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LIPID DOMAINS: EPR DISCRIMINATION BY OXYGEN TRANSPORT

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Discrimination by oxygen transport (DOT) method is a dual-probe saturation recovery EPR approach, in which the observable parameter is a spin-lattice relaxation time (T_1) of spin labels, and the measured value is the bimolecular collision rate between molecular oxygen and the nitroxide moiety of a spin label. The method is based on the variation of the local diffusion-concentration product of oxygen in different membrane domains. Membrane domains can be characterized by profiles of the oxygen diffusion-concentration product *in situ* without the need for physical separation, which provides useful information about internal dynamics in each domain. The DOT method is especially suitable for obtaining time-space characteristics of small transient domains in model and biological membranes. The time scale of the DOT method is approximately 0.1 to 100 µs, and we are able to obtain reliable lipid exchange rates between domains when these rates fit this time window. The sensitivity of the method can be improved by using higher microwave frequencies, such as Q or W-band. Using the DOT method, we are able to discriminate and characterize solid-ordered, liquid-disordered and liquid-ordered domains in membranes made of binary mixtures of phosphatidylcholine and cholesterol or sphingomyelin and cholesterol, and of ternary raft-forming mixtures.

INTRODUCTION

The pulse EPR method of discrimination by oxygen transport (DOT) has been developed at Department of Biophysics, Medical College of Wisconsin, Milwaukee (Ashikawa, Yin, Subczynski, Kouyama, Hyde & Kusumi, 1994), and reviewed in *Current Topics in Biophysics* by Subczynski (2002). In this dual-probe saturation recovery (SR) approach, the measured value is the bimolecular collision rate between molecular oxygen and the nitroxide moiety of spin labels. Molecular oxygen is a unique probe molecule – it has a small size and an appropriate level of hydrophobicity that allows it to partition into different membrane domains. The oxygen transport parameter $W(x)$ was introduced as a convenient quantitative measure of the rate of collision between the spin label and molecular oxygen by Kusumi, Subczynski and Hyde (1982) as:

$$W(x) = T_1^{-1}(air, x) - T_1^{-1}(N_2, x), \qquad (1)$$

where T_1 is the spin-lattice relaxation time of the nitroxides in samples equilibrated with atmospheric air and nitrogen, respectively. The effect of oxygen on the SR signal of a lipid spin label introduced into a model membrane is illustrated in Fig. 1. The oxygen transport parameter is proportional to the local oxygen diffusion-concentration product at a "depth" x in the membrane that is in equilibrium with atmospheric air. It is a useful monitor of membrane fluidity and can describe the three-dimensional dynamic structure of lipid bilayer membranes (for review see Subczynski, 1999; Subczynski & Wisniewska, 2000; Subczynski & Kusumi, 2003; Wisniewska, Draus & Subczynski, 2003).

PROFILES OF OXYGEN AND CuKTSM$_2$ TRANSPORT PARAMETER

In our previous work the profiles of oxygen transport parameter (oxygen diffusion-concentration product) across different model and biological membranes have been obtained, which allowed us to understand the movement of oxygen molecules within the lipid bilayer and to calculate the oxygen permeability coefficient across the membranes (Subczynski, Hyde & Kusumi, 1989; Subczynski, Hyde & Kusumi, 1991; Subczynski, Hopwood & Hyde, 1992; Ashikawa *et al*., 1994; Subczynski & Hyde, 1998). The usefulness of molecular oxygen as a sensitive probe for studying membrane organization and dynamics has been strongly supported by molecular dynamics simulations (Subczynski Pasenkiewicz-Gierula, McElhaney, Hyde & Kusumi, 2003). However, oxygen is such a small molecule that the oxygen transport parameter re-

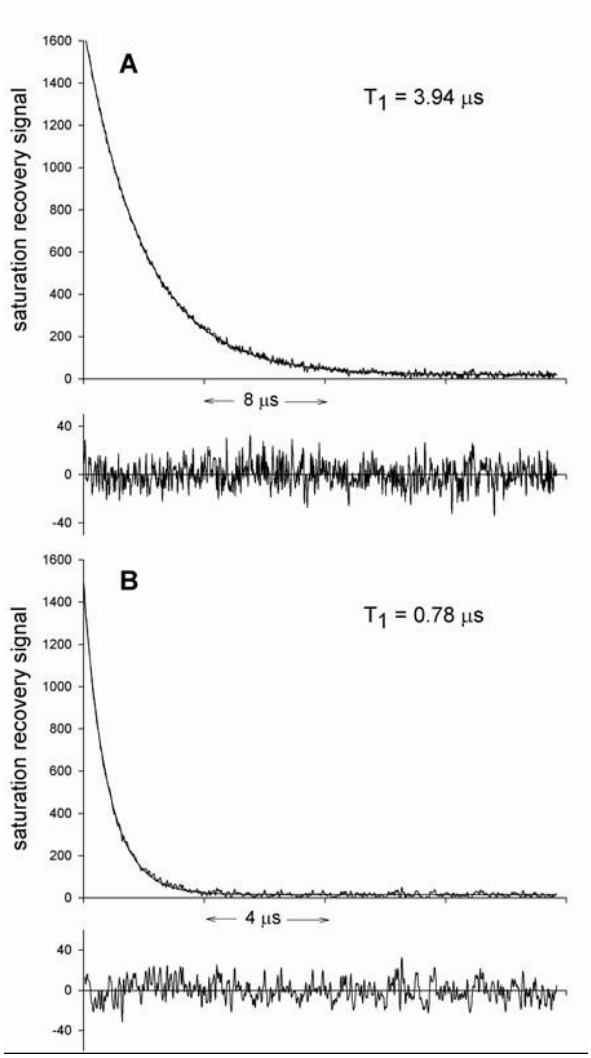

Fig. 1. Representative single-exponential saturation recovery (SR) curves from 14-PC in a DMPC membrane containing 50 mol% cholesterol equilibrated with nitrogen (A) and 40% air (B) measured at 20°C. The experimental and best-fit SR curves are superimposed. The difference between experimental data and the single-exponential fit is shown as a residual under each plot.

flects mainly the distribution and movement of free voids in the membrane. Therefore, in some investigations, another fast-relaxing paramagnetic probe was used, namely the copper complex, (3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato))copper(II) ($CuKTSM_2$) (Subczynski, Antholine, Hyde & Kusumi, 1990). It is highly soluble in the lipid bilayer and because of its larger size (the 8×8 Å square-planar complex) it is more space-filling than oxygen. Fig. 2 demonstrates that both probe molecules, oxygen and $CuKTSM_2$, sense the membrane environment in different ways. Diffusion of both molecules is enhanced in the center of saturated and unsaturated membranes as compared to the near-surface region. Cholesterol increases this enhancement for oxygen diffusion, but not for $CuKTSM_2$. The more fluid region created by cholesterol in the membrane center provides a pathway for lateral transport of very small molecules (Subczynski *et al.*, 1992; Subczynski & Hyde, 1998), but is too narrow to accommodate $CuKTSM_2$. Taken together, profiles of the oxygen and $CuKTSM_2$ transport parameters across the membrane allowed to understand the movement of small hydrophobic molecules within the lipid bilayer (Kusumi, Subczynski & Hyde, 1982; Subczynski *et al.*, 1989, 1990, 1991, 1992) and provided useful information on membrane organization and dynamics (Subczynski *et al.*, 1990; Subczynski *et al.*, 1991; Ahsikawa *et al.*, 1994; Subczynski, Lewis, McElhaney, Hodges, Hyde & Kusumi, 1998; Subczynski *et al.*, 2003).

SPECIFICITY AND APPLICATIONS OF THE DOT METHOD

When located in two different membrane domains, the spin label alone most often cannot differentiate between these domains, giving not distinguishable conventional EPR spectra and similar T_1 values in the absence of oxygen. However, even small differences in lipid packing in these domains affect oxygen partitioning and diffusion, which can be easily detected by observing the different T_1s from lipid spin labels in these two locations in the presence of oxygen. In membranes consisting of two lipid environments with different oxygen transport parameters (BULK domain and slow oxygen transport (SLOT) domain), the SR signal is a double-exponential curve with time constants T_1^{-1} (air, BULK) and T_1^{-1} (air, SLOT), and the oxygen transport parameters in both domains are calculated as follows:

$$W(\text{BULK}) = T_1^{-1}(\text{air, BULK}) - T_1^{-1}(\text{N}_2\text{, BULK}) \quad (2)$$

$$W(\text{SLOT}) = T_1^{-1}(\text{air, SLOT}) - T_1^{-1}(\text{N}_2\text{, SLOT}) \quad (3)$$

Here the x from equation (1) is changed to the two membrane domain, BULK and SLOT, and the depth fixed (the same spin label is distributed between the BULK and SLOT domains).

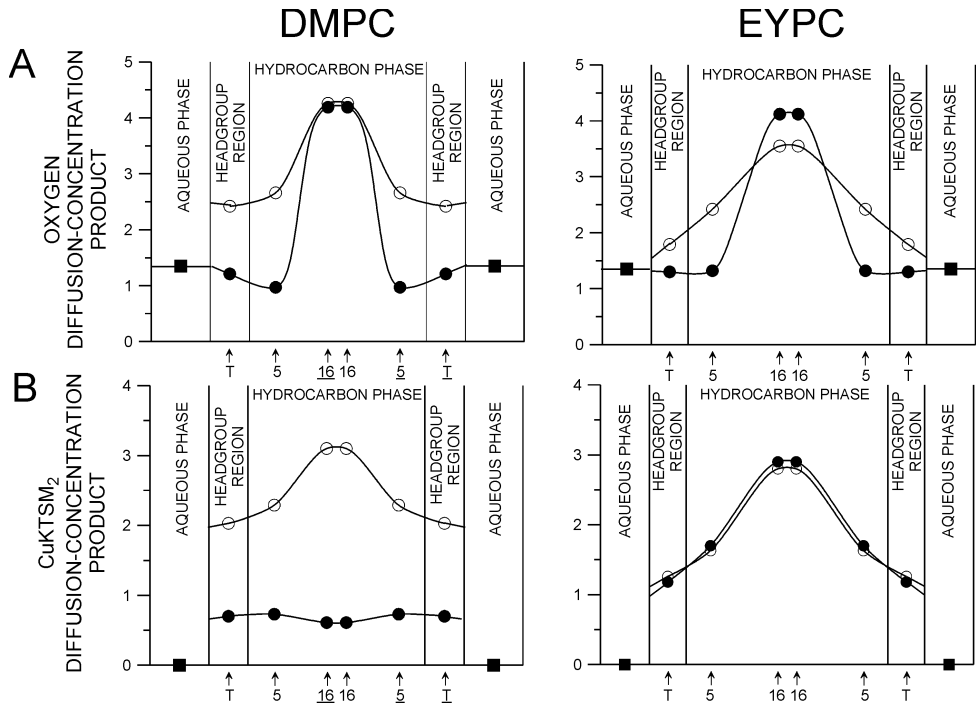

Fig. 2. Profiles of the rate of collision of the nitroxide probe with molecular oxygen (A, oxygen transport parameter, Eq. (1)) and with $CuKTSM_2$ (B, $CuKTSM_2$ transport parameter), observed at 40° C across the DMPC and egg yolk PC (EYPC) membranes in the absence (○) and presence (●) of 30 mol% cholesterol. T indicates a spin label Tempo-PC. The symbol (■) indicates the related value in the aqueous phase.

The SLOT domain was first detected in reconstituted membranes of bacteriorhodopsin (BR) and dimyristoylphosphatidylcholine (DMPC) at a BR/lipid ratio of 1/40. The oxygen transport parameter was there slower by a factor of 5 than in the BULK region (Ashikawa *et al*., 1994). The BULK domain in this case indicates both bulk and protein-boundary regions, because the lipid exchange between these two regions is too fast for the oxygen transport parameter to differentiate them. At a BR/lipid ratio of 1/40, 75% of BR molecules exist as trimers plus oligomers of trimers, and only 25% of BR molecules are monomers. The SLOT domain was thought to be protein rich, with every lipid molecule being in contact with two protein molecules or with a protein and a boundary lipid, and its hydrocarbon chain motion suppressed to the level of the gel-phase membrane. The schematic drawing of the lateral organization of BR and lipid molecules in such membranes is presented in Fig. 3.

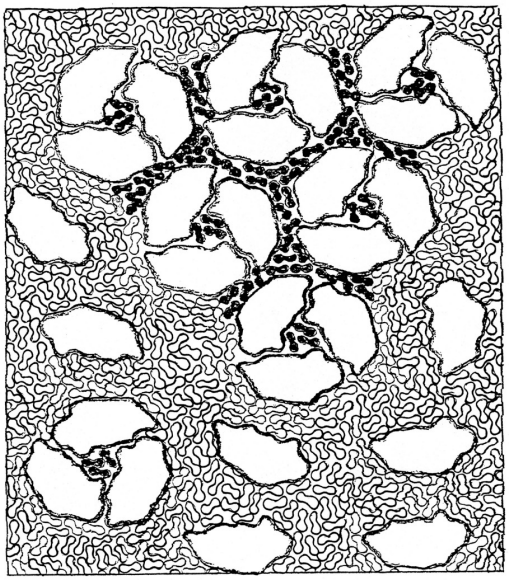

Fig. 3. Schematic drawing of the lateral organization of BR and lipid molecules in the reconstituted membranes of BR and DMPC at a BR/DMPC ratio of 1/40. The coexisting lipid domains, the BULK domain and the SLOT domain, are indicated as open and filled dumbbell-shaped lipid cross sections, respectively.

The first example of the SLOT domain detected in biological membranes by the DOT method was the influenza virus (IFV) envelope membrane (Kawasaki, Yin, Subczynski, Hyde & Kusumi, 2001). The oxygen transport parameter in the SLOT domain in this membrane was slower than in the bulk domain by a factor of 16. The transport parameter was even slower than in the purple membrane, which is a two-dimensional crystal of BR trimers with very small amounts of lipids intercalated in the space between protein molecules. IFV membranes, because of high concentration of

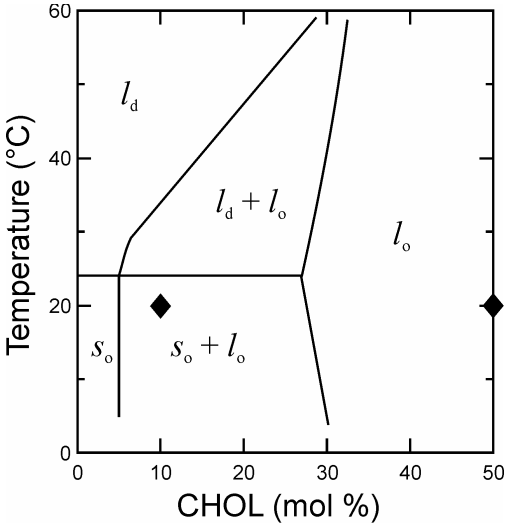

Fig. 4. Phase diagram of the DMPC/cholesterol membrane (adapted from Almeida *et al.,* 1992). Symbols: s_o – solid-ordered, l_o – liquid-ordered and l_d – liquid disordered phase. Diamonds indicate the points for which the SR curves presented in Fig. 1 and 5 were obtained.

cholesterol, are likely to contain a liquid-ordered (l_o) phase, which, according to many reports, is typical for raft domains (Ahmed, Brown & London, 1997; Ge, Field, Aneja, Holowka, Baird & Freed, 1999; Brown & London, 2000; Dietrich, Bagatolli, Volovyk, Thompson, Levi, Jacobson & Gratton, 2001). It was therefore proposed that the SLOT domain in the IFV membrane is a raft domain rich in cholesterol and stabilized by the presence of clustered viral proteins: trimers of hemagglutinin and/or tetramers of neuraminidase (Kawasaki *et al*., 2001).

DOMAINS IN DMPC/CHOLESTEROL MEMBRANES AS A RULER FOR RAFTS

To better understand the lipid organization of raft domains in biological membranes, we have undertaken a thorough investigation of model membrane systems which are known to contain a l_o phase. We have chosen a well defined DMPC/cholesterol system, for which the phase diagram (see Fig. 4) is widely accepted (Ipsen, Karlström, Mouristen, Wennerström & Zuckermann, 1987; Almeida, Vaz & Thompson, 1992). Using the DOT method, we were able to detect coexisting l_o and liquid-disordered (l_d) domains in the fluid phase of the membranes containing cholesterol, and l_o and solid-ordered (s_o) domains in the gel-phase membranes containing cholesterol. An example of a double-exponential SR curve obtained for 14-PC in DMPC membranes containing 10 mol% cholesterol, at 20°C and in the presence of oxygen (40% air), is given in Fig. 5. The point for which this SR curve was obtained, as well as the point for which the single-exponential SR curves presented in Fig. 1 were obtained, are marked as diamonds at the phase diagram (Fig. 4). The single-exponential SR curve should be ascribed to the homogenous l_o phase, while the double-exponential one to coexisting s_o and l_o domains. It is worth to point out that we can not only detect the two coexisting domains, but also ascertain a unique physical property of these domains, namely the oxygen transport parameter. Similarly like in homogenous membranes (Fig. 2), the profiles of the oxygen transport parameter can be acquired, giving the unique information about packing and dynamics of lipid molecules in each domain simultaneously. This physical characteristic obtained for s_o, l_o and l_d domains can

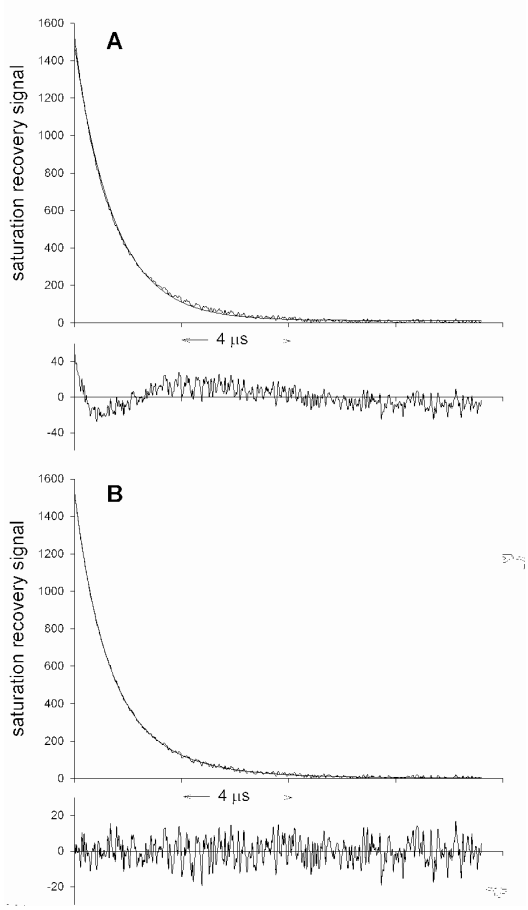

Fig. 5. Typical SR signals from 14-PC in a DMPC membrane containing 10 mol% cholesterol equilibrated with 40% air and measured at 20°C. Fitting the search to a single exponential mode, A, is unsatisfactory, as shown by the residual. The fit B, using a double-exponential mode (time constants of 2.12 and 0.99 μs) is excellent.

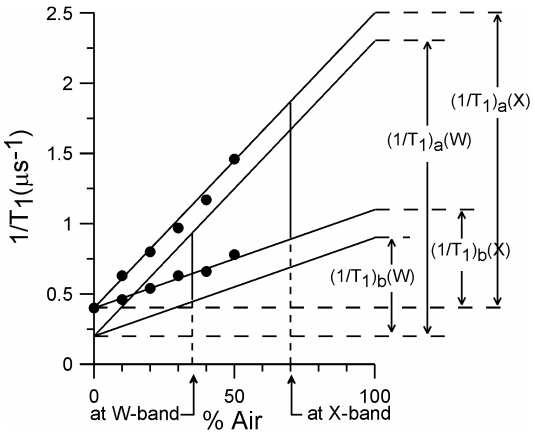

Fig. 6. Plot of $1/T_1$ versus % air for domains a (BULK) and b (SLOT) in BR/DMPC membranes. Experimental points are for X-band. Hypothetical W-band data are also shown. $(1/T_1)_a$ and $(1/T_1)_b$ are oxygen transport parameters in a and b domains, respectively. We apologize for changing the notation of oxygen transport parameter from "W" to $(1/T_1)_a$ and $(1/T_1)_b$, but we wanted to avoid a confusion with W-band.

serve as a ruler with which to classify other domains, e.g. rafts.

FUTURE DIRECTIONS

The results we got for DMPC/cholesterol membranes, as well as preliminary data on sphingomyelin/cholesterol binary mixtures and raft-forming ternary mixtures (phosphatidylcholine/sphingomyelin/cholesterol) support the hypothesis that lipids in model rafts form l_o domains within the bulk l_d environment. In the next step, the physical properties (oxygen and $CuKTSM_2$ transport parameter profiles) of l_o domains in model membranes will be compared with those of raft domains in biological membranes. This should give an ultimate answer whether lipids in rafts are organized like in the l_o phase, and if so, whether the raft l_o domain shows some unique characteristics which may be of biological importance.

The DOT measurements are planned to be extended to higher microwave frequencies, namely Q- and W-bands (35 and 94 GHz, respectively). It has been shown that T_1 values of spin labels in water samples and in membranes increase with microwave frequency raise from 2 to 35 GHz (Hyde, Yin, Subczynski, Camenish, Ratke & Froncisz, 2004). From data obtained by Hyde *et al*. (2004) it can be inferred that at W-band the T_1 values of lipid spin labels will be twice as large as at X-band. Another significant result of this paper is that the oxygen transport parameter measured in aqueous aqueous phase and in membranes is independent of microwave frequency. It seems likely to assume frequency independence of the oxygen transport parameter up to 94 GHz. The sensitivity of the DOT method at W-band should be significantly increased by allowing to differentiate more easily between two components of SR signal. It is schematically illustrated in Fig. 6 assuming that the above mentioned extrapolations are valid (T_1 at W-band is two times longer than at X-band, oxygen transport parameters measured at W- and X-bands are the same). This figure shows data points from Ashikawa *et al*. (1994) for 16-SASL in the BR/DMPC = 1/40 membrane, obtained at X-band (lines and values labeled "X"). Two domains (a and b) can be observed only in the presence of oxygen, with the oxygen transport parameter in each domain equal $(1/T_1)_a(X)$ and $(1/T_1)_b(X)$, respectively. The lines labeled "W" are hypothetical. If T_1 is two times longer and the oxygen transport parameters are the same ($(1/T_1)_a(X) = (1/T_1)_a(W)$, $(1/T_1)_b(X) = (1/T_1)_b(W)$), 50% difference in T_1 comparing the two domains occurs at two times lower concentration of air (compare vertical lines labeled by arrows). For any given experimental limit, the separation will be better at W-band than at X-band by at least an estimated factor of two.

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REFERENCES

Ahmed, S. N., Brown, D. A. & London, E. (1997). On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentration of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. *Biochemistry*, **36**, 10944-10953.

Almeida, P. F. F., Vaz, W. L. C. & Thompson, T. E. (1992). Lateral diffusion in the liquid phases of dimyristoyl/cholesterol bilayers: a free volume analysis. *Biochemistry*, **31**, 6739-6747.

Ashikawa, I., Yin, J-J., Subczynski, W.K., Kouyama, T., Hyde, J.S. & Kusumi, A. (1994). Molecular organization and dynamics in bacteriorhodopsin-rich reconstituted membranes: Discrimination of lipid environments by the oxygen transport parameter using a pulse ESR spin-labeling technique. *Biochemistry,* **33**, 4947-4952.

Brown, D. A. & London, E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.*, **275**, 17221-17224.

Dietrich, C., Bagatolli, L. A., Volovyk, Z. N., Thompson, N. L., Levi, M., Jacobson. K. & Gratton, E.

(2001). Lipid rafts reconstituted in model membranes. *Biophys. J.*, **80**, 1417-1428.

Ge, M., Field, K. A., Aneja, R., Holowka, D., Baird, B. & Freed, J. H. (1999). Electron spin resonance characterization of liquid ordered phase of detergent-resistant membranes from PBL-2H3 cells. *Biophys. J.*, **77**, 925-933.

Hyde, J.S., Yin, J-J., Subczynski, W.K., Camenish, T. G., Ratke, J.J. & Froncisz, W. (2004). Spin-label EPR T_1 values using saturation recovery from 2 to 35 GHz. *J. Phys. Chem. B.*, **108**, 9524-9529.

Ipsen, J.H., Karlström, G., Mouristen, O.G., Wennerström, H. & Zuckermann, M. J. (1987). Phase equilibrium in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta*, **905**, 162-172.

Kawasaki, K., Yin, J.-J., Subczynski, W. K., Hyde, J. S. & Kusumi, A. (2001). Pulse EPR detection of lipid exchange between protein-rich raft and bulk domains in the membrane: Methodology development and its application to studies of influenza viral membrane. *Biophys. J.*, **80**, 738-748.

Kusumi, A., Subczynski, W.K & Hyde, J.S. (1982). Oxygen transport parameter in membranes as deduced by saturation recovery measurements of spin-lattice relaxation times of spin labels. *Proc. Natl. Acad. Sci. USA*, **79**, 1854-1848.

Subczynski, W.K. (1999). Spin-label oximetry in biological and model membranes. *Curr. Top. Biophys*. **23**, 69-77.

Subczynski, W.K. (2002). Pulse EPR detection of membrane domains. *Curr. Top. Biophys*. **26**, 59-64.

Subczynski, W. K., Antholine, W. E., Hyde, J. S. & Kusumi, A. (1990). Microimmiscibility and three-dimensional dynamic structure of phosphatidylcholine-cholesterol membranes: translational diffusion of copper complex in the membrane. *Biochemistry*, **29**, 7936-7945.

Subczynski, W. K., Hopwood, L. E. & Hyde, J. S. (1992). Is the mammalian cell plasma membrane a barrier to oxygen transport? *J. Gen. Physiol*., **100**, 69-87.

Subczynski, W. K. & Hyde, J. S. (1998). Membranes, barriers or pathways for oxygen transport. [In:] Hudetz, A. G. (ed.) *Oxygen Transport to Tissue XX, Advances in Experimental Medicine and Biology* (pp. 95-107). New York: Plenum.

Subczynski, W. K., Hyde, J. S. & Kusumi, A. (1989). Oxygen permeability of phosphatidylcholine-cholesterol membranes. *Proc. Natl. Acad. Sci. USA*, **86,** 4474-4478.

Subczynski, W. K., Hyde, J. S. & Kusumi, A. (1991). Effect of alkyl chain unsaturation and cholesterol intercalation on oxygen transport in membranes: a pulse ESR spin labeling study. *Biochemistry*, **30**, 8578-8590.

Subczynski, W.K. & Kusumi, A. (2003). Dynamics of raft molecules in the cell and artificial membranes: approaches by pulse EPR spin labeling and single molecule optical microscopy. *Biochim. Biophys. Acta*, **1610**, 231-243.

Subczynski, W. K., Lewis, R. N. A. H., McElhaney, R. N., Hodges, R. S., Hyde, J. S. & Kusumi, A. (1998). Molecular organization and dynamics of 1-palmitoyl-2-oleoylphosphatidylcholine bilayers containing a transmembrane α-helical peptide. *Biochemistry*, **37**, 3156-3164.

Subczynski, W. K., Pasenkiewicz-Gierula, M., McElhaney, R. N., Hyde, J. S. & Kusumi, A. (2003). Molecular dynamics of 1-palmitoyl-2-oleoylphosphatidylcholine membranes containing transmembrane α-helical peptides with alternating leucine and alanine residues. *Biochemistry*, **42**, 3939-3948.

Subczynski, W.K. & Wisniewska, A. (2000). Physical properties of lipid bilayer membranes: relevance to membrane biological functions. *Acta Biochim. Polonica*, **47**, 613-625.

Wisniewska, A., Draus, J. & Subczynski, W.K. (2003). Is a fluid-mosaic model of biological membranes fully relevant? Studies on lipid organization in model and biological membranes. *Cell. Mol. Biol. Lett*. **8**, 147-159.