Published in final edited form as:

Anal Chem. 2019 October 15; 91(20): 12942–12947. doi:10.1021/acs.analchem.9b02995.

Mass spectrometry imaging of N-glycans from formalin-fixed paraffin-embedded tissue sections using a novel subatmospheric pressure ionization source

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Abstract

N-linked glycosylation, featuring various glycoforms, is one of the most common and complex protein post-translational modifications (PTMs) controlling protein structures and biological functions. It has been revealed that abnormal changes of protein N-glycosylation patterns are associated with many diseases. Hence, unraveling the disease-related alteration of glycosylation, especially the glycoforms, is crucial and beneficial to improve our understanding about the pathogenic mechanisms of various diseases. In past decades, given the capability of in-situ mapping of biomolecules and their region-specific localizations, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has been widely applied to the discovery of potential biomarkers for many diseases. In this study, we coupled a novel subatmospheric pressure (SubAP)/MALDI source with a Q Exactive HF hybrid quadrupole-orbitrap mass spectrometer for in-situ imaging of N-linked glycans from formalin-fixed paraffin-embedded (FFPE) tissue sections. The utility of this new platform for N-glycan imaging analysis was demonstrated with a variety of FFPE tissue sections. A total of 55 N-glycans were successfully characterized and visualized from a FFPE mouse brain section. Furthermore, 29 N-glycans with different spatial distribution patterns could be identified from a FFPE mouse ovarian cancer tissue section. High-mannose N-glycans exhibited elevated expression levels in the tumor region, indicating the potential association of this type of N-glycans with tumor progression.

Graphical Abstract

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The authors declare no competing financial interest.

Keywords

Mass spectrometry imaging; Subatmospheric pressure ionization source; High resolution and high accuracy mass spectrometry; Glycosylation; Formalin-fixed paraffin-embedded tissue; N-linked glycan analysis

Introduction

N-linked glycosylation is a type of common post-translational modification (PTM), which plays an important role in various physiological processes. Prior studies revealed that aberrant glycosylation was associated with many diseases including Alzheimer's disease,¹ cancers^{2,3} and cardiovascular diseases.⁴ Although electrospray ionization mass spectrometry (ESI-MS)-based glycomic^{5,6} and glycoproteomic analyses^{7–10} have been applied to investigate N-glycan expression changes between healthy and disease specimens, the homogenization of the tissue samples conducted in the vast majority of previous studies inevitably result in the loss of spatial information regarding N-glycan localization. The region-specific N-glycan distribution patterns, especially in the disease area, could be critical in discovering N-glycan biomarkers and revealing disease mechanisms.

Complementary to ESI-MS technique, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) technique has been introduced for *in-situ* biomolecular analysis due to its capability of retaining and directly visualizing biomolecules in tissue sections.^{11–13} Depending on the source pressure, MALDI-MS can be divided into three types: vacuum MALDI-MS, atmospheric MALDI-MS (AP/MALDI-MS) and subatmospheric pressure MALDI-MS (SubAP/MALDI-MS). Vacuum MALDI-MS is the one that commonly used, and generally has higher sensitivity than that of AP/MALDI-MS, enabled by the low pressure (less than 1.33 Pascal (Pa)) in the source.¹⁴ However, to gain the high vacuum in the source, it typically requires a dedicated MALDI MS system to perform these *in-situ* profiling and imaging experiments. Different from the traditional vacuum MALDI source, the AP/MALDI source and SubAP/MALDI source are independent of mass spectrometer and can be switched back and forth with an ESI source on an MS instrument platform, providing greater instrumental versatility and flexibility to allow both ESI-based analysis and MALDI-based analysis on a single mass spectrometer. Furthermore, the SubAP/MALDI source is operated at a subatmospheric pressure (133 Pa–1330 Pa) and offers better sensitivity than AP/MALDI source, making it a more attractive alternative to the traditional vacuum MALDI source.¹⁵

In past decades, MALDI-MS has been successfully applied to map the spatial distributions of various biomolecules on tissue sections, such as lipids,^{16,17} small metabolites^{18,19} and neuropeptides.²⁰ Recently, the scope of the vacuum MALDI-MS, has been successfully extended to the *in-situ* N-glycan imaging analysis,^{21–23} while the application of SubAP/MALDI-MS to N-glycan imaging has rarely been reported, even though its sensitivity has been proven to be sufficient for many biological applications.¹⁵ Considering the advantages of SubAP/MALDI-MS, it could be beneficial to systematically evaluate the potential of SubAP/MALDI-MS, as a complementary tool of vacuum MALDI-MS, in the imaging of N-glycans from tissue sections.

In this study, a new platform was developed by coupling the novel SubAP/MALDI source to a Q Exactive HF (QE HF) hybrid quadrupole-Orbitrap mass spectrometer. After optimization with N-glycan mixtures, this platform was successfully employed to characterize and visualize N-glycans released from formalin-fixed paraffin-embedded (FFPE) tissue sections including mouse brain and murine ovarian cancer tissue sections. Our results, for the first time, demonstrated the applicability of the SubAP/MALDI-MS platform for MS imaging (MSI) of N-glycans with high spatial resolution and mass accuracy.

Experimental section

Chemicals and reagents

Methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), formic acid (FA), sodium chloride (NaCl), trifluoroacetic acid (TFA), citric acid and ammonium bicarbonate were purchased from Fisher Scientific (Pittsburgh, PA). Xylene and microcon-30kDa centrifugal filter unit were purchased from EMD Millipore Sigma (Burlington, MA). Dithiothreitol and PNGase F were purchased from Promega (Madison, WI). Distilled water mentioned in this work was Milli-Q water from a Millipore filtration system (Bedford, MA). α -cyano-4-hydroxycinnamic acid (CHCA), Tris (2-carboxyethyl) phosphine (TCEP), triethylammonium bicarbonate buffer (TEAB) and bovine thyroglobulin (BTG) were purchased from Sigma-Aldrich (St. Louis, MO). 2,5-dihydroxybenzoic acid (DHB) was purchased from Acros Organics (Morris Plains, NJ). All reagents were used without additional purification. Microscope glass slides were purchased from VWR international, LLC (Radnor, PA). Indium tin oxide (ITO)-coated glass slides were purchased from Delta technologies (Loveland, CO).

Preparation of FFPE tissue sections

Mouse studies were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison. For the FFPE mouse brain section, mouse was anesthetized with carbon dioxide. The brain was collected immediately after transcardial PBS perfusion, fixed overnight in 10% neutral buffered formalin, and paraffin embedded using standard techniques. 6 μ m coronal tissue sections were prepared using a microtome (HistoCore MULTICUT, Leica Biosystems).

For the development of ovarian tumor in a mouse model, syngeneic murine ovarian cancer cells (Mouse Ovarian Surface Epithelial Cells, known as MOSEC²⁴) were suspended in

phosphate buffered saline and injected into the peritoneal cavity of 8 to 10-week-old female C57BL/6 mice, 10×10^6 cells/animal. The MOSEC were a gift from Dr. Kathy Roby, Kansas University Medical Center, Kansas City, KS. The mice were housed under controlled conditions in the vivarium and monitored over a period of 2–3 months for the development of ascites as determined by behavioral changes/indications of discomfort, weight gain and presence of a distended belly, and body condition evaluation. Accumulation of ascites typically occurred 10–12 weeks post implantation of the cancer cells. Animals showing significant accumulation of ascites and with worsening body condition were sacrificed and tumors from the peritoneal cavity were obtained during necropsy. The tumors were immediately stored in formalin and embedded in paraffin the following day. 6 μm tissue section was cut from the paraffin block, adhered to the ITO-coated slide, and used for mass spectrometry-based N-glycan imaging.

Preparation of N-glycans released from bovine thyroglobulin

N-glycans were released from glycoprotein standard-bovine thyroglobulin (BTG) with slightly modified filter-aided N-Glycan separation (FANGS) strategy.²⁵ Glycoproteins were dissolved in water to have a concentration of $2 \mu\text{g } \mu\text{L}^{-1}$ and mixed with 5 μL of 0.5 M TCEP. Heat-denaturation was performed by switching sample tubes between 100°C water bath and room temperature for four cycles of 15 seconds each. The mixture was then loaded onto a 30 kDa molecular weight cutoff (MWCO) filter and buffer exchanged with 100 μL 0.5 M triethylammonium bicarbonate buffer (TEAB) by centrifugation at 14000g for three cycles (15 minutes for each) to remove contaminants with low molecular weight. 4 μL of PNGase F in 96 μL of 0.5 M TEAB buffer was added to each filter and incubated at 37°C overnight. N-glycans were separated with deglycosylated proteins and eluted by centrifugation at 14000g for 10 minutes. The filter was then washed with 100 μL 0.5 M TEAB buffer to ensure complete elution of N-glycans. Two fractions were combined and dried *in vacuo*. The released N-glycans were analyzed using the SubAP/MALDI QE HF mass spectrometer as shown in Figure 1.

Pretreatment of FFPE tissue section for SubAP/MALDI N-glycan imaging

SubAP/MALDI imaging of N-glycans from FFPE tissue section was performed using workflow illustrated in Figure S1. 6 μm FFPE tissue sections were placed on a hot plate and heated at 65°C for 20 min. After cooling down, FFPE tissue sections were consecutively washed twice with xylene, ethanol, 95% ethanol and 70% ethanol to remove paraffin. Following that, the antigen retrieval process was performed by boiling tissue sections in 20 mM freshly prepared citric acid buffer for 1 hour. Enzyme and matrix application were performed by a robotic TM sprayer system (HTX Technologies, Carrobo, NC). For enzyme deposition, 20 μL PNGase F dissolved in 330 μL 50 mM ammonium bicarbonate solution was sprayed at a flowrate of 0.02 ml min^{-1} , and in total 16 passes were performed. The nozzle temperature was set to 35 °C with a moving velocity of 800 mm min^{-1} . Then, FFPE tissue sections were incubated in a humidity chamber at 37°C for 12 hours. CHCA dissolved in ACN: H₂O: TFA (v: v: v, 50: 50: 0.1) solution at a concentration of 7 mg ml⁻¹ was used as the matrix for N-glycan imaging. 24 passes of matrix spraying were performed at a flow rate of 0.05 ml min^{-1} , and 30 seconds drying time was set between each pass. The nozzle temperature was set to 80°C with a moving velocity of 800 mm min^{-1} . After matrix

spraying, tissue slides were dried in a vacuum chamber for 30 min and stored at -20°C until use.

Mass spectrometry

The QE HF mass spectrometer is coupled with a novel SubAP/MALDI (ng) ion source equipped with a 355 nm Nd:YAG laser (Figure 1). The laser spot size is 10 μm and the maximum output frequency reaches 10 kHz. Spiral plate motion was used for profiling experiments, while constant speed raster mode was employed for the imaging of tissue sections. A microscopic slide in the sample holder is approximately 2 mm away from the MS inlet capillary. Enabled by two novel design features, the SubAP/MALDI source has better sensitivity than the regular AP/MALDI source. Firstly, it operates at pressures from 1 Torr to 10 Torr instead of regular atmospheric pressure. Secondly, an ion funnel interface is designed to improve the ion collection and transfer into the mass spectrometer. The source is controlled by the Target software (MassTech Inc., Columbia, MD), and the ImageQuest software (Thermo Fisher Scientific, Waltham, MA) is applied to construct the images of target analytes using the XY coordinates of laser spots along with the acquired MS data file. For N-glycan imaging experiments, laser energy of 20% and repetition rate of 200 Hz were used. Ion funnel parameters were set as below: Voltage (V) values for V1 through V7 were set as 0, 0, 5, 10, 170, 200 and 300, respectively; RF amplitude was set as 250 V; QE-HF mass spectrometer was operated at positive full MS scan mode with the mass range of m/z 1100–3000. The capillary temperature and S-lens RF level were set to 200 $^{\circ}\text{C}$ and 50, respectively. Automatic gain control (AGC) of 5e^6 was used to ensure that the maximum ion injection time of 300 ms could be reached at each pixel. Resolution was sample-dependent and will be discussed in Results and discussion section.

Histology staining

H&E staining was performed as described previously.²⁶ Tissue sections were deparaffinized through a xylene and graded ethanol series, then rinsed in hematoxylin, in 1% acid alcohol, and then in eosin. After rinsing in running water to remove excessive staining, slides were dehydrated in graded ethanol and xylene.

Data processing

MS spectra were processed by Xcalibur (Thermo Scientific, Bremen, Germany), and observed N-glycans were annotated by using the GlycoWorkbench²⁷ software with less than 10 ppm mass tolerance. Signal intensities of N-glycans were normalized to total ion chromatogram (TIC) and ImageQuest (Thermo Scientific, Bremen, Germany) was used to construct N-glycan images with the mass tolerance window of 5 ppm. N-glycan compositions were tentatively identified by searching against UniCarbKB database.

Results and discussion

Optimization of operating pressure

SubAP/MALDI source was operated at subatmospheric pressure normally ranging from 133 Pa to 1330 Pa. Given that the ion source pressure is critical to ion transport efficiency, it is necessary to examine the impact of source pressure on the N-glycan signal intensities and

select an optimal pressure for N-glycan imaging analysis. When coupled to QE HF mass spectrometer, a minimum source pressure of 386 Pa was required to maintain the stability of the entire platform. Hence, the pressure optimization was initiated from 400 Pa using N-glycans released from bovine thyroglobulin (A representative MS spectrum is shown in Figure S2). N-glycan mixture was mixed with CHCA, and further spotted onto sample plate for analysis. Ion abundance of 4 dominant N-glycans were normalized to TIC chromatogram, and used for pressure optimization. As shown in Figure 2a–c, TIC-normalized ion abundance and ion abundance of N-glycans significantly dropped following the increase of operating pressure, while no apparent change of TIC intensities was observed. This result suggested that relatively lower pressure could improve the sensitivity of SubAP/MALDI measurements probably due to enhanced transportation of N-glycans. Therefore, to balance the stability and sensitivity of the platform, operating pressure of 400 Pa was selected for N-glycan analysis.

Matrix optimization

DHB and CHCA are two common matrices used for MALDI imaging of small biomolecules. In general, high laser energy is required to ionize analytes co-crystallized with DHB matrix, while lower laser energy is needed for the ionization of CHCA-coated analytes. The performance of these two matrices in N-glycan imaging on the SubAP/MALDI-MS platform was studied. When CHCA was used, increasing laser energy improved TIC intensities (Figure 2f), though the optimal N-glycan ion abundance, either absolute or normalized, was obtained at the laser energy of 6.8 micro Joules per square centimeter ($\mu\text{J}/\text{cm}^2$) (Figure 2d–e). For DHB, higher TIC-normalized ion abundance of N-glycans were observed due to the much lower TIC intensities in comparison to CHCA (Figure 2g). Ion abundance and TIC augmented following the increase of laser energy, while higher energy was required to get similar results as that of CHCA (Figure 2h–i). Thus, to get longer laser lifespan and higher glycan signal intensities, matrix of CHCA and laser energy of $6.8 \mu\text{J}/\text{cm}^2$ were used for SubAP/MALDI-based N-glycan imaging.

High-resolution imaging of N-glycans from FFPE mouse brain sections

Recent N-glycoproteomic studies revealed that N-glycoproteins with heterogeneous N-glycans were highly expressed in mouse brain.^{28–31} Therefore, to test the applicability of the novel SubAP/MALDI-MS platform for high resolution MS imaging acquisition, a 6 μm -thick FFPE mouse brain coronal section was prepared and treated by PNGase F to release N-glycans for imaging analysis with a pixel size of 25 μm . A total of 55 N-glycans including high-mannose N-glycans, fucosylated N-glycans and sialylated glycans were detected and annotated by the accurate mass matching (Figure S3, Table S1). The new SubAP/MALDI-MS platform detected more N-glycans in comparison to previous N-glycan imaging studies of either frozen or FFPE mouse brain section using vacuum MALDI-MS platforms (Figure 3a, Table S2).^{32,33} Also, as shown in Figure 3b, the new SubAP/MALDI source is capable of detecting most N-glycans identified by vacuum MALDI-MS. Furthermore, by matching with the optical image of the mouse brain section (Figure 3c), N-glycans with different distribution patterns on the brain tissue section could be revealed and representative N-glycans were shown in Figures 3d–3h. These results manifested that the new instrument platform had adequate sensitivity for the detection and visualization of N-glycans from

FFPE tissue sections, and therefore could be employed as an alternative to vacuum MALDI-MS platform for *in-situ* N-glycan imaging analysis.

Mapping of N-glycan distribution on FFPE mouse ovarian cancer tissue section

N-glycosylation is a protein PTM that plays an important role in many devastating diseases. Accumulating evidence have indicated that aberrant alteration of N-glycosylation can occur during the onset and progression of ovarian cancer.^{22,34–36} In particular, high-mannose N-glycans, which are synthesized in the endoplasmic reticulum (ER) and are crucial for proper protein folding, have been shown to be dominantly expressed in the cancer area.²² Herein, a mouse model with ovarian cancer was established and a 6 μm FFPE ovarian tissue section containing tumor region was prepared. Following that, the new SubAP/MALDI-MS platform was employed to map the distribution patterns of N-glycans in the tissue section. Considering that the mouse ovarian tissue section was bigger, but less heterogeneous than the mouse brain section, a pixel size of 50 μm was selected without significant compromise on the image quality. In total, 29 N-glycans, including complex N-glycans and high-mannose N-glycans, were successfully detected and annotated by the accurate mass matching (Figure 4, Table S3). More homogenous distributions of complex N-glycans on tissue section were observed and shown in Figures 5a–h. In contrast, high-mannose N-glycans, from $\text{Hex}_4\text{HexNAc}_2$ to $\text{Hex}_{10}\text{HexNAc}_2$, were highly expressed in the cancer region though no apparent accumulation was observed for $\text{Hex}_3\text{HexNAc}_2$ as the simplest N-glycan (Figures 5i–p). This observation is consistent with the previous finding that increasing N-glycan branches could lead to structural and functional changes of N-glycoproteins, contributing to the progression of cancers.^{3,37} Overall, our MSI results revealed that alteration of high-mannose N-glycans was involved in mouse ovarian cancer development, which was consistent with the findings of previous N-glycomic profiling studies,^{22,38,39} and further demonstrating the capability of the new platform for *in-situ* N-glycan imaging analysis.

Conclusions

A novel MS-based imaging platform was developed by integrating a new SubAP/MALDI source with a Q Exactive HF orbitrap mass spectrometer for the *in-situ* N-glycan imaging analysis with high resolution and high mass accuracy for the first time. Several parameters, such as the operation pressure of the SubAP/MALDI source, the type of matrix and laser energy, were optimized with a mixture of N-glycans released from a glycoprotein. After optimization, the new platform was successfully applied to map distribution patterns of N-glycans from FFPE mouse brain and ovarian cancer tissue sections. More N-glycans can be identified on brain section in comparison to that of commonly used vacuum MALDI-MS platforms, and high mannose N-glycans show accumulation in the tumor region. The new platform was demonstrated to have improved sensitivity for N-glycan profiling analysis from biological tissue sections. For future directions, by simply switching the PNGase F to trypsin, it is entirely possible to extend the application of the new platform from N-glycan imaging to peptide imaging, and even *in-situ* glycopeptide identification enabled by the powerful Q Exactive HF orbitrap mass spectrometer or similar high performance MS instrument platforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Dr. Eugene Moskovets and Dr. Vladimir Doroshenko from MassTech, Inc. for access to the SubAP MALDI source and technical support. The research was supported in part by NIH R01 DK071801, R56MH110215, R01AG052324, and U01CA231081. The MALDI Orbitrap instrument was purchased through the support of an NIH shared instrument grant (NIH-NCRR S10RR029531). LL acknowledges a Vilas Distinguished Achievement Professorship and Charles Melbourne Johnson Distinguished Chair Professorship with funding provided by the Wisconsin Alumni Research Foundation and University of Wisconsin-Madison School of Pharmacy.

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Figure 1.

The new SubAP/MALDI-QEHF platform for N-glycan imaging. (a) Schematic illustration of the SubAP/MALDI-QEHF platform. (b) A snapshot showing the QEHF mass spectrometer integrated with the SubAP/MALDI source instead of an ESI source.

Figure 2.

Parameter optimization for N-glycan analysis on SubAP/MALDI MS platform (n=3). The TIC-normalized abundance (a), ion abundance (b) and TIC (c) of four representative N-glycans detected at different operating pressure. The TIC-normalized abundance (d), ion abundance (e) and TIC (f) of four representative N-glycans detected at different laser energy. The TIC-normalized abundance (g), ion abundance (h) and TIC (i) of four representative N-glycans detected at different laser energy.

Figure 3.

(a) More N-glycans were detected and imaged from FFPE mouse brain tissue section by using the novel SubAP/MALDI-MS platform. (b) The Venn diagram showing the overlap of N-glycans detected in this study with N-glycans reported in prior studies (Refs. 32 & 33) using vacuum MALDI-MS platform. (c) H&E stained FFPE mouse brain section post N-glycan imaging. (d-f) MS images of representative N-glycans detected from FFPE mouse tissue section. (g-h) Overlap of different N-glycan images clearly revealed different spatial distribution patterns of N-glycans on mouse brain section.

Figure 4. N-glycans detected from FFPE mouse tissue section with ovarian cancer. The annotated glycan compositions were tentatively identified by searching against UniCarbKB database. H: Hexose; N: N-Acetyl glucosamine; F: Fucose.

Figure 5.

Images of N-glycans showing different spatial distribution patterns on FFPE mouse tissue section with ovarian cancer. (a) H&E stained FFPE mouse tissue section with ovarian cancer. (b-h) Complex N-glycans showed similar distribution in cancer area in comparison to peripheral area; (i-p) High mannose N-glycans accumulated in cancer area except Hex₃HexNAc₂.