. In order to achieve a change of \( M_z \), an energy exchange with the environment is needed which leads to relaxation towards the equilibrium. Assuming first order kinetics the change of \( M_z \) can be expressed by

\[
\frac{d}{dt}M_z = -\frac{1}{T_1} (M_z - M_0)
\]

(2.19)

with the longitudinal relaxation time \( (T_1) \) constant, also called spin-lattice relaxation time. The time range of \( T_1 \) is strongly temperature dependent.

In the equilibrium situation no transverse component of the magnetization is present and it therefore has to decay to zero. Assuming again first order kinetics and the same rate constant for the x- and y-component, the relaxation term can be written by

\[
\begin{align*}
\frac{d}{dt}M_x(t) &= -\frac{1}{T_2} M_x \\
\frac{d}{dt}M_y(t) &= -\frac{1}{T_2} M_y
\end{align*}
\]

(2.20)

with the transverse relaxation time \( (T_2) \), also called spin-spin relaxation time. In general \( T_2 \) is shorter than \( T_1 \) \((T_1 > T_2)\).

The combination of the set of differential equations (equation 2.18) for the precession of the magnetization and the relaxation terms gives rise to the Bloch equations [51]

\[
\begin{align*}
\frac{d}{dt}M_x(t) &= \gamma M_y(t) B_0 - \frac{M_x}{T_2} \\
\frac{d}{dt}M_y(t) &= -\gamma M_x(t) B_0 - \frac{M_x}{T_2} \\
\frac{d}{dt}M_z(t) &= -\frac{M_x - M_0}{T_1}
\end{align*}
\]

(2.21)

which can be solved for the evolution of the magnetization to

\[
\begin{align*}
M_x(t) &= [M_x(0) \cos(\omega_0 t) - M_y(0) \sin(\omega_0 t)] \cdot \exp(-t/T_2) \\
M_y(t) &= [M_x(0) \sin(\omega_0 t) + M_y(0) \cos(\omega_0 t)] \cdot \exp(-t/T_2) \\
M_z(t) &= M_z(0) + (M_x - M_0) \cdot \exp(-1/T_1).
\end{align*}
\]

(2.22)
2.2. Relaxation

2.2.2 Bloembergen-Purcell-Pound Theory

The Bloch equations introduce the relaxation times $T_1$ and $T_2$ as empirical constants whereas in 1948 Bloembergen, Purcell, and Pound developed the basic concept to explain in a quantitative manner the relation of the spin lattice interaction and $T_1$ respectively $T_2$. The formal theory formulation advanced by Wangsness and Bloch [53] and Redfield [54] is applicable to any type of stochastic interaction, provided it is weak and has a sufficiently short correlation time ($\tau_C$). Here I will refer to the basic described concept of taking the random fluctuation of the local magnetic field as a reason of relaxation into account as Bloembergen-Purcell-Pound theory (BPP).

According to BPP, relaxation is affected by the fluctuation of the field at the position of the spin and the main source of relaxation is considered to be the dipole-dipole interaction between spins. The dipole-dipole interaction is randomly modulated because of lattice motion and thus leads to relaxation. The reorientation (tumbling) of the spin-labels during the measurement, which could in principle lead to a fluctuating local magnetic field, is significantly reduced in the considered case of this thesis because pulse EPR experiments are performed at low temperature in frozen solution.

In pulse EPR the motion of the paramagnetic centres with respect to each other in very limited due to the low measurement temperature. The main primary source of relaxation thus is connected to the coupling of the spin system to the vibrations of the atoms. The random fluctuation of the atom positions can influence the terms of the Hamiltonian ($\mathcal{H}$), including the spin-orbit coupling terms

$$H_{SO} = \lambda L \cdot S$$  \hspace{1cm} (2.23)

with $L$ being the orbital momentum of spin system, $S$ the spin quantum number, and $\lambda \propto \frac{1}{r} \left( \frac{2V}{r^3} \right)$ defines the spin-orbit coupling parameter with $V$ the Coulomb potential of the electron in the field of the atom and $r$ is the radius of the potential. The random fluctuation of the atom position therefore can be converted into a stochastic change of the local magnetic field that acts on the spin. This kind of interaction is particularly strong for those paramagnetic centres, where the orbital moment is not equal to zero in the ground state. This includes a number of transition metal complexes and almost all lanthanide ions. An important exception is the paramagnetic Gd$^{3+}$ with $L = 0$ and $S = 7/2$ in contrast to Dy$^{3+}$ featuring $L = 5$ and $S = 5/2$ ($S_{\text{effective}} = 1/2$). In case a slowly relaxing organic radical (e.g. nitroxide radical) is placed in the close vicinity of the fast relaxing species (e.g. Dy$^{3+}$) the relaxation of the 'slow' spin can be enhanced via a dipole-dipole mechanism. In a semi-classical approximation the matrix elements of the spin operators of the fast relaxing species are substituted by a set of random functions with the correlation times being $T_1$ and $T_2$ of the fast relaxing spin. The fluctuation of the fast relaxing spin leads to a fluctuating local field at the position of the slowly relaxing spin, which can be described as a time dependence of terms $\hat{B} - \hat{F}$ of the dipolar alphabet (equation 2.13). The magnetic field fluctuation can be described by a correlation function of a
random process \( (C(\tau)) \)

\[
C(\tau) = \frac{\langle \tilde{B}(0) \cdot \tilde{B}(\tau) \rangle}{\langle \tilde{B}(0) \rangle} \tag{2.24}
\]

where \( \tilde{B} \) is the local magnetic field. \( \tilde{B}(0) \) is determined by the time-independent
spin Hamiltonian \( (\mathcal{H}_0) \) and \( \tilde{B}(\tau) \) representing the field fluctuation is character-
ized by \( \mathcal{H}_1 \), the time-dependent Hamiltonian of the system. Fourier transform-
ation of the correlation function of a random process provides the so called
spectral density function \( (J(\omega)) \)

\[
J(\omega) = \int_0^\infty C(\tau) \exp(-i\omega\tau)d\tau. \tag{2.25}
\]

The spectral density function is normalized and can be pictured as the relative
intensity of a random process with the frequency \( \omega \). The correlation time \( (\tau_C) \)
of the fluctuation provides a measure of the speed of the process. Furthermore, \( ||\mathcal{H}_1|| \) has to be smaller than \( ||\mathcal{H}_0|| \) that second order perturbation theory can
be applied to describe transitions between different spin states (longitudinal
relaxation). In order to operate in the Redfield regime, the dipolar coupling
\( (w_{DD}) \) has be much smaller than \( \tau_C^{-1} \), i.e.

\[
w_{DD} << 1/\tau_C \tag{2.26}
\]

is valid. A detailed mathematical description of relaxation phenomena is pro-
vided e.g. by Slichter [47] or Advanced Magnetic Resonance (Relaxation HS
2009) lecture at ETH Zurich [57].

The scope of this thesis is the investigation of the dipolar coupling between
a relatively slowly relaxing nitroxide radical and a fast relaxing \( \text{Ln}^{3+} \) ion. In
sufficiently diluted samples the Bloembergen-Purcell-Pound theory can be used
as a tool to describe the enhancement of the nitroxide relaxation due to the
presence of a fast relaxing agent. This can be verified by substituting the matrix
elements of the operator representing the fast relaxing spin \( (S_1) \) in equation 2.13
by a random function with the correlation time \( (\tau_C) \). In the Redfield regime
second order perturbation theory provides an expression for the relaxation
enhancement \( (\Delta k) \) can be described by

\[
\Delta k(r, \theta) = \frac{1}{T_{1s}} - \frac{1}{T_{1s,0}} \tag{2.27}
\]

\[
= S(S + 1) \cdot \frac{g_s^2 \mu_0^2 \mu_B^2}{(4 \cdot \pi)^2 h^2 r^6} \cdot [f_B + f_{CD} + f_{EF}]
\]
where $S$ is the effective spin of the fast relaxing species and the $f_{B^*}$, $f_{CD^*}$ & $f_{EF}$-terms, referring to their origin from the dipolar alphabet, are given by

$$f_{B} = \frac{1}{3} \cdot \left(1 - 3 \cos^2 \theta\right)^2 \cdot \frac{T_{2f}}{1 + (\omega_f - \omega_s)^2 \cdot T_{2f}^2}$$

$$f_{CD} = 3 \sin^2 \theta \cdot \cos^2 \theta \cdot \frac{T_{1f}}{1 + \omega_s^2 \cdot T_{1f}^2}$$

$$f_{EF} = \frac{2}{3} \sin^4 \theta \cdot \frac{T_{2f}}{1 + (\omega_f + \omega_s)^2 \cdot T_{2f}^2} \cdot (2.28)$$

$T_1$ relaxation enhancement ($\Delta k$) is defined as the difference of the longitudinal relaxation rates of the slowly relaxing species in the absence ($1/T_{1s,0}$) and presence ($1/T_{1s}$) of a fast relaxing agent.

The terms in equation 2.28 depend on the resonance frequencies of the slowly ($\omega_s$) and fast ($\omega_f$) relaxing species. The relaxation times of the fast relaxing agent ($T_{1f}, T_{2f}$) also have to be taken into account. From equation 2.27 one can see that in the Redfield regime the relaxation enhancement effect scales with $r^{-6}$.

### 2.2.3 Averaged Relaxivity Approximation

In 2008 Jäger et al. [39] introduced an averaged relaxivity approximation based on the Bloembergen-Purcell-Pound theory (BPP). To make the problem traceless further assumptions where added in order to simplify the mathematical description of the problem and reduce the number of parameters. The average relaxivity approximation holds true if the fast relaxing agent can be treated within the Redfield limit. The resonance frequency of an electron spin is defined by

$$\omega = g\mu_B B_0 / \hbar. \quad (2.29)$$

The $g$-tensor for Dy$^{3+}$ ions is strongly anisotropic and this anisotropy influences the amplitude of the relaxation enhancement effect as well as the resonance frequency $\omega_f$. To a good approximation, the $g$ matrix has axial symmetry with principal values $g_{f\parallel} = 14$ and $g_{f\perp} = 4.2$ (figure 2.3), so that $g_{f}$ calculates to

$$g_{f} = \sqrt{g_{f\perp}^2 \sin^2 \theta_f + g_{f\parallel}^2 \cos^2 \theta_f} \quad (2.30)$$

where $\theta_f$ is the angle between the static field and the unique axis of the lanthanide $g$ tensor. A strong $g$ anisotropy leads to an orientation dependent deviation of the quantization axis of the lanthanide spin from the magnetic field direction. By applying the Likhtenshtein approximation [58] the minor effect on the dipole-dipole coupling that arises from this tilt of the quantization axis is neglected while the major effect of the orientation dependence of $g_{f}$ is included. The principal values $g_{f\parallel}$ and $g_{f\perp}$ can be determined experimentally
by CW EPR measurements (see figure 2.3).

While working in the fast relaxation regime for lanthanide ions at temperatures above 20 K the longitudinal and transverse relaxation times can be considered as equal ($T_{1f} = T_{2f}$) and the orientation dependence of $T_{1f}$ can be neglected [39]. By doing so the magnitude of the relaxation enhancement effect depends on $T_{1f}$, the two angles $\theta$ and $\theta_f$, the g values of both spins and the on inter-spin distance r. For given parameters $T_{1f}$, $\theta$ and $\theta_f$ equation 2.27 can be simplified to

$$\Delta k = \frac{C(T_{1f}, \theta, \theta_f)}{r^6}$$  \hspace{2cm} (2.31)

with $C$ being an orientation-dependent relaxivity. The presence of all orientations of the principal axis system of the fast relaxing species in frozen solution allows for an isotopic averaging approach assuming that all lanthanide ion ($\text{Ln}^{3+}$) complexes have the same relaxation time. In case no correlation between the principal axis of the fast and slowly relaxing species occurs, it is possible to sum the calculated $\Delta k$ decays for all orientations and to determine the $1/e$ times of the obtained time trace. Therefore an orientation averaged relaxation enhancement ($\overline{\Delta k}$) can be formulated as

$$\overline{\Delta k}(r, T_{1f}) = \frac{C(T_{1f})}{r^6}$$  \hspace{2cm} (2.32)

with the average relaxivity $\overline{C}$ depending on $T_{1f}$ but not on the orientation or on the distance.

The two remaining variables, first the inter-spin distance ($r$) and second the longitudinal relaxation time of the fast relaxing spin ($T_{1f}$), can be determined almost independently from each other. The value of $T_{1f}$ can be approximated according to an empirical power law

$$\frac{T_{1f}}{T_s} = A \times \left(\frac{T_{\text{max}}}{T}\right)^p$$  \hspace{2cm} (2.33)

where $T_{\text{max}}$ is the temperature showing the strongest relaxation enhancement effect and the power-parameter $p$ is influencing the shape of the temperature dependence of $\overline{\Delta k}$ in terms of the width. The pre-factor $A$ can reach values in the range from $10^{-11}$ to $10^{-12}$ depending on spin-orbit coupling and atom vibrations near the fast relaxing species, its complex formation, and the measurement frequency. The inter-spin distance $r$ vertically shifts the amplitude of the temperature dependent relaxation enhancement and therefore works as a scaling factor.

The average relaxivity approximation was tested by Jäger et al. [39] on statistical distributed Dy$^{3+}$ loaded DOTA complexes and nitroxide radicals in solution and on a model system with a mean distance of 2.72 nm according to molecular dynamics (MD) simulations. An overview of the obtained results is shown in figure 2.6. The temperature dependence of the relaxation enhancement $\overline{\Delta k}$ of TEMPOL by 10 mM Dy$^{3+}$ DOTA complex in a glassy 0.6:0.4 (v/v) mixture of
2.3 Pulse EPR Measurements

Figure 2.6: Relaxation enhancement effect of Dy$^{3+}$ on nitroxide radicals (adapted from Jäger et al. [39]). A) Temperature dependence of the relaxation enhancement $\Delta k$ of TEMPOL by 10 mM Dy$^{3+}$ DOTA in a glassy 0.6:0.4 (v/v) mixture of glycerol and water. Open circles are experimental data, the solid line is a fit based on an empirical power law for $T_1 f$ (Eq. 2.33 and the dashed lines correspond to the contributions of the dipole-dipole alphabet. B) Dependence of the orientation-averaged relaxation rate enhancements $\Delta k$ on distance. Open circles are numerically simulated values while the solid line is a fit according to Eq. 2.32. C) Magnetic field and temperature dependence of relaxivity expected if the field dependence of the relaxation times of the nitroxide and the Dy$^{3+}$-DOTA complex in the absence of dipole-dipole interaction is weak. Simulations were performed with $T_1 f = 1.2 \cdot 10^{-11}(41.5 K/T)^{2.4}$ at S-band (solid line, 2 GHz), X-band (dotted line, 9.5 GHz), Q-band (dashed line, magnified by a factor of 5, 35 GHz), and W-band (dash-dot line, magnified by a factor of 10, 94 GHz).

glycerol and water is shown in figure 2.6 A. The temperature of maximum relaxation enhancement effect in this case is $T_{\text{max}} = 41.5 K$ and with the exponent $p = 2.4$ and the pre-factor $A = 1.2 \times 10^{-11}$ the experimental temperature dependence can be fitted. The contribution arising from the different terms of the dipolar alphabet are illustrated in dashed lines. A direct correlation between the Dy$^{3+}$ concentration, the average distance and the relaxation enhancement ($\Delta k$) magnitude could be observed and the expected relaxation enhancement effect for a broad distance range was simulated. The obtained distance for the model system is 2.65 nm and shows a satisfying agreement with expectations from MD simulations. From the basic BPP theory where the resonance frequency influences the relaxation enhancement effect it can be seen that the external magnetic field is affecting the magnitude of $\Delta k$. The theoretical magnetic field and temperature dependence of the relaxivity is shown in figure 2.6 C and was checked experimentally in this thesis.

2.3 Pulse EPR Measurements

A microwave (m.w.) pulse (short but intense radiation) creates non-equilibrium magnetization. The transient signal arising from this non-equilibrium magnetization is called free induction decay (FID) and can be described by the Bloch equations. In EPR the FID is dephasing very fast so that due to the dead time
of the spectrometer usually no signal is observed. In a spin echo experiment the dephasing of spin packets\(^2\) can partially be reversed. It was first described for nuclear spins by Erwin Hahn [59] and is based on the non-linear behaviour of the spin packets. The observation of an electron spin echo was first reported by Blume [3].

The Hahn echo experiment is shown in figure 2.7, where the first \(\pi/2\) pulse flips the magnetization from the \(z\)-direction into the \(x\)-\(y\) plane. The magnetization can be illustrated in a vectorial picture and the magnetization vector will be aligned with the \(y\)-axis directly after the first pulse (figure 2.7B). During the time \(\tau\) the magnetization is dephasing due to the resonance offset of the excited spin packets. By applying an additional \(\pi\) pulse the spins are flipped and the magnetization is refocused. The echo is formed after the time delay \(\tau\).

\[
\begin{array}{c}
\text{A) The two-pulse echo sequence } (\pi/2 - \tau - \pi) \\
\text{leads to an echo formation after additional time } \tau. \\
\text{The FID directly after the m.w. pulse decays fast.}
\end{array}
\]

\[
\begin{array}{c}
\text{B) Vectorial picture of the magnetization during the Hahn echo sequence.}
\end{array}
\]

The EPR spectrum of a paramagnetic species is usually broader than the excitation bandwidth of a single pulse. That is why even with the hardest hard pulses available often only a fraction of the spectrum can be excited. In the following the main experimental schemes used in this thesis will be introduced.

### 2.3.1 Relaxation Measurements

This section is focused on the experimental determination of the longitudinal relaxation time \((T_1)\) and transverse relaxation time \((T_2)\) of a paramagnetic species. A detailed description of the available experiments is given by Schweiger and Jeschke [1].

\(^2\)A spin packet is an ensemble of spins with the very same resonance frequency and therefore each spin experiences the same time averaged local fields.
2.3.1.1 Longitudinal Relaxation Measurements

Several experimental methods are available in order to measure the longitudinal relaxation time ($T_1$) of a paramagnetic centre, e.g. the saturation recovery or the inversion recovery experiment. In the saturation recovery (SR) experiment a long saturating pulse equalizes the population of the energy levels in the spin system and the recovery to equilibrium magnetization is monitored by detection with a probe pulse. The saturation pulse should not only saturate the transitions detected by the probe pulse but also the ones influencing the detection via spectral or spin diffusion. In the SR experiment only the equilibration of the population difference of the energy levels in the spin system can be observed, whereas the inversion including a strong m.w. pulses makes use of the total population difference and therefore the sensitivity of the experiment can be increased. However, to avoid the influence of spectral and spin diffusion, the detection Hahn echo sequence needs to be much more selective than the inversion pulse. While such detection still observes more spins than detection by a weak probe pulse, the advantage of detecting the whole time evolution in a single shot is lost in the echo-detected inversion recovery experiment. On the other hand, the signal is condensed in time, so that less noise is accumulated. All these conditions influence relative sensitivity of the two experiments, which depends on the system studied, on the temperature and the available hardware.

In this thesis the longitudinal relaxation was measured by the so called inversion recovery experiment which is not fraught with the resonator frequency stability problems of saturation recovery detection. The corresponding pulse sequence is depicted in figure 2.8A. The inversion pulse at the beginning of the pulse sequence is significantly harder than the pulses of the Hahn-echo detection sequence. The inter-pulse delay for the detection is fixed with usually $\tau_2 = 200\ \text{ns}$ and the starting value for the first variable delay $\tau_1$ is set to $2\ \mu\text{s}$. An inversion pulse length of 32ns and observer pulses lengths of 52 and 104ns for the $\pi/2$ and $\pi$ pulse, respectively, is used in the experimental scheme. This ensures a much larger bandwidth of the inversion pulse as compared to the detection sequence, which reduces the effect of spectral and spin diffusion on the obtained experimental data.

The integration window for the echo matches the detection $\pi$ pulse length of 104ns. The $\pi/2$ pulse is phase cycled $[+\langle +x \rangle - \langle -x \rangle]$ to cancel receiver offsets and avoid any contributions from a free induction decay or two-pulse echoes involving the inversion pulse.

It was shown that the relaxation of a nitroxide radical in a diamagnetic environment at temperatures below 100K shows only weak frequency dependence [60]. The experimental inversion recovery traces should show a pure exponential decay in a homogeneous environment. In reality the experimental trace can not be fitted by a mono-exponential decay function. This reflects the inhomogeneity of the local surrounding of the nitroxide radical. Normally, the experimental inversion recovery traces can be fitted by a stretched exponential decay for the
form:

$$I(t_1) = A - B \cdot \exp \left[ - \left( \frac{T}{T_1} \right)^\alpha \right],$$

(2.34)

with $I(t_1)$ being the intensity at the particular time delay $T$ in the experiment. The variables $A$ and $B$ determine the scaling of the fit, whereas $\alpha$ is the stretching exponent and $T_1$ is representing an effective longitudinal relaxation time of the slowly relaxing species. An $\alpha$ parameter of 1 is representing a homogeneous decay with a mono-exponential behaviour and in case $\alpha < 1$ the homogeneity of the decay function is disturbed.

### 2.3.1.2 Transverse Relaxation Measurements

If the lineshape of an EPR transition is dominated by relaxational broadening, the $T_2$ can be determined from the linewidth in a CW EPR spectrum. This situation rarely applies and is not encountered in this thesis. Hence, $T_2$ times were measured by observing decay the two-pulse echo as discussed in the following.

Transverse relaxation corresponds to dephasing of spins in the $xy$-plane. After the excitation of the spins ($\pi/2$ pulse) the phase coherent situation, where all spins precess in a similar manner, is destroyed over time ($t_1$). The phase coherence is lost because of energy exchange between the spins (flip-flop process). The dephasing originating from field inhomogeneity can be reversed by the re-focusing $\pi$ pulse and the pure transverse relaxation in terms of echo intensity after an additional time delay ($t_1$) is measured as a function of the inter-pulse delay. The obtained time domain data (see figure 2.9B) reveal a characteristic
2.3. Pulse EPR Measurements

decay of the magnetization in the xy-plane (transverse magnetization) and the
time at which the echo intensity is decayed to $1/e$ of the initial magnetization
can be approximated as $T_2$ time.

In contrast to the spin-lattice relaxation no energy is dissipated to the environ-
ment in case of the spin-spin relaxation process. $T_2$ is a time constant which
is independent on the strength of the magnetic field $B_0$, since a pure spin-spin
interaction ($T_2'$) can be assumed, where the lifetime broadening ($1/(2T_1)$) is
neglected. Otherwise $T_2$ calculates to:

$$\frac{1}{T_2} = \frac{1}{T_2'} + \frac{1}{2T_1}$$  \hspace{1cm} (2.35)

![Diagram of pulse EPR sequence](image)

**Figure 2.9:** Hahn echo decay experiment. **A)** Hahn echo pulse sequence with
a time varying inter-pulse delay $\tau_1$. The refocusing $\pi$ pulse is shifted by $\Delta t$
and therefore the detection position has to shift with $2 \times \Delta t$. **B)** The obtained
experimental time trace as a function of the varying inter-pulse delay $\tau_1$ re-
veals the decay of the transverse magnetization. The $1/e$ time is approximately
characterizing the transverse relaxation time ($T_2$).

The experimental traces might show additional oscillations on top of the expo-
nential decay function of the form:

$$I(\tau_1) = A \cdot \exp \left[ - \left( \frac{\tau_1}{T_2} \right) \right]$$  \hspace{1cm} (2.36)

with the transverse relaxation time ($T_2$). Additional oscillations are arising from
the resonance frequencies of the surrounding nuclei and are called electron spin
echo envelope modulation (ESEEM). These nuclear modulations do not influ-
ence the obtained relaxation time of the nitroxide radical in case of a protonated
matrix.
2.3.2 Double Electron-Electron Resonance

The double electron-electron resonance (DEER) experiment, also called pulsed electron double resonance (PELDOR), is a method to measure the dipolar coupling between spatially separated unpaired electrons. The time-dependent detection of the dipolar interaction is analogous to the spin-echo double resonance (SEDOR) experiment used in nuclear magnetic resonance (NMR) [61]. The transfer of the idea to the needs of EPR was implemented by Milov et al. [62, 63] and later extended to the four-pulse sequence shown in figure 2.10A which allows for dead time free detection [19, 64]. Nowadays, the DEER techniques are established to measure distance constraints in the range of 1.5 nm to 6 nm [17–19, 63] and are applied in structure and dynamics studies of bio-macromolecules and bio-macromolecular complexes [8, 20–23]. Typically, nitroxide-nitroxide spin pairs are used for this kind of applications and the upper distance limit is restricted by $T_2$ of the nitroxide spin label [8]. Furthermore, two m.w. frequency sources are required and the resonator has to accommodate both frequencies with a typical split of 65 MHz at X-band frequencies.

The refocused echo sequence at the observer frequency is invariant during the experiment and the inversion pump pulse shifts in time ($t$). The echo detected field sweep spectrum of a nitroxide radical depicted in figure 2.10C is broader than the excitation bandwidth of a single pulse. Therefore, a selective manipulation of a fraction of spins is feasible. In the DEER experiment on nitroxide-nitroxide spin pairs the pump frequency is chosen such that the maximum of the spectrum is excited by the m.w. pulse and the detection is performed at the low-field shoulder at X-band frequencies. The inversion of a fraction of spins (B, red) leads to a change of the local field at the position of the observer spins (A, blue) (see figure 2.10B) in case they are coupled to each other.

The resulting time-domain data ($V(t)$) is the product of contributions due to inter- ($B(t)$) and intra-molecular interactions ($F(t)$) of the electron spins. By assuming an exponential decay of the background signal, as it arises from the random distribution of spin systems in a three dimensional space, the inter-molecular contributions can be separated from the so called form factor ($F(t)$) using:

$$V(t) = F(t) \times B(t)$$

(2.37)

with

$$F(t) = 1 - \lambda [1 - \cos(\omega_{DD} t)]$$

$$B(t) = \exp(-kt^{D/3}).$$

(2.38)

The form factor depends on the dipolar frequency ($\omega_{DD}$) and the inversion efficiency ($\lambda$). The exponent $D/3$ allows for homogeneous distributions of spin systems with fractal dimension $D < 3$, as they may occur in confined environments, such as lipid bilayers. The form factor is encoding not only the distance but also the distribution on distances in the system of interest. Each single
2.3. Pulse EPR Measurements

Figure 2.10: Four pulse DEER experiment. A) Pulse sequence. At the observer frequency ($\nu_{\text{obs}}$) the spins are prepared with a three pulse sequence and the pump $\pi$ pulse is shifted in by $t$ during the experiment. B) The local field induced by the presence of spin B (red) is inverted due to the $\pi$ pump pulse at $\nu_{\text{pump}}$, thus the local field at the position of spin A (blue) is changed (dipole-dipole interaction). C) Echo detected field sweep of a nitroxide radical at X-band frequencies. The excited fraction of spin of the spectrum at the pump (red) and observer frequency (blue) for the DEER experiment are split by approximately 65 MHz. D) DEER data analysis of the illustrated compound. Primary four-pulse DEER data $V(t)$ (black line) and fitted background function $B(t)$ (violet line) (left panel) provide the form factor $F(t) = V(t)/B(t)$ with the inversion efficiency ($\lambda$) (middle panel). Distance distribution $P(r)$ obtained by approximate Pake transformation of $F(t)$ using the software package DEER Analysis (right panel). The shown data are extracted from Jeschke et al. [65].

A distance present in the sample leads to a form factor and can be transferred into a Pake doublet (Pake pattern) by Fourier transformation. The distance distribution can be obtained by fitting the experimental time trace as a linear combination of multiple time domain traces, originating from Pake doublets for different distances. Usually, a smoothing procedure is applied in order to remove erroneous oscillations in the distance distribution that arise since conversion of the form factor to the distance distribution is an ill-posed problem with low stability of the solution. This procedure is implemented into the software package DeerAnalysis [66, 67], and uses the Tikhonov regularization with a L-curve criterion. Like that, the 'optimum' smoothness of the distance distribution can be obtained for the ill-posed problem.

Nuclear modulation artefacts can be strongly suppressed by systematic variation of the inter-pulse delay $\tau_1$. For protonated matrices at X-band frequencies adding traces for eight values of $\tau_1$ with an increment of $\Delta \tau_1 = 8$ ns provides a good DEER trace for proteins [65]. For deuterated matrices, eight values with $\Delta \tau_1 = 56$ ns are appropriate to cover the modulation arising from the nu-
clear frequency of $^2$H. At Q-band frequencies proton modulations are negligible, whereas deuterium modulations can be suppressed with $\Delta \tau_1 = 16$ ns. Pronounced oscillations in the F(t) domain will result in a narrow distribution of distances, whereas a broader distance distribution shows a smooth characteristics of the time-domain data. For spin labelled bio-macromolecules usually a rather broad distance distribution is observed, originating from the flexibility of the linker of the label.
3. Materials and Methods

The careful evaluation of new experimental schemes is of great importance and therefore well known model systems have to be studied to be able to interpret the results in a reliable way. The comparison of different approaches for distance measurements is useful in order to get an insight into the structure of the studied system.

In this thesis three model systems all containing a nitroxide radical spin label and a lanthanide ion, which is kept in place by a chelating agent, were studied. Among these systems a terpyridine base system constitutes a rigid model with a well defined distance. In a small membrane incorporated polypeptide, which can show the relevance of the used methods for biological applications, the flexibility of the linker influences the obtained distance. Furthermore we studied gold nanoparticles with a random distribution of the spin label on the surface. Due to the different structural properties of the model systems an extended comparison of the used experimental approaches can be carried out.

As already pointed out before, the topic of determining distances in the nanometre range is addressed by two different pulse electron paramagnetic resonance (EPR) methods. On the one hand we measure distances by obtaining the dipolar interaction between a nitroxide radical and a gadolinium ion (Gd$^{3+}$) with the double electron-electron resonance (DEER) experiment. Furthermore we gain information on the distance by measuring the enhancement of the nitroxide relaxation due to the presence of the fast relaxing species (lanthanide ion).

This chapter will make the reader familiar with the studied model systems and the sample preparation. In addition the experimental details will be presented for the two experimental methods.

3.1 Gadolinium loaded Terpyridine Derivative

In order to validate the prospect of the DEER experiment performed between a Gd$^{3+}$ ion and a nitroxide spin label, a model system with well defined distance between the two different paramagnetic centres is favourable. Such a system can be provided by a chelate complex of Gd$^{3+}$ ion with a spin labelled ligand (2,2,5-dihexyl-1-[2-(4-hydroxyphenyl) ethynyl]-4-[2-(2,2':6',6''-terpyridin-4'-yl) ethynyl]benzene (terpyridine derivative)), where the distance is known for an analogous compound loaded with a Cu$^{2+}$ ion. In this case, Cu$^{2+}$ - nitroxide
distance measurements (2.38-2.46 nm) were reported to agree with the theoretical prediction by density functional theory (DFT, 2.43 nm) [32]. For the Gd$^{3+}$ loaded terpyridine derivative a slightly longer distance is expected due to the bigger ion radius of Gd$^{3+}$ compared to Cu$^{2+}$. Nevertheless, the rigid model system features a well defined and known distance. The experimental setup and the basic performance of the DEER experiment on Gd$^{3+}$-nitroxide spin pairs were investigated with the terpyridine derivative system.

**Materials** Materials and reagents were of the highest commercially available grade and were used without further purification, unless indicated. The terpyridine derivative (2,2,5-dihexyl-1-[2-(4-hydroxyphenyl) ethynyl]-4-[2-(2,2′,6′,6″-terpyridin-4′-yl) ethynyl]benzene) was synthesized by Narr et al. [32] in the group of Prof. A. Godt. All remaining chemicals were purchased from Sigma-Aldrich.

**Sample Preparation for EPR Studies:** The mixture of terpyridine derivative and GdCl$_3$ was dissolved in deuterated ethanol/dichloromethane (1/1) mixture to obtain a Gd$^{3+}$ concentration of approximately 600 µM. A small excess of nitroxide carrying terpyridine derivatives was added to ensure complex formation for all Gd$^{3+}$ ions. After preparation the sample was filled in an EPR quartz tube of a maximum diameter of 3 mm and shock frozen in order to avoid crystallization of the solvent. The sample tubes were stored at liquid nitrogen temperature.

**Figure 3.1:** Chemical structure of the investigated model compound 2,2,5-dihexyl-1-[2-(4-hydroxyphenyl) ethynyl]-4-[2-(2,2′,6′,6″-terpyridin-4′-yl) ethynyl]benzene (terpyridine derivative) with a distance of approximately 2.43 nm between the metal centre and the nitroxide radical calculated by density functional theory (DFT) for a related system [32].

3.2 WALP23 - Polypeptide

The distance determination approach based on dipole-dipole interaction on orthogonal spin pairs has been be tested on a biological model system for mem-
brane spanning domains in proteins [68–70]. The WALP23 peptides are synthetic polypeptides consisting of a repetitive sequence of alanine and leucine in the core. The core sequence is flanked by two tryptophans at each side to anchor to the lipid bilayer [71, 72]. These polypeptides form well defined α - helices [69, 73] and, due to the hydrophobic core sequence, they solubilize in lipid bilayers [69] and show no extensive aggregation in the bilayer[74]. It was shown, that WALP23 matches the width of a 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) bilayer [74, 75] and that the α-helical structure is maintained upon labelling with a nitroxide radical (S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate (MTSSL)) at different positions in the peptide sequence.

The model system is modified with a 1,4,7,10-tetraazacyclododecane N,N',N",N"'-tetraacetic acid (DOTA) - lysine derivative at the N-terminus in order to form a chelate complex with lanthanide ions at a defined position of the WALP23 sequence. Four different WALP23 constructs with the nitroxide spin label located at positions 7, 11, 15 or 19 and each having the DOTA complex at the N-terminus (see 3.2) were investigated.

![Figure 3.2: Ideal α-helical model structure of WALP23 with DOTA lysine linker at the N-terminus and MTSSL labels at the positions 07, 11, 15 or 19. The distance between the Ln³⁺ DOTA complex and the spin labels is indicated by a black dotted line. The helical pitch of 0.54 nm is highlighted in black.](image)

All constructs were studied in a DOPC bilayer. The distance between the loaded lanthanide ion and the nitroxide radical was determined by DEER in case the DOTA-lysine derivative is loaded with Gd³⁺. The obtained distance for the four WALP23 constructs was used as a reference for the distances determined by relaxation enhancement. Furthermore the predicted frequency dependence [39] by measuring the temperature dependence of the relaxation enhancement at S, X and Q band was examined.

Materials Materials and reagents were of the highest commercially available grade and were used without further purification, unless indicated. The DOTA-
lysine derivative [1,4,7,10-Tetra-azacyclododecane-1,4,7-tris(t-butyl acetate)-10-(N-α-Fmoc-N-ε-acetamido-L-lysine), Cat. No. B-275] was purchased from Macrocyclics Inc. (Dallas, USA). MTSSL was obtained from Toronto Research Chemicals Inc. (Toronto, CDN). DOPC solution (20 mg/mL chloroform) was provided by Avanti Polar Lipids Inc. (USA). All remaining chemicals were purchased from Sigma-Aldrich. TPX Capillaries with 0.6 mm inner diameter and a sample volume of 15 µL (Cat. No. TPX-2) were obtained from Molecular Specialties, Inc. (Milwaukee, USA).

**Peptides and Spin Labelling:** The four WALP23 constructs were synthesized by Pepceutical Limited (Nottingham, UK) by solid-phase synthesis (sequences are given in 3.1) with the DOTA-lysine derivative at the N-terminus. For spin labelling the peptide was dissolved in trifluoroethanol (TFE) (3 mg/mL) by stirring at room temperature for approximately 1-2 h, 6 µL triethylamine as well as 100 µL of a 20 mM MTSSL solution in TFE (2 equiv) were added. The mixture was stirred for 2 h at room temperature in the dark. The solution of the spin-labelled peptide was separated into portions of approximately 330 µL and mixed with 50 µL of 10 mM LnCl₃ solution in methanol. We used lanthanide chloride/methanol solutions of LaCl₃, GdCl₃ or DyCl₃ to form the DOTA - Ln³⁺ complex. The solution was stored at room temperature for 1-2 h.

The doubly spin labelled WALP23 was precipitated in 10 mL of -20 °C cold 1:1 mixture of methyl tert-butyl ether/n-hexane to remove unbound MTSSL and lanthanide ions. The solution was allowed to precipitate at -20 °C for 1 h. The precipitate was collected by centrifugation at 4 °C (20 min, 6000 rpm), after decantation of supernatant the precipitate was washed twice with 10 mL of -20 °C cold 1:1 mixture of methyl tert-butyl ether/n-hexane including a storage of the solution for 30 min at -20 °C and centrifugation at 4 °C (20 min, 6000 rpm). The washed precipitate was dissolved in 1 mL of TFE and stored as stock solution at -80 °C.

**Table 3.1:** Sequence of WALP23 constructs for labelling position 07, 11, 15 and 19.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Peptide name</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WLP23_A07R1</td>
<td>DOTA-WALP23-A07C</td>
<td>KWWLALCLALALALALALALALWWA</td>
</tr>
<tr>
<td>WLP23_A11R1</td>
<td>DOTA-WALP23-A11C</td>
<td>KWWLALCLALALALALALALALWA</td>
</tr>
<tr>
<td>WLP23_A15R1</td>
<td>DOTA-WALP23-A15C</td>
<td>KWWLALCLALALALALALALALWWA</td>
</tr>
<tr>
<td>WLP23_A19R1</td>
<td>DOTA-WALP23-A19C</td>
<td>KWWLALCLALALALALALCLA LW</td>
</tr>
</tbody>
</table>

**Sample Preparation for EPR Studies:** EPR samples were prepared by mixing TFE stock solution of the peptide and DOPC solution (20 mg/mL chloroform) under N₂ gas flow to give a peptide-to-phospholipid molar ratio of

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³MTSSL is commonly abbreviated by R1
1/800. The solvent was evaporated under N$_2$ gas flow and the residue was dried under vacuum in the dark for 1 h. The remaining aliquot was rehydrated with 2 mL of 20 mM Tris-HCl buffer (pH = 7.5). Oxygen was removed from the buffer solution by a constant N$_2$ flow for at least 30 min. The suspension was mixed with a vortexer for 1 min and went through three freeze-thaw cycles to form vesicles. Unilamellar vesicles were prepared by extruding the suspension ten times through a filter with a pore size of 400 nm.

During the vesicle preparation it is important to exclude the contact of oxygen (O$_2$) (from the air) with the lipids as good as possible because of potential oxidation of the lipids. Despite a very careful and fast workflow one can not avoid the diffusion of some oxygen into the lipid bilayer. The samples obtained directly after extrusion therefore are called 'O$_2$ containing' samples. To remove residual oxygen from the lipid bilayer N$_2$ gas was flushed for 20 min over the sample and after filling the EPR sample tube (2.9 mm and 5 mm outer diameter) the so called 'oxygen free' sample was shock frozen in liquid N$_2$. The sample tubes were stored at liquid nitrogen temperature. Continuous wave (CW) EPR measurements were performed in TPX capillaries, where a constant nitrogen (N$_2$) flow ensured the elimination of oxygen from the sample. Samples were directly taken from the 'oxygen free' preparation.

**Insertion into Lipid Bilayer:** The $\alpha$-helical structure of WALP23 is maintained upon labelling with MTSSL and the mobility of the nitroxide label is influenced by its environment and should decrease towards the polar head groups of the DOPC bilayer [75]. The CW EPR spectra of all spin labelled constructs incorporated into DOPC and the DOTA complex being loaded with La$^{3+}$ are shown in figure 3.3A. Generally, the spectral shape follows the trend observed by Nielsen et al. [75] and position A11R1 being close to the middle of the bilayer therefore shows the most mobile spectra. By comparing spectra obtained from the La$^{3+}$ loaded WALP23_A07R1 in TFE with the same incorporated into DOPC it can be seen that the spectra of the lipid inserted peptides are broader and thus less mobile. This implies the insertion of the polypeptides into the lipid bilayer, as well as an efficient labelling for all four nitroxide labelled constructs. The same behaviour has been observed with different types of lanthanide ions, like Dy$^{3+}$ or Gd$^{3+}$ loaded WALP23 samples, which reveals no influence of the type of loaded lanthanide ion on the structure of WALP23 peptides.
3.3 Gold Nanoparticles

Validation of the distance determination approaches investigated in this thesis is extended to nanostructured materials. By analysing gold nanoparticles (Au-NPs) the applicability of the methods (1) four-pulse double electron-electron resonance (DEER) between two nitroxide radicals as well as between nitroxide and gadolinium ions and (2) $T_1$ and $T_2$ relaxation enhancement, where the enhancement of the relaxation of a nitroxide radical is induced by dysprosium ion ($\text{Dy}^{3+}$) or gadolinium ion ($\text{Gd}^{3+}$) can be enlarged and the performance can be tested.

EPR spectroscopy in general and spin-labelling methods in particular can be used to study the organization of ligands and chemical reactions at the nanoparticle surface [76–79]. It is possible to introduce different functional ligands at the surface of the gold nanoparticles as illustrated in figure 3.4 and Au-NPs can be treated as functional nanostructures. Different functional ligands can be attached to the surface of Au-NPs via gold-sulfur interaction. In this manner the nitroxide spin label and the chelating agents for binding lanthanide ions were attached to the surface of Au-NPs.
3.3. Gold Nanoparticles

Figure 3.4: Functionalization of gold nanoparticles (Au-NPs). Different functional ligands can be attached to Au-NPs via gold-sulfur interaction. (figure adapted from Victor Chechik)

Functionalized gold nanoparticles were synthesized in the lab of Victor Chechik at the University of York (UK) by Muhammad Farooq Warsi. The prepared Au-NPs are protected by a monolayer of chelator ligands. Monolayer, as opposed to multilayer, protection gives rise to nanoparticles which are structurally well defined. Additionally, the coverage by a monolayer is better suited for preparing multifunctional nanoparticles by co-adsorption of different ligands on the surface of the same nanoparticle. The used chelator ligand is based on a diethylenetriaminepentaacetic acid (DTPA) ligand commonly employed as Gd$^{3+}$ chelators for MRI [80]. Bifunctional Au-NPs were obtained by extra ligands carrying a nitroxide radical. The chelator ligands were loaded with different amounts of La$^{3+}$, Gd$^{3+}$ or Dy$^{3+}$.

The chemical structure of the system is shown in figure 3.5A. The studied Au-NPs have an average diameter of $d = 1.75 \pm 0.4$ nm (TEM) and the length of the nitroxide and DTPA linker differs by approximately 0.6 nm. Both paramagnetic labels are statistically distributed over the sphere (figure 3.5B). The distribution of distances between the labels can be described by a 'particle on two spheres’ model. The distance distribution for such a model is shown in figure 3.5B in black. In the first distance range between 0 nm and 0.6 nm the probability is zero, due to the difference in length of the label linkers. By simplifying the system towards a 'particle on a sphere’ model (green) the mean distances for both distances distributions will coincide at 2.9 nm, and the longest distance present in the distance distribution is as long as 4.3 nm and can also be called cut-off distances.

The empirical formula for Au-NPs is approximately $\text{Au}_{166}\text{Ligand}_{49}$. According to this formula, a 100% load of lanthanide ion (Ln$^{3+}$) leads to the new formula $\text{Au}_{166}\text{Ligand}_{49}\text{Ln}_{49}$. The statistic distribution of the spin labels leads to an average label content per particle, where e.g. a loading with 2% of Ln$^{3+}$ approximately corresponds to an average of one Ln$^{3+}$ per Au-NPs. The preparation conditions were optimised to obtain approximately 0.3 active nitroxide radicals per particle. This allowed to neglect multi-spin effects for Gd$^{3+}$-nitroxide DEER measurements. On such a system the performance of DEER on nitroxide-nitroxide and Gd$^{3+}$-nitroxide spin pairs with similar concentration and distance
distributions can be studied. By comparison of this system to the WALP23 polypeptide and the terpyridine derivative the influence of different chelating agents on the experimental setup can be discussed. Furthermore, the applicability of the relaxation enhancement based distance determination approach has been explored.

Figure 3.5: Gold nanoparticles (Au-NPs). A) Au-NPs are labelled with DTPA ligands and nitroxide spin probes. DTPA forms a complex with $\text{Ln}^{3+}$ ions and its linker is 0.6 nm longer than the one of the nitroxide spin probes. B) Particle on a sphere model scheme for Au-NPs (yellow) with different length of the label linkers (nitroxide-blue, $\text{Ln}^{3+}$-red) and an average sphere diameter of $2 \cdot R_S$. C) The distance distribution for a homogeneous distribution of spin labels for a 'particle on two spheres' model (2sph-black) or for a 'particle on one spheres' model (1sph-green) reveals the same mean distance (red). Therefore the experimental data were analysed according to the simplified 'particle on one spheres' model.

Sample Preparation: The samples were prepared by Muhammad Farooq Warsi [80] and a detailed description of the preparation can be found in [81].

Sample Preparation for EPR Studies: The dry and powder-like $\text{Ln}^{3+}$-loaded gold nanoparticles were dissolved under vortexing in water/glycerol mixture (60/40) to obtain a concentration of approximately 100 $\mu$M. After preparation the sample was filled into an EPR quartz tube of a maximum diameter of 3 mm and shock frozen in order to avoid crystallization of the solvent. The sample tubes were stored at liquid nitrogen temperature.

3.4 Experimental Details

The experimental work mainly focuses on pulse EPR techniques (DEER, measurement of $T_1$ and $T_2$). Nevertheless, also CW EPR measurements have been performed. The experimental details are given in the following.
3.4. Experimental Details

3.4.1 Continuous Wave EPR

All continuous wave (CW) experiments were performed at X-band frequencies (9.3 - 9.4GHz) with a Bruker ElexSys E500 spectrometer equipped with a Bruker super high Q resonator ER4122SHQ. The samples were loaded into a TPX capillary and recorded with 100kHz field modulation, 24dB of 200mW microwave power, 0.10mT modulation amplitude, 10.24ms time constant and 40.96ms conversion time, unless indicated.

3.4.2 DEER

The DEER experiments were carried out at X-band (9.5GHz) and Q-band (34GHz). A Bruker ElexSys II 580 combined X/Q EPR spectrometer was used for the DEER measurements. Additionally the experiments at Q-band with short (12ns) pulses were performed on the home-built spectrometer described elsewhere [82] with a rectangular resonator specially developed for oversized samples [83]. This allowed to use the same sample tube of 2.9mm outer diameter for commercial X- and home built Q-band spectrometers.

Table 3.2: Optimized pulse settings for the DEER experiment at X- and Q-band frequencies for Gd$^{3+}$ - nitroxide and nitroxide - nitroxide spin pairs.

<table>
<thead>
<tr>
<th></th>
<th>NO-NO DEER</th>
<th>Gd$^{3+}$-NO DEER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X-band</td>
<td>Q-band$^a$</td>
</tr>
<tr>
<td>$\Delta \nu$ [MHz]$^b$</td>
<td>+65</td>
<td>-100</td>
</tr>
<tr>
<td>$\pi$ (pump) [ns]</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>$\pi/2$ (obs.) [ns]</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>$\pi$ (obs.) [ns]</td>
<td>32</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$ for the home-built Q-band spectrometer

$^b$ $\Delta \nu = \nu_{\text{obs}} - \nu_{\text{pump}}$

The pulse settings for the DEER experiment at X-band and home-build Q-band spectrometer are given in table 3.2 to perform distance measurements on Gd$^{3+}$ - nitroxide and nitroxide - nitroxide spin pair as a comparison. At the commercial Q-band spectrometer the following pulse shortest lengths could be achieved: $\pi_{\text{pump}} = 52$ns, $\pi_{\text{obs}}/2 = 64$ns and $\pi_{\text{obs}} = 2 \times \pi_{\text{obs}}/2 = 128$ns.

3.4.3 Relaxation Measurements

Before performing relaxation measurements the echo-detected field sweep EPR spectra were measured and the relaxation properties were determined at the
maximum of the echo-detected field sweep spectrum, unless indicated differently. The echo-detected field sweep EPR measurements are performed by applying a Hahn echo sequence and changing the external magnetic field.

Pulse EPR relaxation experiments were performed in a temperature range from 20K to 100K in 10K steps with a continuous flow He cryostat (ESR900, Oxford Instruments) equipped with an Oxford Instruments temperature controller ITC 503S to stabilize the temperature. At X-band frequencies (9.3 - 9.4GHz) measurements were carried out at a Bruker ElexSys 580 spectrometer equipped with a Bruker split-ring or dielectric resonator (ER 4118X - MS3 or ER 4118 MD5).

Q-band experiments (34-35GHz) were performed at a home-built Q-band spectrometer [82] with a rectangular cavity which allows for over sized samples [83]. S-band measurements (4 GHz) were recorded at a home-built S-band spectrometer equipped with a loop-gap resonator [84] hosting samples with an outer diameter of 5 mm.

All pulsed EPR relaxation measurement series were performed as follows:

**Inversion Recovery** ($T_1$ measurement): The inter-pulse delay for the detection was fixed usually at $\tau = 200$ ns and the starting value for the first variable delay $T_0$ was set to $2 \mu$s. An inversion pulse length of 32 ns and observer pulses lengths of 52 ns and 104 ns for the $\pi/2$ and $\pi$ pulse, respectively, were used in the experimental scheme as described before [39]. The use of these pulses with different excitation bandwidths for inversion and detection ensures a sufficient suppression of spectral diffusion effects.

**Two Pulse ESEEM** ($T_2$ measurement): The interpulse delay $\tau$ in the 16 ns $\pi/2 - \tau - 32$ ns $\pi$ sequence starts at 344 ns and is increased by steps of $\Delta t$ of 4 ns or 8 ns.

**Data Fitting:** The fitting algorithm follows the Nelder-Mead downhill simplex method implemented in MatLab (MathWorks, Natick, USA) using a root mean square deviation (rmsd) criterion to judge the quality of the fit. A Monte Carlo method was applied in order to sample the fitting space by 100 trials with random starting values.
4. Double Electron-Electron Resonance

The development of alternative approaches for distance determination in the nanometre range, based on an orthogonal spin pair consisting of a nitroxide radical and a chelate complex of a gadolinium ion (Gd$^{3+}$), is explored in the following chapter. The method of choice is the double electron-electron resonance (DEER) experiment, which is already routinely applied on nitroxide-nitroxide spin pairs. The performance of the DEER experiment on Gd$^{3+}$-nitroxide spin pairs is investigated and the applicability of the methods for different types of model systems, from membrane incorporated macromolecules to system relevant in material science is shown. This work builds a bridge between the two types of spin labels and thus opens up possibilities for more diverse complex experimental schemes, where the distance determination between a pair on nitroxide radicals, a pair of Gd$^{3+}$ ions or a combined spin pair of Gd$^{3+}$ and nitroxide can be performed on the very same sample.$^1$

4.1 Selective Detection

The EPR spectra of a gadolinium ion (Gd$^{3+}$) and a nitroxide spin labels are overlapping, but due to the properties of both species the EPR spectra can be detected almost independently of each other. The $|−1/2⟩ \leftrightarrow |1/2⟩$ transition of Gd$^{3+}$ centers ($S = 7/2$) has a transition moment that is four times bigger than the one of nitroxide radicals ($S = 1/2$). The transition moment ($P_{nm}$) for a paramagnetic species is given by

$$P_{nm} = \sqrt{S(S+1) - m_S(m_S-1)}$$

with the spin quantum number $S$ and the magnetic quantum number $m_S$ for a specific state.

In case of nitroxide radicals the transition moment calculates to

$$P_{nm}(\text{NO}) = \sqrt{\frac{1}{2} \cdot \frac{3}{2} + \frac{1}{2} \cdot \frac{1}{2}} = \sqrt{\frac{3}{4} + \frac{1}{4}} = 1$$

with $S = 1/2$ and $m_S = 1/2$.

In case of Gd$^{3+}$ ions the transition moment for the central transition ($|−1/2⟩ \leftrightarrow$ $|1/2⟩$) gives

$^1$Data shown in section 4.2 are already published [38].
|1/2\rangle with \( m_S = 1/2 \) and a magnetic quantum number \( S = 7/2 \) calculates to

\[
P_{nm}(\text{Gd}^{3+}) = \sqrt{\frac{7}{2} \cdot \frac{9}{2} + \frac{1}{2} \cdot \frac{1}{2}} = \sqrt{\frac{63}{4} + \frac{1}{4}} = 4. \tag{4.3}
\]

Hence, the excitation of the central transition of the \text{Gd}^{3+} spectrum requires sixteen times less power than the excitation of a transition of the nitroxide radical. The \text{Gd}^{3+} species are relaxing much faster than nitroxide radicals and the about three orders of magnitude ratio of the longitudinal relaxation times of the two paramagnetic species, in combination with the difference in the transition moment, provides the possibility to detect \text{Gd}^{3+} centres and nitroxide radicals almost independently from each other. This allows for a selective excitation of the different paramagnetic species by choosing the microwave power and the shot repetition time (srt) accordingly.

**Figure 4.1:** Selective measurement of the echo-detected (ED) field-sweep EPR spectra for the \text{Gd}^{3+} loaded terpyridine derivative at **A)** X-band and **B)** Q-band frequencies. ED EPR spectra are obtained at 10K with microwave (m.w.) pulses and repetition times optimized for the detection of the nitroxide radicals (shown in black; srt = 16000\( \mu \)s) or \text{Gd}^{3+} centres (shown in red; srt = 337\( \mu \)s). Broader range ED EPR spectra are shown in the insets. The arrows mark the position of the pump and observer frequencies for the DEER experiment. The most promising combinations are the settings I and V. The difference between Va and Vb is the excitation bandwidth.

Due to this behaviour the overlap of the nitroxide EPR spectrum with the central peak of the \text{Gd}^{3+} spectrum at X band (figure 4.1) is of no significant consequence for the DEER experiment. At Q-band frequencies the nitroxide spectrum does not overlap with the centre of the \text{Gd}^{3+} spectrum due to the increased resolution. The broad spectrum of \text{Gd}^{3+} is related to the zero-field splitting (ZFS) present for the high electron spin.
4.2 Optimization of Measurement Conditions

A significant modulation depth (M.D.) in the DEER experiment can only be achieved if a substantial fraction of spins is flipped by the pump pulse to induce a change of the local magnetic field for a significant fraction of those spins which participate in the formation of detected spin echo. The Gd$^{3+}$ spectrum is rather broad and even the hardest pulses available (8 ns) are just able to excite a minor fraction of the Gd$^{3+}$ species. In contrast, about 40%-60% of all nitroxide spins can be inverted with current spectrometers. Additionally, the fast relaxation of Gd$^{3+}$, even at very low temperatures (10 K), enables for a fast repetition of the experiment with a favourable Boltzmann population difference. Whereas with nitroxide observer spins the experiment would have to be performed at higher temperature or with lower repetition rate. Therefore, the most efficient experimental scheme turns out to be the one with detection on Gd$^{3+}$ and with pump pulse set on the maximum of the nitroxide radical spectrum. The performance of this method was studied at X- (9.5 GHz) and Q-band (34 GHz) frequencies with the frequency shifts depicted in the figure 4.1. All DEER measurements were performed at 10 K. The Gd$^{3+}$ loaded terpyridine derivative was studied for all pulse setups I – V (comparison figure 4.1 and table 4.1) to investigate the basic performance of the DEER experiment on Gd$^{3+}$-nitroxide spin pairs. The other model systems were studied under the most favourable experimental condition I at X-band and Vb at Q-band frequencies.

Table 4.1: Pulse settings for the DEER experiment at X- and Q-band frequencies for Gd$^{3+}$-nitroxide spin pairs including the corresponding modulation depth for the Gd$^{3+}$ loaded terpyridine derivative.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta \nu$</th>
<th>$\pi$ (pump)</th>
<th>$\pi/2$ (obs.)</th>
<th>$\pi$ (obs.)</th>
<th>M.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-band (I)</td>
<td>-80 MHz</td>
<td>12 ns</td>
<td>12 ns</td>
<td>12 ns</td>
<td>0.38</td>
</tr>
<tr>
<td>Q-band (II)</td>
<td>+212 MHz</td>
<td>52 ns</td>
<td>80 ns</td>
<td>160 ns</td>
<td>0.02</td>
</tr>
<tr>
<td>Q-band (III)</td>
<td>-212 MHz</td>
<td>52 ns</td>
<td>60 ns</td>
<td>120 ns</td>
<td>0.04</td>
</tr>
<tr>
<td>Q-band (IV)</td>
<td>-212 MHz</td>
<td>52 ns</td>
<td>60 ns</td>
<td>120 ns</td>
<td>0.09</td>
</tr>
<tr>
<td>Q-band (Va)</td>
<td>-300 MHz</td>
<td>52 ns</td>
<td>64 ns</td>
<td>128 ns</td>
<td>0.06</td>
</tr>
<tr>
<td>Q-band (Vb)</td>
<td>-300 MHz</td>
<td>12 ns</td>
<td>12 ns</td>
<td>12 ns</td>
<td>0.2</td>
</tr>
</tbody>
</table>

$^a \Delta \nu = \nu_{\text{obs}} - \nu_{\text{pump}}$

The DEER experiment on the Gd$^{3+}$ loaded terpyridine derivative was performed at X-band and Q-band frequencies. The pulse settings are listed in table 4.1, where pulse settings II – Va correspond to the optimum conditions at a commercial Bruker X/Q EPR spectrometer (see chapter 3) and setting Vb can be obtained on a home-built Q-band spectrometer. The detected modulation depth shows a dependence on the excitation bandwidth of applied pulses, on the detection position and on the mode profile of the probe-head. Baseline corrected and normalized time domain DEER traces are depicted in
Figure 4.2 A. All time-domain traces reveal pronounced oscillations which indicate the presence of a very well defined distance as expected for the investigated model system. At X-band frequencies modulation depth (M.D.) is largest followed by the Q-band experiment performed with the same pulse length but different frequency offset between pump and detection position to ensure the excitation at the maximum of both species. The difference in modulation depth is related to the fact that the home-built cavity is not yet 100% optimized and M.D. was depending on the resonator mode. The improvement of the hardware is not part of this thesis and was done by several other people in the group. Later (refined cavity), it could be shown that modulation depth with the settings I and Vb are virtually the same, as expected for the experiment being performed with a pump pulse at the peak of the nitroxide spectrum because in nitroxide-nitroxide spin pairs M.D. is comparable at X- and Q-band frequencies. This dependence of M.D. is related to the resonator profile and its shape near pump and detection frequency. The actual excitation bandwidth of m.w. pulses is a superposition of pulse shape and mode profile. A reduced excitation bandwidth results in a lower modulation depth and sensitivity.

Figure 4.2: Experimental DEER data for the Gd$^{3+}$ loaded terpyridine derivative with different pulses settings I-V (details see table 4.1). A) Background corrected data $F(t)/F(0)$ B) Fourier transformation of the time domain data $F(t)/F(0)$ results in the dipolar spectra. C) Distance distribution $P(r)$ obtained by converting $F(t)/F(0)$ with DeerAnalysis 2009 (red). The artefacts arising from admixing $^2$H nuclear modulation marked with (*).

The modulation depth of the DEER experiment is reduced by the usage of longer pulses with a narrower excitation bandwidth, which accounts for a smaller fraction of spins being flipped by the pump pulse. Therefore, the trace Va shows a smaller M.D. then the trace Vb. The change of the detection position on the
Gd$^{3+}$ spectrum influences the sensitivity of the experiment because of the different echo intensity at these positions ($II, IV - Va$). The optimum sensitivity can be achieved with a detection at the maximum of the Gd$^{3+}$ spectrum. The modulation depth, defining the signal, depends on the pump frequency. The comparison of trace $III$ and $Va$ clearly depicts that and suggests the preferable pump pulse position at the peak of nitroxide radical.

At X band the performance of Gd$^{3+}$-nitroxide DEER in terms of signal-to-noise ratio ($S/N$), modulation depth and measurement time is roughly comparable to nitroxide-nitroxide DEER measurements. The advantages of lower temperature and faster repetition are compensated by the smaller fraction of observed spin pairs, which results from the larger width of the Gd$^{3+}$ EPR spectrum compared to the nitroxide. With the home-built Q-band spectrometer the lengths of pump and detection pulses could be set to approximately the same values as at the commercial X-band spectrometer. In this configuration $S/N$ increases by approximately a factor of 34 at Q band as compared to X band, which implies a speed-up of the measurement by a square of this value, i.e. by approximately a factor of 1156. It is known that sensitivity of EPR measurements increases at Q band because of the larger equilibrium polarization of electron spins and because of the better sensitivity of the detection at higher frequency. In this case the impressive increase is further aided by the narrowing of the central peak of the Gd$^{3+}$ spectrum at higher frequencies [34–37] as well as by the use of the same sample volume as at X band [83]. Another important contribution to the significant sensitivity increase arises from deuterium ($^{2}H$) present in the sample. $^{2}H$ induces strong nuclear modulations of the echo intensity in a two pulse ESEEM experiment at X-band frequencies and only a weak influence on the echo amplitude at Q band. The phase-cycling applied in DEER includes a nuclear modulation averaging over one period of the nuclear frequency dominant in the sample. In the present case at X band the echo intensity for some phase-cycling steps is close to zero and thus only a minor signal is added. This leads to a decrease of $S/N$ at X-band frequencies and does not occur significantly at Q-band frequencies.

Data analysis was performed with the DeerAnalysis 2009 package [66], which is originally written for the detection on $S = 1/2$ species. This is permissible since in the high-field limit the frequency change for each single-quantum transition of the $S = 7/2$ species upon the inversion of the nitroxide spin is the same as for the $S = 1/2$ species. The high-field approximation is not generally applicable to the Gd$^{3+}$ EPR spectrum, but it is feasible for those species that have resonance frequencies in the vicinity of the central peak.

The Fourier transformation (FT) of the time-domain data is shown in figure 4.2B. The dipolar spectrum (Pake pattern) for the experiments done with soft pulses ($II - Va$) reveals a not complete Pake pattern where the high frequency shoulder cannot be excited due to the limited bandwidth of the pulses. The outer shoulders of overlapping Pake patterns are suppressed. The dipolar spectra for the experimental setups with hard pulses (I, Vb) show that the complete Pake pattern can be excited.

Distance distributions $P(r)$ were obtained with DeerAnalysis 2009. The best fit
was obtained with a Tikhonov regularization parameter of 1 for X-band data and 0.01 for Q-band data. The difference in optimum regularization parameter is mainly due to the different noise level of the data. All distance distributions feature a main peak with a mean distance of 2.54 nm, which agrees with the Cu-nitroxide distance of approximately 2.43 nm reported for a related system [32]. Compared to the copper(II) complex with two terpyridine ligands [32], the distance distribution is narrower, as is also apparent from the observation of many more oscillations in the DEER trace. A small increase of the measured distance is most probably related to the bigger ion radius of Gd$^{3+}$ as compared to Cu$^{2+}$. Furthermore, probably more spin density is transferred to the directly coordinated nitrogen atoms in the copper case, and these are somewhat closer to the nitroxide.

Beside the main peak the distance distributions at both X and Q band also show some satellite features. The small peak that appears at around 2.8 nm at X band and shifts to approximately 1.8 nm at Q band can be unambiguously assigned to the incomplete suppression of the deuterium ESEEM oscillations ($\gamma[^2H] = 4.1 \cdot 10^7 \text{rad/T s}$). At X band an 8-step cycle of $\tau_1$ averages with increment $\Delta\tau_1 = 56$ ns was used in order to average out the deuterium ESEEM oscillations [85]. At Q band no such averaging was employed but the oscillations were substantially suppressed due to much bigger split between the detection and the pump frequencies and lower nuclear modulation depth.

Additionally, two more satellite peaks appear in the distance distribution roughly symmetrically around the main peak. Currently we can only speculate on the origin of these features, and attribute them to violation of the high-field approximation and for part of molecules to effects arising from excitation of transitions other than the $|{-1/2}\rangle \leftrightarrow |{1/2}\rangle$ transition of Gd$^{3+}$. Violation of the high-field approximation for only a fraction of molecules may occur due to a broad distribution of zero-field splitting parameters, typical for chelate complexes of Gd$^{3+}$ in frozen solutions [37]. The presence of a fraction of different types of Gd-terpyridine complexes, e.g. with more than one terpyridine ligand, cannot be excluded either. The relative weight of these satellites is apparently smaller at Q band than at X band, which would favour the explanation based on the violation of the high-field approximation. Nevertheless the relative amplitude of satellite signals remained unchanged at different interpulse delays in the DEER sequence, which implies similar relaxation behavior for both main peak and satellite signals. If the excitation of different transitions of Gd$^{3+}$ was the reason of these satellite features, then a different relaxation behaviour would be expected.

The feature at 2 nm as well as the deuterium artefact are not appearing in the distance distribution obtained from the Q-band DEER measurements on the commercial Bruker spectrometer (II-Va). This establishes a lower limit of detectable distances with these pulse settings to somewhere between 2 and 2.5 nm. This lower limit results from suppression of modulation when the excitation bandwidth of pump or observer pulses is smaller than the dipole-dipole coupling [86, 87].

The measurement conditions can be optimized by measuring relaxation times of
4.2. Optimization of Measurement Conditions

the sample at different temperatures (figure 4.3) and analyzing them together with the echo amplitude, which follows Curie law. The signal-to-noise ratio depends on the length of the DEER experiment and can be computed as [8]:

$$ S/N(T) \propto \frac{1}{T} \cdot \exp \left( -\frac{2t_{\text{max}}}{T_2(T)} \right) \cdot \sqrt{\frac{1}{T_1(T)}} \quad (4.4) $$

An additional correction is required if this formula is applied to the Gd$^{3+}$ species. The transverse relaxation of Gd$^{3+}$ was measured to be non-mono-exponential. It is known that a broad distribution of zero-field splitting (ZFS) parameters between different Gd$^{3+}$-complexes is present in shock-frozen solutions [37]. This is expected to result in a distribution of relaxation times. The species with the slowest relaxation times are the ones with the smallest ZFS (normally the most symmetric arrangement of ligands). Apparently, these slowly relaxing species are most relevant in the DEER experiment and the above presented expression should be corrected for this. The $T_2$ relaxation curves for Gd$^{3+}$ were fitted by bi-exponential decay functions and used the relative amplitude and decay time of the slower component of the fit to estimate the sensitivity of Gd$^{3+}$-nitroxide DEER in the temperature range between 5K and 40K. Figure 4.3 shows that an additional improvement in S/N is expected if the measurements would be performed at 5K instead of 10K the temperature that was used in the present work. The corresponding $T_2$ times of Gd$^{3+}$ range between 2 and 3 $\mu$s at the optimum temperatures.

**Figure 4.3:** Optimization of measurement temperature for DEER experiment in Gd$^{3+}$-nitroxide spin pairs. A) $T_1$ (blue) and $T_2$ (red) values for Gd$^{3+}$ centres vs. measurement temperature, for $T_2$ the slow component of the bi-exponential fit is shown. B) Temperature dependence of the expected signal-to-noise ratio in Gd$^{3+}$-nitroxide DEER as calculated according to [8] with additional correction for the partial amplitude of the slow $T_2$ component of Gd$^{3+}$ for an experimental trace with $t_{\text{max}} = 4 \mu$s.
4.3 Spectral Features

The performance of the DEER experiment on Gd$^{3+}$-nitroxide spin pairs does not only depend on measurement conditions like point of the pump and observer frequencies or temperature. An essential factor is also the nature of the Gd$^{3+}$ complex used for the experiment. Gd$^{3+}$ is a high spin system with $S = 7/2$, and its EPR spectrum strongly depends on the parameters of the zero-field splitting (ZFS).

In figure 4.4 the echo detected field sweep of Gd$^{3+}$ centres in all three model systems is shown at X-band and Q-band frequencies. The central transition $|{-1/2}⟩ \leftrightarrow |{1/2}⟩$ narrows at higher fields, according to $\sigma \propto \frac{D^2}{g \mu_B B}$ and the other transitions in the spin system have the same width [37]. This is related to the fact that the system is closer to the high-field approximation and so the central transition becomes more pronounced. From the spectral shape a decrease of the ZFS when going from gold nanoparticles (Au-NPs) (ligand: DTPA) via the WALP23 samples (ligand: DOTA) to the Gd$^{3+}$-terpyridine complex (ligand: terpyridine derivative) can be anticipated. A small ZFS also leads to narrowing of the Gd$^{3+}$ spectra and therefore enhances the sensitivity of the DEER experiment due to excitation of a larger fraction of the Gd$^{3+}$.

Analysis of the DEER data shows that the change of the external magnetic field (X- and Q-band) does not influence the obtained distance distribution (see chapter 4.2 and 4.4).

![Figure 4.4: Echo-detected field sweep spectra of Gd$^{3+}$ obtained at 10 K at A) X-band and B) Q-band for Au-NPs (green), WALP23 (blue) and the Gd$^{3+}$-terpyridine complex (red).](image)

Another interesting point in discussing the performance of the DEER experiment on Gd$^{3+}$-nitroxide spin pairs is the dependence of the echo-detected field sweep spectra of Gd$^{3+}$ on the inter-pulse delay ($\tau$). This is of crucial interest because the detection in the DEER experiment is done after a time $\tau_2$, which is of the order of $T_2$ (see figure 2.10) and only those species that still contribute
4.3. Spectral Features

to the echo at such long $\tau$ can be detected. The time delay $\tau_2$ in the DEER experiment usually reaches $1 - 3 \mu s$. Therefore the dependence of the spectral shape of Gd$^{3+}$ as a function of the inter-pulse delay in this range is shown in figure 4.5.

![Figure 4.5: Echo detected field sweep spectra of Gd$^{3+}$ at 10 K at A) X-band B) and Q-band frequencies for the Gd$^{3+}$ loaded terpyridine derivative (left panel), DTPA (middle panel) and DOTA (right panel) as a function of the fixed inter-pulse delay $\tau$ between $\pi/2$- and $\pi$- pulse.](image)

The Gd$^{3+}$ loaded terpyridine derivative having the smallest ZFS parameters shows the biggest effect upon increase of $\tau$. The width of the Gd$^{3+}$ spectrum decreases dramatically with increased inter-pulse delay. The tendency is preserved at higher fields and the central $|-1/2\rangle \leftrightarrow |1/2\rangle$ transition gets more pronounced, which implies an expected better performance of the DERR experiment in terms of S/N. In case of Au-NPs and WALP23 only a minor influence on the spectral shape is observed due to the increase of the inter-pulse delay. In contrast, for Gd$^{3+}$ loaded DTPA (Au-NPs) the spectra are basically superimposable and for DOTA (WALP23 polypeptides) even a slight increase of the spectral width is observed with an increase of the inter-pulse delay. The narrowing of the Gd$^{3+}$ spectrum might be related to the fact that different transitions in the high spin system show different relaxation properties or that species with bigger ZFS parameters relax faster and can not be detected any more at long $\tau$. The central transition exhibits longer relaxation and therefore is more pronounced with increased delay between excitation and detection. Furthermore, the possibility of interference at the time of detection is decreasing because of a more homogeneous ensemble of spins. This interference manifests
in a reduction of the refocused echo intensity due to the presence of the pump pulse. The pump pulse tuned for nitroxide radicals has a flip angle of $4\pi$ for Gd$^{3+}$. The amplitude of the refocused Gd$^{3+}$ echo reduces in the presence of the pump pulse in the DEER experiment (see figure 4.6). In case of terpyridine derivatives (Au-NPs) the echo intensity is decreased to 30%. The magnitude of the reduction effect is independent of the external magnetic field and the same reduction is observed at Q-band frequencies with the pulse setup Vb as at X-band frequencies (pulse setup I). For Gd$^{3+}$ loaded DOTA complexes (WALP23 polypeptides) the refocused echo dwindles down to 20% of the original intensity due to the presence of the pump pulse. The stronger reduction effect might be related to the slight increase of the spectral width of the Gd$^{3+}$ spectrum for a longer detection sequence (see figure 4.5).

![Figure 4.6](image)

**Figure 4.6:** The amplitude of the refocused Gd$^{3+}$ echo in the presence (dark) and absence (light) of the nitroxide pump pulse in the DEER experiment at **A)** X-band (blue) and **B)** Q-band frequencies (black) with pulse settings Vb for the gold nanoparticles (Au-NPs) and **C)** for WALP23 polypeptides at X-band (green).

In case of terpyridine derivatives, where the Gd$^{3+}$ spectrum narrows significantly with increased inter-pulse delay, only a minor change in the echo intensity is observed ($\approx 5 - 10\%$). This suggests a correlation of the echo reduction effect and the change of Gd$^{3+}$ spectral width. The qualitative tendencies seen so far are supported by all model systems investigated. These findings go in line with further experiments done in our group on further Gd$^{3+}$-nitroxide systems, where the refocused echo intensity is vanishing completely and correspondently the Gd$^{3+}$ spectrum broadens for a longer detection sequence [88]. The reduction effect can be decreased by applying m.w. pulses at the peak of the nitroxide spectrum with a smaller flip angle (less power, same length). This implies a smaller inversion efficiency and therefore a smaller modulation depth which corresponds to the signal measured by DEER.

The echo reduction effect decreases the sensitivity of the DEER experiment on Gd$^{3+}$-nitroxide spin pairs because of a smaller detectable signal. Further systematic investigations have to be done in order to gain a better understanding of the underlying spin dynamics.
4.4 Application to Model Systems

Although the spectral features in Gd$^{3+}$-nitroxide spin pairs are not completely understood to date, the results on the terpyridine derivative model compound (section 4.2) show that the experiments reveal the correct distance. Hence, distance measurements can be performed on such systems and provide consistent information.

4.4.1 WALP23

In WALP23 polypeptides the nitroxide spin labels (MTSSL) are flexible due to conformational distribution of the linker. This distribution can be simulated by a rotamer library approach [8, 89] where possible conformations of specific spin labels are calculated from molecular dynamics (MD) simulations and merged in a library of about 100 to 200 rotamers sufficient for the representation of EPR distance measurements. The rotamer library approach was implemented as module of the Matlab-based protein visualization and modelling program MMM (Multiscale Modelling of Macromolecular Systems) [90]. This open-source program can be downloaded from http://www.epr.ethz.ch/software/index [91].

The lysine linker covalently binding the complexing agent DOTA carries Gd$^{3+}$ at the N-terminus of the polypeptide also has big conformational freedom. The conformational space occupied by the lysine linker can be estimated by a cone model. The resulting model assumes that the core structure of WALP23 can be represented by an ideal $\alpha$-helix and is shown in figure 4.7.

Due to flexibility and conformational distribution of the linkers for both spin labels not a single distance but a rather broad distance distribution is expected. Nevertheless, the distance offset between consecutive labels should be given by the helix pitch for an $\alpha$-helix (0.54 nm).

Figure 4.7: Theoretical description of WALP23 as ideal $\alpha$-helix inserted into a lipid bilayer with calculated MTSSL rotamer distribution for position 11 (rotamer library) and an estimated representation of the DOTA label as a cone model. The polar head group region of the lipid bilayer is represented by the red areas and the middle of the bilayer is shown in green.

The DEER experiment with pumping at the peak of the nitroxide spectrum and detecting at the maximum of Gd$^{3+}$ was performed at X- and Q-band frequencies.
The background corrected experimental time domain data $F(t)/F(0)$ are shown in figure 4.8A. The steepness of the first decay in the time domain already gives a hint on the expected mean distance, where a fast decay indicates a short distances and a prolonged drop of the signal intensity encodes a rather long distance. The experimental time-domain data clearly reveal an elongation of the first decay by going from WALP23_A07R1 to WALP23_A19R1. This trend is expected since the distance between the Gd$^{3+}$ loaded DOTA-linker at the N-terminus and the nitroxide label increases in that sequence.

![Figure 4.8: DEER on Gd$^{3+}$-nitroxide spin pairs in membrane incorporated WALP23 for constructs with the nitroxide label at position 07 (yellow), 11 (orange), 15 (green) and 19 (blue) performed at X-band (light) and Q-band frequencies (dark). A) Background corrected experimental data $F(t)/F(0)$ and the fit from DeerAnalysis 2009 (red/black). B) The rather broad resulting distance distributions ($P(r)$) are independent of the measurement frequency and shift with the $\alpha$-helical pitch of 0.54 nm.](image)

In contrast to DEER traces obtained for Gd$^{3+}$ loaded terpyridine derivative where pronounced oscillations are observed (see figure 4.2), the WALP23 samples show a fast dampening of the oscillations in the DEER trace. This peculiarity points towards a rather broad distribution of distances whereas in case of terpyridine derivative the distance is well defined. This behaviour is related to the fact that every distance appearing in the system features its own defined dipolar frequency ($w_{DD}$) and the presence of a manifold of frequencies induces interference leading to a damping of observed oscillations. The modulation depth (M.D.) for all doubly labelled WALP23 constructs is close
4.4. Application to Model Systems

to 0.5 at X band and indicates a full labelling for both positions (chelating agent and cysteine). At Q-band frequencies modulation depth is significantly smaller and no correlation in terms of signal intensity can be observed. This can be explained by the not yet optimized cavity where the resonator mode is not well adjustable and the performance of the experiment is strongly dependent on the shape of the resonator profile approximately 300 MHz apart from the centre of the mode (pulse setup $V_b$). Nevertheless, the advantage of working at higher bands includes an increase of Boltzmann population when performing the experiment at the same temperature. Hence, signal-to-noise ratio at Q band is comparable to the one obtained at X band although modulation depth ($\alpha$ signal) is significantly smaller. The improvement of detection condition and an expected increase of sensitivity in terms of S/N will be illustrated later on the example of gold nanoparticles.

The background corrected time traces were fitted by DeerAnalysis 2009. The resulting distance distributions (figure 4.8B) are rather broad due to flexibility of MTSSL and the lysine linker of the Gd$^{3+}$ complex. The obtained distribution of the distance does not depend on the measurement frequency and is similar at X and Q band. The distance distributions show small bumps originating from noise present in the time domain data. Especially, data obtained for WALP23_A15R1 at Q band are very noisy, thus the resulting distances distribution shows more pronounced artefacts and the agreement of X- and Q-band data in this case are not perfect. With a validation tool implemented in DeerAnalysis 2009 the influence of background and M.D. can be investigated. The obtained results reveal that the small bumps in the distribution are artefacts and are not within the trust range of the experiment.

Despite a broad width of the distance distribution, the expected shift of the mean distance with the $\alpha$-helical pitch of 0.54 nm is clearly revealed. The distance artefacts seen for the Gd$^{3+}$ loaded terpyridine derivative (figure 4.2) are not visible for the membrane incorporated WALP23 polypeptides. This might be related to the broad distribution of distances. Either the artefacts are hidden in the wings of the distribution or the artefacts also get broadened and due to their decreased intensity the broadening leads to a vanishing of these features. These results demonstrate that DEER on Gd$^{3+}$-nitroxide spin pairs in membrane incorporated polypeptides can be measured in a concentration range of 50 $\mu$M to 200 $\mu$M, which corresponds to concentrations commonly used for DEER on nitroxide-nitroxide spin pairs. Despite the decreased modulation depth at Q-band frequencies the obtained distance distribution remains unchanged. Although the reduction of the refocused echo intensity due to the presence of the pump pulse decreases the echo to 20% of the initial intensity the obtained results are apparently not influenced. This is confirmed first of all by the expected shift of the mean distance. The obtained M.D. of 0.5 for DEER on Gd$^{3+}$-nitroxide spin pairs at X-band frequencies goes in line with expections from nitroxide-nitroxide spin pairs.
4.4.2 Gold Nanoparticles

The gold nanoparticles (Au-NPs) system can be described by a particle on a sphere model (section 3.3). In this case a random distribution of the paramagnetic centres on a spherical shell is assumed. The expected distance distribution for Au-NPs should be rather broad and should feature a triangular like shape [92].

Au-NPs studied here carry 49 ligands able to bind a lanthanide ion (Ln$^{3+}$). The content of Gd$^{3+}$ loading is given in percent (%) and refers to the amount of available ligands. A smaller Gd$^{3+}$ content is connected to a lower probability of having a Gd$^{3+}$ at the surface of a particle. From the modulation depths of DEER experiments on Au-NPs it can be seen that nitroxide radical occurrence is limited to statistically 0.3 nitroxide radicals per particle.

By taking into account a random distribution of paramagnetic species on the surface of Au-NPs and the dependence of the amount of spin pairs on the Gd$^{3+}$ content one can assume that a smaller Gd$^{3+}$ content leads to a smaller fraction of Au-NPs carrying both paramagnetic species. Furthermore, the presence of more Gd$^{3+}$ on the sphere surface might influence the random distribution of paramagnetic species, due to the increased required space occupied by Gd$^{3+}$. This might also have an effect on the cut-off distances in the distance distribution. The cut-off distances is determined by the longest distances present in the model system and is connected to the particle size and the length of the linker for the paramagnetic centres. The impact of the loading on the cut-off distances can not be predicted easily but can be measured by performing DEER on Gd$^{3+}$-nitroxide spin pairs.

![Figure 4.9: DEER on Au-NPs at 10K at X-band frequencies with 2% of loaded Gd$^{3+}$ (dark green) and 4% of loaded Gd$^{3+}$ (light green). A) The background corrected $F(t)/F(0)$ time domain data was fitted by DEERAnalysis (red). The spectra are vertically displaced for better visibility. B) The obtained distance distribution reveals the same mean distance but different cut-off distance.](image)

The DEER experiment on Gd$^{3+}$-nitroxide spin pairs was performed on gold nanoparticles loaded with 2% respectively 4% of Gd$^{3+}$. This corresponds to an average of one and two Gd$^{3+}$ ions per particle, respectively. In figure 4.9 A background corrected time-domain data ($F(t)/F(0)$) show a similar initial decay
indicating the same mean distance. The modulation depth (M.D.) is as small as 0.15 in both cases and is related to only 0.3 active nitroxide radicals per particle. The optimum M.D. of 0.5 could be achieved in case of one nitroxide radical and one Gd$^{3+}$ ion per Au-NPs, as seen for completely labelled WALP23 polypeptides (figure 4.8).

The evaluation with DeerAnalysis 2009 shows that the mean distance of 2.9 nm does not depend on the content of Gd$^{3+}$. The distance distribution obtained for the 2% Gd$^{3+}$ loaded Au-NPs has a shorter cut-off distance (4.3 nm) than the one for 4% Gd$^{3+}$ loading with 4.6 nm cut-off distance.

The performance of the DEER experiment on Gd$^{3+}$-nitroxide spin pairs loaded with 2% Gd$^{3+}$ at different frequencies is shown in the upper panel of figure 4.10 and a comparison with the DEER on nitroxide-nitroxide spin pairs is presented for completeness (figure 4.10, lower panel).

The length of the four-pulse DEER trace determines the available distance range [8], where a longer trace allows for longer reliably assignable distance information. The length of the DEER trace is experimentally restricted by the $T_2$ of the observer spin because in the refocused echo pulse sequence the relevant part of magnetization evolves in the perpendicular plane. The sensitivity of the DEER experiment increases with longer $T_{2,\text{obs}}$. In Gd$^{3+}$-nitroxide spin pairs the detection is performed on the Gd$^{3+}$. Figure 4.10 A shows that $T_2$ is not affected by the measurement frequency. The decay of the transverse magnetization for Gd$^{3+}$ at 10 K is not purely exponential but similar at X and Q band and allows for DEER traces up to $\approx 3.5 \mu s - 4 \mu s$ long.

The background corrected form factor ($F(t)/F(0)$) shown in figure 4.10 B reveals the same modulation depth (M.D.) at X- and Q-band frequencies. In comparison to measurements on Gd$^{3+}$ loaded terpyridine derivative or WALP23 polypeptides the Q-band cavity was optimized and a more broad banded resonator profile could be achieved. The optimization improved the performance of the
experiment and the observable modulation depth increases, therefore also the signal-to-noise ratio improves.

Distance distributions $P(r)$ obtained at different frequencies are approximately the same and show a broad spread (figure 4.10 C). The cut-off distance does not change by measuring at different external magnetic fields. Especially from the less noisy trace at Q band a clear triangular shape of the distance distribution can be extracted. This gives evidence for the random distribution of the paramagnetic species on the surface of a sphere.

Since the two experimental traces were recorded under similar conditions at the same temperature, same srt, and same length (similar $T_2$ given) the noise level can be directly compared. It can be clearly seen that the noise of the DEER trace obtained at Q-band frequencies is smaller than at X band. This improvement is related to the higher Boltzmann population difference at higher fields. The signal-to-noise ratio (S/N) increases by a factor of three by going from X- to Q-band frequencies. This leads to a gain in sensitivity and a reduction of the measurement time needed to obtain the same quality of the experimental data. Despite the echo reduction effect the sensitivity of the DEER experiment on Gd$^{3+}$-nitroxide spin pairs is good and the distance distribution can be determined also in samples where this distribution is very broad as often encountered in material science applications.

The Au-NPs model system opens up the possibility to perform not only DEER on Gd$^{3+}$-nitroxide spin pairs but also on nitroxide-nitroxide spin pairs. Both species are randomly distributed on the surface of a sphere and the expected distance range should be similar. This situation is suitable for the comparison of the performance for both experiments and a short overview on the achieved results will be given in the following.

DEER on nitroxide-nitroxide spin pairs in 2% Gd$^{3+}$ loaded Au-NPs was performed at X and Q band at the standard temperature of 50 K. The $T_2$ at this temperature is comparable to the one of Gd$^{3+}$ at 10 K. The modulation depth is similar at X- and Q-band frequencies ($\approx 0.2$) and confirms an average of 0.3 active nitroxides per Au-NPs. The length of the DEER trace is the same as the one for Gd$^{3+}$-nitroxide DEER traces. Also in the case of nitroxide-nitroxide spin pairs the obtained distance distribution is broad and has a triangular shape. The cut-off distance is somewhat shorter than the one for Gd$^{3+}$-nitroxide because of the difference in the length of the two linkers. Again, normalized Q-band time-domain data show less noise than at X band. The S/N improves by a factor of 4. In the direct comparison of DEER on Gd$^{3+}$-nitroxide and nitroxide-nitroxide spin pairs the detection on the Gd$^{3+}$ center improves the sensitivity by a factor of three at X-band and two at Q-band frequencies, although the sensitivity is reduced by the echo reduction in the presence of the pump pulse. The main reason for the improvement of the DEER experiment can be based on the increased Boltzmann population difference at 10 K compared to 50 K. The time needed for a scan with a similar S/N is further influenced by the longitudinal relaxation time ($T_1$) of the observed species. This additionally favours the Gd$^{3+}$ center because Gd$^{3+}$ ions relaxe faster at 10 K than nitroxide radical at 50 K and therefore the experiment can be repeated more often during
the same available measurement time. All obtained results for DEER on Gd$^{3+}$-nitroxide spin pairs suggest that also in this model system for material science applications the expected distance information can be extracted. Summarizing the performance of the experiment we could detect a change of the distribution width with the content of loaded Gd$^{3+}$. Nevertheless the mean distance stays unchanged and is related to the linker length and the diameter of the Au-NPs. The modulation depth was similar for different samples and implies a reproducibility of the sample preparation. Since the experiment is sensitive to the width of the distance distribution, the satellite features observed for the Gd$^{3+}$ loaded terpyridine derivative are not observable here. The reduction of the echo intensity to 30% of the initial echo amplitude in the presence of the pump pulse is reducing the sensitivity of the experiment. Nevertheless, comparison with DEER on nitroxide-nitroxide spin pairs shows even a slightly better performance at similar conditions.

4.5 Conclusion

The dipole-dipole interaction between Gd$^{3+}$ ions and nitroxide spin labels can be measured directly by using DEER, with detection on Gd$^{3+}$ and pumping on nitroxide radicals. DEER measurements on Gd$^{3+}$-nitroxide pairs provide reliable average distances along with the distance distribution if the experimental time-domain data are analysed with standard tools (DeerAnalysis). This holds true, not only when a narrow distance distribution is present, but also for a broad distribution of distances, as it is often encountered in material science applications and in membrane incorporated systems relevant for structure and structural changes studies.

The transverse relaxation time ($T_2$) of Gd$^{3+}$ at 10K of approximately 2$\mu$s to 3$\mu$s is similar to the one of nitroxide spin labels, and can be prolonged at lower temperatures. The very good performance of the DEER experiment at low temperature and especially at Q-band frequencies provides a valuable alternative to conventional nitroxide-nitroxide distance measurements. The evaluation of the distance distributions obtained from Gd$^{3+}$-nitroxide spin pairs showed unwanted satellite artefacts in case of a narrow distance distribution and in a case where the spectral width of the Gd$^{3+}$ spectrum narrows with increased inter-pulse delay. The usage of different chelating agents gives rise to a variation of ZFS parameters and thus to changes of the shape of the Gd$^{3+}$ spectrum. These variations are connected to a different relaxation behaviour of the different Gd$^{3+}$ transitions and the spectral width of Gd$^{3+}$ can be narrowed down or even broadened for an increased inter-pulse delay in an echo-detected field sweep experiment. Furthermore, a reduction of the refocused echo intensity was observed in the presence of the pump pulse in the DEER experiment. It seem, that the different investigated complexing agents give rise to different strength of the echo reduction, where stronger effects are observed for broadened...
spectra and only a minor effect is present for narrowing spectra.
Relaxation enhancement (Δ$k$) is the change of the relaxation rates of a slowly relaxing paramagnetic species (nitroxide radical) in the presence and absence of a fast relaxing agent (lanthanide ion (Ln$^{3+}$)). In this thesis relaxation enhancement (Δ$k$) is analysed according to the average relaxivity approximation [39], where Δ$k$ is averaged over the orientation dependence of the fast relaxing Ln$^{3+}$ ion. The reference measurements in the absence of the fast relaxing agent are obtained from lanthanum ion (La$^{3+}$) loaded samples. The orientation averaged relaxation enhancement (∆$k$) was analysed for a dysprosium ion (Dy$^{3+}$) or a gadolinium ion (Gd$^{3+}$) in the close vicinity of the detected slowly relaxing nitroxide spin label. In the case of Dy$^{3+}$ an effective spin $S = 1/2$ can be assumed [49]. For Gd$^{3+}$ the high spin system with $S = 7/2$ has to be taken into account. Two model systems were studied, first an α-helical polypeptide (WALP23) with DOTA as N-terminal chelating agent and, second, gold nanoparticles (Au-NPs) functionalised with the complexing agent DTPA.

In the course of this chapter the enhancement of the longitudinal relaxation time ($T_1$) and the transverse relaxation time ($T_2$) of the nitroxide spin label induced by Dy$^{3+}$ or Gd$^{3+}$ will be discussed. The strongest Δ$k$ effect is observed for $T_1$ of nitroxide radicals in the presence of Dy$^{3+}$. The discussion of this type of investigated spin pair represents the main part of this chapter and a detailed analysis of distance extraction, applying the averaged relaxivity approximation is presented. In addition, the estimation of $T_1$ of Dy$^{3+}$ centres from temperature dependent relaxation enhancement measurements, as well as the frequency dependence of the relaxation enhancement effect and an evaluation of short distance suppression (< 1.5 nm), are presented in section 5.1.

In section 5.2 the longitudinal relaxation enhancement induced by Gd$^{3+}$ and the $T_2$ relaxation enhancement by Dy$^{3+}$ or Gd$^{3+}$ are discussed qualitatively. In section 5.3 relaxation enhancement on a three-spin system is investigated for a combination of nitroxide radical, Dy$^{3+}$/Gd$^{3+}$ ion and paramagnetic oxygen (O$_2$) dissolved in a lipid bilayer.
5.1 Longitudinal Relaxation Enhancement Induced by Dysprosium

5.1.1 Temperature Dependent Time Domain Data

The experimental longitudinal relaxation data of the nitroxide spin label are obtained from the inversion recovery experiment introduced in section 2.3.1. The time domain data can be fitted by a stretched exponential function (equation 2.34). As an example, a typical $T_1$ time domain traces at 80 K for WALP23_A11R1 loaded with Dy$^{3+}$ or La$^{3+}$, as diamagnetic reference, are given in figure 5.1A. The stretched exponential fit shows a satisfactory overlap with the experimental traces. A mono-exponential decay is not able to represent the experimental time domain data because already the environment of the nitroxide spin label in the absence of any additional paramagnetic species is not completely homogeneous. The nitroxide radicals in the close vicinity of Dy$^{3+}$ relax significantly faster than in the presence of the diamagnetic La$^{3+}$. The fits of inversion recovery time traces are in good agreement with the experimental data for La$^{3+}$ loaded samples whereas for Dy$^{3+}$ loaded ones some deviations of the fit from the time domain data can be seen, especially at the beginning of the time trace (figure 5.1B). This behaviour implies a better representation of the nitroxide relaxation by the fit in the absence of the fast relaxing agent.

As shown in figure 5.1, the raw inversion recovery time traces can be nicely represented by a stretched exponential fit. However, this is not the only possibility to fit the experimental data. The performance of a bi-exponential fit is only slightly worse according to rmsd criteria. Nevertheless, the resulting mean longitudinal relaxation time for the nitroxide radical ($1/e$ time) is not changing significantly and therefore the type of fit does not influence further analysis of the relaxation enhancement effect. A mono-exponential fit can not represent
the experimental data with a reasonable rmsd tolerance. This suggests a distribution of nitroxide $T_1$ times and can be related to a not totally homogeneous environment of different MTSSL rotamers.

The temperature dependent relaxation rates ($k_1 = 1/T_1$) for all four spin labelled WALP23 constructs obtained between 20 K and 100 K in 10 K steps in the presence of $\text{La}^{3+}$ or $\text{Dy}^{3+}$ are depicted in figure 5.2 A. The temperature dependence of the longitudinal relaxation times of the nitroxide spin label indicates that the magnetization of samples loaded with $\text{Dy}^{3+}$ (squares) decays significantly faster than the magnetization of samples being loaded with $\text{La}^{3+}$ (circles). Furthermore it can be seen that the relaxation times of the slowly relaxing nitroxide spin labels with $\text{La}^{3+}$ in the close vicinity are almost independent of the labelling position and constant for a particular temperature. In contrast the labelling with $\text{Dy}^{3+}$ induces a change of the $T_1$ nitroxide relaxation times, depending on the position of the nitroxide spin label. The slowly relaxing nitroxide spin label reveals the fastest magnetization decay for position 11 at temperatures between 70K and 80K.

The stretching exponent $\alpha$ from the stretched exponential fit (equation 2.34) is a measure for the width of the distribution of relaxation times. An $\alpha$ parameter of one would imply purely mono-exponential rebuilding of the equilibrium magnetization. Figure 5.2 B illustrates the dependence of $\alpha$ on the temperature, on the distance (different labelled constructs) and on the type of loaded $\text{Ln}^{3+}$. All $\text{La}^{3+}$ loaded and spin labelled WALP23 constructs show the same distribution of relaxation times independent from the distance between the slowly relaxing nitroxide radical and the diamagnetic $\text{La}^{3+}$. Nevertheless, even under the reference conditions a non-mono-exponential decay is observed. The width
Chapter 5. Relaxation Enhancement

of the $T_1$ distribution is constant over a broad temperature range and only at lower temperatures ($< 40 \text{K}$) a decrease of the homogeneity can be seen. The presence of Dy$^{3+}$ increases the width of the existing variation of the $T_{1s}$ times. Figure 5.2B also reveals that for shorter inter-spin distances between Dy$^{3+}$ and nitroxide smaller $\alpha$ values are obtained from the stretched exponential fit and therefore a broader distribution of $T_{1s}$ times is present. The strong enhancement of the nitroxide relaxation in combination with the broad distribution of relaxation times might result in the significant residual of the fit with respect to the experimental time trace in the presence of Dy$^{3+}$.

5.1.2 Distance Information from Relaxation Enhancement

Relaxation enhancement in general is defined as the change of the relaxation rate of a slowly relaxing species in the presence and absence of a fast relaxing agent (equation 2.27). By fitting the inversion recovery traces observed for the nitroxide spin label, the relaxation times $T_{1s}$ (with Dy$^{3+}$) and $T_{1s,0}$ (with La$^{3+}$) can be determined as described above. The temperature dependent relaxation enhancement is calculated from the difference of the inverse relaxation times of the nitroxide radical at each temperature.

Distance information is obtained from relaxation enhancement by applying the average relaxivity approximation as introduced in section 2.2.3. The averaged relaxation enhancement $\Delta k$ can be matched with the experimental $\Delta k$ data by a two-dimensional fit depending, first, on the relaxation time of the fast relaxing agent with the fitting parameter $p$ and $T_{\text{max}}$ and, second, on the inter-spin distance ($r$) of the two paramagnetic species (equation 2.32).

In case of Dy$^{3+}$ acting as fast relaxing agent, an effective spin of $S = 1/2$ can be used in order to analyse the acquired and fitted time-domain data. The continuous wave (CW) EPR spectrum of Dy$^{3+}$ (figure 2.3) reveals a strong g-anisotropy. The orientation dependence of $g_f$ is taken into account by summing up the simulated relaxation enhancement traces calculated for each orientation of the Dy$^{3+}$ centre. Furthermore, it is assumed that the longitudinal and transverse relaxation times of Dy$^{3+}$ can be considered as equal ($T_{1f} = T_{2f}$) and the orientation dependence of $T_{1f}$ can be neglected in order to extract distance information.

The relaxation enhancement effect for all four constructs induced by Dy$^{3+}$ is calculated by

$$\Delta k(r, \theta) = \frac{1}{T_{1s}} - \frac{1}{T_{1s,0}}.$$  \hspace{1cm} (5.1)

The obtained temperature dependent $\Delta k$ values are shown in figure 5.3. The experimental data reveal a stronger $\Delta k$ effect for position 11 with respect to position 15 or 19. The vertical scaling of $\Delta k$ is proportional to the distances ($\propto r^{-6}$) and therefore the experimental data go in line with distance information obtained from double electron-electron resonance (DEER) on Gd$^{3+}$ loaded...
5.1. Longitudinal Relaxation Enhancement Induced by Dysprosium

WALP23 polypeptides (figure 4.8). However, position 07 having the shortest distance to the N-terminal loaded Dy$^{3+}$ shows a comparable $\Delta k$ effect as for the nitroxide spin label at position 11 and therefore a similar distance is extracted from the experimental data.

![Figure 5.3: Temperature dependent relaxation enhancement data (circles) for position 07 (yellow), 11 (orange), 15 (green) and 19 (blue) of WALP23 with the corresponding simulation as solid line ($\Delta k$).](image)

From a fit of the temperature dependent $\Delta k$ data the averaged relaxivity ($\overline{C}$) and the inter-spin distance ($r$) can be determined. The two parameters can be extracted almost independently of each other from the experimental data because $\overline{C}$ relies on the relaxation of the fast relaxing agent ($T_{1f}$) and determines the shape of the $\Delta k$ curve and $r$ corresponds to a vertical scaling factor. The temperature dependence of $T_{1f}$ which determines the shape of the curve can be fitted by an empirical power law

$$\frac{T_{1f}(T)}{s} = 1.3 \cdot 10^{-11} \left( \frac{T_{\text{max}}}{T} \right)^p$$

(5.2)

where $T_{\text{max}}$ is the temperature for the optimum enhancement of nitroxide radical relaxation and the power exponent $p$ determines the width of the temperature dependence. $T_{\text{max}}$ can be determined as the maximum of the temperature dependent data set. The temperature maximum ranges within 10K between approximately 70K and 80K for this kind of samples. The pre-factor $A$ can be approximated with $1.3 \cdot 10^{-11}$, as a relaxation time of $1.3 \cdot 10^{-11}$s of the fast relaxing species causes the strongest enhancement of the nitroxide relaxation. Also the power parameter $p$ is not fluctuating substantially. Hence the temperature dependent relaxation time of Dy$^{3+}$ can be determined with reasonable precision from the fit of the temperature dependent relaxation enhancement effect. The resulting fitting parameters are listed in table 5.1 and the fits are shown in figure 5.3 as solid line. The obtained distance information is in line with the trend of the magnitude of the $\Delta k$ effect, as it was discussed before.
Sample WALP23_A07R1 is expected to show the shortest distance and thus the highest $\Delta k$ amplitude, but table 5.1 reports a similar distance for this construct as for WALP23_A11R1. Already the relaxation enhancement effect of WALP23_A07R1 shows irregularities in the temperature dependence shape. The combination of a non-smooth characteristic and a reduced vertical shift of $\Delta k$ reveals a failure of the used distance determination approach for very short distances. The distances obtained for the other three constructs go in line with expectations from available DEER data (section 4.4) with a moderate underestimation of the distance.

Figure 5.3 and table 5.1 show a correlation of the distance information from orientation averaged relaxation enhancement ($\Delta k$) induced by Dy$^{3+}$ with DEER data obtained for the same model polypeptide system with the N-terminal DOTA ligand loaded with Gd$^{3+}$. The comparison of the obtained distance information reveals a systematic underestimation of the inter-spin distance by relaxation enhancement as compared to DEER on Gd$^{3+}$-nitroxide spin pairs. The main difference between distance determination by DEER or relaxation measurements is the averaging, where the DEER technique 'directly' measures the distance distribution and for the single distance $\Delta k$ approach $< r^{-6} >^{1/6}$ is determined. In order to investigate the apparent underestimation of $< r^{-6} >^{1/6}$ due to the $r^{-6}$ averaging, Gaussian distributions with a fixed mean distance of 3.75 nm were simulated and the width of the distribution was varied in the range from 0.2 nm to 0.5 nm. The averaging of the distance was performed as $P_{r^{-6}}(r) = P_{\text{Gauss}}(r) \times r^{-6}$ and the resulting distance distributions are shown in figure 5.4. As a result of $r^{-6}$ averaging of a distance distribution with defined mean distance and width $< r^{-6} >^{1/6}$ will be smaller than $< r >$. The width of the input distance distribution is influencing the apparent mean distance ($< r^{-6} >^{1/6}$) after $r^{-6}$ averaging where a broader width leads to stronger underestimation. The averaged distance distributions ($P_{r^{-6}}(r)$) show a pronounced probability for very short distances. Very short distances are non-physical because such short distances can not appear in the experiment due to steric hindrances of the chelating agent. The short distance peak in $P_{r^{-6}}(r)$ is arising from a non-zero probability for short distances in the assumed Gaussian input distribution and is more distinct for broad distance distributions. For distance distributions with a smaller mean distance the shift towards shorter distances and the appearance

### Table 5.1: Fitting parameters for relaxation enhancement induced by Dy$^{3+}$ on nitroxide spin label at positions 07, 11, 15 or 19 in WALP23 peptides and distances extracted by Gd$^{3+}$-nitroxide DEER for all four constructs

<table>
<thead>
<tr>
<th>sample</th>
<th>$T_{\text{max}}$</th>
<th>$\Delta k$</th>
<th>DEER</th>
</tr>
</thead>
<tbody>
<tr>
<td>WALP23_A07R1</td>
<td>93.5 K</td>
<td>2.21 nm</td>
<td>2.4 nm</td>
</tr>
<tr>
<td>WALP23_A11R1</td>
<td>70.9 K</td>
<td>2.15 nm</td>
<td>2.7 nm</td>
</tr>
<tr>
<td>WALP23_A15R1</td>
<td>79.8 K</td>
<td>2.75 nm</td>
<td>3.3 nm</td>
</tr>
<tr>
<td>WALP23_A19R1</td>
<td>77.4 K</td>
<td>2.98 nm</td>
<td>3.8 nm</td>
</tr>
</tbody>
</table>

Sample WALP23_A07R1 is expected to show the shortest distance and thus the highest $\Delta k$ amplitude, but table 5.1 reports a similar distance for this construct as for WALP23_A11R1. Already the relaxation enhancement effect of WALP23_A07R1 shows irregularities in the temperature dependence shape. The combination of a non-smooth characteristic and a reduced vertical shift of $\Delta k$ reveals a failure of the used distance determination approach for very short distances. The distances obtained for the other three constructs go in line with expectations from available DEER data (section 4.4) with a moderate underestimation of the distance.

Figure 5.3 and table 5.1 show a correlation of the distance information from orientation averaged relaxation enhancement ($\Delta k$) induced by Dy$^{3+}$ with DEER data obtained for the same model polypeptide system with the N-terminal DOTA ligand loaded with Gd$^{3+}$. The comparison of the obtained distance information reveals a systematic underestimation of the inter-spin distance by relaxation enhancement as compared to DEER on Gd$^{3+}$-nitroxide spin pairs. The main difference between distance determination by DEER or relaxation measurements is the averaging, where the DEER technique 'directly' measures the distance distribution and for the single distance $\Delta k$ approach $< r^{-6} >^{1/6}$ is determined. In order to investigate the apparent underestimation of $< r^{-6} >^{1/6}$ due to the $r^{-6}$ averaging, Gaussian distributions with a fixed mean distance of 3.75 nm were simulated and the width of the distribution was varied in the range from 0.2 nm to 0.5 nm. The averaging of the distance was performed as $P_{r^{-6}}(r) = P_{\text{Gauss}}(r) \times r^{-6}$ and the resulting distance distributions are shown in figure 5.4. As a result of $r^{-6}$ averaging of a distance distribution with defined mean distance and width $< r^{-6} >^{1/6}$ will be smaller than $< r >$. The width of the input distance distribution is influencing the apparent mean distance ($< r^{-6} >^{1/6}$) after $r^{-6}$ averaging where a broader width leads to stronger underestimation. The averaged distance distributions ($P_{r^{-6}}(r)$) show a pronounced probability for very short distances. Very short distances are non-physical because such short distances can not appear in the experiment due to steric hindrances of the chelating agent. The short distance peak in $P_{r^{-6}}(r)$ is arising from a non-zero probability for short distances in the assumed Gaussian input distribution and is more distinct for broad distance distributions. For distance distributions with a smaller mean distance the shift towards shorter distances and the appearance
of the short distance peak might coincide. This can lead to a extreme shift of $<r^{-6}>^{1/6}$. In case of intermediate distances the averaging in combination with a suppression of very short distances (discussed later) leads to an apparently longer mean distance (smaller $\Delta k$) and can partially account for the reduced relaxation enhancement effect observed for WALP23_A07R1 as compared to the expectation from the DEER measurements. A more detailed analysis of the short distance suppression will be discussed in section 5.1.4.

5.1.3 Frequency Dependent Relaxation Enhancement Effect

The basic equation describing relaxation enhancement (equation 2.27, 2.28) reveals a frequency dependence of $\Delta k$. The terms of the dipolar alphabet involve the resonance frequencies of the slowly ($\omega_s$) and fast ($\omega_f$) relaxing species and therefore a field dependence is introduced. The strongest $\Delta k$ effect is reached if one of the characteristic frequencies ($\omega_s + \omega_f$, $\omega_s - \omega_f$ or $\omega_s$) equals the inverse relaxation time of fast relaxing agent. Due to the direct proportionality of the resonance frequency to the external magnetic field this correspondence can be achieved at longer $T_{1f}$ for lower fields. This corresponds to a lower temperature and thus to longer $T_{1s,0}$. Hence, the magnitude of the $\Delta k$ effect will increase at lower frequency as discussed by Jäger et al. [39]. The predicted behaviour of $\Delta k$ with the frequency includes two assumptions. First the temperature dependence of the fast relaxing species is only weakly influenced by the external magnetic field (figure 5.6). Second, as shown by Eaton et al. [60], the relaxation of a nitroxide radical in a diamagnetic environment at temperatures below 100 K shows only little frequency dependence. Hence, the enhancement of $\Delta k$ might occur at lower fields. The estimated frequency behaviour is tested in the following on a nitroxide labelled WALP23 polypeptide loaded with Dy$^{3+}$ for a label position where no suppression of distances is expected.
In figure 5.5 the temperature and magnetic field dependence of the $T_1$ relaxation enhancement for WALP23_A15R1 is shown. It can be seen that the relaxation enhancement effect for the same sample is higher at S-band as compared to X-band and Q-band frequencies. An increase of the maximum $\Delta k$ magnitude by approximately 65% at S-band compared to X-band is observed. Furthermore, the relaxation enhancement effect at Q-band frequencies does not reach its maximum of the $\Delta k(T)$ dependence in the measured temperature range and is approaching a saturation regime at 100 K. The data set cannot be extended to much higher temperatures, as then other relaxation channels for the nitroxide radical due to enhanced molecular motion become available. Nevertheless, it can be safely stated that the relaxation enhancement effect at X-band frequencies is approximately 80% higher than at Q-band frequencies. Thus, for the detection at lower frequencies the expected behaviour is verified and $\Delta k$ increases.

![Figure 5.5: Magnetic field and temperature dependence of $\Delta k$ for WALP23_A15R1 at Q-band (dark green), X-band (green) and S-band (light green) frequencies with the experimental data as circles and the simulations as solid lines.](image)

For the evaluation of the frequency-dependent performance of the relaxation enhancement approach not only the magnitude of $\Delta k$ is important but also the reliability of the extracted distance information is of significant interest. The experimental relaxation enhancement data were fitted according to the averaged relaxivity approximation (equation 2.32). The resulting fitting parameters are listed in table 5.2 and the simulated temperature dependent $\Delta k$ is plotted in figure 5.5. The obtained distance for the same Dy$^{3+}$-loaded construct (WALP23_A15R1) deviates slightly for measurements at different m.w. bands and a tendency towards longer extracted distance is observed at lower fields. The mean distance obtained by DEER on Gd$^{3+}$-nitroxide spin pairs for this labelled construct is 3.3 nm. Hence, the mean distance extracted from relaxation enhancement is increasing to some extend towards the expected mean distance by measuring at lower frequencies. This might be connected to the fact that the relaxation enhancement effect is more pronounced at lower fields and therefore
the distance sensitivity is better.

### Table 5.2: Fitting parameter for relaxation enhancement data for construct WALP23_A15R1 at S-, X- and Q-band frequencies induced by Dy$^{3+}$ (DEER distance = 3.3 nm)

<table>
<thead>
<tr>
<th>frequency</th>
<th>(&lt; r^{-6} &gt;^{1/6})</th>
<th>(A)</th>
<th>(T_{\text{max}})</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 GHz (S band)</td>
<td>2.93 nm</td>
<td>(3.1 \cdot 10^{-11})</td>
<td>60.0K</td>
<td>3.0</td>
</tr>
<tr>
<td>9.5 GHz (X band)</td>
<td>2.75 nm</td>
<td>(1.3 \cdot 10^{-11})</td>
<td>79.8K</td>
<td>3.0</td>
</tr>
<tr>
<td>35 GHz (Q band)</td>
<td>2.45 nm</td>
<td>(4.0 \cdot 10^{-12})</td>
<td>(&gt; 100K)</td>
<td>3.0</td>
</tr>
</tbody>
</table>

One of the assumptions for the prediction of the frequency-dependent relaxation enhancement was a minor change of the Dy$^{3+}$ relaxation times at different fields. The temperature dependent \(T_{1f}\) relaxation times of Dy$^{3+}$ can be obtained from the fit of the relaxation enhancement data by applying equation 2.33. The resulting \(T_{1f}(T)\) curves are shown for S-, X- and Q-band frequencies in figure 5.6 and no field dependence is revealed. For further analysis equation 2.33 can be simplified to the following form:

\[
T_{1f}(T) = X \cdot T^{-p} \tag{5.3}
\]

with a factor \(X = A \cdot (T_{\text{max}})^p\). The fitting parameter \(T_{\text{max}}\) and \(A\) from table 5.2 are frequency dependent and the factor \(X\) calculates to \((6.69 \cdot 10^{-6} - 6.57 \cdot 10^{-6})\) s K from S- to Q-band frequencies, which eventually goes in line with the assumption of a field-independent temperature behaviour of the fast relaxing Dy$^{3+}$ centres.

### Figure 5.6: Temperature-dependent \(T_{1f}\) relaxation times of the fast relaxing Dy$^{3+}$ at S-, X- and Q-band frequencies calculated according to equation 2.33 with the parameter given in table 5.2 with \(T_{\text{max}}\) at Q-band frequencies of 118 K. The inset shows the temperature dependence of the Dy$^{3+}$ relaxation rate \((1/T_{1f})\).

The increase of the relaxation enhancement effect at lower fields is therefore exclusively related to the combination of the resonance frequencies of the fast and
slow relaxing species (characteristic frequency for each term in equation 2.28) and its interplay with the relaxation time of the fast relaxing species \((T_{1f})\). Since the characteristic frequency scales with the external magnetic field the enhancement of the nitroxide relaxation reaches its optimum at longer \(T_{1f}\) at lower field, thus the magnitude of \(\Delta k\) increases. However, for short distances the suppression of the relaxation enhancement effect might also be influenced and further work along this line is required.

5.1.4 Suppression of Short Distances

The evaluation of distance information obtained for the four spin labelled WALP23 constructs has shown that the distance extracted from relaxation enhancement for position 07 is the same as for position 11, although DEER data obtained for this labelling position reveal a shorter distance (section 5.1.2). The apparent suppression effect for short distances will be investigated in this section.

There are several factors which could lead to the suppression of short distances. First, the experimental data are acquired by performing an inversion recovery experiment. In the detection sequence the first data point in the time domain is measured after a time of \(T_0 = 2\,\mu s\). This value was chosen arbitrary and could be reduced to approximately 100 ns and is related to the dead-time of the spectrometer. During the time \(T_0\) the system is already evolving and no signal is observed. Second, by applying the average relaxivity approximation an isotropic averaging over all principal axis orientation of Dy\(^{3+}\) is performed. A more detailed analysis of the orientation dependent relaxation enhancement effect is required in order to see if the averaging possibly leads to the suppression of short distances. Even without a comprehensive explanation of the suppression effect, it is present and an evaluation of a distance distribution by applying a suppression function will be performed in this section. This function should take the time offset \(T_0\) for the detection and a possible orientation dependence into account.

The influence of the dead time of the inversion recovery experiment as a possible reason for the suppression of short distances is investigated in the following. A simulation of \(\Delta k\) for different distances is performed at the temperature maximum obtained for WALP23_A11R1, \(T = 70.9\,\text{K}\). The relaxation time of the fast relaxing Dy\(^{3+}\) \(T_{1f}\) is calculated according to equation 5.2 for the same position and is used in the following simulations. The relaxation enhancement decay trace was calculated for a single distance by summing over all decay traces of each orientation of the Dy\(^{3+}\) centre. In figure 5.7 the simulated \(\Delta k\) time trace are shown for distances between 1 nm and 1.6 nm.

Before the first data point of the inversion recovery trace \((T_0 = 2\,\mu s)\) no information about the decay is available. It can be seen that for distances shorter than 1 nm the signal is completely decayed before \(T_0\). This means that very short distances (< 1 nm) can not be detected by relaxation enhancement with
5.1. Longitudinal Relaxation Enhancement Induced by Dysprosium

Figure 5.7: Simulated relaxation enhancement decay for different distances (1.04 nm - red, 1.16 nm - green, 1.46 nm - blue, 1.58 nm - violet) at $T = 70.9$ K with $T_1f(T)$ according to the fit of WALP23_A11R1. The first grey area marks the range before the first accessible experimental point via inversion recovery ($T_0 = 2 \mu s$; vertical line) and the 1/e value for the decay function is marked as well (horizontal line).

the applied measurement scheme. Distances up to 1.6 nm are still relaxing fast and only a minor part (less than 1/e) of the relaxation enhancement decay is detected with $T_0 = 2 \mu s$. Fitting of the time domain data (section 5.1.1) reveals that especially the first part of the experimental time trace is not reproduced with a very high precision by the stretched exponential decay function (equation 2.34) in case Dy$^{3+}$ is present in the close vicinity of the nitroxide radical. This also implies that the missing information of the first 2000 ns might influence the fitted relaxation times of the nitroxide spin label and therefore the magnitude of the relaxation enhancement effect might be affected slightly. For distances above 1.6 nm a significant part of the $\Delta k$ decay is detectable and fitting of the experimental inversion recovery time trace should show a reasonable result. In order to change the suppression limit towards smaller distances the first inter-pulse $T_0$ delay could be decreased. A $T_0$ of 0.1 $\mu s$ could be used and the suppression limited due to the delay of the detection could be decreased. The suggested usage of $T_0 = 0.1 \mu s$ would result in a total suppression of distances smaller than 0.6 nm and the decay of $\Delta k$ to less than 1/e of the initial intensity would be reached for distances bigger 1 nm (data not shown).

The investigation of the relaxation enhancement effect and its suppression at short distances also has to include the discussion of orientation averaging for a single distance. In figure 5.8 the time evolution of $\Delta k$ is calculated for every orientation of the Dy$^{3+}$ for two distances. Case A with an inter-spin distance of $r = 1.22$ nm illustrates the situation where a substantial suppression is expected ($r < 1.6$ nm). For a distance of $r = 1.83$ nm (case B) only a minor suppression for the orientation averaged relaxation enhancement decay is expected because the suppression limit was found to be around $r = 1.6$ nm.
Figure 5.8: Time decay traces of the relaxation enhancement effect for A) 1.22 nm and B) 1.83 nm for Dy$^{3+}$ ‘orientations’ with g-values from $g_\parallel = 4.2$ to $g_\perp = 14$ in green with the parallel orientation in yellow. The simulated full relaxation enhancement decay including the probability of the orientation is illustrated as a red solid line. The grey lines mark the 1/e value (horizontally) and the cut off time of $T_0 = 2 \mu s$.

Starting from $g_\parallel = 4.2$ (yellow trace) $\Delta k$ increases towards $g_\perp = 14$ of the fast relaxing Dy$^{3+}$ centre in both cases. The orientation averaged decay trace (red trace) follows the expected trend of the suppression limit. For the short inter-spin distance all simulated time traces of different Dy$^{3+}$ principal axis orientations decay substantially before the first data point is acquired in the inversion recovery experiment. In case of the intermediate distance $r = 1.83$ nm the individual traces extend significantly beyond the cut-off region of the experiment. In this situation the determination of the decay time for each single orientation can be easily performed and the resulting orientation averaged $\Delta k$ includes the contribution of all orientations. Therefore, the averaged relaxivity approximation can be applied for expected distances above the suppression limit. As depicted in figure 5.8 the slowly decaying individual time traces are originating from orientations in the region of $g_\parallel$ with a lower probability. This implies a smaller influence on the averaged $\Delta k$ and the suppression is only insignificantly affected by orientation averaging. Hence, the orientation dependence of the relaxation enhancement effect is not influencing the obtained distance for distances in the intermediate range ($r > 1.6$ nm) and can be neglected for further discussion.

Still a suppression is observed for short distances and for distances longer than 1.6 nm the suppression will continuously be reduced due to the prolonged evolution time of the relaxation enhancement effect. In order to describe this behaviour a suppression function can be used, where the earlier discussed influence of $T_0$ has to be taken into account. Orientation dependence of the relaxation enhancement will be neglected due to the minor influence for intermediate distances. By showing the influence of the starting value of the inversion recovery experiment $T_0$ on the detectable relaxation enhancement effect the suppression
function \( S(r) \) can be approximated by

\[
S(r) = \exp \left[ -C \cdot \left( \frac{r_0}{r} \right)^6 \right]
\] (5.4)

with a function \( C = T_0 \cdot \Delta k \) including, without losing generality, the calculated relaxation enhancement effect \( \Delta k = 120 \cdot 10^3 \text{s}^{-1} \) at a particular defined distance \( r_0 = 2.01 \text{nm} \) and \( r \) being the evaluated inter-spin distance. The implementation of the suppression function can lead to a more realistic interpretation of distance information obtained in the intermediate distance range. As shown before for distances below 1 nm the usage of \( T_0 = 2 \mu \text{s} \) leads to a total suppression of the observable relaxation enhancement effect in the time domain data of the inversion recovery experiment (figure 5.7), this behaviour can be represented by applying the suppression function. Figure 5.9 illustrates how the distances distribution of an \( r^{-6} \) averaged Gaussian distribution changes where the application of the suppression function reduces the probability of short distances. The consequence on the obtained mean distance is shown for two input distances, one being relatively long and significantly out of any suppression regime \((r = 3.75 \text{nm})\) and the other one being an intermediate distance \((r = 1.7 \text{nm})\) with an orientation independent suppression but an expected influence of the experimental cut-off time \(T_0\).

The shift of \(<r^{-6}>^{1/6}\) towards smaller mean distance due to \(r^{-6}\) averaging was already discussed. For intermediate distances such as 1.7 nm this effect is very significant because of a noticeable probability for very short distances especially for Gaussian distributions with a broader width. By applying the suppression function to the \(r^{-6}\) averaged distance distribution the short distance averaging peak, arising from non-zero probability of short distances in the Gaussian distribution, is cut off and distances up to 1.2 nm are totally suppressed. For long distances (figure 5.9 - left panel) the major shift of the mean distance is related to the \(r^{-6}\) averaging and the suppression function has no significant influence on the obtained distance information. For the intermediate distance \((r = 1.7 \text{nm})\) shows a tremendous change of the distance information is observed when applying the suppression function. This is related to the fact that a significant part of the distance distribution is shifted to the range where the suppression function has the most impact. Therefore, also the probability for

<table>
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<tr>
<th>(&lt;r&gt;)</th>
<th>(\sigma = 0.2 \text{nm})</th>
<th>(\sigma = 0.3 \text{nm})</th>
<th>(\sigma = 0.4 \text{nm})</th>
<th>(\sigma = 0.5 \text{nm})</th>
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<tr>
<td>1.7 nm</td>
<td>1.69 nm</td>
<td>1.70 nm</td>
<td>1.72 nm</td>
<td>1.73 nm</td>
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<tr>
<td>increase of the distance with the width</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.75 nm</td>
<td>3.69 nm</td>
<td>3.60 nm</td>
<td>3.47 nm</td>
<td>3.30 nm</td>
</tr>
<tr>
<td>decrease of the distance with the width</td>
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</table>
Figure 5.9: Analysis of the suppression effect for distance distributions with a mean distance of \( r = 3.75 \text{ nm} \) (left panel) and \( r = 1.7 \text{ nm} \) (right panel) and a width of 0.2 nm (red), 0.3 nm (green), 0.4 nm (blue) and 0.5 nm (violet). The mean distance is marked as dashed line. A) Simulated Gaussian distance distribution for the given parameters. B) Normalized distance distributions after \( r^{-6} \) averaging (\( P(r) \times r^{-6} \)) show a shift of the distances toward smaller values. For the mean distance of 1.7 nm the averaging leads to a significant contribution of very short distances. C) The application of a suppression function \( S(r) = \exp \left[-C \cdot \left(\frac{r_0}{r}\right)^6\right] \) to the averaged distance distribution effects the obtained mean distance and the short, not detectable, distances are neglected.
the detection of such short distances is significantly decreased. Eventually, the broad distance distributions include also longer distances and the mean distance is rising in such a case. The increase of the mean distance for the intermediate distance range can even overcome the down-wards shift by $r^{-6}$ averaging and the resulting mean distance rises above the input distance for broad Gaussian distributions. The mean distance obtained for the presented cases including the $r^{-6}$ averaging and the suppression of short distances are summarized in table 5.3.

Coming back to the experimental results from WALP23 for position 07 and 11, one might be able to explain the extracted distances information in a similar manner. The distance distributions obtained by DEER on the Gd$^{3+}$ loaded WALP23 constructs are rather broad and as shown before the width of the original distance distribution is influencing the shift of the mean distance extensively. A possible reason for the coinciding of the distance might be a reduction of the apparent distance for position 11 due to the $r^{-6}$ averaging and a increase of the distance for position 07 due to the strong suppression of short distances and therefore the shift towards longer distances. In order to get a more detailed understanding of the effects leading to the apparent suppression and consequently the ‘wrong extraction’ of distances for short inter-spin distances, further experiments with better defined distance distribution in the intermediate distance range are needed. To summarize, possible reasons causing the suppression of short distances were discussed and the analysis shows no significant orientation dependence in the range where distances can be extracted. The variation of the detection sequence to $T_0 = 100\, \text{ns}$ sounds promising in order to reduce the suppression effect. Furthermore, it might be possible to influence the suppression limit by working at higher fields, where the $\Delta k$ effect is reduced. Also the usage of other lanthanide ions showing different relaxation behaviour could affect the detectability for short distances.

### 5.1.5 Relaxation Enhancement in Gold Nanoparticles

As described in section 3.3, gold nanoparticles (Au-NPs) carry 49 ligands which can be loaded with Ln$^{3+}$ ions. A rather broad distribution of distance between Gd$^{3+}$ and nitroxide radicals was obtained by DEER and the shape of the distance distribution was slightly affected by the amount (%) of Ln$^{3+}$ loaded. The triangular distance distribution, arising from the particle on a sphere model, contains a substantial amount of short distances which will be suppressed due to the dead time in the inversion recovery detection sequence. By assuming a random distribution of the Ln$^{3+}$ ions, the percental loading of the 49 chelating ligands on the surface of the Au-NPs leads to Au-NPs carrying one, two or even no Ln$^{3+}$ ions. The effect of an incomplete labelling of Au-NPs can result in a different relaxation enhancement effect because nitroxide spin labels having no Dy$^{3+}$ in the close neighbourhood will not show an enhanced relaxation, but will still contribute to the observed inversion recovery trace.
Chapter 5. Relaxation Enhancement

Figure 5.10: Relaxation enhancement effect induced on Au-NPs with different Dy\textsuperscript{3+} loading, 1\% (dark green), 2\% (green), and 4\% (light green). The extracted distance decreases with increased loading. The maximum $\Delta k$ effect is observed at $T_{\text{max}} = 95.2$ K.

The relaxation enhancement effect for Au-NPs loaded with 1\%, 2\%, or 4\% of Dy\textsuperscript{3+} is calculated by equation \ref{eq:5.5} and shown in figure 5.10. The magnitude of $\Delta k$ increases continuously with increased Dy\textsuperscript{3+} content and therefore a shortening of the distance is implied. The experimental data points were fitted by applying the average relaxivity approximation where the Dy\textsuperscript{3+} relaxation time is given by

\begin{equation}
T_{1f} = 1.3 \cdot 10^{-11} \left( \frac{T_{\text{max}}}{T} \right)^p
\end{equation}

with $T_{\text{max}} = 95.2$ K for all samples. The extracted single distance, shown in figure 5.10 varies by about 30\% (0.9 nm) although the distance distribution obtained by DEER on Gd\textsuperscript{3+} loaded Au-NPs reveals an almost constant shape of the distance distribution with the longest detectable distance of 4.6 nm. The apparent increase of the obtained distances might be related to the incomplete labelling of Au-NPs. In table 5.4 the probability for the number of the occupied chelating ligands ($n$) as a function of the loading with Ln\textsuperscript{3+} is given.

It becomes obvious that especially for a small loading content a substantial fraction of nanoparticles does not carry any Dy\textsuperscript{3+} and therefore the relaxation of the attached nitroxide spin labels will not be influenced. This leads to a distortion of the measured inversion recovery trace and thus to a prolonged observed relaxation time. To the first approximation one can assume that the extracted $T_1$ times of the nitroxide for the loaded Au-NPs are non-linear averages of the ones having a Dy\textsuperscript{3+} close by and showing a relaxation enhancement effect and the ones revealing no enhanced relaxation due to the absence of the fast relaxing Dy\textsuperscript{3+}. The relaxation of the undisturbed nitroxide radical is known from the measurement of the diamagnetic reference loaded with La\textsuperscript{3+} ($T_{1s,0}$).
5.1. Longitudinal Relaxation Enhancement Induced by Dysprosium

Table 5.4: Probability of occupied chelating ligands for a random distribution of Ln$^{3+}$ ions on the surface of Au-NPs for different loadings

<table>
<thead>
<tr>
<th>Loading</th>
<th>$P(n)$ of occupied chelating ligands $n$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$n = 0$</td>
</tr>
<tr>
<td>1%</td>
<td>61.1%</td>
</tr>
<tr>
<td>2%</td>
<td>37.2%</td>
</tr>
<tr>
<td>4%</td>
<td>13.5%</td>
</tr>
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</table>

In order to illustrate the potential effect of the incomplete labelling on $\Delta k$, all species are assumed to show mono-exponential decay behaviour with the relaxation time of the nitroxide radical in the presence of Dy$^{3+}$ being estimated as follows:

$$\frac{1}{T_{1s}(\text{Dy}^{3+}+\text{La}^{3+})} = [1 - P(0)] \cdot \frac{1}{T_{1s}(\text{Dy}^{3+})} - P(0) \cdot \frac{1}{T_{1s,0}(\text{La}^{3+})}$$ \hspace{1cm} (5.6)

with $T_{1s}(\text{Dy} + \text{La})$ being the observed longitudinal relaxation time for the loaded Au-NPs, $P(0)$ being the probability of having no Dy$^{3+}$ in the close vicinity of the nitroxide spin label and $T_{1s}(\text{Dy})$ representing the nitroxide relaxation time in the presence of a fast relaxing Dy$^{3+}$ centre. Since, $T_{1s}(\text{Dy} + \text{La})$ and $T_{1s,0}(\text{La})$ can be determined experimentally, the induced relaxation enhancement effect by Dy$^{3+}$ can be calculated using equation 2.27 and the new resulting temperature dependence of $\Delta k$ is shown in figure 5.11 as triangles in combination with the previously extracted data (circles). The calculated relaxation enhancement effect induced by Dy$^{3+}$ is bigger than the one extracted directly for fractionally loaded Au-NPs. Thus, a decrease of the distance towards the expected distance determined by DEER on Gd$^{3+}$-nitroxide spin pairs is implied. The correction of the extracted distance increased with the amount of unloaded nanoparticles from 4% to 1%. The variation of the extracted distance for the differently loaded Au-NPs is decreasing by 50%.

The most striking effect of the corrected $\Delta k$ effect is obtained for 1% Dy$^{3+}$ loaded Au-NPs. In this case only a minor fraction with 2 or more Dy$^{3+}$ per particle is present, whereas for higher percental Dy$^{3+}$ loading also multiple spin interactions exist. The distance distributions determined by DEER show a longest cut-off distances of approximately 4.3 nm and the mean distance can be approximated to 3 nm. The original $\Delta k$ data for 1% Dy$^{3+}$ loaded Au-NPs reveal a distance of 4.2 nm. This is within the cut-off range determined by DEER. Nevertheless, the $r^{-6}$ averaging should shift the extracted $< r^{-6} >^{1/6}$ toward a significant smaller value. By correcting for incomplete labelling and assuming a random distribution of Dy$^{3+}$ a decrease of the distance to 3.6 nm is found. This is a more realistic representation of the situation present in Au-NPs. A refinement for the extraction of the undisturbed $T_{1s}(\text{Dy}^{3+})$ times might therefore lead to a more precise result for the obtained distance in case of incomplete labelling. Another possible disturbance for the relaxation enhancement effect for Au-NPs might originate from the nature of the nanoparticle,
Figure 5.11: Relaxation enhancement effect induced on Au-NPs with different Dy$^{3+}$ loading, 1% (dark green), 2% (green), and 4% (light green). The extracted distance decreases with increases with increased loading and the maximum $\Delta k$ effect is observed at $T = 95.2\,K$. The original data are shown as circles and the corrected data taking into account the incomplete Dy$^{3+}$ labelling (equation 5.6) are shown as triangles.

which can be considered as small metallic particle and could additionally induce a change of the relaxation behaviour of the present paramagnetic species. The relaxation times of Dy$^{3+}$ ($T_1$) do not appear to be significantly influenced because a similar temperature dependence of $T_1$ is obtained for the $\alpha$-helical WALP23 polypeptides and the loaded Au-NPs. However, nitroxide relaxation might change due to the close neighbourhood to a metallic core of the Au-NPs. Furthermore, for the case of Ln$^{3+}$ loaded Au-NPs a very broad distribution of distances between the Ln$^{3+}$ ion and the nitroxide spin label is present and the $r^{-6}$ averaging as well as the suppression of short distances might influence the precision of the extracted distance information. Especially, the very short distances giving rise to a strong $\Delta k$ effect are completely cut-off and do not contribute to the observed inversion recovery traces. The combination of the discussed effects on $\Delta k$ can account for the prolongation of the determined distance using average relaxivity approximation.

In general the incomplete labelling of the system of interest leads to a distortion of the observed $\Delta k$ effect biased by the amount of nitroxide radicals that miss a fast relaxing partner within the same nano-object.

5.2 Other Relaxation Enhancement Processes

5.2.1 Longitudinal Relaxation Enhancement induced by Gadolinium

Bloembergen-Purcell-Pound theory (BPP), based on second order perturbation treatment is applicable to fast relaxing agents satisfying the conditions of the
Redfield regime ($\tau_c << \omega_{DD}$). Relaxation of gadolinium ions (Gd$^{3+}$) is not as fast as for dysprosium ions (Dy$^{3+}$) [93] and the applicability of this lanthanide for relaxation-based distances measurements has to be tested.

![Figure 5.12: Temperature dependence of the relaxation enhancement ($\Delta k$) for $\alpha$-helical WALP23 constructs labelled with Gd$^{3+}$ at the N-terminus and MTSSL at the positions 07 (yellow), 11 (orange), 15 (green) or 19 (blue). The solid lines are not fitted to the data points, but shall indicate the tendency of the temperature dependence.](image)

In figure 5.12 the temperature dependence of $\Delta k$ for all MTSSL labelled constructs of WALP23 loaded with Gd$^{3+}$ is shown. Gd$^{3+}$ has a pronounced effect on $T_1$ times of the nitroxide spin label. Position 07 shows the strongest relaxation enhancement effect, which decreases continuously with the labelling position. The experimental data reveal no consistent distance dependence of $\Delta k$. These findings imply that BPP theory is not applicable in this distance and frequency range for a spin pair consisting of Gd$^{3+}$ and nitroxide radicals. Therefore, the investigated system is not suitable for distance determination. Nevertheless, the presence of Gd$^{3+}$ in the close neighbourhood of a nitroxide spin label is influencing its longitudinal relaxation time ($T_1$). The systematic investigation on a well characterized model system like labelled WALP23 polypeptides could prove the deviation from the $r^{-6}$ distances dependence. Eventually, care has to be taken by examining $\Delta k$ effects on pairs where the relaxation times of the fast relaxing species is not significantly smaller than the dipolar coupling because an enhancement of the nitroxide relaxation can still be observed although the underlying basic conditions for distance determination are not valid. This situation may apply to several studies where Fe(III) ions were used as the fast relaxing species.

5.2.2 Transverse Relaxation Enhancement

Bloembergen-Purcell-Pound theory (BPP) was developed not only for the longitudinal relaxation time ($T_1$) of the slowly relaxing species but is also applicable
to transverse relaxation times ($T_2$). In the following section $T_2$ relaxation enhancement on slowly relaxing nitroxide spin labels induced by Dy$^{3+}$ or Gd$^{3+}$ is investigated on WALP23 polypeptides.

In figure 5.13 temperature-dependent $T_2$ relaxation enhancement data are shown. The experimental data were fitted by a mono-exponential decay and $\Delta k$ was calculated according to equation 2.27:

$$\Delta k(r, \theta) = \frac{1}{T_{2s}} - \frac{1}{T_{2s,0}}$$

(5.7)

The experimental data do not show the expected distance dependence. In cases where Dy$^{3+}$ is in the close vicinity to the nitroxide label, the temperature of maximum relaxation enhancement effect is not reached within the accessible temperature range (20K-100K). The transverse relaxation time ($T_2$) enhancement effect induced by Dy$^{3+}$ shows a tendency of increased magnitude for shorter distance, but the expected $r^{-6}$ dependence can not be observed. This behaviour might be explained by the fact that relaxation properties of Dy$^{3+}$ ($T_{1f}, T_{2f}$) in the investigated temperature range do not match the condition of optimum enhancement. By changing the fast relaxing agent and therefore $\omega_f$, $T_{1f}$ and $T_{2f}$, the relaxation enhancement ($\Delta k$) can be influenced. Further investigation for optimum relaxation enhancement conditions including the choice of fast relaxing agent still has to be done in a systematic manner in order to evaluate $T_2$ relaxation enhancement. Generally, one can say that due to the absence of a temperature maximum the determination of the required $T_{1f}$ is very difficult and furthermore the ratio $T_{2s,0}/T_{2s}$ is much smaller than $T_{1s,0}/T_{1s}$ which reduces the magnitude of $\Delta k$. Therefore, it is more advantageous to use $T_1$ relaxation enhancement then $T_2$ relaxation enhancement induced by Dy$^{3+}$.

![Figure 5.13](image)

Figure 5.13: Temperature dependence of $T_2$ relaxation enhancement ($\Delta k$) for $\alpha$-helical WALP23 constructs labelled with A) Dy$^{3+}$ or B) Gd$^{3+}$ at the N-terminus and MTSSL at the positions 07 (yellow), 11 (orange), 15 (green) or 19 (blue). The solid lines are not fitted to the data points, but shall indicate the tendency of the temperature dependence.

The presence of Gd$^{3+}$ in the close neighbourhood of a nitroxide spin label induces a transverse relaxation enhancement effect. The maximum effect can be...
observed in a range between 70K and 90K. The experimental $\Delta k$ data do not show any distance dependence and the enhancement effect is super-imposable for all four Gd$^{3+}$ loaded WALP23 constructs. Gd$^{3+}$ is a commonly magnetic resonance image (MRI) contrast agent [94] and influences the relaxation rate of protons in the investigated sample. If the relaxation of matrix protons of the DOPC lipid bilayer in WALP23 samples is enhanced also the transverse relaxation of the nitroxide radical can be enhanced significantly. Therefore, the presence of Gd$^{3+}$ might be related to an enhancement of proton relaxation and, consequently, an enhanced nitroxide radical transverse relaxation. One can speculate that if the effect for matrix protons is strong the observed effect is similar throughout the membrane and similar for all four nitroxide labelled WALP23 constructs. In any case, for Gd$^{3+}$, the $T_2$ relaxation enhancement is most probably dominated by some indirect interactions, since no distance dependence is observed.

To conclude, transverse relaxation enhancement can not be used for distance determination in the investigated spin pairs where the nitroxide relaxation times are influenced by the presence of Dy$^{3+}$ or Gd$^{3+}$. For a more detailed analysis a systematic study with different fast relaxing agents at different inter-spin distances is needed in order to rule out suppression effects like the one seen for short distances in $T_1$ relaxation enhancement.

5.3 Three Spin Interaction - Oxygen Effect

In contrast to NMR, where multi-spin systems are investigated on a regular basis, in EPR mainly pairwise interactions are studied. In case more than two paramagnetic species are present the understanding of the interaction scheme becomes more challenging, especially if the interaction is detected indirectly, like in relaxation enhancement. Here, a triad of paramagnetic species consisting of a slowly relaxing nitroxide radical, a fast relaxing Ln$^{3+}$ ion, and a fast relaxing paramagnetic oxygen (O$_2$) is studied and the influence of residual O$_2$ dissolved in the lipid bilayer on the distance obtained by relaxation enhancement is investigated.

Under accessible conditions O$_2$ can not be directly observed by EPR, because it is relaxing too fast on the EPR time-scale. It is known, that O$_2$ is not homogeneously distributed throughout the membrane but a so called O$_2$-profile is present. In figure 5.14A such a O$_2$-profile (blue) is shown for WALP23 in a DOPC bilayer [75], obtained from pulsed saturation recovery measurements on a series of nitroxide spin labelled WALP23 constructs in presence and absence of O$_2$.

The O$_2$-profile reveals a higher oxygen concentration in the middle of the membrane whereas in the region of polar head groups less O$_2$ is dissolved. In this section the effect of O$_2$ in the membrane on the relaxation enhancement effect measured between a nitroxide spin label and a Ln$^{3+}$ ion is investigated on
WALP23 polypeptides. The nitroxide spin label position 11 is chosen for these studies because in the middle of the membrane the biggest effect is expected. It is well-known that the fast relaxation of O$_2$ influences the relaxation time of nitroxide radicals. Furthermore, the relaxation enhancement effect induced on the nitroxide radical by a fast relaxing Ln$^{3+}$ ion has been discussed earlier in this chapter.

In the case of weak interaction between O$_2$ and Ln$^{3+}$, both $\Delta k$ effects (O$_2$ and Ln$^{3+}$) will be additive and the presence or absence of O$_2$ will induce the same $\Delta k$ effect for samples loaded with paramagnetic and diamagnetic lanthanide ions. In this case the extracted distance information for relaxation enhancement should not be influenced if the diamagnetic reference and fast relaxing sample both contain the same amount of residual oxygen. Another possibility is the presence of a strong interaction between O$_2$ and Ln$^{3+}$, where an intertwined effect of O$_2$ and Ln$^{3+}$ on the relaxation of the nitroxide spin label would be observed. If so, $\Delta k$ induced by the presence of O$_2$ is influenced by the presence of a paramagnetic Ln$^{3+}$ and the obtained distance information will be biased by the interaction of both fast relaxing species (O$_2$ and Ln$^{3+}$). In order to investigate the potential influence of O$_2$ in combination with a fast relaxing paramagnetic species on the longitudinal relaxation time ($T_1$) of the slowly relaxing nitroxide radical in a first step, the pure effect of O$_2$ has to be studied and can be compared with the effect present in case of additional fast relaxing species in the close vicinity of the spin system (figure 5.14 B). The
enquiry of the isolated $\text{O}_2$ impact was analysed with WALP23_A11R1 loaded with diamagnetic $\text{La}^{3+}$ where additional pathways for $\Delta k$ are blocked and only the pairwise interaction nitroxide spin label - $\text{O}_2$ is present. As already described in section 3.2, WALP23 samples were prepared with carefully multiply degassed buffer. Nevertheless, during sample preparation the suspension is in contact with air and a fraction of $\text{O}_2$ is dissolving in the lipid bilayer. Therefore these samples contain residual $\text{O}_2$ and will be called ‘$\text{O}_2$ containing’. An additional degassing of the samples directly before shock freezing can remove a substantial amount of $\text{O}_2$ from the membrane and in the following I will refer to this kind of samples as ‘$\text{O}_2$ free’.

In order to evaluate the relaxation enhancement effect induced by oxygen, a new quantity $\Delta \kappa(O_2, \text{Ln}^{3+})$ is introduced analogous to equation 2.27 as

$$
\Delta \kappa(O_2, \text{Ln}^{3+}) = \frac{1}{T_{1s}(\text{Ln}^{3+}, \text{‘O}_2\text{-containing’})} - \frac{1}{T_{1s}(\text{Ln}^{3+}, \text{‘O}_2\text{-free’})}
$$

with $T_{1s}(\text{Ln}^{3+}, \text{‘O}_2\text{-free’})$ being $T_1$ of the slowly relaxing nitroxide radical in the presence of a particular $\text{Ln}^{3+}$ ion under ‘$\text{O}_2$ free’ conditions and $T_{1s}(\text{Ln}^{3+}, \text{‘O}_2\text{-containing’})$ is $T_1$ of the slowly relaxing nitroxide radical in the presence of the same particular $\text{Ln}^{3+}$ ion and additional residual $\text{O}_2$.

The investigation of the temperature dependent relaxation enhancement induced exclusively by oxygen ($\Delta \kappa(O_2, \text{Ln}^{3+})$) is performed on $\text{La}^{3+}$ (diamagnetic) loaded WALP23_A11R1 with the nitroxide spin label in the middle of the lipid bilayer (pairwise interaction [NO-$\text{O}_2$]). In figure 5.15A the temperature dependence of $T_{1s}(\text{La}^{3+}, \text{‘O}_2\text{-containing’})$ is depicted and it can be seen that with a conscientious degassing of the buffer (blue) reproducible conditions are reached. In case the buffer is not carefully (cyan) or not at all degassed (purple) the $\text{O}_2$ concentration in the membrane is higher and an increased relaxation of the nitroxide radical can be observed. Degassing of the samples shortly before shock freezing removes reproducibly the residual $\text{O}_2$ from the membrane and the $T_1$ relaxation times are
similar for repeated experiments under 'O$_2$-free' conditions ($T_{1s}$ (Ln$^{3+}$, 'O$_2$-free')) figure 5.15B). The $\Delta \kappa$(O$_2$, Ln$^{3+}$) is calculated according to equation 5.8 with La$^{3+}$ as particular Ln$^{3+}$ ion and the temperature dependence is shown in figure 5.15C for the different preparation conditions.

Multiple degassing of the buffer before the preparation ensures a reproducibly small $\Delta \kappa$(O$_2$, Ln$^{3+}$) effect on the nitroxide relaxation in the presence of a diamagnetic surrounding (La$^{3+}$). In case of elevated O$_2$ concentration in the buffer the magnitude of $\Delta \kappa$(O$_2$, Ln$^{3+}$) increases dramatically. Hence, one has to work under optimum conditions with a multiply degassed buffer. The investigation concerning the cross effect of three paramagnetic species (nitroxide radical - O$_2$ - paramagnetic Ln$^{3+}$) is performed with multiply degassed buffer solution.

The presence of an additional paramagnetic species (Dy$^{3+}$ or Gd$^{3+}$) opens up new pathways for enhancing the relaxation of the observed slowly relaxing nitroxide radical (figure 5.14B). In the three-spin system, an enhancement induced by paramagnetic Ln$^{3+}$ is present on top of the one induced exclusively by O$_2$. One pathway is the direct interaction of the nitroxide radical with the fast relaxing paramagnetic Ln$^{3+}$ ion, as described previously in this chapter. Furthermore, the nitroxide relaxation is enhanced by the presence of O$_2$. An additional pathway could be provided by a strong interaction of O$_2$ and paramagnetic Ln$^{3+}$, which can change their relaxation properties and consequently the relaxation enhancement effect on $T_1$ of the slowly relaxing nitroxide radical. In order to investigate if an additional strong interaction between O$_2$ and Ln$^{3+}$ takes place, $T_1$ of the nitroxide radical in the presence of the same fast relaxing Ln$^{3+}$ is measured under 'O$_2$-free' and 'O$_2$-containing' conditions and $\Delta \kappa$(O$_2$, Ln$^{3+}$) is calculated according to equation 5.8:

$$\Delta \kappa(O_2, \text{Gd}^{3+}) = \frac{1}{T_{1s} \ (\text{Gd}^{3+}, \ 'O_2\text{-containing}')} - \frac{1}{T_{1s} \ (\text{Gd}^{3+}, \ 'O_2\text{-free}')},$$

or

$$\Delta \kappa(O_2, \text{Dy}^{3+}) = \frac{1}{T_{1s} \ (\text{Dy}^{3+}, \ 'O_2\text{-containing}')} - \frac{1}{T_{1s} \ (\text{Dy}^{3+}, \ 'O_2\text{-free}')},$$

(5.9)

with Gd$^{3+}$ or Dy$^{3+}$ as Ln$^{3+}$ ions. For a weak coupling between Ln$^{3+}$ and O$_2$ the relaxation enhancement effect in the presence of all three paramagnetic species is expected to be additive and $\Delta \kappa$(O$_2$, Ln$^{3+}$) should be the same as for the diamagnetic La$^{3+}$ reference. In this case the influence of O$_2$ on $\Delta k$ could be neglected when measuring the fast relaxing and the diamagnetic reference sample under the same 'O$_2$-containing' conditions. In case of strong coupling, the interaction between Ln$^{3+}$ and O$_2$ is capable to change the relaxation properties of one or both species and non-additive effects in the relaxation enhancement measurements are expected.

Figure 5.16 depicts the temperature dependent $\Delta \kappa(O_2, \text{Ln}^{3+})$ effect in the presence of the diamagnetic La$^{3+}$, and the paramagnetic Gd$^{3+}$, or Dy$^{3+}$. In comparison to La$^{3+}$ a strong $\Delta \kappa(O_2, \text{Ln}^{3+})$ effect can be observed for the paramagnetic Ln$^{3+}$. The magnitude of $\Delta \kappa(O_2, \text{Ln}^{3+})$ is more pronounced in the presence of Dy$^{3+}$. This behaviour implies that the significant change of $\Delta \kappa(O_2, \text{Ln}^{3+})$
results in a change of the lanthanide induced $\Delta k$ effect in presence and absence of $O_2$.

\[ \Delta \kappa \left( O_2, Ln^{3+} \right) = \frac{1}{T_{1f}(Gd^{3+})_{O_2\text{-containing}}} - \frac{1}{T_{1f}(Gd^{3+})_{O_2\text{-free}}} \quad \text{(5.10)} \]

The temperature dependent $\Delta \kappa(O_2, Ln^{3+})$ effect induced by the presence of two fast relaxing paramagnetic species in addition to the nitroxide spin label leads to an intertwined, strong interaction, where $O_2$ and the fast relaxing paramagnetic $Ln^{3+}$ ion apparently influence each others relaxation behaviour. $Dy^{3+}$ and $O_2$ are relaxing too fast in order to be detected with pulse EPR techniques. However, $Gd^{3+}$ can be observed and $T_1$ relaxation times are measured in a temperature range from 40 K to 80 K on Gd$^{3+}$ loaded DOTA complex at the N-terminus of WALP23_A11R1 in the presence and absence of $O_2$. The resulting inversion recovery traces at 40 K are shown in figure 5.17 A. A stretched exponential decay can represent the experimental trace and the fitted longitudinal relaxation time ($T_{1f}$) of Gd$^{3+}$ are shown in figure 5.17 B. It can be seen, that $T_{1f}(Gd^{3+})$ changes due to the presence of $O_2$ and the enhancement of $T_{1f}(Gd^{3+})$ ($\Delta \kappa_{Gd^{3+}}(O_2)$) can be calculated according to equation 5.8:

\[ \Delta \kappa_{Gd^{3+}}(O_2) = \frac{1}{T_{1f}(Gd^{3+})_{O_2\text{-containing}}} - \frac{1}{T_{1f}(Gd^{3+})_{O_2\text{-free}}} \cdot \]

The temperature dependent $\Delta \kappa_{Gd^{3+}}(O_2)$ shown in figure 5.17 C, representing the apparent change of the Gd$^{3+}$ relaxation times, reveals an interaction with $O_2$. This additional interaction gives rise to the observed enhancement of the nitroxide relaxation in the presence of Gd$^{3+}$ under ‘$O_2$-free’ and ‘$O_2$-containing’ conditions (figure 5.16). It can not be excluded that also the relaxation time
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of O$_2$ is changing, but already a one-way change is sufficient to explain the experimental trend. In case of Dy$^{3+}$ the pairwise interaction between O$_2$ and the fast relaxing Ln$^{3+}$ can not be experimentally tested beyond the data shown in figure 5.16.

![Figure 5.17: Relaxation behaviour of Gd$^{3+}$ loaded on N-terminal DOTA in DOPC incorporated WALP23_A11R1. A) Inversion recovery trace of Gd$^{3+}$ at 40K under 'O$_2$-free' (black) and 'O$_2$-containing' (blue) conditions. The residual of the experimental data and a stretched exponential fit (red) is shown in the inset. B) Temperature dependent $T_1f$ relaxation times. C) Relaxation enhancement effect induced by O$_2$ on the Gd$^{3+}$ relaxation time $\Delta \kappa_{Gd^3+}(O_2)$.](image)

The relaxation of O$_2$ estimated to be 7.5 ps at room temperature [95] is very fast and it can be assumed that also at lower temperatures fast relaxation occurs although it was never measured in solution. Therefore, it can be assumed that O$_2$ relaxes faster than relaxation observed for relaxation agents such as Gd$^{3+}$ ($\approx 1 \mu$s - figure 5.17). The effect of a slowly relaxing species on the relaxation behaviour of a significantly faster relaxing centre is minor and can be neglected in a first approximation. Hence, it can be assumed that the very fast relaxing O$_2$ is not influenced by the slower relaxing paramagnetic Gd$^{3+}$ centres in the system. It could be shown, that Gd$^{3+}$ relaxation is enhanced by the presence of O$_2$ and the relaxation times of the two species differ significantly. In case of Dy$^{3+}$ the relaxation time difference reduces dramatically ($\approx 1$ ns - figure 5.6) [93]. Hence, one can not speculate about the direction of interaction, but the resulting additional enhancement of the nitroxide relaxation could be proven (figure 5.16).

The O$_2$-paramagnetic Ln$^{3+}$ interaction might be intertwined and therefore the directionality can not be disentangled. Nevertheless, an additional effect on the nitroxide relaxation could be observed due to the simultaneous presence of a fast relaxing Ln$^{3+}$ centre and O$_2$ dissolved in the lipid bilayer. This increased relaxation enhancement can also affect the determined distance information from $\Delta \kappa$.

By coming back to the original formula describing relaxation enhancement ($\Delta k$) (equation 2.27), where the La$^{3+}$ loaded samples is used as diamagnetic reference and the relaxation enhancement is induced by a fast relaxing paramagnetic species (Dy$^{3+}$ or Gd$^{3+}$), the influence of O$_2$ on the extracted distance can be evaluated. Therefore, both samples (diamagnetic and paramagnetic) are prepared under either 'O$_2$-free' conditions or 'O$_2$-containing' conditions. The temperature
dependent $\Delta k$ for both conditions is shown in figure 5.18 and an increased $\Delta k$ effect due to presence of $O_2$ is obtained. This behaviour implies the extraction of apparent shorter distances under 'O$_2$-containing' conditions as compared to 'O$_2$-free' conditions.

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 5.18:** Influence of residual dissolved $O_2$ in lipid bilayer for WALP23 A11R1 loaded with A) Dy$^{3+}$ or B) Gd$^{3+}$. The temperature dependent $\Delta k$ effect under 'O$_2$-free' conditions is shown in orange whereas the 'O$_2$-containing' conditions are depicted in red (Dy$^{3+}$) and green (Gd$^{3+}$).

In the presented case of WALP23 A11R1 the examined change of $\Delta k$ is clearly visible for Dy$^{3+}$ and Gd$^{3+}$ and the magnitude $\Delta k$ changes in both cases by approximately 20%. In the case of Dy$^{3+}$ the relaxation enhancement effect under 'O$_2$-containing' conditions was fitted by applying the average relaxivity approximation including the assumption that $T_1 \rho (Dy^{3+})$ does not change significantly. The determined distance of 2.07nm is shorter than the one under 'O$_2$-free' conditions with 2.15nm. The distance obtained from $\Delta k$ is already underestimated with respect to DEER data (table 5.1) and the presence of $O_2$ adds to this error.

This shows that nitroxide relaxation enhancement by paramagnetic lanthanide ions and oxygen is not additive. For distance measurements via relaxation enhancement on membrane incorporated systems it is thus important to carefully degas the sample before shock-freezing.

### 5.4 Conclusion

In this chapter the relaxation enhancement ($\Delta k$) effect on the relaxation times of a nitroxide spin label induced by two different fast relaxing paramagnetic lanthanide ions (Gd$^{3+}$ and Dy$^{3+}$) was investigated. Gd$^{3+}$ can not be treated within the Redfield limit due to too slow relaxation on the microsecond time scale compared to the dipolar coupling on the Megahertz frequency scale. The $\Delta k$ effect induced by Gd$^{3+}$ is thus not scaling with the inter-spin distance.
Whether this is related to the non-Redfield behaviour or results from other indirectly induced processes cannot be disentangled. Thus it is important to perform systematic studies on the induced $\Delta k$ effect if a new fast relaxing species is introduced. In contrast to Gd$^{3+}$, Dy$^{3+}$ relaxes much faster on a nanosecond time scale and can be treated within the Redfield regime. The relaxation enhancement effect induced by Dy$^{3+}$ on the longitudinal relaxation time ($T_1$) of nitroxide radicals scales systematically with $r^{-6}$ and the extracted distance information is consistent with distances obtained from DEER on Gd$^{3+}$-nitroxide spin pairs on the same system. The resulting distance obtained by applying the average relaxivity approximation shows a tendency of underestimation due to $r^{-6}$ averaging. The distortion is not related to the isotropic averaging over all principal axis orientations of Dy$^{3+}$.

The systematic analysis of the relaxation enhancement effect of Dy$^{3+}$ on nitroxide radicals according to the averaged relaxivity approximation shows that on $\alpha$-helical polypeptides incorporated into a lipid bilayer distances in a range of 2-3 nm can be extracted. The indirect measurement of the dipolar interaction in Dy$^{3+}$-nitroxide spin pairs with a distance up to 6 nm should still show an enhancement of the $T_{1s}$ relaxation of approximately 20% with respect to a diamagnetic reference measurement $T_{1s,0}$ at X-band frequencies. The determination of this difference is experimentally feasible and therefore such long distance information can potentially be provided by relaxation enhancement. By performing the experiment at lower microwave (m.w.) frequencies the available distance range should increase further.

For short inter-spin distances ($r \leq 2$ nm) suppression effects are observed. The extraction of very short distances is influenced by the length of the first interpulse delay ($T_0$) in the inversion recovery experiment and a reduction of $T_0$ can decrease the discussed suppression effect for short distances. Another possibility of influencing the suppression effect are the usage of different types of Ln$^{3+}$-ions with reduced relaxivity, and experiments at higher fields, where $\Delta k$ is reduced due to the interplay of the resonance frequencies in the spin system ($\omega \propto B_0$) and the relaxation time of the fast relaxing species ($T_{1f}$). In case longer distances have to be determined it is advantageous to perform the relaxation time measurements at lower m.w. frequencies, due to the increased $\Delta k$ effect present. Furthermore, care must be taken in extracting distance information from incompletely labelled systems. This leads to a reduction of $\Delta k$ and thus to a longer apparent distances. If the labelling efficiency is known or can be estimated it is possible to correct for the overestimation to a certain extent by evaluating the time domain data as a superposition of labelled and unlabelled species.

Experimentally a non-additive relaxation enhancement effect on the relaxation time of nitroxide radicals can be observed when two types of fast relaxing paramagnetic species (O$_2$ and Ln$^{3+}$) are present. This reveals the change of the relaxation properties of at least one of fast relaxing agents. In the studied case, the non-additive effect, due to the presence of residual O$_2$, produced only a small distortion of the $\Delta k$ effect and the corresponding distance distortion was on the order of a few percent. Nevertheless, if possible the investigated sample should be degassed in order to obtain more precise distance information. On
the other hand, the relaxation enhancement induced by oxygen ($\Delta\kappa(O_2, \text{Ln}^{3+})$) increased dramatically in the presence of paramagnetic $\text{Ln}^{3+}$. Therefore, it is crucial to take care about the presence of additional paramagnetic species if the effect of $O_2$ on the relaxation properties of a nitroxide is investigated e.g. by accessibility measurements.

The temperature dependent $\Delta k$ behaviour encodes not only the distance between the fast relaxing paramagnetic species and the slowly relaxing nitroxide radical, due to the amplitude, but also the relaxation time of the fast relaxing species ($T_{1f}$) can be extracted from the shape of $\Delta k(T)$. This information is very valuable because the direct detection of e.g. $T_{1f}(\text{Dy}^{3+})$ is challenging, due to the fact that $\text{Dy}^{3+}$ is not detectable in the investigated temperature range by pulse EPR. Therefore, it is important to stress that the extracted relaxation information $T_{1f}(\text{Dy}^{3+})$ from $\Delta k$ goes in line with data shown in literature.
6. Summary and Outlook

The dipole-dipole interaction in orthogonal spin pairs consisting of lanthanide ions \( \text{Ln}^{3+} \) and nitroxide radicals can be detected directly by double electron-electron resonance (DEER) or indirectly by relaxation enhancement \( (\Delta k) \). Both methods provide reliable distance information, if the gadolinium ion \( \text{Gd}^{3+} \) is detected in DEER measurements or the dysprosium ion \( \text{Dy}^{3+} \) is inducing relaxation enhancement on the nitroxide radicals. Both methods can cover a broad range of applications, reaching from biological to material science applications. The choice of the method should be determined by the intrinsic properties of the studied system and the resulting restrictions.

If short distances, approaching the discussed suppression limit \( (\leq 2\text{nm}) \) present for the evaluation of \( T_1 \Delta k \) data, have to be determined it is more advantageous to perform DEER measurements on \( \text{Gd}^{3+} \)-nitroxide spin pairs, where also the distribution of distances is covered by the direct detection of the dipolar frequencies. On the other hand the detection of long distances by DEER is limited due to the transverse relaxation time \( (T_2) \) of \( \text{Gd}^{3+} \), which is in the same order of magnitude than \( T_2 \) of nitroxide spin labels (figure 4.10), where the upper distance limit is approximately 5nm for membrane incorporated biomacromolecules and biomicromolecular complexes. In this situation, it is rewarding to use the determination of the longitudinal relaxation time \( (T_1) \) of nitroxide spin labels in the presence and absence of \( \text{Dy}^{3+} \), where distances up to 6nm can potentially be extracted from orientation averaged relaxation enhancement \( (\Delta k) \). Alternatively, the DEER experiment can be performed at lower temperatures, e.g. at 5K instead of 10K, where the relaxation of \( \text{Gd}^{3+} \) is prolonged and thus longer distances can be detected.

Single-frequency relaxation measurements, at the echo-detected field sweep maximum, feature an enormous sensitivity advantage over two-frequency DEER measurements with detecting at the edge of the resonator profile on a minor fraction of the spin system. Single-frequency measurements are feasible at spin concentrations of \( \approx 1 \mu\text{M} \) on a commercially available X-band pulsed EPR spectrometer [30]. In contrast, nowadays DEER measurements on such low concentrated samples can only be performed on a high-field, high-power, home-built spectrometer with a big sample volume [30]. Therefore, particularly for studies of biological samples that are not available in large amounts or for samples that are very dilute distance determination via relaxation enhancement \( (\Delta k) \) is advantageous on only commercial hardware. Hence, distance measurements by \( \text{Gd}^{3+} \)-nitroxide DEER and by \( \text{Dy}^{3+} \)-induced nitroxide relaxation enhancements are complementary.
Apart from distance information also the relaxation times of fast relaxing paramagnetic species in the close vicinity of the nitroxide spin label can be extracted from the temperature dependence of $T_1$ relaxation enhancement data. This provides the possibility to get an insight into the relaxation behaviour of fast relaxing paramagnetic species in a broad temperature range, where the direct detection of the species is not feasible by pulse EPR measurements. Important steps towards the understanding of the observed echo reduction effect and of the occurrence of satellite features in the distance distribution of the DEER experiment could be made in the future by studying numerous Gd$^{3+}$ complexes with different zero-field splitting (ZFS) parameters and the behaviour of the spectral shape of echo-detected EPR spectra of these complexes upon the increase of the inter-pulse delay. This would provide the possibility to verify or disproof the anticipated correlation between this behaviour and the strength of the echo reduction effect. The simultaneous investigation of environmental influences on especially $T_2$ of Gd$^{3+}$ could provide ways for prolongation of the available distance range in DEER measurements by prolongation of the transverse relaxation times. Therefore, not only new chelating agents have to be studied, but also model systems featuring well defined, long and short distances have to be synthesized. On such systems also the distance range accessible by relaxation enhancement measurements could be validated and the influence of other fast relaxing paramagnetic lanthanide ions on the relaxation behaviour of nitroxide radicals could be studied at different frequencies and distances. A more detailed investigation of the short distance suppression limit should be included.

The application of the developed techniques for determination of the structure and structural changes of bio-macromolecules depends on the advancing field of orthogonal spin labelling. If the system of interest can be labelled in an orthogonal manner, the distances on Dy$^{3+}$-nitroxide, Gd$^{3+}$-nitroxide, nitroxide-nitroxide and Gd$^{3+}$-Gd$^{3+}$ spin pairs can be determined independently from each other. This opens up the possibility to combine several experimental approaches and to increase the information content per sample substantially.
A. Appendix

A.1 DEER traces

The evaluation of experimental time-domain double electron-electron resonance (DEER) traces \( V(t)/V(0) \) includes a background correction. The background is arising from the random distribution of remote spins. In the following the experimental normalized \( V(t)/V(0) \) data for all DEER traces on Gd\(^{3+}\)-nitroxide spin pairs, discussed in this thesis, are shown together with the resulting form-factor and distance distribution.

Figure A.1: Experimental DEER data for the Gd\(^{3+}\) loaded terpyridine derivative with different pulses settings I-V (details see table 4.1). A) Normalized \( V(t)/V(0) \) data with exponentially decaying background (red) arising from the three-dimensional distribution of remote spins. B) Background corrected data \( F(t)/F(0) \) C) Distance distribution \( P(r) \) obtained by converting \( F(t)/F(0) \) with DeerAnalysis 2009 (red).
Figure A.2: DEER on $\text{Gd}^{3+}$-nitroxide spin pairs in membrane incorporated WALP23 for constructs with the nitroxide label at position 07 (yellow), 11 (orange), 15 (green) and 19 (blue) performed at X-band (light) and Q-band frequencies (dark). **A)** Normalized $V(t)/V(0)$ data with exponentially decaying background (red/black) arising from the three-dimensional distribution of remote spins. **B)** Background corrected experimental data $F(t)/F(0)$ and the fit from DeerAnalysis 2009 (red/black). **C)** The rather broad resulting distance distributions ($P(r)$) are independent of the measurement frequency and shift with the $\alpha$-helical pitch of 0.54 nm.

Figure A.3: DEER on gold nanoparticles (Au-NPs) at 10K at X-band frequencies with 2% of loaded $\text{Gd}^{3+}$ (dark green) and 4% of loaded $\text{Gd}^{3+}$ (light green). **A)** Normalized $V(t)/V(0)$ data with exponentially decaying background (red/black) arising from the three-dimensional distribution of remote spins. **B)** The background corrected $F(t)/F(0)$ time domain data was fitted by DEERAnalysis (red). The spectra are vertically displaced for better visibility. **C)** The obtained distance distribution reveals the same mean distance but different cut-off distance.
A.1. DEER traces

Figure A.4: Performance of the DEER experiment on Au-NPs at X band (light) and Q-band (dark) frequencies on Gd$^{3+}$-nitroxide (red - upper panel) and nitroxide-nitroxide (grey - lower panel) spin pairs. **A)** Normalized $V(t)/V(0)$ data with exponentially decaying background (red/black) arising from the three-dimensional distribution of remote spins. **B)** Form factor $F(t)/F(0)$ and **C)** distance distributions fitted by DeerAnalysis.
A.2 Relaxation enhancement

The three spin interaction (Oxygen Effect) on spin labelled and Dy$^{3+}$ loaded WALP23 polypeptides incorporated into oxygen ($O_2$) containing DOPC lipid bilayers was investigated in section 5.3 for position 11. This position was chosen because the $O_2$ content in the middle of the membrane is highest (figure 5.14).

The Influence of the non-additive relaxation enhancement ($\Delta k$) effect of Dy$^{3+}$ and $O_2$ leads to a shorter apparent distance. In case of Dy$^{3+}$ loading for position 11 the magnitude of $\Delta k$ changes by approximately 20% due to the presence of residual $O_2$ and the determined distance changes from 2.15 nm to 2.07 nm.

The study of the three spin interaction was extended to position 15, where the content of residual $O_2$ is reduces compared to position 11 [75]. The influence of residual $O_2$ on the obtained $\Delta k$ effect for position 15 is shown in figure A.5A. The magnitude of $\Delta k$ changes by approximately 15% due to the presence of $O_2$ and the trend observed for position 11 is maintained. The distance changes from 2.75 nm under 'O$_2$-free' conditions to 2.69 nm under 'O$_2$-containing' conditions. The direct comparison to position 11 is shown in figure A.5B.

**Figure A.5:** Influence of residual dissolved $O_2$ in lipid bilayer for WALP23 polypeptides loaded with Dy$^{3+}$ **A** with the nitroxide spin label at position 15 under 'O$_2$-free' conditions (green) and under 'O$_2$-containing' conditions (red) and **B** the comparison to position 11 (orange) (figure 5.18).
Bibliography


List of Publications

Publications


in preparation M. Yulikov, P. Lueders, M. Warsi, V. Chechik and G. Jeschke *Distance measurements in Au nanoparticles functionalized with nitroxide radicals and Gd$^{3+}$-DTPA chelate complexes.*

M. Yulikov, P. Lueders and G. Jeschke *Influence of zero-field splitting parameters on Gd$^{3+}$-nitroxide DEER measurements.*

P. Lueders, M. Yulikov and G. Jeschke *Relaxation-based distance measurement in a WALP23 polypeptide incorporated into a lipid bilayer.*

P. Lueders, M. Yulikov and G. Jeschke *Relaxation enhancement including three spin interactions - Oxygen effect.*

Other Publications


Oral Presentations

2011 31.05. Zurich/Switzerland
Kolloquium für Physikalische Chemie, Frühjarhssemester 2011
*Long Range Distance Information on Orthogonal Spin Pairs by EPR*

03.04.-07.04. York/United Kingdom
The 44th Annual International Meeting of the ESR Spectroscopy Group of the Royal Society of Chemistry
*Distance Measurements on Lanthanide Ion - Nitroxide Radical Spin Pairs in the Nanometre Range by Relaxation Enhancement*

2010 20.09.-23.09. Münster/Germany
32nd Discussion Meeting and Joint Benelux/German MR Conference
*Distance information in the nanometer range obtained from Ln^{3+} - nitroxide radial spin pairs*

Stipends and Awards

2011 JEOL Student Lectures Prize
03.04.-07.04. York/United Kingdom
The 44th Annual International Meeting of the ESR Spectroscopy Group of the Royal Society of Chemistry
*Distance Measurements on Lanthanide Ion - Nitroxide Radical Spin Pairs in the Nanometre Range by Relaxation Enhancement*

SCNAT/SCS Chemistry Travel Award

2010 Poster Prize
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The 43rd Annual International Meeting of the ESR Spectroscopy Group of the Royal Society of Chemistry
*Double Electron-Electron Resonance measured between gadolinium ions and nitroxide radicals*

Poster Presentations

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The 44th Annual International Meeting of the ESR Spectroscopy Group of the Royal Society of Chemistry
Distance Measurements by DEER in Gd$^{3+}$-Nitroxide Radical Spin Pairs

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EPR based distance determination on lanthanide - nitroxide radical spin pairs

04.07.-09.07. Florence/Italy
Joint EUROMAR 2010 and 17th ISMAR Conference
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21.03.-25.03. Cardiff/United Kingdom
The 43rd Annual International Meeting of the ESR Spectroscopy Group of the Royal Society of Chemistry
Double Electron-Electron Resonance measured between gadolinium ions and nitroxide radicals

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Long range distance constraints obtained from the relaxation enhancement in a lanthanide - and nitroxide - labelled WALP23 polypeptide incorporated into a lipid bilayer

2008 22.08.-01.09. St.Andrews/Scotland
4th EF EPR Summer School, COST P15 Training School and SUSSP 64
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