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Reference:

Oliveira Maria Cecilia, Cordeiro Rodrigo M., Bogaerts Annemie.- Effect of lipid oxidation on the channel properties of Cx26 hemichannels : a molecular dynamics study

Archives of biochemistry and biophysics - ISSN 1096-0384 - 746(2023), 109741

Full text (Publisher's DOI): <https://doi.org/10.1016/J.ABB.2023.109741>

To cite this reference: <https://hdl.handle.net/10067/2002820151162165141>

Effect of Lipid Oxidation on the Channel Properties of Cx26 Hemichannels: a Molecular Dynamics Study

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ABSTRACT

Intercellular communication plays a crucial role in cancer, as well as other diseases, such as inflammation, tissue degeneration, and neurological disorders. One of the proteins responsible for this, are connexins (Cxs), which come together to form a hemichannel. When two hemichannels of opposite cells interact with each other, they form a gap junction (GJ) channel, connecting the intracellular space of these cells. They allow the passage of ions, reactive oxygen and nitrogen species (RONS), and signaling molecules from the interior of one cell to another cell, thus playing an essential role in cell growth, differentiation, and homeostasis. The importance of GJs for disease induction and therapy development is becoming more appreciated, especially in the context of oncology. Studies have shown that one of the mechanisms to control the formation and disruption of GJs is mediated by lipid oxidation pathways, but the underlying mechanisms are not well understood. In this study, we performed atomistic molecular dynamics simulations to evaluate how lipid oxidation influences the channel properties of Cx26 hemichannels, such as channel gating and permeability. Our results demonstrate that the Cx26 hemichannel is more compact in the presence of oxidized lipids, decreasing its pore diameter at the extracellular side and increasing it at the amino terminus domains, respectively.

The permeability of the Cx26 hemichannel for water and RONS molecules is higher in the presence of oxidized lipids. The latter may facilitate the intracellular accumulation of RONS, possibly increasing oxidative stress in cells. A better understanding of this process will help to enhance the efficacy of oxidative stress-based cancer treatments.

Keywords: Gap junctions, lipid oxidation, molecular dynamics, oxidative stress.

INTRODUCTION

Cell-cell interactions play a pivotal role in physiological processes, such as cell development, tissue and organ homeostasis, neurotransmission, and immune response [1]. Failure or improper interactions result in a large variety of pathologies, such as skin and hair disorders, cardiomyopathies, sensory defects, psychiatric disorders, and cancers [2]. In vertebrates, cell-cell interactions can be classified into four functional classes: (i) adherens junctions, (ii) desmosomes; (iii) communicating junctions (or gap junctions (GJs)); and (iv) occluding junctions (or tight junctions) [2]. For this work, we will focus on GJs cell-cell interactions, which chemically and electrically couple neighboring cells.

GJs consist of small gaps when two hemichannels (or connexons) of opposite cells interact with each other (Figure 1A), allowing an intracellular communication between these cells. An assembly of six transmembrane proteins called connexins (Cxs) results in the formation of a hemichannel [3] (Figure 1B). The human connexin (Cx) protein family contains 21 members, named according to their relative molecular mass, which can range from 23 to 62 kDa [4]. Each Cx is composed by four transmembrane (TM) domains in the α -helical conformation (TM1 to TM4). These domains are connected by two extracellular loops (EL-1 and EL-2) and one cytoplasmic loop (CL), containing an amino (NT) and carboxyl terminus (CT) in the cytoplasm [5] (Figure 1C).

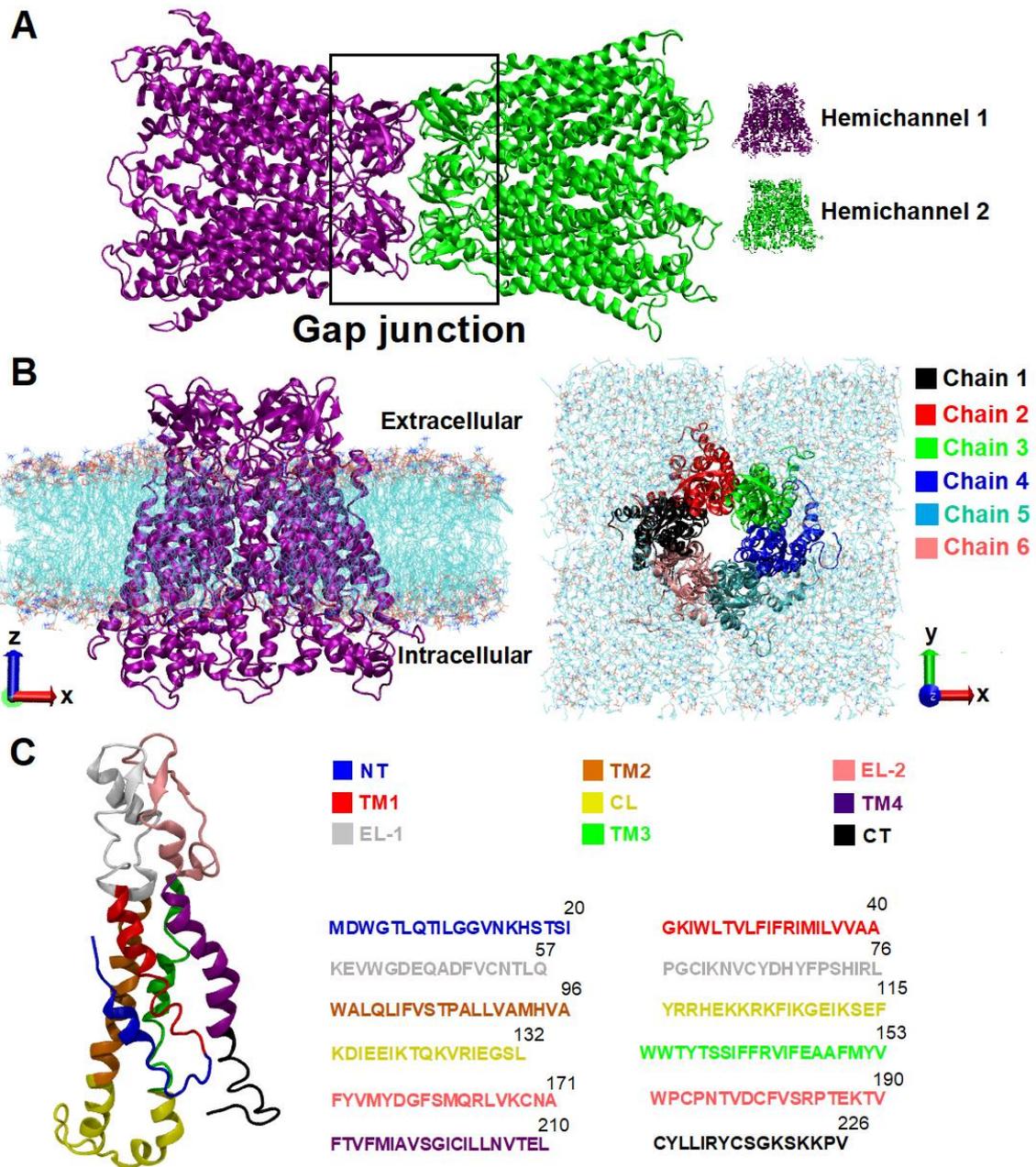


Figure 1. Schematic representation of the Cx structure. **(A)** A gap junction channel of Cx26 proteins. Each hemichannel is represented as ribbons by a different colour (i.e., purple or green). The lipid bilayers (i.e., membranes) were removed for the sake of clarity. **(B)** Side view (left) and top view (right) of a Cx26 hemichannel (consisting of six Cx chains) embedded into a POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) lipid bilayer. The lipid bilayer is represented as lines of different colours. In the top view, each colour chain represents a single Cx26 protein. All chains (chain 1 to chain 6) are identical, and its structure can be seen in **(C)**. It should be noted that there is an open and closed conformation of hemichannels, and the top view represents an open conformation. **(C)** Cx26 protein structure and its domains: transmembrane domains (TM1 (red), TM2 (orange), TM3 (green), and TM4 (purple)), extracellular loops (EL-1 (grey)

and EL-2 (pink)), cytoplasmic loop (CL (yellow)), amino terminus (NT (blue)), and carboxyl terminus (CT (black)). The amino acid sequence of each domain is shown in different colours. The number for each sequence represents the position number of that amino acid residue in the domain.

GJs are present in almost all human cells [6], and play an important role in the maintenance of physiological functions, such as cell growth, differentiation, homeostasis [7], angiogenesis [8], neural migration [9], and stem cell development [10]. They allow the transfer of various species directly from the interior of one cell to a neighbouring cell, including autocrine and paracrine signals (e.g. nicotinamide adenine dinucleotide (NAD⁺) and adenosine triphosphate (ATP)), glutamate, glucose, and ions (e.g. Ca²⁺, K⁺, and Na⁺), but also reactive oxygen and nitrogen species (RONS) [4]. The permeability of GJs is regulated by changes in the membrane potential [11], phosphorylation [12], the presence of unsaturated fatty acids [13], changes in redox potential [14], pH [15], and alterations in extracellular Ca²⁺ [16] and Mg²⁺ [17] concentration. For instance, post-translational modifications, such as phosphorylation, oxidation, carbonylation, and C-terminal cleavage, have been shown to inhibit the GJ and hemichannel permeability [18]. Protein-protein interactions mediated by Cxs have also been able to modulate gap junction (GJ) channel function and regulation [19]. For instance, several reviews have summarized that interactions of Cxs with multiple proteins around it, which take place during Cx trafficking from the endoplasmic reticulum to the plasma membrane, are essential for GJ formation, membrane stabilization, and degradation [20-22]. Thus, knowledge of how biological molecules pass through GJ channels is extremely important.

The importance of GJs for disease induction and therapy development is becoming more appreciated, especially in the context of oncology. *In vitro* studies on solid tumor cells revealed that a lack of cell communication through GJs in certain tumor types results in abnormal cell growth [23]. Interestingly, restoration of GJs in tumor cell lines reduces tumor growth and proliferation [24]. This tendency was corroborated tracking Cx levels in cancer cells: a high Cx expression leads to better cancer prognosis, while a low Cx expression leads to a worse cancer prognosis [25]. For instance, a low expression of Cx46 proteins was found during differentiation of glioblastoma cancer stem cells, enhancing the

cell-cell communication between cancer cells and consequently their self-renewal and tumor growth [26]. Once these outcomes may occur through both GJ-dependent pathways (based on Cx isoform, cancer type and stage) [27] and GJ-independent pathways (based on the CT domain of Cxs) [28-30], these results suggest that GJs and Cxs have anti-tumorigenic properties.

On the other hand, GJs and Cxs have also shown pro-tumorigenic properties. For example, Cx26 and Cx43 proteins were found to form GJs between endothelial cells or astrocytes (i.e., healthy cells) on one hand, and breast and melanoma cancer cells on the other hand, promoting metastasis and growth [31]. Altered levels of Cx26 and Cx43 proteins were also found in some subtypes of metastatic breast and lung cancer cells, when compared to the protein expression in primary tumor cells [32]. Additionally, mutations to the amino acid sequence of Cx26 proteins have also been associated with dysfunctions of hemichannels and GJs to cause many pathologies, such as skin diseases, peripheral and central neuropathic disorders, lens cataracts, and deafness [33,34]. To summarize, GJs and Cxs can have pro- and anti-tumorigenic properties, and its outcome depends on the GJ and Cx properties, tumor factors, and cancer stage [27,28].

Since GJs are able to transport a variety of biological molecules, e.g., RONS, from the interior of one cell to another cell, it is crucial to understand how this transport occurs and its effects on the cell function. For instance, RONS generated during oxidative stress-based cancer treatments (e.g., hydrogen peroxide (H_2O_2), hydroperoxyl radicals (HO_2^\bullet), hydroxyl radicals (HO^\bullet), singlet oxygen (1O_2), and nitric oxide radicals ($^\bullet NO$)) are able to interact with the surface of cancer cells, and kill them by lipid and protein oxidation [35]. Consequently, the effects caused by RONS may also affect GJ function/activity. In fact, an increasing body of experimental evidence demonstrates that RONS can modulate GJs and hemichannels, either via direct effects on channel gating (pore opening/closing), or by affecting the intracellular trafficking of Cxs, impacting the number and stability of the GJ channel at the plasma membrane [36,37]. For instance, photodynamic therapy (PDT)-generated RONS were found to be able to improve Cx hemichannel activity and consequently, ATP release (paracrine signaling) from Cx hemichannels in syngeneic murine melanoma models *in vivo*, inducing bystander cell death and antitumor response via enhanced Ca^{2+}

signaling [38]. These effects were greatly enhanced by combination treatment with S-nitrosoglutathione (GSNO), an endogenous •NO radical donor that biases hemichannels towards the open state [38]. These results highlight Cx hemichannels as a potential target to increase cytotoxic bystander effects in cancer cells.

Likewise, lipid oxidation may also alter the properties of GJs and hemichannels. Studies have shown that GJ formation and disruption are modulated by lipid oxidation-mediated pathways [39,40], but the underlying mechanisms are not well understood and require further investigation. Lipid oxidation has also been found able to induce alteration in Cx expression and in phosphorylation, contributing to the pathogenesis of atherosclerosis [41]. Accordingly, lipid oxidation may also directly affect channel gating. For example, Retamal found that 4-hydroxy-2-nonenal (4-HNE), a reactive aldehyde derived from oxidized lipids, inhibits the Cx46 hemichannel activity in *Xenopus laevis* oocytes [42]. That inhibitory effect of 4-HNE on Cx46 hemichannels occurred through carbonylation of extracellular cysteines, inhibiting GJ formation and pointing out the importance of the disulfide bridges between extracellular cysteines in GJ formation [42]. Polyunsaturated fatty acids, such as linoleic acid and arachidonic acids, which can be easily oxidized, also inhibited the hemichannel activity *in vitro* [13]. Therefore, a plausible mechanism for hemichannel inhibition is through lipid oxidation.

The effect of lipid oxidation on the membrane properties has been largely studied both experimentally [43-45] and by simulations [46-48]. However, how it affects GJs and hemichannel properties still needs to be elucidated. In this study, we applied molecular dynamics (MD) simulations to study the effect of lipid oxidation on the channel properties of Cx26 hemichannels, more specifically the channel gating (opening/closing) and permeability. Several Cx proteins play a crucial role in cancer cell migration and metastasis, such as Cx26 [31,32], Cx32 [49], Cx43 [31,32,50], and Cx46 [51]. Although we are focused solely on Cx26 proteins in this work, we will consider exploring other Cx proteins in future studies.

METHODS

Model Systems and Construction

The initial structure of the Cx26 hemichannel was taken from the Cx26-composed GJ channel (Cx26-GJ) at 3.5 Å resolution X-ray crystal structure, obtained by Maeda et al. [52]. The crystal structure of the Cx26-GJ was proposed to be an open channel, and can be obtained from the Protein Data Bank under the code 2ZW3. Due to the inherent flexibility of some domains in Cx26 hemichannels, several amino acid residues are missing in the crystal structure: residue 1 of the NT domains of the six Cx26 proteins, residues 110-124 of the CL domains, and residues 218-226 of the CT domains [52]. In principle, as our interest is to study the effect of lipid oxidation on the property of the Cx26 hemichannel pore, the missing of these amino acid residues may not affect our MD simulation results as a whole, since the channel pore is lined by amino acid residues in the N-terminal half of the EL-1 domains, the C-terminal half of the TM1 domains, the first half of the NT domains, and the C-terminal half of the TM2 domains [52] (see Figure 1C). However, although the missing amino acid residues may not be pore-lining, Kwon et al. demonstrated later that the completion of the crystal structure affects the channel gating of the Cx26 hemichannel [53]. Thus, we simulated the completed Cx26 hemichannel structure taken from Albano and co-workers [54], based on the crystal structure of Maeda et al. [52].

The Cx26 hemichannel was embedded into either a 100% POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) lipid bilayer (POPC + Cx26) or a 100% oxidized POPC lipid bilayer into hydroperoxide —OOH group (POPCOOH + Cx26). POPC lipids are broadly used in biomimetic computational and experimental studies, since it is the major component of eukaryotic biological membranes [55]. Moreover, the experimental thickness of membranes composed of POPC lipids was measured as 38 Å [56], which is identical to the thickness of the TM domains in the crystal structure of the Cx26 GJ channel [52]. Likewise, lipid hydroperoxides are also widely studied, because they are one of the major products of oxidation of unsaturated fatty acids [57].

Each model system was composed of 500 lipid molecules (250 in each leaflet) solvated with a sufficient number of water molecules, to ensure proper hydration of the lipid bilayer and to prevent the interaction of the Cx26

hemichannel with itself. We packed the lipids around the Cx26 hemichannel using the InflateGRO program [58]. Sodium (Na^+) and chlorine (Cl^-) ions were randomly placed in the water at a concentration of 0.15 M, to neutralize the net charge of the Cx26 hemichannel and to mimic a physiological ionic strength. It should be pointed out that cholesterol molecules also play a very important role in membrane systems, since it regulates the membrane fluidity and permeability, as well as the formation of coexisting phases and domains in the membrane [59]. However, we have excluded cholesterol from our model systems in first instance, because it can also be oxidized, which would lead to a more complex membrane composition in terms of lipids and cholesterol oxidation products.

Figure S1 of the *Supporting Information* (SI) presents the initial structures of the simulated model systems. In Table S1 we summarize the characteristics of each model system described above. The structures of POPC and POPCOOH lipid molecules can be visualized in Figure S2. The —OOH groups are attached at the C9 carbon of oleoyl (*sn*-2) chains of POPCOOH lipid molecules, with *R* stereocenter (see Figure S2).

Molecular Dynamics Simulation Parameters

All MD simulations were performed using GROMACS version 2020.2 [60], applying the united-atom GROMOS 54A7 force field [61]. We used well-validated models for the description of POPC [62] and POPCOOH lipids [63], and adopted interatomic interaction parameters for the amino acid residues from the standard GROMOS 54A7 force field library [61]. Water molecules were modeled with the simple point charge model [64]. Periodic boundary conditions were used in all cartesian directions. Newton's equations of motion were integrated using the leapfrog algorithm with a time step of 2 fs. A cut-off radius of 1.4 nm was used for non-bonded (Lennard-Jones and van der Waals) interactions. Coulomb interactions were treated using the reaction-field method [65]. The covalent bond lengths were constrained using the linear constraint solver (LINCS) algorithm [66].

We performed a steepest descent energy minimization, followed by a thermalization in the canonical ensemble (NVT) for 0.1 ns and an equilibration in the isothermal-isobaric ensemble (NPT) for 500 ns. The temperature was maintained at 310 K to maintain a disordered liquid state of the POPC lipids, by

coupling the system to an external temperature bath using the Nose-Hoover thermostat [67,68]. The temperature coupling constant was 0.5 ps. The pressure was also maintained at around 1 bar by coupling the system to an external pressure bath using the Parrinello-Rahman barostat [69]. The pressure coupling was applied semi-isotropically, with a coupling constant of 2 ps and isothermal compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$ [70].

Visualization and Data Analysis

Visualization of the simulated model systems and generation of the figures were carried out using the VMD software [71]. We used several built-in tools of GROMACS to perform analysis of the stability and dynamics of the Cx26 hemichannel: *gmx rms* for analysis of the root mean square deviation (RMSD), *gmx rmsf* for analysis of the root mean square fluctuation (RMSF), *gmx gyrate* for analysis of the radius of gyration (Rg), and *gmx rdf* for analysis of the radial distribution function (RDF). The bilayer thickness of the lipid bilayers (i.e., membranes) was defined as the average distance along the z-axis between the center of mass of the phosphorus atoms of both leaflets, using the *gmx traj* tool of GROMACS. The area per lipid (A_L) was calculated as:

$$A_L = \frac{L_x \times L_y}{n_L} \quad (1)$$

where L_x and L_y are the box length in the x and y-direction, respectively, and n_L is the number of lipids in each leaflet (i.e., 250).

For analysis of the permeability of the Cx26 hemichannel, we calculated the surface electrostatic potential with the adaptive Poisson-Boltzmann solver (APBS) program [72]. The pore profile was calculated using the Pore-Walker program [73]. Analysis of the density map was calculated using the *gmx densmap* and *gmx xpm2ps* tools of GROMACS. The angles within the Cx26 hemichannel was calculated using the *gmx gangle* tool of GROMACS. The polarity and charge of the Cx26 hemichannel was calculated using the MOLEonline program [74].

The distribution of water molecules along the Cx26 hemichannel pore was calculated using an in-house script, based on the number density of water

molecules at different layers along pore-centered cylindrical cross sections. The cylinder had a radius of 7 Å and was oriented parallel to the z-axis. The free energy change (ΔG) associated with the transport of a water molecule from a distant point in solution to a specific position z inside the pore was calculated using the Boltzmann equation [75]:

$$\Delta G(z) = -k_B T \ln \frac{\rho(z)}{\rho_\infty} \quad (2)$$

where k_B is the Boltzmann constant, T is the temperature, $\rho(z)$ is the distance-dependent number density of water molecules and ρ_∞ is its bulk value.

RESULTS AND DISCUSSION

Stability and Dynamics of Cx26 Hemichannels

In order to assess the structural stability and dynamics of Cx26 hemichannels embedded in native (i.e., non-oxidized) and oxidized membranes, we calculated the RMSD of the protein alpha carbons ($C\alpha$), using as a reference the initial configuration structure of the hemichannel [54], and the Rg of the protein hemichannel in each model system. The RMSD is a measure of how much a structure deviates in position from a reference structure [76], and thus represents the stability of the whole system, whereas the Rg is a measure of the compactness of the system: the higher the Rg of the system is, the less compact is the system (loosest packing) [77]. Figure 2 compares the RMSD and Rg of the Cx26 hemichannel embedded into POPC and POPCOOH membranes. As is apparent from Figure 2, the systems are equilibrated after 300 ns of simulation time. Therefore, we performed all the analysis of the average quantities using the last 200 ns of the simulations, to eliminate any transient effects on the results presented.

The Cx26 hemichannel embedded into the POPCOOH membrane shows a slightly higher deviation from the reference structure than the Cx26 hemichannel embedded into the POPC membrane (Figure 2A), i.e., Cx26 hemichannels embedded into oxidized membranes exhibit more conformational changes. Conformational changes of a protein are critical determinants for its

biological function. For instance, conformational changes of particular proteins (e.g., unfolding, misfolding, and aggregation) have been correlated to many diseases, by toxic gain of a function, loss of a function, improper degradation, and improper localization [78,79]. On the other hand, conformational changes on pro-diseases proteins have also been used to hinder disease progression [80,81]. In Figure S3 we compare the structures of the initial configuration of the Cx26 hemichannel and of our equilibrated Cx26 hemichannels, showing that the intracellular side of the Cx26 hemichannel presents a higher deviation from the initial structure in the presence of oxidized lipids (see Figure S3). This result is in agreement with the higher conformational changes of the Cx26 hemichannel embedded into the POPCOOH membrane.

Interestingly, the R_g of the Cx26 hemichannel in the xy plane (i.e., along the z -axis of the membrane) is lower in the presence of oxidized lipids (Figure 2B), suggesting that the Cx26 hemichannel is more compact in the oxidized membrane. Normally, more compact proteins require a higher folding time, and thus are more susceptible to undergo protein denaturation (unfolding). Indeed, Lobanov et al. performed statistical analysis of the R_g of several proteins, and demonstrated that α -proteins are less compact and fold more rapidly than β - and $(\alpha + \beta)$ -proteins [77].

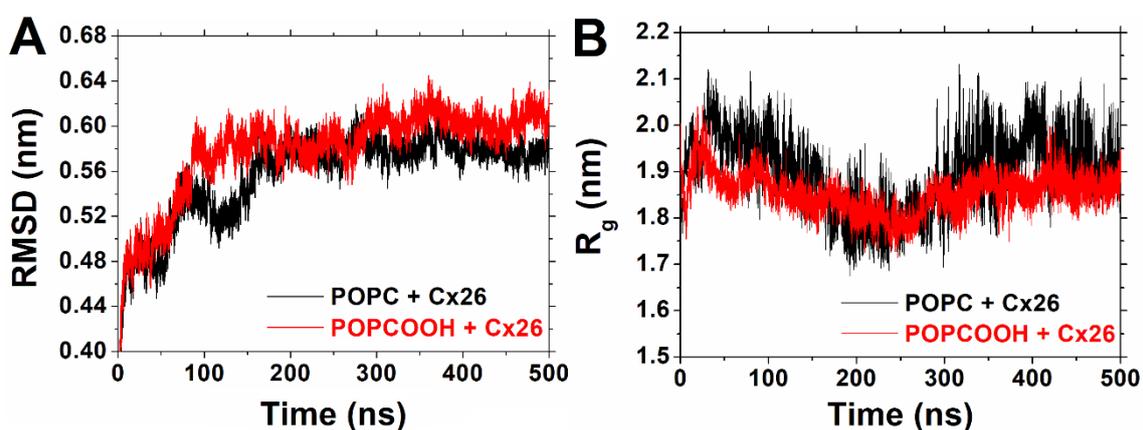


Figure 2. Temporal evolution of the structural properties of the Cx26 hemichannel embedded into POPC and POPCOOH membranes: **(A)** average root mean square deviation (RMSD) and **(B)** average radius of gyration (R_g) in the xy plane of 10 slices along the z -axis. These data are an average of the 6 identical chains of the Cx26 hemichannel.

To gather insight in the relative fluctuation of different regions within each Cx26 protein, we also evaluated the RMSF of each amino acid residue in the six different chains of the six Cx26 proteins that compose the hemichannel (Figure 3). Similar fluctuation patterns are observed for the same Cx domains of the six chains. The highest fluctuations are observed within the NT (residues 1-20), EL-1 (residues 41-76), CL (residues 97-132), EL-2 (residues 154-190), and CT (residues 211-226) domains (see Figure 3). This is expected, since these domains are located outside the membrane, so they are more flexible because of their lack of structural constraints imposed by the membrane. Thus, the TM domains are the most stable regions, since they are embedded in the membrane and organized in α -helices, which restrict their movements. We can notice higher RMSF values when the Cx26 hemichannel is embedded into the POPC membrane than when embedded into the POPCOOH membrane (compare Figures 3A and 3B). This is in agreement with the Rg values, where the Cx26 hemichannel embedded into the POPCOOH membrane is more compact, suggesting that lipid oxidation influences the dynamics properties of Cx26 hemichannels. Figure S4 demonstrates that lipid oxidation increases the area per lipid of the lipid bilayer and decreases the bilayer thickness (cf. POPCOOH vs. POPC). Consequently, it will affect the stability and dynamics of the Cx26 hemichannel.

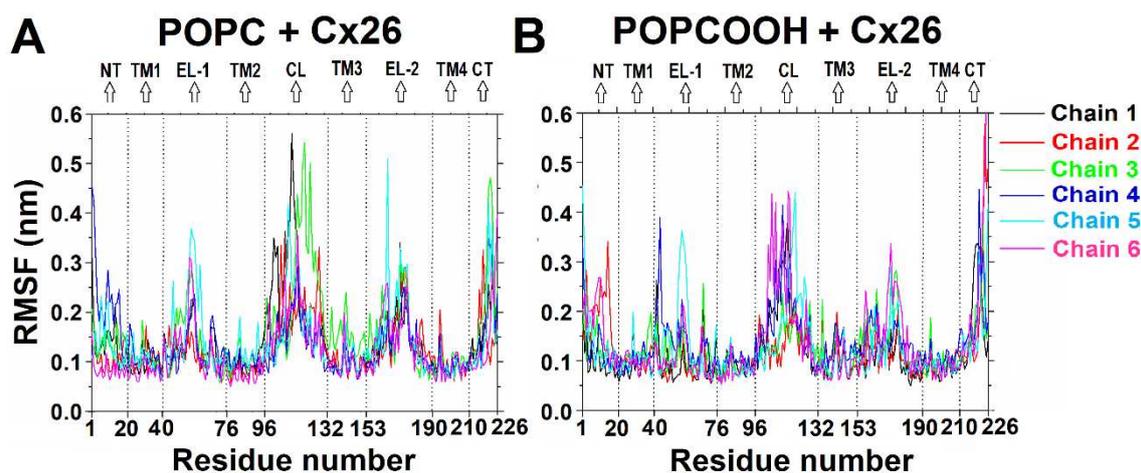


Figure 3. Average root mean square fluctuation (RMSF) of each amino acid residue of the Cx26 hemichannel embedded into (A) the POPC membrane and (B) the POPCOOH membrane, calculated from the last 200 ns of simulation. Each colour chain represents the trajectory of a single Cx26 protein that composes the hemichannel.

To obtain insights into the interactions between the lipids and the Cx26 hemichannel, we calculated the number of contacts between the center of mass of the headgroups, the double bond, and the —OOH group of lipids with the Cx26 hemichannel (Figure 4). The lipid headgroups are defined as the phosphate and carbonyl ester groups of each lipid molecule (see Figure S2). Interestingly, we can note slightly less contacts between the center of mass of the headgroups and the double bond of POPCOOH lipids with the hemichannel compared with the number of contacts for POPC lipids (compare Figures 4A and 4B), suggesting that POPCOOH lipids are interacting more with each other than with the hemichannel. When calculating the number of hydrogen bonds (H-bonds) between the —OOH group of POPCOOH lipids, with water and other groups of POPCOOH lipids, we can see that the —OOH groups are more prone to establish H-bonds with the carbonyl ester group of POPCOOH lipids around it (Figure S5), which is due to a strong dipole-dipole interaction between these groups. This behavior is in agreement with our previous studies for oxidized membranes, causing a migration of —OOH groups toward the headgroup region and thereby increasing the lipid disorder [82]. Since hemichannels are embedded into the membrane, we can expect that lipid-lipid interactions affect the hemichannel properties. In addition, the —OOH group of POPCOOH lipids is more prone to interact with the hemichannel than the double bond of POPCOOH lipids (see Figure 4B). In addition, the increase in the area per lipid caused by lipid oxidation (see Figure S4A) will also favor the higher interaction of the —OOH group of POPCOOH lipids with the Cx26 hemichannel (see Figure 4B).

Altogether, these results suggest that lipid oxidation affects the stability and dynamics of Cx26 hemichannels, and that lipid-lipid and lipid-protein interactions must be taken into account to study the hemichannel properties. It is well known that lipid oxidation increases the membrane permeability [83,84]. In the next section we will discuss the effects of lipid oxidation on the Cx26 hemichannel permeability.

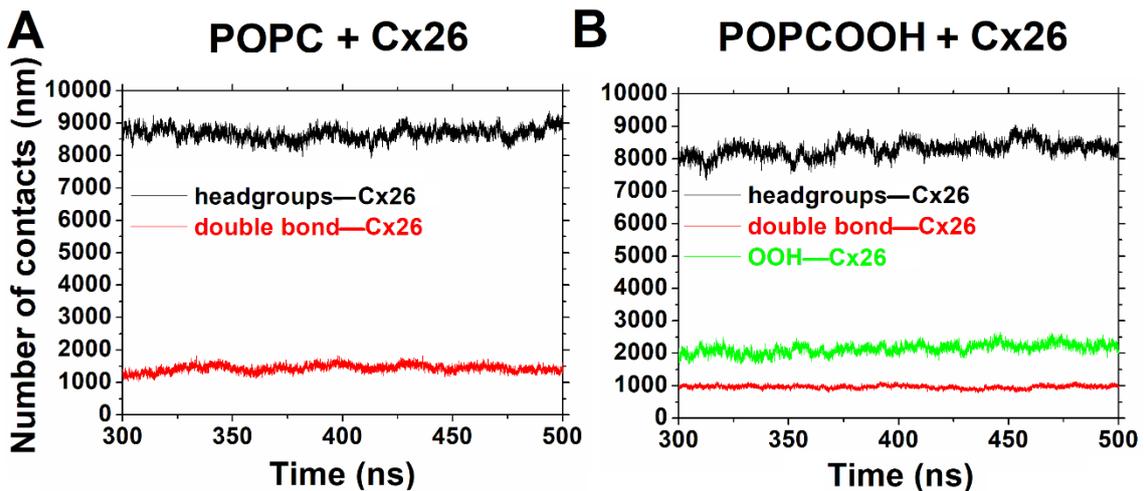


Figure 4. Number of contacts < 0.6 nm between the center of mass of the headgroups, the double bond, and the $-OOH$ group of the lipids and the Cx26 hemichannel embedded into **(A)** the POPC membrane and **(B)** the POPCOOH membrane, calculated from the last 200 ns of simulation.

Permeability of Cx26 Hemichannels

Since lipid oxidation affects the stability and dynamics of the Cx26 hemichannel, we expect it may also affect the hemichannel permeability. In order to evaluate the effect of lipid oxidation on the permeability of the Cx26 hemichannel, we calculated the surface electrostatic potential of the Cx26 hemichannel embedded into either the POPC membrane (Figure 5) or POPCOOH membrane (Figure 6). For both systems, we observed a negative surface electrostatic potential near the extracellular side of the hemichannel (red regions) and a positive surface electrostatic potential near the intracellular side (blue regions). We can note that the total electrostatic energy slightly decreases over time when the Cx26 hemichannel is embedded into the POPC membrane (see Figure 5). Conversely, the total electrostatic energy increases a bit when the Cx26 hemichannel is embedded into the POPCOOH membrane (see Figure 6).

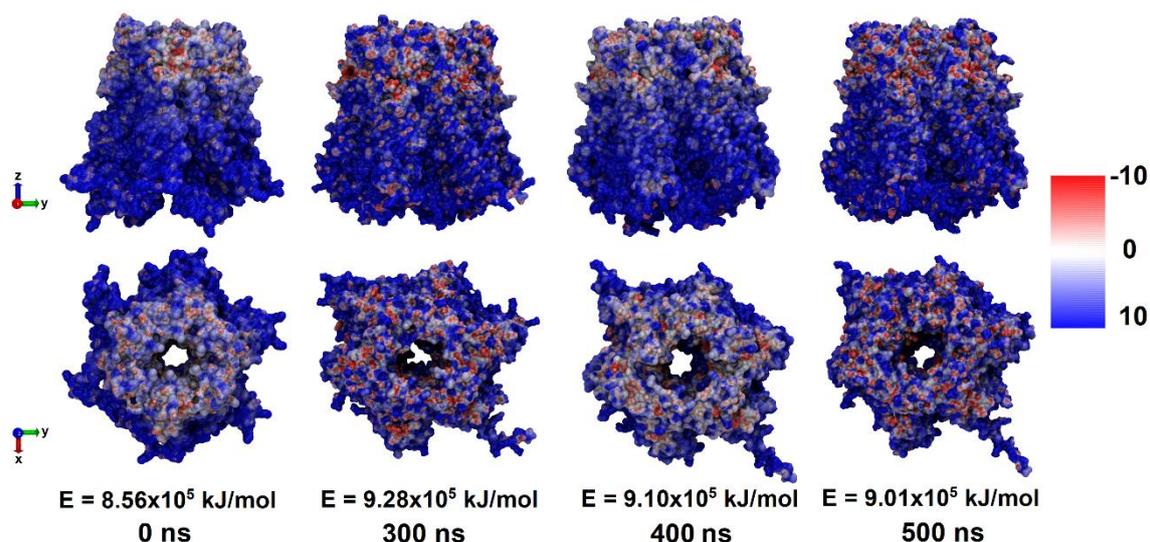


Figure 5. Surface electrostatic potential of the Cx26 hemichannel embedded into the POPC membrane, calculated with the adaptive Poisson-Boltzmann solver (APBS) program. Red colours represent negative charges, and blue colours positive charges. The colour scale represents the range of the average charges. The E values represent the total electrostatic energy. The solute and solvent dielectrics are 2.000 and 78.540, respectively. The solvent probe radius is 1.400 Å and the temperature is 298.150 K.

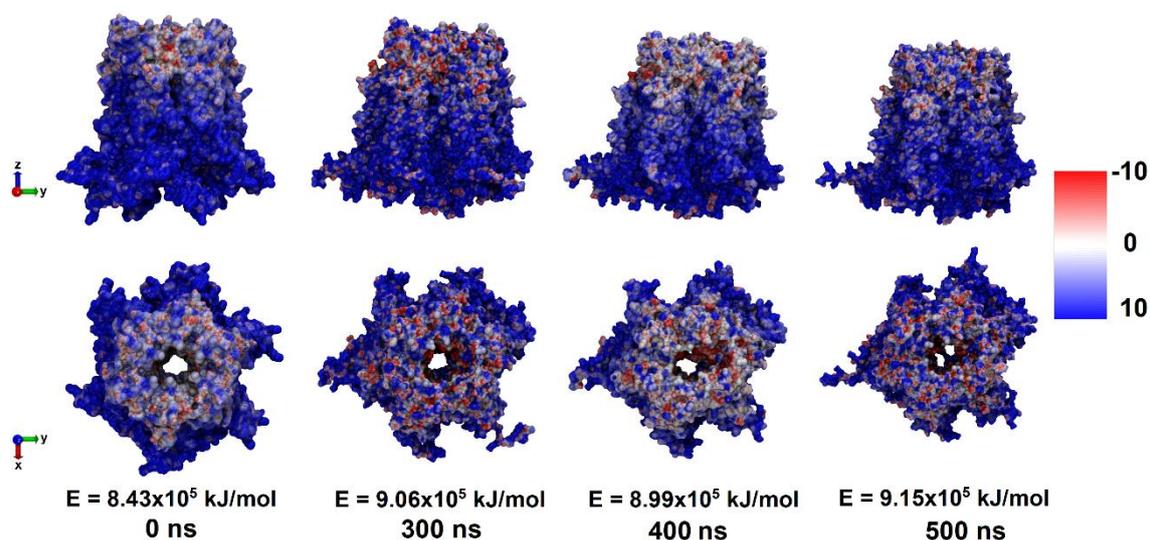


Figure 6. Surface electrostatic potential of the Cx26 hemichannel embedded into the POPCOOH membrane, calculated with the adaptive Poisson-Boltzmann solver (APBS) program. Red colours represent negative charges, and blue colours positive charges. The colour scale represents the range of the average charges. The E values represent the total electrostatic energy. The solute and solvent dielectrics are 2.000 and 78.540, respectively. The solvent probe radius is 1.400 Å and the temperature is 298.150 K.

Changes in the surface electrostatic potential may allow or disrupt the passive diffusion of water, ions, and small molecules across GJs and hemichannels. Indeed, experimental evidence has suggested that the permeability barrier of GJs and hemichannels is modulated by potential differences, through two voltage-regulated gating mechanisms: 1) a transmembrane voltage, i.e., the potential difference between the cytoplasm and the extracellular space; and 2) a transjunctional voltage, i.e., the potential difference between the cytoplasm of two adjacent cells [85,86]. Consequently, an increase in the total electrostatic energy of the Cx26 hemichannel caused by lipid oxidation may affect the hemichannel permeability. For instance, our results demonstrated a higher density of Na⁺ ions close to the intracellular and extracellular regions of the Cx26 hemichannel in the presence of native lipids, whereas these Na⁺ ions are distributed more homogeneously in the presence of oxidized lipids (compare dark blue lines in Figures S6A and S6B).

To test the hypothesis that changes in the surface electrostatic potential of the Cx26 hemichannel caused by lipid oxidation may affect the hemichannel permeability, we investigated the Cx26 hemichannel pore topology using the Pore-Walker program [73]. Through this tool, we evaluated the average pore diameter profile at different simulation times: 300 ns, 325 ns, 350 ns, 375 ns, 400 ns, 425 ns, 450 ns, 475 ns, and 500 ns (Figure 7A). The pore diameter profiles at different simulation times can be seen in Figure S7. We calculated the central pore diameter as the narrowest region of the pore diameter profile, i.e., the minimum region. In our simulations, we found a value for the central pore diameter around 9 Å and 8 Å for the Cx26 hemichannel embedded into the POPC membrane and POPCOOH membrane, respectively (see Figure 7A). Maeda et al. reported a central pore diameter of the Cx26 hemichannel of 14 Å, but their calculation of the central pore diameter was based on minimal center-to-center distances of opposed heavy atoms, and did not consider atom diameters [52]. Thus, our results demonstrate that the central pore diameter of the Cx26 hemichannel is almost the same in the presence of oxidized lipids (see Figure 7A). Conversely, the pore diameter at the extracellular side of the Cx26 hemichannel is smaller when embedded into the POPCOOH membrane: ca. 16 Å when embedded into the POPCOOH membrane *versus* 21 Å when embedded into the POPC membrane (see Figures 7A and 7B), suggesting a more closed

conformation of the hemichannel in the presence of oxidized lipids. This more closed conformation is favored by the decrease in the bilayer thickness caused by lipid oxidation (see Figure S4B), thus the hemichannel should constrict to accommodate in the membrane. The decrease in the bilayer thickness is correlated with membrane pore formation [83] and may affect lipid-protein interactions, such as the stability/activity of the Cx26 hemichannel. The closed conformation of the Cx26 hemichannel embedded into the POPCOOH membrane was also observed when analyzing the Cx26 hemichannel density maps: we can note that the pore diameter at the extracellular side is smaller in the presence of oxidized lipids (compare Figures 8A and 8B).

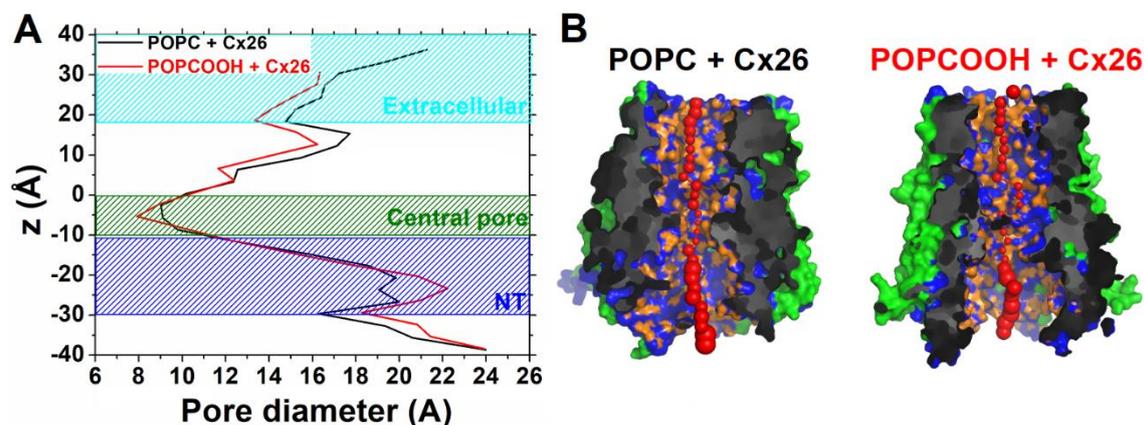


Figure 7. Characterization of the Cx26 hemichannel embedded into POPC and POPCOOH membranes, calculated with Pore-Walker. **(A)** Average pore diameter profile at 3 Å steps. **(B)** Visualization of pore section showing the position of the pore centers (red spheres) at 500 ns of simulation time. The red spheres are proportional to the corresponding measured diameters, i.e., 1/10 of the pore diameter calculated at that point. Pore-lining atoms and amino acid residues are coloured in orange and blue, respectively. The remaining part of the protein is shown in green and black.

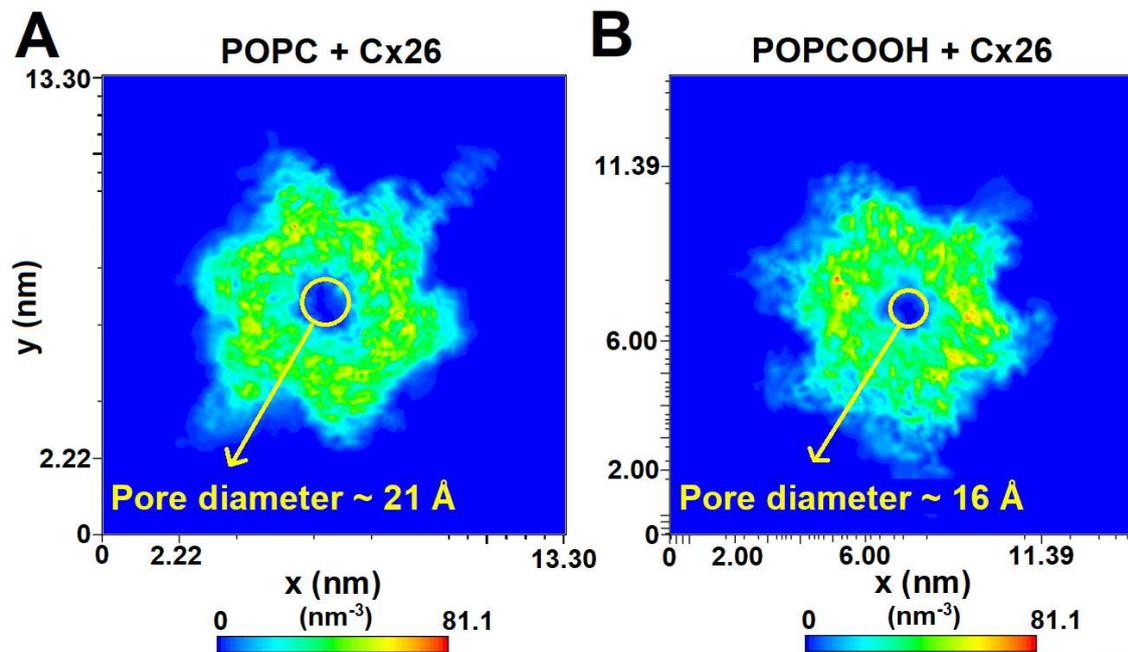


Figure 8. Density maps of the Cx26 hemichannel embedded into (A) the POPC membrane and (B) the POPCOOH membrane, calculated from the last 200 ns of simulation. The dark blue region corresponds to the absence of protein (space occupied by the lipids). The yellow circle represents the pore diameter at the extracellular side, calculated in Figure 7A.

Moreover, our results also demonstrate that the pore diameter at the NT domains is larger for the Cx26 hemichannel embedded into the POPCOOH membrane: ca. 22 Å when embedded into the POPCOOH membrane *versus* 19 Å when embedded into the POPC membrane (see Figure 7A). Experimental evidence has suggested that the NT domains participate in the voltage-regulated gating mechanism of hemichannels and GJs, moving in response to changes in electrical potential [87,88]. Thus, to obtain a quantitative measure of the effect of the NT domains on the hemichannel pore, we introduced a parameter, called eccentricity coefficient (E), defined as the ratio between the maximum and minimum diameter of a hexagon built on the C α of the six asparagine amino acid residues within the NT domains that compose the Cx26 hemichannel (see Figure S8 for more details). We computed E values equal to 1.391 ± 0.303 and 1.436 ± 0.152 for the Cx26 hemichannel embedded into the POPC membrane and the POPCOOH membrane, respectively. Therefore, the Cx26 hemichannel pore at the NT domains is slightly larger in diameter when it is embedded into oxidized membranes. Note that these values are higher than the E value found by Zonta

et al. [89] for the uncompleted crystal structure of the Cx26 hemichannel (i.e., 1.10 ± 0.05), suggesting that the missing amino acids residues in the uncompleted crystal structure are important to evaluate the channel gating properties of the Cx26 hemichannel.

We also analyzed the dynamical behavior of the NT domains by tracking the angles (A) of the hexagon built on the $C\alpha$ of the six threonine amino acid residues within the NT domains that compose the Cx26 hemichannel (see Figure S8 for more details). In Figure S9 we plot the temporal evolution of the angles in the Cx26 hemichannel embedded into the POPC membrane and the POPCOOH membrane. Note that the Cx26 hemichannel embedded into the POPCOOH membrane displays larger angle variations than when it is embedded into the POPC membrane (compare Figures S9A and S9B). This angle analysis is in agreement with our calculated E values, where lipid oxidation slightly increases the pore diameter at the NT domains of the Cx26 hemichannel. It should be pointed out that although the difference between our calculated E values for the Cx26 hemichannel embedded into the POPC membrane and the POPCOOH membrane is very small, we must pay attention to how lipid oxidation influences the Cx26 hemichannel properties, instead of their absolute values. In addition, the difference between our calculated E values is more significant looking at the angle variations of the hexagon (see Figure S9).

We further analyzed how lipid oxidation affects not only the pore diameter profile, but also the physical chemical properties, such as polarity and charge at different pore length and radius, using the MOLEonline program [74]. For both systems, we observe an increase in the polarity at the extra- and intracellular sides of the Cx26 hemichannel (Figures S10 and S11). However, the presence of oxidized lipids increases the polarity in the transmembrane region of the Cx26 hemichannel (compare Figures S10 and S11). Hence, it may facilitate the passage of polar molecules. Indeed, the water density at the transmembrane region of the Cx26 hemichannel is slightly higher in the presence of oxidized lipids (Figure 9A). Likewise, the ΔG for water permeation across the Cx26 hemichannel is overall slightly lower at the transmembrane region in the presence of oxidized lipids (Figure 9B). These results strengthen the idea that lipid oxidation slightly increases the permeability of the Cx26 hemichannel.

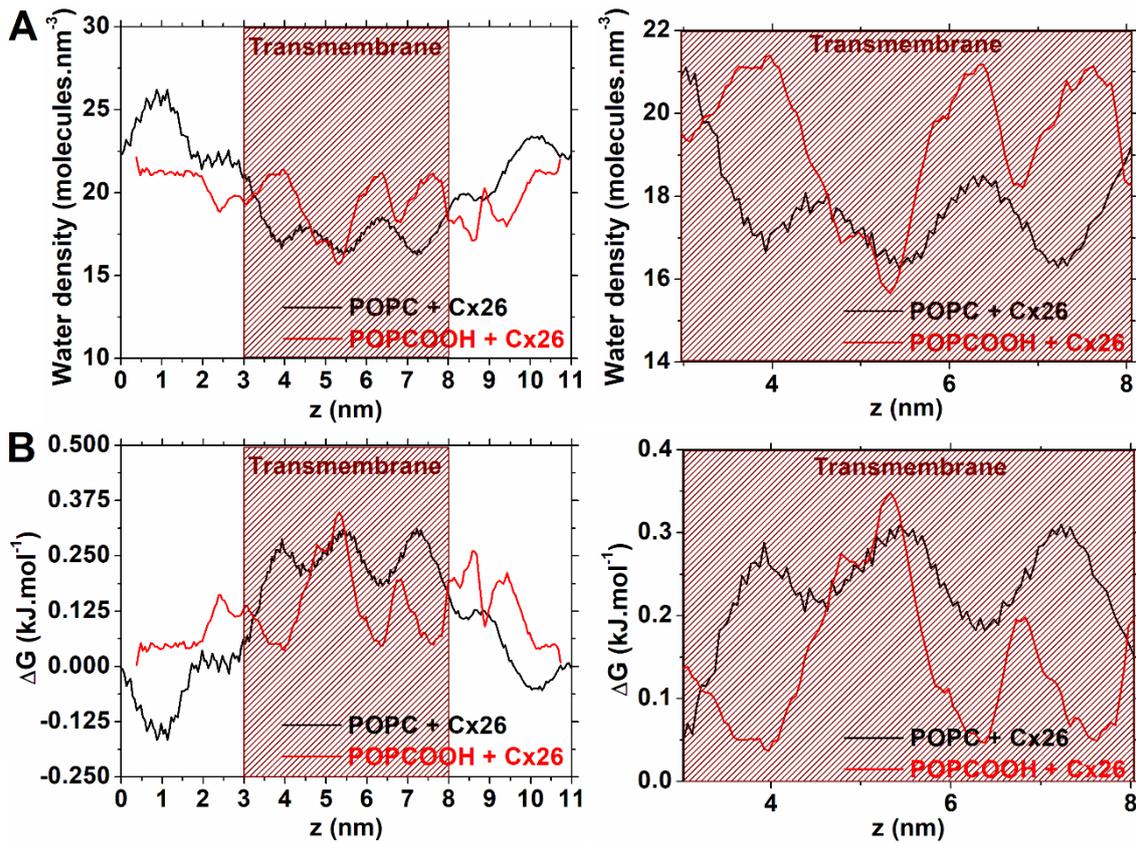


Figure 9. Analysis of water permeation across the Cx26 hemichannel, calculated from the last 200 ns of simulation: (A) Distribution of water molecules along the Cx26 hemichannel (left) and expansion of the transmembrane regions (right); and (B) Free energy (ΔG) for water permeation across the Cx26 hemichannel (left) and expansion of the transmembrane regions (right). The position $z = 5.25$ nm was set at the central pore region.

Distribution and Dynamics of RONS over the Cx26 Hemichannel

To evaluate the permeability of the Cx26 hemichannel more in-depth, we also performed equilibrium simulations of RONS distributed over the Cx26 hemichannel embedded into either a POPC membrane or a POPCOOH membrane, in order to verify whether RONS may permeate the Cx26 hemichannel easier in the presence of oxidized membranes. It is important to understand the distribution and dynamics of RONS at hemichannels, since RONS can modulate the GJ and hemichannel activity [37,90]. For instance, Xu et al. performed reactive MD simulations of the Cx26 hemichannel reacting with HO^\bullet and HO_2^\bullet radicals, and they found that these radicals chemically react with the NT domains of the Cx26 hemichannel, and can structurally damage the Cx26 hemichannel [91].

Thus, we studied the behavior of RONS starting at the water/lipid interface, and how they can permeate the membrane in the presence of the Cx26 hemichannel. For this purpose, 200 HO₂• radicals were initially placed together in the aqueous phase (100 molecules in the upper aqueous phase and 100 in the bottom aqueous phase), surrounding a pre-equilibrated model system of the Cx26 hemichannel embedded into either a POPC membrane or a POPCOOH membrane. This value corresponds to an initial molar fraction of ca. 0.5% for each HO₂• radical in the aqueous phase, which is several orders of magnitude higher than the experimentally measured RONS concentrations in mitochondria [92] but it is needed to obtain reasonable statistics. We chose these RONS based on the work of Xu et al. [91] and the similarity with the —OOH group attached to POPC lipid molecules (oxidized lipids).

Figure 10 shows the trajectory of the HO₂• radicals from the last 50 ns of simulation. At first glance, most of the HO₂• radicals remain at the water/lipid interface when the Cx26 hemichannel is embedded into the POPC membrane (see Figure 10A). On the other hand, most of these radicals permeate the Cx26 hemichannel in the presence of oxidized lipids (see Figure 10B), suggesting a higher interaction of the HO₂• radicals with the hemichannel.

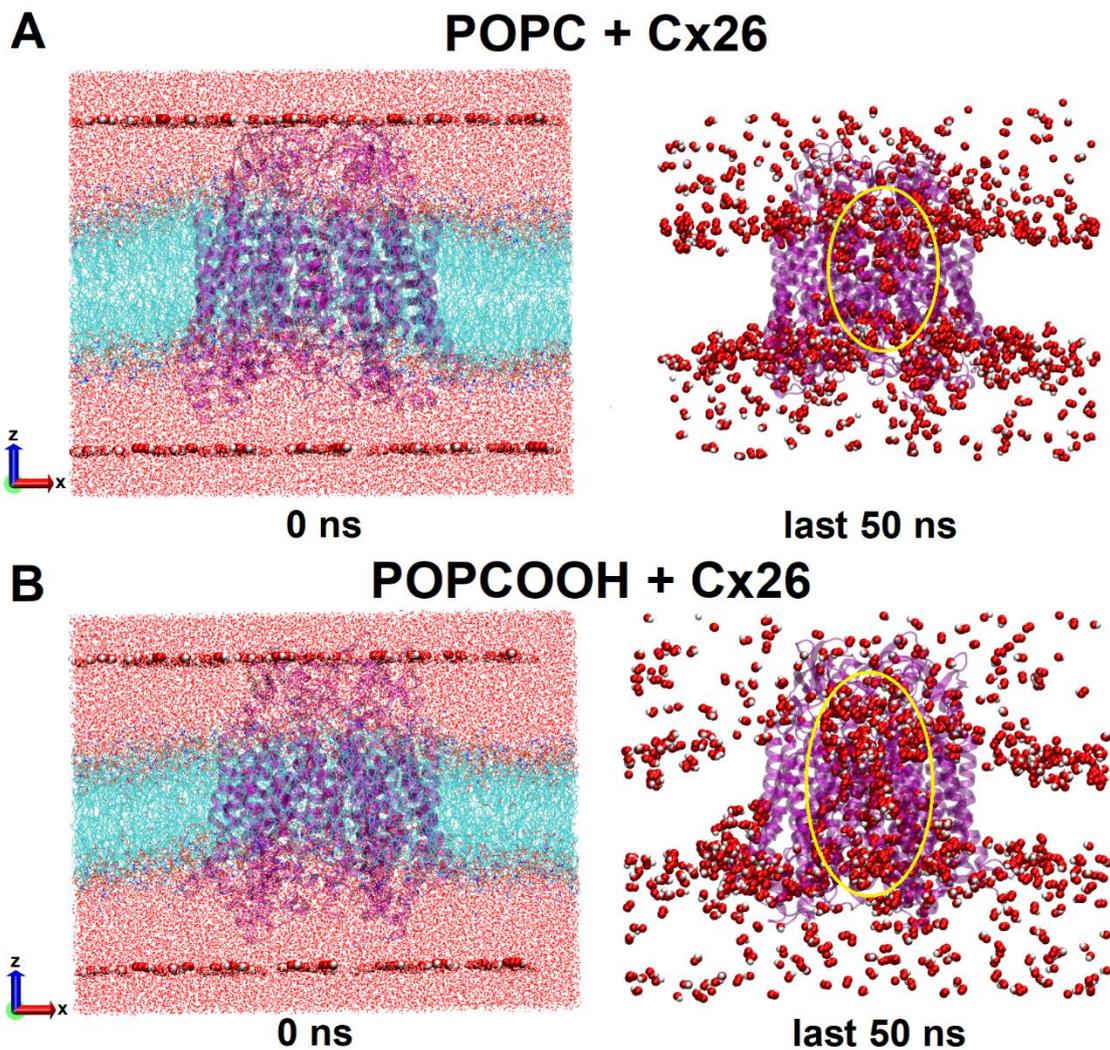


Figure 10. Snapshot of the initial structure (left) and trajectory of the last 50 ns (right) of the equilibrium simulations of HO₂[•] radicals over the Cx26 hemichannel embedded into (A) the POPC membrane and (B) the POPCOOH membrane. Lipid molecules are represented as lines of different colours, water molecules as red points, the Cx26 hemichannel as shadow ribbons, and the HO₂[•] radicals as red and white van der Waals spheres. For the sake of clarity, lipids and water molecules are not represented in the trajectory (i.e., last 50 ns). All frames of the last 50 ns are overlaid in order to highlight the permeation path of HO₂[•] radicals (only HO₂[•] radicals within 0.4 nm of the Cx26 hemichannel are represented). We can see that the HO₂[•] radicals remain more at the water/lipid interface in case of the POPC membrane, while they interact more with the Cx26 hemichannel in the case of the POPCOOH membrane. The yellow circles represent the HO₂[•] radicals that permeate the Cx26 hemichannel pore.

We calculated the radial distribution function (RDF) of the HO₂[•] radicals for each domain of the Cx26 hemichannel, and our results demonstrate that

indeed these radicals interact more with the TM domains of the Cx26 hemichannel embedded in a POPCOOH membrane (compare red, orange, green, and purple lines in Figure 11). Additionally, these radicals also establish more interactions with the NT domains of the Cx26 hemichannel embedded into the POPCOOH membrane (compare blue lines in Figure 11). Specifically, the HO_2^\bullet radicals are more prone to interact with the asparagine Asp_2 and glutamine Gln_7 amino acid residues of the NT domains: When calculating the minimum distances between HO_2^\bullet radicals and each amino acid residue of the NT domains of the Cx26 hemichannel embedded into the POPCOOH membrane, we found that these radicals present a somewhat shorter distance to Asp_2 and Gln_7 amino acid residues (Table S2). In Figure S12 we can see the interaction between the HO_2^\bullet radicals and the NT domains of the Cx26 hemichannel embedded into the POPCOOH membrane. These analyses support the idea that RONS may permeate easier the Cx26 hemichannel in the presence of oxidized lipids.

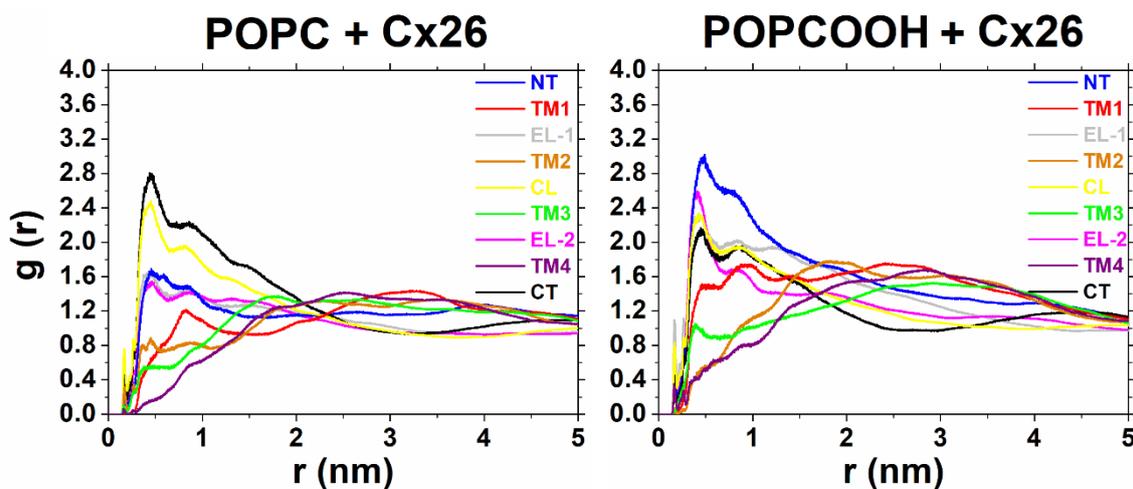


Figure 11. Radial distribution function (RDF) of HO_2^\bullet radicals calculated from the last 50 ns of simulation, for Cx26 embedded into POPC and POPCOOH membranes. The RDFs of the HO_2^\bullet radicals are generally higher in the latter case, indicating that these radicals stronger interact with the Cx26 hemichannel when embedded into the POPCOOH membrane.

The ability of GJs to enhance the intracellular accumulation of RONS has been widely explored experimentally [40,93,94]. For instance, Wu et al. demonstrated that Cx26-GJs increased the level of intracellular RONS in HeLa cells treated with PDT [40], suggesting that GJs may improve the sensitivity of

cancer cells to PDT-induced cell death. Cx26 hemichannels were also found to be able to improve PDT treatment by bystander cell killing [38]. Concurrently, Cx43-composed GJ channels (Cx43-GJs) also increased the sensitivity of melanoma cells to RONS during non-thermal plasma (NTP) treatment, and propagated apoptotic death signals to neighbouring cells (bystander effect) [95]. These results highlight the property of GJs to enhance oxidative stress-induced cell death during cancer treatments, based on e.g., PDT and NTP, but it can also be applicable to other treatments based on oxidative stress. In addition, since mutations in Cx26, Cx30, Cx31, Cx32, and Cx43 proteins have been associated with nonsyndromic or syndromic deafness in humans [96-98], the oxidative damage induced by oxidative stress may affect Cx proteins-mediated deafness and hearing loss [99].

Our results for the distribution and dynamics of HO_2^\bullet radicals over the Cx26 hemichannel reinforce the idea that Cx26 hemichannels could enhance the intracellular accumulation of RONS in cancer cells during oxidative stress-based treatments, and this effect may be stronger in the presence of oxidized lipids. However, additional studies are still necessary to elucidate the mechanism of RONS transportation through GJs and hemichannels. Calculation of the free energy profiles for RONS permeation across the Cx26 hemichannel by means of equilibrium simulations would require unrealistically high concentrations of these species in solution. This could lead to artifacts in solvent properties and hemichannel conformation. Therefore, more accurate techniques should be used, such as umbrella sampling simulations [100]. We will address this issue in our future work.

CONCLUSIONS

The dual property of GJs and Cxs to act as tumor suppressors or tumor promoters allows us to modulate these properties in cancer cells, to hinder cancer progression. Modulation of these properties can assist the activation of anti-cancer immunity, propagation of cell death, and oxidative stress-mediated cell death [101]. One promising therapeutic strategy is the use of these GJ channels to transport RONS to the interior of cancer cells, and kill them by oxidative stress. However, little is known about the mechanism of RONS transportation across GJs and hemichannels. In addition, the effect of lipid oxidation on the properties

of GJs and hemichannels still needs to be elucidated. In this work, we studied how RONS-induced lipid oxidation affects the channel properties of Cx26 hemichannels. Our simulation results demonstrate that lipid oxidation induces changes in the stability and dynamics of Cx26 hemichannels, affecting lipid-lipid and lipid-protein interactions. Interestingly, the presence of oxidized lipids changes the pore diameter profile of the Cx26 hemichannel: the pore diameter decreases at the extracellular side and increases at the NT domains. Since the NT domains are strongly correlated to the gating mechanisms of hemichannels and GJs, a larger pore diameter would improve the entry and accumulation of RONS inside the cell to cause oxidative damage. Indeed, our simulations demonstrate that HO_2^\bullet radicals are more prone to interact with the TM and NT domains of the Cx26 hemichannel in the presence of oxidized lipids, strengthening the idea that lipid oxidation facilitates the intracellular accumulation of RONS through the hemichannel. Our results are in agreement with experimental results for other Cx hemichannels (e.g., Cx46 hemichannels) where lipid oxidation affected the hemichannel activity [13,42]. Our results highlight lipid oxidation as an important factor to be considered when modulating GJ properties during oxidative stress-mediated cell death. A better understanding of these effects may improve the efficacy of oxidative stress-based cancer treatments, such as e.g., PDT and NTP.

It should be pointed out that the number of atoms of GJ channels (> 500,000) restricts full atomistic MD simulations of these systems to a couple of hundreds of nanoseconds, and this simulation time might be insufficient to obtain an equilibrated and fully open GJ channel, given its size and complexity. However, independently whether our equilibrated Cx26 hemichannel is in its open or closed conformation, our results demonstrate that lipid oxidation affects the channel properties of Cx26 hemichannels. Additionally, given the permeability of hemichannels for a large variety of physiological signaling molecules, we will consider exploring in future studies the permeability of ATP, glutamate and other signaling molecules, across Cx26 hemichannels in the presence of oxidized lipids.

ACKNOWLEDGMENT

We thank the University of Antwerp and the Turing HPC infrastructure at the CalcUA core facility of the University of Antwerp, a division of the Flemish Supercomputer Center VSC, funded by the Hercules Foundation, the Flemish Government (department EWI), for providing the computational resources needed for running the simulations. This work was funded by the Research Foundation-Flanders (FWO), project number 42/FA070200/8300.

DECLARATIONS OF INTEREST

None

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