Application Note

Matrix-Assisted Ionization and Tandem Mass Spectrometry Capabilities in Protein Biomarker Characterization—An Initial Study Using the Small Cell Lung Cancer Biomarker Progastrin Releasing Peptide as a Model Compound

Trine Grønhaug Halvorsen,* Maren Christin Stillesby Levernæs, and Cecilie Rosting

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: This initial study evaluates vacuum matrix-assisted ionization (vMAI) mass spectrometry (MS) for identification and determination of tryptic peptides from the biomarker protein progastrin releasing peptide (ProGRP). Similar peptides and charge states were observed as in liquid chromatography (LC) electrospray ionization (ESI) MS. The prolonged ion duration in vMAI with similar charge states as in ESI was advantageous for determining the MS/MS fragmentation conditions compared to MAI. It is assumed that the vacuum ionization conditions lower the detection limits of the experiment. This may be the reason vMAI combined with high resolution MS enabled detection of tryptic peptides from more digested proteins than MAI selected reaction monitoring MS. Additionally, MAI ion mobility spectrometry MS (MAI-IMS−MS) was evaluated for differentiation of intact protein isoforms, successfully enabling differentiation of the isoforms by drift time selection. Examples are both shown for model proteins bovine serum albumin, cytochrome C, and lysozyme and the clinically relevant small cell lung cancer protein biomarker ProGRP, which exists in three isoforms. Coupling with the vacuum ionization conditions using a dedicated vacuum-probe source MAI enables information to be extracted readily as with conventional approaches, just faster.
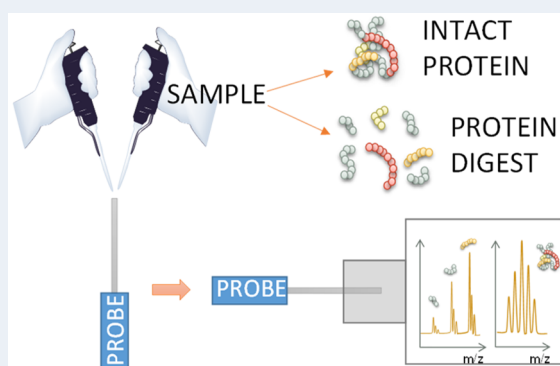
INTRODUCTION

In recent years, mass spectrometry (MS) has been gaining acceptance in clinical environments. It has become an important tool for screening of potential biomarkers and is experiencing increasing use in biomarker analysis for treatment follow-up.[1] Our group is interested in contributing to increased use of MS in clinical environments through innovative solutions for sample handling, treatment, and analysis of clinically relevant protein biomarkers.[2−4] The goal is to develop methods and strategies that reduce costs, increase user-friendliness, and improve throughput and robustness without compromising the accuracy of the analysis.

The simplicity of matrix-assisted ionization (MAI) MS requiring neither a high voltage source, heat, laser, nor ion beam makes it an attractive technique for development of low cost, user-friendly, and high throughput solutions for the clinical laboratory.[5] Hence, we have recently implemented MAI-MS in our lab to, cautiously, explore its use in targeted bottom-up analysis of proteins.[6] In this work, previously established liquid chromatography electrospray ionization selected reaction monitoring (LC-ESI-SRM)−MS transitions of tryptic signature peptides were applied in targeted MAI-SRM-MS analysis from biological matrices.[6]

MAI-MS for proteins is gaining in popularity;[7,8] however, only a few papers have shown applicability with MS/MS in previous work.[6,7,9] One reason may be that for MS/MS analyses, the speed of the MAI ionization event, typically a few seconds depending on the inlet temperature, is challenging. In the MAI-SRM-MS work performed previously, this was circumvented by applying relatively high analyte levels to generate sufficient MS/MS signal.[6]

vMAI is MAI performed from *subatmospheric pressure*.[10] In vMAI-MS, the analyte ion duration is prolonged; hence, a continuous stream of ions is achieved for up to several minutes,[11] similar to ESI but consuming significantly less sample volume. Because the ionization is from vacuum, ion transmission can be expected to be improved.[12] Lu et al. reported in 2016 on the first dedicated vMAI source and demonstrated excellent detection of low analyte levels,
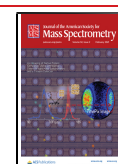
simplicity, and robustness.[11] These advantages may be an important step toward simpler method development for determination of proteins in complex matrices using vMAI-MS/MS, which in combination with high resolution MS analysis may result in better analysis specificity and detection limits. Ion mobility spectrometry (IMS) may assist in additional gas phase separation, pertinent for direct ionization methods where liquid chromatography (LC) is not applicable.[13]

The goal of the present work was to evaluate the applicability of vMAI in analysis of proteins and their tryptic peptides by MS and MS/MS with and without a preceding IMS step. The evaluation was performed using both standard model proteins and a clinical relevant biomarker, progastrin releasing peptide (ProGRP). ProGRP was selected both due to its clinical relevance and because a ProGRP-specific peptide, NLLGLIEAK, already has been shown to be detectable by MAI in a bottom-up proteomics approach.[6] The evaluation was performed during a short visit to Trimpin's lab at Wayne State University.

■ RESULTS AND DISCUSSION

Experimental details for all experiments can be found in the Supporting Information.

Due to limited availability of the ProGRP isoforms, initial method development was performed with well-known standard proteins (bovine serum albumin (BSA), lysozyme, and cytochrome C) and their tryptic digests. vMAI-MS analyses of the tryptic digests of BSA, lysozyme, and cytochrome C showed long ion abundances (4−5 min) but moderate sequence coverage (39−54%). For BSA, the coverage was substantially lower than when analyzing a BSA digest by LC−ESI-MS, probably due to the low ion abundances (see Supplementary Discussion). The peptides identified had similar charges as typically seen for ESI-MS. An overview of detected peptides can be found in Tables S1−S3. No contamination issues were observed between samples.

Successful MS/MS fragmentation was obtained for tryptic peptides of all three model proteins (Figure S3). This was despite low signal intensity for some of the fragment ions (especially seen for the BSA peptide, see Figure S3a). The optimum collision energy (CE) was obtained through 1−3 consecutive matrix/analyte sample introductions and a stepwise increase of the CE across the long, continuous ion duration, similar to ESI. While MAI-MS/MS has been successfully implemented on commercial sources and on various instruments,[6−8] the vacuum probe previously provided lower abundant fragment ions using MS/MS on the same SYNAPT G2.[11] The suspected reason for the difficult fragmentation of doubly charged peptide ions in the previous study was incomplete loss of matrix from the gas phase charged matrix/analyte particles. However, the MS/MS studies performed here demonstrate the possibilities of this new approach, despite the observed lower ion abundances, which likely were related to detector issues of an aging SYNAPT G2 mass spectrometer.

Intact lysozyme and Cyt C were also successfully analyzed by vMAI-MS. The resulting mass spectra are shown in Figure S4. For both proteins, the typical ESI-like protein charge state distributions were seen. The probe approach has previously been demonstrated in intact protein analysis of smaller proteins, with ubiquitin (8.6 kDa) being the largest.[11,14] The protein amounts used in the present work are approximately in
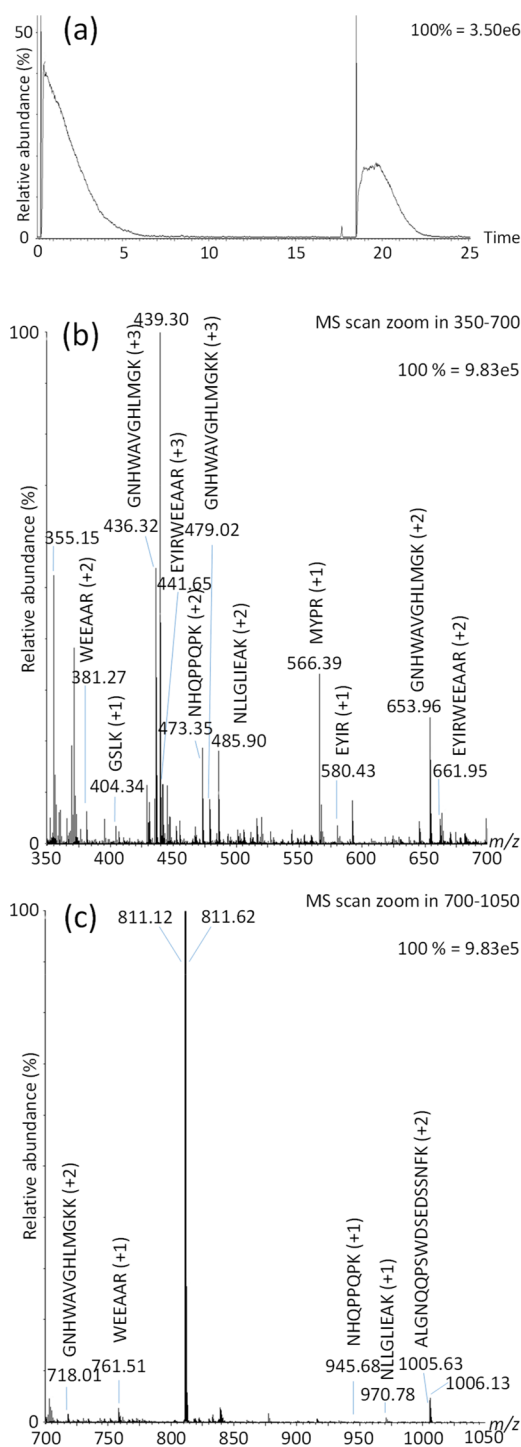

Figure 1. ProGRP isoform 2 in-solution digest analyzed with vMAI-MS. (a) Total ion chronogram and mass spectrum with (b) m/z 350−700 and (c) m/z 700−1050. Identified peaks are indicated. Full scan mass spectrum (no zoom-in) is included in Figure S5.

the same range as those in the study performed using the same mass spectrometer (25 vs 6 pmol, respectively) but significantly larger than with those on the vMAI probe approach on the Thermo Q Exactive Focus.[14] However, both lyzosyme (14.3 kDa) and Cyt C (12.4 kDa) are significantly larger molecules than ubiquitin.

More extensive results and discussions are included in the Supporting Information.
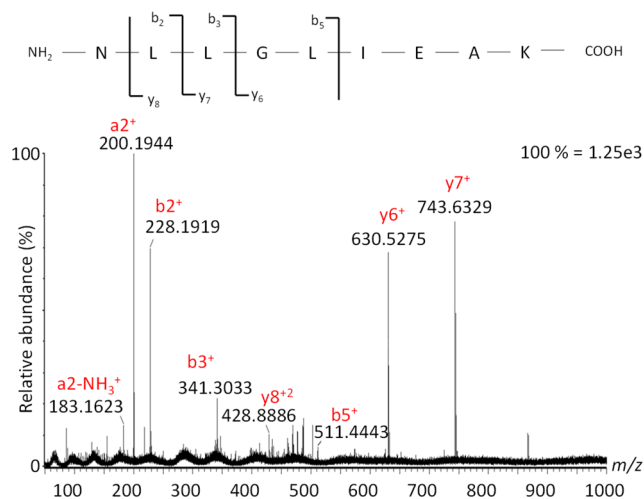
Figure 2. vMAI-MS/MS spectrum of NLLGLIEAK (+2; m/z 485.9) with detected fragment ions indicated and a collision energy of 25 eV. The baseline is associated with problems with the detector.

vMAI-MS of Digested ProGRP Isoforms. After initial method development/evaluation using the standard proteins, vMAI-MS analysis was applied to digests of the three ProGRP isoforms. In Figure 1, the total ion chronogram (TIC) and zoom-in mass spectrum of an in-solution digest of ProGRP isoform 2 are shown. The mass spectrum is dominated by two unknown peaks (m/z 338.42 and 811.12, see spectrum in Figure S5); however, zooming into the lower intensity peaks, several tryptic ProGRP peptides were identified (Figure 1b,c and Table S4). The mass spectra of isoforms 1 and 3 were similar to the one obtained for isoform 2 (Figures S6 and S7). The signal intensity of the isoform 3 digest was lower than those for isoforms 1 and 2; this might be because the digestion had to be downscaled 5 times due to very limited amounts of the isoform available.

Based on two separate sample introductions, each of which lasted around 3.5 min, i.e., until the matrix is sublimed, the protein coverage was calculated to 57, 56, and 36% for isoforms 1, 2 and 3, respectively (allowing for up to one missed cleavage). An overview of the peptides identified after vMAI-MS for all three isoforms can be found in Table S4. The calculated protein coverage for isoforms 1 and 2 is within the range typically seen from in-solution digests of ProGRP analyzed by nanoLC−ESI-MS/MS (54−90% coverage, Table S5).

vMAI-MS/MS of Tryptic ProGRP Peptides. The observed optimum CE (25 eV) for the tryptic bovine serum albumin peptide was used as a starting point for fragmentation optimization of ProGRP peptides. MS/MS was evaluated for two peptides: NLLGLIEAK, commonly used as a signature peptide for ProGRP in bottom-up quantification of total ProGRP by LC−ESI-MS/MS[15] and previously shown to be detectable in MAI-SRM-MS,[6] and GNHWAVGHLMGK. For both peptides, the doubly charged ions were fragmented. The mass spectrum of NLLGLIEAK using a CE of 25 eV is shown in Figure 2. As indicated in the figure, several peptide fragments were detected, and no further optimization of the CE was performed. The other peptide, GNHWAVGHLMGK, did not fragment as easily. The CE was therefore increased stepwise from 25 eV across the ion duration, applying seven different collision energies across three sample introductions. The TICs and MS/MS spectra of GNHWAVGHLMGK (+2)
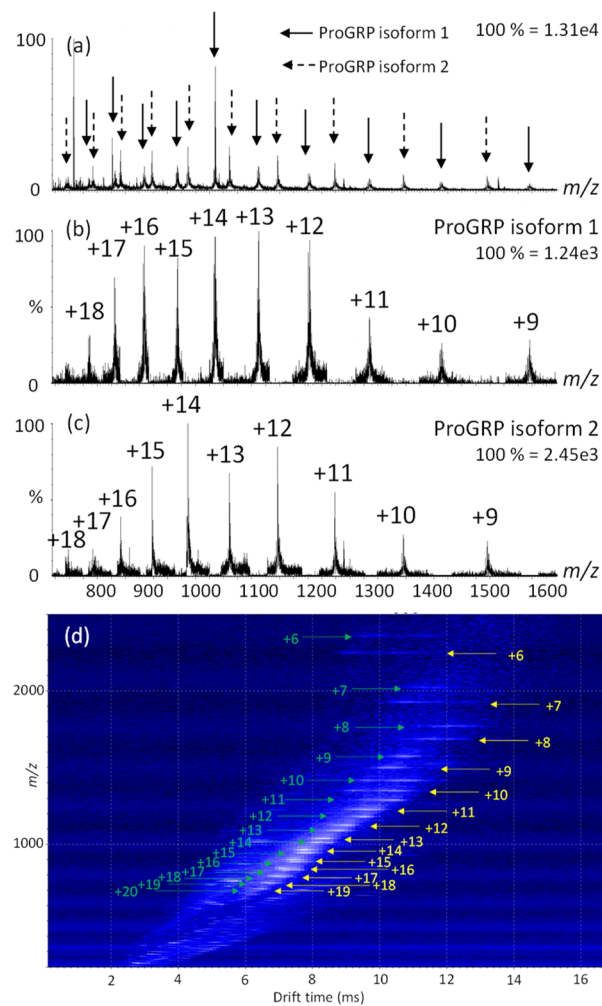

Figure 3. MAI-IMS−MS of ProGRP isoforms 1 and 2 after combined introduction by atmospheric pressure MAI. (a) Total mass spectrum and extracted mass spectra of (b) isoform 1 and (c) isoform 2. Charge states are indicated in the figure. Applied smoothing: SG, 4 × 3.00. For (a−c), % on y axis refers to relative abundance. (d) Two-dimensional plot of drift time versus m/z. Charge states of ProGRP isoform 1 are indicated with green numbers (left), and charge states of ProGRP isoform 2 are indicated with yellow numbers (right).

at different collision energies are shown in Figure S8. Satisfactory fragmentation of GNHWAVGHLMGK was first obtained at a CE of 37 eV, where several peptide fragments were identified (Figure S9).

MAI-IMS−MS of the Intact ProGRP Isoforms. vMAI was successful for two of the intact standard proteins (Figure S6) but not for the intact ProGRP isoforms, although they are similar in size. This might be explained by the 10-fold lower concentration used for the ProGRP isoforms as well as the limitation in the mass range of the vMAI source (briefly described in the Supporting Information).

Atmospheric introduction of MAI was, however, successful for ProGRP isoforms 1 and 2, and by applying IMS, differentiation of the two isoforms in a mixture was readily possible (Figure 3). Differentiation on the intact protein level could be advantageous for ProGRP isoforms, as it has not been possible to establish an isoform-specific signature peptide for isoform 2 through the bottom-up approach.[15]

CONCLUSION

While the fundamentals of the new ionization processes are still discussed,[16,17] the dedicated instruments and methods that are being developed can be used in ways that were unimaginable just a few years ago. Exploring the potential of vMAI in targeted biomarker analysis is an important step toward use of the new ionization processes in clinical analysis. Despite the need for relatively high analyte levels to obtain satisfactory MS and MS/MS signals and a limited mass range currently making determination of intact proteins challenging, the robustness and simplicity of vMAI are features that increase the chances of success outside the research laboratory. Still, for use in clinical settings, more evaluation is necessary, and commercial development is highly desirable, especially for vMAI.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.0c00336.

> Experimental: Overview of the experimental conditions and setup; Supplementary Results and Discussion including supplementary figures and tables (PDF)

AUTHOR INFORMATION

Corresponding Author

Trine Grønhaug Halvorsen — *Department of Pharmacy, University of Oslo, 0371 Oslo, Norway;* orcid.org/0000-0002-2545-3474; Email: t.g.halvorsen@farmasi.uio.no

Authors

Maren Christin Stillesby Levernæs — *Department of Pharmacy, University of Oslo, 0371 Oslo, Norway*

Cecilie Rosting — *Department of Pharmacy, University of Oslo, 0371 Oslo, Norway*

Complete contact information is available at:
https://pubs.acs.org/10.1021/jasms.0c00336

Notes

The authors declare no competing financial interest.
The paper is associated with the "Focus: Ionization Technologies used in MS: Fundamentals and Applications" collection, published in the January 5, 2021, issue of *J. Am. Soc. Mass Spectrom.*

ACKNOWLEDGMENTS

REFERENCES

(1) Lassman, M. E.; McAvoy, T.; Chappell, D. L.; Lee, A. Y.; Zhao, X. X.; Laterza, O. F. The Clinical Utility of Mass Spectrometry Based Protein Assays. *Clin. Chim. Acta* **2016**, *459*, 155−161.

(2) Levernæs, M. C. S.; Brandtzaeg, O. K.; Amundsen, S. F.; Reubsaet, L.; Lundanes, E.; Halvorsen, T. G.; Wilson, S. R. Selective Fishing for Peptides with Antibody-Immobilized Acrylate Monoliths, Coupled Online with NanoLC-MS. *Anal. Chem.* **2018**, *90*, 13860−13866.

(3) McKitterick, N.; Braathen, F.; Switnicka-Plak, M. A.; Cormack, P. A. G.; Reubsaet, L.; Halvorsen, T. G. Magnetic Synthetic Receptors for Selective Clean-up in Protein Biomarker Quantification. *J. Proteome Res.* **2020**, *19*, 3573−3582.

(4) Skjærvø, Ø.; Halvorsen, T. G.; Reubsaet, L. Pre-Lab Proteolysis for Dried Serum Spots − a Paper-Based Sampling Concept Targeting Low Abundant Biomarkers. *Anal. Methods* **2020**, *12*, 97−103.

(5) Trimpin, S.; Inutan, E. D. Matrix Assisted Ionization in Vacuum, a Sensitive and Widely Applicable Ionization Method for Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 722−732.

(6) Skjærvø, Ø.; Trimpin, S.; Halvorsen, T. G. Matrix-Assisted Ionization Mass Spectrometry in Targeted Protein Analysis − an Initial Evaluation. *Rapid Commun. Mass Spectrom.* **2021**, *35*, No. e8437.

(7) Marshall, D. D.; Inutan, E. D.; Wang, B.; Liu, C.-W.; Thawoos, S.; Wager-Miller, J.; Mackie, K.; Trimpin, S. A Broad-Based Study on Hyphenating New Ionization Technologies with MS/MS for PTMs and Tissue Characterization. *Proteomics* **2016**, *16*, 1695−1706.

(8) Chen, B.; Lietz, C. B.; Li, L. Coupling Matrix-Assisted Ionization with High Resolution Mass Spectrometry and Electron Transfer Dissociation to Characterize Intact Proteins and Post-Translational Modifications. *Anal. Bioanal. Chem.* **2018**, *410*, 1007−1017.

(9) Inutan, E. D.; Trimpin, S. Matrix Assisted Ionization Vacuum (Maiv), a New Ionization Method for Biological Materials Analysis Using Mass Spectrometry. *Mol. Cell. Proteomics* **2013**, *12*, 792−796.

(10) Trimpin, S. Novel Ionization Processes for Use in Mass Spectrometry: 'Squeezing' Nonvolatile Analyte Ions from Crystals and Droplets. *Rapid Commun. Mass Spectrom.* **2019**, *33*, 96−120.

(11) Lu, I. C.; Pophristic, M.; Inutan, E. D.; McKay, R. G.; McEwen, C. N.; Trimpin, S. Simplifying the Ion Source for Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2016**, *30*, 2568−2572.

(12) Trimpin, S. "Magic" Ionization Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2016**, *27*, 4−21.

(13) Takyi-Williams, J.; Liu, C.-F.; Tang, K. Ambient Ionization MS for Bioanalysis: Recent Developments and Challenges. *Bioanalysis* **2015**, *7*, 1901−1923.

(14) Trimpin, S.; Pophristic, M.; Adeniji-Adele, A.; Tomsho, J. W.; McEwen, C. N. Vacuum Matrix-Assisted Ionization Source Offering Simplicity, Sensitivity, and Exceptional Robustness in Mass Spectrometry. *Anal. Chem.* **2018**, *90*, 11188−11192.

(15) Torsetnes, S. B.; Nordlund, M. S.; Paus, E.; Halvorsen, T. G.; Reubsaet, L. Digging Deeper into the Field of the Small Cell Lung Cancer Tumor Marker ProGRP: A Method for Differentiation of its Isoforms. *J. Proteome Res.* **2013**, *12*, 412−420.

(16) Trimpin, S.; Inutan, E. D.; Karki, S.; Elia, E. A.; Zhang, W. J.; Weidner, S. M.; Marshall, D. D.; Hoang, K.; Lee, C.; Davis, E. T. J.; Smith, V.; Meher, A. K.; Cornejo, M. A.; Auner, G. W.; McEwen, C. N. Fundamental Studies of New Ionization Technologies and Insights from IMS-MS. *J. Am. Soc. Mass Spectrom.* **2019**, *30*, 1133−1147.

(17) Trimpin, S.; Lu, I. C.; Rauschenbach, S.; Hoang, K.; Wang, B.; Chubatyi, N. D.; Zhang, W.-J.; Inutan, E. D.; Pophristic, M.; Sidorenko, A.; McEwen, C. N. Spontaneous Charge Separation and Sublimation Processes Are Ubiquitous in Nature and in Ionization Processes in Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2018**, *29*, 304−315.