Front. Chem. Sci. Eng. 2019, 13(2): 253–263
https://doi.org/10.1007/s11705-018-1786-8

REVIEW ARTICLE

Plasma for cancer treatment: How can RONS penetrate through the cell membrane? Answers from computer modeling

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Abstract Plasma is gaining increasing interest for cancer treatment, but the underlying mechanisms are not yet fully understood. Using computer simulations at the molecular level, we try to gain better insight in how plasma-generated reactive oxygen and nitrogen species (RONS) can penetrate through the cell membrane. Specifically, we compare the permeability of various (hydrophilic and hydrophobic) RONS across both oxidized and non-oxidized cell membranes. We also study pore formation, and how it is hampered by higher concentrations of cholesterol in the cell membrane, and we illustrate the much higher permeability of H_2O_2 through aquaporin channels. Both mechanisms may explain the selective cytotoxic effect of plasma towards cancer cells. Finally, we also discuss the synergistic effect of plasma-induced oxidation and electric fields towards pore formation.

Keywords plasma medicine, cancer treatment, computer modelling, cell membrane, reactive oxygen and nitrogen species

1 Introduction

In recent years, there is a lot of interest in the use of cold atmospheric plasma for cancer treatment [1]. However, the underlying mechanisms are far from fully understood. It is generally believed that reactive oxygen and nitrogen species (RONS) generated by plasma play an important role in killing the cancer cells [2,3]. A noticeable rise of intracellular reactive oxygen species (ROS) in cancer cells compared to normal cells has been reported, which might subsequently lead to oxidative damage of biomolecules inside the cells, and this might explain the selectivity of plasma towards cancer cells. Indeed, some studies report the selective action of plasma towards cancer cells *vs.* normal cells, although this selectivity is obviously not always observed. In addition, it should be noted that ROS alone are not generally sufficient for cancer cell death. Indeed, cellular damage also needs to be actively translated into a given cell death program by the cellular signaling machinery [4].

However, before the plasma-induced ROS can cause oxidative damage inside the cells, the plasma species first interact with the cell membrane, chemically modifying or oxidizing its lipids. It is therefore important to study the behavior of the oxidized cell membrane and its effect on the penetration of various plasma-induced RONS through this cell membrane. More specifically, it is important to understand whether passive transport of RONS is possible or whether pores or transmembrane protein channels, such as aquaporins (AQPs), must be present. AQPs are transmembrane proteins which are stated to be important for H_2O transport across the cell membrane. Besides H_2O, they can also transport other small molecules, like H_2O_2, NO, and NO_3^- [5,6]. Keidar and colleagues reported that knocking out AQP8 in glioblastoma cells could significantly weaken the toxicity of plasma-treated liquid medium on these cells, which was the first evidence for their role in plasma for cancer treatment [7]. As most cancer tissues express more AQPs in their cytoplasmic membrane than homologous normal tissues [5], this could explain why cancer cells are more sensitive to plasma treatment than normal cells.

Furthermore, it is shown by molecular dynamics (MD) simulations that lipid oxidation yields an overall increase in the membrane permeability [8], a change in the lipid mobility in the phospholipid bilayer (PLB) [9], pore creation and bilayer disintegration [10]. Furthermore, both simulations [11,12] and experiments [13] have revealed that cholesterol can protect oxidized membranes against

Received July 15, 2018; accepted October 14, 2018

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pore formation. Indeed, liposomes containing 50 mol-% cholesterol are resistant against disruption by plasma, whereas cholesterol fractions below 50 mol-% lead to increased disruption of liposomes [13]. This result is also of great interest for plasma-based cancer therapy, as cancer cells typically contain less cholesterol in their plasma membrane, so the above observation might also be one of the explanations for the selectivity of plasma treatment towards cancer cells, as they allow the reactive plasma species to reach the cell interior more easily through pore formation.

Finally, in addition to RONS generation, some plasma sources produce strong electric fields, ranging from a few up to 100 kV/cm [14−16], which may play an important and synergistic role in plasma-cell interactions [14], as they are high enough to create pores in the membranes, either temporarily or permanently, i.e., so-called electroporation [17]. Several MD studies have been devoted to electroporation (e.g., [18−20]), but little is known about the synergy between plasma-induced lipid oxidation and the electric field, and more specifically on how this affects the cell membrane permeability.

Several groups have performed experiments for the interaction of RONS with either synthetic model membranes or the membranes of isolated cells [21−27]. Tai et al. studied the fluidity and structure of model membranes under oxidative attack, using fluorescence correlation spectroscopy and Raman spectroscopy, and they reported that OH radicals cause a significantly higher lateral fluidity of the membranes, while H_2O_2 has little effect [21]. An increase of the membrane disordering was observed in [22,23], while the opposite effect, i.e., an increase of the lipid order and a drop in the membrane fluidity was reported in [24,25]. This contradiction might be associated with the sample preparation method and/or the depth to which the measuring probe enters the bilayer [26]. Besides studying membrane order and fluidity during plasma treatment, Szili et al. investigated mechanisms of transport of reactive plasma species across the membrane of synthetic phospholipid vesicles, suggesting an interplay of concentration gradients of short-lived and long-lived ROS, in combination with electric fields [27].

In spite of the interesting results obtained in these experiments, most experimental techniques lack the resolution needed to track the motion of very short-lived RONS, including their interaction with the membrane, and the complex lipid reorganization dynamics that might result from it. For this reason, molecular level (MD) simulations can be very useful, also called “computational microscope” [28]. For instance, Cordeiro et al. have demonstrated that H_2O_2 and small oxy-radicals typically reside close to the PL head groups and interact with the unsaturations along lipid acyl chains [29,30]. Within our group PLASMANT, we have also performed several MD simulations to study the behaviour of the PLB upon oxidation and/or an electric field, as well as the hampering effect of cholesterol and the role of AQPs, including the consequences for the penetration of RONS [11,12,31−34].

In this review paper, we will first provide a brief explanation of the various molecular scale modelling techniques that can be used for this purpose, to put the simulation results presented here in a broader perspective. Subsequently, we will give an overview of our recent simulation results on the permeation of RONS across oxidized and non-oxidized cell membranes, including the possibility of pore formation and transport through aquaporin channels, and the combined effect of oxidation and electric field, both arising from plasma. Finally, we will identify future research directions, to gain further insight in the underlying mechanisms and make progress in this highly promising research field of plasma for cancer treatment.

2 Modelling techniques at the molecular level

A wide variety of modelling techniques at the molecular level can be applied to study the interaction of plasma species with biomolecules or the subsequent behavior of these biomolecules as a result of such interactions. They are illustrated in Fig. 1, along with the corresponding system sizes and time scales that can be reached nowadays within a reasonable calculation time.

The most accurate computational method is based on first principles, i.e., quantum mechanical (QM)

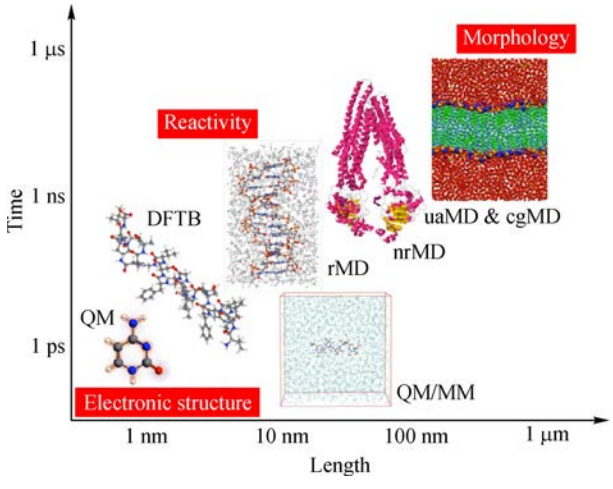

Fig. 1 Overview of the computational methods that allow to obtain atomic/molecular level insight in the interaction of plasma species with biomolecular systems, as a function of the attainable system sizes and time scales (QM = quantum mechanics, DFTB = density functional-tight binding, QM/MM = quantum mechanics/molecular mechanics, rMD = reactive molecular dynamics, nrMD = non-reactive molecular dynamics, uaMD = united-atom molecular dynamics, cgMD = coarse-grained molecular dynamics). Adopted from [35] with copyright permission

calculations. There exist various QM techniques, which vary in their approach to solve the Schrödinger equation and in their level of approximations made (see more details in [35]). For plasma medicine applications, density functional theory (DFT) is the most appropriate, in view of the required system sizes. DFT calculations are, however, still very time-consuming, so they can handle standard system sizes in the order of 100 atoms. DFT-based MD calculations, also called "*ab initio* MD" (AIMD) can handle time scales in the order of picoseconds. Note that in MD simulations, all atoms in the system are followed through space and time, by integrating their equations of motion, and the forces acting on the atoms are obtained as the derivative of some suitable interatomic potential, which can be based on QM data (like in AIMD), but also based on classical fitting parameters (like in classical MD; see below).

Somewhat larger systems can be handled with the density functional tight binding (DFTB) method, which is an approximate DFT method, based on a Taylor series expansion of the DFT total energy expression [36]. Typically, it can handle a few thousand atoms on time scales of tens of picoseconds. DFTB has been applied in the context of plasma medicine to study the interaction of ROS with the head group of the PLB [32], a specific protein (P-glycoprotein) [37] and peptides [38], as well as the behaviour of O and OH in water [39].

Classical reactive MD simulations, which are based on classical force fields, can typically handle much larger systems and longer time scales compared to DFT or DFTB calculations, ranging from 10^4 to 10^6 atoms, at a time scale in the order of 1 ps to 100 ns, depending on the complexity of the interatomic potential. This (classical) potential is typically based on a large number of parameters that can be obtained by fitting against DFT calculations. Two examples of reactive potentials that have been used already for plasma medicine applications, are the Brenner potential [40] and the ReaxFF potential [41]. Reactive MD simulations can describe bond breaking and formation, so they can study chemical reactions of plasma species with biomolecules. This has been applied already for the interaction of ROS with peptidoglycan [42,43], lipid A [44], lipids [45−47], DNA [48,49], a water layer [50] and simple organic molecules in water [51].

While reactive classical MD simulations are already much faster than QM calculations, they still require a long calculation time and are thus limited to relatively short time scales. Non-reactive MD simulations, also called "molecular mechanics" (MM), can handle system sizes and time scales two orders of magnitude larger than reactive MD, hence, in the order of 10^6–10^8 atoms, at time scales of 0.1 ns to 10 μs, for so-called "all-atom force fields". Indeed, in this type of simulations, the molecular connectivity in the system is fixed, so in contrast to reactive MD, the bond order of each bond must not be recalculated in every step. For the same reason, however, it cannot describe bond formation and bond breaking, but it allows to follow the system over a longer time scale, to study conformational changes, stability, etc.

Besides all-atom force fields, where all atoms in the system are treated separately, non-reactive MD simulations can also make use of so-called "united-atom" and "coarse-grained" force fields, which can handle even larger system sizes (typically up to one order of magnitude larger), for the same time scales. In united-atom force fields (e.g., [52]), all heavy atoms are treated separately, but the H atoms bound to a C atom are combined and treated as one (methyl or methylene) group. This is for instance the case for the apolar tails of phospholipids (see next section). Hence, the number of separate particles in the system is reduced, allowing to simulate larger systems. Some well-known non-reactive interatomic potentials are AMBER [53], CHARMM [54] and GROMOS [55]. This review paper mainly presents results from non-reactive MD simulations (see next section).

Moreover, in a coarse-grained method, the atoms comprising entire functional groups, i.e., typically, 3−5 heavy atoms with their H atoms, are represented by coarse-grained particles, which further reduces the number of particles in the system, and thus speeds up the calculations or allows larger system sizes. An example is the Martini force field [56].

Finally, as each of these methods has its strengths and limitations, in terms of accuracy or type of information that can be obtained, as well as time scale and system size that can be handled (see Fig. 1), it is also possible to combine these methods in so-called QM/MM methods [57]. In this case, a small (chemically most relevant) part of the system (e.g., the active site of the biological system) will be described at the quantum chemical (electronic) level, while the surrounding embedding atoms and molecules are treated at a classical (atomic) level.

More details on these methods, and examples of their simulation results for plasma medicine, can be found in [35,58]. In the following, we will focus on typical simulation results, mostly obtained from non-reactive MD, for the permeation of RONS across the cell membrane, which is relevant for cancer treatment by plasma.

3 Permeation of RONS across the cell membrane: Examples of calculation results

3.1 Different behavior of hydrophilic and hydrophobic RONS: Oxidized *vs.* non-oxidized cell membranes

Figure 2 depicts the free energy profiles (FEPs) of various ROS and RNS across both native and 50% oxidized PLBs, assuming oxidation of the lipid tails into aldehyde (see details in [34]). These FEPs are obtained by umbrella sampling simulations, as explained in detail in [34]. The

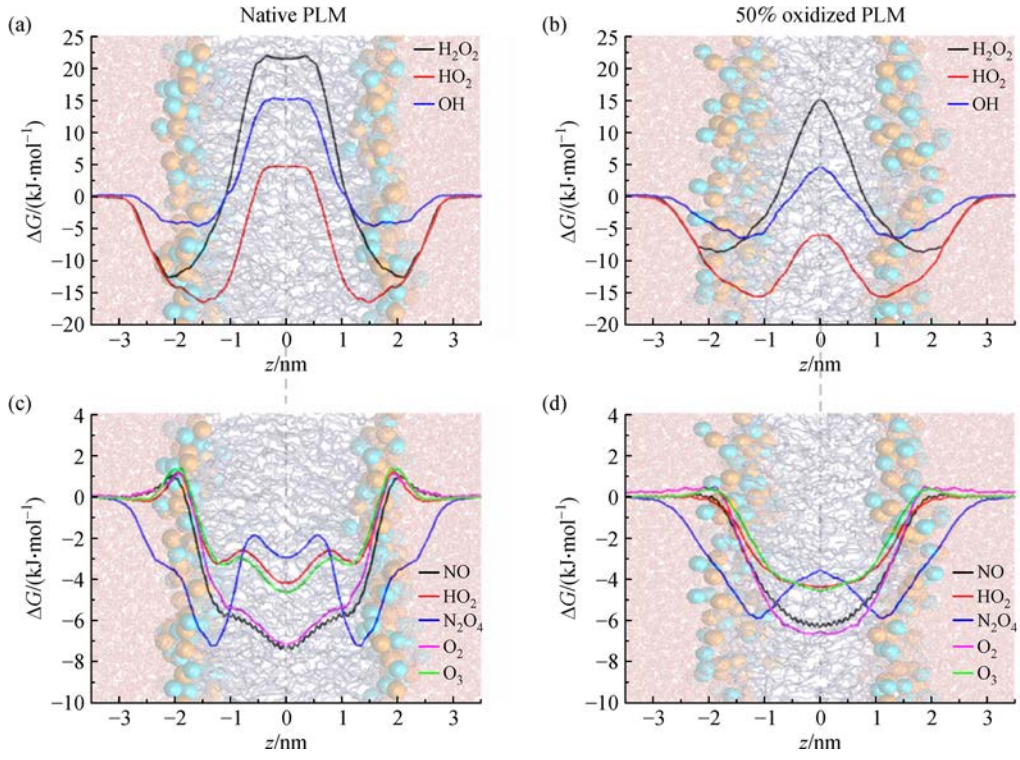

Fig. 2 FEPs of the hydrophilic (a,b) and hydrophobic (c,d) ROS and RNS, across native and 50% oxidized PLBs. The PLB structure is drawn in pale color at the background, to indicate the position of the water layer, head groups and lipid tails

structure of the PLB, as model system for the cell membrane, is drawn in pale color behind the FEPs. The center of the PLB is at $z = 0$, the head groups are around $z = \pm 2$ nm, and beyond this distance is the water phase surrounding the PLB. Although the cell membrane consists of both lipids and proteins, which contribute each for about 50% to the mass of the cell membrane, we only consider the lipids here, as they play a crucial role in the structure of the bilayer.

When the hydrophilic ROS (i.e., OH, HO_2, H_2O_2) move from the water phase to the PLB center, their FEP first decreases, reaching a minimum around the head groups, followed by a steep rise towards the center, hence showing a clear energy barrier when crossing the PLB. This free energy barrier is significantly reduced upon oxidation of the PLB (cf. Figs. 2(a,b)), which is logical because oxidation increases the hydrophilicity of the PLB, thus increasing the permeability of hydrophilic ROS. The differences in the FEPs of these ROS are explained in [34].

It is clear that these hydrophilic ROS prefer to reside close to the head groups, also in case of the oxidized PLB. For this reason, we recently studied oxidation of the head groups of the PLB, by means of DFTB [32], and we found that HO_2 and H_2O_2 molecules do not react with the head groups and only show weak attractive non-bonded interactions, while OH radicals do react with the head groups, leading to detachment of some parts in the PLB, and hence in a drop in the lipid order and rising membrane fluidity, in agreement with experiments [32]. It should be noted that OH radicals react with virtually all biomolecules and this chemistry is thus only relevant if the OH radicals are generated in very close vicinity to the target (here the membrane) due to their small diffusion distance. The drop in lipid order due to detachment of some parts in the PLB might allow RONS to penetrate more easily through the PLB, causing further lipid tail (per)oxidation, which might give rise to pore formation (see next section).

The hydrophobic ROS and RNS exhibit a completely different behavior from the hydrophilic ROS (cf. Figs. 2(c, d)), with very low permeation barriers around the PLB head groups and minima in the center (compared to the water phase). This indicates that these species prefer to reside in the lipid tail region, where they can cause lipid (per)oxidation. This is most pronounced for O_2 and NO, which are virtually non-polar. The small differences between these ROS/RNS are explained in detail in [34]. These FEPs do not change drastically upon oxidation of the PLB, except that they become smoother, which is attributed to the higher membrane fluidity [34]. These simulation results agree well with experimental observations, where the permeability of hydrophobic RONS (NO and O_2) was found to be 3−6 orders of magnitude higher than the permeability of hydrophilic ROS (H_2O_2) [59,60]. It is thus clear that pores or AQP channels are needed for the active transport of hydrophilic ROS in and out of the cell, as will be illustrated in next sections, while for

hydrophobic RONS, transmembrane transport may easily take place even in the absence of AQP channels and pores. Note that hydrophilic RNS, like HNO_2, HNO_3/NO_3^- and ONOOH, might behave the same as the hydrophilic ROS, but their permeability across the PLB could not yet be studied, due to unavailability of the required force field for the simulations.

3.2 Effect of cholesterol in the cell membrane

It is known that the cell membrane of some cancer cells (e.g., leukemic cells) has a lower cholesterol-to-phospholipid ratio compared with normal counterparts (such as lymphocytes) [61]. Hence, to investigate whether this can provide an explanation to the selective action of plasma on cancer cells *vs.* normal cells, we studied the effect of cholesterol, present in various concentrations in the cell membrane, on the FEPs of various (hydrophilic and hydrophobic) ROS [12].

Figure 3 (upper part) illustrates the FEP of H_2O_2 for various cholesterol concentrations. The presence of (higher concentrations of) cholesterol yields a clear rise in both the free energy barrier height and width, and it also causes the formation of a local free energy minimum in the center of the PLB. The latter will hamper the penetration of H_2O_2 towards the intracellular environment, even when it would succeed to penetrate into the PLB center. The same behavior was observed for OH and HO_2 [12]. Nevertheless, even the FEP of the system without cholesterol exhibits a too high barrier for H_2O_2 (and other hydrophilic ROS) to penetrate through the membrane, as shown in previous section, indicating the need for pore formation or the presence of AQP channels in the cell membrane (see below).

The lower part of Fig. 3, however, shows that the FEP of O_2 exhibits a minimum in the center, as was also illustrated in previous section. Nevertheless, some extra free energy barriers are created upon increasing cholesterol concentration at around 1 nm from the center of the PLB. They are attributed to the presence of the bulky sterol rings, and they

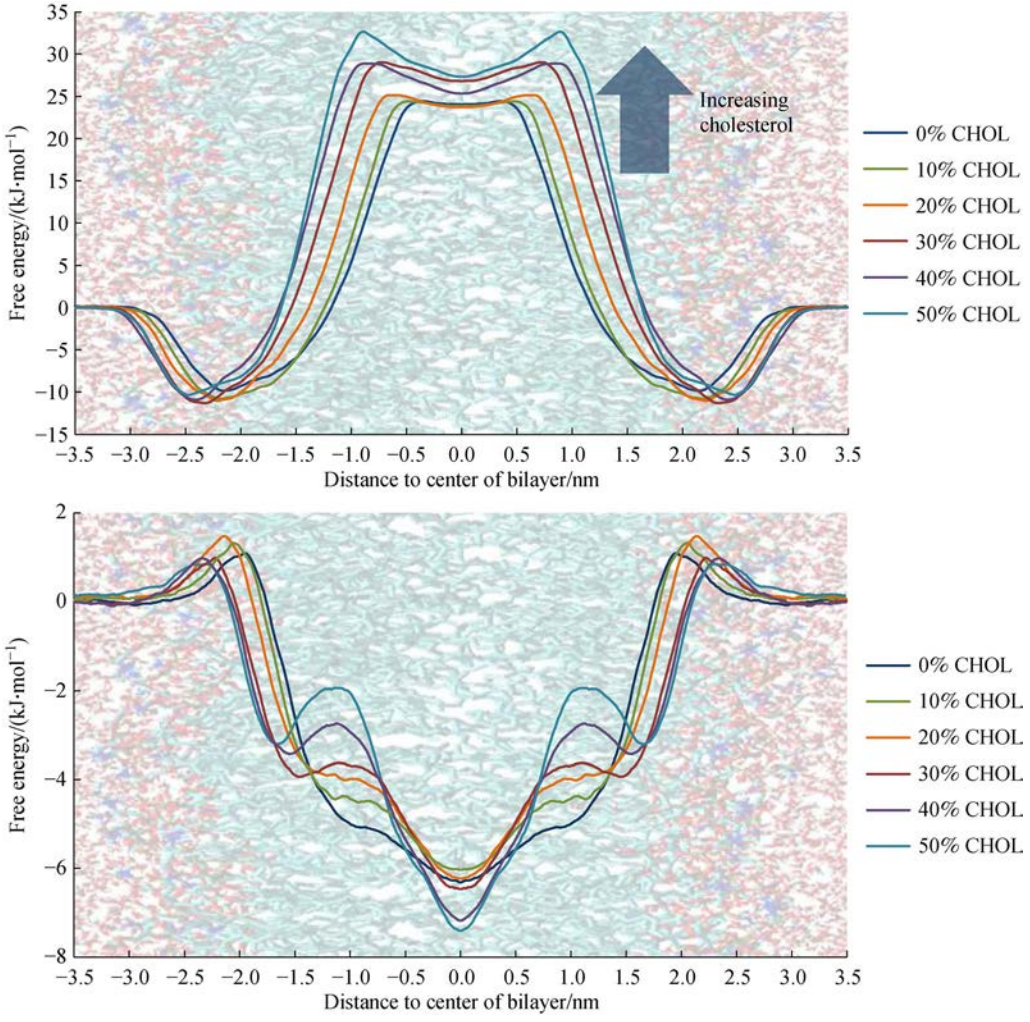

Fig. 3 FEPs of H_2O_2 (upper part) and O_2 (lower part) across the PLB, for various cholesterol concentrations in the cell membrane. Adopted from [12] with permission

will drastically reduce the probability of lipid (per) oxidation of the lipid tails, and thus of pore formation. This might explain why it is more difficult for RONS to reach the cell interior of normal cells, due to their higher cholesterol fraction in the cell membrane, and thus, why plasma treatment is more selective towards cancer cells.

3.3 Pore formation in the cell membrane

It is clear from above that hydrophilic RO(N)S cannot easily penetrate though the PLB, due to their high free energy barriers, so we also investigated pore formation in the cell membrane after lipid (per)oxidation, for various concentrations of cholesterol [11]. To study the effect of lipid (per)oxidation, some lipid (per)oxidation products, based on data from literature [62], were added to the model systems, with concentrations varying between 0 and 100% (see details in [11]).

We analysed typical properties of the PLB, as a function of increasing lipid oxidation degree and increasing cholesterol fraction, such as the surface area per lipid, the thickness of the PLB, the water density inside the PLB (used as a measure for the polarity inside the membrane), and the so-called deuterium order parameter, which is a measure for the order of the lipid tails in the PLB (see details in [11]). The PLB thickness was found to drop upon oxidation, followed by a rise when the oxidation approached 100%, and this was attributed to pore formation. This is illustrated in Fig. 4 for a model system without cholesterol and 100% lipid oxidation. The initial conformation (after 10 ns) does not exhibit water defects (Fig. 4(a)). After 40 ns (Fig. 4(b)), a significant amount of water is present in the center of the PLB, and after 80 ns (Fig. 4(c)), a pore with diameter of 15 Å is formed, which might allow RONS to penetrate and reach the cell interior. The thickness of the PLB is also indicated in the figure. Pore formation allows water to enter the PLB, resulting in swelling, and thus in a somewhat thicker bilayer (cf. Fig. 4 (c) *vs.* Figs. 4(a,b)). Similar conclusions were also drawn from other MD simulations in literature on oxidized PLBs [8−10], reporting an overall increase in the membrane permeability [8], a change in the lipid mobility in the PLB [9], and pore creation and bilayer disintegration [10] upon introduction of oxidized lipids.

When comparing model systems for various cholesterol fractions, to investigate the possible difference between normal and cancer cells, we found that for cholesterol fractions above 15%, the cell membrane fluidity did not increase to the same extent, and no pore formation was observed [11], as is clear from Fig. 5, plotting the water density in the center of the PLB in case of 100% oxidation, as a function of the fraction of cholesterol in the bilayer. For a cholesterol concentration up to 11%, the water density is significant, due to pore formation, while a higher cholesterol concentration results in a significant drop in the water density, indicating inhibition of pore formation. Because some cancer cells contain less cholesterol in their cell membrane than normal cells, as mentioned above, it means that RONS might more easily penetrate through their cell membrane, giving rise to oxidative stress inside the cell. Hence, this might provide one of the explanations why plasma can selectively treat cancer cells, while leaving the normal cells undamaged. Another plausible reason, i.e., the higher expression of AQPs in the cell membrane of cancer cells, will be discussed in section 3.5 below.

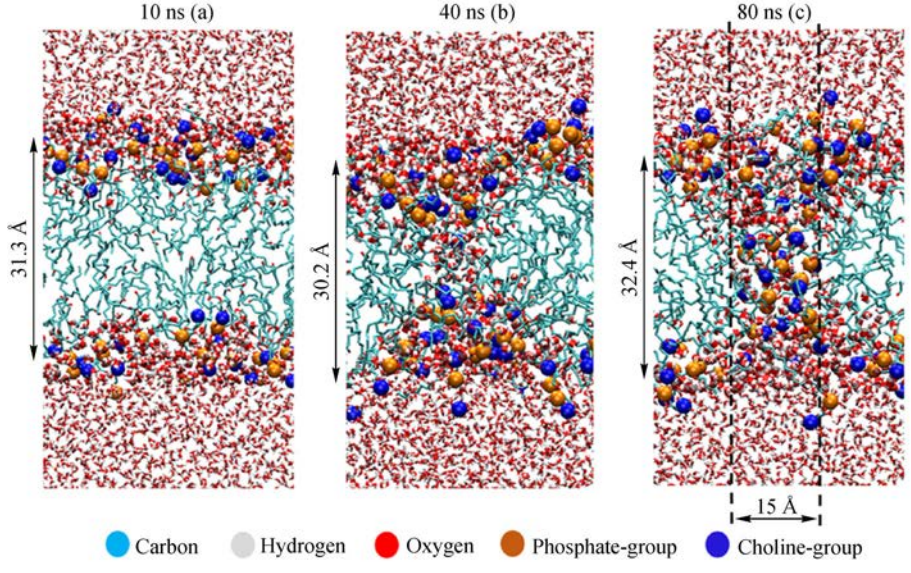

Fig. 4 Snapshot of MD simulations, at (a) 10 ns, (b) 40 ns and (c) 80 ns, illustrating pore formation in a model system of a PLB without cholesterol and 100% oxidation. A pore with diameter of ca. 15 Å is formed in (c). Adopted from [11] with permission

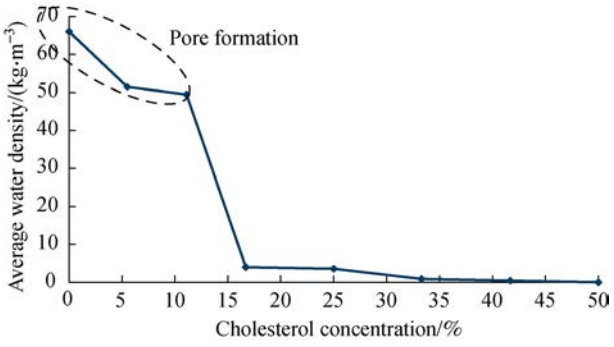

Fig. 5 Calculated average water density in the center of the PLB, for model systems with 100% oxidation, as a function of cholesterol concentration in the PLB, indicating that pore formation occurs more easily in cell membranes containing less cholesterol, which is typical for cancer cells. This might be one of the explanations of the selectivity of plasma treatment for cancer cells *vs.* normal cells. Adopted from [11] with permission

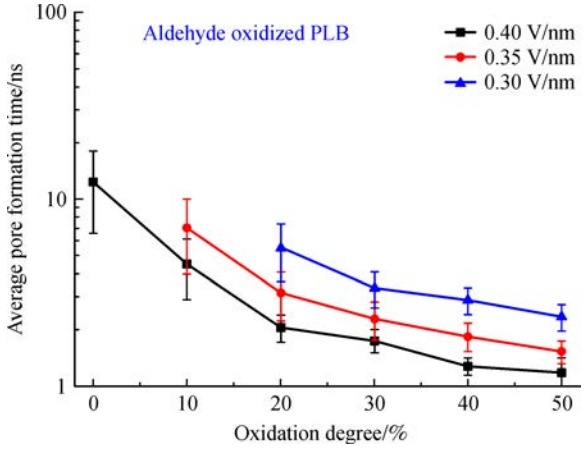

Fig. 6 Average pore formation time for three different electric field values, as a function of the oxidation degree of the PLB, for lipid oxidation into aldehydes. Adopted from [31] with permission

3.4 Synergistic effect of electric field and lipid oxidation on pore formation

Some biomedical plasma sources do not only create RONS, but also strong electric fields, ranging from a few up to 100 kV/cm (see e.g., [14−16]). This might be high enough to induce pore formation in membranes, i.e., so-called electroporation [18−20], and maybe cause synergistic effects in combination with plasma-induced lipid oxidation. As shown in section 3.2 above, lipid oxidation results in a higher permeability of hydrophilic ROS across the PLB, but the permeation free energy barriers are still too high for spontaneous permeation. However, we studied the combined effect of lipid oxidation and electric fields, and we observed that lipid oxidation into aldehydes causes a drop of the electric field threshold needed for pore formation in the PLB, as well as a shorter average pore formation time [31] (Fig. 6). As pore formation is a stochastic process, the pore formation time can fluctuate considerably, explaining the large error bars in the figure, which cannot be reduced by increasing the number of simulations. Nevertheless, the effect of the oxidation degree is clearly visible.

Note that the applied electric fields in electroporation simulations are much higher than the fields used in electroporation experiments or in plasma medicine applications [14−16], which typically vary between 0.1 and 100 kV/cm (or between 0.01 and 10 mV/nm). However, the macroscopic field applied in experiments is not at all equivalent to the field that is felt by the membrane (and which is applied in MD simulations), and thus, these values should not be directly compared, as explained in detail in [31]. Furthermore, the average pore formation times obtained in MD simulations cannot be directly related to the experimental pore formation kinetics either, as also explained in [31]. However, the trends of pore formation times for different values of electric fields and oxidation degrees presented in Fig. 6 clearly indicate that oxidation of the lipid tails in the PLB facilitates pore formation, by lowering the threshold electric field, as well as the pore formation time, thus clearly illustrating the synergistic effect of the electric field together with lipid oxidation on the permeability of cell membranes.

3.5 Permeability through aquaporins

As mentioned above, another plausible explanation for the selective action of plasma on cancer cells *vs.* normal cells is the higher expression of AQPs in the cell membrane of cancer cells, which are known as H_2O_2 channels [4−7]. We therefore calculated the FEPs and the diffusion rate profiles of H_2O_2 across a model AQP, i.e., AQP1, to determine its permeability coefficient through AQP, in comparison with the PLB [33].

Figure 7 depicts the FEPs of H_2O_2 through both AQP1 and the PLB. Details can be found in [33]. The free energy barrier for H_2O_2 transport through AQP1 is ca. three times lower than through the PLB. As a consequence, the permeability coefficient of H_2O_2 across AQP1 was calculated to be more than two orders of magnitude higher than through the PLB, i.e., 2.57 cm/s *vs.* 6.62×10^{-3} cm/s. Thus, AQP creates a more favorable pathway for H_2O_2 permeation, as explained in detail in [33], and this might explain the selectivity of plasma against cancer cells.

4 Conclusions and future research directions

In this feature article, we illustrated some calculation results that provide more insight in the permeability of

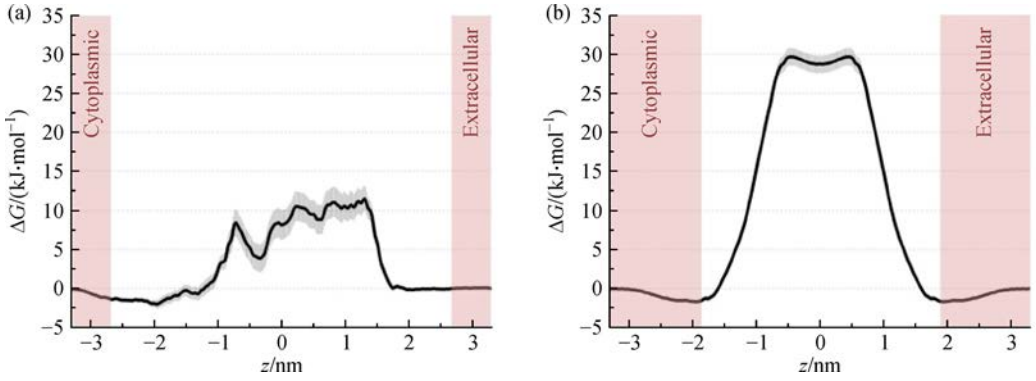

Fig. 7 FEPs of H_2O_2 across (a) AQP1 and (b) the PLB. The cytoplasmic and extracellular water layers are shown in pink color. The associated standard deviations of the FEPs are shown in grey

various RONS across the PLB, as model system for the cell membrane, for both native and oxidized structures, as well as the effect of cholesterol present in the cell membrane, the synergistic effect of lipid oxidation and electric fields, and the different permeability across AQP channels *vs.* the PLB.

We showed that hydrophobic RONS, like NO, NO_2, N_2O_4, O_2 and O_3, can significantly better penetrate across both native and oxidized PLBs, compared to hydrophilic ROS, such as OH, HO_2 and H_2O_2, as they have much lower free energy barriers. Oxidation of the PLB does not strongly affect the FEPs of the hydrophobic RONS, but it significantly reduces the barriers of OH, HO_2 and H_2O_2, thus increasing their translocation probability across oxidized PLBs. However, the energy barriers for permeation of these hydrophilic ROS across the PLB still remain relatively high, indicating the need for specific protein channels (e.g., AQPs) or pores created by an electric field, to allow their penetration into the cytoplasm, eventually to cause oxidative damage.

We also demonstrated that lipid oxidation can lead to pore formation, and it also reduces the threshold electric field needed for pore formation, as well as the characteristic poration time, pointing towards the synergistic effect of lipid oxidation and electric fields, which are both induced by plasma.

In addition, we illustrated that cholesterol, which might be present in higher concentrations in the cell membrane of normal cells than cancer cells, causes a drop in the RONS permeation ability, as well as in the probability of pore formation in the cell membrane. This might be one of the explanations for the selectivity of plasma towards cancer cells *vs.* normal cells, as some cancer cells have lower cholesterol in their cell membrane than their normal counterparts.

Finally, we compared the FEP of H_2O_2 across both AQP and the PLB. Our calculations predict three times lower energy barriers for penetration of H_2O_2 through AQP than through the PLB, resulting in a permeability coefficient across AQP1 being more than two orders of magnitude higher than through the PLB. This clearly illustrates that AQP creates a more favorable pathway for H_2O_2 permeation, and thus, it can also explain the plasma selectivity towards cancer cells, as the latter have a higher AQP expression in their cell membrane. Nevertheless, it needs to be mentioned that the selective action of plasma towards cancer cells is not always observed. Furthermore, although H_2O_2 is an important molecule generated by plasma, it is not the only important RONS, and it is the cocktail of RONS (including both long-lived and short-lived species), combined with other plasma-effects, which makes plasma promising as new anti-cancer therapy.

These simulations give more insight in how RONS can penetrate through the cell membrane, either by passive transport (for hydrophobic RONS) or through pores or AQP channels (for hydrophilic RONS), as well as the combined effects of lipid oxidation and the electric field, both induced by plasma. Hence, they are very valuable for a better understanding of plasma treatment of cancer cells.

However, more research is obviously needed to elucidate all mechanisms how plasma-induced RONS can enter the cell. In future work, we want to investigate the behaviour of more complex cell membrane structures, including the role of other membrane proteins besides AQP, such as antiporters. We also want to study in more detail the combined effect of plasma-induced oxidation and electric fields on the permeability across AQP, to further understand the selective action of plasma towards cancer cells. Moreover, we need to gain more insight in the permeability of other RONS, not investigated up to now, across the cell membrane. Indeed, we could not yet describe the behaviour of hydrophilic RNS, like HNO_2, HNO_3/NO_3^- and ONOOH, because no accurate force fields are available yet for these simulations. Hence, there is a clear need to develop such force fields, to obtain a more comprehensive picture of the behaviour of all possible RONS in the cell membrane. In addition, these various RONS might create a myriad of different lipid oxidation

(and nitration) products in the cell membrane, and more knowledge is needed on these different products formed, and how they affect the biophysical properties of the cell membrane, and thus its function. Indeed, we already showed that different oxidation products may affect the degree of packing of the cell membrane in different ways [32]. Up to now, simulations of oxidized membranes were based on rather simplified descriptions of membrane composition, while it has been demonstrated that lipid peroxidation might lead to liquid ordered-liquid disordered phase separation in membranes [63], and this might favor pore formation. Hence, in future work, we want to look in more detail at phase-separated membranes.

Finally, it would be interesting if experiments can be designed to provide molecular-level validation of these model predictions. To realize this, very controlled conditions would have to be pursued, generating for instance only a beam of OH radicals, instead of a complex mixture of RONS and other plasma effects, as well as well-defined model systems of biomolecules, gradually mimicking the more complex tissues. Various labs are performing such experiments, investigating for instance the separate and synergistic effects of plasma-generated radicals and UV/VUV photons at the cellular and molecular level for various kinds of biomolecules, or experiments with simple model systems for the cell membrane, based on synthetic phospholipid membrane vesicles or liposomal model membranes (e.g., [64−74]). We believe that the combination of such experiments and modelling is needed to obtain a deeper understanding of the underlying mechanisms of plasma medicine.

Acknowledgements We acknowledge financial support from the Research Foundation–Flanders (FWO; Grant Nos. 1200216N and 11U5416N). The computational work was carried out using the Turing HPC infrastructure at the CalcUA core facility of the Universiteit Antwerpen (UA), a division of the Flemish Supercomputer Center VSC, funded by the Hercules Foundation, the Flemish Government (department EWI) and the UA. We are also very thankful to R. Cordeiro for the very interesting discussions.

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