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# Trityl Radicals: Spin Labels for Nanometer-Distance Measurements

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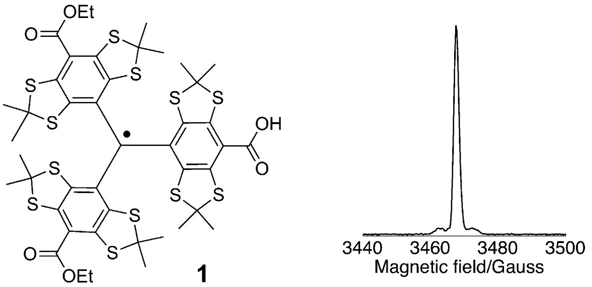
Structural biology and material sciences are engaging ever-larger complexes, either isolated, in composites or within whole cells. Thus, methods that can provide structural information for these molecular architectures on the relevant length scale are needed. EPR methods, especially pulsed electron–electron double resonance (PELDOR or DEER), have emerged as very powerful tools to quantitatively measure nanometer distances in the range of approximately 1.4 to 8 nm.[1–4] Because many of the systems are diamagnetic, site-directed spin labelling is required, usually with nitroxides.[5,6,7] Although nitroxides are relatively stable radicals that can be readily incorporated into molecules to be studied by EPR, they have the disadvantage of rather low sensitivity for EPR-based nanometer-distance measurements and require measurements at cryogenic temperatures. Furthermore, they are only stable for minutes under the reducing environment that exists within cells,[8] which makes in-cell distance measurements with nitroxides very demanding.[9]

Herein, an approach that may overcome some of these limitations by using carbon-centred triarylmethyl (trityl) radicals instead of nitroxides for nanometer-distance measurements is introduced. Specifically, the tetrathiatriarylmethyl radical **1**[10] was used as the spin label (Figure 1), which gave an EPR spectrum with one line only (plus very weak $^{13}C$ satellite lines; Figure 1) and has a transverse relaxation time $T_M$ in the microsecond regime, even at room temperature in the liquid state.[11,12] The trityl radical is stabilized against dimerization by the substituted aryl groups[13] and the in-cell survival time is in the range of hours.[14,15]



**1**

Figure 1. The chemical structure of the trityl spin label **1** and it's field-swept X band EPR spectrum.

Poly(*para*-phenyleneethynylene)s (PolyPPEs) was chosen to evaluate the potential of trityls as spin labels for nanometer distance measurements. The structure and conformational flexibility of this class of polymers, which have found wide use in material science,[16,17] have previously been studied by PELDOR in combination with nitroxide labelling.[18,19] Two compounds (**2** and **3**, Scheme 1) were prepared for distance measurements with trityl spin labels. Compound **2** contains one trityl and a typical nitroxide, whereas compound **3** has two trityl groups. The reason for synthesizing a compound with both a trityl and a nitroxide had two purposes: the combination of a trityl label with a nitroxide spin label may be useful for biological heterodimers, and because it enables a direct comparison to PELDOR measurements on bisnitroxides. In both compounds **2** and **3**, the two radicals are connected by a linear tether consisting of aryl and acetylene units that were linked through a series of Sonogashira cross-coupling reactions. Synthesis of the key-linking unit **4** (Scheme 1 a), which contains heptyl groups to facilitate adequate solubility of the biradicals, is outlined in Scheme S1 in the Supporting Information. Synthesis of the trityl-nitroxide biradical **2** (Scheme 1 a) started with conjugation of nitroxide **12**, prepared by coupling 4-iodobenzoic acid and 4-amino TEMPO (see the Supporting Information), to linker **4** to give compound **13**. The monoacid trityl radical **1**, prepared by limited alkaline hydrolysis of trityl alcohol **14** and subsequent treatment with TFA (see the Supporting Information), was coupled with nitroxide **13** to give the trityl-nitroxide biradical **2**. For synthesis of trityl biradical **3**, complex **1** was coupled with linker **4** to give trityl radical **16** (Scheme 1 b), followed by a Pd-catalyzed di-

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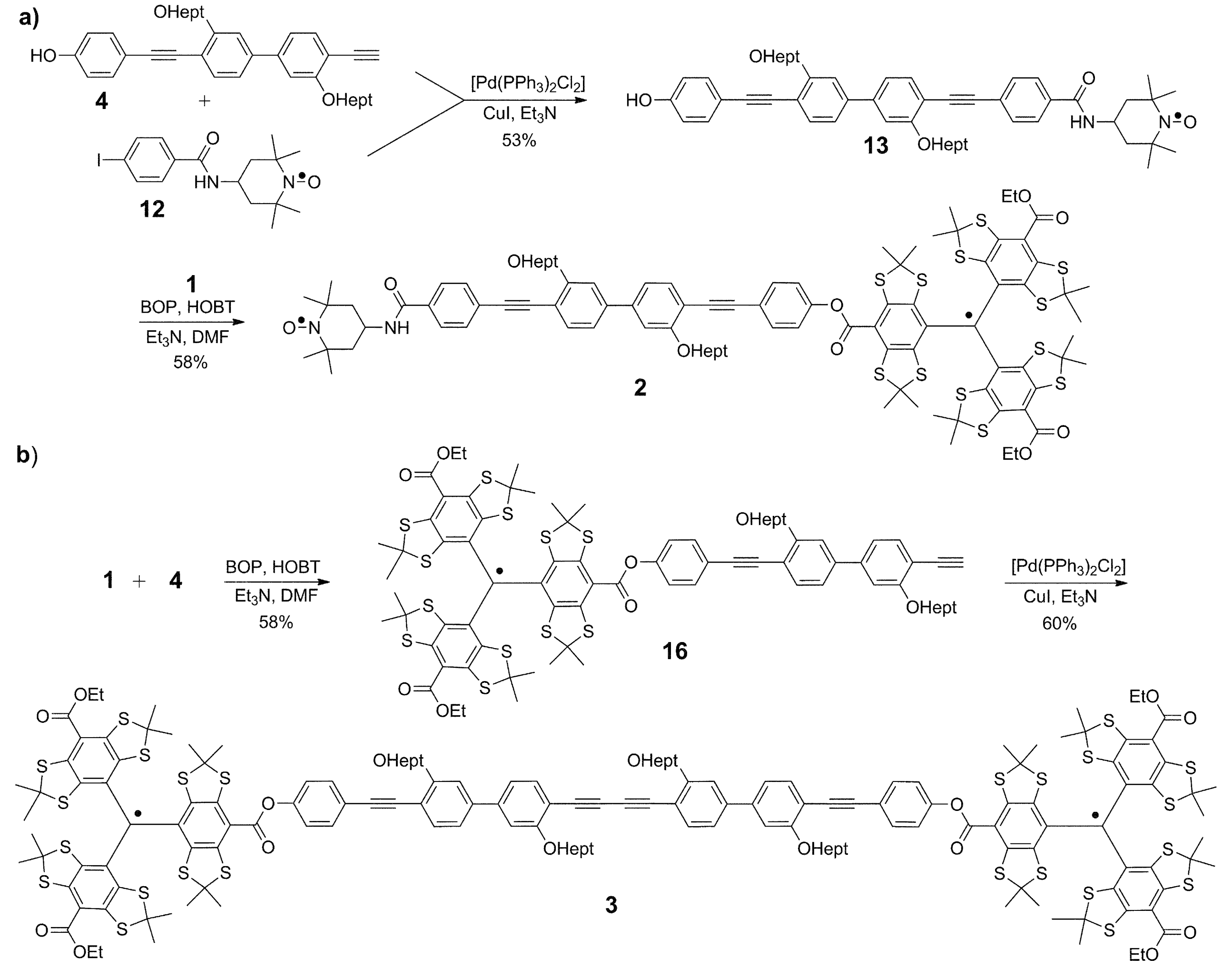

Scheme 1. Synthesis of a) the trityl-nitroxide biradical **2** and b) the trityl–trityl biradical **3**. HOBT = hydroxybenzotriazole; BOP = benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate.

merization in the presence of CuI under atmospheric oxygen, which gave biradical **3**.

Two-pulse Hahn echo field-swept spectra of compounds **2** and **3** confirmed the presence of the radicals (Figure 2). For compound **2**, the width of the nitroxide spectrum enabled placement of the detection pulses on the nitroxide spectrum, at 30–90 MHz higher frequencies (Figure 2a) than the frequency of the inversion pulse on the centre of the trityl resonance. Because the trityl peak has a linewidth of only 2 *G*, the inversion pulse with an excitation bandwidth of 11 *G* should invert all of the trityl spin centres.

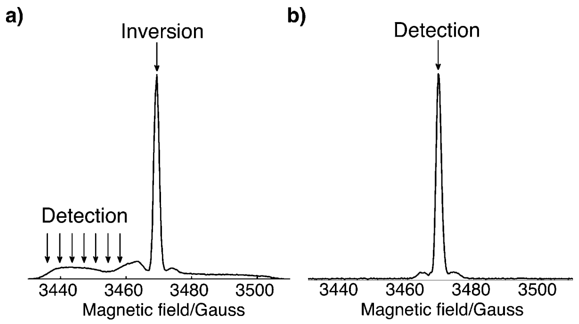

Figure 2. The two-pulse Hahn echo field swept EPR spectra of a) compound **2** and b) compound **3**.

Experimentally, a modulation depth *Δ* of 90% was observed independent of the frequency offset for the detection pulses (Figure 3a). This is twice the modulation depth observed for bisnitroxide systems at X band[20,21] and indicates that excitation of the trityl is almost complete. Deviation from the expected 100% is attributed to the inversion pulse not having an excitation profile of an ideal *π* pulse (see the Supporting Information). Fourier transforms of the time traces showed that the intensity of the perpendicular component ($\theta = 90°$) of the dipolar coupling increases, whereas the intensity of the parallel component ($\theta = 0°$) decreases, if the detection frequency is moved from 30 to 90 MHz offset (Figure 3b). This indicates that orientation-selective PELDOR measurements can still be performed on such mixed trityl/nitroxide-labelled systems, but with much improved modulation depth compared to bisnitroxides.[22] To extract the distance distribution, the time traces of the dif-

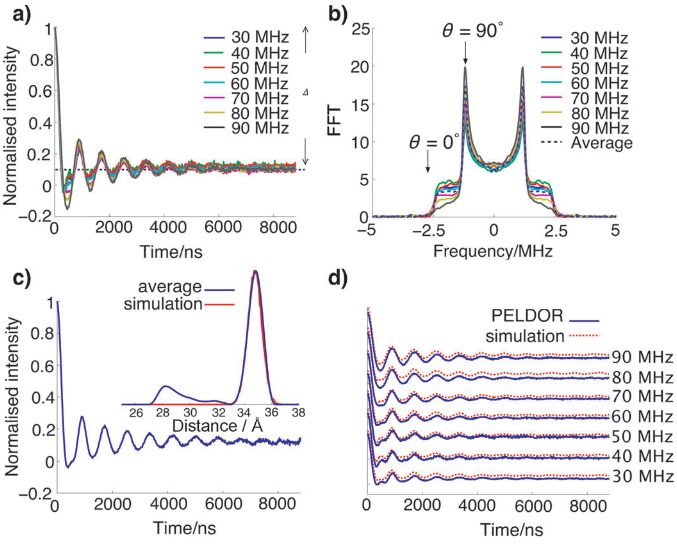

Figure 3. PELDOR data obtained from **2**. a) Background-subtracted time traces. b) Fourier transformed time traces in a). c) Orientation-averaged time trace and distance distributions obtained from Deer analysis (blue line) and by simulations (red line). d) Background-corrected time traces (blue line) and simulated time traces (broken red line). Experimental and simulated time traces have been displaced on the vertical axis for clarity.

ferent frequency offsets (Figure 3 a) were added together, and the resulting orientation-averaged time trace (Figure 3 c) was analysed with Deer analysis[23] (Figure 3 c, inset). The obtained mean distance fits to the distance calculated with molecular mechanics (Table 1). The small peak in the distance distribution from the orientation averaged time trace is attributed to incomplete orientation averaging, because its peak value corresponds to the over weighted parallel component of the dipolar spectrum.

Table 1. Interspin distances for **2** and **3** obtained from molecular mechanics, simulations and from PELDOR and DQC, both in combination with Deer analysis. All distances are given as a mean value ± two standard deviations.

| | $r_{\text{sim}}$ [Å] | $r_{\text{PELDOR}}$ [Å][a] | $r_{\text{DQC}}$ [Å][a] | $r_{\text{MM}}$ [Å] |
|---|---|---|---|---|
| **2** | 34.8 ± 1.1 | 34.8 ± 1.0 | 34.8 ± 1.2 | 35 |
| **3** | 48.8 ± 1.3 | – | 48.9 ± 1.4 | 51 |

[a] The mean distance ± two standard deviations is given for the most probable peak.

To obtain more information on the conformational dynamics of the system, the PELDOR time traces for **2** were also simulated with a home-written Matlab program, which uses a conformational model and takes orientation selectivity into account.[24] The conformational model for **2** was generated by treating the backbone and spin labels as a chain of rigid segments linked by joints (see the Supporting Information).[18,19] This dynamics model and one set of EPR parameters were used to reproduce the orientation-selected PELDOR time traces (Figure 3 d) and provided an excellent fit to the distance distribution from the orientation-averaged time trace (inset in Figure 3 c and Table 1). Distributions in length and flexibility of segments that resulted in the best fit between simulations and experiment are summarized in Table S2 in the Supporting Information. These results are in excellent agreement with those from Jeschke et al., which studied similar polymers by nitroxide labelling and PELDOR.[18] This indicates that the size of the trityl-spin label has no influence on the dynamics of the PolyPPEs, and that the spin density distribution within the trityl label can be neglected.

The narrow spectral width of the trityl centre also prompted us to perform double-quantum coherence (DQC) measurements on compound **2** (Figure 4).[25,26] DQC EPR is an elegant way to solely detect the dipolar electron–electron coupling in a time-domain experiment by introducing a double-quantum coherence filter into the pulse sequence. It was first introduced by the lab of Freed,[27] and it only requires one microwave source. In contrast to bisnitroxide biradicals, for which we were not able to obtain DQC time traces on a commercial EPR spectrometer (data not shown), DQC gave very good time traces for compound **2** with almost complete excitation of the whole Pake pattern (Figure 4 b). This indicates that it might be sufficient for DQC to completely excite the dipolar coupling on one of the two spin centres. The distance distribution obtained from Deer analysis has a mean value and a standard deviation that is in agreement with the result obtained by PELDOR (Table 1). In addition, the DQC time trace and distance distribution were simulated with the same dynamics model as was used for the PELDOR time traces and gave an excellent fit (Figure 4 a and c).

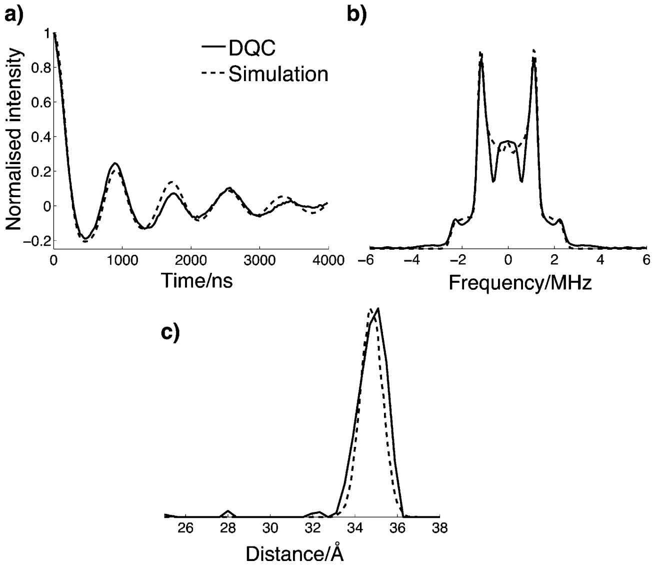

Figure 4. Six-pulse DQC data obtained from **2**. a) Background-corrected time trace (solid line) and simulated time trace (broken line). b) Fourier transformation of the time traces in a). c) Distance distributions from the DQC time trace by using Deer analysis (solid line) and the simulation model (broken line).

Bistrityl radical **3** gave an EPR spectrum with a width of only 2 *G* (Figure 2b), which is about a factor 30 narrower compared to a typical bisnitroxide spectrum with a width of about 70 *G*. This reduced width translates into increased signal intensity for the bistrityl system by roughly the same factor. DQC measurements on **3** gave a time trace with excellent signal-to-noise ratio that is a factor of two better than for a PELDOR time trace obtained for a bisnitroxide under the same conditions (Figure 5a and the Supporting Information). Measurement on **3** with PELDOR gave only electron-spin echo envelope modulation (ESEEM) artefacts due to a strong pulse overlap (see the Supporting Information). The DQC time trace for **3** was simulated by using the same segmented dynamics model as for **2** (Figure 5a and Table S2 in the Supporting Information).

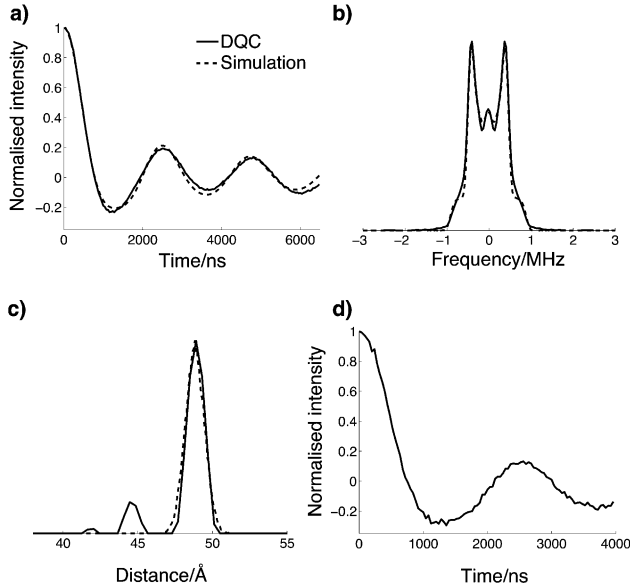

Figure 5. Six-pulse DQC data obtained from **3**. a) Background-corrected time trace (solid line) and simulated time trace (broken line). b) Fourier transform of the time traces in a). c) Distance distributions from the DQC time trace in a) by using Deer analysis (solid line) and the model-based simulation (broken line). d) Background-corrected DQC time trace obtained from **3** at 100 K.

The distance distributions, obtained from Deer analysis and from the model-based simulation are in very good agreement (Figure 5c). The average distance agrees with the distance obtained from molecular-mechanics modelling (Table 1).

The high signal-to-noise ratio and the $T_\mathrm{M}$ relaxation behaviour of **3** prompted us to perform DQC measurements also at higher temperatures. Because the spin system has to be immobile on the time scale of the coupling to be observed, the solvent (toluene) still had to be frozen. Therefore, a temperature of 100 K, at which a time trace with an excellent *S*/*N* of 60:1 could be obtained within 20 h, was chosen (Figure 5d). Compared to 50 K, the $T_\mathrm{M}$ was reduced by a factor of 1.5 and the signal-to-noise ratio was reduced by a factor of 2.7 only. In contrast, no spin echo was observed for a bisnitroxide radical at 100 K, making PELDOR measurements at this temperature for nitroxide labels with *gem*-dimethyl structure or in matrices others than *ortho*-terphenyl unfeasible.[28,29]

In summary, we have demonstrated that trityl radicals can be successfully used as spin labels for nanometer-distance measurements by using pulsed EPR. They provided deep PELDOR modulations in experiments with mixed trityl/nitroxide labels and improved sensitivity in trityl/trityl DQC experiments. The trityl label is certainly more bulky than a nitroxide, but whether this leads to structural distortions, for example, in biological systems will depend on the specific molecular structure and will have to be checked in each individual case as for nitroxides. Please note that a paper from the laboratories of Freed and Hubbell on trityl–trityl distance measurements in the liquid state on proteins appeared, while this paper was being reviewed.[30]

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**Keywords:** dipolar coupling · double-quantum coherence · EPR spectroscopy · nanostructures · pulsed electron–electron double resonance

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## EPR Spectroscopy

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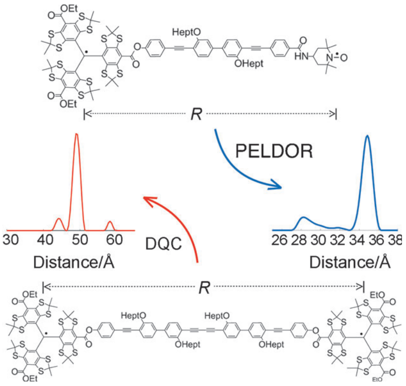
**Trityl Radicals: Spin Labels for Nanometer-Distance Measurements**



**Spin labelling with trityls**: To gather information about structure and dynamics of trityl radicals, spin-labelled polymers were measured with pulsed electron–electron double resonance (PELDOR) and double-quantum coherence (DQC). This study demonstrates that trityl radicals have great potential as spin labels that eliminate some limitations of nitroxide spin labels (see figure).