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RESEARCH ARTICLE

Distance determination from dysprosium induced relaxation enhancement: a case study on membrane-inserted WALP23 polypeptides

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Abstract. Membrane incorporated synthetic α -helical polypeptides labeled with Dy(III) chelate complexes and nitroxide radicals were studied by the inversion recovery (IR) technique and Dy(III)-nitroxide distances were obtained. A comparison of obtained distances with the previously reported Gd(III)-nitroxide pulse double electron-electron resonance (DEER or PELDOR) calibration data was performed and revealed reliability of the IR-based technique for the distance determination in membrane-incorporated biomacromolecules. The presented distance determination technique is 'spectroscopically orthogonal' to DEER-based distance measurements and can be potentially combined with DEER to study multiply spin-labeled biomacromolecules. The key steps of the data processing, the types of obtained distance information and the areas of possible application of the technique are discussed.

Keywords: lanthanide ions; EPR/ESR; inversion recovery; biomacromolecules; dipolar spectroscopy; distance measurements

1. Introduction

Nanometer range distance constraints obtained from dipolar electron paramagnetic resonance (EPR) spectroscopy are broadly used in studies of structure and conformational changes of large biomolecules [1–11]. The distance measurements by EPR can supplement other methods, like NMR [12] or crystallography [13], or can be used as a stand-alone technique [14–16]. The EPR-based techniques do not require sample crystallization and do not depend strongly on the size of studied biomolecules, thus providing tools applicable to very big biomacromolecules or macromolecular complexes. On the other hand, as most biomolecules are diamagnetic, the use of EPR relies on site-directed labeling with paramagnetic probes [17]. The use of conventional nitroxide radicals as spin labels in most cases restricts the information extracted from a single sample to one distance distribution measured between two labeled sites. Combining different types of spin labels can enhance the information content accessible per one spin-labeled biomolecular sample [18–26]. The combination of nitroxide radicals with lanthanide ions, which provides good

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sensitivity, excellent spectroscopic selection and robust performance, appears to be particularly advantageous [22–25].

All EPR work mentioned was performed with the direct detection of static dipolar interaction between two paramagnetic centers [27–30]. The corresponding pulse double electron-electron resonance (DEER or PELDOR) method has developed into a ‘semi-routine’ approach with well established experimental schemes [30], data processing algorithms [31, 32] and open source data analysis software [33]. In contrast to NMR spectroscopy, where relaxation based distance measurements are of major importance for structural studies, only a limited number of relaxation based structural studies with EPR have been reported [34–41]. The principle of measurement for the stochastic dipolar interaction is based on the change of the relaxation of a slowly relaxing paramagnetic probe in the presence of a fast relaxing agent [42–45]. This is different from the detection of a static dipolar interaction, which relies on the modulation of electron spin echo amplitude for one paramagnetic species upon an enforced flip of the magnetic moment of a neighboring paramagnetic species. These two methods could potentially be combined, leading to further increase of information content in EPR-based structural studies.

The lanthanide family offers a perfect playground to test the relaxation based methods *versus* the more conventional DEER technique. Due to slow relaxation of Gd(III) ions they can be used in Gd(III)-nitroxide DEER experiment [22–25]. On the other hand, due to very similar chemical properties, other lanthanide ions can substitute Gd(III) in most chelate complexes [46] and relaxation based distance measurements can thus be performed on nearly the same model systems.

It has been demonstrated that orientation-averaged relaxation enhancement can provide a good measure of the lanthanide-nitroxide distances [40]. The introduction of a fast relaxing agent may in principle affect other relaxation channels in addition to the one driven by the interspin dipolar interaction [41]. With respect to this, two important remarks should be made, both favoring the use of lanthanide ions. First, it is advantageous to use very fast relaxing species to induce the relaxation enhancement. The relaxation of most lanthanide ions, except of Gd(III) is strongly dominated by spin-orbit coupling related mechanisms. Other mechanisms, which may depend on the surrounding (like interaction with magnetic nuclei or other paramagnetic centers) do not contribute strongly to the relaxation of such ions. The relaxation properties of lanthanide based labels are thus very similar for different samples, which helps making the distance measurement approach systematic and reproducible. Second, in addition to direct dipole-dipole interaction with nitroxide spin probes, fast relaxing agents interact with any other magnetic species in the sample, which induces additional indirect enhancement of the relaxation of nitroxide spin probes. While other unwanted *paramagnetic* species, like membrane dissolved oxygen, can usually be removed [41], the presence of magnetic nuclei is unavoidable for biological samples. The fast relaxing lanthanides ($T_f \sim 1 - 10$ ps) are not very efficient in enhancing nuclear relaxation. Thus, the additional relaxation channels only weakly affect distance measurements. Regarding the interaction with other relaxation channels, the measurements based on observing T_1 relaxation of nitroxide radicals are more favorable as compared to the T_2 -based methods, because longitudinal relaxation is much less affected by the interaction with surrounding magnetic nuclei. In addition, smaller absolute relaxation time changes can be detected with T_1 -based approaches, thus increasing the accessible distance range.

In this paper we present evaluation of the relaxation based distance measurements in dysprosium-nitroxide spin pairs on paramagnetically labeled membrane-inserted WALP23 polypeptide. We use Dy(III) ions as an example of efficient fast relaxing agent, but other lanthanide ions, such as Holmium [47], can provide good

relaxation enhancement (RE) as well. We demonstrate the performance of the method in a difficult case, when a high local density of ^1H nuclei is present in the vicinity of nitroxide spin labels. We describe in detail the measurement procedure and the data evaluation. The relaxation based distance measurements are compared to reference Gd(III)-nitroxide DEER data [25]. We discuss the type of obtained distance constraints, restrictions and possible areas of applications for the presented distance measurement approach.

2. Materials and methods

2.1. Sample preparation

The samples were prepared in full accordance with the previously published procedure [41]. Four WALP23-based constructs were synthesized by Pepceutical Limited (Nottingham, UK) by solid-phase synthesis with one cysteine at position 7, 11, 15 or 19. The cysteine residue was labeled with a nitroxide spin label MTSSL (S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate). In each construct a lysine-DOTA 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 purchased from Macrocyclics Inc. (Dallas, USA)] was added at the N-terminus. The DOTA moiety was loaded either with paramagnetic Dy(III) or with La(III) as a diamagnetic reference. In the following the Dy(III)- or La(III)-loaded samples are abbreviated accordingly as WALP23-XXDy or WALP23-XXLa, where XX stands for the cysteine/nitroxide site (07, 11, 15 or 19). **The sequences of the four WALP23 constructs are listed in Table 1.**

Table 1. Sequence of WALP23 constructs for labeling position 07, 11, 15 and 19.

Sample	peptide sequence
WALP23-07La/Dy	K ^a WWLALCLALALALALALWVA
WALP23-11La/Dy	K ^a WWLALALALCLALALALALWVA
WALP23-15La/Dy	K ^a WWLALALALALALCLALALWVA
WALP23-19La/Dy	K ^a WWLALALALALALALCLWVA

^aLysine-DOTA derivative.

In each case the WALP23-based constructs were incorporated into DOPC bilayers with a WALP23/DOPC ratio of 1/800. The volume concentration of labeled WALP23 molecules of about 200 μM was estimated from intensity comparison of room temperature continuous wave EPR spectra of nitroxide radicals in WALP23 samples and of a standard 300 μM nitroxide radical solution. This corresponded to approximately 280 nm² of the membrane area per WALP23 molecule [25]. Vesicles with an average size of 400 nm were prepared by extrusion. As the presence of membrane dissolved oxygen can change the value of Dy(III)-induced relaxation enhancement for nitroxide radicals, all samples were prepared with additional removal of oxygen by blowing dry nitrogen above the sample just before its freezing by immersion of the sample tube into liquid nitrogen [41]. In the cited reference these conditions were referred to as 'oxygen free'. Once prepared, all samples were stored in liquid nitrogen in order to exclude possible diffusion of oxygen into the bulk of the samples.

2.2. EPR Measurements

Pulsed EPR measurements were carried out at X band (9.3 - 9.4 GHz) as well as at S band (4 GHz) and at Q band (35 GHz). X-band measurements were per-

formed on a Bruker ElexSys 580 spectrometer equipped with a Bruker split-ring or dielectric resonator (ER 4118X - MS3 or ER 4118 MD5). Q-band measurements were performed on a home-built high power spectrometer [48, 49] equipped with a custom resonator for samples with 3 mm outer diameter [50]. The S-band measurements were performed on a home built spectrometer [51]. In each case temperature stabilization was performed with a continuous flow He cryostat (ESR900, Oxford Instruments) equipped with an Oxford Instruments temperature controller ITC 503S. Pulse EPR experiments were performed in a temperature range from 20 K to 100 K in 10 K steps. At S band only measurements up to 80 K were performed and at Q band the 20 K measurements were omitted. The RE data were obtained from T_1 relaxation measurements with an IR pulse sequence as described earlier [41] with $T_0 = 2000$ ns, an inversion pulse of 32 ns and the Hahn echo detection sequence with 52 ns ($\pi/2$) - 104 ns (π) pulses and interpulse delay of 344 ns. Rather weak influence of spectral diffusion has been observed with these settings for nitroxide-Dy(III) pairs [40]. Echo detected (ED) EPR spectra were recorded with a 16 ns ($\pi/2$) - 32 ns (π) Hahn echo sequence with an interpulse delay of 400 ns. The shapes of the nitroxide ED EPR spectra at different bands and the detection positions in IR experiment are shown in Figure 1.

2.3. Data analysis

2.3.1. Theoretical background

The determination of Dy(III)-nitroxide distances from RE is based on the second order perturbation equations for the stochastically modulated dipolar interaction [42–45]. The relaxation enhancement Δk is defined as the change of the relaxation rate of the nitroxide radical in the presence of fast relaxing Dy(III) center:

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

In Eq. 1 the T_{1s} and $T_{1s,0}$ times correspond to the longitudinal relaxation of slowly relaxing nitroxide radicals in the presence and in the absence of fast relaxing agent (Dy(III)). The relaxation of Dy(III) centers is much faster compared to the characteristic evolution times upon dipole-dipole interaction, thus up to the second order in perturbation series the relaxation enhancement is given by [45]:

$$\Delta k(r, \theta) = S(S+1) \frac{g_s^2 g_f^2 \mu_0^2 \mu_B^4}{(4\pi)^2 \hbar^2 r^6} (f_B + f_{CD} + f_{EF}) = \frac{C(T_{1f}, T_{2f}, \theta, \theta_g)}{r^6} \quad (2)$$

with terms f_B , f_{CD} and f_{EF} originating from the corresponding terms of the dipolar alphabet [43, 44]:

$$\begin{aligned} f_B &= \frac{1}{6} (1 - 3 \cos^2 \theta)^2 \frac{T_{2f}}{1 + (\omega_f(\theta_g) - \omega_s)^2 T_{2f}^2} \\ f_{CD} &= 3 \sin^2 \theta \cos^2 \theta \frac{T_{1f}}{1 + \omega_s^2 T_{1f}^2} \\ f_{EF} &= \frac{3}{2} \sin^4 \theta \frac{T_{2f}}{1 + (\omega_f(\theta_g) + \omega_s)^2 T_{2f}^2}. \end{aligned} \quad (3)$$

Here g_s and g_f stand for the g-values of the slowly and fast relaxing paramagnetic

species, μ_B is the Bohr magneton and μ_0 is the magnetic permeability of vacuum. The angle θ defines the orientation of the spin-spin vector with respect to the static magnetic field and the angle θ_g defines the orientation of the g-tensor of the fast relaxing species (Dy(III)) with respect to the static magnetic field. For [Dy(DOTA)] complexes we assumed an axial g-tensor with $g_{\perp}=1.4$ and $g_{\parallel}=4.2$ (see electronic supplementary information). In case of Dy(III) the difference between T_{1f} and T_{2f} can be neglected [40]. It has been demonstrated that by averaging over all possible orientations of the Dy(III) g-tensor, the averaged relaxation enhancement still follows the same distance dependence [40]:

$$\overline{\Delta k} = \frac{1}{\tau_1} = \frac{\overline{C}(T_{1f})}{r^6} \quad (4)$$

Under these assumptions, the average relaxivity $\overline{C}$ depends on a single value T_{1f} , which changes with temperature. By measuring the temperature dependent RE one can simultaneously fit the Dy(III)-nitroxide distance and the temperature dependence of T_{1f} of Dy(III) ions. The latter temperature dependence can be approximated as a phenomenological power law of a form [40]:

$$T_{1f} = \Lambda \cdot T^{-p} = A \cdot \left(\frac{T_{\max}}{T} \right)^p \quad (5)$$

For a more clear physical interpretation the prefactor Λ (former representation) can be separated into the temperature $T_{\max}$, at which the highest RE is observed, and the optimum value of relaxation time for the fast relaxing species $T_{1f}(T_{\max}) = A$. At X band $A \approx 1.2 \times 10^{-11}$ s [40], at other bands the values of A and $T_{\max}$ would change, but, if T_{1f} does not depend on the applied magnetic field, then the product $A \cdot T_{\max}^p$ should stay constant.

The relaxation behavior of lanthanide ions is dominated by different mechanisms in different temperature ranges [52]. Nevertheless, the presented approach only requires fitting the T_{1f} in a relatively narrow temperature range, where a simplified phenomenological Eq. 5 suffices. Importantly, for the correct distance determination the most crucial is the $T_{\max}$ value, which is determined experimentally from the maximum of RE temperature dependence. We tested experimentally the applicability of this equation by performing the RE measurements on the same sample at three different detection frequencies and by fitting them to a single temperature dependence of T_{1f} (see below in the Results section).

The used approach, thus, assumes fast relaxation of paramagnetic Dy(III) centers and a uniform distribution of g-tensor orientations with respect to the external magnetic field and to the spin-spin vector. It additionally neglects the anisotropy of the resonance frequency of nitroxide radicals [40]. Furthermore, we assume in our analysis that the relaxation contributions can be factorized [39, 40]. One can represent the longitudinal relaxation trace of nitroxide radicals in the absence of RE agent as a sum of monoexponential decays for each individual radical species:

$$V_0(t) = \sum_{i=1}^N \exp\left(-\frac{t}{T_{1s,0,i}}\right) \quad (6)$$

If relaxation of individual spins is not monoexponential, then each spin contributes multiple monoexponential terms with fractional amplitudes in the above sum, but the following considerations remain valid. In the presence of fast relaxing

Dy(III) centers the enhanced relaxation can be described as:

$$V(t) = \sum_{i=1}^N \exp \left(-t \cdot \left(\frac{1}{T_{1s,0,i}} + \Delta k_i \right) \right) \quad (7)$$

In the following we assume that the local environment of the nitroxide radical, which determines its original non-perturbed longitudinal relaxation, is not correlated with the Dy(III)-nitroxide distance. This implies that one can separate slowly relaxing species into subensembles with respect to T_{10} such that the corresponding subensemble distributions for Δk are the same as the Δk distribution for the full ensemble. At the same time it is assumed that the separation into Δk subensembles results in identical distributions of T_{10} times for each Δk subensemble. With these assumptions Eq. 7 can be transformed into a product of a non-perturbed decay function $V_0(t)$ and a time trace $V_k(t)$, which contains only the RE contributions:

$$\begin{aligned} V(t) &= \sum_i \sum_j \exp \left(-\frac{t}{T_{1s,0,i}} \right) \exp (-t \cdot \Delta k_j) \\ &= \left(\sum_i \exp \left(-\frac{t}{T_{1s,0,i}} \right) \right) \cdot \left(\sum_j \exp (-t \Delta k_j) \right) \\ &= V_0(t) \cdot \sum_j \exp (-t \cdot \Delta k_j) \stackrel{\text{def}}{=} V_0(t) \cdot V_k(t) \end{aligned} \quad (8)$$

The data analysis can thus be done by dividing the longitudinal relaxation traces of Dy(III)- and La(III)-loaded samples and analyzing the obtained $V_k(t)$ traces:

$$\frac{V(t)}{V_0(t)} = V_k(t) = \sum_j \exp (-t \cdot \Delta k_j) \quad (9)$$

2.3.2. Key steps of data processing

The main data processing steps are illustrated in Figure 2. The primary IR time traces were inverted and offset corrected to decay towards the value of 0 at infinite time limit (Figure 2(a, b)). The offset correction was obtained by fitting the data with a multiexponential function that included a constant offset as a fitting parameter, followed by the subtraction of the fitted offset value. The characteristic decay times of such overdetermined multiexponential fits do not have direct physical meaning, but the shape of decay curves could be reproduced perfectly and the extracted offset values could be computed with fairly good precision. For each particular nitroxide label position and measurement temperature the IR time trace obtained for the Dy(III)-loaded sample was divided by the corresponding data for the La(III)-loaded sample (Figure 2(c)). The resulting time traces (RE time traces) contained contributions from the intramolecular and intermolecular RE. The intramolecular RE was manifesting itself as a fast initial decay of the time trace. In an ideal case of 100% Dy(III) labeling this contribution should lead to the signal intensity decaying to zero. In practice we observed decays down to the signal level between 0.4 and 0.2 (Table 2), indicating incomplete Dy(III) labeling in the range of 60-80%. The remaining signal came from the WALP23 molecules not loaded with Dy(III) ions and thus reflected the intermolecular RE. Due to rather long distances between WALP23 molecules in the lipid bilayer, the separation of

the RE time trace into the intramolecular and intermolecular contributions was unambiguous.

In our earlier work on this system [41], we made a conclusion of 100% Dy(III)-labeling efficiency from the fact that very slow relaxation observed in the samples labeled with diamagnetic La(III) was not present in the Dy(III)-labeled samples. With the presented, more detailed analysis, we can see that intermolecular relaxation enhancement hides the presence of unloaded complexes by increasing the relaxation rate also for those nitroxide radicals, attached to WALP23 without Dy(III)-label. The fact of incomplete labeling does not affect main conclusions of our previous publication, as they were exclusively based on qualitative considerations.

A small fraction of WALP23 aggregates has been confirmed by the nitroxide-nitroxide DEER measurements (see electronic supplementary information for details). These aggregates should have contributed to the measured intramolecular RE. On the other hand the distance distribution in aggregates and their fraction was similar in WALP23 samples loaded with different lanthanide ions. Thus, the comparison of Dy(III)-induced RE and Gd(III)-nitroxide DEER data should not be significantly affected by this sample imperfectness. The slowly decaying intermolecular contributions in the RE time traces were fitted by a polynomial function and subtracted from the RE time traces. The final intramolecular RE time traces were rescaled to an initial value of 1.0 and fitted to obtain $1/e$ decay times that were used as a measure of the average relaxation enhancement.

2.3.3. Background correction

In order to obtain an unambiguous background correction procedure, we employed a truncated cost function approach [53]. The cost function $\phi(x)$ was defined as

$$\phi(x) = \begin{cases} x^2 & \text{if } x \leq s, \\ s^2 & \text{otherwise.} \end{cases} \quad (10)$$

The cost function Eq. 10 allows to treat positive 'peaks', i.e. the regions where the signal deviates from the background function by the value x that exceeds the threshold parameter s , differently from the 'background' regions, i.e. the regions, where $x < s$. Due to strong oversampling, the actual precision in the RE time trace measurement was higher than the one deduced from the apparent noise magnitude. High frequency noise was removed by the fitting procedure with multiexponential functions and the fitted smooth functions have been used for the intermolecular background fits. The obtained low-order (first to third) polynomial background functions were subtracted from the RE time traces. With the described procedure the selection of the region for the background fit and the separation into the background and form factor was done automatically.

Due to the division by the diamagnetic reference data, the signal-to-noise ratio in the RE time trace decreases towards longer times. In cases, when the end of the RE trace was too noisy, such a trace was cut at around the region of signal-to-noise equal to 0.3-0.5. It was tested that variation of the cutoff time did not strongly change the resulting Δk value. Observed small variations were included into the error estimate for Δk and for the loading efficiency of Dy(III) ions.

2.3.4. Error estimates

Two important values can be computed from the RE time traces: the overall labeling efficiency for the Dy(III) chelate complexes and the averaged Dy(III)-nitroxide distance. The former value relies on the quality of the background fit for

the RE time trace, on the sufficiently different relaxation in the presence and in the absence of Dy(III) and on the signal-to-noise ratios of the primary IR traces. For our RE data we estimate an error bar of about 15% for this type of measurement. Such an error is similar to a typical error in the labeling efficiency measurements from the DEER modulation depth. The latter value (average Dy(III)-nitroxide distance) is computed from the average $1/e$ decay time of the intramolecular RE data. Due to the very strong ($\propto r^6$) dependence of the $1/e$ time on the interspin distance, the error in the distance is much smaller than the error in the average decay time. With the obtained data quality we can estimate the error in the $1/e$ time to be about 10-15% which results in about 2-3% error for the average distance.

3. Results

The temperature dependencies of Δk for the four different Dy(III)-nitroxide-labeled WALP23 constructs are shown in Figure 3 for X and Q band, and the fit parameters for the $T_{1f}(T)$ are given in the electronic supplementary information. One can see that at X band the maximum in the relaxation enhancement curve is observed at around 70-80 K. This maximum corresponds to the optimum relaxation enhancement conditions: $T_{1f}(T_{max}) \approx 1/\omega_{res}$. One can see that this optimum RE temperature does not change with the change of the nitroxide labeling site. The extraction of distances from RE data relies on the independent fit of the temperature dependence of the T_{1f} . This fit is most stable if the region around T_{max} can be experimentally accessed, which is true for the X-band RE measurements. At Q band the optimum enhancement temperature shifted above 100 K due to the increase of the resonance frequency. At temperatures above 100 K longitudinal relaxation of nitroxide radicals in the absence of Dy(III) ions rapidly increased with the increase of temperature and thus the relative contribution of the Dy(III) induced RE, as compared to other relaxation mechanisms, was reduced. As a result, we could not unambiguously determine the T_{max} for those Q-band RE data and the $T_{1f}(T)$ fits from X-band measurements were used in the analysis. Figure 4 presents the values of the RE contrast calculated as a ratio between Δk and inverted longitudinal relaxation time of nitroxides in the La(III) loaded WALP23 constructs $k_0 = 1/T_{1s,0}$. Because the longitudinal relaxation of nitroxide spin label changes depending on its position in the lipid bilayer, the RE contrast figures do not exactly reproduce the figures for the absolute RE values. The optimum RE contrast was observed in the temperature range 40-60 K in the X-band measurements. The RE contrast did not change strongly with temperature in the Q-band measurements for the temperature range 30-100 K, for higher temperatures it rapidly decreased. The optimum RE contrast at X band was about two times better than at Q band (Figure 4).

The single distances, extracted from the fits of X-band RE time traces are listed in Table 2 and compared to the Gd(III)-nitroxide DEER data, for which both the mean distances and the widths of distance distributions are given. For all four samples the RE-extracted distances are shorter than the mean DEER distances. The deviation increases from $\sim 11\%$ for the shortest measured distance (WALP23-07Dy sample) to $\sim 30\%$ for the longest measured distance (WALP23-19Dy sample) with two other samples in line with this trend.

The RE time traces were fitted with an assumption that the relaxation of Dy(III) centers can be described by a single temperature dependent relaxation time $T_{1f}(T)$. To verify this assumption we measured temperature dependence of RE for a single nitroxide labeling site (samples WALP23-15Dy/WALP23-15La) at three different microwave bands (S, X and Q-band). The correspondence between the $\Delta k(T)$ de-

Table 2. Comparison between Dy(III)-nitroxide distances obtained from X-band RE (RE_X) and Gd(III)-nitroxide distances obtained from DEER measurements.

Sample	RE_X	DEER ($r \pm \sigma$) ^a	Dy(III) loading ^b
WALP23-07Dy	2.15 nm	(2.4 $\pm$ 0.5) nm	56%
WALP23-11Dy	2.28 nm	(2.7 $\pm$ 0.6) nm	79%
WALP23-15Dy	2.69 nm	(3.3 $\pm$ 0.6) nm	70%
WALP23-19Dy	2.89 nm	(3.8 $\pm$ 0.5) nm	69%

^aMean distance (r) and FWHM (2σ) from the Gd(III)-nitroxide DEER[25].

^bAveraged over all RE traces detected at different temperatures.

pendencies and a single function $T_{1f}(T)$, obtained from the X-band data fitting according to Eq. 5 is shown in Figure 5. One can see that fairly good agreement with experimental data could be achieved for all three bands, and thus the single T_{1f} -time approximation is sufficient for describing multiple frequency RE measurements. The presented fit assumed no field dependence of the T_{1f} , which is in line with the Raman relaxation mechanism that typically dominates in the studied temperature range [52].

4. Discussion

X band provides the best detection conditions for the RE measurements. The drawback, as compared to Q band, is the lower sensitivity. But the possibility to observe the T_{max} region and the obtained RE contrasts indicate that the RE-based distance measurements would be best performed at X band rather than at Q band. At S band even better RE contrast was observed and the T_{max} region could be experimentally detected as well. Nevertheless, regarding yet lower sensitivity, it seems disadvantageous to use S band for RE detection. Besides, the increase in the RE at S band leads to only very little increase of the accessible distance range, due to the very steep distance dependence of the Dy(III)-induced RE.

The data in Table 2 show that, while the averaged distances from RE measurement are within the range of distances observed in the reference DEER experiments, there is a discrepancy between RE and DEER mean distances. One reason for such a discrepancy could come from the extraction of single distance from the RE data, measured for a sample with distribution of Dy(III)-nitroxide distances. The RE value depends on the inverted sixth power of the interspin distance. If the function of the form C/r^6 is averaged over a distance distribution, the averaged distance $\bar{r} = \sqrt[6]{1/\langle 1/r^6 \rangle}$ would be shorter than the mean distance $\langle r \rangle$ for the given distribution. In case of RE experiments, the actual averaging has a more complicated mathematical form (the overall 1/e decay time for a sum of multiple decaying exponents is computed). In contrast, the DEER experiment allows to determine the distance distributions and, thus, the inverse cubic dependence of the dipolar frequency on the interspin distance does not affect the mean distance determination. Still, the direct calculations (data not shown) reveal that only minor shift of the average distance could be achieved if RE traces are simulated for a distribution of distances similar to the experimentally detected ones.

Notably for the four measured samples the difference between RE distance and the DEER mean distance increases with the increase of the Ln(III)-nitroxide distance. This trend, if confirmed on further systems, suggests an underlying physical mechanism responsible for the discussed difference. As reported previously [41], additional relaxation pathways are possible if Dy(III)-nitroxide pairs are surrounded by other magnetic species. In our experiments the oxygen concentration in the sam-

ples was strongly depleted, thus removing the reported indirect relaxation pathway. But in biological samples one cannot avoid the presence of magnetic nuclei. It is possible that, while Dy(III) ions are not the most efficient relaxing agents (in particular, for protons), the indirect relaxation enhancement via the proton-channel is not negligibly small and its influence increases as the RE value in the direct channel gets smaller for longer distances. If this or similar mechanism indeed dominates the discrepancy between RE and DEER data, a calibration scaling of RE distances could significantly improve the agreement between RE and DEER. But, even in its present form, the RE method allows for a reasonably good distance estimation and shows a systematic behavior on the studied series of WALP23 constructs.

An important output of this study is, thus, that if Dy(III) centers are used as RE agents, then reliable distance information can be extracted for membrane incorporated biomolecules. Regarding possibilities of non-additive relaxation effects between the two paramagnetic centers and the protons densely distributed in the lipid bilayer, this result would not be generally expected. It appears that fast relaxation of Dy(III) centers decouples them sufficiently strong from the protons and the non-additive relaxation effects induced on nitroxide radicals are small enough (even though, possibly, not negligible) to allow for reliable distance determination.

Some remarks can be made regarding the requirements for the RE based distance measurements. First, the division approach we used for the data analysis relies on a sufficiently good RE contrast. At the ratios $\Delta k/k_0 \approx 0.5$ (with $k_0 = 1/T_{10}$) the time traces need to be measured at least 3-4 times longer than T_{10} with a good signal-to-noise ratios till the end of the time trace, where the normalized IR signal decayed to $\sim 1\%$ of its original value. While such measurement is still feasible experimentally, yet lower RE contrasts would already be very difficult to detect. The value of 0.5 can thus be considered as a good estimate of the minimal RE contrast required to obtain distance information. With that value the upper limit of detectable distance for the presented approach should be about 4 nm. In cases of 100% Dy(III) labeling the requirements for the RE contrast are weaker, as no separation between intramolecular and intermolecular contributions is needed. In such cases distances up to ~ 5 nm should be accessible with the RE technique.

A valuable feature of the presented approach is that it is 'spectroscopically orthogonal' to the DEER-based distance measurements, i.e. DEER distance measurements between two slowly relaxing paramagnetic species can also be performed in the presence of fast relaxing Dy(III) ions. In fact, as Dy(III) reduces T_1 but only weakly affects T_2 of nitroxides, such measurements could be repeated faster and would thus have enhanced sensitivity compared to measurements in the absence of the Dy(III) label. The type of distance information (underestimation of the mean distance) must be considered in structure modeling applications and may potentially cause consistency problems between several distributions in the model. On the other hand the approach should be valuable in studies of conformational changes, association/dissociation of biomolecules and other applications where qualitative information is of major importance. The RE technique allows to determine Ln(III) labeling efficiency, which is not accessible from Gd(III)-nitroxide DEER experiments. The error estimate for the determination of the labeling efficiency from RE is about the same as for nitroxide-nitroxide DEER-based approaches.

5. Conclusions

Our study demonstrates that RE-based distance measurements with lanthanide ions as relaxing agents are feasible for membrane-incorporated biomacromolecules. An upper distance limit of ~ 4 nm should be achievable for moderate Dy(III) la-

being efficiency and it should expand up to 5 nm for samples with 100% Dy(III) labeling efficiency. Because the RE technique underestimates the Dy(III)-nitroxide distances, its straightforward application can be foreseen mainly in conformational change studies and studies of association/dissociation of macromolecular complexes. In case if the RE-based distances can be scaled up in a systematic way, the reported approach should also be useful in structure determination studies. An important feature of the presented distance measurement approach is that it is spectroscopically orthogonal to the DEER-based distance measurements and could thus be used in combination with DEER on multiply labeled biomacromolecules.

6. Supplementary Material

CW EPR spectrum of $[\text{Dy}(\text{DOTA})]^-$; selected RE data; nitroxide-nitroxide DEER measurements. This material is available free of charge in the online version of this article.

7. Acknowledgments

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Figure 1. ED EPR spectra for sample WALP23-15La at 50K for S, X and Q band. The detection positions are marked with arrows.

Figure 2. Illustration of the data processing procedure for sample pair WALP23-11Dy/WALP23-11La. IR traces were measured at $T = 80$ K at X band. **(a)** primary IR data, rescaled to the interval $[-1, 0]$ (blue - Dy(III) loaded, black - La(III) loaded); **(b)** offset corrected and inverted IR traces (blue - Dy(III) loaded, black - La(III) loaded) and the multiexponential fit functions (green) according to the offset fitting procedure, described in the text; **(c)** the RE time trace, obtained by division of the two offset corrected IR traces (black), and the background fit function (green); **(d)** the background corrected form factor RE trace (black) together with the multiexponential fit function, used for the $1/e$ time determination (green).

Figure 3. Temperature dependent RE for the four studied sample pairs: WALP23-07Dy/WALP23-07La (black), WALP23-11Dy/WALP23-11La (blue), WALP23-15Dy/WALP23-15La (green) and WALP23-19Dy/WALP23-19La (magenta). The experimental data points are shown with circles and the fitted functions according to the described procedure are shown as solid lines. Data are shown for X band (left figure) and Q band (right figure).

Figure 4. Temperature dependent RE contrast for the four studied sample pairs: WALP23-07Dy/WALP23-07La (black), WALP23-11Dy/WALP23-11La (blue), WALP23-15Dy/WALP23-15La (green) and WALP23-19Dy/WALP23-19La (magenta). The experimental data points are shown with circles, the solid lines are connecting the data points for better visualization. Data are shown for X band (left figure) and Q band (right figure).

Figure 5. Temperature dependent RE for the sample pair WALP23-15Dy/WALP23-15La at three detection bands: S band (green), X band (blue) and Q band (magenta). The experimental data points are shown with circles and the fitted functions according to the described procedure are shown as solid lines.