Plasma-induced species deposited in liquids: On the Sulphur oxidation path to elucidate predominant chemical dynamics

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Abstract: In contrast to gas phase plasma chemistry, liquid phase chemistry induced by cold plasma is underexplored but essential for understanding biomedical applications of plasma, e.g. in the concepts of wound healing or cancer treatment. In this study, the oxidation of the small molecule tracer cysteine by an argon plasma jet was investigated in order to generate a dynamic model reflecting the reactive species deposition in relation to the plasma treatment conditions. In particular, the role of short and long-lived species was clarified, as well as the role of UV radiation produced by the discharge.

Keywords: thiols, plasma liquid chemistry, redox signaling, mass spectrometry

1. Introduction

The effectiveness of cold atmospheric plasma (CAP) in various applications, such as microbial inactivation, seed germination, cancer treatment, or would healing, is addressed to the action of reactive species formed in the gas phase; able to act directly on the target or via prior diffusion in liquid [1].

Specific mechanisms are still under study, mostly considering the complex non-equilibrium reactions occurring in the liquid phase before reaching the (biological) target. Indeed, the journey of species produced in the gas and going into and through the liquid includes many variables, such as solubility, reactivity, and consequently lifetime in the liquid or in the cellular environment. The CAP derived chemistry in the liquid phase is a result of transport processes and reactions occurring in the gas phase, at the interphase and in the bulk of the target under study, where further secondary or tertiary species may be formed [2].

It was shown how the composition of a liquid, such as diverse cell culture media or buffer solutions generate a different liquid chemistry which reflect back on the plasma effectiveness, for example at the cellular level [3].

Similarly, the deposition of reactive species can be modulated by the composition of the working gas, the gas flow dynamics, or the plasma source itself. Treatment conditions, such as distance and duration, are further influencers. Techniques used for species quantification are various chemical assays or ion chromatography for stable species, as well as electron paramagnetic resonance (EPR) for radicals. In addition, small molecule tracers can be used as tracers for the reactive species deposited in liquids or other targets. Structure elucidation techniques, e.g. mass spectrometry, can be applied to determine the role of the respective species by observing the covalent chemical modifications occurring on the tracer molecules. The observable product pattern facilitates understanding of the dynamics in plasma liquid chemistry by mirroring the reactive species deposition [4, 5].

Here, the potential of cysteine, containing a thiol moiety, as tracer compound was evaluated. Indeed, the sulphur atom is very sensitive to redox reactions, leading to a significant reactivity of cysteine towards CAP derived reactive species [6]. Furthermore, cysteine is an essential element in cell culture media and is a key amino acid determining protein folding and functionality, as well as it is relevant in redox-signalling pathways (thiol switch) [7].

2. Methods

Plasma treatments. The argon-driven plasma jet kinpen09 was used in these experiments. Different gas compositions consisting in 3 slm of argon with or/and without 1% molecular admixture (molecular oxygen, molecular nitrogen, or the mixture of both) was chosen for the treatments. In some of the experiments, isotopically labelled molecular oxygen ($^{18}O_2$) was introduced in the working gas. The distance between jet-nozzle and liquid surface was constant at 9 mm, as well as the treated volume (750 µL) in 24 well plates. In contrast, different treatment time were performed, going from short (30 sec) to longer treatments (10 min). Aqueous solutions of cysteine at different concentrations were treated, but in the present study treatments of 300 µM cysteine were mostly reported.

Direct and indirect treatments of cysteine solutions were performed; while in the first case the plasma impacts directly on the liquid, in the second case aqueous solutions were treated in absence of cysteine. Right after the treatment, cysteine concentrated solutions were diluted into the plasma-treated water; subsequently, the solution was incubated for 1 minute.

Sulphur derivatives characterization & long-life species quantitation. The cysteine-derived structures after plasma impact were characterized via mass spectrometry using a negative polarity. In particular, direct injections in a high

resolution mass spectrometer (tripleTOF) was used for the structural identification at the MS^2 level. In this case, normalization of the data for each spectra was performed introducing an internal standard (IS). In case of the isotopically labelled cysteine derivatives, the subtraction of the natural isotopic occurrence was performed.

For the quantification, Hydrophilic interaction liquid chromatography (HILIC) was coupled to a mass spectrometric detection (qTRAP). A Multiple Reaction Monitoring modality was set, leading to a more accurate quantification based on MS^2 transitions specific for each derivative. The hydrogen peroxide (H_2O_2) was quantified via ferrous oxidation-xylenol orange (FOX) assay.

3. Results & Discussion

Cysteine product pattern as descriptive model. The plasma-induced cysteine derivatives, having different oxidation states (OS), were firstly characterized via high resolution mass spectrometry. Information about the structures were obtained thought the MS/MS analysis, and the resulting identified peaks and compounds are shown in **Figure 1**. In the same figure, it is shown the possible pathway of formation of these derivatives, based mostly on literature research. In general, two are the principal ways in which cysteine (RSH, OS -2) can be oxidized: the first occurs via oxygen addition, by forming cysteine sulfenic acid (RSOH, OS 0), then cysteine sulfinic acid (RSO_2H, OS +2), and finally cysteine sulfonic acid (RSO_3H, OS +4).

This compound can further be oxidized in the ending product sulfate (SO_4^-, OS +6). While the first two steps are reversible, the formation of RSO_3H and SO_4^- is an irreversible reaction. In parallel, two molecule of cysteine could be oxidized in cystine (RSSR, OS -1) by forming a disulphide bond. This compound can be further oxidized in biologically active sulfoxides, by addition of one to four oxygen on the two sulfur atoms. These derivatives, as well as the cysteine sulfenic acid, are labile compounds, difficult to observe in such oxidative conditions.

As by-product of the oxidation of the sulfoxides, it was possible to observe the cysteine *S*-sulfonate ($RSSO_3H$, OS +4), which in turn can be converted into RSO_3H.

From these results we could firstly conclude that the plasma-induced oxidation of cysteine is mostly on the sulphur atom and oxygen-driven. Consequentially, the model is well suitable for the investigation of reactive oxygen species generate by cold plasma. Regarding reactive nitrogen species, it was not possible to observe covalent modifications of cysteine with nitrogen derived species, except for the *S*-nitrosocysteine (RSNO, OS 0).

This compound is highly unstable, and it was observed mostly only by tuning the working gas and shielding gas composition to specific conditions [8].

As the few observations regarding the action of nitrogen species could be explained with the prevalent action of plasma-generated oxygen species, it is necessary to take in consideration that the cysteine model could be not suitable for the detection of nitrogen reactive species. **Figure 1** also shows the higher production of each cysteine derivative under certain plasma treatment conditions. The differential production of cysteine derivatives reflects back the differential deposition of reactive species in liquids in diverse conditions. For this reason, the cysteine product pattern could be considered as a dynamic and reactive model to describe the liquid chemistry of plasmas.

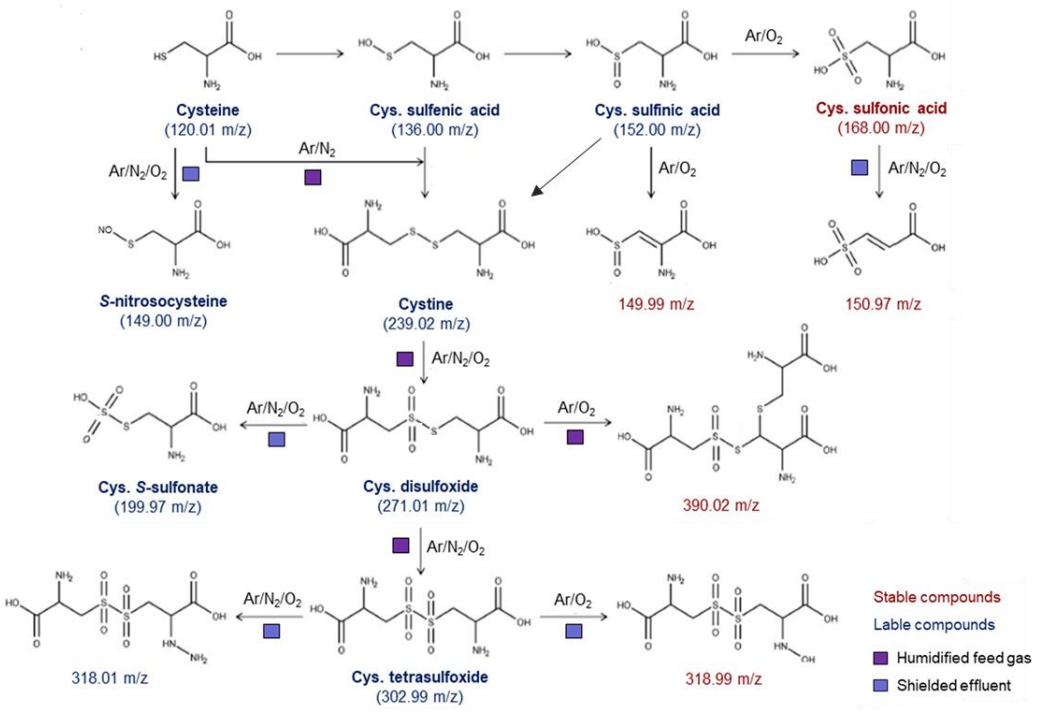

Fig. 1. Plasma-induced cysteine derivative pattern and conditions to obtain maximum production.

Additionally, it was possible to group the cysteine derivatives in stable and labile compounds, since the first resulted to be mostly produced in "stronger" oxidative conditions (presence of oxygen in the working gas and long treatments), while the second shown the opposite behaviour in the same conditions.

Indeed, their amount was higher in "softer" oxidative conditions (absence of oxygen in the feed gas, use of the shielding gas, short treatments).

Reactive short-lived species as major effectors. To confirm the dominant activity of reactive species in the oxidation of cysteine, and therefore in the potential effectiveness of cold plasma on other biological substrates, further investigations were performed. Firstly, direct treatments were compared with indirect treatments.

Indeed, while in the first case the liquid received the direct effect of all the plasma components (e.g. reactive species, radiation), in indirect treatments the cysteine may interact with long-lived species only. **Figure 2** shows data obtained via HILIC-MRM analysis, clearly proofing this assumption. Attention was given predominantly to cystine and cysteine acids production.

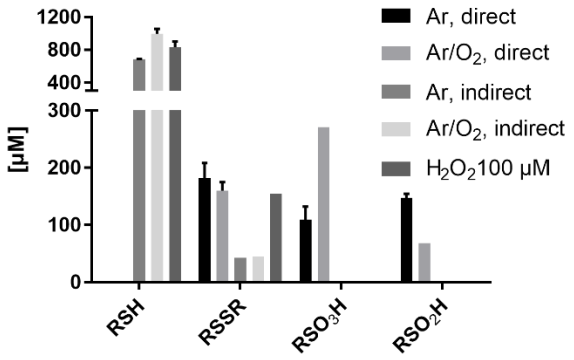

Fig. 2. Impact of direct *vs* indirect treatments on the cysteine thiol moiety (cysteine 1 mM, 10 min).

The results show that in case of direct treatments a stronger oxidation occurs on the thiol moiety of cysteine. In case of indirect treatment, no oxidized compounds are produced, except for cystine. From this experiment it can be concluded that, while in case of direct treatments cysteine interacts with short-live species and possibly other short-lived plasma elements such as radiation, in case of indirect treatment the long-lived hydrogen peroxide dominate the product portfolio. When quantifying H_2O_2 (**Figure 3)**, it can be observed that in the presence of cysteine the deposited amounts are reduced. Considering the results shown in **Figure 2**, where the incubation with hydrogen peroxide shows only limited oxidation capabilities on the cysteine structure, this observation can be explained by a consumption of hydrogen peroxide precursors, e.g. hydroxyl radicals (•OH).

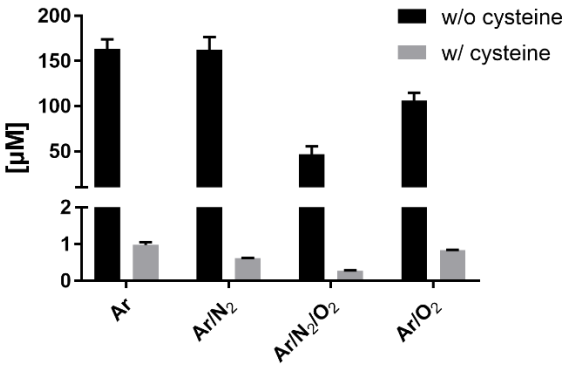

Fig. 3. Hydrogen peroxide deposition using different working gases (cysteine 0.3 mM, 3 min).

To further confirm the role of short-lived species and investigate the role of the UV radiation, experiments were performed by using a MgF_2 window, transparent to light above 115 nm in a micro-chamber. The isolated effect of the VUV radiation produced by plasma was observed on the thiol moiety. The results are shown in **Figure 4**, indicating that the major products were cystine and, to a lesser extent, cysteine sulfonic acid.

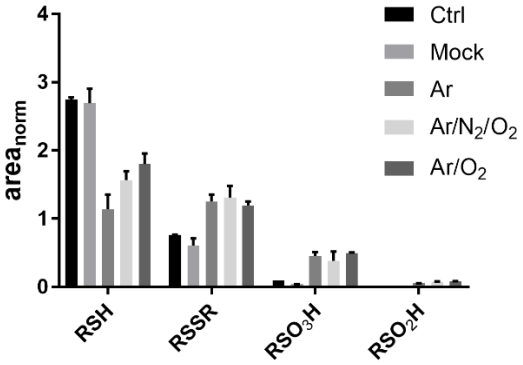

Fig. 4. Impact of VUV radiation on the cysteine thiol moiety (cysteine 0.3 mM, 1 min).

This result is independent from the chosen working gas for the treatments. Probably species generated by the photolysis of water under VUV radiation, such as •OH, hydrogen radicals (•H) and solvated electrons, promote the formation of thiyl radicals (RS•), which in presence of other RS• and lack of reactive oxidative species, react predominantly together to form cystine [9, 10, 11]. This would explain also the low amount in the production of cysteine sulfonic acid, in this case possibly depending by the reaction with •OH.

Principal Component Analysis (PCA) performed of the data (**Figure 5)** reveals significant differences between the controls, the treatments with VUV radiations and the direct treatments with cold plasma.

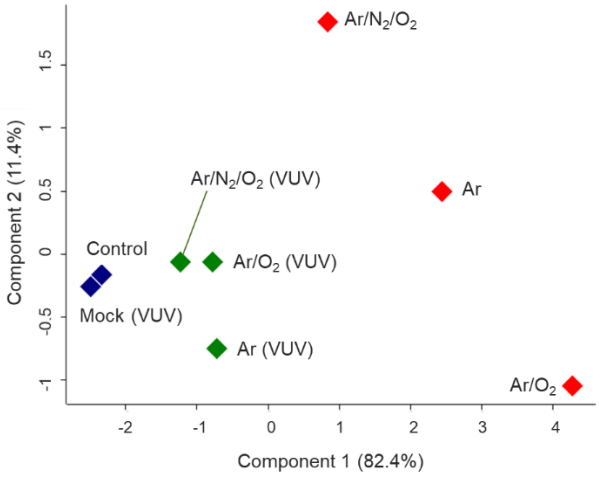

Fig. 5. PCA of cysteine derivatives produced under different conditions (cysteine 0.3 mM, 1 min).

Gas-phase species deposition in aqueous liquids. The results of the experiments performed by using isotopically labelled oxygen (^{18}O) in the working gas are shown in **Figure 6**. In particular, among all derivatives, cysteine sulfonic acid was chosen as representative compound, since most of the derivatives follow the same behaviour.

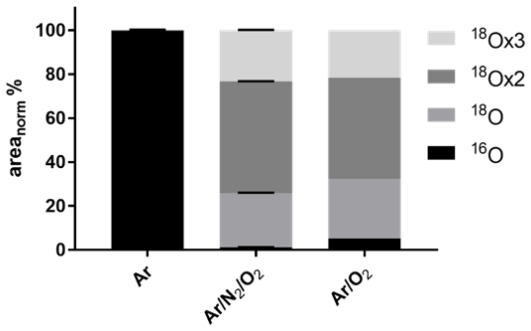

Fig. 6. Incorporation of gas phase oxygen species in the cysteine sulfonic acid (cysteine 0.3 mM, 1 min).

Figure 6 shows the incorporation of zero or more oxygen from the gas phase. Data were normalized on the total amount of cysteine sulfonic acid generated using different working gas compositions, in order to compare the results.

These data confirm the chemical activity of gas phase reactive species in liquid-gas interphase or liquid bulk, leading to the direct oxidation of the cysteine. In particular, the gas phase-species contribute with up to one single oxygen, suggesting the involvement of reactive species as atomic oxygen (•O), singlet oxygen ($^1O_2$), which follow fast reaction kinetics toward cysteine [9, 10].

4. Conclusions

Cysteine derivatives pattern is a potential model to describe and standardized the oxidative potential in liquids and biological substrates of different plasma sources, as well as a tool to optimize and modulate the treatment conditions in relation to the chosen application. Regarding the kinpen09, a greater impact is shown by short-lived oxygen species produced in the gas phase and directly deposited into the aqueous liquid, possibly acting directly on the cysteine structure.

A partial impact is given also by the water-derived species (tertiary species) induced by the isolated impact of VUV radiation generated by plasma.

Further investigations regarding the specific product of cysteine reactions with reactive species as •OH, •O and $^1O_2$ will be investigated, both under the plasma treatments and as isolate reactions.

5. Acknowledgements

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6. References

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