


FULL PAPER

Phosphatidylserine flip-flop induced by oxidation of the plasma membrane: a better insight by atomic scale modeling

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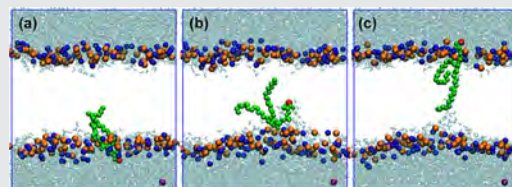
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We perform molecular dynamics simulations to study the flip-flop motion of phosphatidylserine (PS) across the plasma membrane upon increasing oxidation degree of the membrane. Our computational results show that an increase of the oxidation degree in the lipids leads to a decrease of the free energy barrier for translocation of PS through the membrane. In other words, oxidation of the lipids facilitates PS flip-flop motion across the membrane, because in native phospholipid bilayers this is only a “rare event” due to the high energy barriers for the translocation of PS. The present study provides an atomic-scale insight into the mechanisms of the PS flip-flop upon oxidation of lipids, as produced for example by cold atmospheric plasma, in living cells.

**KEYWORDS**

membranes, molecular dynamics, oxidation, permeability, peroxides

1 | INTRODUCTION

The cellular antioxidant mechanism maintains a redox homeostasis by preventing a build-up of reactive oxygen species (ROS), which endanger cellular health due to their oxidizing properties.^[1] Whenever the redox homeostasis is compromised, either by antioxidant depletion or the increased presence of ROS, the cell enters a state of oxidative stress.^[2] If excessive amounts of oxidative stress are presented to the cell, it will undergo programmed cell death, that is, apoptosis. This is exploited for example when treating cancer cells with radiotherapy,^[3] or with an emerging technique that uses ionized gases at room temperature, so-called non-thermal atmospheric pressure plasmas (NTAPPs).^[4]

Apoptosis can be the result of many different harmful attacks to cellular health, of which oxidative stress is one example, and it results in the phagocytic uptake of dead cells, which are fragmented into apoptotic bodies with intact plasma membranes.^[5] To facilitate the uptake, apoptotic cells

will present various markers on their external plasma membrane, which function as “come get me” and “eat me” signals.^[6] One of these markers is phosphatidylserine (PS), a lipid normally situated in the inner leaflet of the plasma membrane.^[7] If the apoptotic pathway is activated, PS will flip from the inner leaflet into the outer leaflet through a flip-flop mechanism and act as an “eat me” signal for lymphocytic cells.^[8] While the effect of PS flip-flop is known, and it is suggested to occur due to the action of scramblases,^[9] the mechanism remains to be unraveled,^[10] although there are some studies trying to explain this mechanism by means of so-called push-in, sliding or rotational flip-flop models^[11] (see below).

Apart from activating apoptosis, excessive oxidative stress can also oxidize the membrane lipids,^[12] which increases the rate of PS flip-flop, as shown by Volinsky et al.^[13] Through molecular dynamics (MD) simulations, they observed a reduction of $20 \pm 5 \text{ kJ mol}^{-1}$ in the flip-flop energy barrier in the presence of 20% oxidized

phosphatidylcholine in the lipid bilayer.^[33] The same effects were also qualitatively observed by means of fluorescence spectroscopy of oxidized liposome bilayers.^[13] However, although this study presents valuable knowledge on the influence of oxidized membranes on the PS flip-flop, the authors only compared non-oxidized membranes to a membrane with a fixed amount of oxidation.^[13] Hence, the impact of increasing levels of membrane oxidation on the PS flip-flop energy barrier has not been characterized by atomic level simulations up to now.

In this paper, we therefore study the flip-flop motion of PS across the plasma membrane as a function of increasing oxidation degree of the membrane. For this purpose, we calculate the free energy profiles of PS flip-flop across the membrane. The aim of this study is to reveal the atomistic details of the PS flip-flop mechanism, and to link it to the oxidative damage of the plasma membrane, which eventually may facilitate the flip-flop motion of PS.

2 | SIMULATION METHOD

2.1 | Simulation setup

To study the flip-flop motion of PS across the (oxidized) membrane, we perform MD simulations based on the GROMOS (43A1-S3) force field.^[14] The parameters for peroxide groups in the oxidized phospholipids (PLs, see Figure 1(b)) are obtained from ref.^[15] As a model system we use the phospholipid bilayer (PLB) shown in Figure 1(a), representing the cell membrane. It consists of 128 PLs arranged in two leaflets and covered with 5120 water molecules on top and bottom layers. The PL investigated is palmitoyl-oleoyl-phosphatidylcholine (POPC) presented in Figure 1(b).

The bottom leaflet contains a single palmitoyl-oleoyl-phosphatidylserine (POPS) molecule replacing one of the PLs, so it contains 63 POPC molecules and one POPS molecule (see Figure 1[a]). Note that we simply write PS instead of POPS in the discussion below, for the sake of simplicity.

To study the effect of the oxidized PLs, the oxidation product of POPC, that is, a peroxide, is included in the simulated systems (see Figure 1[b]). This peroxide was chosen because it is one of the two major oxidation products of POPC^[16] and it is the end product of the main lipid peroxidation reaction. To create membranes oxidized to various degrees, 12.5, 25, 37.5, or 50% of the POPC molecules in the native PLB structure are replaced by the peroxidized PLs.

The initial configuration of each simulation system is created using the Packmol package.^[17] For each peroxidation degree (i.e., 12.5, 25, 37.5, and 50%), six different structures are created, placing PS at a random position in the x and y direction, while keeping its z position unchanged (cf. Figure 1

[a]). In order to neutralize the negatively charged PS, a sodium cation shown in Figure 1(a) is placed in the water phase of the system and is fixed in all three directions to avoid its interaction with PS. Periodic boundary conditions are applied in all three directions.

The simulations are performed in the NPT ensemble by applying the semi-isotropic Parinello-Rahman barostat^[18] and the Nose-Hoover thermostat.^[19] The temperature of the systems is kept fixed at 300 K using a relaxation time of 0.2 ps.^[20] The applied reference pressure is one atmosphere combined with a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$ and a coupling constant of 1 ps. For the non-bonded interactions, a 1.1 nm cut-off is applied for the van der Waals interactions. The long range electrostatic interactions, on the other hand, are described by the particle mesh Ewald (PME) method,^[21] using a 1.1 nm cut-off for the real-space interactions in combination with a 0.15 nm spaced-grid for the reciprocal-space interactions and a fourth-order B-spline interpolation.

All simulation systems (i.e., in total 30, including the native PLB) are optimized using the steepest descent algorithm and then equilibrated for 150 ns in the NPT ensemble. Subsequently, umbrella sampling (US) simulations^[22] (see below) are run for 90 ns applying again the NPT ensemble, of which the last 20 ns is used for further analysis. In all simulations a time step of 2 fs is used. All simulations and analyses mentioned in this study are performed using the GROMACS 5.1 package.^[23]

2.2 | Umbrella sampling simulation

As mentioned above, the US simulations are performed in order to determine the free energy profiles of PS translocation across the native as well as peroxidized PLBs. For each energy profile, we extract 42 windows along the z-axis, which are separated by 0.12 nm. These windows are obtained by pulling one of the oxygen atoms on the head group of PS (see red color in Figure 1[a] and cf. Figure 1[b]) along the z-axis for 500 ps, using a harmonic bias between this atom and the center of mass of the PLB, with a force constant of $1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ and a pulling rate of 0.01 nm ps^{-1} . Each window is then equilibrated for 90 ns, and the last 20 ns are used for analysis, that is, to collect the US histograms. Free energy profiles are constructed using a periodic version of the weighted histogram analysis method (WHAM),^[24] as implemented in GROMACS.^[25] The final energy profiles are obtained by averaging over six US runs for each system, which differ from one another based on their starting structure, to allow for some statistical variations. Subsequently, the energy barriers with associated standard deviations (see Figure 4[a] below) are obtained by calculating the difference between energy minimum and maximum in each energy profile and averaging them again over six US simulations. Thus, in total 1260 US simulations are performed for the calculation of the free energy profiles.

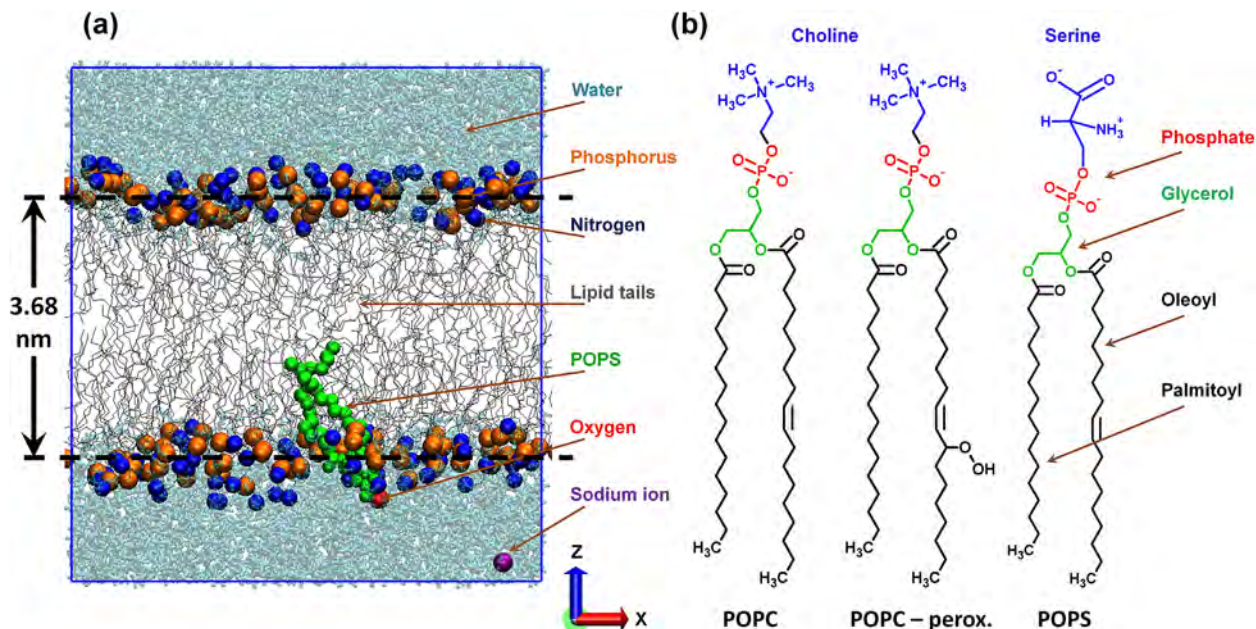


FIGURE 1 (a) Native POPC PLB, together with a single POPS at the bottom layer. The P and N atoms of POPC are depicted with bigger beads, for the sake of clarity. The simulation box is presented by the blue rectangle. (b) Schematic illustrations of the native (POPC) and peroxidized (POPC-perox.) PLs as well as POPS. The color legend also applies to the other similar figures below

3 | RESULTS AND DISCUSSION

Figure 2 shows an example of the pulling simulation, that is, the PS transition from the inner leaflet to the outer leaflet under the applied force. It is clear that the transition of PS from one leaflet to the other is accompanied with the formation of a narrow water channel (see Figure 2[b,c]). This is in agreement with other MD studies,^[26] where the authors showed the lipid flip-flop motion to occur by means of water channels, that is, along hydrophilic pores created in the membrane. Analysis of the mechanism of the PS flip-flop motion shows that the translocation of PS begins with changing of the orientation of its hydrophilic head, followed by the rotation of PS, entering into the hydrophobic core of the bilayer, which is beyond its equilibrium position (see Figure 2[b]). This is accompanied

with the formation of spontaneous water defects around the headgroup of PS. Finally, this bidirectional motion of PS ends up with joining the opposite leaflet (see Figure 2[c]).

The observed trajectory of the PS translocation corresponds to one of the three mechanisms suggested in,^[11] that is, to the “rotational flip-flop” mechanism. In summary, the flip-flop motion of PS takes place in the presence of water pores in the PLB. This indicates that the occurrence of spontaneous lipid flip-flop is energetically unfavorable, as also evidenced below. In general, the flip-flop motion of lipids directly depends on the acyl chain length and the structure of the headgroup of the PLB.^[27] Moreover, the oxidation of lipids in the PLB also plays an essential role in the occurrence of lipid flip-flop motion.^[13] This is what we will now study in more detail.

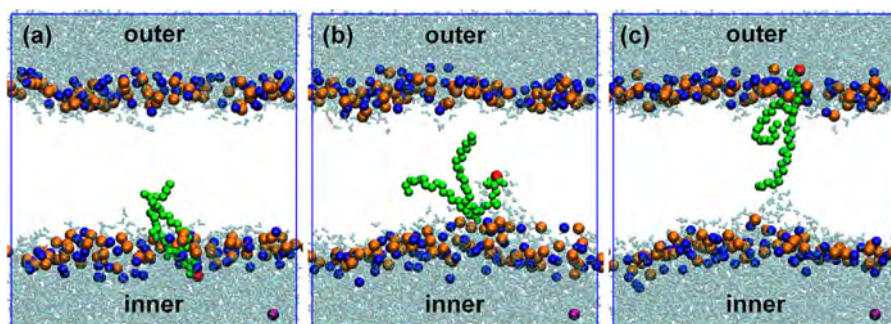


FIGURE 2 Snapshots from MD simulations, illustrating the PS flip-flop motion from the inner leaflet to the outer leaflet of the native PLB in time frames of (a) 0 ps (cf. Figure 1[a]), (b) 268 ps, and (c) 500 ps. The lipid tails of the POPC molecules are removed, to illustrate more clearly the presence of water molecules around PS during its flip-flop motion in (b,c). The color code is the same as in Figure 1(a). Note that (a), (b), and (c) correspond to the beginning, intermediate and final snapshots of a single pulling simulation

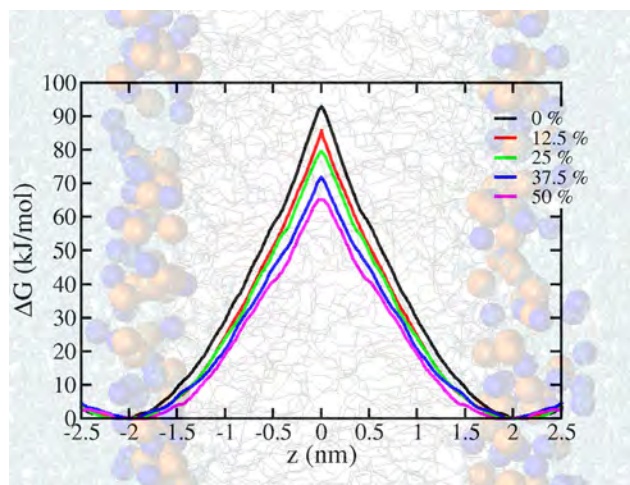


FIGURE 3 Symmetrized free energy profiles for the translocation of PS across the PLB with different peroxidation levels

The effect of lipid oxidation on the PS flip-flop motion is depicted in Figure 3. It illustrates the free energy profiles of PS translocation across a PLB with different peroxidation levels. Note that these profiles are calculated for the PS flip-flop motion from inner to outer leaflet (cf. Figure 2). However, our test simulations showed similar profiles for the flip-flop of PS from outer to inner leaflet, that is, within the standard deviations (the results are not given). This is probably attributed to the equal concentration of oxidized lipids assumed in both sides of the bilayer in our model. As is clear, the ΔG starts to rise when the PS moves toward the hydrophobic core and reaches its maximum at the center of the membrane. Moreover, the free energy barriers decrease by increasing the oxidation degree (see Figure 3). This is also obvious from Figure 4(a), where the calculated free energy barriers are plotted as a function of the oxidation degree. For the native PLB the calculated free energy barrier is $93 \pm 4 \text{ kJ mol}^{-1}$, which is within the range of free energy barriers given in literature (i.e., 75–120 kJ mol^{-1}).^[13,26a,26c,28]

As is evident, the free energy barriers linearly decrease upon increasing the oxidation degree. The energy barrier in the case of a 50% peroxidized PLB is $65 \pm 6 \text{ kJ mol}^{-1}$ (see

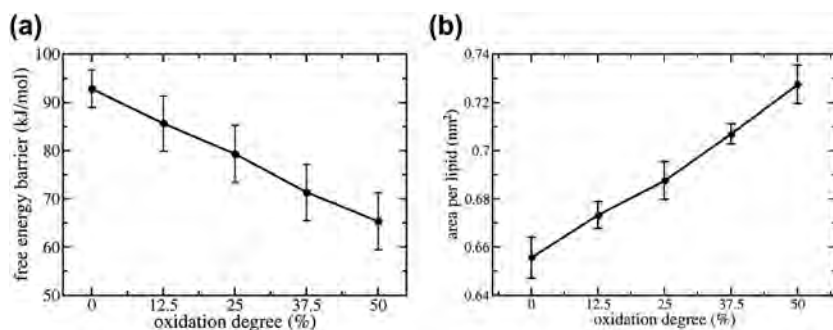


FIGURE 4 (a) Free energy barrier for the translocation of PS across a PLB, and (b) average area per lipid, both calculated as a function of the peroxidation degree of the PLB

Figure 4[a]), which means that it drops by $\sim 30\%$ compared to the native PLB. Moreover, even in low concentration (i.e., 12.5%, which is close to experimental values^[29]) a drop of the energy barrier by $\pm 7 \text{ kJ mol}^{-1}$ is observed, which indicates an increase of the flip-flop rate. Thus, we can conclude that the oxidation of the lipids in the PLB facilitates the flip-flop motion of PS by decreasing its free energy barrier. This is also observed in ref.,^[13] where the authors showed a drop in free energy barrier when the lipids are oxidized by 20%, although for a different oxidation product. Moreover, fluorescence measurements indicated that the flip-flop time of PS significantly decreases in oxidized POPC liposomes, that is, from >2 weeks (native) to ~ 9 h (oxidized).^[13] The authors suggested that the creation of transient pore-like defects is induced by lipid oxidation, which in turn facilitates the PS flip-flop motion.^[13] Thus, the bidirectional motion of lipids must be a pore mediated process, that is, it should occur through the water pores in protein free membranes.^[13] As is obvious from Figure 3, the calculated free energy barriers in our US simulations are still too high for PS translocation to occur across the bilayer, which indicates that hydrophilic pores are indeed necessary to enable the flip-flop motion of PS. Similar considerations were made in ref.[26c] It was reported that the calculated free energy barrier required for lipid flip-flop motion (i.e., 80 kJ mol^{-1}) is in good agreement with the energy of pore formation in experiments (i.e., 75–100 kJ mol^{-1}).^[26c] In summary, despite the fact that the oxidation of the PLs leads to a reduction of the energy barrier for the translocation of PS, there is still a need for water pores in order to traverse PS from the inner leaflet to the outer leaflet.

The drop in free energy barrier upon oxidation of the PLs can be explained by analyzing the calculated area per lipid in the PLB (see Figure 4[b]). It can be seen from the figure that the area per lipid increases linearly upon increasing the oxidation level. This is in good agreement with experimental as well as theoretical data.^[15,30] Indeed, Itri et al. clearly demonstrated by means of giant unilamellar vesicles that fully oxidized lipids lead to an increase of the total surface area of the bilayer by $\sim 14.5\%$.^[30] This was also shown in ref.^[15]

applying MD simulations, where a $\sim 15\%$ increase in area per lipid was observed for a 100% peroxidized POPC membrane.^[15] Our test simulations for a 100% peroxidized PLB also showed a 15% increase in area per lipid, which is in line with the above mentioned results.

The main reason for enlargement of the bilayer surface (and thus the area per lipid) is the bending of the polar oxidized groups from the hydrophobic core toward the water interface, as also described in literature.^[15,31] This increases the chance for deeper penetration of water molecules,

that is, toward the hydrophobic part of the membrane,^[32] thereby increasing the permeability of the bilayer. The latter leads to an increase of the probability of pore formation, as well as a decrease of the barrier for PS flip-flop, thereby enhancing the PS translocation rate.^[13]

4 | CONCLUSION

We performed US MD simulations, in order to study the PS flip-flop motion in the presence of different concentrations of lipid peroxides in a POPC membrane. We showed an expansion of the membrane area upon increasing the oxidation level of the lipids, which causes a noticeable change in the membrane permeability, in line with previous modeling results from our group^[32] and experimental results from literature.^[13,33] As a result, the energy barrier for PS flip-flop across the membrane drops upon increasing peroxidation level, which plays a vital role in apoptosis signaling. Note that PS translocation occurs quite naturally and spontaneously in a timescale of days, but this is far too long to be observed during our MD simulations. However, based on our MD results we can conclude that the drop of free energy barrier upon oxidation of the PLB makes PS flip-flop a “more frequent” event across the PLB.

This study is particularly interesting for plasma medicine, as plasma generates reactive oxygen species and electric fields, both of which can lead to (a) oxidation of the lipids, as well as (b) pore formation (i.e., electroporation) in the cell membrane, thereby increasing the PS flip-flop rate, which eventually results in apoptosis of cancer cells. In general, this study is of interest for applications where reactive oxygen species and strong electric fields both come into play.

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