Glycopeptide Analysis of Antibodies by Capillary Electrophoresis and Q-TOF Mass Spectrometry

Application Note

Biopharm

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Abstract

Glycosylations of monoclonal antibodies (mAb) can impact biological activity and immunogenicity. Due to the importance of mAb as therapeutic agents, there is a growing demand for monitoring the carbohydrate structures attached to mAb. Improvements in capillary electrophoresis (CE) technology have made CE-MS a widely used tool for protein characterization. Here, we have analyzed the glycopeptides of an mAb using an Agilent 7100 Capillary Electrophoresis system coupled to an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) system with a coaxial sheath liquid interface. A tryptic digest of the mAb was subject to CE-MS analysis and the glycopeptides were assigned using accurate mass measurement. In addition, CE-MS/MS analysis was performed to search for diagnostic oxonium ions generated from a glycan moiety to identify the glycopeptides. The CE-MS platform, combined with the powerful data processing capabilities of the Agilent MassHunter and BioConfirm software, identified the glycan modification attached to mAb complex.

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Introduction

Glycosylation is an important post-translational modification (PTM) of proteins. Glycosylation of recombinant antibodies has a significant impact on biological activity and is relevant to therapeutic applications[1]. Due to different degrees and variety of glycosylation modifications, there is a growing demand for monitoring the carbohydrate structures attached to monoclonal antibodies (mAb).

Capillary electrophoresis (CE) has an enormous potential for the analysis of biopharmaceuticals due to its high resolution separation capabilities for large molecules. In addition, there is a growing interest in exploring CE coupled to mass spectrometry (MS) for higher sensitivity and better compound identification with accurate mass measurements. Coupling CE to quadrupole time-of-flight (Q-TOF) provides high mass accuracy and resolution at high acquisition rates.

This Application Note shows the usefulness of CE-Q-TOF/MS for the separation of the monoclonal antibody glycopeptide and identification of the glycan modifications. The tryptic peptide map of the mAb was generated and the glycopeptide was assigned using accurate mass measurement on a Q-TOF mass spectrometer. Subsequently, Agilent MassHunter BioConfirm software was used to map the glycosylation sites on the mAb.

Materials and method

Immunoglobulin G (IgG) (Mab-hmAb100) was obtained from ProMab Biotechnologies, Inc (USA). 2,2,2-trifluoroethanol (TFE), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, acetic acid and solvents were purchased from Sigma Aldrich. High quality sequence grade trypsin was obtained from Stratagene, a division of Agilent Technologies, Inc.

CE materials: water (5062-8578), polyvinyl alcohol (PVA) coated capillary (G1600-67219), sample vials (5183-4623) were obtained from Agilent Technologies, Inc.

Trypsin digestion

Before the digestion of the mAb with trypsin, the disulfide bonds were reduced (DTT) and alkylated (IAA) under denaturing conditions (TFE). This pretreatment ensured that the monoclonal antibody was completely denatured and soluble, allowing the protease to efficiently access its substrate[2].

The mAb (200 µg) was lyophilized, reconstituted in ammonium bicarbonate containing TFE and DTT, and then incubated at 95 °C for 20 min. IAA was added to this solution and incubated at room temperature in the dark for 60 min. To neutralize the excess IAA, DTT was added again and incubated at room temperature for 60 min. The solution was adjusted to pH 7–8 and trypsin digestion (20:1, protein to protease w/w) was performed overnight incubating at 37 °C. The samples were either immediately analyzed by CE-MS or stored at –20 °C until use.

Experimental

Equipment

- Agilent Capillary Electrophoresis system (G7100)
- Agilent CE-MS Adapter Kit (G1603A)
- Agilent CE-ESI-MS Sprayer Kit (G1607A)
- Agilent 1200 Series Isocratic HPLC Pump (automated sheath liquid delivery) (G1310A)
- Agilent 6520 Accurate-Mass Q-TOF LC/MS with Dual Electrospray Source
- Agilent ChemStation and MassHunter software packages

Instrumentation

The CE-ESI-MS analysis was performed using the CE system with a CE-MS capillary cassette coupled to the Accurate-Mass Q-TOF equipped with dual electrospray source and orthogonal coaxial sheath liquid interface. CE system was interfaced with MS via a CE-MS adapter kit and CE-ESI-MS Sprayer Kit. The CE system was controlled by the ChemStation software package. In ChemStation, the external detector (MS) configuration was used to perform CE-MS analysis. The sheath liquid was delivered by an Agilent 1200 Series Isocratic Pump equipped with a 1:100 flow splitter. The sheath liquid mainly serves to electrically connect the CE outlet to ground potential at the sprayer and ensures that voltage is constantly applied across the length of the separation capillary with stable electrospray at constant flow rates. ChemStation software was used for CE instrument and isocratic pump control.

Q-TOF parameters were optimized automatically through MS tuning programs and the MS system was calibrated using an ESI tuning mixture. An internal mass calibration sample was infused continuously during the CE-MS runs using the second spray needle in the dual spray ESI-source. This internal reference mass system allowed accurate, and automated mass calibration correction during the CE-MS runs. Spectra were recorded in positive ion and centroid modes. The data obtained from MS and MS/MS were analyzed using features contained in the following software packages: Agilent MassHunter Qualitative Analysis and Agilent MassHunter BioConfirm (version B.04.00). The CE-MS raw data were processed using the Molecular Feature Extractor (MFE) algorithm and matched with a theoretical peptide digest list with predicted glycosylation modifications.

The CE-MS parameters are shown in Table 1.

Capillary Electrophoresis (CE)	
CE:	7100 CE
Sample:	mAb digest (200 µg)
Injection:	10 s at 50 mbar (~0.4 pmoles)
Capillary:	PVA, total length 60 cm, 50 µm id
Buffer:	2% acetic acid
Voltage:	27 kV
External pressure:	10 mbar
Temperature:	20 °C
Mass Spectrometry (MS)	
MS:	Agilent 6520 Accurate-Mass Q-TOF LC/MS System
Ionization mode:	Dual ESI
Acquisition mode:	MS (mass range 300-3200 *m/z*)
Sheath liquid:	0.5 % acetic acid in 50 % methanol at 4 µL/min
Drying gas flow:	5 L/min
Nebulizer:	10 psi
Drying gas temperature:	150 °C
Fragmentor:	175 V
V_{cap}:	3500 V
Accumulation time:	333.3 ms/spectrum
Accumulation rate:	3 spectra/s
MS/MS:	automatic, no masses excluded
Precursor threshold:	1000
Isolation width:	~4 *m/z*

Table 1
CE-MS conditions

Results and discussion

The tryptic digest of the mAb was applied to CE-MS analysis. Figure 1A shows the base peak electropherogram (BPE) obtained with an orthogonal coaxial sheath liquid CE-MS interface using a PVA coated capillary. All the peptides migrated before 20 min with very good resolution between the peaks. Peptide mapping of heavy and light chains resulted in 93% of protein sequence coverage with mass accuracy of 10 ppm. The glycopeptides migrated around 14.28 min (Figure 1A) and the corresponding mass spectrum is shown in Figure 1B with sequence. Using the MFE and the BioConfirm sequence editor, peptide masses from the CE-MS run were matched with the theoretical digest at a 10 ppm error with preferred glycosylation modifications included in the sequence of an antibody. Figure 2 shows a snapshot of the BioConfirm window for the trypsin digest of a mAb and the matched glycopeptide. The major form of modification corresponds to G0F.

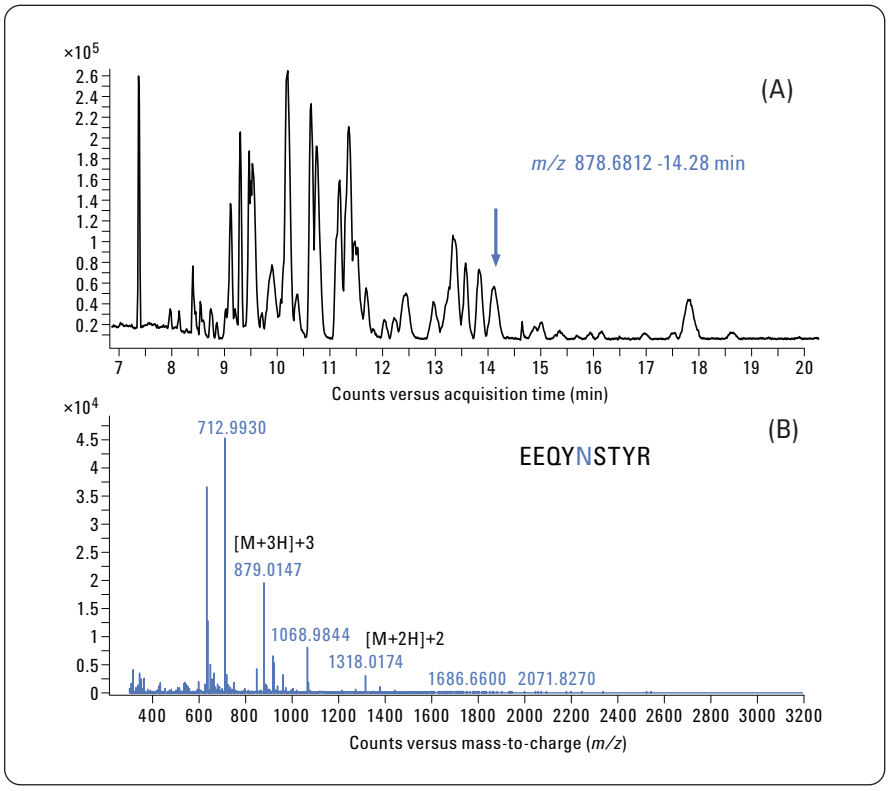

Figure 1
(A) Base peak electropherogram (BPE) of a trypsin-digested mAb using a PVA coated capillary. The arrow indicates the elution of the glycopeptide. (B) Mass spectrum of the glycopeptide with sequence.

Compound List

Show/Hide	RT	Mass	Height	Sequence	Seq Loc	Seq Name	Tgt Seq Mass	Pred Mods
☑	14.92	2957.1244	707	EEQYNSTYR	A(301-309)	Heavy Chain	2957.1443	1*G2F (NA2F)(+1768.6395)
☑	14.599	2795.0725	6223	EEQYNSTYR	A(301-309)	Heavy Chain	2795.0914	1*G1F(+1606.5867)
☑	14.282	2633.0226	18992	EEQYNSTYR	A(301-309)	Heavy Chain	2633.0386	1*G0F (NGA2F)(+1444.5339)
☑	3.544	1465.9302	184	ALPAPIEKTISKAK	A(335-348)	Heavy Chain	1465.8868	
☑	4.437	1266.7376	172	ALPAPIEKTISK	A(335-346)	Heavy Chain	1266.7547	
☑	5.275	1676.7697	177	FNWYVDGVEVHNAK	A(283-296)	Heavy Chain	1676.7947	
☑	5.904	1807.023	196	VVSVLTVLHQDWLNGK	A(310-325)	Heavy Chain	1806.9992	
☑	8.811	1188.4915	300	EEQYNSTYR	A(301-309)	Heavy Chain	1188.5047	

Minor forms

Major form

Figure 2
BioConfirm window in MassHunter Qualitative Analysis software showing the compound list of the matched tryptic digest with theoretically generated peptides of the mAb. The matched major glycopeptide is highlighted in the compound list with a red arrow. The orange colored arrows point to the minor forms of glycopeptides (G1F and G2F) observed in the tryptic digest of mAb.

Tracing the characteristic diagnostic sugar oxonium ion fragments (b-type oxonium ions) confirms the N-linked carbohydrate attachment. CE-MS/MS runs were performed to identify and confirm the presence of glycopeptides. With a mass error limit of 1 ppm, the glycopeptides were confirmed using the intense extracted ion electropherogram (EIC) at *m/z* 204.085 corresponding to the diagnostic sugar oxonium fragment ions as shown in Figure 3A. The CE-MS and CE-MS/MS runs show the same migration time for the peptide peak, which confirms the glycopeptides assignment. Figure 3B shows the product ion spectrum of the triply charged precursor of the glycopeptides. The MS/MS spectrum contains major carbohydrate ion fragments in addition to minor peptide ions. The intense peaks at the predicted *m/z* values of 204 and 366 confirm the glycan attachment. The peptide sequence (EEQYNSTYR) has only one asparagine residue/NXT motif, which is a known site of glycosylation in mAbs. Therefore, it was deduced that the glycan is attached to the asparagine residue of the NST motif in the peptide sequence. Further analysis of the MS/MS spectrum reveals the minor forms G1F and G2F modifications of this mAb (Table 2).

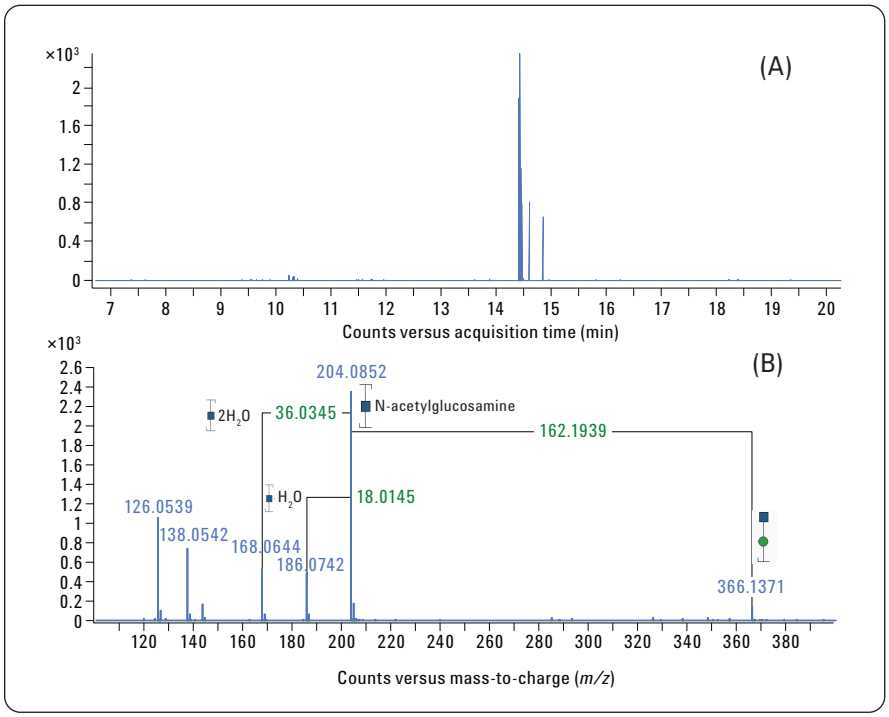

Figure 3
CE-MS/MS analysis of glycopeptides. (A) Extracted ion chromatogram MS (all) at *m/z* 204.085 for the trypsin-digested mAb using a PVA coated capillary. (B) Product ion mass spectra (*m/z* 878.68) of the glycopeptide. Observed major oxonium ions are annotated.

Code	Oligosaccharide structure	Average mass	Comments
G0F		1445.35	Major form found in this mAb
G1F		1607.49	Minor form found in this mAb
G2F		1769.64	Small amount found in this mAb

Galactose Mannose Fucose N-acetylglucosamine

Table 2
Structures of N-linked glycans identified by CE-MS analysis of mAb.

Conclusions

This Application Note demonstrated:

- Feasibility of the G7100 Agilent 6520 Accurate-Mass Q-TOF MS setup for biopharmaceutical analysis
- Rapid characterization of an mAb glycopeptide at low pmole levels using CE-MS
- Identification of major and minor glycan modifications of mAb with CE-MS/MS analysis
- The combination of CE technology with Q-TOF mass spectrometer is a valuable tool for peptide mapping of small quantity biopharmaceuticals, providing analytical power that enhances protein characterization studies

References

1.
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2.
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January 31, 2011
Publication Number 5990-7138EN

Agilent Technologies