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Continuous wave electron paramagnetic resonance of nitroxide biradicals in fluid solution

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Abstract

Nitroxide biradicals have been prepared with electron-electron spin-spin exchange interaction, J , ranging from weak to very strong. EPR spectra of these biradicals in fluid solution depend on the ratio of J to the nitrogen hyperfine coupling, A_N , and the rates of interconversion between conformations with different values of J . For relatively rigid biradicals EPR spectra can be simulated as the superposition of AB splitting patterns arising from different combinations of nitrogen nuclear spin states. For more flexible biradicals spectra can be simulated with a Liouville representation of the dynamics that interconvert conformations with different values of J on the EPR timescale. Analysis of spectra, factors that impact J , and examples of applications to chemical and biophysical problems are discussed.

1. Introduction

Interactions between nitroxides moieties in biradicals report a wide range of physical phenomena, including stereochemistry and collisions between parts of a molecule,¹ intramolecular spin delocalization,² and spin physics phenomena such as half-field transitions.³ The observation of modulation due to electron-electron spin-spin interaction in electron spin echo decays of nitroxide biradicals (dinitroxides)⁴ evolved into the widely-used double electron-electron resonance (DEER) method for determining distances in spin labeled molecules.⁵

A variety of dinitroxides have been designed for use as polarizing agents in dynamic nuclear polarization (DNP).^{6–10} Dinitroxides are used in DNP in preference to mono-radicals due to the cross-effect (CE) process.¹¹ Dipolar interactions between the two nitroxides can be designed to match the nuclear Zeeman frequency. Studies of many dinitroxides demonstrate that the largest DNP enhancements are achieved with rigid linkages that orient the g_{yy} tensors orthogonal to one another.^{8,11–18} Spiro linkages assisted in creating orthogonal arrangements of the g tensors. DNP enhancement is increased by increasing the saturation factor that depends on the T_1T_2 product. Many dinitroxides were developed for DNP at ca. 100 K, at which temperature the saturation factor can be increased by deuteration, replacement of methyl groups to increase T_2 , and by increasing molecular weight to increase T_1 . Some dinitroxides that are currently used extensively in DNP are discussed in Refs.^{19–22}

The criteria for optimum biradicals for solid state DNP at high NMR frequency are distinct from the criteria for optimum biradicals for in vivo imaging at low EPR frequency. As part of the characterization of the dinitroxides developed for DNP many were studied in dilute fluid solution.^{8,11,17} However, the simulations performed for spectra at X-band or higher frequencies are inadequate for low-frequency spectra. An innovation in the present report is a practical way to simulate spectra that will be needed for interpreting the impact of physiology on various dinitroxides designed for in vivo imaging at EPR frequencies such as 250 MHz.

There is a large literature concerning EPR of dinitroxides and their spin-spin interactions. Rozantsev reviewed the literature prior to 1970.¹ Parmon et al.²³ reviewed the interpretation of spectra of dinitroxides in fluid and frozen solution prior to 1977. There is a separate, and extensive, literature on biradicals produced photochemically, such as spin-correlated radical pairs, in which dynamic effects on the exchange interaction, J , have been analyzed.²⁴ The focus of this overview is on spin-spin interaction in molecular dinitroxides in fluid solution, and their application in chemical and biophysical studies.

2. EPR Spectra of dinitroxides

2.1 Dependence of spectra on exchange interaction, J

EPR spectra of dinitroxides that are tumbling rapidly in fluid solution depend strongly on two factors: (i) the ratio of the exchange interaction, J , to the nitrogen hyperfine, A_N ,²⁵ and (ii) the rate of interconversion between conformations with different values of J .²⁶ In this article the Hamiltonian for the exchange interaction is written as $J \mathbf{S}^{(1)} \mathbf{S}^{(2)}$, where 1 and 2 denote the two unpaired electrons, the exchange is assumed to be isotropic, and J is in magnetic field units. The trace of the dipolar interaction tensor is zero, so in the rapid tumbling regime the dipolar interaction averages to zero and does not impact the EPR spectra unless the interaction is so large that it is not fully averaged by the tumbling. The spectra for relatively rigid dinitroxides with a single average observable conformation are discussed in this section. Spectra of relatively flexible dinitroxides in which the rate of interconversion of conformations with different values of J has a strong impact on the spectra are discussed in section 2.2.

Electron-electron spin-spin interaction results in 'AB' splitting patterns^{27,28} that are analogous to the nuclear spin-spin splittings observed in NMR.²⁹ The positions and relative intensities of the four lines in an AB pattern are shown in Table 1, where B_{avg} is the average of the resonance fields for the two spins in the absence of interaction, C is the separation of the resonance fields in the absence of spin-spin interaction, $C = 0.5(J^2 + A_N^2)^{1/2}$ and $\sin 2\theta = J/2C$.²⁹ A stick diagram for the case of $J = 0.5 A_N$ is shown in Figure 1. As J/A_N increases (i) lines a' and b' move away from B_{avg} and decrease in intensity and (ii) lines a and b move toward B_{avg} and increase in intensity.^{25,29,30} The splitting between lines a' and a is equal J , which is also equal to the splitting between b and b'. The lines that move away from the center of the spectrum as J increases (a' and b') are sometimes called outer lines.³¹

Calculated spectra for a dinitroxide at X-band are compared in Figure 2 for $J = 0, 0.5 A_N, 2 A_N$ and $\gg A_N$. Stick diagrams calculated with the locally written program CuNO³¹ are

shown in Figure 2A and full line shapes calculated with a MatLab version of dynbir³⁰ are shown in Figure 2B. The details of the simulations are discussed in section 2.4. For each molecule there is a single value of the nuclear spin state for each nitroxide nitrogen, which is designated as $m_I^{(1)}$ or $m_I^{(2)}$ for the first and second nitrogens, respectively. For a dinitroxide with normal isotope distribution, >99% of the nitrogen is ^{14}N so the contributions from ^{15}N to the spectra are not considered in the following discussion. For ^{14}N there are nine combinations of $m_I^{(1)}$ and $m_I^{(2)}$. Independent of the value of J the resonances for $(m_I^{(1)}, m_I^{(2)}) = (+1, +1)$, $(0, 0)$, and $(-1, -1)$ occur at $B_{\text{center}} - A_N$, B_{center} , and $B_{\text{center}} + A_N$, respectively, where B_{center} is the center field for the spectrum. In Figure 2A the positions of these lines are marked as $M_I = 2, 0$, and -2 , where $M_I = \sum_i m_I^{(i)}$. For $J > 0$ and $m_I^{(1)} \neq m_I^{(2)}$ interaction between the two spin states results in 4-line AB splitting patterns. In Figure 2A the AB splitting patterns are labeled as **c** for $(m_I^{(1)}, m_I^{(2)}) = (+1, -1)$ or $(-1, +1)$; **d** for $(m_I^{(1)}, m_I^{(2)}) = (1, 0)$ or $(0, 1)$; and **e** for $(m_I^{(1)}, m_I^{(2)}) = (0, -1)$ or $(-1, 0)$. For the AB pattern labeled as **c** the positions of the four lines move from 3488.5, 3496.5, 3521.5, and 3529.5 for $J = 0.5 A_N$ to 3470.4, 3502.4, 3515.6, and 3547.6, respectively, for $J = 2 A_N$ (Figure 2A, marked with dashed lines). Since J is greater for AB pattern **c** than for patterns **d** or **e**, the intensities of the **a'** and **b'** lines for pattern **c** are greater than for **d** or **e**. The progression in values of J relative to A_N is commonly denoted as weak, intermediate, and strong exchange, although the distinction between weak and intermediate is not clearly delineated.

An example of a biradical for which $J \sim 2 A_N$ is the carbonate-linked dinitroxide **I**,^{25,26,32} for which the room temperature spectrum in deoxygenated toluene is shown in Figure 3. The spectrum can be simulated with $J = 28.9$ G, and $A_N = 15.6$ G, which corresponds to $J/A_N = 1.85$.²⁵ In Figure 3 the positions of the outer lines are marked with 'o'. The positions of these lines are strongly dependent on J , so the observation of relatively sharp outer lines indicates that dynamic processes that interconvert conformations with different values of J are fast enough on the EPR timescale to fully average differences in J . Simulation of the positions of the outer line accurately defines the value of J . Replacement of the carbonate linkage in **I** by an oxalate linkage decreases the value of J by about a factor of 7.³³

In the limit of $J \gg A_N$ the positions of line **a** and **b** of each AB pattern converge to B_{avg} and the intensities of lines **a'** and **b'** go to zero. The dinitroxide spectrum then reduces to 5 lines with relative integrated intensities of 1:2:3:2:1 (Figure 2 $J \gg A_N$) and hyperfine splitting of $A_N/2$. The total M_I for each of the five lines is the sum of $m_I^{(1)}$ and $m_I^{(2)}$ for the two interacting spin states as listed in Table 2. The differing numbers of combinations of $m_I^{(1)}$ and $m_I^{(2)}$ that contribute to each of the 5 lines is the reason for the 1:2:3:2:1 relative intensity pattern. In Figure 2A the line labeled 'c' for the case of $J \gg A_N$ is the sum of contributions

from $(m_I^{(1)}, m_I^{(2)}) = (0,0)$ for which the position is independent of J and the strong exchange limit of the AB pattern designated as c.

Dinitroxides **II** - **IV** are examples of biradicals with $J \gg A_N$ that give well-resolved 5-line spectra as shown in Figure 4 for **II**.^{25,32} Simulations of the spectrum of **II** required $J/A_N > 40$. The line widths are different for different values of M_I so the amplitudes of the peaks do not exhibit the expected 1:2:3:2:1 ratio, although the double integral (Figure 4) shows the expected relative areas under the five peaks. Analysis of the EPR spectra of **III** and **IV** in glassy toluene at low temperatures found values of J of $>0.12 \text{ cm}^{-1}$ and 0.05 cm^{-1} , respectively.³⁴

$J \gg A_N$ was observed for dinitroxide **V**.³⁵ However, for this biradical the interspin distance is so short and the dipolar interaction so large, that even in low viscosity solutions molecular tumbling is not fast enough to fully average the electron-electron dipolar splitting. As a result, the fluid solution EPR spectrum is a single very broad line.

2.2 Dependence of spectra on dynamic processes that interconvert conformations on the EPR timescale

For processes with rates that are significantly slower than the energy differences in Hz, separate EPR signals are observed. These processes are called 'slow' on the EPR timescale. For example, if two resonances are separated by 5 gauss (about 14 MHz), processes with rates less than about $1.4 \times 10^7 \text{ s}^{-1}$ would be too slow to average the signals. Processes for which rates are much faster than the energy differences give spectra with average EPR observables and are called fast on the EPR timescale.

The spectra in Figure 2 – 4 are characteristic of dinitroxides with relatively rigid linkages between the paramagnetic centers. Interconversion between the limited range of thermally-accessible conformations is fast enough that the spectrum is characteristic of a single value of J and relatively narrow lines are observed. For dinitroxides with more flexible linkages between the two paramagnetic centers, the EPR spectra are strongly dependent on the kinetics of interconversion between conformations, on the populations of the conformations, and on the values of J for the conformations.²⁶ Conformations with strong exchange may occur via through-space interaction if the two paramagnetic centers are in close spatial proximity or through bonds if pathways are short or delocalized. Weaker interactions occur when through-space interspin distances are longer or when delocalization through bonds is weaker. Molecular motions may interconvert conformations with large and small values of J . The positions of the lines with $M_I = \pm 2$ and the contribution to $M_I = 0$ from $(m_I^{(1)}, m_I^{(2)}) = (0,0)$ (Table 2) are independent of the value of J so these lines have constant width, independent of the rate of interconversion of conformations and independent of differences

in J for various conformations. However, the widths of the lines with $M_I = \pm 1$ and contributions to $M_I = 0$ from $(m_I^{(1)}, m_I^{(2)}) = (+1, -1)$ and $(-1, +1)$ are strongly dependent on the rates of interconversion and values of J. Early in the EPR studies of dinitroxides,^{26,36,37} this effects was called the 'alternating line width' effect because lines 1, 3, and 5 were relatively narrow and lines 2 and 4 of the 5-line spectrum were much broader. For example, dinitroxides connected via a phenyl ring display ortho-, meta-, para- effects, with negligible exchange in the para position, and relatively strong exchange in the ortho position.¹ The much stronger interaction between nitroxides at the ortho positions is attributed to spatial proximity and ease of collision. The conformational interconversions can be described by the model shown in Figure 5 that is based on three subsets of molecules.³⁸ Some conformations permit strong collisional exchange between the two nitroxide groups. The population for that subset of molecules is p_2 . Conformations with weak interactions and a high barrier to interconversion are designated as population p_1 . Other conformations, with population p_3 , have weak spin-spin interaction but interconvert rapidly with the strong exchange conformation(s).

Since separate signals are not seen for individual conformations p_2 and p_3 of these flexible molecules, the dinitroxides are designated as being in the 'fast' exchange regime. Increasing the rate of interconversion leads to narrower widths for lines 2 and 4. The literature uses, somewhat interchangeably, terms such as slow and weak, fast, and strong. However, the rates of interconversion and strengths of interaction are separable concepts and the words should not be used equivalently.²³ The widths of lines 2 and 4 can be analyzed to characterize the motions that interconvert conformations p_2 and p_3 , with different values of J.^{23,26,39,40} In the rapid tumbling regime line widths for nitroxide monoradicals are approximately independent of m_I . Contributions to T_2 combine as the sum of reciprocals. The contribution to the line width that results from modulation of the exchange interaction, $T_2^{-1}(\text{exch})$ is then given by Eq. (1)^{40,41}

$$T_2^{-1}(\text{exch}) = T_2^{-1}(2, 4) - T_2^{-1}(1, 5) \quad (1)$$

where $T_2^{-1}(2,4)$ and $T_2^{-1}(1,5)$ are values of T_2^{-1} for lines 2 and 4 which are impacted by modulation of the exchange interaction, and lines 1 and 5 are not impacted by modulation of exchange (see Figure 6). The width of line 3 is not used in this analysis because it is the superposition of lines for which the positions are independent of J and ones that are dependent on J (Table 2 and Figure 2A). For a Lorentzian line

$$T_2^{-1} = \frac{\sqrt{3}}{2} \gamma_e \Delta B_{pp} \quad (2)$$

where γ_e is the electron gyromagnetic ratio and B_{pp} is the peak-to-peak line width in magnetic field units. The shapes for lines 2 and 4 in the fast exchange regime are close to Lorentzian. The shapes for lines 1 and 5 may deviate from Lorentzian due to contributions from unresolved proton hyperfine splitting but approximating as Lorentzian is unlikely to introduce large error in the following calculation. Eq. (1) can then be rewritten as

$$T_2^{-1}(exch) = \frac{\sqrt{3}}{2} \gamma_e [\Delta B_{pp}(2, 4) - \Delta B_{pp}(1, 5)] \quad (3)$$

The contributions to the widths of lines 2 and 4 from modulation of the exchange interaction can be expressed as in Eq. (4).^{23,26,39,40}

$$T_2^{-1}(exch) = \frac{A_N^2 \tau_{eff}}{4} \quad (4)$$

where τ_{eff} is 'a complex combination of modulation parameters and its value is close to the longest of the characteristic times for the modulations'.³⁹ It has also been described as the approximate time between interconversions.²³ Combining Eq. (3) and (4) gives Eq. (5).³⁹

$$A_N \tau_{eff} = \frac{3.46 \gamma_e}{A_N} [\Delta B_{pp}(2, 4) - \Delta B_{pp}(1, 5)] \quad (5)$$

Eq. (5) has been used to characterize motion of dinitroxides in a variety of solvents. Measurement of the temperature dependence of $A_N \tau_{eff}$ has been used to characterize the thermodynamics of the conformational interconversions.⁴²⁻⁴⁴

Ref. ⁴¹ gives examples of the use of Eq. (5) to analyze the temperature dependence of spectra for a dinitroxide with a di(ethylene glycol) linkage. The equilibrium between molecules outside a solvent cage (p_1) and inside a solvent cage ($p_2 + p_3$) is described by $H = 16.8 \pm 1.4$ kJ/mole and $S = 48.5$ J/mole K in water and $H = 6.3 \pm 1.9$ kJ/mole and $S = 9.2$ J/mole K in 1:1 water:isopropanol.⁴¹ The kinetics of interconversion between conformations within the solvent cage (p_2 and p_3) are described by $E_a = 22.7$ kJ/mole in water and 23.4 kJ/mole in 1:1 water:isopropanol.

The temperature dependence of spectra for dinitroxides with flexible linkages such as ethylene glycol,⁴¹ (CH₂)₄³⁹ or adipic acid⁴⁵ can be modeled with dynbir or dynbirm (see section 2.4) by allowing the populations to change and the rates of interconversion to increase with increasing temperature. Figure 6 shows simulations at 295 and 353 K, based on the thermodynamic parameters for the di(ethylene glycol) linked dinitroxide reported in ref. ⁴¹. At 295 K the p_1 of molecules outside the solvent cage is substantially larger than the combined population ($p_2 + p_3$) so the spectrum is dominated by lines characteristic of $J = 0$. At 353 K the temperature dependence of the equilibrium constant shifts to favor large $p_2 + p_3$. The rate constant for conversion of p_1 to p_2 is slow on the EPR timescale ($k \sim 10^4$) for all of the spectra shown in Figure 6. The rate constants for conversion of p_2 to p_3 were in the range of 5×10^7 to 3.3×10^8 , and increased with increasing temperature. The model reported in Ref. ⁴¹ does not specify the separate values of p_2 or p_3 or the values of J for each conformation. The values used in the simulations are shown in the figure caption. The details of the simulation are discussed in section 2.4.

2.3 ^{15}N isotopically substituted dinitroxide

The ^{15}N isotope has $I = 1/2$. When a dinitroxide is labeled with ^{15}N the EPR spectrum is substantially simplified as shown in Figure 7A and B and as demonstrated in the early work of Briere et al.²⁵ There are only four combinations of $m_I^{(1)}$ and $m_I^{(2)}$. For $(m_I^{(1)}, m_I^{(2)}) = (1/2, 1/2)$ or $(-1/2, -1/2)$, which have $M_I = \pm 1$, the positions of the lines are independent of J . There is one AB pattern for $(m_I^{(1)}, m_I^{(2)}) = (1/2, -1/2)$ or $(-1/2, 1/2)$ for which $M_I = 0$. As J/A_N increases the positions and intensities of the lines for the AB pattern change as shown in Table 1. In the limit of $J \gg A_N$ the spectrum of a ^{15}N labeled dinitroxide is a three-line pattern with relative intensities 1:2:1 (Table 3). Only the transitions for $M_I = 0$ are broadened by interconversion of conformations with differing values of J . The smaller number of lines for the ^{15}N dinitroxides and the simpler spectra are advantageous for in vivo applications of dinitroxides.^{46,47}

2.4 Simulations

Interpretation and simulation of dinitroxide spectra in polycrystalline samples and fluid solution are reviewed in Ref. ²³. Analysis of dinitroxide spectra in a rigid lattice requires taking into account the anisotropies of Zeeman and hyperfine interactions and the orientation of the interspin vector relative to the magnetic axes of the two interacting centers, as well as the magnitude of J and the interspin distance r .⁴⁸

Simulations of fluid solution spectra for relatively rigid dinitroxides (section 2.1) are a special case of the more general problem of exchange interaction between inequivalent paramagnetic centers in fluid solution that is calculated in the locally-written program CuNO.³¹ The Hamiltonian includes the g and A interactions in the monoradicals in addition to the interaction term $J S^{(1)}S^{(2)}$. The spectra are superpositions of AB patterns for each combination of $m_I^{(1)}$ and $m_I^{(2)}$ as discussed in section 2.2. In this program it is possible to use broader line widths for outer lines of the AB patterns than for other transitions to account for the greater impact of small variations in J or distributions in J on the energies for these transitions. This was done in the simulations shown in Figure 3. For dinitroxides with flexible linkages simulation of spectra in fluid solution requires inclusion of both the exchange interaction (J) and the dynamics of interconversion of conformations with different values of J . A fast computational approach was developed by Sankarapandi et al.³⁰ using a Liouville density matrix analysis implemented in Fortran in a program called dynbir. The program has options for conformations with different values of J and populations as shown in Figure 5. The isotropic g , A , and populations may be different for each conformation. The Fortran code, graciously provided by the authors of Ref. ³⁰, has been translated into MatLab by members of the Eaton group and is compatible with EasySpin⁴⁹ which permits least-square fitting of spectra.

Most EPR studies of dinitroxides have been performed at X-band so in Ref. ³⁰ it was reasonable to assume that A_N is much smaller than the external magnetic field B_0 , which is called the high-field limit. At lower magnetic field and resonance frequency, the ratio of A_N to B_0 increases, and the high-field limit expressions are no longer valid.⁵⁰ When the limiting expressions are not valid, the spacings between the three nitrogen hyperfine lines are not equal as is seen in nitroxide spectra at 250 MHz.⁵¹ When spectra are not in the high-field

limit, the positions for the nitrogen hyperfine lines can be found more accurately by including perturbation corrections to third-order as given by Rieger.⁵² In addition, when spectra are not in the high-field limit, the ratio of A_N to B_0 may change substantially as the magnetic field is scanned over ranges that are not small relative to B_0 . To address this issue, the MatLab modification of dynbir calculates the electron spin energy levels at each point along the magnetic field axis of the scan. From the resulting frequency swept spectrum the segment is selected that matches the microwave frequency (or radio frequency) for the incident radiation. This program is referred to as dynbirm and is used in conjunction with EasySpin.

The recently reported spectra of dinitroxide **VI** in tris buffer (pH = 7.2) at 20° C at 258 MHz, L-band (1.0 GHz), and X-band (9.8 GHz)⁴⁷ are reproduced in Figure 8. Absorption spectra at 250 MHz and 1.0 GHz were obtained by rapid scan. The underlying rapid scan background signal⁵³ was removed by subtraction of a polynomial. The X-band spectrum was obtained by CW spectroscopy and the absorption spectrum was obtained by integration. The simulations in Figure 8 were performed with dynbirm and EasySpin. The simulations assume the model shown in Figure 5 and were initially performed independently for spectra at each frequency. The average of the best-fit parameters at the three frequencies were then used to generate the simulations shown in Figure 8. For these samples there is no slowly interconverting conformation, so p_1 is 0. To match the observed line shapes two conformations with different values of J , p_3 and p_3' , were included, both of which are interconverting rapidly with p_2 and with each other. It is probable that these two populations are an approximation for a relatively broad range of interconverting conformations. The populations (Figure 5) and corresponding values of J are p_3 (13%, $J = 0$ G), p_2 (36%, $J = 28$ G), and p_2' (51%, $J = 710$ G). The first order rate constant for conversion of p_3 to p_2 is $4.8 \times 10^6 \text{ s}^{-1}$ and for p_2 to p_2' is $3.6 \times 10^6 \text{ s}^{-1}$. Other rate constants are derived from the equilibrium constants. The value of J for p_2' is large enough to give a strong exchange spectrum ($J \gg A_N$) as shown in Figure 2. The intrinsic line widths are frequency dependent: 1.3 G at X-band, 1.5 G at L-band, and 1.9 G at 250 MHz. The dynbirm simulations do not include the effects of incomplete motional averaging of g and A anisotropy that causes the high-field $M_I = -2$ line to be broader than the low-field $M_I = 2$ line.

Bogdanov and Vorobiev⁵⁴ simulated the X-band spectra of dinitroxide **VII** from the rigid limit to freely tumbling using the stochastic Liouville operator to account for motional averaging of g , A , and dipole-dipole interactions. To test their model spectra that span the motional range from rigid lattice to rapid tumbling were analyzed in squalene between 98 and 370 K and in the aligned liquid crystal *n*-octylcyanobiphenyl from 100 to 298 K. The

exchange, J , decreased with increasing temperature from ca. 620 G to ca. 520 G. For this relatively rigid dinitroxide the spectra could be simulated with a single conformation, without inclusion of the dynamics that are included in dynbir and dynbirm.

For **III** and **IV** DFT calculations of spin density show that the unpaired electron is predominantly on the NO moiety, but there is non-zero spin density on all atoms.³⁴ For **VIII** 43% of the spin density is calculated to be on the nitrogen of the NO moiety.⁵⁵ ENDOR has been used to measure the 0.48 MHz hyperfine coupling to the isotopically enriched ^{13}C in the bridge of **IX**⁵⁶ in toluene at 80 K. DFT calculations with various models and PBE0 functionals found values of the hyperfine coupling ranging from 0.33 to 0.52 MHz. The best results were obtained with PBE0/N07D or PBE0/EPR-II. Molecular orbital calculations of J are challenging because of the dependence on small spin delocalization. To our knowledge the work of Neese and co-workers to calculate the relative contributions of exchange and dipolar interaction in a delocalized system² is the only example of this type of calculation. Trends in J are therefore based on empirical observations as discussed in the next section.

3. Impact of molecular structure on J

Many linkages between pairs of nitroxides have been synthesized, resulting in a wide range of spin-spin interactions.¹ The concept of an 'attenuation coefficient' was introduced to provide a metric that describes the diminution of the through-bond exchange interaction by various linkages between two nitroxides. It is the ratio of J for a biradical without a particular bridging moiety to J for the analogous biradical with that bridge. Kokorin

tabulated the attenuation coefficients of a variety of bridge atoms and groups between piperidiny, pyrrolidiny, and imidazoline nitroxides.³³ Some representative linkages and their attenuation coefficients are in Table 4. For many dinitroxides values of J and attenuation coefficients are strongly dependent on solvent, presumably due to conformational changes. The comparisons in Table 4 provide starting points for design of dinitroxides with desired ranges of values of J for chemical and biophysical applications.

Dinitroxides have also been made with phosphorus linkages (-O-PO(alkyl)-O-).⁵⁷ The X-band EPR spectra obtained at room temperature in toluene solution exhibited intermediate exchange. Spectra characteristic of $J/A_N \approx 2$ was observed for isoindoline dinitroxides by Giroud et al.⁵⁸ The two phenyl rings of the isoindoline groups significantly attenuate the spin-spin interaction.

A series of dinitroxides with one N atom between the rings of the two nitroxides was prepared by Kavala et al.⁵⁹ The pyramidal secondary amine provides opportunity for the nitroxide moieties to be very close together. When the H in the dinitroxide linkage is replaced by a methylsulfonamide, the nitroxide-nitroxide collisions are restricted. The spectra were simulated with $J > A_N$ using EasySpin, but for a static case with an isotropic Hamiltonian, which does not reflect dynamic spin-spin interaction.

For disulfide-linked dinitroxides **X** and **XI** the nitrogens in the imidazoline rings result in additional hyperfine splitting.^{60,61} The spectrum of **X** is particularly significant because it is of a dinitroxide with a short (-S-S-) through-bond distance between rings, and a double bond in the ring, which should increase delocalization of the nitroxide spin toward the S-S bridge. Even with these advantages, the spectrum of **X** in room temperature solution is not in the limit with $J \gg A_N$.⁶⁰

4. Dependence of spin-spin interaction on the environment of the molecule

In selecting dinitroxides for physical and biophysical applications it is important to consider environmental factors that impact the exchange interaction. This section highlights some examples. Studies of the effects of solvent on J prior to 2004 are reviewed in Ref. ³³. For relatively rigid dinitroxides the impact of solvent polarity and temperature on J depends on the dinitroxide studied, which suggests that changes are due to specific solvation interactions with portions of the molecule.^{33,36}

Relatively rigid dinitroxides **VIII** and **XII** both have $|J/A_N|$ about 2.5, but in **XII** there are polar groups in the bridging linkage that might interact with polar solvents, whereas these are not present in **VIII**.⁶² For **VIII** the value of J decreased with increasing temperature to a greater degree in dioxane or acetonitrile than in toluene, which is attributed to interaction of polar molecules with the NO moiety. By contrast the value of J for **XII** increased with increasing temperature, and the temperature dependence was larger than for **VIII**, which is attributed to interaction of the solvent with the polar sulfate group.⁶² J is more strongly temperature dependent for **XIII** than for **XII**, although the value of J is about 7 times larger for **XII** than for **XIII**.⁶³

In radical **XIV** there are low barriers to rotation around several of the bonds in the ethylene diamine linkage.⁶⁴ Values of J vary from 4.2 G at pH = 2.47 to 22 G at pH 11.10, which is attributed to changes in populations of conformations as the amino nitrogens are protonated or deprotonated. In nonaqueous solutions values of J for **XIV** range from 15 G in 1-butanol to 31 G in DMF. Values of J increased with increasing temperature. In all cases there is only one EPR distinguishable species and the lines are relatively narrow. It is proposed that conformations are interconverting rapidly relative to the differences in resonance positions for the interconverting conformations.⁶⁴ Based on density matrix simulations the lifetimes of the conformations that were required to match the spectral lineshapes were estimated to be of the order of 10^{-9} to 10^{-10} s.⁶⁴ The spectra of these species do not exhibit the alternating line widths that characterize flexible dinitroxides in which the rates of interconversion are designated as fast on the EPR timescale (section 2.2). pH dependence of J was also observed for imidazoline dinitroxides with $-NH-$ linkages, with the protonated form exhibiting negligible exchange.⁶⁵

The impact of temperature on spectra of flexible dinitroxides is strongly dependent on the linkage.³⁹ For dinitroxides **XV** and **XVI** with relatively short, aliphatic linkages the values of J increase with increasing temperature. However ΔH for interconversion of conformations does not correlate with solvent viscosity,⁶⁶ which suggested that the exchange interaction was intramolecular and therefore would not correlate with bulk macroscopic properties of

the solvent. Chachaty et al.⁴⁵ studied the conformational dynamics of longer-chain adipic acid bridged **XVII**. ¹³C NMR of the reduced diamagnetic analog provided detailed information on conformations to aid in interpretation of the EPR data. The widths for lines 2 and 4 are much broader than for lines 1, 3, 5 (analogous to Figure 6) and become sharper at higher temperatures.⁴⁵ The jump rate between conformations was calculated to be 5 to $10 \times 10^9 \text{ s}^{-1}$, which corresponds to lifetimes of about $2 \times 10^{-10} \text{ s}$.

The longer chain dinitroxides **XVIII** and **XIX** have been studied in multiple solvents.^{38,40} In five alcohols lines 2 and 4 were broader than lines 1, 3, 5 and became sharper at higher temperatures. Thermodynamic parameters for the nitroxide-nitroxide exchange were calculated using a model of a cage within which the nitroxides have two conformations, one with $J = 0$ (p_3) and another with $J \gg A_N$ (p_2) that are in fast exchange, plus an out-of-cage conformation in which $J = 0$ (p_1) (Figure 5). Exchange between conformations inside and outside the solvent cage is slow. The activation energy for motion inside the solvent cage correlates with solvent viscosity.⁴⁰

When the external pressure was varied for an acetonitrile solution of **XX** it was concluded that the intramolecular dynamics did not follow the Stokes-Einstein-Debye law, but the Arrhenius dependence of rates was followed.⁶⁷ For **XIX** and related radicals dissolved in ionic liquids asymmetry is observed in the spectra at low temperatures, which will require development of new models for motions.⁶⁸

5. Examples of Applications

The effect of proximity of two nitroxides on their EPR spectra was used to demonstrate threading of a spin-labeled molecule through a spin-labeled cyclodextrin.⁶⁹ In this case, collisions between the two nitroxides on different molecules result in broad lines between the three lines of the ¹⁴N nitroxides. The EPR spectra in the cyclodextrin - rotaxane system are similar to that of flexible dinitroxides. This pairwise collision effect is different from the general broadening due to fast collisions among multiple radicals in bulk solution, demonstrating that the exchange interaction between the two nitroxides is specific pairwise interaction.

Sawant et al.³² studied the coordination of dinitroxides **I** and **II** to paramagnetic Cu(II) and vanadyl complexes. When one N-O moiety coordinates to the paramagnetic metal, interaction of the metal with that nitroxide spin result in strong antiferromagnetic coupling, and the spectrum for the biradical bound to the metal complex becomes the 3-line pattern of monoradical, which permitted calculation of the equilibrium constants for the complex formation.

When disulfide biradical **XI** reacted with sulfhydryl groups in the zinc metalloenzyme porphobilinogen synthase, two spin labels attached to the protein within 7.6 Å of one another, based on the intensity of the $m_S = 2$ “forbidden” transition.⁷⁰ This transition is due to dipolar interactions. Fluid solution spectra showed that the exchange interaction was weak. This example demonstrates that conformations of dinitroxide with static distances as close as 7–8 Å are in the “weak exchange” limit unless there is significant orbital delocalization along the linkage between them.

5.1 Measurement of redox status with disulfide linked dinitroxides

Glutathione (GSH) is the dominant reducing species in the brain and the concentration in the human brain is about 1.9 mM.⁷¹ The use of dinitroxides to measure redox status is based on the reaction of glutathione with a –S-S– linkage between two nitroxides.^{46,70} Disulfide-linked dinitroxides, designated as RSSR, exhibit characteristic biradical spectra. Reaction with glutathione cleaves the S-S bond and results in distinctive changes in the EPR spectrum.^{1,60,72–74} Khramtsov et al.⁶⁰ monitored enzymatic activity with dinitroxide **X** which had been shown^{72,73,75} to react with thiols to quantitatively produce monoradicals. The products of the cleavage reaction were assumed to be the nitroxides with SH and SSG

(glutathione) substituents. Roshchupkina et al. published spectra of both ^{14}N and ^{15}N **XI** and showed the simplification of the spectra that was observed when ^{15}N was used.⁴⁶

Elajaili et al. performed 2D spectral-spatial imaging of the conversion of ^{15}N -labeled disulfide-linked biradical **VI** to monoradicals upon equilibration with glutathione in a phantom at 250 MHz (Figure 9).⁴⁷ Experiments at 250 MHz were made possible by the improvement in signal-to-noise that can be achieved with rapid scan EPR.⁵³ Imaging of the nitroxide signals showed that BSO (L-buthionine sulfoximine), a glutathione depleting agent, decreased the reducing equivalents in a tumor.^{76,77} In vivo imaging by Epel et al. showed that the cleavage rate is proportional to the intracellular glutathione concentration, and that BSO markedly changed the kinetics.⁷⁷ In these experiments the monoradical products diffused apart and were unlikely to recombine. An approach to a reversible redox indicator is sketched in Figure 10. In the disulfide bridged form the two nitroxides are too far apart to permit significant interaction and a monoradical EPR spectrum is observed. When the disulfide linkage is cleaved molecular motion permits collisions that give the characteristic intermediate exchange spectrum. By tethering the two radical-containing fragments, there is the possibility for reversible formation of the dinitroxide.

6. Electron Spin Relaxation

Fluid solution CW power saturation studies at 298 K show linear response of lines 2 and 4 in the signal for RSSR **VI** as a function of square root of power up to at least 80 mW incident in a high-Q Bruker cavity, the power limit for the size of aqueous sample measured. The signal for the monoradical formed after reaction of RSSR with glutathione does power saturate under the same conditions. This demonstrates that the T_1T_2 product for lines 2 and 4 in the spectra of dinitroxide with intermediate exchange rates is much shorter than for nitroxide monoradicals under the same conditions. The relaxation rates of the exchange-broadened lines in fluid solution at room temperature are too fast to measure by pulsed EPR with available equipment.⁴⁷ These changes may be largely due to the impact of dynamic processes on T_2 . The significance of rapid relaxation of the flexible dinitroxide at 298 K is that pulsed EPR imaging will not be feasible, and rapid scan imaging^{51,77} will be the method of choice for this type of biradical.

The only pulsed EPR relaxation studies of dinitroxide are by Sato et al. at cryogenic temperatures.⁷⁸ For inter-radical distances of about 9 Å, the spin-lattice relaxation rates over a wide range of temperatures were only slightly faster than for the monoradical with analogous structure. The more flexible the structure, the faster the spin relaxation rates. For shorter distances, relaxation rates for biradicals were faster than for the analogous monoradical. The enhanced relaxation in frozen solutions and glasses was analyzed in terms of contributions from the direct, Raman, and local mode processes.

7. Summary

In a few dinitroxide molecules strong ($J \gg A_N$) coupling between electron spins occur via through-bond pathways. These molecules exhibit 5-line EPR spectra with 1:2:3:2:1 intensity ratios due to coupling of the electrons to two equivalent nitrogen nuclei (^{14}N $I=1$). However,

in many dinitroxides there is dynamic interconversion between conformations with weak exchange and strong exchange. For these molecules alternating line widths are observed, with broad lines (lines 2 and 4) between the three sharper lines (lines 1, 3, and 5) at the positions that are characteristic of a monoradical. At the low magnetic fields that are used for in vivo imaging the spectra are not in the high-field limit. Spectra can be simulated by inclusion of third-order perturbation corrections and calculation of the electron spin energy levels at each point along the magnetic field axis. In vitro⁴⁷ and in vivo⁷⁷ imaging of redox status has been performed using nitroxide disulfide radicals, RSSR. New imaging methods⁴⁷ make possible low-frequency (low magnetic field) imaging of multiline dinitroxide spectra. The understanding of disulfide-linked dinitroxides is sufficiently mature that it can be applied to solving many biomedical problems in which measurement of redox status is a crucial parameter. The organic chemistry of creating new, targeted, dinitroxides will lead to applications not yet attempted.

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Figure 1.

Stick diagram for an AB splitting pattern with $\Delta = 22$ G and $J = 0.5$. The spacings between lines a' and a and between b and b' equal J. As J increases lines a' and b' move away from the center of the spectrum and decrease in intensity while lines a and b move toward the center of the spectrum and increase in intensity.

A.

B.

Figure 2.

Changes in EPR spectra of a dinitroxide as J increases. A) Stick diagram calculated with CuNO^{31} using $\nu = 9.852$ GHz, $g = 2.006$, $A_N = 16.0$ G, and Gaussian absorption line width of 0.2 G for $J = 0$, $J = 0.5 A_N$, $J = 2 A_N$, and $J \gg A_N$. The AB splitting patterns are labeled as c for $(m_I^{(1)}, m_I^{(2)}) = (+1, -1)$ or $(-1, +1)$; d for $(m_I^{(1)}, m_I^{(2)}) = (1, 0)$ or $(0, 1)$; and e for $(m_I^{(1)}, m_I^{(2)}) = (0, -1)$ or $(-1, 0)$. For each of these AB pattern there are 4 lines centered at B_{avg} with positions and relative intensities as shown in Table 1 and Figure 1. The resonances for $(m_I^{(1)}, m_I^{(2)}) = (1, 1)$; $(0, 0)$, and $(-1, -1)$ are independent of J and occur at the positions labeled $M_I = 2, 0$, and -2 , respectively. The changes in the positions of the lines as J increases are discussed in the text and shown with dashed lines for AB splitting pattern c. For $J \gg A_N$ the line labeled as 'c' is the sum of contributions from $(m_I^{(1)}, m_I^{(2)}) = (0, 0)$ for which the position is independent of J and the strong exchange limit of the AB pattern designated as c. B) First derivative spectra calculated with dynbirm³⁰ for the same series of values of J .

Figure 3.

X-band (9.87 GHz) EPR spectrum of 1.7 mM dinitroxide **I** in deoxygenated toluene at room temperature obtained with non-saturating microwave power and 0.1 G modulation amplitude. The positions of the 'outer' lines are marked with the letter 'o'. The simulated spectrum (red dashed line) was calculated with CuNO^{31} using $J = 27.5$ G and $A_N = 15.4$ G.

Figure 4.

X-band (9.87 GHz) EPR spectrum of 1.0 mM dinitroxide **II** in deoxygenated toluene at room temperature obtained with nonsaturating microwave power and 0.2 G modulation amplitude. The simulated spectrum (red line) was calculated with CuNO³¹ using $J \gg A_N$. The double integral of the CW spectrum shows the expected 1:2:3:2:1 ratio of relative intensities of the five lines.

Figure 5.

Model for interconversion of conformations with differing strengths of nitroxide-nitroxide exchange interaction. Exchange is weak for conformations with population p_1 that interconvert slowly with conformations with strong exchange and population p_2 . Other conformations with weak exchange and population p_3 interconvert rapidly with p_2 .

Figure 6.

Simulations of spectra at 295 and 353 K calculated for $\nu = 9.519$ GHz and the thermodynamic parameters reported for a dinitroxide with the flexible di(ethylene glycol) linkage.⁴¹ The values of J are p_1 ($J = 0$), p_2 ($J = 2000$ MHz, which is 714 G), p_3 ($J = 0$). In water at 295 K $p_1 = 0.73$, $p_2 = 0.25$, and $p_3 = 0.01$ and rate constants are $7.3 \times 10^4 \text{ s}^{-1}$ for p_1 to p_2 and 7.3×10^7 for p_2 to p_3 . In water at 353 K $p_1 = 0.47$, $p_2 = 0.53$, and $p_3 = 0.001$ and rate constants are $3.3 \times 10^4 \text{ s}^{-1}$ for p_1 to p_2 and 3.3×10^8 for p_2 to p_3 . In 1:1 water:isopropanol at 295 K $p_1 = 0.81$, $p_2 = 0.17$, and $p_3 = 0.02$ and rate constants are $5.5 \times 10^4 \text{ s}^{-1}$ for p_1 to p_2 and 5.5×10^7 for p_2 to p_3 . In 1:1 water:isopropanol at 353 K $p_1 = 0.73$, $p_2 = 0.26$, and $p_3 = 0.001$ and rate constants are $2.6 \times 10^4 \text{ s}^{-1}$ for p_1 to p_2 and 2.8×10^8 for p_2 to p_3 .

A.

B

Figure 7.

Changes in EPR spectra of an ^{15}N isotopically labeled dinitroxide as J increases. A) Stick diagram calculated with CuNO^{31} using $\nu = 9.852$ GHz, $g = 2.006$, $A_{\text{N}} = 22.4$ G, and Gaussian absorption line width of 0.2 G for $J = 0$, $J = 0.5 A_{\text{N}}$, $J = 2 A_{\text{N}}$, and $J \gg A_{\text{N}}$. For the AB pattern resulting from $(m_{\text{I}}^{(1)}, m_{\text{I}}^{(2)}) = (-1/2, 1/2)$ or $(1/2, -1/2)$ there are 4 lines centered at B_{avg} with positions and relative intensities as shown in Table 1. The resonances for $(m_{\text{I}}^{(1)}, m_{\text{I}}^{(2)}) = (1/2, 1/2)$ and $(-1/2, -1/2)$ are independent of J and occur at the positions labeled $M_{\text{I}} = 1$ and -1 , respectively. The changes in the positions of the lines as J increases are discussed in the text and shown with dashed lines for AB splitting pattern c. B) First derivative spectra calculated with dynbirm with the same parameters.

Figure 8.

Absorption EPR spectra (solid lines) of **VI** at 20°C in aqueous tris buffer recorded at A) 258 MHz and B) 1.09 GHz by rapid scan, and C) at 9.854 G by CW EPR. The spectrum in C) is the first integral of the CW spectrum. Spectra were obtained with RF or microwave power that is small enough that it does not impact the line shape. The dashed lines are simulations obtained with dynbirm using $g = 2.0051$ and $A_N = 44.95$ MHz (16.05 G). Other parameters are discussed in the text.

Figure 9.

2D spectral-spatial images of **VI** and the corresponding monoradical **VIa** in a two-compartment phantom with a 10 mm spacer between compartments. Left: the upper compartment contains 0.5 mM **VI** and the lower compartment contains 1 mM **VIa**, generated by the reaction of 0.5 mM **VI** with 1 mM glutathione; right: slices through the upper (blue) and lower (red) compartments of the image. Reproduced with permission from Ref. ⁴⁷.

Figure 10.

Schematic representation of the reaction of the closed (oxidized) form of the redox indicator with a thiol reducing agent, dithiothreitol (DTT_{red}) to generate the open (reduced) form of the indicator and oxidized DTT (DTT_{ox}). EPR spectra of the indicator before and after the reaction are shown. Indicator concentration was 100 μ M in 100 mM K-HEPES (pH 7.20) containing 100 μ M DTPA; the concentration of DTT_{red} was 10 mM (G. Rosen and J. P. Y. Kao, unpublished results, 2017).

Table 1

Positions and relative intensities of the four lines in an AB splitting pattern.^{29,30}

line	offset from B_{avg} ^a	Relative intensity
a'	$-J/2 - C$	$1 - \sin 2\theta$
a	$J/2 - C$	$1 + \sin 2\theta$
b	$-J/2 + C$	$1 + \sin 2\theta$
b'	$J/2 + C$	$1 - \sin 2\theta$

^a B_{avg} is the average of the resonance fields for the two spin states in the absence of interaction and is different for each combination of interacting nitrogen nuclear spin states of a dinitroxide, ΔB is the separation between the resonance fields for the two nuclear spin states in the absence of spin-spin interaction, $C = 0.5(J^2 + \Delta B^2)^{1/2}$, and $\sin 2\theta = J/(2C)$.²⁹

Table 2

Contributions to the five lines in the EPR spectra of an ^{14}N dinitroxide with $J \gg A_N$

M_I	$(m_I^{(1)}, m_I^{(2)})$
-2	(-1, -1)
-1	(0, -1), (-1, 0)
0	(0, 0), (-1, 1), (1, -1)
1	(0, 1), (1, 0)
2	(1, 1)

$M_I = \sum_I m_I^{(i)}$, where $m_I^{(1)}$ and $m_I^{(2)}$ are the nuclear spin state for nitrogens 1 and 2, respectively.

Table 3

Contributions to the 3 lines in the EPR spectrum for an ^{15}N dinitroxide with $J \gg A_N$

M_I	$(m_I^{(1)}, m_I^{(2)})$
-1	$(-1/2, -1/2)$
0	$(-1/2, 1/2), (1/2, -1/2)$
1	$(1/2, 1/2)$

$M_I = \sum_I m_I^{(i)}$, where $m_I^{(1)}$ and $m_I^{(2)}$ are the nuclear spin state for nitrogens 1 and 2, respectively.

Table 4Attenuation of J for dinitroxides through various linkages ^a

linkage	attenuation coefficient
-N=	1.27
-S-	~ 1.5
-P-	~1.7
-CH=CH-	1.7
=N-N=	1.76
-C≡C-	2.2
-O-	< 3.2
-NH-	~4
p-C ₆ H ₄ -	5.5 to 6.4
-CO-	6.1
-CH ₂ -	>8
Hg	20–25
m-C ₆ H ₄ -	>70

^aData reported in Ref.³³