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Measurements of short distances between trityl spin labels with CW EPR, DQC and PELDOR[†]

Nitin C. Kunjir,^{‡a} Gunnar W. Reginsson,^{‡ab} Olav Schiemann^{*bc} and Snorri Th. Sigurdsson^{*a}

Trityl based spin labels are emerging as a complement to nitroxides in nanometer distance measurements using EPR methods. The narrow spectral width of the trityl radicals prompts us to ask the question at which distance between these spin centers, the pseudo-secular part of the dipolar coupling and spin density delocalization have to be taken into account. For this, two trityl-trityl and one trityl-nitroxide model compounds were synthesized with well-defined interspin distances. Continuous wave (CW) EPR, double quantum coherence (DQC) and pulsed electron–electron double resonance (PELDOR) spectra were acquired from these compounds at commercial X-band frequencies. The data analysis shows that two of the compounds, with distances of up to 25 Å, fall into the strong coupling regime and that precise distances can only be obtained if both the spin density delocalization and the pseudo-secular part of the dipolar coupling are included in the analysis.

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Introduction

Pulsed Electron Paramagnetic Resonance (EPR) methods and site-directed spin labelling have been established as versatile tools to determine structural information regarding large bio-molecular complexes.^{1,2} The most widely used pulsed EPR methods for the study of structure and function of biomolecules are pulsed electron–electron double resonance (PELDOR or DEER)^{3,4} and double quantum coherence (DQC).^{5,6} These methods are capable of quantitatively measuring distances in the range of 14 to 80 Å.^{7,8} The aminoxyl (nitroxide) radical is by far the most frequently used spin label for EPR-based distance measurements in bio-macromolecules and polymers.⁹ To further improve and extend the application of pulsed EPR to structural studies, it is important to develop a wide range of spin labels with complementary properties. Paramagnetic metal ions, such as copper(π),¹⁰ manganese(μ)¹¹ and gadolinium(π),¹² have been used as alternative spin labels.

^b Biomedical Sciences Research Complex, Centre of Magnetic Resonance, North Haugh, St. Andrews, KY16 9ST, UK In addition, the carbon-centered triarylmethyl (trityl) radical¹³ has emerged as a promising spin label.^{14,15} The trityl radical has a half-life of several hours in reducing environments,^{16,17} and a transverse relaxation time ($T_{\rm M}$) of microseconds in the liquid state at room temperature.^{18,19} These properties could become useful for in-cell distance measurements and at ambient temperatures.¹⁵

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Recently, two groups independently reported the use of trityl spin labels for distance measurements, either in spin-labeled polymers¹⁴ or immobilized proteins in liquid solution.¹⁵ Since the trityl radical has a very narrow EPR spectrum $(\sim 2 \text{ G})^{18}$ and about 30% of the unpaired spin is delocalized across several atoms,²⁰ effects from the pseudo-secular part of the dipoledipole coupling and spin delocalization might be appreciable at longer distances than for the more common nitroxide spin labels, which have an EPR spectral width of \sim 70 G and the majority of the spin density localized on the nitrogen and oxygen atoms.²¹ Distances in the 15–30 Å range are often used in biological systems and within this distance range both effects might be important if working with trityl radicals. Therefore, we synthesized three model compounds with well defined trityl-trityl and trityl-nitroxide distances in the range of 17 to 24 Å, recorded X-band DQC and PELDOR spectra and analyzed the data taking both contributions into account. The results show that for this distance range, the pseudo-secular part of the dipole-dipole coupling and the spin delocalization of the trityl radical have to be taken into account for the extraction of accurate interspin distances.

^a University of Iceland, Department of Chemistry, Science Institute, Dunhaga 3, 107 Reykjavík, Iceland. E-mail: snorrisi@hi.is

^c Institute of Physical and Theoretical Chemistry, University of Bonn, Wegelerstr. 12, 53115 Bonn, Germany. E-mail: schiemann@pc.uni-bonn.de

[†] Electronic supplementary information (ESI) available: General procedures and detailed organic synthesis procedures for compounds 1–3, 7, 11–13, 15–16. NMR, EPR, MS spectra and HPLC chromatograms. Simulation parameters for 1–3. Original DQC and PELDOR time traces. Histograms of the ratio, $|\omega_{\rm A} - \omega_{\rm B}|$ to $|\omega_{\rm AB}|$. Average spin densities of the trityl radical. See DOI: 10.1039/c3cp52789a [‡] These authors contributed equally to this work.

Results and discussion

To evaluate the use of EPR spectroscopy to measure short trityltrityl and trityl-nitroxide distances, compounds **1–3** were prepared (Fig. 1). Each of these compounds contains two radicals that are linked by a single phenyl group (compound **1**) or two phenyl groups connected through an acetylene unit (compounds **2** and **3**) (Fig. 1). These linkers were chosen because of their rigid or semi-rigid structure and known dynamics.^{14,22,23} The trityl and nitroxide radicals were connected to the linkers *via* ester- or amide bonds, which serve as insulators to prevent exchange coupling through the π -system of the linker.^{24–26} Compounds 1 and 2 contain two trityl spin labels, with an interspin distance of about 18 and 25 Å, respectively, according to molecular mechanics modelling. These compounds served as model systems for the measurement of short distances. Compound 3 contains both a trityl spin label and a typical nitroxide and has an interspin distance of about 25 Å. This compound was synthesized to evaluate the effects of having spin centers with both a narrow and broad spectrum within the same system. Compound 4 (Fig. 1), previously prepared for DQC studies, was also used in the present study for PELDOR measurements.



Fig. 1 Structures of biradicals 1–4. The synthesis of compound 4 has been previously reported.¹⁴

This compound has a mean interspin distance of 48.9 Å, according to DQC measurements.¹⁴

Synthesis of compounds 1-3

In the synthesis of biradical **1** (Scheme 1a), 1,4-hydroquinone was coupled with monoacid trityl alcohol **6** (ref. 14) using benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP) and 1-hydroxybenzotriazole (HOBT) as coupling reagents. The resulting bi-alcohol 7 (ESI \dagger) was converted to biradical 1 upon subsequent treatment with trifluoroacetic acid (TFA).

The synthesis of biradical 2 (Scheme 1b) started with a Sonogashira cross-coupling of acetylene 8 with 4-iodophenol to yield linker 10. Compound 10 was coupled to the monoacid trityl alcohol 6, followed by deprotection with *p*-toluene-sulphonic acid (PTSA) to give trityl conjugate 12. Compound 12 was coupled to trityl alcohol 6 to give 13 (ESI[†]) and



Scheme 1 Syntheses of (a) trityl-trityl biradical **1**, (b) trityl-trityl biradical **2** and (c) trityl-nitroxide biradical **3**. BOP = benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate, HOBT = 1-hydroxybenzotriazole, Et_3N = triethylamine, PTSA = p-toluenesulphonic acid, THP = tetrahydropyran.

subsequent treatment of **13** with (TFA) generated biradical **2** in a good yield.

In the synthesis of biradical 3 (Scheme 1c), compounds 14 (ref. 14) and 8 were linked by a Sonogashira cross-coupling reaction, followed by deprotection with PTSA to yield nitroxide 16. Compound 16 was coupled with monoacid trityl radical 17,¹⁴ prepared by treatment of trityl alcohol 6 with TFA, to yield the trityl-nitroxide biradical 3.

EPR spectroscopy

Dipole-dipole coupling. The PELDOR and DQC techniques can be used to determine distances between a pair of spin centers through the measurement of the dipole-dipole coupling ω_{ab} between the unpaired electrons. The dipole-dipole coupling between two unpaired electrons A and B, can be described by the Hamiltonian operator shown in eqn (1).²⁷

$$\hat{H}_{AB} = \frac{\mu_0}{4\pi\hbar} g_A g_B \beta_e^2 \left[\frac{\hat{\mathbf{S}}_A^T \cdot \hat{\mathbf{S}}_B}{r^3} - \frac{3\left(\hat{\mathbf{S}}_A^T \cdot \mathbf{r}\right)\left(\hat{\mathbf{S}}_B^T \cdot \mathbf{r}\right)}{r^5} \right]$$
(1)

Here μ_0 is the permeability of vacuum, \hbar the Planck constant divided by 2π , β_e the Bohr magneton, **S** the spin operator, **r** the spin–spin distance vector, while g_A and g_B are the average of the *g*-matrix principal values for spin A and B, respectively. For two S = 1/2 spins, eqn (1) can be simplified to eqn (2) if the following approximations are valid:^{1,28} (i) the dipole–dipole coupling is much smaller than the electron Zeeman interaction of the electron spins. (ii) The *g* anisotropy is small. (iii) The unpaired electron is localized to a single point (point-dipole approximation).²⁹

$$\hat{H}_{AB} = \frac{\mu_0}{4\pi\hbar r^3} g_A g_B \beta_e^2 (a+b)$$

$$a = S_z^A S_z^B (1 - 3\cos^2\theta)$$

$$b = -\frac{1}{4} (S_+^A S_-^B + S_-^A S_+^B) (1 - 3\cos^2\theta)$$
(2)

In eqn (2), θ is the angle between the interspin vector and the direction of the magnetic field **B**₀. The *a* and *b* terms are known as the secular and pseudo-secular parts, respectively. If the absolute resonance difference between spins A and B in the absence of dipole–dipole coupling is large compared to the absolute

dipole–dipole coupling, $|\omega_{\rm A} - \omega_{\rm B}| \gg |\omega_{\rm AB}|$, the spin pair is in the weak-coupling regime and the pseudo-secular part in eqn (2) can be neglected. The dipole–dipole coupling, in angular frequency ($\omega = \nu 2\pi$), is then described by eqn (3).^{30,31}

$$\omega_{AB} = \frac{D}{r^3} (1 - 3\cos^2\theta)$$

$$D = \frac{\mu_0 g_A g_B \beta_e^2}{4\pi\hbar}$$
(3)

In the case of a pair of coupled nitroxides, eqn (3) is valid as long as the interspin distance exceeds ~ 15 Å.³² On the other hand, if the absolute resonance difference between spins A and B is small compared to the absolute dipole–dipole coupling $|\omega_{\rm A} - \omega_{\rm B}| \ll |\omega_{\rm AB}|$ the spin pair is in the strong-coupling regime and the pseudo-secular part has to be included. The dipole– dipole coupling constant *D* is then described by eqn (4).^{30,31}

$$D = \frac{3}{2} \frac{\mu_0 g_A g_B \beta_e^2}{4\pi\hbar} \tag{4}$$

In the intermediate case, when the dipole–dipole coupling is approximately equal to the resonance difference of spins A and B, $|\omega_{\rm A} - \omega_{\rm B}| \sim |\omega_{\rm AB}|$, the extraction of interspin distances from EPR data requires careful simulations and becomes less reliable.^{30,31,33}

If the unpaired electron spin is not localized to a welldefined point, the point-dipole approximation fails and the dipole–dipole interaction between each spin-bearing atom of spin centers A and B has to be considered.³⁴ The dipole–dipole coupling is then described by eqn (5),^{35,36}

$$\omega_{\rm AB} = D \sum_{n,m} \frac{\left(1 - 3\cos^2\theta_{nm}\right)}{r_{nm}^3} \rho_n \rho_m \tag{5}$$

where *n* and *m* denote the atoms of spin centers A and B respectively, carrying spin density of ρ . The axial symmetry of the dipolar tensor is still assumed to be valid.

We show below that both the pseudo-secular part and spindelocalization need to be accounted for in the simulation of the EPR data for compounds **1** and **2**.

CW-EPR of compounds 1–3. Compounds **1–3** were measured with continuous wave electron paramagnetic resonance (CW-EPR) at 120 K to confirm the presence of the radicals and to assess the



Fig. 2 Experimental and simulated CW-EPR spectra. CW-EPR spectra for 1–3, at 120 K, are shown in (a), (b) and (c), respectively. The broken lines show the simulated spectra and have been displaced on the vertical axis for clarity.

dipole-dipole coupling. **1** yielded an EPR spectrum with a width of about 33 MHz (Fig. 2a), which is the first derivative of the dipolar Pake pattern. Since the peak-to-peak linewidth of a single trityl radical in deuterated toluene at 120 K is about 5.6 MHz,¹⁴ this large spectral width indicates a strong dipole-dipole coupling between the trityl radicals in **1**.

The separation of the Pake pattern singularities in the EPR spectrum of **1** equals ~12 MHz. Using eqn (3) (with $\theta = 90^{\circ}$), this dipole–dipole coupling is equal to an interspin distance of 16.3 Å, but using eqn (4), yields an interspin distance of 18.6 Å, which is in good agreement with the distance of 18.4 Å, calculated from a molecular mechanics model of **1** (Table 1). To obtain a better estimate of the interspin distance of **1** the CW-EPR spectrum was simulated using the EasySpin toolbox³⁷ (Table S1, ESI[†]). To obtain a good fit between the simulated and experimental spectra, a dipole–dipole coupling of 9.5 MHz had to be included (Fig. 2a). Using eqn (3) (with $\theta = 90^{\circ}$), this dipole–dipole coupling is equal to an interspin distance of 17.7 Å, which is in good agreement with the distance of 18.4 Å, calculated from a molecular mechanics model of **1** (Table 1).

CW-EPR measurement of compound **2** gave an EPR spectrum with a peak-to-peak linewidth of about 5.6 MHz (Fig. 2b). Although the peak-to-peak linewidth of **2** is similar to that of a single trityl radical, the full width of the main peak was larger by about 5 MHz. A good fit was obtained in the simulation of **2** by including a dipole–dipole coupling of 3 MHz, which is equal to an interspin distance of about 26 Å according to eqn (3) (with $\theta = 90^{\circ}$). This distance is also in good agreement with the distance of 25.2 Å from a molecular mechanics model of **2** (Table 1).

As the EPR spectral width of trityl is a factor of 30 narrower than for a nitroxide, the strong-coupling regime is valid for trityls at longer distances than for a pair of nitroxide radicals.³¹ Compound **3** is, therefore, useful for estimating the effects from the narrow spectral width of the trityl radical. CW-EPR measurement of **3** at 120 K shows that the trityl spectrum has a peak-to-peak width of about 8.4 MHz (3 G), which is about 2.8 MHz wider than for a single trityl (Fig. 2c). The dipole–dipole coupling for **3** is, therefore, observed as a slight broadening of the trityl peak. Simulating the line-broadening in a CW-EPR spectrum for a twospin system with unlike spin centers is not as straightforward as for a pair of identical spin centers. The CW-EPR spectrum of **3** was,

 Table 1
 Interspin distances for 1–3 obtained from CW-EPR, DQC, PELDOR, simulations and molecular mechanics

	$r_{\text{CW-EPR}}^{a}$ (Å)	$\mathbf{r}_{\mathrm{DQC,DA}}{}^{b,c}\left(\mathrm{\mathring{A}}\right)$	$r_{\text{PELDOR,DA}}{}^{b,c}\left(\text{\AA}\right)$	$r_{\sin}^{d,e}$ (Å)	$r_{\mathbf{M}\mathbf{M}}^{d}$ (Å)
1	17.7	15.4 ± 0.6	15.5 ± 0.6	17.4 ± 0.4	18.4
2	26	21.7 ± 0.8	_	24.2 ± 0.8	25.2
3	24	24.4 ± 1.4	24.3 ± 1.6	$\textbf{23.8} \pm \textbf{0.8}$	24.5

 a Interspin distance read from the perpendicular component of the dipole–dipole frequency. b The mean distance \pm two standard deviations, is given for the most probable peak. c Distance distributions were obtained from DeerAnalysis 2011 (DA). d For 1 and 2, the distance is between the center carbon atoms of the trityl radicals. For 3 the distance is from the center carbon atom of the trityl radical to the center of the N–O bond of the nitroxide radical. e The simulated distance distributions were used to simulate both the DQC and PELDOR time traces.

The CW-EPR spectra and simulations for compounds 1–3 show that for trityl radicals in this distance range, the interspin distance can be estimated from the CW-EPR spectrum with good accuracy. This is a considerably larger distance range than for nitroxides.

DQC of compound 1. DQC measurement of **1** at 50 K provided a good quality time trace with a clear modulation (Fig. 3a). Fourier transforming the background corrected time trace resulted in a dipolar spectrum with perpendicular components ($\theta = 90^{\circ}$) at about ±13.7 MHz and parallel components ($\theta = 0^{\circ}$) at about ±27.4 MHz (Fig. 3a, inset). The same is observed for compounds **2** and **3**. Analyzing the background corrected DQC time trace of **1** with Tikhonov regularization, as implemented in DeerAnalysis³⁸ gives a distance distribution with a mean value of 15.4 Å (Fig. 3d and Table 1). This distance is 3 Å shorter than determined from a molecular mechanics model of **1**.

As can be seen from the frequency spectrum obtained from the DQC time trace (Fig. 3a, inset), the dipole–dipole coupling for **1** is larger than the spectral width of a single trityl radical. It is, therefore, expected that **1** is within the strong-coupling regime, which requires inclusion of the pseudo-secular part of the dipole–dipole coupling.^{8,31} Since DeerAnalysis is not optimized for analyzing DQC experiments, and especially not in the strong-coupling regime, it is not surprising that the distance distribution from DeerAnalysis is incorrect.

To include the pseudo-secular coupling in the analysis of the DQC data, a home-written Matlab program was used.³⁹ The program uses a model-based approach that includes distributions of conformers to simulate the time traces. To simulate the dynamics and distance distribution of **1**, a dynamics model was made by representing **1** as a chain of rigid segments with flexible joints. This rigid-segment model has previously been shown to accurately represent the interspin distance and conformational dynamics of structurally similar biradicals with both trityl and nitroxide spin labels.^{14,23}

The rigid segment model of **1** yields a distance distribution with a mean interspin distance of **17.4** Å (Table 1). Using this model in combination with eqn (3) the time trace in Fig. 3a was obtained. As can be seen, the modulation frequency of the simulation does not fit the experimental time trace. On the other hand, simulating the DQC time trace using eqn (4), *i.e.* including the pseudo-secular part, yields a time trace with a modulation frequency closer to the experimental time trace (Fig. 3b). Although the inclusion of the pseudo-secular part to the dipole–dipole coupling improved the fit between the experimental and simulated DQC time traces for **1**, the modulation frequency of the simulated time trace is still slightly higher compared to the experimental time trace (Fig. 3b, inset).



Fig. 3 DQC measurements of **1**. Experimental (solid line) and simulated (broken line) DQC time traces. (a) Simulation using only the secular part of the dipole–dipole coupling (eqn (3)). (b) Simulation including the pseudo-secular part of the dipole–dipole coupling (eqn (4)). (c) Simulation including the pseudo-secular part of the dipole–dipole coupling (eqn (4)). (c) Simulation including the pseudo-secular part of the dipole–dipole coupling (eqn (4)). (c) Simulation including the pseudo-secular part of the dipole–dipole coupling and the spin delocalization of the trityl radical. (d) Distance distribution obtained from DeerAnalysis (solid) and simulated distance distribution (broken line) from the dynamics model of **1**. The insets in (b) and (c) show a magnified view of the time traces. The inset in (a) shows the Fourier transform of the DQC time trace and simulation in (c). The original DQC time trace is shown in Fig. S1 (ESI†).

Given the short interspin distance and narrow distance distribution for **1**, it is possible that spin delocalization of the trityl radicals has an appreciable effect on the interspin distribution. To estimate the spin delocalization of the trityl radical, density functional theory (DFT) calculations were carried out on the trityl radical moiety. The DFT results showed that about 70% of the spin density is on the center carbon atom, 26% distributed across the carbon atoms in the phenyl groups and 4% across the rest of the molecule (Table S2, ESI[†]). These spin densities are also in agreement with previous CW-EPR measurements and DFT calculations on structurally similar trityl radicals.²⁰

To account for the spin delocalization in the simulation, only the center carbon atom and the phenyl carbons were considered. To simplify the modelling of the spin delocalization, the spin centers in the dynamics model for **1** were split up into four points. The positions of the spin-bearing points were approximated from the DFT geometry-optimized structure of **1**. The center carbon atom is the major spin-bearing atom and the spin-bearing carbon atoms in each phenyl group were combined into a single point, located at the center of the phenyl group (Fig. 4). The spin density of this point was found by adding the spin densities of the phenyl carbon atoms and averaging over the three phenyl groups (Table S2, ESI[†]). Adding the four-point



Fig. 4 Molecular structure of the trityl radical moiety in **1**. The position of the four spin centers, used to represent the spin delocalization, in the dynamics model of **1**, are shown by black dots. The numbers show the spin density for each of the spin-bearing centers, obtained from DFT calculations. The simulated distance distribution, shown in Fig. 3d, only represents the distance between the center carbon atoms of the trityl biradical.

spin centers to the rigid segment model of **1** and using spin densities from the DFT calculations yields a simulated DQC time trace with a very good fit to the experimental time trace (Fig. 3c, inset). Comparing the simulated DQC time trace,

before and after including the spin delocalization shows that the spin delocalization increases the mean interspin distance by 0.3 Å.

In summary, inclusion of the pseudo-secular part of the dipole–dipole coupling and spin delocalization shows that the experimental DQC time trace corresponds to a mean interspin distance of 17.4 Å. This distance is in good agreement with the distance of 17.7 Å, obtained from the CW-EPR spectrum. It should be noted that the CW-EPR spectrum is simulated with the pseudo-secular part of the dipole–dipole coupling taken into account. The distance of 17.4 Å is only 1 Å shorter than the distance from the molecular mechanics model of 1, which only represents a single conformer and, therefore, not the mean interspin distance. Since compound 1 is in the strong-coupling regime we cannot see from the Pake pattern if there is also an exchange coupling.³¹

To obtain a better measure of the relative strength of the dipole-dipole coupling of 1, 20 000 conformers from the rigidsegment model and EPR parameters from a CW-EPR spectrum of a single trityl radical,¹⁴ were used to simulate a powder CW-EPR spectrum of 1, without dipole-dipole coupling. The absolute resonance difference between spins A and B, $|\omega_A - \omega_B|$ and the absolute value of the corresponding dipole-dipole coupling $|\omega_{AB}|$, according to (eqn (3)), were calculated for each spin pair. Computing the ratio of $|\omega_{\rm A} - \omega_{\rm B}|$ to $|\omega_{\rm AB}|$ reveals that about 82% of the spin pairs have a ratio that is less than 1 and about 18% of spin pairs have a ratio that is 1 or larger (Fig. S2 and S3, ESI[†]). The dipole-dipole coupling in 1 is therefore predominantly larger than the resonance difference between spins A and B. This is in agreement with the observation that the experimental DQC time trace predominantly oscillates with a frequency that is given by eqn(4). For comparison, calculating the ratio of $|\omega_{\rm A} - \omega_{\rm B}|$ to $|\omega_{\rm AB}|$ for the structurally analogous trityl biradical 4 (Fig. 1) with a mean interspin distance of about 48.9 Å (ref. 14) revealed that only 18% of spin pairs have a ratio that is less than or equal to 1. The dipole-dipole coupling of 4 is, therefore, predominantly much smaller than the resonance difference between spins A and B. This is also not surprising since the analysis of the DQC time trace for this long trityl biradical did not require taking the pseudo-secular part into account.¹⁴ Although the dipole-dipole coupling of **1** is not completely in the strong-coupling regime, the DQC data can be correctly analysed without taking into account the complications due to the intermediate-coupling regime.

DQC of compound 2. DQC measurements of **2** (Fig. 5) and subsequent analysis of the background corrected time trace with DeerAnalysis yield an average distance of 21.7 Å, which is about 2.5 Å shorter than from the rigid segment model of **2** (Table 1). Fourier transforming the background corrected DQC time trace gives a dipolar spectrum with perpendicular components at about ± 4.9 MHz and parallel components at about ± 9.8 MHz indicating the absence of an exchange coupling contribution. Calculating the ratio of $|\omega_A - \omega_B|$ to $|\omega_{AB}|$ from the rigid segment model of **2**, as was done for **1**, shows that about 65% of the spin pairs have a dipole–dipole coupling that is larger than the resonance difference (ratio less than 1) and



Fig. 5 DQC measurement of **2**. (a) Experimental (solid line) and simulated (broken line) DQC time traces. The simulation used the secular part of the dipoledipole coupling (eqn (3)) and spin delocalization of the trityl radical. (b) A simulation including the pseudo-secular part of the dipole-dipole coupling and spin delocalization. The inset shows the distance distributions from DeerAnalysis (solid line) and simulated distance distribution from the rigid segment model of **2** (broken line). The inset in (a) shows the Fourier transform of the experimental time trace and simulation in (b). The original time trace is shown in Fig. S1 (ESI+).

about 35% of the spin pairs have a dipole–dipole coupling that is similar or larger than the resonance difference (Fig. S2 and S3, ESI[†]). Simulating the DQC time trace with the rigid segment model of **2** and only accounting for the spin delocalization results in a poor fit between the experimental and simulated time traces (Fig. 5a). However, inclusion of the pseudo-secular part of the dipole–dipole coupling results in a very good fit between the simulated and experimental DQC time traces (Fig. 5b). Therefore, it can be concluded that even at distances in the range of 20–24 Å, a trityl biradical is predominantly in the strong-coupling regime and the pseudo-secular part of the dipole–dipole coupling, as well as the spin delocalization of the trityl, have to be included for extraction of correct distances from the EPR data.

DQC of compound 3. Calculating the ratio of $|\omega_A - \omega_B|$ to $|\omega_{AB}|$ for each spin pair from a rigid segment model of the trityl-nitroxide compound 3, as was done for 1 and 2, shows that only about 10% of the spin pairs have a dipole-dipole coupling that is larger than, or in the range of, the resonance difference (ratio of 0 to 1). About 90% of the spin pairs have a dipole-dipole coupling that is substantially smaller than the resonance difference between spins A and B (Fig. S2 and S3, ESI[†]). For a pair of nitroxides this same ratio is about 95%.



Fig. 6 DQC measurement of **3**. Experimental (solid line) and simulated (broken line) DQC time traces. (a) Simulation using the secular part of the dipole–dipole coupling (eqn (3)). (b) Simulation including the spin delocalization of the trityl radical. The inset shows the distance distributions from DeerAnalysis on the experimental time trace (solid line) and simulated distance distribution (broken line) from the rigid segment model of **3**. The inset in (a) shows the Fourier transform of the experimental time trace and simulation in (b). The original time trace is shown in Fig. S1 (ESI†).

These observations suggest that compound **3** will be predominantly within the weak-coupling limit. Measuring **3** with DQC and analyzing the background corrected time trace with Deer-Analysis yielded an interspin distance distribution with a mean value of 24.4 Å for the major peak, which is only 0.6 Å longer than the interspin distance from the rigid segment model of **3** (Fig. 6a and Table 1). Fourier transforming the background corrected DQC time trace gave a dipolar spectrum with perpendicular components at about ± 3.5 MHz and parallel components at about ± 7 MHz (Fig. 6a, inset), indicating negligible exchange coupling.

Although a good fit between simulated and experimental DQC time traces was obtained already when only the secular part of the dipole–dipole coupling (eqn (3)) was included (Fig. 6a), further improvement in the simulation of the time trace was achieved by including the spin delocalization of the trityl radical (Fig. 6b). Thus, the simulated DQC time traces show that compound **3**, where only about 9% of the spin pairs have a dipole–dipole coupling that is larger than the resonance difference, is within the weak-coupling regime. For this trityl–nitroxide biradical, the spin delocalization of the trityl radical has to be included for the extraction of correct distances from the EPR data.

PELDOR of trityl-trityl biradicals 1 and 4 and trityl-nitroxide 3. Although the narrow width of the trityl EPR spectrum makes single frequency techniques, *e.g.* DQC, the preferred method for the measurement of the dipole–dipole coupling, we also wanted to investigate the use of the two-frequency method PELDOR to measure the dipole–dipole coupling between two trityl radicals. PELDOR measurements on compound **1** were performed by placing the inversion pulse on the center of the EPR spectrum and the detection pulses on the region of the ¹³C satellite, 15 MHz (5.4 G) away from the inversion pulse (Fig. 7a).

To minimize spectral overlap of the pulses, the length of the inversion pulse was set to 60 ns and the $\pi/2$ and π detection pulses to 32 and 64 ns, respectively. The excitation width (width at half-height) of the inversion and detection pulses is about 13 and 8 MHz, respectively. The excitation width for both the inversion and detection pulses is, therefore, in the range of the dipole–dipole coupling for **1**, which was determined as 9.5 MHz from the CW-EPR spectrum.

This PELDOR setup for **1** resulted in a time trace with a clear modulation but a rather poor signal to noise ratio and low modulation amplitude compared to the DQC time trace of **1** (Fig. 8a). When the dipole–dipole coupling is in the range of or larger than the excitation width of the microwave pulses, low modulation depth and distortions to the time trace are expected.^{40,41} To confirm whether the observed modulation is due to the



Fig. 7 2-Pulse echo-detected field sweeps of compounds 1 (a), 3 (b) and 4 (c). Arrows show the positions of the PELDOR inversion and detection pulses.



Fig. 8 PELDOR measurements of compounds **1** and **4**. (a) Background-corrected experimental (solid line) and simulated (broken line) PELDOR time traces of **1**. The inset shows the Fourier transformed PELDOR time trace. (b) Distance distributions from DeerAnalysis on the experimental time trace of **1** (solid line) and simulated distance distribution (broken line) from the rigid segment model of **1**. (c) Background corrected experimental PELDOR time trace of **4** (solid line) and the simulated time trace obtained from a rigid segment model of **4** (broken line). The Fourier transformed PELDOR time trace is shown in the inset. (d) Distance distributions from the rigid-segment model of **4** (broken line) and DeerAnalysis on the PELDOR time trace (solid line).

dipole-dipole coupling, the PELDOR time trace was simulated using the same rigid segment model as for the simulation of the DQC time trace. The pseudo-secular part of the dipoledipole coupling as well as spin delocalization was included. The modulation frequency of the simulated time trace is in good agreement with that of the experimental time trace (Fig. 8a), which suggests that the observed modulation is the dipoledipole coupling between the trityl radicals.

To investigate if the above mentioned PELDOR setup could be used to measure the dipole-dipole coupling in the case of a longer interspin distance and hence a narrower EPR spectrum, we also measured compound 4 (Fig. 1), which has a mean interspin distance of 48.9 Å,¹⁴ with PELDOR. We have previously shown that measuring this compound with the inversion and detection pulses positioned on the center of the EPR spectrum does not yield a PELDOR time trace with discernible dipole-dipole coupling.¹⁴ Measuring 4 using the same PELDOR setup as for the measurement of 1, with the exception that the detection pulses were placed 13 MHz (4.6 G) away from the inversion pulse (Fig. 7b) on the ¹³C satellites, resulted in a PELDOR time trace with a clear modulation and a good fit to the simulated time trace (Fig. 8c). Analyzing the time trace with DeerAnalysis yielded a distance distribution in very good agreement with that obtained from a rigid-segment model of 4 (ref. 14) (Fig. 8d). These PELDOR measurements on 1 and 4 show that it is possible to use PELDOR to measure the dipoledipole coupling between trityl radicals. However, the narrow spectral width of the trityl radical requires PELDOR pulses with a narrow excitation bandwidth, which limits the inversion efficiency, signal to noise ratio and accessible distance range of the PELDOR measurement.⁴¹

The larger spectral width of the nitroxide radical makes it more ideal to use PELDOR to measure the interspin distance for 3.¹ The inversion pulse was placed on the maximum of the echo-detected field-swept spectrum, *i.e.* on the center of the trityl spectrum. The detection pulse sequence was placed on the nitroxide at 30–90 MHz higher frequency relative to the inversion pulse (30–90 MHz offset) (Fig. 7c). PELDOR measurements on 3 gave time traces with about 80% modulation depth and pronounced modulation (Fig. 9a). The modulation frequency of the PELDOR time traces depends on the field position of the detection pulse sequence, indicating that the orientation of the nitroxide spin label is correlated with the orientation of the interspin vector.

The dipolar spectra show that the parallel components ($\theta = 0^{\circ}$), at about ± 7 MHz, have a higher intensity when the



Fig. 9 PELDOR data from 3. (a) Background-corrected PELDOR time traces. (b) Fourier-transformed time traces in (a). (c) Orientation-averaged PELDOR time trace. Distance distributions from the orientation-averaged PELDOR time trace (blue) and from the rigid segment model of 3 are shown in the inset. (d) Experimental (solid line) and simulated (broken line) PELDOR time traces. The simulated time traces have been offset on the *y*-axis for clarity. Original time traces are shown in Fig. S1 (ESI†).

detection frequency mostly excites the *x*- and *y*-components of the ¹⁴N hyperfine coupling matrix (30 MHz offset). On the other hand, when the detection sequence excites mainly the *z*-component of the ¹⁴N hyperfine coupling matrix (90 MHz offset) the perpendicular components ($\theta = 90^{\circ}$) at about ± 3.5 MHz have the highest intensity (Fig. 9b). This trend between the dipolar spectrum and frequency offset of the detection pulses was also observed for a nitroxide–trityl biradical in a previous study using a longer linker between the trityl and the nitroxide.¹⁴

An orientation-averaged time trace is obtained by adding together the individual time traces (Fig. 9c). Analyzing the background-corrected and orientation-averaged time trace with DeerAnalysis yields the distance distribution in Fig. 9c, inset. The major peak in the distribution has a mean value of 24.3 Å, in good agreement with the mean interspin distance from the rigid segment model of 3 (Table 1). The smaller peak, at about 19–20 Å is due to incomplete orientation averaging since its mean value corresponds to the parallel component of the dipolar spectrum. To obtain a more accurate evaluation of the mean interspin distribution and to gain insight into the conformational distribution of the spin labels, the PELDOR time traces for 3 were simulated using the same rigid segment model for 3 as used for the simulation of its DQC time trace. The optimum fit between the simulated and experimental PELDOR time traces, at all offsets, was obtained by using eqn (3), *i.e.* the secular part of the dipole–dipole coupling, and spin delocalization for the trityl radical (Fig. 9d). The PELDOR measurements are, therefore, in full agreement with the results from DQC.

Conclusions

We have synthesized three model systems with distances in the range of 17 to 24 Å. Using CW-EPR, DQC and PELDOR we have demonstrated that for trityl-trityl distance measurements in this distance range, the pseudo-secular part of the dipoledipole coupling and the spin density distribution of the trityl radical have to be taken into account to obtain correct interspin distances. PELDOR measurements on compounds 1 and 4 showed that it is also possible to use PELDOR to measure the distance between two trityl spin labels, despite the narrow spectral width of the trityl radical. The work presented here shows that trityl radicals are well suited as spin labels and even for short distances, where the dipole-dipole coupling exceeds the linewidth of the trityl radical. More importantly, these results show that for measurements of unknown interspin distances between trityl radicals, special care should be taken if the width of the EPR spectrum of a dipole-dipole coupled trityl radical is broader than for the single trityl radical. Here, it was shown that the strong-coupling regime was reached when the full width of the bistrityl EPR spectrum was larger than 5.6 MHz. If the width of the bistrityl EPR spectrum is equal to 5.6 MHz, the dipoledipole coupling is either in the intermediate or weak-coupling regime. Calculating the ratio of $|\omega_{\rm A} - \omega_{\rm B}|$ to $|\omega_{\rm AB}|$ for a pair of trityl radicals shows that the percentage of spin pairs with a ratio of 1 ± 0.05 is maximum for interspin distances of ~ 30 Å. Therefore, the range of trityl-trityl distances that would fall within the intermediate regime, which is more difficult to analyze and thus to extract precise distances, is estimated to be 28–32 Å. For a pair of nitroxides, this intermediate regime is valid for distances in the range of 10-15 Å.31 If experimental conditions are considerably different from the ones used here, it can be anticipated that the full width of the single trityl radical and the distance limit for the high-coupling regime will be different than determined here.

Experimental

EPR spectroscopy

Continuous wave EPR measurements were performed on a Bruker EMX spectrometer equipped with a liquid nitrogen setup from Oxford Instruments. Pulsed EPR measurements were done on a Bruker ELEXSYS E580 X-band EPR spectrometer equipped with a SpecJet-II and PatternJet-II combination. All pulsed experiments were performed at 50 K using a standard flex line probe head with a dielectric ring resonator (MD5) together with a continuous flow helium cryostat (CF935) and a temperature control system (ITC 502) from Oxford Instruments. Samples of 1-3 were dissolved in deuterated toluene to yield a spin concentration of 80 µM and a final volume of 100 µL. The samples were frozen in liquid nitrogen before the EPR measurements at cryogenic temperatures. Field swept spectra were acquired by detecting the echo from a $\pi/2-\tau-\pi$ pulse sequence and varying the field magnitude. The pulse lengths were 12 and 24 ns for the $\pi/2$ and π pulse, respectively, and τ was set to 380 ns. The whole echo was recorded with a 450 ns acquisition window. A double microwave frequency setup was used for PELDOR measurements. Microwave pulses were amplified with a 1 kW TWT amplifier (117X) from Applied Systems Engineering.

PELDOR experiments were done using the 4-pulse sequence, $\pi/2(\nu A) - \tau_1 - \pi(\nu A) - (\tau_1 + t) - \pi(\nu B) - (\tau_2 - t) - \pi(\nu A) - \tau_2$ -echo. To eliminate receiver offsets the $\pi/2(\nu A)$ pulse was phase-cycled. The length of the detection pulses (νA) were 16 ns ($\pi/2$) and 32 ns (π), unless otherwise stated. The frequency of the inversion pulse (νB) was set at the maximum of the trityl field sweep spectrum and the length was 18 ns, unless otherwise stated. The amplitude and phase of the pulses was set to optimize the refocused echo. All PELDOR spectra were recorded with a shot repetition time of 3000 µs, a video amplifier bandwidth of 20 MHz and an amplifier gain of 51 to 57 dB. τ_1 was 380 ns, which corresponds to a blind spot for deuterium Larmor frequency. Deuterium modulation was suppressed by incrementing τ_1 8 times by 56 ns and adding the consecutive spectra. The position of the inversion pulse was incremented by 8 ns. The orientation-averaged PELDOR time trace was obtained by normalizing the original time traces and adding them together. The individual time traces were recorded with the same settings including video amplifier gain and number of scans.

DQC EPR measurements were also performed on a Bruker ELEXSYS E580 X-band EPR spectrometer, using the six-pulse sequence, $\pi/2-\tau_1-\pi-\tau_1-\pi/2-\tau_3-\pi-\tau_3-\pi/2-\tau_2-\pi-\tau_2$ -echo. The DQC pulses were positioned on the center of the trityl peak. The dipolar signal was filtered out by a 64-step phase-cycling program.⁸ To suppress nuclear modulation, τ_1 and τ_2 were incremented 4 times by 108 ns and the spectra added together. The initial value of τ_1 was 50 ns and the initial value of τ_2 was between 2000 and 3000 ns. τ_3 was held constant at either 30 or 50 ns. The DQC time traces were recorded by increasing τ_1 and decreasing τ_2 in steps of either 4 or 8 ns. The $\pi/2$ and π pulse length was 8 and 16 ns, respectively, for the DQC measurements on nitroxide–trityl sample and 12 and 24 ns for the DQC on trityl biradicals. The DQC echo was recorded with an acquisition window of 40 ns.

Data analysis and simulations

For the simulation of CW-EPR data the 'pepper' function in EasySpin-5.4.0 was used. A two-spin system was used and the resonance fields were computed using the 'matrix' method. The simulated spectra were fit 'by eyes'. DQC time-traces were background corrected by fitting an exponential function (eqn (6)) to the experimental time traces using the function exponfit from the EasySpin toolbox³⁷ and then subtracting the fitted function from the experimental time trace. It has previously been shown that the background function of the DQC time trace can be successfully removed by subtracting a fitted exponential function from the experimental DQC time trace.⁴²

$$a + b \times e^{-kx} \tag{6}$$

DQC time traces were recorded on each side of the zerotime, and the data points on each side of the zero-time added together to make a single time trace from zero time to the maximum of the time window. To remove the background from the experimental DQC time traces, the latter part of the time traces were fitted by the exponential function (eqn (6)). The starting point of the experimental DQC time trace was chosen such that the fit between the background corrected and simulated time trace was optimized. Dipolar spectra and distance distributions from PELDOR and DQC data were then obtained from the background corrected time traces using DeerAnalysis2011.³⁸

PELDOR and DQC time traces for 1–3 were simulated from a conformational dynamics model using a lab-written Matlab program.³⁹ The simulations of the DQC time traces were done assuming no orientation selection and complete excitation of the EPR spectrum. The simulation of PELDOR, DQC and distance distributions were obtained by generating 20 000 biradical conformers from a harmonic segmented chain (HSC) model (Fig. 10).^{22,23} Segments were defined in the following way: each benzene ring, the bonds between the center of the nitroxide NO bond and the carbon atom of the amide group, the bonds between the trityl radical center and the oxygen atom of the ester group and each remaining bond between atoms in the molecules. These rigid segments are allowed to bend with a



Fig. 10 Segmented chain model. Each rigid segment s_i is allowed to bend and rotate about its joint with θ_i and ϕ , respectively. The bending angles θ_i are the bending angles for the molecular linker, nitroxide spin label and trityl spin label segments θ_{S_i} , θ_{N_i} , θ_{T_i} respectively. They are described by a normal distribution and the torsion angle ϕ is uniformly distributed between 0 and 2π .

Table 2 Dynamics parameters used for conformational model of 1-3

	$\sigma_{ m r}{}^a$	$\theta_{s}{}^{b}$	$\theta_{\mathbf{N}}^{\ \ b}$	$\theta_{\mathrm{T}}{}^{b}$
1	0.1(0.03)	$0 \pm 2.9(1)$	_	$12.7(3) \pm 2.9(1)$
2	0.13(0.03)	$0 \pm 2.9(1)$	_	$12.7(3) \pm 2.9(1)$
3	0.13(0.03)	$0 \pm 2.9(1)$	$24(3)\pm2.9(1)$	$12.7(3) \pm 2.9(1)$

 a σ_r describes the average standard deviation in the length of each segment. b $\theta_S, \ \theta_N, \ \theta_T, \ describe the bending angle <math display="inline">\theta_i$ of the molecular linker-, nitroxide spin label- and trityl spin label-segments, respectively. All segments within the molecular linker have the same bending distribution θ_S . The degree of bending for each segment is described as the mean value \pm two standard deviations. The number in brackets is the error of the corresponding parameter.

normal distribution θ_i and rotate uniformly between 0 and 2π (ϕ) about the atoms, which act as joints. Mean length of the segments and bending angles θ_i were obtained from molecular mechanics calculations. The mean lengths of the segments are: 2.8 Å (benzene), 1.5 Å (benzene-benzene bond), 1.4 Å (benzene-acetylene bond), 1.2 Å (acetylene), 1.5 Å (benzene-oxygen bond), 1.4 Å (benzene-carbon bond), 5.8 Å (nitroxide spin label), 6 Å (trityl spin label). The mean bending angle for all segments within the molecular linkers is 0°. For the nitroxide and trityl spin label segments the mean bending angles are 24° and 12.7°, respectively. Distributions in length and flexibility of segments that resulted in the best fit between simulations and experiment are summarized in Table 2.

Molecular mechanics

Geometry optimized structures of **1–3** were calculated using molecular mechanics and MMFF force field, as implemented in the Spartan software from Wavefunction, Inc.

DFT

A geometry optimized structure and Mulliken atomic spin densities were obtained using density functional theory (DFT) as implemented in the program Orca.⁴³ DFT calculations were done using unrestricted Kohn–Sham methods, the B3LYP/G functional and the 6-31G* basis set.

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