Characterization of Glycosylation in the Fc Region of Therapeutic Recombinant Monoclonal Antibody

Application Note

BioPharma

Authors

James Martosella, Phu Duong, and Alex Zhu
Agilent Technologies, Inc.

Abstract

Biotherapeutic drugs, such as monoclonal antibodies, constitute the most rapidly growing drug class, and have become a major clinical success in human therapeutics over the past decade. However, the process of discovery and development of biotherapeutics poses throughput challenges that are different from those set by traditional small molecule drugs. They require more complicated manufacturing and characterization processes that need faster data turnaround times, yet they must be well characterized with regard to potency, identity, quality, purity, and stability. Thus the need for rapid and accurate profiling techniques is necessary. In this note we describe a fast and efficient liquid chromatography-TOF mass spectrometry approach to characterize an IgG derived from CHO cells. An Agilent Poroshell 300SB-C3, 5 µm superficially porous IgG1 column was used to obtain accurate glycoform masses of an intact IgG1, with a subsequent analysis of papain-digested IgG1 to obtain site-specific glycosylation profile information of the Fc region for further characterization of IgG1 heterogeneity. Additionally, a subsequent mAb-glyco chip LC/MS analysis was used to build a glycan accurate mass database for additional validation of the glycans identified in the TOF mass analysis.

Agilent Technologies

Introduction

Monoclonal antibodies (mAbs) are used increasingly as therapeutic agents to target various diseases, and form a large fraction of therapeutic proteins commercially approved or currently under development. With an increasing number of mAbs moving to market, the need for rapid analytical methods for their detailed characterization has dramatically increased [1]. For example, during mAb development it is important to find the recombinant production system that offers optimal productivity with rapid turnaround times to meet process development demands. Assessing the glycosylation profiles in shortened analysis times is vital to mAb development and the need to have rapid and reliable liquid chromotography-mass spectrometry (LC/MS) glycoprofiling methods are growing.

This application note describes a rapid online reversed-phase (RP) LC/MS approach to profile glycosylation for a therapeutic mAb (IgG1). Separations of intact IgG1 and papain-cleaved IgG1, to yield Fc and Fab fragments, were optimized for rapid analysis using an Agilent Poroshell 300SB-C3, 5 µm superficially porous column. Specifically, intact IgG1 was characterized to obtain glycoform and accurate glycan mass information. The IgG1 was then subjected to papain digestion and the glycosylation containing Fc portion analyzed by time-of-flight (TOF) mass spectrometry to determine glyco-specific modifications. The methods were developed to provide essential glycoform profile information using rapid LC/MS procedures for achieving quick data turnaround times. For further validation of the Fc glycan-specific assignments from the TOF analysis, the intact mAb was then subjected to a fully automated rapid glycan analysis. This was achieved using an mAb-glyco chip kit to establish a glycan accurate-mass database. The methods described herein are useful for cell line and clone selection processes, and cell culture process optimizations where absolute quantitation is not necessary, but where rapid, reliable and efficient characterizations are desired.

Experimental

Humanized monoclonal antibody derived from a Chinese hamster ovary (CHO) cell line was purchased from Creative Biolabs, Shirley, NY. Triflouroacetic acid was purchased from Sigma-Aldrich Corp., St. Louis, MO, and isopropanol, *n*-propanol and acetonitrile were supplied from Honeywell Burdick & Jackson, Muskegon, MI. The 1-propanol was purchased from VWR (p/n BJ322-4). Dialysis cassettes had a 3,500-Da protein MW cut-off (MWCO) and were purchased from Thermo Scientific (p/n 66330).

Papain digestion of mAb

To prepare the Fc and Fab fragments, a Pierce Fab Micro Preparation Kit (Thermo Scientific p/n 44685) was used. Final Fab and Fc cleanup used microcentrifugation with spin columns to separate the immobilized papain from the fragments. The final concentration was 2 µg/µL.

Intact IgG1 chromatographic conditions

Column:	Agilent Poroshell 300 SB-C3, 2.1 × 75 mm, 5 µm (p/n 660750-909)	
Eluent:	A: water (5% AcOH, 1.0% FA, 0.05% TFA) B: 70/20/10 IPA/ACN/water (5% AcOH, 1.0% FA, 0.05% TFA)	
Injection volume:	2 µL	
Flow rate:	1.0 mL/min	
Gradient:	Segmented	
	Time (min)	% B
	0	20
	4	45
	8	45
	9	90
	10	20
Temperature:	80 °C	
Detector:	UV, 280 nm	

Fc fragment chromatographic conditions

Same as intact IgG1 separation, except:

Injection volume:	5 µL (2 µg/µL)
Gradient:	20 to 50% B, 7 min, 50 to 90% B, 4 min, 90 to 20% B, 1 min (re-equilibration)
Detector:	DAD, 225 nm

Instrumentation

LC/MS

Agilent 1200 Infinity Series with auto injector (High Performance Autosampler), binary pump, thermostatted oven (TCC) and diode array detector (DAD) coupled to an Agilent 6224 TOF LC/MS.

Glycan library

Agilent 1200 Infinity HPLC-Chip/ MS system (Agilent mAb-Glyco Chip Kit) coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS.

6224 TOF instrument parameters

Spectra were recorded in positive ion and centroid modes.

Gas temperature:	350 °C
Drying gas:	10 L/min
Nebulizer:	45 psi
Vcap:	5,500 V (intact), 5,000 V (Fc)
Octupole 1 RF:	750 V
MS:	4 Hz
Mass range:	100 to 7,000 *m/z*
Ref. mass:	149.02332, 922.009798
Acquisition mode:	Extended dynamic range
Range mode:	2 GHz

MS data analysis

Data was processed using Agilent MassHunter Qualitative Analysis and MassHunter BioConfirm software.

Agilent mAb-Glyco Chip Kit

The kit incorporates the mAb-glyco chip (p/n G4240-64021), reagent pack, mAb-glyco chip content disk, and a quick-start guide. The reagent pack provides all chemicals needed for ready chip operation, that is, system conditioning reagent for flow path deactivation and carry over minimization, glycan standards for chromatographic checkout and method development, antibody standard for functional checkout and troubleshooting, and deglycosylation buffer for dilution of standards and samples, and for loading mAb samples onto the chip's enzyme reactor. Conditions for the glyco chip were; reactor (310 nL PNGase F), separation column (43 mm PGC, 5 µm), and enrichment column (160 nL PGC, 5 µm).

Results and Discussion

Analysis of intact IgG1

Reversed-phase separation of intact IgG1 can be challenging due to the large molecular size, diffusion limitations and increased hydrophobicity. For this reason, obtaining high speed separations with narrow and symmetrical peak shapes has become more challenging, primarily due to the diffusion paths of these molecules within the particle pore structure. More recently, superficially porous materials have gained attention to circumvent the problems associated with faster, large-molecule separations afforded by the thin layer of porous packing around a solid core particle. Decreasing the diffusion time and limiting the diffusion path will result in narrower peaks and more efficient separations for macromolecules such as mAbs. Here, the migration path of the protein (mAb) is shortened compared to that of a fully porous particle. Figure 1 shows how this migration can improve mAb separation during rapid run times.

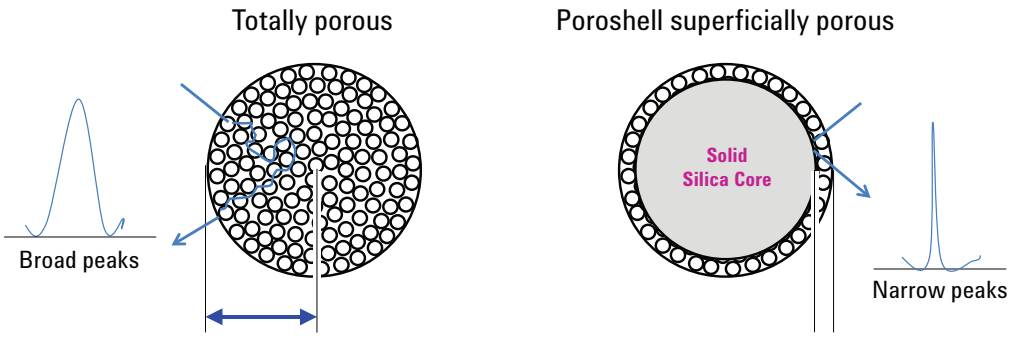


Figure 1. Biomolecule migration paths in totally porous and superficially porous particles.

For the separation of IgG1, a Poroshell 300 SB-C3 superficially porous column was used at elevated temperature, with carefully optimized conditions for highest MS analytical sensitivity, to enable a rapid and efficient separation (Figure 2A). Positive mode electrospray ionization (ESI) MS showed multiple peaks from 2,800 to 4,800 *m/z* corresponding to isoforms at different charge states (Figure 2B). These multiple charged envelopes were deconvoluted by the Agilent MassHunter software to obtain the molecular weight values of the different intact IgG1 glycoforms and to identify the accurate glycan masses derived from the IgG1 sequence data shown in Table 1.

The MS spectrum peaks are 140 to 150 Da apart. These data indicate that other sources of IgG1 heterogeneity are present and that the difference in mass observed is a sum effect of these different modifications, along with the various glycosylation forms.

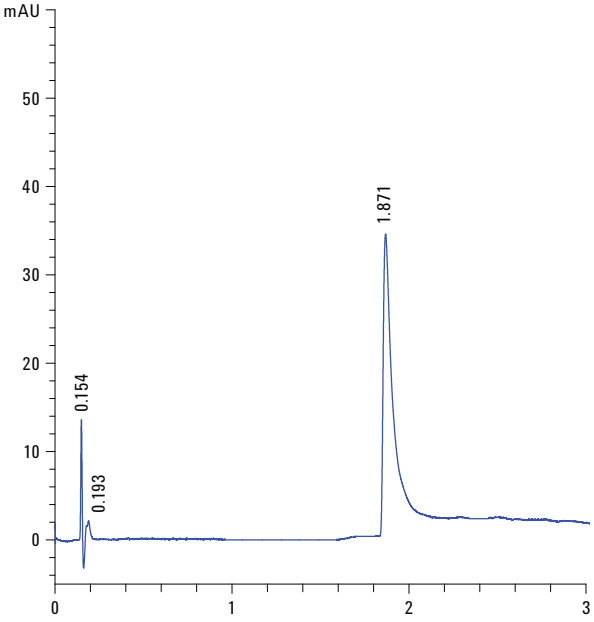


Figure 2A. LC/MS analysis of intact IgG1, with rapid reversed-phase separation using an Agilent Poroshell 300 SB-C3 LC column.

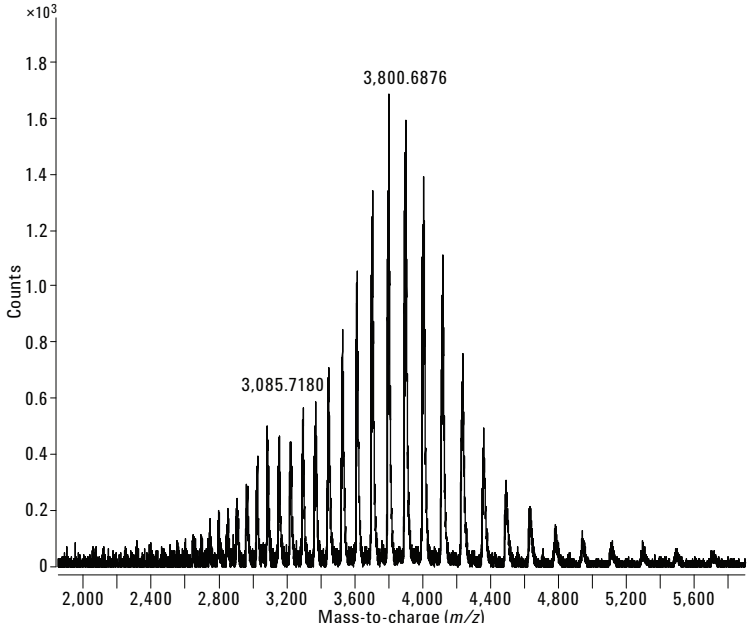
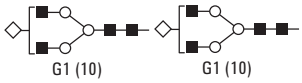
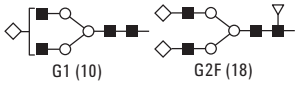
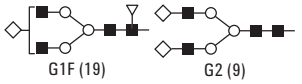
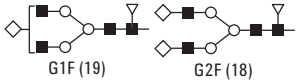


Figure 2B. ESI mass spectrum generated by averaging the MS scans across the main peak of the UV chromatogram in Figure 2A.

Table 1. Three major IgG1 glycoforms identified using MassHunter maximum entropy deconvolution from Figure 2B.

Intact mAb glycoform mass	Gylcan assignment	Structure	Accurate glycan mass
148253.5366	G1/G2		(+3084.8581)
	or G1/G2F		(+3231.0019)
148400.7426	G1F/G2		(+3231.0019)
148549.6014	G1F/G2F		(+3377.1458

Papain-digested IgG1 and Fc fragment analysis

To further profile the nature of the heterogeneity from the intact IgG1 analysis, the IgG1 was cleaved into its Fc and Fab fragments by papain digestion. Analysis of the Fc region bears a highly conserved N-glycosylation site. Glycosylation of the Fc fragment is essential for Fc receptor-mediated activity, and the N-glycans attached to this site are predominantly core-fucosylated biantennary structures of the complex type [2]. IgG1 cleavage with papain was used to clip below and above the hinge region to generate Fc and Fab fragments. Figure 3 shows the separation, and the conditions after cleavage in the reversed-phase chromatogram. In this separation, performed in under 3 minutes, two primary peaks at 2.4 and 2.7 minutes are shown, representing the Fc and Fab fragments, respectively. Additionally, there are smaller sets of partially resolved peaks at 2.1 to 2.5 minutes among the Fc region, representing variants of the Fc fragment.

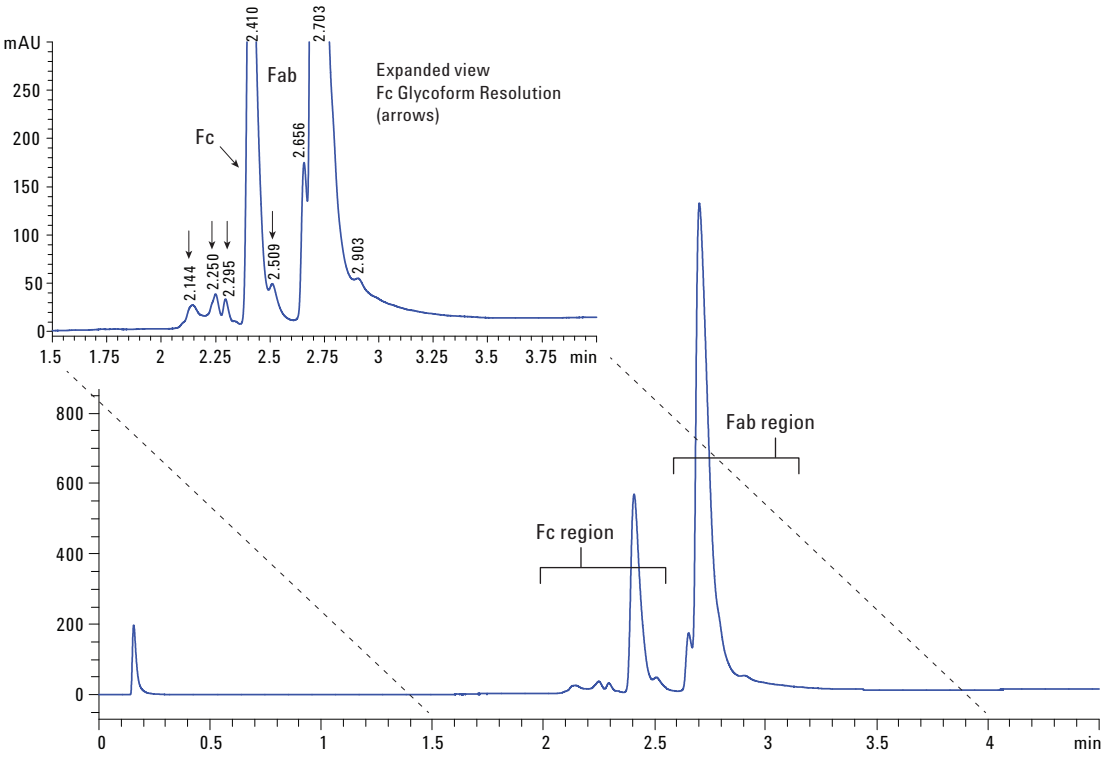


Figure 3. Reversed-phase separation of IgG1 after papain digestion showing two primary peaks of the Fc and Fab fragments. The inset details partially resolved peaks representing variants of the Fc and Fab fragments (arrows).

Figures 4A and 4B show the average mass spectrum and deconvoluted spectra of the primary Fc peak at 2.4 minutes. Fc with different glycans on it differ by a mass of 162 Da. Table 2 lists the glycosylation forms identified in the Fc fragmented IgG1. Fractionation of the intact IgG1 into Fab and Fc by papain digestion and the optimized rapid chromatographic reversed-phase separation of Fc have thus enabled a more efficient and rapid analysis of the glycoform distribution, demonstrating the value of the method for gaining relative estimations that can be used to assess glycosylation profiles.

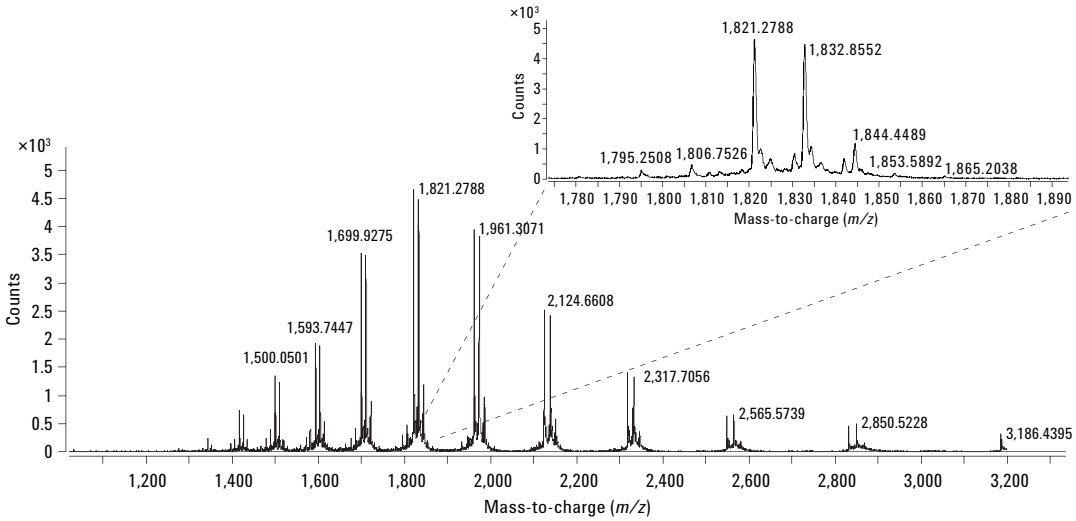


Figure 4A. Fc peak average mass spectrum showing charge distribution profile from 1,400 to 3,200 *m/z*. The inset is a zoomed spectrum of the +14 charge state. The main peaks correspond to different glycoforms.

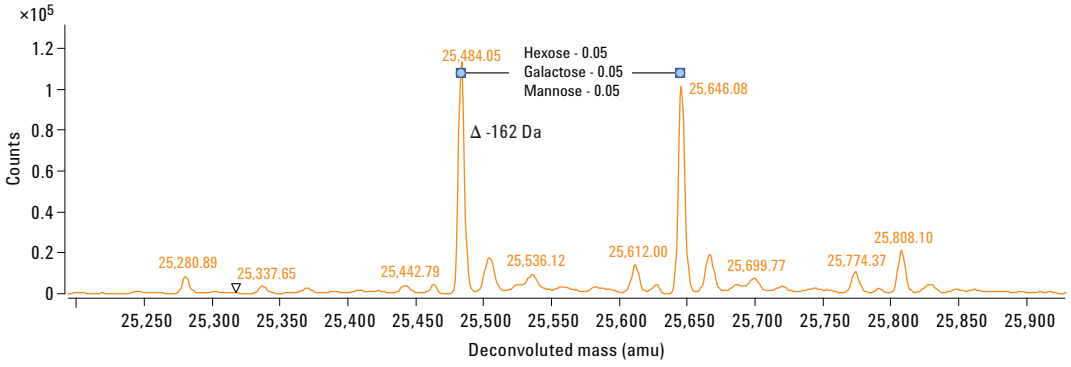


Figure 4B. Deconvoluted ESI mass spectra of an IgG1 Fc fragment generated from the chromatographic peak at 2.4 minutes in the chromatogram shown in Figure 3, representing glycoforms regularly spaced by hexose units (Δ *m/z* = 162).

Table 2. TOF LC/MS Fc fragment glycan assignments.

Glycan assignment	Accurate glycan mass
2010 0A 0G (G1)	(+1,461.3574)
2010 0A 0G (G2) (NA2)	(+1,623.5007)
2110 0A 0G (G1F) (NA2)	(+1,607.5012)
2110 0A 0G (G1F) (NA2)	(+1,769.6445)
2030 0A 0G	(+1,785.6439)
1010 0A 0G	(+1,258.1613)
1000 0A 0G	(+1,096.0181)

To validate the glycan assignments obtained from the Fc fragment analysis, we performed rapid chip-based online glycan analysis that enriches the released glycans cleaved from the intact mAb and then separates them prior to nanospray ionization-TOF analysis. A glycan accurate mass database was then established (Table 3) allowing quick assignment and validation of the glycans identified in the TOF mass analysis performed on the Fc region of the papain digested mAb (Table 2). This additional investigation provided a fast complementary structure analysis while giving a higher confidence in the glycan characterizations.

Table 3. Intact glyco-chip glycan results.

Glycan assignment	Accurate glycan mass
2010 0A 0G (G1)	1,461.3574
2110 0A 0G (G1F)	1,607.5012
2020 0A 0G (G2)	1,623.5007
2120 0A 0G (G2F)	1,769.6445
2030 0A 0G	1,785.6439
1000 0A 0G	1,096.0181
1010 0A 0G	1,258.1613
2000 0A 0G	1,299.2141

Conclusions

A rapid LC/MS-based approach was developed for the glycosylation profiling of a recombinant therapeutic monoclonal antibody. Optimized high resolution fast separation by an Agilent 5 µm PoroshellI 300SB-C3 column enabled sensitive characterization of an intact mAb (IgG1) and its Fc fragment after papain digestion. Coupled with TOF MS analysis, this application note demonstrates fast and effective characterization of mAb glycosylation. Additionally, we have validated the TOF Fc region glycan analysis using mAb-glyco chip LC/MS analysis on the intact mAb through glycan cleavage.

Although this method provides only relative estimation and not absolute quantitation, it gives sufficient information and is useful for assessing the glycosylation profile of a monoclonal antibody. The rapid separation and data turnaround time afforded by the LC/MS TOF analysis can facilitate biopharma product quality analyses, such as glycosylation profiling, during process optimization studies, and additionally provides an alternative to peptide mapping validation that can slow productivity times.

References

1. A. Lim, A. Reed-Bogan, B. J. Harmon. *Anal. Biochem*. **375**, 163 (2008).

2. H. S. Gadgil, P. V. Bondarenko, G. D. Pipes, T. M. Dillon, D. Banks, J. Abel, G. R. Kleemann, M. J. Treuheit. *Anal. Biochem*. **355**, 165 (2006).

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

For Research Use Only. Not for use in diagnostic procedures.

Agilent shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance, or use of this material.

Information, descriptions, and specifications in this publication are subject to change without notice.

© Agilent Technologies, Inc., 2013, 2017
November 2, 2017
5991-2323EN

Agilent Technologies