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Influence of Plasma Treatment on the Structure and Function of Lipids

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ABSTRACT: The membrane of both pro- and eukaryotic cells is the cell's interface with the environment. It is the first interaction site of any substance that is externally applied, including reactive species in the liquid cell environment created by plasma medical treatments. Therefore, the liquid surrounding the cell is, due to its influence on the chemical paths, an important mediator for plasma-borne reactive species, and the cellular membrane is their primary target structure. A cellular membrane consists, according to the Singer–Nicolson model, of a lipid bilayer with embedded proteins. Here, we describe experiments of plasma treatments of lipids and liposomal model membranes. The investigations show membrane activity of plasma-borne reactive species against lipids and lipid structures. The methods applied are Raman microscopy and chromophore-based light spectroscopy. Results of dynamic light scattering (DLS) and fluorophore-based assays show that, during the applied plasma treatment, neither macroscopic collapse of the lipid superstructure nor liposome fusion was observed. Raman spectroscopy reveals increased fluidity of lipid layers due to plasma treatment. The results are discussed based on our observations and published results. We propose a detailed molecular mechanism for the formation of lesions that allow a "self-mediated in- and efflux" of plasma-borne reactive species and cell signaling molecules. Resulting consequences for cellular membranes and the cell as a whole are discussed.

KEY WORDS: plasma medicine, plasma treatment, nonthermal plasma, membrane, bilayer, lipids, lipid oxidation, plasma poration, lesions, self-mediated in- and efflux

I. INTRODUCTION

Plasma medicine is a new field of research in which physical atmospheric-pressure plasmas are used in medical applications.[1–3] A major goal of this emerging field is the plasma-supported healing of skin wounds.[4] For an optimized healing process, effects on both eu- and prokaryotes are relevant. For bacteria, the case is relatively simple: Lethal or inhibiting effects should be maximized for an optimized medical treatment. From a great variety of the published research results, it is clear that most plasma sources show some antibacterial activity.[5–8] For human prokaryotes, the case is more complicated; malicious cellular reactions like necrosis or irreparable DNA damage must be avoided or at least minimized, while benign effects must be maximized. The latter can include promoted growth and increased proliferation of skin cells. In the literature, many of these effects are

described, but detailed explanations are rare. However, to optimize medical treatment, in-depth knowledge is required to find the optimal balance between these effects.

Recently, the membrane of plasma-treated cells and bacteria have become the objects of more and more general scientific focus[9] because every externally applied substance has to initially interact with or overcome this outer interface of cells. Thus, the membrane is the first target for plasma-created reactive species.

According to the Singer and Nicolson model,[10] the cell membrane is composed of two lipid monolayers with embedded proteins. Roughly half of the area is occupied both by lipids and proteins. Lipids of the class of phospholipids—or glycerophospholipids—are the most relevant lipids of eukaryotic membranes. Phosphatidylcholine (PC) accounts for more than half of them.[11] The hydrophilic zwitterionic headgroup of the molecule consists of a choline group bound to a phosphate residue, which binds to a glycerol group. To the last group, two acyl chains (hydrocarbon chains, "fatty acids") are coupled. Their composition differs in length (number of C atoms in the acyl chain) and in the number of double bonds of C atoms (–C=C–), the so-called degree of unsaturation.

By this classical view, proteins are the functional elements in a membrane, and lipids are the structural ones. Due to physical properties and organization, mixtures of different lipids can form microdomains,[12] also called rafts in biological membranes, which are areas with different lipid distribution in an inhomogenous bilayer. With the dynamic expansion of the membrane model and the widely accepted lipid raft/domain theory of Kai Simons,[13] it is clear that lipids are also involved in signaling processes via the membrane by providing the "right" microdomain environment ("rigid" or "fluid" like) for the proteins by raft formation.[14] For example, lipid rafts are involved in the signal transduction processes of proteins such as T-cell antigen receptor (TCR),[15,16] integrins,[17,18] and eNOS (endothelial nitric oxide synthase).[19,20] Therefore, lipids not only provide the structural elements of membranes but are also involved in the functional part. Considering a normal structure–function relationship, it is widely accepted that modification of the lipid (chemical) structure will also affect its function.

One observed phenomenon on membranes caused by plasma treatment is the so-called "plasma poration." Leduc et al.[21] showed that the plasma source APGD-t is capable of inducing transient pores or lesions that allow dextran molecules, which normally cannot overcome cell membranes, to enter the cells.[22] The observed maximum radius of the lesions was less than 6.8 nm. The authors concluded that "reactive species of the plasma reaching the cell membrane react with the lipid bilayer and ultimately create transient pores" initiated by lipid peroxidation most probably by hydroxyl radical •OH. Leduc et al. believe that cross-linked fatty acid side chains lead to the formation of transient pores. Furthermore, they mentioned that mechanical forces of the gas stream could also have an impact on the permeability of membranes.

Lipid peroxidation is a widely known process[23,24] and creates exceptional conformational changes of membranes.[25] For most prominent active agents of plasma-treated liquids, their potential to initiate the oxidation of lipids is described: peroxynitrate $ONOO^-$ and/or its decomposition products (i.e., hydroxyl •OH and nitrogendioxide $•NO_2$),[26]

hydrogenperoxide H_2O_2,[27] ozone O_3,[28,29] and superoxide $\bullet O_2^-$ and singlet oxygen O_2^*.[30] Notably, a recently published review mentions that H_2O_2 and superoxide $\bullet O_2^-$ are not able to directly initialize lipid oxidation[23] but are merely indirectly involved. The most widely proposed mechanism for the interaction of H_2O_2 and O_2^- with biological samples is the creation of hydroxyl radicals by the iron-catalyzed Haber-Weiss reaction.[31] The described lipid peroxidation is initiated by the detachment of a hydrogen atom by a radical. Because the double bond weakens the adjacent C–H bond, lipids with (poly-) unsaturated fatty acids (PUFAs) are more susceptible to lipid oxidation than lipids with saturated ones.[32] The resulting lipid radical undergoes structural rearrangements while being oxidized by O_2. Because the lipid is still radical-like, it is capable of initiating lipid peroxidation in a nearby lipid. Consequently, a single lipid peroxidation process can result in a chain reaction. In addition to hydroperoxides, the primary products of lipid peroxidation, a continued oxidation or degradation creates aldehydes that are, in part, very reactive.[23] Besides these second toxic messengers, several lipid peroxidation products–e.g. oxidized PC– have been identified as damage-associated molecular patterns (DAMPs) and therefore provoke a response of the innate immune system.[33]

Apart from the process of lipid peroxidation, an oxidation of lipids by hydroxyl radicals is described in the literature.[34] The •OH reaction results in cleavage of acyl chains. In the proposed model by Tai et al., an acyl chain of the lipid is cracked at a –C=C– bond. Finally, a carbohydrate chain with an end-standing newly bound oxygen atom is split off the lipid. Now the lipid has a shorter acyl chain with a terminal O atom. This kind of lipid modification results in significantly increased fluidity of the lipid membrane.[34]

In this study, we investigated the modification of the lipid structure and its function by plasma treatment. Lipid stacks/films and liposomes composed of PC, the most representative lipid for eukaryotic cells,[11] were used as artificial membrane models. Liposomes have been shown to be a suitable model for cells also in the context of plasma treatment.[35]

Using Raman spectroscopy, the influence of plasma treatments on lipids was investigated, especially its effect on the lipids' state of phase ("fluidity"). Dynamic light scattering (DLS) measurements were performed to determine plasma-induced changes of liposome size and their zeta potential. By chromophore-based fusion assay, the ability of plasma treatment to induce liposome merging was tested. The observations led to a model for lesion formation by plasma-borne reactive species based on their ability to change the phase state of the lipid bilayer. Possible dose-dependent consequences for cellular reactions are discussed.

II. METHODS AND MATERIALS

A. Plasma Source and Treatment

The non-equilibrium atmospheric-pressure plasma source kinpen (neoplas GmbH, Greifswald, Germany), which produces a non-thermal tissue-tolerable plasma effluent, was

used for all experiments in this study. A detailed description can be found elsewhere.[36] The plasma source was equipped with a shielding device as described elsewhere[37] to keep experimental conditions constant. Argon was used as working gas at a flow rate of three standard liters per minute (slm); shielding gas composition was oxygen and nitrogen in a ratio of 1:3 to mimic air, with a flow rate of 5 slm. Treatments were conducted with the effluent touching the treated surface, corresponding to a distance of 9 mm from the plasma outlet to the surface. Lipid stacks were treated in a point-like manner, while liposomal solutions were treated with a constantly moving plasma jet. All presented measurements were performed immediately after treatment. Treatment times were 3 minutes, if not mentioned otherwise.

B. Preparation of Lipid Stacks

For Raman investigations, dioleoylphosphatidyl-choline (DOPC) lipids were prepared as a lipid film or lipid stack. A volume of 30 µl of 10 mg/ml lipids in chloroformic solution was transferred to a glass slide. Chloroform was evaporated under a nitrogen stream until only a dried lipid film remained. This dehydrated lipid stack was kept under normal room conditions, leading to an at least partial rehydration.

C. Preparation of Liposomes

Liposomes, which can be described as spherical bilayers enclosing an aqueous volume, were prepared as described in the following section. DOPC dissolved in chloroform (10 mg/ml; Avanti Polar Lipids Inc., Alabaster, Alabama, USA) was transferred into a glass vial that was then filled with chloroform to a final concentration of lipids of 2.5 mM. A volume of 400 µl of this solution was transferred into a new glass vial. Evaporation of chloroform under a N_2 stream resulted in a thin and large lipid film at the side wall of the vial. A volume of 4 ml of an aqueous buffer (100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES, and pH = 7) was added to reach a final lipid concentration of 250 µM. The liposomes were then produced by heating up the lipid-containing liquid for 30 min in a 70°C water bath under ultra-sonic irradiation. Afterward, the vials were cooled down to 4°C. This thermo cycle was repeated two more times.

D. Raman Spectroscopy

Using a Raman spectrometer capable of Raman microscopy in combination with an inverted microscope (inVia with Leica DMI 3000M, Renishaw, Pliezhausen, Germany), lipid films were investigated. Detailed machine parameters were argonlaser (532 nm) 50%, exposition time 10 s, grating with 2400 grooves/cm. The spectra were smoothed and the baselines were corrected. Phase states of lipid films can be determined by the ratio of different CH_x bands: CH_2 at 2852 cm^{-1} and CH_2 of ordered all-*trans* chains at 2882 cm^{-1}.[38,39] The fluid control was kept at room temperature; the rigid control was created by cooling down a lipid film with an –80°C metal block, resulting in a temperature

of the lipid film on the glass slide of approximately –60°C to –40°C, clearly below the main phase transition temperature of –20°C.

E. Dynamic Light Scattering

Using dynamic light scattering (DLS) or photon correlation spectroscopy, the hydrodynamic diameter and the zeta potential (an electric surface potential) of untreated and plasma-treated liposomes were determined. Briefly, the zeta potential is the electrostatic potential at the plane of sheer and determines whether a colloidal dispersion is stable or not. A commonly accepted threshold value for stability is ±25mV. Measurements were performed on a ZetaSizer Nano (Malvern Instruments GmbH, Herrsching, Germany) at 25°C. (For further details, see Hunter.[40])

F. Liposome Fusion Assay

For the liposome fusion assay, 200 µl of the liposome solution described above was transferred into a vial containing 2.3 ml of the liposome buffer. The whole solution was incubated with 10 µl of Rh-18 dye according to the manufacturer's protocol (Life Technologies GmbH, Darmstadt, Germany). After storage at 4°C in the dark for 1 h, the labeled liposomes were mixed with 4.6 ml liposome buffer and 400 µl unlabeled liposomes solution, resulting in a total volume of 7.5 µl buffer containing 20 µM liposomes with a ratio of 1:2 labeled to unlabeled liposomes. After gentle mixing, 2 ml of this solution was used for each experiment and the two controls, respectively. The plasma treatment was conducted with the same gas mixture used for the lipid films. An untreated sample was used as a negative control. For a positive control, a sample was treated in an ultrasonic bath for 15 minutes. We assumed that this resulted in complete fusion of liposomes. To overcome bleaching issues, the measured plasma-treated sample was ultrasonificated (15 min) afterward to enforce fusion and its fluorescence spectrum was measured again. We assumed that liposomes that did not fuse during plasma treatment fused during the consecutive ultrasonification.

Measurements were conducted using a spectrometer also capable of emission spectroscopy (Fluorolog-3, Horiba Jobin Ivon GmbH, Bensheim, Germany) with excitation wavelength of 360 nm, emission range 450–650 nm. The intensity of 590-nm emission maximum of the dye was determined and normalized to the measured Raman water peak at 410 nm. In each of the three independent measurement setups, the relative intensities of the samples were normalized to the corresponding plasma-treated sample.

Because just a third of the liposomes were labeled with this dye, the fusion of labeled and unlabeled liposomes led to a dilution of the dye density on the resulting liposomes or lipid aggregates compared with the unfused labeled liposomes. The self-quenching characteristic of this dye results in an increased emission intensity for the fused liposomes if the dye is excited. Therefore, the emission intensity of Rh-18 was used as a measure for liposome fusion in complete agreement with manufacturer's proptocol.

III. RESULTS

A. Raman Spectroscopy

Here, we present the results of Raman spectroscopy on the influence of plasma treatment on lipid films. PC, the most abundant phospholipid of plasma-membranes of cells, was used with an acyl chain length of 18 C atoms containing one –C=C– double bond in the –9 position, which is in *cis* configuration (methyl ester of oleic acid, a monounsaturated omega-9 fatty acid (18:1); DOPC).[41] DOPC has a main phase transition temperature of –20°C and is therefore fluid at room temperature.

The first identifications of Raman bands of acyl chains were conducted by recording Raman spectra of fatty acyl esters and polymethylene chain compounds and comparing them with the spectra of lipids. CH_x stretching vibrations result in a strong Raman band in the region of 2800–3100 cm^{-1}.[42] A study on oxidation of oleic acid, the non-estered form of the acyl chains of DOPC, has reported Raman bands and their corresponding CH_x groups.[41] Other authors have reported values that differ up to five wavenumbers.[42] Dehydration is known to shift the bands of lipids toward higher energies and, therefore, wavenumbers.[43] These inaccuracies can sum up to 10 wavenumbers.

The influence of plasma treatment on the global properties of lipid bilayers was investigated by determining the fluidity state of plasma-treated dehydrated lipid films in comparison with untreated films at different phase states induced by temperature. The ratio of peaks of CH2 and all-trans CH2 at ~2850 cm^{-1} and ~2882 cm^{-1}, respectively, determined by Raman spectroscopy, is a measure of the state of phase in which a higher ratio indicates higher fluidity.[38,39] Due to these shifts in wavelength, the wavenumbers to use can differ slightly.

The Raman spectra of untreated and plasma-treated DOPC were recorded. They showed no significant differences in the wavelength region up to ~2800 cm^{-1}, where nearly all peaks correspond to the lipid's head group. Peaks corresponding to (hydrogen-) carbon bonds are exceptions, but the effects are also visible in the CH-stretch region of ~2800–3000 cm^{-1}.

The spectra of untreated and plasma-treated DOPC as well as untreated rigid DOPC, which was brought into rigid state by cooling, differ in the wavenumber region of 2800–3000 cm^{-1} (Fig. 1). The spectra were normalized to the PO_4^{3-} group of the lipid head group, resulting in normalization of the numbers of lipids.

As clearly shown in Fig. 1, the Raman CHx bands of untreated DOPC are more intense compared with both the plasma-treated and the cooled DOPC lipid films. The spectrum of cool, rigid-state DOPC is comparable to that of the plasma-treated one. The ratios of wavenumbers 2852 cm^{-1} and 2882 cm^{-1}, indicating the phase state, are 1.099 for cool, rigid-state DOPC, 1.169 for untreated fluid DOPC at room temperature, and 1.130 for plasma-treated DOPC at room temperature. Measurements were started immediately after plasma treatment.

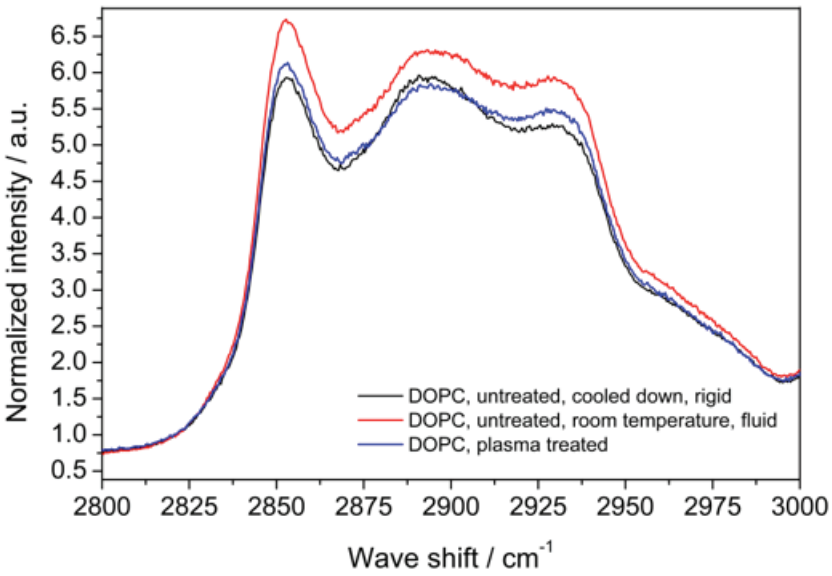

FIG. 1: Comparison of CHx Raman bands of untreated DOPC in fluid state (red) as well as in rigid state (black) and plasma-treated DOPC (blue). The state of phase can be determined by ratios of 2850 cm^{-1} and 2882 cm^{-1}.[38]

B. Liposome Fusion and Aggregation

A liposome is a self-assembled superstructure of lipids that can be described as a spheroidal bilayer enclosing an aqueous volume. In the following experiment, we used a fluorescence-based assay to determine whether the observed lipid modifications by plasma-treatment have an influence on liposomes.

The results of the liposome fusion studies are presented in Fig. 2. The fluorescence intensity of the dye Rh-18 was normalized to the fluorescence intensities of Rh18 in plasma-treated liposomes because of the bleaching effects of the plasma treatment indicated by the reduced fluorescence intensity (~6%) of the plasma-treated (PT) sample in comparison to the untreated one (1.00 to 1.06). As shown in comparison to the fusion controls (3 min plasma treatment and 15 min ultrasonification (PT+US), and 15 min US, respectively), fused liposomes show an increase in emission at 590 nm, as described by the manufacturer's protocol. In our case, emission intensity of PT+US sample was 1.12, which was 12% higher than the PT sample. Notably, the relative increase of the US sample compared with the untreated sample was remarkably greater (1.50 to 1.06 [42%]; 1.12 to 1.00 [12%]).

To determine whether plasma-treated liposomes aggregate, which, as well as fusion processes, leads to an unstable colloidal suspension, the hydrodynamic radius of untreated and treated liposomes was measured using the DLS technique. The mean values and their standard deviations resulting from three independent experiments are shown in Fig. 3.

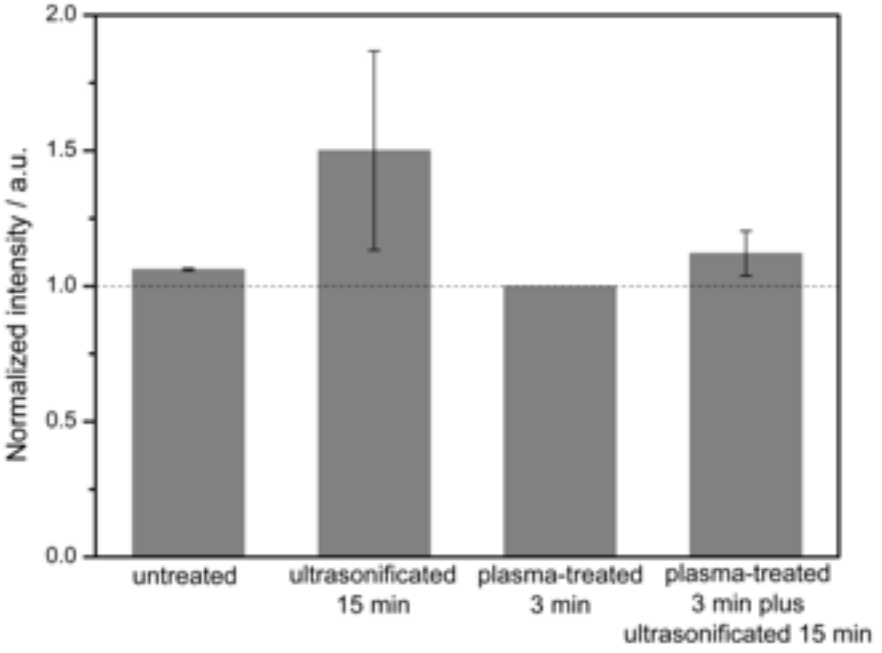

FIG. 2: No or incomplete fusion of liposomes during direct plasma treatment. Liposomes were immersed in buffered saline solution and were treated for 3 min. The emission intensity of the fluorescent dye Rh-18 was measured at 590 nm after excitation. The measured spectra have been corrected to the maximum of the measured Raman water peak at 410 nm and normalized to Rh-18 intensity of plasma-treated liposomes. As a positive control, a combined 3-min plasma treatment and ultrasonic treatment of 15 min was applied. The experiment was performed three times independently; mean values and standard deviations are shown.

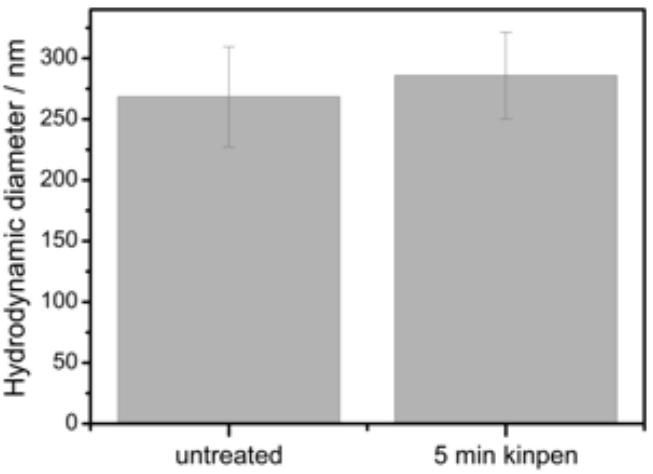

FIG. 3: No change of liposome size in buffered saline solution during 5 min direct plasma treatment. Mean values and corresponding standard deviations resulting from three independent experiments are shown.

As shown in Fig. 3, no significant increase in size of the liposomes was observed after 5 min of direct plasma treatment compared with untreated liposomes. The hydrodynamic diameter was 268 nm ±41 nm for untreated liposomes versus 286 nm ±35 nm for treated liposomes. The zeta potential, a parameter for dispersion stability, was measured for untreated as well as liposomes treated for 5 min by direct plasma treatment. The results of three independent experiments are shown in Fig. 4 as mean values and standard deviations.

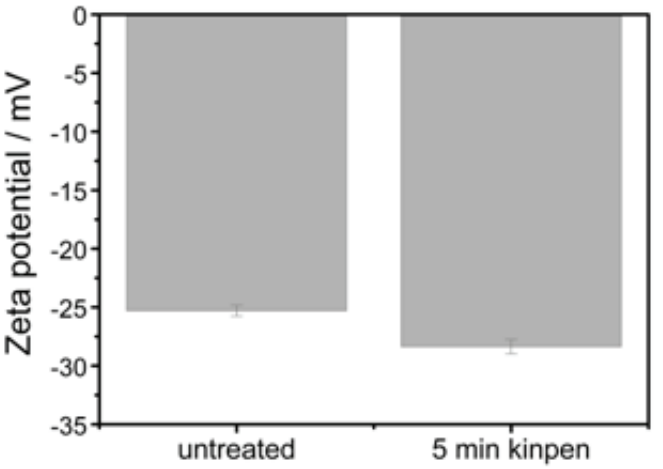

FIG. 4: Zeta potential of untreated and 5 min directly plasma treated liposomes in buffered saline solution. Mean and standard deviations result from three independent experiments are shown.

In parallel to the size measurements, a slight but not significant increase in the (strength of the) zeta potential of liposomes by plasma treatment was observed: –25.3 mV ±0.5 mV for untreated liposomes versus –28.4 mV ±0.6 mV for plasma-treated liposomes.

IV. DISCUSSION

The Raman data (Fig. 1) indicate that plasma treatment of a fluid lipid layer leads to rigidification, as shown by the nearly overlaying spectra of cooled, rigid-state DOPC and plasma-treated DOPC. Furthermore, the decreased ratio of wavelength of 2852 cm^{-1} and 2882 cm^{-1} of treated DOPC compared with untreated DOPC also indicates rigidification. Notably, the frozen DOPC film was still more rigid than the treated DOPC film, as shown by the wavelength ratios. The observation of the rigidification of lipid films agrees with the known rigidification observed in oxidized membranes.[44] A possible explanation for this behavior is that chemical modifications of the acyl chains by plasma-borne reactive species produce polar groups. The normal acyl chain is nearly perfectly apolar due to its symmetric chemical structure; therefore, nearly every modification will increase the polarity of the acyl chain. This is especially true for a modification caused by electrophilic

radicals. This greater polarity can lead to lipid rearrangement with enhanced lateral forces between the acyl chains. This can even result in direct lipid–lipid crosslinking, which has been described in literature.[25] Furthermore, the secondary peroxidation products of lipids, such as 4-hydroxy-2, 3-nonenal (HNE), increase membrane rigidity, possibly by forming complexes with lipids.[45] The fluidity of a lipid bilayer is determined by the lateral diffusion of the lipids. Therefore, enhanced lipid–lipid interaction forces, especially cross-linking of lipids, will reduce the lateral mobility of lipids and thus reduce the fluidity. The observed reduced fluidity of liposomal membranes originates from the alterations in lipid arrangement and lipid cross-linking.[44] The result of the present study show rigidification of lipids due to plasma treatment. Our result contrasts with Tai et al.,[34] who report that chemical oxidization of DOPC by 0.05 mM •OH radicals results in a more fluid lipid bilayer. This qualitative difference could be explained by different applied doses. As reported by Tresp et al. in the article, "Effect of atmosphere shielded plasma jet on different biological relevant liquids" in this special issue of *Plasma Medicine*, treatment with the plasma source and conditions used here leads to an •OH concentration in the low μM range, which is 10 times lower than the concentration used by Tai et al. Based on the common knowledge that the dose causes the toxin (or side effects), a working hypothesis is that cleavage of the oxidized acyl chains occurs if lipid oxidation continues due to higher radical concentration. Lipids with shorter acyl chains have an increased fluidity, so it is very reasonable to think that, at some degree of oxidation, this effect overwhelms the fluidity-reducing effects of enhanced lateral interaction forces and cross-linking. This biphasic behavior of lipid oxidation on fluidity was observed in membranes.[44] This effect will be further tested in studies that measure the long-term effect of plasma treatment on the state of phase of lipid bilayers.

The results of the fusion assay clearly show that no (or just an incomplete) fusion of liposomes occurs during plasma treatment, compared with the plasma-treated sample with consecutive ultrasound treatment that leads to fusion and the reduced intensity values of treated liposomes (Fig. 2). The reduced intensity of the plasma-treated liposomes cannot be easily explained by molecular rearrangement but rather by the bleaching of dyes due to the intense (UV) light of the plasma effluent. This bleaching of dyes was previously observed for different dyes and is an inherent challenge for investigations of plasma-treated liquids or lipids. A direct comparison of the intensity increase of ultrasonification of the plasma-treated/-bleached with the untreated sample was not possible due to the nonlinear quenching characteristic of the dye. In other words, the observed 41% increase by ultrasonification in the case of non–plasma-treated control does not necessarily indicate more complete fusion than the 12% increase achieved by subsequent ultrasonification in the plasma-treated sample.

Measurement of the liposome size of untreated and treated liposomes showed a slight but not significant increase in size due to plasma treatment (Fig. 3). Clearly, the plasma treatment did not induce aggregation of liposomes.

The zeta potential of treated liposomes, which was even slightly stronger than before treatment, can explain why no fusion or aggregation took place (Fig. 4); the

repulsive part of DLVO interaction between liposomes was unchanged or even slightly stronger. Therefore, the liposomes could not overcome the repulsive barrier to get in contact, which was necessary for fusion and aggregation of liposomes. Notably, in the MD simulations, oxidation of the acyl chains resulted in chain reversal that decorated the membrane interface with negatively charged groups.[46] This could explain the slight but not significant increase in the size and zeta potential of the treated liposomes due to increased head-group area and surface charge (Figs. 3 and 4).

Taking these observations together, we conclude that the plasma treatment investigated here does not induce fusion or aggregation of liposomes due to the present of a repulsive potential of investigated liposomes, even if an influence on the phase state of lipids was observed.

Our finding that, under these conditions, the plasma treatment did not result in complete fusion or aggregation of liposomes contrasts with the finding that a guided streamer plasma source leads to aggregation and destruction of liposomes.[35] Different plasma sources and treatment conditions, including time, were used in both studies; therefore, the concentrations of the reactive species cannot be compared. Svarnas et al.[35] mentioned that the liposome integrity was substantially affected by their plasma treatment in a time-dependent manner, especially when lipid concentration was very high. In contrast to their concentration of 60 mg/ml lipids (~100 mM), in this study we used a concentration of 250 μM, which corresponds approximately to the amount of lipids in a typical cell culture solution. As mentioned by Svarnas et al., the observed effects were weak (5 min treatment time) or even not significant (shorter treatment times) when lipid concentration was lower, but still at a high concentration of 10 mg/ml. Taking this lipid concentration dependency into account, it is reasonable to conclude that, with our experimental setup, which was closer to typical biological conditions, no influence on liposome integrity was observed.

A. Model for Lesion Formation

Plasma treatment is known to induce pores or lesions;[22] however, the exact molecular mechanism remains unclear. Based on the described experiments and on the available literature, we propose the following molecular mechanism of lesion formation in lipid bilayers and cellular membranes based on lipid oxidation initiated by reactive species created by plasma treatment (Fig. 5). This preliminary model was presented at the ICPM in Orleans in June 2012:

1. A reactive species binds and oxidizes the acyl chain part of a lipid (indicated by black drawn lipid).
2. Modification of the acyl chain leads to a change of molecular shape due to changed state of phase (red lipid). Due to the changed shape of the lipid, mechanical stress/strain is induced in the lipid monolayer. The hydrophobic mismatch weakens the membrane.
3. Propagation of lipid oxidation leads to growing areas of oxidized lipids.
4. If a threshold value of oxidized lipids is reached, the induced stress is too

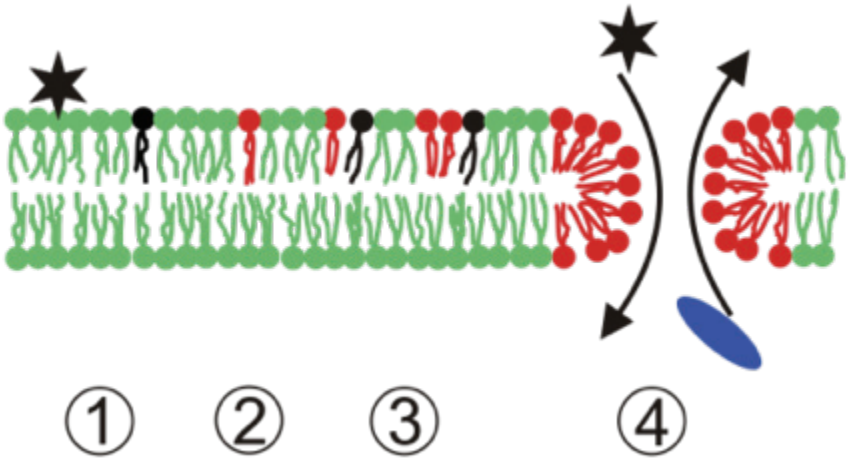

FIG. 5: Formation of a lesion in the lipid bilayer (green) by reactive species (star). Oxidized lipids (black) transform into lipids with changed molecular shape (red), inducing mechanical stress. After reaching a threshold, induced stress leads to local rupturing of the bilayer, thus a lesion is formed. The formed lesion allows a "self-mediated in- and efflux" of membrane-impermeable substances, either extracellular molecules like other plasma-borne reactive species or intracellular ones (blue oval).

> strong and the weakened bilayer ruptures. A rearrangement of the lipids results in (at least dynamically) a stable lesion in the lipid bilayer. After formation of the lesion, it is presumed that the lateral diffusion of oxidized lipids out of the lesion region reduces the induced stress, which finally leads to closing of the lesion ("healing" of bilayer).

Through these lesions, other reactive species can diffuse into the cell, where they can attack intracellular targets like mitochondria or DNA. Furthermore, this could explain the observation that fibroblast growth factor-2 is released by endothelial cells after plasma treatment.[47] Therefore, we name this lesion-forming process, which results in a permeabilization of the membrane, "self-mediated in- and efflux." Essentially, the lesion-forming ability of reactive species allows them and other substances to cross the membrane when they could not do so otherwise. Notably, a similar principle of pore-forming peptides exists, called "self-promoted uptake".[48,49] Our model not only pronounces the possibility that the lesion-forming molecule itself and other non–lesion-forming extracellular substances can enter the cell through the lesion, but also the possibility of an efflux of intracellular molecules like cytokines.

B. Discussion of the Model

The first steps in the proposed mechanism, the lipid oxidation by a reactive species, is well known.[24] Many species that oxidize lipids, including reactive oxygen species like

•OH or singlet oxygen, are identified in the literature,.[26–28] A variety of different lipid oxidation pathways are known, depending not only on the type of reactive species but also on the exact kind of acyl chain of the lipid.[24,34,50] In general, (poly-) unsaturated fatty acids are more susceptible to reactive oxygen species than saturated ones.[51] Most, if not all, types of reactions result in a modification of the acyl chain. Because the hydrophobic force[52] between the two leaflets of a bilayer strongly depends on the molecular orientation and apolarity of the hydrocarbon chains, oxidation introduces polar moieties that change the biophysical parameters dramatically.[53] A reduced hydrophobic moment of the chains is the result. A reorientation of the chains of the oxidized monolayer results in a hydrophobic mismatch between the two leaflets that weakens the bilayer.[54] The polarized chain moves to the water interface,[54] leading to a molecular shape modification with increase in head-group area. All these acyl chain modifications lead to a changed hydrophobic interaction between the two monolayer leaflets of the bilayer, resulting in a hydrophobic mismatch of the formerly energetically favorable state. Therefore, for many different oxidation reactions, a common effect is an increase in head-group area (and therefore a more inverted cone like shape of the lipid), which has been confirmed by molecular dynamics (MD) simulations. Because the shape of lipids determines the curvature of its superstructure, the oxidized lipids tend to be in a monolayer of negative curvature. This induces lateral mechanical stress or strain in the outer monolayer as described in step 2 of our model.

The propagation of lipid oxidation (step 3 in our model) is well known.[24] Therefore, we assume that an accumulation of oxidized lipids occurs over time, leading to growing areas of modified lipids.

Step 5 describes the induced stress due to the mismatch between neutral curvature of an unoxidized bilayer and the fact that the rigidized oxidized lipids tend to form negative curved layers and therefore induce bending energy. The bending energy increases with increasing area of oxidized lipids. It has been proposed that the bilayer ruptures locally if the bending energy reaches a threshold due to increasing areas of oxidized lipids. At concentrations of oxidized lipids higher than 5%, formation of water-filled lesions is observed.[54] In the field of peptide-bilayer interaction, a similar mechanism called “carpet mechanism” includes the accumulation of peptides that insert into the bilayer and induce stress.[55,56] The lesion formation results in a leakage of the bilayer. In the literature, MD simulations are described in which a concentrated oxidation of lipids leads to pore (or lesion) formation.[53] Notably, this resulted in cases of massive oxidation in a collapse of the bilayer. These MD investigations assumed different modifications by oxidation, but the results were always similar. For ozone, its leakage-inducing properties in membranes are known.[28] Little is known about the stability of the lesions, but Leduc et al. reported that transient lesions close after some time in a cellular membrane.[22] The stability of the lesions formed by oxidizing species in lipid bilayers can only be speculated. Svarnas et al. reported pore or lesion formation, aggregation, and fusion of plasma-treated liposomes,[35] but their plasma source differed from the one used in this study. Assumedly, the differences can be attributed to the applied dose, if only lesion formation or a total

collapse of the bilayer occurs. Similar lesions formed by peptides are transient, with an expected lifetime of seconds to minutes for the lesions in lipid bilayers discussed here. This effect should be investigated in future studies.

Here, the mechanism discussed above is proposed as an important step toward understanding the membrane effects of plasma treatment. Further studies using plasma treatment should investigate this model in detail. The transfer of the effects in lipid bilayers to cellular membranes is still a great step, but in the field of lesion-forming peptides, this transfer has already been successful. For the effects of oxidized lipids, especially by plasma treatment, this should be investigated further. Furthermore, the effect of electroporation occurring at areas of oxidized lipids in membranes[57] can also play a role in a synergistic effect between reactive species and electromagnetically fields created by plasma treatments.

Consequences of a dose-dependent oxidization of lipids by plasma treatment of cellular membranes could include the following:

- Very low amounts of oxidized lipids lead to no cellular effects. Repair mechanisms of the cell can control the damage.
- Low amounts of oxidation have an influence on cellular signaling due the influence of oxidized lipids on raft formation or due to secondary messengers of lipid oxidation.
- Medium amounts of oxidized lipids results in transient lesion, as proposed here. This allows a self-mediated in- and efflux. Reactive species created by plasma in the cellular environment could gain intracellular access and induce diverse cellular reactions, e.g., oxidation of mitochondria, including cytochrome c release. This could lead to apoptotic effects.
- High amounts of oxidized lipids lead to too many lesions or to too much stress on the membrane, resulting in a total collapse of the membrane. This leads finally to necrotic cell death.

This proposed dose-response is in good agreement with a previously published speculative dose effect,[58] which proposes cell proliferation, growth factor release, etc. as effects of intermediate doses, and normal cell death and necrotic cell death as a result of high or very high doses, respectively. In contrast, our dose-dependency explanation includes more detailed mechanisms and is based completely on lipid effects.

To summarize our findings, we showed that a 3-minute plasma treatment with the nonthermal plasma jet kinpen does not induce macroscopic breakdown of lipid systems like fusion or aggregation due to the fact that the repulsive potential between liposomes hinders direct contact between the liposomes.

A more subtle change of the state of phase due to plasma treatment was detected, which should lead to a reorganization of the lipid membrane. To explain the known phenomena of "plasma poration," we proposed a molecular mechanism based entirely on lipid interaction that results in transient lesion formation, which allows a "self-mediated in- and efflux" of both extra- and intracellular substances.

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