

Advances in spin label oximetry

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Abstract – Spin label oximetry is defined as the use of nitroxide radical spin labels to monitor oxygen transport. Bimolecular collisions of oxygen with spin labels alter both the longitudinal and transverse relaxation times of the spin label. Various experimental approaches can be used to observe these effects. Two of these are discussed here. (1) Saturation–recovery time–domain ESR using high observing power, rather than, as is customary, a low value, is shown to improve the sensitivity to bimolecular collisions with oxygen. First results obtained with a recently constructed S–band saturation–recovery apparatus are presented. (2) The use of electron–electron double resonance (ELDOR) as an oxygen sensitive display is considered. It is found to have substantial advantages over continuous wave (CW) saturation studies. Because it is instrumentally a simpler technique than saturation recovery, it can be useful in laboratories where acquisition of pulse equipment is unrealistic.

INTRODUCTION

The subject of spin label oximetry has recently been reviewed (ref. 1). Bimolecular collisions of molecular oxygen with spin labels alter both the spin–lattice relaxation time and the linewidth. In Ref. 1, the various possible methods for observation of these collisions were classified as T_1 –sensitive or T_2 –sensitive. For spin labels in low viscosity solutions, T_2 –sensitive methods are preferred because they are instrumentally easy. For spin–labeled macromolecules or for spin labels in high viscosity environments such as membranes, T_1 –sensitive methods are preferred. This is because the bimolecular collision rate needs to be comparable either to T_1^{-1} or T_2^{-1} for T_1 or T_2 –sensitive techniques, respectively. Since $T_1^{-1} \ll T_2^{-1}$ when rotational diffusion is slow, much lower and more easily managed concentrations of oxygen can be used with T_1 –sensitive methods.

It can be argued that the continuous wave (CW) saturation technique is an adequate and instrumentally easy T_1 –sensitive method (ref. 2). This is true when relative rather than absolute measurements in a series of samples of the same linewidth are to be made. But if absolute values of the bimolecular collision rate are to be determined and T_2 is not known or not well defined, CW saturation is inadequate.

By far the best T_1 –sensitive method is saturation–recovery ESR. An article has been written describing the use of this method to measure bimolecular collisions of spin–labels with oxygen (ref. 3). The energy–level diagram of Fig. 1 was used. The physical interaction of spin labels (SL) with

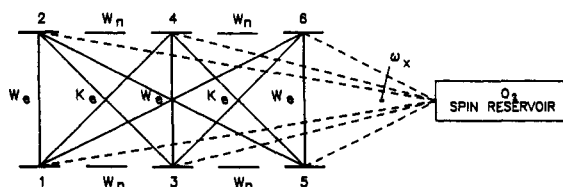


Fig. 1 Energy level diagram.

O_2 is Heisenberg exchange, but the spin–lattice relaxation of O_2 is so short that every collision of SL with O_2 results in a coupling to the lattice and a decrease in the effective T_1 . Rate equations connecting the various energy levels of Fig. 1 were set up and solved. In general, triple exponentials are predicted that depend on the various relaxation rates in the problem. The theory predicts that each of the three time constants is shortened by the same amount,

$$\tau_1^{-1} = 2W_e + 2\omega_x \quad (1)$$

$$\tau_2^{-1} = 2W_e + 2\omega_x + W_n + K_e[\text{SL}] \quad (2)$$

$$\tau_3^{-1} = 2W_e + 2\omega_x + 3W_n + K_e[\text{SL}] \quad (3)$$

Here $(2W_e)^{-1} = T_1$, the spin-lattice relaxation time, ω_x is the bimolecular collision rate of oxygen with spin labels, W_n is the nitrogen nuclear spin-lattice relaxation rate, and $K_e[\text{SL}]$ describes Heisenberg exchange between spin labels.

A problem in the saturation-recovery method is that after perturbing the populations of the levels by a strong saturating microwave pulse, the recovery to equilibrium is monitored with a weak observing microwave source. Thus one does not directly observe the time evolution of the m_x component of magnetization, which is the quantity calculated using the rate-equation method; m_x is observed indirectly by monitoring m_x or m_y . Since the observing power is low, the signal-to-noise ratio suffers.

If the observing power is increased, the apparent T_1 is artificially shortened. Percival and Hyde calculated this effect for a single homogeneous line, Eq. 4. (ref. 4).

$$2W_e(\text{eff}) = 2W_e + \gamma_e^2 B_1^2 T_2 \quad (4)$$

Because of possible complications, all previous workers therefore kept the observing power low and accepted the accompanying loss in sensitivity. However, we have recently shown (ref. 5) that bimolecular collision frequencies of ^{15}N substituted nitroxides with ^{14}N -containing labels can reliably be obtained using high observing power.

Our first subject in this presentation is a study of the use of high observing power in saturation-recovery ESR spectroscopy to measure bimolecular collision rates of oxygen with spin labels. The goal of the research is to improve the signal-to-noise ratio.

Saturation-recovery ESR spectroscopy is demanding from an instrumental point-of-view. Relaxation times are short, state-of-the-art microwave pulse techniques are required, and digitization and accumulation must be at high rates. It has occurred to us that CW ELDOR is an easier T_1 -sensitive oximetric method. To our knowledge, ELDOR experiments intended to quantitate oxygen concentration and diffusion have not previously been reported. The second half of this presentation is directed to a study of ELDOR effects in the presence of oxygen.

ELDOR spectroscopy has recently been reviewed (ref. 6). There are two rationales for revisiting this field, which was initiated by Hyde *et al* (ref. 7) and Benderskii *et al* (ref. 8). The first is the introduction of the loop gap resonator into ELDOR spectroscopy (ref. 9). Because the Q of this resonator is low, one can simply introduce the two microwave frequencies directly into the resonator. In addition, the signal-to-noise ratio improved by more than an order of magnitude with a loop gap resonator relative to what could be achieved using a bimodal cavity. The other rationale lies in the rate equation analysis of Hyde and Yin (refs. 10,11). A rigorous theory applicable over a wide range of experimental situations was developed.

METHODS AND MATERIALS

The saturation-recovery spectrometer is based on the design of Husjen and Hyde (ref. 12), upgraded by the addition of high speed signal acquisition (ref. 13) and a loop gap resonator (ref. 14). Our previous experiments were all at X-band. Recently, an octave bandwidth 2-4 GHz (S-band) saturation-recovery microwave bridge has been completed, and first results are presented here.

The ELDOR apparatus has been described by Yin and Hyde (ref. 9). It consists of a second pumping microwave source, spaced a hyperfine interval apart from the observing source, a loop gap resonator, and a microwave filter to keep the pumping microwave power from interfering with detection of the observing microwave signal. The oxygen tension in the sample was controlled using the gas exchange method of Popp and Hyde (ref. 15).

The notation $^{14}\text{NC16}$ and $^{15}\text{NC12}$, stands for stearic acid spin labels, nitrogen isotope 14 or 15, doxyl moiety at the 16th or 12th carbon, respectively. ^{14}N -containing stearic acid spin labels were from Aldrich (Milwaukee) and ^{15}N -containing compounds were synthesized in this laboratory by the method of Joseph and Lai (ref. 16). Perdeutero ^{15}N CTPO was from Merck (Canada) (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy).

Liposomes containing stearic acid spin labels were prepared from dimyristoylphosphatidylcholine (DMPC) as described by Feix *et al* (ref. 17).

SATURATION-RECOVERY EXPERIMENTS AT HIGH OBSERVING POWER IN THE PRESENCE OF OXYGEN

For stearic acid spin labels in membranes, nitrogen nuclear relaxation is substantially more rapid than electron spin lattice relaxation. The transitions corresponding to the nitrogen ESR hyperfine lines are effectively shorted together, and the saturation-recovery signal when a long saturating pulse is employed is a single exponential. The bimolecular collision rate of oxygen with the spin label is given by the Smoluchowski equations, Eq. 5:

$$\omega_x = p4\pi R \{D(SL) + D(O_2)\} [O_2] \quad (5)$$

Here R is the interaction distance (about 4 Å) and p is the probability that an observable event occurs when collision does in fact occur. From Ref. 3, the low power saturation-recovery time is

$$\tau_1^{-1} = 2W_e + 2\omega_x, \quad (6)$$

whereas according to Ref. 2, it is

$$\tau_1^{-1} = 2W_e + 2/3 p(T_1)\omega_x. \quad (7)$$

The numerical factors of the order of unity depend on the detailed model for bimolecular collision. Data were presented in Ref. 1, Fig. 3, indicating that some variation is observed experimentally. There remain unanswered questions, and one approach is to define the experimental oxygen transport after Kusumi *et al*, Ref. 18.

$$W = T_1^{-1}(\text{air}) - T_1^{-1}(N_2) \quad (8)$$

extrapolated to 1 atm air. We then can write

$$\tau_1^{-1} = 2W_e + Wf \quad (9)$$

where f is the fraction of air in the N₂-air mixture. Figure 2 shows data obtained at S-band for ¹⁴NC16 in DMPC using low observing microwave power demonstrating the linear relationship predicted by Eqs. 6,7,9 and Table I gives data at X-band (Ref. 3) and S-band.

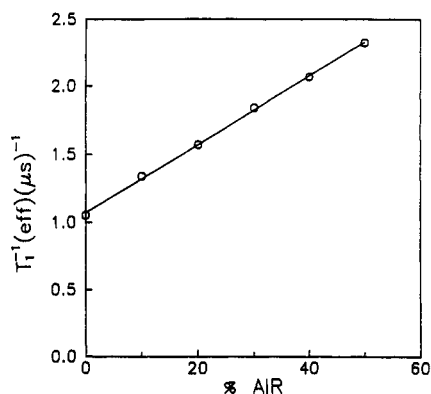


Fig. 2 S-band saturation-recovery data on 0.5 mol% ¹⁴NC16 in DMPC at pH 9.5, 27°C.

Table I. Oxygen relaxivity

	T_{1e} (μs)	W (μs ⁻¹)
X	2.54	2.67
S	0.94	2.52

The decrease in T_{1e} on going from X- to S-band is a notable and unexpected result that will be studied in greater depth in the future. Many experiments were performed on different samples on different days. The T_{1e} -values reported in Table I are reliable.

A second result is that W is independent of microwave frequency. A possible concern with the entire field of spin-label oximetry is that not only Heisenberg exchange but also dipole-dipole interactions contribute to the shortening of the spin-label T_1 . The latter are expected to be microwave-frequency dependent. Table I gives evidence that Heisenberg exchange is dominant.

Figure 3 shows values of the observed single-exponential time constant as a function of oxygen concentration and microwave power at S-band for $^{14}\text{NC16}$ in DMPC. The data are consistent with Eq. 10.

$$2W_{\text{eff}} = 2W_0 + Wf + \gamma_0^2 B_1^2 T_2 \quad (10)$$

where it is noted that

$$T_2^{-1} = T_2^{-1}(\text{No Air}) + p(T_2)\omega_x \quad (11)$$

Here $p(T_2)$ is the probability that a T_2 -sensitive event occurs when a collision occurs. From the data of Fig. 3, $p(T_2)\omega_x \ll T_2^{-1}$, as expected based on the relatively large ESR linewidths of the spin label.

However, the lines in Fig. 3 are not quite parallel. If we introduce the notation

$$S = \gamma_0^2 \Lambda^2 T_2; \Lambda^2 = B_1^2 / P_0 \quad (12)$$

where P_0 is the incident power, Λ is the resonator efficiency parameter (ref. 14), and S stands for slope in Fig. 3, Eq. (11) can be rewritten

$$S^{-1} = S^{-1}(\text{No Air}) + p(T_2)\omega_x(\gamma_0^2 \Lambda^2 T_2)^{-1} \quad (13)$$

The slopes in Fig. 3 are plotted in Fig. 4 and are consistent with Eq. 13.

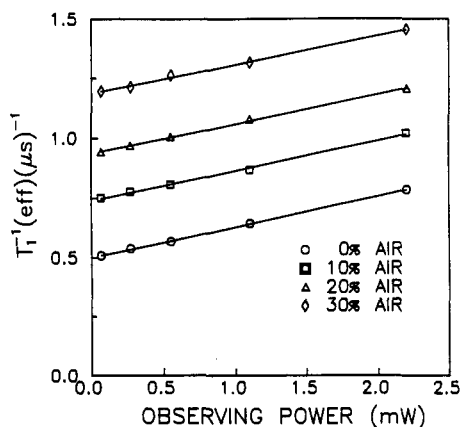


Fig. 3 S-band saturation-recovery data on 0.5 mol% $^{15}\text{NC12}$ in DMPC at pH 9.5, 27°C.

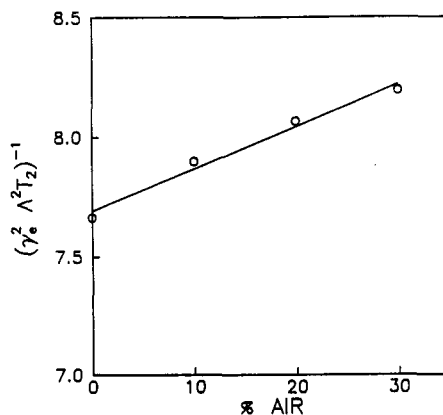


Fig. 4 Plot of the slopes from Fig. 3 as a function of air concentration in the gas-exchange mixture.

The microwave efficiency parameter Λ of the S-band resonator is 6 in free space and drops to about 4 when the sample is inserted. Combining Eqs. 7–13, we can calculate a ratio $p(T_2)/p(T_1)$ of about 3. In Ref. 3, Fig. 3, values for the ratio of this order of magnitude were reported in very viscous solvents and attributed to dipolar effects of oxygen on T_2 . However, we may be guilty of over interpreting our data. The points in Fig. 4 are very sensitive to small instrumental errors.

It is concluded from our experiments that Eq. 10 adequately describes our experiments. If one wants to measure the oxygen transport parameter, W , it is generally sufficient to measure the effective T_1 at high observing power at two concentrations of air. Because bimolecular collisions also contribute to T_2 , which appears in the last term of Eq. 10, somewhat refined values of W can be obtained by collecting data at two or more observing microwave power levels.

THE USE OF ELDOR TO MEASURE BIMOLECULAR COLLISIONS OF OXYGEN WITH SPIN LABELS

Transfer of saturation from one hyperfine line to another depends on the transverse relaxation rate relative to the electron spin-lattice relaxation rate. If T_1 becomes shorter, the ELDOR effect is diminished. Introduction of molecular oxygen shortens the effective T_1 and diminishes the ELDOR

effect. If we want to study this process and learn something about oxygen transport, we must have conditions such that a strong ELDOR effect is observed in the absence of oxygen.

The ELDOR reduction at low observe powers extrapolated to infinite pump powers for an ¹⁵N-containing nitroxide is

$$R_{\infty} = \frac{W_n + (N/2) K_x}{2W_n + W_n + N/2 K_x} \quad \text{or} \quad (R_{\infty}^{-1} - 1) = \frac{2 W_n}{W_n + \omega_x} \tag{14}$$

where $\omega_x = (N/2)K_x$, the Heisenberg exchange frequency, N is the total concentration of spin labels and W_n is the nitrogen nuclear relaxation rate.

In a particular system where oximetric studies are to be performed, one in general will not have control of W_n . An option for achieving a strong ELDOR signal is to adjust the concentration. It is easy to show from Eq. 14 that the greatest sensitivity of R_{∞} to changes in W_n occur when

$$(W_n + \omega_x) = 2W_n \tag{15}$$

If we assume that W_n is independent of concentration of spin label and W_n is negligible, $\omega_x(10^{-3}M)/\omega_x(10^{-4}M) = 9.6, 9.5$ at 27 and 37°C respectively. The Table and Eq. 15 demonstrate that a concentration of about $10^{-3}M$ is optimum for CTPO in water.

We expect from Eq. 14 that

$$(R_{\infty}^{-1} - 1) = \frac{2 W_n}{\omega_x} + \frac{K_o[O_2]}{\omega_x} \tag{16}$$

where K_o is the rate constant for Heisenberg exchange between oxygen and spin label. Fig. 5 demonstrates the expected linear dependence for CTPO in water at 37°C, pH 7.4, $10^{-3}M$. The experiment was done at low observing power. The display demonstrates good sensitivity to oxygen tension.

Table 2 shows ELDOR data for perdeutero ¹⁵N CTPO in water.

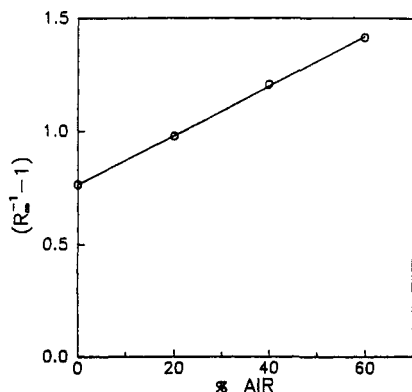


Fig. 5 ELDOR effect for $10^{-3}M$ ¹⁵N-CTPO in water at 37°C, pH 7.4.

Table 2. ELDOR reduction R_{∞}

Temp	$10^{-3}M$	$10^{-4}M$
27°C	54.5%	11.1%
37°C	56.8%	12.3%

Just as for saturation recovery, it is customary to use low observing microwave power in ELDOR. As the observing microwave power increases, the signal-to-noise improves but the theoretical equations predicting the ELDOR effect become very complicated (ref. 5). However at quite high observe powers, a simplification occurs (refs. 5, 7), and an expression similar to Eq. 14 is obtained.

$$(R_{\infty}^{-1} - 2) = \frac{2 W_n}{W_n + \omega_x} \tag{17}$$

We expect, based on Eq. 17, that oximetric studies can be carried out using ELDOR high observing microwave, but no experiments have yet been performed.

It is well established that nitrogen nuclear spin-lattice relaxation times for spin labels in membranes or for spin-labeled proteins can be very short (ref. 10). In this limit, $R_{\infty} = 1$ (Eq. 14) or $1/2$ (Eq. 17). Strong ELDOR signals are observed, but they are insensitive to changes in $W_e(\text{eff})$ that might be caused by bimolecular collisions with oxygen. Thus the use of ELDOR for oximetric studies in many systems of widespread interest and importance might appear to be negated. We suggest a way around this difficulty: The value of W_e can be made approximately equal to W_n by introduction of oxygen. In Ref. 3 it was observed, for example, that $T_{1e}(\text{eff})$ of a stearic acid spin label was shortened by a factor of 2 by introduction of a 16% air mixture in DMPC at 27°C. Introduction of 29% oxygen mixture would drop $T_{1e}(\text{eff})$ by 10 times. This is the range required to satisfy Eq. 15. Having decreased $W_e(\text{eff})$, one then studies oxygen transport by controlled introduction of still higher concentrations of oxygen. Thus, for example, oximetric data could be collected by comparing ELDOR reductions at 30 and 40% oxygen. The loop gap resonator (refs. 9,14) make this idea feasible because of the very high values of Λ . It could be difficult to measure R_{∞} in cavity resonators with values of $W_e(\text{eff})$ 10 times greater than the intrinsic spin label W_e .

The data of Table 1 showing that intrinsic values of W_e are substantially greater at S- than at X-band suggest that oximetric studies in membranes at S-band using ELDOR might be favored.

It is remarked that we have successfully carried out oximetric studies of $^{14}\text{NC16}$ in DMPC above the main phase-transition temperature. Under these conditions W_n is comparable to W_e and one has good sensitivity to small change in W_e that are induced by bimolecular collisions with oxygen.

The basic rationale in studying oximetry using ELDOR is that only the changes in W_e that occur when bimolecular collisions take place affect the signal. Changes in T_2 do not enter the problem. Nor does one need to know about T_2 of the spin label. The effect depends only on transfer of saturation and analysis of the m_x component of magnetization using rate equations is rigorous.

It is concluded that the ELDOR method is less advantageous than saturation recovery but superior to CW saturation for oximetric investigations. The use of ELDOR to measure small changes in W_e is a new perspective; previously it has been used to measure changes in the transverse relaxation rates that give rise to transfer of saturation.

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