COMBINATION OF COLD PLASMA AND BIOMIMETIC LIPOSOMES AND MEMBRANES AS A VERSATILE TOOLBOX IN REDOX MEDICINE

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Abstract: Cold plasmas expel reactive oxygen species (ROS), that have a significant biomedical impact. Beside a direct effect via receptors, the chemical modification of biomolecules is relevant. Especially lipids are prone to oxidation via plasma-generated ROS, forming lipoxidation products. Here, we compared the impact of short-lived reactive species on phospholipids organized in biomimetic self-organized systems with and without access to the gas-liquid interphase in order to understand plasma chemistry and cellular redox processes.

Keywords: lipid oxidation, plasma liquid chemistry, solid-supported lipid bilayers

1. Introduction

Physical plasmas operated at ambient pressure and body temperature are of particular interest in biomedical research and cosmetic or medical application, e.g., skin treatment, wound management, and cancer [1-3]. To the current knowledge, the major biologically active component of plasma is the variety of reactive oxygen species (ROS; including those containing nitrogen atoms) formed in the discharge or the afterglow from molecular components such as oxygen (O_2), nitrogen (N_2), and water (H_2O) present in the ambient air or used as and admix to the feed gas. Among the plasma-generated reactive species are long-lived species like hydrogen peroxide (H_2O_2), ozone (O_3), nitrous and nitric acid (HNO_2/HNO_3), and nitric oxide (NO), that can cross barriers and diffuse into the treated system (a liquid, or a tissue). In contrast and in its diversity a selling point of plasma in redox biology are the short-lived species, that are highly reactive and confined close to its point of formation, e.g., the gas-liquid interface. Among those, atomic oxygen (O), singlet (delta) oxygen ($^1O_2$), hydroxyl radicals (•OH), superoxide ($O2^{\bullet-}$), nitrogen dioxide radical ($\bullet NO_2$) and peroxynitrite ($ONOO^-$) are most dominant. Secondary and tertiary species may form at interfaces or in the liquid/tissue bulk [4, 5].

Mainly the long-lived species are recognized by cellular receptor molecules, mimicking the impact of physiologically derived ROS during redox signal processes active during mitochondrial activity or for maintaining normoxic conditions [6]. The short-lived species are in presence of biomolecules (proteins, lipids, small metabolites) too reactive to survive transport via diffusion. Instead, they react with biomolecules in their vicinity forming covalent bonds and modified molecules with different physico-chemical properties and a potentially modulated perception by biological systems[7-15]. Dissecting the chemical contribution of each individual ROS is hampered by the fast conversion between the species and the low specificity of the molecular probes available. Using free amino acids and peptides as targets, a strong influence of the discharge conditions and the availability of short-lived species was determined [16-18]. To further extent the knowledge on plasma liquid chemistry with a special regard towards phospholipids as major component of cellular membranes, we conducted experiments using either solid-supported lipid bilayers (SLBs) or liposomes as target structures. In addition, the role of sphingolipids, cholesterol, and other intra- and extra-membrane antioxidants as protecting agents was investigated [19].

In conclusion, we observed significant differences in membrane stability and the appearance of lipid oxidation products between liposomes (access to the gas-liquid interphase) and SLBs (access to liquid bulk only) and the structure of the lipid membrane, confirming the protective role of sphingomyelins and supporting the idea that adaptive processes in the body during aging and inflammation modify the impact of (plasma-generated) ROS.

2. Methods

POPC, PE, PLPC, and SM (d18:1/18:1) were obtained from Avanti Polar Lipids (USA). MTBE and chloroform were purchased from Sigma Aldrich (Germany). Buffer components were obtained from Merck (Germany). UPLC-grade methanol, acetonitrile, formic acid, and isopropanol were obtained from Biosolve VB (Netherlands). Hydrogen peroxide and ammonia solutions are from Carl Roth (Germany). 50 mM phosphate buffer solution (pH=7.4) was used in the experiments.

Plasma source and treatment procedure: A kINPen® (neoplas, Germany) using 3 slm argon plus molecular admixtures (0.5 %, N_2/O_2) was used. For electrochemistry, mass spectrometry, and AFM, buffer layer of 1.8 cm thickness covered the SLB during plasma treatment. Liposomes were treated in 100 µL buffer. The distance between jet nozzle and liquid-air interface was 9 mm.

Liposome and SLB preparation: Liposomes (PLPC:SM or POPC:SM:PE in various ratios) were prepared from chloroform solutions, brought to dryness under inert gas and resuspended in 50 mM phosphate buffer (pH 7.4), and liposomes were prepared by sonication on ice. The size distribution of the obtained vesicles was measured by dynamic light scattering. SLBs were formed on ultra-flat

gold surfaces using the vesicle fusion method. The remaining liposomes was replaced by buffer before plasma treatment.

Membrane permeability after treatment was determined by voltammetric measurements in 10 mM $K_4[Fe(CN)_6]$/ 50 mM phosphate buffer in the potential range from −0.3 V to +0.7 V vs. Ag/AgCl (Autolab PGSTAT 20 and Eco Chemie IME 303, Metrohm, Germany). The SLB covered gold electrode, a platinum electrode and an Ag/AgCl electrode were used as working electrode, counter electrode and reference, respectively.

Mass spectrometry analysis: Plasma treated liposomes or lipid bilayers were extracted by 2 mL MTBE. After solvent removal by nitrogen stream, the dried lipids were dissolved in isopropanol. Liquid chromatography-tandem mass spectrometry analysis was performed by a Vanquish UHPLC (Thermo Germany) system equipped with an Accucore C18 column and an iHILIC-Fusion column, respectively. The column temperature was 50°C and the flow rate was 300 µL/min. Eluent A was acetonitrile:water (50:50), and eluent B was isopropanol:acetonitrile:water (85:10:5), containing 0.1 % formic acid and 5 mM ammonium formate. The elution gradient was: 0−20 min ramp from 10% to 86% B, 20−22 min ramp to 86% to 95% B, 22−26 min 95% B, and the column was then re-equilibrated at 10% B for 8 min. The LC was coupled to a QExactive Plus high-resolution mass spectrometer (Thermo Germany) operated in positive ion mode. Data-dependent acquisition (DDA) settings: in full-scan mode m/z 100 to 1200) was acquired at a resolution of 140,000. The MS/MS spectra were acquired at a resolution of 17,500 (top 10). LPPtiger, LipidSearch, and FreeStyle/manual inspection were applied to identify lipid oxidation products.

Atomic force microscopy (AFM) and scanning electrochemical microscopy (SECM) were performed as described earlier[19-21].

3. Results & Discussion

Plasma treated biomembrane-mimetic liposomes (POPC:SM:PE) showed oxidation of the side chain and the headgroup, depending on the type of molecule and the treatment conditions. Side chain oxidation was observed in POPC and to some extent in SM. Most obvious oxidation occurred in PE, whose ethanolamine headgroup is a good target for chlorination. A strong increase of the signal intensity for chlorinated PEs were observed already after 30 s (Fig. 1). Up to two chlorine atoms were added to the headgroup by the plasma-driven chemistry, and elimination reactions yielded to further variants including a nitrile group. Highest increase in abundance was found for both PE-Chloramines and PE-Nitrile. In contrast, when in addition to O_2 also N_2 was present in the feed gas, the formation of modified lipids decreased. In the case of pure argon, the occurrence of modified lipids decreased further. Especially the PE-Dichloramine represents a decisive molecule, as it was not detected in control and pure argon plasma. Of note, despite the high number of double bonds in PE only negligible side chain oxidations were observed. The side chain of POPC, containing one double bond and two protons in allyl position showed some limited oxidation. Beside side chain breaks (PC 16:0/9:0 + 1O), some oxygen addition products were detected (+2O, +1O atom). The modification of SM in the fatty acid chains was even less pronounced.

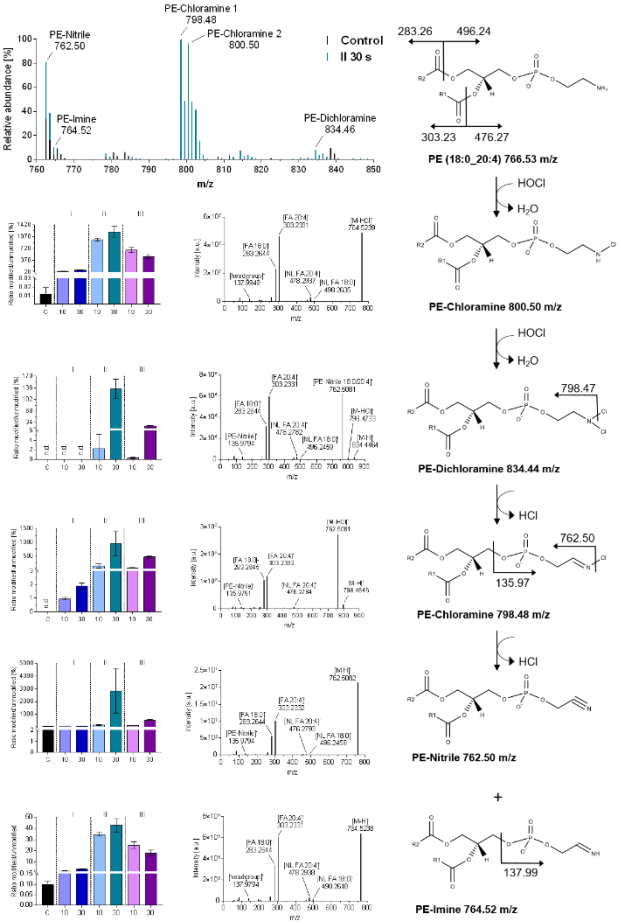

Fig. 1. Increased headgroup oxidation observed after kINPen treatment of biomembrane-mimetic liposomes. PE (18:0/20:4) is an excellent target for chlorination and various variants were observed (details see text).

Taken together, the biomembrane-mimetic liposomes are less sensitive towards plasma-derived chemistry than their mono-lipid class counterparts. The high degree of organization due to the SM content renders the surface of the liposome less penetrable for small molecules including ROS. Therefore, oxidation occurs at the outside, where the charged headgroups are located. The amino group of PE is an excellent “suicide” target, actively attracting the plasma-derived reactive species with a strong focus on active chlorine species that are formed from by the reaction of short lived atomic oxygen with chloride ions at the interface[22-24]. If chloride-free phosphate buffer (PB) is used in contrast to standard phosphate buffered saline (PBS), chlorination of the lipids does not occur. This indicates the

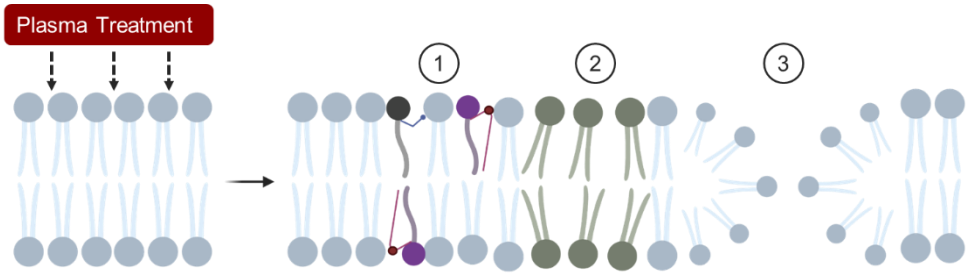

Fig. 2. Plasma-driven oxidation of lipids (1) – increased fluidity (2) – pore formation (3)

strong role of atomic oxygen formed in the plasma in biomolecule oxidation.Plasma treated biomembrane-mimetic SLBs (PLPC:SM, varying ratios) confirmed the results obtained in biomembrane-mimetic liposomes and extended its relevance. Using plasma-derived chemistry as a research tool to mimic the formation of multi-ROS in inflammatory processes of the body, we tested the hypothesis that cells adapt to ROS stress by changing either the capability to turnover ROS (e.g., catalase, SOD, etc.) or to increase the protection of critical structures. In the cornea of the eye, the epithelial cells are critical for the eye sight, since a failure of the cell's membrane would lead to the entrance of water into the stroma and subsequently to the reduction of transparency. During aging, the impact of ROS increases, and membrane composition changes, favouring a higher presence of SM [25]. We tested, how the oxidation of SLBs by plasma changes with increasing part of SM in the membrane. As observed for the liposomes, a protection by SM was detected. The higher degree of organisation within the two sheets of the membrane prevented the penetration of reactive species towards the fatty acid side chains. Consequently, the membrane permeability kept near control levels in the case of PLPC:SM 1:3, in contrast to PLPC:SM 3:1 or PLPC alone (Fig. 3). Using AFM, we could provide additional proof on the integrity of the plasma-treated PLPC:SM 1:3 membrane.

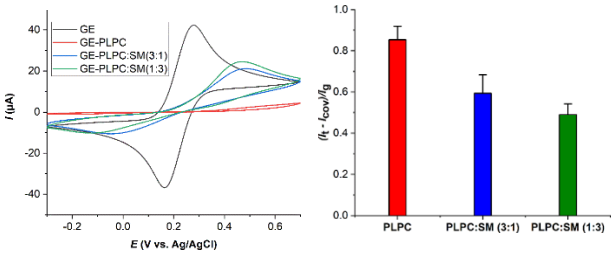

Fig. 3. Membrane permeability of biomemrane-mimetic SLBs is challenged by kINPen treatment (electrochemical measurements). Sphingomyelin (SM) provides protection via changes in self-organisation of the membrane sheets.

Using high-resolution mass spectrometry, a significant decrease of PLPC oxidation products is observed in the presence of SM. SM itself is barely attacked by the plasma derived species, resembling results seen on the liposomes. However, in contrast to the liposomes, treatment time for the SLBs was 10 to 30 min, ranging an order of magnitude higher. Since the buffer used did not contain chloride ions, the formation of reactive chlorine species is impossible and the SLBs have no contact to the gas-liquid interface. Regarding the attacking species there are three options to discuss: peroxynitrite (ONOO-), ozone (O_3), or "traveling" hydroxyl radicals. Hydrogen peroxide is not a sufficient oxidant and has been excluded by control experiments. The formation of $ONOO^-$ has been shown for several plasma sources, and also for the kINPen [17, 26, 27]. However, ideal conditions would be a strongly acetic pH in contrast to the neutral buffer used. Ozone is a strong oxidant, but poorly soluble in water. By "blowing" it over the surface its small Henry´s constants limit its solvation into the aqueous buffer. Hydroxyl radicals are a very strong oxidant and an important part of plasma liquid chemistry. However, since they are very reactive the path of diffusion is strictly limited [28]. Therefore, it is mainly active at the gas-liquid interface. Some authors assume a bond-flipping mechanism that allow the OH radical to "migrate" into the liquid bulk [29]. We have adopted this idea in an experiment on cysteine oxidation and the origin of the atoms incorporated in the products [16]. Since these experiments were missing, it remains to be clarified if the discussed mechanisms could explain the impact observed in bulk-protected SLBs. First pilot experiments using liposomes and heavy oxygen isotopes indicated a role for OH radicals, suggesting that this notion is correct.

4. Conclusion

Lipids are an excellent target for plasma-derived chemistry and a different side chain and head group oxidations can be observed. Depending on the discharge conditions, the impact can be controlled. Extra- and intra-membrane antioxidants and structural optimization further modulate the integrity of the membrane during and after plasma treatment, ultimately leveraging the physiological impact in biological systems The results clearly emphasize the relevant role of cold plasma as a biomedical research tool.

5. Acknowledgement

This work was funded by the DFG (German Research Foundation) 231396381/GRK1947 for M.R, H.K. The EU (H2020 project MASSTRPLAN 675132 to M.F) and the German Federal Ministry of Education and Research (BMBF) (03Z22DN12 and 03Z22D511) to K.W.

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