Separation of Native Monoclonal Antibodies and Identification of Charge Variants:

Teamwork of the Agilent 3100 OFFGEL Fractionator, Agilent 2100 Bioanalyzer and Agilent LC/MS Systems

Application Note

Biosimilar and Biotherapeutics

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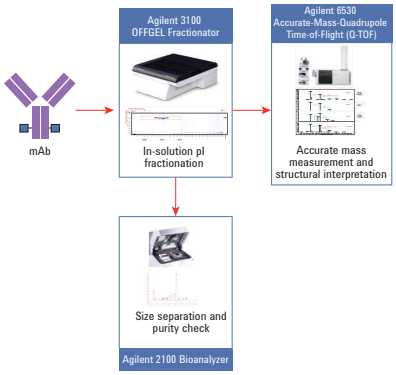
Abstract

Charge variants due to post-translational modifications (PTM) are introduced at various stages of monoclonal antibody (mAb) production and storage. This heterogeneity affects the efficacy, stability, and may also cause adverse immunological reactions. In this work, Agilent equipment was combined to create a workflow solution for the analysis of charge variants of mAb. We used the Agilent 3100 OFFGEL Fractionator, an isoelectric focusing device, to separate mAb variants in solution. The Agilent 2100 Bioanalyzer system was employed to observe the size and distribution of proteins in the resulting fractions. Ultimately, the fractions yielded on the OFFGEL were individually subjected to LC/MS analysis on an Agilent 6530 Accurate-Mass-Quadrupole Time-of-Flight (Q-TOF) instrument to identify potential charge variants. The combination of fractionation, intermediate controls, and accurate mass determination at high resolution makes this solution a valuable tool for characterization and quality control (QC) analysis of mAbs for the biopharmaceutical research and development.



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Introduction

Monoclonal antibodies (mAbs) are the most rapidly growing class of biotherapeutics for the treatment of various diseases. To use a mAb as a therapeutic substance, biological safety, quality, purity, and performance characteristics must be assessed during manufacturing[1]. During biosynthesis and subsequent processing, mAbs can undergo various modifications such as lysine truncation, deamidation, oxidation, glycosylation, and so forth, that lead to heterogeneity, such as charge variants. These changes may impact their stability and activity, with the potential to cause immunologically adverse reactions. Thus, there is a substantial need for powerful analytical methods. These methods are necessary to fully characterize the mAb charge variants in drug development and production while also serving in biopharmaceutical industry[2] quality control (QC) steps.

Although cation exchange chromatography is a method of choice to monitor charge variants, higher order heterogeneity could be illustrated using a panel of techniques. Earlier studies have demonstrated the use of analytical capillary isoelectric focusing (cIEF) for the characterization of mAb charge variants[3,4,5]. This Application Note describes the resolving power of the Agilent 3100 OFFGEL Fractionator using IPG strips. It permits pI-based separation (first dimension) of larger amounts of mAb charge variants in liquid phase samples[6]. In contrast to alternative methods, it can be customized to work with native proteins and preparative characterization. In the second dimension, a microfluidic lab-on-chip protein sizing method was used with the Agilent 2100 Bioanalyzer system to assess the OFFGEL fractions. The fractions generated by OFFGEL separation were subjected to LC/MS analysis using an Agilent 6530 Accurate-Mass-Quadrupole Time-of-Flight (Q-TOF) to identify the charge variants with high confidence.

Materials and Methods

Chemicals

The mAb was a proprietary therapeutic molecule. The focusing IPG buffer pH 6–11 and 24-cm long Immobiline DryStrip, pH 6–9 were obtained from GE Healthcare (Sweden). All the chemicals and solvents used were HPLC grade, and high purity water from Milli Q water purification system (Millipore Elix 10 model, USA) was used.

3100 OFFGEL Fractionator

For IEF, the 3100 OFFGEL Fractionator was used[7], with a setup giving high resolution (3100 OFFGEL 24-well frame set) in the employed pI range of 6–9 with customized conditions. The mAb sample (10 mg) was added to the OFFGEL focusing buffer containing 5 % glycerol and 0.25 % IPG buffer pH 6–11 (sample load). Neither DTT, urea, nor thiourea were added to maintain native conditions because mAbs can easily disintegrate under urea/thiourea denaturing conditions. A 150 µL amount of this sample was loaded on each of the 24 wells. OFFGEL fractionation was performed using the default method for protein samples and 24 fractions (OG24PR01). The recovered fractions were subjected to the 2100 Bioanalyzer and 6530 Accurate-Mass-Quadrupole Time-of-Flight analysis. Fractions obtained after the OFFGEL runs were compatible with an Agilent Protein 230 kit (P230) on the 2100 Bioanalyzer system and for LC/MS analysis without a further desalting step.

2100 Bioanalyzer system

Protein analysis with the 2100 Bioanalyzer system running 10 samples at a time (25 minutes total) on the Protein 230 kit was performed as described in the Kit Guide[8]. In brief, 4 µL of OFFGEL fractions were mixed with 2 µL of sample buffer (nonreducing conditions), heat denatured (95° C, 5 minutes), and then diluted with 84 µL of water applied to the protein chip (6 µL) for analysis.

LC/MS

LC/MS analyses were performed on an Agilent 1290 Infinity LC System coupled to a 6530 Accurate-Mass-Quadrupole Time-of-Flight (Q-TOF) LC/MS. OFFGEL fractions were subjected to reduction with 50 mM DTT (55° C for 30 minutes), and analyzed by LC/MS. Table 1 summarizes the LC and MS parameters used for analysis of the reduced mAb. The Peak Modeling (pMod) deconvolution algorithm was used to obtain a zero-charge spectrum of the mAb. The pMod deconvolution algorithm first deconvolutes the raw spectrum using maximum entropy deconvolution. In addition, it automatically allows different peak models to fit and validate the maximum entropy deconvolution results[9].

Software

- Agilent MassHunter Acquisition, Qualitative Analysis software and MassHunter BioConfirm (LC/MS)
- Agilent 2100 Expert (Bioanalyzer)

Results and Discussion

Figure 1 shows a Bioanalyzer gel-like image for OFFGEL fractions along with the sample load. Intact, nonreduced mAb at approximately 150 kDa were found in multiple fractions around well 8. This represents the most abundant amount, and likely the main charge variant. Fractions detected between 3 to 7 were termed as acidic, and those between 9 to 12 were termed as basic variants respectively.

Given other charge variants are separated by OFFGEL fractionation, they span a pI range of approximately 6.2 to 7.4, and are available at concentrations suitable for LC/MS analysis.

The results demonstrate the feasibility and utility of OFFGEL fractionation of proteins under native conditions, combined with subsequent Bioanalyzer analysis. This approach enables good separation in two dimensions and convenient detection of charge variants and potential impurities.

Table 1. LC/MS parameters used for reduced mAb analysis.

LC parameters	
Column	Agilent Poroshell 300 SB-C8 2.1 × 75 mm, 5 µm
Flow rate	1.2 mL/min
Temperature	65 °C
Mobile phase A	0.1 % formic acid in water
Mobile phase B	80 % *n*-propyl alcohol, 10 % ACN, 9.9 % water, and 0.1 % formic acid
Injection volume	2 µL
Pump mode	Gradient Time (min) %B Flow (mL) 1 25 1.2 5 28 1.2 8 40 1.2 9 40 1.2 10 80 1.2 10.1 20 1.2
Q-TOF MS parameters	
MS	Agilent 6530 Q-TOF MS
Ion source	AJS (ESI)
Polarity	Positive
Mass range	500–3,000 *m/z* (1 GHz)
Drying gas temperature	350 °C
Drying gas flow rate	8 L/min
Nebulizer	40 psig
Sheath gas temperature	350 °C
Sheath gas flow rate	11 L/min
Vcap	4,200 V
Fragmentor	300 V
Nozzle voltage	1,000 V

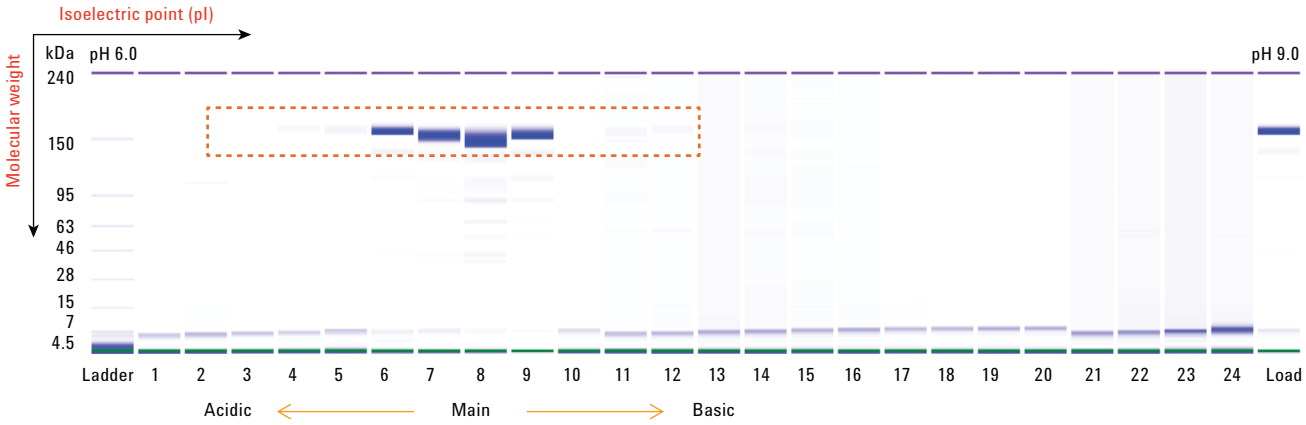

Figure 1. Protein OFFGEL fractionation analysis of mAb followed by Agilent 2100 Bioanalyzer analysis with the Agilent Protein 230 Assay.

To further characterize the charge variants, the OFFGEL Fractions (3 to 12) were subjected to LC/MS analysis after reduction. Figure 2 shows the deconvoluted spectra of the heavy chain for Fractions 6, 8, and 12 along with sample load. The Fraction 8 (major mAb form) and sample load show similar mass spectral profiles. However, a mass difference of ~128 Da observed in Fraction 12 indicated the presence of untruncated C-terminal lysine. In the acidic region, Fraction 6 showed an ~291 Da shift, corresponding to the presence of glycan-containing sialic acid. This is assigned to mono-sialylated-, galactosylated biantennary, substituted with fucose (G1FS1). These results demonstrate the enrichment of exemplary acidic and basic charge variants using the OFFGEL Fractionator.

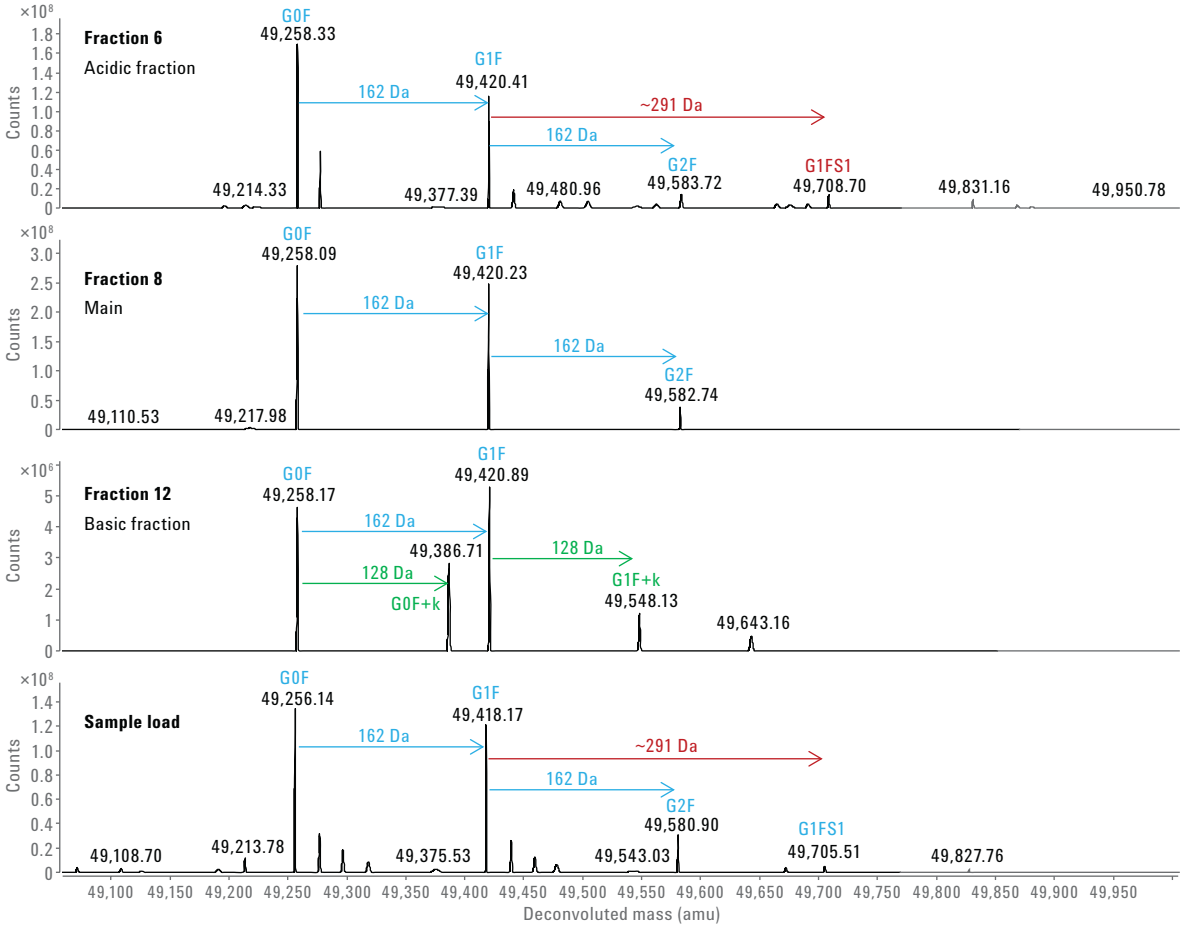

Figure 2. Deconvoluted spectra (pMod) of heavy chain (Fractions 6, 8, 12, and sample load).

Conclusion

This Application Note demonstrates a workflow solution employing various Agilent platform technologies to achieve a higher order characterization of charge variants in mAb.

- The resolving power of the Agilent 3100 OFFGEL Fractionator allows separation and enrichment of mAb charge variants, such as C-terminal lysine truncation and mAb with sialylated glycan into different fractions following a modified OFFGEL protocol. The preparative method provides fractionation in the liquid phase, and the fractions can be directly analyzed by Bioanalyzer and LC/MS for further characterization of charge variants.

- The Agilent 2100 Bioanalyzer system provides a quick QC tool for molecular weight determination of antibody variants in the OFFGEL fractions.

- The Agilent 6530 Accurate-Mass-Quadrupole Time-of-Flight (Q-TOF) provides detailed characterization of charge variants at the molecular level.

References

1. http://www.fda.gov/cder/guidance/

2. Khawli, L. A; *et al.* Charge variants in IgG1. *mAb* **2010**, *2:6*, pp 613-624.

3. Meert. C. D; *et al.* Characterization of Antibody Charge Heterogeneity Resolved by Preparative Immobilized pH Gradients. *Anal. Chem*. **2010**, *82*, pp 3510-3518.

4. Wenz, C. Monoclonal Antibody Charge Heterogeneity Analysis by Capillary Isoelectric Focusing on the Agilent 7100 Capillary Electrophoresis System, *Agilent Technologies Application Note*, publication number 5991-1142EN.

5. Wenz, C. Monoclonal Antibody Charge Heterogeneity Analysis by Capillary Isoelectric Focusing on Fluorocarbon Coated Capillaries, *Agilent Technologies Application Note*, publication number 5991-2885EN.

6. Hörth, P; *et al.* Efficient fractionation and improved protein identification by peptide OFFGEL electrophoresis. *Mol. Cell Proteomics* **2006**, *5*, pp 1968-1974.

7. Suresh Babu, C. V. Monitoring antibody charge variants using a combination of Agilent 3100 OFFGEL Fractionation by isoelectric point and high sensitivity protein detection with the Agilent 2100 Bioanalyzer, *Agilent Technologies Application Note*, publication number 5990-6521EN.

8. Agilent Protein 230 Kit Quick Start Guide, *Agilent Technologies Manual*, publication number G2938-90055.

9. Ning, T; *et al*. pMod – An Advanced Protein Deconvolution Algorithm with Automated Peak Modeling for Charge Deconvolution of Mass Spectrometry Data, *Agilent Technologies Application Note*, publication number 5991-2225EN.

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