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Small unilamellar liposomes as membrane model for cell inactivation by cold atmospheric plasma treatment

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Abstract

Cold atmospheric plasma is thought to be a promising tool for numerous biomedical applications due to its ability to generate a large diversity of reactive species in a controlled way. In some cases, it can also generate pulsed electric fields at the zone of treatment, which can induce processes such as electroporation in cell membranes. However, the interaction of these reactive species and the pulse electric field with cells in a physiological medium is very complex and still need a better understanding in order to be useful for future applications. A way to reach this goal is to work with model cell membranes such as liposomes, with the simplest physiological liquid and in a controlled atmosphere in order to limit the number of parallel reactions and processes. In this work, where this approach has been chosen, 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) small unilamellar vesicles (SUV) has been synthesized in phosphate buffered aqueous solution and this solution has been treated by a nanosecond pulsed plasma jet under a pure nitrogen atmosphere. Only the composition of the plasma gas has been changed in order to generate different cocktails of reactive species. After the quantification of the main plasma reactive species in the PBS solution, structural, surface charge state, and chemical modifications generated on the plasma treated liposomes, due to the interaction with the plasma reactive species, has been carefully characterized. These results allow going further in the understanding of the effect of plasma reactive species on model cell membranes in physiological liquids. Permeation through the liposomal membrane and reaction of plasma reactive species with molecules encapsulated inside the liposomes has also been evaluated. New processes of degradation are finally presented and discussed, which come from the specific conditions of plasma treatment under pure nitrogen atmosphere.

1. Introduction

Disinfection of liquids or wet surfaces by means of cold atmospheric plasma (CAP) treatment is seen as a promising field and market [1,2]. In particular, CAP treatments are envisaged for the disinfection or treatment of biological media due to their biocompatible working conditions i.e. ambient pressure and ambient temperature, and the use of low quantities of biocompatible gases [3–5]. For therapeutic applications, the behavior of human or plant cells can also be affected or controlled by their interaction with plasma reactive species, which are often naturally present in living organisms. Therefore, numerous teams have studied the interaction of CAP with biological samples and have investigated mechanisms which could explain the antibacterial properties of plasma treatments on solutions or gels containing bacteria or other living cells. The disinfection or therapeutic efficiency of the CAP processes was shown to be due to the generation of several reactive oxygen and nitrogen species (RONS): hydrogen peroxide, peroxyxynitrite, ozone or hydroxyl and perhydroxyl radicals among others, with the potential contribution of plasma-issued (V)UV photons [6–12] and pulsed electric fields. Recently, hypochlorite ions and chloramine were shown to play a role in the disinfection process at neutral pH when a nanosecond pulsed cold atmospheric microplasma was in contact with a phosphate buffered saline solution under a N₂ atmosphere [13]. A way to go further into the understanding of plasma disinfection processes is to plasma treat suspensions of model cells as it was already made in [14]. It is well known that unsaturated phospholipids such as DOPC are more present in eukaryotic

cells membrane compare to bacteria membrane more rich in saturated lipids. Unsaturated lipids are also more sensitive to oxidation promoted by UV or RONS species especially compare to saturated phospholipids such as DPPC. However, the presence of a high concentration of cholesterol in eukaryotic cells drastically decrease the damage induced by DOPC oxidation. Even if bacteria have less amount of unsaturated lipids, oxidation of them will results into much higher impact than eukaryote cells due to the relative low concentration of cholesterol in bacteria membrane. Investigations focusing on the interaction between RONS generated by CAP in liquids and DOPC liposomes could also help to better understand the selectivity of CAP treatment during disinfection processes promoted by CAP. A first study using this approach has shown that CAP can induce a disruption of liposomes and the fusion of small liposomes into bigger ones [14]. However the origin of these liposome modifications are still not known and can be due to physical effects (temperature, electric field) or / and chemical effects such an alteration of the phospholipid chemical structure by reactive species generated in solution. In this work, we have investigated how CAP can chemically and structurally impact the DOPC phospholipids bilayers using SUV vesicles loaded with carboxyfluorescein molecules as a model of cell. Liposome stability under CAP processes has been investigated using Dynamic Light Scattering and fluorescence spectroscopy. Chemical modification on DOPC induced by CAP has been characterized using MALDI-Orbitrap mass spectrometry methods. Impact of CAP processes has been compared with the effect of chemical compounds just added in liposome suspensions in the same conditions used for the treatment of bacteria in our previous work [13] with the main difference being the addition of 5mM of Mg^{2+} to stabilize the liposomes [15]. Plasma CAP treatment duration was also increased from 90 to 120 s.mL⁻¹, in order to exacerbate the structural and chemical alteration of the DOPC liposomes.

2. Methods

The following chemicals were purchased and used without further purification: 1,2-dioleoyl-sn-glycero-3-phosphocholine 2.5 mg.mL⁻¹ in chloroform (Avanti Polar Lipids), 5(6)-carboxyfluorescein (Sigma-Aldrich). During the treatment of liposome suspensions, a controlled N₂ atmosphere was generated all around the microplasma jet to favor the generation of reactive nitrogen species as shown in Figure 1. Liposomes were prepared and treated in a phosphate buffered saline (PBS) solution also containing 5 mM of MgCl₂. Interaction between custom-built CAP, with a plasma reactor made at UPPA, in a controlled nitrogen atmosphere or chemicals mimicking reactive species produced by CAP, PBS solutions and liposomes are presented and discussed. He, He + 0.50% N₂ and He + 0.50% O₂ process gas are used here because these gas mixtures were shown to present very different bacterial inactivation efficiencies [13]. Effect of hypochlorite (ClO⁻) – in this paper, hypochlorite will refer to both hypochlorite ion and its acidic counterpart hypochlorous acid, hydrogen peroxide (H₂O₂), nitrites (NO₂⁻), peroxyxynitrites (ONOO⁻), monochloramine (NH₂Cl), ammonium (NH₄⁺) and nitryl chloride (NO₂Cl) reactive species on the liposome chemical structure were also assessed independently and compared to the CAP treatments in order to better understand the inactivation mechanism discussed in our previous work [13] and to better predict plasma reactive species interaction with eukaryotic cells for future biomedical applications.

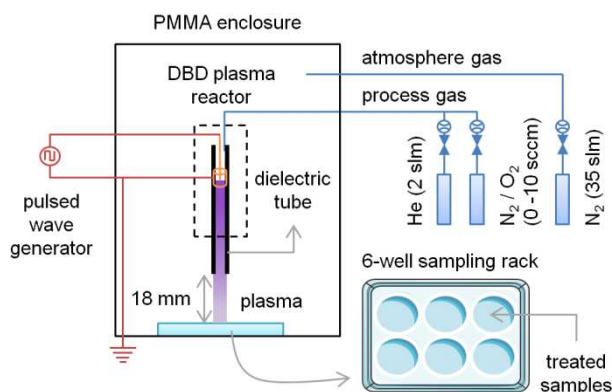


Figure 1: Experimental setup

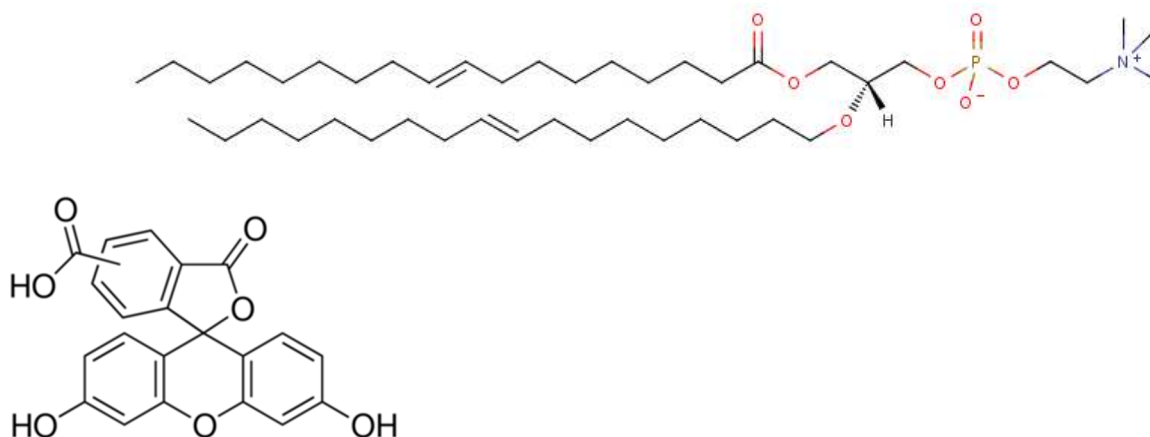


Figure 2: Chemical structures of 1,2-dioleoyl-sn-glycero-3-phosphocholine and (5)6-carboxyfluorescein

The experimental setup we used for plasma treatments is depicted in figure 1. It has already been described in previous publications [13, 16]. Briefly, the dielectric barrier discharge (DBD) plasma reactor consisted of a tungsten filament powered by a pulsed HV and inserted in an alumina dielectric tube. A metallic grounded ring was enclosed outside the tube. Process gas, fed into the reactor through MKS mass flow controllers, was either He at a 2 standard litres per minute (slm) flow rate, He (2 slm) / N₂ (10 standard cubic centime per minute (sccm)) mixture or He (2 slm) / O₂ (10 sccm) mixture. Plasma was generated by applying a 5 kV, 15 kHz, 1.5% duty cycle, positive nanosecond pulsed wave potential between the two electrodes. Control over the atmosphere composition during plasma treatments was obtained by providing a constant N₂ flow (35 slm) inside a poly(methyl methacrylate) enclosure surrounding the DBD plasma reactor without affecting the plasma treatment zone, thus creating a low overpressure in the chamber. The DBD reactor sat on a 3-axis robot to ensure reproducible plasma treatments. Liquid samples to be plasma-treated were poured in a 6-well sampling rack (5 mL per well) and placed in the enclosure, below the DBD plasma reactor. The vertical distance between the tip of the alumina dielectric tube and the liquid interface was kept constant at 18 mm.

All liposome suspensions were prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) commercial stock solution in chloroform (2.5 mg.mL⁻¹). 786 μ L of this stock was transferred into 4-mL Wheaton sample vial. By tilting and rotating regularly the vial under constant N₂ gas flow, chloroform solvent evaporated to give a white DOPC film on the vial's wall. 2.5 mL of degassed PBS buffer – with or without (5)6-carboxyfluorescein 50 mM – and a stirring magnet were then added. The solution was stirred twice for 30 min at 1000 rpm, with a 60 min break at 4°C in between. Thereafter, the vial was submitted to a 30-min sonication. The solution is then filtered through a 200-nm polycarbonate filter (one pass) before extrusion through a 100-nm polycarbonate filter (9 passes). The extruded suspension finally undergoes two filtration steps on PD-10 desalting columns filled with Sephadex G25 medium (GE Healthcare) to remove non-encapsulated (5)6-carboxyfluorescein molecules. Before plasma or wet chemistry treatments, the DOPC liposome suspension is diluted 20 times in PBS + Mg²⁺ 5 mM to reach the desired DOPC concentration of 50 μ M.

For chemical treatments, 2 mL of liquid samples in 12-well sampling rack were used. Measurements were carried out 4 hours after adding the chemical compounds matching the reactive species generated by CAP in solution, in order to ensure a full reaction between phospholipids and reactive species.

Concentration of main reactive species in PBS solutions treated by CAP were determined by photometric rapid Spectroquant® tests from Merck (1.18789 test for H₂O₂, 1.14776 test for NO₂⁻, 1.14773 test for NO₃⁻, 1.14752 test for NH₄⁺ and 1.00599 test for HClO and NH₂Cl). When necessary, plasma treated PBS solutions

were diluted to avoid interference effects with other reactive species. When the dilution approach was not possible, a chemical compound has been added before the colorimetric test to eliminate the reactive species generating interferences. Therefore, for some measurements, catalase and sulfamic acid have been added to eliminate H₂O₂ and NO₂⁻, respectively.

Size distribution and zeta potential of liposomes were respectively measured by dynamic light scattering and laser Doppler electrophoresis, before and after treatment, with a Zetasizer Nano ZS (Malvern Instruments). Samples were poured in DTS1070 disposable cells without further preparation, measurements were performed at 25 ± 1°C.

Fluorescence intensity measurements of 5(6)carboxyfluorescein-loaded samples were performed on an Infinite M1000 Pro UV/vis spectrophotometer (Tecan), with 200 µL of each sample poured in a 96-well sampling rack. In this work, fluorescence intensity of a sample was defined as the area under the curve of fluorescence emission between 500 and 650 nm when this sample is excited at a wavelength of 490 nm; its value was normalized against a reference untreated sample.

Some treated liposome solutions were analyzed with atmospheric pressure matrix-assisted laser desorption/ionization-Orbitrap mass spectrometry (AP-MALDI-Orbitrap). For analysis, treated samples were diluted 10 times in a methanol / water mixture (50/50, volume/volume). 1 µL droplets of these diluted samples were laid down on a gold-plated steel plate. After evaporation of solvents, remaining liposomes and salts from the droplets were rinsed twice with 1 µL of milliQ water to desalt as much as possible the samples. Then, dried desalted liposome samples were mixed to 1 µL of a matrix, made of 2,5-dihydroxybenzoic acid 15 mg.mL⁻¹ in methanol / trifluoroacetic acid mixture (50/50, volume/volume), to protect DOPC lipid from excessive photon irradiation and favour its ionization during AP-MALDI analysis. All measurements were made in triplicate.

3. Results

Under constant N₂ atmosphere, we performed a set of 120 s.mL⁻¹ plasma treatments on PBS + Mg²⁺ 5 mM solution at neutral pH and ambient temperature. Colorimetric tests on the plasma treated solutions allowed for the evaluation of their concentration after plasma treatment, as shown on Table 1.

	Conc. (µM) He + O ₂ plasma	Conc. (µM) He plasma	Conc. (µM) He + N ₂ plasma
H ₂ O ₂	150±50	550±50	350±50
NO ₂ ⁻	500±50	2000±100	4800±200
NO ₃ ⁻	not measured, conc. < 1000 expected (plasma jet quenching due to ROS)	1400±100	2000±100
NH ₄ ⁺	not detected	not detected	65±3
NH ₂ Cl	not detected	not detected	8±2
HClO	not measured, conc. < 2 expected (plasma jet quenching due to ROS)	5±2	5±2

Table 1: Evaluation of the quantity of reactive species generated in PBS + Mg²⁺ solutions at neutral pH after 120 s.mL⁻¹ plasma treatments

For all the plasma treatments, a low $\text{H}_2\text{O}_2/\text{NO}_x$ ratio was observed due to the pure nitrogen atmosphere used. Due to the plasma jet quenching induced by the highest density of reactive oxygen species generated in the discharge zone, the quantity of reactive species generated in PBS solution by He + 0.50% O_2 plasma is much lower compared with the two other plasma treatments (Table 1). Except for HClO and H_2O_2 , He plasma treatments generated reactive species in lower amounts compared with He + 0.50% N_2 plasma ones. For He + 0.50% N_2 plasma treatments, much higher concentrations in NO_x and the generation of additional reactive species (i.e. NH_4^+ and NH_2Cl) were detected.

Then, we performed a set of 120 s.mL^{-1} plasma treatments on 100-nm small unilamellar DOPC vesicles (also called liposomes), containing 50 mM of 5(6)-carboxyfluorescein, suspended in PBS + Mg^{2+} 5 mM. Dynamic light scattering measurements of these samples allowed for the determination of their size distribution profile, before and after plasma treatment, as shown on Figure 3.

One wide peak centred on 1000 nm, appeared after He + 0.50% N_2 120 s.mL^{-1} plasma treatment (Figure 3, blue line), while the initial peak centred on 100 nm decreased. The liposomes coalescence phenomenon observed under He + 0.50% N_2 plasma treatment would explain the peak centred on 1000 nm and the decrease of the one centred on 100 nm (liposomes before treatment). The similar profile of the peak centred at 100 nm for the EtOH chemical treatment (10% addition (volume / volume) to the liposome suspension) and the one of He + 0.50% N_2 120 s.mL^{-1} plasma treatment indicates that liposomes leaked a significant part of their 5(6)-carboxyfluorescein molecules. Indeed, the addition of ethanol is known to increase the permeability of liposomes to hydrophilic molecules and in our case it favours the disruption of the small unilamellar vesicles (SUV) and the delivery of the fluorescent molecules [17,18].

He 120 s.mL^{-1} plasma treatments (Figure 3, black line) result in a gentler decrease of the peak centred on 100 nm indicating that the major part of the SUVs do not break and do not leak. The peak centred on 500 nm is thought to be mainly due to of the agglomeration of several SUVs because no significant leak of fluorescent molecules is detected (i.e. peak at 100 nm similar to non-treated reference one). 100-nm DOPC liposomes form 500-nm liposomal clusters through weak interaction, without rupture of liposome and leakage of the fluorescent dye. He + 0.50% O_2 120 s.mL^{-1} plasma treatment (Figure 3, red line) did not change significantly the size distribution profile of the DOPC liposomes. No structural degradation of liposomes is then generated with these milder plasma treatments.

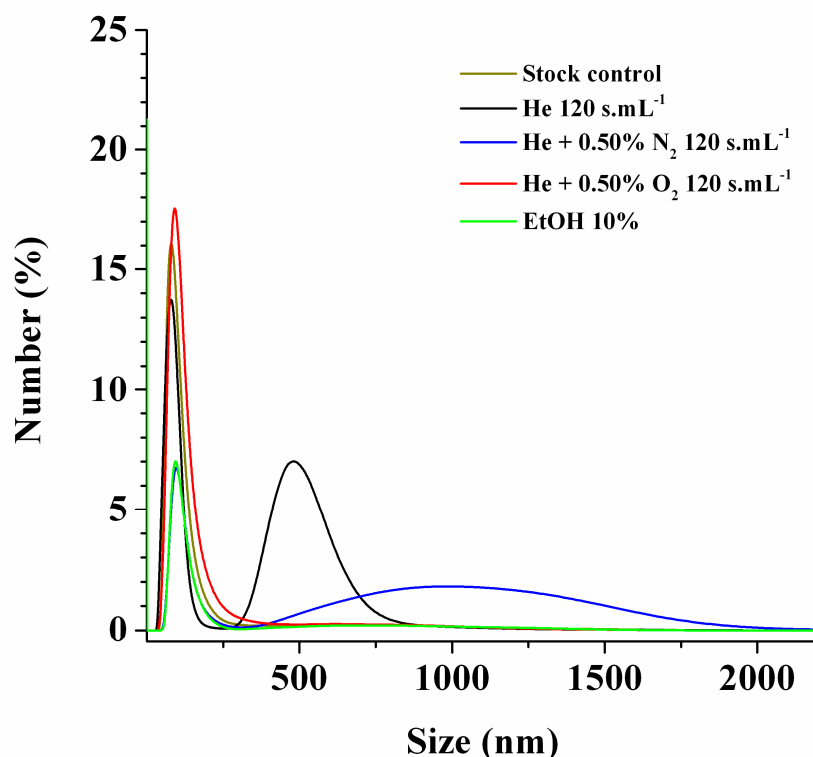


Figure 3: Size distribution profile of DOPC liposomes, encapsulating (5)6-carboxyfluorescein 50 mM, in PBS + Mg^{2+} 5 mM after different 120 s.mL⁻¹ plasma treatments or after addition of 10% (v/v) ethanol

The zeta potential of DOPC liposomes was also determined from their electrophoretic mobility measured on a Malvern Zetasizer Nano ZS (Figure 4). While untreated DOPC liposomes in PBS showed an almost neutral zeta potential (-1.0 ± 2.6 mV), zeta potentials after He 120 s.mL⁻¹ and He + 0.50% N₂ 120 s.mL⁻¹ plasma treatments both decreased to more negative values (respectively -11.8 ± 4.1 mV and -21.4 ± 4.4 mV). The more negative zeta potential value obtained after He + 0.50% N₂ 120 s.mL⁻¹ plasma treatment indicates that the surface of the treated liposomes is more negatively charged due to a larger number of chemical modifications generated on the phospholipids.

The treatment of DOPC liposomes by He + 0.50% O₂ 120 s.mL⁻¹ plasma and ethanol 10% did not modify significantly the zeta potential (-3.0 ± 1.4 mV). It means that no significant chemical modifications are generated on the phospholipids for these two treatments.

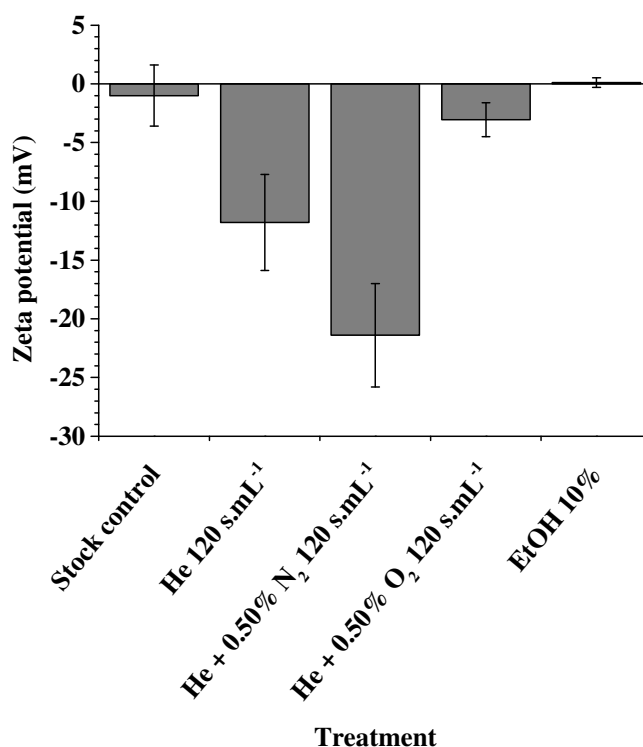


Figure 4: Zeta potential of DOPC liposomes, encapsulating (5)6-carboxyfluorescein 50 mM, in PBS + Mg²⁺ 5 mM after 120 s.mL⁻¹ plasma treatments and after addition of 10% (v/v) ethanol

The fluorescence intensity of 5(6)-carboxyfluorescein, encapsulated at a concentration of 50 mM in the DOPC liposomes, was measured after 120 s.mL⁻¹ plasma treatments and normalized against untreated samples. It has to be noted that the fluorescence intensity of non-treated samples (i.e. stock control) is already low due to the self-quenching phenomenon of the fluorescent molecules in liposomes.

All three 120 s.mL⁻¹ plasma treatments unexpectedly led to a significant decrease of the fluorescence of carboxyfluorescein (Figure 5) down to very low values (close to background). The plasma treatments generate a degradation of the fluorescent dye whatever the gas mixture used. This fluorescent probe cannot resist to our plasma conditions even when they are encapsulated in liposomes. The use of this fluorescent probes is not a good approach in our case to detect the dye release from liposomes due to the novel cocktail of species generated by plasma. However, it shows that reactive species can permeate through the liposomes to react with the molecular probe inside liposomes, which is an interesting information for drug delivery or disinfection applications.

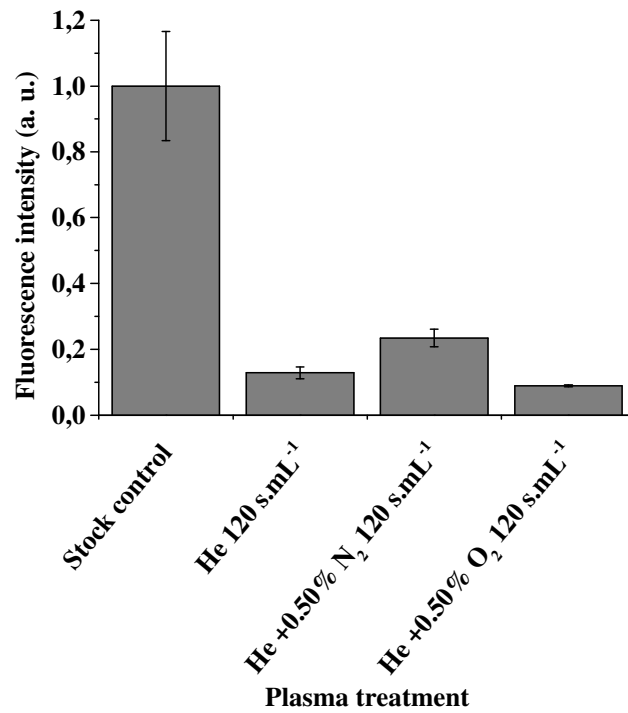


Figure 5: Normalized fluorescence intensity of (5)6-carboxyfluorescein 50 mM, encapsulated in DOPC liposomes suspended in PBS + Mg²⁺ 5 mM after 120 s.mL⁻¹ plasma treatments (n = 3)

Erreur ! Source du renvoi introuvable. Figure 6 shows the fluorescence intensity, normalized against untreated samples, of a (5)6-carboxyfluorescein 200 μM solution (not encapsulated in liposomes) in PBS + Mg²⁺ 5 mM after 0 – 120 s.mL⁻¹ He, He +0.50% N₂ and He + 0.50% O₂ plasma treatments. The fluorescence decrease of non-encapsulated 5(6)-carboxyfluorescein due to the fluorescent molecule degradation is observed for all the plasma treatments even if it is less pronounced for the oxygen-containing one.

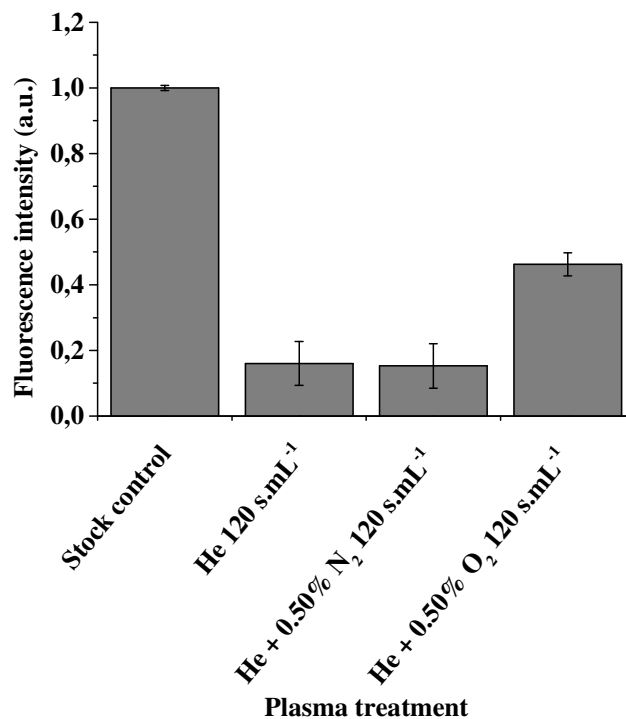


Figure 6: Normalized fluorescence intensity of (5)6-carboxyfluorescein 200 μM in PBS + Mg^{2+} 5 mM after 120 s. mL^{-1} plasma treatments showing the degradation of dye (i.e. bleaching) by plasma reactive species.

In order to identify which kind of plasma-generated RONS are involved in the degradation of both liposomes and fluorescent dye, we have investigated the degradation of liposomes after 35 min exposure to HOCl (5 – 500 μM), NH_2Cl (5 – 500 μM), H_2O_2 (500 – 5000 μM), NH_4^+ (500 μM), NO_2^- (500 μM), NO_2Cl (500 μM) and ONOO^- (500 μM) as shown on Figure 7. These concentration ranges correspond to the quantities generated by the plasma treatments.

From all those species, only HOCl was able to induce an effect on 5(6)-carboxyfluorescein-loaded DOPC liposomes: the fluorescence intensity decreased to 31 (HOCl 5 μM) and 6% (HOCl 50 and 500 μM) of the original value. Although NH_2Cl is potent enough to inactivate *E. coli* bacteria (5.2 \log_{10} reduction after 35 min exposure to 45.2 μM) [13], its degradation power (i.e. fluorescence intensity decrease) on carboxyfluoreceine seemed negligible up to 500 μM , which is a much higher amount than the one generated by our plasma treatment (at least 10 times lower). The same observation was made for NH_4^+ , NO_2^- , NO_2Cl and ONOO^- up to 500 μM and for H_2O_2 up to 5000 μM [19–22].

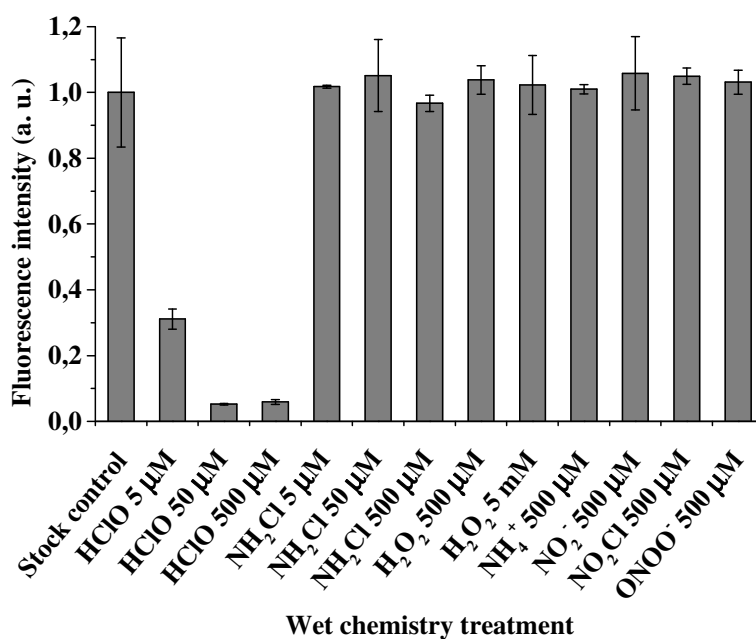


Figure 7: Normalized fluorescence intensity of (5)6-carboxyfluorescein 50 mM, encapsulated in DOPC liposomes suspended in PBS + Mg^{2+} 5 mM after 35 min chemical treatments (n = 2)

In order to determine more precisely the chemical modifications induced on the phospholipids by the plasma and chemical treatments, we used atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI) mass spectrometry to identify the degradation products of DOPC. After a He + 0.50% N_2 plasma treatment of 120 s. mL^{-1} (Figure 7), the most potent bactericidal treatment with our set of parameters, the oxidation and the formation of chlorohydrin by electrophilic addition of HOCl on DOPC unsaturated C=C bonds (Figure 8) was observed [23, 24]. The direct contribution of nitrating species (e.g. peroxyxynitrite) is negligible, as no NO nor NO_2 functionalization was observed on the phospholipids. This last result shows that HOCl is not only involved in fluorescent dye bleaching but it is also involved in the liposome membrane chemical modification, in addition to other reactive oxygen species.

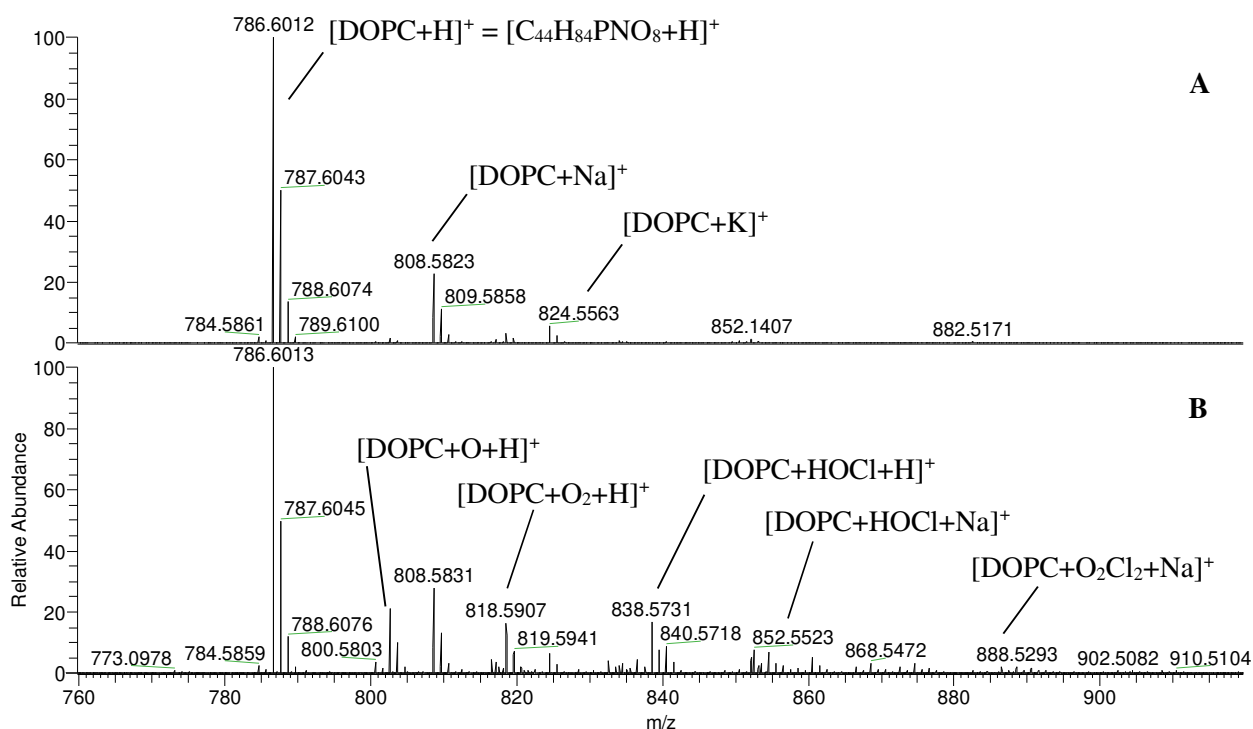


Figure 7: Positive ion AP-MALDI-Orbitrap mass spectra of DOPC liposomes in PBS + Mg²⁺ 5 mM before (A) and after He + 0.50% N₂ 120 s.mL⁻¹ plasma treatment (B).

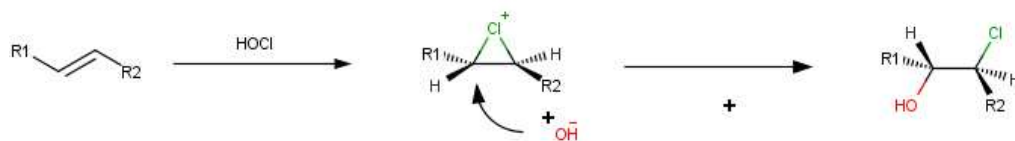


Figure 8: Formation of chlorohydrin by electrophilic addition of HOCl on DOPC unsaturated C=C bonds. R1 and R1 stand for carbon chains of fatty acid.

In order to identify the plasma-generated RONS responsible for the O, OH and Cl functionalizations of DOPC lipid during He + 0.50% N₂ plasma treatment, AP-MALDI mass spectra of DOPC liposome suspension in PBS + 5 mM Mg²⁺ were carried out after 35 min exposure to HOCl (5 – 500 μM), H₂O₂ (500 – 5000 μM), NH₄⁺ (500 μM), NO₂⁻ (500 μM), NO₃⁻ (500 μM) and HOCl / H₂O₂ mixture (500 μM) as previously done with fluorescence measurements. Figure 9 shows the positive ion AP-MALDI mass spectra of DOPC liposome suspension in PBS + 5 mM Mg²⁺ before and after exposure to HOCl. The degradation products detected match closely those observed after He + 0.50% N₂ 120 s.mL⁻¹ plasma treatments: DOPC phospholipids mostly underwent one to two electrophilic additions of HOCl molecules, when it was in contact with HOCl solutions.

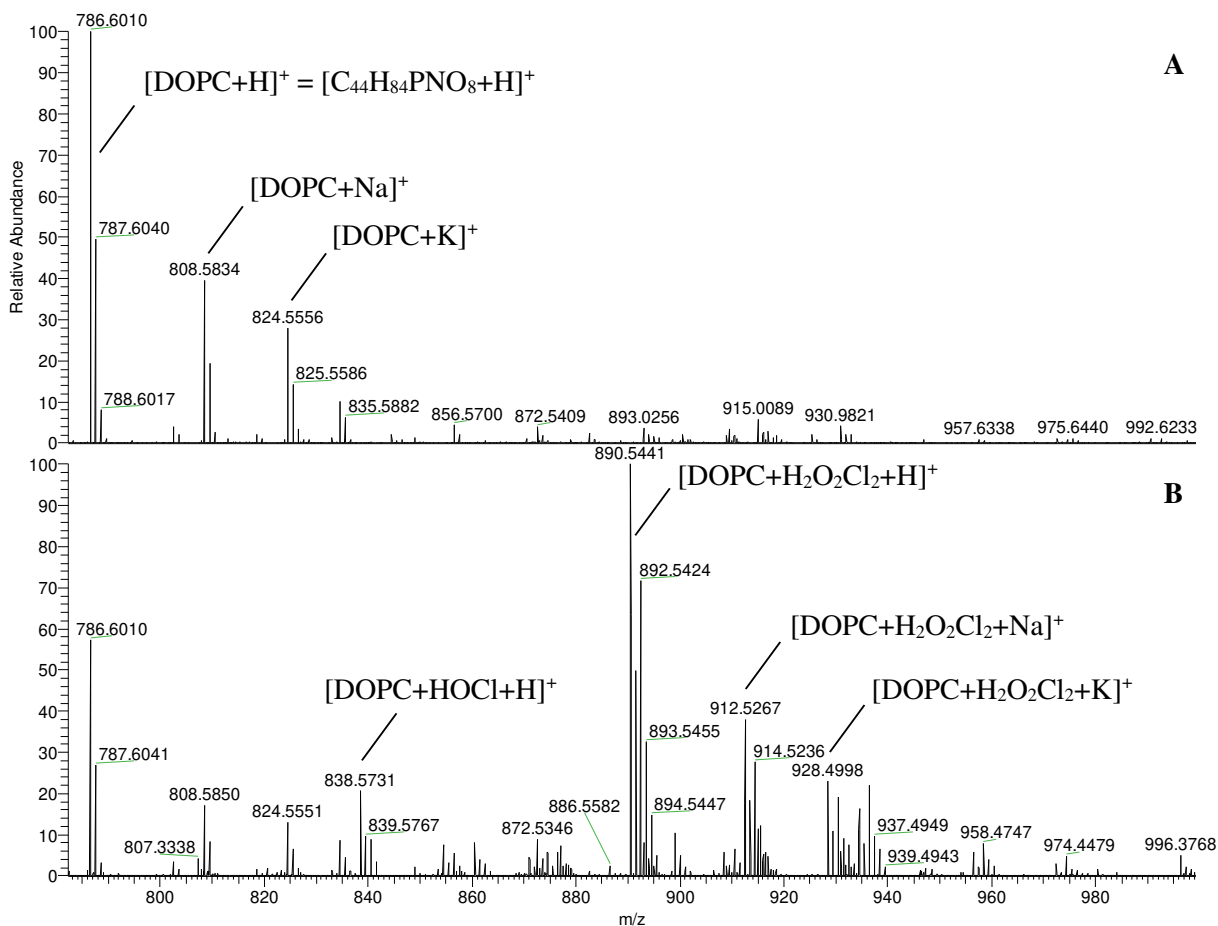


Figure 9: Positive ion AP-MALDI-Orbitrap mass spectra of DOPC liposomes in PBS + Mg²⁺ 5 mM before (A) and after HOCl wet chemistry treatment (B)

Positive ion AP-MALDI-Orbitrap mass spectra of DOPC liposome suspension in PBS + 5 mM Mg²⁺ after exposure to H₂O₂ (500 – 5000 μM), NH₄⁺ (500 μM), NO₂⁻ (500 μM), NO₃⁻ (500 μM) and HOCl / H₂O₂ mixture (500 μM) did not show any degradation of DOPC phospholipids. In the case of exposure to a HOCl / H₂O₂ mixture (500 μM), oxidation products due to the addition of 3 to 6 oxygen atoms to DOPC phospholipids were detected. These oxidation products were not found after He + 0.50% N₂ 120 s.mL⁻¹ plasma treatments. In conclusion, AP-MALDI-Orbitrap measurements show that HOCl-containing solutions give the closest results to the He + 0.50% N₂ plasma treated ones in term of chemical modification on the DOPC structure.

4- Discussion:

In our conditions where a nanosecond pulsed discharge, a solution buffered to neutral pH and an atmosphere of pure N₂ are used, energy/density of electrons and reactive species generated is high, we have observed original cocktails of reactive species formed in solution (Table 1). HOCl and NO₂⁻ are generated in higher quantities compared with more conventional discharges, except for He + O₂ plasma treatments. In the specific case of He + 0.50% N₂ plasma treatments, NH₄⁺ are also generated in significant quantity leading to the formation of NH₂Cl through its reaction with HOCl, which is favoured at neutral pH. These novel cocktails of reactive species generated in pH buffered saline solutions are generating novel degradation processes both on bacteria [13], liposomes and fluorescent probes as discussed below.

- a- Impact of CAP treatments on the structure and zeta potential of liposomes

The amount of chemical modifications on the phospholipids, induced by the reactive species generated by plasma, is proportional to the density of negative charges present at the surface of liposomes. Negative charges are generated on the liposomes mainly due to the nature of chemical groups added on the unsaturations of phospholipid tail i.e. OH and Cl (Figure 8 and 9). NO_x^- anions generated in large amount in solution by plasma treatment (Table 1) may also be trapped in the liposomal membrane and participate to the decrease of zeta potential. The higher amount of chemical modifications on phospholipids induced by He + 0.50% N_2 plasma treatments favour the fusion of liposomes to form larger liposomes, probably due to a higher fluidity and reactivity of the plasma modified liposomal membranes. The lower degradation of liposomes after the He plasma treatments due to the lower amount of reactive species generated in solution, compared with He + 0.50% N_2 plasma (Table 1), are leading to the aggregation of the liposomes. It is thought that the fluidity/reactivity of liposomes is not increased enough in this case to lead to a fusion process. These results may be correlated with our previous results on bacteria inactivation by plasma, where He plasma treatments have almost no effect but He + 0.50% N_2 ones show a fast and intense bacterial inactivation [13]. In the case of He + 0.50% O_2 plasma treatments, no significant modification of the liposomes (i.e. phospholipids) is observed. This results also correlates well with our previous bacterial inactivation results [13]. In summary, critical amounts of reactive species are necessary to lead to the aggregation and then the fusion of liposomes, which are reached only by the He plasma treatment in the first case and by the He + 0.50% N_2 in the second case. The generation of high quantities of NO_x^- (> 6 mM) and the presence of HClO (> 4 μM), NH_4^+ and NH_2Cl species are responsible for the coalescence of liposomes. The generation of intermediate quantities of NO_x^- (> 3 mM) and the presence of HClO (> 4 μM) species are responsible for the aggregation of liposomes. The generation of lower quantities of NO_x^- (< 2 mM) and HClO (< 2 μM) does not lead to structural modifications of liposomes.

b- Molecular mechanisms involved in the degradation processes of liposomes and fluorescent molecules:

Hypochlorites (like peroxyxynitrite) are typical reactive species produced in the organism under pathological conditions or during plasma treatment of water solutions and having a large range of biological effects such as erythrocyte oxidative damage [25]. HClO can penetrate into cells and be transformed through a nonenzymatic reaction into other ROS (e.g. OH and $^1\text{O}^2$) [26] to generate local oxidation processes. The degradation effects of HClO are also explained by the formation of chloramines due to their reactions with the amino group of lipids or proteins [27, 28,] and also by the formation of chlorohydrins (Figure 9) [23, 24]. Cell exposure to HClO was shown to lead to structural rearrangement of the membrane, cell swelling and pore formation [27, 28].

When using a plasma touching saline water solutions, HClO can be generated by the reaction between OH radicals generated by plasma reactive species in contact with water and Cl^- ions present in our solution at a concentration of 154 mM. In our specific conditions, it is shown that HOCl seems to play a major role in fluorescent dye and liposome degradations even at very low concentration of a few μM . For the liposome degradation, the formation of chlorohydrin by electrophilic addition of HOCl on DOPC unsaturated C=C bonds is one reaction pathway as shown from our high resolution mass spectrometry results (Figure 8 and 9) [23, 24]. The generation of ROS in the vicinity of phospholipids from HOCl degradation [26] can also lead to oxidation processes mainly on the C=C unsaturated bond of the phospholipid tail as shown on Figure 8 with peaks at 802.6 (O addition), 818.6 (2 O additions) and 888.5 (2 O + 2 Cl_2 additions). The formation of chloramine from the reaction of HOCl with amine of the phospholipid head was not observed probably due to the low reactivity of the tertiary amine for DOPC. Concerning the (5)6-carboxyfluorescein, the molecule is degraded whatever the plasma treatment and whatever if encapsulated or not. HClO seem to be the main reactive species involved in the degradation of the fluorescent probe (Figure 7), which explain why He + 0.50% O_2 plasma treatments induces less (5)6-carboxyfluorescein degradation and the other plasma treatments higher and similar rates of degradation (figure 6) because HOCl concentrations generated are similar (Table 1). Bleaching of fluorescein by oxygen molecules and ROS has already been reported under light irradiation [29] but the conditions and degradation processes are different to the ones studied in this

work. In particular, no significant amount of photons are generated at the vicinity of dye molecules in our case. The (5)6-carboxyfluorescein plasma degradation processes observed here, probably by HOCl species, have been better documented in [30]. The (5)6-carboxyfluorescein degradation by HOCl, can be either due to an electrophilic addition of HOCl on an aromatic C—C bond or an oxidation of C—C aromatic bonds, both processes leading to C—C aromatic bond breaking. Future mass spectrometry measurements will allow us to determine the degradation process in our conditions.

NO_2^- are known to generate oxidation processes through the formation of reactive oxygen intermediates, which can attack biomolecules present in their vicinity and can lead to lipid peroxidation, protein modification, DNA damage, etc. However, it is shown in our case that nitrite ions are not generating as much damage as expected (e.g. case of He plasma treatments). This last observation can be partly explained by our neutral pH conditions, which inhibit the formation of NO_2H and NO_2 intermediate species from NO_2^- species and therefore inhibit the addition of NO_2 on the C in α position of the C=C unsaturated bond in the phospholipid tail as explained by Mendiara et al. [31]. The lack of molecular fragments with an NO_2 addition confirmed this hypothesis (Figure 8). However, a part of oxidation processes observed on Figure 8 (already mentioned as a possible HOCl effect above) can be generated indirectly by nitrite ions, which explain why He + 0.50% O_2 plasma treatments are generating higher degradation effects on liposomes. These oxidations processes can also be involved in the (5)6-carboxyfluorescein degradation.

Finally, effects of NH_2Cl and NH_4^+ species, only produced by He + 0.50% N_2 plasma treatments, on liposomes and (5)6-carboxyfluorescein degradation are more difficult to discuss because they are masked by the effect of nitrite ions generated in much higher quantities for He + 0.50% N_2 plasma treatments. There is also no pertinent work describing their possible role in the degradation of liposomes and dye molecule similar to (5)6-carboxyfluorescein. More experiments will be carried out to determine their role in the degradation processes.

Conclusion

In this work, where novel cocktails of reactive species are generated by cold atmospheric plasma in pH buffered aqueous solution containing liposomes loaded with a fluorescent dye, it has been shown that several and novel plasma degradation mechanisms of DOPC small unilamellar liposomes and carboxyfluorescein dye are generated in suspensions touched by the plasma. HOCl generated in the micromolar range combined with nitrite ions generated in the mM range is mainly responsible of the degradation of the carboxyfluorescein dye molecules, which make the use of this fluorescent probe not so pertinent in these conditions. It is probable that no fluorescent dye can resist to these plasma treatments and another method than fluorescence spectroscopy is needed to follow the permeation of molecular probe through the liposomal membrane. When a critical quantity of reactive species (i.e. HOCl, NO_2^- and other ROS generated locally from HOCl and NO_2^-) generated by the plasma in the suspension is reached, the aggregation of liposomes and the increase of negative charges on liposomes is observed (case of He + 0.50% O_2 plasma treatments). When another higher critical quantity of reactive species (i.e. HOCl, NO_2^- , NH_4^+ , NH_2Cl and ROS) generated by plasma in the suspension is reached, the fusion of liposomes, an higher increase of negative charges of liposomes and the leakage of fluorescent dye from liposomes is observed (case of He + 0.50% O_2 plasma treatments). Reactive species are shown to react with the liposomal membranes through HOCl electrophilic addition and oxidation processes mainly on the unsaturated C=C bond of the phospholipid tail. These chemical modifications make liposomes more permeable leading to the permeation of encapsulated dye through the membrane, and also more reactive and more flexible leading to a point where liposome fusion is observed. The reactive species (e.g. HOCl, NO_2^-) generated by plasma are also able to cross the liposomal membrane to react with the fluorescent molecules inside the liposomes without the failure of the liposomes. This work bring novel information about degradation processes of encapsulated fluorescent molecules and model membranes that can partially explain previous results on bacterial disinfection in similar conditions [13]. These results will be also very useful for future studies dealing with the selectivity of plasma treatments

for therapeutic applications and the association of plasma treatments and nanomedicine for novel therapeutic and diagnostic applications.

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