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Review Article

Diffusion of nitric oxide and oxygen in lipoproteins and membranes studied by pyrene fluorescence quenching

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Abstract

Oxygen and nitric oxide are small hydrophobic molecules that usually need to diffuse a considerable distance to accomplish their biological functions and necessarily need to traverse several lipid membranes. Different methods have been used to study the diffusion of these molecules in membranes and herein we focus in the quenching of fluorescence of pyrenes inserted in the membrane. The pyrene derivatives have long fluorescence lifetimes (around 200 ns) that make them very sensitive to fluorescence quenching by nitric oxide, oxygen and other paramagnetic species. Results show that the apparent diffusion coefficients in membranes are similar to those in water, indicating that diffusion of these molecules in membranes is not considerably limited by the lipids. This high apparent diffusion in membranes is a consequence of both a favorable partition of these molecules in the hydrophobic interior of membranes and a high diffusion coefficient. Altering the composition of the membrane results in slight changes in diffusion, indicating that in most cases the lipid membranes will not hinder the passage of oxygen or nitric oxide. The diffusion of nitric oxide in the lipid core of low density lipoprotein is also very high, supporting its role as an antioxidant. In contrast to the high permeability of membranes to nitric oxide and oxygen, the permeability to other reactive species such as hydrogen peroxide and peroxynitrous acid is nearly five orders of magnitude lower.

Keywords

Nitric oxide, oxygen, pyrene, fluorescence, membrane permeability, diffusion, partition

Abbreviations: O_2, oxygen; •NO, nitric oxide; PCA, 1-pyrenecarboxylate; PMTMA, (1-(1-pyrenyl)methyl)-trimethylammonium; PUTMA, 11-(1-pyrenyl)undecyl-trimethylammonium; PDA, 1-pyrenedodecanoate; β-Py-C10-HPC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine; PMCho, 1-(pyrenyl)-methyl-3-(9-octadecenoyloxy)-22,23-bisnor-5-cholenate; EYPC, egg yolk phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; RBC, red blood cell; LDL, low density lipoprotein; VLDL, very low density lipoprotein; I_0, fluorescence intensity in the absence of quencher; I, fluorescence intensity with quencher; K_{SV}, Stern-Volmer constant; k_q, quenching bimolecular rate constant; τ_0, fluorescence lifetime; D_{app}^{q}, apparent diffusion coefficient; D_m^q, "true" diffusion coefficient in the membrane; K_P, partition coefficient; D_w^{NO}, diffusion coefficient of •NO in water; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; P_m, permeability coefficient of the membrane; P_w, permeability coefficient of an equally thick layer of water; $•NO_2$, nitrogen dioxide; H_2S, hydrogen sulfide; H_2O_2, hydrogen peroxide; ONOOH, peroxynitrous acid.

Introduction

Oxygen (O_2) is the fundamental molecule for aerobic life, and is also the precursor of important reactive species, such as superoxide, hydrogen peroxide and hydroxyl radical [1, 2]. Nitric oxide (•NO) is involved in several physiological functions, including vasodilation, neurotransmission and immune response [2, 3].

To accomplish their functions these molecules must traverse several lipid membranes. This is evident for O_2, that travels from the air through the plasma membranes of epithelial cells in the alveoli, through endothelial cells in capillaries and then through the membranes of red blood cells to be stored in hemoglobin and then do the reverse path to the peripheral cells' mitochondria. Nitric oxide also has to cross several plasma membranes to accomplish its function. In vasodilation, for instance, •NO that is produced intracellularly in endothelial cells has to exit the cell and reach soluble guanylate cyclase in the cytosol of smooth muscle cells to trigger relaxation [3-5].

Plasma membranes separate the cell from its surroundings and act as selective permeability barriers. Both O_2 and •NO are small slightly hydrophobic molecules that diffuse across lipid membranes very rapidly and this conditions the methods that can be used to study this

process. Stopped-flow, for instance, cannot be used to study their diffusion in membranes because of unstirred layer effects [6]. Both •NO and O_2 are paramagnetic species that deactivate fluorescent molecules in the excited state, so that fluorescent probes inserted in the membrane were used to study diffusion by these molecules.

Fluorescent probes: pyrene derivatives

Pyrene itself is a hydrophobic molecule that locates preferentially in lipid membranes relative to water, and several derivatives are available to control how deep in the membrane they are inserted (Figure 1A). In the absence of membranes, 1-pyrenecarboxylate (PCA) can be used to measure diffusion of O_2 in water [7]. In lipid bilayers, pyrene itself locates preferentially in the acyl chain region [8, 9], (1-(1-pyrenyl)methyl)trimethylammonium (PMTMA) locates near the headgroup region, whereas 11-(1-pyrenyl)undecyltrimethylammonium (PUTMA), 1-pyrenedodecanoate (PDA) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (β-Py-C10-HPC) locate in the acyl chain region closer to the middle of the bilayer (Figure 1A)[7, 10-13]. The cholesteryl ester derivative 1-(pyrenyl)-methyl-3-(9-octadecenoyloxy)-22,23-bisnor-5-cholenate (PMCho) was used to probe the lipid core of the low density lipoproteins [14, 15].

Pyrene derivatives show a structured absorption spectrum with a maximum at 345 nm that is sensitive to the solvent (Figure 1B). The extinction coefficients for these derivatives are around 40000 $M^{-1}cm^{-1}$ at 345 nm [16]. The fluorescence spectrum shows well defined emission peaks between 377 and 397 nm called vibronic bands, the most intense numbered 1 to 5 and clearly visible in apolar solvents such as hexadecane. Vibronic band 3 corresponds to an allowed transition and its intensity is independent of solvent polarity, whereas band 1 corresponds to a forbidden transition and its intensity depends on the solvent polarity. The intensity ratio 3/1 has been extensively used to study solvent polarity and also micellar aggregation [17-19]. Care must be taken to use pyrenes at relatively low concentrations to avoid the formation of excimers that emit at 470 nm and provide an alternative route to deactivate the excited state of pyrenes.

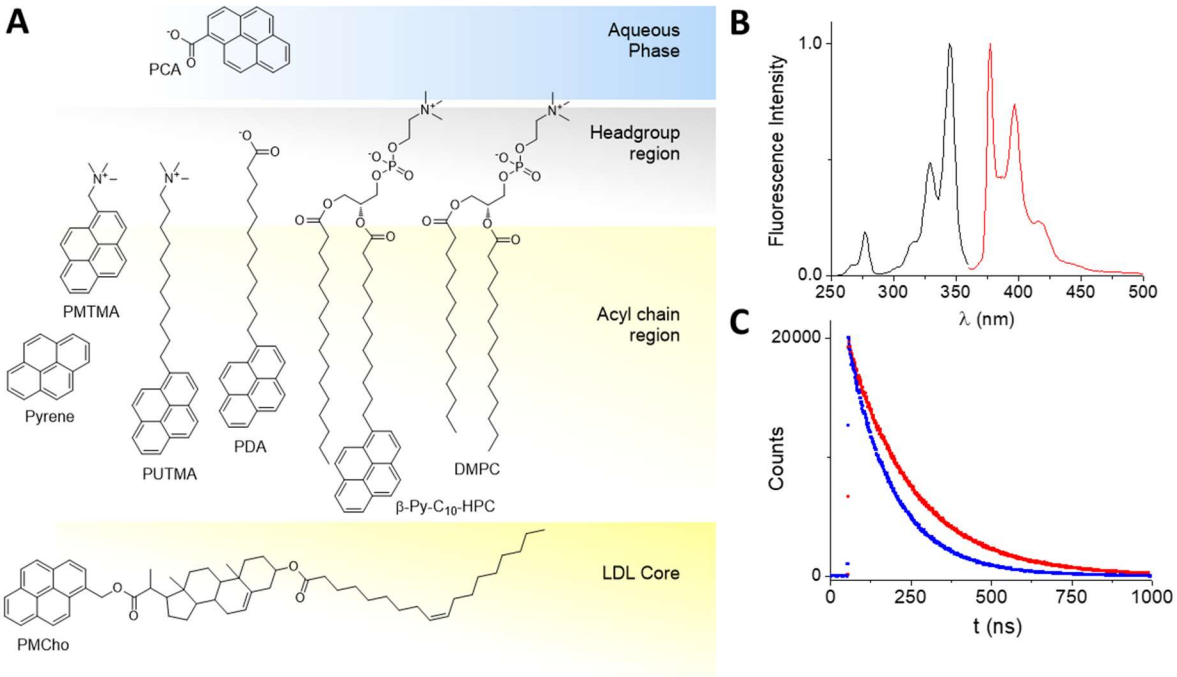

Figure 1. A. Pyrene derivatives used to measure •NO and O_2 diffusion. The preferential locations in phospholipid bilayers and lipoproteins are indicated. Dimyristoylphosphatidyl-choline (DMPC) is shown for reference. **B**. Fluorescence spectra of PDA inserted in DMPC membranes. The emission (red) and excitation (black) spectra were obtained using λ_{ex} = 345 nm and λ_{em} = 377 nm, respectively, that correspond the observed maxima in the spectra. **C.** Pulse experiments show that the fluorescent lifetime of PDA in DMPC with (blue) and without (red) 21% O_2 is 140 and 207 ns, respectively [7].

Paramagnetic species such as O_2 and •NO can deactivate the excited state of pyrene and other fluorescent molecules at diffusion-controlled rates [20]. The long fluorescent lifetimes of pyrenes in the range 50-200 ns make them highly sensitive to O_2 and •NO that are only sparingly soluble in water [10], and pyrene probes inserted in lipid membranes allow us to study the diffusion of •NO and O_2 in this environment by following changes in fluorescence intensity or lifetime [9-11]. Figure 1C shows a representative determination of the lifetime of PDA in dimyristoylphosphatidyl-choline (DMPC) liposomes, where a short pulse of light is used to excite the pyrene and then the decay in fluorescence is measured. In this case the monoexponential decay indicates that in the absence of O_2 the lifetime is 207 ns, whereas it shortens to 140 ns in the presence of air [7].

Quenching of pyrenes by •NO and O_2

From relatively simple experiments, valuable information concerning the molecular interaction between •NO or O_2 with lipids can be obtained.

The quenching experiment is performed using a suspension of membranes preincubated with the pyrene probe that has been purged of O_2 with nitrogen or argon, and then increasing concentrations of •NO or O_2 are added. The addition of the quenchers can be done from a saturated solution using gastight syringes to a septum-sealed fluorescence quartz cuvette with no headspace or by bubbling the gas directly in the suspension of membranes [7, 10]. The final concentration can then be determined electrochemically or by ensuring saturation of the sample [7, 10]. Nitric oxide can also be added using a very short lifetime •NO donor such as PROLI NONOate [21]. Note that •NO donors of longer lifetimes will yield a fluorescence decrease with time that is going to reflect the lifetime of the donor rather than diffusion properties of •NO [14]. Further details and explanations on how to set up the experiment are provided in the *Supplementary material*.

The decrease in fluorescence is analyzed using the Stern-Volmer relation:

$$I_0/I = 1 + K_{SV}[Q]_{buffer} \qquad \text{Eq. 1}$$

Where I_0 is the intensity of fluorescence in the absence of quencher, and I is the intensity in the presence of a given concentration of quencher $[Q]_{buffer}$. The slope of a plot of I_0/I vs. $[Q]_{buffer}$ is the Stern-Volmer constant K_{SV} (Figure 2). In the case of studying quenching in the membrane, the apparent Stern-Volmer constant K_{SV}^{app} is obtained, because the actual concentration of the quencher in the membrane is not known. This K_{SV}^{app} is the product of the apparent quenching bimolecular rate constant (k_q^{app}) and the lifetime of the fluorophore (τ_0):

$$K_{SV}^{app} = \tau_0 k_q^{app} \qquad \text{Eq. 2}$$

In Figure 2, the different slopes indicate that K_{SV} for the quenching of PCA in water by O_2 is lower than K_{SV}^{app} for the quenching of PDA in DMPC and dilauroyl phosphatidylcholine (DLPC) membranes. However, the accessibility of the fluorophore to the quencher is given by k_q that is obtained using the corresponding τ_0 (39.2, 207, and 205 ns respectively), so the order changes: DMPC < water < DLPC, k_q^{app} = 6.2, k_q = 9.9 and k_q^{app} = 14.6 × 10^9 $M^{-1}s^{-1}$, respectively [7].

This is sometimes corrected to include an efficiency factor, but in the case of pyrenes and O_2 or •NO, the quenching is known to be limited only by diffusion and this efficiency is 1 [10, 20]. The apparent diffusion coefficient of the molecules in the lipid environment (D_{app}^q) can then be calculated from k_q^{app}, using the Einstein-Smoluchowski equation:

$$k_q^{app} = \left(D_{pyr} + D_{app}^q\right) 4\pi R N_A / 1000 \qquad \text{Eq. 3}$$

Where R is the sum of molecular radii of pyrene and quencher, (R = 5 × 10^{-8} cm for O_2 [7]), N_A is Avogadro's number, and D_{pyr} is the pyrene's diffusion coefficient. In membranes, D_{pyr} is much lower than D_{app}^q and can be

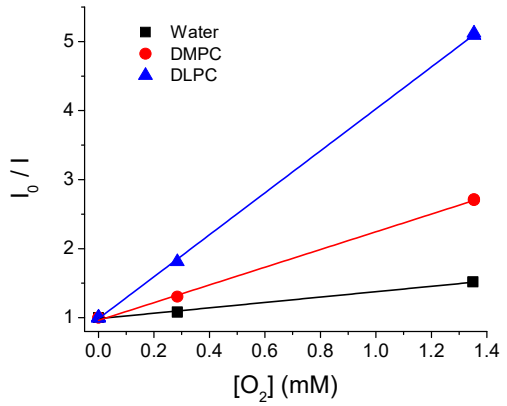

Figure 2. Stern-Volmer plots for pyrene quenching by O_2. These results were obtained at 20°C [7]. PCA was used for the quenching experiments in water, whereas PDA was used for the experiments in phospholipid liposomes of dimyristoylphosphatidyl-choline (DMPC) and dilauroylphosphatidylcholine (DLPC).

considered insignificant [9]. Quenching experiments in membranes yield an apparent diffusion coefficient D_{app}^{q} because the concentration of the quencher inside the membrane is generally not known and the concentration in water is taken as reference. This D_{app}^{q} then depends on both the "true" diffusion coefficient in the membrane D_m^q and on the partition coefficient of the quencher in the membrane (K_P):

$$D_{app}^{q} = D_m^q \, K_P \qquad \text{Eq. 4}$$

Independent measurements of K_P allow us to calculate D_m^q and gain insight at the mechanisms that control diffusion itself in lipid membranes, as discussed below.

Diffusion of •NO and O_2

Diffusion and transport across lipid membranes

Fluorescence quenching experiments have been very important to understand how O_2 and •NO can diffuse across lipid membranes and into other lipid-containing structures such as lipoproteins. As mentioned before, the apparent diffusion coefficent D_{app} obtained from quenching experiments is the product of D_m by K_P (Eq. 4) and D_m itself is difficult to obtain. Nonetheless, the processes that interest us such as transport and reactivity depend on both D_m and K_P. The permeability coefficient P_m is related to the diffusion coefficient, in its simplest form, by:

$$P_m = D_m \, K_P / \delta \qquad \text{Eq. 5}$$

Where δ is the thickness of the membrane, usually around 4 nm. Therefore, D_{app} provides the necessary information to assess the transport properties of a molecule in a given membrane.

In EYPC liposomes it was found that D_{app}^{NO} in the acyl chain region measured by quenching of PUTMA was 1.5 × 10^{-5} cm^2s^{-1}, and that D_{app}^{NO} near the headgroup region probed by PMTMA was 1.7 × 10^{-5} cm^2s^{-1}, [10], comparable to the diffusion coefficient of •NO in water D_w^{NO} = 2.2 × 10^{-5} cm^2s^{-1} [22] (Table I). The results indicate there was almost no resistance to the diffusion of •NO by lipid membranes compared to water. In membranes from red blood cells D_{app}^{NO} was higher in the acyl region (D_{app}^{NO} = 1.5 × 10^{-5} cm^2s^{-1}) than near the headgroup region (0.5 × 10^{-5} cm^2s^{-1}), indicating a higher resistance to permeation than in EYPC membranes [10].

Considering this heterogeneity in D_{app} at different depths in the membrane a more complicated formula than Equation 5 is used to calculate P_m, that considers the contributions of different D_m and K_P at the different depths in the membrane (z), as well as the resistance found at the lipid-water interfaces (r_i) [23].

$$P_m = \left(\int_{-\delta/2}^{\delta/2} \frac{1}{K(z)D(z)} dz + 2r_i \right)^{-1} \qquad \text{Eq. 6}$$

It is based on considering the membrane as a set of resistors in series. The resistance at a given depth in the membrane is calculated as the inverse of the permeability at that region. The inverse of the sum of resistances at all depths in the membrane gives P_m. Therefore, even though the headgroup region usually shows a higher resistance to the transport of •NO, the overall contribution to limit •NO transport across the membrane is small.

Further detail was offered by EPR probes at different depths in the membrane that showed D_{app}^{NO} in the middle of the bilayer of EYPC was 1.8 times higher than in the region close to the interface, and the addition of 30% cholesterol resulted in D_{app}^{NO} increasing in the middle region and decreasing at the interface [24]. In any case, the D_{app}^{NO} of the membrane was similar to that of water, meaning that membranes posed virtually no barrier to the transport of •NO [10, 24].

Table I. Apparent diffusion coefficients of •NO and O_2 obtained using pyrene probes in different systems.

System	T (°C)	$D_{app}^{NO} \times 10^5$ (cm^2s^{-1})	$D_{app}^{O_2} \times 10^5$ (cm^2s^{-1})	References
Water	22	2.2[a]	2.1	[7, 22]
EYPC / PMTMA	20	1.7	2.9	[10]
EYPC / PUTMA	20	1.5	2.7	[10]
RBC / PMTMA	20	0.5	1.2	[10]
RBC / PUTMA	20	1.3	2.6	[10]
LDL / PMTMA	22	2.3	-	[14]
LDL / PMCho	22	1.7	2.0	[14, 15]
DLPC / PDA	20	-	3.9	[7]
DLPC / PDA	40	-	7.2	[7]
DMPC / PDA	20	-	1.6	[7]
DMPC / PDA	37	-	6.5	[7]
DPPC / PDA	25	-	0.9	[7]
DPPC / PDA	37	-	2.0	[7]

[a]This diffusion coefficient was not obtained by fluorescence quenching [22].

In the case of O_2, using pyrenes probes that located at different depths in the membrane of red blood cells it was found that $D_{app}^{O_2}$ increased toward the center of the bilayer [11]. In RBC, EYPC and other phospholipid fluid membranes $D_{app}^{O_2}$ was on average twice as much as that observed for •NO and higher than $D_w^{O_2}$ [7, 10], supporting the conclusion that membranes do not limit transport of O_2 either [7, 9, 10]. This conclusion is further supported by several experiments using EPR probes [25].

Changes in membrane fluidity have an effect on the diffusion of •NO and O_2 in membranes, but it is not very pronounced. In DPPC membranes in the gel phase, $D_{app}^{O_2}$ is only half that of $D_w^{O_2}$ [7](Table I). In contrast, a five orders of magnitude decrease in O_2 diffusion compared to water was observed in compressed DPPC monolayers [26]. Cholesterol decreases the fluidity of membranes and also decreases $D_{app}^{O_2}$ and D_{app}^{NO} [9, 12, 13, 24, 27], but the effect is not very important (~30% decrease) except in pure cholesterol bilayer domains (10-fold decrease) [28].

This knowledge that cellular membranes present virtually no resistance to the transport of •NO has been applied to model the diffusion of •NO in tissues in different situations, such as in inflammation [29], vasodilation [4, 5], neurotransmission [30], and neurovascular coupling [31, 32]. Using electrodes inserted in tissue slices it has been shown that •NO can indeed diffuse very far from the producing cells [33, 34].

To give some perspective, in the vascular system •NO is produced by the endothelial cells that are located facing the lumen of blood vessels, but it acts on smooth muscle cells that are a few µm apart [35]. Because the lipid membranes do not limit •NO diffusion, •NO produced in the endothelial cells spreads in every direction and part of it reaches the underlying smooth muscle cells, activate the soluble guanylate cyclase and then cause vasorelaxation. An important part of the •NO is consumed by red blood cells in the lumen of the arteries [36, 37] but this inefficiency in •NO utilization is likely compensated by the unimpeded diffusion through membranes, in that there is no need for special transport proteins. Although aquaporins have been proposed to transport •NO through the membrane [38], it is very improbable. The transport of water through the membrane of RBC occurs mostly by aquaporin 1 and has a P_m = 5 × 10^{-3} cm s^{-1} in humans [39]. If you consider this value as the maximal possible P_m for •NO through a membrane with aquaporins, it is five orders of magnitude lower than P_m for •NO through the lipids in the membrane [24, 40] and thus it is unlikely that aquaporins contribute significantly to •NO transport.

Another related example to illustrate the complexity of •NO diffusion and its interaction with cellular membranes derives from the red blood cells (RBC), which are considered the most important sinks of •NO in the vascular system [36, 37]. The consumption of •NO by free oxyhemoglobin is very rapid, but occurs 650 times more slowly when oxyhemoglobin is encapsulated in RBC [41]. Although there is still some debate about the role of changes in membrane permeability in regulating •NO uptake by RBC, Liu *et al.* showed that the main reason was actually unstirred layer effects [6, 41]. In short, the high rate of reaction of •NO with oxyhemoglobin, and the high P_m to •NO result in a concentration of •NO at the surface of the cell of essentially zero, with a steep gradient extending a short distance outward (the "unstirred layer"). The rate of •NO consumption by red blood cells was independent of the internal oxyhemoglobin concentration, indicating that the rate-limiting step is the entry into the cell through this layer and not a low membrane permeability to •NO [6, 41].

Diffusion in lipoproteins

Low density lipoproteins (LDL) are important in cholesterol transport and its oxidation is considered to be a key event in the development of atherosclerosis [42, 43]. Nitric oxide was found to inhibit LDL oxidation by preventing lipid oxidation [44, 45]. Using a cholesteryl-ester pyrene probe (PMCho) it was shown that •NO could easily reach and diffuse unhindered through the LDL's core [14]. The values for D_{app}^{NO} were slightly smaller in the core than near the interface, but still very similar to D_w^{NO} (Table I). In oxidized LDL, D_{app}^{NO} increased two-fold both at the core and near the surface, indicating that the oxidation resulted in a less packed lipid structure, facilitating diffusion and probably increasing solubility [14].

It was hypothesized that an increase in omega-3 unsaturated fatty acids in the diet could lead to a more fluid LDL that would allow an easier diffusion of •NO and an improvement of its antioxidant activity, but no differences in •NO diffusion rates were observed in lipoproteins (LDL + VLDL) obtained from rats fed with either maize oil or fish oil [46]. Although omega-3 fatty acids were significantly higher in the lipoproteins from fish oil-fed rats, the total content of PUFAs was higher in the maize oil-fed rats' lipoproteins, and this may explain the observed results [46].

Partition coefficients

Until 2005 there was only data concerning •NO solubility in organic solvents, that suggested the solubility of •NO in lipids could be as much as 10 times higher than in water [34, 47]. Some studies indicated that this resulted in lipids accelerating the rate of •NO reaction with O_2 [48] and also

suggested a partition coefficient between the lipid fraction and water of nearly 10.

The actual partition coefficients (K_P) were then measured using an equilibrium-shift method that depended on electrochemical measurement of either •NO or O_2 [15]. These experiments showed that •NO and O_2 were 4.4 and 3.9 times more soluble in the hydrophobic region of EYPC membranes than in water (Table II). The solubility in the LDL core was slightly lower, 3.4 and 2.9 more soluble than in water for •NO and O_2, respectively [15] (Table II). The lower solubility in LDL than in membranes was probably because cholesteryl-esters are tightly packed in the LDL core, resulting in a lower free volume.

These values of K_P were confirmed by experiments that showed that PC liposomes and LDL could accelerate •NO autoxidation approximately 30 times, supporting that both •NO and O_2 are ~3 times more soluble in the lipids than in water [21]. The consequences of this accelerated production of reactive nitrogen species have already been discussed [49].

Experiments on O_2 solubility in phospholipid membranes of different composition have shown that K_P depends on temperature and even more on the physical state of the membrane [7, 50, 51]. With DLPC membranes, that are fluid above 0°C, K_P increased with temperature, whereas with DMPC membranes, there was a sharp increase in K_P from 1 to 3 at the main transition temperature [7, 50, 51] (Table II).

Table II. Partition coefficients of •NO and O_2 between different hydrophobic phases and water.

Molecule	Hydrophobic phase	T (°C)	K_P	Reference
•NO	hexane	25	9.2	[47]
	1-octanol	37	6.5	[34]
	EYPC	25	4.4 ± 0.4	[15]
	LDL	25	3.4 ± 0.7	[15]
O_2	EYPC	25	3.9 ± 0.4	[15]
	LDL	25	2.9 ± 0.3	[15]
	RBC membranes	37	5 ± 4	[52]
	DLPC	25	2.7 ± 0.2	[7]
	DLPC	40	3.9 ± 0.5	[7]
	DMPC	20	1.0 ± 0.1	[7]
	DMPC	37	3.2 ± 0.3	[7]
	DPPC	25	0.3 ± 0.1	[7]
	DPPC	37	1.0 ± 0.2	[7]

K_P values are presented as the ratio of the molar concentration of the molecule in the hydrophobic phase and in water

Molecular diffusion in lipids

The independent determination of K_P allowed the determination of D_m for •NO and O_2 in the different systems. It was calculated that D_m^{NO} in EYPC and in LDL were 6.4×10^{-6} and 7×10^{-6} cm^2s^{-1}, respectively [15]. These results were striking at first because even though these lipid structures are 100 times more viscous than water, the diffusion of •NO in lipids was only 10 times slower than water[15]. It is usual to assume that diffusion should always be "Stokesian", meaning that diffusion is inversely proportional to viscosity [53]. However, this is true for molecules that are much larger than the solvent molecules, such as a proteins in water. On the other hand, the phospholipid molecules from membranes are much larger than O_2, •NO and other reactive species, and Stokes-Einstein relation does not apply directly [53].

There was a recent attempt to study true diffusion of O_2 in phospholipid membranes using pyrenes fluorescence quenching to measure $D_{app}^{O_2}$ and independent determinations of K_P at different temperatures and at different physical states of the membrane. Surprisingly, it was found that $D_m^{O_2}$ had the same value for all the different conditions [7]. The null response to changes in temperature and membrane fluidity suggested that the bulky pyrenes probes are disturbing the packing of the surrounding lipids [8], creating a similar microenvironment even at different physical states of the membrane [7]. Therefore, using pyrene probes provide values of $D_{app}^{O_2}$ in that semi-fluid membrane, but data regarding the effect of membrane fluidity on $D_m^{O_2}$ cannot be extracted [7].

Alternative methods

There are other methods by which diffusion of •NO and O_2 in the membrane can be studied, like electron paramagnetic resonance EPR spectroscopy [54, 55], molecular dynamics simulations [56, 57] and more recently nuclear magnetic resonance NMR spectroscopy [58, 59].

EPR methods are based on using spin probes that have been designed to locate at different depths in the membrane. Both •NO and O_2 are paramagnetic but are not visible by EPR when in solution. Their collisions with the spin probes lead to changes in EPR spectral parameters and this can be quantified and used to calculate D_{app} at different depths in the membrane [24]. Nitroxyl groups are smaller than pyrene and therefore provide

higher resolution of D_{app} across the membrane.

Most of the studies using EPR to measure D_{app} for O_2 and •NO across the membrane come from Subczynski *et al*. These studies succinctly show that in fluid membranes D_{app} in the headgroup region is similar to D_w, and it increases toward the acyl chains in a bell-shaped manner [24, 60]. Cholesterol has been observed to narrow the bell-shaped profile in the mid-bilayer and decrease D_{app} near the headgroup region, where the packing effect of cholesterol is larger [24, 27]. The bell-shaped profile was lost in gel-state membranes, where a flat profile was observed [61]. In every case, Subczynski *et al* have observed that membranes present virtually no barrier to the diffusion of either O_2 or •NO [24, 27].

Molecular dynamics of O_2 in membranes was pioneered by Marrink and Berendsen [57]. These calculations provide molecular details of the interaction between these molecules and the lipids and both solubility and diffusion can be calculated separately at different depths in the membrane. All studies show that in phospholipid membranes in the fluid phase the solubility of both •NO and O_2 increase toward the center of the bilayer and a similar profile is observed for the diffusion profile [28, 56, 57, 62-64]. This allows the calculation of the permeability coefficient of the membrane. However, care should be taken to cross-validate the model with experimental results and in interpreting the results, because so far the interaction of O_2 with alkanes cannot be modelled entirely correctly, so that the results do not reproduce the experimental data quantitatively [65].

More recently a ^{13}C NMR method has been used to study the profile of D_{app} of O_2 in a lipid membrane. It uses *sn*-2-perdeuterio-1-myristelaidoyl, 2-myristoyl-*sn*-glycero-3-phosphocholine as a probe and has the advantage that all the carbon atoms are sensitive to O_2 and so a high resolution profile of O_2 across the membrane can be obtained [58, 59]. The disadvantages are that so far only this lipid can be used, pressures of 50 atm O_2 should be kept in the NMR tube and molecular dynamics calculations are needed to interpret the results [58, 59].

Comparison with other reactive species

The very high permeability of membranes to •NO and O_2 can be better appreciated when compared to the permeability to other reactive species like nitrogen dioxide, hydrogen peroxide and peroxynitrite, among others [40].

In the case of either O_2 or •NO, there is no resistance to the transfer from the water to the lipid phase in most membranes [7, 10, 24], and it was calculated that P_m in EYPC and RBC membranes for •NO was 73 and 18 cm s^{-1}, respectively [24, 40], and for O_2 was 62 and 32 cm s^{-1}, respectively [24, 40].

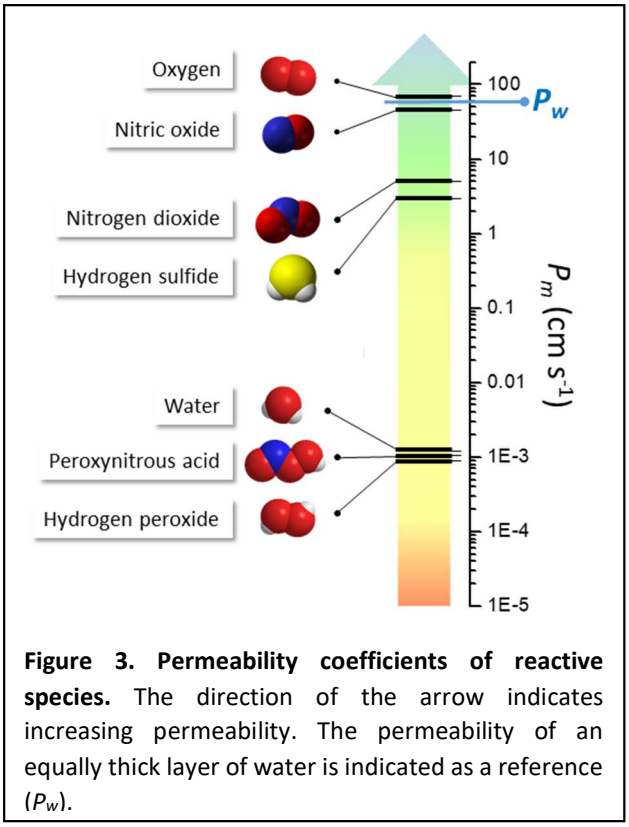

Figure 3. Permeability coefficients of reactive species. The direction of the arrow indicates increasing permeability. The permeability of an equally thick layer of water is indicated as a reference (P_w).

It is useful to compare these P_m values with the permeability coefficient of an equally thick layer of water. Considering that the diffusion coefficients of •NO and O_2 in water are similar (D_w = 2.2 × 10^{-5} cm^2s^{-1}, Table I [7, 22]), and those of the other reactive species are similar P_w = 55 cm s^{-1} will be used as a reference (Figure 3).

Most of the reactive species' diffusion in lipids cannot be studied by fluorescence quenching either because they are not paramagnetic or because they are too short-lived. Nitrogen dioxide (•NO_2), for instance, is a radical but has a very short lifetime in water [66]. In this case, experimental solubility data [67] in conjunction with quantum calculations were used to calculate its solubility in organic solvents and membranes [68]. It was estimated that the permeability of membranes to •NO_2 should be relatively high, P_m = 5 cm s^{-1}, only 10 times lower than that of •NO [68].

Hydrogen sulfide (H_2S) has attracted a lot of attention lately because it can be formed endogenously and exert several physiological responses [69]. The diffusion of H_2S across membranes proved to be too fast to be studied by either stopped-flow or electrochemical approaches [70, 71]. Based on its favorable partition in lipid membranes it was estimated that H_2S could diffuse across membranes

relatively unimpeded, P_m = 3 cm s^{-1}, approximately 10 times more slowly than •NO [70, 71].

The diffusion of hydrogen peroxide (H_2O_2) across membranes is slow enough so that enzyme-dependent techniques can be used to determine the permeability [72]. Recent studies have shown that some aquaporins may facilitate diffusion of H_2O_2 across membranes [73-75]. Plasma membranes from human cells have shown a relatively low permeability to H_2O_2 , $P_m = 2 \times 10^{-4}$, 4.4×10^{-4} and 1.6×10^{-3} cm s^{-1} for Jurkat, HeLa and HUVEC cells respectively [72, 76, 77].

The diffusion of peroxynitrous acid (ONOOH) across lipid membranes is also slow enough that stopped-flow approaches can be used. In pure phospholipid membranes it was observed that P_m for ONOOH was on average $\sim 1 \times 10^{-3}$ cm s^{-1} [78, 79], nearly 10^5 times lower than the permeability to •NO. In red blood cells, it was observed that apart from ONOOH diffusion through the lipid fraction of the membrane, peroxynitrite ($ONOO^-$) could use the band 3 anion transport proteins, and enter the red cells at fluxes similar to ONOOH [80].

Average values of P_m for the different reactive species are shown in Figure 3 and summarized values can be found in [40]. The reactive species under consideration can be separated into three groups according their P_m. The permeabilities of lipid membranes to •NO and O_2 are very high and similar to the permeability of an equally thick layer of water, indicating that most membranes are not limiting the diffusion of •NO and O_2. The diffusion of $•NO_2$ and H_2S is only slightly limited by membranes whereas the permeability of membranes to H_2O_2 and ONOOH is similar to that of water, indicating that there is a much higher barrier to the diffusion of these molecules across membranes. This does not necessarily mean that permeation of the latter group is slow, but that it is amenable to experimental determination by stopped-flow and other fast-kinetic methods that are too slow to study the diffusion of •NO or H_2S. As low reference points, the permeability of phospholipid membranes to other polar molecule such as glycerol is $P_m = 5.4 \times 10^{-6}$ cm s^{-1} [81], and the ion chloride have even lower $P_m = 10^{-11}$ cm s^{-1} [82]. In these cases of low P_m, the role of transport proteins is more important.

Conclusions and Perspectives

Both •NO and O_2 can diffuse across membranes unhindered and this fast diffusion can be studied by pyrene probes inserted in the membrane. The high permeability of membranes to •NO and O_2 is because of a) favorable partition into membranes, and b) high diffusion coefficient in lipids. The diffusion of both •NO and O_2 to the core of LDL is also very fast and similar to that in water.

In comparison, the diffusion of $•NO_2$ and H_2S across membranes occurs 10 times more slowly, whereas that of ONOOH and H_2O_2 occurs nearly 10^5 times more slowly.

Pyrene fluorescence quenching offers a simple experimental approach to study diffusion of these reactive species, but pyrenes as diffusion probes have some drawbacks that should be mentioned. They are bulky molecules that affect the packing of the lipids in membranes [8], thus the effects of membrane fluidity on diffusion cannot be observed [7]. A fluorescent probe that is more similar to acyl chains may avoid affecting the packing of the lipids and may provide more detailed information on the molecular mechanisms of diffusion of O_2 and •NO in membranes. The available probes such as parinaric acid have short lifetimes, so they are not as sensitive to •NO or O_2. The other problem is that pyrenes inserted in plasma membranes other than red blood cells generally present multiexponential decay, indicating that the probe is located in several different microenvironments. The presence of multiple lifetimes preclude the calculation of quenching rate constants and thus of apparent diffusion coefficients.

The ultimate experiment would be to use a fluorescent probe that incorporates in cell membranes causing minimal distortion in its physical properties and that can report the apparent diffusion of •NO and O_2 with high sensitivity using fluorescence microscopy such as fluorescence-lifetime imaging microscopy.

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