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**Reference:**

Van der Paal Jonas, Hong Sung-Ha, Yusupov Maksudbek, Gaur Nishtha, Oh Jun-Seok, Short Robert D., Szili Endre J., Bogaerts Annemie.- How membrane lipids influence plasma delivery of reactive oxygen species into cells and subsequent DNA damage : an experimental and computational study  
Physical chemistry, chemical physics / Royal Society of Chemistry [London] - ISSN 1463-9076 - 21:35(2019), p. 19327-19341  
Full text (Publisher's DOI): <https://doi.org/10.1039/C9CP03520F>  
To cite this reference: <https://hdl.handle.net/10067/1627820151162165141>

# PCCP

Physical Chemistry Chemical Physics

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: J. Van der Paal, S. Hong, M. Yusupov, N. Gaur, J. Oh, R. Short, E. Szili and A. Bogaerts, *Phys. Chem. Chem. Phys.*, 2019, DOI: 10.1039/C9CP03520F.



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# How membrane lipids influence plasma delivery of reactive oxygen species into cells and subsequent DNA damage: An experimental and computational study

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## Abstract

The mechanisms of plasma in medicine are broadly attributed to plasma-derived reactive oxygen and nitrogen species (RONS). In order to exert any intracellular effects, these plasma-derived RONS must first traverse a major barrier in the cell membrane. The cell membrane lipid composition, and thereby the magnitude of this barrier, is highly variable between cells depending on type and state (e.g. it is widely accepted that healthy and cancer cells have different membrane lipid compositions). In this study, we investigate how plasma-derived RONS interactions with lipid membrane components can potentially be exploited in the future for treatment of diseases. We couple phospholipid vesicle experiments, used as simple cell models, with molecular dynamics (MD) simulations of the lipid membrane to provide new insights into how the interplay between phospholipids and cholesterol may influence the response of healthy and diseased cell membranes to plasma-derived RONS. We focus on the (i) lipid tail saturation degree, (ii) lipid head group type, and (iii) membrane cholesterol fraction. Using encapsulated molecular probes, we study the influence of the above membrane components on the ingress of RONS into the vesicles, and subsequent DNA damage. Our results indicate that all of the above membrane components can enhance or suppress RONS uptake, depending on their relative concentration within the membrane. Further, we show that higher RONS uptake into the vesicles does not always correlate with increased DNA damage, which is attributed to ROS reactivity and lifetime. The MD simulations indicate the multifactorial chemical and physical processes at play, including (i) lipid oxidation, (ii) lipid packing, and (iii) lipid rafts formation. The methods and findings presented here provide a platform of knowledge that could be leveraged in the development of therapies relying on the action of plasma, in which the cell membrane and oxidative stress response in cells is targeted.

## 1. Introduction

Targeted cell membrane therapy has the potential to shape the future of how we treat and manage chronic diseases such as cancers. In order to continue improving cell membrane therapies, it is necessary to improve our fundamental understanding of how the unique features of diseased *versus*

1 healthy cell membranes can be exploited for treatment of the disease with minimal damage to healthy  
2 cells and tissue. Indeed, in a wide range of diseases, the cell membrane composition is altered and  
3 membrane function is perturbed.[1,2] New approaches for the treatment of chronic diseases, including  
4 cancers, target these perturbations in membrane composition and function.[2] For example, cancer  
5 cells display a higher phosphatidylethanolamine (PE) content in the outer leaflet of the bilayer  
6 membrane, as they lose their ability to maintain PE asymmetry in both leaflets of the plasma  
7 membrane.[3] Raised PE content enhances the susceptibility of the cell membrane to perforation in  
8 response to peptide-based anticancer therapy and has been shown to lead to eventual cell death.[4]  
9 Another relatively new strategy is the use of molecular drugs, such as Minerval® (2-hydroxyoleic acid),  
10 which interfere with lipid structures within the cell membrane to kill cancer cells.[5]

11 When focussing on targeted membrane therapy, a major issue still to be addressed is to increase our  
12 understanding of how the complex interplay between the array of membrane lipids and other  
13 membrane components influences the cell response to the treatment. This problem is exacerbated by  
14 the fact that there are more than 1000 different lipids in any eukaryotic cell.[6,7] On one hand, the  
15 overall structure of the membrane is determined by the chemical structure of each of these individual  
16 lipids. The fluidity of the membrane, for example, increases when introducing lipids which contain  
17 shorter lipid tails, unsaturated cis-double bonds, and decreases when cholesterol is added to the  
18 system.[8] On the other hand, the overall composition of a membrane is only one part of the picture.  
19 As the lipids are free to move throughout the plane of the membrane, lipids with long saturated carbon  
20 tails and cholesterol molecules tend to cluster together due to strong van der Waals interactions,  
21 leading to the formation of transient microdomains or rafts.[8]

22 In the development of therapeutics that leverage on differences in cell membranes, the starting  
23 point has been to focus on the (currently known) few global differences in the membrane lipid  
24 composition between a diseased (e.g. cancerous) cell and its healthy equivalent cell.[1] Indeed, it is  
25 widely known that the development of cancerous cells is accompanied with alterations in the lipid  
26 composition of the plasma membranes of these cells. Multiple studies already revealed a significant  
27 overlap between the fluidity of plasma membranes and the malignancy of cells.[9–11] More  
28 specifically, cancerous cell membranes, in comparison to their healthy counterpart are typically  
29 characterised by (i) a higher number of aquaporin channels [12,13], (ii) altered levels of cholesterol  
30 [9,11,14], (iii) increased levels of unsaturated lipids [9,14], and (iv) increased expression of certain  
31 lipid types in the outer membrane (e.g. PE as mentioned above).[3,11] These unique features of cancer  
32 cells are currently being investigated as potential targets for anticancer therapies. This is particularly  
33 applicable for therapies that induce oxidative stress in cancer cells (e.g. chemotherapy, radiation  
34 therapy and in the context of this study, plasma therapy) through the production of reactive oxygen  
35 species (ROS), which (once inside the cell) can lead to biochemical changes in the cell such as  
36 irreversible DNA damage, resulting in eventual cell death.[15]

37 An exciting feature of plasma as a cancer treatment therapy, versus radiation therapy, is that plasma  
38 is non-ionising and is therefore more likely to be “gentler” on bystander (non-cancerous) cells.[16,17]  
39 Furthermore, it is possible to scale down the plasma beam to the single-cell-level, enabling highly-  
40 targeted treatment.[18,19] Plasma devices are also much more affordable: for instance, current

1 commercial medical plasma devices retail as low as few tens of thousands of USD, which is  
2 significantly less than the costs of new linear accelerators of around 1M USD.

3 Identifying the exact mode of action of cold atmospheric pressure plasmas (CAPs) on tumours or  
4 individual cells (healthy and cancer cells) remains, however, one of the largest challenges in the field  
5 of plasma medicine.[20] On the biological side, a large number of parameters must be taken into  
6 account, including e.g., intracellular processes and the extracellular matrix. On the physical side,  
7 CAPs can be seen as a very reactive cocktail of many different ingredients, including highly reactive  
8 species, electric fields, neutral atoms or molecules, ions, photons and electrons. The main mode-of-  
9 action of plasma jets (a class of CAPs) in treating cancers is thought to arise from the rich mixture of  
10 ROS and reactive nitrogen species (RNS) delivered by plasma jets into cells and tissue, targeting the  
11 destruction of cancer cells and tumours through oxidative stress.[20–22] These include, e.g., the  
12 hydroxyl radical ( $\cdot\text{OH}$ ), oxygen atoms (O), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), peroxyxynitrite ( $\text{ONOO}^-$ ) and the  
13 superoxide anion ( $\text{O}_2^-$ ). In order to induce a certain cellular response, these molecules need to either (i)  
14 modify the extracellular matrix, which subsequently interacts with cells, (ii) modify the cell membrane  
15 itself, or (iii) enter the cell directly to induce intracellular modifications. Whichever mode-of-action, it  
16 is clear that the outer cell membrane is involved in the transmission of the signal into cells.

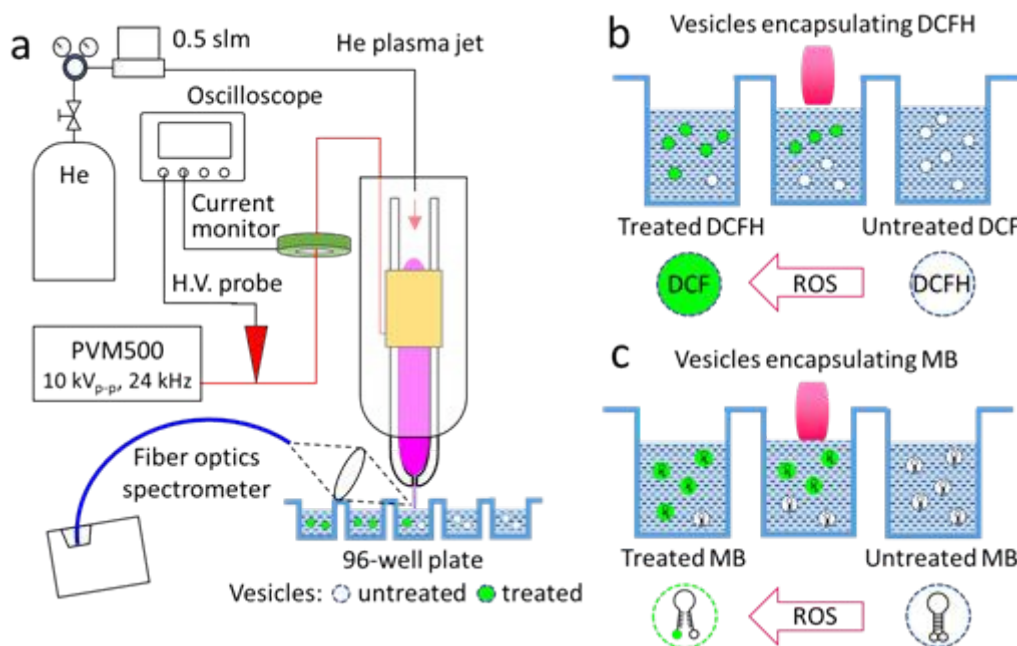
17 Owing to the complexity of cell membranes, simple models usually comprising one or two  
18 phospholipid types have been developed to gain new insights into the role of specific cell membrane  
19 components in regulating cellular processes, both experimentally, e.g. with phospholipid vesicle cell  
20 models, and by computational simulations of lipid bilayers.[23] These model systems have also been  
21 utilised to study the response of specific components of the cell membrane to oxidative stress. For  
22 example, it was observed that the membrane of phospholipid vesicles is more prone to destabilisation  
23 through oxidation processes when the membrane cholesterol content is below 50 mol% [24], and  
24 molecular dynamic (MD) simulations of lipid peroxidation products have revealed that at reduced  
25 levels of cholesterol, membranes are less capable of self-repair from physical damage induced by  
26 oxidation of the membrane lipids.[25]

27 This present study aims to provide new insight into how plasma-derived RONS interact with  
28 vesicles comprising different lipid components of relevance for treating diseases such as cancers. By  
29 experiment and computational modelling, we investigate how ROS interact and penetrate vesicles  
30 comprising different proportions of saturated and unsaturated lipids, and how the addition of  
31 cholesterol and changes in phospholipid (PL) head group influence these interactions and the transport  
32 of ROS to the vesicle interior. The subsequent ability of ROS to induce DNA damage within the  
33 vesicles is measured using a molecular beacon (MB). Vesicles were subjected to a mixture of ROS,  
34 generated using a CAP jet. These jets are being intensively investigated for cancer therapies with  
35 promising patient outcomes already seen in initial clinical case studies.[26] Vesicle experiments are  
36 supported by umbrella sampling (US) MD simulations, investigating the effect of the membrane  
37 composition on the permeation of  $\text{H}_2\text{O}_2$ , which is known to be one of the major longer-lived ROS  
38 generated by CAP jets in aqueous liquids [27], and is itself an important cellular signalling  
39 molecule.[28]

## 1 2. Experimental

## 2 2.1 General set-up

3 Figure 1 illustrates the CAP jet treating vesicles in solution with the location of the voltage probe,  
4 current monitor and the spectrometer for optical emission spectroscopy (OES).



5  
6 **Figure 1:** (a) Illustration of the CAP jet configuration used for the treatment of the vesicles, and the  
7 location of the voltage and current probes and OES for the plasma diagnostics.  
8 Illustration of the induction of fluorescence in vesicles containing (b) encapsulated 2,7-  
9 dichlorodihydrofluorescein (DCFH) or (c) MB.

10 The CAP jet was described in detail elsewhere.[29] A grounded plate was placed below the 96-well  
11 plate, which served as a counter electrode for the powered ring electrode. Helium (He) gas (BOC, high  
12 purity grade) flow was fixed at 0.5 standard litres per minute (slm) throughout all experiments using  
13 a digital mass flow controller (Apex, 0–2 slm flow range). A sinusoidal voltage of 10 kVp–p (peak-  
14 to-peak) at 24 kHz was applied to the external electrode with a PVM500 power supply (Information  
15 Unlimited). The treatment distance, measured between the nozzle of the CAP jet and the top of the 96-  
16 well plate, was fixed at 3 mm.

17 The applied voltage and current were measured using a ground-referenced high voltage probe  
18 (Pintek Electronics, HVP-18HF) directly connected to the HV cable and a current monitor (Pearson  
19 2877) placed around the insulated part of the high voltage cable. The discharge current was  
20 determined by subtracting the current measured during operation of the CAP jet from the baseline  
21 current measured with applied voltage but without helium flow (i.e. without CAP discharge).

22 A fibre optic spectrometer (OceanOptics, Model – Flame-TX-R1-ES, Grating – #31-500/250) was  
23 used for measuring the optical emission between 200–1000 nm. All the measurements were taken in a  
24 custom-built “dark box” to prevent interference from ambient light.[29] The optical emission from the

1 plume of the CAP jet was recorded during treatment of the vesicle solutions. The integration time of  
2 the spectrometer (T) was set to 100 ms.

### 3 2.2 Phospholipid vesicle synthesis

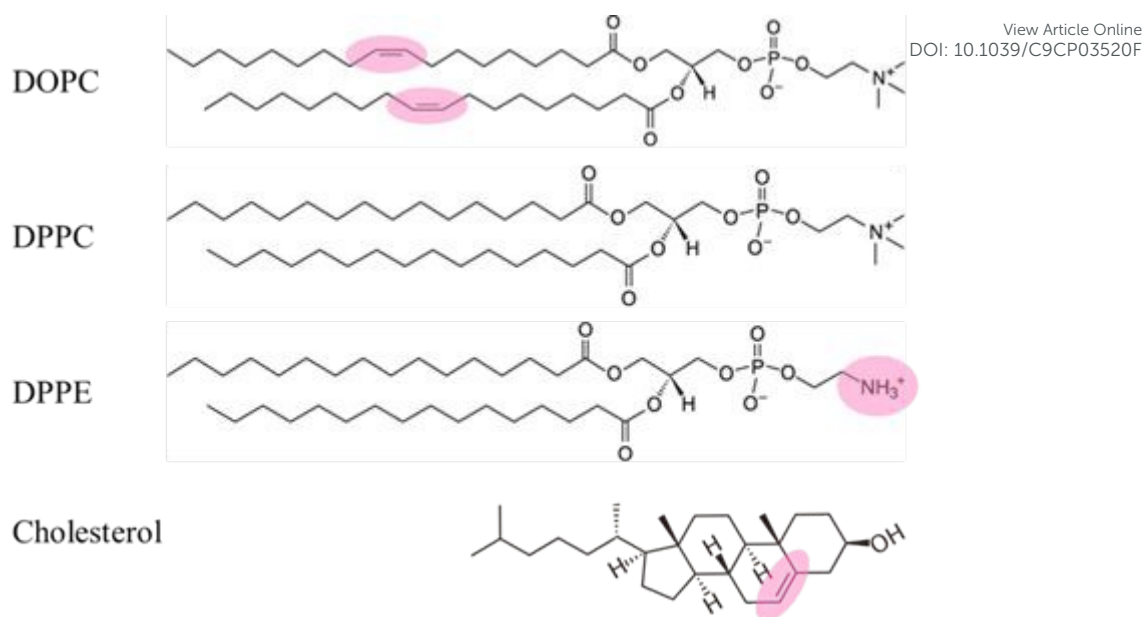
4 Ten different vesicle compositions were synthesized, which all contained different concentrations of  
5 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine  
6 (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-ethanolamine (DPPE), and cholesterol. All compositions are  
7 shown in Table 1.

8 **Table 1:** Composition of the synthesised phospholipid vesicles

Mixture	Cholesterol (mol%)	DOPC (mol%)	DPPC (mol%)	DPPE (mol%)
1	10	0	85	5
2	15	0	80	5
3	25	0	70	5
4	10	85	0	5
5	15	80	0	5
6	25	70	0	5
7	25	0	75	0
8	25	0	50	25
9	25	75	0	0
10	25	50	0	25

9  
10 These specific lipids were chosen, as lipids containing a phosphatidylcholine (PC) head group are the  
11 most common lipids in all cellular membranes (up to almost 60 mol% in specific  
12 membranes).[14,30,31] Moreover, C18 lipids are also one of the most abundant fraction of lipids  
13 found in cellular membranes, both in healthy cells, as well as in cancerous cells.[14,31] Furthermore,  
14 lipids containing a phosphatidylethanolamine (PE) head group were added to this study as certain  
15 diseased cells, such as cancer cells, display a higher phosphatidylethanolamine (PE) content in the  
16 outer leaflet of the bilayer membrane.[3,11] Lastly, by investigating DOPC, DPPC and DPPE, we  
17 were able to systematically alter one lipid parameter (head group or saturation degree), while keeping  
18 all others fixed.

19 The concentrations of DPPE were chosen because the PE concentration in the cell membrane is  
20 approximately 25%. [30] In healthy cells, all PE lipids are in the inner leaflet, but cancer cells lose the  
21 ability to maintain this asymmetry, with PE being expressed also in the outer leaflet. [4] In this study,  
22 we assume that the RONS will affect the outer leaflet first. Therefore, we chose to mimic the  
23 increasing concentration of PE in the outer leaflet of cancer cells up to 25 mol%. The concentration of  
24 cholesterol investigated in this study is also based on concentrations observed in human cells. [32]



1  
2 **Figure 2:** Schematic representation of the structures of DOPC, DPPC and DPPE lipids, and  
3 cholesterol. The parts highlighted in pink indicate key differences in the structure of each  
4 molecule that was tested in this study.

5 The different lipid compositions used in the ten vesicle types synthesized allowed us to tailor (i) the  
6 saturation degree of the phospholipid membranes with DOPC and DPPC, (ii) the cholesterol fraction  
7 within the membranes, and (iii) the size of the lipid head group with DPPE (which has a relatively  
8 small lipid head group compared to DOPC and DPPC). The key features of DOPC, DPPC, DPPE and  
9 cholesterol that were tested in this study are shown in Figure 2.

10 The vesicles were synthesized by mixing 100  $\mu\text{L}$  of a 0.1 mM stock solution of each vesicle  
11 mixture with 200  $\mu\text{L}$  of chloroform, after which the solutions were dried with nitrogen gas until only a  
12 thin film of the lipids on the bottom of the vial was left. Afterwards, 2.5 mL of the pre-prepared MB or  
13 DCFH solution was added, and the mixture was heated to 70  $^{\circ}\text{C}$  for 10 min for DCFH or 50  $^{\circ}\text{C}$  for the  
14 MB (lower temperature to prevent unwanted conformational changes to the MB). Subsequently, the  
15 mixture was extruded 15 times through two 100 nm polycarbonate membranes in a hand-held syringe  
16 extruder (Avanti Polar Lipids, model # 610000). This extruded solution was then transferred to a  
17 Sephadex G25 column, to purify the mixture and remove non-encapsulated dye. Finally, the eluate  
18 was stored overnight at 4 $^{\circ}\text{C}$ . Before CAP jet treatment, the vesicles were diluted by adding 4 mL of 4-  
19 (2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) to the solution. The details of the buffers  
20 used in this synthesis can be found in Appendix A1.

### 21 **2.3 2,7-dichlorodihydrofluorescein (DCFH): assessment of ROS ingress into vesicles**

22 A 1 mg/mL DCFH solution was prepared by dissolving 5 mg of DCFH-diacetate (Sigma-Aldrich,  
23 catalogue number D6883) in 5 mL of neat ethanol. This solution was stored at -18  $^{\circ}\text{C}$  until use. Ester  
24 hydrolysis was induced by adding 500  $\mu\text{L}$  of the DCFH solution to 2 mL of a 10 mM sodium  
25 hydroxide solution and incubating at 25  $^{\circ}\text{C}$  for 30 min at ambient temperature, before adding 10 mL of  
26 HEPES buffer to neutralise the solution.



## 1 **2.4 Molecular beacon (MB): assessment of DNA strand breaks**

2 The MB consisted of a single-stranded loop and double-stranded stem structure with a fluorophore and  
3 quencher moiety at the end of each DNA strand. With the MB intact, the quencher inhibits  
4 fluorescence of the fluorophore. If the DNA strand of the MB breaks, the quencher is separated from  
5 the fluorophore, resulting in a switch-on of fluorescence (as a positive indication of DNA strand  
6 breaks).[33] A 37 nM of MB (Sigma Aldrich, 3017672631, FAM-5-  
7 GCACTGAAGCGCCGACGCCATGTCGACGCG-CTTCAGTGC-3'-BHQ-1), as supplied in each  
8 bottle, was dissolved in 372  $\mu\text{L}$  of 10 mM Tris-base buffer (pH 7.6), giving a final concentration of 0.1  
9 mM. To ensure the correct configuration of the MB, the solution was first heated to 95  $^{\circ}\text{C}$  to  
10 (reversibly) denature the DNA, after which the MB was incubated overnight in the dark at ambient  
11 temperature, allowing the MB structure to slowly reform to its correct configuration. The MB solution  
12 was further diluted (1:5) in HEPES leading to a final working concentration of 16.7  $\mu\text{M}$  for  
13 encapsulation within the vesicles.

## 14 **2.5 Microplate reader measurements**

15 After CAP jet treatment of the 200  $\mu\text{L}$  test solutions, 100  $\mu\text{L}$  of the solution was transferred to a fresh  
16 96-well plate for absorbance or fluorescence measurements on a BMG Labtech Fluostar Omega  
17 microplate reader. Fluorescence of the MB or DCFH was recorded at an excitation wavelength of 485  
18 nm and emission wavelength of 520 nm. Absorbance of ortho-phenylenediamine (OPD) was measured  
19 at a wavelength of 450 nm for determination of  $\text{H}_2\text{O}_2$  concentrations (Appendix A2). Unless otherwise  
20 stated, all samples were measured directly after treatment. Where applicable, the normalised intensities  
21 were calculated by dividing the measured intensity of the test (CAP jet treated) solution by the non-  
22 treated solution.

## 23 **2.6 Simulation: $\text{H}_2\text{O}_2$ delivery across different membrane compositions**

24 We performed MD simulations to study the permeation of  $\text{H}_2\text{O}_2$  across lipid bilayers with various  
25 compositions. Although CAPs generate many different RONS, we choose to focus on  $\text{H}_2\text{O}_2$  as (i) we  
26 demonstrated in our previous work [34,35] that all polar RONS behave in the same way as  $\text{H}_2\text{O}_2$ , and  
27 (ii) as the vesicles are treated in a liquid solution, so a large fraction of the short-lived RONS  
28 generated by the plasma jet (e.g.,  $\cdot\text{OH}$ ) will recombine before reaching the vesicles, making  $\text{H}_2\text{O}_2$  the  
29 most abundant reactive species present in the solution. As in the experiments, ten different bilayers  
30 were constructed, of which the composition was chosen to mimic the composition of the vesicles  
31 studied experimentally, i.e., the bilayers contained different concentrations of DPPE and cholesterol in  
32 DPPC or DOPC systems. All these bilayers contained a total of 128 lipids, hydrated by 6000 water  
33 molecules surrounding the bilayer structure on both sides. The MD simulations thus only included a  
34 small patch of a membrane, which then served as a model system for the entire vesicle. The  
35 construction of the systems, as well as the equilibration procedure and the details of the US  
36 simulations, are provided in Appendix B1 and B2, respectively.

## 37 **2.7 Simulation: Area per lipid calculation**

1 The surface area per lipid was determined by dividing the total surface area of the membrane  
2 (averaged over the 200 ns, by sampling the data after every 200 ps) by the number of lipids of one  
3 leaflet of the phospholipid bilayer (PLB) (i.e.,  $L_x \times L_y/64$ , where  $L_x$  and  $L_y$  are the x and y  
4 dimensions of the PLB, respectively, and 64 is the number of lipids present in one layer).

## 5 **2.8 Simulation: Radial distribution function calculation**

6 The radial distribution function (RDF) determines the probability of finding a particle at a certain  
7 distance from a fixed reference particle. We calculated the RDF between phosphorus atoms of  
8 neighbouring DOPC molecules in membranes containing either 0 or 25% DPPE, using the last 200 ns  
9 of the equilibration run (by sampling the data after every 200 ps) and applying the *gmx rdf* tool of  
10 GROMACS.

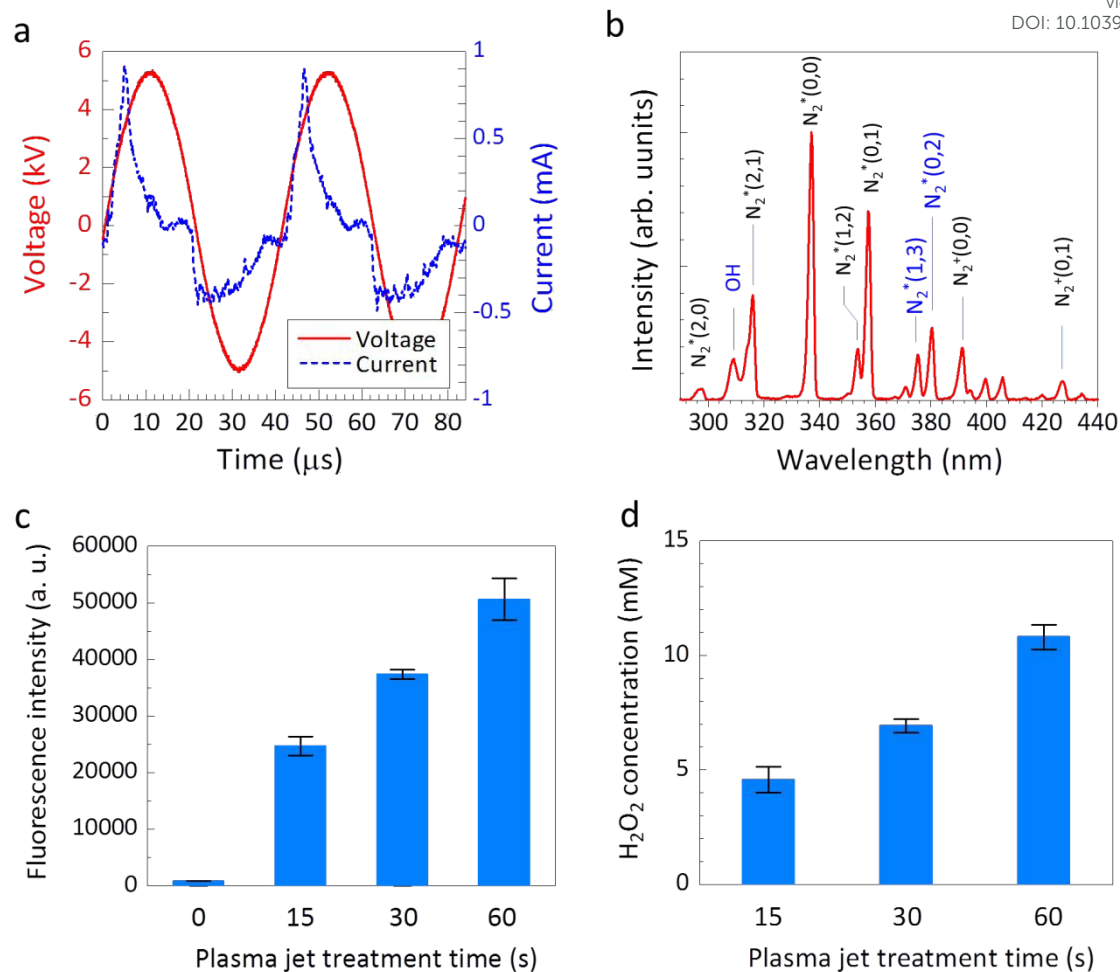
## 11 **2.9 Statistical analysis**

12 Statistical analysis was performed on the data presented in figures 4, 6 and 7, using an unpaired  
13 Student t-test assuming unequal variances. A *p* value of less than 0.05 or 0.001 was considered to be  
14 significantly different (95% and 99.9% confidence, respectively).

# 15 **3. Results and discussion**

## 16 **3.1 CAP jet as a source of oxidative molecules**

17 The basic electrical and optical properties of the CAP jet were characterised in order to determine how  
18 the physicochemical properties of the CAP jet relate to the delivery of ROS into the vesicle solution.  
19 Figure 3a shows the voltage and current waveforms during CAP jet treatment of a vesicle solution.  
20 The same voltage waveform with amplitude of 10 kV<sub>p-p</sub> at 24 kHz was maintained for all of the  
21 experiments (Figure 3a). Figure 3a also shows the typical positive and negative discharge current  
22 peaks expected for a dielectric barrier discharge CAP jet.[36] The average discharge power was  
23 calculated to be 0.79 W.



1  
2 **Figure 3:** Electrical and optical characteristics of the CAP jet and its generation of oxidative  
3 molecules in HEPES buffer. (a) Applied voltage and current waveforms at 10 kV<sub>p-p</sub> and  
4 24 kHz; (b) optical emission spectrum profile between 280–440 nm; (c) ROS and RNS  
5 generated in HEPES with the CAP jet, as measured by fluorescence of the DCFH  
6 molecular probe after oxidation by ROS and RNS; (d)  $\text{H}_2\text{O}_2$  concentrations generated in  
7 HEPES with the plasma jet as measured by the OPD/HRP molecular probe system.

8 Optical emission spectroscopy (OES) was used to determine the high-energy components of the CAP  
9 jet that facilitate ROS (and RNS) chemistry. Figure 3b shows a typical optical emission spectrum  
10 profile of the CAP jet during treatment of the vesicle solution including the  $\text{N}_2$  2nd positive system  
11 ( $\text{C}^3\Pi_u-\text{B}^3\Pi_g$ ) and the  $\text{N}_2^+$  1st negative system ( $\text{B}^2\Sigma_u^+-\text{X}^3\Sigma_g^+$ ) between 300–400 nm, and the  $\cdot\text{OH}$  at 308  
12 nm.[37] Using the theoretical calculation of the  $\text{N}_2$  2nd positive system [ $\text{N}_2^*(1,3)$  and  $\text{N}_2^*(0,2)$ ] with  
13 the spectrum simulator of the  $\text{N}_2^*$  2nd positive system kindly provided by Professor Hiroshi Akatsuka  
14 at Tokyo Institute of Technology [38], we obtained the best fitted spectra with the rotational  
15 temperature ( $T_r$ ) of 0.028 eV.  $T_r$  is considered the gas temperature because of the rapid rotational  
16 relaxation through inelastic collisions between molecules and atoms[39], and was calculated to be 290  
17 K. Here, the  $T_r$  of  $\text{N}_2$  molecules is in very good agreement with our previous calculations.[40–42] The  
18 vibrational temperature ( $T_v$ ) of 0.28 eV was higher than our previous studies. This is probably due to  
19 the higher frequency of 24 kHz used to operate the CAP jet in this study compared to our previous  
20 studies (below 10 kHz), which probably resulted in increased inelastic collisions between electron and

1 N<sub>2</sub> molecules in the ambient air. In Figure 3b, a prominent peak is seen at 308 nm representative of the  
2 •OH, which is a key species that can produce longer-term ROS chemistry in solution, particularly  
3 through recombination of neighbouring •OH molecules producing H<sub>2</sub>O<sub>2</sub> [43,44]. H<sub>2</sub>O<sub>2</sub> can persist in  
4 solution on the timescale of days or longer.[45,46]

5 We used DCFH as a broad range molecular probe for ROS (and RNS) molecules to qualitatively  
6 determine the CAP jet production of these molecules in the buffered solution. As shown in Figure 3c,  
7 the fluorescence of DCFH (after oxidation by ROS and RNS) increased as function of the CAP jet  
8 treatment time. As mentioned above, H<sub>2</sub>O<sub>2</sub> is usually the most abundant longer-lived ROS generated  
9 by CAP jets in solution. Figure 3d shows that the concentration of H<sub>2</sub>O<sub>2</sub> increased as function of CAP  
10 jet treatment time in the concentration range 4-12 mM. We anticipated that this H<sub>2</sub>O<sub>2</sub> concentration  
11 range would be sufficient to observe changes in the vesicles.[47]

### 12 3.2 ROS delivery into vesicles with different membrane compositions

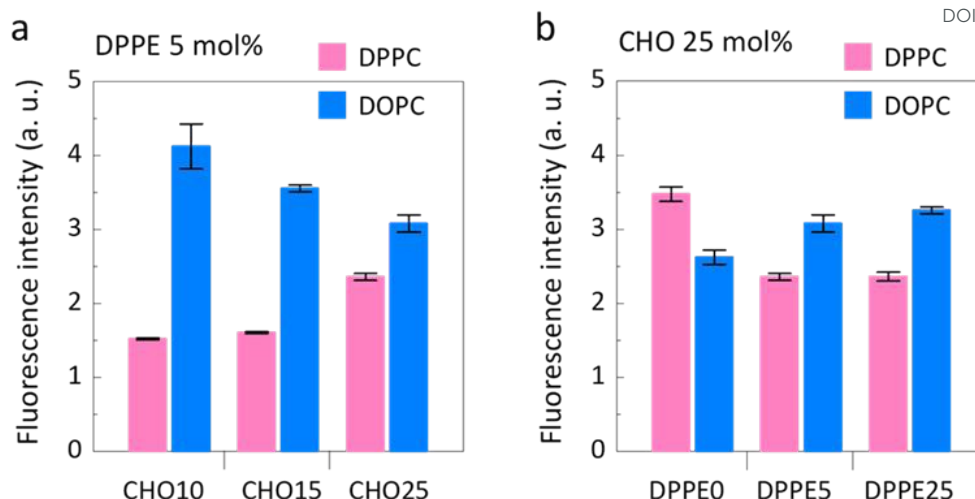
13 Having established that the vesicles remained intact after plasma jet treatment (Appendix A4), we next  
14 set out to study how different components of the vesicle membranes influence the ingress of ROS into  
15 the vesicle interior. Enhanced ingress of ROS into biological cells induces a higher level of oxidative  
16 stress on the cells, and excessive or sustained levels of ROS are toxic to all cells, which for some  
17 applications can be used for the targeted destruction of cells (e.g. in cancer therapy).[48] The specific  
18 membrane components studied were: (i) unsaturated and saturated phospholipids; (ii) cholesterol  
19 fraction; and (iii) DPPE. For these experiments, DCFH encapsulated within the vesicles was used as a  
20 fluorescence probe for detection of ROS (and RNS).

### 21 3.3 ROS delivery across cell membranes

22 Oxidation of non-fluorescent DCFH to fluorescent DCF was used to qualitatively measure the amount  
23 of ROS that had entered vesicles after CAP jet treatment. In the context of the CAP jet used herein,  
24 H<sub>2</sub>O<sub>2</sub> is produced in abundance in the mM range; other molecules such as nitrate (NO<sub>3</sub><sup>-</sup>) and nitrite  
25 (NO<sub>2</sub><sup>-</sup>) are produced at much lower concentrations in the μM range. We looked at (i) the effect of the  
26 saturation degree of the phospholipids, (ii) the cholesterol fraction and (iii) the amount of DPPE  
27 included within the membrane. Below, the experimental results of synthetic vesicles are combined  
28 with MD simulations for all three elements separately.

#### 29 ▪ Effect of lipid tail saturation degree

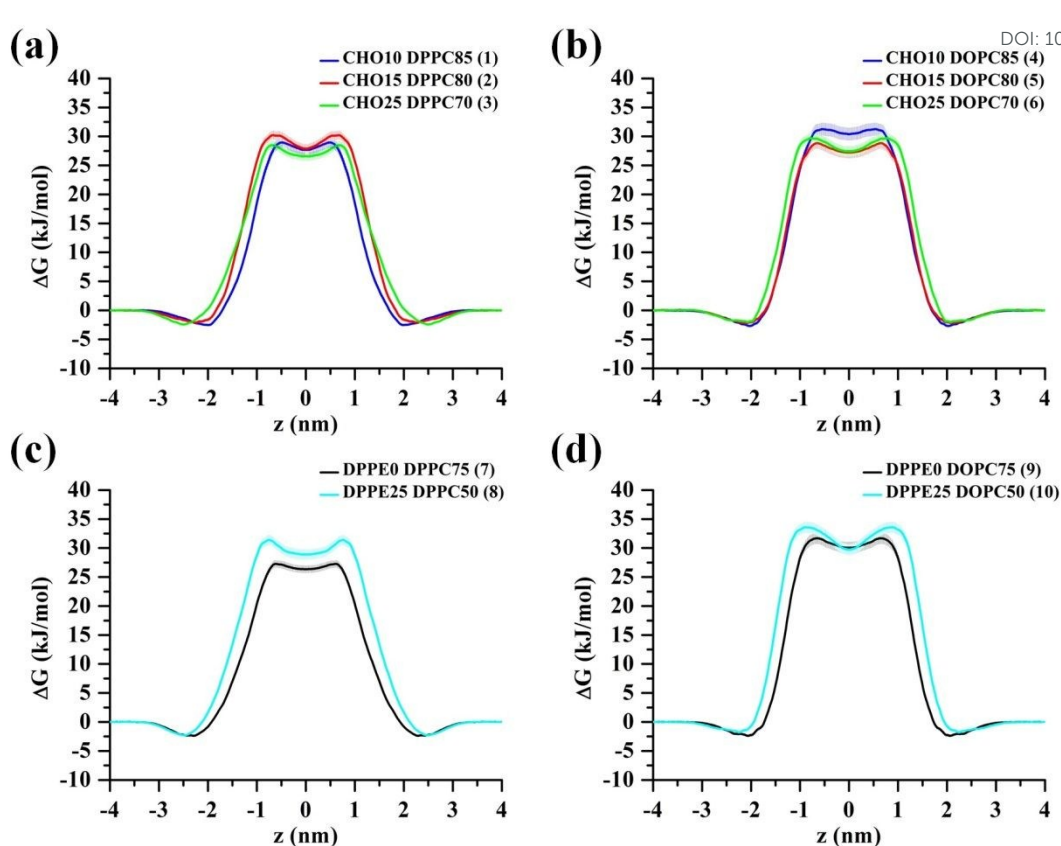
30 To examine the effect of the saturation degree of the phospholipids on the ROS ingress in vesicles,  
31 vesicles were synthesised to incorporate equal amounts of cholesterol and DPPE, and contain either  
32 DOPC (C18:1) or DPPC (C16:0); i.e., we compare vesicle systems 1-3, 7 and 8 (containing DPPC)  
33 with systems 4-6, 9 and 10 (based on DOPC) (Table 1). The results are illustrated in Figure 4.



1  
2 **Figure 4:** Effect of either DPPC or DOPC on the ingress of ROS into vesicles following 60 s of CAP  
3 jet treatment. The vesicles in the left graph contain different concentrations of cholesterol  
4 (10, 15 or 25 mol%) with fixed concentration of DPPE (5 mol%), whereas the vesicles in  
5 the right graph contain different concentrations of DPPE (0, 5 and 25 mol%) at fixed  
6 cholesterol concentration (25 mol%).

7 With a single exception in the case where vesicles contain no DPPE (discussed in following  
8 paragraph), Figure 4 shows that there is greater ingress of ROS in the vesicles containing DOPC (i.e.,  
9 the unsaturated lipids) when compared to those containing DPPC ( $p < 0.001$ ), for which two likely  
10 explanations can be found. First, DOPC has a lower phase transition temperature of 256 K compared  
11 to DPPC (314 K).[49] Therefore, at an average temperature of approximately 298 K, the membrane  
12 containing DOPC will be much more fluid compared to that containing DPPC, which could facilitate  
13 the passive diffusion of ROS into the vesicle interior. The second explanation is that the double bond  
14 within the lipid tails of DOPC presents a potential site for lipid oxidation, with highly-reactive ROS  
15 that reach the vesicle membrane (the oxidation rate of unsaturated hydrocarbons by  $\cdot\text{OH}$  is 10 to 20  
16 times higher compared to that of saturated hydrocarbons).[50] Oxidation increases (i) the polarity of  
17 the membrane interior (in extreme cases even aqueous pores can be formed [34,51]) and (ii) the  
18 fluidity of the membrane; both are expected to facilitate passive diffusion of polar ROS and RNS, such  
19 as,  $\text{H}_2\text{O}_2$ ,  $\text{NO}_2^-$  or  $\text{NO}_3^-$ , as previously demonstrated.[34] Figure 4 also indicates one exception, i.e., for  
20 membranes containing the highest amount of cholesterol (25 mol%) and the lowest amount of DPPE  
21 (0 mol%). In this case, the DPPC-containing membrane was more susceptible to ROS permeation than  
22 the DOPC membrane. This is attributed to the opposite effects that cholesterol and DPPE exert in  
23 either saturated (DPPC) or unsaturated (DOPC) membranes, and will be discussed in more detail in  
24 the later sections, in which we focus on the effect of both cholesterol and DPPE separately.

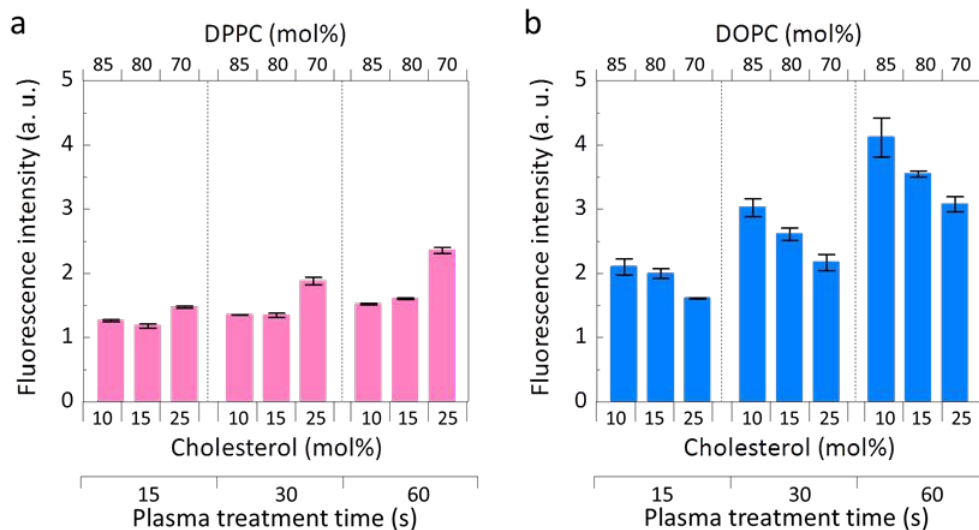
25 MD simulations of PLBs were performed to investigate the importance of DOPC and DPPC on  
26 ROS ingress. The obtained free energy profiles (FEPs) for  $\text{H}_2\text{O}_2$  (major ROS produced by the CAP jet  
27 in this study) are shown in Figure 5, which are a measure for the barrier of passive diffusion of  $\text{H}_2\text{O}_2$   
28 across the membrane.



**Figure 5:** FEPs obtained from the US simulations for all different membrane compositions. The numbers mentioned in the legend indicate the molar fraction of each component in the lipid bilayer. (a) and (b) compare the effect of DPPC and DOPC, respectively, when varying the cholesterol content (keeping the DPPE content fixed at 5 mol%), whereas in the membranes investigated in (c) and (d) the DPPE content is varied, keeping the cholesterol content fixed at 25 mol%, for either (c) DPPC or (d) DOPC.

To understand what these Figures show, we take Figure 5a as an example. The y ordinate shows the increase in free energy ( $\Delta G$ ) as a single molecule of  $\text{H}_2\text{O}_2$  is introduced to the system.  $z = 0$  nm is defined as the centre of the PLB, whereas the head groups of the lipids can be found at  $z \approx \pm 2$  nm. At  $z \approx \pm 4$  nm there is no change in free energy anymore, as the  $\text{H}_2\text{O}_2$  molecule is located in the bulk of the liquid. As we move the  $\text{H}_2\text{O}_2$  molecule from the bulk of the liquid towards the lipid membrane, the free energy of the system increases, which is due to the hydrophilic nature of  $\text{H}_2\text{O}_2$ , in comparison to the hydrophobic environment of the lipid membrane. A local minimum in free energy is observed in the centre of the membrane, which is due to a decreased lipid density in this region.[51]

Figure 5 shows almost no difference between the height of the FEPs of bilayers containing either DOPC or DPPC (comparing Figure 5a with 5b, or Figure 5c with 5d). This means that the passive diffusion rate across all these membranes would be almost identical. This indicates that the differences observed in the vesicle experiments are more likely to arise from lipid oxidation occurring at the unsaturated bonds in DOPC, and not due to differences in fluidity (arising from differences in phase transition temperature). An important note, however, is that these simulations only focus on the diffusion of  $\text{H}_2\text{O}_2$ , thereby ignoring other smaller species (e.g. the  $\cdot\text{OH}$ ), which will be discussed later in more detail.

1 ■ **Effect of cholesterol**

2  
3 **Figure 6:** Effect of cholesterol membrane content on the ingress of ROS during CAP jet treatment of  
4 (a) DPPC or (b) DOPC vesicles. The DPPE content is fixed at 5 mol%.

5 To determine the effect of variations in the cholesterol content on ROS delivery, vesicle systems 1 to 6  
6 (Table 1) were compared; these vesicles contained 10, 15 or 25 mol% cholesterol in combination with  
7 5 mol% DPPE, and either DOPC or DPPC. The results of the DCFH measurements are shown in  
8 Figure 6.

9 First, these graphs illustrate opposite trends in vesicles containing mostly either (a) DPPC or (b)  
10 DOPC. In the DPPC-containing vesicles (pink bars), ROS ingress is increased with increasing  
11 cholesterol fraction, while in the DOPC membranes (blue bars), the opposite trend is seen. Specifically,  
12 at lower cholesterol fractions of 10 and 15 mol%, a significantly higher amount of ROS was detected  
13 in the DOPC vesicles ( $p < 0.001$ ). On the other hand, the difference in the amount of ROS detected in  
14 both vesicles was insignificant ( $p > 0.05$ ) at the highest cholesterol fraction of 25 mol% (due to ROS  
15 ingress increasing for DPPC vesicles and decreasing for DOPC vesicles at the highest cholesterol  
16 fraction). Moreover, Figure 6 shows that in the case of the DOPC vesicles, in comparison to the DPPC  
17 vesicles, (i) the overall ROS ingress is higher, as was also clear from Figure 4; but also that (ii) the  
18 increase in ROS ingress upon increasing treatment time is much more pronounced. Indeed, the  
19 fluorescence intensity in each of the DPPC vesicles is almost constant as a function of treatment time,  
20 while the DOPC vesicles show a clear increase with increasing treatment time from 15 s up to 60 s.  
21 This corresponds with our conclusion above; i.e., that the ingress in the DOPC vesicles is mostly  
22 governed by lipid oxidation, which facilitates permeation of the membrane, and the latter increases  
23 upon longer treatment time, while in the DPPC-containing vesicles, only a small accumulation of ROS  
24 due to passive diffusion is observed.

25 An explanation as to why opposing trends are observed upon altering the cholesterol fraction in  
26 either DPPC or DOPC vesicles can also be found in the ability of CAP jet species to oxidize these  
27 different lipids.[50] In DPPC vesicles lacking cholesterol, oxidation is not likely to occur.[52]

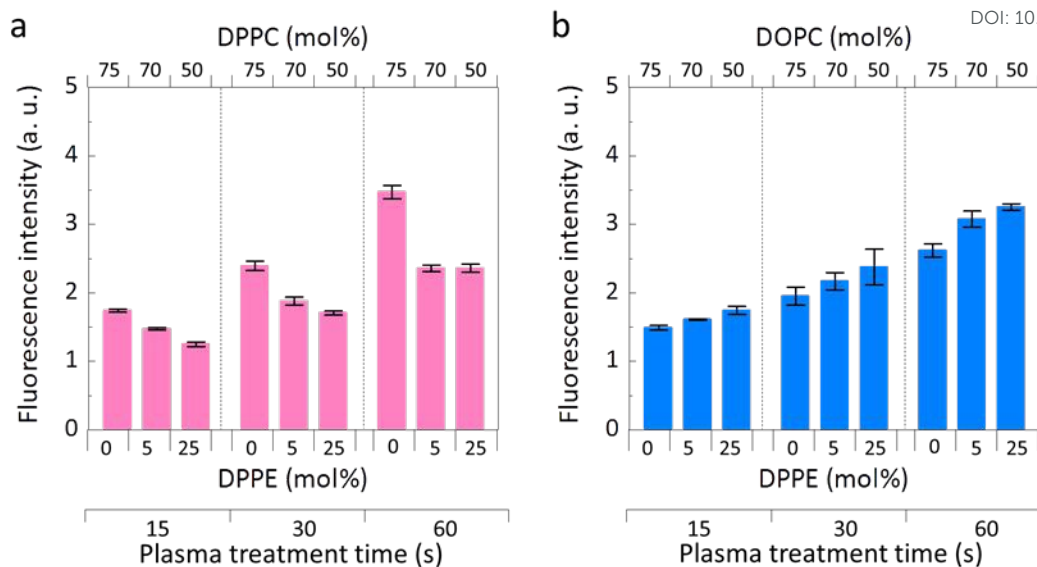
1 However, when cholesterol is added to the membrane, the unsaturated bonds present in these  
2 molecules can be oxidized by impinging ROS. Due to this oxidation, the polarity of the membrane  
3 core would increase slightly, which facilitates further permeation of CAP jet-derived ROS.[53]  
4 Therefore, if the concentration of cholesterol increases in the DPPC vesicles, the permeation of ROS  
5 increases as well, as is observed in Figure 6. In DOPC vesicles, on the other hand, even without the  
6 presence of cholesterol, lipid oxidation already occurs due to the double bond in the lipid tails.  
7 Replacing DOPC by cholesterol reduces the total number of reactive sites per vesicle present in the  
8 lipid core (each molecule of DOPC contains two double bonds, whereas cholesterol only contains one  
9 double bond), thereby lowering the overall reactivity of the membrane. This explains why a reduced  
10 amount of intracellular ROS is measured in the DOPC vesicles upon increasing cholesterol fraction  
11 (Figure 6). Furthermore, the rigid nature of cholesterol also strongly increases the lipid packing in  
12 unsaturated membranes, which would hamper passive ROS permeation further, as previously  
13 shown.[25]

#### 14 ■ **Effect of DPPE content**

15 The effect of the concentration of DPPE on the ingress of plasma-derived ROS, was examined using  
16 vesicles containing 0, 5 or 25 mol% of DPPE. Throughout these experiments, the cholesterol content  
17 was fixed at 25 mol%. The results of the broad-range ROS measurements are shown in Figure 7.

18 Adding DPPE to the vesicles clearly exerts the opposite effect to that of adding cholesterol (cf.  
19 Figure 6 to 7); i.e., adding DPPE leads to a decreasing permeability in the DPPC vesicles, but an  
20 increase of ROS ingress in the DOPC vesicles. The effect of DPPE on ROS ingress is particularly  
21 noticeable when comparing vesicles with 5 mol% and 25 mol% DPPE. At 5 mol% DPPE, ROS  
22 ingress between DPPC and DOPC is not significant ( $p > 0.05$ ), whereas a significantly ( $p < 0.05$ )  
23 higher amount of ROS ingress was observed for DOPC vesicles at 25 mol% DPPE (compared to  
24 DPPC vesicles). In the DPPC vesicles, lipid tail oxidation effects are not expected to play any role, as  
25 DPPC and DPPE contain identical lipid tails (Figure 2). Therefore, differences observed may be  
26 explained by structural changes between DPPC and DPPE, i.e., a different head group of the lipid.  
27 Indeed, DPPC contains a phosphatidylcholine (PC) group ( $\text{PO}_4^- \text{-N}(\text{CH}_3)_3^+$ ), whereas DPPE contains a  
28 phosphatidylethanolamine (PE) group ( $\text{PO}_4^- \text{-N}(\text{H}_3)_3^+$ ) (Figure 2).





**Figure 7:** Effect of DPPE content on the ingress of CAP-jet derived ROS into (a) DPPC or (b) DOPC vesicles. The cholesterol content was fixed at 25 mol%.

The PE head group of DPPE is less bulky than the PC head group in DPPC. Thus, when replacing DPPC by DPPE, the lipids can be packed closer together, which makes passive permeation of ROS more difficult, as indeed observed in the DCFH measurements. This is also confirmed in the MD simulations, by comparing the average area per lipid in the different membrane structures, as shown in Table 2.

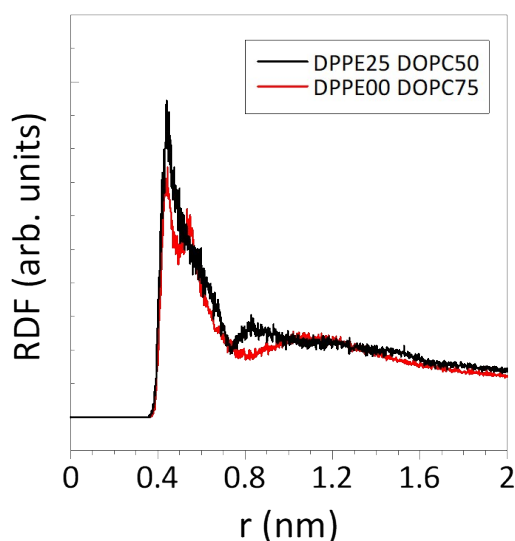
**Table 2:** Calculated values of the area per lipid for the different simulated membranes.

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

Increasing the DPPE content in either the DPPC or the DOPC vesicles increases the packing of the lipids, leading to a decreasing area per lipid (Table 2). Upon increasing the DPPE fraction, the passive diffusion of ROS should be hampered in both systems. For the DPPC vesicles, this is also observed in the US simulations, which show that upon increasing DPPE fraction, the free energy barrier of H<sub>2</sub>O<sub>2</sub> permeation increases (see Figure 5c).

However, in the DOPC vesicles, the DCFH measurements indicate an increasing permeability upon increasing DPPE fraction, which does not correlate with the results in Table 2. The explanation for this is not straightforward, even more so when we consider that when DOPC is replaced by DPPE, the total number of reactive sites in the membrane decreases, which should result in a decrease in the likelihood of pore formation due to lipid oxidation, and thereby also the chances of ROS permeating the membrane. Therefore, we suspect that a different effect is taking place, which counteracts the effects of both an increase in lipid packing density and a decrease in lipid oxidation.

1 One possibility is that the presence of DPPE leads to the creation of lipid rafts in the membrane  
2 structures, i.e.; lipids of the same type pack together to form patches with an enhanced local  
3 concentration of this lipid inside the membrane. It is known that cholesterol tends to form such rafts  
4 together with sphingolipids and other saturated lipids.[8,54] These saturated lipids contain an aliphatic  
5 tail, which is also present in DPPE (see Figure 2), but not in DOPC (which contains a double bond in  
6 each lipid tail). Therefore, upon increasing the DPPE concentration, lipid rafts could be generated,  
7 containing elevated levels of DPPE and cholesterol. As the overall molar fraction of each lipid type is  
8 fixed in the vesicles, this would mean that other parts of the vesicle membrane are enriched in DOPC.  
9 Thus, due to the generation of lipid rafts, enriched in DPPE and cholesterol, other parts of the  
10 membrane might be enriched in DOPC, which serve as the 'weak spot' of these membranes, being  
11 extremely vulnerable to pore formation upon lipid oxidation. In previous research, we have shown  
12 that in order to create pores in lipid membranes, very high lipid (local) oxidation degrees are required<sup>16</sup>,  
13 which can indeed only occur if these oxidised lipids are grouped together in membrane patches.



14  
15 **Figure 8:** RDFs of the P-P distance of neighbouring DOPC lipids in systems containing 0 or 25 mol%  
16 DPPE. The numbers mentioned in the legend indicate the molar fraction of each  
17 component in the vesicles. The cholesterol content is fixed at 25 mol%.

18 To analyse possible lipid raft formations in these membrane structures, we compare the radial  
19 distribution functions (RDFs) of the P-P distance of neighbouring DOPC lipids, in systems containing  
20 0 and 25 mol% DPPE in Figure 8. These RDFs are a measure for the probability of finding a particle  
21 (in this case, any P-atom) at a certain distance from a fixed reference particle (the reference P-atom).

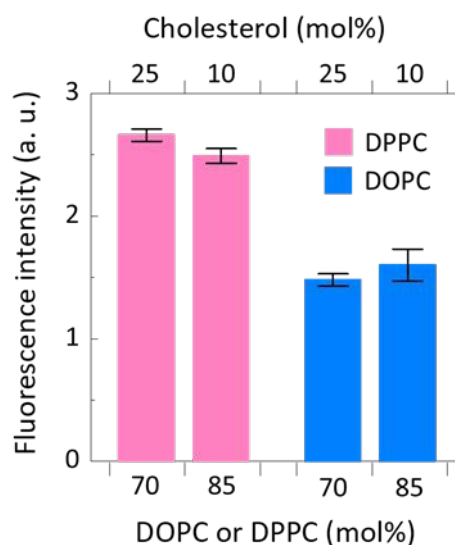
22 In the system containing only DOPC and cholesterol (red curve), the first peak (at a distance of  
23 0.45 nm) corresponds to two DOPC molecules placed next to each other. The second peak, at a  
24 distance of 0.55 nm, most likely occurs from DOPC molecules separated by a cholesterol molecule.  
25 Upon adding DPPE to the system, the black curve clearly shows that the peak of neighbouring DOPC  
26 molecules increases, which means that more DOPC molecules are grouped together. At first sight, this  
27 is counterintuitive, as there are more non-DOPC lipids in the system, so the chance of DOPC being

1 organised directly next to each other should decrease. Therefore, these results might indicate that lipid  
2 raft formation does occur in these systems, creating rafts that are either enriched in DOPC (explaining  
3 this higher peak) or in DPPE/cholesterol. As mentioned before, these DOPC rafts serve as the  
4 membrane's 'weak spot' for lipid oxidation and pore formation, which explains the increased ROS  
5 ingress in Figure 7 upon increasing DPPE content, for the DOPC vesicles. In DPPC vesicles, such  
6 rafts will not be created as both DPPC and DPPE contain the same aliphatic lipid tails. Therefore, their  
7 interaction with cholesterol will be equally strong. It has to be kept in mind, however, that the model  
8 membranes only contain 64 lipids in each leaflet, which makes it difficult to mimic the actual raft  
9 formation process that could take place in the vesicle systems.

10 A combination of different processes discussed above (lipid oxidation and raft formation) can also  
11 explain the anomaly observed in Figure 4; i.e., why are DPPC vesicles containing 0 mol% DPPE and  
12 25 mol% cholesterol are more vulnerable to ROS ingress compared to the DOPC equivalent? As  
13 discussed above, increasing the cholesterol fraction reduces oxidation in DOPC vesicles, whereas it  
14 increases oxidation in DPPC vesicles. Moreover, because the DOPC vesicles lack DPPE, raft  
15 formation will not occur. This means that the cholesterol molecules are dispersed homogeneously  
16 through the membrane, protecting it from pore formation after lipid oxidation of DOPC. On the other  
17 hand, in DPPC vesicles, cholesterol itself is oxidised due to impinging ROS, thereby losing its ability  
18 to strengthen the membrane.

### 19 **3.4 CAP jet induced DNA strand breaks**

20 Finally, we investigate the ability of CAP to induce DNA strand breaks of a MB encapsulated within  
21 vesicles, focusing on the effect of cholesterol in combination with the saturation degree of the lipids.  
22 Therefore, only vesicles 1, 3, 4 and 6 were taken into account, which contain either 10 or 25 mol%  
23 cholesterol, 5 mol% DPPE, combined with either DPPC or DOPC. The fluorescence intensity of the  
24 MB encapsulated in these vesicles was measured after 60 s of CAP jet treatment. A higher  
25 fluorescence intensity correlates with an increased number of DNA strand breaks induced by the CAP  
26 jet treatment. The results of these measurements are shown in Figure 9.



**Figure 9:** Relative amount of DNA strand breaks within vesicles following 60 s CAP jet treatment of DPPC (pink bars) or DOPC vesicles (blue bars), with a membrane cholesterol content of 10 mol% and 25 mol%, and a fixed DPPE content of 5 mol%.

Upon altering the saturation degree of the main lipid that comprises the vesicle, cholesterol was observed to have opposite effects on the ability of the CAP jet to induce DNA strand breaks within the vesicles. Although the differences are rather small, this observation is in line with that of the ROS measurements discussed above. In the vesicles comprising mainly of a saturated lipid, i.e., containing mostly DPPC, increasing the cholesterol content increases the number of DNA strand breaks, just as it increases the total ROS ingress, due to passive ROS diffusion. On the other hand, in the DOPC vesicles, increasing the cholesterol content leads to a decrease in the number of DNA strand breaks, attributed to the lower DOPC content, and thus less lipid oxidation, yielding less pore formation.

A perhaps somewhat surprising result, is that the total number of DNA strand breaks is significantly lower in the DOPC vesicles compared to the DPPC vesicles, which does not correlate with the total ROS ingress measured in the DCFH vesicle experiments (see Figure 4). Short-lived ROS (e.g. the  $\cdot\text{OH}$  or O-atoms, with an intracellular half-life of  $10^{-9}$  s) are much more effective species to induce DNA strand breaks, rather than long-lived ROS (e.g.  $\text{H}_2\text{O}_2$ , with an intracellular half-life of  $10^{-3}$  s). [29,55] Therefore we need to consider how these short-lived, reactive species are likely to interact with the different components of the vesicle membrane. In DOPC vesicles, the double bonds of the DOPC lipids serve as a highly-reactive site, effectively scavenging these short-lived ROS before they can reach the vesicle interior. [50] The ROS measurements discussed above, measure the total number of ROS (and RNS) entering the vesicles, without differentiating between species with different lifetimes. The combination of both assays (DCFH and MB) indicates that in DOPC vesicles, more ROS are able to penetrate the vesicle membrane, but we surmise these are mostly longer-lived ROS, while in DPPC vesicles, a lower number of ROS is able to diffuse through the membrane, but these are mostly shorter-lived ROS. Generally, the latter are much smaller, which facilitates their ability to diffuse through the membrane. Indeed, previous research has shown that the energy barrier

1 for passive diffusion across a membrane of short-lived ROS, such as OH or HO<sub>2</sub> radicals, is  
2 significantly lower compared to that of e.g., H<sub>2</sub>O<sub>2</sub>. [51]

### 3 4. Conclusion

4 CAP jet treatment of lipid vesicles was combined with MD simulations to study the effect of different  
5 membrane lipid compositions on the ability of plasma-derived ROS to diffuse through these  
6 membranes. We varied the saturation degree of the lipid tails, the cholesterol content of the membrane,  
7 and the type of lipid head group (i.e., PE vs. PC head group). We selected these characteristics because  
8 different cell types, including healthy and cancerous human cells, sometimes also show variations in  
9 these types of lipid characteristics, as explained in the Introduction. [9,11–14,56]

10 Our results indicate that CAP jets can potentially be utilised to induce specific cellular effects  
11 (oxidative stress and DNA damage) by targeting interactions of plasma-derived ROS with specific cell  
12 membrane lipid components. The ROS measurements show that generally vesicles containing more  
13 unsaturated lipids (DOPC) are significantly more vulnerable to ROS ingress compared to those  
14 containing more saturated lipids (DPPC). However, adding cholesterol to both types of vesicles makes  
15 the DOPC vesicles less vulnerable, while it increases the vulnerability of DPPC vesicles to ROS  
16 ingress. Similarly, the addition of DPPE, which contains a smaller head group compared to DOPC and  
17 DPPC, also exerts opposite effects in either the DOPC or the DPPC vesicles. In DOPC membranes,  
18 the addition of DPPE makes the vesicles more susceptible to ROS permeation, whereas in DPPC  
19 membranes, it has a ‘protecting’ role. To determine possible underlying mechanisms causing these  
20 observations, we performed MD simulations. Although these simulations cannot be seen as absolute  
21 evidence for the underlying mechanisms, they show that multiple effects can potentially play a role,  
22 including (i) lipid oxidation of unsaturated phospholipids and cholesterol, (ii) lipid packing due to the  
23 head group volume, and (iii) formation of lipid rafts due to strong interactions between lipids of the  
24 same type.

25 We also assessed the ability of a CAP jet to induce DNA strand breaks within vesicles  
26 encapsulating a MB. This led to the surprising observations that, although DOPC vesicles are more  
27 susceptible to ROS permeation compared to DPPC vesicles, the MB within DPPC vesicles is more  
28 susceptible to CAP jet-derived ROS damage. This can be explained by the fact that the double bonds  
29 present in DOPC serve as scavenging sites for short-lived ROS, thereby preventing them from  
30 entering the vesicle interior. On the other hand, due to the reaction of the double bonds with these  
31 short-lived species, the polarity of the bilayer interior increases, facilitating the overall permeation of  
32 other impinging ROS. In DPPC vesicles, no reactive sites are present, which allows short-lived ROS  
33 to diffuse through the membrane. Once they reach the vesicle interior, these species are very efficient  
34 in inducing DNA strand breaks. However, without lipid oxidation occurring, the membrane’s  
35 hydrophobic environment is sustained, thereby hindering the overall ingress of impinging larger and  
36 more stable ROS (e.g., H<sub>2</sub>O<sub>2</sub>).

37 In the past decades, numerous claims have been made for the potential of plasma medicine to help  
38 alleviate a wide range of healthcare challenges, which include the selective treatment of cancers[16,57]

1 However, the full clinical benefits of CAP are yet to be realised, possibly due to our lack in  
2 understanding of how CAP can be used to target desired effects in specific cell types. This paper  
3 aimed to provide new insights into how different membrane lipid components, of relevance for  
4 treatment of certain diseases such as some cancers, can be exploited by CAP technology for targeted  
5 membrane therapy. As the oxidative stress provoked by CAP is considered the main mode-of-action to  
6 induce cellular effects, it is important to develop our understanding of how the cell membrane  
7 landscape influences the cell's response to oxidative stress. To aid this research, more advanced  
8 models will need to be developed both experimentally and computationally, as a means of  
9 scientifically testing the potential of CAP technologies in biology and medicine.

## 10 Acknowledgements

11 We acknowledge financial support from the Research Foundation – Flanders (FWO), grant numbers  
12 11U5416N, 1200216N and 1200219N, as well as an FWO travel grant to the University of South  
13 Australia. The computational work was carried out using the Turing HPC infrastructure at the CalcUA  
14 core facility of the Universiteit Antwerpen (UA), a division of the Flemish Supercomputer Centre  
15 VSC, funded by the Hercules Foundation, the Flemish Government (department EWI) and the UA.  
16 JSO acknowledges the MEXT-Supported Program for the Strategic Research Foundation at Private  
17 Universities (S1511021). The authors wish to thank Prof. Hiroshi Akatsuka at Tokyo Institute of  
18 Technology for providing a spectrum simulator of the N<sub>2</sub> second positive system. S.-H.H, N.G. and  
19 E.J.S acknowledge financial support from the Australian Research Council through Discovery Project  
20 Number DP16010498.

## 21 Appendix A: Experimental details

### 22 A1. Preparation of buffers

23 The 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was prepared by dissolving  
24 the following components in 100 mL of purified water:

- 25       ▪ HEPES—238.3 mg
- 26       ▪ NaCl—624 mg
- 27       ▪ NaOH—22.4 mg
- 28       ▪ EDTA—29.22 mg

29 The pH of the solution was adjusted to 7.4 with 10 mM NaOH solution. The buffer was filtered  
30 through a 0.2 μm syringe filter before use. HEPES buffer was used for the preparation and dilution of  
31 the vesicles for the experiments.

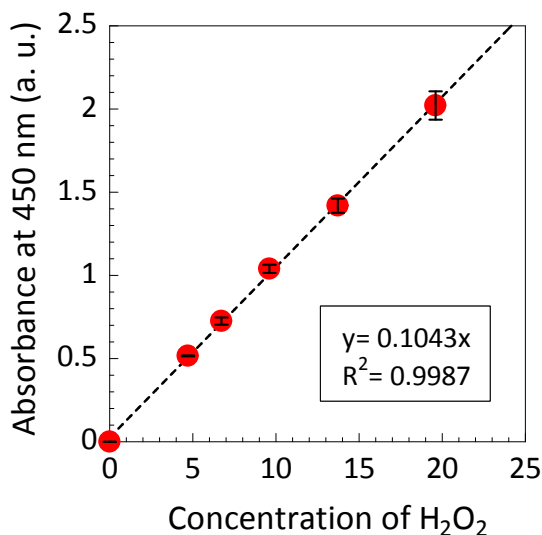
32 A concentrated buffer of 1M Tris buffer was prepared by dissolving 12.11 g of Tris-base in 80 mL  
33 water. The pH was adjusted to 7.6 by adding concentrated HCl (6.2 M), after which the final volume  
34 was adjusted to 100 mL. The concentrated Tris buffer was diluted to 10 mM in water for the  
35 preparation of the molecular beacon (MB) stock solution.

### 36 A2. ortho-phenylenediamine (OPD)/horseradish peroxidase (HRP) H<sub>2</sub>O<sub>2</sub> indicator

1 Ortho-phenylenediamine OPD (Sigma-Aldrich, P9187) was dissolved in 20 mL of PBS supplemented  
2 with 125 ng /ml of horseradish peroxidase (HRP) (Sigma-Aldrich, catalogue number P6782). The  
3 solution was stored at 4 °C until use. The OPD/HRP solution was used to determine the concentration  
4 of H<sub>2</sub>O<sub>2</sub> generated by the CAP jet in solution. This involved adding 200 μL of the OPD/HRP solution  
5 to wells of a 96-well plate and then treating the solution with the CAP jet. After 10 min of incubation  
6 at ambient temperature (to allow time for the HRP to catalyse the oxidation of the OPD in the presence  
7 of H<sub>2</sub>O<sub>2</sub>, into a yellow coloured product), 100 μL of the OPD/HRP solution was transferred to a fresh  
8 96-well plate for measurement. The H<sub>2</sub>O<sub>2</sub> concentration in the plasma jet treated was determined  
9 according to a calibration curve (see below).

### 10 A3. Determination of H<sub>2</sub>O<sub>2</sub> concentration

11 The H<sub>2</sub>O<sub>2</sub> concentration generated by the CAP jet treatments in the HEPES buffer (presented in Figure  
12 3d in the manuscript) was determined according to a calibration curve. The calibration curve was  
13 determined by adding 10 μL of known H<sub>2</sub>O<sub>2</sub> concentrations to 80 μL of OPD/HRP solution (see above)  
14 and incubating for 10 min at ambient temperature (same incubation time as is used in the manuscript,  
15 which was determined to be sufficient to develop the solution). Afterwards, absorbance of the OPD  
16 solution was recorded on a microplate reader, as described in section 2.5 of the manuscript. The  
17 absorbance data were plotted as function of the H<sub>2</sub>O<sub>2</sub> concentration as shown in Figure A1.1 below.  
18 The data covered the full range of absorbance data (both below and above) achieved by the CAP jet  
19 treatments. The calibration curve was near linear ( $R^2 = 0.9987$ ) with the equation from the curve ( $y =$   
20  $0.1043x$ ) used to derive the unknown H<sub>2</sub>O<sub>2</sub> concentration produced by the CAP jet treatments.



21  
22 **Figure A1.1.** Calibration curve used to derive the H<sub>2</sub>O<sub>2</sub> concentration produced in the CAP jet treated  
23 HEPES solution, as shown in Figure 3d in the manuscript.

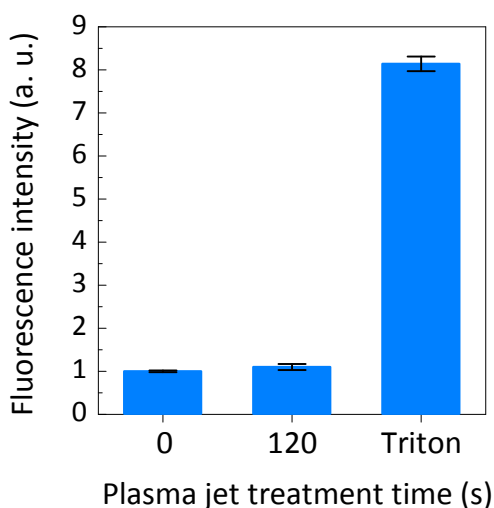
### 24 A4. Carboxyfluorescein (CF): assessment of vesicle rupture

25 Vesicles encapsulating CF were used to analyse the possibility of the vesicle membrane being ruptured  
26 by the CAP jet treatment. In these experiments, 50 mM of CF was encapsulated within the vesicles, at  
27 which concentration CF is self-quenched inside the intact vesicles. However, if the vesicles were  
28 ruptured by the CAP jet treatment, the CF would be released and diluted into the HEPES buffer

1 containing the vesicles, resulting in a switch-on of fluorescence (i.e. a positive indication of vesicle  
2 rupture). To prepare a 50 mM concentration of the carboxyfluorescein (CF) encapsulation buffer, the  
3 following components were dissolved in 13 mL of purified water:

- 4       ▪ CF — 244 mg
- 5       ▪ NaCl — 7.6 mg
- 6       ▪ NaOH — 70.2 mg
- 7       ▪ HEPES — 31 mg
- 8       ▪ EDTA — 3.7 mg

9 The pH was adjusted to 7.4, and the solution was stored shielded from ambient light at 4 °C until use.  
10 Figure A1.2 shows the results of a typical vesicle rupture assay for vesicles encapsulating CF. This  
11 figure shows the results of a 2 min CAP jet treatment of a vesicle system 10 (50 mol% DOPC, 25 mol%  
12 DPPE and 25 mol% cholesterol - see Table 1 in manuscript), which is harsher than any treatment  
13 conditions used in the paper. As seen in Figure A1.2, there was no increase in the CF fluorescence  
14 signal even after this more pro-longed plasma CAP jet treatment. As a positive control, Triton X-100  
15 was added to completely lyse the vesicles, which was seen by a prominent increase in fluorescence  
16 (Figure A1.2).



17  
18 **Figure A1.2.** Result of a typical assay to assess vesicle rupture by CAP plasma jet treatment. The  
19 vesicle membrane composition comprised of 50 mol% DOPC, 25 mol% DPPE and 25  
20 mol% cholesterol (vesicle system 10, Table 1 in manuscript). Positive control of vesicle  
21 rupture was Triton X-100.



## 1 Appendix B: Computational details

### 2 **B1. System set-up and equilibration**

3 All simulations, i.e., energy minimization, equilibration and umbrella sampling runs, were performed  
4 using the GROMACS-5.1 package [58]. For the interatomic interactions the GROMOS 54A7 force  
5 field parameters were applied [59] in combination with the SPC water model [60]. Specifically, the  
6 GROMOS-type parameters for the DOPC, DPPC, DPPE, cholesterol and H<sub>2</sub>O<sub>2</sub> molecules were  
7 adopted from [61], [62], [63], [53] and [64], respectively. After constructing the initial configurations  
8 using the Packmol package [65], an energy minimization of all systems was performed using the  
9 steepest-descent algorithm. After the energy minimization, all systems were equilibrated for 400 ns in  
10 the NPT ensemble (i.e., constant number of particles, pressure and temperature). The temperature at  
11 which the systems were equilibrated was different for systems containing either DOPC or DPPC, i.e.,  
12 at 298 K or 323 K, respectively. These temperatures were chosen based on the transition temperatures  
13 of both lipids (253 K for DOPC and 314 K for DPPC), to ensure that all simulated bilayers were in the  
14 liquid state, as was the case with the vesicles studied experimentally. [66] The reference pressure used  
15 in the simulations was the same for all bilayers, i.e., 1 atmosphere. The NPT ensemble was employed  
16 using the Parrinello-Rahman barostat [67] with compressibility and coupling constant of  $4.5 \times 10^{-5}$   
17 bar<sup>-1</sup> and 2 ps, respectively, as well as the Nose-Hoover thermostat [68] with a coupling constant of  
18 0.5 ps. Considering the non-bonded interactions, the particle mesh Ewald method was used to treat  
19 electrostatic interactions with cut-off radius of 1.0 nm for Coulomb interactions. [69] The same cut-off  
20 radius was applied for the van der Waals interactions. This cut-off radius was chosen after scanning  
21 different values and evaluating the results based on the agreement of the obtained area per lipid with  
22 experimental values (0.63 nm<sup>2</sup> for both DOPC and DPPC bilayers in native form, see [70–73] and  
23 references therein). All simulations were carried out using a time step of 2 fs, and periodic boundary  
24 conditions were applied in all three directions.

### 25 **B2. Umbrella sampling (US) simulations**

26 To investigate the ability of H<sub>2</sub>O<sub>2</sub> permeation through the different bilayers, US simulations were  
27 performed to determine free energy profiles (FEPs) of H<sub>2</sub>O<sub>2</sub> across all systems. [74,75] From the last  
28 40 ns of the equilibration run, five membrane structures were extracted, which served as the initial  
29 configurations for the US runs. In each US simulation, 32 H<sub>2</sub>O<sub>2</sub> molecules were added to the structure,  
30 distributed over eight layers along the bilayer normal (i.e. four molecules in each plane). These  
31 molecules were restrained to move along the *z*-axis by a harmonic bias with force constant of 2000  
32 kJ.mol<sup>-1</sup>.nm<sup>-2</sup>. Furthermore, their movement in the *xy*-plane was also restrained by using the so-called  
33 flat-bottomed position restraint, with a radius of 0.5 nm and a force constant of 500 kJ.mol<sup>-1</sup>.nm<sup>-2</sup>. To  
34 obtain a FEP, 160 US windows were used, which were separated by 0.5 Å. The US simulations were  
35 performed in the NPT ensemble, with the same parameters as in the equilibration runs. The total  
36 simulation time of these runs was 5 ns, of which the last 2 ns were used as sampling time. The  
37 convergence of the free energy results was evaluated based on the symmetry of the profiles to the  
38 centre of the bilayer. From each initial structure, four different FEPs were obtained (one for each H<sub>2</sub>O<sub>2</sub>  
39 molecule placed in each US window). By using five different initial structures (see above), a total of

20 FEPs was obtained for each bilayer composition. These profiles were averaged, leading to the FEPs shown in the Figure 5 of the manuscript. Each FEP was calculated using a periodic version of the weighted histogram analysis method (WHAM), by utilizing the *gmx wham* tool of GROMACS. [76]

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