Mass spectrometry-based investigations of cold atmospheric plasma-induced PTMs in model peptides

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Abstract: Post-translational modifications (PTMs) of peptides or proteins are covalent changes and important for many signaling pathways capable of inducing potentially drastic changes in structure and activity of participating molecules by distinct chemical groups [1]. The investigation of these modifications is still a difficult task due to the high complexity and in part low abundances of the modifications. Using high-resolution mass spectrometry, this study wants to investigate the modifications for model peptides triggered by CAP.

Keywords: Post-translational modification, mass spectrometry, peptides.

1. Introduction

To date, more than 200 different biologically relevant types of PTMs have been identified [2]. The database Unimod (http://www.unimod.org/) contains protein modifications for mass spectrometry applications [3] and has currently over 1400 entries of protein modifications. These numbers show that the study of the PTMs is extremely complex and that special systems are necessary for a detailed study. A large part of these modifications can be caused by reactive oxygen or nitrogen species (RONS) such as H_2O_2, $^1O_2$ or ONOO- , which can be found in cold atmospheric plasma. By using plasma sources in clinical practice, chronic wounds can be successfully treated today [4] and even in the field of cancer research, the first promising studies are already available [5].

Many of these modifications have a major influence on signaling pathways or enzyme activity in the human body. An example is the 3-nitrotyrosine: the nitration of tyrosine can lead to an enzyme activity loss in e.g. cyclooxygenase (COX) which is a regulator of inflammatory processes [6]. 3-nitrotyrosine can thus be used as a biomarker for nitrosative/nitrative stress. The investigation, as well as the exact characterization and quantification of the PTMs represents a new important field in plasma research. The questions that need to be answered here are among others: what types of PTMs are generated by a plasma and which amino acids are how modified after plasma treatment? Finally, it is the goal to control the production of oxidative PTMs in signaling pathways in order to achieve activation or inactivation of specific reactions in the cell.

In recent years, mass spectrometry has distinguished itself as the ideal analytical method for these questions due to its increasingly high-resolution and high-speed instruments. With the combination of these powerful measurement devices and, on the bioinformatic side, software capable of identifying many modifications simultaneously (Byonic Protein Metrics), it is possible to answer these questions.

Three model peptides (Angiotensin 1-7, Bradykinin and a synthetic peptide LYTFAHD) were selected for investigation in order to be able to make as accurate statements as possible about the existing modifications (see Figure 1).

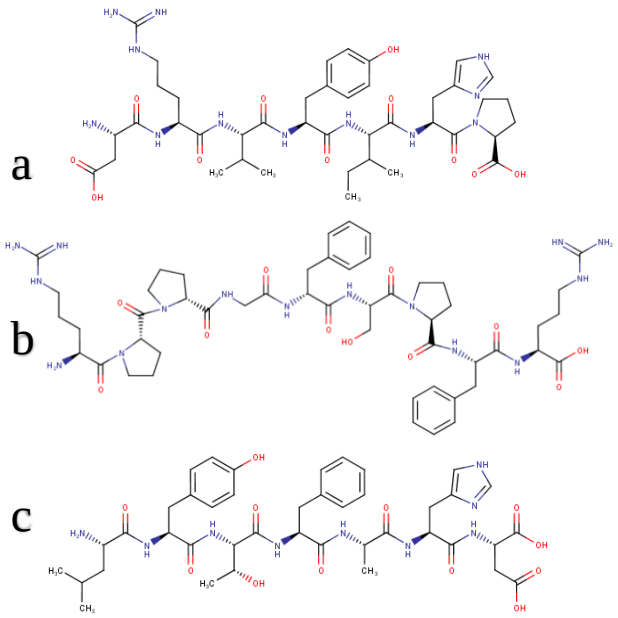

Fig. 1: Peptide sequence of the three model peptides from top to bottom: a) Angiotensin 1-7, b) Bradykinin and c) synthetic peptide LYTFAHD.

Based on the modifications found for these and later other model peptides, a PTM library will be created to predict which modifications can be expected after plasma treatment.

2. Methods

For the plasma treatment of the peptides, in this study two different plasma sources were used: the kINPen [7] developed by the INP Greifswald and the COST reference Jet [8] developed by the chair for experimental physics II from the University of Bochum. The treatment parameters were as follows: 500 µl peptide solution with a concentration of 0.2 mg/ml were treated up to 5 min with 3 slm argon + 0.5% O_2, 9 mm distance (kINPen) and 1 slm helium + 0.5% O_2, 4 mm distance COST-jet in a 24-well plate from Sarstedt. The plasma-modified peptides were analysed by nano liquid chromatography (nLC) and high resolution mass spectrometry measurements (HRMS) (Dionex UltiMate 3000 RSLCnano and Q Exactive™) from ThermoFisher by direct infusion (DI) electrospray ionization (ESI) or by LC-ESI coupling using a PepMap column (C18 material, particle size 2µm, 75 µm width x 150 mm length). The software Byonic from Protein Metrics was used to evaluate the data and identify the PTMs.

3. Results

In the first step, the treated samples were injected directly into the MS device without any separation. As an example, this is shown for the COST-jet treatment and Angiotensin 1-7 in Figure 2.

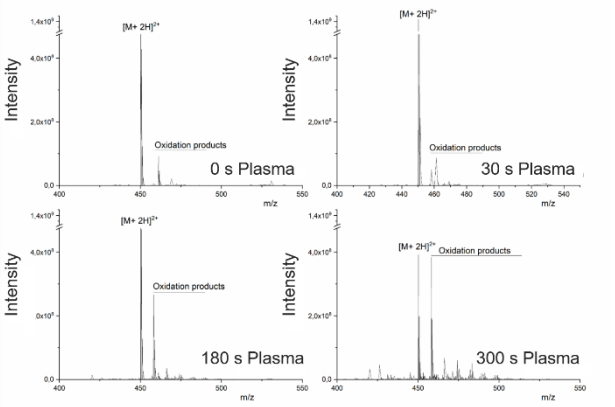

Fig. 2: DI/MS-data of Angiotensin 1-7 treated with the COST-jet for 0-300 s. The intensity is plotted against the mass to charge ratio of observed species.

The oxidation products and other modifications increase with longer treatment times. After this direct injection, an LC method was developed to separate the modified peptides from each other. In order to detect the modifications, the Byonic software had to be used to search for the various modifications such as oxidation or deamidation (a complete list of all the modifications searched for can be found in Table 1).

Table 1. List of searched modifications in Byonic with monoisotopic mass shift and chemical composition.

Name	Monoisotopic mass	Composition
Oxidation	+ 15.994915	+ O
Dioxidation	+ 31.989829	+ O(2)
Trioxidation	+ 47.984744	+ O(3)
Nitrosylation	+ 28.990164	+N +O –H
Nitration	+ 44.985078	+N +O(2) –H
Amidation	– 0.984016	+N +H –O
Deamidation	+ 0.984016	–N –H +O
Carbonylation	+ 13.979265	+O –H(2)
Quinonylation	+ 29.974179	+O(2) –H(2)
Quinonylation + Oxidation	+ 45.969094	+O(3) –H(2)
Didehydration	– 2.01565	–H(2)
Trioxidation - HCNO	+ 4.97893	+O(2) –N –C –H

After the successful search of the different modifications, it was interesting to investigate differences in the modification patterns of kINPen and COST-jet after different treatment times. To this end, we have quantified and compared the different modified peptide fragments for the Bradykinin after 1 and 5 minutes (see Figure 3a and b).

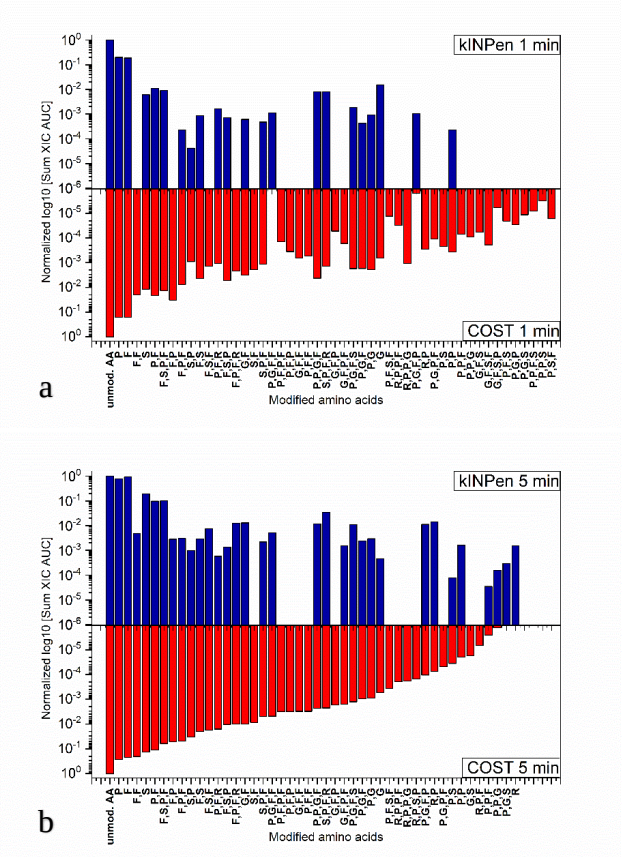

Fig. 3: Modification pattern for Bradykinin after 1 (a) and 5 (b) minutes plasma treatment with the kINPen (blue bars) and COST-jet (red bars). Shown are the normalized intensities for the different modified peptide fragments identified by Byonic.

In addition, the data were further investigated in order to be able to make statements about the modifications on each individual amino acid. As a criterion for the correctness of a modification on an amino acid, all results with a Byonic score lower than 300 and a Delta Mod score lower than 20 were filtered out. These score values have proved to be the best for evaluating the data. Table 2 shows the found modifications for a 30 s kINPen treatment for the synthetic peptide.

Table 2. List of found modifications after 30 s of plasma treating the synthetic peptide LYTFAHD with kINPen.

Position in peptide	Modification
L 1	Didehydration
Y 2	Quinonylation
Y 2	Oxidation
Y 2	Dioxidation
Y 2	Didehydration
T 3	Oxidation
T 3	Didehydration
F 4	Oxidation

In further experiments, it was also investigated whether the surrounding matrix of the peptides in solution has an influence on the found modifications. In addition to glucose, phosphate buffered saline (PBS) was also added to the solution and treated. It has been shown that these additives have very little or no effect. However, additional chlorination was found in the PBS samples.

4. Discussion and Outlook

The investigation of the model peptides has shown that there are differences in the PTM pattern between the two plasma sources. The results indicate that shorter treatment times lead to a more diverse modification pattern, whereas this seems to become smaller with longer treatment times. A possible theory for this observation could be that too long treatment times of the peptides leads to a decomposing of the peptide structure and thus fewer peptide fragments can be detected in general. It is also noticeable that the modification pattern for the COST-jet seems to be slightly more diverse than for the kINPen. However, it has not yet been possible to determine exactly what caused this observation. One assumption here is that a higher diversity is produced in the eluent of the COST-jet and that these show a broader reaction spectrum with the peptides [9].

One problem that still has to be solved in the future are the unspecific modifications that are also detected in untreated peptide samples (see Figure 2 top left: 0 s plasma treatment). By purging the solvent with argon before dissolving the peptides or adding small amounts of ammonium acetate and acetic acid, these modifications can be reduced. The exact knowledge of the modification state of the peptides without plasma treatment is therefore absolutely necessary in the future. It is not only decisive which types of modifications are present, but also in which quantity, since otherwise no reliable statements about low abundant modifications can be made in later experiments.

For the purpose of quantification, derivatives of the peptides labelled with heavy isotopes were synthesized and will be used in future in quantification experiments. The exact number and amount of reactive species in the two plasma sources kINPen and COST-jet is still not fully known and the list of ROS, RNS and associated chemical reaction in solution found, is regularly updated [7,10].

Another important step in the near future will be cell experiments with cellpermeable peptides (CPPs). This special class of peptides is able to penetrate the cell membrane through various mechanisms [11] and after a short time these peptides can be easily detected by e.g. fluorophore labeling [12]. By comparing the found PTMs of plasma-treated peptides outside and inside the cell, it is hoped to gain a deeper understanding of plasma interactions in the cell.

5. Acknowledgements

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6. References

[1] Karve T. M., Cheema A. K., Small changes huge impact: the role of protein posttranslational modifications in cellular homeostasis and disease, *J. Amino Acids*. 2011, 1-13

[2] Walsh C. T., Garneau-Tsodikova S., Gatto G. J., Jr. Protein posttranslational modifications: the chemistry of proteome diversifications, *Angewandte Chemie*. 2005, 44:7342-7372.

[3] Creasy D. M., Cottrell J. S., Unimod: protein modifications for mass spectrometry, *Proteomics.* 2004, 4:1534-6.

[4] Bekeschus S., Schmidt A., Weltmann K.-D., von Woedtke Th., The Plasma Jet kINPen – A Powerful Tool for Wound Healing, *Clinical Plasma Medicine*. 2016, 4:19-28

[5] Metelmann H.-R. *et al*, Clinical experience with cold plasma in the treatment of locally advanced head and neck cancer, *Clinical Plasma Medicine*. 2018, 9:6-13

[6] Dimitrios T., Analytical methods for 3-nitrotyrosine quantification in biological samples: the unique role of tandem mass spectrometry, *Amino Acids.* 2012, 42:45-63

[7] Reuter S., von Woedtke Th., Weltmann K.-D., The kINPen—a review on physics and chemistry of the atmospheric pressure plasma jet and its applications, *J. Phys. D: Appl. Phys.* 2018, 51:233001

[8] Golda J. *et al*, Concepts and characteristics of the 'COST Reference Microplasma Jet', *J. Phys. D: Appl. Phys.* 2016, 49:084003

[9] Waskoenig J., Niemi K., Knake N., Graham L. M., Reuter S., Schulz-von der Gathen V., Gans T., Atomic

oxygen formation in a radio-frequency driven micro-atmospheric pressure plasma jet, *Plasma Sources Science & Technology*. 2010, 19:045018 (11pp)

[10] Verlackt C., Van Boxem W., Bogaerts A., Transport and accumulation of plasma generated species in aqueous solution, *Physical Chemistry Chemical Physics*. 2018, 20:6845-6859

[11] Kauffmanm W. B., Fuselier T., He J., Wimley W. C., Mechanism matters: a taxonomy of cell penetrating peptides, *Trends Biochem Sci*. 2015, 40(12):749-764

[12] Birch D., Christensen M. V., Staerk D., Franzyk H., Nielsen H. M., Fluorophore labeling of a cell-penetrating peptide induces differential effects on its cellular distribution and affects cell viability, *BBA – Biomembranes.* 2017, 1859:2483-2494