Following O and OH in He/O\textsubscript{2} and He/H\textsubscript{2}O gas mixtures—from the gas phase through the liquid phase to modifications on a biological sample

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Abstract
Applied cold atmospheric plasma allows for the controlled delivery of reactive oxygen and nitrogen species tailored for specific applications. Through the manipulation of the plasma parameters, feed gases, and careful consideration of the environment surrounding the treatment target, selective chemistries that preferentially influence the target can be produced and delivered. To demonstrate this, the COST reference microscale atmospheric pressure plasma jet is used to study the generation and transport of O and OH from the gas phase through the liquid to the biological model target cysteine. Relative and absolute species densities of OH and O are measured in the gas phase through laser induced fluorescence (LIF) and two-photon absorption LIF respectively. The transport of these species is followed into the liquid phase by hydrogen peroxide quantification and visualized by a fluorescence assay. Modifications to the model biological sample cysteine exposed to OH and H\textsubscript{2}O\textsubscript{2} dominated chemistry (He/H\textsubscript{2}O (0.25%)) and O dominated chemistry (He/O\textsubscript{2} (0.6%)) is measured by FTIR spectroscopy. The origin of these species that modify cysteine is considered through the use of heavy water (H\textsubscript{18}O) and mass spectrometry. It is found that the reaction pathways differ significantly for He/O\textsubscript{2} and He/H\textsubscript{2}O. Hydrogen peroxide is formed mainly in the liquid phase in the presence of a substrate for He/O\textsubscript{2} whereas for He/H\textsubscript{2}O it forms in the gas phase. The liquid chemistry resulting from the He/O\textsubscript{2} admixture mainly targets the sulfur moiety of cysteine for oxidation up to irreversible oxidation states, while He/H\textsubscript{2}O treatment leads preferentially to reversible oxidation products. The more O or OH/H\textsubscript{2}O\textsubscript{2} dominated chemistry produced by the two gas admixtures studied offers the possibility to select species for target modification.

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(Some figures may appear in colour only in the online journal)

1. Introduction

Atmospheric pressure plasma jets (APPJs) are a source of a variety of reactive species which can be delivered to various targets, e.g. water for plasma agriculture applications or biological targets for plasma medicine. In both applications, reactive oxygen and reactive nitrogen species (RONS) play a key role. Thus, the controlled delivery of RONS from the plasma to the target is one of the key challenges for plasma agriculture and plasma medicine. Biological targets are typically surrounded by a liquid environment, which offers another layer of complexity for following the generation and transport of reactive species. In particular, reactive oxygen species (ROS) like atomic oxygen \( \text{O} \) or the hydroxyl radical \( \text{OH} \) are of interest as they are highly reactive and have been shown to cause major modifications on different organic targets, e.g. cancer cells [1, 2], proteins and amino acids [3–7], and phenol [8, 9]. To produce \( \text{O} \) or \( \text{OH} \) inside an APPJ, a noble gas like helium or argon is typically used with some admixtures, e.g. \( \text{O}_2 \) or water vapor. Reactive species can be produced inside the APPJ, either due to the process gas used or due to impurities, or in the effluent when high-energetic and/or reactive species interact with ambient air. Reactive species produced in the gas phase can then undergo further reactions in the gas phase before approaching the liquid interface. In case of atomic oxygen, the main loss reaction is the formation of ozone by the three-body reaction \( \text{O} + \text{O}_2 + \text{M} \rightarrow \text{O}_3 + \text{M} \). The major loss mechanism of \( \text{OH} \) is the recombination to hydrogen peroxide in the gas phase [10]. Once \( \text{O} \) and \( \text{OH} \) have entered the liquid, further reactions occur in the liquid phase. If a biological target is present, the target itself becomes part of the reaction pathway.

The plethora of APPJ’s developed in different laboratories makes it difficult to compare results and to gain fundamental understanding of the generation and transport of the species produced. To overcome this, the COST radio frequency (RF) plasma jet was developed in the frame of an EU COST action as a reference APPJ, introduced and described in detail in Golda et al [11]. The production of species by the COST-jet and similar devices has been studied in the gas phase using, e.g. optical emission spectroscopy [12], molecular beam mass spectrometry [13–15], two-photon absorption laser-induced fluorescence (TALIF) [15–18], and cavity ringdown laser absorption spectroscopy [15]. Further investigations have been performed in the liquid phase with electron paramagnetic resonance (EPR) spectroscopy, ion chromatography, and colorimetric assays [6, 19, 20]. These studies lay a framework for detailing the initial plasma parameters to deliver specific reactive species from the plasma to the target and help contextualize the results presented here.

We will follow the generation and transport of \( \text{O} \) and \( \text{OH} \) from the gas phase in the presence of a liquid target, through the liquid to the model biological substrate cysteine. Fourier transform infrared (FTIR) spectroscopy of cysteine has previously been introduced as a ready-to-use method for the investigation of plasma-induced modifications [21] and has been used to compare chemical modifications caused by different plasma sources and gas admixtures [3, 6, 22]. The amino acid cysteine is a simple model, containing each chemical group only once which allows identifying chemical modifications easily. Its amino (\( \text{R–NH}_2 \)), carboxyl (\( \text{R–COOH} \)) and thiol (\( \text{R–SH} \)) groups can be found in almost every biologically relevant macromolecule. Especially the thiol group is of biological relevance as the importance of thiol modifications has been recognized in redox biology [23–26]. We compare the modifications on cysteine exposed to \( \text{O} \) and \( \text{H}_2\text{O}_2 \) dominated chemistry and an \( \text{O} \) dominated chemistry in the liquid. Further, we link the chemical modifications observed with liquid phase and gas phase chemistry, in the presence of a liquid interface.

The paper is organized as follows: first, the experimental set-up and the diagnostic set-ups for the gas phase, the liquid phase, and the biological samples are briefly described. The results section is split in four parts: first, the gas phase results obtained by laser induced fluorescence (LIF) and two-photon absorption laser induced fluorescence (TALIF) are presented; in the next part, the transport of atomic oxygen and \( \text{OH} \) is discussed with liquid phase measurements in correlation to the gas phase measurements. In the third part, the transport of the species to a biological target is explored by investigating modifications occurring on the model biological sample cysteine by means of FTIR spectroscopy and mass spectrometry. The origin of the reactive species responsible for the chemical modifications is explored by using heavy water (\( \text{H}_2\text{O}^\text{18} \)) for the cysteine solution. Finally, conclusions are drawn.

2. Experimental setup

2.1. Plasma source

The plasma source used for the experiments is the atmospheric pressure COST reference microplasma jet, described in more detail in Golda et al [11]. Briefly, the COST-jet is a capacitively coupled RF plasma, driven at a frequency of 13.56 MHz. The electrodes are 30 mm long and 1 mm apart from each other, resulting in an active plasma region of 1 mm × 1 mm × 30 mm. Helium is used as the feed gas with small admixtures of either oxygen (0.6%) or water (0.25% equivalent to 2500 ppm). The gas flow is kept constant at 1 slm for all experiments. Helium and oxygen (ARC3, 5.0 purity) is delivered via stainless steel tubing to reduce impurities. For
the He/H₂O mixture, dry helium is passed through a bubbler and mixed back with dry helium downstream in the appropriate ratio. Power is kept constant at 750 ± 10 mW and continuously monitored by integrated current and voltage probes.

2.2. Gas phase diagnostics

Atomic oxygen and OH were measured in the effluent with and without a liquid target present. Atomic oxygen densities in the effluent were measured using two-photon absorption laser induced fluorescence (TALIF) [27] and OH was determined using laser induced fluorescence (LIF). For the measurements with a liquid surface present, a cylinder with a volume of 60 ml is filled with DI water and placed at a 4 mm distance to the jet’s nozzle. The experimental setup is depicted in figure 1.

2.2.1. TALIF for atomic oxygen. Details of the TALIF setup and the density calibration can be found in Myers et al [27]. Briefly, a Nd:YAG laser (EKSPLA) with a 30 ps pulse width and a 50 Hz repetition frequency was used to produce 10 µJ pulse energies. The TALIF signal is recorded with an Andor iSTAR Gen III intensified CCD camera. The ICCD camera is used in conjunction with an 840 nm filter (Andover Corporation, FWHM 10 nm). For spatial scans of the effluent, the laser and detector setup is fixed, while the plasma jet and liquid surface are moved on an x − y − z translational stage. Densities of atomic oxygen found using absolutely-calibrated TALIF are estimated to have an experimental uncertainty of 37% [27].

2.2.2. LIF for hydroxyl radicals. Laser induced fluorescence (LIF) of OH relies on exciting the ground state of the molecule using 283 nm photons tuned to the OH(X²Π(ν = 0) → A²Σ⁺(ν = 1)) transition. From the OH(A²Σ⁺(ν = 1)) excited state one of two processes can occur to produce emission in the wavelength region of interest, as shown in figure 2. The molecule can either de-excite to the OH(X²Π(ν = 1)) state, emitting a 315 nm photon, or undergo a vibrational energy transfer (VET) to the OH(A²Σ⁺(ν = 0)) state, subsequently transitioning to the ground state and emitting a 309 nm photon. Alternatively, the laser-excited OH radical can be collisionally quenched, resulting in no emission. Due to the relatively long radiative lifetime of OH(A²Σ⁺(ν = 1)) (more than 20 times longer than laser-excited O in TALIF measurements), collisional quenching is the dominant de-excitation pathway of the excited state [28, 29]. While helium does not collisionally quench laser-excited OH, the molecular components of the admixtures do so efficiently [30]. As a first-order approximation, the collisional quenching coefficients of H₂O and O₂ with OH(A²Σ⁺(ν = 1)) were multiplied by the density of their respective admixtures (0.25% and 0.6%), and used for calculating the emission probability at all points in the effluent. Additionally, helium does not produce VETs in laser-excited OH; hence, the VET rate coefficients were applied in the same way for the two admixtures. After accounting for collisional quenching and VETs [31, 32], the resulting emission probabilities of OH(A²Σ⁺(ν = 1)) in the He/H₂O effluent are 0.0214 for 315 nm emission and 0.0034 for 309 nm. For the He/O₂ admixture, emission probabilities of 0.0423 at 315 nm and 0.0101 at 309 nm were calculated. Two-dimensional spatial mapping of the OH LIF signal was plotted in Matlab (Mathworks).
Laser induced fluorescence (LIF) measurements of hydroxyl radicals in the effluent of the COST-jet were performed using the same laser as the TALIF measurements, an EKSPLA PL2230 Nd:YAG laser. A slightly lower pulse energy of 9 µJ was used for LIF. A linear relationship between laser power and fluorescence signal, indicative of no interference from laser-induced particle generation, was verified for pulse energies up to 12 µJ. The laser fluorescence was collected using an Andor iStar Gen III ICCD with an A-3 cathode, giving quantum efficiencies of approximately 10% for both fluorescence wavelengths. The ICCD was used along with a 310 nm filter (Andover Corporation, FWHM 10 nm), providing transmittance values of 0.152 at 309 nm and 0.124 at 315 nm. A gate width of 100 ns was used for all LIF measurements, while the acquisition time was varied between the two admixtures to maintain similar intensities of the raw LIF signal on the ICCD. Experimental uncertainty for LIF measurements was estimated to be 30%, owing primarily to the uncertainty in the applicability of collisional quenching rates in the effluent [27]. Variability in the fluorescence signal was considerably lower, generally less than 10% between measurements.

2.3. Liquid diagnostics

2.3.1. Hydrogen peroxide quantification. A xylenol orange based assay (Pierce™ Quantitative Peroxide Assay Kit 23280, Thermo Scientific) was used to detect hydrogen peroxide in 1 ml plasma-treated HPLC grade water, with and without 100 µg ml⁻¹ cysteine present in the solution. Solutions were treated in 12-well plates with the jet nozzle placed 4 mm from the surface of the liquid. Mock treatments were performed by adding hydrogen peroxide (Fisher Scientific, 30% stock solution (v/v) at 8.8 M) in concentrations equivalent to the investigated treatment times to the cysteine solution to determine the consumption of hydrogen peroxide by cysteine. Samples were diluted depending on treatment and left to incubate, with 400 µl of working reagent mixture at room temperature, for 20 min. The absorbance of the dye was detected photometrically at 560 nm through the use of a deuterium-halogen lamp (Ocean Optics DH-2000-BAL) and the Ocean Optics QE Pro spectrometer. Average and standard deviation are plotted in OriginLab.

2.3.2. pH Measurements. pH was monitored for each treatment condition. The cysteine solutions, for both gas admixtures, at 0 min (control), 0.5 min, 1 min, and 5 min had corresponding pH values of 5.5, 4.5, 4.1, and 3.5, respectively. All measurements were taken in triplicate with a pH probe (Hanna Instruments Laboratory Research Grade Benchtop pH/mV/ISE Meter, HI5522-01, probe HI1083B) and have a standard deviation of less than 0.16.

2.3.3. Visualization of atomic O in liquid. The terephthalic acid (TA, 99+%, Acros Organics) assay was used to visualize the transport of species in the liquid. TA is supposed to react selectively with OH to form HTA, which is fluorescent at 425 nm. Although we previously found the TA assay not to be selective in the presence of atomic oxygen [19], the assay is used here only to visualize the transport of the HTA product in the liquid when treated with atomic oxygen-dominated chemistry. Cuvettes were filled, all the way to the top, with 50 mM TA solution and placed at 1 mm gap distance from the COST-jet. Pictures of a He/O₂ plasma treatment were taken in a dark room with a Canon-EOS 6D digital camera (DSLR, 24–105 mm lens) while the cuvette was exposed to 310 nm light coming from a broadband laser-driven light source (Energetiq EQ-99XFC), located at 90° from the camera, and collimated through a fisheye lens. Resulting pictures were cropped and enhanced at 100% sharpness, 25% brightness, and 40% contrast.

2.4. Diagnostics on biological samples

The amino acid cysteine is used as a model biological sample. Here, we use FTIR spectroscopy and mass spectrometry to investigate the chemical modifications occurring on cysteine after plasma treatment.

2.4.1. Sample treatment. L-cysteine was obtained from Alfa Aesar (L-Cysteine A10435, ≥98%). Cysteine was dissolved in either HPLC grade water or heavy water (H₂O, Medical Isotopes O2006, 97%) for a final concentration of 100 µg ml⁻¹. One milliliter of the solutions were transferred into 12-well plates and treated with the COST-jet at 4 mm distance. For both gas admixtures used, gas controls were taken where the cysteine solution was exposed to the respective gas flow for 5 min without igniting the plasma. All samples were treated in triplicate.

2.4.2. Fourier transform infrared spectroscopy. Dried samples were analyzed by FTIR spectroscopy. Aliquots of 20 µl were removed from each 1 ml sample and desiccated for 48 h on transmission FTIR silicon wafer prior to analysis. Spectra were obtained using the LUMOS FTIR microscope (Bruker) and recorded with a spectral resolution of 4 cm⁻¹ with 64 scans per measurement. The spectra were baseline corrected by the rubberband correction method, and normalized through the vector normalization method in the Bruker OPUS software (Bruker Optic GmbH). Background spectra were taken for each silicon wafer before data collection. The experiments were performed in triplicates and for each sample, ten spectra were recorded at different positions and averaged. Standard deviation was calculated and is plotted as shadows Matlab.
2.4.3. Mass spectrometry. 100 µL of each 1 ml sample was transferred to an autosampler vial for analysis by UPLC-MS/MS. The analysis was performed using a Thermo Vanquish LC instrument (Thermo Fisher Scientific, San Jose, CA) coupled to a Thermo Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) with a heated electrospray ionization (HESI) source. Chromatographic separation was achieved on a Waters BEH Amide column (2.1 × 100 mm, 1.8 µM) maintained at 45 °C. The following linear gradient of mobile phase A (H₂O + 0.1% FA) and mobile phase B (MeCN + 0.1% FA) was used: 0–0.1 min (99% B, 0.4 ml min⁻¹), 0.1–7 min (99–30% B, 0.4 ml min⁻¹), 7–10 min (99% B, 0.4 ml min⁻¹). Samples were analyzed (2 µL injections) in positive ion mode (spray voltage 3.5 kV, ion transfer tube temperature 300 °C, vaporizer temperature 350 °C, sheath gas 50 a.u., aux gas 10 a.u., sweep gas 1 a.u.) with a mass range of m/z 60–1000. MS1 data was collected with a resolving power of 60 000 and an AGC target of 1 × 10⁻⁶. MS2 data was collected with a resolving power of 30 000, cycle time of 0.6 s, and stepped HCD collision energy (30, 50, 150).

Raw data files were imported into Skyline [33] for peak picking and integration using a targeted data processing. Peak areas per sample for each target compound were exported from Skyline for figure generation in Origin.

3. Results

3.1. Generation of atomic oxygen and the hydroxyl radical in the gas phase in the presence of a liquid interface

Spatially resolved distributions of OH were recorded in the effluent of the COST-jet using LIF for He/O₂ and He/H₂O admixtures in both an open effluent and in the presence of a water surface. The gap distance was set to 4 mm from the nozzle of the jet to the liquid interface, corresponding to previous liquid phase measurements [19]. In conjunction with absolutely-calibrated TALIF measurements of atomic oxygen under identical conditions [27], a fairly complete picture of the progression of OH and O in the effluent can be attained.

LIF measurements of OH radicals, calibrated for the differing rates of collisional quenching and VETs of laser-excited OH between admixtures, are shown in figure 3 for an unobstructed effluent. Unsurprisingly, the fluorescence signal was more than an order of magnitude higher for the He/H₂O admixture than its He/O₂ counterpart. This is a result of the much higher water concentration in the gas flow (water is only present as an impurity in the He/O₂ case), as H₂O dissociation in the active plasma is the dominant formation mechanism of OH [34]. In addition to the relative fluorescence signal, the spatial distribution of OH in the effluent is similar to that observed for O in previous work [27]. However, without measurements of the decay rates of laser-excited OH(A₂Σ⁺), it is difficult to make definitive statements about the evolution of OH densities throughout the effluent. The fluorescence signal appears to increase out to a few mm in the He/H₂O effluent—a trend also observed for O in the He/H₂O case. Nonetheless, this is likely not a reflection of the OH density distribution but rather the result of differences in the decay rates between the two positions. This assertion is supported by density measurements of OH radicals in a similar APPJ at comparable water admixtures. Benedikt et al used two independent diagnostic techniques, cavity ring-down spectroscopy (CRDS) and molecular beam mass spectrometry (MBMS), which showed no increase in density with increasing distance from the nozzle [15]. Similar to atomic oxygen, the decay rate of laser-excited OH is very sensitive to the background gas composition and a variety of species may contribute to a higher quenching rate close to the nozzle resulting in the lower apparent signal there. A second factor may be the temperature dependence of the collisional quenching of OH(A₂Σ⁺). The gas temperature across the effluent is relatively uniform but highest (around 330 K) at the nozzle before slowly thermalizing towards the effluent edge. Experiments have shown a T⁰·⁵ relationship between the background gas temperature and quenching rate of laser-excited OH, affecting
both the \( \nu = 1 \) and \( \nu = 0 \) vibrational states [31, 35]. As a result, the elevated gas temperature close to the nozzle likely reduces the LIF signal in this region via increased collisional quenching.

The LIF measurements were repeated in the presence of a liquid surface at 4 mm. Figure 4 shows the relative LIF signal along the axis of the effluent plotted with the atomic oxygen densities [27] under the same conditions. Interestingly, the precipitous decline in the LIF signal observed near the water surface mirrors the density trend recorded for atomic oxygen. Although the LIF measurements cannot discern differences in quenching rates, determinations of the decay rates of laser-excited atomic oxygen indicate the background gas composition remains unchanged from the open effluent case for both admixtures [27]. This implies that the decrease in LIF signal corresponds to a lower OH density rather than an artifact of faster decay rates in this region. Furthermore, the similarities between the O and OH density trends near the liquid surface strengthens our previous assertion that this reduction in density is a product of diminished flow velocity. As O and OH are thought to be similarly reactive in the gas phase, a slowing flow rate should manifest as a proportional decrease in density.

3.2. Transport of atomic oxygen and the hydroxyl radical in the liquid phase

Figure 4. OH LIF signal in He/O\(_2\) (red) and He/H\(_2\)O (blue) plasma effluents with a water surface present at 4 mm. Atomic O density detected through TALIF is overlayed for comparison. Reproduced from [27]. © IOP Publishing Ltd. CC BY 3.0.

Measurements of OH and O in the gas phase are critical for understanding the delivery of these reactive species to plasma-treated liquid. The importance of using gas phase measurements to contextualize those performed in the liquid is amplified by the numerous shortcomings of liquid-phase diagnostics, especially in the presence of atomic O. These include a lack of selectivity when both OH and O are present [9, 19], the propensity of atomic oxygen to degrade chemical probes [8, 36], and an absence of aqueous phase reaction rate data for O [37].

Table 1 shows a comparison of OH and O fluxes measured at the liquid interface using gas phase densities obtained by LIF (OH) and TALIF (O) [27] along with liquid diagnostics for H\(_2\)O\(_2\) in DI water, which is mainly formed through OH recombination, and TEMPO, a product of the EPR spin trap TEMP with atomic oxygen [19]. The ratios between He/O\(_2\) and He/H\(_2\)O for the O flux to the liquid and TEMPO formation are in very good agreement. Discrepancies of a factor of 4.2 between the ratios of relative OH flux to the liquid and H\(_2\)O\(_2\) concentration in the liquid for He/O\(_2\) and He/H\(_2\)O can be observed. This is most likely due to the abundance of atomic oxygen in the He/O\(_2\) discharge with consecutive transport into the liquid, as visualized in figure 5.

The solvation of atomic O leads to efficient reactions between O\(_{aq}\) and H\(_2\)O\(_2\) via the reaction (\( k = 1.6 \times 10^9 \)) [38]:

\[
\text{H}_2\text{O}_2 + \text{O} (^{1}\text{P}) \rightarrow \text{OH} + \text{HO}_2
\]

which reduces the H\(_2\)O\(_2\) concentration in He/O\(_2\) plasma-treated water.

Taking a closer look at the H\(_2\)O\(_2\) concentrations for both gas admixtures, it has been shown previously that H\(_2\)O\(_2\) forms to a large extent in the gas phase for the He/H\(_2\)O gas admixture, while it is formed primarily in the liquid for the He/O\(_2\) gas admixture [19]. This has important implications when biological samples are treated as the precursors to form H\(_2\)O\(_2\) (OH, HO\(_2\)) are typically more reactive than H\(_2\)O\(_2\) itself. One possible pathway of H\(_2\)O\(_2\) formation in He/O\(_2\)-treated liquid containing organic molecules is the H abstraction by atomic oxygen [19] so that the organic molecules in the liquid become part and may play a crucial role in the liquid phase chemistry. Previous research has shown that H\(_2\)O\(_2\) production in DI water compared to a solution of the spin trap DMPO and a TA solution remains the same for He/H\(_2\)O, but differs significantly for He/O\(_2\) plasma-treated solutions [19]. An excess H\(_2\)O\(_2\) production in the He/O\(_2\) case has also been reported for phenol solutions [8]. It was postulated that O abstracted hydrogen atoms reacted with aqueous O\(_2\) to produce HO\(_2\) radicals (\( k = 10^{10} \text{M}^{-1}\text{s}^{-1} \)) [8, 19]. The HO\(_2\) radicals can subsequently self-react to form H\(_2\)O\(_2\) (\( k = 10^6–10^7 \text{M}^{-1}\text{s}^{-1} \)) [39] or react with H to form H\(_2\)O\(_2\) (\( k = 10^{10} \text{M}^{-1}\text{s}^{-1} \)) [40].

In order to understand the possible biological implications of O and OH in the aqueous phase, cysteine was used as a model substrate later in this publication. However, given that H abstraction could occur on cysteine from atomic oxygen leading to increases in H\(_2\)O\(_2\) concentrations, H\(_2\)O\(_2\) measurements were taken for He/O\(_2\) and He/H\(_2\)O plasma-treated DI water and cysteine solutions, depicted in figure 6, to elucidate any additional reaction pathways resulting in cysteine modifications. Furthermore, the concentration of H\(_2\)O\(_2\) alone
corresponding to the \( H_2O_2 \) concentrations produced in a He/H\(_2\)O discharge at each treatment time was investigated to check if \( H_2O_2 \) is consumed by cysteine in the liquid. Finally, the pH of the treated cysteine solutions were similar across both admixtures, indicating that the pH did not contribute to the difference in \( H_2O_2 \) concentrations.

For 0.5 min and 1 min He/H\(_2\)O treatment, the DI water and mock-treated cysteine solutions show good agreement. For 5 min treatment time, the \( H_2O_2 \) concentrations differ significantly (note the axis breaks in figure 6). While this has not been observed for phenol [8] or DMPO and TA solutions [19], it indicates that cysteine indeed consumes \( H_2O_2 \), after an initial reaction with more reactive species had to occur since the \( H_2O_2 \) mock treatment does not show a decreased \( H_2O_2 \) concentration, which will be discussed in more detail later in this publication. In case of He/O\(_2\) plasma-treated solutions, the \( H_2O_2 \) concentrations are much lower, as expected (note the axis break). Surprisingly, the \( H_2O_2 \) concentration is higher in the DI water case and lower for the cysteine solution. This indicates that cysteine indeed consumes \( H_2O_2 \), as shown in previous work by using MD simulations due to the lack of available timescales than the 10 ps studied [19].

Note that \( H_2O_2 \) was not included in the MD simulations in previous work, as the reactions occur on significantly longer timescales than the 10 ps studied [3]. Atomic oxygen could not be included in the MD simulations due to the lack of available rate constants.

Figure 8 depicts FTIR spectra of cysteine treated in aqueous solution for 1 min with He/O\(_2\) plasma, He/H\(_2\)O plasma, and H\(_2\)O\(_2\) in the same concentration as produced in the He/H\(_2\)O plasma settings after 1 min treatment time. As control, the gas control for He/O\(_2\) is shown representative for the control substrate cysteine were investigated by means of FTIR spectroscopy and mass spectrometry. Several common and unique modifications were observed, as described in the following. Figure 7 depicts the structures of the native cysteine together with the most important modifications observed after plasma treatment. As shown in previous work by using MD simulations, one initial reaction is the H-abstraction from the thiol group (-SH) by OH with a probability of 30%, yielding a reactive sulfur [3]. From there, two reactive Cys(-H) can combine to form cystine. Another pathway is the addition of OH to the native cysteine with a probability of 50%, yielding Cys-SOH\(_2\) as intermediate. Upon further reaction with OH, \( O_3 \), or \( O_3 \), the transient cysteine sulfenic acid is formed, followed by the higher oxidation states cysteine sulfinic and sulfonic acid. Note that \( H_2O_2 \) was not included in the MD simulations in previous work, as the reactions occur on significantly longer timescales than the 10 ps studied [3]. Atomic oxygen could not be included in the MD simulations due to the lack of available rate constants.
Figure 7. Structures of the unmodified cysteine and the most prominent modifications observed after plasma treatment. Cysteine can oxidize to the intermediates cysteine sulfenic acid and cysteine sulfinic acid, with the last oxidation stage being cysteine sulfonic acid. Once the H-abstraction from the thiol (-SH) group occurred, cystine can form. Different pathways can lead to cysteine-S-sulfonate formation (see text).

Figure 8. FTIR spectra of cysteine after 1 min treatment with He/O$_2$, He/H$_2$O, and H$_2$O$_2$ in the same concentration as produced in the He/H$_2$O plasma settings. As control, the gas control for He/O$_2$ is shown representative for the control spectra taken. He/O$_2$ plasma treatment of the cysteine solution leads to a general loss in signal. While the SH bending signal decreases, S=O stretching and SO$_2$ stretching signals appear. For He/H$_2$O treated cysteine, the SH bending signal vanishes, S=O stretching and SO$_2$ stretching signals appear, as well as a distinct N–O stretching. A general impact on the cysteine backbone structure could be observed as well. The signals between 1300 cm$^{-1}$ and 1650 cm$^{-1}$ which correspond to the amino, carboxyl, and α-C regions were sharp and well defined in the controls and became less defined after plasma treatment. In case of the He/O$_2$ treatment, a distinct loss in the -SH bending signal and a distinct increase of the S=O stretching with a general loss of signal and definition of the bands can be observed. On the other hand, He/H$_2$O treated samples show several peaks and structures in addition, as well as a larger standard deviation for the treated samples, indicating a variety of modifications occurring upon exposure to the reactive species. Compared to the H$_2$O$_2$ mock treatment, where cysteine was exposed to the same concentration of hydrogen peroxide as produced by the He/H$_2$O plasma treatment after 1 min, the SH bending signal vanishes and the S=O stretching and SO$_2$ stretching signals appear in a similar manner. The standard deviation in the
He/H\textsubscript{2}O plasma-treated samples is larger, in particular in the backbone region indicated by the signals between 1300 cm\textsuperscript{-1} and 1650 cm\textsuperscript{-1} which correspond to the amino, carboxyl, and \(\alpha\)-C regions. At the same time, the rise of the N–O stretching signal at 848 cm\textsuperscript{-1} and the N=O stretching at 1490 cm\textsuperscript{-1} may indicate the formation of S-nitrosothiols or oxidation of the amino region \[21, 41\].

Mass spectrometry (figure 9) revealed that the native cysteine (Cys-SH) disappears completely after 5 min He/H\textsubscript{2}O treatment and is still present, if drastically reduced, after 5 min He/O\textsubscript{2} treatment.

The primary modification from He/H\textsubscript{2}O treatment is the formation of cystine (Cys-S-S-Cys), whereas He/O\textsubscript{2} treatment results in oxidation to sulfinic (Cys-SO\textsubscript{2}H) and sulfonic (Cys-SO\textsubscript{3}H) acids. This is supported by the FTIR spectra, which show S=O stretching for He/O\textsubscript{2} plasma treatment. Due to its chemical instability, no traces of sulfenic acid (Cys-SOH) could be detected. Cysteine-S-sulfonate (Cys-S-SO\textsubscript{3}H) is produced to a greater extent in the He/O\textsubscript{2} discharge. Wende \textit{et al} reported the production of cysteine-S-sulfonate, sulfate, sulfide in He/O\textsubscript{2} (COST-jet) and Ar/O\textsubscript{2} (kINPen) \[7\], with a preference for production under the He/O\textsubscript{2} COST-jet conditions. Different pathways can lead to the production of cysteine-S-sulfonate, discussed in more detail in the next section. As mentioned above, the initial reaction is either the H abstraction of (30% probability) or OH addition (50% probability) to the thiol moiety of cysteine \[3\], as observed with MD simulations. The MD simulations did not include H\textsubscript{2}O\textsubscript{2} and O. Assuming that the initial reaction indeed is caused by OH \((k = 1.9 \times 10^{10} \text{M}^{-1}\text{s}^{-1})\), the consecutive reactions must defer between He/O\textsubscript{2} and He/H\textsubscript{2}O conditions. The H abstraction yields a reactive sulfur which can form cystine as observed to a large extent for the He/H\textsubscript{2}O case, or be further oxidized to form sulfenic, sulfonic, and ultimately sulfonic acid, the major modifications observed with He/O\textsubscript{2}. An alternative pathway that was not included in MD simulations is the H addition to cysteine, yielding a reactive sulfur radical as well:

\[
H + \text{Cys-SH} \rightarrow \text{Cys-S}^- + H_2. \tag{2}
\]

There are no rate constants available for this process, thus it is difficult to determine if this process plays a major role in the initiation of the reactions. As a significant amount of H is produced in the He/H\textsubscript{2}O discharge and transported into the liquid \([18, 20]\) it may contribute to the production of cystine in the He/H\textsubscript{2}O case. To further elucidate the reaction pathways, cysteine was treated in heavy water H\textsubscript{2}\textsuperscript{18}O to determine the origin of species responsible for the observed modifications.

3.4. \textit{The origin of species}

Heavy water H\textsubscript{2}\textsuperscript{18}O was used for cysteine solutions to determine the origin of species responsible for plasma-induced modifications on cysteine. The graph shows ratios of oxidative modifications occurring on cysteine after 0.5 min, 1 min, and 5 min treatment with He/H\textsubscript{2}O (top) or He/O\textsubscript{2} (bottom), normalized to 1 for the total oxygen contribution for each molecule. The incorporation of liquid-derived \textsuperscript{18}O into Cys-SO\textsubscript{2}H, Cys-SO\textsubscript{3}H and Cys-S-SO\textsubscript{3}H indicates that reactive species are produced in the liquid phase. It is apparent that Cys-SO\textsubscript{2}H and Cys-SO\textsubscript{3}H are produced to a greater extent by ROS created in the gas phase, while Cys-S-SO\textsubscript{3}H shows the incorporation of liquid-derived oxygen for both gas admixtures. This is in good agreement with a study performed by Wende \textit{et al} \[7\] for the kinpen in comparison to a He/O\textsubscript{2} jet treatment.

In the case of 1 min, He/O\textsubscript{2} treatment, 72.4% and 65.7% of the in Cys-SO\textsubscript{2}H and Cys-SO\textsubscript{3}H incorporated oxygen originates in the gas phase. Our results indicate a slightly higher...
percentage of gas phase oxygen compared to Wende et al. [7] which could be explained by the difference in oxygen admixture in the feed gas (0.6% O₂, which has been shown to produce the highest concentration of atomic oxygen [43] compared to 1% O₂ in [7]), thus a higher flux of atomic oxygen generated in the gas phase is entering the liquid. For 1 min, He/H₂O, only 42% and 54% of the in Cys-SO₂H and Cys-SO₂H incorporated oxygen originates in the gas phase, thus the ratio of liquid phase and gas phase oxygen is closer to 1:1. For the 5 min treatment time, 33% and 62% of the in Cys-SO₂H and Cys-SO₂H incorporated oxygen originates in the gas phase for He/O₂ and only 14% and 52% in the case of He/H₂O. After 5 min treatment time, the ratios of 16O to 18O for Cys-SO₂H remain relatively constant for both gas admixtures while the contributions of 18O rise significantly in Cys-SO₂H for both gas admixtures (He/H₂O: 14% originate in gas phase, He/O₂: 33% originate in gas phase).

Cysteine-S-sulfonate shows a high levels of 18O incorporation for both gas admixtures, with the ratios of contribution being relatively similar for all time points investigated. To compare the two gas admixtures in more detail, at 1 min treatment time Cys-S-SO₂H in the He/H₂O case incorporated 11% gaseous 16O, 14% were detected with one 18O, 22% with two 18O, and 50% with 16O. For He/O₂, only 7% gaseous 16O were detected (compared to 9% in [7]), 10% were detected with one 18O, 8% with two 18O, and 82% with three 18O. There are several pathways to create Cys-S-SO₂H, as described and discussed in detail by Wende et al. [7]. Wende et al. concluded that Cys-S-SO₂H is produced in the bulk liquid by OH generated from the liquid following reaction (5), see below.

There are several possible pathways for the production of 18O in heavy water prior to further reaction with cysteine:

\[
\text{H}_2^{18}\text{O} + h\nu/\text{He}^* \rightarrow \text{OH} + \text{H} \quad (3)
\]

\[
\text{H}_2^{18}\text{O} + h\nu \rightarrow \text{O}^{(3}\text{P}) + \text{H} + \text{H} \quad (E_{\text{th}} = 9.513 \text{ eV}) \quad (4)
\]

\[
\text{H}_2^{18}\text{O} +^{16}\text{O}_{\text{gas}} \rightarrow ^{18}\text{OH} + ^{16}\text{OH}. \quad (5)
\]

Reaction (3) describes water cleavage by the impact of photons or metastables. He metastables are unlikely to reach the water interface 4 mm from the active plasma region when molecular gas admixtures are used, as He metastables are easily quenched [44]. As water absorbs radiation below 200 nm [45], VUV photons would be required for this reaction. Hefny et al. reported that the addition of just 100 ppm of any molecular gas quenches the He excimer emission and concluded that the effect of energetic photons is negligible [8]. Radiances of atomic oxygen lines (115 nm and 130 nm) have been measured at a distance of 4 mm in He/O₂ (0.6%) in controlled helium atmosphere [46]. The 10 ìW mm⁻² sr⁻¹ reported present thus an upper limit for our open air effluent in contact with a liquid interface. The three-body dissociation reaction (4) has a threshold energy of 9.513 eV which corresponds to the atomic oxygen line emission at 130.2 nm (9.5 eV). Further atomic oxygen lines (94.9 nm (13 eV), 98.9 nm (12.5 eV), 115.2 nm (10.72 eV)) as well as atomic hydrogen lines were recently reported for the COST-jet for He/O₂ in controlled helium atmosphere by Golda et al. [47], again providing an upper limit due to the open atmosphere and the liquid interface used in this study. Nonetheless, liquid water has a local energy absorption cross section maximum at 9.8 eV (σ = 8 × 10⁻¹⁸ cm²/molecules), increasing to about σ = 20 × 10⁻¹⁸ cm²/molecules for 13 eV [48]. Although the number of VUV photons reaching the liquid interface is most likely low under the experimental conditions used, it may be enough to initiate reactions in the water by hydrolysis. Reaction (5) has been suggested as another potential reactant for water cleavage [9, 49], but in conditions with the highest atomic O density (He/O₂ compared to He/H₂O), the lowest inclusion of 18O was observed. This aligns well with the results presented in [7]. Protonated water clusters H⁺(H₂O)ₙ have been shown to reach distances of up to 10 mm, with a preference for heavier clusters (n = 6–8) further away from...
the active plasma [50]. Ions are recombining quickly in the effluent and the expected densities at 4 mm distance are low [50] but their impact can not be ruled out. Another possibility would be that gaseous $^{16}\text{OH}$ enters the liquid and undergoes H transfer with $\text{H}_2\text{O}_2$ [51, 52], so that gas phase $^{18}\text{OH}$ would be able to create liquid phase $^{18}\text{OH}_2$.

Once $^{18}\text{OH}$ or $^{18}\text{O}$ has been created, they can modify cysteine directly or undergo a variety of liquid reactions forming other reactive species, e.g. $\text{H}_2^{18}\text{O}^{16}\text{O}$, $\text{H}^{18}\text{O}^{16}\text{O}$, thus transporting and accumulating $^{18}\text{O}$ in reactive species.

4. Discussion

The data taken together depict the outcome of species generated in the gas in the presence of a liquid interface, their delivery into the liquid phase, reactions with molecules in the liquid and ability to modify a model substrate. The plasma-generated OH from He/$\text{H}_2\text{O}$ was shown to mainly form $\text{H}_2\text{O}_2$ in the gas phase prior to solvating in the liquid phase, whereas the He/$\text{O}_2$ admixture generated O which crosses the liquid interface and requires a substrate in the liquid for $\text{H}_2\text{O}_2$ production through H abstraction [9, 19]. Regardless of where the $\text{H}_2\text{O}_2$ is produced, an initiating reaction from H, O or OH is required for the modification of cysteine [3], as evidenced by the unaltered differences (sulfinic acid, sulfonic acid) $\cdot \text{Cysteine-S-sulfonate}$, which can also be formed by the attack of $\cdot \text{Cysteine-S-sulfonate}$, which can also be formed by the attack of $\cdot $

\[
\text{O} + \text{H}_2\text{O}_2 \rightarrow \text{HO}_2^- + \text{OH}^-(k = 1.6 \times 10^9) \quad (6)
\]

\[
\text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{HO}_2^- + \text{H}_2\text{O} \quad (k = 2.7 \times 10^7) \quad (7)
\]

can produce the hydroperoxyl radical. The production of the hydroperoxyl radical is very efficient in the He/$\text{O}_2$ case with the $\text{H}_2\text{O}_2$ concentration being the rate-limiting factor. In case of the He/$\text{H}_2\text{O}$ case, the rate constant is two orders of magnitude lower but there is an abundance of $\text{H}_2\text{O}_2$ and OH present in the liquid. In addition, as mentioned above, HO$_2$ is produced in the gas phase and transported into the liquid. The backbone modifications observed could thus be explained by the production of hydroperoxyl radicals which are able to attack the amino acid backbone, leading eventually to backbone fragmentation.

The $\text{H}_2\text{O}_2$ measurements give further insights into the reaction pathways. In case of the He/$\text{H}_2\text{O}$ plasma treatment, the $\text{H}_2\text{O}_2$ concentrations are comparable in DI water and cysteine solutions for 1 min treatment time and significantly lower in the cysteine solution after 5 min treatment time. In previous studies, TA and DMPO solutions did not alter the $\text{H}_2\text{O}_2$ concentrations in He/$\text{H}_2\text{O}$ treated solutions [19]. As most of the hydrogen peroxide is already generated in the gas phase, this indicates that cysteine reduces $\text{H}_2\text{O}_2$. Compared to the $\text{H}_2\text{O}_2$ mock treatment, where no reduction of $\text{H}_2\text{O}_2$ was observed, it indicates that an initial reaction has to be performed with a more reactive species generated in the plasma or the liquid.
e.g. O, OH, or H. While it has been observed for ring structures [9, 19] that H$_2$O$_2$ is produced in He/O$_2$ plasma-treated solutions in the presence of organic matter by H abstraction, in case of cysteine solutions the H$_2$O$_2$ was observed to be higher in the DI water case, indicating that no additional H$_2$O$_2$ is produced by H abstraction. In fact, the H$_2$O$_2$ concentration is lower when cysteine is present, implying that cysteine reduces H$_2$O$_2$ or reacts with precursors before they can form H$_2$O$_2$. This confirms the previously performed MD simulations [3], in which it was predicted that H abstraction is not the major initial step for cysteine modifications. Instead, the addition of OH was found to be the more prevalent initial modification. There is no data available if O would add in a similar manner to cysteine. Since OH can be produced in He/O$_2$-treated liquids via reaction (1) as well, it is not possible to identify the initial reaction with the data available.

The observed modifications to cysteine show that both admixtures effectively alter the structure of the model substrate. For biological applications of non-thermal plasma, the resulting effects from treatment will depend on how the structures are modified and if these modifications are reversible. Typically, plasma treatments degrade proteins and chemical probes [9, 19, 59], however Krewing et al could show reversible activation of oxidized cysteine residues [59]. In combination with the results presented in this paper, this suggests that the resultant plasma-induced modifications, whether it is activation or degradation, could be managed at the plasma parameter input level. An efficient oxidation method for a biological target may be desired, however the overoxidation of these targets may be outside of the preferred outcome and occur on time scales faster than those of biological responses. For these reasons, a tailored approach for the delivery of plasma-generated species in biological applications must consider the generation of these species in the gas and liquid phase, the flux of the species across the gas–liquid interface, and the resulting chemistry and modification to the biological target.

5. Conclusions

He/O$_2$ (0.6%) and He/H$_2$O (0.25%) gas admixtures in the COST atmospheric pressure plasma jet were used to study the generation and transport of O and OH from the gas phase to the liquid to the biological model target cysteine. The COST-jet is an efficient source of O in He/O$_2$ gas admixtures and an efficient source of OH/H$_2$O$_2$ in the He/H$_2$O gas admixture. By using heavy water (H$^{18}$O) and mass spectrometry, the impact of liquid-derived reactive species was studied. The liquid chemistry in He/H$_2$O discharges appears more complex as the incorporated $^{18}$O was found to be higher in He/H$_2$O treated cysteine. Taken together with FTIR analysis of cysteine after treatment, it can be concluded that an initial reaction with plasma-derived OH, O, or H must take place before further reactions follow. The reaction pathways differ significantly for He/O$_2$ and He/H$_2$O; while He/O$_2$ mainly targets the sulfur moiety of cysteine for oxidation up to irreversible oxidation states, He/H$_2$O treatment leads preferentially to reversible oxidation products. This highlights that atomic oxygen is unknown to biological systems, while H$_2$O$_2$ and OH are physiologically relevant ROS. The more O or OH/H$_2$O$_2$ dominated ROS produced by the two gas admixtures studied offers the possibility to select species for target modification. By tailoring where the species are produced and how many initiating reaction molecules are required will determine the approach for biological targets or agricultural applications.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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