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Intact Mass Analysis of Therapeutic Proteins

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Mass Analysis is routinely performed during the characterization of proteins or as an identity test in the pharmaceutical industry to confirm the structure of a biotherapeutic protein. To achieve maximum accuracy, mass analysis is most often performed by Mass Spectrometry (MS) using instruments such as a Quadrupole Time-of-Flight (QTOF) or an Extended Mass Range Orbitrap™ after either Matrix Assisted Laser Desorption Ionization (MALDI) or Electrospray Ionization (ESI). The latter technique is more commonly used because it produces highly-charged ions that can be scanned at lower m/z values, providing superior mass accuracy and resolution with minimal sample preparation. Large proteins such as Monoclonal Antibodies (mAbs) and Antibody Drug Conjugates (ADCs) can easily be analyzed using ESI-MS giving information concerning the integrity of the amino acid sequence, the identity of various isoforms and glycoforms, and the presence of molecular modifications.

Mass Analysis is usually done after a chromatographic separation is used to isolate the protein from salts and detergents that may be present in the formulation. Sample preparation normally requires only a dilution if the protein is at a sufficient concentration (approximately greater than 10 mg/mL). However, if the sample is at a low concentration and if a surfactant is present, a cleanup step and enrichment using Solid Phase Extraction (SPE) or Molecular Weight Cut off Filters (MWCO) may be required. A reversed-phase separation is most commonly used with aqueous mobile phases of water and an organic phase of either acetonitrile or a short chain alcohol such as n-propanol. Usually either Formic Acid or Trifluoroacetic Acid (TFA) is added to aid in ionization, or in the latter case, for ion pairing to aid in retention and to increase peak sharpening. Although TFA aids in improving chromatographic separation and is more amenable to UV detection, it suppresses electrospray ionization and causes a loss of response. Size Exclusion Chromatography (SEC) and Hydrophilic Interaction Chromatography (HILIC) can easily be applied since mobile phases used for those separations are usually compatible with ESI-MS.

Although reversed-phase chromatography can be used to isolate intact proteins, it offers little help in reducing the heterogeneity of a mAb and in resolving various isoforms that comprise the intact molecule. If smaller fragments of the mAb or subunits are present, they can usually be resolved from the main peak by using a shallow gradient. Reversed-phase chromatography also has the disadvantage of containing an organic solvent that will denature the protein and can even cause fragmentation or precipitation.

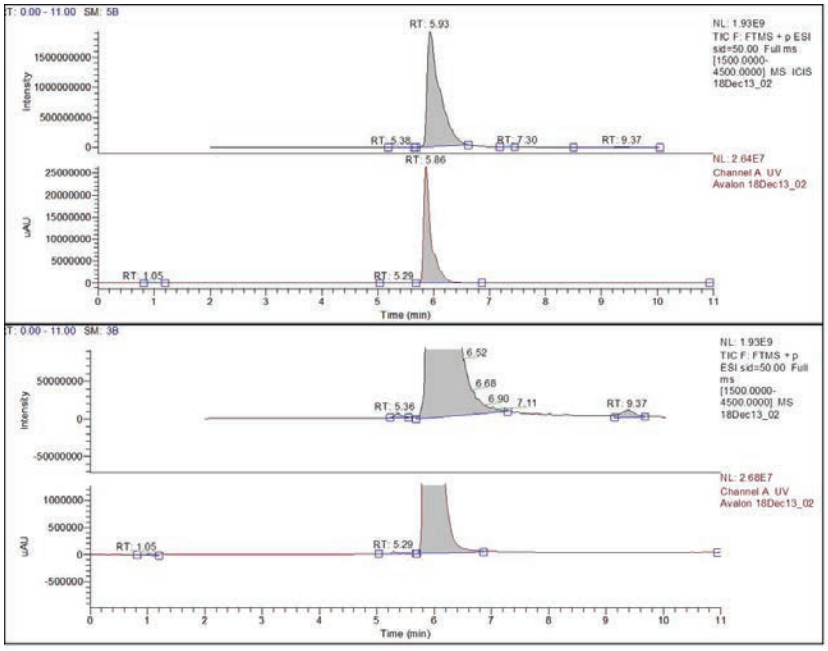

Figure 1 Example Chromatogram of Innovator Monoclonal Antibody Adalimumab (Humira)

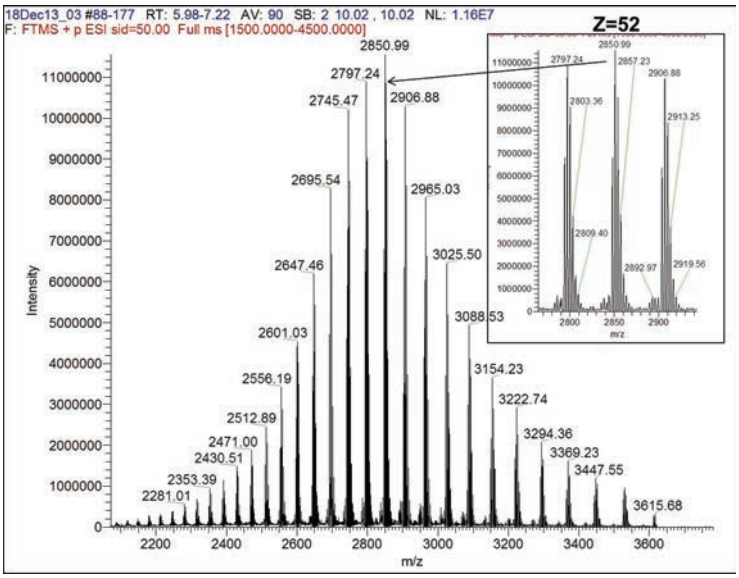

Figure 2 Example Electrospray Spectrum of NIST Reference mAb 8671; Acquired on Lumos Orbitrap at 15000 Resolution

Following the separation, Electrospray Ionization occurs, and it is necessary to acquire a quality electrospray spectrum as shown in Figure 2 in order to produce meaningful data.

The charge envelopes in this spectrum are centered on ions having a charge (z) of 53. Each charge envelope is rich in information about the structure. The NIST mAb has four major glycoforms that can be observed in the inset of Figure 2. When carried through deconvolution to obtain a neutral charge spectrum of the monoclonal antibody, this profile will be replicated. Deviations to this pattern are considered to be artifacts of the deconvolution algorithm. The modifications to the molecule and isoforms that can be detected are dependent on the resolution and sensitivity of the mass spectrometer, which work against each other. As resolution increases, sensitivity decreases. Small modifications such as deamidation or even oxidation may not be detected during intact mass analysis depending on the size of the protein and the resolution of the mass spectrometer. An example deconvoluted spectrum of the NIST mAb showing the neutral average masses of the glycoforms of the NIST mAb is presented in Figure 3.

Identification of glycoforms, modifications and other isoforms in a protein are based on the mass measurement and matching the observed mass to the theoretical mass of the isoform or modification. Monoclonal antibodies are large (approximately 150 kDa) heterogeneous molecules and can demonstrate a myriad of combinations of modifications and glycoforms making mass identification tedious and difficult. For example, the NIST mAb contains glutamine (Glu) at the N-terminus of both heavy chains and contains more glycosylation than most IgG1 mAbs. The confidence in identification also depends on mass accuracy that is dependent on the resolution and sensitivity of the mass spectrometer. High resolution mass spectrometers can obtain mass accuracies of less than 10 ppm for a major glycoform. Mass accuracy generally drops off for minor and trace glycoforms, especially when small peaks in the spectrum are not fully resolved, thus lowering the confidence in identifications and making them more subjective. It should also be mentioned that some isoforms are isobaric (equal masses) and cannot be differentiated by mass analysis. For example, in Figure 3 the peak labeled as G1F/G1F (148363 Da) could as be identified G0F/G2F, which is isobaric. Table 1 shows intact mass data for several common mAbs and compares the major and minor glycoforms observed in each of these mAbs.

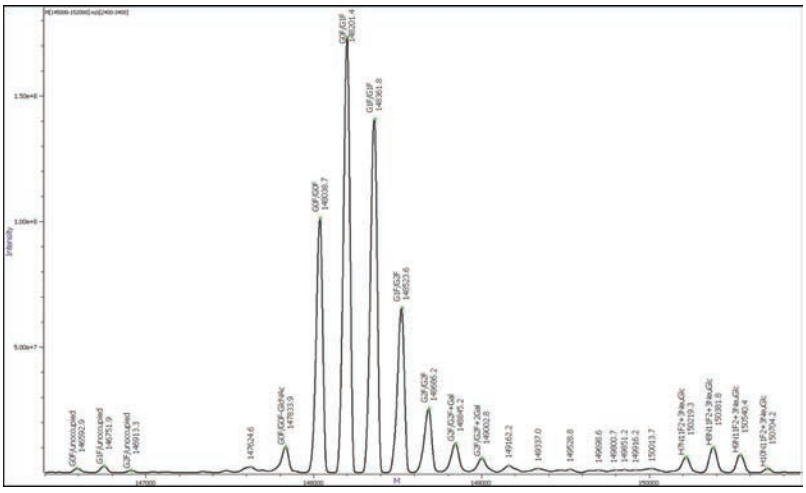

Figure 3 Example Neutral Spectrum of NIST Reference mAb 8671; Acquired on Lumos Orbitrap Mass Spectrometer with Resolution at 15000

The complexity caused by heterogeneity of the intact mAb can be alleviated by reducing the mAb to subunits, using an enzyme such as Ides or Papain to further fragment the protein, or by deglycosylating the intact protein with Peptide: N-glycosidase F (PNGase F). An example spectrum of the deglycosylated NIST Reference mAb is presented in Figure 4.

As can be observed, the N-linked glycans have been removed from the mAb by the PNGase F enzyme, leaving the intact protein structure. The observed mass of 145148.9 Da in this spectrum was within ± 1 Da of the theoretical mass for the deglycosylated NIST mAb (145149.4 Da), confirming the primary structure and amino acid sequence of the protein.

In conclusion, mass analysis of intact proteins using high resolution mass spectrometry is a rapid method for confirming the identity and the primary structure of proteins. Mass analysis can provide information for evaluating and comparing the isoforms, glycoforms, and molecule wide modifications associated with a protein. The analysis can readily compare manufacturing batches or can be used to compare a biosimilar to the innovator. Intact mass analysis provides a sweeping view of the mAb and can detect molecular level modifications that a bottom-up technique such as peptide mapping cannot detect.

Table 1 Comparison of Relative Abundances (%) of Glycoforms Identified in Common IgG1 Monoclonal Antibodies Using Intact Mass Analysis by LC/MS

Glycoform	Trastuzumab	Bevacizumab	Adalimumab	NIST 8671
G0F/G0F	100	100	100	58.3
G0F/G1F	83.8	20.6	37.1	100
G1F/G1F	42.7	6.2	8.5	81.2
G1F/G2F	12.1	1.7	NI	37.9
G2F/G2F	NI	0.6	NI	14.8
G2F/G2F+Gal	NI	NI	NI	6.7
G2F/G2F+2Gal	NI	NI	NI	3.4
G0F/G0F-GlcNAc	5.3	5.6	NI	6.0
G2F/G2F-GlcNac	NI	NI	2.4	NI
Man5/Man5	3.0	NI	0.2	NI
G0F/unoccupied	2.3	3.7	2.7	1.0
G1F/unoccupied	1.6	1.1	0.7	1.6

NI–not identified; Gal-Galactose; GlcNac-N-Acetylglucosamine; Isobaric glycoforms not differentiated; Tentative Identifications based on mass

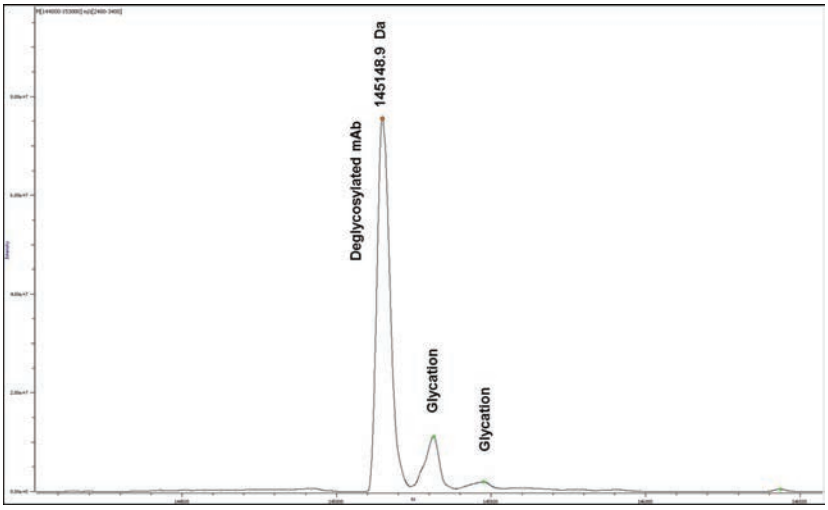

Figure 4 Example Neutral Spectrum of Deglycosylated NIST Reference mAb 8671; Acquired on Lumos Orbitrap Mass Spectrometer with Resolution at 15000