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Diffusion and transport of reactive species across cell membranes

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Abstract

This chapter includes an overview of the structure of cell membranes and a review of the permeability of membranes to biologically relevant oxygen and nitrogen reactive species; oxygen, singlet oxygen, superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide, nitrogen dioxide, peroxynitrite and also hydrogen sulfide. Physical interactions of these species with cellular membranes are discussed extensively, but also their relevance to chemical reactions such as lipid peroxidation. Most of these species are involved in different cellular redox processes ranging from physiological pathways to damaging reactions against biomolecules. Cell membranes separate and compartmentalize different processes, inside or outside cells, and in different organelles within cells. The permeability of these membranes to reactive species varies according to the physicochemical properties of each molecule. Some of them, such as nitric oxide and oxygen, are small and hydrophobic and can traverse cellular membranes practically unhindered. Nitrogen dioxide and hydrogen sulfide find a slightly higher barrier to permeation, but still their diffusion is largely unimpeded by cellular membranes. In contrast, the permeability of cellular membranes to the more polar hydrogen peroxide, is up to five orders of magnitude lower, allowing the formation of concentration gradients, directionality and effective compartmentalization of its actions which can be further regulated by specific aquaporins that facilitate its diffusion through membranes. The compartmentalizing effect on anionic species such as superoxide and peroxynitrite is even more accentuated because of the large energetic barrier that the hydrophobic interior of membranes presents to ions that may be overcome by protonation or the use of anion channels. The large difference in cell membrane permeability for different reactive species indicates that compartmentalization is possible for some but not all of them.

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Cellular membranes

Lipid membranes are selective barriers that compartmentalize different cellular functions. They separate the cells from its surroundings and also separate organelles within cells. Cell membranes are commonly represented by the

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fluid mosaic model of Singer and Nicolson [1] where a fluid lipid bilayer accommodates different types of proteins. The model serves as a basic framework to understand the function of different membranes, but additional factors need to be considered to acknowledge the complexity of cell membranes. For instance, the lipid and protein composition of membranes varies depending on the cell type and the subcellular location [2]. The most abundant lipids in mammalian cells are phosphatidylcholine, phosphatidylethanolamine and cholesterol, whereas some lipids are more abundant in particular locations, such as cardiolipin in mitochondria [2]. Furthermore, the lipids in cellular membranes are distributed asymmetrically, for instance, glycolipids locate exclusively on the outer leaflet of plasma membranes, whereas phosphatidylserine is mostly found in the inner leaflet [3].

Proteins account from 18 to 76 % in weight of the total membrane in myelin or mitochondria respectively, whereas in erythrocytes and liver cell plasma membrane they account for 49-45% of the total weight [2]. The proteins are responsible for most of the functions of particular membranes, thus, different membranes also show different protein composition, i.e. the proteins found in plasma membrane are different from those found in mitochondria. The proteins are also asymmetrically located in the membrane, always oriented in the same direction. Some plasma membrane proteins are glycosylated, and these modifications are located on the external face of the membrane. These carbohydrates on the surface are used in cell-cell recognition, communication and adhesion [4].

Unlike the classical representation of the fluid mosaic model, cell membranes contain a high density of transmembrane proteins that perturb lipid packing, transmembrane proteins often contain bulky extramembranous protein domains and protein-protein oligomers are a common feature in membranes, resulting in restricted lateral diffusion in membranes. As a consequence of the presence of transmembrane proteins, the thickness of the membrane is not homogenous. The lipids have to accommodate slightly different lengths of hydrophobic α-helices in the transmembrane domains of proteins, usually by increasing or decreasing the proportion of *gauche* rotamers, which can decrease or increase the length of the alkyl chains. As a result, some regions of the membrane may be thinner than others [3]. Furthermore, lateral heterogeneities are found in membranes, where domains enriched in certain lipids and proteins are formed. Also, the lipids may arrange themselves in other phases beside the liquid-crystalline lamellar phase [3].

The passage of molecules and ions across these membranes is regulated by different mechanisms. Small nonelectrolytes are capable of crossing the membrane by diffusion through the lipids, with rates that depend mostly on their size and hydrophobicity. Oxygen, that is a small molecule and mildly hydrophobic, is able to diffuse across cell membranes at high rates [5]. For polar molecules this rate may be very low and membrane proteins are involved in their transport (i.e. glucose). Because of the amphipathic nature of fatty acids, they also rely on transporters in the membrane to be internalized [6]. Because of the apolar nature of membrane´s interior, an electric charge faces a huge thermodynamic barrier that effectively limits the permeability of membranes to ions and ionic species and this rely on specific transport proteins [7].

Diffusion across membranes

Many small molecules can cross membranes through the lipid fraction virtually unhindered. Such is the case of dissolved gas molecules involved in respiration, such as oxygen and carbon dioxide. It is also the case of the so called “gasotransmitters”, like nitric oxide, carbon monoxide and hydrogen sulfide, and of other reactive species such as nitrogen dioxide (see below). These molecules are gases in their standard state and most of them are only sparingly soluble in water. The unfavorable interactions with water give these molecules a slightly hydrophobic character in solution that

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translate into a favorable partitioning into membranes and very high permeation rates. The permeability to more polar or ionic species by simple diffusion is rather limited, and protein channels facilitate the diffusion across the membrane. No active transport has been reported for reactive species.

Simple diffusion requires that the molecule first enters the membrane, and then diffuses across the acyl chain region of the membrane to the other side. For polar molecules, the highest resistance to permeation is given by the low solubility at the acyl chain region. Actually, a correlation between solubility in organic solvents and permeation was observed by Overton more than 100 years ago and confirmed several times since then [8-10]. This indicates that hydrophobic molecules can traverse the membrane more easily than polar molecules by overcoming the thermodynamic barrier presented by the acyl chains. Furthermore, the tight packing of lipids presents a further selectivity to small molecules, so that small size also makes the molecules to traverse the membranes more easily than expected from polarity alone [5, 9, 11, 12]. Since diffusion coefficients are similar among small molecules, the correlation between permeability and solubility suggests that the largest barrier presented by membranes is thermodynamic rather than kinetic for small molecules.

Two regions may be identified in the membrane that show different barriers to diffusion. The first one is the interface, where the water molecules meet the polar headgroups in the lipid bilayer. The headgroups of phospholipids are bulky, ionic and polar so that there is a tight packing and high capacity to form hydrogen bonds. The other region would be the acyl chain region, characterized by a lower atom density and a high hydrophobicity.

The main barrier lies in different regions depending on the physicochemical properties of the molecule. For small hydrophobic molecules this barrier is usually located in the interface region, whereas for small polar molecules this barrier is located in the acyl chain region [13]. As a general rule, the diffusion coefficient of small molecules increases towards the center of the bilayer, regardless of its physicochemical properties [14]. However, solubility is very different for either hydrophobic or polar molecules, and pose an important thermodynamic barrier that slows down the passage of polar molecules. In the case of hydrophobic molecules, the headgroup region presents a small thermodynamic barrier, whereas for polar molecules the acyl chain region presents a large thermodynamic barrier [13, 14].

Membranes may be thought of as a selective filter where individual molecules cross the membrane at basically the same speed, but there are millions more holes for hydrophobic than for polar small molecules.

The permeability coefficient

The permeability coefficient (P_m) indicates how fast molecules cross a membrane. The permeability coefficient can be used to compare the ability of different molecules to traverse membranes and therefore identify groups of molecules that are more or less capable to diffuse across a membrane [15].

Basically, P_m (expressed in cm s^{-1}) is related to the flux of molecules through the membrane. For uncharged small molecules, it is related to the diffusion coefficient of the molecule in the membrane (D_m), as well as its solubility in the membrane (represented by the partition coefficient between membrane and water, K_P).

In its simplest form, P_m is related to the diffusion coefficient, by:

$$P_m = D_m K_P / \delta \qquad \text{Eq. 1}$$

Where δ is the thickness of the membrane, usually approximated to 4 nm. A more complex equation can be used to take into account that both diffusion and solubility change at different depths in the membrane and that interfacial resistance may be significant [16].

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Interactions of reactive species with membranes

Reactive oxygen and nitrogen species (ROS and RNS) are very general and vague terms that include a wide range of molecules with different physicochemical properties. Most ROS and RNS derive from oxygen and nitric oxide (Fig. 1) and they do have in common the ability to directly or indirectly cause chemical damage to biomolecules. Many of these reactive species are produced under controlled conditions by specialized proteins to accomplish physiological functions but they can also derive from environmental sources such as smog, smoke, xenobiotics and exposure to ultraviolet radiation.

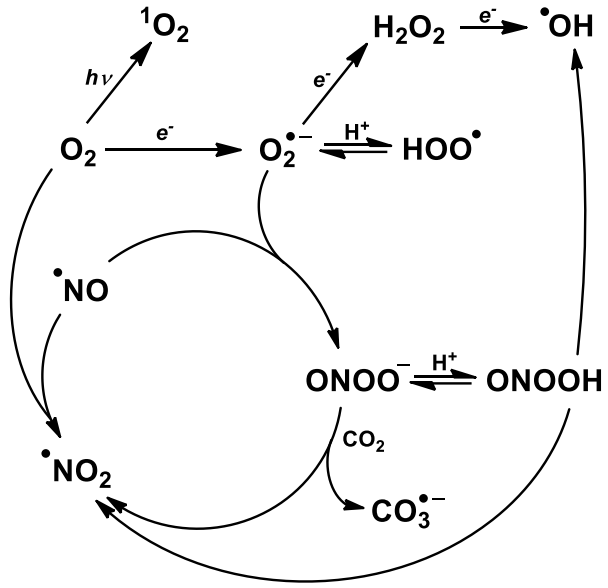

Figure 1. Reactive Oxygen and Nitrogen Species. Oxygen (O_2) and nitric oxide ($^\bullet NO$) are the precursors of ROS and RNS. Photosensitization can give rise to singlet oxygen ($^1O_2$) whereas partial reduction of O_2 produces superoxide ($O_2^{\bullet -}$), that can be protonated to give the hydroperoxyl radical ($HOO^\bullet$) or further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$). The reaction of $^\bullet NO$ with O_2 (autoxidation) generates nitrogen dioxide ($^\bullet NO_2$), while the reaction with $O_2^{\bullet -}$ generates peroxynitrite anion ($ONOO^-$) that can either protonate to the peroxynitrous acid (ONOOH) that can then homolyze and give $^\bullet NO_2$ and hydroxyl radical ($HO^\bullet$) as products (5-30% yield) or react with carbon dioxide (CO_2) and give $^\bullet NO_2$ and carbonate radical ($CO_3^{\bullet -}$ 35% yield).

Accomplishing either a physiological function or exerting a toxic effect, will depend largely on the ability of the reactive species to cross the cellular membranes, so a directionality and compartmentalization of action may be established. Some of these reactive species are able to cross membranes rapidly by simple diffusion, so that they can diffuse practically unhindered in a large volume of tissue. In this case, chemical reactions rather than membranes will be responsible for decreasing its diffusion distance. Other reactive molecules, however, find high energy barriers to traverse membranes thus their reactions and actions can be more effectively compartmentalized.

Furthermore, cellular membranes are not just spectators of the flow of reactive species, but are also targets of their reactivity. The most reactive targets are polyunsaturated fatty acids, which oxidation may start a lipid peroxidation chain reaction, but other lipids may also be oxidized by specific reactive species. The permeability of membranes to the different reactive species also indicates how easy it is for these reactive species to oxidize the lipids and embedded proteins in a membrane.

The following section will focus on membrane interactions and potential reactions with particular reactive species, the most relevant in biology. The parameters used to evaluate these interactions will allow us to compare and classify them according to how easily they can travel across membranes.

Oxygen

Molecular oxygen (O_2) is one of the essential molecules that define aerobic life. Because of oxygen, much more energy can be extracted from organic matter such as glucose and sustain the metabolism of larger lifeforms. Its usefulness comes with the associated risk of forming reactive species in mitochondria by partial reduction of oxygen. It is calculated that 0.1-0.5% of all the oxygen consumed by mitochondria is converted to reactive oxygen species such as superoxide and hydrogen peroxide [17].

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Oxygen is only sparingly soluble in water (1.28 mM / atm at 25°C for pure O_2 and 0.27 mM / atm at 25°C for air [18]) and therefore larger animals need proteins to transport it through the body such as hemoglobin. On the other side, oxygen is around 10 times more soluble in organic solvents than in water [19]. A slight hydrophobic character combined with a small size allow it to simply diffuse through lipid membranes [5]. Oxygen also plays an essential role in lipid oxidation. The initial formation of a lipid radical leads to the addition of an O_2 molecule and the formation of the longer lived lipid peroxyl radical, that then can oxidize another molecule of lipid and propagate the oxidative damage [20]. These chain reactions that occur in the membrane are usually inhibited by the membrane's main antioxidant, α-tocopherol [21].

The diffusion of oxygen through membranes has been studied by several approaches including fluorescence quenching, electron paramagnetic resonance, nuclear magnetic resonance and molecular dynamics [13, 22-24]. Each technique has different advantages and disadvantages (discussed in [16]), but taken together yield a complete picture of the diffusion of oxygen through membranes at a molecular level.

Apparent diffusion coefficients (D_{app}) are experimentally accessible, and provide useful information about the rate of permeation and reactions of oxygen in the membrane [16]. These values derive from quantifying the collisions between probes inserted in the lipid membrane and oxygen, that depend on both the diffusion coefficient inside the membrane and the local concentration of oxygen in the membrane. Most times the concentration of O_2 inside the membrane is not known, so the aqueous concentration of O_2 is taken as a reference [16]. The apparent diffusion coefficient is thus the product of the true diffusion coefficient in the membrane times the partition coefficient ($D_{app} = D_m.K_P$). The permeability coefficient can then be easily obtained from Eq.1. On the down side, apparent diffusion coefficients yield an incomplete mechanistic picture of the permeation process, because diffusion cannot be separated from partition. Nevertheless, some attempts have been done in this direction [5].

Different methods have been developed to determine the partition coefficients for membranes. Most of them rely on quantifying how much O_2 can be dissolved in membrane suspensions relative to the aqueous buffer alone [5, 25-27]. To summarize, it was found that O_2 is 3-4 times more soluble in fluid lipid membranes than in water, and that the solubility depends on the temperature but more importantly on the physical state of the membrane and the available free volume [5]. Phospholipid membranes in the gel state showed a very low solubility to O_2 ($K_P < 1$), with a sharp increase above the transition temperature [5, 25, 26]. Although no results are yet available for actual plasma membranes, there is evidence suggesting that some domains in cellular membranes that are more rigid and tightly packed may prevent the partition of O_2 and limit the permeability.

The independent determination of apparent diffusion coefficients and partition coefficients permitted the calculation of the diffusion coefficient of O_2 in the membrane [5]. Unexpectedly, a virtually unique $D_m = 1.6 \times 10^{-5}$ cm^2 s^{-1} was found for membranes of different compositions, in different physical states. Those studies were conducted by pyrene fluorescence quenching and a plausible explanation is that the bulky pyrene probe is disrupting the local lipid structure, creating a similar microenvironment in the different conditions and preventing the study of D_m by this approach [5].

A more detailed picture of the interaction of O_2 with membranes can be obtained from molecular dynamics simulations. In this case the solubility and diffusion can be obtained separately at different depths in the membrane, and show that both the solubility and diffusion have a bell-shaped profile with its maximum in the middle of the bilayer, with only a low barrier at the headgroups region [13, 14, 28].

The permeability coefficients determined so far indicate that the diffusion of O_2 through lipid

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membranes is extremely rapid (P_m ~ 40 cm s^{-1}), similar to diffusing through an equally thick layer of water (Table I) [5, 15]. Therefore, no protein channels are needed to facilitate O_2 diffusion that will diffuse unhindered over a large number of cells. The only case where O_2 diffusion through membranes decreased more than 2 orders of magnitude was in compressed phospholipid monolayers [29]. We proposed that lung surfactant may behave in a similar way, creating a "closed valve effect" when compressed during respiration [5], but this remains to be proven.

Singlet Oxygen

The main difference between singlet oxygen ($^1O_2$) and oxygen at the basal triplet state is the electronic configuration. In the singlet state $^1\Delta_g$ the two electrons at the highest energy state are in the same molecular orbital with paired (opposite) spins, whereas in the triplet state $^3\Sigma$ the electrons are located in separated orbitals with the same spin. This leads to a dramatic change in reactivity. Triplet oxygen reacts exclusively with paramagnetic species, such as some metals and radicals, but singlet oxygen can react with other singlet state, non-radical, and electron-rich molecules containing double bonds [30, 31]. Singlet oxygen is produced mostly by photochemical reactions involving energy transfer from a sensitizer in the excited state to triplet oxygen. There are endogenous sensitizers that can produce $^1O_2$ when exposed to UV light, such as protoporphyrin IX, FMN, FAD, NADH and NADPH, whereas exogenous sensitizers can be found in cosmetics, food additives and drugs [30].

In lipid membranes, singlet oxygen is expected to share most of O_2 physicochemical properties, except for reactivity. Singlet oxygen reacts with double bonds in the acyl chains to produce lipid hydroperoxides directly [30, 31], and with membrane proteins to produce protein hydroperoxides and other reaction products [32, 33]. These oxidative reactions can be prevented by carotenoids that are very effective $^1O_2$ quenchers [34, 35].

Nitric oxide

Nitric oxide ($^\bullet$NO) is synthesized by the enzymes nitric oxide synthases (NOS) of which three isoforms are known: endothelial, neuronal and inducible. Nitric oxide is used as a signaling molecule in vasorelaxation and also in neurotransmission. The signaling is accomplished mostly by interacting with the hemeprotein soluble guanylate cyclase in the target cells [36]. Moreover, when produced in large quantities by the inducible NOS it has cytostatic and cytocidal activity. The toxicity depends mostly on the formation of secondary more reactive species such as nitrogen dioxide and peroxynitrite [37].

Nitric oxide has a low dipole moment (0.159 D [38]), so it has weak intermolecular interactions and it is a gas at 1 atm and 25°C. It shares some similarities with oxygen in that it is only sparingly soluble in water (1.94 ± 0.03 mM [39]), but about ten times more soluble in organic solvents [40]. Because of packing effects that decrease the solubility in membranes, the partition coefficient in EYPC liposomes and human low density lipoprotein is 4.4 and 3.4, respectively at 25°C [27].

The diffusion through membranes is very rapid and requires the use of either fluorescence or EPR probes inserted in the membrane that change their parameters upon collision with $^\bullet$NO. The use of fluorescent pyrene probes to study $^\bullet$NO diffusion has recently been reviewed [16]. These measurements have shown that lipid membranes do not limit $^\bullet$NO diffusion significantly and at most can decrease the diffusion to a half that in water [27, 41, 42]. This is because of the combination of a favorable partition coefficient and a high diffusion in the membrane. The permeability coefficients of lipid membranes to $^\bullet$NO range from 18 to 73 cm s^{-1} [41, 42], in the order of an equally thick layer of water (Table I).

The favorable partitioning of $^\bullet$NO into lipids leads to an acceleration of the autoxidation reaction, to form the oxidizing and nitrosating nitrogen dioxide and dinitrogen trioxide [43, 44].

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Table I. Permeability coefficients for different membranes and reactive species.

Permeant	Membrane	T (°C)	P_m (cm/s)	Reference
O_2	4 nm slab of water	20	53*	
O_2	DMPC	18	12	[118]
	DMPC	29	125	[118]
	DOPC	30	114	[118]
	POPC	35	157	[119]
	POPC:Chol (50% Chol)	35	50	[119]
	RBC (human)	20	38	[41]
	CHO cells	20	21	[120]
	CHO cells	37	42	[120]
$^{\bullet}NO$	EYPC	20	73	[42]
	EYPC:Chol (30% Chol)	20	66	[42]
	RBC (human)	20	18	[41]
$^{\bullet}NO_2$	EYPC	25	~ 5	[19]
H_2S	E.coli lipids	23	≥ 0.5	[78]
	DLPC	25	~ 3	[77]
H_2O	EYPC	25	3.3×10^{-3}	[121]
H_2O_2	RBC (horse)	20	6×10^{-4}	[99]
	Peroxisome (rat liver)		3×10^{-3}	[122]
	RBC (rat)		1.2×10^{-2}	[103]
	Jurkat T cells	37	2×10^{-4}	[100]
	Chara corallina		3.6×10^{-4}	[107]
	Escherichia coli	37	1.6×10^{-3}	[102]
	PC12 cells		4×10^{-4}	[123]
	HUVEC cells		1.6×10^{-3}	[123]
	IMR-90 cells		1.1×10^{-3}	[123]
	HeLa cells		4.4×10^{-4}	[124]
$HOO^{\bullet}$	EYPC	23	4.9×10^{-4}	[88]
$O_2^{\bullet-}$	SBPC	25	2.1×10^{-6}	[125]
	EYPC	23	7.6×10^{-8}	[88]
ONOOH	DMPC	23	8×10^{-4}	[83]
	EYPC	21	1.3×10^{-3}	[60]
	DMPC	21	6.3×10^{-4}	[60]
	DPPC	21	4×10^{-4}	[60]

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; Chol, cholesterol; RBC, red blood cells; DLPC, dilauroylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.
*calculated $P_w = D_w^{O2} / \delta = (2.1 \times 10^{-5}\ cm^2\ s^{-1}) / 4 \times 10^{-7}$ cm [5].

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Nitric oxide is extremely efficient at inhibiting lipid peroxidation [45-47]. The antioxidant properties of •NO in lipids are supported by the diffusion-controlled reaction with peroxyl radicals [48], aided by the favorable partitioning in lipids and the very high diffusion [27]. The products include nitrosated and nitrated lipids that show several biological effects [46, 49].

Nitrogen dioxide

Nitrogen dioxide ($^{\bullet}NO_2$) is one of the oxidizing products of •NO, either from autoxidation or from peroxynitrite decomposition [43, 50]. Alternatively it can be formed from the oxidation of nitrite by myeloperoxidase [51] and it has been proposed to be an important component in the new therapeutics by cold atmospheric plasma [52]. It is also an important pollutant in urban areas and one of the main components of smog. It is paramagnetic and reactive with lipids, proteins and DNA bases [50]. It can undergo different types of reactions, including radical recombination, addition to double bonds, hydrogen abstraction and electron transfer [53]. The characteristic products include nitrated proteins and lipids, which have been dealt in detail elsewhere [54, 55].

Nitrogen dioxide has an angle of 134.4° and a low dipole moment (0.29-0.58 D [56]) and is also a gas at 25°C and 1 atm. It is more soluble than •NO in water (12 ± 4 mM at 1 atm, 20°C) [57, 58], but rapidly decomposes because of a fast dimerization and reaction with water to produce nitrite and nitrate [57, 58]. The solubility has been measured in a few organic solvents, including decane, chloroform and carbon tetrachloride (reviewed in [59]) and it has been estimated by quantum mechanical calculations that $^{\bullet}NO_2$ is 2.7 times more soluble in octanol than in water, and 1.5 times more soluble in lipid membranes than water [19]. It was estimated a permeability coefficient of 5 cm s^{-1} (Table I) [19], in the upper range of that estimated by Khairutdinov *et al.* [60]. Molecular dynamics has provided a more detailed picture of $^{\bullet}NO_2$ interacting with lipid membranes and shown a favorable interaction between $^{\bullet}NO_2$ and the lipid membrane interior, in agreement with previous estimations, with the permeation barrier located at the headgroups region [52, 61].

Hydrogen sulfide

Hydrogen sulfide (H_2S) has been related to the origin and evolution of life [62]. Not only it is necessary for cysteine synthesis in plants and microorganisms but it can also be a source of electrons for respiration in chemolithotrophy and anoxygenic photosynthesis. Nonetheless, high levels of hydrogen sulfide represent a threat to mammals because it can inhibit cytochrome c oxidase and mitochondrial respiration [63]. Surprisingly, it was found that hydrogen sulfide is synthesized in mammals from cysteine or homocysteine at moderately high rates by three enzymes: cystathionine β-synthase, cystathionine γ-lyase and 3-mercaptopyruvate sulfurtransferase, whose relative contribution is tissue-dependent [64]. Hydrogen sulfide is also efficiently detoxified by an enzymatic system located in the mitochondria [65]. Physiological effects of administering low levels of H_2S include neuromodulation, vasodilation, protection in ischemia-reperfusion injury events and even induction of a suspended animation state in mice [66-69]. In spite of the diversity of observed physiological effects, the mechanisms for sulfide signaling are still elusive and a subject of intensive research [70].

The molecular structure of hydrogen sulfide is analogous to the molecule of water, but with a smaller angle between bonds (92°) and a lower dipole moment (0.97 D) because of the lower electronegativity of sulfur relative to oxygen. Moreover, H_2S is not able to form hydrogen bonds, so, under normal temperature and pressure conditions, it is a gas (boiling temperature: -60 °C). It is fairly soluble in water (101.3 mM / atm at 25 °C) hydrated like a hydrophobic solute [71] and, as weak acid, is able to donate protons to the solvent. Hydrogen sulfide is in fast equilibria with hydrosulfide anion (HS^-) and sulfide anion (S^{2-}) with pK_as of 6.98 at 25 °C for the first dissociation step and > 17 for the second one [72]. Hydrosulfide anion is the main species at pH 7.4 (~70%) while H_2S accounts for

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the remaining and the concentration of sulfide anion is negligible.

Hydrosulfide anion is a nucleophile able to react towards biological oxidants. It reacts with two electron oxidants (hydrogen peroxide [73], peroxynitrite [74] and hypochlorite [73, 75]), as well as with one electron oxidants (hydroxyl radical, carbonate radical, superoxide and $^{\bullet}NO_2$ [70]), forming diverse sulfur oxidation products. Among other cellular targets, disulfides (RSSR) and sulfenic acids (RSOH) are candidates for signaling transduction [76]. These reactions produce persulfides (RSSH), reactive sulfane derivatives of thiols, of increasing interest [70].

As a first approach to the hydrophobicity of H_2S, partition coefficients of 2.1 ± 0.2 and 1.9 ± 0.5 at 25 °C were reported for H_2S in octanol and hexane [77]. Due to the pK_a of H_2S, dissociation to form HS^- determines a distribution coefficient of 0.64 ± 0.05 in octanol at pH 7.4 and 25 °C. Using multilamellar liposomes of DLPC as a model for biological membranes, a partition coefficient of 2.0 ± 0.6 at 25 °C for H_2S was determined [77]. This affinity of H_2S for hydrophobic milieu suggests a high permeability in membranes.

The ability of H_2S to traverse biological membranes has been assessed through different approximations. Mathai et al. studied the resistance imposed to H_2S transport by planar bilayers prepared from *E. coli* lipids [78]. They concluded that the diffusion through the membrane was not the rate-limiting step, allowing the estimation of a lower boundary for the permeability coefficient of 0.5 cm s^{-1}, probably limited by the diffusion through the unstirred water layers. Moreover, the inclusion of cholesterol or sphingomyelin in the composition of the bilayer did not restrict the diffusion through the membrane. This estimation lead to the conclusion that neither aquaporins nor other protein channels are needed to facilitate the transport of H_2S. Accordingly, permeability of H_2S was also too rapid to be observed by stopped-flow using fluorophores encapsulated in DMPC:cholesterol 1:1 unilamellar liposomes [77]. Semi-theoretical approaches comparing partition and permeability coefficients for similarly sized molecules, enabled the estimation of a permeability coefficient ≥ 3 cm s^{-1} (Table I)[77]. Molecular dynamics simulations on lipid membranes of DPPC showed that the barrier for H_2S permeation was negligible according to the Gibbs energy profiles, and a permeability coefficient of 11.9 ± 0.7 cm s^{-1} was estimated [79].

Although H_2S shows higher solubility in biological bilayers than in water, most of the reactions involving this molecule are triggered by HS^-, the nucleophile species, which is essentially not soluble in hydrophobic environments. Unless a relevant biological reaction is being considered for undissociated H_2S within the bilayer, it is concluded that the favorable partition does not assist the acceleration of reactions but contribute to permeation and spreading through tissues.

Peroxynitrite anion and peroxynitrous acid

Peroxynitrite ($ONOO^-$) is formed by the diffusion-controlled reaction between nitric oxide and superoxide [80]. It is continually generated under basal conditions, but its formation increases with higher rates of superoxide and $^{\bullet}NO$ production such as those found in inflammation [80]. It is considered one of the effector molecules in the cytotoxic effect of $^{\bullet}NO$ [37]. Peroxynitrite has a pK_a of 6.8, and the peroxynitrous acid is unstable and either isomerizes to nitrate or homolyzes to yield $^{\bullet}NO_2$ and $^{\bullet}OH$. The most rapid reactions of peroxynitrite in a biological context are with CO_2, in a reaction that yields an intermediary that homolyzes to $^{\bullet}NO_2$ and carbonate radical with a 35% yield. In addition, rapidly reacts with peroxidatic cysteines of the antioxidant proteins peroxiredoxins (Prx), yielding nitrite and an oxidized Prx that can then be reduced by thioredoxin [80]. Peroxynitrite is also able to nitrate proteins [54] and oxidize and nitrate lipids [81, 82].

It has been well established that peroxynitrite is able to cross lipid membranes through different pathways. Peroxynitrous acid can cross directly through the lipid fraction, and this has been shown in pure phospholipid membranes [60, 83]. Estimated permeability coefficients of

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phosphatidylcholine membranes to ONOOH range from 4×10^{-4} in gel state membranes to 1.3×10^{-3} cm s^{-1} in fluid state membranes (Table I) [60, 83]. Recent molecular dynamics studies show that peroxynitrous acid interacts favorably with the headgroups in phosphatidylcholine membranes but finds an energetic barrier in the acyl chain region, and the free energy barrier experienced by ONOOH is similar to that of ethanol [61].

Peroxynitrite anion has to deal with a very large energetic barrier to diffusion through the hydrophobic fraction of the membrane, but it can cross membranes through protein channels. In erythrocytes it was demonstrated that peroxynitrite could use the anion exchanger 1 (band 3) to traverse the membrane [7] and that this path accounted for 50% of the transport of peroxynitrite into red blood cells.

Carbonate radical may be an important product derived from peroxynitrite. Based on its short life given by its high reactivity, its negative charge, and that CO_2 decreases lipid peroxidation by peroxynitrite, the permeability of membranes to carbonate radical is expected to be very low, even through anion channels [50].

Superoxide and hydroperoxyl radical

Superoxide ($O_2^{\bullet -}$) is the one-electron reduction product of O_2 and can be produced enzymatically by NADPH oxidases, xanthine oxidases, and also as a byproduct in mitochondrial respiration [84]. Superoxide by itself is not very reactive, but is a precursor of more reactive species, such as peroxynitrite and hydrogen peroxide (Figure 1).

Superoxide has a pKa of 4.7, and the conjugated acid, the hydroperoxyl radical ($HOO^{\bullet}$) is more oxidizing than superoxide [85]. Both superoxide and $HOO^{\bullet}$ have been observed to initiate lipid peroxidation [86], leading to cell lysis (erythrocyte ghost membranes) [87].

The permeability of phospholipid membranes to superoxide was studied by Gus'kova *et al.*, who found that egg yolk phosphatidylcholine liposome membranes had a permeability of 4.9×10^{-4} cm s^{-1} to $HOO^{\bullet}$, and 7.6×10^{-8} cm s^{-1} to superoxide at 23°C (Table I) [88].

Because of its charged nature, superoxide does not interact favorably with membranes. Molecular dynamics show that superoxide remains in the aqueous phase and is excluded even from the headgroup region [13], as expected for a charged particle. Even though the passage through the lipid fraction is highly unfavorable, superoxide can use anion channels in the membrane such as anion exchange 1 (band 3) in erythrocytes [89] and thus also traverses plasma membranes. Unlike superoxide, $HOO^{\bullet}$ is not charged and interacts favorably with the headgroup region, by means of hydrogen bonds with acyl chain carbonyls, and has a lower energy barrier to traverse the membrane than hydrogen peroxide [13, 90]. It can reach the unsaturated bonds in the lipids and therefore it may be the main responsible for superoxide-initiated lipid peroxidation [13, 90].

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is generated from many sources, but mainly NADPH oxidases, xanthine oxidase and from the dismutation of superoxide. Cellular sources are therefore located in the plasma membrane, mitochondria, endoplasmic reticulum peroxisomes and phagolysosomes. Although mitochondria is considered the major site of production under physiological conditions, it is generated in large amounts during neutrophil activation and the respiratory burst by the activation of NADPH oxidases [84]. The role of H_2O_2 as a signaling molecule is starting to be unveiled. It was found that H_2O_2 affects cell proliferation, growth, migration, apoptosis and survival [91]. The reactivity of H_2O_2 is limited to metal centers, selenoproteins and specific thiol proteins [92]. The most reactive proteins include catalase, glutathione peroxidase and peroxiredoxins [92, 93]. Peroxiredoxins are present in cells at high concentrations, thus keep intracellular levels of H_2O_2 very low [93][94, 95]. The molecular mechanisms of H_2O_2-induced signaling are under study but suggest that peroxiredoxin play a key

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role in relaying oxidation equivalents to secondary proteins [95, 96].

Hydrogen peroxide itself is not able to oxidize lipids, but can yield other products such as hydroxyl radical, that can initiate lipid peroxidation [97]. It is also the fundamental substrate of peroxidases such as myeloperoxidase, which uses it to make more reactive species such as hypochlorous acid [92].

The idea of a permeability barrier imposing a limit to the entry of hydrogen peroxide into the cell was first introduced by Clayton [98] and Nicholls [99], by studying the catalase activity in intact and disrupted *Rhodopseudomonas spheroides* cells and horse erythrocytes, respectively. Later on, the presence of gradients across the membranes from mammalian cell lines, bacteria and yeast was reported, confirming that biological membranes generally prevent the free diffusion of H_2O_2 [100-102]. Notably there are very few reports of permeability of pure lipid membranes to H_2O_2. Although changes in diffusion rates depending on composition, temperature and compressibility have been observed, no permeability coefficients are reported for liposomes [103-105].

Most of the reported H_2O_2 permeability coefficients come from studies with cell membranes and lie between 1×10^{-3} and 4×10^{-4} cm/s, very similar to the values reported for water (Table I). Water and H_2O_2 have similar molecular properties, almost the same dipole moment, dielectric constant, molecular diameter and ability to form hydrogen bonds [106]. In fact, it is believed that H_2O_2 passes into the cell the same way as water, both by simple diffusion and by facilitated diffusion through specific aquaporins. The first study that showed that aquaporins facilitate H_2O_2 diffusion was done by Henzler and Steudle, in the plant cell model *Chara corallina* [107].

Aquaporins are tetrameric proteins, where each monomer acts as a functional channel composed of six membrane-spanning helices connected by five loops (A to E). Loops B and E contain the highly conserved asparagine-proline-alanine (NPA) sequence and these motifs meet in the middle of the membrane, forming a narrow hydrophobic pathway. A second selectivity filter is provided by the aromatic/arginine (ar/R) region, formed by four aminoacids, which works as a size exclusion barrier and generates the adequate environment for the establishment of the hydrogen bonds necessary to transport the substrate [106].

The importance of aquaporins in facilitating the diffusion of H_2O_2 was further established by Bienert et al., who used *Saccharomyces cerevisiae* to express different aquaporins. Overexpression of human aquaporin 8 (hAQP8) and TIP1;1 and TIP1;2 from *Arabidopsis thaliana* lead to a decrease in growth and survival of the yeasts exposed to H_2O_2 [108]. In contrast, hAQP1, hAQP2, rAQP3, rAQP4, hAQP5, hAQP9 and other aquaporins from *Arabidopsis thaliana* did not affect the survival in the presence of H_2O_2, suggesting that these isoforms were not permeable to H_2O_2 [106, 108]. Similar assays identified AQP1 from rat and multiple other aquaporins from plants, including PIP2;1 from *Arabidopsis thaliana* [106, 109]. It was suggested that PIP2 type aquaporins are efficient H_2O_2 channels, and the main regulation of H_2O_2 passage is given by the ar/R region [110]. The idea that H_2O_2 is transported across the cell membrane by aquaporins is also supported by computational simulations, using bovine AQP1 and PIP2;1 from *Spinacia oleracia* as models [111].

Studies in mammalian cell lines were also conducted and they showed further evidence that confirmed the role of hAQP8 in H_2O_2 transport. Human aquaporin 3 was also proposed as a transporter with the characteristic that it can endure changes in its expression levels in order to modulate the accumulation of H_2O_2 and thus regulate cellular signaling cascades [112-114]. On the other hand, studies carried out in *S. cerevisiae* and erythrocytes suggest that human aquaporin 1 is unable to transport H_2O_2 [103, 108]. In these experiments it is shown that the H_2O_2 permeability of the cells does not change in the absence or presence of mercurial compounds, which are well known aquaporin 1 inhibitors. This potential selectivity for different aquaporins

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suggests that the expression profiles of aquaporins could dictate the susceptibility of a particular cell or tissue to external H_2O_2 signaling [112].

A decrease in the capacity of red blood cells to metabolize exogenously added H_2O_2 during storage for transfusion has recently been described that was independent of the loss of antioxidant thiols [115]. It is proposed that the decrease in H_2O_2 metabolism is related to changes in the permeability of the membrane to H_2O_2.

Hydroxyl radical

Hydroxyl radical ($^{\bullet}OH$) is one of the most reactive molecules [85]. It can be formed from H_2O_2 by reaction with a reductant such as Fe^{2+} (Fenton reaction) and from the homolysis of ONOOH [54, 116]. This radical reacts at diffusion-controlled rates with most organic compounds by electron transfer or addition [117]. Hydroxyl radical is responsible for starting lipid peroxidation in metal catalyzed reactions, and also in peroxynitrite induced lipid peroxidation [81].

The diffusion of $^{\bullet}OH$ is limited by its very high reactivity and therefore extremely short lifetime. Molecular dynamics show that it can interact with the membrane headgroup region, and suggest that $^{\bullet}OH$ has a lower energy barrier to permeation than H_2O_2 [13, 52]. As pointed out before, the high reactivity of $^{\bullet}OH$ will prevent its diffusion across membranes, and rather favor reactions near the headgroup region.

Considerations about the permeability of membranes to reactive species

Cell membranes are not equally permeable to all reactive species (Table I). The small and more hydrophobic molecules can freely diffuse across cell membranes whereas the more polar molecules find significantly higher barriers to diffusion that permit their compartmentalization (Figure 2).

The high permeability of cellular membranes to O_2, $^{\bullet}NO$ and H_2S has the biological advantage that no specific transport proteins are needed, and that these molecules will be able to diffuse large distances in tissues unrestrictedly [77]. The limiting factor will be given by the chemical reactions that consume them and decrease their concentration and activity range. Therefore, the diffusion of O_2 in tissues is limited mostly by its consumption by mitochondria. For $^{\bullet}NO$, one of the most relevant reactions decreasing its lifetime and diffusion distances in tissues is with oxyhemoglobin in red blood cells [126, 127]. On the other side, the same properties that make cell membranes permeable to beneficial molecules may also make cells more susceptible to damage by $^{\bullet}NO_2$. Although the lifetime of $^{\bullet}NO_2$ is short because of rapid reactions with endogenous antioxidants and even hydrolysis of the $^{\bullet}NO_2$ dimer by water [50], diffusion of $^{\bullet}NO_2$ through lipid membranes will be fast and likely contribute to cellular damage.

In contrast to the aforementioned reactive species, the permeability of lipid membranes to hydrogen peroxide, peroxynitrite and superoxide is significantly lower (Figure 2), and fast reactions inside the cells will create a steep concentration gradient across the membrane [100]. Furthermore, these molecules may use protein channels to access the cells, and specific aquaporins have been found to facilitate the transport of H_2O_2, whereas anion channels such as band 3 in erythrocytes have been found to facilitate the transport of the anions peroxynitrite and superoxide (Figure 2).

A low membrane permeability allows for true compartmentalization so that higher concentrations of one of these reactive species can be achieved in a membrane-enclosed organelle such as lysosome, phagosomes, peroxisomes or endoplasmic reticulum. Furthermore, a low membrane permeability will allow only a small fraction of the reactive species to leak into the cytosol, so that antioxidant defenses are not overwhelmed. There will also be a clear directionality in the production of a given

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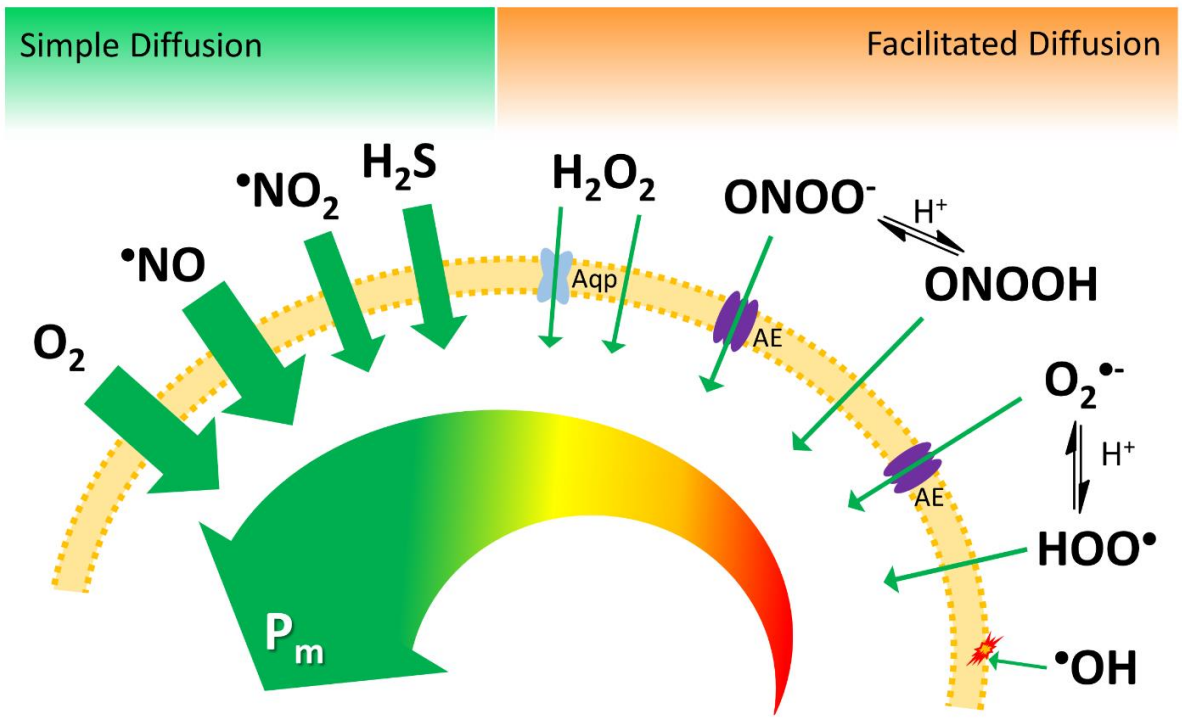

Figure 2. Permeability of lipid membranes to different reactive species. The size of the arrows indicates how permeable cell membranes are to the different molecules. Two groups may be differentiated based on the permeability of the membrane. 1) Oxygen, •NO, $•NO_2$ and H_2S can traverse lipid membranes virtually unhindered by simple diffusion, 2) molecules which permeation rates have been observed to increase by the presence of specific proteins that facilitate their diffusion across cellular membranes: H_2O_2 (can use specific aquaporins, Aqp), $ONOO^-$ and $O_2^{•-}$ (can use anion exchange protein channels, AE). The permeability (P_m) to these species in their electrically neutral form through pure phospholipid membranes is five orders of magnitude lower than that of O_2. Cellular adaptations to decrease simple diffusion by H_2O_2 had been observed, indicating that cell membranes could tightly regulate the transport of this group of reactive molecules. Hydroxyl radical is in a different category because it is so reactive that reacts with basically any component near the surface of the cellular membrane and therefore cannot cross it.

reactive species to a given compartment. For instance, the production of H_2O_2 by NADPH oxidases in the plasma membrane occurs towards the extracellular space, and in the absence of specific aquaporins the H_2O_2 will tend to diffuse away from the cell rather than consume its antioxidant defenses. Another example is given by the formation of peroxynitrite. Conversely to the freely diffusible •NO, the low membrane permeability of superoxide limits the formation of peroxynitrite to the same compartment [80]. The concentration of peroxynitrite will therefore be significantly higher in intracellular sites of superoxide formation such mitochondria and phagosomes [80].

There is still much to uncover about how cell membranes regulate the transport, especially of this last group of reactive species. The membrane permeability is a fundamental parameter when trying to understand the metabolism or signaling properties of these molecules. For instance, the steady state concentration of H_2O_2 in the vascular system (still a matter of debate), will be determined by both the rate of formation and the rate of decomposition that will largely depend on the permeability of red blood cells to it, that is still not known in human cells.

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