Mass spectrometric analysis of non-enzymatic post-translational modifications introduced by cold plasma-derived reactive species in peptides and proteins

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List of Abbreviations

CAP .... cold atmospheric-pressure plasma
DNPH .... 2,4-dinitrophenyl hydrazine
Ar .... argon
He .... helium
EPR................... electron paramagnetic resonance
ECM .... extracellular matrix
HPLC .... high-pressure liquid chromatography
ESI .... electrospray ionization
ELISA............... enzyme-linked immunosorbent assay
PD.................... proteome discoverer
SILAC.............. stable isotope labeling with amino acids in cell culture
AQUA .... absolute quantification
LFQ .... label-free quantitation
HR .... high resolution
HIF1α.............. hypoxia-inducible factor 1-alpha
MALDI............. matrix-assisted laser desorption ionization
MD.................... molecular dynamic
MMP................ matrix metalloproteases
MS .... mass spectrometry
MS/MS............. tandem mass spectrometry
oxPTM .... non-enzymatic oxidative modifications
PLA_2 .... phospholipase A2
RNS .... reactive nitrogen species
ROS .... reactive oxygen species
RSS .... reactive sulfur species
slm................... standard liter per minute
3-Tyr-NO......... nitrosotyrosine
VUV .... vacuum ultraviolet
VHL.................. von-hippel-lindau
TALIF .... two-photon absorption laser-induced fluorescence
3-Tyr-NO_2 .... 3-nitrotyrosine

In addition to the abbreviations mentioned so far, the one and three-letter codes for proteinogenic amino acids and for the SI units were used.

Objectives & Outline

In the context of newly proposed biomedical tools, the objective of this thesis is to contribute to understand the mechanisms of action and the biological effects of cold atmospheric-pressure plasmas (CAPs). In this work, the applied strategy consisted in characterizing non-enzymatic oxidative modifications, occurring after various plasma treatments in proteins, peptides, and amino acids, developing and applying mass spectrometry-based methods. The main hypothesis is that CAPs can modulate downstream redox signaling pathways by changing the molecular structure of key biomolecules, like proteins and lipids.

The specific objectives are i) to identify all the potential oxidative modifications introduced by cold plasmas in proteins; ii) to compare and characterize the chemistry induced by different plasma sources and treatment parameters; iii) to improve the controllability of the treatments and their effects by identifying main variables; iv) to correlate results obtained from model solutions of peptides and proteins to those obtained treating complex matrices. The thesis contributes to the resolution of these objectives, finally supporting the overall understanding of cold plasmas biological effectiveness, and the possibility to tune plasma sources for targeted biomedical applications in future. In particular, objectives i), ii), and iii) are covered in Section 2.1, while objective iv) is investigated in Section 2.2.

The thesis consists in an introduction, major results obtained from 5 articles published in peer-reviewed journals, a conclusion and outlook section, and a summary. Regarding the results, Articles 1 and 2 (A1 and A2) investigated which type of oxidative modifications can be driven by cold plasmas in model peptides solutions. Articles 3, 4, and 5 (A3, A4, and A5) applied the model systems gained knowledge to plasma treatments of more complex models, such as protein solutions (A3), and epidermal tissues (A4 and A5). **Article 1** examined which modifications could be introduced by plasmas on two peptides (an angiotensin derivate and bradykinin), additionally comparing the produced experimental data with in silico data obtained by previous works. Here, the differential chemistry induced by varying plasma source and treatment conditions have been discussed. **Article 2** deepened and extended this to a library of peptides to compare the amino acids reactivities also based on their position in the peptide primary structure. In both articles, a classification of the reactivity of all proteinogenic amino acids towards plasma sources and the identification of key responsible plasma components, such as reactive species, have been investigated, resulting in a modulation of the effects in relation to variables (e.g., modulating source-design or gas composition).

These findings have been used and applied from model peptide solutions to more complex environments, such as intact protein solutions (**Article 3**), and in the proteome of wound exudates and skin tissues, as investigated respectively in **Article 4** and **Article 5**. An overview of the biological impact on proteins was given also for complex matrices, reflecting the various mechanism of action of plasma components in presence and in absence of a water vehicle. The results were finally discussed in the conclusion and outlook section, where optimized tunable conditions for plasma treatments have been associated to defined proteins effects and proposed for adjusting plasma conditions in relation to the biomedical aim. Finally, still unsolved criticalities in applying cold plasmas in clinical settings have been discussed.

1. Introduction & Motivation

1.1. Current applications and challenges in plasma medicine

Plasma medicine is 25 years since its first application [1, 2], a relatively young medical field. However, the use of cold atmospheric-pressure plasmas has undergone significant progress in recent years. After the realization that this form of plasma could be useful for biological applications, various progress has been made in the field of decontamination [3, 4], dentistry [5, 6], chronic wound healing in both animal model and clinical studies [7-12], and more recently in cancer remediation [13, 14], indicating that cold plasmas can be used in many different research areas. Plasma, which is in the 4th state of matter, is formed when the main gas, for example, the noble gases argon or helium, is provided with energy. Finally, the main gas gets ionized, releasing free electrons, thus forming ions, excited metastable molecules, and other chemical species. In addition to physical components, such as radiation from the vacuum ultraviolet (VUV) to the infrared region, visible light, electromagnetic fields, and thermal radiation, cold atmospheric-pressure plasmas (CAPs) consist of most importantly, a varying type and number of reactive oxygen and nitrogen species, including radicals and neutral or charged species (Figure 1.1) [15]. CAPs differ from thermal plasmas as they are generated by applying electrical fields, resulting in a low ionization degree of the gas, with generation of a rich gas-phase chemistries rather than increase in the amount of hot electrons [16]. Indeed, the temperature of CAPs is between 300 and 370 Kelvin, allowing their usage on mammalian tissues, where temperatures above 310 Kelvin should not be exceeded.

These unique properties make CAPs suitable tools to possibly manipulate biological processes by the exogenous delivery of reactive components, and therefore they are currently proposed for various biomedical applications, such as the treatment of chronic wounds [17] or cancer [14]. In clinical applications, cold plasmas are already used for treating head and neck cancer patients [18, 19]. Here, the bacterial load was reduced due to plasma treatments, in addition to tumor regression and accelerated healing of the wound.
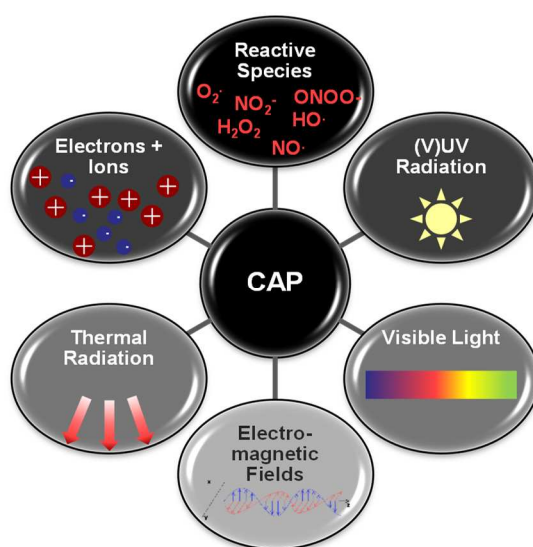

Figure 1.1. Overview of CAP components. CAPs consist of different components, such as reactive species, VUV radiation, visible light, electromagnetic fields, thermal radiation, electrons, ions, radicals, and neutral species.

For the use in medical applications, it is crucial to prove that plasmas do not pose any risk [20, 21] and that the application is beneficial for the patient. One plasma source that has received approval as a certified medical device is the argon-driven plasma jet kINPen MED [22]. This plasma source consists of a pin-type electrode, which is housed in a dielectric ceramic tube (Figure 1.2B). The effluent of the kINPen plasmas forms, among other reactive components, excited argon molecules which can react, due to the turbulent flow, with ambient air molecules (e.g, H_2O, N_2, and O_2), generating secondary gas species, e.g. short-lived atomic oxygen ($^\bullet O$), singlet oxygen ($^1O_2$), hydroxyl radicals ($^\bullet OH$) and nitrogen oxide radicals ($^\bullet NO$) [23-25]. Due to the relatively long distance of the kINPen effluent to the target (9-10 mm), more stable long-lived species can be produced, such as ozone (O_3), hydrogen peroxide (H_2O_2), or nitrate (NO_3^-) and nitrite (NO_2^-) in the liquid phase[26-28]. The gaseous chemistry of the kINPen 09 has been investigated by varying conditions related to the gas composition, e.g., in presence of gas admixtures (with a maximum of 1% O_2 and/or N_2). Dominant oxygen chemistry in short (mostly $^\bullet O$ and $^1O_2$) and long distances (mostly O_3) has been observed by admixing O_2 to the working gas [26, 27, 29]. Typically, this jet is operated with 3 to 5 standard liters per minute (slm). With these parameters, the kINPen is distinguished from the European COST reference micro-plasma MP1101 jet or in short COST-Jet (Figure 1.2A) [30], which has been developed as a standard device to compare other cold physical plasma sources and to allow comparability of results gained by different plasma medicine science groups.

Although this device is not currently certified as a medical tool, it is used as a reference plasma source. In particular, $^\bullet O$ can be produced until the target surface, due to the short distances of the effluent. In contrast to the kINPen, this plasma source is operated with 1.0 slm helium gas and the distance to the target is significantly shorter (2-4 mm). These differences, based on another source design and the use of helium as a driven gas, generate plasmas differentially, leading to a significantly altered plasma chemistry and thus cocktails of plasma components, such as reactive species [23]. The presented plasma sources are those mainly investigated in this thesis.
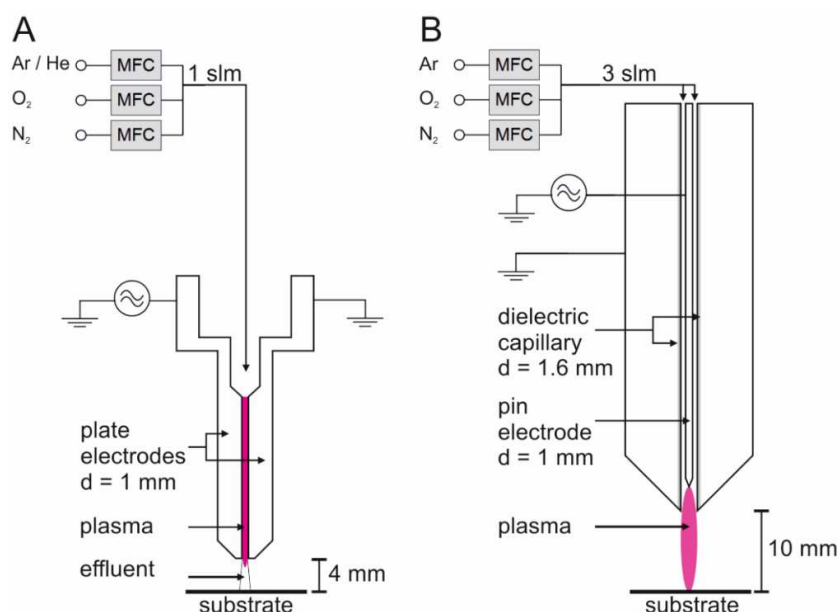

Figure 1.2. Schematics of the COST-Jet (A) and kINPen (B). MFC: mass flow controller, slm: standard liter per minute. *Illustration adapted with permission according to* [31], https://creativecommons.org/licenses/by/4.0/.

These differences may lead to variable medical effects of plasmas, for example in biological modifications occurring on cell proteins. Further studies are required to standardize and optimize the selectivity of biological effects induced by controlled plasma chemistries.

Even though CAP consists of many different components, reactive oxygen (ROS) and nitrogen (RNS) species are probably the most relevant actors, besides H_2O_2, in plasma medical effects [32-38]. These effects can be potentially a source for several oxidative modifications of cell biomolecules and are therefore presumably involved in modulating redox signaling pathways. To date, the complex gas phase, liquid phase, and interphase chemistry between these two is still under investigation. In particular, it has not been fully characterized, which is the amount and type of short-lived (nanoseconds to milliseconds) or long-lived species (seconds to days) [39] generated especially in liquid compartments surrounding biological cell structures. It becomes even more sophisticated as species that are well studied in the gas phase can differ significantly in the plasma-liquid interphase and in the aqueous phase [40].

Similarly, new species can be formed from gaseous precursor molecules or by following other chemical reaction pathways. As an example, $\cdot$NO can be produced in the gas phase, interphase, and liquid phase. In the gas phase, it is formed by the reaction of oxygen (O_2) with a nitrogen radical ($\cdot$N). In the interphase, it can be generated by the reaction of $\cdot$O and $\cdot$NO$_2$, while in the liquid phase, among other possible mechanisms, by the reaction of O_3 with $\cdot$NO$_2$ [41]. To date, more than 25 different reactive species have been identified in the gas and liquid phase in combination with plasma treatments (e.g. HO_2, H_2O_2, OH, O_3, $^1O_2$, NO_2^-, NO_3^-, $ONOOH$, O_2^-) [36], which can potentially react with each other [42], or with target biomolecules, such as proteins and lipids, in a variety of ways [43, 44]. However, the type and amount of these reactive species can also be altered by changing certain plasma parameters, such as treatment time, gas plasma composition [25, 29], solvent, or distance to the treated object [45, 46]. To date, most of the literature focused on the investigation of reactive species in the gas phase due to the detection methods like two-photon absorption laser-induced fluorescence (TALIF) spectroscopy, which allows even absolute quantification of some species like atomic oxygen [26, 47]. Since most processes relevant for biological effects take place in the liquid phase, it is important to gain a deeper knowledge on the characterization of the plasma-stimulated liquid phase and the interphase. Some of these informations have been so far obtained using dynamic simulations [48, 49]. In contrast to short-lived species, long-lived species like H_2O_2 [50], NO_2^- or NO_3^- are relatively easy to measure via colorimetric assays [51].

To detect short-lived species in plasma-stimulated liquids, such as highly reactive radicals, more sophisticated methods are needed. Electron paramagnetic resonance (EPR) spectroscopy is an example [52] which has been used especially to detect ozone, singlet oxygen [53], and nitrogen oxide radicals [41] in treatments with kINPen 09 (a precursor of the kINPen MED) and other plasma sources [54]. However, a direct quantitative detection, especially of extremely short-lived species, is only partially achievable using EPR or other optical spectrometric methods, as they do not produce absolute quantitative results [24].

Different assays exist for the detection of species. As an example, the highly reactive and biologically relevant peroxynitrite ($ONOO^-$) can be detected by using the luminescence of a lanthanide probe [55]. However, knowledge about the mode of action of a set of species for a given condition must be further investigated to standardize biomedical processes, especially in liquid. Therefore, alternative strategies are needed to enable fast and complete detection of the overall chemical reactions and biological effects of plasma sources and treatment

conditions. One possibility to characterize the plasma liquid chemistry of diverse treatment conditions and plasma sources is to investigate the reacting species in liquid by monitoring modifications occurring in proteins, peptides, or amino acids. The detection of these modifications can be carried out by using a wide variety of methods. Spectrophotometric methods are mainly used for the detection of protein carbonylation, using 2,4-dinitrophenylhydrazine (DNPH) as a reagent reacting with the protein in the analysis [56].

Fluorescence measurements of oxidized amino acids have become established in many laboratories, as this is a very robust method with good sensitivity and just small instrumentations are required [57]. However, the disadvantage is that the selectivity of this method is low compared to other methods. Immunochemical methods such as immunoblotting or ELISA represent another large group of techniques for the detection of oxidative modifications using antibodies [58]. However, it is necessary to overcome different problems with these methods.

For example, the exact epitope, which can be bound by the antibody, is often not known; additionally, most antibodies detect more than one oxidation product. Finally, absolute quantifications are difficult to achieve. Thus, the gold standard for the determination of modified amino acids in peptides or proteins is mass spectrometry (MS) [59-61]. This technique can be either coupled with high-pressure liquid chromatography (HPLC) or carried out via direct infusion of the sample. The proteomics MS analysis is generally made by two approaches, called top-down and bottom-up [62, 63]. In the top-down approach, the entire protein of interest gets ionized as a whole and is analyzed and fragmented only in the MS.

In contrast, in the bottom-up approach, the protein of interest is first digested into smaller peptides and examined afterward. The detection of oxidative modifications using HPLC-MS methods in model solutions and tissues is the goal of this work. Main questions addressed in this work are "*How does the impact of various plasma sources and treatment parameters differ on peptides and proteins in form of oxidative modifications, and can these results be used to characterize, foresee, and define the effects of specific treatment conditions?*"

1.2. Reactive species: key actors in cell signaling and oxidative stress

ROS and RNS are generic terms for a large variety of different molecules. It is known for almost 30 years, that a large number of these reactive species is formed in the cell, especially in mitochondria [64], from complex I and III of the electron transport chain [65], as well as from different oxidases [66-68], and that they also have important roles in physiology [69] and disease processes [70]. The principal mechanism of action of reactive species is to lead redox reactions in the cell by inducing non-enzymatic post-translational modifications (PTMs) in proteins, as well as structural modifications of many other biomolecules (e.g., DNA, lipids, sugars). Over the last years, various modifications like acetylation, carbonylation, methylation, and also oxidative modifications like hydroxylation and nitration were found to have a biological impact [71]. Some of the induced modifications are very complex and are present in large numbers in different tissue areas and cell types. These modifications can be reversible (e.g., phosphorylation or hydroxylation) or irreversible (e.g., deamidation).

Smaller chemical groups can be added (e.g., methylation), or even large and complex molecular structures (e.g., ADP-ribosylation) [72]. Many of these modifications play a crucial role in various signaling pathways. One of the most studied modifications is the phosphorylation of proteins, which was already discovered in the 1950s [73]. About 1 to 2/3 proteins in eu-

karyotes [74], thus about 13000 proteins in the entire human proteome, are temporarily phosphorylated. The most common amino acids phosphorylated by protein kinases are serine, threonine, and tyrosine [75]. Through these phospho-modifications, various signaling pathways can be directly activated or inactivated. Interestingly, as described later, the tyrosine kinases themself can be activated by reactive species, via induction of structural modifications.

This shows how reactive species can change, modulate, and regulate signaling pathways at different levels, being part of a very complex and complicated network. Helmut Sies has done outstanding progress in this field, as he was able to clarify that oxidative stress is not necessarily negative for the organism themselves (Figure 1.3) [76]. Only when uncontrolled reactions occur in presence of very large amounts of reactive species (e.g., exogenously produced), a disturbance of the redox signaling occur and it is possible to speak about the so-called distress [77], or negative stress. However, if the amounts of reactive species are controllable and not in excess, these can induce targeted modifications on specific target molecules, acting on physiological pathways. This phenomenon is referred to as oxidative eustress and could be used to induce beneficial effects by the exogenous introduction of species, e.g., in the case of cold plasmas. The balance between these two oxidative stresses is extremely important for the cell, as many signaling pathways depend on ROS and RNS levels [79-81].

Important signaling pathways, such as the transcription factor NF-kB, which is responsible for cellular processes like immune response, inflammatory response, cellular adhesion, differentiation, proliferation, autophagy, and apoptosis [82], depend on ROS levels. ROS can stimulate NF-kB-dependent signaling pathways in the cytoskeleton, and on the other hand, inhibit them in the nucleus. Another example depends on the cellular levels of H_2O_2, which lead to the activation of NF-kB by alternative phosphorylation of a second factor, IkBα.

Inhibition of NF-kB can also be induced when glutathionylation led by ROS action occurs on a cysteine in IkBα [83]. Other examples of signaling pathways in which ROS plays a regulatory role are the mitogen-activated protein kinase (MAPK) cascades, Keap1-Nrf2-ARE, and phosphoinositide-3-kinase- (PI3K-) Akt [84].
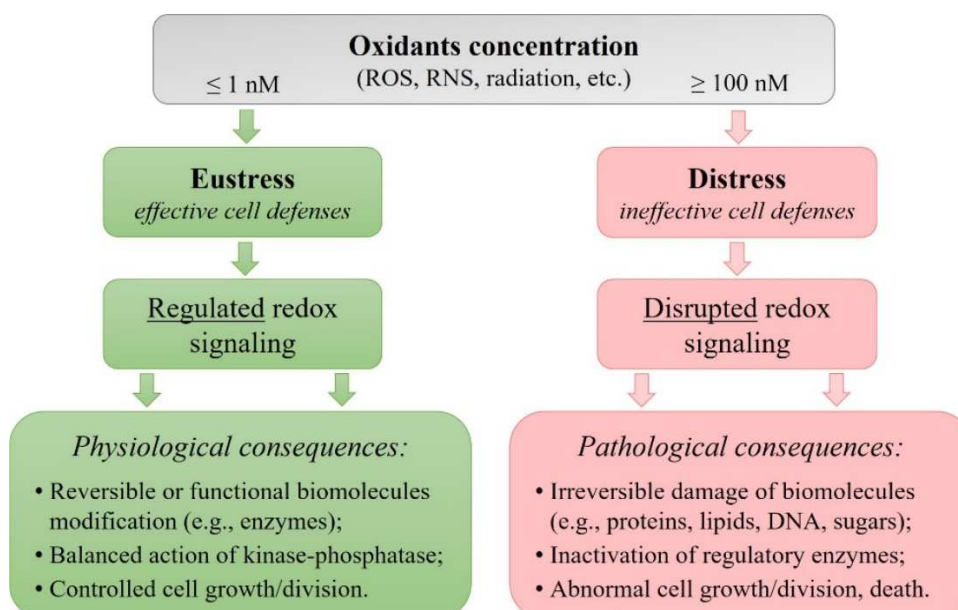

Figure 1.3. Representation of the theory concerning the endogenous homeostasis of reactive species, leading to oxidative eustress and cell stimulation if in small amounts, and to oxidative distress and cell death if highly concentrated. *Illustration redesigned with permission according to* [78].

The direct effect of reactive species as second messengers can be demonstrated also by the example of tyrosine kinases, as shown in Figure 1.4. Endogenous ROS, for example, H_2O_2, selectively oxidize tyrosine kinases at various cysteine residues, like the apoptosis signal-regulating kinase 1 (ASK1), inducing its activation. This activated kinase can phosphorylate and activate further target enzymes, important in other downstream processes.

Examples are p38 mitogen-activated protein kinase and cJun NH_2-terminal kinase (JNK), which are responsible for apoptosis, cell cycle control, and growth inhibition. Being a regulated process, the oxidized form of kinases can be reduced by the action of thioredoxin reductase (TrxR), which oxidizes thioredoxin-1 (Trx1) in a redox reaction [85]. Hydrogen peroxide (H_2O_2) is one of the most studied reactive species in biological redox reactions [86].

Endogenously, it is formed in the body as a by-product of various biochemical reactions, and it was recognized for a long period only as a harmful species. Sources of H_2O_2 are for example the NAD(P)H oxidases, the mitochondrial respiratory chain, and other oxidases. In total, 31 human intracellular enzymes can form peroxides [87]. The xanthine oxidase, for example, forms hydrogen peroxide during the degradation of hypoxanthine to uric acid [88], which is then converted to water and oxygen by catalase. H_2O_2 was one of the first reactive species to be identified as a messenger molecule [89, 90]. H_2O_2 plays an important role in processes such as cell proliferation and migration. In particular, a model study with injured larvae of a zebrafish was able to show that an H_2O_2 gradient was newly formed after wounding, which then led to increased recruitment of leukocytes [91]. This was an important indication that H_2O_2 plays an important role in wound healing processes, by recruiting cytokines and epidermal growth factors. As anticipated, H_2O_2 mainly acts in signal transduction by adding reversible oxidative modifications to proteins, thus modulating their structure and function [92]. A major mechanism for intracellular signaling pathways is the reversible cysteine oxidation, mediated by H_2O_2 [93]. It must be emphasized that cysteine residues are modified specifically in the cell [94]. Interestingly, between 5 and 12% of all cysteines in the proteome are oxidized under physiological conditions. By adding various oxidative reagents, this value can be increased to over 40% [95]. These oxidations occur on specific cysteine residues.
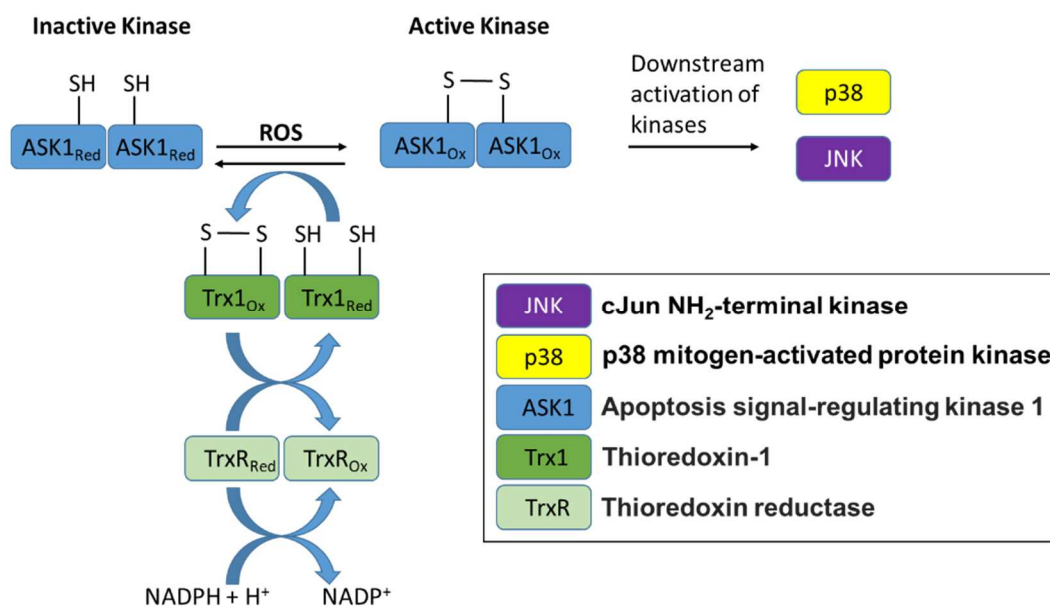

Figure 1.4. Exemplary scheme for the reversible activation of the Apoptosis signal-regulating kinase 1 (ASK1) via cysteine oxidation by reactive oxygen species (ROS).

The local pH, the hydrophobicity of the amino acids close to cysteine residues, and the overall protein structure, influence and regulate oxidative modifications occurring on thiol groups in the cell [96]. Hydrogen peroxide, however, is not the only reactive oxidative messenger molecule. Nitric oxide radical ($^\bullet$NO) is another reactive species released in human cells that can act as a signaling molecule. It has recently been shown that $^\bullet$NO plays an essential role in embryonic wound healing [97]. This molecule is also known to cause modifications in proteins, mainly *S*-nitrosylation [98]. $^\bullet$NO is also a precursor molecule for several other RNS, including nitrogen dioxide radicals ($^\bullet$NO$_2$), peroxynitrite (ONOO$^-$), nitrite (NO$_2^-$), nitrate (NO$_3^-$), nitroxyl (HNO), and dinitrogen trioxide (N$_2$O$_3$) [99, 100]. Finally, superoxide anion (O$_2^-$) and its radical form ($^\bullet$O$_2^-$) are other reactive compounds that are formed in the cells and act as signal molecules in the redox signaling processes [101].

The relevance of various ROS and RNS, such as hypochlorite anion (ClO$^-$) [102] and ozone (O$_3$) [103] in biological processes has also been shown. Although the ROS and RNS lists are not complete, the mode of action is commonly to induce specific structural changes in target molecules, e.g., enzymes or proteins, whose activity or stability gets altered triggering in turn further effects in the cell. Oxidized proteins have reduced activity in most cases [104]. For this reason, these oxidations were often classified as harmful or deleterious in the past. In recent times, however, it has become increasingly apparent that oxidative modifications on proteins can also have beneficial effects for the cell or the organism [105], for example when enzymes have a toxic increase of activity in certain diseases which can be reduced by oxidative modifications. The regulation of the extracellular matrix (ECM) by matrix metalloproteases (MMP) is, for example, important for different types of cancer diseases. This class of enzymes is normally responsible for the extracellular matrix reorganization and angiogenesis [106], but in tumor cells, the activity is increased to the point that malignant cells can spread unhindered and thus form metastases. Inactivation of these enzymes through, for example, oxidative modifications, would therefore mean a better prognosis for the patient.

Today's medicine is already using the effects of targeted ROS and RNS release in different approaches. Cancer-based therapies such as photodynamic therapy, sonodynamic therapy, or chemodynamic therapy, are based on the endogenous production of ROS, e.g., singlet oxygen. Similarly, this mechanism is also used for medical treatments against bacterial infections or cardiovascular and inflammatory diseases [107]. In the same way, CAPs mechanism of action may be based on the introduction of oxidative conditions on the biological target, delivering exogenous reactive species and can lead to mild (e.g., short treatment times, only helium or argon as working gas) or stronger (e.g., long treatments, presence of oxygen in the working gas) oxidation. As observed on biological targets, softer stimulations can increase inflammation, cell proliferation, promoting tissue regeneration and blood coagulation via platelet activation [108]. In contrast, CAPs can also generate a larger amount of highly oxidative reactive species under high oxidative conditions, inducing apoptosis and necrotic cell death reactions on the targeted cells, effects highly beneficial for oncologic treatments [109, 110]. Similarly, strong oxidative conditions have been used in applications involving the antimicrobial effects of plasmas [3, 111], as well as in cancer regression studies *in vitro* and animal models [13, 112-115]. Relevant results have been obtained in controlling the gas-phase chemistry, distance, treatment time, and reaction solution since these are the most important parameters to consider for the production of ROS and RNS in the liquid phase [45]. It has already been shown that plasma treatment can stimulate and promote the Nrf2 signaling pathway [10, 116], which

is relevant for wound healing processes. It was also successfully shown *in vitro* that key tumor suppressor proteins, such as p53 [117], can be modulated by plasmas [118, 119]. Although different physiological effects have been observed, key molecular reaction mechanisms of plasmas are still under investigation. The ability to modify biological structures of the cell and the extracellular matrix (sugars or proteins) was proven as a direct effect of CAPs [36]. Even more importantly, this mode of action, involving the induction of oxidative additions on biomolecules, was also proven *in vitro* and *ex vivo* experiments [120-123]. These modifications, which can occur on proteins, can both lead to a loss or a gain of function, modulating redox signaling pathways [33, 124]. The molecular mechanisms connecting the action of short-lived gaseous species and the long-time scales of the biological effects have been investigated in this thesis. In this context, additional knowledge about the qualitative and quantitative in liquid deposition/formation of species reaching biological targets under defined plasma setups has been clarified. By identifying non-enzymatic oxidative modifications (oxPTMs) in peptides, the present work contributed to the understanding of the nature of molecular biological responses in different amino acids after plasma treatments.
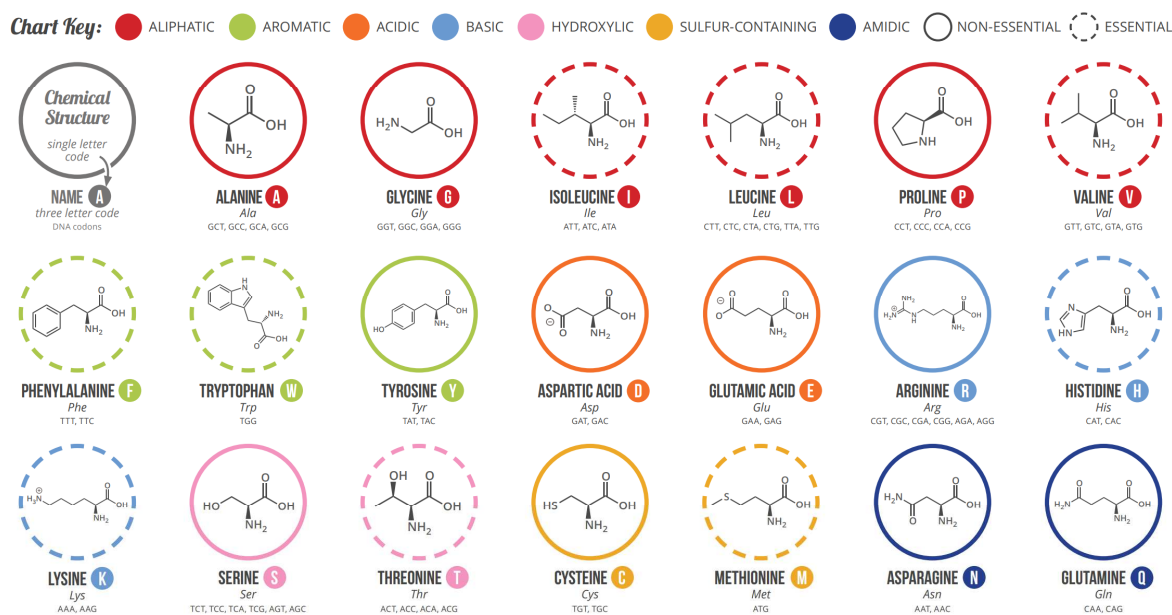
1.3. Amino acids as a target of bioactive reactive species

Proteins and enzymes consist of 22 proteinogenic amino acids, although two non-canonical amino acids, selenocysteine and pyrolysine, only occur in some bacteria and archaea. The other 20 amino acids (Figure 1.5) form the basic structure of all proteins and enzymes in humans, which are a part of the cell signaling pathways. These alpha-amino acids have an amino group ($-NH_2$), which is located at the second carbon atom ($C\alpha$) and is also connected to the carbon atom of the carboxyl group (-COOH). In addition, there is a hydrogen atom at the $C\alpha$, and a variable side chain (-R), which is specific for each amino acid and defines their reactivity. The individual amino acids can be grouped in a variety of ways in relation to the physical-chemical properties, for example in non-polar and polar amino acids, hydrophobic and hydrophilic amino acids, and acidic and basic amino acids. In addition, a distinction can be made based on the side chains, for example in aromatic or aliphatic amino acids, or based on the presence in the side chain of special atoms, such as sulfur or oxygen. The differences in the side chains are the main cause of different amino acids reactivity, which for some amino acids have been already reported towards reactive species [125, 126]. For example, aromatic amino acids like tryptophan have higher reactivity than the simplest and smallest amino acid glycine. Amino acids such as tyrosine, methionine, tryptophan, and phenylalanine are highly reactive towards singlet oxygen [125], while free cysteine and methionine rapidly react with peroxynitrite, as well as tyrosine through a two-step reaction [100].

The complexity increases again significantly if peptides are considered rather than individual amino acids. Peptides consist of a small number (up to 50) of amino acids, linked by a peptide bond (-OC-NH-). This bond is formed when the C-atom of the carbonyl group of an amino acid X connects with the N-atom of the amino group of a second amino acid Y, with the elimination of a water molecule. The reactive -COOH and $-NH_2$ groups are then no longer available for a reaction, thus the reactivity of the peptide is defined especially by the different side chains (-R). The reactivity of each -R in a peptide, however, is specific towards certain reactive species and is furthermore influenced by neighboring amino acids.

Figure 1.5. Classification, chemical structure, and codes of the 20 proteogenic amino acids. *Illustration reprinted according to* https://www.compoundchem.com/2014/09/16/aminoacids, https://creativecommons.org/licenses/by-nc-nd/4.0/.

For example, the reactivity of an amino acid surrounded by aromatic amino acids may be reduced due to the local steric hindrance or increased hydrophobicity. In the same way, the reactivity of an amino acid could be modified by the presence of acidic or basic amino acids, which will modulate the local pH [127]. The complexity and reactivity increase further in proteins and enzymes, where amino acid sequences form highly complex structures (from primary to quaternary), being assembled in variable ways (e.g., hydrophilic or hydrophobic interactions, Van der Waals interactions, ionic or covalent bonds). For various oxidative modifications, the modulation of single amino acid reactivity in peptides or proteins has been not fully investigated. However, these knowledges become essential in medical therapies based on the exogenous delivery of reactive species, as oxidative modifications on amino acids can have a direct influence on signaling pathways. Previous studies have already shown that specific amino acids are targets of plasma-derived reactive species [120, 121, 126, 128].

In biological contexts, cysteine is the most sensitive amino acid to reactive species, together with tyrosine [129-131] and tryptophan [132, 133]. However, amino acids which are modified less often, can have an important physiological influence.

Hypoxia-inducible factor (HIF)-1α is a master regulator in response to hypoxia and is significantly hydroxylated at proline P564 and P402 under normal conditions. Under pathophysiological conditions, such as stroke and therefore hypoxic conditions, proline becomes dehydroxylated, thus the von Hippel-Lindau (VHL) protein cannot bind (HIF)-1α. As a result, (HIF)-1α translocates into the cell nucleus and combines with HIF-1β. This complex finally binds a DNA region which regulates various processes like erythropoiesis and

angiogenesis. The discovery of this oxidative modification, having such a major physiological impact, was crucial enough to be rewarded with the Nobel Prize in Physiology and Medicine in 2019 [134, 135]. The nitration of tyrosine is also a well-studied oxidative modification. In the 1990s, it was found that peroxynitrite is the main species leading to protein nitration [136, 137]. The ONOO$^-$ dissociates in aqueous solutions with a pH < 7.4 into the radicals $^\bullet$OH and $^\bullet$NO$_2$ [138, 139]. The radicalization of tyrosine would then occur in the cell (generating a metastable tyrosyl radical) because of its rapid reaction with $^\bullet$NO$_2$ and finally generate the nitro-tyrosine [130]. At that time, this modification was considered a damaging outcome of nitrosative stress. Over the last decades, however, it has been recognized that tyrosine is not randomly modified in cell proteins, and that specific nitrated proteins can be used as biomarkers for certain diseases. Firstly it was associated with different cancer types, and nowadays also with neurodegenerative diseases [140], atherosclerosis [141], and systemic lupus erythematosus [142]. In addition, there are other areas in which nitration plays an important role such as the cell signaling cascade or immune response [130]. However, the high abundance of nitrated proteins in biological samples makes it difficult to identify and quantify the exact and specific protein site which is modified [143].

Generally, oxidative modifications can be grouped into reversible and irreversible. Even though nitration is irreversible, and therefore mostly associated with negative impact for a biological system, it has been recently shown that it can occur under normal physiological conditions, such as during pregnancy [144]. Similarly, carbonylation is also an irreversible modification induced by ROS or RNS in proteins under physiological conditions.

Protein carbonylation is produced in various signal transduction pathways [145, 146], being involved in ischemic preconditioning, thus protecting blood flow restoration induced by tissue damage [147]. In addition to these irreversible modifications, there are many reversible ones, such as hydroxylation, acetylation, methylation, glycosylation, and ubiquitylation, influencing cell biology [72, 148]. As anticipated, some of the most relevant oxPTMs are found in proteins cysteines. The sulfur moiety of this amino acid was identified as the most reactive structure towards short-lived reactive species, e.g., $^\bullet$O, ^{1}O$_2$, $^\bullet$OH, ONOO$^-$, $^\bullet$NO, $^\bullet$NO$_2$, also produced from CAPs [126]. Long-lived reactive species, e.g., H$_2$O$_2$, O$_3$, react with cysteine especially at higher pH (higher thiolate form) or with the support by cell enzymatic activity.

It was possible to demonstrate that the plasma-driven oxidation of cysteine residues can lead to an inactivation of the RNase A in bacteria [120]. Most cysteine modifications are reversible, allowing a fine regulation of various signaling pathways, such as in the case of *S*-nitrosothiol (-SNO), and sulfenic acid (-SOH), which can be both induced by ONOO$^-$ for example. Cysteine sulfenic acid can be also produced by reaction with H$_2$O$_2$, $^\bullet$O, $^\bullet$OH, while S-nitrosocysteine can be produced by reaction with a radical (thiyl radicals, -S$^\bullet$) followed by reaction with $^\bullet$NO or $^\bullet$NO$_2$ [96]. However, stronger oxidation defined by stronger oxidants (e.g., ^{1}O$_2$, O$_3$) or high oxidants amounts leads to the production of sulfinic acid (-SO$_2$H) and finally the highly oxidized sulfonic acid (-SO$_3$H), which is an irreversible structural change [149, 150].

Some reversible modifications have a major influence on the regulation of relevant enzymes, e.g., pyruvate kinase M2 [151]. The regulation is possible by preventing the active tetrameric form from nitration and oxidation at specific cysteine residues. The cysteine modifications are also essential in cellular defense mechanisms, where redox signaling cascades counteract stress stimuli [152]. Due to the variety of modifications and their relevance in redox biology, cysteine solutions have been already used to trace the production

of reactive species derived from cold plasma [31, 149, 153]. Finally, further investigations, partially fulfilled in this work, need to be made to underlie which modifications can be introduced by plasma species in peptides and proteins, highlighting the influence that these can have on enzymes reactivities and signaling pathways.

1.4. Mass spectrometric detection of oxidative modifications in proteins and peptides to trace plasma-derived reactive species

Among all plasma components, reactive species, and in part radiation, are responsible of cold plasmas' modulatory effects. As already shown in amino acid solutions, oxidative modifications may be introduced due to the action of reactive species on proteins [126].

Although it is challenging, the precise detection of these modifications in proteins is a key step to investigate the chemistry and the biological mechanisms of action of plasmas. As previously described, the best method for detecting oxidative modifications is mass spectrometry (MS) [154], which is the main technique applied in the present work. MS is used in proteomic studies since the 1980s when electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) have been developed. The advantages of a proteomics bottom-up MS investigation are various, but mainly, several thousand proteins and corresponding modifications can be identified in a single sample run [155]. Moreover, over being identified precisely, proteins can be also quantified with this technique [156]. Relative quantification can be achieved in a differential proteomic analysis when the abundancies of a protein in a certain condition are compared to the profiles measured in a reference sample.

Typical absolute quantification strategies include the use of stable isotope labeling by amino acids in cell culture (SILAC) [157], using synthetic isotopically labeled peptides (AQUA), or label-free quantification (LFQ) calculations [158]. Due to the low abundances of modified proteins or peptides in comparison to the entire proteome in a cell, different enrichment protocols can be applied to improve their detection. However, this strategy has not been yet implemented for all known oxidative modifications, a reason why this must be further investigated in the future. Currently, only specific oxidative modifications like nitration at tyrosine [159], or oxidation at cysteine [160] can be enriched, e.g., using beads with specific antibodies, or targeting the exact bonds for the $-NO_2$ or $-O_x$ groups with specific reagents [96].

Enrichment is an effective way to detect low abundant modifications, having as a downside the potential loss of any other kind of modification from the detected spectra. In an MS analysis, the detection of PTMs occurs on entire or digested proteins (peptides) via the so-called tandem MS (MS/MS or MS^2) [61]. By using an ionization source (e.g., ESI, MALDI), intact proteins or peptides are ionized. At this point, two identification steps based on the biomolecule's mass can take place, one on the MS level, another on the MS/MS level.

The MS spectrum is acquired first, to identify the molecular mass of the entire protein or the peptides (if the protein was digested previously), which is called parent ion. To increase the accuracy of the detection, each protein or peptide can be fragmented via MS/MS by energy impact or transfer with a collisional inert gas. Here, the fragmentation of the peptide bond produces a set of ions (daughter ions) overlapping the sequence of the parent ion in analysis.

If there is a PTM on a certain amino acid, the mass of the modified amino acid will change, and a mass shift (Δm) will be carried in both the parent ion and in one or more daughter ions, as shown in Figure 1.6.
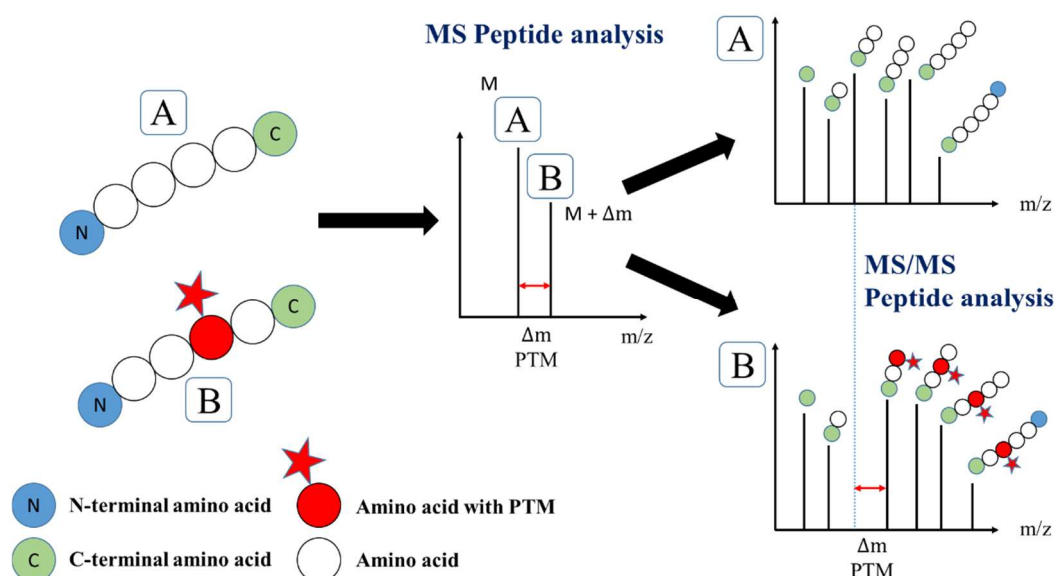
Figure 1.6. Detection of post-translational modifications using tandem mass spectrometry. A peptide (A) consisting of six amino acids produces an MS and an MS/MS fragment spectrum with six peaks. The same peptide with a modification (B) produces shifted MS and MS/MS spectra due to the modified amino acid. The determination of the exact mass shift (Δm) at the MS level allows the identification of the modification, while at the MS/MS level allows the precise localization of the modification on the amino acid level. *Illustration redesigned according to* [61], https://creativecommons.org/licenses/by/4.0/.

Analyzing a single peptide manually is possible, but often the proteomics analysis of many complex samples, having tens of thousands of different modified or non-modified proteins, is required (e.g., in case of the analysis of various cancer cell lines).In these cases, the manual data analysis becomes very complicated and time-consuming. Therefore, data are analyzed by using software that can search for several thousand proteins simultaneously, screening additionally for different modifications.

Here, all possible theoretical fragment spectra are generated in the software (in silico) for all identified proteins, and these are compared with the experimentally measured spectra. If the match between the theoretical and experimental spectrum is high enough (score threshold), the protein or peptide is considered to be correctly identified. An example is the software Proteome Discoverer (Thermo Fisher) [161], which is largely used for this purpose in the proteomics field. The software Byonic (Protein Metrics) [162] has been used in the present work as additional software to identify a wide range of oxidative modifications occurring on specific amino acids, overcoming the limits of enrichment techniques.

The latter, indeed, rather focuses on a single modification and requires long and sensitive experimental protocols. Another advantage of Byonic is that an unlimited amount of PTMs can be overall screened, also allowing the identification of more modifications for each single amino acid in a peptide. Furthermore, the so-called Wildcard Search supports the untargeted analysis of all possible modifications found in a sample, without the necessity to plan and focus the study on a previous hypothesis [162].

A relatively small number of studies has been performed on the impact of reactive species in relation to the proteins' structures. Thanks to the described techniques, one of the novelties of the present study include the characterization of the reactivity of amino acids based on the peptide sequence, highlighting competing reactions with other amino acids.

The complex interplay of plasma-induced reactive species, which can also react with each other, and their effects on target molecules, such as proteins, have been also studied in this work, with further focus on modulating plasma jets and conditions to produce a different pattern of plasma components to monitor their biological effects.

For these reasons, the general workflow consisted of 1) a systematic investigation of the biochemical impact of different plasma conditions and sources, 2) the identification of amino acids modifications in peptides via mass spectrometric methods, and 3) the transfer of gained knowledge from model systems to complex molecules and sample-types. Further details about these points are listed below:

1) The used plasma sources were the argon-driven kINPen 09, due to the well-characterized gaseous chemistry, and the helium-driven COST-Jet as a reference source (Figure 1.2). Modulated conditions were treatment duration (15 to 60 sec), distance from the jet nozzle to the target surface (from 4 to 9 mm), solvent-type if solutions treated (water, PBS), molecular admixtures in feed gas (0.5% O_2 and/or N_2) (methods applied from A1 to A5).

2) The pattern of plasma-induced modifications in every amino acid was identified via nanoliters-flow liquid chromatography (nanoLC) coupled to MS detection, using Orbitrap-type MS systems. The identification of modifications was performed by tandem mass spectrometry (MS/MS). Quantification of the identified modifications was performed via label-free peptide spectrum match counting [163] (methods applied from A1 to A5).

3) Modifications occurring in more complex structures were investigated, in particular in i) phospholipase A2 (PLA_2) in aqueous solutions (applied in A3), due to its relevance in lipid metabolism, ii) in patients' wound exudates (applied in A4), and iii) in porcine epidermis skin tissues (applied in A5), due to the application of cold plasmas in would healing acceleration [7-12].

2. Cold Plasma Effects on Proteins: from Peptides Solutions to Tissue Proteomes

This chapter summarizes the main results of the thesis. First, it focuses on the detection of possible oxidative modifications, induced by cold plasmas in peptides, which have been discussed in **Section 2.1**. Considering a liquid environment surrounding cells, tissues, and molecular structures, the plasma-driven biochemistry induced in aqueous solutions containing peptides has been discussed. The modulation of parameters and sources (kINPen and COST-Jet), which may lead to differences in oxidation potentials of plasmas, are also a part of this chapter.

The generation of plasma species in aqueous solutions has been investigated by screening the resulting oxidative modifications in the peptides. The results obtained from Article A1 have been discussed, where aqueous solutions of two model peptides angiotensin 1-7 and bradykinin have been used. Experimental data were additionally compared with in silico data obtained from previous works which applied dynamic simulations, currently widely used in plasma medicine (**Section 2.1.1**). The main findings of Article A2 have been also included to discuss the biochemistry induced on an artificial peptides' library in solutions. Here, an evaluation of the most reactive amino acids in relation to their position in each peptide has been elaborated (**Section 2.1.2**). In **Section 2.1.3**, the knowledge gained in the first part of the work has been applied in aqueous solutions containing the protein phospholipase A2 (PLA_2), as shown in Article A3. Here, the differences in oxidative modifications concerning the treatment of a more complex structure have been discussed and compared with previous results, giving additional insights on how plasma can alter protein functionality. **Section 2.2** finally resumes results obtained on proof-of-concept experiments carried out to verify if plasma-driven modifications occur also in cell proteomes, such as in wound exudates of patients' skin (Article A4, **Section 2.2.1**), and in intact porcine skin (Article A4, **Section 2.2.2**). Due to the relevance of cold plasmas in applications promoting wound healing, the obtained results have been discussed also in terms of clinical safety and effectiveness of plasma treatments.

2.1. Screening structural changes of peptides in solution

Despite the increasing use of plasma in medical applications [164, 165], various questions regarding its mode of action are still unanswered. The effects of CAPs on biological molecules like lipids [123], small molecules [166], and DNA [43, 167] have been previously studied. Still, it is unclear whether plasma can modulate cell signaling pathways through these effects. It is known that reactive species can add a variety of oxidative modifications to proteins and enzymes, but there is no direct link on how CAPs can modify specific amino acids in a peptide or a protein. To investigate these questions, oxidative modifications were characterized after treatment with cold plasmas using mass spectrometry. Firstly on a library consisting of 10 peptides with variable conformations (Fig. 1, A2), then on 2 biologically relevant peptides (angiotensin 1-7 and bradykinin, Fig. 4/5, A1) and finally in aqueous solutions of PLA_2 (A3).

Due to the heterogeneity of plasma sources and their tunable parameters currently proposed for biomedical applications, various conditions (e.g., driven gas composition, treatment duration) and plasma jets (kINPen 09, COST-jet) have been used for these studies.

The results obtained in this thesis support previous works, treating amino acids in aqueous solutions, where it has been shown that different conditions reflect a differential production and generation of plasma components, mainly reactive species [126, 128, 149].

To screen a bigger number of possible modifications on each amino acid, the software Byonic was used to fulfill this task (A1, A2, A3). The software compared real mass shifts (Δm) with theoretical ones calculated in silico, finally producing a list of modifications occurring on each amino acid after plasma treatment, as shown in Table 2.1. Each mass shift represents the addition or loss of a certain number in Da, which could be correlated to the addition or loss of a defined molecular group. Due to the high accuracy of the used MS, the monoisotopic mass shift in Da could be calculated, reflecting the precise identification of the funcional group added or lost on each amino acid structure. In this thesis, peptides (A1, A2) or a mixture of peptides derived from proteins digestion (A3, A4, A5) have been analyzed via mass spectrometry methods (bottom-up approach).

Spectra of peptides containing each modification on each amino acid were counted and eventually normalized on the number of peptides containing the same unmodified amino acid. After training the software, due to the long calculation times, the presented list of oxidative modifications induced by varying plasma parameters have been applied for target analysis of possible modifications occurring on specific amino acids in Articles A3, A4, and A5, where the analysis of entire proteins or complexes of proteins was performed. More details about the plasma treatment conditions where these modifications occurred, and on which specific amino acids, are discussed in the following sections.

Table 2.1. Main oxidative modifications were detected via nanoLC-MS after treatment with different plasma conditions on peptides amino acids. *Modified and adapted from Articles A1, A2, and A3.*

Δm [Da]	Elemental Composition	Chemical Modification / Potential Product
+ 15.99	+ O	hydroxylation, oxidation
+ 31.98	+ 2O	dihydroxylation, peroxide, dioxidation
+ 47.98	+ 3O	hydroxylation + peroxide, trihydroxylation, trioxidation
+ 28.99	+ N + O - H	Nitrosylation
+ 44.98	+ N + 2O - H	Nitration
+ 60.98	+ N + 3O - H	nitration + oxidation
+ 76.97	+ N + 4O - H	nitration + dioxidation
+ 0.98	- N - H + O	deamidation
- 0.98	+ N + H - O	Amidation
+ 13.98	+ O - 2H	carbonylation (oxo group)
+ 29.97	+ 2O - 2H	oxo group + hydroxylation
+ 45.97	+ 3O - 2H	oxo groups + two hydroxylations or peroxide
- 2.02	- 2H	didehydrogenation (double bond)
- 4.03	- 4H	two didehydrogenation (two double bonds)
+ 4.98	+ 2O - N - C - H	ring cleavage (histidine: formylasparagine)
- 3.05	+ O - 5H - N	oxidative deamination (lysine) [126]
+ 33.96	+ Cl - H	chlorination

2.1.1. Biochemical modulation with treatment condition (A1)

To investigate if and how the modulation of plasma parameters could generate specific protein structural changes, a study has been performed on solutions of peptides in A1. In particular, the impact of CAPs on two peptides, angiotensin 1-7 and bradykinin has been investigated. Indeed, the oxidative modifications induced on these peptides using the COST-jet as a plasma source have been already previously studied using molecular dynamic simulation (MD) [168].

This is a method currently proposed in plasma medicine to contribute to the characterization of molecular mechanisms induced by cold plasmas in aqueous solutions or other biological models [34]. Therefore, in A1 it has been discussed if experimental data would support or correct the mechanisms highlighted by in silico simulations. Here, in addition to the COST-jet, the kINPen 09 was used as a plasma source. Differences and similarities in the liquid chemistry of the two plasma sources have been identified, as shown in Figure 2.1.

Tyrosine (Y) was one of the most reactive amino acids in the peptide angiotensin 1-7, being mostly modified when further gases (e.g., N_2, O_2, or both) were added. Another aromatic amino acid, phenylalanine (F), was mostly modified in bradykinin. Regarding Y, this result could be reproduced for both plasma sources. In particular, the addition of $-NO_2$ (nitration), 1 or 3 -OH groups (hydroxylation) could be observed on the benzene ring on tyrosine (Fig. 3 and 4, A1). The modifications changed qualitatively and quantitatively in relation to the gas composition, reflecting a differential active liquid chemistry. For example, nitration and one hydroxylation occurred mainly with $Ar \pm N_2/O_2$, while a high degree of hydroxylation occurred mainly in $Ar + O_2$. To support the investigation of bioactive species in liquid, long-lived species (H_2O_2, NO_2^-, and NO_3^-) have been quantified in A1 (Fig. 2, A1). H_2O_2 was more significantly produced in Ar plasmas of kINPen 09 compared to the He plasmas of COST-Jet, as the excimers forms of argon in kINPen 09, and the consequential production of VUV radiation [34, 169], may lead to a higher water (H_2O) dissociation into $^\bullet OH$ and $^\bullet H$ [170].

The $^\bullet OH$ can quickly recombine to form H_2O_2. However, in presence of another substrate like tyrosine, $^\bullet OH$ can react in a direct reaction with Y to hydroxylate it. This mechanism would explain the overall higher production of OH-tyrosine in conditions with Ar-only.

Regarding tyrosine nitration, the presence of both N_2/O_2 produced higher amounts of NO_2^- and NO_3^-, according to previous studies [41, 171]. These RNS are reactive towards tyrosine mainly at low pH (<3) [137], which was not reached after treatment. However, these plasma conditions may produce other species more reactive towards Y, such as gaseous $^\bullet NO$ and $^\bullet NO_2$, or $ONOO^-$, whose oxidized forms are NO_2^- and NO_3^- (Fig. 3, A4) [130, 172].

Although the lowest amount of long-lived species was measured in conditions with O_2, the highest number of modifications was detected (Fig. 3, 6, and 7, A1). To explain this is the fact that gaseous short-lived species like $^\bullet O$ and $^1O_2$ can be formed [26, 166], acting in liquid directly with the amino acids [173, 174] or with water molecules, producing tertiary species such as those resulting from water homolysis and ionization ($^\bullet OH$, $^\bullet H$, and H_2O_2) [175].

Overall, most amino acids in the model peptides showed strong differences between the two plasma sources, showing ROS-dominated chemistry mostly driven by O_2 or $^\bullet O$ respectively in kINPen 09 and COST-jet, as shown previously [53, 153, 176, 177]. The reactivity of each amino acid was related to its position in the peptide, as shown for prolines (P) in positions 3 or 7 in bradykinin, or F in positions 5 or 8 in bradykinin (Figure 2.1).
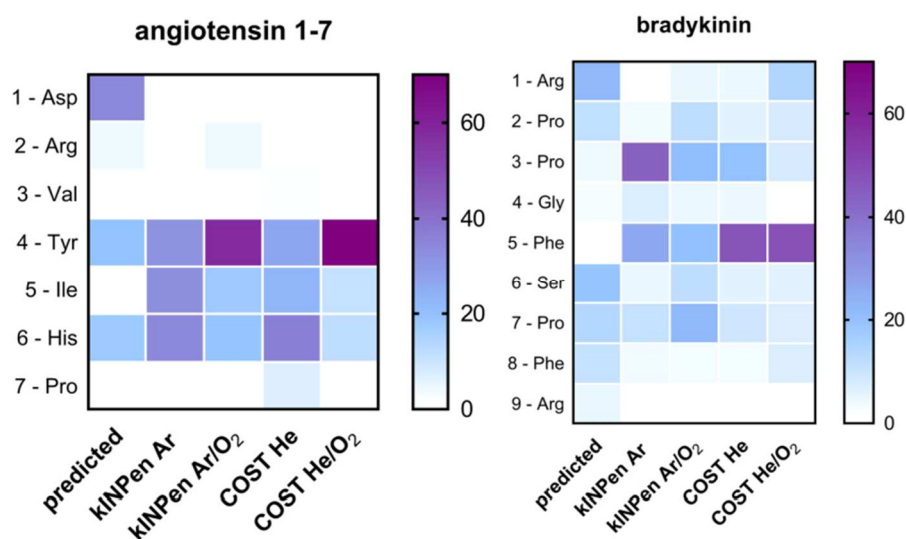
Figure 2.1. Comparison of oxidative modifications (normalized on the untreated control peptide) identified in angiotensin 1-7 and bradykinin after plasma treatments (kINPen 09, Ar ± O_2; COST-jet, He ± O_2), and using *in silico* dynamic simulations [168]. *Adapted from Article A1.*

Further investigations about the influence of the peptide sequence in receiving plasma-driven modifications have been performed in A2, which is resumed in the following section. Finally, differences between experimental and *in silico* data [168] have been identified (Figure 2.1). The major reason for these differences could be connected to the introduction of only ˙OH in the MD simulation, while real plasma treatments presented a qualitatively and quantitatively different cocktail of plasma-generated reactive species [25, 178].

2.1.2. Influence of position on amino acids' reactivity (A2)

A systematic approach with an artificial peptide library was used to understand how plasmas can differentially modify amino acids, additionally by varying peptide sequences. The library was created to contain all 20 canonic amino acids and included 10 peptides (Fig. 1, Tab. S1, A2) where each amino acid occurred with equal frequency either at the C-terminal, central, or N-terminal of a peptide. A various chemical environment was generated for each amino acid by diversifying the neighboring amino acids. Peptides have been treated in solutions using two plasma sources (kINPen 09 and COST-jet) and modulating various parameters to investigate their influence on the peptides, as in A1. Additionally, the solvent type was modulated in A2, using water-only and PBS solutions and direct vs indirect treatments have been compared.

These conditions were considered as PBS simulates the neutral pH which can be found in physiological applications, and which could be essential in defining the overall chemical dynamics. Indirect treatments, which means treating the solution only, with the following incubation with the peptide in analysis, have been used to simulate the chemistry occurring *in vitro* applications, where cells are incubated with plasma-treated media [179, 180]. The used approach and the follow-up bioinformatic processing of the data enabled specific statements about the reactivity of amino acids in peptides after plasma treatment, in contrast to previous studies focusing on single amino acids solutions and longer treatments [126]. Additionally, in these experiments, the result of competitive reactions possibly occurring also in complex biological systems between different amino acids could be observed. These results supported previously found evidence in A1, extending and completing them for all amino acids.

In particular, sulfur-containing amino acids showed most modifications after plasma treatments, mainly consisting in the addition of oxygen groups to generate highly oxidized forms of sulfenic and sulfonic acid (Fig. 3 and 4, A2), especially in presence of oxygen in the plasma. Also, aromatic amino acids were modified in a higher degree, e.g., nitration or hydroxylation, confirming the results observed in A1. Independently from the applied plasma source, the amino acids cysteine, methionine, tryptophan, tyrosine, and phenylalanine were those overall mostly modified. Other amino acids, quantitatively less modified, showed a bigger difference between sources. Lysine, proline, and serine, for example, were significantly more chlorinated by COST-Jet than kINPen 09 (Fig. 9, A2), and chlorination was the modification overall mainly found with the COST-Jet. This result, which could be observed only due to the presence of sodium chloride (NaCl) in PBS solutions, confirmed the predominant action in this jet of atomic oxygen when oxygen is added to the plasma, according to [181, 182], generating the chlorinating agent hypochlorite (OCl^-) by reaction with chloride ions in water (Fig. 5, A2). Interestingly, in presence of tyrosine close to serine in the peptide chain, a reduction in chlorination was observed in serine in favor of tyrosine chlorination, suggesting a higher reactivity of the aromatic ring in comparison to the aliphatic structure of serine. Nitration, on the other hand, was exclusively found using kINPen 09. The plasma generated by this source is characterized by a turbulent flow with consequent major target liquid admixture and plasma interaction with ambient air molecules (e.g., N_2, O_2, H_2O) [166].

The interaction of excited forms of Ar with ambient air is responsible for an increased production of nitrogen chemistry observed in kINPen 09 plasmas. The generation of gaseous $\cdot NO$, in combination with gaseous $\cdot O_2^-$, generated peroxynitrite in the liquid target especially in conditions with Ar $\pm$ N_2/O_2 [183], as described in A4, which is a strong nitrating reagent (Fig. 8, A2; Figure 2.2) [130]. Interestingly, tryptophan modifications consisted mainly in its cleavage to form kynurenine derivatives (Fig. 6 and 7, A2), which in the cell environment can be produced by the presence of singlet oxygen. Plasmas could induce them when oxygen was present in the driven gas, a condition which was already previously shown to produce gaseous $^1O_2$ directly acting in the liquid target, especially using kINPen 09 as a source (Fig. 9, A2) [53, 153]. Further results identified the modification pattern for each amino acid under specific conditions, e.g., by variating working gas composition (Fig. 6, A2), treatment time, direct or indirect mode, and solvent type (Fig. 10, A2). This highlights similarities and differences between amino acids also in relation to their position in a peptide structure (Figure 2.2).

This phenomenon could be already observed in A1 (e.g., in Figure 2.1), and has been systematically implemented and discussed in A2 due to the presence of more peptides. The presence of variable neighboring amino acids and the N-, or C-terminal proximity influenced the nitration of tyrosines belonging to different peptides, as shown in Figure 2.2.

In particular, in contrast to solutions with PBS, major nitration has been observed in unbuffered solutions. The presence of chloride anions, indeed, generated rather a major chlorination of amino acids. Possibly, reactive oxygen species are necessary, as previously discussed, to induce both protein chlorination and nitration. The latter modifications could be therefore favored in absence of chloride anions. Furthermore, the terminal position of tyrosine in peptide 1 promoted its nitration, which was quite lower in peptide 6, possibly due to the presence of an aliphatic (valine), and basic (lysine) amino acids. This conformation, indeed, potentially reduced the reaction rate by mechanisms driven by pH modulation and/or steric hindrance.
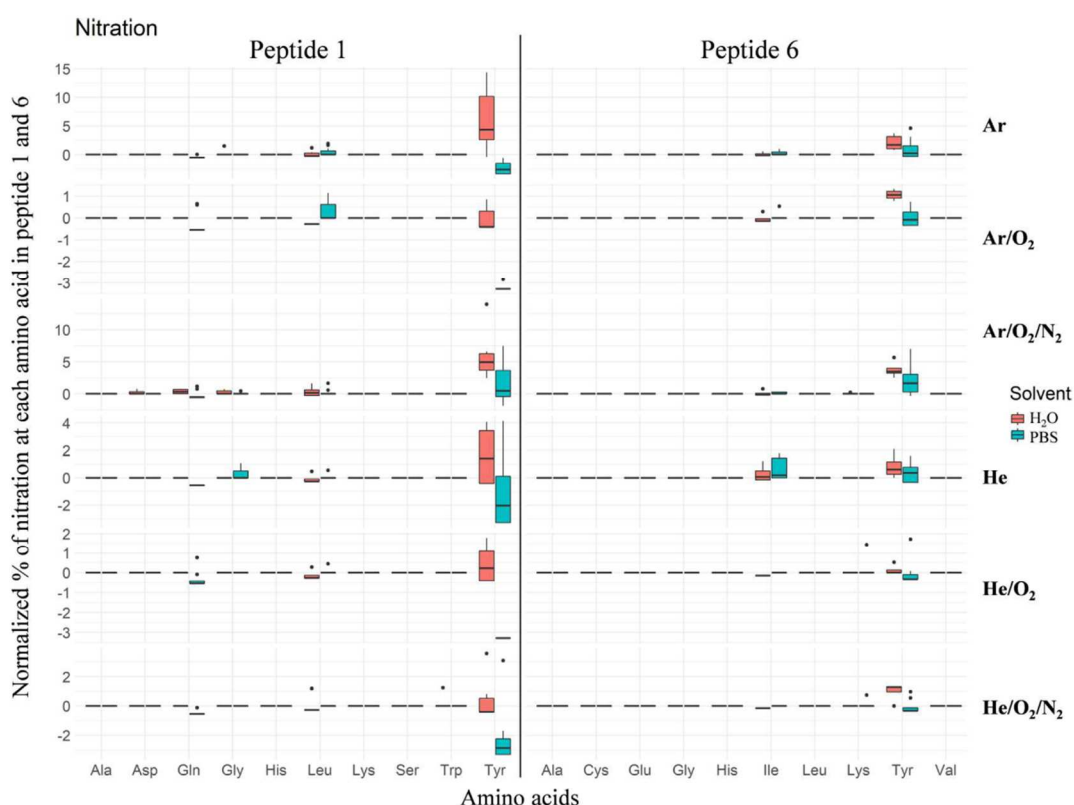
Figure 2.2. Example of differential nitration occurring on tyrosines in having N-terminal position in peptide 1 (left), and internal position in peptide 6 (right). Treatments with kINPen 09 (Ar-driven) or COST-Jet (He-driven). Values in treated peptides normalized on untreated peptides. *Adapted from Article A2.*

It has been shown, indeed, that higher or lower pH may favor tyrosine nitration [137, 172, 184]. Another example involved peptides 3 and 7, having both cysteine (Cys) and tryptophan (Trp) in the chain (Fig. 1, A2). In peptide 7 these two amino acids were separated by only one amino acid, whereas the entire peptide was in between Cys and Trp in peptide 3. While various oxidative modifications (e.g., oxidation, deoxidation; Fig. 7, A2) have been observed at the Trp in peptide 3, treatments with pure argon of peptide 7 solutions showed a definitively lower degree of structural changes in Trp (supporting information in paper A2). In peptide 7, major oxidative modifications have been observed in Cys than Trp, which in this case was the main target of oxidation, dioxidation, and trioxidation mediated by plasmas. Through these results, it was confirmed that cysteine is one of the most sensitive amino acids towards cold plasmas, not only in amino acid solutions but also in peptides and proteins, and could mediate the alteration of redox signaling pathways observed after plasma treatment of cell cultures and other biological samples [11, 12, 118, 180, 185-187]. Through these results, it was confirmed that the peptide structure is an important factor defining the resulting effects of plasma.

Therefore, it can be stated that not only the plasma parameters and the chemistry generated in both the plasma sources must be considered, but also the target must be considered. Finally, deeper studies are necessary to develop targeted applications in plasma medicine.

2.1.3. Consequences on protein functioning (A3)

The identification of oxidized amino acids in different peptides was the initial key step to understanding if and how proteins can be modified by plasmas. From previous results, it was

clear that plasma can interact with amino acids and, in relation to their position in the peptide sequence, induce oxidative modifications. The latter could be introduced based on the used plasma sources and treatment parameters. A further investigation, connecting the hypothesis that plasmas act through biomolecules modifications, was deepened in Article A3. Indeed, the treatment of peptides enriched the information about the reactivity of amino acids in complex biomolecules, however, it must be still clarified whether these structural changes have a demonstrable influence on the activity or stability of a protein and could therefore potentially lead to a modulation of cell signaling pathways mediated by plasmas. For this purpose, an experiment based on the argon plasma treatment with kINPen 09 of buffered aqueous solutions of phospholipase A2 (PLA$_2$) has been performed and the results have been published in Article A3 and discussed in this section.

PLA$_2$ is an enzyme capable of cleaving acyl ester bonds from glycerophospholipids, releasing free fatty acids and lysophospholipids. A massive overexpression and thus increased activity of this enzyme occurs in various diseases, such as cancer, asthma, autoimmune disorders, and autism [188-190]. In this context, reducing the activity of this enzyme could be an advantageous strategy to target these diseases. Although the applicability of plasmas in various diseases is still in evolution, the potential of these tools in introducing oxidative modifications able to reduce the functionality of PLA$_2$ has been tested as an exemplary experiment. After plasma treatment and MS-based analysis of the digested protein, the most frequently modified amino acid was tryptophan (Trp) in position 128 (Figure 2.3). In contrast, another Trp in position 8 did not show modifications at this position. On the other hand, trioxidation at cysteine next to the latter tryptophan was found. As discussed in the previous section, this result may be generated due to the higher reactivity of cysteine in comparison to tryptophan, which results in the preservation of the tryptophan structure in position 8.

Additionally, the two tryptophans were both located at the N- or C-terminal end of the peptide, confirming that, rather than the position, neighboring amino acids, such as cysteine, played a key role in the structural changes of tryptophan. Overall, the presence of PLA$_2$ in a gold electrode supported lipid bilayer showed that the permeability of the membrane increased in comparison with the lipid bilayer alone (Fig. 2, A3). The tryptophan dioxidation of PLA$_2$ at position 128 was the most common detected modification. With this knowledge, it was verified via simulations if this structural change could influence the stability and the activity of the protein in the context of a cellular membrane. As a result, it was shown that the modification did not influence the macromolecular folding and structure of the enzyme (Fig. 5, A3), but rather altered the interaction and the stability of the protein in the phospholipid membrane (Fig. 6, A3). In particular, as shown from 2D and 3D simulations of the interaction of PLA$_2$ with the lipid bilayer, the plasma-induced dioxidation of tryptophan prevented the anchoring of the enzyme on the membrane, which is mediated by the tryptophan carbonyl group connected to the phospholipids via two hydrogen bonds.

The loss of interaction with the membrane may lead to the loss of function of the PLA$_2$ in biological contexts. Plasmas could finally be applied as a strategy to reduce the activity of similar enzymes having interfacial tryptophan, and therefore exposing this structure to the oxidants generated by plasmas. As an example, tryptophan in enzymes is a locus that many viruses (e.g., Ebola, Influenza) use to stabilize in host cells, confirming the potential target use of plasmas for disinfection purposes [191-194].
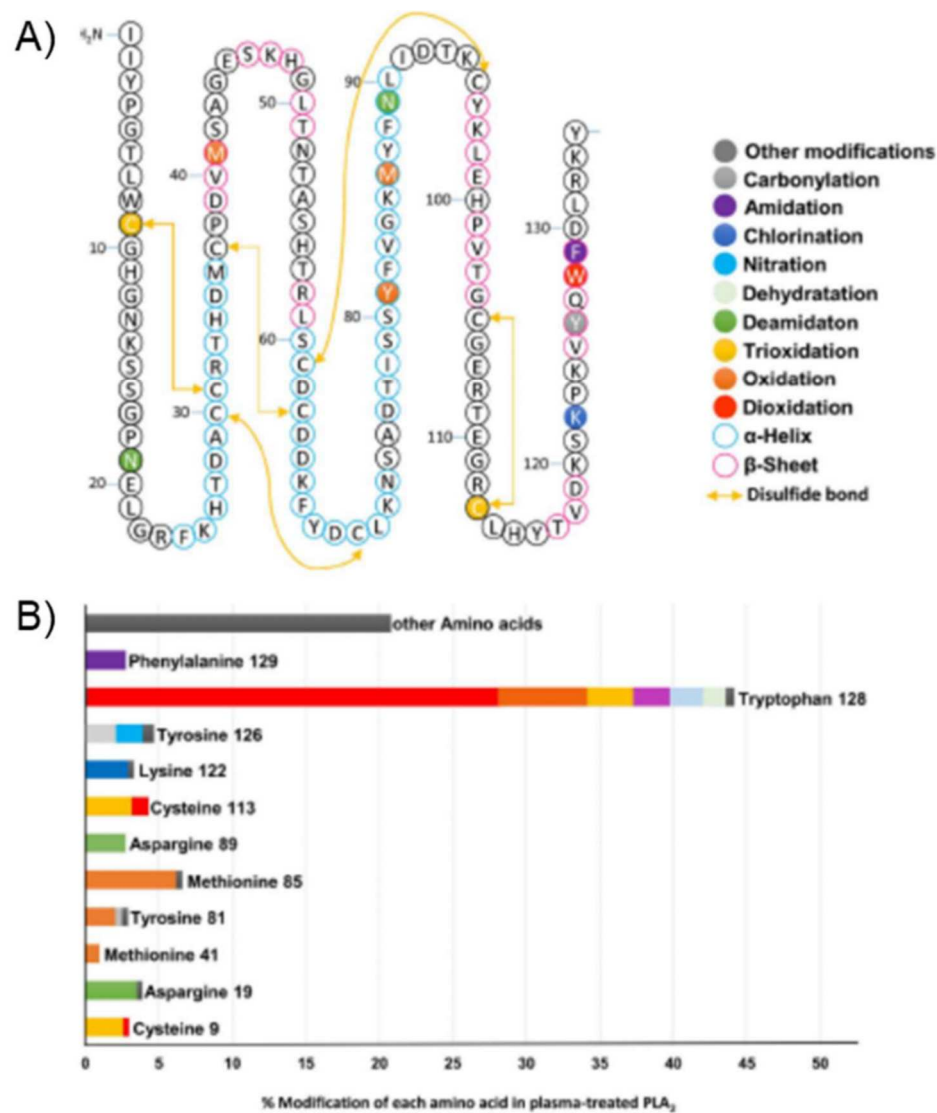
Figure 2.3. The sequence of PLA$_2$ with location (A) and relative amount (B) of ox-PTMs after plasma treatment (kINPen 09, Ar). Values in treated peptides normalized on untreated peptides. *Adapted from Article A3. The illustration was created in collaboration with Seyedali Memari.*

2.2. Identification of oxidative modifications in tissue proteomes

In the previous sections, it could be shown that plasma treatments can induce very different structural modifications for the analyzed proteins or peptides in liquid solutions, in terms of both amount and type of reactive species, potentially reflecting tunable effects also in cells and tissues. In particular, various variables, such as the used plasma source and gas composition, defined the type of modification, which was also specific in relation to the targeted amino acids and their position in the peptide sequence. The liquid chemistry, generated by a plasma, was similarly differentially modulated with the plasma source and plasma treatment. Further open questions include the verification of the plasma effects in complex matrices rather than model solutions, such as cell cultures or even intact or wounded skin.

Additionally, it must be further investigated if the presence of a water compartment acts as a key amplifier of plasma-induced reactions, or if plasma components present predominantly in the gas phase may also impact dry biological matter, such as intact skin.

This topic is of particular interest considering that, due to the easy accessibility and treatability of human skin, cold plasmas are currently applied in various skin-related diseases (e.g., infections, wound healing, cancer, pigmentation diseases), both in in vitro research than in clinical experimental stages [11, 12, 118, 180, 185, 187]. To contribute to the understanding of how plasmas react with complex matrices, results and main conclusions published in Article A4 and Article A5 have been summarized in the following sections. These experiments applied plasma treatments of wounded skin, with consequent oxo-proteomics analysis of the wound exudates (A4), and of intact porcine skin (A5).

2.2.1. Human skin wound exudates (A4)

The deregulation of pathways connected to wound healing following plasma treatments has been already described, showing that the activity and expressions of transcription factors, such NF-kB or HIPPO [118, 180], could be altered. These alterations have been correlated to the promotion of wound healing induced by plasma treatments using kINPen MED with Ar-only. Although downstream effects such as pathways deregulation and visible acceleration of the healing have been observed, the molecular mechanism of plasma effects is still under investigation. In Article A4 it has been performed a proof-of-concept experiment to verify if cold atmospheric plasma can induce oxidative modifications in human wound proteomes. Oxidative modifications, as for enzymatic modifications, are key pathways regulators, and plasma could trigger a signaling modulation through proteins modifications. Although the effects of plasmas in amino acids, peptides, and proteins solutions have been proven, a screening of oxidative modifications occurring in wound proteomes has been investigated only for the first time in Article A4. Here, patients with diabetic-related complications leading to large wounds have been swabbed before and after plasma treatments (Figure 2.4A), and the wound exudates have been submitted to proteomic analysis as previously described.

A targeted analysis of oxidative modifications on proteins has been performed using Byonic, applying the information previously obtained by screening possible modifications occurring on peptides and proteins in solutions. As a result, 330 proteins have been identified, and by comparing the oxidation state before and after plasma treatments, kINPen MED induced an increase in 63% of oxidation (-OH group addition), 44% more nitration (-NO$_2$ group addition), and 69% more nitrosylation (-NO group addition), as shown in Figure 2.4B. These results confirmed, those protein modifications may be the first mechanism of action of CAPs, which could justify their visible clinical effects. In particular, blood proteins (e.g., haptoglobin, albumin, hemoglobin) and proteins belonging to the connective tissue (e.g., fibronectins, keratins) were mainly modified, suggesting that external structural proteins could protect deeper skin layers. This aspect must be considered for targeted approaches and further studies must be planned to define how other proteins could transfer the obtained oxidation potential and translate into downstream effects. Interestingly, treatments with plasma not only induced oxidative modifications but also nitrosative modifications, which in the cell environment finely regulate various pathways, in contrast to reactive oxygen species, which generally lead to necrosis or apoptosis pathways. This aspect highlights the potential of cold atmospheric plasm to be finely tuned for targeted aims, which can promote or inhibit different pathways.
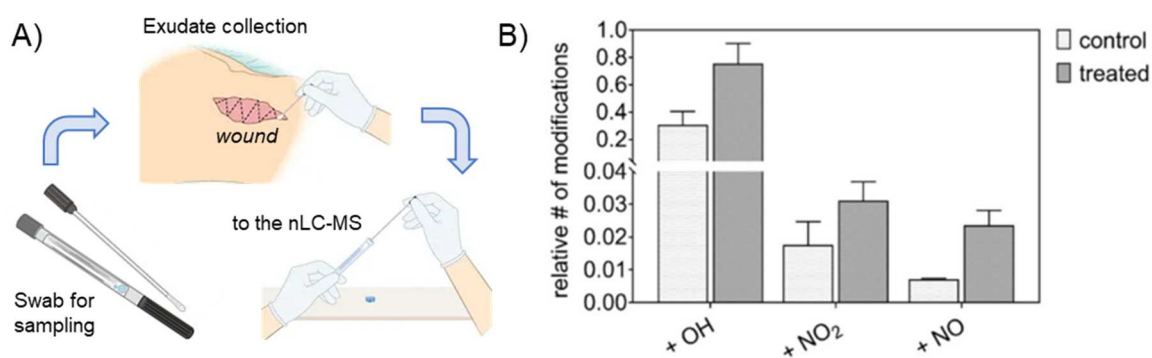
Figure 2.4. Ox-PTMs were identified after plasma treatment (kINPen MED, Ar) of wounds belonging to diabetic patients. Wound exudates were collected and analyzed. Values in treated peptides normalized on untreated peptides. *Modified and adapted from Article A4.*

2.2.2. Porcine epidermal tissues (A5)

The eventuality of changing plasma treatment parameters led to a differential chemistry, and consequentially different biochemical and functional effects on proteins. However, to date, the only certified and clinically approved conditions to be applied using kINPen MED plasmas is the use of Ar-only at 5 slm. The use of other gas compositions is still under investigation, especially in relation to the safety and risks aspects. The eventual change of gas composition will be only possible after reaching some priority milestones, which include the full understanding of which effects Ar-only kINPen MED plasmas can have on human tissues.

In all the studies performed up to date, a major focus has been on the action of plasma-generated reactive species, while the isolated impact on biological structures of plasma radiation has been just studied partially [169]. Additionally, many experiments and studies have been performed in liquid models, an aspect which helped the researcher to highlight that a liquid compartment can be used as a plasma effect amplifier. However, results on how proteins can be modified in dry skin have been not yet elaborated.

To fill this gap, this section includes a summary of the main results shown in Article A5, where porcine skin tissues have been treated with Ar-only kINPen MED. Over treatments with full plasmas, however, which included the impact of reactive species and other plasma-derived particles, the effects of an isolated range of radiation have been evaluated by using various filtering windows between the plasma source and the porcine skin surface. Normally, kINPen MED plasmas can emit from the vacuum UV region (VUV, 100-200 nm) to the infrared region (> 1000 nm), including the UV range (200-400 nm) (Fig. 3, Fig. 4, A5) [169, 195].

As radiation with a shorter wavelength are more ionizing and therefore possibly the most dangerous, the impact of VUV radiation was evaluated by using different windows, such as magnesium fluoride windows (MgF_2), which are permeable to radiation starting from 120 nm, Suprasil-1 windows, permeable from 200 nm, and Borofloat-33 windows, transmitting from 300 nm. In this work, the major focus was centered on the modifications occurring on cysteine, being one of the most sensitive amino acids towards oxidative modifications. Additionally, the effects of plasma in cysteine solutions have been intensely characterized, in previous works and Article A5, and these data allowed a final comparison of effects observed in aqueous or dry models. Additionally, different skin layers have been sampled before and after treatments, allowing the comparison of plasma effects between skin layers.

In comparison to the oxidative modifications observed in cysteine solutions (Fig. 6, A5), where aqueous chemistry was included (Fig. 5, A5), cysteines in the dry tissue proteome were generally less, as reported in Figure 2.5. In particular, the main observed modifications in the tissue proteomes were the addition of another cysteine on a cysteine (forming cystine), cysteine trioxidation (forming sulfonic acids), the breakage of the cysteine C-S bond (forming alanine), the addition of cysteine of a sulfone group (-SO_3, forming cysteine *S*-sulfonate), and cysteine dehydrogenation (Fig 8, A5). Main effects in terms of modified protein cysteines were observed using the full plasmas Ar-only, especially the formation of *S*-sulfonate especially in the third layer, and the C-S bond breakage in both the first and third layer. Here, argon metastables and excimers are highly produced in relation to all other conditions and could be therefore responsible for the observed effects [169].

In contrast, Ar + O_2 conditions, which mainly produced gaseous ROS (e.g., $^\bullet$O, $^1O_2$, O_3) [53, 153], induced mostly cystine formation in the first skin layer, and dehydrogenation in the third layer. These products may be the result of a radical attack to the C-S or C-C bonds, inducing respectively thyil radicals' formation and recombination in cystine, or loss of 2 hydrogens with double bond formation. By filtering only the impact of radiation, dehydrogenation of cysteine was mildly observed in the first and third layer, with a weak increase of *S*-sulfonate in the third layer. The production of *S*-sulfonate overall in the third layer suggests that this modification occurs predominantly not for the direct action of reactive species and other gaseous plasma components, but rather as secondary structural changes triggered by plasmas.

This phenomenon may be connected to the excitation and loss of electrons from the thiol moiety induced by radiation [196, 197]. While especially the impact of VUV radiation produced water dissociation in cysteine solutions or solutions without cysteine (monitored by the production of $^\bullet$OH and H_2O_2, Fig. 5, A5), the effects were minor on dry tissues, as well as for treatments with full plasma. This result suggested that overall, the safety of plasmas can be controlled for various reasons. One reason could be that possibly the scavenging effects of biomolecules could not only mediate the effects of plasmas but also control the negative effects leading to oxidative distress rather than eustress. It could be concluded that the presence of liquid could be used as a compartment to control the further generation of new species, which in turn can be used as an oxidizing cocktail on biological targets. Finally, the effects of VUV radiation could be observed even less in the deeper layers. This shows that although their higher ionization energy, they do not undermine the safety of the plasmas.
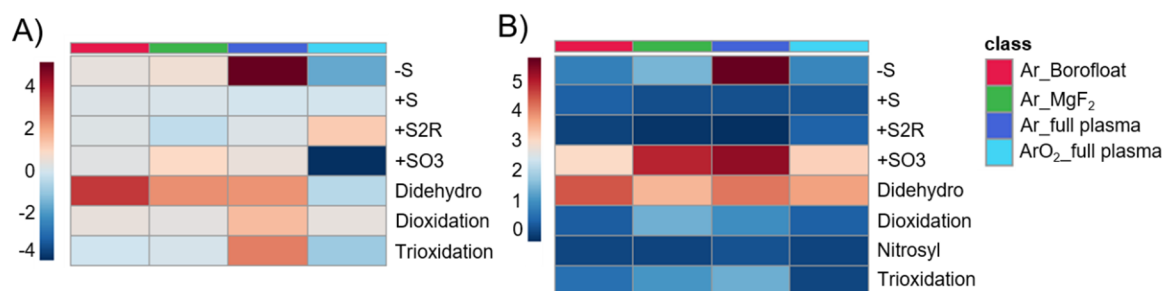

Figure 2.5. Oxidative modifications were identified on the sulfur moieties of the proteome of porcine epidermal skin after plasma treatments (kINPen 09). The first layer (A) and third layer (B) are represented. Values in treated peptides normalized on untreated cysteine-containing peptides. *Adapted from Article A5.*

3. Conclusions & Outlook

Cold atmospheric-pressure plasmas are powerful tools generating oxidative modifications on biomolecules, through the generation in the plasma of bioactive components, especially reactive oxygen and nitrogen species, which production can be further amplified in liquid compartments, generally surrounding cells and tissues. It has been shown that peptides are sensitive structures that can be modified in solutions and biological samples by plasmas.

Oxidative modifications and the consequential structural changes can lead to loss or gain of function of proteins, suggesting that plasmas can be used to modulate complex redox signaling pathways in biomedical applications such as wound healing and cancer treatment. In this work, a screening of possible modifications induced by different CAP sources (kINPen and COST-jet) have been presented and discussed, addressing the objectives i) and ii), regarding the identification of the potential oxidative modifications introduced by cold plasmas in proteins, and the comparison and characterization of the chemistry induced by different plasma sources and treatment parameters. Additionally, different variables, such as gas composition, treatment time, and protein structure, have been identified, and their influence in determining the resulting biochemistry has been presented to improve the controllability of plasma effects in future clinical applications, addressing objective iii). Further conclusions on this objective have been deepened in **Section 3.1**.

In particular, it has been shown that the presence of a liquid vector amplifies the production of species and modifications after plasma treatments, as the water molecules themselves become part of the plasma-chemistry machinery. In contrast, dry biological targets are shown to be modified in a much minor way, supporting the usage of plasma-treated water as a tool itself to be used in various applications (e.g., disinfection). Indeed, in this case, the major target may be rather the open wound, which usually presents a higher level of humification in comparison to the healthy surrounding skin. These results were obtained through the comparison of model solutions containing peptides and proteins with plasma treatments performed on intact porcine skin, in order to address objective iv), regarding the correlation of results obtained from model solutions of peptides and proteins to those obtained treating complex matrices. The present study, finally, enriched the current knowledge in plasma medicine, by connecting in part the translational gap between what is known about plasma chemistry (plasma components produced in the gaseous phase) and plasma biology (deregulated pathways, visible wound improvements).

3.1. Adjusting cold plasma parameters for biomedical applications

A major conclusion supported by this thesis is, that cold plasma can be fine-tuned to affect biological targets differently, inducing specific oxidative modifications in proteins and consequentially, has the potential to regulate in various ways the resulting redox signaling.

Further details about the dependency of peptide modifications on the plasma source and other parameters have been discussed along with all published articles (Articles A1 to A5) and are unified in Figure 3.1. Here, it is possible to assign dependencies from the various parameters for the individual modifications and modified amino acids, in order to be able to predict the influence of a parameter on a certain amino acid for future investigations on other model peptides. In particular, the modulated plasma treatment parameters, as well as different

targets have been used. In Articles A1 and A2, the treatment time, the solvent type, the treatment modality, and the plasma sources have been modulated in treatments of peptide solutions. In Article A3 similar modulations have been applied for treatments of protein solutions. Finally, in Article A4 and A5 plasma parameters have been modulated for the treatment of human wound exudates and porcine skin.

The present work contributed to confirming the results found so far about the analyzed plasma sources in literature and their chemistries (e.g., reactive species on free amino acids) and to assign biological targets in a biological environment, which resulted in the potential biochemical mediator of the visible plasma effectiveness. The results clearly show that not only the treatment time and the distance can influence molecules modification, but also the liquid-type where treatments are performed, the driven gases, and the specific chemistry of target molecules can determine differential plasma effects (see article A2). CAPs are currently used in clinical applications for dentistry and wound healing disorders (e.g., diabetes) and plasma medicine is recently spreading further in cancer treatment, where first successes have been recorded in animal model studies. For the future of plasma medicine and in order to avoid side effects from plasma treatments, it must therefore be ensured that all necessary plasma parameters which affect differently the chemistry and biochemistry of target biomolecules are accurately identified and characterized. Only then it is possible, that these fundamental findings contribute to ensuring, that each pathophysiological problem can eventually receive the optimal plasma treatment condition.

3.2. Outlook: CAPs and clinical applications

As the one focus of the plasma research is to underlie relevant molecular mechanisms of CAPs, the present work intended to fill this knowledge gap regarding CAPs interaction and effects with key biomolecules in the cells, which are proteins. Proteins play a crucial role in many biochemical signaling pathways, especially in the redox signaling. They can stimulate the activation or inactivation of these circuits, being also key reactions accelerators in form of enzymes. Activities and stabilities of proteins can be changed after structural modification, leading to the alteration of relevant pathways. These processes are of interest in various bio-medical fields, such as to promote healing processes or induce apoptosis/necrosis pathways in cancer cells. Regarding redox signaling, these modifications can be attached to proteins by chemically reactive species, such ROS and RNS. With the results obtained in the present thesis, it could be concluded that CAPs are a source of ROS and RNS, which pattern can be differentially produced by varying plasma treatments.

Using the modifications induced on protein structures, the liquid chemistry of CAPs was elucidated, proposing a tool to tune plasmas for biomedical applications. To deepen the research connected to the molecular mechanisms of plasmas, a different pattern of oxidative modifications induced in proteins was identified for different conditions, suggesting a potential bridge connecting CAPs with the previously observed physiological effects. Indeed, plasma-modified proteins are candidate biomolecules able to alter the redox signaling and induce defined effects. The results obtained in the model amino acids aqueous solutions have been applied to complex structures, confirming the potential controllability and effectiveness of plasmas in clinical applications. However, the oxidative modifications found are only a first indication of the interaction of plasma and proteins, peptides, and amino acids.
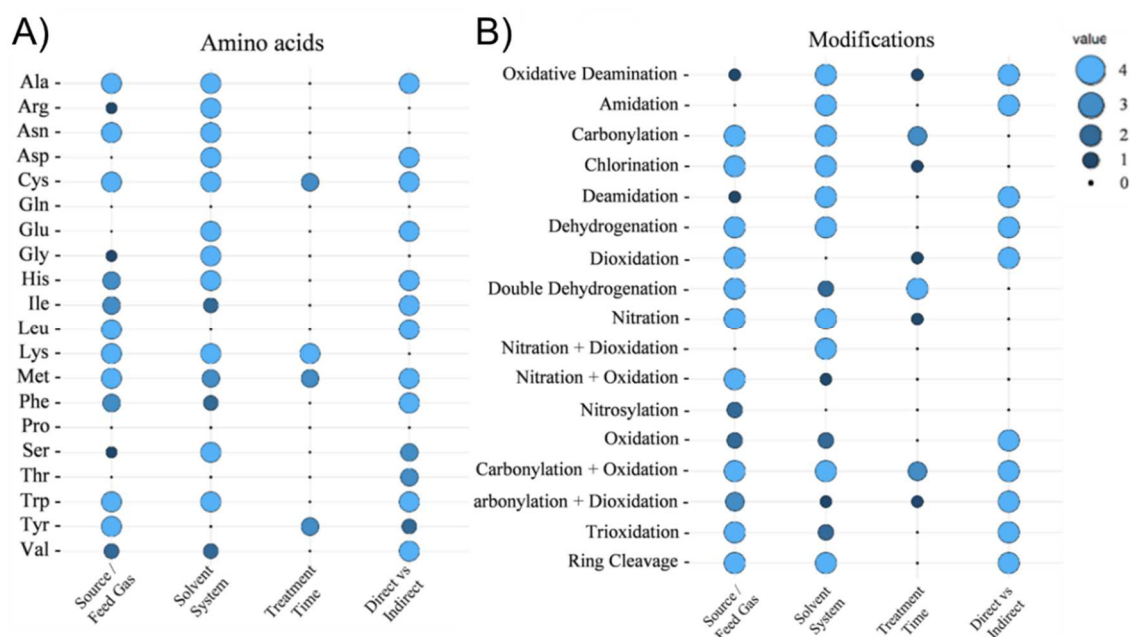
Figure 3.1. Using ANOVA-test calculated dependencies of amino acids (A) and modifications (B) related to the gas composition of the plasma, solvent, treatment time, and treatment conditions (direct or indirect). Significance levels: "4" $p \leq 0.001$; "3" $p \leq 0.01$; "2" $p \leq 0.05$; and "1" $p \leq 0.1$. *Adapted from Article A2.*

The targeted search for the modifications and their influence on the various biochemical signaling pathways remains an exciting field to be investigated. The open search for modifications at model structures such as known peptides after plasma treatment has been an important tool to screen unknown modifications that CAPs can introduce into proteins. However, this untargeted search could not be expanded in complex biological samples due to long measurement times, reason why in these cases targeted analyses have been performed.

In the future, mass spectrometry will be able to assert itself as the ideal method, since new, faster, and higher-resolution mass spectrometers are constantly being developed. The remaining difficulty will be the correct analysis and interpretation of the obtained data and to do this within a reasonable time period since a wildcard search with a mass range of just 100 Dalton increases the search time approximately 100-fold. The progress of the MS field and connected method must be considered in future also to support a better interpretation of the acquired data, potentially being able to identify isomeric and isobaric structures, and to discriminate the position and the elemental composition of specific functional groups, even when in low amounts. These types of mixed modifications must be considered and accurately defined, due to the complexity of the plasma chemistry, to better optimize and standardize processes and consequences of CAPs, and to finally certify them in clinical applications.

In this context, the present thesis filled a relevant research gap by applying the strategy of carrying out firstly experimental studies on suitable models' solutions of protein structures, which could be transferred to more complex samples. The obtained results, indeed, could be in future used also for the analysis of proteomes after biomedical treatments of patients' skin with plasmas, which could be itself a tool to monitor the progression and the effectiveness after defined treatments in clinical settings.

Of course, there are still several open questions that need to be clarified before then. So far, there is no exact information on how deep the different species can penetrate tissue or cells or up to which layer depth modifications can be detected and thus the impact of plasma.

Various model experiments are already available for this [198-200], but these usually only concern the direct detection of the species and not possible downstream reactions. With the results shown in this thesis it could be shown that plasma-generated reactive species and radiation have not massive effects in dry skin layers, highlighting the importance of the aqueous vehicle to amplifying the gaseous plasma chemistry. However, further studies must be performed to complement these results, as the influence of plasma is often only detectable after days or weeks and thus only long-term changes in the tissue or cell can be detected.

Another important aspect is the influence of the other plasma parameters (for example radiation) on the identified changes (modifications) at proteins and enzymes. The data obtained here provide the basis for the identification of atmospheric plasma-induced oxidative modifications in highly complex samples such as wound samples or whole proteome samples with thousands of proteins per sample. Finally, these results can be helpful for the plasma biomedical community to clarify further the mechanisms of action of CAP. As different conditions have been associated with the production of a specific pattern of reactive species and relative amino acids and protein modifications, these results represent the base to support in future clinicians in the application of specific tuned plasma conditions to achieve desired biological effects. To conclude, part of the gap observed between the knowledge regarding plasma physics/gas chemistry and observed downstream physiological effects of plasmas has been fulfilled with the present work.

4. Summary

This thesis had the stated aim of investigating which oxidative modifications can be found on proteins and peptides after they have been treated with different cold atmospheric plasma conditions, in order to give possible explanations of how these modifications can affect downstream signaling processes. All structural changes induced on two model peptides in solutions were first investigated after treatment with different plasma conditions. Experimental data have been evaluated and compared with existing in silico simulations, which are currently the major method applied in the plasma field to study the chemistry in liquids of CAPs (Article A1). In experimental results, plasmas could generate a broad range of modifications at different amino acids, especially different degrees of oxidation, nitration, and nitrosylation.

These results have been complemented with a systematic investigation of ten artificially generated peptide library (Article A2). It could be observed that the general reactivity of an amino acid can be affected by the position or by the neighboring amino acids. Finally, the target chemistry and the amino acids positioning resulted to be relevant aspects influencing plasma-mediated protein oxidation. Cysteine, tyrosine, and tryptophan were the amino acids mostly modified, assuming the potential role in the plasma treated proteome of redox signaling modulators. Additionally, it could be demonstrated that there is unique plasma chemistry in the respective used plasma source and treatment conditions, which can be specifically modulated by different variables (e.g., driven gases, admixture gases, treatment duration, target liquid composition, target chemistry). Therefore, unique modifications, such as carbonylation, chlorination, oxidation, nitration, were detected almost exclusively in certain conditions. The production of specific reactive species cocktails under defined conditions was finally associated with a specific oxidative modification pattern in proteins, potentially leading to controllable biological effects in more complex environment.

The potential of plasmas in inducing oxidative modifications was investigated also in solutions of intact protein (Article A3). PLA_2 was treated with CAPs and examined for oxidative modifications that may explain the reduced activity found after such plasma treatment. It could be shown that one particular amino acid, tryptophan 128, was strongly oxidized, leading to a structural change in the enzyme with reduced functionality.

Therefore, it was shown that the chemical activity of an enzyme could be specifically altered by plasma-induced oxidative modifications, providing an important building block for the elucidation of the mode of action of CAPs on the biological target proteins. In the last part of the work (Articles 4 and 5), the results found so far in model experiments were successfully transferred to real protein samples, respectively analyzing wound exudates and skin proteomes. In particular, modifications identified in model solutions of amino acids were analyzed in the cell proteomes in a targeted manner. Here, it was shown that plasma can induce modifications at defined sites of certain proteins, inducing both nitrosative and oxidative stress in a controllable manner, and inducing downstream pathways modulation. With the studies performed in A5, it has been shown that reactive species mainly influence the modifications of proteins, while plasma radiation play only a minor role, also in dry sample types such skin.

To conclude, specific plasma conditions producing characteristic chemical cocktails have been associated to defined protein modifications, consisting in a further important step for the understanding and controllability of cold atmospheric plasma in medical applications.

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6. Original Publications

The present work is based on peer-reviewed articles published or submitted in international scientific journal. These are listed below together with the authors' contribution.

Article A1

S. Wenske, J.-W. Lackmann, S. Bekeschus, K.-D. Weltmann, T. von Woedtke and K. Wende. "Nonenzymatic post-translational modifications in peptides by cold plasma derived reactive oxygen and nitrogen species". *Biointerphases*, 15 (6), 061008, 2020.

The experiment was designed by SW, JWL and KW. The measurements, data evaluation and visualization were performed by SW and JWL. The manuscript was written by SW, JWL and KW and edited by all co-authors.

Article A2

S. Wenske, J.-W. Lackmann, L.-M. Busch, S. Bekeschus, T. von Woedtke and K. Wende." Reactive species driven oxidative modifications of peptides—Tracing physical plasma liquid chemistry". *J. Appl. Phys.*, 129 (19), 193305, 2021.

The experiment was designed by SW, JWL and KW. The measurements, data evaluation and visualization were performed by SW, LMB and KW. The manuscript was written by SW and KW and edited by all co-authors.

Article A3

Z. Nasri, S. Memari, S. Wenske, R. Clemen, U. Martens, M. Delcea, S. Bekeschus, K.-D. Weltmann, T. von Woedtke and K. Wende. "Singlet Oxygen-Induced Phospholipase A2 Inhibition: a Major Role for Interfacial Tryptophan Dioxidation", *Chem.* Eur. J., 27 (59), 14702, 2021.

The experiment was designed by ZN and KW. The electrochemical experiments and data evaluation were performed by ZN and SM. The mass spectrometric experiments and data evaluation was performed by SW and SM. The manuscript was written by ZN, SM, SW, RM and edited by KW.

Article A4

G. Bruno, S. Wenske, J.-W. Lackmann, M. Lalk, T. von Woedtke, and K. Wende. "On the liquid chemistry of the reactive nitrogen species peroxynitrite and nitrogen dioxide generated by physical plasmas". *Biomolecules*, 10 (12), 1687, 2020.

The experiment was designed by GB and KW. The experiments and data evaluation were performed by GB and SW for the proteomics data elaboration. The manuscript was written by GB and edited by SW, JWL and KW.

Article A5

G. Bruno, <u>S. Wenske,</u> H. Mahdikia, T. Gerling, T. von Woedtke, and K. Wende. "Radiation driven chemistry in biomolecules – is (V)UV" involved in the bioactivity of argon jet plasmas?". *Frontiers in Physics*, 9, 759005, 2021.

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Article A1

Nonenzymatic post-translational modifications in peptides by cold plasma-derived reactive oxygen and nitrogen species

S. Wenske, J.-W. Lackmann, S. Bekeschus, K.-D. Weltmann, T. von Woedtke and K. Wende.
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Nonenzymatic post-translational modifications in peptides by cold plasma-derived reactive oxygen and nitrogen species

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ABSTRACT

Cold physical plasmas are emerging tools for wound care and cancer control that deliver reactive oxygen species (ROS) and nitrogen species (RNS). Alongside direct effects on cellular signaling processes, covalent modification of biomolecules may contribute to the observed physiological consequences. The potential of ROS/RNS generated by two different plasma sources (kINPen and COST-Jet) to introduce post-translational modifications (PTMs) in the peptides angiotensin and bradykinin was explored. While the peptide backbone was kept intact, a significant introduction of oxidative PTMs was observed. The modifications cluster at aromatic (tyrosine, histidine, and phenylalanine) and neutral amino acids (isoleucine and proline) with the introduction of one, two, or three oxygen atoms, ring cleavages of histidine and tryptophan, and nitration/nitrosylation predominantly observed. Alkaline and acidic amino acid (arginine and aspartic acid) residues showed a high resilience, indicating that local charges and the chemical environment at large modulate the attack of the electron-rich ROS/RNS. Previously published simulations, which include only OH radicals as ROS, do not match the experimental results in full, suggesting the contribution of other short-lived species, i.e., atomic oxygen, singlet oxygen, and peroxynitrite. The observed PTMs are relevant for the biological activity of peptides and proteins, changing polarity, folding, and function. In conclusion, it can be assumed that an introduction of covalent oxidative modifications at the amino acid chain level occurs during a plasma treatment. The introduced changes, in part, mimic naturally occurring patterns that can be interpreted by the cell, and subsequently, these PTMs allow for prolonged secondary effects on cell physiology.

I. INTRODUCTION

Cold physical plasma is increasingly used for various biomedical applications. Besides decontamination and surface optimization for medical products such as implants,[1,2] acute and chronic wound management,[3] and cancer treatment[4,5] are major fields of research and (pre)clinical use. Specifically, the controllable mix of short- and long-lived reactive oxygen and nitrogen species (ROS/RNS) in combination with electrical fields and UV radiation allows plasmas to interfere with cellular signaling processes on different levels.[6–8] Besides a direct interaction of ROS with cellular signal receptors, e.g., peroxiredoxins (reviewed in Ref. 9), short-lived reactive species such as singlet oxygen $[O_2(^1\Delta_g)]$, atomic oxygen O, or peroxynitrite $(ONOO^-)$ may introduce covalent chemical changes in a range of biomolecules, thereby influencing their structure and activity.[10–13] Assumingly, such covalent modifications contribute to the observed physiological impact of plasma specifically when the resulting

structure resembles biologically active post-translational modifications (PTMs). However, mechanisms and extent remain to be elucidated.

Post-translational modifications are versatile "customizations" of proteins and peptides, controlling their activity, fate, and lifetime.[14,15] Often, they are enzymatically introduced after protein translation, and more than 200 biologically relevant types of PTMs have been identified,[16] with phosphorylations, ubiquitination, and glycosylations being the most frequent.[17] However, the database Unimod (http://www.unimod.org/) contains 1500 entries of protein modifications (May 2020), many of those with unknown impact in biological systems, showing the complexity of the research area. Also, nonenzymatic routes have been described and were found relevant for biological processes,[18,19] this time focusing on oxidative structures including hydroxylation, carbonylation, and nitration.[20] The proteome-wide identification of enzymatic and nonenzymatic PTMs by high-resolution mass spectrometry is a challenging approach receiving increasing attention.[21,22]

Here, the impact of the cold plasma treatment on two model peptides, angiotensin 1–7 and bradykinin, was investigated. The widely used argon based plasma jet kINPen and the helium based plasma jet COST-Jet were tested for their ability to introduce nonenzymatic post-translational modifications and to infer on the underlying chemical reactions and reactive species, respectively. Both plasma sources vary significantly in design and reactive species output.[23–26] Bradykinin is a small pro-inflammatory peptide in mammals, which is composed of nine amino acids (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), and angiotensin 1–7 (Asp-Arg-Val-Tyr-Ile-His-Pro) is the amino-terminal fragment of the angiotensin I/II involved in blood pressure regulation. The amino acids present contain hydrophobic side chains (valine and isoleucine), aromatic side chains (tyrosine and phenylalanine), charged (arginine and histidine) or uncharged side chains (asparagine and serine), and some special cases (glycine and proline) and represent a suitable range of target structures. Of note, no sulfur-containing amino acid is present in both peptides since previous experiments revealed a strong oxidation of thiol groups by reactive species, precluding an unbiased view of the other amino acids' reactivity.[27,28]

Recent work by various authors rose awareness to discriminate between interface and bulk reactions in liquids that are in contact with a plasma discharge or the active effluent,[29–33] indicating that the short-lived species such as hydroxyl radicals dominate the downstream chemistry. This is addressed in a molecular dynamics simulation study on the impact of hydroxyl radicals on bradykinin and angiotensin published by Verlackt et al.,[34] allowing to pre-estimate target structures for the current approach and to identify relevant reactive species by comparison between theoretical and experimental results. Using the peptides as target molecules that preserve the impact of reactive species in a covalent chemical bond allows taking snapshots of the plasma chemistry at the interface and bulk simultaneously, augmenting electron paramagnetic resonance spectroscopy data present for the kINPen.[35,36]

In conclusion, two different plasma sources are used to tackle the following questions: First, which PTMs are induced nonenzymatically by ROS/RNS and which amino acids are the major targets? Second, which reactive species are responsible for these modifications? And third, are the experimental results congruent with the simulation results? High-resolution mass spectrometry coupled to nanoflow liquid chromatography (nanoLC-MS/MS) along with bioinformatics tools was applied to identify and quantify the oxidative post-translational modifications resulting from the treatment by the two applied plasma sources. The results confirm the relevance of secondary effects in cold plasma effects.

II. EXPERIMENT

A. Treatment conditions and plasma sources

Angiotensin 1–7 and bradykinin (Sigma-Aldrich-Chemie GmbH, Germany) were dissolved in $500\,\mu$l of double distilled H_2O (Millipore) with a concentration of 0.2 mg/ml. The water was thoroughly degassed by bubbling with argon gas for 30 min to reduce background reactions. For the treatments, we used the kINPen (neoplas tools GmbH, Germany)[37] and the COST Reference Microplasma Jet (COST-Jet is the outcome of the European COST action MP 1101 "Biomedical Applications of Atmospheric Pressure Plasma Technology").[38] The kINPen consists of a grounded ring electrode and a ceramic capillary, where a centered rod electrode is located inside (Fig. 1 right). A voltage of $2–6\,kV_{pp}$ is applied to the central electrode with a frequency of 1.1 MHz. Argon gas (purity 99.999%) was used as a working gas with a flow rate of 3 standard liters per minute (sLm).[39] For some treatments, 0.5% of the working gas was replaced with N_2, O_2, or with a mixture of both gases (15 SCCM each). The COST-Jet is a device that was developed by the European COST action MP 1101 as a reference device for plasma sources. It consists of two 1 mm thick metal plate electrodes spaced 1 mm apart in between which the plasma is ignited (Fig. 1 left). A Tektronix DPO 4104 Digital Phosphor Oscilloscope was used to measure the time resolved voltage for the plasma. For pure helium experiments, the plasma was sustained to 143 mV, for helium/oxygen to 145 mV, for helium/nitrogen to 159 mV, and for helium/oxygen/nitrogen to 165 mV. The treatments were performed at a sample distance of 4 mm and 1 sLm helium. If desired, 0.5% oxygen, nitrogen, or both gases were added (5 SCCM each). All treatments were done in 24-well plates and placed 4 mm (COST-Jet) or 9 mm (kINPen) away from the liquid surface for 30 or 60 s. After each individual treatment, samples were placed on wet ice and immediately analyzed by nanoLC-MS/MS to minimize postdischarge reactions (see Sec. II B). Three independent experiments were performed.

For further characterization of the two plasma sources, the long-lived species H_2O_2, nitrate (NO_3^-), and nitrite (NO_2^-) (Fig. 2) were also determined for the different compositions of gases. The quantification of nitrite and nitrate was performed with ion chromatography (ICS-5000, Thermo Fisher Scientific). For the separation, an IonPac AS23 anion exchange column (2×250 mm, Thermo Fisher Scientific) and an isocratic mobile phase (4.5 mM Na_2CO_3/0.8 mM $NaHCO_3$) with $250\,\mu$l/min were used. Hydrogen peroxide was quantified via the colorimetric reaction with xylenol orange using a commercially available assay (Pierce Quantitative Peroxide Assay Kit, Thermo Scientific) according to the manufacturer's protocol.

B. nanoLC-MS/MS acquisition

An UltiMate 3000 RSLCnano system equipped with an Acclaim Pepmap C18 column (150 mm $\times 75\,\mu$m, $2.0\,\mu$m particle
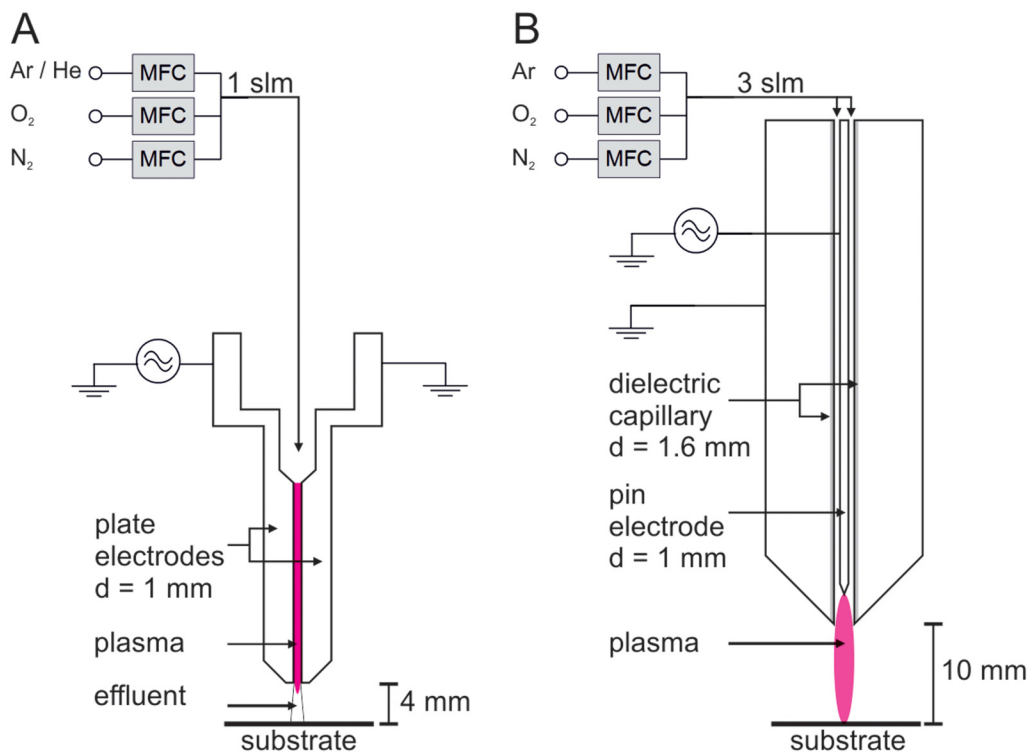
FIG. 1. Schematics of the COST-Jet (a) and kINPen (b). MFC, mass flow controller; sLm, standard liter per minute. Reprinted with permission from Lackmann *et al.*, Sci. Rep. **8**, 7736 (2018). Copyright 2018 Author(s), licensed under a Creative Commons Attribution (CC BY) License.
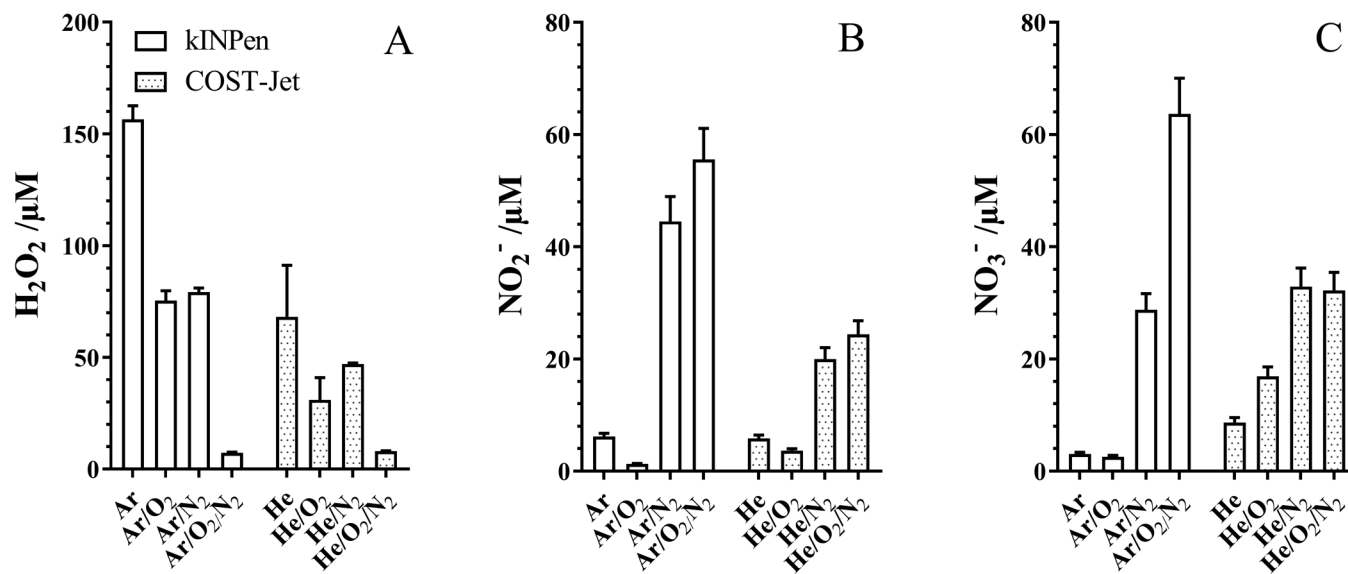

FIG. 2. Measured concentrations of H_2O_2 (a), nitrite (b), and nitrate (c) in ultrapure MS water after 60 s kINPen or COST-Jet treatment for various gas compositions (0.5% of molecular gases added in part). For the exact determination of the concentration, see text. Mean of three replicates + SD.

TABLE I. Flow gradient for LC separation of model peptides.

Eluent B (%)	Time (min)	Flow (μl/min)
12	0	0.400
12	8	0.400
20	10	0.300
22	26	0.400
80	29	0.500
80	31	0.500
12	32	0.400
12	35	0.400

size, Thermo Fisher Scientific) and corresponding precolumn and a QExactive Hybrid-Quadrupol-Orbitrap mass spectrometer from Thermo Fisher was used. The injection volume was $1\,\mu$l, equaling 200 ng of sample. A step gradient (see Table I) consisting of water/ 0.1% v/v acetic acid (A) and acetonitrile/0.1% v/v acetic acid (B) was applied to achieve separation of modified peptides. All samples were injected twice.

MS acquisition was performed in a data dependent Top 10 mode (full MS/dd-MS/MS) at resolving powers of 70.000 in MS mode and 17.500 in MS/MS mode. A Nanospray Flex electrospray ion source was used in positive mode, and the spray voltage was set to 2.30 kV using stainless steel emitters. The temperature of the transfer capillary was set to 250 °C. The collision energy in the higher-energy collisional dissociation cell was fixed at 27.5 V (HCD fragmentation). The observed mass shifts allowed identification of the peptide sequence and to pinpoint modified amino acids.

C. Peptide and post-translational modification identification

Raw data analysis was achieved by the Proteome Discoverer 2.2 software (Thermo Fisher Scientific) using Byonic (Protein Metrics),[40] Version 3.4.1, as a plug-in to identify post-translational modifications. This tool significantly extents search options for peptide modifications. Based on the previous work,[12,41,42] an inclusion list of expected oxidative modifications was compiled (Table II). In the search, precursor mass tolerance was set to 3 ppm and fragment mass tolerance to 10 ppm. A false discovery rate of 1% was accepted. Due to calculation power restraints, maximum three PTMs were allowed per peptide. The spectra were examined manually to ensure the presence of specified modification. To obtain semiquantitative data, the number of spectra matching a detected modifications summed up (PSM counting).[43]

III. RESULTS AND DISCUSSION

Previous investigations on isolated amino acids determined a broad range of chemical structures to be susceptible to the impact of plasma-derived reactive species.[12,29,44] For one, it remained to be clarified whether similar covalent changes occur in a peptide chain, since the competition for targets or species may occur. For another, it should be determined whether these modifications relate to physiological PTMs since they may contribute to the observed physiological consequences of cold plasma.[8,45]

TABLE II. List of preferred native (oxidative) post-translational modifications searched in angiotensin 1–7 and bradykinin. A given mass shift may reflect different chemical structures depending on the target structure.

Monoisotopic mass shift (Da)	Elemental composition	Name/potential product
+15.994 915	+O	Hydroxy
+31.989 829	+2O	Hydroperoxide/dihydroxy
+47.984 744	+3O	Trihydroxy/sulfonic acid/ hydroxyformylkynurenin
+28.990 164	+N + O − H	Nitrosylation
+44.985 078	+N + 2O − H	Nitration
+0.984 016	−N − H + O	Deamidation
−0.984 016	+N + H − O	Amidation
+13.979 265	+O − 2H	Oxo, carbonyl
+29.974 179	+2O − 2H	Dione (quinone)
+ 45.969 094	+3O − 2H	Dione (quinone) + hydroxy
−2.015 65	−2H	Didehydro
+4.978 93	+2O − N − C − H	Formylasparagine
+33.961 028	+Cl − H	Chlorination

A. Oxidative PTMs observed for kINPen treatments

The argon-driven kINPen showed a significant impact on the amino acids in both angiotensin 1–7 and bradykinin (Figs. 3 and 4). The reactivity toward the plasma-derived species differs by amino acid, its position within the peptide chain, the gas phase composition, and the plasma source (kINPen or COST-Jet). The highest numbers of post-translational modifications were observed for tyrosine (Y), isoleucine (I), and histidine (H) in the angiotensin 1–7 and phenylalanine (F) and proline (P) in bradykinin. Interestingly, the proline in angiotensin 1–7 (position 7) remains almost unmodified, while at the positions 3 and 7 of bradykinin, it is significantly attacked. In the same molecule, proline at the position 2 remains scarcely attacked by the plasma-generated species. A similar behavior was observed for phenylalanine of bradykinin at positions 5 (center) and 8 (carboxy terminal end). Assumingly, steric hindrances and local variations of the electron densities of heteroatoms or aromatic rings contribute to the observed divergence in reactivity. In Fig. 4, the most commonly observed modifications for angiotensin 1–7 (A) and bradykinin (B) are shown. For pure argon discharge, the most frequent modifications are the oxidation (Δm +15.99 Da) and nitration (Δm +44.99 Da) at the amino acids tyrosine and isoleucine of angiotensin and oxidation of phenylalanine at position 5 of bradykinin. Additionally, dioxidations (Δm +31.99 Da) were found at histidine (angiotensin) and proline (bradykinin, positions 3 and 7). A ring cleavage forming formylaspargine (Δm +4.98 Da) was found for histidine according to the literature driven by singlet oxygen.[46] Almost no modifications of the arginine moiety were observed. With respect to the positively charged guanidinium group, a significant impact of the electron-rich (and in part negatively) charged reactive oxygen species was assumed. However, in accordance with qualitative
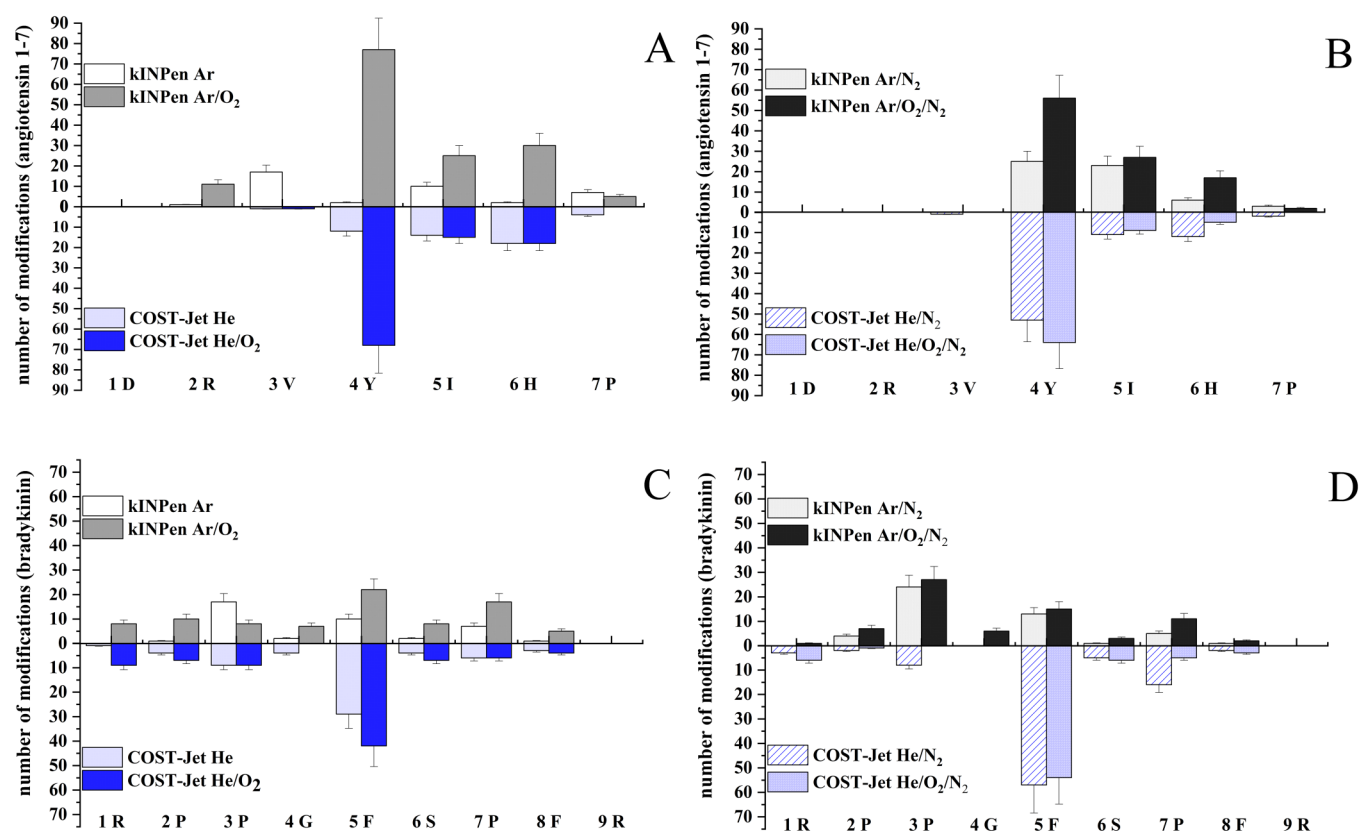
FIG. 3. Observed extent of modifications at angiotensin 1–7 (a) and (b) or bradykinin (c) and (d) after plasma treatment (60 s of kINPen/COST-Jet). Argon/helium was enriched with 0.5% molecular gases where necessary. Mean of three replicates/two injections + SD.

observations,[12] arginine shows a remarkably resilience to reactive species that is lifted to some extent by ROS-rich Ar/O_2 discharges.

If O_2 is added to the working gas, nitrations disappear in favor of oxygen-dominated PTMs, reflecting changes in the gas phase and liquid phase chemistry.[47,48] Oxygen enriched plasmas are known to produce O_3, $^\bullet O$, and $^1O_2$,[49] species that are strong oxidants[50] with high prevalence in the gas-liquid interface.[29] Experimental evidence is the observation of a modification on tyrosine of Δm +47.98 Da with three oxygen atoms and the increase in Δm +31.99 Da mass shifts (e.g., isoleucine hydroperoxide and double oxidized histidine). Interestingly, it is the only condition where arginine is oxidized, implying a role for atomic and singlet oxygen.

When nitrogen was added to the working gas, the aromatic amino acids become the major target. The nitration of phenylalanine occurs only under these conditions in bradykinin, and mono-hydroxylation of the aromatic rings in either Phe or Tyr was found. The oxidation of proline (Δm +15.99 Da) at position 3 of bradykinin was observed only here, indicating that this modification is destroyed by harsher oxidizing conditions as in Ar/O_2.

For the kINPen argon/O_2/N_2 plasma, nitrogen-containing modifications were frequent, with nitrosylation of phenylalanine as

a marker for the presence of nitric oxide standing out in bradykinin. In the angiotensin 1–7 peptide, nitrotyrosine is formed via reaction with peroxynitrite $ONOO^-$,[51,52] which yields at the gas-liquid interface from the reaction of superoxide ($O_2^{\bullet -}$) and nitric oxide ($^\bullet NO$),[53] and it is already shown that this molecule also occurs in physical plasma.[54] This nitrotyrosine is not formed when oxygen or nitrogen alone is used as an admixture. This supports the notion that nitric oxide is formed at the interphase by the reaction of nitrogen dioxide radicals and atomic oxygen and that it serves as a reactant for the formation of peroxynitrite,[55] and such requires both ROS and RNS.

B. Oxidative PTMs observed for COST-Jet treatments

For COST-Jet, similar amino acid modifications were observed (Fig. 3). Their overall number is lower than for the kINPen. This is particularly evident for the 60 s helium plasma treatment of angiotensin [Fig. 3(a)]. While the same three amino acids, tyrosine, isoleucine, and histidine, are modified, its total extent is only 25%–30% of the kINPen treatments. The main modifications of tyrosine are quinone formation (Δm +29.97 Da) and monoxidation (Δm +15.99 Da). The structure is shown in Fig. 5(a). In bradykinin,
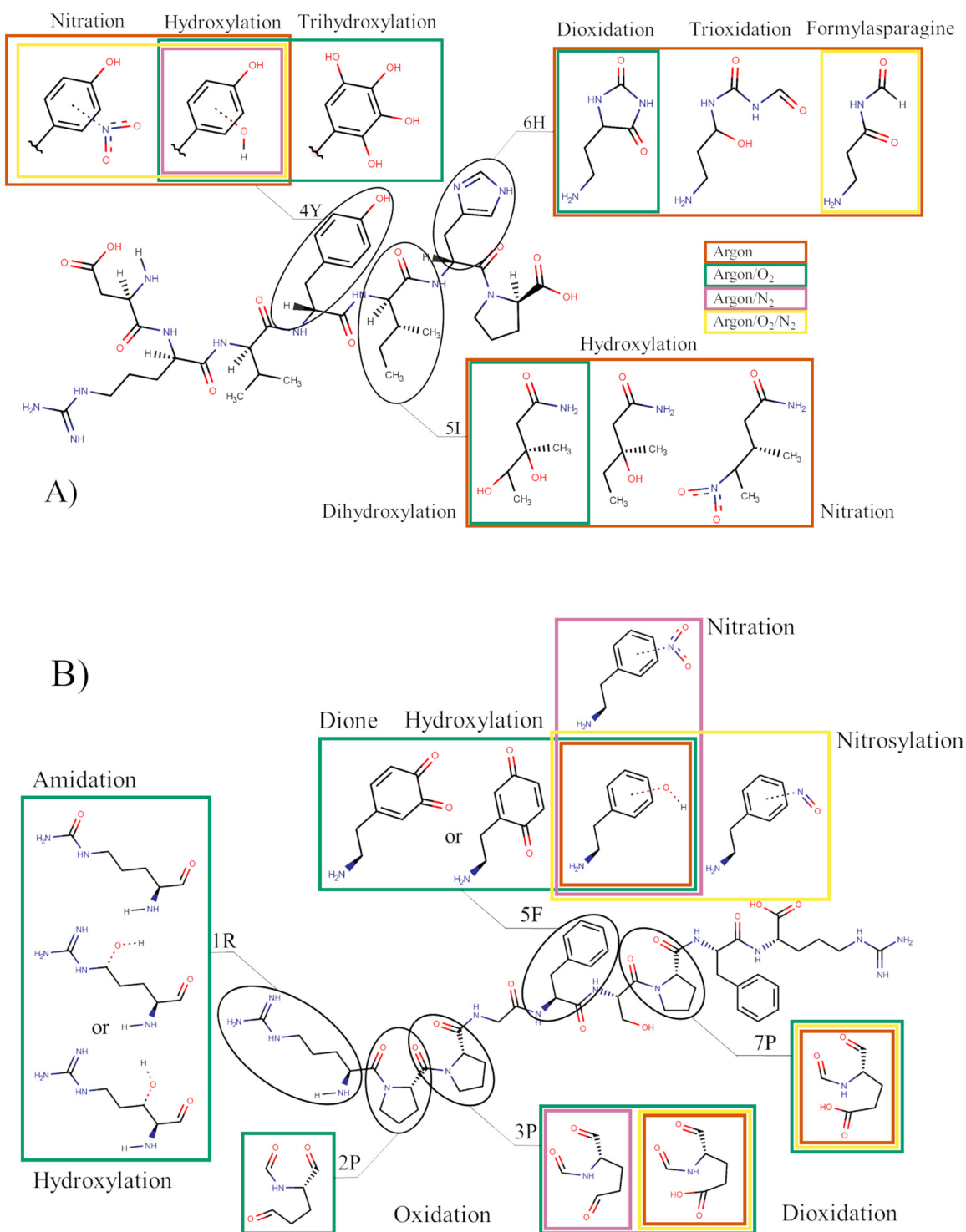
FIG. 4. Most common oxidative modifications for angiotensin 1–7 (a) and bradykinin (b) after 60 s kINPen treatment. The peptides amino acid sequence is drawn in full, the colored rectangles indicate the observed changes to the molecule. The gas phase compositions have a significant impact: argon (orange), Ar/0.5% O_2 (green), Ar/ 0.5% N_2 (pink), or Ar/0.5% O_2 and 0.5% N_2 (yellow). Not all modifications are present at the same time, see text.
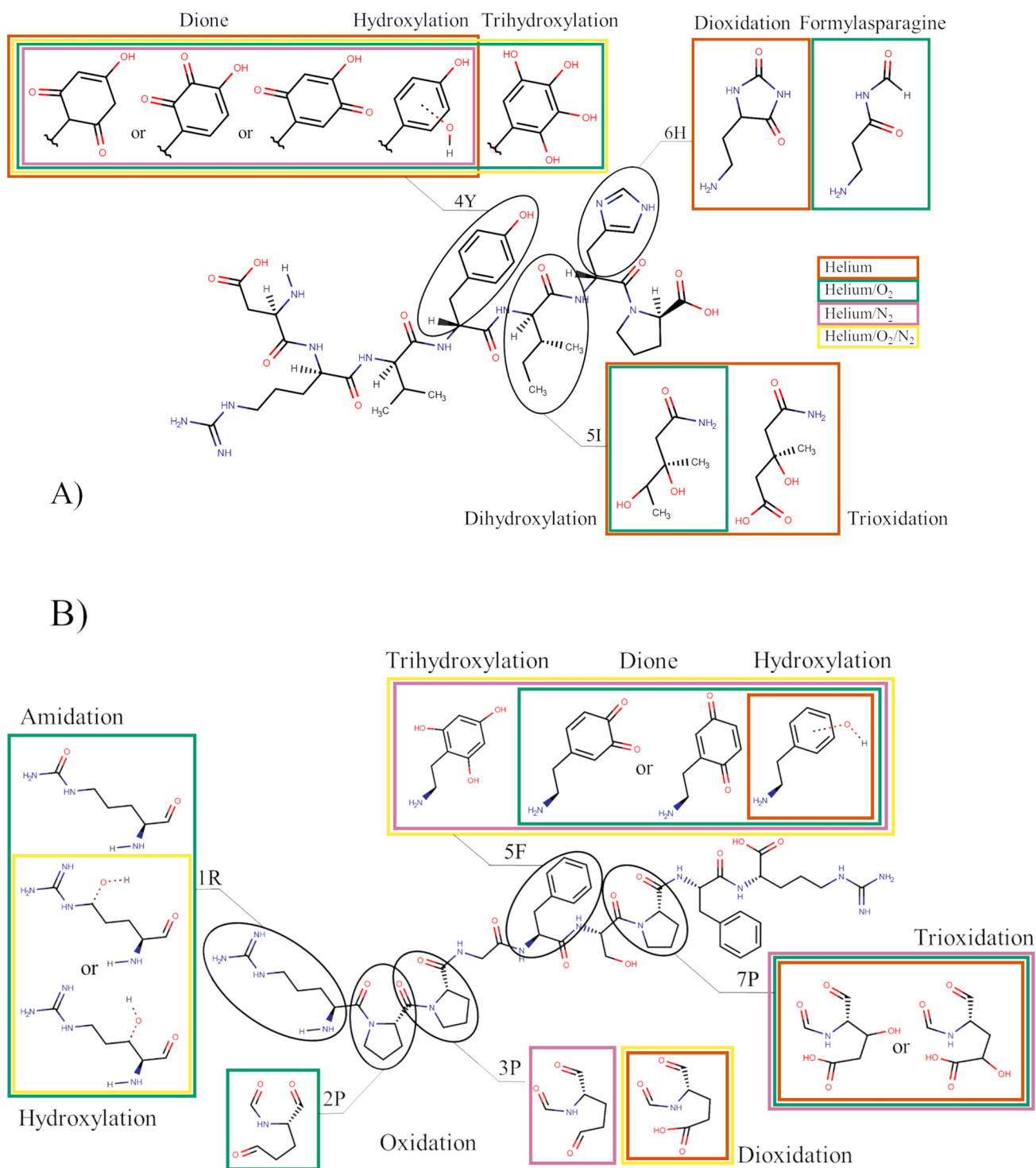
FIG. 5. Most common oxidative modifications for angiotensin 1–7 (a) and bradykinin (b) after 60 s COST-Jet treatment. The peptides amino acid sequence is drawn in full, the colored rectangles indicate the observed changes to the molecule. The gas phase compositions have a significant impact: helium (orange), He/0.5% O_2 (green), He/0.5% N_2 (pink), or He/0.5% O_2 and 0.5% N_2 (yellow). Not all modifications are present at the same time, see text.
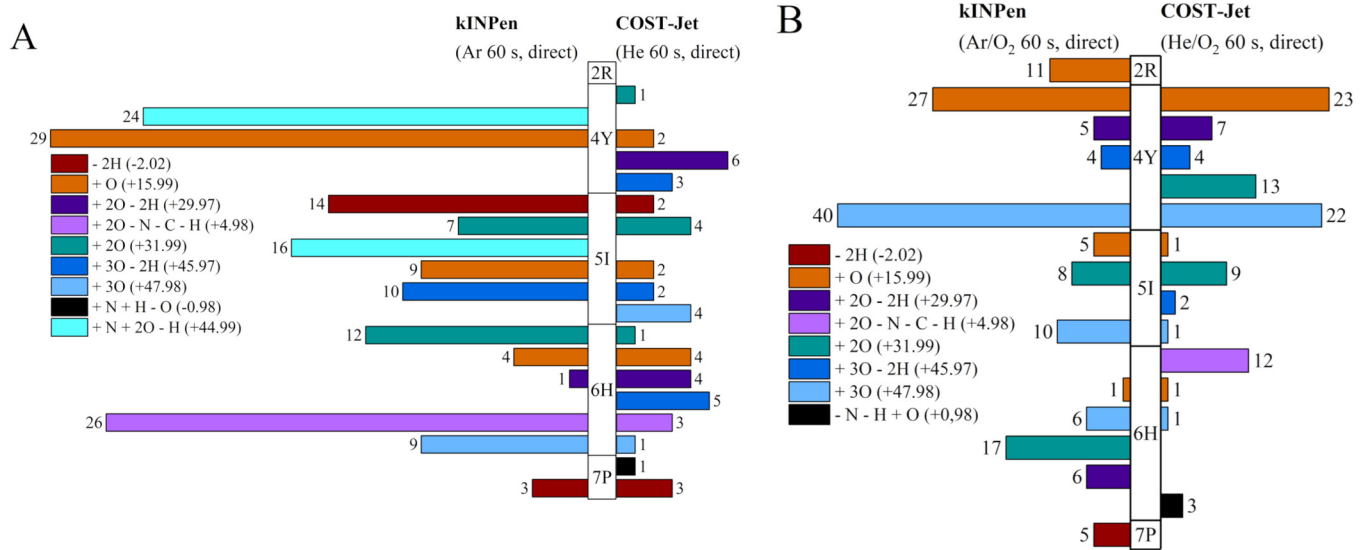
FIG. 6. Angiotensin 1–7: Absolute number and type of modifications observed as peptide spectrum matches in five of the seven amino acids that differ by a plasma source. (a) For kINPen, hydroxylation (+O) and nitration (+N + 2O − H) of tyrosine (4Y) and ring cleavage (+2O − N − C − H) of histidine (6H) dominate. The COST-Jet was less effective but more versatile. (b) Oxygen admixture increased the effectivity of the COST-Jet, reduced impact of RNS (no +N + 2O − H), and yielded in one (+O), two (+2O), or three (+3O) added oxygen as dominant products. Error bars are not shown for the sake of clarity, and the experimental error is ≈20% (see Fig. 3).

phenylalanine at position 5 is the major target [Figs. 3(c) and 3(d)]. With the pure helium discharge, hydroxylations (+O, Δm +15.99 Da) dominate by far (Fig. 7). Two proline residues at positions 3 and 7 are modified to carry two or three oxygen atoms (Δm +31.99 or Δm +47.98 Da). The oxygen admixture to the feed gas led in a massive increase of tyrosine oxidation (one to three oxygen atoms added) in angiotensin. This result mirrors observations using phenol as a target molecule[32] assumingly due to atomic oxygen and/or singlet oxygen that are produced in high levels by COST-Jet in these conditions.[26] Of note, a modification of the arginine residue at position 1 occurs in the same kind and amount as with the kINPen. Phenylalanine was oxidized to a 1,2- or 2,5-quinone [Δm +29.97 Da, Fig. 5(b)].

By adding nitrogen to COST-Jet, the modification of phenylalanine is further increased, yielding, especially, trihydroxylation (+3O, Δm +47.98 Da). However, under these conditions, RNS were expected, yet no nitration or nitrosylation was observed for any

COST-Jet conditions. This is in accordance to the literature, with oxygen species dominating in the discharge. If both molecular gases were added, tyrosine at position 4 of angiotensin 1–7 and phenylalanine at position 5 of bradykinin remain the main targets for the ROS/RNS in the plasma [Figs. 3(b) and 3(d)].

C. Similarities and differences between the plasma sources

In general, the treatment of a peptide by COST-Jet led to a significantly lower total number of PTMs than the kINPen (Fig. 3). This is due to a lower gas flow rate and the significantly lower specific density of helium. The combination of heavy argon and a higher gas flow rate create an entirely different gas dynamics and mechanical impact of the kINPen,[56] yielding a richer interface chemistry. Both plasma sources yielded different modification patterns at the two model peptides (Figs. 6 and 7) reflecting their
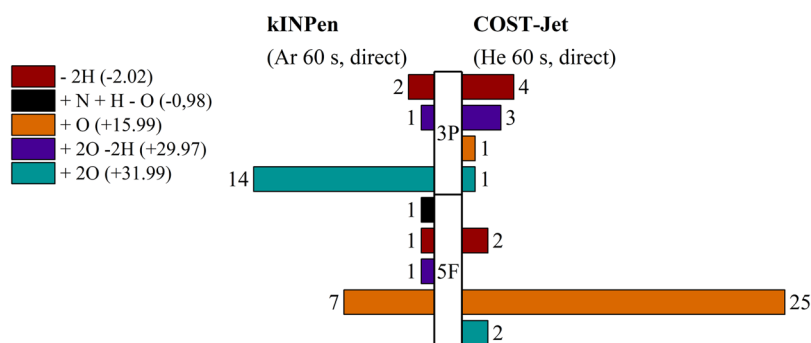

FIG. 7. Bradykinin: Absolute number and type of modifications observed at the amino acids phenylalanine (5P) and proline (3P) after kINPen (Ar) or COST-Jet (He) treatment (60 s). The differences reflect distinct species production by each source, see text. Error bars are not shown for the sake of clarity, and the experimental error is ≈20% (see Fig. 3).

different gas-phase and liquid-phase chemistries that were observed for cysteine and glutathione model systems.[27,28]

Nitration and nitrosylations were found for conditions rich in RNS deposition as reported for kINPen (Ar, Ar/N$_2$/O$_2$).[55,57,58] When atomic oxygen was a major species (COST-Jet), quinone formation at tyrosine or histidine was observed. As well, the dioxidation of tyrosine does occur predominantly for variants rich in atomic oxygen [Figs. 4(a), 5(a), and 6][32,59] but not in the singlet oxygen rich kINPen that favored the formylasparagine modification ($+2O - H - C - H$) at histidine [ninefold higher than COST-Jet, Fig. 6(a)]. Other modifications attributed to hydroxyl radicals were found in both plasma sources in the same extent as the monoxidation of histidine or the didehydration of proline (Δm −2.02 Da). A strong variation in the modification rate of the same amino acids at a different position (=a different chemical environment) was observed at the bradykinin's phenylalanine residue at position 5. For COST-Jet, this amino acid was oxidized particularly strong (Figs. 3 and 7). When treated with He/N$_2$, 5 F modification by the COST-Jet exceeded that of the kINPen by a factor of 5. In contrast, the phenylalanine residue at position 8 remained without detected modifications. Bradykinin's proline (3P) is the main target for the kINPen derived reactive species with dioxidation as the most common modification. The same amino acid loses two hydrogen atoms (didehydration) by the COST-Jet treatment, assumingly via an addition-subtraction reaction mechanism driven by hydroxyl radicals. These observations fit to previous results,[29] showing that the kINPen liquid chemistry is influenced by singlet oxygen O$_2$($^1\Delta_g$), leading to the dioxidation of thiols. On the other hand, the role of atomic oxygen in case of COST-Jet[38] leads to hydroxyl radical driven oxidations such as hydroxylations (+O) or H abstractions (−2H). Besides the short-lived species, both plasma sources deposit long-lived species in liquids (see Fig. 2). Hydrogen peroxide is a major product in kINPen and COST-Jet for argon or helium only discharges while nitrite and nitrate dominate conditions with

N$_2$ and N$_2$/O$_2$ admixtures. Despite the significant concentrations (up to $150 \mu M$), only a very limited impact on the peptides could be attributed to their presence. In indirect treatments (peptides were added after the plasma was switched off), only a negligible number of PTMs was observed. This is in accordance with previous results using cysteine as a tracer.[27,42,44]

D. Comparing MD simulations with experiment data

Verlackt et al. published in 2017 a study investigating the oxidation of bradykinin and angiotensin 1–7 by OH radicals[34] using a combined approach of reactive and nonreactive long time scale molecular dynamics simulation. In angiotensin, the four amino acids, asparagine, tyrosine, histidine, and proline, were identified as major targets; for bradykinin, the corresponding targets were arginine, serine, and proline. Predominantly, an H-atom abstraction was predicted as an initial step. Only for bradykinin, a minor 3% of the reactions were OH additions at phenylalanine 8, yielding predominantly tyrosine or positional isomer thereof. The current experimental data, comparing kINPen and COST-Jet, revealed both similarities and differences with the model (Fig. 8). For angiotensin 1–7, a good agreement between expected and observed chemical reactivity toward kINPen (Ar)- and COST-Jet (He)-derived reactive species was found for the amino acids valine, tyrosine, and histidine. The aromatic amino acids tyrosine and histidine are electron-rich and intermediates stabilize via electron delocalization, accordingly the model and the experiment coaligned on their intense modification. In the simulation, valine and isoleucine are inert to the OH attack in the tested time frame (10 ps), while experimentally, valine and, especially, isoleucine were oxidized by both plasma sources. This is in agreement with Takai et al. who reported the formation of hydroxylated dicarbonic acids.[12] The longer side chain of isoleucine protruding from the peptide backbone and the presence of an additional secondary C-atom discriminate it from
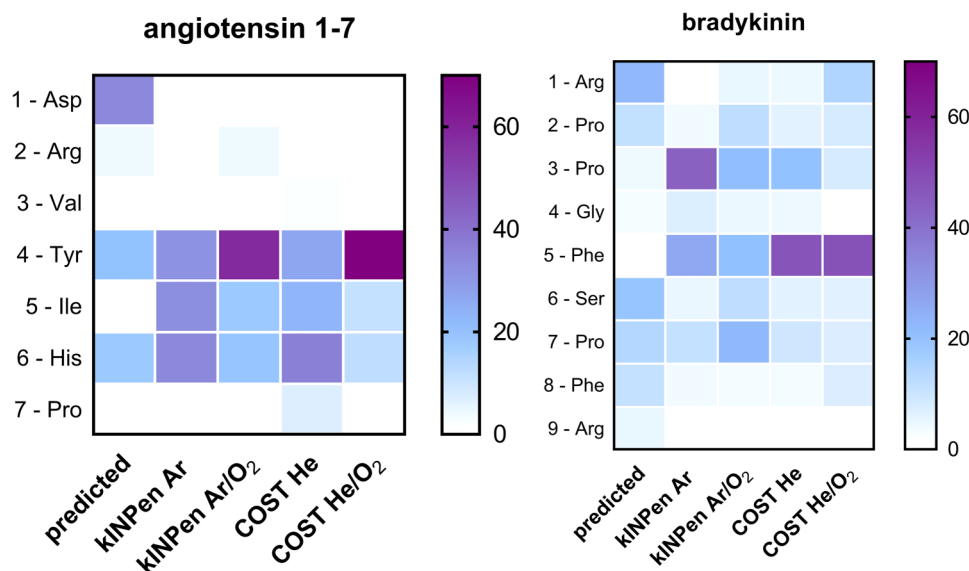

FIG. 8. Comparison of the extent of modifications found in angiotensin (left) and bradykinin (right) with simulations by Verlackt et al.[34] kINPen Ar or Ar/O$_2$ and COST He or He/O$_2$ were considered, and relative values (%) are shown. Significant differences between the model and the experiment and between plasma sources/discharge conditions are obvious, see text.

the less modified valine. Presumably, time scales >10 ps or reactive species not considered in the simulation are relevant in the attack. Atomic oxygen, the major species produced by COST-Jet in the He/O_2 regime, did not yield to a stronger isoleucine oxidation than singlet oxygen rich kINPen. A discord between the model and the experiment was also observed for aspartic acid: in contrast to predictions, no modifications were detected, which is again in agreement with Takai *et al.* It may be concluded that the assumed abstraction of an H-atom at the side chain carbonic acid moiety is not relevant for subsequent covalent modifications since the resulting radical is resonance stabilized at low energy. Additionally, with a pKa of 3.9, the β-COOH moiety is quite acidic and deprotonates in near neutral solutions. The resulting electron-rich COO^- moiety deters electron-rich and negatively charged reactive species and thereby protecting the carbon backbone from modification. A similar observation was made for Pro at carboxy terminus position 7 in angiotensin. With a pKa value of 1.99, the COOH moiety deprotonates under the experimental conditions, deterring the attack of electron-rich reactive species. In contrast, for bradykinin, where proline's carboxyl group is masked as an amide, modifications (dioxidations/ring cleavages) were frequent. Finally, arginine (Arg) was expected to be a major target, but neither source led to observed modifications, in good agreement with Takei *et al.* In the model, bradykinin's phenylalanine at position 5 was expected to be inert, yet the experimental observations showed that the amino acid is indeed a good target due to its large and resonance stabilized aromatic moiety. The discrepancy with simulation can be explained by technical restraints in the model and, additionally, by the relevant reactive species (assumingly atomic oxygen) that the model did not consider. Regarding the comparison of the experimental data obtained for the oxygen admixture with the models' prediction, a gradual improvement could be observed. Specifically, for COST-Jet He/O_2 and peptide bradykinin, a good correlation between the prediction and the observation—with the exception of phenylalanine—can be stated. Under this condition, the COST-Jet is known to produce reactive oxygen species, with atomic oxygen as major species. In contrast, reactive nitrogen species have low abundancy. Such model, taking OH radicals into account that easily yield from the reaction of atomic oxygen with water molecules, has a higher prediction power for this source. The kINPen, with its significant production of reactive nitrogen species, highlights the limits of the simulation.

In conclusion, the observed divergence between the model and the experiment is multifaceted: beyond OH radicals, further species such as atomic oxygen, singlet oxygen, and the triad nitric oxide/superoxide/peroxynitrite contribute to liquid chemistry.[35,44,55,60–62] Technical constraints of simulation studies limit the ability to address, e.g., local electron density changes or the degree of solvation. Experimental limitations include that natural occurring PTMs were focused, limiting the detection of unexpected modifications. However, the significant overlap with the plasma treatment of isolated amino acids suggests their extent to be minor.

E. Implications for cold plasma effectivity in biomedicine

Despite the known efficacy of cold plasma in biological systems *in vitro* and *in vivo*, a gap between the ns/µs time scales of the gaseous plasma chemistry and the short lifetime of the reactive species involved compared to the substantial longer time scales of cellular processes (seconds to hours) must be acknowledged. In addition to direct effects of long-lived species (e.g., hydrogen peroxide), secondary processes resulting from the modifications of biomolecules by the short-lived species may modulate cell response and signaling processes. It was observed that the cold plasma treatment interferes with the protein structure and subsequently its function.[11,63–67] More recently, oxidative modifications to proteins have been connected to changes in the immune response, possibly attenuating cancer cell immunoediting. The current results reveal parts of the underlying molecular basis showing that cold plasma can introduce nonenzymatically post-translational modifications in a number of amino acids and that most of these PTMs belong to the natural subset that can be interpreted by the cellular machinery. Heavy oxidation directs a protein toward protein ubiquitinylation and subsequent degradation, or activates chaperons-like heat shock proteins to regain its normal function. Along with the significant oxidation of cysteine, seminal processes like the unfolded protein response that control cell fate and function can be triggered.[68] Less extensive modifications like nitrations or nitrosylations at tyrosine can be involved in kinase signaling cascades, influencing phosphorylation events as has been observed in the nitric oxide research community for the human aldolase A.[69] In this light, a strong link between the short-lived species in the discharge and the cells prolonged response can be found in the covalent modifications of proteins and peptides.

IV. SUMMARY AND CONCLUSION

Utilizing the model peptides bradykinin and angiotensin 1–7, nine different post-translational modifications (PTMs) were observed after cold plasma treatment (kINPen and COST-Jet). Major targets were the amino acids tyrosine, tryptophan, histidine, and phenylalanine as well as the aliphatic amino acids proline and isoleucine. In contrast, the amino acids valine, arginine, and aspartic acid remained mostly unchanged. The observed PTM patterns indicate the activity of the short-lived species hydroxyl radicals, atomic and singlet oxygen, and peroxynitrite. Frequent observed PTMs were nitration, oxidation (plus one/two oxygen atoms), and ring cleavage (e.g., histidine). Experimental data only in part overlap with a previously published molecular dynamics simulation, indicating that hydroxyl radicals are not the only—or major—oxidant. The chemical environment and steric restriction within the peptide chain significantly influenced the amino acids' reactivity, a fact requesting consideration when analyzing complex 3D structures like proteins. Since the observed PTMs belong to naturally occurring modifications, it can be assumed that CPPs derive their impact on biological systems in part via protein modification. The presented data pave the way for further studies to evaluate their impact on cellular signaling pathways in inflammatory processes in cancer and wound biology in full.

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Article A2

Reactive species driven oxidative modifications of peptides—Tracing physical plasma liquid chemistry

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Reactive species driven oxidative modifications of peptides—Tracing physical plasma liquid chemistry

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ABSTRACT

The effluence of physical plasma consists of a significant share of reactive species, which may interact with biomolecules and yield chemical modifications comparable to those of physiological processes, e.g., post-translational protein modifications (oxPTMs). Consequentially, the aim of this work is to understand the role of physical plasma-derived reactive species in the introduction of oxPTM-like modifications in proteins. An artificial peptide library consisting of ten peptides was screened against the impact of two plasma sources, the argon-driven MHz-jet kINPen and the helium-driven RF-jet COST-Jet. Changes in the peptide molecular structure were analyzed by liquid chromatography–mass spectrometry. The amino acids cysteine, methionine, tyrosine, and tryptophan were identified as major targets. The introduction of one, two, or three oxygen atoms was the most common modification observed. Distinct modification patterns were observed for nitration ($+N + 2O–H$), which occurred in kINPen only (peroxynitrite), and chlorination ($+Cl–H$) that was exclusive for the COST-Jet in the presence of chloride ions (atomic oxygen/hypochlorite). Predominantly for the kINPen, singlet oxygen-related modifications, e.g., cleavage of tryptophan, were observed. Oxidation, carbonylation, and double oxidations were attributed to the impact of hydroxyl radicals and atomic oxygen. Leading to a significant change in the peptide side chain, most of these oxPTM-like modifications affect the secondary structure of amino acid chains, and amino acid polarity/functionality, ultimately modifying the performance and stability of cellular proteins.

I. INTRODUCTION

The 2019 Nobel Prize in Physiology and Medicine was awarded to the discovery that the degradation of the protein hypoxia-inducible factor 1α is accelerated by its hydroxylation under normal oxygen levels, while in lack of oxygen—such as stroke—the protein is not hydroxylated and accumulates, launching physiological countermeasures.[1,2] This example illustrates the important role of (oxidative) post-translational modifications (oxPTMs) in the regulation, protein functionality, and relevance for most cellular signaling pathways.[3,4] More than 200 different (ox)PTMs are known, dominated by phosphorylation, acetylation, and ubiquitinylation with a massive impact on a proteins function and lifetime.[5] Kinase-driven protein phosphorylation[6] activates or prevents downstream signals[7] and was found relevant in the response of mammalian cells toward cold physical plasma treatment (CAP).[8]

The role of oxPTMs[9,10] on cellular signaling is in the focus of research activities, fostered by the improvement of high-resolution mass spectrometry instrumentation and workflows.[11–13] For a long time, (ox)PTMs had been considered as signs of destruction only, such as aging processes.[14] A complex regulation network is currently being revealed, it has been proven that downregulation (distress) and stimulating effects (eustress) coexist,[15,16] and the cellular processes are influenced by various modifications at the same time. Apoptosis, for example, is regulated by S-nitrosylation,[17] N-acetylation,[18] and N-myristoylation.[19]

Controlled production of reactive oxygen and reactive nitrogen species (ROS/RNS) is also found in cold physical plasma, generating short- and long-lived reactive species, which are capable of reacting with most biomolecules and representing an emerging field of redox therapy. Due to its unique properties, physical plasma serves many applications. For long, it has been used for decontamination[20] or the refinement of various surfaces.[21] Recently, physical plasma treatments have been proven to be useful in medical applications, e.g., the treatment of chronic wounds[22,23] or cancers.[24–27] However, the molecular mechanisms are not yet fully understood. It remains elusive, i.e., which species penetrate sufficiently in biological systems and which of those are (chemically) active to trigger downstream signals. While the long-lived plasma-derived species as H_2O_2, nitrite, and nitrate can be easily quantified are used as fundamental markers for CAP treatment, the short-lived species are difficult to access. However, they appear to be of significant importance.[28–35] Given their high reactivity, a penetration seems unlikely. Alternatively, it may be hypothesized that exposure of biomolecules to short-lived species yields secondary products, which have biological effects on themselves and ultimately modulate the physiological processes in a cell or tissue.

A number of reports indicate that amino acids in proteins are excellent targets for plasma-derived reactive species.[36–40] In addition, their regulation via physiological (ox)PTMs offers an excellent intersection with physical plasma. The aim of this work is, therefore, to determine the impact of plasma-derived reactive species on model peptides, focusing on the formation of artificial non-enzymatic post-translational modifications. To gain an overview of a variety of gas phase chemistries, the argon-driven kINPen was compared to the helium-driven COST-Jet[41] while modulating the discharge parameters including the gas phase composition. To investigate these modifications, high-resolution mass spectrometry coupled to nanoflow liquid chromatography and subsequent bioinformatical analysis were used.

II. EXPERIMENTAL

A. Development of the artificial peptide library

A peptide library of ten peptides with a length of ten amino acids each was designed and synthesized (ProteoGenix, Schiltigheim, France) in such a way, that the 20 major proteinogenic amino acids occur with equal incidence (Fig. 1 and Table S1 in the supplementary material). In addition, the peptide sequence was designed to locate each amino acid at the N-terminus, the C-terminus, and the center region and to have as many different neighboring amino acids as possible, exemplified for alanine (A, red) and tryptophan (W, blue) in Fig. 1.
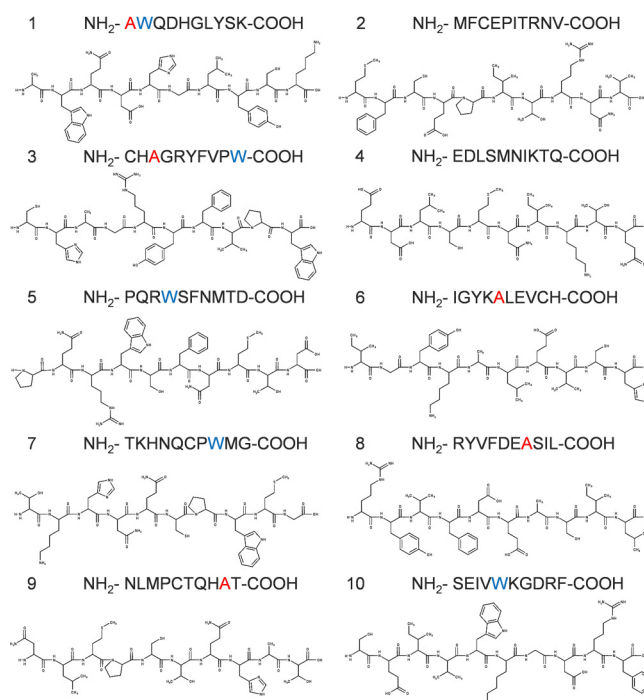

FIG. 1. Expanded peptide sequence of the ten-peptide library challenged with physical plasma-derived reactive species. Alanine (A, red) and tryptophan (W, blue) exemplify the varying positions adopted by each amino acid in the peptides.

B. Treatment conditions and plasma sources

The peptides were dissolved in double distilled water (Merck Millipore, Darmstadt, Germany) or phosphate buffered saline (PBS, Sigma Aldrich, Deisenhofen, Germany) at a concentration of 0.1 mg/ml. Both solvents were degassed by bubbling with argon gas for at least 30 min to reduce background reactions. The treatments with cold atmospheric pressure plasma were performed with an argon plasma jet (kINPen, neoplas tools GmbH, Greifswald, Germany) and a helium plasma jet (COST-Jet, a standard device identified by the European COST action MP 1101 "Biomedical Applications of Atmospheric Pressure Plasma Technology"). The kINPen[42] [Fig. 2(b)] consists of a grounded ring electrode enclosing a ceramic capillary, where a powered central rod electrode is located inside (2–6 kV$_{PP}$ at 1.1 MHz). A 3000 standard cubic centimeters per minute (SCCM) flow of argon (Air Liquide, 99.999%) served as the main feed gas. For some treatments, 0.5/1% of the feed gas was replaced by oxygen (15 SCCM) or by oxygen + nitrogen (15 sccm each). The COST-Jet [Fig. 2(a)] consists of two 1 mm thick metal plate electrodes leaving a 1 mm gap, where the plasma is ignited. The capacitively coupled electrodes are driven by an AC voltage at 13.56 MHz. The dissipated power was held constant at 330 mW by using a Tektronix DPO 4104 Digital Phosphor Oscilloscope in accordance with Ref. 41. The helium feed gas flow was kept at 1000 SCCM and if desired, enriched with 0.5% oxygen (5 SCCM), or 0.5% oxygen + 0.5% nitrogen (5 SCCM each).
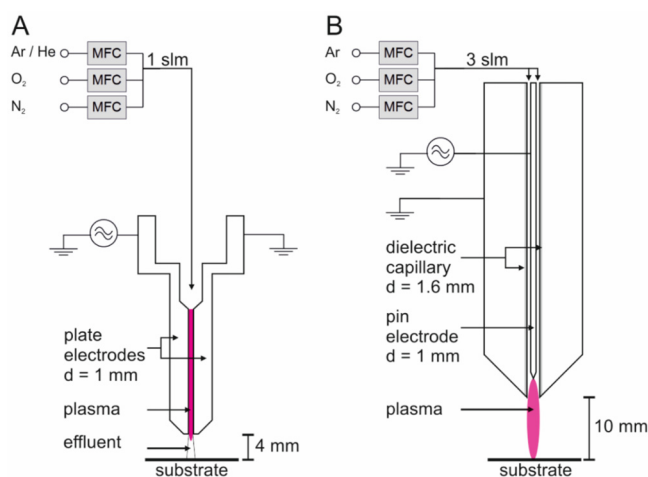
FIG. 2. Schematics of the COST-Jet (a) and kINPen (b). MFC: mass flow controller, sLm: standard liter per minute. For details, see text. Reprinted with permission from Lackmann et al., Sci. Rep. **8**, 7736 (2018). Copyright 2018 Author(s), licensed under a Creative Commons Attribution (CC BY) License.

750 μl of each peptide solution (water/PBS) was placed in 24-well plates and treated 15 or 60 s with a nozzle to a liquid distance of 4 mm (COST-Jet) or 9 mm (kINPen) for each gas mix (feed gas + O_2 or O_2/N_2). For indirect treatments, the procedure was adapted: only the solvents were plasma-treated and the peptides were added 1 min after the plasma was switched off as a bolus solution. After each individual treatment, samples were placed on ice, and immediately analyzed by nanoflow liquid chromatography–mass spectrometry (nano-LC-MS/MS), to minimize post-discharge reactions. Three independent experiments and measurements were performed. The long-lived species H_2O_2, nitrate (NO_3^-) and nitrite (NO_2^-) were determined according to the previously published approach.[38]

C. Nano-LC–MS/MS acquisition

For nanoflow liquid chromatography mass spectrometry, an UltiMate 3000 RSLCnano (Thermo Scientific, Dreieich Germany) system was used with an Acclaim Pepmap C18 column (150 mm × 75 μm, 2.0 μm particle size, Thermo Scientific, Dreieich Germany) with the corresponding precolumn (20 mm × 100 μm, 5.0 μm particle size). The injection volume was 1 μl, equaling 100 ng of the sample. A gradient (see Table I) consisting of water/0.1% v/v acetic acid (A) and acetonitrile/0.1% v/v acetic acid (B) was applied to achieve separation.

Mass spectrometry acquisition was carried out in a data dependent mode with a full scan range of 86.7–1300 m/z (resolution 70 000) and with the fragmentation of the top ten most abundant precursor ions (QExactive Hybrid-Quadrupol-Orbitrap, Thermo Scientific, Dreieich Germany). Precursor ions were fragmented using normalized higher energy collisional dissociation (HCD, 27.5 eV) and detected at a mass resolution of 17 500 at m/z 200. The automatic gain control (AGC) target for full MS was set

TABLE I. Flow gradient for nano-LC separation of peptides on a PepMap100 C18 column. A = water/0.1% v/v acetic acid, B = acetonitrile/0.1% v/v acetic acid.

Eluent B (%)	Time (min)	Flow (μl/min)
15	0	0.700
18	4.5	0.700
22	10.5	0.700
85	11	0.700
85	15	0.700
15	17	0.700
15	20	0.700

to 1×10^6 ions and for MS/MS to 2×10^5 ions with a maximum ion injection time of 120 ms for both modes. Dynamic exclusion was set to 30 s and ions with charge state one or above eight were excluded.

D. Raw data analysis, data handling, and statistics

To identify the peptides and their potential post-translational modifications, the MS raw data were analyzed by the software Proteome Discoverer 2.4 (Thermo Scientific, Dreieich Germany). For the detection of covalent modifications in the amino acid chain, the software Byonic (Protein Metrics), version 3.6.0, was used as a plug-in tool. In contrast to the Proteome Discoverer, this program is able to perform unlimited searches for multiple modifications in a single peptide. The precursor mass tolerance was set to 3 parts per million (ppm) and fragment mass tolerance to 10 ppm. The false discovery rate (FRD) was set to 1%. For technical limitations, the allowed maximum of modifications per peptide was set to three. The dataset was pre-filtered using different scores from Byonic.[43] The first filter was the Byonic score with a cut-off of 250 (range 0–1000), and the Delta Mod score was set to 5 (range from 0 to 100). Both scores result from the match of the theoretical fragment spectrum of a peptide and the experimental one. Following vendor recommendations, these cut-offs can be considered significant. To obtain semi-quantitative data, the number of peptide spectra matches (PSMs) that were detected for a specific modification were counted (PSM counting.[44])

In total, 103 497 PSMs were analyzed with R (v4.0.2)[45] using RStudio (v1.3.1093) and the Tidyverse (v1.3.0)[46] package. The observation data were normalized on the total number of spectra matches for each peptide to account for variances in the ionization efficiency after background subtraction, yielding the occurrence of de novo modifications in percent. Linear models [later referred to as n-way analysis of variance (ANOVA)] were used to analyze the effects of discharge parameters on peptide modification. This statistical method was chosen due to its simplicity, the existence of negative values in the dependent variable (percentage of modifications), and the assumption that in general modification percentages in similar treated samples show a Gaussian distribution. The percentage of one particular PTM was modeled as a result of the categorical effects: (1) amino acid, (2) peptide, (3) solvent, (4) plasma, (5) indirect or direct treatment, and (6) treatment time. To address the issue, that the amino acid sites of the modification (not only the

type of modification) might be dependent on the effects (2) to (6) as well, models were additionally fitted considering those interactions. Fitted models with and without interactions were compared for each modification and the models with interaction were significantly more fitting (F-Test; p-value $<2.2 \times 10^{-16}$). This indicates an important impact of the "biochemical" background for the occurrence of modifications.

III. RESULTS AND DISCUSSION

A. Sulfur-containing and aromatic amino acids are the major targets of plasma-derived reactive species

The dominant targets within the investigated peptide library were the sulfur-containing amino acids cysteine (Cys) and methionine (Met) and the aromatic amino acids tryptophan, tyrosine, and phenylalanine (see Fig. 3), corroborating previous studies using cold physical plasma (CAP).[36–39] The thiol group of cysteine and the thioether group of methionine are excellent targets for oxidation, carrying predominantly one oxygen (Met) or even three (Cys), in this case forming the strongly acidic sulfonic acid moiety. This oxidation is irreversible; prohibiting the formation of sulfide bridges and consequently interferes with protein folding and signaling processes. Alongside, modifications containing only one (cysteine sulfenic acid) or two oxygen atoms (cysteine sulfinic acid) are possible and have been found using cysteine as a model compound during CAP treatment.[28,30] These oxidation states are still reversible in physiologic conditions, e.g., via sulfiredoxin activity, but under CAP impact quickly convert into the stable sulfonic acid form. Further oxidation yielding sulfate and a residual alanine has been found for CAP-driven cysteine oxidation; however, a conversion from cysteine to alanine was not observed in the studied peptide library. Methionine monoxidation is prevalent *in vivo* and is a marker for oxidative stress.[47] It occurs during sample preparation by oxidation via triplet oxygen as an artifact, indicating the high reaction probability at the thioether. Methionine oxidation is

linked to conformational changes of proteins, modulating their functionality.[48]

From the group of aromatic amino acids, tryptophan (Trp) is the major target. The possible chemical reactions are far more complex than for Cys or Met, with special regards to the heterocyclic nature of one of the two annealed rings.[49] The position of the nitrogen within the rings increases the electron density in both rings, facilitating electrophile substitution reactions introducing oxygen, nitrogen, or chloride atoms that subsequently allow elimination or oxidation reactions enriching the observable product portfolio. In addition, the N-containing 5-ring substructure is prone to ring cleavages via a variety of reaction pathways (see below). Due to its bulkiness and relative hydrophobicity, Trp is typically found in the inner and/or hydrophopic parts of a protein, impeding access by reactive species from the outside. The impact on protein function is hard to predict due to the diversity of the products.

Next to Trp, tyrosine (Tyr), and phenylalanine (Phe) as hydroxybenzene resp. benzene derivatives of α-amino propionic acid belong to the most relevant targets of CAP-derived reactive species. However, due to the higher electron density in the aromatic ring of Tyr resulting from the hydroxyl group, this amino acid is far more often modified than Phe. Again, electrophile substitution reactions dominate, and downstream reactions yield a variety of products. While one or two additional hydroxyl groups and the corresponding oxidation products (quinones, semi-quinones) dominate, an interesting role can be attributed to nitrogen-derived modifications, especially nitration. Nitrated tyrosine residues are reported as markers for oxidative stress. The presence of the electron-pulling nitro group in the aromatic system decreases the pKa value of the amino acids hydroxyl group, subsequently increasing the probability for its phosphorylation by regulatory proteins (tyrosine kinases).[50,51] Therefore, a significant impact on protein functionality can be assumed.

The other 15 amino acids are modified by CAP-derived species to a (far) lesser extent. However, some of the observed modifications are indicative of the presence of specific reactive species and may serve as a diagnostic marker (see below). Ahead of all, lysine (Lys) with its ϵ-positioned primary amino group is sensitive toward hypochlorite, a species that is generated from atomic oxygen when conditions allow. The products comprise simple chlorination, and addition–elimination reactions ultimately yielding multiple bonds in the Lys molecule.

Somewhat unexpectedly, most of the aliphatic amino acids like valine (Val), leucine (Leu), and isoleucine (Iso) were often targeted, indicating a significant role of primary or secondary generated OH radicals. The products were hydroxylated molecules that in the physiologic context may be relevant by increasing the hydrophilicity of the respective amino acid yielding a modulation of protein conformation and interaction with binding partners. The polar amino acids aspartic acid (Asp), asparagine (Asn), glutamic acid (Glu), glutamine (Gln), arginine (Arg), and serine (Ser) rarely bore modifications. Since these amino acids carry electron-rich structures such as hydroxyl and carboxyl groups or a guanidinium moiety, a significant reactivity was expected.[52] However, most bonds in the respective moieties are resonance stabilized, reducing their reactivity. In addition, they can reform without a permanent change in the chemical composition exemplified in the following reaction (1):
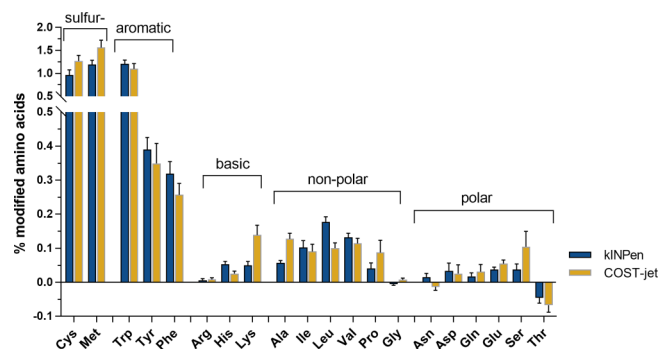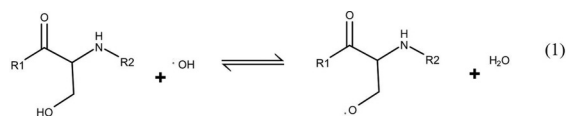

FIG. 3. Occurrence of amino acid modifications in all direct treatment conditions for kINPen (Ar, in part w/ admix, blue) or COST-Jet (He, in part w/ admix, yellow). Amino acids are sorted according to their major chemical characteristics. Data presented relative to the untreated peptide control. Note the split y axis.

The formation of an alkoxy radical via H-atom abstraction, shown in (1) for a serine moiety, is a typical reaction of an OH radical.[53] The radical may return to ground state by reaction with water. Alkoxy radicals are not very reactive, yet dimerization forming a peroxide or hydroperoxide can occur.[54] Since this structure is not stable, the reverse reaction yields to the initial hydroxyl group. Alternatively, atomic oxygen, $O(^3P)$, can replace the OH radical, as shown in (2),[55]

$$R-OH + O(^3P) \rightarrow \ ^\cdot OH + R-O^\cdot, \qquad (2a)$$

$$H_2O + O(^3P) \rightarrow 2^\cdot OH. \qquad (2b)$$
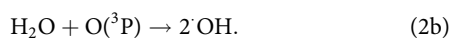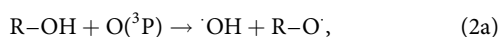
When applying the reaction (1) to an aliphatic amino acid, a two-step reaction yields hydroxylated amino acids. The resulting product 3-hydroxy valine (3b) is stable and yields a permanent change that is annotated as hydroxylation (+15.99 Da),

The annular amino acids histidine (His) and proline (Pro) remain predominantly unchanged (Fig. 3), except when Pro has an exposed amino group in position one in the peptide (peptide 5, NH_2–PQRWSFNMT–COOH). His has a special reactivity toward singlet oxygen, yielding ring-open products (see below).

B. Reactive oxygen-derived modifications dominate in the product profile

In all, 17 different modifications were observed in the investigated peptide library. Assumingly, all of these derived from the attack of reactive species contained a differing number of oxygen atoms. In some cases, oxygen is eliminated during formation or
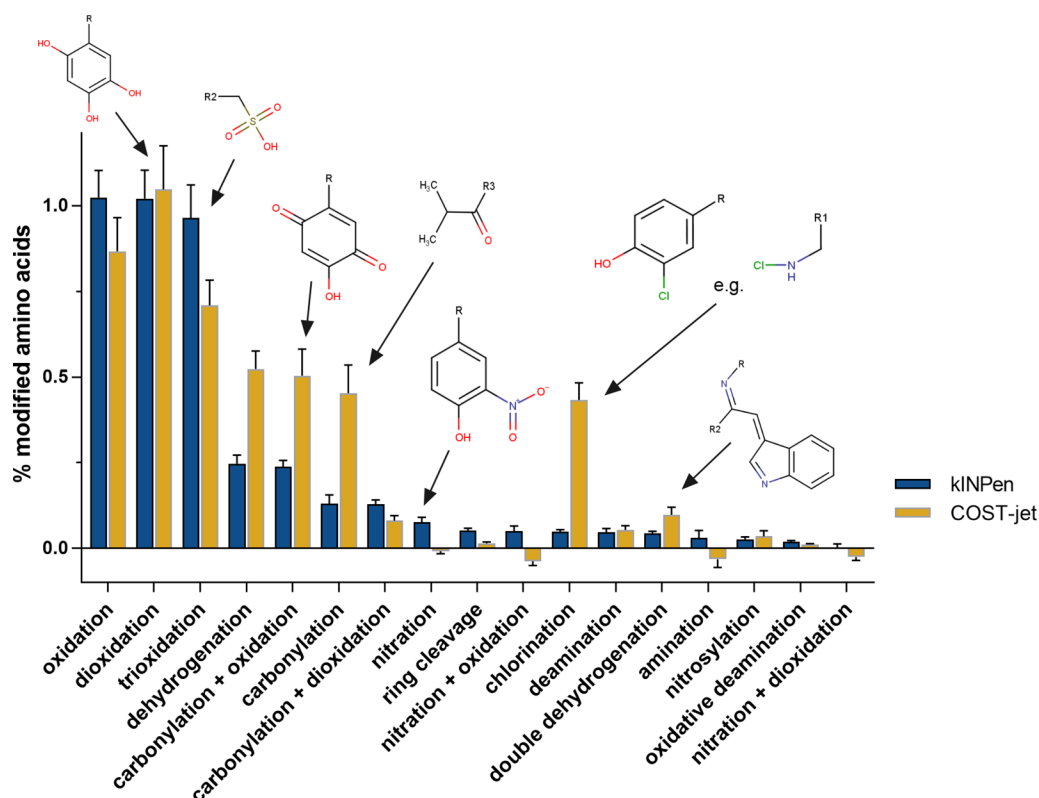

FIG. 4. Most relevant modifications after direct treatment within the peptide library. Data presented relative to the untreated peptide control. Background artifacts were removed. Chemical structures exemplify modifications with high incidence, basing on tyrosine, cysteine, isoleucine, lysine, or tryptophan.

TABLE II. Major amino acid modifications observed within the ten-peptide library after plasma treatment.

Monoisotopic mass shift (Da)	Elemental composition	Chem. mod./(potential product)
+15.99	+O	Oxidation (e.g., hydroxyl group)
+31.98	+2O	Dioxidation (two hydroxyl groups, peroxide)
+47.98	+3O	Trioxidation (hydroxyl group + peroxide, three hydroxyl groups, sulfonic acid)
+28.99	+N + O–H	Nitrosylation (nitroso group)
+44.98	+N + 2O–H	Nitration (nitro group)
+60.98	+N + 3O–H	Nitration + oxidation
+76.97	+N + 4OH	Nitration + dioxidation
+0.98	−N–H + O	Deamination (loss of amino group)
−0.98	+N + H–O	Amination (amino group)
+13.98	+O–2H	Carbonylation (oxo group)
+29.97	+2O–2H	carbonylation + oxidation (oxo group + hydroxyl group)
+45.97	+ 3O–2H	Carbonylation + dioxidation (oxo group + two hydroxyl groups or peroxide)
−2.02	−2H	Dehydrogenation (loss of two hydrogen atoms, creating a double bond)
−4.03	−4H	Double dehydrogenation (two double bonds)
+4.98	+2O–N–C–H	Ring cleavage (histidine: formylasparagine)
−3.05	+O–5H–N	Oxidative deamination (lysine)[36]
+33.96	+Cl–H	Chlorination (chlorine atom replaces hydrogen)

downstream reactions. Of the detected modifications, 13 contained oxygen atoms, up to three (Fig. 4 and Table II). Two modifications (dehydrogenation/double dehydrogenation) were solely characterized by a loss of atoms, forming multiple bonds. In one modification (chlorination), chlorine apparently replaces one hydrogen atom in the initial molecule. Nitrogen, as the second most relevant contributor to the CAP-derived reactive species, contributed to five modifications. However, the abundance of such modifications is significantly lower than that for the oxygen counterparts. Besides a lower prevalence of reactive nitrogen species in the investigated discharges, subsequent elimination reactions further reduce their presence as indicated by the deamination of free amino groups that are observed under some conditions. Some of these modifications are created by enzymatic processes during physiological post-translational processing and via redox-controlled signaling events,[3,18,56] pointing at the potential to interfere with protein and ultimately cell functionality via CAP-derived reactive species. The major modifications base on the introduction of gas phase derived hydroxyl radicals or atomic oxygen as shown for the aliphatic amino acids [reaction (3)].[30,32] The hydroxylation observed in the aliphatic amino acids, according to (3a) and (3b), may be driven by hydroxyl radicals. However, due to the slow reaction of atomic oxygen with water ($2.2 \times 10^{-23}\,\mathrm{m^3\,s^{-1}}$), yielding to hydroxyl radicals [reaction (2)],[57] their role may be limited while the direct attack of atomic oxygen at the peptide amino acid moieties is in favor ($1.1 \times 10^{-17}\,\mathrm{m^3\,s^{-1}}$).[32] This is in contrast to experiments using Fenton's reaction to generate hydroxyl radicals in liquids, indicating that this model suffers limitations when used to mimic plasma-derived reactive species.[58] A significant role of singlet oxygen is indicated by the fact that by far the insertion of two oxygen atoms, yielding a dihydroxylation or peroxidation, prevails over monoxidation (hydroxylation) or triple hydroxylation. Since the peroxides are unstable during sample preparation and analysis, two hydroxyl groups are the oxidative modification observed.[59]

In addition, ring cleavage reactions in histidine and tryptophan (see below) are indicative for 2 + 2 or 4 + 2 cycloadditon reactions known for singlet oxygen. In the presence of chloride ions, CAP-derived atomic oxygen leads to the formation of hypochlorite (OCl^-).[31,33] The OCl^- ion is unstable; it acts as a chlorination agent and an oxidant, yielding a number of prominent modifications, above all chlorination, carbonylation, and dehydrogenation. The molecule is reactive and reacts both with primary amines, yielding N-chloro- or N,N-dichloro compounds (chloramines)[60] and with aromatic systems,[61] yielding mono-, di-, or trichloro derivatives (electrophilic substitution). Accordingly, lysine and tyrosine were the main targets observed (Fig. 5 and Fig. S2 in the supplementary material). Surprisingly, no significant chlorination could be found at phenylalanine; although its aromatic ring is similar to Tyr, it lacks the electron-pushing hydroxyl group. Further modifications related to the presence of atomic oxygen are dehydrogenations and the formation of oxo groups (carbonylation). In both cases, addition–elimination reaction occurs. The peptide backbone (amide bonds) was insensitive toward hypochlorite and no fragments have been detected. Since chloride ions are ubiquitous in biological systems, hypochlorite and its downstream products (ClO_2^-, ClO_3^-)[62] and described peptide modifications must be considered for all plasma discharges creating atomic oxygen.

C. The type of plasma source and the working gas composition determine the introduction of peptide modifications

While it was already shown that the feed gas composition of a given plasma source influences the presence of modifications for some amino acids bound in a peptide,[37–39] the current approach using a peptide library should provide a more general insight. Despite a general concordance in the main target amino acids and
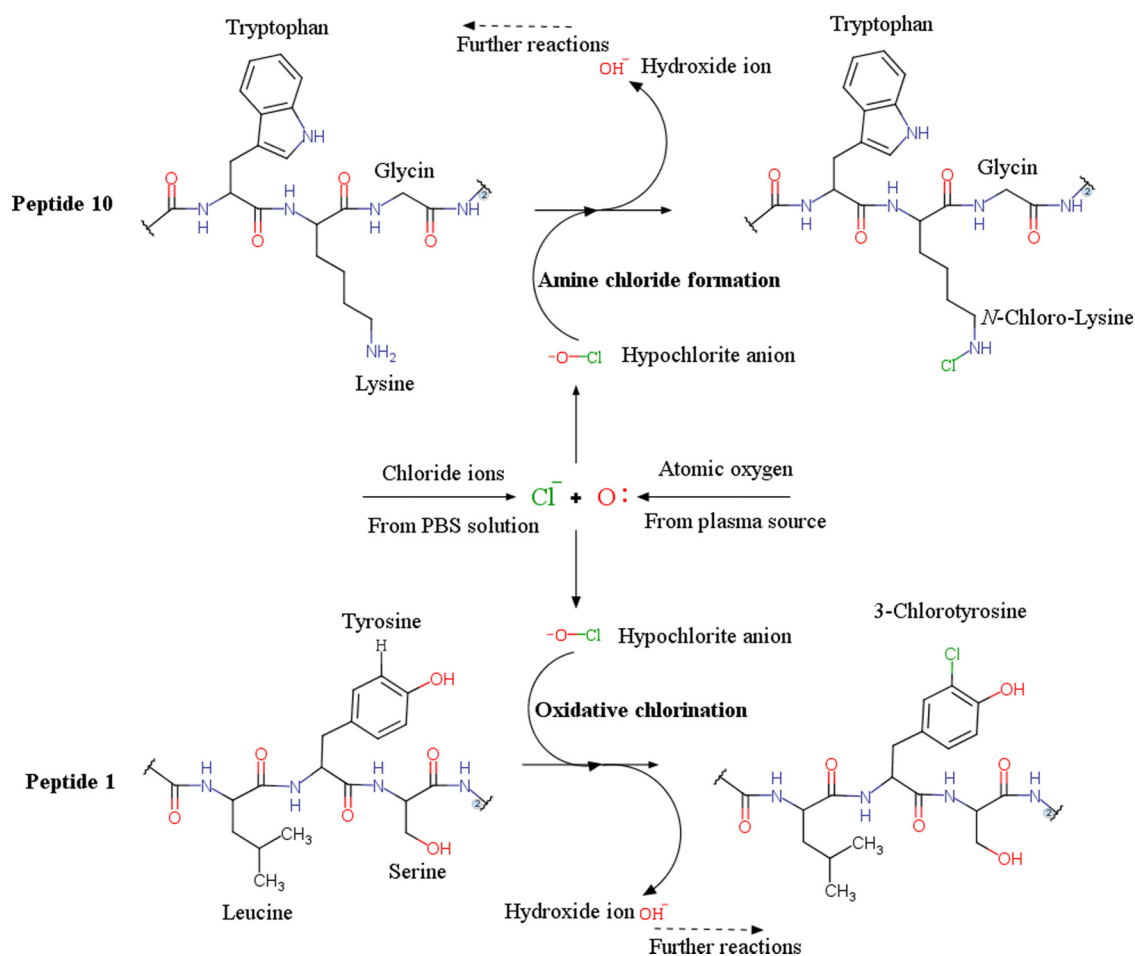
FIG. 5. Suggested reaction mechanism for the generation of *N*-chloro-lysine in peptide 10 (NH$_2$–SEIVWKGDRF–COOH) and 3-chlorotyrosine in peptide 1 (NH$_2$–AWQDHGLYSK–COOH). Shown is the structure of lysine and tyrosine and the two respective neighboring amino acids tryptophan and glycine as well as leucine and serine. Hypochloride is formed by the atomic oxygen from the plasma source and chloric ions in solution from PBS. The amine chloride formation and oxidative chlorination take place with the elimination of a hydroxyl anion for further reactions like carbonylation.

the type of introduced modifications (Figs. 3 and 4), explicit differences can be found for a number of specific gas plasma conditions [Figs. 6(a) and 6(b)]. The most obvious difference between the two plasma sources is the introduction of chlorination, carbonylation, and additional double bonds (one/two dehydrogenations) that were observed for the COST-Jet (He/O$_2$) mainly and are related to the prominent generation of atomic oxygen by this device. For example, the amino acid lysine that was not attacked by kINPen was chlorinated extensively. However, when the COST-jet is driven in the He-only mode, the reactivity is almost completely lost. Instead, kINPen treatment yielded trioxidation (e.g., three hydroxyl groups) and the cleavage of heterocyclic rings that are related to the reactivity of singlet oxygen. In contrast to the COST-Jet, the kINPen also introduced nitrogen-containing modifications, especially nitration, in part combined with oxidation. Both plasma sources did not differ in the total number of the most common

modification, the simple hydroxylation (+16 Da). Some amino acids were modified by the COST-Jet modified to a larger extent, e.g., serine, methionine, and cysteine, probably due to the atomic oxygen density of this device. In contrast, phenylalanine was a better target for the kINPen-derived species. Most aliphatic amino acids (e.g., valine and isoleucine) are modified to a similar extent by both plasma sources, and no clear correlation with the working gas composition was found.

The observations reflect the differences of the working gases argon and helium regarding the formation and lifetime of higher energy states and the different electrode configuration/driving power, yielding differences in reactive species formation and dynamics, which is further modulated by the addition of molecular gases.[63,64] For the helium-only COST-Jet, an almost complete absence of any modifications was noticed, indicating the inability of He higher energy states to reach the surface of the target and a
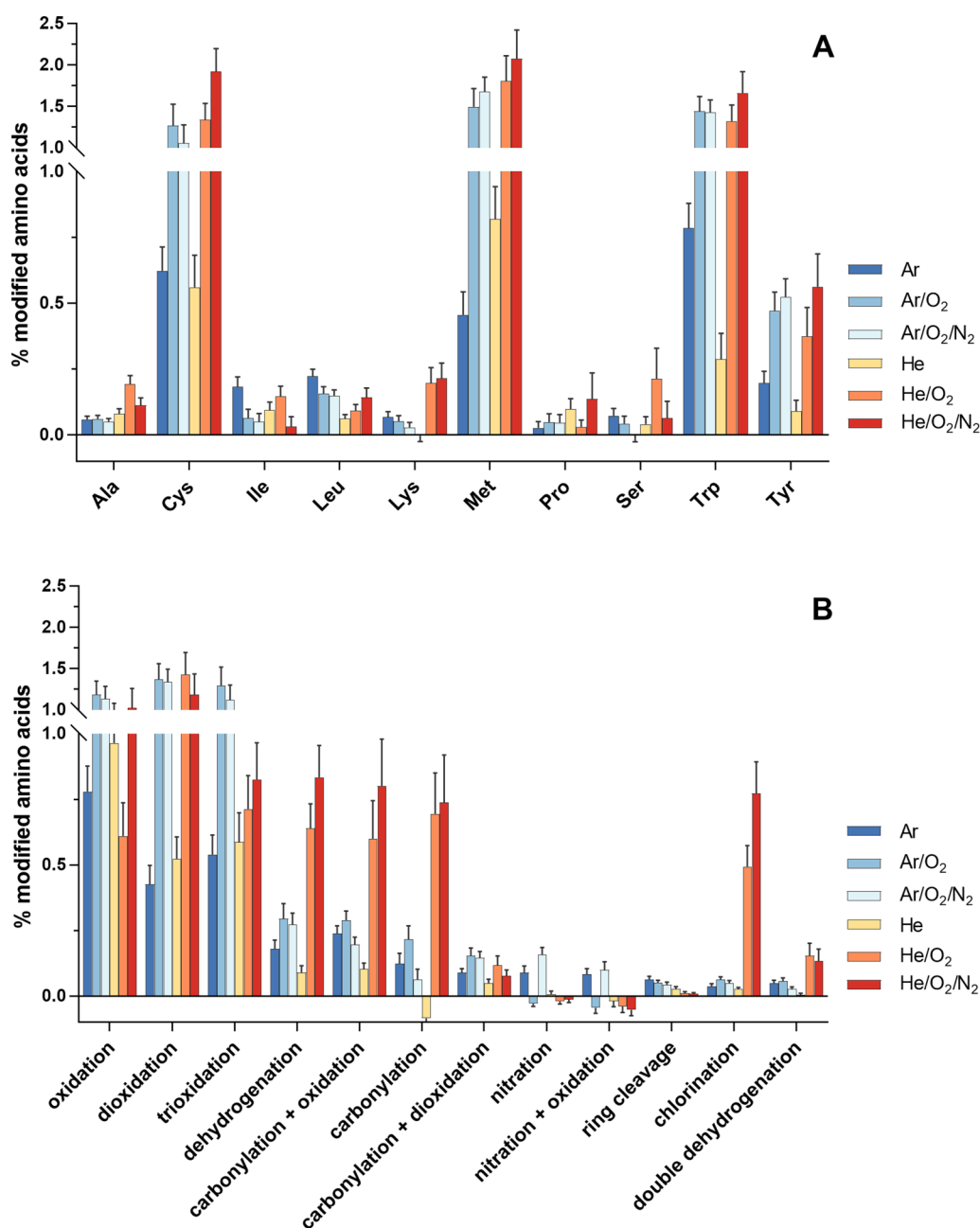
FIG. 6. Most frequently modified amino acids (a, in alphabetical order) and major modifications (b) for kINPen (blue, Ar) or COST-Jet (yellow-red, He) when modulating the working gas composition (+0.5% oxygen or +0.5% oxygen and +0.5% nitrogen). The occurrence relative to the untreated peptide control is given.

very limited impact of ambient air species due to a laminar gas flow leading to a minimum of reactive species.

kINPen in the Ar-only mode retained a high basal generation of ROS and RNS due to the turbulent effluent and the subsequent interaction with the ambient air, and the higher mobility of argon higher energy states. The device showed an interesting modification pattern regarding carbonylation, ring cleavage reactions, and nitration. Carbonylation, which is dominant for the COST-jet and induced by atomic oxygen, was detected for Ar/O_2 treatment and decreases again with N_2-admixture. This indicates that kINPen forms atomic oxygen to a limited extent, but the observed chemistry is dominated by singlet oxygen[35] and N_xO_y molecules.[65]

FIG. 7. Tryptophan cleavage by singlet oxygen yielding kynurenine derivatives (+2O–N–C–H, +4.98 Da). For explanation, see text.

Second, ring cleavage reactions at tryptophan or histidine, which are maximal in argon only conditions and decrease with each additional gas, are exclusively for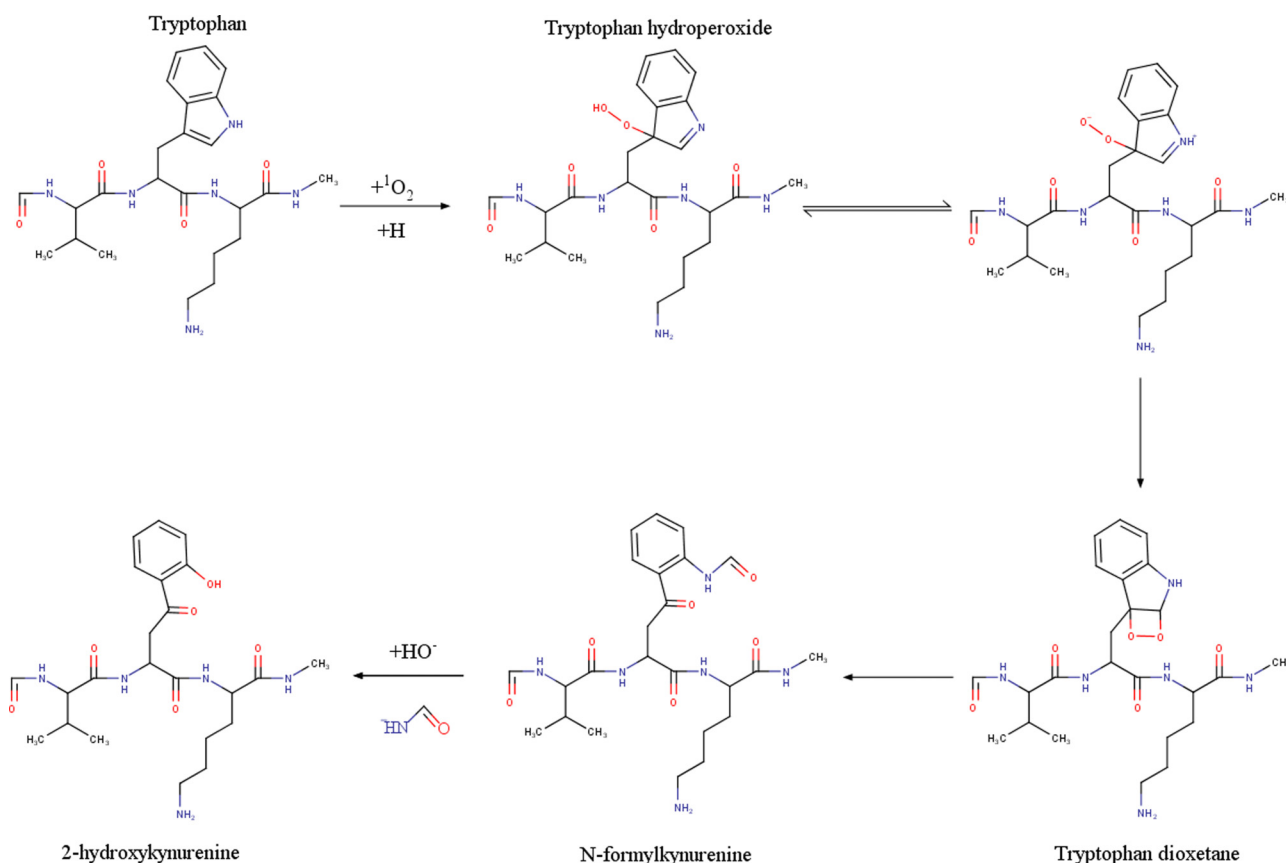med after kINPen treatments. The reaction is attributed to singlet oxygen and the potential mechanism for the reaction with tryptophan is shown in Fig. 7.[49,66] Initially, singlet oxygen attacks the carbon atom and forms tryptophan hydroperoxide, which converts to the tryptophan dioxetane. Alternatively, a 2 + 2 cycloaddition yields directly the dioxetan ring. Subsequent rearrangement forms the *N*-formylkynurenine. In the last step, the final product 2-hydroxykynurenine is formed under the attack of a hydroxyl ion. It remains elusive why the addition of oxygen does not favor an increase of Trp cleavage, since the generation of singlet oxygen increases in this condition.[35] It may be speculated that either changes in reactive species dynamics at the boundary layer and the liquid bulk reduced the fraction of the chemically active singlet oxygen (e.g., ozone formation) or subsequent secondary reactions after the initial cleavage of the Trp moiety led to non-recognizable modifications. Third, the introduction of nitro groups into aromatic amino acids (nitration, nitration + oxidation), which is exclusive for the kINPen, appears for Ar or Ar/O$_2$/N$_2$ admixtures but not in Ar/O$_2$, supporting the above conclusion. Nitration is mainly found in peptides 1 (NH$_2$–AWQDHGLYSK–COOH) and 6 (NH$_2$–IGYKALEVCH–COOH) at tyrosine (Fig. 11). An assumed mechanism for the nitration of the peptide is shown in Fig. 8.

Assumingly, peroxynitrite is the main responsible reactive species. Its generation by plasmas is well-recognized[68,69] and production at the interface from nitric oxide and superoxide anion radicals can be assumed.[70] If only oxygen is available in the discharge, NO· can no longer be generated in sufficient quantity and thus ONOO⁻ generation ceases, yielding a reduced number of nitrations [Fig. 6(b)]. Additionally, secondary reactions remove the bulky nitro groups in favor of hydroxyl groups or quinones.[71] In Fig. 9, the amino acids Cys, Lys, Pro, Ser, Trp, and Tyr are compared, displaying strong differences in type and amount of modifications in dependence on the used plasma source and between the different amino acids. This is specifically obvious for amino acids that can be modified by chlorination (Tyr, Lys), or singlet oxygen (Trp).

D. Short-lived vs long-lived species—The remaining plasma chemistry after the plasma is switched off

It remained questionable, which contribution from long-lived reactive species can be expected for the above-described
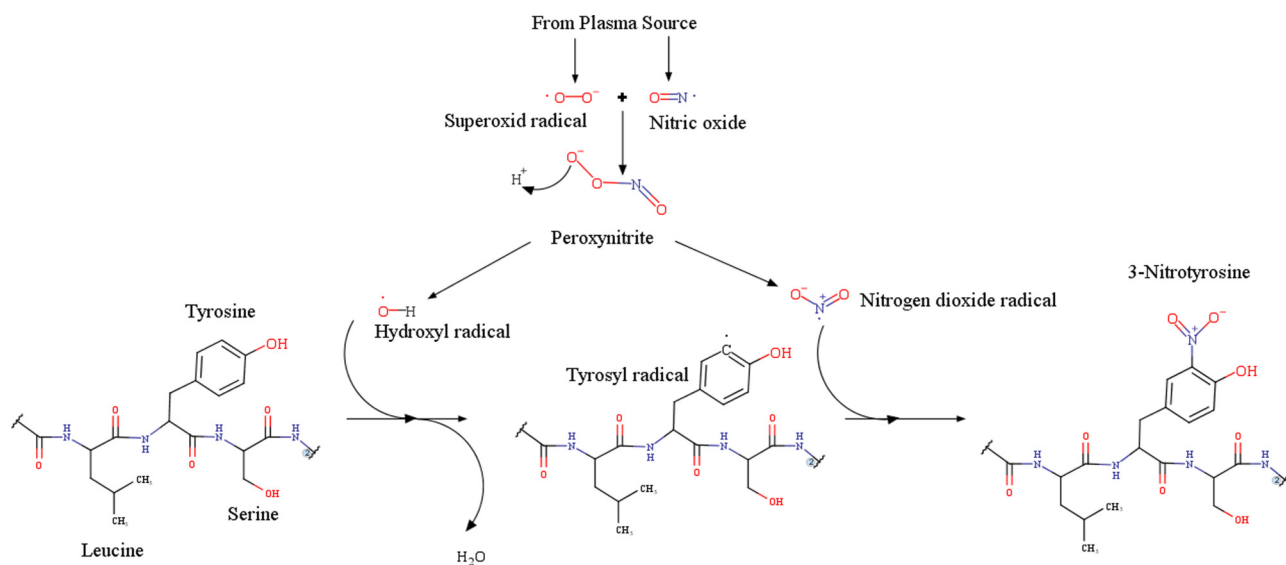
FIG. 8. Suggested reaction mechanism for the production of 3-nitrotyrosine in peptide 1, showing the structure of tyrosine and the two neighboring amino acids leucine and serine. Peroxynitrite is formed from the superoxide radical and nitric oxide and decomposes into an OH- and NO_2-radical. In a first intermediate step, a tyrosyl radical is formed, which is then attacked by the nitrogen dioxide radical to form nitrotyrosine in a second step. Adapted from Ref. 67.

modifications. Hydrogen peroxide, nitrite, nitrate, or ozone are formed to a varying degree during the treatment.[38] The medium-lived OCl^- chemistry preserves the short-lived atomic oxygen and requires to be considered likewise. Therefore, the peptides were exposed to CAP in two orthogonal approaches: the peptides were present either during the discharge was ignited (direct treatment) or were added to the liquid after the discharge had been switched off (indirect treatment). A significant difference between direct and indirect treatment regimens was observed for the two plasma sources [Figs. 10(a) and 10(b)], predominantly inflicted by the hypochlorite chemistry [Figs. 10(c) and 10(d)]. Amino acids sensitive to OCl-driven chlorination (Tyr, Lys) or carbonylation (Ser, Trp) were major targets of the indirect COST-Jet treatment. The co-occurrence of the two modifications strongly suggests that the hypochlorite ion is an active agent for both cases. In contrast, kINPen favored modifications generated during direct treatments, driven by the chemical impact of hydroxyl radicals, atomic oxygen, or singlet oxygen (Cys, Trp, Tyr). Therefore, nitration and the ring cleavages that occur only in direct treatments emphasize the role of the short-lived singlet oxygen and NO_2 radicals.[72]

Perpetuating investigations on the role of the environment during the impact of plasma-derived ROS, the modification of peptides dissolved in water or a buffered, chloride ions containing system (PBS) was compared. In addition to the discussed hypochlorite chemistry, PBS favored reactions that benefit from higher pH such as the oxidation of thiol groups and aminations. Direct oxidations by gas phase species (atomic oxygen, singlet oxygen) decrease in PBS since the abundant chloride ions depress species density at the boundary layer significantly. Therefore, the number of ring cleavages at Trp is reduced in PBS. Reactions, which accelerate at low pH, e.g., nitrations, were found in water predominantly.

E. The chemical environment influences the modification pattern of an amino acid in the peptide chain

The role of the chemical environment of a given amino acid in the peptide chain determined by its position and neighboring amino acids is considered. To exemplify the situation, the nitration of tyrosine is considered (+44.98 Da; + N + 2O–H). While mostly observed for the kINPen (Ar, Ar/N_2/O_2), its appearance fluctuated for the different tyrosine residues present in the library (see Fig. S4 in the supplementary material). Clearly, the sequence and the spatial structure of the peptides are relevant: in peptide 3 (NH_2–CHAGRYFVPW–COOH) and peptide 8 (NH_2–RYVFDEASIL–COOH), phenylalanine (Phe) is in close neighborhood to the modified tyrosine. The weakly reactive Phe has a large aromatic ring, interfering either by reducing access to the tyrosine moiety by steric hindrance, or by reducing the reactivity of the tyrosine's aromatic ring via hydrogen bonds or charge-transfer complexes.[73] In contrast, the tyrosine moieties of peptide 1 (NH_2–AWQDHGLYSK–COOH) and 6 (NH_2–IGYKALEVCH–COOH) do not have amino acids with bulkier or aromatic side chains nearby and can, therefore, be nitrated unhindered (Fig. 11). A similar observation has been made for complete proteins.[74] A second example is the chlorination (+33.96 Da, +Cl–H) (Fig. S2 in the supplementary material). Comparing all peptides in the library, the tyrosine in peptide 6 showed the highest sensitivity to chlorination. A possible explanation is that tyrosine is the exclusive target for oxidative chlorination in this peptide. The only other aromatic structure in the peptide is the imidazole ring of histidine, but this is located far from the targeted tyrosine and not prone for oxidative chlorination. Besides the ϵ-amino groups of lysine

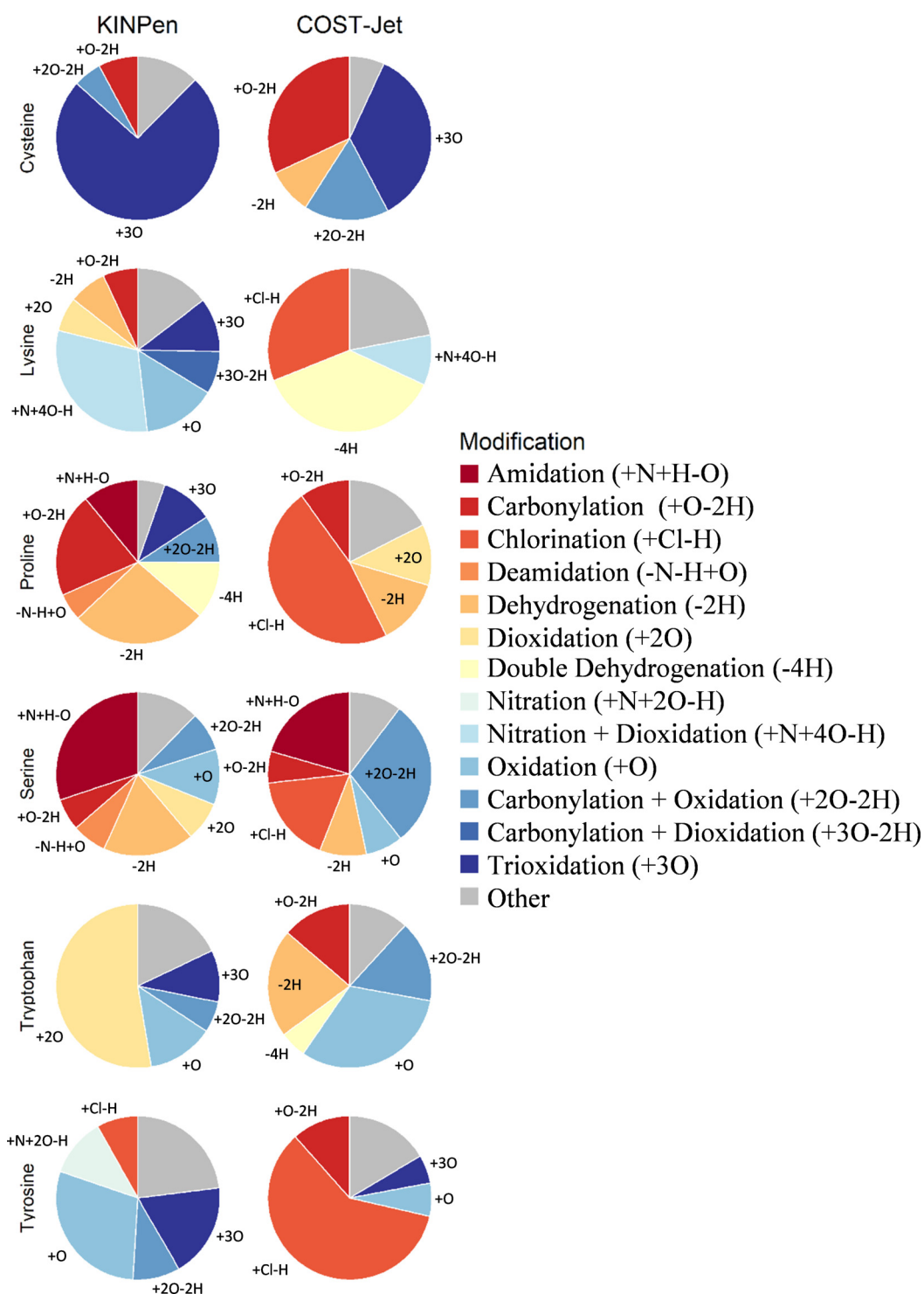**FIG. 9.** Relative distribution of the most frequently identified modifications at the amino acids Cys, Lys, Pro, Ser, Trp, and Tyr after kINPen (left column) or COST-Jet treatments (right column). Treatment variations were not distinguished to reduce complexity. Shown are those modifications that contribute with at least 5% to the total amount. All remaining modifications are shown as "other."

within the peptide chain, the amino groups at the N-terminus of all peptides were chlorinated in appropriate conditions. The α-amino groups incorporated in the peptide chain as part of the peptide bond are not attacked. Cysteine is among the most frequently modified amino acids in the set and shows distinct differences in type and frequency of modifications (Fig. S5 in the supplementary material). The amino acid occurs at different positions in the peptides 2 (NH$_2$–MFCEPITRNV–COOH), 3 (NH$_2$–CHAGRYFVPW–COOH), 6 (NH$_2$–IGYKALEVCH–COOH), 7 (NH$_2$–TKHNQCPWMG–COOH), and 9 (NH$_2$–NLMPCTQHAT–COOH). It is worth mentioning that the cysteine in peptides 3 and 6 both have histidine as a neighboring amino acid and in peptides 7

and 9, proline is next to the cysteine. However, the modifications at cysteine in peptides 3 and 6 differ significantly.

While mono-/di-/trioxidation was found on the cysteine in peptide 6 in most treatment conditions, this is not the case for the cysteine in peptide 3. Here, cysteine is located at the N-terminus, while it is close to the C-terminal in peptide 6. Potentially, the local pH, which modulates the thiol group reactivity drastically, differs. The pKa value of the thiol group may range between pKa = 3.5 and 10, spanning several log-steps.[75] A high degree of dissociation aligns with a reaction probability. Yet, the isoelectric points (pI) of peptides 3 (pI = 8.23) and 6 (pI = 6.74) do not suggest a higher probability of cysteine oxidation in peptide 6. However, peptide 3
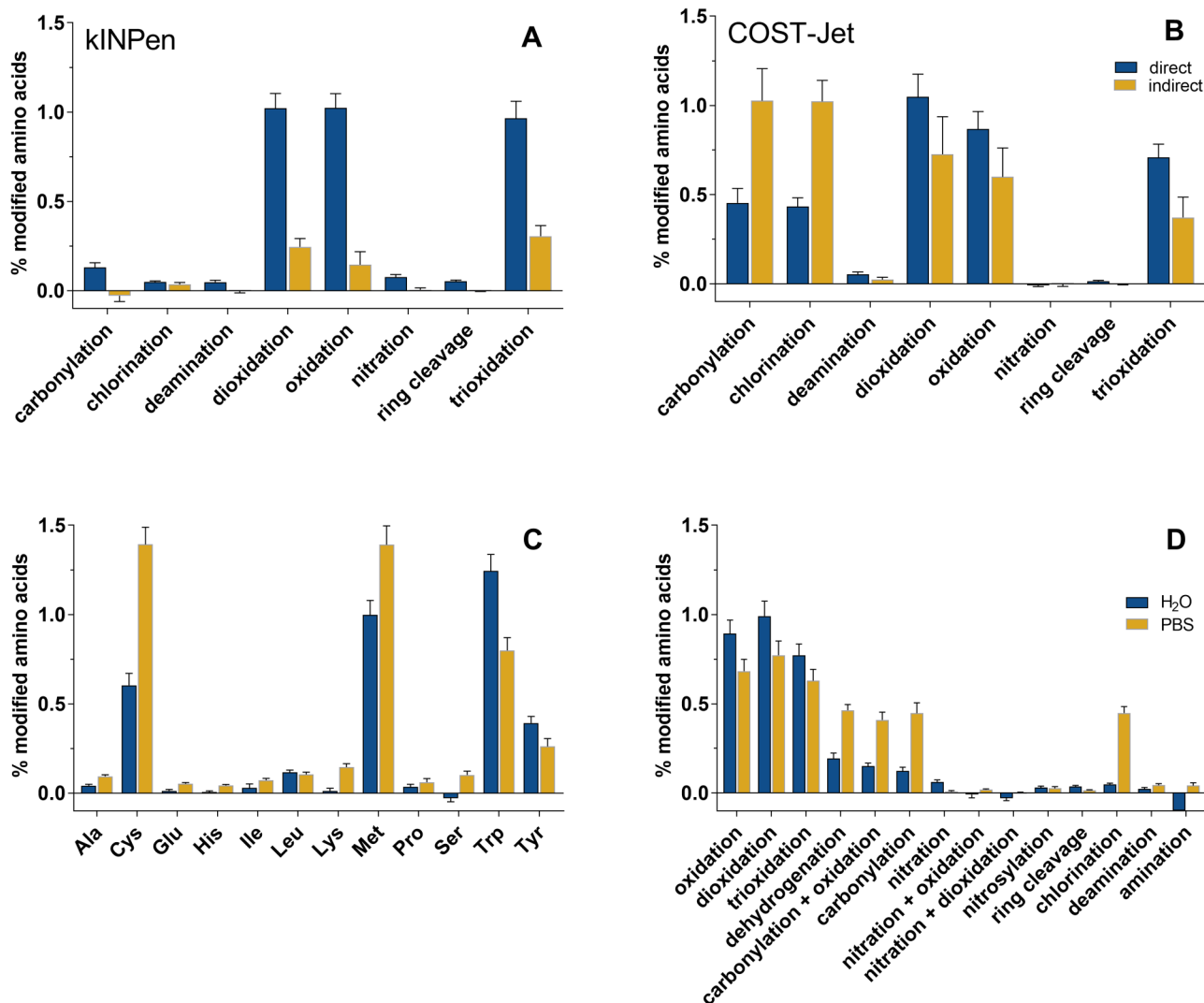
FIG. 10. The impact of timing: figures (a) (kINPen) and (b) (COST-Jet) compare the occurrence of plasma-driven modification after a direct treatment (peptide present in solution during plasma ignited, blue bars) or indirect treatment (yellow bars, peptide added to a plasma-treated solution after discharge was switched off). Impact of pH and chloride ion availability on target amino acids (c) and occurrence of modifications (d) indicate a strong role of atomic oxygen driven hypochlorite and are the basis of the strong impact of the COST-jet in indirect plasma treatment (PBS, phosphate buffered saline, pH 7.4).

has a *C*-terminally located tryptophan, which is an excellent scavenger for the plasma-generated species. It was shown that tryptophan reacts with a rate constant of $3.2 \times 10^7 \, M^{-1} \, s^{-1}$[76] with singlet oxygen ($^1O_2$), which is the fastest compared to other amino acids. In addition, since Trp is hydrophobic and the N-terminus protonated, the peptide adopts a protecting inward position of cysteine at the highly reactive interface toward the liquid bulk. Positively charged amino acids stabilize the resulting thiolate anion and contribute to the lower pKa, in parallel increasing the thiol group reactivity of the cysteine.[77] A similar observation was made for the cysteine residues the peptides 7 and 9. Redox-signaling proteins, e.g., peroxiredoxins, exploit this diversity in reactivity.[56] In addition, there is increasing evidence that oxidative modifications are in crosstalk with other regulatory post-translational modifications, controlling cells performance.[51]

F. Relationship of modifications, amino acids, and treatment conditions

Using statistical tools, the relationship between the plasma source, the mode of treatment, the treatment time, and the solvent system was analyzed [Figs. 12(a) and 12(b)]. The best correlation between the targeted amino acids or observed modifications and the conditions under test was found for the mode of treatment (direct vs indirect). Corroborating, the solvent system (water or PBS) had a strong impact on the target. Both indicators point toward short-lived reactive species including the atomic oxygen-derived hypochlorite as major ROS/RNS. Of note, the treatment time exerted a very limited impact compared to the other parameters. This indicates to some degree the technical limitations of the adopted approach of PSM counting to quantify the observed modifications. It even more emphasizes the relevance of interface reactions, where the full amount of gas phase reactive species is present at any given time point during the treatment process. Whether a depletion of the boundary layer by fast gas phase species–target molecule reactions might be of relevance, leading to an increasing inefficacy of the treatment with longer treatment time, might be debatable.

A boundary layer depletion was observed for hypochlorite formation, and cysteine or glutathione thiol group oxidation.[28,33,39,62] Experiments segregating the target peptides and the boundary layer yielded to a different pattern of modifications, indicating again that
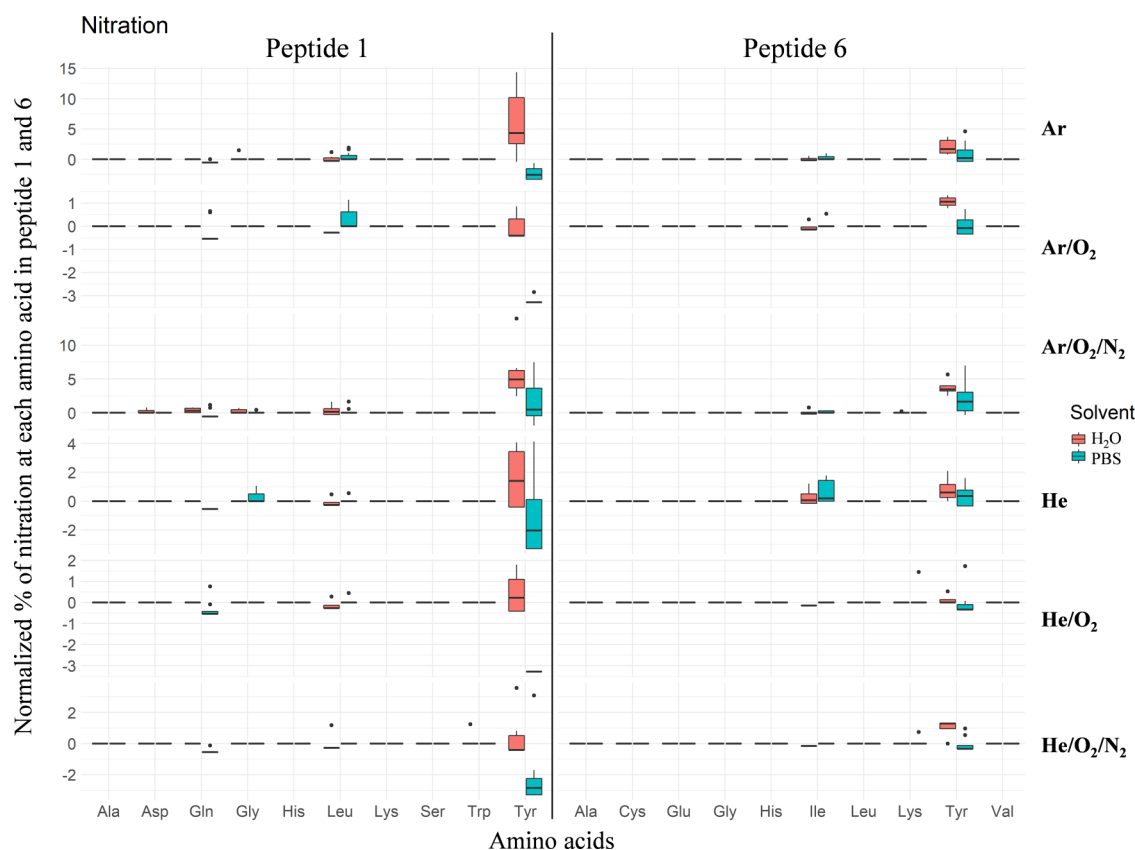

FIG. 11. Overview of every identified nitration in peptides 1 and 6. Divided according to the peptides in which the nitration was identified, and according to the different plasma treatment conditions. The percentage value of the nitrations both in water (orange) and PBS (green) are given and normalized to the amount of nitration found for the corresponding untreated peptide.
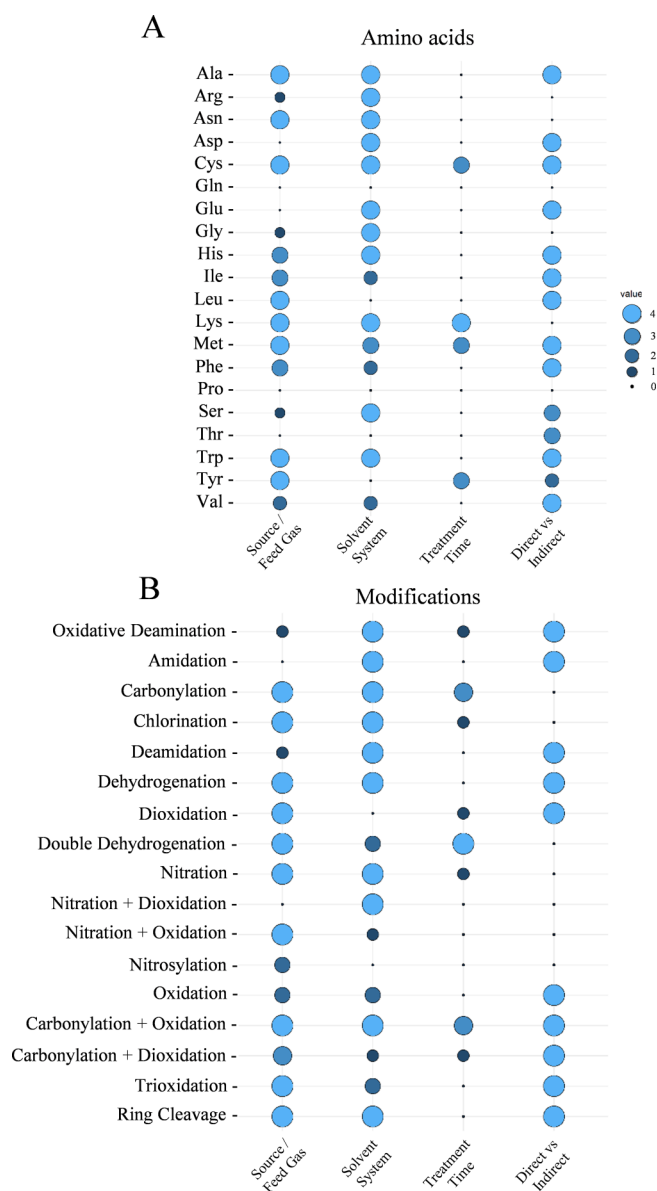
FIG. 12. Using ANOVA-test calculated dependencies of amino acids (a) and modifications (b) related to the gas composition of the plasma, solvent, treatment time, and treatment conditions (direct or indirect). Significance levels: "4" $p \leq 0.001$; "3" $p \leq 0.01$; "2" $p \leq 0.05$; and "1" $p \leq 0.1$.

histidine on the modification pattern. In the human skin, urocanic acid, a catabolite of histidine, may serve the same function.[78]

IV. SUMMARY AND CONCLUSIONS

This work focused on the detection of covalent modifications introduced by reactive oxygen and nitrogen species from cold physical plasmas in a model peptide library. Major targets were the amino acids cysteine, methionine, tyrosine, and tryptophan bearing additional oxygen, nitrogen, or chlorine atoms forming additional hydroxyl, oxo, nitro, or chloro groups. The type of the modification and their extent depend on the CAP source, treatment conditions, the environment of the peptide or protein, and the local chemical environment of each amino acid. Dominant short-lived reactive species were atomic oxygen, singlet oxygen, and to a lesser extent hydroxyl radicals. The formation of hypochlorite and peroxynitrite showed a significant contribution to peptide modification. Long-lived species like hydrogen peroxide, nitrite, or nitrate were irrelevant. The cleavage of histidine and tryptophan pointed on singlet oxygen, a major product of the Ar-driven kINPen. Atomic oxygen, a major product of He/O_2 discharges (COST-Jet), left a significant mark via chlorination when favored by conditions.

Most of the newly introduced groups are identical or equivalent to the enzymatically introduced post-translational modifications (oxPTMs) in physiologic protein processing or cell signaling. They are stable enough to withstand sample preparation and mass spectrometry analysis, suggesting that such *de novo* introduced peptide or protein modifications occur *in vitro* and *in vivo* after a CAP treatment and may subsequently modulate local signaling or protein functionality. Reports on the impact of myeloperoxidase-derived products on the fibrous matrix protein fibronectin emphasize this notion,[61,79] underlining the unique potential of CAP to interfere with biological processes via the modification of biomolecules.

SUPPLEMENTARY MATERIAL

In the supplementary material, a complete overview of all oxidation (Fig. S1) and chlorination events (Fig. S2) for all peptides in the library is shown. The modifications are normalized and divided between the different peptides, gas compositions, and treatment conditions (Fig. S1) or the solvent system (Fig. S2). Figure S3 shows the impact of the different treatment times (15 and 60 s) for nine different amino acids (S3A) and ten different modifications (S3B). Figure S4 gives a similar overview, like Fig. S1, for all modifications identified at cysteine in the five peptides that contained this amino acid. Finally, Table S1 displays the peptide sequence (single letter amino acid code) for the whole library together with the corresponding molar mass and the calculated isoelectric point.

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interface reactions are dominant for all conditions where either atomic oxygen is irrelevant or chloride ions are not present. For future studies, it seems to be worth extending the investigated solvent systems, since the dominant role of chloride ions depends according to the literature on their concentration, modulating the hypochlorite formation. In addition, the presence of scavengers common to complex biological systems influences the modification pattern. Pilot experiments indicated a fundamental impact of free

DATA AVAILABILITY

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

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Article A3

Singlet Oxygen-Induced Phospholipase A2 Inhibition: a Major Role for Interfacial Tryptophan Dioxidation

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Singlet-Oxygen-Induced Phospholipase A₂ Inhibition: A Major Role for Interfacial Tryptophan Dioxidation

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Abstract: Several studies have revealed that various diseases such as cancer have been associated with elevated phospholipase A₂ (PLA₂) activity. Therefore, the regulation of PLA₂ catalytic activity is undoubtedly vital. In this study, effective inactivation of PLA₂ due to reactive species produced from cold physical plasma as a source to model oxidative stress is reported. We found singlet oxygen to be the most relevant active agent in PLA₂ inhibition. A more detailed analysis of the plasma-treated PLA₂ identified tryptophan 128 as a hot spot, rich in double oxidation. The significant dioxidation of this interfacial tryptophan resulted in an N-formylkynurenine product via the oxidative opening of the tryptophan indole ring. Molecular dynamics simulation indicated that the efficient interactions between the tryptophan residue and phospholipids are eliminated following tryptophan dioxidation. As interfacial tryptophan residues are predominantly involved in the attaching of membrane enzymes to the bilayers, tryptophan dioxidation and indole ring opening leads to the loss of essential interactions for enzyme binding and, consequently, enzyme inactivation.

Introduction

Phospholipase A₂ (PLA₂) is a group of membrane proteins that specifically cleaves the acyl ester bond at the *sn*-2 position of glycerophospholipids, releasing fatty acids and

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lysophospholipids.[1] The mammalian genome encodes more than 30 PLA₂ isoforms or related enzymes. The PLA₂ superfamily has three primary classification: secretory PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂), and Ca²⁺-independent PLA₂ (iPLA₂). The activity of PLA₂ isoforms leads to the release of a mixture of bioactive lipids or lipid mediators. Lysophosphatidic acid (LPA) is one such bioactive phospholipid produced by the enzymatic action of PLA₂. It has been found to induce many cancer hallmarks, including cellular processes such as proliferation, growth, survival, migration, invasion, and angiogenesis promotion.[2] PLA₂ overexpression is not only associated with numerous cancers,[3] but also with inflammation in pathological processes such as asthma or allergy.[4] Moreover, increased PLA₂ activity has been reported in autoimmune disorders,[5] schizophrenia,[6] autism,[7] and bipolar disorders.[8] Therefore, elevated PLA₂ activity may play a role in the aforementioned disease states, and the regulation of its activity is of great interest. Several PLA₂ inhibitors have been developed so far: Nevertheless, there has been no reported clinical successes to date and no treatments have reached the market.[9] Hence, novel strategies for the effective and safe inhibition of PLA₂ are highly desirable.

Two regions are known to be involved in the PLA₂ activity; first, the interface site responsible for the enzyme binding to the membrane and the catalytic site.[10] Accordingly, the lack of efficient binding to the lipid membrane leads to a reduction of enzymatic activity. Interfacial bindings are formed primarily by hydrophobic amino acids, of which tryptophan plays a significant role.[11] Leslie et al. reported that the association of the catalytic domain of human cPLA₂ with the membrane is mediated in part by a tryptophan residue located at the membrane-exposed face of the enzyme.[12] Mutation of this tryptophan residue significantly reduced the membrane association of the enzyme.[13] It is also known that tryptophan plays

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an essential role in the interfacial binding and activity of other PLA$_2$ isoforms.[14] Han et al. reported that tryptophan 31 in the binding surface of human group V PLA$_2$ was essential for membrane penetration of the enzyme.[15] Hence, a mutation of tryptophan 31 reduced enzyme activity 7-fold.[16] Notably, Beers et al. reported that the addition of only a single tryptophan to the membrane binding surface of human group IIA significantly enhanced the enzyme activity.[17] PLA$_2$ enzymes which contain the tryptophan residue in the interfacial binding surface are reported to display the highest activity toward neutral phospholipid substrates.[18] Gaspar et al. found that the non-steroidal anti-inflammatory drugs (NSAIDs) inhibit PLA$_2$ activity due to the disturbance of the enzyme binding efficiency to the membrane, possibly by shielding the tryptophan residues of the enzyme.[19] Based on that, we considered the destruction of the tryptophan-lipid interactions beneficial for the enzyme inhibition.

It is reported that the indole ring of the tryptophan has efficient interactions with the membrane's interfacial region.[20] We aimed to determine if the cleavage of the indole ring leads to the loss of effective enzyme-membrane binding. Tryptophan oxidation has been shown to be responsible for the opening of the indole ring.[21] To this end, cold physical plasma (CPP) was used as a source of reactive oxygen and nitrogen species (RONS) for tryptophan oxidation. CPP is a promising medical tool, which has been widely studied in different fields of medicine, such as cancer cell treatment, blood coagulation, wound healing, and skin disease treatment.[22] CPP is a body-temperature ionized gas produced at atmospheric pressure by applying energy to neutral gases.[23] CPP produces a mixture of active agents, including free charged particles, radicals, RONS, UV radiation, and electromagnetic fields.[24] It is assumed that plasma generated RONS, such as hydrogen peroxide (H$_2$O$_2$), ozone (O$_3$), hydroxyl radical ($^\bullet$OH), superoxide ($^\bullet$O$_2^-$), singlet oxygen (^{1}O$_2$), atomic oxygen (O), peroxynitrite (ONOO$^-$), and nitric oxide ($^\bullet$NO) are the critical elements in the above mentioned medical applications of CPPs.[25]

Herein, we report the effective inhibition of bee venom PLA$_2$ by direct plasma treatment. The choice of enzyme was based on the existence of a tryptophan residue on its interfacial region. Supported lipid bilayers (SLBs) were prepared on the surface of a gold electrode, and the activity of the wild-type and plasma-treated enzymes to cleave the SLBs was monitored by electrochemical techniques. Moreover, a detailed analysis of the effect of plasma-produced RONS on the enzyme peptide chain was performed using high-resolution liquid chromatography-tandem mass spectrometry (HR-LC-MS). Finally, the impact of tryptophan oxidation on the enzyme-lipid interactions was identified by molecular dynamics (MD) simulation and molecular docking studies.

Results and Discussion

Electrochemical studies to access the activity of PLA$_2$

To study the PLA$_2$ activity to cleave the lipids, lipid bilayers from phosphoethanolamine (PE) and phosphatidylserine (PS) with ratio of PE:PS (70%:30%) were transferred onto the gold electrode surface by Langmuir-Blodgett and Langmuir-Schaefer deposition techniques. Optimum deposition pressure was assessed at 38 mN m^{-1}, where significant changes in the plot of surface pressure as a function of area per lipid molecule (π-A isotherm) were observed. It is possible to decrease the bilayer thickness by lowering the deposition pressure. As a result, thicker bilayers will be transferred at higher deposition pressures.[26]

Electrochemical measurements were performed to confirm the successful transfer of the lipid bilayer to the electrode surface. As shown in Figure 1, the cyclic voltammogram (CV) and differential pulse voltammogram (DPV) of [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe, in the case of gold supported lipid bilayer had no significant peak current. This indicated that the gold electrode surface was covered with the lipid bilayer and the barrier properties of the transferred lipid bilayer were blocking the probe's access to the electrode surface. The enzymatic activity of PLA$_2$ to cleave the lipid bilayer was studied by injecting 10^{-4} mg mL^{-1} of the enzyme into the electrochemical cell containing the gold supported lipid bilayer working electrode. It is reported that the bee venom PLA$_2$ molecule is Ca^{2+} dependent and highly basic (pI=10.5±1.0), which its
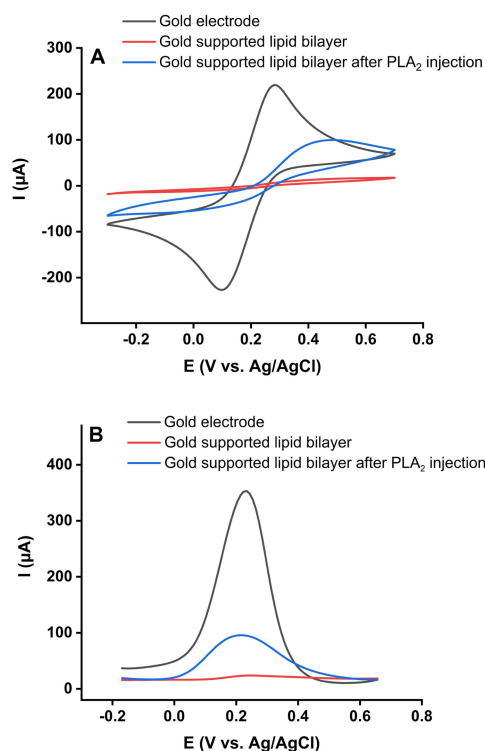

Figure 1. CVs (A) and DPVs (B) of the gold electrode, and gold supported lipid bilayer electrodes before and after injection of PLA$_2$ enzyme.

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optimal activity occurs at alkaline pH.[27] Based on that, measurements were performed in Tris buffer (pH 8.9) containing 5 mM CaCl$_2$.

The ability of the enzyme to hydrolyze the phospholipids damaged the lipid bilayer. It led to the gold electrode surface being more accessible for the redox probe, facilitating electron transfer between the probe and the electrode. As a result, the observed increase in the current and area of the DPV and CV peaks of [Fe(CN)$_6$]$^{3-/4-}$ (Figure 1) after two hours was related to PLA$_2$ activity.

Balashev et al. have visualized the degradation of lipid bilayer due to the action of PLA$_2$ by atomic force microscopy. They found that hydrolysis of the lipid bilayer is initiated at specific regions where traces of structural bilayer defects or holes are distinguished or depressions in the bilayer have been identified. After PLA$_2$ injection, the existing structural defects were enlarged, and new holes appeared.[28]

The changes in lipid bilayer permeability after injection of PLA$_2$ to the electrochemical cell were calculated based on Equation (1).

$$\% \text{ Changes in permeability} = [(A_1 - A_2)/A_3] \times 100 \quad (1)$$

A$_1$, A$_2$ and, A$_3$ were the area of the DPV curves of 10 mM [Fe(CN)$_6$]$^{3-/4-}$ for gold supported lipid bilayer after two hours of PLA$_2$ injection, gold supported lipid bilayer, and gold electrode, respectively.

The effect of plasma treatment on the activity of the PLA$_2$ enzyme was investigated (Figure 2). A solution of 60 s Ar plasma-treated PLA$_2$ was added to the electrochemical cell containing a gold supported lipid bilayer working electrode. Interestingly, we found that lipid bilayer permeability changes were negligible (Figure 2A), which illustrated that the plasma-treated enzyme was no longer able to hydrolyze the phospholipids. To better understand the plasma-mediated enzyme inhibition, Tris buffer (pH 7.4) was exposed to Ar plasma, and the PLA$_2$ enzyme was added immediately after. The results of which demonstrated that this 'indirectly'-treated enzyme cleaved the lipid bilayer to increase its permeability: as such, indirect plasma treatment could not inhibit the enzyme activity. We concluded that short-lived reactive species from plasma were responsible for the observed enzyme inhibition, and the responsible agents for enzyme inactivation were lost in indirect treatment. Accordingly, the concentrations of deposited reactive species during 60 s Ar plasma treatment were quantified (Figure S2). We found •OH and ^{1}O$_2$ as short-lived and H$_2$O$_2$, nitrite (NO$_2^-$), and nitrate (NO$_3^-$) as long-lived plasma-produced reactive species in our measurement. Further experiments were conducted by adding a similar amount of identified long-lived reactive species to the PLA$_2$ solution. The obtained results are shown in Figure 2A, which indicated that none of these species were able to inactivate the enzyme.

^{1}O$_2$ was one of the short-lived reactive species found only within direct plasma treatment (Figure S2) and is reported to be related to the anti-tumor effects of CPP.[29] Bauer et al. introduced 2 mM of histidine as a standard concentration of ^{1}O$_2$ scavenger.[30] To assess the effect of ^{1}O$_2$, histidine was added to
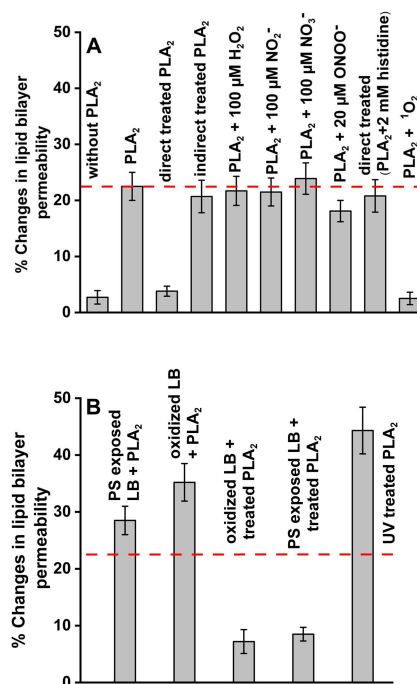

Figure 2. The changes in the PE: PS (70%: 30%) lipid bilayer (LB) permeability after incubation of bilayer with PLA$_2$ enzyme for 2 h (A). The changes in the lipid bilayer permeability after incubation of PE: PS lipid bilayers with different physical properties with PLA$_2$ for 2 h (B). The dashed red line indicated the activity of wild-type PLA$_2$ to cleave PE: PS (70%: 30%) lipid bilayer.

the PLA$_2$ solution during direct plasma treatment. It was found that in the presence of histidine, the direct plasma-induced abrogation was abolished. This suggested ^{1}O$_2$ to be the responsible agent for the enzyme inactivation. To verify the role of ^{1}O$_2$ in enzyme inhibition, a solution of PLA$_2$ in D$_2$O was irradiated in the presence of 10 µM of rose bengal as photosensitizer with continuous oxygenation. Then, the appropriate amount of the irradiated enzyme was injected in the electrochemical cell containing gold supported lipid bilayer to have 10^{-4} mg mL^{-1} PLA$_2$. After two hours, the changes in the permeability of the lipid bilayer were monitored. We found that the irradiated enzyme was inactivated and was not able to cleave the phospholipids. The control experiment was repeated similarly by mixing PLA$_2$ and rose bengal in D$_2$O without irradiation. In the control experiment, the enzyme was able to cleave the bilayer and increase the permeability. These results confirm our suggestion that ^{1}O$_2$ is involved in the inactivation of the enzyme by plasma treatment. Moreover, the effect of substrate physical properties on the PLA$_2$ activity was investigated and is shown in Figure 2B. The obtained results indicated that wild-type PLA$_2$ had a higher activity for cleaving oxidized and PS exposed lipid bilayers (PE: PS (50%: 50%)) due to the altered structure of the membrane, which facilitated adsorption and access of the PLA$_2$ to the sn-2 ester bond of the phospholipids.[31] However, cleavage of the oxidized and PS exposed membranes by treated PLA$_2$ was sluggish. Furthermore, the impact of UV radiation from the plasma on the

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activity of PLA$_2$ was investigated. The enzyme was treated in a chamber with an MgF$_2$ window to eliminate the effects of plasma-generated RONS and ensure only UV radiation can affect the enzyme. As shown in Figure 2B, the UV radiation increases the enzyme activity of lipid cleavage and as such is unlikely to be an influential factor in enzyme inhibition.

Dynamic light scattering (DLS) and circular dichroism (CD)-spectroscopy measurements

DLS was performed to analyze possible enzyme degradation or aggregation by investigating any changes in size and heterogenicity of the enzyme after plasma treatment. Comparing the correlograms of PLA$_2$, there were no significant changes in the light scatter decay time (Figure 3A) and the total area under the curve (AUC) (Figure 3B) for the enzyme after plasma treatment. The high polydispersity index (wild-type = 0.85, plasma-treated = 0.87) suggested a significant level of heterogenicity that did not change following plasma treatment. Additionally, the size of the enzyme before and after plasma treatment was measured as 4.97 ± 0.85 nm (wild-type) and 4.58 ± 0.72 nm (plasma-treated) (Figure 3C), which revealed no notable changes in diameter after plasma treatment. Collectively, these findings suggest no enzyme degradation or aggregation as a result of plasma treatment.

Consequently, we performed CD-spectroscopy analyses to track the changes in the secondary structure of the enzyme that may appear after plasma treatment. For this purpose, the CD spectra of the PLA$_2$ enzyme before and after plasma treatment with the Tris buffer spectrum used as the control measurement were recorded. As is shown in Figure 3D, the enzyme α-helix is depicted in the CD-spectra without considerable changes. Assuming a constant enzyme concentration in both measurements, no significant differences appeared in the enzyme's secondary structure after plasma treatment.
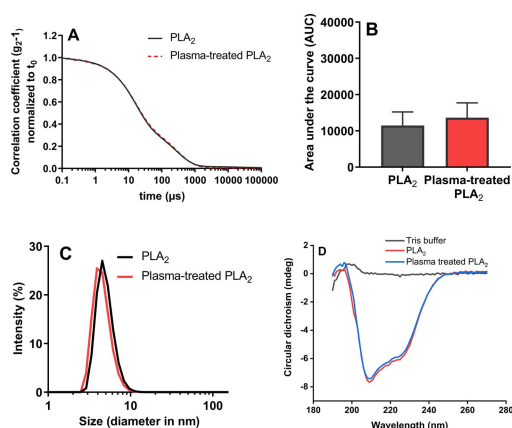
HR-LC-MS analysis

The inactivation of the plasma-treated PLA$_2$ prompted us to analyze the oxidative modifications of the enzyme induced by RONS using HR-LC-MS. The coverage of the protein sequence was more than 90% before filtering the peptide spectrum matches with Byonic and delta mod score and 75% after filtering. The comparison of the chromatograms revealed that cysteine and methionine, as very reactive amino acids,[21e,32] did not show significant oxidative modifications after plasma treatment. However, tryptophan 128 at the exterior of the enzyme appeared as a hot spot. A detailed view of the RONS-induced oxidative modification sites found in the amino acid sequence of the enzyme after plasma treatment and the relative amount of each modification compared to the total number of modifications is shown in Figure 4. Obviously, among all modifications, tryptophan 128 dioxidation constituted more than 25% of all the identified modifications. This number indicated the high susceptibility of tryptophan to dioxidation, possibly due to the oxidation by $^1O_2$ produced from plasma. The origin of other detected plasma-induced modifications of amino acids was studied extensively.[33]

Dioxidation of tryptophan resulted in the formation of different products.[34] Li et al. have studied the chemical basis of tryptophan oxidation using an Ar plasma jet.[35] In addressing this issue, they fully characterized each tryptophan-derived product using LC-MS^2. They demonstrated that the plasma-induced oxidation of tryptophan gave rise to a mixture of hydroxyl derivatives and hydroperoxides, which were decom-

Figure 3. Correlation coefficient (A), AUC (B), and the size (C) of untreated and plasma-treated PLA$_2$ in Tris buffer (pH 7.4) measured by DLS (n = 3), and CD spectra of Tris buffer (pH 7.4) and PLA$_2$ enzyme before and after plasma treatment (D) (n = 2).
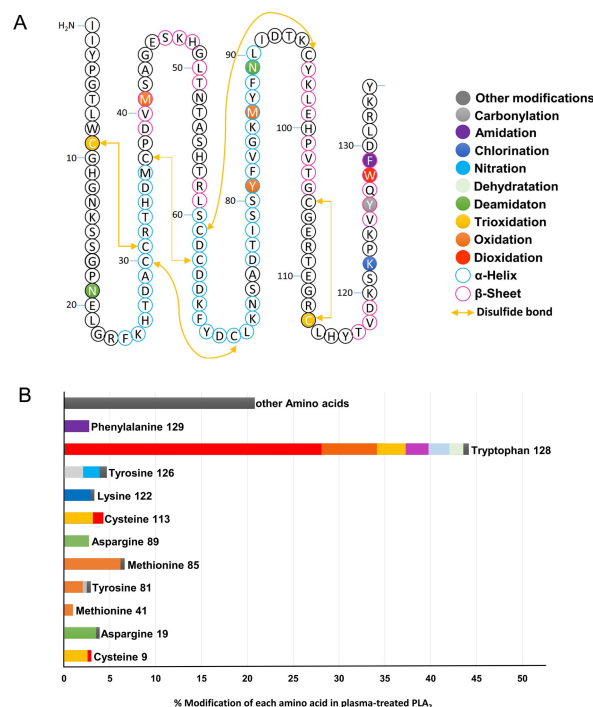

Figure 4. The complete amino acid sequence of PLA$_2$ with the specific location of each identified RONS-induced modification (A), and the relative amount of each amino acid modification in plasma-treated PLA$_2$ compared to the total number of modifications (B).

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posed into N-formylkynurenine (NFK) under physiological conditions. Furthermore, they proposed a mechanism for the tryptophan reaction with the plasma-generated RONS, which introduced NFK as the final product of tryptophan oxidation by the Ar plasma jet. Moreover, Ronsein et al. have studied the mechanism of tryptophan oxidation by $^1O_2$.[21b] They characterized two cis- and trans-tryptophan hydroperoxide (WOOH) isomers as the major tryptophan oxidation products. They have shown that WOOHs were highly unstable and rapidly decomposed under heating or basification, leading to the formation of NFK. A similar mechanism was proposed by Gracanin et al. for tryptophan oxidation to NFK.[36] As plasma is a body-temperature ionized gas, and the treatment was done at slightly basic solutions (pH 7.4) and based on the UV absorption spectra (Figure 5S), we assigned NFK as the major product of tryptophan dioxidation and the main oxidative product in the plasma-treated PLA$_2$ sequence.

The oxidation of tryptophan to NFK in proteins has been known. Kuroda et al. found that inactivation of lysozyme occurred only when one specific tryptophan residue was oxidized to NFK.[37] It was the first evidence that oxidation of a particular tryptophan residue can impair enzyme activity. They reported this oxidative inactivation is accompanied by the loss of the ability of lysozyme to form an enzyme-substrate complex. Moreover, tryptophan dioxidation to NFK has been detected in a variety of other proteins, including mitochondrial ATP synthase,[38] photosystem II subunits,[39] apoliprotein B-100,[40] myoglobin,[41] aconitase-2 (mitochondria),[42] frataxin,[43] troponin I and actin.[44] Furthermore, Kasson et al. introduced NFK as a new marker identify ROS generation sites in photosystem II and other proteins.[45]

It is known that the interactions of the tryptophan indole ring with biomembranes are highly remarkable in their solvation.[20,46] As a result, tryptophan dioxidation and the indole ring-opening can prevent membrane proteins from efficiently anchoring into the lipid bilayer, which leads to enzyme inactivation. To prove our hypothesis, MD simulation and molecular docking were done to measure the interactions between wild-type PLA$_2$ containing tryptophan 128 residue and plasma-treated PLA$_2$ with the NFK group and a PE lipid.

MD simulation

To further highlight that tryptophan dioxidation and NFK formation had no effect on the size and structure of the enzyme, but only altered the interactions with phospholipids, MD simulation was applied. For this purpose, structures and the surface electrostatic potentials indicating the positively and negatively charged regions for the wild-type and plasma-treated PLA$_2$ were generated with PyMol and APBS and are compared in Figure 5. As is shown, there were no significant changes in the enzyme's structure and size after plasma treatment; especially in the active site of the enzyme, supports the finding of the obtained DLS and CD-spectroscopy experimental data. The most apparent structural change was observed in the region where tryptophan 128, tyrosine 126, and
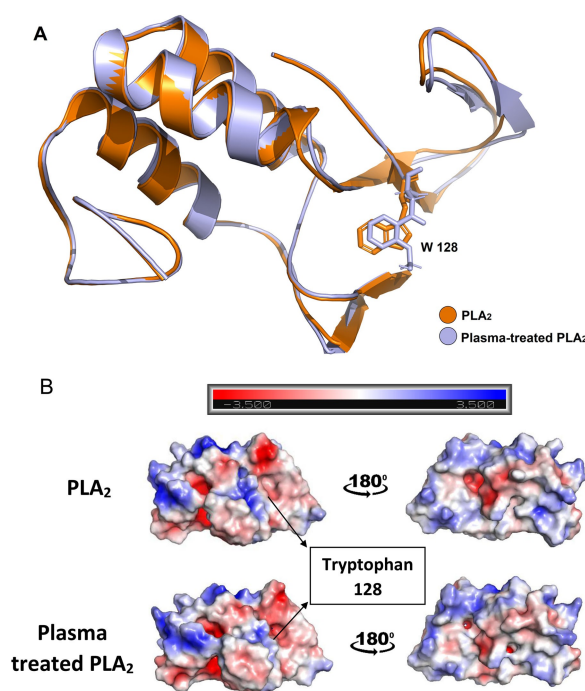

Figure 5. Structural alignments (A) and surface electrostatic potentials (blue for positively charged, red for negatively charged), before and after rotation by 180° about the vertical axis (B) for wild-type and plasma-treated PLA$_2$.

phenylalanine 129 were modified. Moreover, root-mean-square-deviation (RMSD) for untreated against plasma-treated PLA$_2$ was calculated as 0.585. This small RMSD suggests only partial differences between the structure of enzyme before and after plasma treatment.

As mentioned, tryptophan plays an essential role in the binding of membrane enzymes. In this regard, FTMap[47] was performed to identify if tryptophan 128 could be a hotspot for different ligands. It was found that tryptophan 128 is able to establish hydrogen bonds with 2.46% ligands (Figure S6), which was a significant percentage for the outer region of the enzyme active site. Hydrogen bonds and electrostatic-π interactions are essential for indole solvation, and these interactions are responsible for tryptophan's role in anchoring proteins into the membrane.[46] As a result, any changes in the indole ring structure due to plasma treatment could affect tryptophan's ability to bond with the membrane. As such, molecular docking was performed to simulate the interactions between PE lipid and wild-type or plasma-treated PLA$_2$ with tryptophan or NFK residues, respectively.

The molecular docking results indicate that PE lipids can establish two groups of hydrogen bonding via its carbonyl groups with tryptophan 128 of wild-type PLA$_2$. The first hydrogen bonding was between the lipid carbonyl group and NH$_2$ of tryptophan 128, which had an angle of 14.5 degrees to the x-axis, and 143.8 degrees to the y-axis at a distance of 3.0 Å. The other hydrogen bond formation was between the N atom of the tryptophan 128 indole ring and the second carbonyl group of PE, with an angle of 17.9 degrees to the x-axis and 116.8

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degrees to the y-axis at a distance of 2.8 Å (Figure 6A and B). After plasma treatment and dioxidation of tryptophan, these hydrogen bond and the other electrostatic-π interactions of tryptophan 128 with phospholipid were eliminated (Figure 6C and D). Dioxidation of tryptophan and opening of the indole ring decreased the total interactions between the phospholipid and the enzyme. The enzyme's affinity for ligand binding was calculated as -4.3 kcal mol^{-1} (docking score) for PLA$_2$, which was reduced to -3.4 kcal mol^{-1} after plasma treatment. The molecular docking results indicated that in the case of plasma-treated PLA$_2$, the absence of sufficient interactions between the enzyme and lipid membrane prevented enzyme localization in the lipid bilayer, which resulted in the inactivation of the enzyme.

Kubiak et al. studied the products of bovine eye lens protein gB-crystallin (GCS) oxidation by MD modeling.[48] They induced mutations at tryptophan residues converting them into NFK. According to quantum chemical calculations, they found the rearrangement of the charge distribution in the tryptophan moiety after its oxidation to NFK is localized in the opened indole ring. They demonstrated the increased local electric dipole moment of the NFK residue decreased the hydrophobicity of the molecule. Furthermore, they reported the NFK residue significantly more polar than tryptophan. Thus, under favorable conditions, new hydrogen bonds with water were created. They presented a snapshot from the GCS and NFK trajectories, showing that NFK penetrated the water further than tryptophan. Considering their results, oxidation of tryptophan to NFK was decreasing the lipophilicity of the treated enzyme. Thus, the plasma-treated enzyme had less affinity to form hydrogen bonds with phospholipids, leading to the loss of efficient interactions between the enzyme and phospholipids and enzyme inactivation. This could represent a novel treatment strategy for many diseases that involve PLA$_2$ over-expression.

Conclusion

In this study, we demonstrated that the plasma-produced $^1O_2$ led to the efficient inactivation of the PLA$_2$ enzyme, which is highly desirable due to its increased activity in many diseases. We verified that the dioxidation of interfacial tryptophan 128 residue to NFK led to the decay of functional interactions between the enzyme and phospholipid, preventing the enzyme from anchoring in the membrane and consequently inhibited the enzyme efficiently. The introduced inhibition mechanism is not limited to the PLA$_2$ enzyme, but can be extended to those proteins in which interfacial tryptophan plays a role in their membrane anchoring and functionality. Influenza virus haemagglutinin,[49] Ebola virus matrix protein,[50] multidrug resistance protein 1,[51] and Escherichia coli α-Hemolysin[52] are only a few examples in which tryptophan residues are known to be required for their stabilizing and proper function in cells. This study deepens our knowledge of the structure-function relationship of the PLA$_2$ enzyme and introduces the possibility of using this inactivation strategy based on pro-oxidant
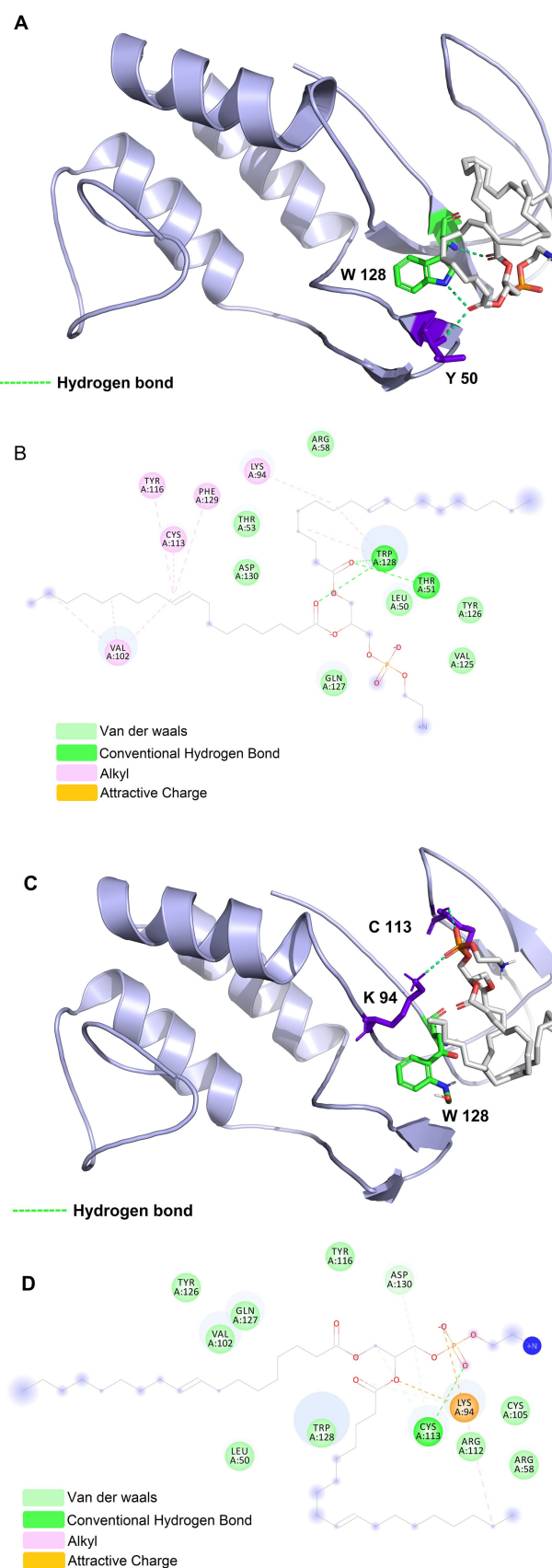

Figure 6. 3D and 2D interactions of PLA$_2$ and PE lipid before (A and B) and after (C and D) plasma treatment. Abbreviations for amino acids: W: tryptophan, Y: tyrosine, C: cysteine, K: lysine.

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therapies for all the proteins in which tryptophan has a role in their membrane anchoring and functionality.

Experimental Section

Reagents

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (18:1 (Δ9-Cis) PE (DOPE)) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (18:1 PS (DOPS)) were from Avanti Polar Lipids and purchased from Otto Nordwald (Otto Nordwald GmbH, Germany) and used without further purification. Chloroform and ethanol were HPLC grade from Carl Roth (Carl Roth GmbH+Co. KG, Germany). Methanol (99.95%) was from Th. Geyer (Th. Geyer GmbH & Co. KG, Germany). Phospholipase A_2 from honey bee venom (Apis mellifera, UniProt P00630), rose bengal, potassium hexacyanoferrate(II) trihydrate, potassium hexacyanoferrate(III), and hydrogen peroxide (30% w/w) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Germany). Peroxynitrite was from Merck (Merck Chemicals GmbH, Germany). Water used for cleaning and measurements was purified with ultrapure MilliQ water (Milli-Q® Merck KGaA, Germany). All other reagents were of analytical grade and used without further purification.

Preparation of gold supported lipid bilayer

To prepare the gold-supported lipid bilayers, Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) deposition techniques were applied for the transfer of the first and second lipid monolayers onto the Au(111) (arrandee metal GmbH+Co. KG, Germany) substrates, respectively. For this purpose, surface pressure-area isotherms were recorded using a Langmuir trough (KSV NIMA, LOT-QuantumDesign GmbH, Germany). The Au (111) substrate was flame-annealed and cleaned in piranha solution (3:1 mixture of concentrated sulfuric acid and 30% hydrogen peroxide solution) before being transferred into the trough.[53] A sufficient quantity of 1 mg mL^{-1} lipid solution was dispersed onto the surface of the water subphase in a dropwise manner. The lipid solvent was allowed to evaporate for 15 min, and then the barriers were closed to obtain the target pressure of 38 mN m^{-1}. The Au substrate was raised vertically through the monolayer film at a speed of 2 mm min^{-1}, while the pressure was maintained at the target pressure and then dried in argon for 30 min. A transfer ratio of 1.0±0.1 was accepted as a successful transfer of the lipid to the substrate. To transfer the second monolayer, the Langmuir-Schaeffer (horizontal dip) was performed at a speed of 0.5 mm min^{-1}. The Au substrate-supported lipid bilayer was placed immediately into the electrochemical cell.

Electrochemical measurements

Electrochemical measurements were carried out using a potentiostat AUTOLAB PGSTAT302 N (Deutsche METROHM GmbH & Co. KG, Germany) electrochemical system. All experiments were performed in a three-electrode system consisting of a leakless Ag/AgCl miniature reference electrode and a platinum wire auxiliary electrode (eDAQ Europe, Poland), and a gold substrate supported lipid bilayer as the working electrode. DPVs and CVs were recorded in a solution of 10 mM [Fe (CN)$_6$]$^{3-/4-}$ in Tris buffer [0.010 M Tris (pH 8.9), 0.150 M NaCl, 0.005 M CaCl$_2$].

CPP treatment

The kINPen09 (Neoplas tools GmbH, Germany) was used as a well-characterized plasma source[54] running at 1.1 W and a frequency of 1 MHz. Argon was used as feed gas at a flux of 3.0 standard liters per minute (slm). Volumes of 750 µl of PLA$_2$ enzyme in Tris buffer [0.010 M Tris (pH 7.4), 0.150 M NaCl, 0.005 M CaCl$_2$] were treated with the kINPen at a distance of 9 mm between the jet nozzle and the sample surface for 60 seconds.

Singlet oxygen generation

Samples of 0.01 mg mL^{-1} PLA$_2$ enzyme in D$_2$O were irradiated for 30 minutes in the presence of 10 µM of rose bengal as photosensitizer with PL201 compact laser module 520 nm wavelength (Thorlabs GmbH, Germany) with continuous oxygenation.

DLS measurements

Samples of untreated and plasma-treated PLA$_2$ (1 mg ml^{-1}) in Tris buffer (pH 7.4) were loaded in low-volume disposal cuvettes (ZEN0040). Spectra were recorded using a ZS90 dynamic light scattering device (Malvern Instruments, UK) equipped with a helium-neon laser light source (632 nm). The samples were measured at 25°C, with an equilibration time of 120 seconds. Attenuator was set to 9, backscatter angled detection was performed at 173° with a scattering collection angle of 147.7°. Measurement was performed once in three technical replicates, with a measurement duration of 25.2 sec (1.68 sec per run) for each replicate. Data were evaluated with ZS XPLORER Software 1.3.0.140 and GraphPad prism 9.0.2.

CD-spectroscopy

Circular dichroism data were monitored with a Chirascan CD spectrometer (Applied Photophysics, UK) combined with a temperature controller (Quantum Northwest, USA). Each measurement was done with samples in a concentration of 25 µg ml^{-1} diluted in Tris buffer (pH 7.4) and loaded in 5 mm-pathlength cuvettes (Hellma Analytics, Germany). CD spectra were recorded at 25°C in the range of 190 to 270 nm with a bandwidth of 1.0 nm, a scanning time of 1.5 s per point, and five repetitions. All spectra were blank corrected and repeated at least two times.

HR-LC–MS measurements and data analysis

The PLA$_2$ samples were heated for 10 min to 95°C. After cooling down to room temperature, the protein was digested with trypsin with an enzyme to protein ratio of 1:25 for 2 h at 37°C. After digestion, the peptides were ready for measurement by HR-LC-MS. The peptides were analyzed on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific, Germany) coupled to an UltiMate 3000 RSLCnano UHPLC system (Thermo Scientific, Germany). Peptides were loaded on a trap column (Acclaim PepMap100 C18 material, 5 µM 0.1×20 mm, Thermo Fisher) with a flow rate of 5 µL per minute for 6 minutes. The loaded peptides were resolved at 300 nL min^{-1} flow rate using a linear gradient of 4 to 45% solvent B (0.1% acetic acid in 95% acetonitrile) over 42 minutes on an analytical column (150 mm×75 µm, 2.0 µm particle size, Thermo Fisher Scientific). Attached was an EASY-Spray ion source operated at 1.9 kV voltage, and the temperature was 250°C, with a stainless steel emitter.

Mass spectrometry analysis was carried out with the peptide application mode peptide and in a data-dependent manner with a

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full scan range of 350 to 1200 m/z with top N where N was set to 15 most abundant ions. Both MS and ddMS^2 were measured using an Orbitrap mass analyzer. Full MS scans were measured with a resolution of 120,000 at 200 *m/z*. Precursor ions were fragmented using fixed normalized higher-energy collisional dissociation (HCD) collision of 30% and detected at a mass resolution of 15,000 at m/z 200. Normalized Automatic gain control (AGC) target for full MS was set to 300% (3e6 ions), and for MS^2 was set to standard with an automatic maximum ion injection time for full MS and 50 ms for ddMS^2. Dynamic exclusion was set to 30 seconds, and singly charged ions were excluded. The raw data analysis to identify the peptides and the oxidative modifications was performed with the Proteome Discoverer 2.4.1.15 software (Thermo Fisher Scientific) using Byonic (Protein Metrics)[55] Version 3.8 as a plug-in tool. The peptide spectrum matches were filtered via Byonic score of 250 and delta mod score of 5. A list of expected oxidative modifications was used to screen and detect the modified peptides in Byonic (Table S2) can be found in the supplemented information of the paper.

MD simulation

MD simulation and molecular docking were applied to investigate the structure of PLA$_2$ and the interactions between tryptophan 128 residue of wild-type and plasma-treated PLA$_2$ and PE lipid. MD simulation was performed with GROMACS[56] program package (version 5.0) OPLS-AA/L all-atom force field. The coordinate file of PLA$_2$ was obtained from the protein data bank (PDB ID: 1POC). The structure of PLA$_2$ was placed in the simulation box, and the box type was defined as a cube with a size of 5.5 nm. The box was filled with water molecules (the SPC water model) surrounding PLA$_2$, sodium ions were added to the system. Afterward, the energy of the system was minimized. Eventually, a 100 ps equilibration run was performed employing the NVT ensemble (i.e., a system with a constant number of particles N, volume V and temperature T). Finally, a 100 ns production run was accomplished using NPT dynamics (i.e., a system with a constant number of particles N, pressure P, and temperature T). The reference pressure was set to 1 bar. The calculated values were not identical for wild-type and plasma-treated PLA$_2$, and the treated enzyme showed less stability.

Molecular docking was performed by AutoDock Vina.[57] The model mentioned above was used as the target, and PE was obtained from PubChem[58] web server as the ligand. For docking, tryptophan 128 was chosen as the center of the grid box. According to affinity (kcal mol^{-1}) and root-mean-square-deviation, which were given by AutoDock Vina, the best model for wild-type PLA$_2$ was selected. The interactions between untreated PLA$_2$ and PE ligand were analyzed using AutoDockTools-1.5.6 and Discovery Studio.[59] First, all the modifications detected by HR-LC-MS were applied to the wild-type PLA$_2$ using Avogadro[60] software. Afterward, energy minimization was performed to optimize the modified structure using the steepest descent algorithm. After that, according to the study done by Kubiak et al.,[48] angles and distances were investigated for tryptophan. Finally, the mentioned experiments were repeated to study the effect of modifications on the structure and interactions of treated PLA$_2$. Subsequently, the best model for the plasma-treated PLA$_2$ was selected, and by PyMOL,[61] the structures of wild-type and plasma-treated PLA$_2$ were compared.

Conflict of Interest

The authors declare no conflict of interest.

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Article A4

On the Liquid Chemistry of the Reactive Nitrogen Species Peroxynitrite and Nitrogen Dioxide Generated by Physical Plasmas

G. Bruno, S. Wenske, J.-W. Lackmann, M. Lalk, T. von Woedtke, and K. Wende. *Biomolecules*, 10 (12), 1687, 2020.

On the Liquid Chemistry of the Reactive Nitrogen Species Peroxynitrite and Nitrogen Dioxide Generated by Physical Plasmas

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Abstract: Cold physical plasmas modulate cellular redox signaling processes, leading to the evolution of a number of clinical applications in recent years. They are a source of small reactive species, including reactive nitrogen species (RNS). Wound healing is a major application and, as its physiology involves RNS signaling, a correlation between clinical effectiveness and the activity of plasma-derived RNS seems evident. To investigate the type and reactivity of plasma-derived RNS in aqueous systems, a model with tyrosine as a tracer was utilized. By high-resolution mass spectrometry, 26 different tyrosine derivatives including the physiologic nitrotyrosine were identified. The product pattern was distinctive in terms of plasma parameters, especially gas phase composition. By scavenger experiments and isotopic labelling, gaseous nitric dioxide radicals and liquid phase peroxynitrite ions were determined as dominant RNS. The presence of water molecules in the active plasma favored the generation of peroxynitrite. A pilot study, identifying RNS driven post-translational modifications of proteins in healing human wounds after the treatment with cold plasma (kINPen), demonstrated the presence of in vitro determined chemical pathways. The plasma-driven nitration and nitrosylation of tyrosine allows the conclusion that covalent modification of biomolecules by RNS contributes to the clinically observed impact of cold plasmas.

Keywords: cold physical plasmas; kINPen; redox signaling; reactive nitrogen species; nitrosative stress; oxidative post-translational modifications

1. Introduction

Reactive nitrogen and oxygen species (RNS/ROS) are unstable compounds prone to react rapidly with cellular molecules. In biological systems, they may be involved in redox signaling pathways (e.g., oxygen sensing, muscles and vascular physiology, immune defense, inflammatory processes) [1], mostly by covalently changing the structure of biomolecules such as lipids and proteins [2–5]. When their homeostasis is impaired, they become markers or drivers of pathological conditions like cancer progression as well as metabolic and neurodegenerative diseases. While some species are constantly formed as by-products of cell metabolism, others are generated for dedicated purposes (second messengers, oxidative burst) [4,6–9]. Well-known endogenous radicals are hydroxyl

radicals ($^.$OH); superoxide anion ($^.$O$_2{}^-$); nitric oxygen radicals ($^.$NO); nitric dioxide radicals ($^.$NO$_2$); and other reactive species like singlet oxygen (^{1}O$_2$), hydrogen peroxide (H$_2$O$_2$), peroxynitrite (ONOO$^-$), dinitrogen trioxide (N$_2$O$_3$), and the nitrite ion (NO$_2{}^-$). The dual role of ROS and RNS is exploited by various strategies aiming to control or modulate redox-signaling pathways [10–13]. Among these, cold plasma discharges, ionized gases with complex physical and chemistry properties, are being investigated [14,15]. Most prominent components are small reactive species, many of which are also endogenously generated. A large number of gas plasma sources with different gas phase chemistry have been developed [16,17]. One of the best-characterized plasma sources is the kINPen, approved as certified medical tool in the medical version (kINPen MED) [18], currently applied in research (cancer immunology) and in clinics (wound care, skin-related diseases) [19–24]. To the current knowledge, by tuning central parameters of the discharge including working gas composition, distance, or power, the variation of the resulting plasma chemistry is responsible for the observed biological and biochemical effects [25–29]. In addition to direct signaling events triggered by long-lived reactive species like H$_2$O$_2$, e.g., via peroxiredoxins, plasma-derived short-lived reactive species can covalently modify biomolecules in model systems [26,30–35]. It remains to be clarified whether extracellularly modified molecules yield to intracellular physiological consequences or if only changes in cellular structures (e.g., cell membrane proteins) are relevant.

Gas plasmas can be tuned to create high levels of oxygen species, such as singlet oxygen ^{1}O$_2$; atomic oxygen $^.$O; ozone O$_3$; and, to a lesser extent, hydrogen peroxide H$_2$O$_2$ and hydroxyl radicals $^.$OH. These conditions are applied for disinfection or cancer regression [36–40]. In contrast, other conditions foster an N$_2$-driven chemistry, yielding species like $^.$NO, $^.$NO$_2$, N$_3$O$_5$, HNO$_2$, and HNO$_3$ [41,42]. The nitrogen species are assumed to play a role in bactericidal [43,44] and virucidal effects [45], as well as cell stimulation, e.g., in wound healing [11,46–52]. In contrast, data on their transport, solubilization, or production in liquids, as well as their impact on biological systems, are limited. While the long-lived species nitrite and nitrate can be easily accessed by the Griess assay or the ion chromatography and are almost inevitably observed for plasma discharges, both ions are in physiologic conditions not reactive enough to contribute significantly to the effects seen after plasma treatments. Nitration [28,53,54] and nitrosylation [35] of biomolecules have been observed, indicating the presence (and activity) of more reactive species at least under certain conditions. Potential candidates are, e.g., $^.$NO$_2$, N$_2$O$_3$, and ONOO$^-$. The presence of peroxynitrite [43,54] and peroxynitrate [55] has been reported for liquid systems under acidic conditions (pH < 4.5), as well as in alkaline conditions (pH 12) in the case of peroxynitrite [56], while information on neutral environments is missing. However, using an peroxynitrite sensitive europium probe, intracellular peroxynitrite was recently detected in human cell lines following a plasma treatment [57]. Natural sources of RNS are enzymes of the nitric oxide synthase (NOS) complex, generating $^.$NO. This RNS can react with O$_2$ yielding oxidized RNS such as $^.$NO$_2$ and N$_3$O$_5$, or with superoxide anion radicals produced by mitochondria, NADPH-oxidases, or xanthine oxidase, forming peroxynitrite [58]. Because of the capability of reactive nitrogen species to modify biological structures and to modulate signaling pathway, e.g., via acting as a second messenger like nitric oxide, a significant contribution of RNS can be expected [59].

Tyrosine is a key amino acid reactive towards RNS and protein tyrosines are significant targets in biological environments [60–62]. The principal roles of tyrosine are the formation of hydrophobic cores in proteins, as well as the transduction of signals via phosphorylation events. In this, tyrosine has a superior relevance compared with serine or threonine because of the high specificity of some protein kinases [63]. The presence of the aromatic hydroxyl group also allows the reaction with non-protein structures, in contrast to phenylalanine. Therefore, modifications of the tyrosine substructure yield a gain or loss of function of proteins and subsequently alter signaling pathways or immunological responses [61,64]. Protein nitration also acts as a signal for negative feedback regulation, leading to ubiquitination and protein degradation [65,66]. Some studies suggest the involvement of tyrosine nitration in the pathogenesis or as marker of nitro-oxidative stress [65,67–69]. Nitric oxide and its oxidation products are also involved in wound healing processes, although the mechanisms are

still under investigation [70,71]. For example, tyrosine nitration occurring on metalloproteinase-13 (MMP-13) promoted its release by endothelial cells, triggering a faster cell migration, angiogenesis, and wound healing [67]. In the same way, it was shown that protein tyrosine nitration by exogenous ˙NO donors inhibited the degranulation processes in mast cell lines [72]. The release of exogenous ˙NO is a possible strategy to stimulate skin healing [73], potentially beneficial in diabetic patients, where the increased hyperglycemia leads to decreased ˙NO bioavailability and delays the wound healing process [74]. The effectiveness of cold plasmas was shown in the treatment of wounds in both diabetic and normal subjects, as well as in wound models [22,23,75,76]. The responsible species are not completely known, but recent observations emphasized the role of the local cell stimulation over wound bed disinfection—suggesting that reactive nitrogen species may be involved. Therefore, this work intends to further investigate the N_2-driven biochemistry induced by the kINPen plasma source, aiming to define the principal formed bioactive reactive species and their mechanisms of action. To reach this aim, tyrosine was chosen as a tracer molecule and modifications occurring as a result of the impact of plasma-derived reactive nitrogen species were identified and quantified via high-resolution mass spectrometry. Finally, the ability of kINPen plasmas in modulating the RNS-mediated pathways in complex models was tested. Overall, a significant impact of nitrosating and nitrosylating agents, assumingly peroxynitrite, was observed, leading to a covalent chemical modification of sensitive target structures. Given this result, functional consequences will arise in both pro- and eukaryotic cells, highlighting a possible key mechanism in promoting wound healing, modulation of cancer immunology, and antimicrobial effects.

2. Materials and Methods

2.1. Sample Preparation

2.1.1. Model Solutions

L-tyrosine (Merck KGaA, Darmstadt, Germany) solutions with a concentration of 0.3 and 30 mM were prepared in 5 mM ammonium formate buffer pH 7.4 (Merck KGaA, Darmstadt, Germany) fresh and kept on ice until use. The singlet oxygen and superoxide scavenger ergothioneine (Enzo Life Sciences GmbH, Lörrach, Germany) and the nitric oxide scavenger cPTIO (Lot. PK822, Dojindo Molecular Technologies, Kumamoto, Japan) were dissolved in double distilled water to create a 10× stock solution with 3 mM concentration [77–79].

2.1.2. Wound Exudates

Wound exudates were obtained from seven diabetic patients with chronic wounds of the Klinikum Kalsburg, Germany according to an existent ethics approval. After removal of the wound bandage material, the patient wound was rinsed with sterile natural saline followed by blotting with a dry gauze. Afterwards, Copan eSwab samples (Mast Diagnostica GmbH, Reinfeld, Germany) were taken in a circular, inside-out motion across the treated wound surface, and rotated during sampling. Samples were placed in 1000 µL of serum-free Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Scientific, Rockford, IL, USA). Subsequently, wounds were plasma treated (see Section 2.2), rinsed again, and sampled as before using eSwabs. The swabs were vortexed for the maximal elution of wound exudates into the medium, and the supernatant was centrifuged at 4500 rpm for 5 min and sterile-filtered (0.22 µm). All swabs and supernatants were kept at −80 °C until further use.

2.2. Cold Plasma Treatments and Incubation with Control Oxidants

For the model solutions, the kINPen09 (neoplas tools GmbH, Greifswald, Germany) with a shielding device was used as a source of reactive species, as shown in Figure 1. Briefly, 750 µL of tyrosine solutions was treated in 24-well plates at a distance of 9 mm for 30 or 180 seconds. Argon (±1% of molecular gas admixture, Air Liquide, Paris, France) served as working gas using 3

standard liters per minute (slm). Admixtures were 1% oxygen, 1% nitrogen, or a mix of both (0.7% N_2 and 0.3% O_2, Air Liquide, Paris, France). For some treatments, isotopically labelled working gas admixtures ($^{15}N_2$, or $^{18}O_2$, Merck KGaA, Darmstadt, Germany) or solvent (water, $H_2^{18}O$, Merck KGaA, Darmstadt, Germany) were used. The plasma effluent was shielded against the ambient air by a gas shielding of 5 slm nitrogen. For some treatments, the working gas was enriched with water (320 ppm) by guiding 1% of the total flow through a gas wash bottle containing double distilled water [41,80,81]. Chronic wounds were treated in the Klinikum Karlsburg using the kINPen MED (neoplas tools GmbH, Greifswald, Germany) under standard medical conditions (30 s per 1 cm^2 of wound area and 5 slm of Argon (Air Liquide, Paris, France), without a shielding device.
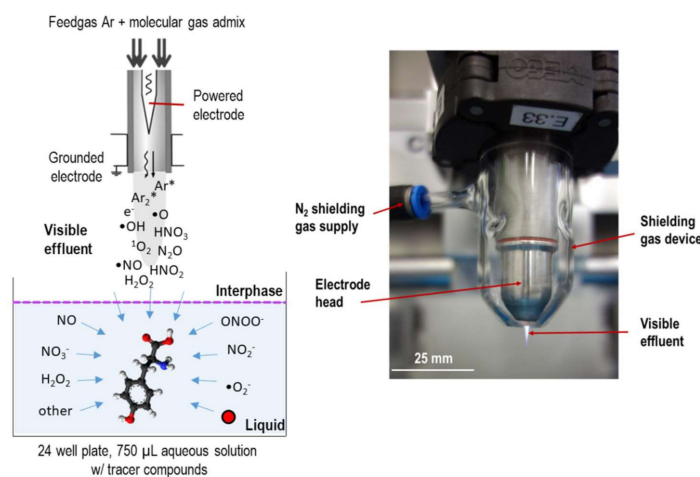
Figure 1. Schematic layout of treatment of tyrosine model solutions (**left**), and kINPen device w/gas shield installed (**right**).

Buffered solutions (pH 7.4) of tyrosine 0.3 mM were incubated for 2 min at room temperature under constant mixing with equimolar amounts of control oxidants (300 µM). Those were (1) peroxynitrite (Merck KGaA, Darmstadt, Germany) at pH 14; (2) peroxynitrite at pH 6.4, yielding 30% peroxynitrous acid dissociation in nitric dioxide radicals and hydroxyl radicals [54,82,83]; (3) the nitric oxide donor DEA NONOate (Biomol GmbH, Hamburg, Germany); (4) nitrite and nitrate (Merck KGaA, Darmstadt, Germany); (5) hydrogen peroxide (Merck KGaA, Darmstadt, Germany); and (6) mixed solutions of nitrite, nitrate, and hydrogen peroxide. For solutions (5) and (6), 300 µM of each control oxidant was included. After reaction, samples were put on ice and immediately subjected to mass spectrometry analysis. An overview of all the plasma treatment conditions (working gases and treated solutions) and of the solutions incubated with control oxidants is shown in Figure 2.
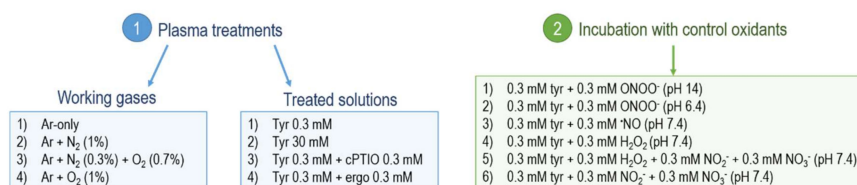
Figure 2. Overview of the solutions treated with plasmas (1) and incubated with control oxidants (2). Here, 3 slm of every working gas applied, together with 5 slm N_2 shielding gas. Prepared solutions were buffered with 5 mM ammonium formiate. Tyrosine (Tyr); ergothioneine (ergo).

2.3. Cold Plasma-Induced Modifications of Tyrosine

2.3.1. Qualitative Screening via Direct Infusion High-Resolution Mass Spectrometry

All samples were analyzed by high-resolution mass spectrometry using a TripleTOF 5600 (AB Sciex GmbH, Darmstadt, Germany). Samples were diluted 1:1 with acetonitrile/0.1% formic acid to stimulate ionization and to facilitate evaporation and injected using a syringe pump (50 μL/min). Ionization was achieved by positive mode electro spray ionization with the following settings: +5.5 kV probe voltage, 300 °C, +30 V declustering potential, 40 psi curtain gas, 20 psi gas 1, and 25 psi gas 2 (Turbo Ion Source). Concentrated sample solutions (30 mM) were diluted 1:100 before injection. All spectra were acquired in the m/z range from 40 to 600 m/z. After a first qualitative scan of the produced compounds, their structures were elucidated by acquisition in tandem mass spectrometry (MS^2 spectra) and collisional energies for optimal fragmentation were tuned for each compound of interest.

2.3.2. HPLC-MS^2 Quantitation of Tyrosine and 3-Nitrotyrosine

Considering their relevance, L-tyrosine and 3-nitro-L-tyrosine (Merck KGaA, Darmstadt, Germany) were absolutely quantified by coupling a chromatographic separation (Infinity II 1290, Agilent Technologies, Berlin, Germany) to the mass spectrometry detection (qTRAP 5500, AB Sciex GmbH, Darmstadt, Germany). A hydrophilic liquid chromatography (HILIC) strategy was adopted, using a 2.1 mm × 5 mm Acquity Amide VanGuard Pre-column followed by a 2.1 mm × 100 mm Acquity Amide Column (both 130 Å pore size, 1.7 μm particle size, Waters Corporation, Berlin, Germany). For the separation, eluent A (85% acetonitrile, 0.15% of formic acid, and 10 mM ammonium formate) and eluent B (HPLC water, 0.15% formic acid, and 10 mM ammonium formate; pH 3), were used. A linear gradient at a flow rate of 800 μL/min was applied (time, B): 0 min, 99%; 4 min, 92%; 4.1 min, 99%; 5 min, 99%. After a 1:5 dilution in buffer A, 20 μL of sample was injected. Ionization was achieved in positive mode, using the following parameters: +5.5 kV probe voltage at 150 °C, +100 V declustering potential, 35 psi curtain gas, 25 psi gas 1, and 30 psi gas 2. Compounds of interest were quantified by multiple reaction monitoring. The transitions and collisional energies (CEs) were tuned differently for each compound: tyrosine 182 → 136 m/z (quantitative), CE 18 V and 165 m/z (qualitative), CE 10 V. Nitro-tyrosine 227 → 181 m/z (quantitative) and 158 m/z (qualitative), both CE 10 V. For the quantification, external calibration curves were generated.

2.3.3. Data Analysis and Visualization

All experiments were performed three times each with technical duplicates. Statistical analysis was performed using GraphPad Prism 7. The MarvinSketch software (version 18.8.0) was used to identify the exact molecular weight and the formula of each compound, as well as to predict the reactivity of tyrosine and control oxidants in different pH. The spectra produced through direct infusion high-resolution mass spectrometry were calibrated and analyzed with PeakView (version 1.2.0.3, AB Sciex GmbH, Darmstadt, Germany). The peak areas of each observed derivative were normalized on the peak area of the control (untreated tyrosine), giving a relative estimate of the conversion. For treatments involving isotopes, the measurement data were corrected according to [36], taking into account the natural distribution of isotopes and the purity grade.

2.4. Characterization of Plasma-Induced Protein Modifications in Wound Exudates

Proteins were precipitated by incubating with 80% ice-cold acetone (Carl Roth GmbH, Karlsruhe, Germany) overnight. The pellets were washed twice and dissolved in 100 μL resuspension buffer (10 mM Tris/HCl with 1 mM EDTA, pH 8, all Merck KGaA, Darmstadt, Germany). Protein concentrations were determined by Bradford assay following the vendor's high sensitivity protocol (Roti-Nanoquant, Roth, Germany). Single dimension electrophoresis was achieved by loading 60 μg of each sample onto 10% precast protein gels (Bio-Rad Laboratories, Hercules, CA, USA), followed by in-gel digestion [30].

Briefly, gel slices were dried and incubated for 5 min with 50 μL of 10 mM tris(2-carboxyethyl)phosphine (Merck KGaA, Darmstadt, Germany) at 60 °C followed by an incubation with 50 μL of 50 mM iodoacetamide to alkylate reduced thiols at room temperature (RT) for 30 min (Merck KGaA, Darmstadt, Germany). After brief drying, 2.5 μg trypsin (sequencing grade, Promega GmbH, Mannheim, Germany) was added and samples were incubated at 37 °C for 16 h. Peptides were eluted into ultra-pure water by ultra-sonication for 30 min. The peptides were further purified using Pierce C18 tips (Thermo Fisher Scientific, Hennigsdorf, Germany) following the included protocol for peptide purification and desalting. Eluted peptides were subjected to nanoLC/HRMS. An UltiMate 3000 nanoLC (Dionex Corp., Sunnyvale, CA, USA) was coupled to a QExactive mass spectrometer using electrospray ionisation (both Thermo Fisher Scientific, Hennigsdorf, Germany). Sample aliquots of 1 μg were loaded onto an Acclaim PepMap 100 precolumn (2 cm × 100 μm, 5 μm particle size, 100 Å pore size) for 6 min at 5 μL/min flow followed by separation on a PepMap RSLC column (25 cm × 75 μm, 2 μm particle size, 100 Å pore size). The following gradient was used at 200 nl/min: 2% to 35% in 6 min, to 50% B in 15 min, to 90% B in 15 min, keeping at 90%for 15 min, and equilibration at 2% B for 20 min (A: H_2O + 0.1% acetic acid, B: acetonitrile + 0.1% acetic acid, both Merck KGaA, Darmstadt, Germany). Each sample was injected twice. The QExactive was run in Top10 DDA mode with a dynamic exclusion of 30 s. MS1 spectra were acquired with a resolution of 70,000, whereas MS2 spectra were acquired in 17,500 resolution. Raw data files were analyzed with the Proteome Discoverer 2.2 (Thermo Fisher Scientific, Hennigsdorf, Germany) software. At least two unique peptides had to be identified with a maximal mass divergence of 5 ppm (MS1) and 0.02 Da (MS2) for the corresponding protein to be accepted. A maximum false discovery rate (FDR) of 5% was accepted for the datasets. Afterwards, abundances were normalized on individual trypsin intensities and resulting relative intensities taken for label free quantification using two-fold cut offs. In a second step, samples were analyzed using the Byonic (Protein Metrics Version 3.6) plug-in for Proteome Discoverer. Here, a list of oxidative chemical modifications was identified using a machine-learning algorithm with a database, which was previously acquired using model peptides [84]. For normalization, the peptide spectrum matches with a modification were counted and divided by the total number of peptide spectrum matches in each of the samples. The modifications found with Byonic were filtered and scored to separate nonsense peptide spectrum matches from those correctly identified.

3. Results and Discussion

3.1. Tyrosine Modification Induced by Plasma-Generated Reactive Oxygen and Nitrogen Species

Tyrosine solutions were treated by cold plasma with varying parameters or incubated with control oxidants. The generated products were identified via the accurate monoisotopic mass and on the tandem-MS level identifying molecule substructures. All the identified structures are listed in Table 1, independently from the condition in which they were produced. According to [33,34], where tyrosine solutions have been treated with plasma sources operated with air or helium as ionized gases, structures 2, 3, 10, and 22 were also identified in the current work using kINPen plasmas. Besides, more than 20 other types of functionalization were observed. The dominant modifications observed were all localized on the aromatic ring: hydroxylations, nitrosylations, nitrations, and a combination of different groups (up to four groups). Here, the addition of a functional group via electrophilic substitutions is stabilized by resonance effects [85,86]. To a lesser extent, the dimerization of tyrosine to dityrosine and the functionalization of this structure with other groups were also detected. While hydroxylations were mostly driven by oxygen species, the presence of N-containing functional groups indicated an active RNS chemistry. This was partially shown previously using cysteine as tracer, yielding S-nitroso-cysteine [30].

Table 1. Overview of modifications introduced in the tyrosine (Y) structure by tuning kINPen cold plasmas. * Assignment of a group letter in relation to the results obtained from the experiment performed in the presence of scavengers (details in Paragraph 3.5, Figure S4). The areas of compounds scavenged in the same way were summed up to generate six groups (from a to f).

Functional Group(s) on Tyrosine	Formula	[M+H]$^+$	Compound Code	Group Letter *
None	$C_9H_{11}NO_3$	182.081725	1	-
$1 \times OH$	$C_9H_{11}NO_4$	198.0766	2	-
$2 \times OH$	$C_9H_{11}NO_5$	214.071525	3	-
$3 \times OH$	$C_9H_{11}NO_6$	230.066425	4	-
$4 \times OH$	$C_9H_{11}NO_7$	246.061425	5	-
$1 \times NO$	$C_9H_{10}N_2O_4$	211.071925	6	e
$2 \times NO$	$C_9H_9N_3O_5$	240.062025	7	a, d
$3 \times NO$	$C_9H_8N_4O_6$	269.052225	8	b, d
$4 \times NO$	$C_9H_7N_5O_7$	298.042325	9	b, d
$1 \times NO_2 + 1 \times OH, 1 \times NO$	$C_9H_{10}N_2O_5$	227.066825	10	c, e
$2 \times NO_2 + 2 \times OH, 2 \times NO$	$C_9H_9N_3O_7$	272.051825	11	b, e
$3 \times NO_2$	$C_9H_8N_4O_9$	317.036925	12	b, e
$4 \times NO_2$	$C_9H_7N_5O_{11}$	362.022025	13	b, e
$1 \times Y$	$C_{18}H_{20}N_2O_6$	361.139925	14	-
$1 \times Y, 1 \times OH$	$C_{18}H_{20}N_2O_7$	377.134925	15	-
$1 \times Y, 2 \times OH$	$C_{18}H_{20}N_2O_8$	393.129825	16	-
$1 \times Y, 3 \times OH$	$C_{18}H_{20}N_2O_9$	409.124725	17	-
$1 \times Y, 1 \times NO$	$C_{18}H_{19}N_3O_7$	390.130125	18	c, d
$1 \times Y, 1 \times NO_2 + 1 \times Y, 1 \times OH, 1 \times NO$	$C_{18}H_{19}N_3O_8$	406.125025	19	e
$1 \times OH, 2 \times NO$	$C_9H_9N_3O_6$	256.056925	20	a, f
$1 \times OH, 3 \times NO$	$C_9H_8N_4O_7$	285.047125	21	b, f
$1 \times OH, 1 \times NO_2 + 2 \times OH, 1 \times NO$	$C_9H_{10}N_2O_6$	243.061725	22	c, f
$2 \times OH, 1 \times NO_2 + 3 \times OH, 1 \times NO$	$C_9H_{10}N_2O_7$	259.056625	23	f
$3 \times OH, 1 \times NO_2$	$C_9H_{10}N_2O_8$	275.051525	24	f
$1 \times OH, 2 \times NO_2$	$C_9H_9N_3O_8$	288.046825	25	f
$2 \times OH, 2 \times NO_2$	$C_9H_9N_3O_9$	304.041725	26	a, f
$1 \times OH, 3 \times NO_2$	$C_9H_8N_4O_{10}$	333.031825	27	f

However, the detected amounts were low, suggesting that cysteine is not an optimal target for the intended downstream analysis technique. The number and amount of identified N-containing modifications of tyrosine show a good suitability to study the RNS output of cold plasma discharges. An overview of oxidative modifications induced on tyrosine by different reactive species, possibly also formed by kINPen plasmas, is shown in Figure 3. When considering a radical-driven reaction mechanism, the first step for tyrosine derivatization is the formation of tyrosyl radicals by different one-electron oxidants (·NO$_2$, ·OH). ·NO$_2$ is a candidate species able to form tyrosyl radicals via a slow reaction (k = 3.2×10^5 M^{-1} s^{-1}), while hydroxyl radicals react at rates $\geq 1 \times 10^9$ M^{-1} s^{-1}. Consequential further direct reactions of tyrosyl radicals with tyrosine yield dityrosine (k = 2.3×10^8 M^{-1} s^{-1}); those with ·NO yield nitrosotyrosine (k = 1.0×10^9 M^{-1} s^{-1}); those with ·NO$_2$ yield nitrotyrosine (k = 3.0×10^9 M^{-1} s^{-1}); and those with ·O$_2^-$ yield tyrosine hydroxyquinone (k = 1.5×10^9 M^{-1} s^{-1}) (only on free tyrosines). This last product rapidly loses O$_2$ from the structure to reform tyrosine [59,65,87]. After the formation of those derivatives, a further addition of groups led by reactive species was assumed. The conversion of NO-tyrosine to NO$_2$-tyrosine can occur in oxidative conditions firstly by formation of an iminoxyl radical (·NO-tyrosine) and, further,

oxygen addition. This two one-electron oxidation step process is a slow one, promoted by the presence of metals, which are not included in the used liquid model. In contrast, a direct reaction with ·OH (and possibly ·O) leads to the formation of tyrosine hydroxyl radicals (·OH-tyrosine) ($k = 1.2 \times 10^{10}$ M^{-1} s^{-1}), which rapidly lose an electron to become OH-tyrosine [65].
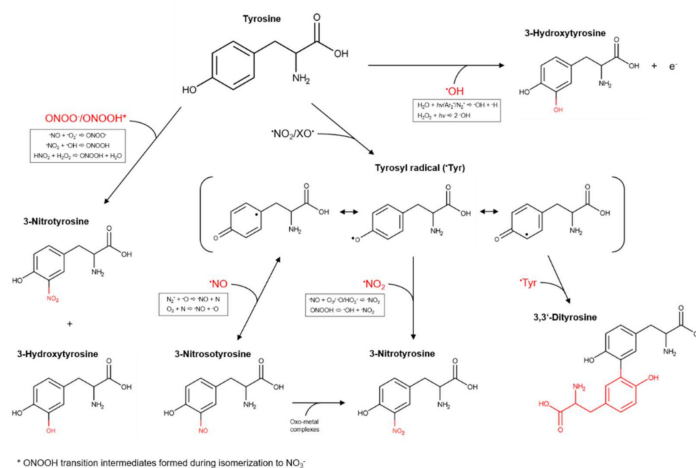

Figure 3. Formation pathways of tyrosine derivatives considering a neutral pH. The hypothesized possible responsible species generated by kINPen plasmas and the reactions leading to their origin are highlighted in the box [15,65,81,88,89].

A non-radical mechanism driven by peroxynitrous acid and peroxynitrite has also been proposed by [88], leading to tyrosine hydroxylation and nitration by formation of ONOOH transition intermediates during the H$^+$ driven isomerization of peroxynitrous acid to NO$_3^-$ and H$^+$. Indeed, the energy and the rate for the isomerization (18 kcal mol^{-1} and 1.3 s^{-1}, respectively) are equivalent to those necessary to achieve the nitration or hydroxylation of aromatic compounds, occurring independently from their concentrations. At very low pH (<2.5), the formation of nitryl anion (NO$_2^+$) by peroxynitrous acid or peroxynitrite heterolysis could occur and lead to tyrosine nitration [89]. However, this mechanism can be excluded in our system, because of the controlled pH at 7.4.

3.2. Gas Composition: A Crucial Parameter to Regulate the NO$_x$ Generation

Tyrosine solutions were treated using different working gas compositions and treatment times, yielding various products. While tyrosine and 3-nitrotyrosine were absolutely quantified by a multiple reaction monitoring approach (Figure 4), all other tyrosine derivatives were relatively quantified (Figure 5—dry working gas, Figure 6—humidified working gas).

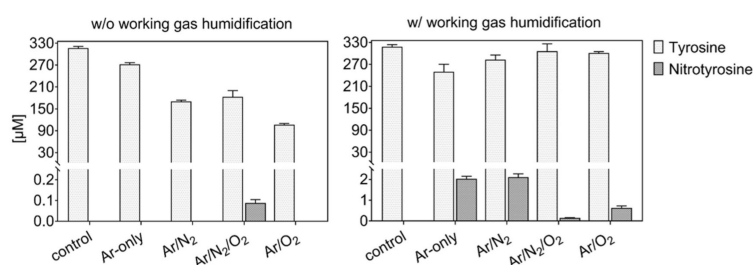**Figure 4.** Tyrosine consumption and 3-nitrotyrosine formation by plasma-derived reactive oxygen species (ROS). The presence of water in the working gas reduced gas phase oxidation of reactive nitrogen species (RNS) and yielded higher activity of RNS in the liquid (3 min, 0.3 mM tyrosine in 5 mM ammonium formate, pH 7.4). Plasma is formed from dry (left) or humid (320 ppm H$_2$O, right) working gas.
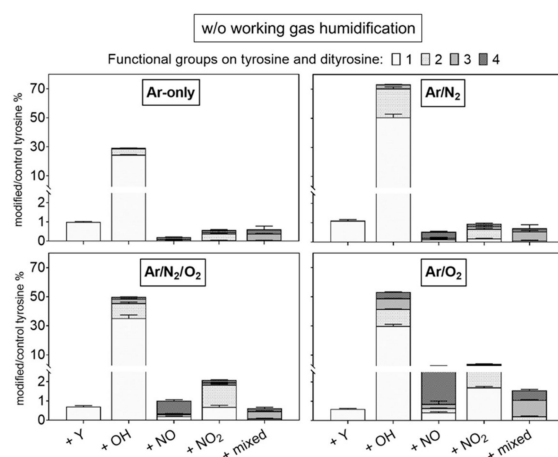
Figure 5. Modifications of tyrosine observed after plasma treatment using dry working gas are dominated by hydroxylation. Multiple nitrations/nitrosylations were observed predominantly when O_2 was present in the working gas. Labels: +Y (tyrosyl, +180.066 Da), +OH (hydroxyl, +15.9949 Da), +NO (nitroso, +28.9902 Da), and +NO$_2$ (nitro, +44.9851 Da). Up to four groups were observed per molecule (indicated by light to dark grey). The introduction of diverse groups is represented as "mixed" (Compounds 20 to 27, Table 1). Treatment time 3 min, 0.3 mM tyrosine in 5 mM ammonium formate, pH 7.4. Relative compound intensities are given (tyrosine ~4445 counts in control).
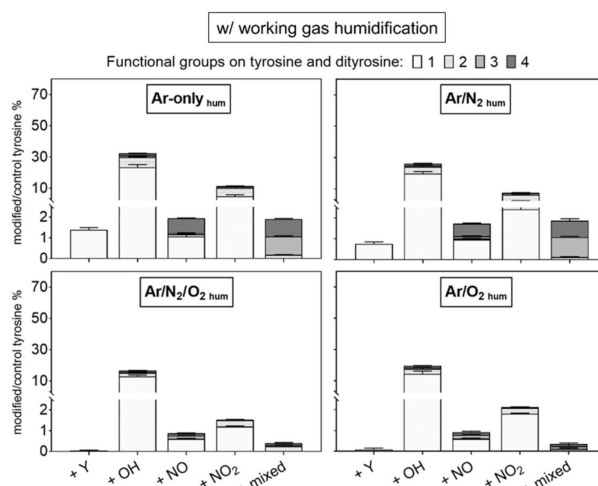
Figure 6. Modifications of tyrosine observed after plasma treatment using humidified working gas show a substantial introduction of one nitroso or nitro group. Labels: +Y (tyrosyl, +180.066 Da), +OH (hydroxyl, +15.9949 Da), +NO (nitroso, +28.9902 Da), and +NO$_2$ (nitro, +44.9851 Da). Up to four groups were observed per molecule (indicated by light to dark grey). The introduction of diverse groups is represented as "mixed" (Compounds 20 to 27, Table 1). Treatment time 3 min, 0.3 mM tyrosine in 5 mM ammonium formate, pH 7.4. Relative compound intensities are given (tyrosine ~4445 counts in control).

The highest tyrosine consumption (68%) was observed for a discharge regime rich in short-lived ROS (dry Ar/O$_2$) [25]. In contrast, hydrogen peroxide rich conditions (dry Ar) [81] yielded only 14% tyrosine conversion.

Using admixtures of molecular gases, more than 50% of tyrosine was converted into hydroxytyrosine. Target water ionization, homolysis, or photolysis is promoted by excited states of Ar$_2$ or N$_2$ (excimers), as well as from radical reactions [36,90]. The direct reaction with those species (or for excimers, with their radiation) leads to the predominant formation in water of ˙OH and ˙H [91–93], which would react directly with tyrosine to form hydroxytyrosine. The higher OH-tyrosine production

in conditions with Ar/N_2 (up to 70% converted tyrosine) confirmed the synergistic action on the target of Ar_2 and N_2 excimers [15]. In contrast, in conditions including oxygen, gaseous radicals (e.g., $\cdot$O) are predominantly formed, and could be responsible for tyrosine hydroxylation (up to 4 −OH groups) via direct reaction with tyrosine or via water dissociation and $\cdot$OH formation in liquid [94,95]. In the presence of both N_2 and O_2 in the working gas, a substantial consumption of tyrosine was observed ($\approx$5%), alongside the detection of only small amounts of 3-nitrotyrosine in dry conditions (Figure 4).

Accordingly, the formation of primary or secondary nitrosative NO_x species can be assumed for this condition, corroborating previous results determining S-nitrosocysteine formation. Here, the presence of $\cdot$NO in the liquid did not yield in *S*-nitrosylation, and $\cdot$NO oxidation products (e.g., $ONOO^-$, N_2O_3) were assumed to be of relevance. Considering the strong accumulation of nitrate in this discharge condition], an end product that can derive from the decomposition of peroxynitrite, $ONOO^-$ could be responsible for both S-nitrosylation and nitrotyrosine formation [96]. The incorporation of two −NO_2 (%) and four −NO (up to 5%) groups was detected in major amounts in admixtures including oxygen (Figure 5), confirming an active nitrogen chemistry in the liquid also using shielding N_2 as a source [42,97]. Considering quantitative data, an isobaric structure for nitrotyrosine (e.g., hydroxy-nitrosotyrosine, see Table 1) was detected in conditions with oxygen only as admix. Previous simulation studies showed the highest gas phase formation of $\cdot NO_2$ (~8×10^{13} cm^{-3}) and N_2O (~5×10^{12} cm^{-3}) using dry working gases (N_2 shielded) containing 1 % N_2/O_2 with O_2 in less than 50%. Those species decreased by increasing the O_2 %, with the increase of highly oxidized species, such as O_3 (~1.5×10^{15} cm^{-3}) and N_2O_5 (~3×10^{13} cm^{-3}) [81]. Considering the higher production of nitrotyrosine in conditions with 1:1 N_2/O_2 admixtures (Figure 4), $\cdot NO_2$ could be a direct nitrating agent of tyrosyl radicals in the target. However, it must be considered that only a minimal amount of $\cdot NO_2$ would be able to diffuse from the gas phase into the bulk of the liquid, owing to its low solubility in water ($H^{cp} = 3.4 \times 10^{-2}$ Pa^{-1}) [98]. Most likely, a direct nitration due to gaseous $\cdot NO_2$ could occur at the interface. These limitations could justify the overall low production of nitrotyrosine.

Alternatively, the production of other nitrating agents that have $\cdot NO_2$ as precursor can be considered. The reaction between $\cdot NO_2$ and water molecules generates HNO_2, which is highly soluble [35]. However, at a pH of 7.4, the nitrous acid cannot be considered as a nitrating agent. $\cdot NO_2$ could form N_2O_3, a nitrating agent, by reaction with $\cdot NO$, which is present in gas and liquid phase [81]. However, because of the low solubility of N_2O_3 ($H^{cp} = 6.0 \times 10^{-1}$ Pa^{-1}) [98], its penetration to the target is unlikely. With that, the well soluble peroxynitrite could be a prominent candidate for the effective nitrating species, acting on tyrosine via dissociation in $\cdot NO_2$ [65], or transition intermediates of peroxynitrous acid formed during its isomerization in nitrate [99]. $ONOOH/ONOO^-$ production is promoted by reaction of gaseous $\cdot NO$ and $\cdot O_2^-$ [81], but considering $\cdot NO_2$ as major gaseous precursor, interface/bulk reactions of $\cdot NO_2$ (gas) with $\cdot OH$ (in liquid) or HNO_2 with H_2O_2 (in liquid) are possible formation pathways [99–101]. The production in liquid of H_2O_2 and $\cdot OH$ by water dissociation/ionization driven by radicals and vacuum UV radiation was shown previously [36,90]. The formation of peroxynitrite by the reaction of HNO_2 with H_2O_2 is favored by low pH (<4), which may be achieved in the gas–liquid interface [99,101] and not in the liquid bulk (Figure S1) [102].

As shown in Figures 4 and 6, the addition of humidity in the working gas reduced the general tyrosine oxidation in conditions containing oxygen, increasing drastically in Ar and Ar/N_2 the production of nitrotyrosine (up to 2 μM), nitrosotyrosine (one and four −NO groups, 2%), mixed modifications (2%), and multiple nitration events (two −NO_2 groups, 8%). Conditions with Ar-only in the working gas became slightly more oxidative (25% oxidized tyrosine) than dry conditions. In contrast, tyrosine oxidation was lower than in dry conditions with molecular admixtures (7% and 5% for Ar/O_2 and Ar/N_2/O_2, respectively), as well as the production of tyrosine derivatives (Figure 6).

This can be explained by the interaction of water molecules in the effluent with gaseous species formed using Ar/O_2 and Ar/N_2/O_2 (e.g., $\cdot$O, $^1O_2$, $\cdot NO_2$). In parallel, it was shown that this interaction, mostly with Ar_2^* and $\cdot$O, generated $\cdot$OH, $\cdot$H, and $\cdot O_2^-$ in the effluent area, which partially diffuse into the target, forming high amounts of H_2O_2 by recombination of $\cdot$OH [40,103]. In the gas phase of

humidified kINPen plasmas with low O_2%, a boost of gaseous nitrogen chemistry was also detected, with higher production of HNO_3 ($\sim4 \times 10^{13}$ cm^{-3}) and $^{\cdot}NO$ ($\sim6 \times 10^{13}$ cm^{-3}), rather than $^{\cdot}NO_2$, the formation of which depended on O_2% densities [41].

Considering the 10-times higher production of nitrotyrosine in humid conditions with Ar-only or Ar/N_2 than in conditions with Ar/O_2 and Ar/N_2/O_2, a higher formation of nitrogen species in liquid is key. Rather than a direct impact of gaseous $^{\cdot}NO_2$, the formation of peroxynitrite via different pathways is facilitated. The reaction of gaseous or dissolved $^{\cdot}NO$ with $^{\cdot}O_2^-$ at the interface or in the bulk of the liquid is a substantial pathway. Additionally, the high amounts of HNO_3 in the gas phase favor an attack of $^{\cdot}OH$, forming $^{\cdot}NO_3$, which is an unstable species generating H_2O_2 and $^{\cdot}NO_2$ in contact with water. Alternatively, $^{\cdot}NO_2$ could form HNO_2 by reacting with water molecules. The subsequent reaction with H_2O_2 yielding ONOOH is not favored at the pH of 7.4, precluding this as a major reaction route [102]. At the interface, $^{\cdot}NO_2$ could form peroxynitrite by the reaction with $^{\cdot}OH$ radicals. Table 2 shows an overview of the major modifications induced on tyrosine during plasma treatment, together with the related plasma-derived species in the gas phase and the proposed reactions occurring in liquid to form the attacking species.

Table 2. Overview of the most relevant tyrosine derivatives observed in solution, potentially related plasma-derived species [41,81,97], and proposed reactions occurring in the liquid [15,81,99–101].

	Added Functional Groups on Tyrosine	Plasma Components in the Gas Phase	Proposed Reactions in Liquid
Ar-only	$1 \times OH$	Ar_2^*, VUV	$H_2O \rightarrow {}^{\cdot}H + {}^{\cdot}OH$
Ar + N$_2$	1–$2 \times OH$	Ar_2^*, VUV	$H_2O + h\nu \rightarrow {}^{\cdot}H + {}^{\cdot}OH$
Ar + N$_2$ + O$_2$	1–$2 \times OH$; $1, 4 \times NO$; 1–$2 \times NO_2$	$^{\cdot}NO_{2(g)}$, $^{\cdot}O_{(g)}$	$^{\cdot}O + H_2O \rightarrow {}^{\cdot}OH + {}^{\cdot}OH$ $^{\cdot}NO_2 + {}^{\cdot}OH \rightarrow ONOOH$
Ar + O$_2$	1–$4 \times OH$; $1, 4 \times NO$; 1–$2 \times NO_2$; mixed	$^{\cdot}NO_{2(g)}$, $^{\cdot}O_{(g)}$, $O_{3(g)}$ $N_2O_{5(g)}$	$^{\cdot}O + H_2O \rightarrow {}^{\cdot}OH + {}^{\cdot}OH$ $^{\cdot}NO_2 + {}^{\cdot}OH \rightarrow ONOOH$
Ar-only hum	$1 \times OH$; $1, 4 \times NO$; 1–$2 \times NO_2$; mixed	$^{\cdot}NO_{(g)}$, $HNO_{3(g)}$, $H_2O_{2\,(aq)}$, $^{\cdot}O_2^-{}_{(aq)}$, $^{\cdot}OH_{(aq)}$	$^{\cdot}NO + {}^{\cdot}O_2^- \rightarrow ONOOH$ $HNO_2 + H_2O_2 \rightarrow ONOOH$
Ar + N$_2$ hum	$1 \times OH$; $1, 4 \times NO$; 1–$2 \times NO_2$; mixed	$^{\cdot}NO_{(g)}$, $HNO_{3(g)}$, $H_2O_{2\,(aq)}$, $^{\cdot}O_2^-{}_{(aq)}$, $^{\cdot}OH_{(aq)}$	$^{\cdot}NO + {}^{\cdot}O_2^- \rightarrow ONOOH$ $HNO_2 + H_2O_2 \rightarrow ONOOH$
Ar + N$_2$ + O$_2$ hum	$1 \times OH$; $1 \times NO$; $1 \times NO_2$	$^{\cdot}NO_{2(g)}$, $N_2O_{5(g)}$, $H_2O_{2\,(aq)}$, $^{\cdot}O_2^-{}_{(aq)}$, $^{\cdot}OH_{(aq)}$	$^{\cdot}NO_2 + {}^{\cdot}OH \rightarrow ONOOH$ $HNO_2 + H_2O_2 \rightarrow ONOOH$
Ar + O$_2$ hum	$1 \times OH$; $1 \times NO$; $1 \times NO_2$	$^{\cdot}NO_{2(g)}$, $N_2O_{5(g)}$, $H_2O_{2\,(aq)}$, $^{\cdot}O_2^-{}_{(aq)}$, $^{\cdot}OH_{(aq)}$	$^{\cdot}NO_2 + {}^{\cdot}OH \rightarrow ONOOH$ $HNO_2 + H_2O_2 \rightarrow ONOOH$

3.3. Tyrosine Modification Induced by Control Oxidants

Incubations with control oxidants (NO donor, NO_2^-/NO_3^-, $H_2O_2/NO_2^-/NO_3^-$) were performed at neutral pH (7.4). Peroxynitrite was tested at two different pH (pH 14 and 6.4) [83,84]. The observed tyrosine derivatives are shown in Figure 7 (quantitative) and 8 (qualitative). Peroxynitrite was most efficient in modifying tyrosine (46% and 37% at pH 14 and 6.4, respectively). In particular, nitrotyrosine (~10 μM) was formed (Figure 7). The substantial functionalization observed for ONOO$^-$ at near neutral pH is due to the protonation of the ion, yielding instable peroxynitrous acid (ONOOH, 58.7% considering pKa = 6.8). This rapidly isomerizes into NO_3^- and H+, and dissociates in $^{\cdot}OH$ and $^{\cdot}NO_2$ (30% yield) [62,66,70]. The incorporation of up to four $-NO_2$ groups in the tyrosine structure emphasized the favored formation of $^{\cdot}NO_2$ by peroxynitrite dissociation. In addition, the single nitration of tyrosine could be induced by the indirect reaction of ONOOH via the formation of a transition intermediate generated in the isomerization of ONOOH to NO_3^- [88]. According to [88,89], the hydroxylation was stronger at lower pH because of the formation of a peroxynitrous acid intermediate that facilitates the cleavage of the ions O-O bond.
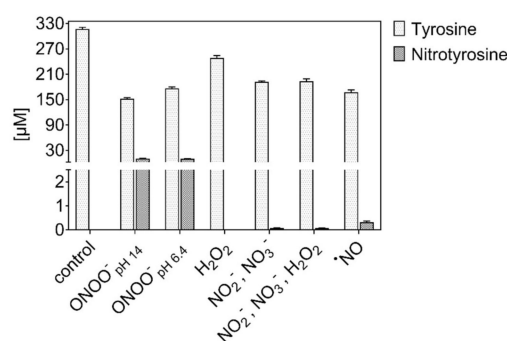
Figure 7. Tyrosine consumption and 3-nitrotyrosine formation by control oxidants. Peroxynitrite efficiently introduced a nitro group, but independently from pH (3 min incubation of 0.3 mM tyrosine in 5 mM ammonium formate, pH 7.4, 300 μM of each control oxidant).

However, at pH 14, a similar nitration yield was observed. According to [99], different products generated during $ONOO^-$ homolysis ($\cdot NO$ and $\cdot O_2^-$) and decomposition (O_2 and NO_2^-) could be involved in the direct or indirect functionalization of biomolecules, including the peroxynitric anion (O_2NOO^-). The functionalization of tyrosine via non-radical processes was confirmed, also inducing a higher incorporation of up to four different functional groups into the tyrosine moiety (+2 −OH groups, two and four mixed groups) (Figure 8). Incubations with any other compound (mix) yielded very low amounts of nitrotyrosine (<0.5 μM), and only the $\cdot NO$ donor could induce nitroso-groups on tyrosine, confirming a direct reaction with the aromatic ring (Figure 8) [65]. The low formation of nitrosotyrosine indicates the low reactivity of $\cdot NO$ itself with the ring and a limited oxidation of $\cdot NO$ to nitrite ions' respective nitrous acid. A partial oxidation to $\cdot NO_2$ in the presence of O_2 could justify the formation of nitrotyrosine.
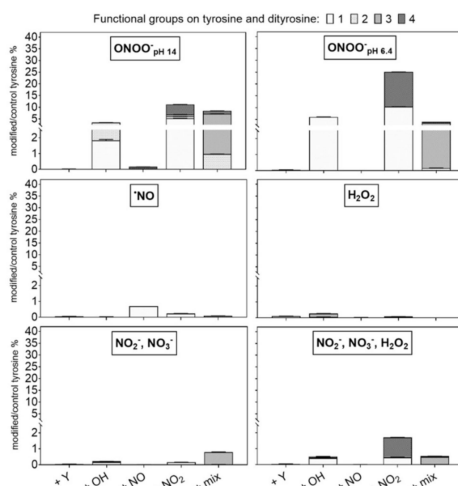

Figure 8. Modifications of tyrosine observed after treatment using control oxidants. Peroxynitrite was most efficient, with higher pH boosting the introduction of multiple groups. Labels: +Y (tyrosyl, +180.066 Da), +OH (hydroxyl, +15.9949 Da), +NO (nitroso, +28.9902 Da), and +NO_2 (nitro, +44.9851 Da). Up to four groups were observed per molecule (indicated by light to dark grey). The introduction of diverse groups is represented as "mixed" (Compounds 20 to 27, Table 1). Treatment time 3 min, 0.3 mM tyrosine in 5 mM ammonium formate, pH 7.4, direct infusion high-resolution mass spectrometry (MS). Relative compound intensities are given (tyrosine ~4948 counts in control).

By the incubation with H_2O_2, only 11% of the tyrosine was consumed, and yielded hydroxylated products. When nitrite and nitrate ions were available at the same time, a few nitrations were observed. Of note, no nitrosylation occurred, indicating that peroxynitrite, formed from H_2O_2 and NO_2^-,

was responsible. However, because of the pH of 7.4 in the experiment, this reaction was not favored. Likewise, the pH in the plasma treatment never reached below pH 7, even after 10 min of treatment (Figure S1 in SI), excluding a substantial formation of peroxynitrite in the bulk. When nitrite/nitrate were the sole available compounds, no modification of tyrosine occurs. The apparent consumption of tyrosine in conditions with a high sodium content was overestimated as a result of the formation of tyrosine salts that evaded detection in the applied experimental conditions.

3.4. Both Gas Phase- and Liquid Phase-Derived Species Contribute to Tyrosine Modification

To investigate the role of solvent-derived reactive species, higher concentrated solutions of tyrosine (30 mM) were treated. The identified products were similar to the more diluted solutions (see Figure S2/dry working gas and Figure S3/humidified working gas for a complete overview). While the total number of oxidized molecules increased, the conversion was proportionally lower and indicated a limitation of the produced ROS/RNS in liquid. In conditions with Ar-only, Ar/N_2, and Ar/O_2, a conversion of 3.2×10^{18}, 3.0×10^{18}, and 5.0×10^{18} molecules per second occurred respectively (Figure 9). This corresponds to a total of 24%, 22%, and 36% tyrosine conversion in derivatives. In comparison, treatments of 0.3 mM tyrosine solutions yielded a conversion rate of 1.8×10^{16}, 6.6×10^{16}, and 9.5×10^{16} molecules per second, corresponding to 12.8% (Ar-only), 45% (Ar/N_2), and 66% (Ar/O_2) converted tyrosine, respectively. On average, the conversion in concentrated tyrosine solutions was a factor of $\approx$50 fold higher than in diluted solutions, while their concentration was 100-fold higher. This would suggest that, using these working gases and a high tyrosine concentration, the amount of induced modifications was reduced as a result of limited production/action of species in liquid. In parallel, gaseous species were still effective on the target.
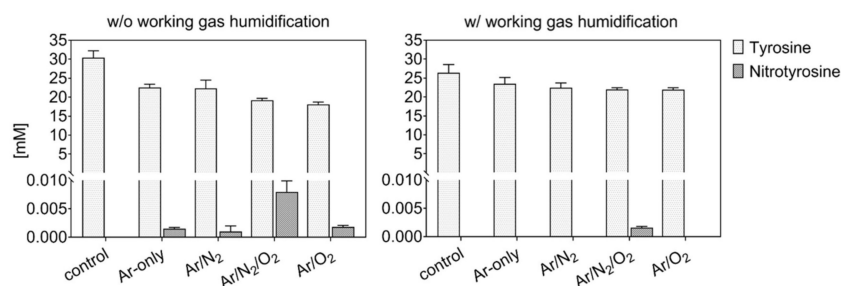

Figure 9. Tyrosine consumption and 3-nitrotyrosine formation by plasma-derived ROS in concentrated tyrosine solutions. The activity of peroxynitrite (humid conditions) is quenched in favor of nitrogen dioxide (dry conditions). Three minutes, 30 mM tyrosine in 5 mM ammonium formate, pH 7.4. Plasma is formed from dry (left) or humid (320 ppm H_2O, right) working gas.

In contrast, conditions with both N_2 and O_2 in the dry working gas led to an identical proportional formation of nitrotyrosine (0.026% of converted tyrosine) for both concentrations. In this case, the high amounts of generated ·NO_2 in the gas phase could directly modify tyrosine molecules at the interface or in the underlying water layers of both high and low concentrated solutions. The apparent reaction probability remained constant, indicating that the interface occupation by the tyrosine molecules did not change with the concentration or that a corresponding decay reaction (e.g., formation of hydroxytyrosine from nitrotyrosine) increased proportionally.

The role of gaseous ·NO_2 in forming nitrotyrosine was determined using heavy isotopes ($^{15}N_2$ or $^{18}O_2$, or $H_2{}^{18}O$). Almost 80% ^{18}O and 100% ^{15}N originated from the gas phase (Figure 10), confirming the direct nitration of tyrosine by ·NO_2, rather than other species (e.g., peroxynitrite) originating from liquid chemistry.
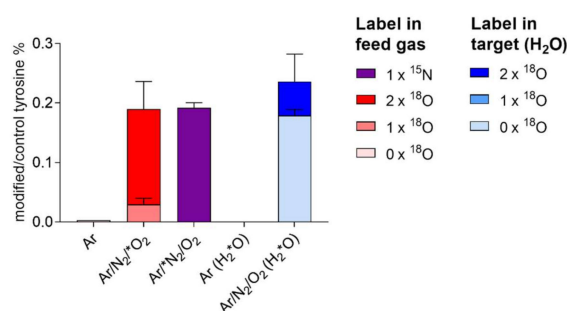
Figure 10. Nitrotyrosine formed from plasma treatment of tyrosine incorporates predominantly gas phase-derived atoms (100% N, ≈80% O). Here, 20% of oxygen atoms are introduced from the solvent (water), indicating a role for water-derived OH radicals (for details, see text). Dry argon working gas contained $^{18}O_2$ and $^{15}N_2$. In independent experiments, tyrosine was dissolved in labelled water ($H_2^{18}O$). Measurements performed via direct infusion mass spectrometry in triplicates.

By introducing humidity in the working gas, the oxidation of tyrosine, as well as the production of nitrotyrosine (Figure 9) and other tyrosine derivatives, such as dityrosine and nitrosotyrosine (Figure S3), were drastically reduced in relation to dry conditions (Figure S2). This suggests that, in humidified conditions, relevant species act and/or are formed in the bulk of the liquid. It was observed that, for working gases with <0.5 O_2%, the presence of water molecules induced mostly the formation of species such as HNO_3 and ˙NO, along with water-derived species H_2O_2, ˙OH, ˙H, and O_2^{-}˙, rather than highly oxidized gaseous species, such as N_2O_5, O_3, and ˙NO_2 [41]. When treating 0.3 mM tyrosine solutions with humid plasmas, nitrotyrosine was detected in a 10-fold higher amount than for the respective dry discharges. Considering that high concentrated solutions limited the formation/action of reactive species in liquid, gaseous species formed in humidified working gases (<0.5 O_2%) acted only in the bulk liquid or most likely as precursors of other nitrating species (e.g., peroxynitrite) formed in liquid.

3.5. Identification of Plasma-Derived Reactive Nitrogen Species

In presence of the ˙NO scavenger cPTIO or the peroxynitrite scavenger ergothioneine, the formation of N-containing tyrosine derivatives was differential.

A general overview of all identified compounds containing nitrogen is given in Figure S4, while Figure 11 shows quantitative data achieved by quantitative mass spectrometry (HILIC-MRM) for nitrotyrosine. As shown in Figure 11, in conditions with humidified working gases, the production of nitrotyrosine was almost abolished in the presence of cPTIO or ergothioneine. Further, almost all other N-containing tyrosine derivatives were inhibited (Figure S4). These results are in good agreement with the hypothesis that the addition of humidity to the working gas favors the production of peroxynitrite, predominantly originating by the reaction of ˙NO with O_2^{-}˙ derived by water dissociation. The conversion of HNO_3 in ˙NO_2 in liquid and its further reaction with ˙OH could still be an additional route for peroxynitrite formation.

The formation and activity of reactive nitrogen species in liquids could not be excluded for dry working gases (Figure S4). Indeed, the formation of some compounds that bear nitroso-groups was prevented only by cPTIO (<0.5% in Ar/N_2/O_2, Figure S4a), indicating a role for a direct or indirect role of ˙NO, reformed in the liquid phase from gaseous ˙NO_2 [81]. Furthermore, almost 2.5% of tyrosine was converted in conditions with dry Ar/N_2/O_2 in derivatives scavenged by ergothioneine (Figure S4b,c). These data confirm the formation and chemical activity of peroxynitrite in dry working gas conditions. Finally, conditions with dry working gases produced the maximal amount of nitrogen-containing derivatives in the presence of gas admixtures (3% oxidized tyrosine). Their formation was due paramount to the direct action of gaseous ˙NO_2 and, to a lesser extent, to a reformation of nitrosative species in the liquid (e.g., H^+/NO_2^-, $ONOO^-$). For humidified working gas, an increase of

nitrogen-containing derivatives was evident, provided when no oxygen was added (>14% oxidized tyrosine). Here, the nitrosative peroxynitrite is formed in liquid (Figure S4).
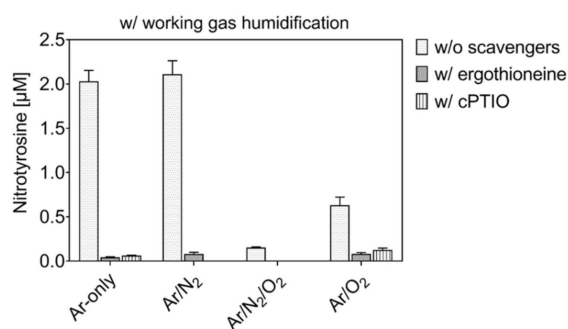

Figure 11. Nitrotyrosine formation by plasma treatment is prevented in the presence of scavengers. Three minute treatment, 0.3 mM tyrosine in 5 mM ammonium formate, pH 7.4, with and without ergothioneine or cPTIO, using Ar ± 1% N_2/O_2 as humidified working gas and N_2 as shielding gas.

However, non-specific reactions must be considered. It was reported that the NO scavenger cPTIO partially reacts with $^{\cdot}NO_2$ [78], while ergothioneine is able to scavenge $^1O_2$ and $^{\cdot}OH$ [79,104]. No discriminative derivatives produced by the reaction of scavengers with control oxidants could be identified in this work.

3.6. Plasma Induces Oxidative and Nitrosative Protein Modifications in Wounds

In order to verify that nitrosative modifications are introduced in vivo as well, the wound proteome of human patients before and after plasma treatment was analyzed. A special emphasis was given to modifications at the tyrosine moiety.

Overall, 330 proteins have been identified and were searched for oxidative post-translational modifications (PTMs). PTMs were detected at 80 proteins; 27 of these were modified at tyrosine moieties (Table 3). An increase of 63% (oxidation), 44% (nitration), and 69% (nitrosylation) compared with the control was observed (Figure 12). Target proteins were highly abundant blood plasma and blood cell components (transferrin, albumin, hemoglobin, and so on), or belonged to the wound bed/wound margins (keratins, fibronectin). The modified tyrosine residues were located at the protein surface or otherwise exposed structures. As samples were taken immediately after treatment, regular physiologic reactions were unlikely and the increase of modifications was attributed directly to the impact of plasma-derived reactive species. The oxidation of tyrosine and cysteine residues in blood plasma proteins was already observed for inflammatory levels of hypochlorous acid [105]. Target proteins were, among others, complement C3 and apolipoprotein A-I, and albumin was affected in this study as well. Cold plasma-derived atomic oxygen forms hypochlorite anions upon reaction with chloride ions locally, which subsequently serves as oxidants yielding di-tyrosine, hydroxylations, and chlorinations (rare). The introduction of nitro and nitroso groups into tyrosine occurs by local formation of peroxynitrite and NO_2 radicals, which are only effective at or close to the treated surface. Yielding a higher reactivity of the tyrosine's phenolic hydroxyl group, significant effects on the regulation of cell migration, angiogenesis, and mast cell degranulation [67,72] were described for physiologic conditions (nitric oxide pathway). The proportional strong oxidation/nitration of haptoglobin, a protein protective against hemoglobin related oxidative stress, and of bulky structural proteins like fibronectin and keratins, confirms a significant presence of reactive species from the plasma discharge and the proteins' roles as scavenger molecules.

Table 3. Twenty seven proteins were identified from wound exudates carrying oxidative modifications on tyrosine (oxidation/+15.99 Da, nitrosylation/+28.99 Da, nitration/+44.99 Da). The total number of proteins found with modification was 80 out of 308 proteins identified. Blood-derived proteins dominate the list. Modifications occur predominantly at the protein surface.

| Uniprot Identifier	Abbreviated Protein Name	Protein Name	Modified Tyrosine Residue (Bold Denotes Significant Site)
P02768	ALBU	Albumin	**Y54**, Y172, Y174, **Y287**, **Y377**, Y425, Y356, **Y365**, Y521
P01009	A1AT	Alpha-1-antitrypsin	Y184
P01023	A2MG	Alpha-2-macroglobulin	Y544, Y818, Y1152, Y1264
O43299	AP5Z1	AP-5 complex subunit zeta-1	**Y344**
P02647	APOA1	Apolipoprotein A-I	Y124, Y190
P00915	CAH1	Carbonic anhydrase 1	Y21
P00450	CERU	Ceruloplasmin	Y55, Y539
P01024	CO3	Complement C3	Y139, Y1447, Y1620
P0C0L4	CO4A	Complement C4-A	Y1612
P02751	FINC	Fibronectin	Y841, Y2362
P00738	HPT	Haptoglobin	Y224, **Y242**, **Y280**, Y386, Y389
P69905	HBA	Hemoglobin subunit alpha	**Y25**, **Y43**
P68871	HBB	Hemoglobin subunit beta	**Y36**, **Y131**
P02042	HBD	Hemoglobin subunit delta	Y36, Y131
P01876	IGHA1	Immunoglobulin heavy constant alpha 1	Y276
P01857	IGHG1	Immunoglobulin heavy constant gamma 1	**Y161**, Y319
Q14624	ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	Y157
P13645	K1C10	Keratin, type I cytoskeletal 10	Y172, Y325
P02533	K1C14	Keratin, type I cytoskeletal 14	Y46
P35527	K1C9	Keratin, type I cytoskeletal 9	Y330, Y345
P04264	K2C1	Keratin, type II cytoskeletal 1	Y266, Y295, Y358, Y373
Q7Z794	K2C1B	Keratin, type II cytoskeletal 1b	Y361
P35908	K22E	Keratin, type II cytoskeletal 2	Y563, Y589
P02788	TRFL	Lactotransferrin	**Y211**, **Y545**
P32119	PRDX2	Peroxiredoxin-2	Y126
P00747	PLMN	Plasminogen	Y66
P02787	TRFE	Serotransferrin	Y114, Y333, Y533
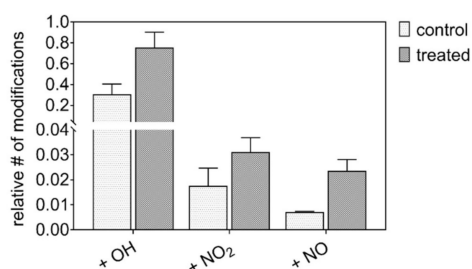
Figure 12. Cold plasma induced post-translational modifications in the proteome of wound exudates of diabetic patients. Proteome analysis performed via nanoLC-MS, with the detection of mass shifts corresponding to the introduced functional group (OH = 15.9949 Da; NO = 28.9902 Da, NO_2 = 44.9851 Da), according to [80].

The introduction of nitro groups into proteins by plasma-derived species suggests at least a contributing role in the observed crosstalk to cellular redox signaling pathways involved in acute and chronic wound healing, well in line with a recent study that emphasized the role of target cell stimulation over anti-microbial effects [22]. The results of this study suggest that the clinical effectivity of cold plasmas would benefit from a switch from the current condition (argon only) to argon/N_2/O_2 as a working gas to increase the impact of the plasma-derived RNS on biomolecules and subsequent signaling events.

4. Summary and Conclusions

This work studied the liquid chemistry of argon plasmas generated by the kINPen, with a special focus on reactive nitrogen species. Assuming that the liquid chemistry is the bridge between the gaseous plasma and biological systems, we looked for the impact of nitrogen species on the model biomolecule tyrosine. Using a mass spectrometry driven approach, 26 different compounds were identified. Their respective pattern was exploited to determine the dominant reactive species formed in the gas or in/at the liquid phase.

Nitration reactions were significant for dry Ar/O_2/N_2 plasma. Gaseous ·NO_2 was found to be responsible for a direct nitration of tyrosine at the gas–liquid interface. Additionally, it is involved in the formation of peroxynitrite and nitric oxide radicals. While the relevance of nitric oxide for the product formation was limited, peroxynitrite contributed to a substantial extent. When water molecules were present in the working gas, its role was further emphasized owing to a higher peroxynitrite formation via additional pathway; that is, the reaction of gaseous nitric oxide with superoxide anion radicals and the reaction of hydrogen peroxide and nitrous acid/nitrite at the gas–liquid interface. In conditions with a high prevalence of ROS (e.g., dry Ar/O_2), the impact of RNS was minimized. Here, stable species like N_2O_5 that do not penetrate the interface evolve from ·NO_2 in the gas phase and the formation of peroxynitrite decreases. Because of the high activity of ROS at the interface and the liquid bulk, potentially formed nitrated/nitrosylated tyrosine products are eliminated in favor of hydroxylated compounds. Finally, the introduction of nitroso and nitro groups into proteins in vivo by cold plasma treatments was confirmed in human diabetes 2-related chronic wounds. The tyrosine moiety was particularly attacked, allowing for changes in the protein functionality. This suggests a contribution of plasma-derived RNS via covalent changes to the observed efficacy in wound healing.

In conclusion, this work verified the controllability of kINPen plasmas to achieve a relevant production of reactive nitrogen species (Figure 13). The dissolved or at the interface generated species, especially ·NO_2 and $ONOO^-$, led to nitrosative reactions on biomolecules, in both complex and model conditions. A relevant contribution to the observed biomedical effects of plasmas must be assumed.
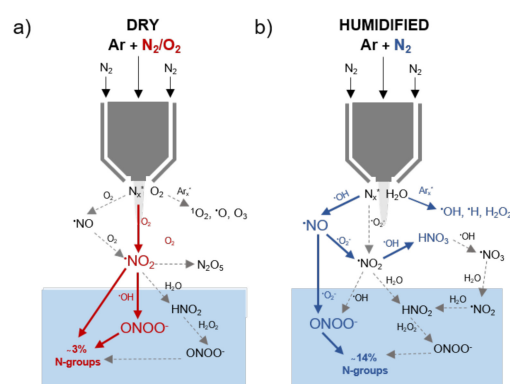

Figure 13. Suggested chemical pathways for the production of bioactive RNS species in plasma treated tyrosine solutions in dry (**a**) and humidified (**b**) conditions. Represented species were previously detected for the kINPen [41,82,100], except for peroxynitrite. Pathway confirmed in liquids (compact lines) by use of scavengers or isotope labels.

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Article A5

Radiation driven chemistry in biomolecules – is (V)UV involved in the bioactivity of argon jet plasmas?

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Radiation Driven Chemistry in Biomolecules—is (V)UV Involved in the Bioactivity of Argon Jet Plasmas?

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Cold physical plasmas, especially noble gas driven plasma jets, emit considerable amounts of ultraviolet radiation (UV). Given that a noble gas channel is present, even the energetic vacuum UV can reach the treated target. The relevance of UV radiation for antimicrobial effects is generally accepted. It remains to be clarified if this radiation is relevant for other biomedical application of plasmas, e.g., in wound care or cancer remediation. In this work, the role of (vacuum) ultraviolet radiation generated by the argon plasma jet kINPen for cysteine modifications was investigated in aqueous solutions and porcine skin. To differentiate the effects of photons of different wavelength and complete plasma discharge, a micro chamber equipped with a MgF_2, Suprasil, or Borosilicate glass window was used. In liquid phase, plasma-derived VUV radiation was effective and led to the formation of cysteine oxidation products and molecule breakdown products, yielding sulfite, sulfate, and hydrogen sulfide. At the boundary layer, the impact of VUV photons led to water molecule photolysis and formation of hydroxyl radicals and hydrogen peroxide. In addition, photolytic cleavage of the weak carbon-sulfur bond initiated the formation of sulfur oxy ions. In the intact skin model, protein thiol modification was rare even if a VUV transparent MgF_2 window was used. Presumably, the plasma-derived VUV radiation played a limited role since reactions at the boundary layer are less frequent and the dense biomolecules layers block it effectively, inhibiting significant penetration. This result further emphasizes the safety of physical plasmas in biomedical applications.

Keywords: cold physical plasma, redox signaling, porcine skin model, VUV radiation, tape stripping model, kINpen

1 INTRODUCTION

Emerging therapies for the treatment of chronic wounds and cancerous lesions involve the administration of exogenous reactive species directly delivered on the target (e.g., cold physical plasmas) [1–4]; or produced *in situ* by administration of specific drugs (e.g., nanoprodrugs) [5,6]. For example, the formation of singlet oxygen by irradiation (600–800 nm) of a photosensitizer, is the molecular mechanism behind the effectiveness of the photodynamic therapy in use for cancer regression [7]. Among the emerging therapies, cold physical plasmas are multi-function tools comprising of reactive species, ions, metastables, electrons, magnetic fields, and photons [8,9]. These, synergistically acting on the target, are effective in cancer regression [10–12]; and wound healing

[13–15]. Furthermore, the use of plasmas is considered in other fields, such as sterilization [16–18]; and dentistry [19–21].

While downstream effects have been detected [11,22], many are the open questions regarding the working mechanism of plasmas on biological target. Therefore, the variable production of plasma elements has been studied, with focus on reactive species, at date considered the predominant responsible of plasma effectiveness [23]. In particular, their amounts on the target can be regulated by tuning the plasma parameters (e.g., treatment duration, distance, working gases) [2,24], achieving bivalent aims such as promoting cell proliferation and migration in would healing, or inducing cell death and apoptosis for cancer treatment and biological decontamination. Cold plasmas can be generated by a multitude of different designs, yielding differences in species output and biomedical impact [25]; [26].

One plasma source is the kINPen, an argon-driven jet which gas phase has been already well characterized. The production of primary reactive species such as excited states of argon (e.g., metastables, excimers) were observed in the effluent area (or gas phase). Those reactive species react with others gases (e.g., N_2, O_2, H_2) present in the surrounding atmosphere or in the core gas to generate secondary species, such as atomic oxygen ($\cdot O$), singlet oxygen ($^1O_2$), ozone (O_3), hydroxyl radicals (OH), superoxide anions radicals (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide radicals (NO_x), acids containing nitrogen (HNO_x). Finally, a tertiary chemistry is stimulated directly in the target. Using biochemical models, covalent modification of biomolecules were predicted and observed, especially in amino acids and proteins [27–30]; and lipids [31–33].

In the cellular environment, plasma-induced biomolecules modifications could be the responsible event for the deregulation of redox signaling pathways. Indeed, it was shown that kINPen plasmas led to an abnormal production/ functioning of e.g., transcription factors (e.g., Nrf2 and p53), which modulates differentially gene expressions, cellular organization and apoptosis processes [15,34–36]. Together with reactive species, radiation generated by cold plasmas could cover important synergistic effects in stimulating these processes.

In kINPen plasmas, their relevance in inducing oxidative stress must be considered, since the emission region goes from the vacuum UV region (105 nm) (emitted by argon excimers) to the near infrared (1,000 nm) [9,37]. The ultraviolet radiation (100–400 nm) emitted by kinpen plasmas could have a synergistic role in their effectiveness, e.g., for antibacterial purposes as shown also for other plasma sources [38–41].

Radiation can be generally classified in ionizing (10–125 nm), which have short wavelength, high frequency and energy, and non-ionizing (>125 nm), which oppositely are longer wavelengths with lower frequency and energy. Therefore, even if measured in low levels [42,43], vacuum UV radiation (100–200 nm) emitted by argon metastable produced by kINPen plasmas could have enough energy to impact strongly on the biological matter, leading to DNA damage, protein denaturation and cell death. Indeed, it is well known that high levels of ionizing radiation can be harmful for the living matter, generally disrupting and damaging molecular structures (e.g.,

lipids, proteins, nucleic acids, carbohydrates) [44–48]. However, in relation to the quantity and time of exposure, UV radiation (mostly UV-C and UV-B, 200–315 nm) can also increase the general oxidative stress, induce indirectly or directly structural changes in biomolecules, and consequentially modulate redox signaling pathways [49,50]. The mechanism of action of radiation is still under clarification in biology, but generally they can generate biomolecules modifications by being directly absorbed (e.g., amino acids cysteine, tyrosine, tryptophan), or by stimulating sensitizing compounds (e.g., exogenous or endogenous). In both cases, excited forms will be generated, which starts photo-oxidation reactions. Indeed, radicals can be formed by hydrogen abstraction or one electron oxidation (Type I mechanism) and reactive species can be formed by energy transfer to molecular oxygen, which forms singlet oxygen (Type II mechanism) [50,51]. Generally, an increased release of reactive species from mitochondria was measured after radiation exposure, as well as the activation of a calcium dependent NOS-1 with increase of peroxynitrite levels. The chain reaction induced by photo-oxidation can be harmful in long and intense exposure, but for short and not severe exposure a transitory effect was observed, leading to a cytoprotective response mediated by MAPK1/2 activation [51]. The UV and vacuum UV light produced by different plasma sources has been considered as essential elements for the antimicrobial activity [38–41,52,53].

In this work, the role of ultraviolet radiation generated by the argon plasma jet kINPen for cysteine modifications was investigated. Cysteine is easily oxidized, and served to investigate plasma chemistry in liquids before [24,54–56]. Modifications occurring on cysteine in aqueous solutions or porcine epithelium were identified via mass spectrometry and major derivatives were quantified using it coupled to high-pressure liquid chromatography. To isolate the effects of photons from complete plasma discharge, a micro chamber equipped with VUV, UV-C, and UV-A windows was used. Alongside, optical emission spectrometry and aqueous chemistry was applied to characterize reactive species formation in the gas and liquid phase. A significant contribution of plasma derived UV radiation on cysteine chemistry was observed if water molecules were present.

2 MATERIALS AND METHODS

2.1 Sample Preparation

Crystalline cysteine (Sigma Aldrich) was solubilized in double distilled water (ddH$_2$O) to a final concentration of 2 mM on a daily basis. For short-term storage, blue ice was used to avoid pH value distortions during freeze-thawing cycles [57]. After respective plasma or irradiation treatments, solutions were immediately subjected to high-resolution mass spectrometry, multiple reaction monitoring mass spectrometry, or ion chromatography. Fresh porcine ears were received from Landmetzgerei Urich (Bad Koenig, Germany) on blue ice, serving as a well-accepted replacement model for human skin [58]. The ears were washed carefully, shaved, and the superficial

FIGURE 1 | Micro-chamber used to apply UV radiation emitted kINPen plasmas **(A, B)**. Cysteine solutions **(A)** or porcine skin sections **(B)** were introduced into the chamber. The light transmission of the three windows is indicated in **(C)**.
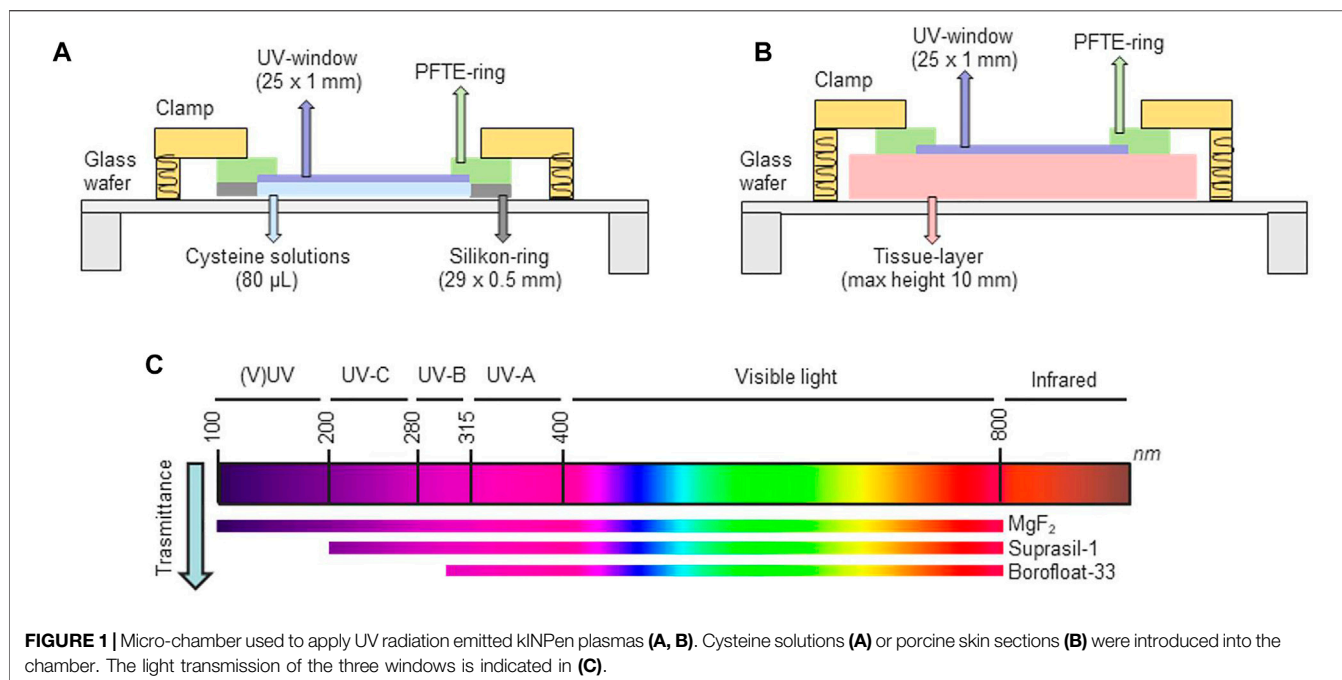
corneocyte layer was removed with a single CorneoFix strip (Courage and Khazaka electronic GmbH, Cologne, Germany) to increase homogenicity. Plasma treatment was performed in selected clean and homogenous areas of a 2 cm × 2 cm dimension.

2.2 Plasma Treatments

The kINPen, an argon-driven (99.999%, Air Liquide) dielectric barrier plasma jet with a flow rate of 3 standard liters per minute (slm) served as plasma source. If desired, the working gas was modified by 1% admixture of molecular oxygen (99.998, Air Liquide). Its central electrode is powered by an alternating current with a sinusoidal waveform, 2–6 kV peak–peak voltage, and a frequency of around 1 MHz. The outer electrode is insulated by a ceramic tube. The dissipated electrical power is around 1.1 W. For most experiments, a gas curtain created by a nozzle and 5 slm nitrogen (99.999%, Air Liquide) shielded the effluent from the ambient air. For details about the design and working principle of the jet, refer to Reuter and colleagues [9] and citations therein. To investigate the plasma-derived products and emitted radiation by optical emission spectroscopy, the plasma jet was positioned on axis at a distance of 9 mm to the front of a spectrometer (AvaSpec-2048; Avantes, Germany) allowing the observation of both UV and VIS/NIR range (195–980 nm) with a spectral resolution of 0.7 nm. For the VUV spectral measurements, a single grating monochromator (Acton VM-502, grating 1200 g/ mm) was used. This system was set to a spectral resolution of 0.2 nm and the spectral range of 100–200 nm was observed. Furthermore, the system was under low pressure ($2.2*10^{-6}$ mbar) and connected via an MgF_2 window for VUV transmissions down to 100 nm. The kINPen was placed at 9 mm distance in front of the MgF_2 window.

The distance between the nozzle and the target was kept at 9 mm. Targets were either 750 µL aqueous solution in a 24 well-plate, fresh porcine skin prepared as described in 2.1, or a 25 mm diameter radiation chamber. The chamber could be equipped with windows that transmit different parts of the VUV/UV radiation and was filled with 80 µL solution forming a 500 µm thick layer or sections of porcine skin (**Figure 1A,B**). After treatments (20 s–180 s) samples were submitted to reactive species analysis (**Section 2.3**). Liquids containing the tracer molecule cysteine were also analyzed via liquid chromatography coupled to mass spectrometry for the detection of oxidative modifications (**Section 2.4**). The first 3 layers of treated porcine skin tissues were sampled using three consecutive CorneoFix strips that were immediately deposited in a protein solubilization buffer and subjected to shotgun proteomics (as described in **Section 2.5**). Each experiment was performed in triplicate.

2.3 OH and H₂O₂ Quantification via Colorimetric Assays in Liquids

For the quantification of plasma-generated, short-lived ROS (hydroxyl radicals, atomic oxygen) solution of 5 mM terephthalic acid in 25 mM phosphate buffer, pH 7.4 was used despite the limited selectivity [59]. The reaction yield to the fluorescent compound 2-hydroxyterephthalic acid (HTPA) that could be quantified at 318 nm excitation and 426 nm emission using an external calibration curve. Hydrogen peroxide deposited in treated liquids was determined using the ferrous oxidation–xylenol orange (FOX) assay according to the manufacturer's protocol (Thermo Scientific, Dreieich, Germany). The reaction yield to a purple product, which absorbance at 595 nm was

measured in a spectrophotometer (Tecan M200 multi-plate reader, Männedorf, Switzerland).

2.4 Characterization of Plasma-Induced Sulfur Chemistry in Liquids
2.4.1 Cysteine Derivatives.
Cysteine, cystine, cysteine sulfonic acid, cysteine sulfinic acid, alanine, cysteine-S-sulfonate were quantified by coupling a chromatographic separation (Agilent 1,290 Infinity II, Waldbronn, Germany) to targeted mass spectrometry (Q-Trap 5500, Sciex, Darmstadt, Germany). Analytes were separated on a 2.1 mm × 100 mm Acquity Amide Column with 130 Å pore size and 1.7 µm particle size and a corresponding VanGuard precolumn (Waters, Manchester, England) at a column temperature of 35°C. Mobile phase A consisted of 10 mM ammonium formate in water plus 0.15% formic acid while B consisted of 10 mM ammonium formate in acetonitrile plus 0.15% formic acid. The flow rate was 0.8 ml/min. A linear gradient was applied (0.0 min–99% B; 4.0 min–85% B; 7.0 min–30% B; 7.1 min–99% B; 9.0 min–99% B). Prior to injection, samples were diluted 1:5 in mobile phase B. Compounds were determined via multiple reaction monitoring in positive mode. The electrospray (ESI) source parameters were the following: curtain gas 35 psi, gas 1 20 psi, gas 2 25 psi, temperature 150°C, 5.5 kV probe voltage, 50 V declustering potential. The transitions and the correspondent collisional energies (CE) used for each compounds were for cysteine 122 → 76 m/z, CE 20; cystine 241 → 152 m/z, CE 10; cysteine sulfinic acid 154 → 74 m/z, CE 20; cysteine sulfonic acid 170 → 124 m/z, CE 10; cysteine-S-sulfonate 202 → 120 m/z, CE 10; alanine 90.1 → 44.1 m/z, CE 8. External calibration curves allowed the absolute quantification of the listed compounds in plasma-treated or irradiated samples.

2.4.2 Hydrogen Sulfide.
To quantify the formation of hydrogen sulfide (H_2S) from cysteine, the monobromobimane (mBB) assay was optimized [60]. A solution of 100 mM MBB in acetonitrile was freshly prepared. First, 25 µL of analyte solutions was mixed with 2 µL MBB and 65 µL of 100 mM phosphate buffer at pH 7.8. After vigorous mixing, samples were incubated at 37°C for 10, 30, or 60 min using a thermomixer, yielding sulfodibimane (SDB) in the presence of H_2S. The reaction was stopped by adding 5 µL formic acid 50% and cleared by centrifugation. SDB was quantified by targeted mass spectrometry. Analytes were separated on a 2.1 × 50 mm Zorbax RRHD Eclipse Plus C18 column (Waters, 95 Å pore size, 1.8 µm particle size) and corresponding guard column. Mobile phases were water (A; Th. Geyer, Renningen, Germany) and acetonitrile (B; ibid). A linear gradient was applied (0 min–5% B; 2.1 min–40% B; 5 min–40% B; 5.1 min–98% B; 6 min–98% B; 6.1 min–5% B; 8 min–5% B). The flow rate was of 0.8 ml/min. Atmospheric pressure chemical ionization (APCI) was applied with the following source parameters: curtain gas 20 psi, gas 1 50 psi, temperature 500°C, 3 kV needle current, 100 V declustering potential, 32 V collision energy. The transitions used

for the analyzed compounds were for MBB 272 → 193 m/z; SDB 415.1 → 193.3 m/z.

2.4.3 Sulfite and Sulfate.
Ion chromatography (ICS-5000, Dionex Corp., Sunnyvale, United States) was used for the quantification of sulfite (SO_3^-) and sulfate (SO_4^-) anions. These were separated on a IonPac® AS23 pre-column (2 × 50 mm) coupled to an IonPac® AS23 anion exchange column (2 × 250 mm, Thermo Fisher Scientific Inc., Waltham, United States). Isocratic separation was achieved using a carbonate buffer (4.5 mM Na_2CO_3/0.8 mM $NaHCO_3$) and the flow rate of 0.25 ml/min.

2.5 Investigation of Protein Modifications in Tissues
2.5.1 Protein sample preparation.
After sampling via tape stripping controls and plasma treated porcine skin layers, proteins were solubilized by introducing the tape strip in 500 µL of SDS-based lysis buffer (5% SDS, 50 mM TEAB pH 7.55) and by vortexing the vials for 2 min at room temperature. A S-Trap midi spin column digestion protocol from ProtiFi was applied according to the manufacturers protocol. The solutions were sonicated to disrupt cells, dissolve proteins, and shear DNA and clarified by centrifugation at 4000g for 10 min at 4°C. The supernatant was transferred to a clean vial. The reduction and alkylation of sulfhydryl groups was performed by incubating respectively with 5 mM tris(2-carboxyethyl) phosphine (TCEP, 55°C, 15 min) and subsequently with 20 mM methyl methanethiosulfonate (MMTS) at RT for 10 min. The reaction was stopped by adding 12% phosphoric acid. Next, 300 µL S-Trap buffer (90% methanol, 100 mM TEAB, pH 7.1) was added, and samples were transferred to the spin column. After centrifugation (2 min × 4000 g), the column was washed with 300 µL S-Trap buffer three times. Protein digestion was achieved in column by adding 1:25 wt:wt sequencing grade trypsin (Promega, Madison, United States) in 50 mM TEAB and incubating for 1 hour at 47°C. In this case, the columns were sealed with a lid to avoid solution evaporation. Finally, peptides were eluted with 500 µL of acetonitrile containing 0.2% formic acid. The solutions were dried using a SpeedVac and resuspended in 10 µL of water containing 0.1% formic acid before nanoLC-MS analysis.

2.5.2 LC-MS analysis.
The proteomes and the oxidative modifications occurring in porcine skin layers after treatments were analyzed by nanoflow liquid chromatography, using an UltiMate 300 RSLCnano coupled to a QExactive Hybrid-Quadrupol-Orbitrap from Thermo Fisher Scientific, Dreieich/Germany. The technical details of the separation and detection are described in Wenske et al., 2021. Raw data were analyzed with the Proteome Discoverer 2.4 (Thermo Fischer Scientific) and the Byonic 3.6.0 node (Protein Metrics) for searching protein modifications. A list of 15 modifications previously identified for gas plasma treatments of thiol moieties was used to reduce

TABLE 1 | Analyzed thiols oxidative modifications occurring in the proteome of plasma treated porcine skin samples.

Mass shift (Da)	Composition	Modification	Acronym	Label
−2.02	−2H	Dehydrogenation	Didehydro	A
+15.99	+O	Oxidation	Oxidation	B_1
+21.98	+2O	Dioxidation	Dioxidation	B_2
+47.98	+3O	Trioxidation	Trioxidation	B_3
+28.99	+N + O -H	Nitrosylation	Nitrosyl	C1
+44.98	+N +2O -H	Nitration	Nitro + O	C2
−15.01	−N −H	Deamination	−NH	D_1
−17.03	−N −3H	Deamination + Dehydrogenation	−NH3	D_2
−31.97	−S	Sulphur loss	−S (alanine)	E_1
+31.97	+S	Sulphur addition	+S	E_2
+79.96	+S +3O	Sulfonylation	+SO3	E_3
+119.00	+S +3C +5H + N +2O	Cysteine addition	+S2R	F_1
+150.99	+S +3C +5H + N +4O	Cysteine addition + Dioxidation	+S2O4R	F_2
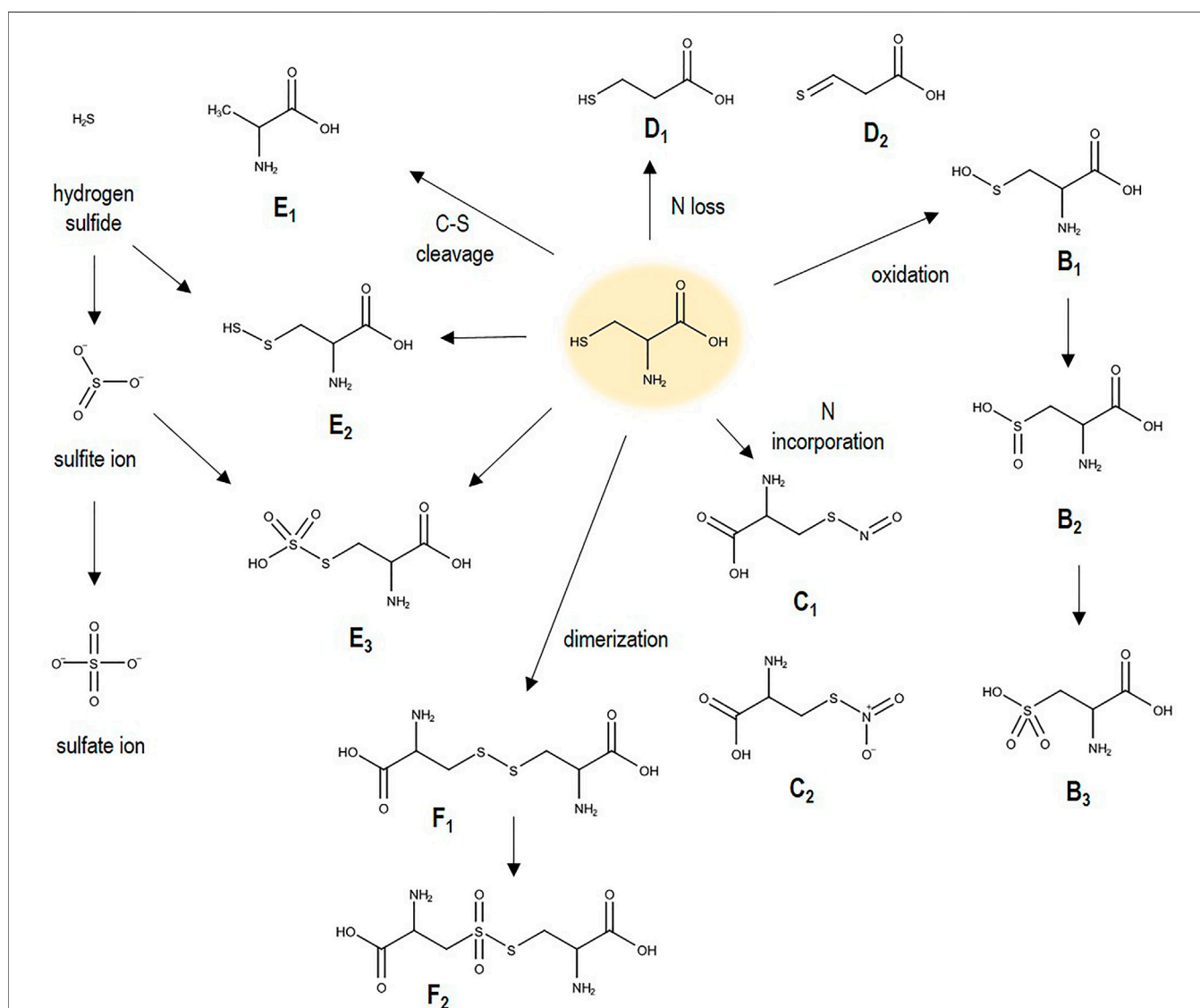
FIGURE 2 | Cysteine oxidation and cleavage products (see **Tables 1**, **2**). When incorporated into a protein, the carboxyl and amino group are incorporated in the peptide bond.

FIGURE 3 | OES spectra registered at 9 mm distance from jet nozzle in the range 195–465 nm (UV range, **(A and C)** and 430–980 nm (VIS/NIR range, **(B and D)**. **(A and B)**: kINPen09, 3 slm Ar, **(C and D)**: kINPen09/3slm Ar + 0.5% oxygen. Free plasma jet, and radiation filtered through MgF_2 (transparent >125 nm), Suprasil (quartz based, > 195 nm), and Borofloat glass (>300 nm) were compared (see legend in spectra). Due to limitations in setup (no measurements <195 nm), only slight changes between 200 and 300 nm were observed. The VIS/NIR region remained unchanged by the different windows except for a minor reduction in intensity.

calculation times (**Table 1**) [24,55]. A maximum of three modifications per peptide was set, and to ensure result validity, only peptides with a Byonic score >250 and a Delta Mod score of >5 were accepted for downstream data analysis. A normalization on the total peptides containing cysteine was performed, yielding a percentage of modified thiols on the total proteome. Furthermore, the oxidative modifications found in controls were subtracted as background from all other samples and data are shown as difference from the control. One-way ANOVA statistical test was performed in order to detect significant modifications, allowing a comparison between different conditions.

FIGURE 4 | OES spectra registered at 9 mm distance from kINPen09 jet nozzle in the range 100–200 nm. Argon excimer lines were observed around 120–130 nm and 180–200 nm. A significant reduction of these emissions lines was observed for 0.5% oxygen admixture to the argon working gas **(A)**. The VUV range was blocked for some experiments using borosilicate or suprasil windows **(B)**, or allowed to access the target (MgF$_2$ window).

FIGURE 5 | Formation of hydroxyl radicals **(A)**, and hydrogen peroxide **(B)** after plasma treatments using 3 slm Ar ± O$_2$ and 5 slm shielding gas (N$_2$). Solutions were treated in either 24 well plates or radiation chamber (see **Figure 1**) with different volumes due to technical constraints (see text). Quantification performed *via* colorimetric assays [•OH according to [72]]; experiment performed in triplicates. Error bars (range) are in some cases smaller than data points and not visible.
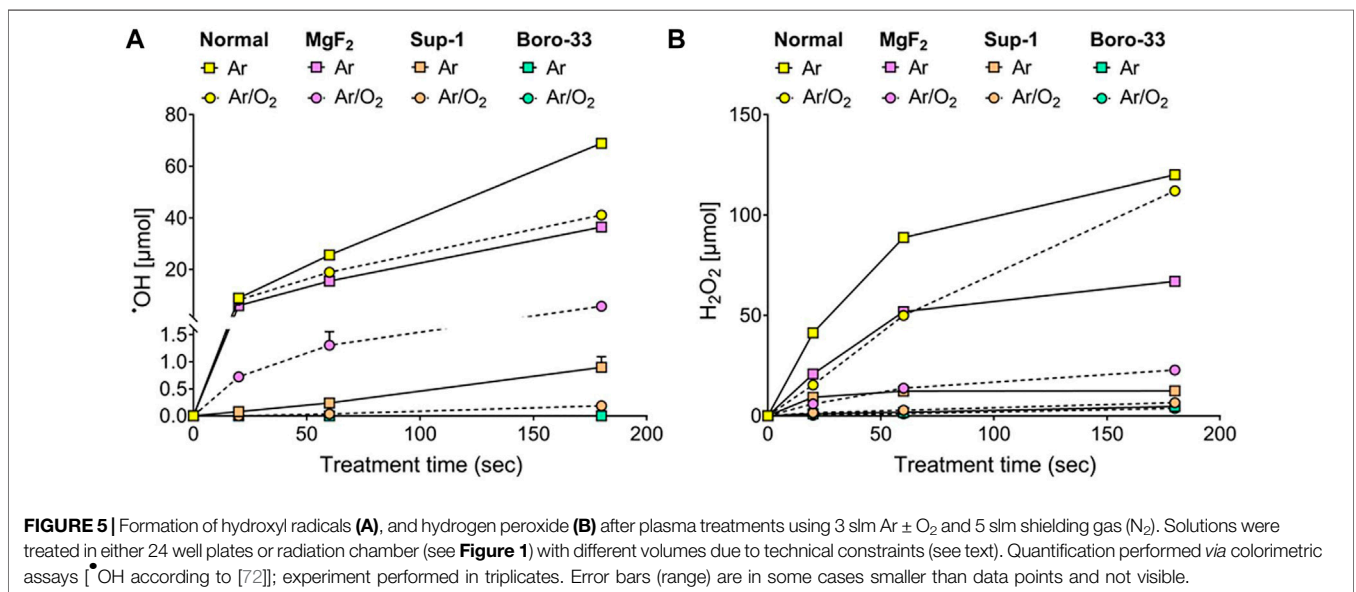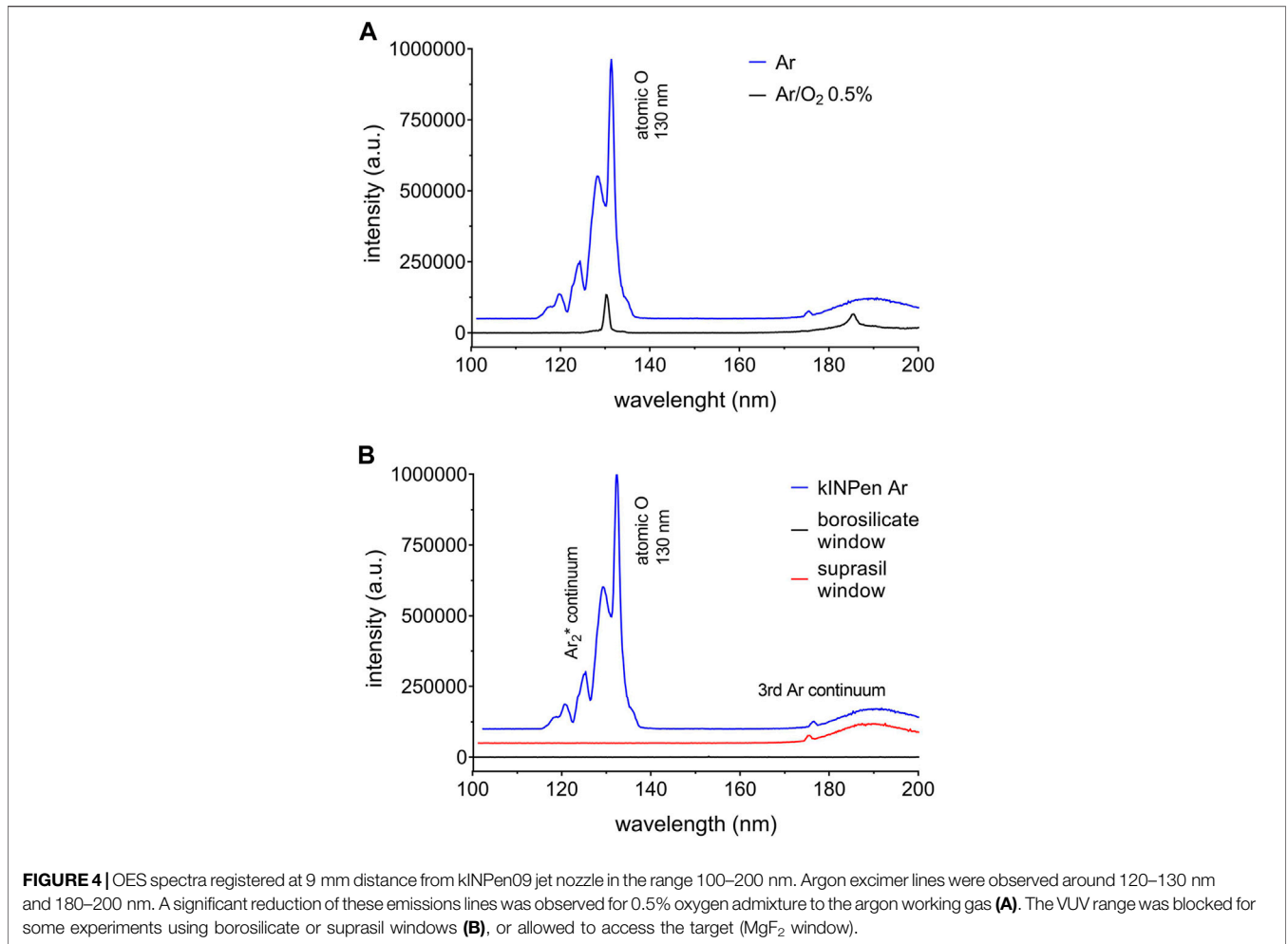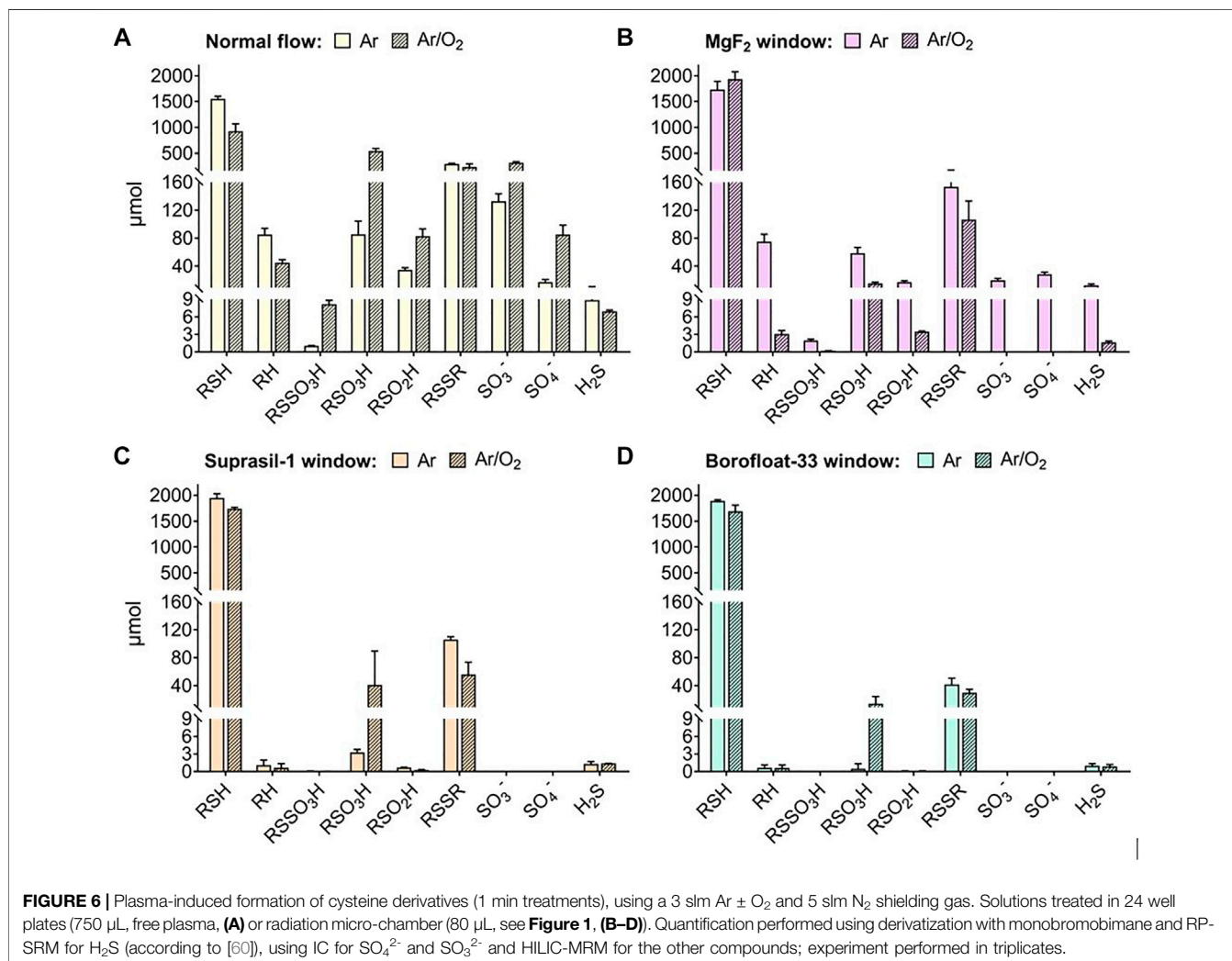
TABLE 2 | Analyzed cysteine derivatives, acronyms, formulas, quantification method and responsible plasma element.

Name	Acronym	Hill notation	Analysis	Plasma element
Cysteine	RSH	$C_3H_6NO_2S$	HILIC-MRM	None
Cystine	RSSR	$C_6H_{12}N_2O_4S_2$	HILIC-MRM	Radicals
Alanine	RH	$C_3H_7NO_2$	HILIC-MRM	(V)UV
Sulfonic acid	RSO_3H	$C_3H_7NO_5S$	HILIC-MRM	˙OH, $^1O_2$, ˙O
Sulfinic acid	RSO_2H	$C_3H_7NO_4S$	HILIC-MRM	˙OH, $^1O_2$, ˙O
S-sulfonate	$RSSO_3H$	$C_3H_7NO_5S_2$	HILIC-MRM	˙OH, ˙O (indirect)
Sulfite	SO_3^-	O_3S	IC	(V)UV, ˙OH, $^1O_2$, ˙O
Sulfate	SO_4^-	O_4S	IC	(V)UV, ˙OH, $^1O_2$, ˙O
Hydrogen sulfide	H_2S	H_2S	MBB, RP-MRM	(V)UV

FIGURE 6 | Plasma-induced formation of cysteine derivatives (1 min treatments), using a 3 slm Ar ± O₂ and 5 slm N₂ shielding gas. Solutions treated in 24 well plates (750 µL, free plasma, **(A)** or radiation micro-chamber (80 µL, see **Figure 1**, **(B–D)**). Quantification performed using derivatization with monobromobimane and RP-SRM for H₂S (according to [60]), using IC for SO_4^{2-} and SO_3^{2-} and HILIC-MRM for the other compounds; experiment performed in triplicates.

3 RESULTS AND DISCUSSION

3.1 Fingerprinting the kINPen Plasma Radiation

The kINPen spectra of emission goes from the vacuum UV (100 nm) to the near infrared (1,000 nm) [61]. The intensity of the UV irradiation was determined to be around 100 µJ cm^{-2}.

Optical emission spectroscopy was performed in order to characterize the emission spectra of the applied plasma treatment conditions (**Figure 1**). In this case, the emission spectra were measured applying the three different windows filtering various radiation ranges (**Figure 2**). Additionally, the vacuum UV range was recorded using a vacuum setup (**Figure 3**) [9,37]. In pure argon, emission lines from impurities of •OH and

N_2 were measured between 300 and 350 nm, and small atomic oxygen lines at 777 and 844 nm. The infrared region was dominated by argon emission lines, while between the range 400–700 nm no emission was observed. In the vacuum UV region (<195 nm), a dominant continuum centered at 126 nm can be measured for argon excimer (Ar_2^*), which includes also small absorption lines from ozone and O_2. The modulation of working or shielding gases, induced changes in the emission spectra, e.g., the addition of molecular oxygen in the working gas leads to a reduction of Ar_2^* emission lines and increase those oxygen-based [9,37]; (**Figure 2**). The presence of molecular O_2 in the working gas interfered with the gas phase chemistry and UV emission of various species, such as $^\bullet$OH, N, $^\bullet$NO, and Ar_2^*. Suprasil and Borofloat-33 scavenged the emission of argon excimers, while in the range between 195 and 465 more subtle changes were observed, reflecting the characteristics of normal glass (**Figure 2**). Particularly in conditions with pure argon, which are certified for medical applications with kINPen MED, the role of vacuum UV radiation was dominant (**Figure 3**). As discussed previously, this event could be due to the lower formation of gaseous reactive species and therefore less reactions of argon metastable and excimers with surrounding gases to form further species, e.g., ozone, atomic oxygen, singlet oxygen, etc. [9,37]; (**Figures 2**, **4**).

3.2 Vacuum UV as Source of Water-Derived $^\bullet$OH and H_2O_2 Production

The formation of $^\bullet$OH and H_2O_2 in water was compared using full plasma treatments (3 slm Ar and Ar + 1% O_2) and treatments using windows filtering different radiation ranges (**Figure 5**). The dissociation of water yielding OH radicals was favored in full argon plasma treatments and when VUV radiation was admitted (MgF_2-window). For short treatments only small differences in $^\bullet$OH production was observed, but enlarging with treatment time. Full argon plasma produced around 30 μmol of $^\bullet$OH more than Ar plasma/MgF_2-window. It may be argued that either OH radicals from the gas phase contributed here or atomic oxygen generated from impurities. A recent report showed the sensitivity of terephathalic acid to this reactive short-lived species. Also liquid dynamic effects increasing the contact area between emitted radiation and target in contrast to the static treatments performed in the micro-chamber can contribute. Upon addition of molecular oxygen to the working gas, a decrease in $^\bullet$OH and H_2O_2 production was observed, especially when the MgF_2-window was used, blocking interphase chemistry. In the near-complete absence of VUV radiation from the argon excimers, water dissociation did not occur. Corroborating this observation, almost no OH/H_2O_2 formation was sparked by the longer UV ranges (UV-C and B and UV-A, respectively Suprasil-1 and Borofloat 33). Therefore, oxygen-base emission lines (atomic oxygen in NIR, ozone and O_2 in VUV), which increases in presence of molecular oxygen in the working gas, did not led to water dissociation. However, their potential direct impact on cysteine structures was investigated [55]. Overall, being highly energetic, VUV radiation emitted by Ar_2^* were able to induce water ionization, in contrast to the other UV ranges.

Therefore, the direct and indirect (e.g., water-derived species production) impact of VUV radiation resulted as predominant responsible of the effects induced on the liquid target. In the presence of oxygen in the gas phase, species derived from interphase chemistry and/or chemistry in liquid bulk are dominant, while VUV radiation is eliminated (**Figure 3A**).
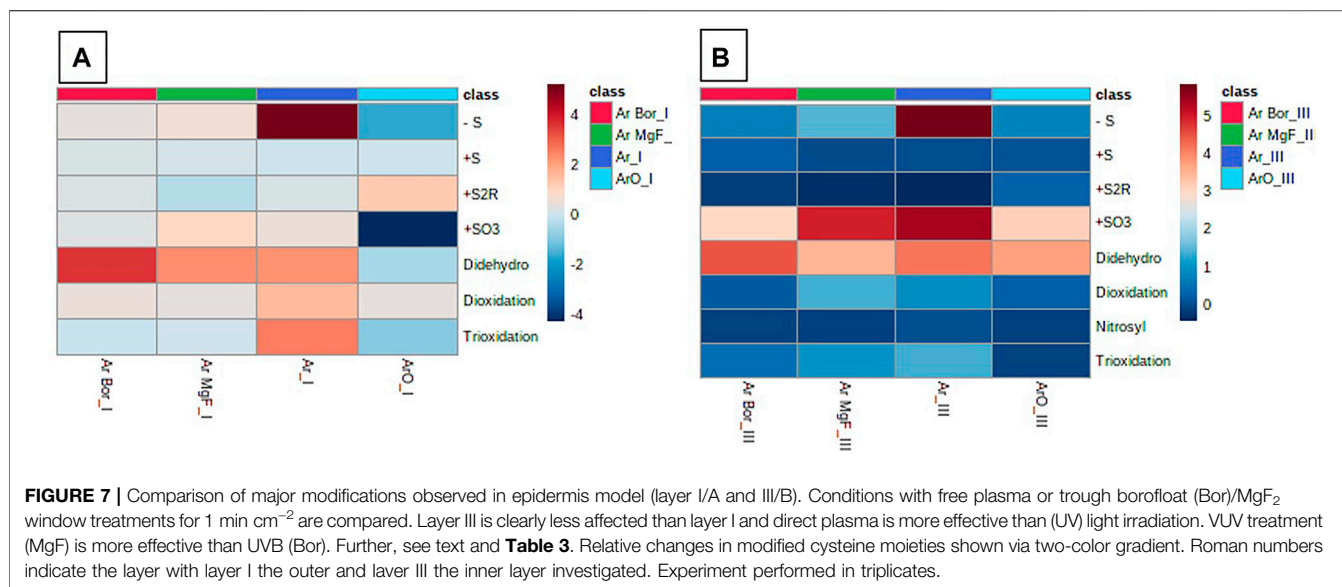
3.2 Direct and Liquid-Mediated Effects of Radiation on Cysteine Solutions

The impact of full plasma treatments and plasma-generated UV/VIS/NIR light on cysteine molecules is shown in **Figure 6**. **Table 2** shows the structures, the quantification methods, and the major plasma components involved in the generation of each cysteine derivative. As discussed, argon plasma conditions stimulated predominantly pathways in liquid via impact of VUV radiation emitted by argon excimers, leading to $^\bullet$OH and H_2O_2 production. The same experiments were performed with cysteine containing solutions. The formation of products that derive from the loss of the thiol group in cysteine (alanine, cysteine-*S*-sulfonate, sulfite ions, sulfate ions, and hydrogen sulfide) was almost identical in treatments with full argon plasmas and filtered VUV radiation of argon plasmas (MgF_2). In contrast, these products almost disappeared when VUV-impermeable oxygen was introduced to the working gas and gas–liquid phase chemistry was blocked by an MgF_2-window. This confirmed the key role of VUV radiation, leading to a cleavage of the carbon-sulfur bond [63], that was not observed by using other windows/radiation ranges. The energy of this bond is 272 kJ mol^{-1}, weaker than other bonds in cysteine or water (H-O 465 kJ mol^{-1}, C-C 347 kJ mol^{-1}, S-H 347 kJ mol^{-1}). The energy of the impinging UV photons of the argon excimer lines is much higher, 949 kJ mol^{-1} (126 nm), allowing the cleavage of all bonds present in the target, including the oxygen-hydrogen bond in water molecules. Accordingly, it might be argued that OH radical formation is the first step ultimately yielding in thiol moiety abstraction. However, the presence of hydrogen sulfide (H_2S) clearly indicates that a direct cleavage of the C-S bond contributes significantly or is even dominant considering the weaker bond energy compared to the H-O bond. Interestingly, no indications of a C-C bond breakage in cysteine, leading e.g. to the formation formic acid, was observed. Summarizing, conditions with pure argon showed a significant contribution of the VUV radiation, stimulating i) the production of OH radicals, and ii) C-S bond breakages. Under VUV radiation, derivatives such as cystine and cysteine acids were also produced in consistent amounts in relation to full argon plasmas. The origin of the oxygen incorporated in structures such as sulfite, sulfate, cysteine acids and *S*-sulfonate, in this case, are water-derived species [55].

As previously discussed, due to the controlled pH (7.2), only low amounts of thiolate were available to react with H_2O_2 since the pKa of the cysteine thiol group is 8.18—allowing less than 5% deprotonation. Therefore, a minimal role in thiol oxidation could be attributed to hydrogen peroxide. Furthermore, a two-step reaction would be needed to form cystine: a first reaction of H_2O_2 with thiolate, with formation of cysteine sulfenic acid, and a second reaction of RSOH with another thiolate [63]; [64]. More

TABLE 3 | Cysteine focused protein modifications observed in porcine epidermis model (ANOVA and Post-hoc analysis Fisher's LSD, p-value and FDR ≤0.05).

	$p < 0.05$ (n)	Type of modification
Overall	5	sulphur loss (-S), Trioxidation, Sulfonylation (+SO3), Cysteine addition (+S2R), Didehydrogenation (didehydro)
Layer I	5	Sulphur loss (-S), Trioxidation, Sulfonylation (+SO3), Cysteine addition (+2SR), Didehydrogenation (didehydro)
Layer II	3	Cysteine addition (+S2R), Nitrosylation (nitrosyl), Sulphur loss (-S)
Layer III	3	Sulphur loss (-S), Cysteine addition (+S2R), Trioxidation
Ar/O2 plasma	5	Nitrosylation (nitrosyl), Sulphur loss (-S), Sulfonylation (+SO3), Trioxidation, Didehydrogenation (didehydro)
Ar plasma	4	Cysteine addition (+S2R), Sulfonylation (+SO3), Didehydrogenation (didehydro), Sulphur loss (-S)
Ar MgF2 window	1	Nitrosylation (nitrosyl)
Ar Borofloat window	1	Cysteine addition (+S2R)

FIGURE 7 | Comparison of major modifications observed in epidermis model (layer I/A and III/B). Conditions with free plasma or trough borofloat (Bor)/MgF$_2$ window treatments for 1 min cm^{-2} are compared. Layer III is clearly less affected than layer I and direct plasma is more effective than (UV) light irradiation. VUV treatment (MgF) is more effective than UVB (Bor). Further, see text and **Table 3**. Relative changes in modified cysteine moieties shown via two-color gradient. Roman numbers indicate the layer with layer I the outer and layer III the inner layer investigated. Experiment performed in triplicates.

likely, the reaction of $^\bullet$OH with cysteine generates cystine by first formation of thiyl radicals (RS$^\bullet$), which rapidly recombine to form cystine. The formation of cysteinyl radicals was detected using BMPO/EPR spectroscopy earlier [54].

The reaction of RS$^\bullet$ with $^\bullet$OH would lead to cysteine sulfenic acid, which immediately is oxidized into sulfinic and further to sulfonic acid by $^\bullet$OH or H$_2$O$_2$ [65–67]. The production of S-sulfonate (RSSO$_3$H), in absence of atomic oxygen, could be promoted by first cystine oxidation by two $^\bullet$OH, with following C-S breakage promoted by VUV and final incorporation of another oxygen. Sulfite can be generated by cut of the C-S bond in cysteine sulfonic acids, or most likely, cysteine S-sulfonate (S-S bond dissociation energy 414.6 kJ mol^{-1}) [68]. Supplementary experiments have been performed by treating cysteine sulfonic acid in the radiation chamber, as shown in the supplementary material (**Supplementary Figure S1**). Despite the high concentration of the reference compound cysteine sulfonic acid, only small amounts of sulfite and sulfate ions are formed, indicating that the majority of C-S bond cleavages takes place at the cysteine or cystine level. With that, most sulfite and sulfate ions were generated by the oxidation of H$_2$S. This pathway is favored in the presence of reactive oxygen species produced by the cleavage water molecules (e.g. H$_2$O$_2$, $^\bullet$OH). Clearly, a number

of chemical pathways were active in liquids under the influence of vacuum UV radiation, in contrast to radiation >195 nm (UV-C).

The cysteine products generated by UV-C (Suprasil) or UVB/UVA (Borofloat) were clearly different. In these cases, almost no production of the water-derived species OH/H$_2$O$_2$ (**Figure 5**) and cysteine derivatives generated by C-S bond cleavage (SO$_4^{2-}$, SO$_3^{2-}$, alanine/RH; **Figure 6**) were observed.

The formation of cystine via hydrogen abstraction in cysteine (type I photo-oxidation mechanisms) and RS$^\bullet$ recombination [50,51]; was observed independent from the window, but with lower extent when vacuum UV is blocked (Borofloat/Suprasil). The production of sulfonic acid (RSO$_3$H, **Figure 6**) was measured in conditions with oxygen in the working gas, suggesting the potential role of radiation emitted in the near infrared and depending by the presence of O$_2$. The origin of oxygen incorporated by sulfonic acid, in this case, could be due to the reaction of thiyl radical with water, generating $^\bullet$OH by hydrogen abstraction. Even though the radiation emitted in these ranges are not ionizing, due to the lower energy, it was shown that also the UV radiation >200 nm can induce oxidative stress in cellular compartments in relation to the exposure time, with increase in the cellular production of reactive species [49,50].
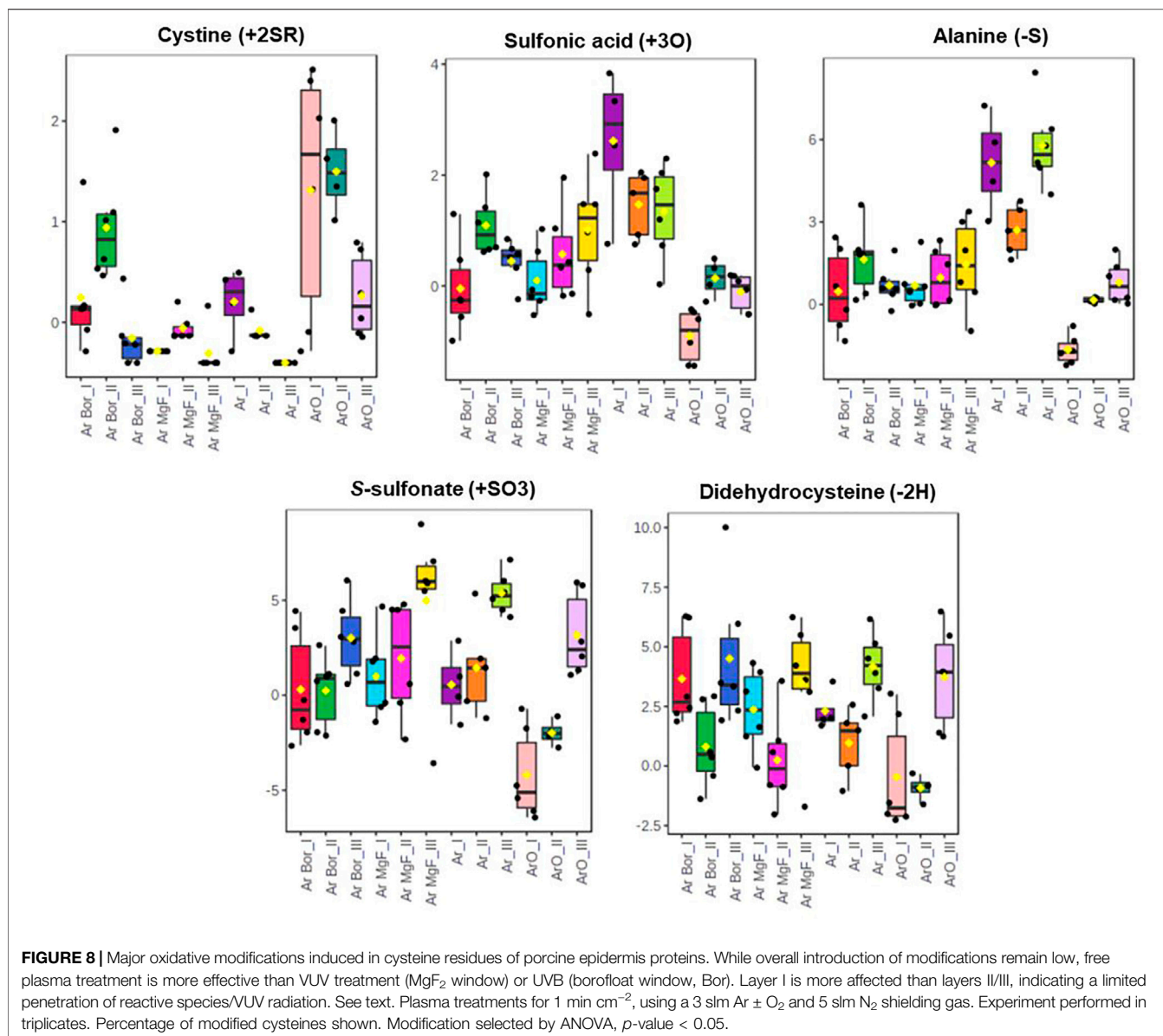
FIGURE 8 | Major oxidative modifications induced in cysteine residues of porcine epidermis proteins. While overall introduction of modifications remain low, free plasma treatment is more effective than VUV treatment (MgF_2 window) or UVB (borofloat window, Bor). Layer I is more affected than layers II/III, indicating a limited penetration of reactive species/VUV radiation. See text. Plasma treatments for 1 min cm^{-2}, using a 3 slm Ar $\pm$ O_2 and 5 slm N_2 shielding gas. Experiment performed in triplicates. Percentage of modified cysteines shown. Modification selected by ANOVA, p-value < 0.05.

3.3 Plasma Radiation Impact on Epidermal Protein Structures

The analysis of the oxidative modifications of cysteine belonging to the porcine epidermal proteome was performed via tape stripping assay in combination with high-resolution mass spectrometry. The oxidation was monitored in the first three stratum corneum layers of the porcine epidermis, reflecting a penetration depth of 5–7 μm. **Table 3** and **Figure 7** give an overview of the detected modifications, while **Figure 8** shows a quantitative comparison of the most relevant oxidative modifications. Unstable oxidative modifications, such as S-nitrosylation, may have been underestimated. According to proteomics standard procedures, thiols were reduced and alkylated during sample workup. Some losses to the plasma chemistry on protein thiols (e.g., sulfenic acids,

S-nitrosylation) cannot be excluded although stable modifications (e.g., cysteine sulfonic acid, cysteine-S-sulfonate) were retained. Overall, five types of modifications were found to be introduced into the epidermal proteins with a statistical significance. These are the apparent replacement of cysteine by alanine (sulphur loss), the formation of cysteine sulfonic acid (trioxidation), the formation of cysteine-S-sulfonate (sulfonylation), the formation of a disulfide with other cysteine moieties (+S2R), and the loss of two hydrogen atoms forming an unsaturated cysteine molecule (didehydrogenation) (**Table 3**). Alongside depth in the epidermis, the intensity and number of detected modifications decrease (**Figure 7**). The impact of plasma-derived UV or VUV light was lower than expected from the *in vitro* experiments (**Table 3**). Only nitrosylation was found to be significantly elevated by MgF_2 filtered kINPen irradiation, pointing at a limited role of (V)UV photons *in vivo*.

The conversion of cysteine in cysteine S-sulfonate (+SO_3) and dehydrogenated cysteine (-2H) (**Figure 8**), was promoted especially in the third skin epidermis layer, regardless of the applied treatment condition. The results indicate that protein modifications result from complex dynamics and are not corresponding to the action of only one plasma components produced in specific conditions.

Some modifications appear in special conditions only. For example, the conversion of cysteine into cystine was observed in treatment with Ar/O_2 full plasmas, with a maximum in the first layer and a progressive decrease in the second and third. In this condition, the effluent contains significant amounts of singlet oxygen and atomic oxygen, especially at short distances from the nozzle. Atomic oxygen is able to form thyil radicals by hydrogen abstraction to the protein cysteines, which rapidly may recombine forming a disulfide bond. This event causes conformational changes of the protein, alongside a potential gain or loss of function. The oxidation of cysteine into sulfonic acid by the incorporation of three oxygen atoms, and the breakage of the C-S bond with conversion of cysteine to alanine were events observed predominantly in treatments with full argon plasma in the first layer of the skin. When using windows, even in case of the VUV-transmitting MgF_2 and more prominent in suprasil and Borofloat-33 windows, only few such modifications were detected. This indicates that the VUV radiation, although most prominent in Ar plasma, does in soft targets not contribute in the same manner to biomolecule modification than in liquid targets. In this case, the direct impact of argon metastables and other gas phase species and the subsequent formation of secondary reactive species is more prominent. This is in line with a report investigating the oxidation of human skin lipids that showed a limited impact of the argon plasma on lipid side chain oxidation in the absence of water [33]. Obviously, the highly energetic VUV radiation is unable to penetrate deeper into layers of biomolecules such as the model described here or the sebum lipids, limiting its ability to contribute significantly to the plasma chemistry *in situ*.

4 CONCLUSION

In aqueous targets (**Sections 3.2, 3.3**), plasma-derived VUV radiation is an effective component in plasma-liquid chemistry. The liquid phase acts as compartment amplifying the plasma chemistry by the *de novo* formation of secondary water-derived reactive species (e.g., hydroxyl radicals, hydrogen peroxide) at the gas-liquid interphase, that subsequently allow the modification of sensitive targets such as thiol moieties. In contrast, in complex targets like the intact skin the plasma-derived VUV radiation is blocked effectively by the dense biomolecules layers and plays a limited role only. While this might be disappointing from the scientific

viewpoint it emphasizes the safety of physical plasmas which has been a significant concern for years. Corroborating a number of reports proofing the safety [69]; [70]; [71], our results further support the safe application of physical plasmas. Even in humid wounds where resident water molecules allow the formation of secondary species by the UV radiation increasing the effectiveness of plasma while the protein layer in the wound bed protects the local tissue.

Proteomics Data
The proteomics data connected to this paper have been uploaded to the ProteomeXchange servers under the project name "Gas plasma and (V)UV impact on porcine epidermis using a tape strip assay approach" (Project accession: PXD028915, username: reviewer_pxd028915@ebi.ac.uk/Password: OPKS8hii).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ProteomeXchange with accession PXD028915.

AUTHOR CONTRIBUTIONS

GB, KW, and TvW devised the experiments, wrote an corrected the manuscript HM and TG performed OES measurements and discussed the data GB, SW, and KW performed mass spectrometry analysis and discussed the data/incorporated it into the manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphy.2021.759005/full#supplementary-material

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7. Eigenständigkeitserklärung

Hiermit erkläre ich, Sebastian Wenske, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

_____   _____

Date   Sebastian Wenske

8. Curriculum Vitae

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10.2012 - 09.2015   Bachelor Studium Biochemie an der Universität Greifswald
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10.2010 - 09.2012   Bachelor Studium Physik an der Universität Rostock

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_____   _____

Date   Sebastian Wenske

9. List of Publications & Conference Contributions

Publications in Peer-Reviewed Journals

- G. Bruno, <u>S. Wenske</u>, J.-L. Lackmann, M. Lalk, T. von Woedtke, K. Wende. "On the liquid chemistry of the reactive nitrogen species peroxynitrite and nitrogen dioxide generated by physical plasmas". *Biomolecules*, 10 (12), 1687, 2020.

- <u>S. Wenske</u>, J.-W. Lackmann, S. Bekeschus, K.-D. Weltmann, T. von Woedtke and K. Wende. "Nonenzymatic post-translational modifications in peptides by cold plasma-derived reactive oxygen and nitrogen species". *Biointerphases*, 15 (6), 061008, 2020.

- M.-C. Sommer, M. Balazinski, R. Rataj, <u>S. Wenske</u>, J. F. Kolb and K. Zocher. "Assessment of Phycocyanin Extraction from *Cyanidium caldarium* by Spark Discharges, Compared to Freeze-Thaw Cycles, Sonication, and Pulsed Electric Fields". *Microorganisms,* 9, 1452, 2021

- <u>S. Wenske</u>, J.-W. Lackmann, L.-M. Busch, S. Bekeschus, T. von Woedtke and K. Wende. "Reactive species driven oxidative modifications of peptides—Tracing physical plasma liquid chemistry". *J. Appl. Phys.*, 129 (19), 193305, 2021.

- Z. Nasri, S. Memari, <u>S. Wenske</u>, R. Clemen, U. Martens, M. Delcea, S. Bekeschus, K.-D. Weltmann, T. von Woedtke and K. Wende. "Singlet Oxygen-Induced Phospholipase A2 Inhibition: a Major Role for Interfacial Tryptophan Dioxidation ". *Chem. Eur. J.*, 27 (59), 14702, 2021.

- J. Striesow, J.-W. Lackmann, Z. N. Ni, <u>S. Wenske</u>, K.-D. Weltmann, M. Fedorova, T. von Woedtke and K. Wende. "Oxidative modification of skin lipids by cold atmospheric plasma (CAP): A standardizable approach using RP-LC/MS^2 and DI-ESI/MS^2". *Chem. Phys. Lipids*, 223, 104786, 2019.

- G. Bruno, <u>S. Wenske</u>, H. Mahdikia, T. Gerling, T. von Woedtke, and K. Wende. "Radiation driven chemistry in biomolecules – is (V)UV involved in the bioactivity of argon jet plasmas?". *Frontiers in Physics,* 9, 759005, 2021.

Contributions to Workshops and Conferences

Oral presentations

- <u>S. Wenske</u>, J.-L. Lackmann, T. von Woedtke, K.-D. Weltmann, K. Wende. "Oxidative PTMs in peptides and proteins – Identification strategies and physiological implications". *Baltic Redox Workshop*. Virtual Conference. September, 2020.

- <u>S. Wenske</u>, J.-L. Lackmann, T. von Woedtke, K. Wende. "Creation of a cold atmospheric plasma (CAP) generated post-translational modification (PTM) library of amino acids and investigation in artificial skin model system". *Plasma-Biomed-Workshop*. Neumünster, Germany. October, 2019.

- <u>S. Wenske</u>, J.-L. Lackmann, T. von Woedtke, K. Wende. "The investigation of posttranslational protein modifications (PTMs) in artificial skin after cold atmospheric plasma treatment". *8th Young Professionals Workshop on Plasma Medicine - Frontiers in Redox Biochemistry and Medicine (FiRBaM)*. Rostock, Germany. August, 2019.

- <u>S. Wenske</u>, J.-L. Lackmann, T. von Woedtke, K.-D. Weltmann, M. Lalk, K. Wende. "Mass spectrometry-based investigations of cold physical plasma-induced PTMs in peptides". *7th Young Professionals Workshop on Plasma Medicine - Frontiers in Redox Biochemistry and Medicine (FiRBaM)*. Rostock, Germany. September, 2018.

Poster presentations

- <u>S. Wenske</u>, J.-L. Lackmann, G. Bruno, S. Bekeschus, T. von Woedtke, K.-D. Weltmann, K. Wende. "MS-based investigations of cold physical plasma-induced PTMs in peptides". *22nd International Mass Spectrometry Conference (IMSC)*. Florence, Italy. August, 2018.

- <u>S. Wenske</u>, J.-L. Lackmann, T. von Woedtke, K.-D. Weltmann, K. Wende. "MS-based investigations of cold physical plasma-induced PTMs". *52nd Tagung der deutschen Gesellschaft für Massenspektrometrie (DGMS)*. Rostock, Deutschland. März, 2019.

- <u>S. Wenske</u>, J.-L. Lackmann, T. von Woedtke, K.-D. Weltmann, K. Wende. "MS-based investigations of cold physical plasma-induced PTMs". *Advances in the Study of Lipid and Protein Oxidation: From Methods to Targets*. Gent, Belgien. März, 2019.

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- <u>S. Wenske</u>, J.-L. Lackmann, T. von Woedtke, K.-D. Weltmann, K. Wende. "Oxidative PTMs in peptides – Understanding the physiological consequences of physical plasma". *19. Human Proteome Organization World Congress (HuPo)*. Stockholm, Schweden. Oktober, 2020 (online).

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