EPR Spectroscopy

Deutsche Ausgabe: DOI: 10.1002/ange.201606335
Internationale Ausgabe: DOI: 10.1002/anie.201606335

Selective High-Resolution Detection of Membrane Protein–Ligand Interaction in Native Membranes Using Trityl–Nitroxide PELDOR

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Abstract: *The orchestrated interaction of transmembrane proteins with other molecules mediates several crucial biological processes. Detergent solubilization may significantly alter or even abolish such hetero-oligomeric interactions, which makes observing them at high resolution in their native environment technically challenging. Dipolar electron paramagnetic resonance (EPR) techniques such as pulsed electron–electron double resonance (PELDOR) can provide very precise distances within biomolecules. To concurrently determine the inter-subunit interaction and the intra-subunit conformational changes in hetero-oligomeric complexes, a combination of different spin labels is required. Orthogonal spin labeling using a triarylmethyl (TAM) label in combination with a nitroxide label is used to detect protein–ligand interactions in native lipid bilayers. This approach provides a higher sensitivity and total selectivity and will greatly facilitate the investigation of multimeric transmembrane complexes employing different spin labels in the native lipid environment.*

The cell membranes of living cells contain numerous hetero-oligomeric protein complexes that mediate diverse cellular processes such as transport, secretion, motility, cell–cell recognition, and response to external signals. The multiple subunits in those large complexes dynamically interact in a complicated manner dictated by external factors, including the membrane environment. Though X-ray crystallography and cryo-EM can provide high-resolution structures of large protein complexes, obtaining similar information in the native lipid environment is yet to be realized. Dipolar EPR spectroscopy techniques such as the pulsed electron–electron double resonance (PELDOR or DEER)[1,2] and double quantum coherence (DQC)[3] have emerged as unique methods to measure long-range distances with high precision in proteins[4,5] at physiological temperatures[6,7] or even in the cellular environment.[8–12] Conventional PELDOR employs nitroxide spin labels (NO) incorporated using site-directed spin labeling[13] or genetic encoding.[14–16] The methanethiosulfonate spin label (MTSL), which forms the side chain R1, is the most popular nitroxide label; and Gd^{III} also has been shown to be a suitable spin label for structural studies, especially in the cellular environment.[10] The interspin distances obtained using PELDOR can reveal conformational changes or could be used as constraints for modeling and visualization of alternate states in biomolecules.

In general, PELDOR or DQC-based distance measurements employ identical spins labels. This simplifies the labeling procedure; however, it severely limits the investigations of larger systems, such as the multi-subunit hetero-oligomeric complexes. Placing an identical spin label on different subunits in an oligomeric protein can lead to artefacts and poor resolution from overlapping distances.[17] By labeling individual subunits with different spin labels, for example, in the case of a hetero-oligomeric complex, the inter-subunit interaction can be resolved through the selective detection of the distances between non-identical spin pairs, whereas the distances between identical spin labels can provide conformational changes within a subunit, all using the same sample. In such situations, the modulation depth parameter (λ) between non-identical spin pairs can provide direct information on the dynamics of complex formation. Gd^{III} and Cu^{II} have been site-specifically incorporated into biomolecules as an orthogonal spin pair for PELDOR measurements.[17–21] In other cases, the paramagnetic center was already a part of the biomolecule,[22–24] which limits their applications to specific problems.

Labeling two sites in a protein with different spin labels poses a challenge and requires particular strategies, such as temporary protection of one of the sites[25] or introduction of chemically selective labeling sites through genetic encoding.[14] The situation is less complicated when the biomolecule binds a paramagnetic metal ion or the monomer/ligand in a complex can be selectively labeled prior to complex formation. The carbon-centered triarylmethyl (trityl or TAM) radical is emerging as an alternative spin label to measure distances in biological systems. TAM has a narrow EPR line, it is resistant to reduction, and has a long transverse relaxation time (T_2) at room temperature,[26,27] which allows distance measurements in biomolecules even in the physiological temperature range.[6,28–30] As demonstrated on model compounds, TAM can be used as an orthogonal spin pair with NO.[31,32] For proteins, the application of TAM so far has been limited to T4 lysozyme for many reasons, such as the

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201606335.

instability of the labeled protein and the lack of functionalized TAM with a high reactivity to cysteine.

Previously, we have shown that the outer membrane cyanocobalamin (CNCbl) transporter BtuB in *E. coli* can be labeled with MTSL in whole cells and native membranes.[8,9] Though spin labeling directly in the native membranes produces non-specific labeling, those spins are random in spatial distribution and did not give any particular distances in the PELDOR. To test the feasibility of labeling a membrane protein with TAM, we isolated the native outer membranes (OM) from *E. coli* following over-expression of BtuB wild-type (WT) or BtuB 188C mutant, which is located on the second extracellular loop (Figure 1 a). We used a functionalized TAM radical containing a methanethiosulfonate group (Figure 1 b), which reacts with the SH group of a cysteine to make the side chain TAM1.[30] The membranes containing BtuB 188C or WT BtuB were incubated with the TAM at room temperature for an hour and subsequently washed to remove the free TAM. Room temperature continuous wave (RT CW) EPR spectroscopy revealed an unusually broad spectrum for the TAM attached to the membranes containing BtuB 188C (Figure 1 c) or BtuB WT (Figure S1 a in the Supporting Information). The TAM spin label concentration in this sample was 250 ± 25 μM. The concentration of BtuB in the membrane preparation was estimated using a NO-labeled cyanocobalamin (TEMPO-CNCbl), which was shown to bind BtuB with high affinity[8] (Figure S1 b in the Supporting Information) and was approximately 30 μM. Thus, the higher concentration of TAM suggested that a large fraction of it binds non-specifically, similar to what was previously observed with MTSL.[8,9]

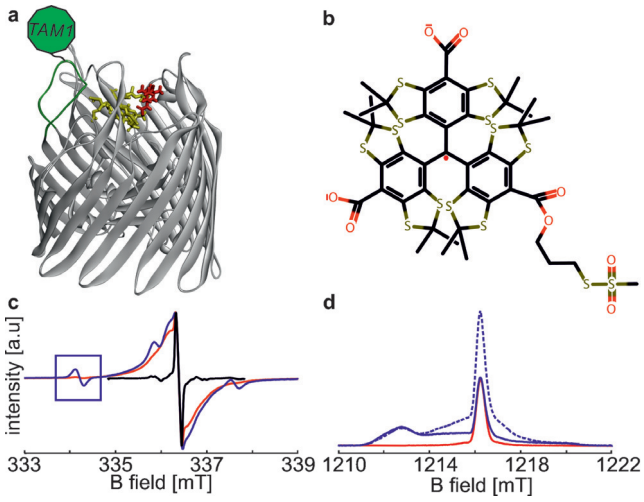

Figure 1. a) Representation of the TAM1 side chain attached to the second extracellular loop (green) of BtuB (PDB 1NQH). The TEMPO-CNCbl was modeled using the open source package Multiscale Modeling of Macromolecules (MMM),[33] with the attached TEMPO in red. b) Structure of the TAM spin label. c) Normalized RT CW EPR spectrum of free TAM (black), TAM attached to OM containing BtuB 188C (red), and TAM attached to OM containing BtuB 188C with 30 μM TEMPO-CNCbl (blue). The blue square shows the fraction of the unbound TEMPO-CNCbl (8 ± 2 μM). d) Echo-detected field-swept spectra of the corresponding samples at 50 K (red and blue solid lines) or 5 K (blue dotted line).

To test the feasibility of performing PELDOR, we added 30 μM TEMPO-CNCbl to the TAM-labeled membrane preparation. RT CW EPR measurement of the sample showed that 8 ± 2 μM TEMPO-CNCbl is free and the rest is bound (Figure 1 c). We performed echo-detected field-swept (FS) experiments at the Q-band (33 GHz) frequency. As the TAM gives a rather broad echo,[31] the entire signal was integrated in the FS for a relative comparison of the TEMPO-CNCbl and the TAM signal intensities. Interestingly, the FS spectrum revealed a significant reduction of the TAM signal (Figure 1 d, blue solid line) when compared to the RT CW EPR measurement. Further quantitative analysis taking the spin relaxation rates into account showed that the sample contains only circa 20 μM TAM, which is about 13-fold lower than the concentration detected using RT CW EPR (Figure S2 in the Supporting Information).

We found that most of the TAM labels could not be detected in the FS because they relax too fast at 50 K (Figure 1 d and Figure S3 in the Supporting Information), likely owing to the aggregation favored in the membrane environment. The TAM molecule is made soluble by the three carboxylic acid groups, whereas the rest of the molecular surface is very hydrophobic (Figure 1 b). Protonation of these carboxylic groups at a pH below 4.0 can induce aggregation of the TAM molecules and broadening of the RT CW EPR spectra[34], as we observed experimentally (Figure 1 c). The biological membrane has a low dielectric constant, which could considerably increase the pK_a of the TAM carboxylic groups. This would favor the protonated TAM molecules, which are very hydrophobic and could attach to the membranes to form the aggregates.

Having a single narrow EPR line, these TAM aggregates, in which the molecules are coupled by sub-nanometer distances, can lead to strong dephasing and very fast relaxation. Interestingly, performing the FS at 5 K (with a short repetition time [SRT] of 1 s) significantly increased the relative signal intensity of TAM with an enhanced presence of the broad features near the TAM peak (Figure 1 d, dotted line). Such broad features might arise from a distribution of the TAM clusters containing a varying number of molecules coupled to each other. In FS experiments at 50 K, the presence of these broad wings gradually decreased with increasing τ values (inter pulse delay) with a complete absence at $\tau = 1500$ ns (Figure S3 in the Supporting Information). Thus, if most of the TAM aggregates relax too fast to be detected at 50 K, this may facilitate a selective observation of the TAM molecules that are attached mostly to the proteins within the membrane.

In the FS spectrum, the maxima of the TEMPO-CNCbl and the TAM spectra are separated by circa 100 MHz (Figure 1 d). In principle, the PELDOR experiment can be performed by observing the TEMPO-CNCbl while pumping the TAM or vice versa. In the first case, a higher λ would be expected because of the narrow features of the TAM spectrum. Though the λ is reduced when pumping TEMPO, the S/N ratio may be compensated by the larger signal when TAM is observed. As the control sample, we used TAM-

labeled outer membranes containing WT BtuB (Figure S1 a in the Supporting Information). PELDOR performed on this preparation by observing TEMPO-CNCbl gave only an exponentially decaying signal devoid of any particular distances (Figure 2 a). This result confirmed that all of the

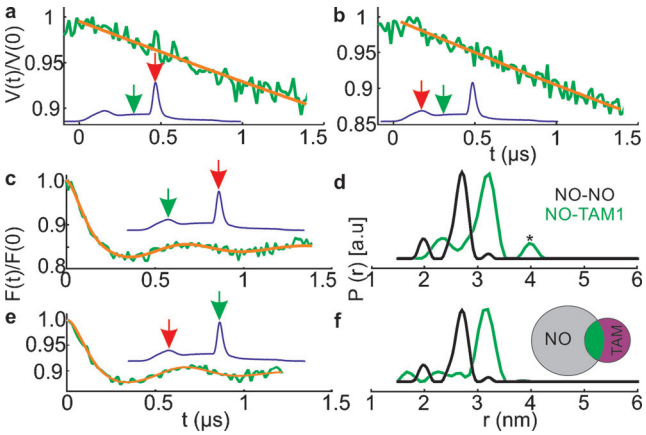

Figure 2. Q-band PELDOR at 50 K in TAM-labeled membranes containing BtuB WT or 188C and 30 μM TEMPO-CNCbl. a) NO(observer)–TAM1(pump) PELDOR with BtuB WT. Insets (a–c,e): FS spectra indicating the position of the pump (red arrow) and observer (green arrow) pulses. b) NO–NO PELDOR for BtuB 188C. c) NO(observer)–TAM1(pump) PELDOR for BtuB 188C and d) the corresponding distance distribution (green). e) NO(pump)–TAM1 (observer) PELDOR for BtuB 188C and f) the corresponding distance distribution (green). For (d) and (f) the distance distribution from 188R1–TEMPO-CNCbl PELDOR[8] is overlaid (black) for comparison. Inset (f): Relative amounts of the NO (gray circle) and TAM1 (violet circle) and fraction of the coupled spins (green intersection) in the sample. Data analysis was performed with DeerAnalysis[35] software.

non-specifically bound TAM molecules observed at 50 K are far enough from the TEMPO-CNCbl labels to be not detected in the PELDOR.

In the next step, we attempted to perform PELDOR on the TAM1-labeled membranes containing BtuB188C and TEMPO-CNCbl. We performed a PELDOR measurement with both the pump and the observer pulses placed on the nitroxide spectrum (with a 60 MHz offset) to rule out that no distances arise from a dimer or an oligomer of the TEMPO-CNCbl (Figure 2 b). Next, we placed the pump pulse on the TAM and observed at the maximum of the TEMPO-CNCbl with a 100 MHz offset. Unlike with WT BtuB, we could obtain a well-oscillating PELDOR trace with a 15 % λ (Figure 2 c,d, Figure S4 in the Supporting Information) revealing the interaction of TEMPO-CNCbl with TAM bound to BtuB. Changing the frequency offset between the pump and the observer pulses in the range of 40–100 MHz did not change the PELDOR data, which suggested the lack of any orientation selectivity (Figure S4 in the Supporting Information). We used a 20 ns pump pulse, which could excite nearly the whole TAM spectrum (Figure S5 in the Supporting Information). Having 30 μM TEMPO-CNCbl and only circa 20 μM TAM in the sample, the maximum possible λ while pumping TAM is about 66 %. Hence, the 15 % λ indicates that about one fourth of the TEMPO-CNCbl (ca. 7 μM) is coupled with TAM attached to BtuB, or approximately 25 % of the BtuB molecules in the membrane might be labeled with TAM. Thus, with circa 14 μM spin labels coupled to each other (ca. 7 μM TEMPO-CNCbl and TAM, each) the sample contains about 28 % of BtuBTAM1–TEMPO-CNCbl pairs.

Subsequently, we switched the pulses to observe the TAM1 while pumping TEMPO-CNCbl at a 50 or 100 MHz offset. This gave PELDOR signals similar to the first experiment. Pumping TEMPO-CNCbl at a 50 MHz offset gave 8 % λ (Figure S6 in the Supporting Information), which was increased to 10 % when the pump pulse was placed on the maximum of the TEMPO-CNCbl spectrum at a 100 MHz offset (Figure 2 e). Though the spectrum of TEMPO-CNCbl is much broader than that of TAM1 (ca. 280 vs. ca. 28 MHz), λ was reduced only by 33 % of the value obtained while pumping TAM (10 % and 15 %, for the TEMPO- and TAM-pumped experiments, respectively). Though unexpected, this is due to the different amounts of TEMPO-CNCbl and (observable) TAM in the sample (inset in Figure 2 f). In a NO–NO PELDOR under our experimental set up, a 10 % λ while pumping at the maximum of the NO arises when 40 % of the spins are coupled ($\lambda_{max} \approx 25$ % for 100 % coupling). The FS experiments have shown that the amount of detectable TAM is circa 20 μM (and 30 μM for TEMPO-CNCbl) at 50 K (Figure 1 d, Figure S2 in the Supporting Information), and the sample contains about 7 μM coupled spin labels (see the discussion at the end of the preceding paragraph). This fraction of circa 7 μM coupled spin labels is approximately 35 % of the TAM concentration in the sample, which should give 8–9 % λ in the PELDOR, close to the 10 % λ we observed. Further relaxation of more of the TAM during the much longer dipolar evolution (Figure S3 in the Supporting Information) in the PELDOR experiment, which would effectively reduce the observable TAM concentration, may also contribute to an increase in λ. These results further confirm that most of the TAM in the membranes relaxes too fast at 50 K and that only a fraction (ca. 20 μM out of 250 ± 25 μM) contributes to the final PELDOR signal. The uncoupled fraction of the TAM (ca. 60 %) constitutes the observable non-specific labeling, which is slightly lower than that observed with MTSL (ca. 70 %) in these membranes.[8]

Though we could overcome the problem of protein solubility by labeling the protein in the membranes, the effect of the relatively larger size of TAM1 (Figure 1 a) on the protein structure and function is another concern. The second extracellular loop carrying 188C is involved in CNCbl-ligand binding through a conformational change.[9] The observation that the labeled BtuB can bind TEMPO-CNCbl reveals that TAM does not interfere with the conformational change required for the ligand binding. Furthermore, we compared the 188TAM1–TEMPO-CNCbl distances to the previously measured 188R1–TEMPO-CNCbl distances in the same membranes.[8] The 188TAM1–TEMPO-CNCbl PELDOR gave a distance distribution similar to that of 188R1–TEMPO-CNCbl. Despite the larger size, TAM1 shifted the mean distance only by < 5 Å (Figure 2 d,f). For the major peak, the mean distance shifted from 2.7 ± 0.14 nm to 3.14 ± 0.19 nm. Additionally, the width of the distance distribution is comparable to that of the NO–NO distances, suggesting that

TAM1 may not induce any structural perturbation or broadening at the loop sites and that the distribution of the spin density within TAM1 can be neglected.

Transverse relaxation measurements revealed that the TAM1 radical has a T_m of circa 0.7 μs at 150 K (Figure S2 in the Supporting Information), which offers the possibility to perform PELDOR at elevated temperatures. In this case, the PELDOR experiment might be limited mostly by the T_1 of the TEMPO-CNCbl. In the FS, the TEMPO-CNCbl signal was significantly reduced at higher temperatures (Figure S7 in the Supporting Information). Although the TEMPO-CNCbl is spectroscopically invisible, it is still possible to pump it while observing TAM to perform PELDOR at 150 K or even 175 K (Figure 3, Figure S8 in the Supporting Information). The obtained distance distributions are similar to what was observed at 50 K, suggesting that an increase of 125 K did not

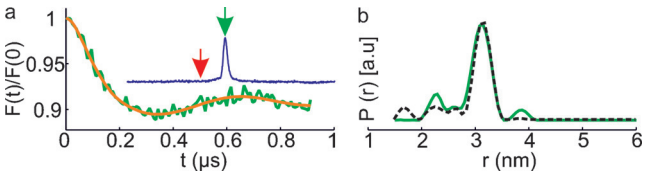

Figure 3. Q-band PELDOR at 150 K in native outer membranes. a) NO-(pump)–TAM1 (observer) PELDOR on BtuB 188C–TEMPO-CNCbl. Inset: FS spectrum indicating the position of the pump (red arrow) and observer (green arrow) pulses placed at a 50 MHz offset. b) Distance distributions obtained at 150 K (green) and 50 K (black dotted line, see Figure 2 f).

make any significant changes to the second extracellular loop conformation or TEMPO-CNCbl binding. Above 175 K it was difficult to obtain PELDOR data owing to other reasons, such as the fast (T_1) relaxation of the TEMPO and the microwave losses in the resonator.

Compared to a NO–NO pair, the narrow line width of the TAM provides higher λ (2–4-fold depending on the set up) and sensitivity for TAM–NO PELDOR. As it can be fully excited, TAM is an ideal spin label for samples with very high background signals, such as the native membranes. The sample used presently is a highly concentrated membrane suspension, hence a concentration of circa 200 μM TAM as aggregates may not cause significant membrane perturbation. With orthogonal labeling any possible distances that could arise from non-specific labeling can be selectively eliminated. Thus, TAM–NO PELDOR can be used for the unambiguous observation of the oligomerization of or ligand interaction among several membrane proteins in their native environments. By observing TAM, distances can be measured at higher temperatures, which also considerably reduces running costs. This approach could work with bacterial inner membranes and eukaryotic organellar or cellular membranes, provided that the membranes can be concentrated to achieve micromolar protein concentrations. The availability of TAM as an additional spin label together with MTSL and Gd^{III} will greatly facilitate the investigation of hetero-oligomeric membrane protein complexes employing multiple spin labels.

Acknowledgements

This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 807) to B.J. and T.F.P., Russian Science Foundation (14-14-00922) to E.G.B. and Russian Foundation for Basic Research (14-03-93180) to V.M.T. B.J. would like to thank Dr. Andriy Marko and Philipp Schöps for the fruitful discussions, Prof. Enrico Schleiff for providing the protein expression facilities, and Prof. David Cafiso for the plasmids.

Keywords: EPR · membrane proteins · PELDOR or DEER · spin labeling · trityl

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 11538–11542
Angew. Chem. **2016**, *128*, 11710–11714

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Received: June 30, 2016
Published online: August 11, 2016

