MSIA Workflow for Therapeutic Antibodies: Qualitative, Quantitative, and Functional Verification Data from HR/AM Detection of Intact, Reduced, and Peptide-level Forms of Adalimumab

Application Note

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Goal

To illustrate an innovative and versatile MSIA Workflow approach to therapeutic antibody analytics, using adalimumab in human donor plasma as a model biological system and subsequent analysis by three distinct mass spectrometry detection formats: Intact, Reduced, and Bottom up.

The Therapeutic Monoclonal Antibody

The biopharmaceutical industry has adopted biologics as a major component of their drug portfolios and developing pipelines. At the forefront of this drug class are monoclonal antibodies (mAb), highly discriminative molecules that can be engineered for specific targets. The rapid adoption of this class of biologics is due to their high affinity for their targets, selective biological actions, predictability of common side effects, and standardization of manufacturing processes.

With individual mAb therapeutics having annual sales in the billions of dollars, biotechnology and pharmaceutical companies are naturally working to develop the next blockbuster. However, as mAb therapeutic innovation and biological complexity increase, it is evident that classical protein analytical methods are no longer able to meet the demanding data requirements for pharmacokinetics, biotransformation assessment, and antibody functional determination studies. For instance, a new class of mAb based therapeutics, Antibody-Drug Conjugates (ADCs), has unique data requirements for the establishment of Drug-Antibody Ratios (DAR). By convention, this data would be generated using a Ligand Binding Assay (LBA), the gold standard in therapeutic assessment.

However, historical biological issues, compounded by complex data generation and analysis has made the LBA deficient in application and drug sponsors are looking for alternative analytical methodologies for mAb analysis.

Challenges with the Traditional Ligand Binding Assay (LBA) for Therapeutic Antibody Analysis

First established in the small molecule world, the LBA has become the universally accepted tool for drug analytics. With the growth of biologics, the ELISA format has been adopted for LBA analyses and requires high quality affinity reagents. Specifically needed is a highly selective antibody pair that works in tandem as a part of a sandwich assay system. However, the adoption and now evolution of the mAb therapeutic has rendered the traditional LBA data deficient, biologically challenging and cost prohibitive. There are fundamental limitations to antibody selectivity, making the generation of an affinity reagent for these subtle, yet critical modifications of the mAb based therapeutics challenging, if not impossible.

The above challenges are exacerbated by the biological process of protein complex formation - known as neutralization events. The development of anti-drug antibodies by the treated organism is the most common side effect of any biologic and a major debilitating factor for effective analytical performance of a conventional LBA. The formation of these antibodies decreases therapeutic function and blocks access to the epitopes needed for effective analysis. Secondly, protein complexation results from the natural function of the mAb therapeutic if a soluble form of the target exists. The engineered drug function takes place *in vivo*, but will continue once the blood sample is collected. This continuous binding is not indicative of *in vivo* drug action, but a simple artifact of time. This has historically been ignored as the therapeutic dosing has been in vast excess over the soluble target concentration. However, as many of the next generation mAb therapeutics target higher concentration ligands, this analytical challenge is moving to the forefront.

The last challenge of the conventional LBA is the inability to provide characterization information for the therapeutic mAb. In order for sponsors to develop novel antibody therapeutics, structural information including variants and sites of glycosylation and PTMs (post-translational modifications) are required for drug safety, efficacy, and stability. In the case of the development of ADCs, DAR determinations are critical. Furthermore, as the regulatory process around biologics too evolves, innovators of these therapeutics are challenged to disclose the comprehensive sequence and structure to achieve BLA (Biologic License Application) status.

This regulatory challenge transcends the innovative biologic market, and, as the patent window for first-in-class therapeutic antibodies narrows, manufacturers of biosimiliars must demonstrate their molecule is identical to the innovative drug or risk the creation of a new and

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unintended biological entity. If the biosimilar reveals any signs of structural inconsistency with the innovative therapeutic comparator, the molecule will be considered a new drug and will require a full IND (Investigational New Drug) submission.

Limitations of current LC-MS methods

To address these recognized deficiencies of the traditional LBA methods, drug sponsors are looking to mass spectrometric (MS) detection as the solution. MS is an analytically sensitive and robust detection method that provides the added benefit of specificity over traditional immunoassays. By identifying biomolecules by their intrinsic property of molecular mass, a highly specific and accurate analysis may be achieved by mass spectrometry.

To date, selected reaction monitoring (SRM) performed on triple quadrupole mass spectrometers has been the preferred technique for quantitative analysis. To accommodate the mass-to-charge (m/z) limitations of these instruments, the therapeutic antibodies must be proteolytically digested, usually with trypsin, to produce surrogate peptides that are subsequently analyzed for mAb quantification. Although peptide-level analysis offers robust quantification, traditional MS methods for peptide analysis are not ideal for therapeutic mAb analysis.

The majority of the developing MS methods universally follow a process that involves total protein crash, followed by global digestion and then some form of LC-MS/MS peptide analysis. In some methods, the full complement of digestion peptides is injected onto the LC-column, while others utilize SPE or an immuno-capture (IC) step(s) for an additional level of analytical selectivity. Even though this global digestion process remedies issues concerning therapeutic mAb neutralization events, the resultant peptide mixtures are exponentially more complex than the original plasma samples. Moreover, even though the use of MS detection does provide desired structural data, this data content is still limited. This limitation stems from lack of analytical sensitivity of the method, and inherent issues around the generated peptides, which may demonstrate poor ionization efficiency or high hydrophobicity characteristics. These properties are detrimental to LC-MS/MS analysis and thwart the generation of comprehensive sequence coverage data. Improvements are seen with the implementation of IC, but if key structural information does not reside within the purified peptides, the developed method is still blind to the desired structural data.

Even though there are immediate benefits observed through the adoption of such MS based methods, great improvement is still required. Incorporation of anti-peptide immunoaffinity purification improves sensitivity through selective peptide purification.

However, generation of unique peptides from mAb therapeutics is challenging because these mAb are recombinant proteins with human, or humanized, frameworks. As a result, their differentiation from endogenous IgG (immunoglobulin), except in the variable region, is an analytical challenge. There are some unique sequences that are conserved within these therapeutic mAbs that can be used as a unique identifier, however, if the desired structural area for characterization is not within these regions, the information is ultimately lost. Therefore, therapeutic mAb methods that circumvent these presented issues are still of great desire to the industry, and due to the biology, comprehensive methodologies to mAb analytics are needed.

MSIA Workflow for Therapeutic Antibody Analysis

To address the specific data generation requirements for mAb therapeutics, the MSIA Workflow for Therapeutic Antibodies was developed with the potential to measure three key properties – antibody quantity, quality and functionality – simultaneously.

This unique workflow is enabled through MSIA Streptavidin D.A.R.T.'S, a proprietary product that contains microcolumns that are covalently derivatized with streptavidin and enable the capture of biotinylated antigen ligands. By forming an immobilized streptavidin-biotin complex, desired therapeutic antibodies are purified by binding to their immobilized antigen binding partner and are then subjected to downstream MS analysis.

The MSIA Workflow for Therapeutic Antibodies offers advantages over traditional ligand binding assays and MS based methods:

- **No anti-drug antibodies are required** – The therapeutic antibody's target antigen is used as the affinity ligand for a highly selective purification.
- **Therapeutic antibody functionality is verified** – The ability of the therapeutic antibody to bind to the antigen for purification directly confirms its biological activity and function.
- **Applicable to human plasma systems** – functional purification circumvents interference from endogenous IgG.
- **Provides a remedy for neutralization events** – The functionality of the MSIA Streptavidin D.A.R.T.'S easily accommodates plasma samples pre-treated for protein complex disruption.
- **Automated** – The workflow is amiable to high through-put automation for standardized routine application.
- **Peptide sequence coverage** - The use of bottom up MS detection in the workflow provides access to sequence coverage of both the heavy and light chains.
- **Complete sequence information is preserved** – Ability to perform intact analyses for comprehensive characterization analysis with the Thermo Scientific™ Q Exactive™ mass spectrometer for high resolution accurate mass (HRAM) detection.
- **Quantitative** – Amiable to MS quantitation.

In this study, human donor plasma samples were spiked with adalimumab – a mAb therapeutic that inhibits anti-tumor necrosis factor-alpha (TNF-α). The therapeutic antibody was purified using TNF-α-derivatized MSIA Streptavidin D.A.R.T.'S and analyzed on a Thermo Scientific™ Q Exactive™ mass spectrometer.

Methods

The MSIA Workflow is illustrated in Figure 1. The first step in the workflow is the derivatization of the MSIA Streptavidin D.A.R.T.'S with biotin-conjugated TNF-α. Next, the prepared TNF-α-derivatized MSIA Streptavidin D.A.R.T.'S are incubated with donor plasma to capture and purify adalimumab. Purified adalimumab is then eluted from the functionalized MSIA Streptavidin D.A.R.T.'S into a micro-titer plate, where the eluent may be (1) reduced, alkylated and digested, as the case for the

Figure 1. MSIA Workflow for Therapeutic Antibodies.

bottom-up workflow, or (2) reduced and injected onto LC-MS for analyses of the reduced therapeutic antibody heavy and light chains, or (3) injected onto the LC-MS by autosampler for intact mAb analyses. The resulting LC-MS raw data are then processed by software.

Preparation of TNF-α-derivatized MSIA Streptavidin D.A.R.T.'S

MSIA Streptavidin D.A.R.T.'S were treated with a solution of biotin-conjugated TNF-α, enabling the immobilization of the affinity ligand onto the streptavidin surface. The prepared TNF-α-derivatized MSIA Streptavidin D.A.R.T.'S then underwent a series of brief rinses in phosphate buffered saline (PBS) to remove unbound biotin-conjugated TNF-α prior to its subsequent use in the capture of adalimumab from human donor plasma samples.

Sample Preparation

A variety of samples were prepared with adalimumab spiked into human donor plasma, listed in Table 1. For analyses of intact and reduced adalimumab, human donor plasma was spiked with 1.69 pmol to 67.6 pmol of adalimumab, resulting in final concentrations ranging from 500 ng/mL to 20 µg/mL. For bottom-up analyses, 6.76 fmol to 13.5 pmol of adalimumab was spiked into human donor plasma, spanning a final concentration range of 5 ng/mL to 10 µg/mL. Prior to immuno-capture of adalimumab, the adalimumab spiked plasma samples were diluted at a 1:1 ratio for bottom-up analysis and at a 2:1 ratio for intact and reduced analysis with PBS pH 7.4.

Sample Analysis	Adalimumab Concentration	Sample Volume	Total Adalimumab per Sample	Volume of PBS Diluent
Intact	500 ng/mL to 20 µg/mL	500µL	1.69 pmol to 67.7 pmol	250 µL
Reduced	500 ng/mL to 20 µg/mL	500µL	1.69 pmol to 67.7 pmol	250 µL
Bottom-Up	5ng/mL to 10 µg/mL	200µL	6.76 fmol to 13.5 pmol	200 µL

Table 1: Samples prepared for three different analytical methodologies: Human Plasma spiked with adalimumab.

Affinity Purification of Adalimumab

The purification of adalimumab was achieved through incubation of spiked plasma samples with the TNF-α-derivatized MSIA Streptavidin D.A.R.T.'S. Using a Thermo Scientific™ Versette™ automated liquid handler, or for individual samples, a Thermo Scientific™ Finnpipette Novus i electronic pipette, incubation was carried out by repetitively pipetting (aspirating and dispensing) the sample solutions through the TNF-α-derivatized MSIA Streptavidin D.A.R.T.'S. The purification step was followed by rinses with buffer and water to remove non-specifically bound proteins and salts that would affect the downstream mass spectrometric detection of adalimumab.

Adalimumab Elution and Pre-MS Sample Processing

The MSIA Streptavidin D.A.R.T.'S containing bound adalimumab were treated with the elution solvent (0.4% trifluoroacetic acid containing 33% acetonitrile) to release the purified adalimumab. The purified adalimumab samples were analyzed by three different analytical methodologies that required separate pre-MS processing steps (Table 2). For bottom-up analysis, the eluent was buffered to pH 5 with ammonium bicarbonate containing TCEP to carry out reduction. Reduced adalimumab was alkylated and then digested using 100 ng of trypsin. The resulting tryptic digest was then loaded onto the LC-MS for analysis. For reduced, the eluent was treated similarly to the bottom-up approach except for the omission of the alkylation and digestion steps. The eluent was diluted with ammonium bicarbonate containing TCEP to reduce the adalimumab into its heavy and light chains. The intact heavy and light chains of adalimumab were then loaded onto the LC-MS for their intact analyses. For intact analyses, the acetonitrile concentration of the eluent was reduced to 25% with water, allowing the analytical liquid chromatography column to retain the adalimumab during loading. The intact adalimumab was then injected onto the LC-MS and analyzed.

Liquid Chromatography-Mass Spectrometric (LC-MS) analyses

Demonstrating the MSIA workflow on three distinct mass spectrometry detection formats required two separate and independent settings for the LC-MS, one for bottom-up and another for the analyses of reduced and intact adalimumab.

Bottom-up LC-MS Workflow

Liquid Chromatography

Tryptic peptides from digested adalimumab were separated on a Thermo Scientific™ Hypersil GOLD™ Syncronis™ aQ LC column (2.1 x 100 mm) with an UltiMate 3000 XRS Pump running at 150 µL/min with a gradient of 2%–35% B (0.2% formic acid in acetonitrile) in 45 minutes. Mobile phase A comprised of 0.2% formic acid in water. The column was heated to 70 °C and the electrospray voltage was set to 3.5 kV.

Mass Spectrometry

Mass spectrometry data were acquired in positive-ion mode on a Thermo Scientific™ Q Exactive™ mass spectrometer operated in data-dependent mode, with the

Sample Analysis	Elution Solvent	Pre-MS Sample Processing	Reduction	Alkylation	Digestion
Intact	0.4% Trifluoroacetic Acid in 33% Acetonitrile (v/v)	Dilute Acetonitrile from 33% to 25% with Water	–	–	–
Reduced	0.4% Trifluoroacetic Acid in 33% Acetonitrile (v/v)	Buffered with Ammonium Bicarbonate, pH 5.0	✓	–	–
Bottom-Up	0.4% Trifluoroacetic Acid in 33% Acetonitrile (v/v)	Buffered with Ammonium Bicarbonate, pH 5.0	✓	✓	✓

Table 2: Elution of adalimumab from derivatized MSIA D.A.R.T.'S and Pre-MS Sample Processing for Intact, Reduced and Bottom-up Analyses.

top 10 ions selected for MS/MS analysis. Full-scan MS data were acquired with a resolution of 70,000 (at m/z 200). MS/MS data were acquired with a resolution setting of 17,500 (at m/z 200). Automatic gain control (AGC) targets were set to 1e6 and 1e5 for MS1 and MS2, respectively. Normalized collision energy for the Orbitrap HCD cell was 27, and dynamic exclusion length was 20 sec.

Intact and Reduced LC-MS Workflow

Liquid Chromatography

Intact adalimumab and reduced heavy and light chains of adalimumab were separated on a Thermo Scientific™ ProSwift™ RP-4H 500 µm x 10-cm column running at 100 µL/min with a gradient of 20%–35% acetonitrile in 0.2% formic acid in 15 minutes. Mobile phase A comprised of 0.2% formic acid in water. The column was run at 60 °C and the electrospray voltage was set to 3.0 kV.

Mass Spectrometry

Full-scan MS data were acquired in positive-ion mode on a Thermo Scientific™ Q Exactive™ mass spectrometer over the mass range of m/z 1000–4,500 with a resolution of 17,500 (at m/z 200). S-lens RF level was set to 80 while the Automatic gain control (AGC) was set to 3.00e6. Deconvolution analysis was performed with Thermo Scientific™ Protein Deconvolution™ software version 3.0.

Results and Discussion

The MSIA Workflow yields a highly purified concentration of target molecules – in this case, adalimumab – thereby reducing background and enhancing sensitivity. The resulting antibody material is amenable to mass spectrometric analysis either as intact molecules, intact heavy and light chain molecules, or peptide fragments.

The bottom-up workflow provides high peptide coverage

The peptide digestion workflow is perhaps the most widely accessible because, though the data presented here were acquired on a Thermo Scientific™ Q Exactive™ Orbitrap mass spectrometer, the method only requires a triple-quadrupole mass spectrometer.

In the bottom up workflow the intact protein is digested into peptides, each of which is then analyzed (i.e., sequenced) in the mass spectrometer to reveal the structure of the starting molecule. In this case, antibody molecules – comprised of two heavy and two light chain polypeptides covalently linked by disulfide bonds – are treated to break disulfide bonds, alkylated and treated with trypsin to generate a population of peptides. These peptides are then separated by liquid chromatography and sequenced by MS/MS to assess post-translational modifications, biotransformations that may have occurred post-injection, antibody-drug ratios, and other derivatized forms.

Shown in Figure 2A is a total ion chromatogram of adalimumab peptides eluting from an LC column, with a selected area at approximately 21 minutes highlighted. Representing multiple peptides, this area in the chromatogram was selected for deeper analysis. As shown in Figure 2B, the Thermo Scientific Q Exactive is capable of resolving even isotopic forms of the same peptide one of which was then fragmented for peptide sequencing analysis (Figure 2C).

Peptide coverage refers to the total number of amino acids in the starting protein molecule recovered by the bottom-up process. As shown in Figure 3, the enhanced sensitivity and lower background afforded by purification with MSIA Streptavidin D.A.R.T.'S yields 91.1% peptide coverage of the adalimumab heavy chain sequence and 100% for the light chain.

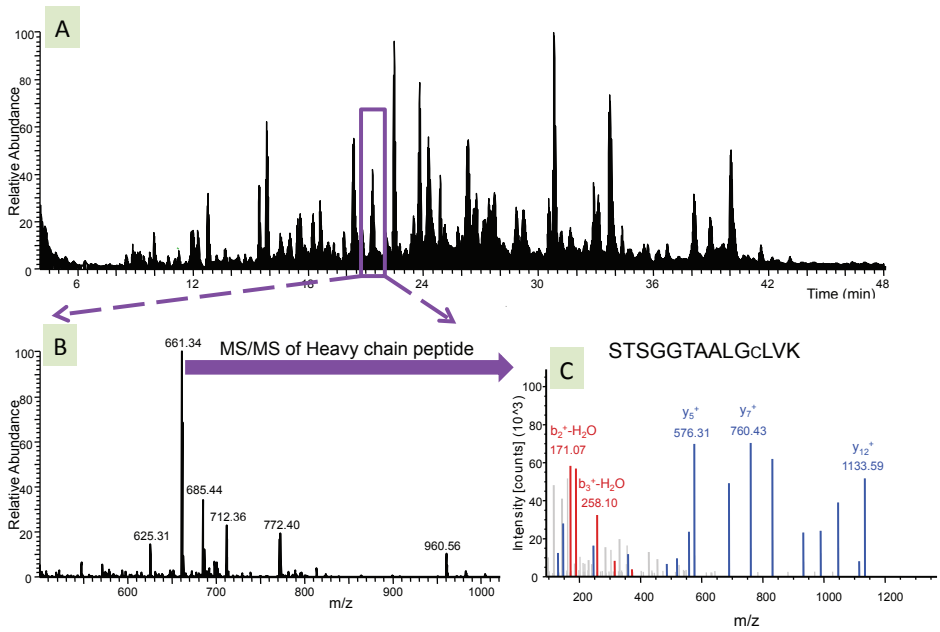
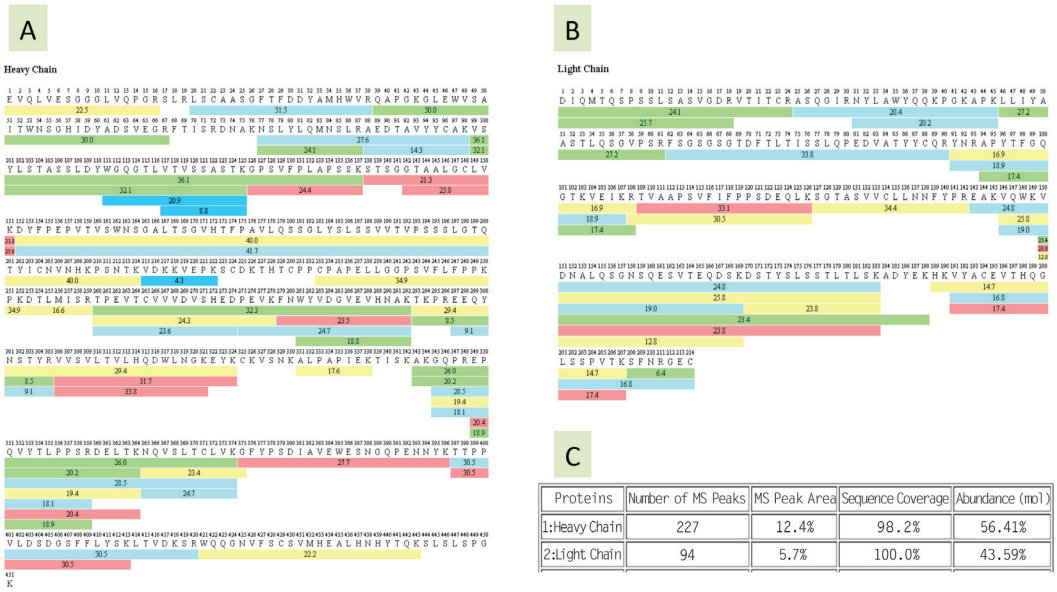


Figure 2. LC-MS analysis of trypsin digested adalimumab (5ng/mL) extracted from 200 µL of plasma. (A) Total ion chromatogram, (B) mass spectrum of peptide ions that were eluted around 21 minutes in the chromatogram shown in (A), and (C) MS/MS and amino acid sequence of heavy chain peptide at m/z 661.34.



Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:Heavy Chain	227	12.4%	98.2%	56.41%
2:Light Chain	94	5.7%	100.0%	43.59%

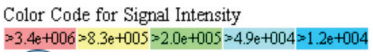


Figure 3. Bottom up sequence coverage for A) heavy chain and B) light chain of adalimumab at 5 ng/mL. The sequence underlined in red was found to be heavily glycosylated based on MS full scans.

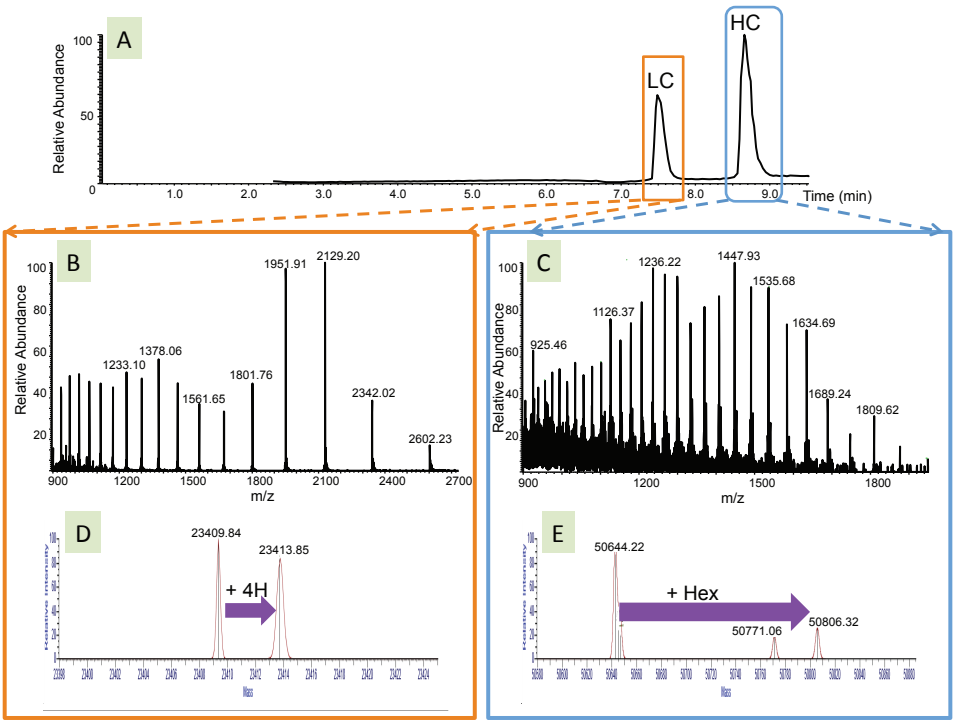


Figure 4. Intact analysis of reduced adalimumab A) Base peak chromatogram of reduced adalimumab showing the elution profiles of light chain (LC) and heavy chain (HC). B) and C) Raw MS spectra for LC and HC, respectively. D) The deconvolved average mass (M+H) of LC. E) The deconvoluted average mass (M+H) of HC.

The reduced and alkylated workflow resolves intact heavy and light antibody chains

The advantage of the bottom-up approach is its accessibility – triple quadrupole mass spectrometers are relatively inexpensive and ubiquitous. But the data are highly complex, and the workflow rarely recovers the entire protein sequence. An alternative approach is to study the antibody heavy and light chain polypeptides as intact molecules.

Adalimumab purified from human plasma with MSIA Streptavidin D.A.R.T.'S was reduced to detach antibody heavy and light chains proteins from one another. These were then separated on an LC column and analyzed in the Thermo Scientific Q Exactive mass spectrometer.

As shown in the base peak chromatogram in Figure 4A, the heavy and light chains are easily resolved in significantly shorter LC run times, with the light chain eluting at 7.49 minutes and the heavy chain at 8.66 minutes. (The corresponding mass spectra are shown in Figure 4B (light chain) and Figure 4C (heavy chain) while their deconvolved average masses are shown in Figure 4D (light chain) and Figure 4E (heavy chain)).

The masses of the split light-chain peaks in Figure 4D are 23409.33 and 23413.68. This 4 Da separation is indicative of incomplete reduction of the two disulfide linkages of the light chain. The mass at 23413.72 is within 1 Da of the theoretical light chain average mass of adalimumab. The deconvolved average mass at 50644.22 in the heavy chain spectrum in Figure 4E is suggestive of a modification by loss of the C-terminal lysine and the addition of one N-linked glycan. The mass at 50806.32 represents the addition of a hexose group. The Orbitrap's HRAM capabilities allow for characterization of these modifications and enable researchers to, for instance, easily identify different

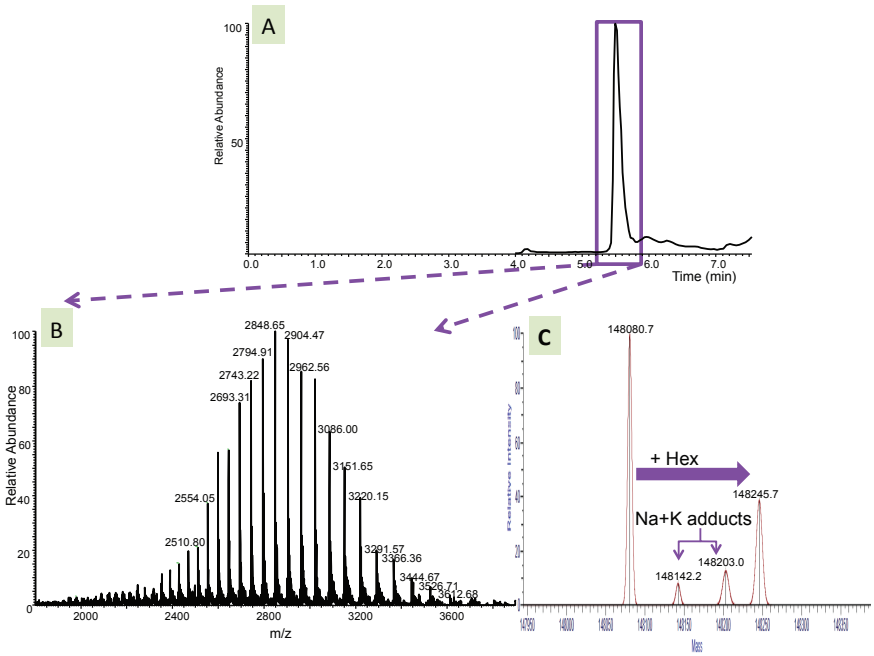


Figure 5. Intact analysis of adalimumab A) total ion chromatogram of intact adalimumab with data acquisition beginning at 7 minutes. B) and C) are Raw MS spectrum and deconvolved average mass (M+H) of intact adalimumab, respectively.

post-translational modifications or measure drug-antibody ratios in antibody-drug conjugates.

The intact molecule workflow can characterize all molecular variants simultaneously

The reduced workflow provides cleaner data and more comprehensive coverage than the bottom-up workflow, but still requires reduction step, which can decrease yield and require more hands-on time. Additionally, by breaking intact molecules into pieces, information about changes that may occur in different parts of individual molecules can be lost. But given a mass analyzer with sufficiently high mass accuracy and resolution, researchers can simply inject antibody molecules intact and let the mass spectrometer sort them out. This is the intact molecule workflow.

In this case, adalimumab molecules were separated by LC and resolved in a Thermo Scientific Q Exactive mass spectrometer. As shown in Figure 5, the Q Exactive easily resolves the different isoforms of this ~150 kDa molecule, enabling researchers to quantify and detect, with essentially no sample preparation, drug-antibody ratios, post-translational modifications, and other physical changes to the molecules in a single pass.

Conclusion

As mAb therapeutic innovation and biological complexity increase, it is becoming evident that the classical protein analytical methods, Ligand Binding Assays, are no longer able to meet simple data content needs (i.e, pharmacokinetics, biotransformation assessment, and antibody functional determination) required by the industry. To address the deficiencies of the traditional LBA methods, drug sponsors are looking to mass spectrometric (MS) detection as the solution.

MS methods provide greater analytical specificity over LBA and a remedy to neutralization effects that plague traditional LBA methods. Though there are immediate benefits observed through the adoption of MS based methods, these methods are labor intensive, lack standardization, and the peptide selectivity is limited. Therefore, improvements to MS methods are still needed. As demonstrated here, the MSIA Workflow for Therapeutic Antibodies provides an improvement over existing MS methods.

These benefits include:

- A reproducible and robust workflow for analysis of therapeutic antibodies.
- The sensitivity to provide an analytical detection limit of 5 ng/mL for adalimumab from human donor plasma, using high flow LC.
- The ability to overcome cross reactivity issues that plague traditional generic Fc targeting capture Ab based methods by using the target antigen as an affinity ligand.
- The workflow provides highly specific characterization data. In the adalimumab model, multiple glycoforms were detected, setting the foundation for other high value characterization methods, such as Drug-Antibody Ratio determination of Antibody-Drug Conjugates.
- The MSIA Workflow provides a remedy to chronic issues observed with therapeutic neutralization events and protein complexation.
- The described method sets the foundation for the simultaneous quantification, characterization and functionality determination of the targeted therapeutic molecule.

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