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SPIN-LABEL OXIMETRY IN BIOLOGICAL AND MODEL SYSTEMS

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Over the last two decades, spin-label oximetry methods were developed and applied to study oxygen consumption and evolution in different biological and biochemical systems, as well as oxygen transport in better-defined model systems. I will briefly review the early history of spin-label oximetry in which the Biophysics Department of Jagiellonian University has been actively involved. Although molecular oxygen is paramagnetic, the direct detection of oxygen in biological systems using the electron paramagnetic resonance technique is not possible. However, indirect methods exist in which bimolecular collisions of oxygen with paramagnetic molecules alter the resonance characteristics of the paramagnetic molecule. Previously, the term *spin-label oximetry* described the application of nitroxide radical spin labels to oximetry measurements. This term should now be broadened to include any paramagnetic substance that is sensitive to collisions with oxygen, because new, stable free radicals and solid-state paramagnetic probes have been introduced, especially for *in vivo* oximetry measurements. I will indicate applications of these new oxygen-sensitive probes. Finally, I will describe the use of oxygen as a probe to study three-dimensional molecular organization and dynamics in membranes.

INTRODUCTION

Applications of spin-label oximetry to biological systems were first emphasized by Backer, Budker, Eremenko & Molin (1977). They pointed out that the effect of oxygen on the resolution of the proton superhyperfine structure of spin label I (see Fig. 1) could be a useful oximetric method. Spin label II, which was obtained from Rozantsev's laboratory in Moscow, was introduced in our department in early 1978 (Pajak, Cieszka, Gurbiel, Subczynski & Lukiewicz, 1978). This spin label, later named CTPO (Popp & Hyde, 1981), was first used by us to measure oxygen consumption in cell suspensions with special attention paid to cells containing melanin (Pajak, Subczynski, Panz & Lukiewicz, 1979a; Pajak, Subczynski, Panz & Lukiewicz, 1979b; Pajak, Subczynski, Panz & Lukiewicz, 1980; Panz, 1979). At that time, we also performed the first *in vivo* spin-label oximetry measurements on an intact bean leaf (Cieslikowska, 1980). The calibration curve was produced using parameterization of superhyperfine structure of electron paramagnetic resonance (EPR) spectra of CTPO with the parameter $\alpha = b/a$ (see Fig. 2 and Subczynski (1984) for details). Later, Sarna, Duleba, Korytowski and Swartz (1980), introduced a new parameter, $K = (b + c)/2a$ (see Fig. 2), which became widely accepted. Finally, Lai, Hopwood, Hyde and Lukiewicz (1982), presented numerous calibrations of K as a function of oxygen concentration, temperature, spin-label concentration, and microwave power. Because of its high polarity, CTPO samples oxygen in the aqueous phase. There have been few attempts to introduce other spin labels for oximetry measurements that use superhyperfine structure (Morse & Swartz, 1985; Chan, Glockner & Swartz, 1989), but only CTPO (with the K parameter and Lai's calibration curves) has been widely accepted and used for oximetry measurements in many laboratories.

OTHER SPIN-LABEL MOLECULAR PROBES

The partially deuterated CTPO analog (spin label III) was described by Halpern at the 29th Rocky Mountain Conference in August 1987. This spin label has just two resolved superhyperfine lines, which arise from the ring proton. This spectral feature is used to distinguish broadening associated with self-interaction from that due to environmental oxygen (Halpern, Perik, Nguyen, Spencer, Teicher & Lin, 1990). These authors also report a 20-fold increase in the sensitivity of the EPR spectrum to oxygen exchange broadening.

The spin label TEMPONE (spin label IV), is to my knowledge the only one that shows no evidence of proton coupling, and in water solution, pos-

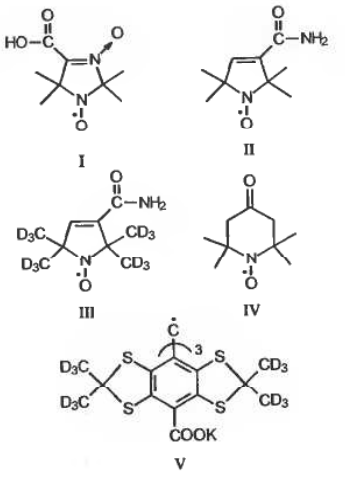

Fig. 1 Chemical structures of spin-label molecular probes.

sesses very narrow EPR lines of 0.25 G. Narrower lines of 0.15 G can be obtained after deuteration of this spin label. TEMPONE was used to measure oxygen consumption in biochemical reactions (Reszka & Sealy, 1984; Kalyanaraman, Feix, Sieber, Thomas & Girotti, 1987) to record fast changes of oxygen concentration during photosynthesis (Strzalka, Sarna & Hyde, 1986), oxygen transport in hydrocarbons (Subczynski & Hyde, 1984), and intracellular oxygen concentration (Wood, Dobrucki, Glockner, Morse II & Swartz, 1989).

Recently, a new class of oxygen-sensitive molecular probes, named TRITYLS (structure V in Fig. 1), were introduced (Andenkjaer Larsen, Laursen, Leunbach, Ehnholm, Wistrand, Peterson & Golman, 1998). The minimum linewidth is approximately 25 mG. TRITYLS offers the possibility of an order-of-magnitude improvement in signal to noise, spatial resolution, and physiologic sensitivity of *in vivo* spectral–spatial EPR imaging. Two-spatial-dimension images of a mouse tumor were presented using this new type of stable free radical (Halpern, Chandramouli, Williams, Barth & Galtsev, 1998).

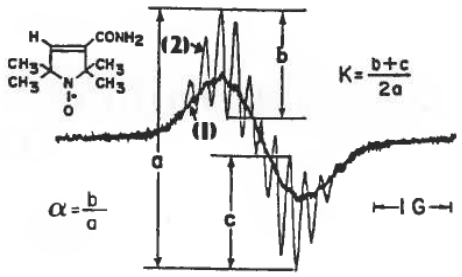

Fig. 2 The central field component of the EPR spectrum of the spin probe CTPO ($1.1 \cdot 10^{-4}$ M) in air-saturated (1) and nitrogen-saturated (2) water at 37°C. Definitions of the α parameter and the K parameter are indicated.

BIMOLECULAR COLLISIONS

Applications of nitroxide spin labels and other free radical molecules dissolved directly in investigated systems can be considered molecular probe methods because every molecule senses collisions with oxygen that depend on their local environment. If it is possible to put a spin label at specific labeled sites or in restricted domains such as membranes, the bimolecular collision frequencies with oxygen in these specific environments will be recorded.

The Smoluchowski equation forms the basis of the molecular probe method:

$$\omega = 4\pi R\{D(\mathrm{SL}) + D(\mathrm{O_2})\}[\mathrm{O_2}] \qquad (1)$$

Molecular oxygen is paramagnetic and during collisions it affects spectral characteristics of the spin probe. To measure the collision rate, an experimental observable, ω_{exp}, should be related to the actual collision frequency, ω:

$$\omega_{exp} = 4\pi pR\{D(\mathrm{SL}) + D(\mathrm{O_2})\}[\mathrm{O_2}] \qquad (2)$$

Here, p is the probability that an observable event occurs when a collision does in fact occur. For Heisenberg exchange between spin labels and oxygen, $p \approx 1$ (see Hyde and Subczynski (1989) for details). It is this fact that is responsible for making the method quantitative.

A useful simplification occurs by neglecting the diffusion coefficient of the spin label, D(SL), relative to that of oxygen, $D(O_2)$:

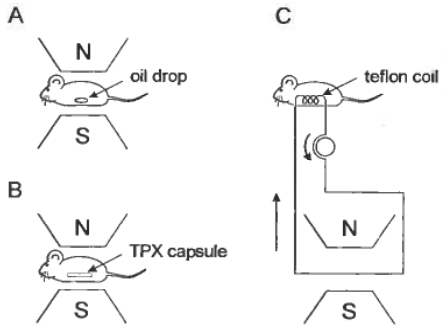

Fig. 3 Diagram illustrations of *in vivo* oximetry measurements. (A) and (B). Mice (2-3 weeks old, weight approx. 20 g) were inserted into a cylinder of an L-band resonator 25 mm in diameter and 30 mm long. (C). The oxygen-permeable teflon coil was slipped into the peritoneal cavity of the animal. EPR measurements were carried out using an X-band spectrometer with a glass capillary crossing the spectrometer cavity.

$$\omega_{exp} = 4\pi pRD(O_2)[O_2] \qquad (3)$$

If this is possible, the experimental observable yields the oxygen diffusion-concentration product, $D(O_2)[O_2]$. The interaction distance, R (4 to 5 Å), can in principle be adjusted to force the agreement of $D(O_2)$ obtained from measurements of diffusion against a concentration gradient (macroscopic diffusion) and measurements from the Smoluchowski equation (self-diffusion) (Hyde & Subczynski, 1984; Subczynski & Hyde, 1984).

MICROSCOPIC AND MACROSCOPIC SPIN-LABEL PROBES

The effect of oxygen on EPR spectral characteristics of spin labels depends on the oxygen diffusion-concentration product in the solvent surrounding the spin labels. Spin labels dissolved in solvents with high oxygen solubility and a high oxygen diffusion coefficient will be more sensitive to changes in oxygen tension. Hydrocarbons like paraffin oil or hexane are such solvents: they dissolve oxygen 4 to 10 times better than water, while diffusion of oxygen is about as facile as in water.

In 1984, together with Prof. Lukiewicz, we tried to use this methodological approach to increase the sensitivity of spin-label oximetry for *in vivo* measurements. First we used perdeutero ^{15}N TEMPONE solution in light paraffin oil injected into the peritoneal cavity of a mouse (Fig. 3A). However, the spin label leaked out of the oil drops into the aqueous environment of the mouse body. To protect the spin label from leaking and against reduction, we enclosed the paraffin oil solution in a gas-permeable TPX capsule. The TPX capsule was placed in the peritoneal cavity of a mouse (Fig. 3B) and *in vivo* oximetry measurements were performed at L-band using an EPR spectrometer with a loop-gap resonator (Subczynski, Łukiewicz & Hyde, 1986).

Another variation of this methodological approach for *in vivo* oximetry measurements is schematically presented in Fig. 3C (Łukiewicz, Zarnowska & Lackowska, 1985a). An oxygen-permeable teflon coil can be placed in the area of interest in an animal body. A closed system of oxygen-impermeable glass and rubber tubes with a peristaltic pump is used for the circulation of the filling solution. After about 30 min. the solution (light paraffin oil containing the spin label TEMPONE) is equilibrated with oxygen partial pressure which is surrounding the teflon coil. EPR measurements are performed outside the animal, so the size of the animal is not critical. The methods mentioned above, which we can call the macroscopic approach in spin-label oximetry, were further developed and applied to measure the steady-state concentration of oxygen in solid tumors - a subject of critical importance for radiation therapy and experimental oncology (Łukiewicz, 1985; Łukiewicz, Sochanik & Łukiewicz, 1985b).

A similar idea was used to develop oxygen-sensitive microscopic spin-label probes. Here bovine serum albumin (BSA)-coated light paraffin oil particles containing cholestane spin label were used (Ligeza, Wisniewska & Subczynski, 1992; Ligeza, Wisniewska, Subczynski & Tikhonov, 1994). Similarly, Liu, Greenstaff, Jiang, Suslick, Swartz & Wang (1994), used BSA-coated hexane particles containing stearic acid spin label. In this way it is possible to isolate nitroxides from water-soluble reductants and paramagnetic ions that might interfere with spin-label oximetry measurements. In these particles, spin labels are always surrounded by the same hydrocarbon solvent, which dissolves oxygen very well. Therefore, oxygen partial pressure is the only factor that can in-

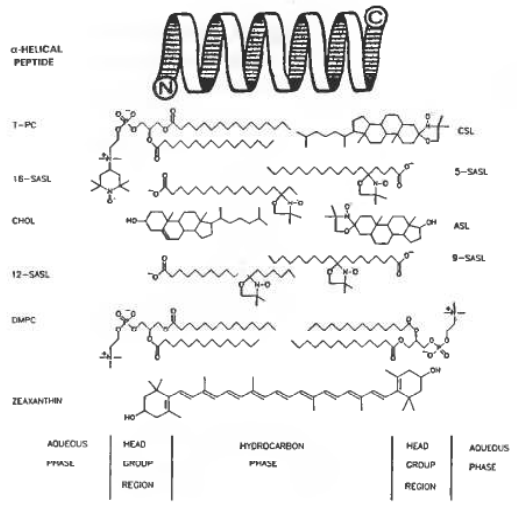

Fig. 4 Cross-sectional drawing of the DMPC bilayer including membrane modifiers (cholesterol (CHOL), zeaxanthin, and α-helical peptide) and spin labels. Locations across the membranes are illustrated.

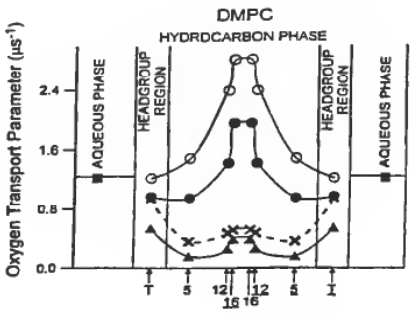

Fig. 5 Profiles of the oxygen transport parameter across DMPC membranes without BR (○), in reconstituted membranes of BR/DMPC ratio of 1/40 (●, X), and in purple membranes (Δ). Data for SLOT domain (●), and for bulk-boundary domain (X). Data obtained at 26°C. Approximate locations of the nitroxide moieties of spin labels are indicated. (Profiles made on the basis of the data presented by Ashikawa *et al.*, 1994).

fluence the EPR spectrum of the spin label in the microscopic probe. Such microscopic probes (a few micrometers in diameter) are readily and uniformly distributed within the sample, thus giving a rapid response to changes in oxygen partial pressure. All these properties of microscopic probes make it possible to measure oxygen production and consumption by chloroplasts *in situ* and *in vitro* (Ligeza *et al.*, 1994) as well as changes of oxygen tension in skeletal muscle of the mouse *in vivo* (Liu *et al.*, 1994).

Other microscopic solid-state probes have also been used for oximetry measurements, such as certain crystalline forms of lithium phthalocyanine (Liu, Gest, Moussavi, Norby, Vahidi, Walczak, Wu & Swartz, 1993), a derivative of coal termed fusinite (Swartz, Boyer, Gest, Glockner, Hu, Liu, Moussavi, Norby, Vahidi, Walczak, Wu & Clarkson, 1991), and India ink (Goda, Liu, Walczak, O'Hara, Jiang & Swartz, 1995). These probes were used for measurement of oxygen partial pressure in the tissues of intact animals (Swartz & Glockner, 1991) as well as an intact leaf (Ligeza, Tikhonov & Subczynski, 1997).

Table 1. Oxygen Permeability Coefficients for Different Membranes

Membrane	Temp. (°C)	P_m (cm s^{-1})
DMPC*	8	5.3
DMPC	25	105.0
DMPC	38	185.0
DMPC — 10% zeaxanthin	25	59.0
DMPC — 50% cholesterol	38	38.0
EYPC**	25	119.0
EYPC	40	201.5
EYPC — 10% zeaxanthin	25	88.0
EYPC — 50% cholesterol	40	73.4
CHO plasma membrane†	37	42.0
Thylakoid membrane	20	39.5
SLOT‡	35	34.7
Purple membrane	35	21.3

* gel-phase membrane
** egg yolk phosphatidylcholine
† Chinese hamster ovary
‡ measurements for slow oxygen-transport domain in reconstituted membranes of BR and DMPC (BR/DMPC=1/40).

OXYGEN PERMEABILITY OF MODEL AND BIOLOGICAL MEMBRANES

Spin-label oximetry makes it possible to measure the transport of oxygen within and across lipid bilayer model membranes and in the lipid portion of biological membranes. This approach can be used to obtain the profile of the oxygen diffusion-concentration product across the membrane because the nitroxide free-radical moiety of spin labels can be located at different depths in the membrane (see schematic, Fig. 4). In these types of measurements, samples (liposome suspensions) should be precisely equilibrated with the given partial pressure of oxygen at defined temperatures. This was possible with the use of capillaries made of a plastic called TPX, which is permeable to oxygen, nitrogen, and other gases and is substantially impermeable to water (Popp & Hyde, 1981; Subczynski & Hyde, 1981). Popp & Hyde (1981) acknowledged Prof. Lukiewicz, who first brought the high gas permeability of TPX to our attention. The importance of the TPX gas exchange sample cell to the development of spin-label oximetry has been very great.

Typical profiles of the oxygen diffusion-concentration product (also called the oxygen transport parameter) across fluid-phase dimyristoylphosphatidylcholine (DMPC) membranes in the absence and presence of bacteriorhodopsin (BR) are shown in Fig. 5. Other fluid-phase model and biological membranes show similar, bell-shaped profiles with the oxygen diffusion-concentration product in the membrane center a few times greater than that in and near the headgroup region. Membrane modifiers (see Fig. 4 for their structures and locations) affect oxygen transport within the lipid bilayer differently in different membrane regions. Cholesterol decreases oxygen transport in the polar headgroup region and in hydrocarbon near the polar headgroup region, and increases it in the membrane center (Subczynski, Hyde & Kusumi, 1989; Subczynski, Hyde & Kusumi, 1991a). Polar carotenoids (zeaxanthin and violaxanthin) decrease the oxygen diffusion-concentration product in saturated and unsaturated membranes (Subczynski, Markowska & Sielewiesiuk,1991b). The effect is negligible in the headgroup region and the strongest in the membrane center. The transmembrane α-helical peptide, Ac-$K_2L_{24}K_2$-amide, also decreases oxygen transport in the lipid bilayer, however, its effect is minimal in the membrane center and increases towards the headgroup region (Subczynski, Lewis, McElhaney, Hodges, Hyde & Kusumi, 1998).

Knowledge of the profile of the oxygen diffusion-concentration product makes it possible to calculate the membrane oxygen permeability coefficient, P_m, using the procedure developed by Subczynski *et al.* (1989). Obtained data are collected in Table 1. P_m depends on membrane composition. Cholesterol at high concentration decreases the value of P_m of model membranes by 3 to 5 times and polar carotenoids at 10 mol% by 2 times. The greatest effect is observed in lipid domains crowded with integral membrane proteins. The lipid domain of the purple membrane shows an oxygen permeability coefficient about 6 to 10 times smaller than that is fluid-phase lipid bilayers (Ashikawa, Yin, Subczynski, Kouyama, Hyde & Kusumi, 1994). Evaluations presented by Subczynski, Hopwood and Hyde (1992), and Ligeza, Tikhonov, Hyde and Subczynski (1998), show that even with these low oxygen permeability coeffi-

cients, the possible oxygen concentration differences across the cell plasma membrane, the mitochondrial membrane, and the thylakoid membrane at physiological conditions are very small (0.012 μM, 0.12 μM, and 1 μM, respectively). The overall conclusion of these studies is that membranes are not barriers to oxygen transport, and oxygen concentration differences across membranes at physiological conditions are negligible.

THREE-DIMENSIONAL DYNAMIC STRUCTURE OF MEMBRANES AS REVEALED BY THE STUDY OF OXYGEN TRANSPORT

Molecular oxygen has a unique characteristic as a membrane probe: its small size and appropriate level of hydrophobicity allow it to enter the small vacant pockets that are transiently formed in the lipid bilayer membranes. Therefore, the molecular collision rates between oxygen and nitroxide spin labels placed at specific locations in the membrane are sensitive to the dynamics of *gauche-trans* isomerization of lipid alkyl chains and to the structural nonconformability of neighboring lipids. Using this approach, reconstituted model membranes and biological membranes were investigated, with special attention paid to the membranes crowded with integral proteins (Ashikawa *et al.*, 1994; Kawasaki, Yin, Subczynski, Ohnishi, Hyde & Kusumi, 1999) or single transmembrane α-helices (Subczynski *et al.*, 1998). A new pulse EPR spin-labeling method was developed to detect and characterize local domains in these membranes. This method is based on variations of the local oxygen transport parameter (oxygen diffusion-concentration product) in various membrane domains, thus called "the method of discrimination by oxygen transport (DOT method)." More specifically, this method is sensitive to the product of the (local) translational diffusion coefficient and the (local) concentration of oxygen in the membrane.

In reconstituted membranes of BR and DMPC, the presence of a specific lipid region that appears only in protein-rich membranes has been indicated where oxygen transport is five times slower than in the bulk-boundary domain (Fig. 5). This domain is called the "slow oxygen transport (SLOT) domain," and its oxygen transport properties are similar to those in the lipid domain of the purple membrane (Ashikawa *et al.*, 1994). The rate of lipid exchange between the SLOT domain and the bulk-boundary domain is slower than 10^5 to 10^6 s^{-1}. It is speculated that the SLOT domain consists of lipids in contact with two proteins and lipids in contact with protein and boundary lipids. Alkyl chains and BR are closely packed in the SLOT domain with few vacant pockets to allow entrance and movements of even small molecules such as molecular oxygen.

In the influenza virus membrane, two membrane domains with slow and fast oxygen transport were indicated (Kawasaki *et al.*, 1999). A 16-fold difference in the oxygen diffusion-concentration product was observed between these two domains. The SLOT domain in viral membranes may be the membrane region corralled in the trimers of hemagglutinin, in which oxygen transport is greatly reduced.

In the case of membranes reconstituted with a transmembrane α-helical peptide, Ac-$K_2L_{24}K_2$-amide, results from the DOT method indicated that the peptide is highly miscible in the lipid bilayer, even at high concentrations with any indications of SLOT domains (Subczynski *et al.*, 1998).

It is concluded that molecular oxygen makes a particularly useful probe for studies of molecular organization and dynamics in protein-rich membranes.

OXIMETRY MEASUREMENTS IN LIPID BILAYER MEMBRANES—COMPARISON WITH MOLECULAR DYNAMICS SIMULATION RESULTS

The model presented on the basis of molecular dynamics (MD) simulations splits the lipid bilayer membrane into four regions, each of which has its own special characteristics. It is stressed that the exact locations of the boundary regions are somewhat arbitrary, however, the qualitative idea of the four-region model is considered to be applicable to different bilayer membranes (Marrink & Berendsen, 1994). The first two regions belong to the headgroup region of the membrane (low and high headgroup density regions); the other two regions describe the interior of the membrane (high and low tail density regions).

On the basis of oxygen transport parameter measurements, three regions of the lipid bilayer membrane could be distinguished: the headgroup region with a low oxygen diffusion-concentration product; the near headgroup region (the alkyl chain region up to the depth of ~ the ninth carbon) with a low oxygen diffusion-concentration product; and the central region where the oxygen transport parameter is a few times greater than that of the other two regions (Subczynski *et al.*, 1989, 1991a).

MD simulation also indicated that small molecules (smaller than benzene) experience enhanced diffusion in the lipid bilayer membranes, and the enhanced diffusion rate is a few times greater in the bilayer center (Stouch & Bassolino, 1996). This is also in agreement with oximetry measurements, which show that oxygen diffusion in membranes is as facile as in water (Subczynski & Hyde, 1981).

Diffusion of small molecules in membranes has been related to the creation and movement of kinks due to rapid *gauche-trans* isomerization of alkyl chains (Träuble, 1971; Pace & Chan, 1982). The idea of vacant pockets in the membrane presented by Subczynski *et al.* (1991a) is similar to these kink models, but the vacant pockets cover a wider range of packing defects in the membrane and vacant pockets formed by a variety of mechanisms contribute to the oxygen transport in the membrane. Oxygen molecules jump from one pocket to an adjacent one or move with the movement of the pocket itself. These hypotheses are supported by MD simulations, which show that free voids exist in the hydrocarbon interior of the bilayer that are commonly of a size large enough to accommodate small molecules. These free voids are most common in the bilayer center (Stouch & Bassolino, 1996). Profiles of the oxygen diffusion-concentration product reflect the distribution of voids across lipid bilayers, which also confirms that oxygen is a good probe of membrane organization.

Kusumi, Subczynski and Hyde (1982) showed that oxygen diffusion in the fluid-phase DMPC membrane is isotropic, based on measurements of collision rates between oxygen and lipid-soluble spin labels with a different orientation of the π-orbital of the nitroxide radical relative to the membrane normal. This statement was the subject of criticism because the membrane is described as an axially symmetric anisotropic environment. MD simulation of translational diffusion of NO in the hydrocarbon region of the DMPC bilayer led to the same conclusion—that NO diffusion in fluid-phase membranes is isotropic (Pasenkiewicz-Gierula & Subczynski, 1996). The calculated average coefficients of NO translational diffusion in both lateral and transversal directions appear to be the same. Similarly, the lateral diffusion coefficient profile of water molecules in a DMPC bilayer obtained from MD simulation was very similar to the transverse profile (Marrink & Berendsen, 1994). These observations confirm the experimental statement that diffusion of small molecules in the membrane looks essentially isotropic.

Finally, I would like to point out that because oxygen and NO are paramagnetic (oxygen has a triplet ground state, while NO has one unpaired electron making it a free radical), a similar approach can be used to study NO concentration and transport in biological and model systems (Singh, Hogg, Mchaourab & Kalyanaraman, 1994; Clarkson, Norby, Smirnov, Boyer, Vahidi, Nims & Wink, 1995; Subczynski & Hyde, 1998). It has been shown that this method, called "spin-label NO-metry", is also quantitative, giving a local NO diffusion concentration product (Lomnicka & Subczynski, 1996; Subczynski, Lomnicka & Hyde, 1996).

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