How the cell membrane composition influences plasma-induced cellular effects

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Abstract: Lipid vesicle experiments were combined with molecular dynamics simulations to study the effect of variations in membrane lipid composition on the ability of plasma-generated ROS to (i) diffuse through these membranes and (ii) induce intracellular DNA damage. We show that the lipid composition strongly affects the intracellular effects induced during CAP treatment, which underscores the importance of taking the lipid composition of cell membranes into account when studying plasma-cell interactions.

Keywords: Lipid vesicles, molecular dynamics, ROS permeation, DNA oxidation

1. Introduction

In the last decades, cold atmospheric plasmas (CAPs) have been investigated for numerous medical applications, ranging from decontamination of wounds and accelerating wound healing [1], to the use of CAPs in cancer therapy [2]. Highly promising results have been obtained in different stages of research, ranging from *in vitro* results to randomized clinical trials. [3–6] This suggests that cold plasmas might be used in the future as a selective and effective technique, supplementing the existing set of treatment modalities. However, the need for more fundamental research to identify the exact mode of action by which cold plasma operates in these applications is also shown by the large variation of results when treating, *e.g.*, different types of cancer cells. [7,8]

Up until now, it has been shown that highly reactive oxygen and nitrogen species (ROS and RNS, or RONS), generated by adding oxygen and nitrogen into the feed gas, or by contact of the discharge/afterglow with ambient air, are the most important elements to induce biological effects. [9,10] These include, *e.g.*, hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and superoxide anions (O_2^-). In order to induce a certain cellular response, these RONS need to either (i) modify the extracellular matrix which subsequently interacts with cells, (ii) modify the cell membrane itself or (iii) enter cells directly. Whichever mode of action, it is clear that the outer cell membrane is involved in the transmission of the signal into cells. Hence, it is very important to study the effect CAP exerts on this plasma membrane.

Although the plasma membrane of a cell is a very complex mixture of many different proteins surrounded by thousands of lipids, research has shown that a few global differences can be identified in the lipid composition of cell membranes of *e.g.* healthy human cells, cancerous cells and bacterial cells. Therefore, to obtain a more general overview of how the lipid composition of the cell membrane influences ROS permeation and intracellular processes such as DNA damage, we combine lipid vesicle experiments with molecular dynamics simulations, looking at differences in lipid composition that are observed between healthy cells and either cancerous cells or bacterial cells. The composition of the phospholipid vesicles is tuned to investigate the effect of different cholesterol concentrations, lipid saturation degrees and DPPE-lipid content, both individually as well as the combined effects of multiple factors.

2. Methods

2.1. Vesicle experiments

Ten different vesicle compositions were synthesized, which all contained different molar fractions of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol. After synthesizing and purifying the vesicles, 200 µL of a HEPES solution containing the vesicles was treated with an in-house plasma jet set-up [11], using treatment times up to one minute.

Two different assays, both using encapsulated reporter molecules, were used to assess the effect of the different lipid types on plasma-induced cellular effects. In the first assay, 2,7-dichloro-dihydrofluorescein (DCFH) was encapsulated in the vesicles. As ROS are able to oxidize non-fluorescent DCFH into fluorescent 2,7-dichlorofluorescein (DCF), encapsulated DCFH can be used as an indicator for the amount of broad-range ROS that is able to penetrate the vesicle membrane. Secondly, to assess the ability of CAP-generated ROS to induce DNA-strand breaks inside vesicles, encapsulated molecular beacon (MB) was used. The MB is an oligonucleotide comprising of double-stranded DNA which contains at one end a 5'-fluorescent moiety and a 3'-quenching moiety. If double-strand breaks are induced during CAP treatment, this leads to the separation of the quencher and fluorophore which again leads to the switch-on of a fluorescence signal.

2.2. Molecular dynamics simulations

Molecular dynamics (MD) simulations were performed to study the permeation of H_2O_2 across lipid bilayers with various compositions. Similar to the vesicle experiments, ten different bilayers were constructed, of which the lipid composition was chosen to mimic the composition of the vesicles studied experimentally. Each bilayer contained a total of 128 lipids, hydrated by 6000 water molecules surrounding the bilayer structure on both sides. In these simulations, the GROMACS 5.1 package [12] in combination with the GROMOS 54A7 united atom force field [13] was used. After constructing and equilibrating the membranes, umbrella sampling (US) simulations were performed to calculate the free energy profiles (FEPs) of H_2O_2 across the different membranes. We also calculated the area per lipid in each system, together with the radial distribution function (RDF) between phosphorus atoms of neighbouring phospholipids. Both properties were used to provide more insight into the experimental observations.

3. ROS delivery across cell membranes

3.1 Lipid saturation degree

To examine the influence of the saturation degree of the phospholipids on the ROS ingress in vesicles, we compared vesicles containing different amounts of cholesterol and DPPE, in combination with either DOPC (C18:1) or DPPC (C16:0). The results after 60s CAP treatment are shown in **Fig. 1**.

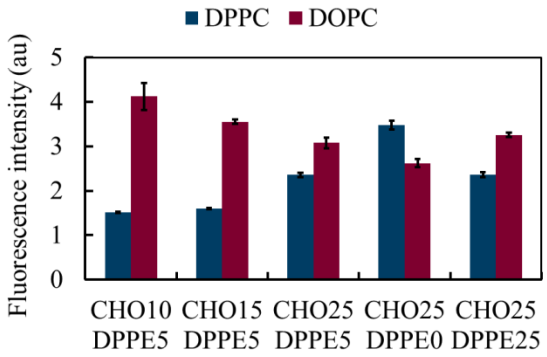

Fig. 1. Effect of lipid saturation degree on ROS ingress. The numbers indicate the mol% of each lipid. In each case, the remainder of the lipids is either DPPC or DOPC.

As is clear from these results, in general, DOPC containing vesicles are more vulnerable to ROS ingress following CAP treatment. However, the FEPs obtained from the US simulations did not show a significant difference between DOPC or DPPC membranes, which indicates that the passive diffusion rate through both types of systems should be similar. This contradiction between experiments and simulations indicated that the main reason for ROS ingress in the DOPC vesicles is lipid oxidation. Indeed, the double bond present in the lipid tails makes these lipids vulnerable to oxidation by impinging ROS. Because oxidized lipids were not included in the simulations, these differences could not be observed. In addition, **Fig. 1** shows that the exact difference in fluorescence intensity between DPPC and DOPC vesicles depends on the concentration of cholesterol and DPPE as well. This indicates that those lipids also play an important role in determining the total ROS ingress. Therefore, in the sections below, these elements are discussed in more detail.

3.2 Cholesterol concentration

The effect of cholesterol was examined by synthesizing vesicles that contain either 10, 15 or 25 mol% cholesterol. Again, DCFH was used to quantify the ROS ingress in all treated vesicles (see **Fig. 2**).

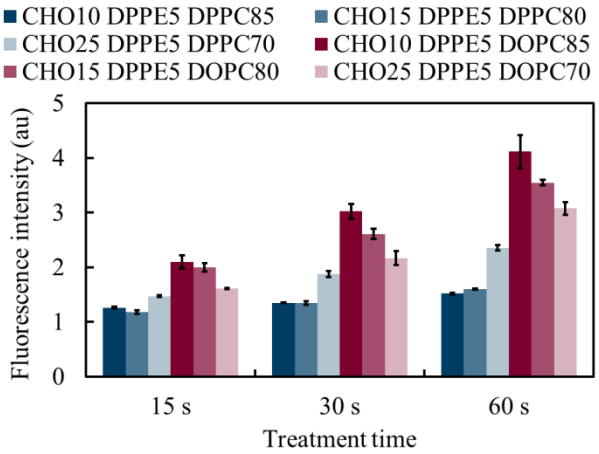

Fig. 2. Effect of the cholesterol content on ROS ingress.

We observed that the same variation of the cholesterol content leads to opposite effects in vesicles containing either DPPC or DOPC. This difference can be explained by looking at the ability of CAP to oxidize the different membranes. In DPPC vesicles lacking cholesterol, oxidation is not likely to occur. However, when cholesterol is added to the membrane, the unsaturated bond present in cholesterol can be oxidized by impinging ROS. Due to this oxidation, the membrane core's polarity increases slightly, which facilitates further permeation of plasma generated ROS. Therefore, if the concentration of cholesterol is increased in the DPPC vesicles, the permeation of ROS increases as well.

In DOPC vesicles, on the other hand, even without the presence of cholesterol, lipid oxidation already occurs due to the double bond in the lipid tails. Replacing DOPC by cholesterol reduces the total number of reactive sites present in the lipid core (each molecule of DOPC contains two double bonds, whereas cholesterol only contains one double bond), thereby lowering the overall reactivity of the membrane. This explains why a reduced amount of intracellular ROS is measured in the DOPC vesicles upon increasing the cholesterol fraction. Furthermore, the rigid nature of cholesterol also strongly increases the lipid packing in unsaturated membranes, which hampers passive ROS permeation even more, as was shown in previous research. [14]

3.3 DPPE concentration

The third factor we investigated is the DPPE content of the vesicles, of which the results are shown in **Fig. 3**.

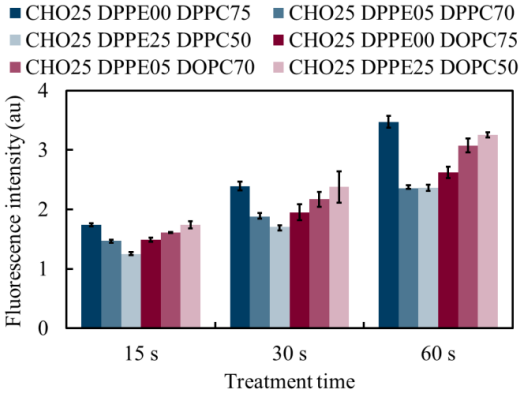

Fig. 3. Effect of the DPPE content on ROS ingress.

The effect of the DPPE concentration on ROS ingress is opposite to that of cholesterol, *i.e.*, adding DPPE inhibits ROS permeation in DPPC vesicles whereas in DOPC vesicles it facilitates ROS permeation. In the DPPC vesicles, oxidation effects are not supposed to play a role, as both DPPC and DPPE contain the same lipid tails. Thus, the difference observed must be caused by the size of the head groups. Indeed, the head group of DPPE (PO_4^--$N(H_3)_3^+$) is significantly smaller than that of DPPC (PO_4^--$N(CH_3)_3^+$), allowing the lipids to be packed closer together in the DPPE systems. Due to this tight packing, the free space in between neighbouring lipids decreases, which makes passive diffusion more difficult. By comparing with the calculated area per lipid of the different membranes, the MD simulations confirmed this observation (see **Table 1**).

Table 1: Effect of DPPE on the area per lipid, calculated by the MD simulations.

Vesicle type	Area per lipid (nm^2)	
	0 mol% DPPE	25 mol% DPPE
DPPC	0.451 ± 0.004	0.435 ± 0.001
DOPC	0.506 ± 0.002	0.449 ± 0.001

Moreover, as oxidation effects do not play a role in this particular case, we expected to observe the same trend in the results of the US simulations. The FEPs of DPPC membranes containing either 0 or 25 mol% DPPE are shown in **Fig. 4**. These profiles show that, upon adding DPPE to the system, passive permeation of H_2O_2 is hampered (*i.e.*, higher free energy of permeation). Important to note is that, because size is the determining factor in this process, the exact numbers will be different for other ROS (*e.g.*, OH radicals).

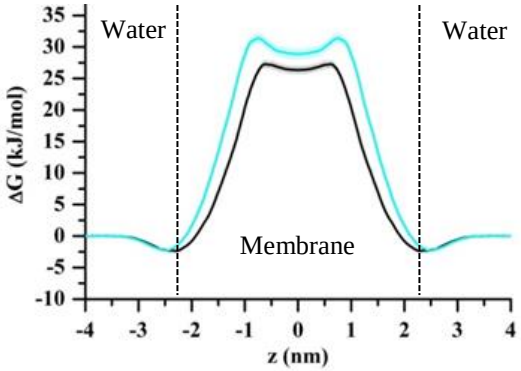

Fig. 4. FEPs of H_2O_2 across a DPPC membrane containing 0 or 25 mol% DPPE (black and blue lines, respectively), calculated by the MD simulations.

To explain the trend observed in the DOPC vesicles, we have to take another phenomenon of lipid membranes into account, which is the formation of lipid rafts. These rafts are patches in the membrane in which certain type of lipids are grouped closely together. This means that the overall membrane is inhomogeneous, containing multiple parts with elevated concentrations of different lipids. Indeed, the literature shows that cholesterol is known to create lipid rafts with lipids containing aliphatic, *i.e.*, saturated lipid tails. [14] Therefore, when increasing the DPPE concentration, lipid rafts could be generated which contain elevated levels of DPPE and cholesterol, which in the meantime creates other patches with elevated DOPC levels. A possible theory is thus that due to the generation of lipid rafts, the parts of the membrane that are enriched in DOPC serve as the 'weak spot' of these membranes, being extremely vulnerable to pore formation due to lipid oxidation. In previous research, we have shown that, in order to create pores in lipid membranes, very high lipid oxidation degrees are required locally [15], which can indeed only occur if these oxidized lipids are grouped together in membrane patches.

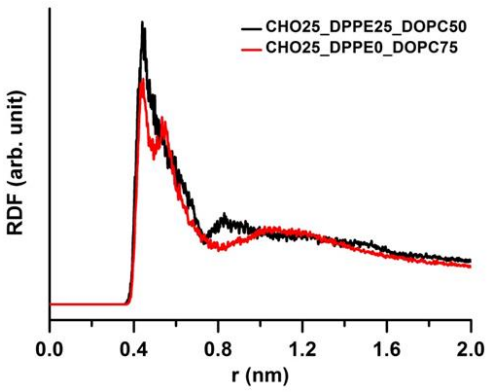

Fig. 5. : RDF graphs of the P-P distance of neighbouring DOPC lipids in systems containing 0 or 25 mol% DPPE, calculated by the MD simulations.

To analyse possible lipid raft formations in these membrane structures, we extracted the RDF graphs of the

P-P distance of neighbouring phospholipids in DOPC systems containing 0 or 25 mol% DPPE (see **Fig. 5**).

In the system containing only DOPC and cholesterol (red curve), the first peak (at a distance of 0.44 nm) corresponds to two DOPC molecules placed next to each other. Upon adding DPPE to the system (black curve), the peak of neighbouring DOPC molecules increases, which means that more DOPC molecules are grouped together. This is counterintuitive, as there are more non-DOPC lipids in this system, so the chance of DOPC lipids being organized directly next to each other should decrease. Therefore, due to the increase in number of neighbouring DOPC molecules, it can be derived that lipid raft formation does occur in this system, creating rafts that are either enriched in DOPC or in DPPE/cholesterol.

4. CAP-induced DNA double strand breaks

Encapsulated MB was used to assess the effect of cholesterol on the ability of CAP to induce intracellular DNA damage. Opposite to the broad range ROS measurements, these results show that the total number of DNA strand breaks is significantly lower in the DOPC vesicles compared to the DPPC vesicles (see **Fig. 6**).

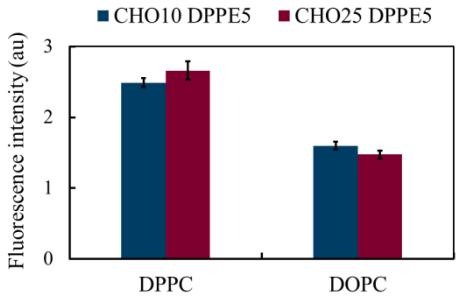

Fig. 6. : Effect of the cholesterol content on CAP induced intracellular DNA damage.

A possible explanation for this starts from the fact that short-lived ROS (e.g. OH radicals) are the most effective species to induce DNA strand breaks, compared to longer-lived ROS (e.g. H_2O_2). [11] In DOPC vesicles, the double bonds serve as reactive sites, scavenging these short-lived ROS before they can reach the cell interior. The combination of both assays (DCFH and MB) could thus indicate that in DOPC vesicles more species are able to penetrate the vesicle membrane, but that these mostly include longer-lived species. In DPPC vesicles, on the other hand, a lower number of species is able to diffuse through the membrane, but these mostly include short-lived species. These species are in general much smaller, which facilitates their ability to diffuse through the membrane. Indeed, previous research has shown that the energy barrier for passive diffusion across a membrane of short-lived species such as OH or HO_2 radicals is significantly lower compared to that of, *e.g.*, H_2O_2.[16]

5. Conclusion

In this research, we have clearly shown that intracellular effects induced during CAP treatment strongly depend on the cell membrane lipid composition. Indeed, both the broad-range ROS measurements, as well as the measurements of DNA damage show that the outcome of a CAP treatment strongly depends on the exact lipid composition of membranes, as small differences can have opposing results. Moreover, these results also emphasize how important it is to identify a correct model system to be used in MD simulations, as the choice of a certain lipid type in the model could completely reverse the message obtained.

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