

Small molecule analytics to elucidate the impact of plasma derived chemical species on biological targets

K. Wende¹, G. Bruno¹, S. Wenske¹, S. Bekeschus¹, T. von Woedtke², M. Lalk³, K.-D. Weltmann² and J.-W. Lackmann¹

¹Center for Innovation Competence plasmatis, Leibniz Institute for Plasma Science and Technology (INP), Greifswald, Germany

²Leibniz Institute for Plasma Science and Technology (INP), Greifswald, Germany

³University of Greifswald, Institute for Biochemistry, Greifswald, Germany

Abstract: Cold atmospheric plasmas (CAPs) are successfully applied in chronic wound treatment and tested in the concept of cancer control and other conditions under the control of redox signalling processes. So far, the underlying biochemical mechanisms are not determined, especially with respect to the role of short lived species. Here, the (bio-) chemical impact of CAP derived species on various tracer molecules, in part using heavy isotopes as feed gas additive or liquid phase modulators (e.g. $^{18}\text{O}_2$) was investigated. Via high-resolution mass spectrometry coupled to liquid chromatography, resulting covalent modifications were identified. In particular, the roles of the short-lived species atomic oxygen and singlet oxygen compared to OH radicals and the long-lived species hydrogen peroxide were addressed using cysteine as relevant tracer molecule. A significant difference product pattern between a direct and indirect treatment regimen was found for two plasma jets. Additionally, mass spectrometry revealed that a considerable amount of the reactive species attacking the tracer molecules derive from the liquid phase. In conclusion the question whether plasma treated liquids can indeed be used as alternative for direct CAP treatment in plasma medicine is addressed.

Keywords: plasma medicine, mass spectrometry, short lived species, cysteine model

1. Introduction

The effectiveness of cold atmospheric plasma (CAP) in various applications, such as microbial inactivation, plant seed germination, cancer treatment, and healing of chronic or acute wounds, is predominantly addressed to the action of reactive species[1-4]. In the core region of molecular gas-doped noble gas jet plasmas and especially in their effluent, various reactive oxygen and reactive nitrogen species, alongside with electrons and noble gas ions or metastables, have been detected. The specific composition is determined by the plasma source design, the working gas composition (if any) and further modulated by distance from the active plasma zone or ambient conditions. Significant input from the plasma physics community seeks to describe and understand the resulting multi-dimensional dynamics of the active species composition. From the biomedical point of view only species transferred into the cell environment or tissue are relevant. Upon entering the biological system, plasma-derived species may compromise cellular integrity, interact with cellular signalling processes, or trigger chemical reactions that relay the impact of the plasma source from the point of entrance to other parts of the model or body. Yet, the molecular basis of these processes is not determined and it can be assumed that the primary or secondary species generated by the plasma are relayed via biomolecules (metabolites, lipids, proteins). Which reaction of the biological system follows the treatment depends on the number and chemical properties of the species hitting the

target (“plasma dose”). Therefore, a thorough understanding of the plasma derived species trajectories is desired in order to broaden CAP treatment options in plasma medicine, foster plasma source design, and safe use.

So far, a wealth of studies shows either clinical impact of CAP, with chronic or acute wounds as the major topic, or in vitro results using different primary or immortalized cell lines[5-10]. Such, while we have good knowledge on the functional outcomes resulting from CAP treatment, we lack a connection between the gas phase species generated by the plasma, their path into the biological system, and their primary targets (Fig. 1). In liquids treated by CAP the long lived species hydrogen peroxide (H_2O_2), protons (H_3O^+), nitrite (NO_2^-), and nitrate (NO_3^-) are found frequently. Short lived species, such as peroxynitrite (ONOO^-) was determined to be present, but direct detection of ONOO^- remains elusive[11]. Using electron paramagnetic resonance spectroscopy, hydroxyl radicals (OH), superoxide anion radical (O_2^-) and nitric oxide (NO) were detected, but could not be quantified due to unknown reaction probabilities of the used spin traps with the respective radicals[12, 13]. With that, an unsatisfying and incomplete picture of plasma – liquid respective plasma – cell interaction is the current status. While H_2O_2 is clearly a major product of some plasma discharges especially when working gases are not absolutely dry, other do not produce it (or do so only transiently) and nonetheless have a massive impact on biological systems[14, 15].

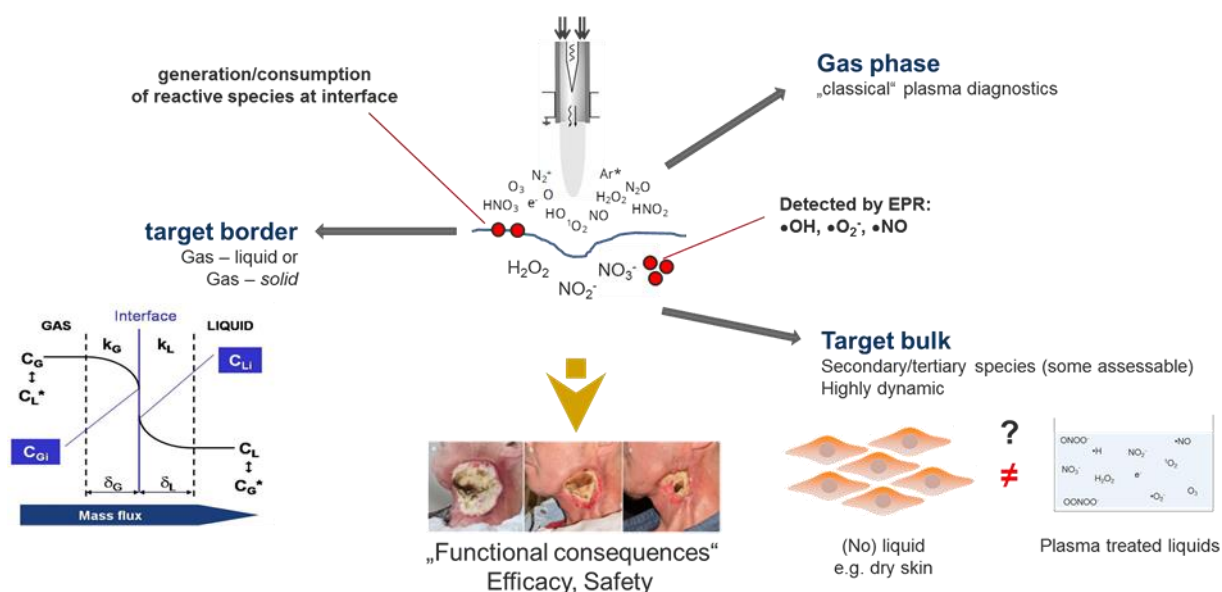


Fig. 1: Cold atmospheric plasmas in biomedical applications – which species can reach the ultimate target? A significant discrepancy between in vitro experiments using aqueous buffer systems with large volumes and in vivo situation (highly concentrated, small amounts of liquids) exists.

Here, experimental data are presented that for plasma discharges in direct proximity to aqueous systems containing organic compounds short lived species dominate the long lived hydrogen peroxide. Using cysteine as small organic tracer compound and the peptide bradykinin as biological macromolecule prototype the chemical impact of the argon jet kINPen (Greifswald) and the helium jet COST jet (Bochum, York) was evaluated using high resolution mass spectrometry as tool to elucidate plasma derived covalent modifications. While in the case of cysteine the thiol group is the preferred target of plasma derived reactive oxygen species, peptides such as bradykinin are targeted by both ROS and RNS. Major differences between a direct CAP treatment of the target compounds and the indirect treatment by a plasma treated liquid were observed. These results emphasize the role of short lived plasma derived species and allow the conclusion, that in the clinical setting a large proportion of the chemical energy applied is scavenged by the organic molecules outside the cells. Future work must seek to understand the further fate of the newly generated chemical groups and their biological impact.

2. Methods

Plasma treatments: The argon-driven plasma jet kINPen09 or the helium driven COST jet was used in these experiments[16, 17]. The working gas flow (3 or 1 slm, respectively) was in part enriched with 0.5 or 1 % molecular gas (oxygen, nitrogen, or a 1:1 mixture of both). In some of the experiments, heavy molecular oxygen ($^{18}\text{O}_2$) was introduced in the working gas or the liquid (H_2^{18}O , 99 %). The distance between jet-nozzle and liquid surface

was constant at 9 mm (kINPen) and 4 mm (COST jet), as well as the treated volume (750 μL) in 24 well plates. Treatment times between 30 s and 600 s were used. Aqueous solutions of cysteine at different concentrations were treated, but in the present study 300 μM cysteine were predominantly used. Direct and indirect treatments of cysteine solutions were performed; while in the first case the plasma impacts directly on the buffer containing the tracer, in the second case the buffer was treated in absence of cysteine. Immediately after the treatment, cysteine was added to the plasma treated liquid and allowed to react for 1 minute.

Cysteine derivative elucidation: cysteine-derived structures after plasma impact were characterized via high resolution mass spectrometry (Sciex TripleTOF 5600) via direct infusion. If desired, MS2 experiments were performed to achieve substructure information. For the quantification, hydrophilic interaction liquid chromatography (HILIC) was used for some experiments.

Bradykinin derivative elucidation: Solution with a concentration of 0.2 mg/ml were treated up to 5 min with 3 slm argon + 0.5% O_2 , 9 mm distance (kINPen) and 1 slm helium + 0.5% O_2 , 4 mm distance COST-jet in a 24-well plate from Sarstedt. The plasma-modified peptides were analysed by Nano-LC-HRMS/MS-measurements (Dionex UltiMate 3000 RSLCnano and Q Exactive™) from ThermoFisher by direct infusion (DI) electrospray ionization (ESI) or by LC-ESI coupling using a PepMap column. The software Byonic from Protein Metrics was used to evaluate the data and identify the PTMs.

3. Results & Discussion

Numerous plasma-induced cysteine derivatives were observed, being in good agreement with DFTB simulations[18]. Information about the structures were obtained by MS/MS analysis, hyperfine mass data and reference spectra. Prominent cysteine products derived from the impact of CAP species are shown in Figure 2.

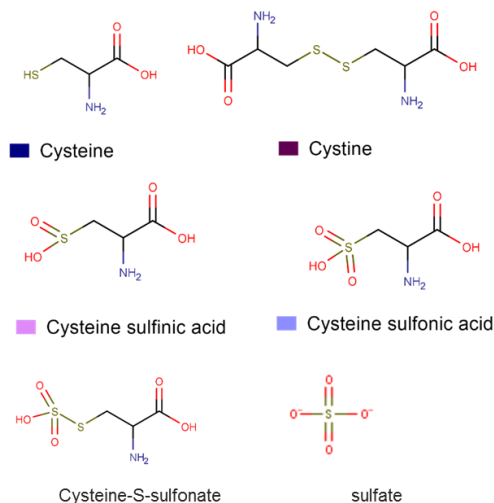


Fig. 2: prominent derivatives of cysteine after direct plasma treatment

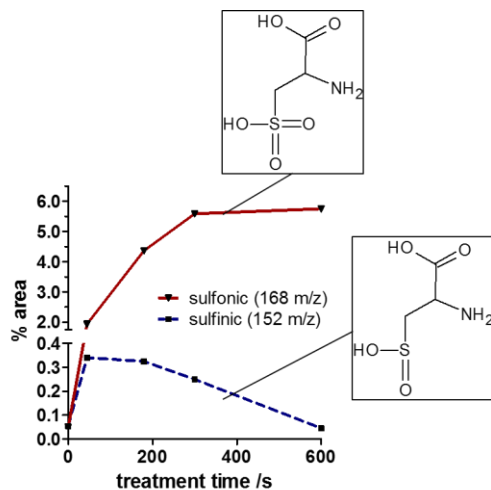


Fig. 3: Cysteine is consumed during plasma treatment. Major products are cysteine sulfinic and sulfonic acid. Cysteine sulfinic acid is a transient product, and oxidation culminates in cysteine sulfonic acid. Ultimate species is the sulfate ion (oxidation number of sulphur +6)

While most compounds are transient and disappear with increasing treatment time (e.g. cystine), ultimately cysteine sulfonic acid (Cys-SO₃H) and sulphate ions (SO₄⁻) accumulate as highly oxidized apex species (Fig. 3). Intermediates of interest are sulfenic acid (Cys-SOH) that is biological active but could not be detected due to its

instability, and cysteine sulfinic acid (Cys-SO₂H) that was found in considerable amounts if conditions were not highly oxidizing as in Ar-O₂ or He-O₂, and various oxidized cystine derivatives. The latter are assumed to be precursors of cysteine-S-sulfonate, a biologically active compound. Only traces of RNS derived compounds, such as S-nitroso cysteine, could be detected. From these results it could be concluded that the plasma-induced oxidation of cysteine occurs mostly at the sulphur moiety and is oxygen-driven.

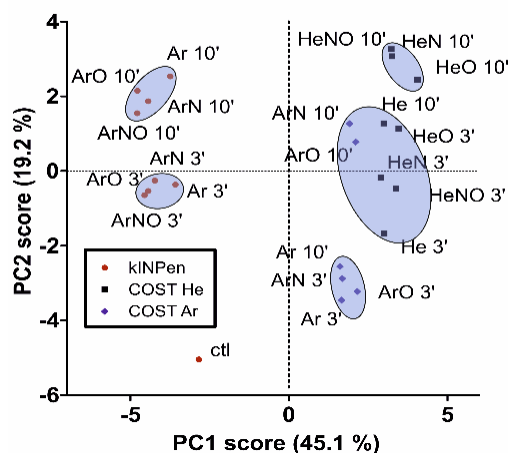


Fig. 4: PCA analysis reveals differences in kINPen and COST jet plasma deposited (or chemically active) species reacting with the cysteine tracer compound. Overall, 129 cysteine derivatives contribute to the analysis[18].

The concentration and composition of the detected cysteine derivatives are highly dynamic and allow conclusions on the plasma source and discharge parameters. A clear distinction between kINPen and COST jet could be detected by the model system, further modulated by the composition of the working gas (Fig 4). To confirm the predominant action of short lived reactive species in the oxidation of cysteine, direct treatments were compared with indirect treatments. Indeed, while in the first case the liquid receives the direct effect of all the plasma components (e.g. reactive species, radiation), in indirect treatments the liquid interacts mostly with long-lived species. Figure 5 shows experiments performed via HILIC-MRM separation and quantification of the cysteine derivatives. In this case, the attention was given mostly for the production of cystine (RSSR, primary product) and cysteine sulfinic (RSO₂H) or sulfonic acid (RSO₃H, secondary/tertiary products). The results clearly show that in case of direct treatments a strong oxidation on the thiol occurs, leading to the presence of both cysteine sulfenic and cysteine sulfonic acid. In case of the indirect treatment, no oxidized compounds except cystine are produced. Cystine is also the product that occurs when only hydrogen peroxide is used as control experiment, indicating that in the indirect treatment H₂O₂ is the dominant active species.

Both the impact of ROS and RNS was observed, with a clear majority of ROS driven PTMs. While targets, specificity and biological significance are still under investigation, a comparison of the direct plasma treatment (= CAP) and indirect treatment (= plasma treated liquid, PTL) was recorded. Figure 7 compiles the found PTMs at the different amino acids of bradykinin. As could be expected were the phenylalanine (F) and the arginine (R) targets easily modifies resp. oxidized. Under direct

treatment conditions, numerous further positions of the peptide were found to be attacked (Fig. 7A). Additionally, under direct treatment conditions, not only “oxidation” (+16 Da) but predominantly “trioxidation” (+48 Da) was observed. These data align with the observations in the cysteine model under direct and indirect treatment conditions and further emphasize the role of short lived species like atomic oxygen (O) or singlet oxygen ($^1\text{O}_2$). When taking the two approaches frequently used for the application of CAP in the clinics and in the biomedical research into account, the data obtained and presented here strongly suggest that direct and indirect treatment cannot be compared from the chemical point of view. This is valid for the small molecule cysteine, but also for the larger and more complex peptide bradykinin. Accordingly, care must be taken when transferring experiences from in vitro settings into in vivo.

4. Conclusion and outlook

Using cysteine and the peptide bradykinin as model substances, the derivatives generated by the plasma treatment form distinctive patterns enabling description and standardization of the chemical potential of different plasma source and discharge parameters in liquids. It is suitable as a tool to understand the liquid chemistry of the chosen treatment conditions. For the plasma sources tested here, a strong impact of short-lived oxygen species produced in the gas phase and that are directly deposited in the aqueous liquid or at its surface and directly acting on the cysteine thiol moiety, was shown. In contrast, hydrogen peroxide dominated liquid chemistry produced by indirect plasma treatment failed to return strongly oxidized cysteine derivatives and it must be assumed that the direct plasma treatment performed in the clinical applications of CAPs are not well described by many (cell) models using plasma treated liquids (“plasma activated medium, PAM”). By using isotope pattern, the transition of gas phase species into bulk liquid residing cysteine molecules was determined, and a significant amount of liquid phase oxygen was detected in the oxidized cysteine derivative. The biological relevance must be addressed by future research as the deposition of plasma derived species in the in vivo setting was so far not studied.

5. Acknowledgements

This work is funded by the German Federal Ministry of Education and Research (BMBF) (Grant No. 03Z22DN11&12).

6. References

- [1] S. Reuter, T. von Woedtke, K.-D. Weltmann, T. J. Phys. D: Appl. Phys., 51 (2018) 233001.
- [2] A. Schmidt-Bleker, J. Winter, A. Bösel, S. Reuter, K.-D. Weltmann, Plasma Sources Sci. Technol., 25 (2015) 015005.
- [3] D. B. Graves, Coupling Plasma Physics, Biochemistry and Biology, PP (2017) 1-1.
- [4] D.B. Graves, Clinical Plasma Medicine, 2 (2014) 38-49.

- [5] A. Schmidt, S. Dietrich, A. Steuer, K.-D. Weltmann, T. von Woedtke, K. Masur, K. Wende, J. Biol. Chem., 290 (2015) 6731-6750.
- [6] M. Keidar, D. Yan, Beilis, II, B. Trink, J.H. Sherman, Trends Biotechnol., 36 (2018) 586-593.
- [7] A. Schmidt, S. Bekeschus, K. Wende, B. Vollmar, T. von Woedtke, A Exp. Dermatol., 26 (2017) 156-162.
- [8] A. Schmidt, S. Bekeschus, H. Jablonowski, A. Barton, K.-D. Weltmann, K. Wende, R Biophys. J., 112 (2017) 2397-2407.
- [9] H.-R. Metelmann, C. Seebauer, V. Miller, A. Fridman, G. Bauer, D.B. Graves, J.-M. Pouvesle, R. Rutkowski, M. Schuster, S. Bekeschus, , Clinical Plasma Medicine, (2017).
- [10] K.R. Liedtke, S. Bekeschus, A. Kaeding, C. Hackbarth, J.P. Kuehn, C.D. Heidecke, W. von Bernstorff, T. von Woedtke, L.I. Partecke, , Sci. Rep., 7 (2017) 8319.
- [11] P. Lukes, E. Dolezalova, I. Sisrova, M. Clupek, Plasma Sources Sci. Technol., 23 (2014) 015019.
- [12] S. Reuter, A. Schmidt-Bleker, H. Tresp, J. Winter, S. Iseni, M. Hammer, M. Duennbier, K.D. Weltmann, Plasma Physics and Technology, 1 (2014) 55-57.
- [13] Y. Gorbanev, D. O'Connell, V. Chechik, Chemistry (Easton), 22 (2016) 3496-3505.
- [14] S. Bekeschus, K. Wende, M.M. Hefny, K. Rodder, H. Jablonowski, A. Schmidt, T.V. Woedtke, K.D. Weltmann, J. Benedikt, Sci. Rep., 7 (2017) 2791.
- [15] K. Wende, P. Williams, J. Dalluge, W. Van Gaens, H. Aboubakr, J. Bischof, T. von Woedtke, S.M. Goyal, K.-D. Weltmann, A. Bogaerts, P. Bruggeman, Biointerphases, 10 (2015) 029518.
- [16] K.D. Weltmann, E. Kindel, R. Brandenburg, C. Meyer, R. Bussiahn, C. Wilke, T. Von Woedtke, Contributions to Plasma Physics, 49 (2009) 631-640.
- [17] S. Kelly, J. Golda, M.M. Turner, V. Schulz-von der Gathen, , Journal of Physics D-Applied Physics, 48 (2015).
- [18] J.W. Lackmann, K. Wende, C. Verlackt, J. Golda, J. Volzke, F. Kogelheide, J. Held, S. Bekeschus, A. Bogaerts, V. Schulz-von der Gathen, K. Stapelmann, Sci. Rep., 8 (2018) 7736.
- [19] H. Jablonowski, J.S. Sousa, K.-D. Weltmann, K. Wende, S.J.S.r. Reuter, , Sci. Rep., 8 (2018) 12195.
- [20] T. Murakami, K. Niemi, T. Gans, D. O'Connell, W.G. Graham, Plasma Sources Sci. Technol., 23 (2014).
- [21] K. Niemi, D. O'Connell, N. de Oliveira, D. Joyeux, L. Nahon, J.P. Booth, T. Gans, Appl. Phys. Lett., 103 (2013).
- [22] C. Verlackt, W. Van Boxem, D. Dewaele, F. Lemiere, F. Sobott, J. Benedikt, E. Neyts, A. Bogaerts, J. Phys. Chem. C, 121 (2017) 5787-5799.