Published in final edited form as:

Free Radic Biol Med. 2009 March 15; 46(6): 707–718. doi:10.1016/j.freeradbiomed.2008.11.024.

Physical properties of lipid bilayers from EPR spin labeling and their influence on chemical reactions in a membrane environment

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Abstract

The influence of a variety of micro-environmental factors on the inherent reactivity of membrane located reagents is poorly understood. A goal of this review is to provide detailed profiles of membrane properties, including hydrophobicity, oxygen and nitric oxide solubility and diffusion rates, bilayer penetration of metal ions and metal-ion complexes, and membrane order and fluidity, which can be obtained with EPR spin-labeling methods. These properties can drastically vary with membrane composition membrane depth, and membrane domain formation, influencing the fate of chemical reactions that occur in a lipid bilayer environment.

Keywords

lipid bilayer; spin labeling; EPR; hydrophobicity; polarity; fluidity; oxygen; nitric oxide

Introduction

Electron paramagnetic resonance (EPR) spin labeling methods provide a number of unique approaches for determining several important membrane properties as a function of bilayer depth. These include hydrophobicity, diffusion-concentration products for oxygen and nitric oxide, penetration depths of metal ions and metal-ion complexes, as well as structural and dynamic parameters such as order parameters and alkyl chain bending. Using spin labeling, the transmembrane profiles of these properties can be obtained for homogenous membranes and, in some cases, for coexisting membrane domains or coexisting membrane phases without the need for their physical separation. Profiles differ in saturated and unsaturated membranes and are affected by cholesterol, membrane-bound peptides, integral membrane proteins, and other membrane components. Thus the microenvironment in which membrane-located reagents are immersed can change drastically with membrane composition and depth in the lipid bilayer. This suggests that micro-environmental factors presented by the membrane should be taken into account in the analysis of chemical reactions occurring in a lipid bilayer environment. In this paper we provide a brief description of how EPR spin labeling methods can be used to obtain the above mentioned profiles, and present examples of how these properties depend on membrane composition. We conclude by presenting examples where knowledge of the membrane microenvironment provided a better understanding of chemical processes occurring within the lipid bilayer.

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EPR spin-labeling approaches for profiles of membrane properties

To obtain a detailed profile across the membrane, a variety of lipid-analog spin labels are incorporated in the lipid bilayer that place the nitroxide moiety, which gives rise to the observed EPR signal, at specific depths and in specific membrane domains (Fig. 1). The physical/chemical properties of the microenvironment in the immediate vicinity of the nitroxide are then characterized using EPR spectroscopic methods that include both conventional and time-domain techniques. Information obtained from conventional EPR spectra includes profiles of the order parameter and hydrophobicity, whereas time-domain EPR methods (primarily saturation recovery) are highly sensitive to collisions between the nitroxide and other paramagnetic molecules – allowing direct measurement of nitroxide-nitroxide, nitroxide-oxygen, nitroxide-NO, and nitroxideparamagnetic metal ion interactions. A strength of the EPR method is that it is applicable to a wide variety of physical states, including membranes under physiological conditions. All of the profiles presented in this review were obtained for fluid-phase membranes with the exception of hydrophobicity profiles, which were based on measurements of frozen membrane suspensions in order to separate motional and solvent effects. The magnetic parameters (including hyperfine interaction tensors used to characterize hydrophobicity) of spin labels in membranes at low temperatures have been shown to correlate well with those at physiological temperatures [1].

To correctly determine the depth-dependent profile of a given property across the lipid bilayer based on EPR spin labeling, one must know the position (depth) at which the nitroxide moiety is located. Lipids are highly dynamic, especially in the fluid phase, where they undergo both lateral diffusion as well as rapid *trans-gauche* isomerization about the carbon-carbon single bonds of their alkyl chains. This is reflected both in spin label order parameters [2] and in the observation of alkyl chain vertical fluctuations [3,4,5,6]. Such studies indicate that a distribution in the vertical positions of the nitroxide exists for *n*-SASL and *n*-PC spin labels, with the mean value of each distribution shifting toward the center of the bilayer as *n* increases (see Figure 1). The positions of carbon atoms in phospholipid alkyl chains have been determined by neutron diffraction [7], which indicated that their mean positions (or the position of an attached nitroxide) can be defined with an accuracy of ± 1 Å, even in the liquid-crystalline state. Assuming a Gaussian distribution of the labeled segments in the projection on the bilayer normal, these authors reported that the time-averaged positional fluctuations increase from 1.5 Å for the C4 position to 3.4 Å for the C12 position. Thus it can be concluded that the time-averaged position of a nitroxide is accurate to within 1.5 – 3.4 Å.

In the following sections we will first describe transmembrane profiles for hydrophobicity and for the diffusion-concentration products of oxygen and nitric oxide, along with data showing how these profiles are influenced by cholesterol and integral membrane peptides. Profiles for Ni^{2+} and $\text{Fe}(\text{CN})_6^{3-}$ will be given as examples of membrane penetration by ionic species. Finally, profiles of alkyl chain bending (vertical fluctuation), which are relevant to the chemical reactivity and scavenging of lipid peroxidation products, and profiles of the alkyl chain order parameter, the most often used parameter for characterizing the physical state of lipid bilayers, will be presented.

Hydrophobicity

Hydrophobicity of the membrane interior is largely determined by the extent of water penetration, giving rise to a polarity gradient across the bilayer in which the environment becomes increasingly nonpolar as one moves from the membrane surface to the terminus of the lipid alkyl chains. This has obvious implications for chemical processes requiring water, as well as for reactions influenced by solvation. In the absence of water the hydrocarbon environment of the membrane is highly nonpolar, and it has been shown that dehydration

abolishes the hydrophobicity gradient [8]. Four types of techniques have been employed to monitor spatial variations in hydrophobicity across the membrane, including neutron diffraction [9,10], capacitance measurements [11], fluorescence methods [12], and EPR spin-labeling [8,13,14,15]. More recently these experimental data have received strong support from molecular dynamics simulations of the distribution of water molecules across the lipid bilayer [16].

The EPR spin-labeling method is based on the dependence of unpaired electron spin density at the nitrogen nucleus on solvent polarity. Polar solvents enhance the unpaired electron spin density at the nitrogen atom, thereby increasing hyperfine interaction between the unpaired electron spin and the nitrogen nuclear spin. With an increase in solvent polarity the z component of the hyperfine interaction, A_z , increases, as does the motionally averaged isotropic hyperfine coupling constant, A_0 (see Figure 1 in Ref. [13]). The pioneering studies of Griffith et al. [8] using this approach were the first to demonstrate the existence of a hydrophobicity gradient across the membrane bilayer. In isotropic solvents, A_0 can be used as a measure of hydrophobicity. However, in a membrane environment this parameter may also be influenced by spin label motion. Since spin labels at different depths in the bilayer will have different mobilities in the fluid phase, hydrophobicity measurements are typically performed on samples in the frozen state to eliminate the motional contribution, using $2A_z$ (see Figure 1 in Ref. [13]) as a convenient experimental observable.

Effect of cholesterol

Figure 2 shows hydrophobicity profiles across saturated DMPC and unsaturated POPC bilayers in the presence and absence of cholesterol. Note that the horizontal axis is compressed for DMPC to accurately reflect its smaller hydrophobic transmembrane distance as compared to POPC [17,18]. In such figures, $2A_z$ data (or other observable) are presented as a function of the approximate position of the nitroxide moiety of the spin label across the lipid bilayer, with 14-PC and 16-PC nearest the center of the bilayer, 5-PC and 7-PC near the membrane surface, Tempo-PC (T) located in the headgroup region, and values for spin labels in the bulk aqueous phase (-x-) indicated for comparison. Stearic acid spin labels (especially 16-SASL) are somewhat soluble in water at high pH, when their carboxyl groups are ionized. This allows evaluation of the isotropic hyperfine coupling constant, A_0 , in the bulk aqueous phase and, using eq. (1) in Ref. [13], evaluation of A_z . Smaller $2A_z$ values (upward changes in the profiles) indicate a more hydrophobic (i.e. more nonpolar) environment. In the absence of cholesterol, both systems show small, gradual increases in hydrophobicity toward the bilayer center. Note that the hydrophobicity in POPC is greater than that in DMPC at all locations in the membrane, although these differences are small in the absence of cholesterol. Cholesterol has dramatic effects on the hydrophobicity profiles of both saturated and unsaturated PC membranes [13, 19]. The presence of cholesterol (30–50 mol%) induces an *increase* in polarity in the polar headgroup region and in the hydrocarbon region up to approximately the seventh-to-ninth carbon of the lipid alkyl chain, and a significant *decrease* in polarity in the central region of bilayer (Figure 2). For 50 mol% cholesterol the profiles show a sharp increase in hydrophobicity between the ninth and tenth carbon atoms. Thus, this very sharp increase occurs within one carbon-carbon bond (i.e. 1.34–1.53 Å) along the alkyl chain. At positions below (i.e. deeper than) this transition $2A_z$ values are almost constant, indicating a region of homogeneously low polarity.

To put these values in context, it is convenient to relate hydrophobicity as observed by $2A_z$ at a selected depth in the membrane to the polarity (or dielectric constant, ϵ) of bulk organic solvents, as shown in Fig. 2C. Thus, for POPC the addition of 50 mol % cholesterol increases polarity near the membrane surface (at the 5 – 7 positions) from that of 2-propanol ($\epsilon = 15$) to that of methanol ($\epsilon = 50$), although this is still considerably less polar than the bulk aqueous

phase ($\epsilon = 80$). Conversely, incorporation of 50 mol% cholesterol decreases polarity (increases hydrophobicity) in the middle of the bilayer from the level of 2-pentanol or 1-octanol ($\epsilon = 10$ –20, saturated PCs) or 1-decanol ($\epsilon = 10$, POPC) to the level of hexane ($\epsilon = 2$) [19].

Effect of peptides

In addition to cholesterol, integral membrane proteins make important contributions to the physical state of the membrane bilayer. In eukaryotic cells membrane proteins are comprised primarily of transmembrane α -helices, and we have modeled these effects by incorporating transmembrane α -helical peptides into lipid bilayers. The effects of two such peptides, Ac-K₂L₂₄K₂-amide (L₂₄) and Ac-K₂(LA)₁₂K₂-amide (LA)₁₂, on the hydrophobicity profile across the POPC membrane bilayer are shown in Fig. 3A [20,21]. Both peptides, when incorporated at a concentration of 10 mol%, increase the hydrophobicity of the membrane center from the level of 1-decanol ($\epsilon = 10$) to a level between hexane and dipropylamine ($\epsilon = 2$ –3), i.e. an effect similar in magnitude to that observed with 50 mol % cholesterol. However, hydrophobicity profiles in the presence of transmembrane peptides retain a bell-like shape, without the sharp transition observed upon incorporation of cholesterol, and there is no region of increased polarity near the membrane surface. The effects seen with (LA)₁₂ are smaller than with L₂₄, perhaps because the polarity of the peptide surface is higher for (LA)₁₂. In (LA)₁₂, half of the leucine residues that form the hydrophobic α -helical core of the L₂₄ peptide are replaced by alanines. When all leucines are replaced by alanines (A₂₄), the polarity of the peptide is such that it remains in the water phase outside the membrane [22].

Dramatically different effects are seen upon addition of the peptide ionophore, gramicidin A. When added to DMPC bilayers at a concentration of 15 mol%, gramicidin A almost eliminates the polarity gradient across the membrane (Fig. 3B). Similarly, addition of 15 mol% gramicidin A to egg yolk PC (EYPC) bilayers decreases hydrophobicity at all depths in the membrane and substantially reduces the difference in polarity between the bilayer surface and membrane interior (Fig. 3C) [23]. In the presence of gramicidin the polarity of the hydrocarbon phase just below the membrane surface is similar to that of methanol or ethanol, and the polarity near the membrane center is similar to that of ethanol or propanol. It is also notable that the hydrophobicity profiles of DMPC and EYPC, which alone are quite different, become very similar in the presence of gramicidin. The presence of gramicidin A also induces a decrease in hydrophobicity (increase in polarity) in the polar headgroup region occupied by Tempo-PC (Figure 3B and 3C). These data all indicate increased water penetration throughout the bilayer in the presence of gramicidin A.

Because the results presented above were obtained at relatively high peptide concentrations, they provide information about the membrane environment close to the peptide or protein surface. They indicate that the hydrophobicity of the peptide-lipid interface (and, by inference, protein-lipid interface) will strongly depend on the amino acid composition of a given peptide or integral membrane protein. This data also suggest that the hydrophobicity of the peptide-lipid interface is only weakly affected by the degree of lipid alkyl chain unsaturation.

The Oxygen diffusion-concentration product

Bimolecular collisions of a nitroxide spin label with molecular oxygen (which is a fast relaxing paramagnetic species not directly observed under standard experimental conditions) induces spin exchange, enhancing spin-lattice relaxation of the nitroxide. This effect can be measured indirectly by conventional EPR methods [15,24,25,26], or directly using saturation-recovery EPR [17,27,28,29,30]. An oxygen transport parameter was introduced as a convenient quantitative measure of the collision rate between the spin label and molecular oxygen by Kusumi et al. [27], and is given by the difference between the spin-lattice relaxation rates of spin labels in samples equilibrated with air and nitrogen (relaxation rate being the inverse of

the spin-lattice relaxation time). The oxygen transport parameter is proportional to the product of the local translational diffusion coefficient and the local concentration of oxygen at the depth in the membrane where the nitroxide moiety is located. By convention, the oxygen transport parameter is normalized to an oxygen concentration corresponding to the sample equilibrated with atmospheric air. To obtain the oxygen transport parameter, in principle, two saturation-recovery measurements should be performed: one for the sample equilibrated with nitrogen and the other for the sample equilibrated with air (see eq. (1) in Ref. [27]). Here we focus on oxygen transport parameter profiles obtained with the lipid analog spin labels shown in Figure 1. A similar profile was also obtained with spin labels attached at different sites along a transmembrane α -helical peptide [31].

Effects of cholesterol

Transmembrane profiles of the oxygen transport parameter across (saturated) DMPC and (unsaturated) POPC membranes in the presence and absence of 50 mol % cholesterol are presented in Fig. 4. In the absence of cholesterol both the saturated and unsaturated membrane profiles have bell-like shapes with a gradual increase of the oxygen transport parameter toward the membrane center [17,18,,20,21,32]. Note that the oxygen transport parameter is greater at all positions in the bilayer, including the headgroup region, than in the surrounding aqueous phase (values of the oxygen transport parameter in aqueous phase are indicated in all profiles as (-x-)), consistent with the known favorable partitioning of oxygen into membranes [33,34, 35]. The oxygen transport parameter is also slightly greater in saturated membranes (Fig. 4B) as compared to unsaturated membranes (Fig. 4A). Interestingly, measured phenomena which depend on translational diffusion always indicate slower motion in unsaturated membranes. This was observed not only for oxygen (MW = 32) [36], but also for a membrane soluble square planar copper complex (MW = 394) [37]. Similarly, measurements of lipid lateral diffusion based on the collision frequency between n-SASL or n-PC spin label pairs are always greater in saturated than in unsaturated membranes [4,6,38,39] (see also Fig. 9). Thus, reactions in the bilayer which depend on translational diffusion of the reacting molecules should be expected to proceed at a slower rate in unsaturated membranes.

As with hydrophobicity, cholesterol affects the oxygen transport parameter differently in different regions of the membrane (Fig. 4) [15,17,18,19,32,36]. Cholesterol markedly decreases oxygen transport in the polar headgroup region and in the hydrocarbon region near the bilayer surface, while having little or no effect near the membrane center. The profiles at 50 mol% cholesterol have a remarkable feature – an abrupt increase of the oxygen transport parameter between the C9 and C10 positions of the lipid alkyl chain. The oxygen diffusion-concentration product just below the membrane surface is ~ 3-fold *less* than in the surrounding aqueous phase, and near the bilayer center it is 2 – 3 fold *greater* than in the aqueous phase, giving differences in the oxygen diffusion-concentration product as large as 5 – 6 fold at different bilayer depths. The oxygen transport parameter from the membrane surface to the depth of the ninth carbon is as low as in gel-phase membranes, and at locations deeper than the ninth carbon, as high as in fluid-phase membranes [17,18,19,32,36]. These profiles present a marked contrast with the typical bell-shaped profiles across membranes that are observed in the absence of cholesterol for which the oxygen transport parameter increases gradually toward the membrane center without any abrupt change. As with hydrophobicity, the region of low oxygen transport corresponds to that occupied by the rigid tetracyclic sterol ring [13,36,40], and the abrupt change from a gel-phase-like membrane to a fluid-phase-like membrane occurs within the distance of one carbon-carbon bond along the alkyl chain.

The oxygen diffusion-concentration profiles in the presence of high cholesterol concentrations (i.e. > 30 mol%) lead to some remarkable conclusions regarding the environment for chemical reactions with oxygen (and presumably other small hydrophobic molecules). From the

membrane surface to the depth of the ninth carbon of the phospholipid alkyl chains, diffusion-controlled reactions with oxygen should be 3–4 times slower than in water. However, in the center of the membrane bilayer, diffusion-controlled reactions with oxygen should be accelerated more than two-fold as compared to the aqueous phase. Thus, the precise localization of a substrate within the membrane can have a profound influence on its reaction with molecular oxygen.

Effect of peptides

Effects of the two transmembrane α -helical peptides L_{24} and $(LA)_{12}$ on the oxygen transport parameter profile across POPC membranes are shown in Fig. 5 [20,21]. With L_{24} , decreases in the oxygen transport are observed only near the membrane surface and little or no effect is seen near the center of the bilayer. In contrast, incorporation of 10 mol% $(LA)_{12}$ induces large decreases in the oxygen transport parameter across the entire lipid bilayer. This striking difference between the effect of L_{24} and $(LA)_{12}$ on oxygen transport is not a result of different packing in the lipid bilayer, as both peptides were found to be well dispersed in the POPC membrane [20,21]. This difference is believed to be a consequence of differing organization of the L_{24} and the $(LA)_{12}$ hydrophobic surfaces as well as differing interaction of peptide side chains with the lipid hydrocarbon chains. In L_{24} all amino acids that form the hydrophobic α -helical core of the peptide are leucines, and their isobutyl side chains are packed tightly, giving a rather hard, smooth, hydrophobic surface. In $(LA)_{12}$, half of the leucine residues are replaced by alanine, and these amino acids are placed alternately with leucine residues in the transmembrane α -helical segment. Alternately positioning the small methyl side groups of alanine and the large isobutyl side chains of leucine results not only in significant roughness of the hydrophobic surface of $(LA)_{12}$ but also in significant freedom of motion of the isobutyl side chains of leucine (a flexible, brush-like surface). With this new organization of the hydrophobic surface, the isobutyl side chains of $(LA)_{12}$ can protrude between the hydrocarbon chains of lipid molecules. Flexibility of both side chains of the peptide and hydrocarbon chains of lipids allows for better mixing and stronger interaction of these two components. In this model, both the frequency of *gauche-trans* isomerization of hydrocarbon chains and the concentration of vacant pockets (voids) in the lipid bilayer are reduced in the presence of $(LA)_{12}$, resulting in a decrease in the oxygen transport parameter. It can be concluded that the properties of the peptide-lipid interface (and possibly protein-lipid interface), including the solubility and diffusion of small hydrophobic molecules, is strongly dependent on the amino acid composition of the peptide (protein).

Effect of integral membrane proteins

Figure 6 presents results of an investigation of the oxygen transport parameter in the lipid phase of reconstituted membranes containing the seven-transmembrane helical protein bacteriorhodopsin (BR) in DMPC, as well as in native purple membranes from the *Halobacterium halobium* JW3, which contains a very high BR concentration and exhibits gel-like fluidity parameters [30]. BR molecules are monomers in reconstituted membranes with a lipid/BR molar ratio of 80:1 (80-rec), and are 25% monomers and 75% trimers plus oligomers of trimers when the lipid/BR ratio is 40:1 (40-rec) [29,41,42]. A single homogeneous phase is observed in 80-rec membranes and values of the oxygen transport parameter are smaller than in pure DMPC by a factor of ~ 1.6 , independent of the depth in the hydrocarbon region. In contrast, two distinct domains can be observed in 40-rec bilayers, each with a distinct oxygen transport parameter profile. One domain of the 40-rec membrane (Figure 6, closed triangles) exhibits an oxygen transport parameter profile essentially identical to that observed for 80-rec bilayer (Figure 6, open triangles), corresponding to a bulk-plus-boundary lipid region of the membrane where BR is monomeric. The other domain of the 40-rec bilayers (Figure 6, closed squares) exhibits an oxygen transport parameter which is smaller by factor of ~ 5 than in the bulk-plus-boundary region and close to that for native purple membranes (Figure 6, open

squares), reflecting a special lipid environment with greatly diminished oxygen concentration-diffusion (referred to as the slow oxygen transport domain). This latter domain, likely due to self-association of BR, consists of lipids in contact with two (or more) proteins and/or lipids in contact with protein and boundary lipids. The alkyl chains and BR are closely packed in the slow oxygen transport domain, with few vacant pockets to allow entrance and diffusion of even small molecules such as molecular oxygen.

Many biological membranes and membrane domains are crowded with integral membrane proteins. These model studies with BR demonstrate that in protein-rich membranes, regions of the bilayer can exist in which the diffusion of small solutes, including molecular oxygen, is decreased to the level of gel-phase membranes [28,30,37]. The EPR spin-labeling methods described allowed the characterization of these domains without their physical separation [43]. Similarly, profiles of the oxygen transport parameter can be obtained in coexisting lipid phases such as liquid-ordered and liquid-disordered or liquid-ordered and solid-ordered [32, 44]. Such methods may prove extremely valuable in the study of raft domains [30,43].

The NO diffusion-concentration product

Reactions involving nitric oxide (NO) are of keen interest in medicine and biology. Both nitric oxide (NO) and oxygen (O₂) are paramagnetic. However, they cannot be directly monitored in solution using routine EPR techniques. Fortunately, both molecules can be observed in solution using indirect EPR methods [45,46,47]. As with oxygen (described above), bimolecular collisions of NO with nitroxide radical spin labels alter relaxation times and EPR spectral parameters of the spin label. These effects are due to physical (as opposed to chemical) interaction, and do not alter the concentrations of colliding species (either NO or spin label). This is in contrast to the widely applied EPR detection of nitric oxide by spin-trapping in which NO is chemically “trapped” and removed from the system via reaction with a nitroxide precursor, forming a stable, EPR-detectable spin adduct [48]. Various experimental approaches can be used to observe the effects of NO on EPR spectral characteristics of spin label [46]. Because of technical difficulty (NO is poisonous, and cannot be introduced into the sample by simply passing a stream of NO gas over a gas-permeable sample capillary open to the laboratory, as is done with oxygen) the line-broadening EPR method is mostly used [46,47].

Because NO and oxygen closely resemble each other in solubility and diffusion properties [47], existing profiles for oxygen can be used for guidance regarding the expected local NO diffusion-concentration product in specific regions of the membrane or membrane domains of interest. Nonetheless, it is important to verify the similarity of oxygen and NO diffusion-concentration profiles. The rate of chemical reactions involving NO will depend on the collision frequency of NO with substrate and, thus, on the local NO diffusion-concentration product.

Effect of cholesterol

The results of NO-induced line broadening measured as a function of the position of the nitroxide moiety are shown in Fig. 7 for both EYPC and DOPC in the presence and absence of 30 mol% cholesterol [49,50]. Samples were equilibrated with 1 atm NO and sealed. Such studies allow determination of the NO diffusion-concentration product, analogous to the oxygen transport parameter described above. However, because NO-induced line broadening is an empirical parameter results are expressed as the ratio of the local NO diffusion-concentration product in the membrane to that in water. Actual values of the NO diffusion-concentration product at a given depth in the membrane equilibrated with 1 atm partial pressure of NO at 20°C can be obtained by multiplying the ratio found in Fig. 7 by the NO diffusion-concentration product in water ($4.7 \times 10^{-11} \text{ mol} \times \text{cm}^{-1} \times \text{s}^{-1}$, that was determined by classical diffusion measurements and tables of NO solubility in water [51]). The data in Figure 7 show that for pure EYPC and DOPC bilayers, the NO diffusion-concentration product in the

hydrocarbon region of the membrane is greater than in water, especially near the hydrophobic center of the membrane. In EYPC bilayers, upon addition of 30 mol% cholesterol NO diffusion-concentration decreases in and near the polar headgroup region while increasing in the membrane center. These profiles are very similar to those obtained for oxygen in the same PC membranes in the presence and absence of cholesterol [36]. It can be inferred that chemical reactions of NO with oxygen, lipid peroxy radicals, or other substrates will proceed 2.5 – 3 times more readily in the membrane center than in the region close to the membrane surface and 2 – 2.5 times more readily in the membrane center than in the aqueous phase. The membrane environment may also protect NO from scavenging by numerous water-soluble compounds, which can significantly limit its lifetime [52]. Thus, these studies provide a physical basis for the concept that the membrane provides a protected environment for NO (i.e. from scavenging by water soluble compounds), and that the reactivity with NO is facilitated for reactants that are able to partition into the hydrophobic center of the membrane (where there is an increased NO diffusion-concentration product).

Penetration of metal ions and metal-ion complexes

Effects of cholesterol and peptides

The penetration of paramagnetic metal ions and their complexes into the membrane can be evaluated with saturation-recovery EPR and with the continuous-wave power-saturation method involving conventional EPR technique [13,14,53]. Bimolecular collision of paramagnetic metal ions or metal-ion complexes (fast-relaxing species) with nitroxide spin labels (relatively slow-relaxing species) leads to spin exchange and an effective shortening of the nitroxide spin-lattice relaxation time, T_1 , and the spin-spin relaxation time, T_2 , that is in proportion to the collision frequency. The accessibility parameter, which is proportional to this collision frequency, and thus, proportional to the product of the local concentration and the local translational diffusion coefficient, can be estimated directly from the saturation-recovery measurements of T_1 for a spin label in the presence and absence of given metal ions in a manner identical to that used for molecular oxygen. In this case the accessibility parameter is given by the difference between the spin-lattice relaxation rates of spin labels in samples with and without a given paramagnetic metal ion or complex. This parameter ($P(x)$ in Fig. 8A) was introduced in analogy to the oxygen transport parameter (see eq. 2 in Ref. [13]). Profiles of the accessibility parameter for the paramagnetic iron complex, $\text{Fe}(\text{CN})_6^{3-}$ in (unsaturated) DOPC membranes in the presence and absence of cholesterol obtained with the saturation-recovery EPR technique are shown in Fig. 8A. In the absence of cholesterol $\text{Fe}(\text{CN})_6^{3-}$ penetration gradually decreases toward the membrane center. In the presence of 30 mol% cholesterol, the penetration of $\text{Fe}(\text{CN})_6^{3-}$ is increased up to the depth of ninth carbon, while it is decreased in the membrane center.

Accessibility profiles for another paramagnetic metal ion, Ni^{2+} , across EYPC membrane in the absence and presence of 5 mol% gramicidin A are presented in Fig. 8B. These profiles were obtained using a continuous-wave power-saturation EPR method. In this method the parameter $P_{1/2}$ is measured, which is the incident microwave power at which the EPR signal is half as great as it would be in the absence of saturation. Collisions with paramagnetic metal ion increase spin-lattice relaxation and thereby increase $P_{1/2}$. Fig. 8B shows that both in the absence and presence of gramicidin A, penetration of Ni^{2+} into the membrane gradually decreases toward the membrane center. However, Ni^{2+} penetration is greater at all depths of the membrane in the presence of gramicidin A (Figure 8B, closed circles). This is consistent with studies on the effects of gramicidin A on the membrane hydrophobicity profile (Figure 3C), which indicate that this peptide increases water penetration throughout the bilayer. Thus, gramicidin A perturbs membrane integrity in such a way as to increase penetration of polar/ionic substances.

All accessibility profiles presented in Fig. 8 were obtained for fluid-phase membranes. Their excellent correlation with the hydrophobicity profiles presented in Figure 2 and Figure 3 (which were obtained for frozen samples) further validates the use of frozen samples to characterize membrane polarity. It should also be noted that to obtain measurable effects on EPR spectral characteristics for spin labels located in membranes, the concentration of metal ions and metal-ion complexes in the water phase were as high as 10–50 mM. Thus, at the concentrations normally found in biological systems, the membrane penetration of metal ions (and their hydrophilic complexes) will be low although, as these studies show, perhaps not negligible. Both methods, saturation-recovery using a pulse EPR spectrometer and continuous-wave power saturation employing a conventional EPR spectrometer, give similar results and can be used interchangeably (see also Ref. [54]). Recently, Bruker started production of EPR spectrometers capable of saturation-recovery measurements at X-band, although $P_{1/2}$ can be measured, reported, and duplicated in many laboratories already possessing conventional EPR spectrometers.

Alkyl chain bending (vertical fluctuations)

Effect of cholesterol

Although membrane phospholipids are usually drawn in an all-*trans* configuration, the carbon-carbon bonds of their alkyl chains actually undergo rapid *trans-gauche* isomerization. This leads to a bending of the lipid alkyl chains, with their terminal methyl groups fluctuating towards the membrane surface. The frequency of such “vertical fluctuations” depends on membrane composition (i.e., alkyl chain unsaturation, cholesterol content, etc.) and can influence chemical reactivity – for example the quenching of lipid peroxyl radicals by vitamin E [55,56]. These types of fluctuations are the basis of the transmembrane fluidity gradient, and *trans-gauche* isomerization in the alkyl chains of lipid spin labels gives rise to the well known dependence of order parameters and rotational correlation times on membrane depth [2]. Vertical fluctuations of phospholipid and stearic acid spin label alkyl chains can be directly observed by electron-electron double resonance [3,4,57,58,59] and saturation recovery [6,58,60] EPR techniques in which the collisions between ^{14}N : ^{15}N lipid spin-label pairs in fluid-phase membranes are measured. Early studies revealed that a nitroxide moiety at the 16-position of a stearic acid alkyl chain undergoes collisions with a nitroxide at the 5-position with a surprisingly high frequency [3,4].

Transmembrane profiles for bimolecular collisions between spin labels at different positions along the alkyl chain in EYPC and DMPC bilayers are shown in Figure 9 [6]. For this data, the ^{15}N nitroxide moiety was always attached at the C16 position of stearic acid and the position of the ^{14}N nitroxide moiety was varied between C16, C10, C7, and C5. The interaction (collision) between the ^{14}N C16: ^{15}N C16 pair is dependent primarily on lateral diffusion of the stearic acid spin labels in the plane of the membrane, whereas the interaction between ^{14}N C10: ^{15}N C16, ^{14}N C7: ^{15}N C16 and ^{14}N C5: ^{15}N C16 pairs require vertical fluctuation of the nitroxide moiety at the C16 position toward the polar surface of the bilayer. Note that in the absence of cholesterol (Figure 9, open circles) the collision rate for the 16:5 pair is ~ 50 – 60 % of that for the 16:16 pair in DMPC and EYPC, respectively, demonstrating that vertical fluctuation is indeed a common event. The addition of cholesterol (Figure 9, closed circles) reduces collision frequencies near the membrane surface (collisions of C16 with C5 and C7) and increases collision frequencies in the membrane center (collisions of C16 with C10 and C16). Comparison of Figure 4, Figure 7, and Figure 9 suggests the possibility of enhanced lateral transport of small non-polar molecules along the inner core region of the membrane (parallel to the membrane surface).

Interestingly, the polar carotenoid lutein (incorporated at 10 mol%) reduces the collision frequency for all spin-label pairs [6]. The observed differences in the effects of lutein and

cholesterol on alkyl chain bending result from differences in both their structure and their localization within the membrane. As noted above, the rigid sterol ring of cholesterol extends only to a depth of approximately the 9th carbon of lipid alkyl chains, as evidenced by sharp transitions in the transmembrane profiles for hydrophobicity (Figure 2) and the oxygen diffusion-concentration product (Figure 4), and a given molecule of cholesterol is confined to only one leaflet of the bilayer. Additionally, the cross section of the isooctyl chain of the cholesterol molecule located in the membrane center is much smaller than the cross section of the rigid sterol ring, and therefore produces additional possibilities for undulation and *trans-gauche* transitions of alkyl chains in the membrane center. In contrast, the rigid lutein molecule extends across both leaflets of the lipid bilayer, with polar groups on opposite ends interacting with opposite hydrophilic surfaces of the membrane. Also, lutein has the same cross sectional area along its entire length, allowing for efficient packing with the lipid alkyl chains at all depths in the hydrocarbon region of the membrane. Thus vertical fluctuations of the ends of the lipid alkyl chains, as well as oxygen and NO transport parameters, are reduced [61].

Membrane order

The most commonly used EPR method for evaluating the effects of different membrane components or membrane modifiers on the structure and dynamics of the lipid bilayer is comparison of alkyl chain order parameters, using *n*-SASL or *n*-PC spin labels with the nitroxide moiety attached at the *C_n* position (see Fig. 1) [2]. In the membrane, such spin labels undergo rapid anisotropic motion about the long axis of the alkyl chain, superimposed on which is a wobbling motion (arising from *trans-gauche* isomerizations as described above) such that the nitroxide moiety is confined within a cone-shaped region imposed by the membrane environment. A more highly ordered (i.e. more rigid) membrane imposes greater restriction on the rotational mobility of the nitroxide, resulting in an increase in the order parameter. For brevity, an increase in the order parameter is most often described as a decrease in spin label mobility, and thus, as a decrease in membrane fluidity.

Effect of cholesterol

For comparison with the profiles of different membrane properties presented in earlier sections of this review, Fig. 10 shows the effect of 50 mol% cholesterol on the profile of the order parameter in POPC membranes [19,20]. Note that in the absence of cholesterol there is a marked fluidity gradient, with membrane order decreasing continuously as one goes deeper into the lipid bilayer. This profile indicates that the alkyl chains are strongly ordered close to the membrane surface (the less fluid region) and strongly disordered in the membrane center (the more fluid region), and is characteristic of all fluid-phase bilayers. The addition of cholesterol increases order at all depths of the bilayer (Figure 10). Although this effect is somewhat more pronounced near the membrane center, no abrupt transition in the order parameter is observed, in contrast to profiles of other membrane properties such as hydrophobicity and the oxygen transport parameter. This seeming discrepancy between the well-established ordering effects of cholesterol, which extend the whole length of the alkyl chain, and the enhancement of oxygen transport near the bilayer center, is discussed in [19].

Effect of peptides

The effects of two transmembrane α -helical peptides, L₂₄ and (LA)₁₂, on the profile of alkyl chain order across the POPC membrane are shown in Figure 11 [20,21]. Comparison of these profiles with the effects of L₂₄ and (LA)₁₂ on the oxygen transport parameter and hydrophobicity profiles [20,21] gives additional insight into the mechanism of the interaction of these peptides with the POPC bilayer. In POPC bilayers alone, the overall shape of the hydrophobicity profile is similar to the shape of the oxygen transport parameter profile. Both are bell-shaped, with a maximum at the membrane center. This correspondence is expected

because a higher hydrophobicity should produce better solubility of hydrophobic molecules such as oxygen. Also, as seen in Figure 11, the membrane is the most fluid at the membrane center, which should also enhance oxygen diffusion in this region.

Concluding remarks

For understanding chemical reactions involving oxygen and/or NO, the most important parameter is the product of their local diffusion coefficient and local concentration. Spin-label oximetry and spin-label NO-metry allow measurements of these products in membranes at specified depths, and in restricted domains such as membrane rafts.

In membranes, NO is protected against reactions with water soluble compounds, which limit its life-time [55]. Thus, in the absence of oxygen or at a very low oxygen concentration, the membrane could be considered as a short-term storage environment for NO within the cell [49] that supplements the long-term NO-buffering provided by S-nitrosothiols [62,63]. It can be inferred, however, that reactions of NO with oxygen will proceed more readily in the membrane than in aqueous phase, given that the concentrations of both reactants are elevated in the lipid bilayer. The second order reactions (with respect to NO) with oxygen are dramatically accelerated within the hydrophobic region of model and biological membranes as compared to the reactions in the surrounding water [64]. The acceleration factor for the reaction of NO with oxygen within the hydrophobic core of membranes, detergent micelles and low-density lipoproteins has been recently modified from 300 as reported earlier [64] to 30 [65]. This acceleration is explained by ~3 times higher solubility of NO and oxygen in these hydrophobic phases, relative to water, which results in a higher local concentration of reactants and, therefore, a higher rate of reaction. This explanation is also consistent with the transmembrane profiles of NO and oxygen solubility and diffusion presented here (Figure 4 – Figure 7). Thus, in contrast to the situation in the absence of oxygen noted above, in the presence of oxygen biological membranes and other hydrophobic compartments of the cell should be considered as important sites for NO consumption.

For oxygen, important chemical reactions occurring within the membrane include lipid peroxidation and the formation of reactive oxygen species. For these reactions the knowledge of profiles of the oxygen diffusion-concentration product across the membrane is highly significant. In addition, other membrane properties described in this review are also important. For example, Barclay and Ingold [55] emphasized the significance of the vertical fluctuation of lipid peroxy radicals in the termination of lipid peroxidation chain reactions by vitamin E. The demonstrated propensity of alkyl chains to undergo rapid vertical fluctuations suggests that other processes involved in lipid peroxidation could also occur at the membrane-water interface, such as initiation, which requires hydrogen abstraction from unsaturated lipid carbon-carbon bonds, and secondary initiation by water-soluble hydroperoxides. Both are promoted by reactions with certain metal ions and water-soluble free radicals [56]. These reactions can occur at the membrane-water interface during vertical fluctuations of lipid alkyl chains toward the membrane surface. They can also occur in the membrane core because metal ions and other polar molecules can, to a certain degree, penetrate the lipid bilayer. Both vertical fluctuations of lipid alkyl chains towards the membrane surface and penetration of polar molecules into the bilayer should thus be considered in the investigation of chemical reactions involved in lipid peroxidation.

Variations in membrane hydrophobicity are also expected to influence chemical reactions that are dependent on solvent polarity. For example, nitration of a tyrosyl probe in the hydrophobic phase is preferred over the nitration of tyrosine in the aqueous phase [66]. The nitration of transmembrane tyrosyl-containing peptides in the phospholipid bilayer is about 10-fold greater

than that of tyrosine nitration in the aqueous phase, and the extent of tyrosine nitration was observed to be dependent on its membrane depth [67].

The striking similarities between the transport profiles of small hydrophobic molecules (the oxygen transport parameter in Fig. 4 and the NO transport parameter in Fig. 7) and the hydrophobicity profiles (Fig. 2) in membranes suggest the possibility of lateral transport of non-polar molecules along the inner core region of the membrane (parallel to the membrane surface), which is referred to as “hydrophobic channeling.” Skulachev [68] previously noted the possibility of lateral transport of small hydrophobic molecules along the filamentous extended network of mitochondria. We advanced this hypothesis, showing that in membranes a high cholesterol content is responsible for creating hydrophobic channels for oxygen transport parallel to the membrane surface [18,19,69]. At the same time a high cholesterol content is also responsible for creating a barrier to oxygen transport across the membrane which is located near the membrane surface. Thus, transport of small hydrophobic molecules (reactants), including oxygen and NO, is facilitated in membrane hydrophobic channels as compared with transport in water.

The studies discussed above are just a few of many examples in the literature indicating that various microenvironmental factors such as hydrophobicity, order, membrane concentrations and diffusion rates of oxygen and nitric oxide, and the extent of alkyl chain vertical fluctuations can play a critical role in determining the mechanisms and selectivity of chemical reactions occurring within the membrane environment. Transmembrane profiles of these properties can be obtained not only for simple model membranes, but also for bilayers composed of lipids extracted from biological samples, as in case of the fiber cell plasma membranes of eye lenses [18,19,69], as well as for intact biological membranes, as in the case of the CHO plasma membrane [70]. Given the degree of variation in these properties with membrane composition, it will be important to extend this area of research to additional native membrane systems. Nonetheless, the parameters given here for well-defined model systems provide useful guidelines that can aid the understanding of processes involving reactive-oxygen, reactive-nitrogen, and other radical species in biological membranes.

Acknowledgment

This work was supported by grants EY015526, GM068829, and HL003119 of the National Institutes of Health.

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Fig. 1.

Chemical structures of selected spin labels. 1-palmitoyl-2-(n-doxylstearoyl) phosphatidylcholine (n-PC), tempocholine-1-palmitoyl-2-oleoylphosphatidic acid ester (T-PC), n-doxy stearic acid spin labels (n-SASL), androstane spin label (ASL) and cholestane spin label (CSL). Chemical structures of dimyristoylphosphatidylcholine, (DMPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and cholesterol (CHOL) are shown for comparison. Approximate locations of these molecules across the lipid bilayer membrane are also illustrated. Shown are all-*trans* configurations; however it should be noted that lipid alkyl chains will have multiple *gauche* conformations in fluid phase bilayers, somewhat shortening chain-length projections along the membrane normal.

Fig. 2. Hydrophobicity profiles ($2A_Z$) across (A) POPC and (B) DMPC membranes in the absence (○) and presence (●) of 50 mol% cholesterol. Upward changes indicate increases in hydrophobicity. Numbers across the bottom of the figures correspond to the approximate locations of the nitroxide moieties for the lipid-analog spin labels shown in Figure 1. Note that the DMPC membrane (possessing 14 carbon chains) is significantly thinner than the POPC membrane (possessing 16 and 18 carbon chains). Method of the calculating membrane thickness and evaluating positions of nitroxide moieties is described in Refs. [17,18]. (C) Relationship between hydrophobicity as measured by $2A_Z$ or $2A_0$ and the dielectric constant (ϵ) for a series of bulk organic solvents. The solvents are numbered as follows: (1) hexane, (2) dipropylamine, (3) N-butylamine, (4) ethyl acetate, (5) acetone, (6) dimethylformamide, (7) acetonitrile, (8) methylpropionamide, (9) 1-decanol, (10) 1-octanol, (11) 2-propanol, (12) ethanol, (13) methanol, and (14) water. Closed circles and open circles represent data for solvents that can and cannot form hydrogen bonds with the nitroxide N-O group, respectively. Because water play a key role in determining $2A_Z$, the curve for the hydrogen bond-forming

solvents (upper curve) is used as a reference, except for cases in which the local membrane hydrophobicity is high (for further details, see Refs. [8,13]).

Fig. 3.

Effects of peptides on membrane polarity gradients. (A) Hydrophobicity profiles ($2A_z$) across POPC membranes in the absence ($\circ$) and presence of ($\bullet$) 10 mol% L_{24} or ($\blacklozenge$) 10 mol% $(LA)_{12}$. Hydrophobicity profiles across (B) DMPC and (C) EYPC membranes in the absence ($\circ$) and presence ($\bullet$) of 15 mol% gramicidin. Upward changes indicate increased hydrophobicity. To relate hydrophobicity as observed by A_z at a selected depth in the membrane to hydrophobicity (or ϵ) of bulk organic solvent, see Fig. 2C. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 4. Profiles of the oxygen transport parameter across (A) POPC and (B) DMPC membranes in the absence ($\circ$) and presence ($\bullet$) of 50 mol% cholesterol at 25°C. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 5. Profiles of the oxygen transport parameter across POPC membranes in the absence ($\circ$) and presence of ($\blacklozenge$) 10 mol% L₂₄ or ($\bullet$) 10 mol% (LA)₁₂ at 25°C. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 6. Profiles of the oxygen transport parameter across DMPC membranes in the absence (○) and in the presence of bacteriorhodopsin (BR), with a DMPC/BR molar ratio of 80 (△), with a DMPC/BR molar ratio of 40 (▲,■), and across the purple membrane (□) All data taken at 26°C. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 7.

Profiles of the relative NO diffusion-concentration product across (A) DOPC and (B) EYPC membranes in the absence ($\circ$) and presence ($\bullet$) of 30 mol% cholesterol at 20°C. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 8.

(A) Profiles of the relative accessibility parameter, $P(x)$, for the $\text{Fe}(\text{CN})_6^{-3}$ complex obtained at 25°C for DOPC membranes without ($\circ$) or with ($\bullet$) 30 mol% cholesterol in equilibrium with buffer containing 50 mM $\text{K}_3\text{Fe}(\text{CN})_6$. (B) The relative accessibility parameter for Ni^{+2} obtained at 25°C for EYPC membranes in equilibrium with buffer containing 10 mM NiCl_2 without ($\circ$) and with ($\bullet$) addition of 5 mol% gramicidin A. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 9.

Profiles of the bimolecular collision rate for a nitroxide moiety at the C16 position of the stearic acid alkyl chain with other SASLs in (A) EYPC and (B) DMPC membranes in the absence (○) and presence (●) of 20 mol% cholesterol at 27°C. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 10.

Profiles of the order parameter across POPC membranes in the absence (○) and presence (●) of 50 mol% cholesterol at 25°C. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.

Fig. 11.

Profiles of the order parameter across POPC membranes in the absence (○) and presence of (◆) 10 mol% L_{24} or (●) 10 mol% $(LA)_{12}$ at 20°C. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows.