Analysis of N-glycans from a Monoclonal Antibody by Capillary Electrophoresis and Mass Spectrometry

Application Note

Biopharma

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Abstract

Glycosylation is one of the most important post-translational modifications involving the attachment of glycan moieties to proteins. Alterations in the glycosylation patterns have a profound impact on the immunogenicity and overall biological activity. Glycan characterization is of crucial importance to biopharma-based applications. Various analytical techniques have been widely used to profile the N-glycans and face significant challenges in accurate detection of low levels of glycan species.

This Application Note demonstrates the analysis of recombinant monoclonal antibody glycans (mAb) by employing capillary electrophoresis and mass spectrometry (CE-MS). This method involves enzymatic cleavage of glycans from mAb by PNGase F followed by fluorescent (8-aminopyrene-1,3, 6-trisulfonic acid trisodium salt–APTS) labeling of glycans, and analysis of the glycans by using an Agilent 7100 CE system coupled to an Agilent 6520 Accurate Mass Q-TOF LC/MS. We identified seven glycans from a recombinant mAb and the relative percentage ratio of individual glycan moieties reveals the presence of both the major and minor forms of glycan modifications. This Application Note demonstrates faster separation of CE along with the sensitive detection limits of MS indicating the coupling of CE-MS to be a successful and promising alternative analytical solution to LC/MS for the characterization of glycans from mAbs/or glycoproteins.

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Introduction

In recent years, monoclonal antibodies (mAb) have become the emerging potential protein drug candidates for biopharmaceutical industries. The drug discovery pipeline comprises a series of meticulously controlled and evaluated steps, demanding careful and critical monitoring of the therapeutic stability and efficacy of the target compounds. Therefore, a comprehensive characterization of mAbs at every stage is highly beneficial prior to commercialization. Among various well studied protein post-translational modifications, glycosylation is known to play an important fundamental role in several biological processes, for example, protein degradation and transcription, influencing health and disease progression[1]. A reliable, robust and sensitive analytical technique is needed to identify the glycans attached to protein molecules of interest.

Recently, capillary electrophoresis (CE) has gained much attention in analyzing the glycoproteins, delivering high efficiency separations in shorter run times. Enzymatically released glycans are labeled with a fluorescent chromophore (8-aminopyrene-1,3, 6-trisulfonic acid trisodium salt–APTS, anionic) and subjected to CE separation with high sensitivity laser-induced fluorescence (LIF) or mass spectrometry (MS) detection. The APTS labeling imparts negative charge to glycans, aiding in enhanced electrophoretic separations and is also amenable to electrospray ionization (negative mode), making the entire separations and detection process highly compatible with the fluorescent labeling chemistry. Coupling CE to MS is beneficial in identifying unknown and assigning the glycan modification or mass information[2].

This Application Note demonstrates the CE-QTOF MS analysis of N-glycans derived from recombinant mAb. The CE-MS method with APTS labeling helps to identify all glycan moieties attached to mAb. Further relative percentage ratios of each glycan were presented to show the major and minor forms of glycan modifications.

Materials and method

Chemicals

Monoclonal antibody, PNGase F and PVA coated capillaries were from Agilent Technologies, Inc. ε-aminocaproic acid and 1 M sodium cyanoborohydride in tetrahydrofuran (THF) from Sigma-Aldrich (St. Louis, MO, USA). APTS was obtained from Molecular Probes, Invitrogen (Eugene, OR, USA). Glycan standards were purchased from Prozyme (Hayward, CA, USA). PhyTip Columns were from PhyNexus (San Jose, CA, USA).

Enzymatic deglycosylation, glycan extraction and APTS labeling

Deglycosylation of mAb were performed using PNGase F. The mAb (15 mg/mL) was treated with PNGase F in 0.25 M Tris buffer (pH 7.6) overnight at 37 °C. The deglycosylated protein was heat precipitated and centrifuged. The supernatant containing released glycans were dried and labeled with APTS by reductive amination. To the dried glycan sample, 2.5 µL of 50 mM APTS in 1.2 M citric acid and 2.5 µL of 1 M sodium cyanoborohydride in THF were added and incubated overnight at 37 °C in a water bath. The reaction mixture was diluted with 50 µL water to stop the reaction. Unbound excess dye was removed using PhyNexus normal phase polyamide resin containing PhyTip which was conditioned with 95% ACN. After sample application, the column was washed with 95% ACN and finally N-glycans were eluted with 20% ACN.

CE-MS instrumentation

The CE-ESI-MS analysis was performed using the 7100 CE system with a CE-MS capillary cassette (G1603A) coupled to the 6520 Accurate-Mass Q-TOF equipped with dual electrospray source and orthogonal coaxial sheath liquid interface (G1607B)[3]. Separations and spray stability were optimized using the blank buffers and a standard. A sheath-liquid CE-MS interface with a low flow rate (5 µL/min) is maintained to preserve the high efficiency separation of CE and to provide a stable flow and spray conditions essential for electrospray ionization. Q-TOF parameters were optimized automatically through MS tuning programs and the MS system was calibrated using an ESI tuning mixture.

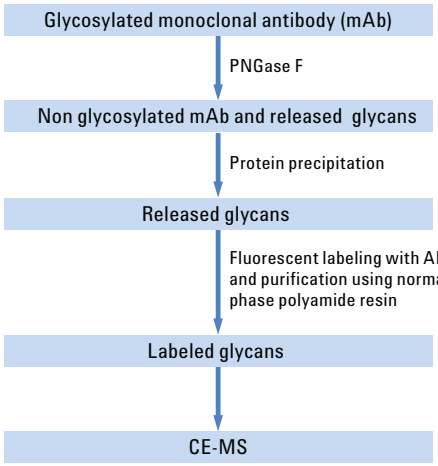
Table 1 shows the CE-MS parameters. The total compound list was extracted using the MassHunter molecular feature extractor (MFE) algorithm.

Capillary Electrophoresis (CE)	
CE:	Agilent 7100 Capillary Electrophoresis System
Sample:	Released N-glycans from mAb
Injection:	40 seconds at 30 mbar
Capillary:	PVA, total length 60 cm, 50 µm id
Buffer:	40 mM ε-aminocaproic acid pH 4.5
Voltage:	-25 kV
External pressure:	10 mbar
Temperature:	20 °C
Mass Spectrometry (MS)	
MS:	Agilent 6520 Accurate-Mass Q-TOF LC/MS
Ionization mode:	ESI
Acquisition mode:	MS (mass range 400–3,200 *m/z*)
Sheath liquid:	Isopropanol:water (1:1 v/v) with 0.2% NH_3 at 5 µL/min
Drying gas flow:	5 L/min
Nebulizer:	8 psi
Drying gas temperature:	250 °C
Fragmentor:	175 V
Vcap:	3,200 V
Accu time:	980.3 ms/spectrum
Acc rate:	1.02 spectra/s

Table 1
CE-MS conditions.

Results and discussion

Athough CE-LIF is routinely used for glycan analysis, CE-MS has an advantage in identifying the unknown migrating species present in the electrophoretic run providing molecular weight information. In this Application Note CE was coupled to Q-TOF MS using sheath-liquid interface for assignment of glycans mass information from mAb. Scheme 1 outlines the glycan profiling steps for mAb using CE-MS. Briefly, the released glycans from antibody were labeled with APTS, followed by CE-MS analysis. Figure 1 shows the CE-MS total compound profile for N-linked glycans released from recombinant mAb. Using a PVA-coated capillary, all the mAb glycans were migrated within a 15-minute separation time. We successfully identified the uncharged N-linked glycan species G0, G0F, G1, G1F, G2, and G2F in replicate runs. In addition, mono-sialylated glycan moiety G2F+1NANA was also observed. All the peak assignments were based on accurate mass measurements from Q-TOF analysis.



Scheme 1
Schematic overview of the glycoprofiling of mAb using CE-MS.

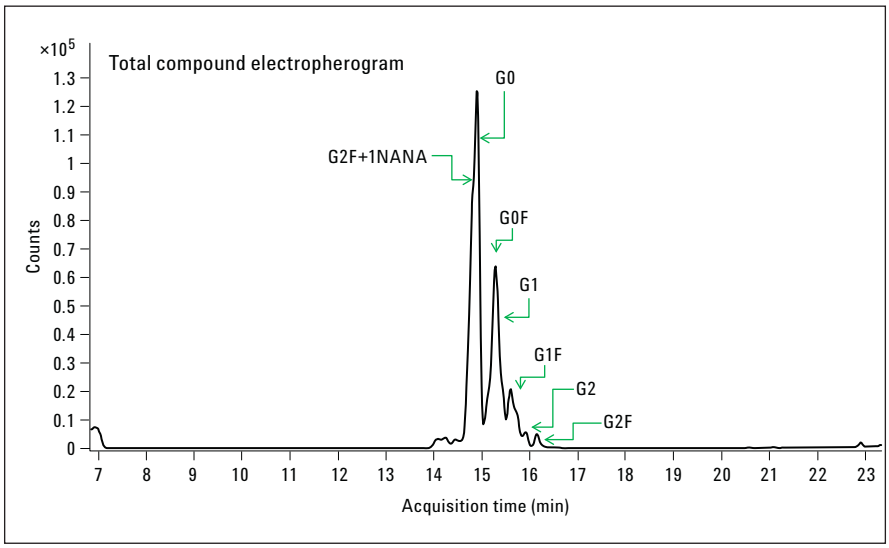
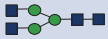
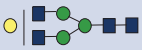
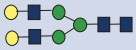
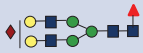


Figure 1
CE-MS of APTS labeled N-glycans released from mAb.

Table 2 summarizes the glycans identified in this study. Note that the G2 species was successfully identified by CE-MS, but not with LC/MS analysis (data not shown) indicating the significant advantage of CE-MS based analysis (complementary value of CE-MS).

Glycan abbreviation	Monoisotopic measured mass	Most abundant charge state measured	Glycan structures	Monoisotopic theoretical mass	Relative percentage
G0	1,757.4575	877.7212 (-2)		1,757.4512	20.8
G0F	1,903.5121	950.7484 (-2)		1,903.5091	34.0
G1	1,919.5071	958.7389 (-2)		1,919.5040	2.3
G1F	2,065.5649	1,031.7756 (-2)		2,065.5619	4.2
G2	2,081.5666	1,039.7751 (-2)		2,081.5568	3.1
G2F	2,227.6228	1,112.8041 (-2)		2,227.6147	1.2
G2+1NANA	2,518.7191	838.5664 (-3)		2,518.7101	34.4

Galactose Mannose Fucose N-acetylglucosamine Sialic acid

Table 2
Summary of mAb N-glycans identified using CE-MS.

The mass spectrum for individually resolved glycans is depicted in Figure 2. The most abundant charge states observed in the mass spectrum are indicated. Estimating the absolute/relative glycan quantity levels is of paramount importance in the therapeutic product development pipeline. Therapeutic products are sensitive to degradation and any minimal modifications. The percentage volume relative to total compound peaks volume was estimated and relative quantification of mAb released glycans are shown in Table 2. In the present case, the major form of the glycoform was found to be G2+1NANA.

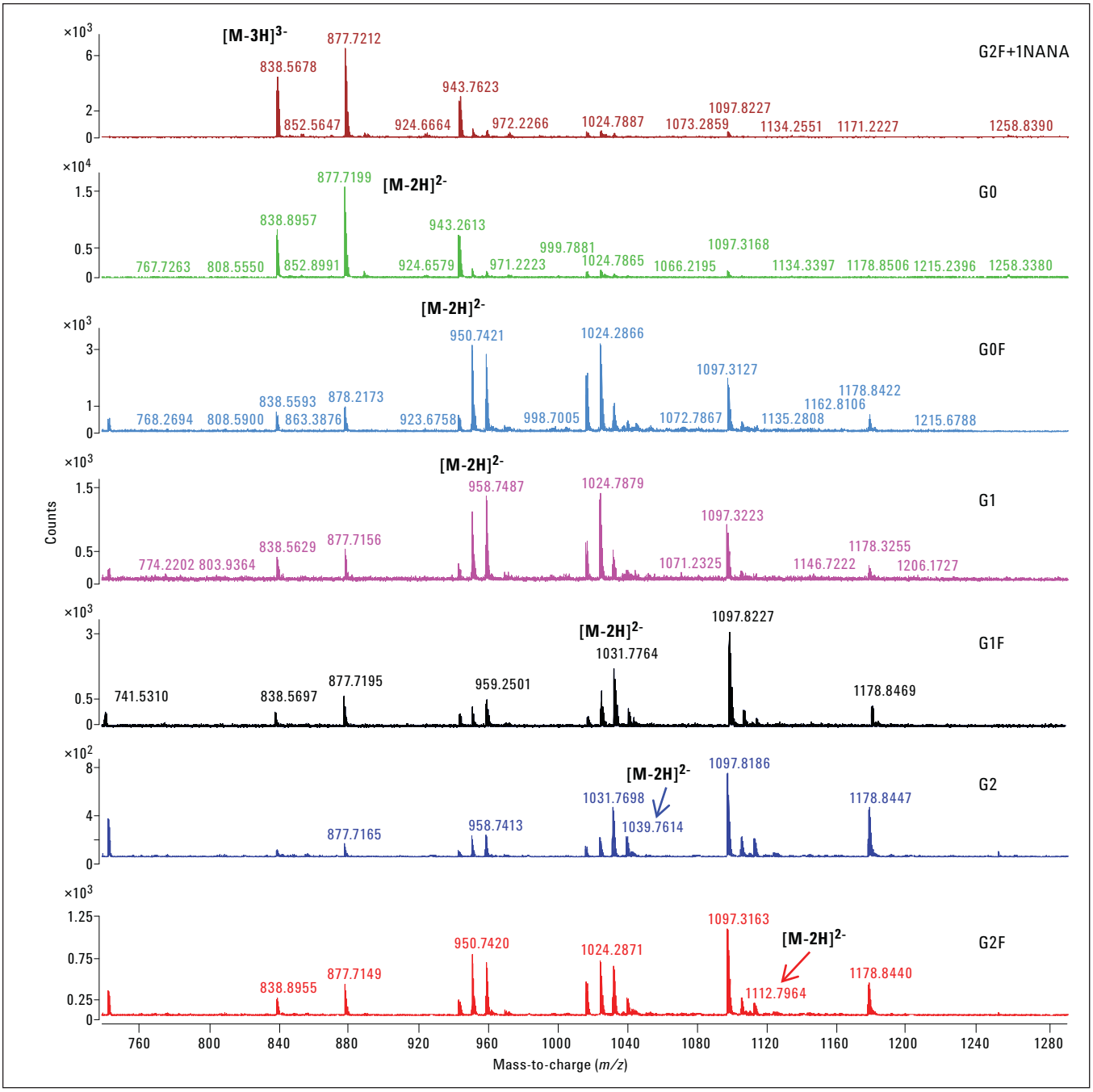


Figure 2
Mass spectrum of APTS labeled mAb N-glycans.

These results clearly indicate that CE-MS can be used effectively as an alternative analytical tool to monitor glycan profiles. CE-MS coupled separations avoid contaminations, due to minimal sample handling steps and the buffers used for CE separations are highly compatible with the electrospray ionization. The current approach is sensitive, accurate in glycan profiling as compared to conventional CE-LIF.

Conclusions

This Application Note provides an Agilent CE-MS based solution for mAb glycan profiling. The powerful data processing capabilities of Agilent MassHunter and BioConfirm suite enable in successful and detailed identification/profiling of the glycoforms of mAb. The glycan pattern was reasonably resolved allowing separation of major and minor forms of glycans.

References

1.
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