N-glycosylation analysis of mAbs by CESI-MS at the glycopeptide and released glycan levels

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ABSTRACT

Analysis of the N-linked carbohydrates of therapeutic glycoproteins can be routinely done by capillary electrophoresis at the glycopeptide[1] and/or released glycan level.[2] While glycopeptide level studies requires tryptic digestion only, N-linked glycan analyses necessitate endoglycosidase based release of the carbohydrate moieties and charged fluorophore labeling. CE provides high resolution separation of glycopeptides and released, labelled (usually by aminopyrene trisulfonate, APTS) glycans, while coupling with MS detection offers additional information. Integration of CE and electrospray ionization (ESI) into a single dynamic process (CESI, Figure 1) provides the capability of performing CE separation and MS ionization with ultra-low flow rates, resulting in reduced ion suppression and improved sensitivity. We present a set of proof-of-principle experiments as an alternative workflow to RPLC-, HILIC-, and MALDI-MS for N-linked glycan characterization on therapeutic proteins. High resolution separation was obtained using acetate-based buffers at the glycopeptide and APTS-glycan level by CESI-MS. The comprehensiveness, sensitivity, and confidence of the MIDAS™ workflow (MRM Initiated Detection And Sequencing) was applied to CESI-MS glycopeptide separations. Notably, glycopeptides and glycans can be analyzed in an automated fashion using the same capillary column and instrumentation by simply loading different separation buffers and switching the MS polarity.

INTRODUCTION

The rapidly increasing usage of glycoproteins as biopharmaceutical products has created a demand for fast, efficient, and reliable bioanalytical techniques for glycosylation analysis. One of the fastest growing groups of these new generation drugs are monoclonal antibodies (mAbs). In most instances mAbs possess a conserved N-linked glycosylation site in each of the CH2 domains of the Fc portion of the heavy chain of the molecule, but may also have additional attached sugar structures on the Fab domains. Increasing evidence shows that the carbohydrate moieties of therapeutic antibodies play important roles in their biological activity, physicochemical properties, and effector functions. Even minor changes in the carbohydrate structures (linkage, position, and site occupancy) can influence the bioactivity of these products. The extremely high diversity of glycosylation makes their structural elucidation very difficult and in most instances only the combination of various methods can provide the desired information. The most frequently used analytical methods for the structural analysis of complex carbohydrates include capillary electrophoresis (CE), high performance liquid chromatography (HPLC), and mass spectrometry (MS), often combined with exoglycosidase digestion techniques.

MATERIALS AND METHODS

Sample Preparation: Immunoglobulin G was denatured using iTRAQ® reagent kit and digested with TPCK treated trypsin overnight at 37ºC. A reaction was stopped by applying liquid nitrogen. The APTS-labeled maltooligosaccharide ladder, IgG glycans, and RNAse B glycans were prepared by PNGase release from proteins, APTS-labeling, and dialysis.

CESI 8000 Plus MS Mode Conditions: CESI-MS experiments were carried out with a SCIEX CESI 8000 Plus system equipped with a temperature controlled separation capillary and autosampler. CESI separations were achieved using an OptiMS bare fused-silica capillary cartridge with 30 kV applied in either normal or reverse polarity modes by a high voltage power supply. Acetate-based background electrolytes (BGE) were used for the glycopeptide and the APTS-labeled glycan separations.

MS/MS Conditions: Data were collected on a QTRAP® 6500+ System (SCIEX) system with a NanoSpray® III source and a CESI adapter. Glycopeptide analysis was performed in positive ion mode using multiple reaction monitoring (MRM) with independent data acquisition (IDA) triggered enhanced product ion scan (EPI) in the trap. Low curtain gas (5.0 mL/min) and orifice temperature (50ºC) were applied to improve spray stability of 50 nL/min. The MRM table was built using the +2, +3, +4 charge states of the target glycopeptides including the G0, G0F, G0FB, G1F, G1FB, G2, G2F, and G2FB glycoforms and monitoring the 204.10 daughter ion. The released glycans were analyzed using negative ESI with 250 ms acquisitions from 250 - 2000 m/z.

Data Analysis: MS, MS/MS, and MS3 spectra were analyzed using SCIEX PeakView® software.

Figure 1. CESI 8000 Plus High Performance Separation-ESI Module coupled to a QTRAP® 6500+ System. The core of the CESI-MS technology is an etched porous capillary tip that allows for electrospray ionization without dilution of the BGE and sample by a make-up liquid. Instead a conductive liquid is used to apply the ESI voltage to and through the porous tip to increase sensitivity and reduce ion suppression.

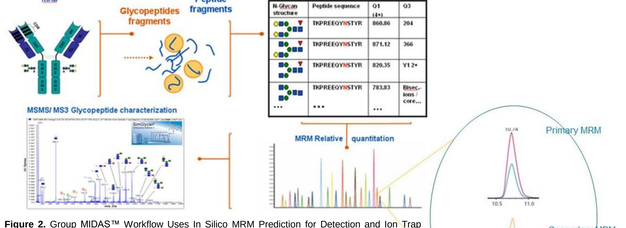
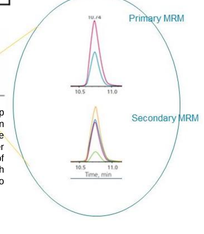


Figure 2. Group MIDAS™ Workflow Uses In Silico MRM Prediction for Detection and Ion Trap MS/MS for Confirmation. As the glycans on IgG proteins are constructed in a known way on known peptide sites, the glycopeptides formed during digestion can be easily predicted. This enables the in silico prediction of MRM transitions to these potential glycopeptides. Multiple transitions per glycopeptide (both primary and secondary MRM transition) are used to increase the specificity of detection with group triggered MRM workflow in the Scheduled MRM™ Pro Algorithm. Both additional MRMs and full scan MS/MS spectra can be collected in a targeted manner to characterize and quantitatively profile glycopeptides from IgG.



RESULTS

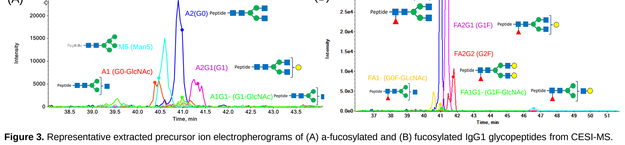


Figure 3. Representative extracted precursor ion electropherograms of (A) a-fucosylated and (B) fucosylated IgG1 glycopeptides from CESI-MS.

Sample preparation using trypsin digestion was optimized to obtain better quality MS data. The six targeted glycan (G0, G0F, G0BF, G1F, G2, G2FB) containing glycopeptides were separated and analyzed by MIDAS™ workflow. The MIDAS Workflow combines an MRM scan with a full MS/MS product ion scan to allow examination of all fragment ions in the same spectrum for sequence confirmation resulting in high intensity peaks of the targeted glycopeptides. The high data quality of TIC, XIC and MS/MS spectra (shown in Figure 4) allowed for complete evaluation from a single run.

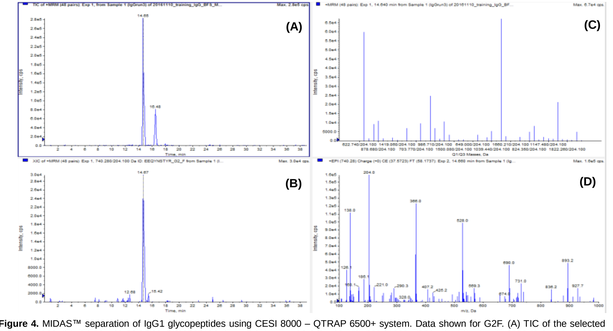


Figure 4. MIDAS™ separation of IgG1 glycopeptides using CESI 8000 – QTRAP 6500+ system. Data shown for G2F. (A) TIC of the selected glycopeptides. (B) XIC of the selected glycopeptide (G2F). (C) MRM data of the first peak (see Panel A). (D) MS/MS spectra of the selected glycopeptide containing the G2F feature.

A carbohydrate ladder (Figure 5A), mAb glycan standards (Figure 5B), and RNAse B glycans (Figure 6) were analyzed to evaluate the general capabilities of CESI-MS for the analysis of released, APTS-labeled glycans from proteins of interest. Separation and detection of all released glycans illustrated efficient separations (~6 sec peak widths) and sensitive detection in negative ESI mode. MS/MS fragmentation of RNAse B glycans illustrates the ability to identify and differentiate fucosylated and a-fucosylated glycans, along with high mannose glycans.

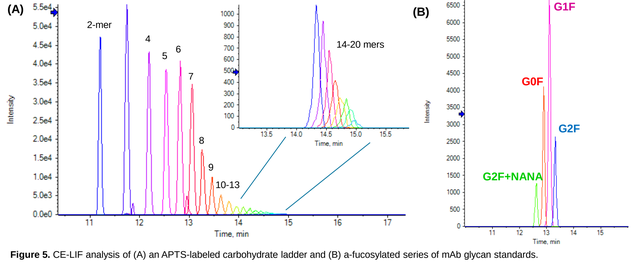


Figure 5. CE-LIF analysis of (A) an APTS-labeled carbohydrate ladder and (B) a-fucosylated series of mAb glycan standards.

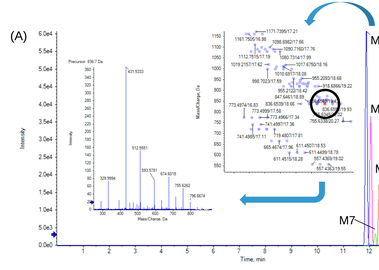
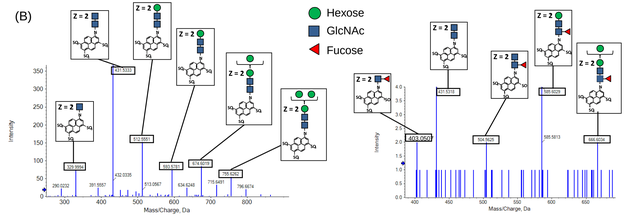


Figure 6. (A) Extracted ion electropherogram of released, APTS-labeled mannose 5, 6, 7, 8, and 9-mer glycans from RNAse B. IDA analysis generated multiple spectra for glycan peaks to confirm expected glycan structures.

(B) The combined MS and MS/MS spectra of APTS-glycans can be annotated to identify fucosylated and a-fucosylated glycan structures.



CONCLUSIONS

Through these proof-of-principle experiments, CESI-MS proved to be a powerful toolset to characterize and quantify glycopeptides and released N-glycans from therapeutic proteins. Both analyses delivered high resolution separations and structural characterization options for fucosylated, a-fucosylated, and high mannose glycans. When coupled with the QTRAP® 6500+ system, high sensitivity CESI-MS analyses were performed in an automated fashion by simply switching the separation BGEs and the MS polarity. Analyses by MS/MS within the MIDAS workflow™ facilitated further confirmation of glycopeptide and released glycan structures.

REFERENCES

1. Lies, M. *et al.*, SCIEX Technical Note 10440114-01.
2. Lew, C. *et al.*, SCIEX Technical Note RUO-MKT-02-1896.
3. Albanese, J. *et al.*, SCIEX Technical Note RUO-MKT-02-2590-A.

TRADEMARKS/LICENSING

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