

Process Monitoring of Monoclonal Antibody Subunits for Fast, Critical Quality Attribute Monitoring Using the ACQUITY QDa Detector

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Abstract

In this work, we demonstrate the applicability of the ACQUITY QDa Detector for reliable mass determination during subunit analysis of protein-based therapeutics. Routine monitoring of subunit data offers timely detection of variations in manufacturing processes, and can be used to screen for batch-to-batch consistency.

Benefits

- Complementary mass data for subunit analysis using MaxEnt deconvolution algorithm in MassLynx. Software
- Routine monitoring of subunit data offers timely detection of variations in manufacturing processes for added cost savings
- Profiling of subunit glycoforms for rapid screening of batch-to-batch consistency

Introduction

Protein-based therapeutics represent a continuously growing class of pharmaceutical products. Because their production is a complex process, a variety of testing is required to monitor critical quality attributes (CQAs) throughout the development and manufacturing process to ensure product quality and safety. Studying protein glycosylation has become an important quality attribute, because galactosylation has been linked to cytotoxicity in some marketed recombinant monoclonal antibodies (mAbs).¹ Analysis methods can include fluorescence and MS-based release-and-label techniques, and “bottom-up” approaches to monitor individual glycans or glycopeptides, respectively. Alternatively, a “middle-down” strategy can be used to assess major glycoforms of mAb subunits. This latter approach is of particular importance in a manufacturing environment, where identifying variations in the glycosylation pattern could ideally be reported and corrected in a timely and cost-effective manner.

The ACQUITY QDa Detector has been established as a cost-effective tool for obtaining mass spectral data within existing LC workflows when used as an orthogonal detection technique. The scope of the current study is to evaluate the applicability of the ACQUITY QDa Detector for subunit analysis. Trastuzumab, an IgG1, can be partially reduced using dithiothreitol (DTT) to its ~25 kDa light chain and ~50 kDa heavy chain fragments (Figure 1A). Acquisition using MassLynx Software provides the ability to interpret complex spectra through the MaxEnt1 deconvolution algorithm in order to determine the molecular weight for each subunit. An IdeS digest and reduction can be used to produce three fragments, each ~25 kDa (Figure 1B). Because an IdeS digest provides specific and efficient cleavage at a single site over the antibody hinge region, it has become a popular characterization tool for therapeutic mAbs – and in this case, provides an alternative route for monitoring the major glycoforms.

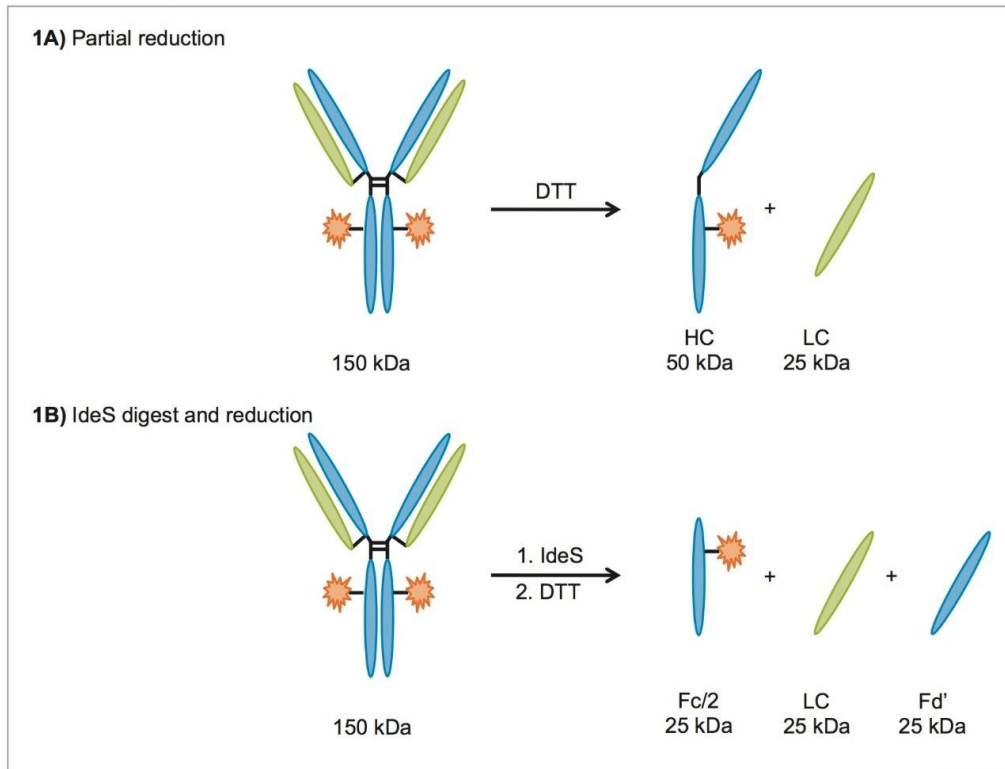


Figure 1. Formation of subunits. 1A) Partial reduction using DTT reduces the bonds holding the heavy chain (HC) and light chain (LC) together. 1B) IdeS digest and reduction results in three fragments: Fc/2, LC, and Fd', each of which is approximately 25 kDa in size. Glycosylation takes place in the Fc region, thus allowing the glycosylation profile to be monitored through mass detection of the heavy chain or Fc/2 fragments.

Experimental

LC Conditions

LC system:

ACQUITY UPLC H-Class System*

| | |
|------------------------|-----------------------------------------------------------------------------------|
| Absorption wavelength: | 214 nm |
| Sampling rate: | 5 Hz |
| Column: | ACQUITY UPLC Protein BEH C ₄ , 1.7 μm, 2.1 mm x 50 mm (p/n: 186004495) |
| Column temp.: | 80 °C |
| Mobile phase A: | Water with 0.1% (v/v) formic acid |
| Mobile phase B: | Acetonitrile with 0.1% (v/v) formic acid |
| Sample temp.: | 10 °C |
| Injection volume: | 3 μL |

better separation could be achieved with an ACQUITY Premier System

Gradient:

| Time (min) | Flow rate (mL/min) | %A | %B | %C | %D |
|------------|--------------------|----|----|----|----|
| Initial | 0.4 | 95 | 5 | 0 | 0 |
| 15 | 0.4 | 40 | 60 | 0 | 0 |
| 20 | 0.4 | 20 | 80 | 0 | 0 |
| 23 | 0.4 | 20 | 80 | 0 | 0 |

| | | | | | |
|----|-----|----|---|---|---|
| 27 | 0.4 | 95 | 5 | 0 | 0 |
| 30 | 0.4 | 95 | 5 | 0 | 0 |

QDa settings

| | |
|--------------------|-----------------|
| Sample rate: | 5 Hz |
| Mass range: | 350–1250 Da |
| Ionization mode: | ESI+, continuum |
| Cone voltage: | 15 V |
| Capillary voltage: | 1.5 kV |
| Probe temp.: | 600 °C |

Data management

MassLynx v. 4.1 Software with MaxEnt1

Partial reduction of trastuzumab:

A 10 µL aliquot of 21 mg/mL trastuzumab was added to 190 µL of 25 mM Tris-HCl, pH 7.5 to give an approximate protein concentration of 1 mg/mL. Concentrated DTT was added to the sample to give a final DTT concentration of 1.0 mM in solution. The sample was then incubated at 37 °C for 20 minutes. An equal volume of 3% acetonitrile and 0.1% formic acid in water was added to yield an estimated final protein concentration of 0.5 mg/mL. The sample was injected immediately because this preparation is not intended for long-term storage.

IdeS digest and reduction of trastuzumab:

A 1 mg/mL sample of trastuzumab in 25 mM Tris buffer was incubated with IdeS protease (FabRICATOR; Genovis, Cambridge, MA) at 37 °C for 30 minutes. Concentrated DTT was added to the IdeS digested sample to

give a final DTT concentration of 1.0 mM in solution. The resulting sample was incubated at 37 °C for 20 minutes. An equal volume of sample to 3% acetonitrile and 0.1% formic acid in water was prepared prior to injection to give an estimated final protein concentration of 0.5 mg/mL.

Results and Discussion

To obtain data for the light and heavy chains of trastuzumab, the antibody was partially reduced as described above. The optical trace can be seen in Figure 2A. To determine the mass of each fragment, the MaxEnt1 algorithm of the MassLynx Software was used to deconvolute the mass data to a zero charge state. MaxEnt1 resolution was set to 0.5 Da/channel with a peak width of 0.7 Da using the uniform Gaussian model. Spectra were combined from the middle of the peak of interest to the baseline. The maximum number of iterations used was 20 in order to avoid over-processing the data. Figures 2B and 2C show the deconvoluted spectra of the light chain and heavy chain subunits, respectively. The deconvolution of the light chain collapses the charge envelope to a single peak while the deconvolution of the heavy chain identifies four major glycoforms.

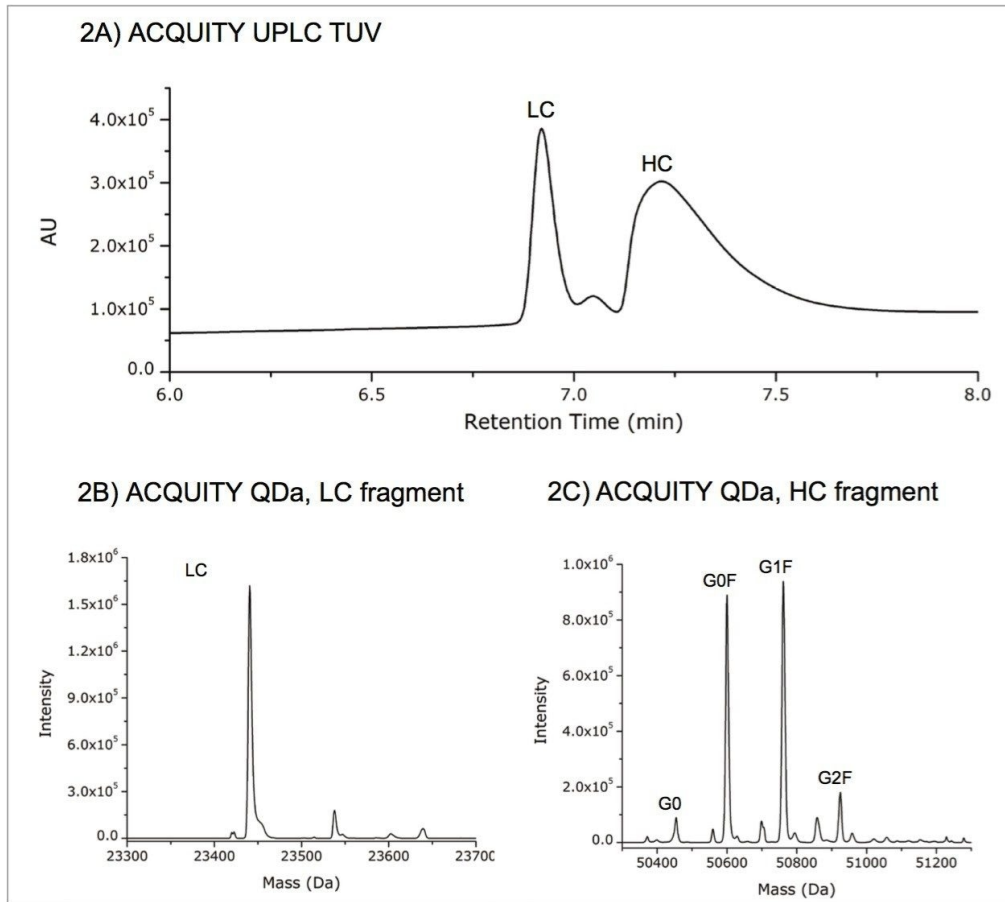


Figure 2. Partial reduction of trastuzumab. 2A) Optical trace showing separation of the light chain (LC) and heavy chain (HC) fragments. 2B) and 2C) Corresponding mass data acquired from an ACQUITY QDa Detector. The MaxEnt1 algorithm was used for deconvolution of the light chain and heavy chain fragments. The light chain collapses to a single charge state while the heavy chain contains four glycoforms.

Because the mass range of the ACQUITY QDa Detector is 30–1250 m/z , the lower charge states for both the light chain and heavy chain are outside of this detection window. Figure 3 overlays the charge envelope of the light chain from the ACQUITY QDa data with a simulated charge envelope from a high-resolution MS having a greater scan range. From the overlay, the ACQUITY QDa data represents only a portion of the charge envelope produced from a high-resolution instrument. To determine the bearing this has on mass determination, the results generated by MaxEnt1 for the light chain, as well as for the heavy chain, are reported in Table 1 as experimental mass, and are compared to the theoretical average molecular weight.

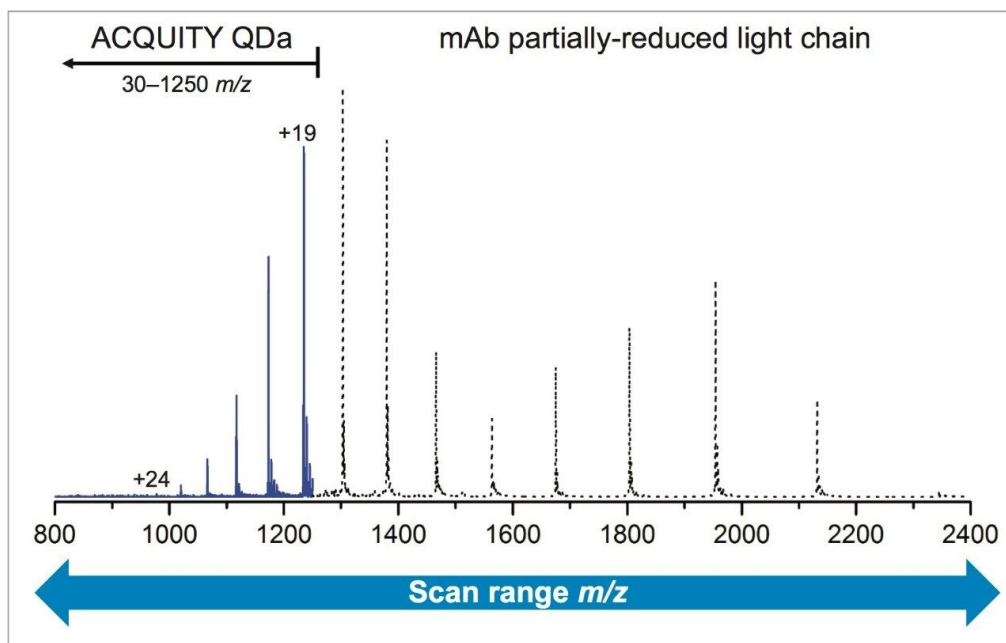


Figure 3. Overlay of light chain charge envelopes. The ACQUITY QDa Detector (blue) covers a fraction of the charge envelope generated from a high-resolution MS (dotted). The ACQUITY QDa data covers the +19 to +24 charge states.

| | Light chain | Partial reduction | | | | IdeS digest and reduction | | | |
|------------------------|-------------|-------------------|---------|---------|---------|---------------------------|---------|---------|---------|
| | | Heavy chain | | | | Fc/2 | | | |
| | | G0 | G0F | G1F | G2F | G0 | G0F | G1F | G2F |
| Average mass (Da) | 23439.1 | 50447.7 | 50593.8 | 50756.0 | 50918.1 | 25090.2 | 25236.3 | 25398.4 | 25560.6 |
| Experimental mass (Da) | 23440.5 | 50454.5 | 50600.0 | 50761.5 | 50925.0 | 25090.0 | 25235.0 | 25397.5 | 25560.0 |
| Δ mass (Da) | 1.4 | 6.8 | 6.2 | 5.5 | 6.9 | -0.2 | -1.3 | -0.9 | -0.6 |
| Mass accuracy (ppm) | 60 | 135 | 123 | 108 | 136 | -8 | -52 | -35 | -23 |

Table 1. Comparison of average molecular mass to experimental mass as determined by deconvolution using MaxEnt1 for the partial reduction of trastuzumab and an IdeS digestion and reduction of trastuzumab. Mass accuracy is given by $(\Delta \text{ Mass}/\text{Average Mass}) * 106$.

Instrument contributions to mass error are proportional to charge state where increased error is associated with higher charge states. For example, consider the charge states spanning from +19 to +50, which covers the range

of observed charge states for both the light chain and heavy chain charge envelopes acquired using the ACQUITY QDa Detector. The instrument mass error associated with these charge states ranges from ± 3.8 Da to ± 10 Da ($\pm 0.2 \times$ charge state). If we consider the light chain of trastuzumab, which has charge states ranging from +19 to +24, the expected mass accuracy ranges from 150 ppm to 200 ppm. Because the reported experimental mass accuracy of the light chain is 60 ppm (Table 1), this value easily falls within the instrument's expected range. Likewise, the mass accuracy of the heavy chain should fall within 150 to 200 ppm, and all fragments meet these criteria as well (Table 1).

To further test the applicability of the ACQUITY QDa Detector for subunit analysis, the commonly-used IdeS enzyme was employed to provide a second method for generating subunit fragments. By performing an IdeS digest and reduction, three fragments – Fc/2, LC, and Fd' – were produced, each ~25 kDa in size. Figure 4A shows the optical trace of the IdeS digested and reduced sample of trastuzumab, with each of the three fragments clearly resolved. Figure 4B shows deconvoluted mass data of the Fc/2 fragment which contains the glycosylation site, and thus shows the same four major glycoforms previously identified from the heavy chain data. Table 1 reports the mass accuracy of the glycoforms from the Fc/2 fragment, which also fall within the expected range of the instrument. Like partial reduction, an IdeS digest and reduction allows for product confirmation of the desired product, and variations to the production processes can be more readily determined.

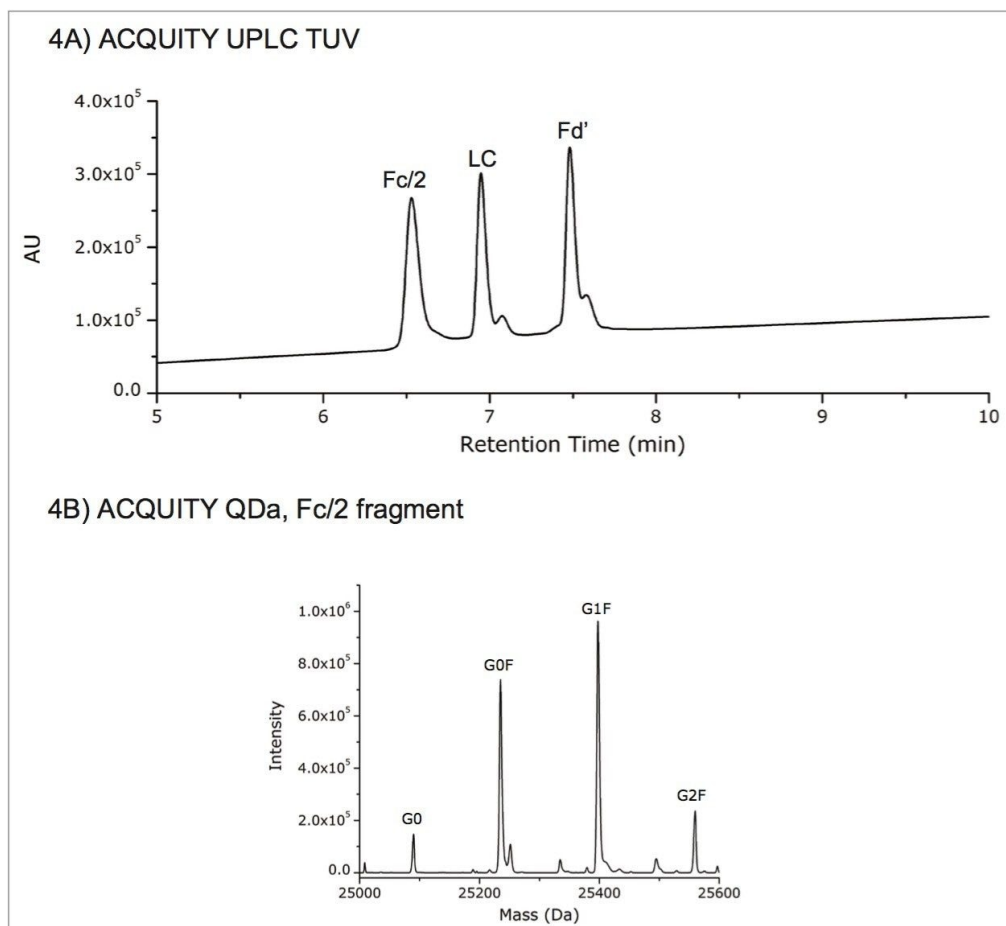


Figure 4. IdeS digest and reduction of trastuzumab. 4A) Optical trace showing separation of Fc/2, LC, and Fd' fragments. 4B) Corresponding mass data acquired from an ACQUITY QDa Detector. Deconvolution identifies four Fc/2 glycoforms.

Conclusion

It was demonstrated that the ACQUITY QDa Detector can be used for reliable mass determination at the subunit level via two different avenues. A sample of trastuzumab was partially reduced to yield light chain and heavy chain fragments that fell well within the expected limits of mass accuracy for the instrument. An IdeS digest and reduction was used to generate smaller antibody subunit fragments with lower charge states. By monitoring

major glycoforms on both the ~50 kDa heavy chain fragment and the ~25 kDa Fc/2 fragment from the IdeS digest and reduction, results can be compared between the two treatments. Both methods take advantage of the ACQUITY QDa Detector for providing a quick and effective way to collect subunit data, which could ultimately be used in a screening protocol for verifying product consistency and identity.

References

1. Raju T.S. and Jordan R.E. Galactosylation variations in marketed therapeutic antibodies. *mAbs*. 2012. 4(3):385–391.
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