Analysis of Monoclonal Antibodies and Their Fragments by Size Exclusion Chromatography Coupled with an Orbitrap Mass Spectrometer

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Overview

Purpose: Demonstrate SEC-MS as a characterization platform for MAb and its fragments.

Methods: Coupling size-exclusion chromatography with high resolution Orbitrap mass spectrometer (SEC-MS) enables accurate mass measurement of MAb and its fragments.

Results: 1. MAb intact mass can be measured by SEC-MS under non-denaturing condition using near neutral pH eluent; 2. Exactive Plus EMR mass spectrometer enables the accurate detection of MAb at m/z 350- 20,000; 3. Thermo Scientific™ MAbPac™ SEC-1 column successfully separates the HC and LC, and partially separates Fab and Fc fragments using denaturing eluent.

Introduction

The biopharmaceutical industry has continued its focus on the development of biotherapeutic monoclonal antibody (MAbs) drug [REF 1]. For the final biopharmaceutical product approval and subsequent manufacturing processes, a comprehensive characterization of MAb purity, aggregate forms, and charge variants is required by the regulatory agency. MAbs produced from mammalian cell culture may contain significant amounts of dimers and higher-order aggregates. Studies show that these aggregates present in drug products may cause severe immunogenic and anaphylactic reactions. Size exclusion chromatography (SEC) is a well-accepted technique for the detection and accurate quantification of protein aggregates in biological drug products. It is routinely used for the characterization and quality control of MAb products.

There is a growing trend to obtain intact mass information as well as the glycan profile in the QC of monoclonal antibody using high resolution mass spectrometer. The most commonly employed LC/MS method is to desalt MAb via reversed phase chromatography following by the MS analysis. However, the extreme low pH and organic solvent used in the reverse phase chromatography often denatures the MAb. In the case of antibody-drug conjugate (ADC) with interchain cysteine linked drugs, the harsh solvent condition will dissociate the heavy and light chains of ADC and prevent the measurement of intact mass. Non-denaturing SEC based desalting mass spectrometry method enables mass measurement of MAb at its native state. The volatile ammonium formate buffer is compatible with MS and preserves intact protein structure.

Full characterization of MAb includes determination of mass of the MAb fragments, such as heavy chain and light chain generated by reduction of interchain disulfide bonds, as well as Fab and Fc generated by papain digestion (figure 1). Using denaturing eluent containing 20% acetonitrile, 0.1% TFA, and 0.05% formic acid, SEC can baseline separate HC and LC, as well as partially separate Fab and Fc. It serves as a platform method for MAb fragment analysis.

The Thermo Scientific™ Exactive™ Plus EMR Mass Spectrometer combines high-resolution accurate-mass with the extended mass range (EMR). It has a m/z range of 350 to 20,000 and improved transmission of higher-mass ions for stronger signals. All these features make the Exactive Plus EMR mass spectrometer a superb tool for accurate intact mass measurment of MAb and high-performance screening of MAb glycosylation profile.

The MAbPac SEC-1 is a size exclusion chromatography (SEC) column designed for monoclonal antibody (MAb) analysis, including monomers, aggregates, and fragments. Its stable surface bonding leads to low column bleed and compatibility with MS detection. In this study, we demonstrate the compatibility of MAbPac SEC-1 with Exactive Plus EMR Mass Spectrometer. SEC-MS enables intact mass detection of MAb under non-denaturing condition and fragments (including heavy chain, light chain, Fab, and Fc) under denaturing condition.

Methods

Chemicals and reagents:

High purity ammonium formate (≥99.995%) and ammonium acetate (≥99.99%) were purchased from Sigma®. Other reagents were purchased from reputable suppliers. Monoclonal antibodies were gifts from a local biotech company.

Columns
MAbPac SEC-1, 5 µm, 4 × 300 mm (P/N 074696)

Liquid Chromatography
HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System equipped with:
SRD-3400 Membrane Degasser
NCS-3500RS dual-gradient pump and column compartment
WPS-3000TPL Rapid Separation Thermostatted Autosampler

Reduction of MAb to heavy chain (HC) and light chain (LC) subunits
Reduction of inter-chain disulfides in a MAb (1 mg/mL) was achieved by incubation of MAb with 20 mM DTT at 50 °C for 30 min. The reduced sample was acidified with formic acid to final concentration at 0.1%.

Papain digestion of MAb to generate Fab and Fc subunits
The digestion was carried out by incubating MAb (1 mg/mL) with papain (0.04 mg/ml) in 100 mM Tris-HCl, pH 7.6, 4 mM EDTA and 5 mM Cysteine buffer at 37 °C. After 4 hours, the digestion was stopped by addition of formic acid to final concentration at 0.1%.

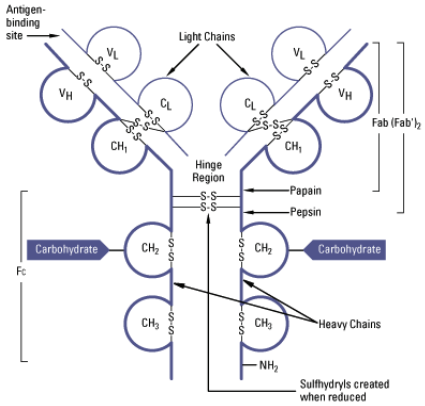
Non-denaturing SEC mobile phase
20 mM ammonium formate (pH 6.3) or 20 mM ammonium acetate (pH 6.8).

Denaturing SEC mobile phase
20% acetonitrile, 0.1% formic acid, and 0.05% trifluoroacetic acid acid (TFA).

MS Conditions
The Exactive Plus EMR Orbitrap mass spectrometer was used for this study. Intact MAb or MAb fragments were analyzed by ESI-MS for intact molecular mass. For non-denaturing LC-MS analysis, EMR mode was turned on. Full scan with mass range from 400-20,000 m/z was employed for data acquisition, HCD was set as 10 to help desolvation, microscans was 5 with AGC target set at 1E6, maximum IT was 300ms at resolution 35,000, probe was heated to 400 °C. For denaturing analysis, MS was operated at non-EMR mode. The spray voltage was 4.3 kV. Sheath gas flow rate was set at 30. Auxiliary gas flow rate was 10. Capillary temperature was 275 °C. S-lens level was set at 200. In-source CID was set at 100 eV. Resolution was 17500. The AGC target was set at 1E6. Maximum IT was set at 200 ms, heater temperature 200 °C, microscans 1, mass range from 400-6,000 m/z.

Data processing
Full MS spectra of intact MAbs, HC, LC, Fab, and Fc fragments were analyzed using Thermo Scientific™ Protein Deconvolution 2.0 software that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the MAb or their fragments. The averaged spectra were subsequently deconvoluted using an input m/z range of 4000 to 6000 for spectra acquired under non-denaturing conditions (or 2000 to 4000 m/z for spectra acquired under denaturing conditions), an output mass range of 140,000 to 160,000 Da with a target mass of 150,000 Da for MAb, or an output mass range of 45,000 to 55,000 Da with a target mass of 50,000 Da for MAb fragments, and a minimum of at least eight consecutive charge states from the input m/z spectrum was used to generate the deconvolution results.

Figure 1: IgG structure.



Results

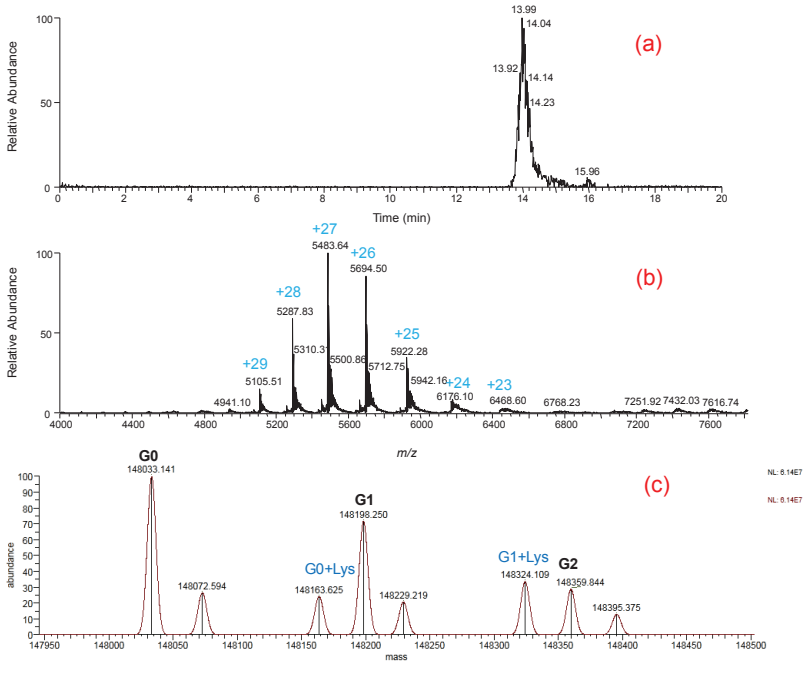
Analysis of MAb by non-denaturing SEC-MS

The analysis of MAbs by SEC is typically performed under non-denaturing conditions at physiological pH range (6-8). The commonly used buffer is phosphate buffer with 300 mM NaCl. However, the non-volatile nature of phosphate buffer and half salt content makes this buffer non-compatible with online mass spectrometry detection. Therefore, we explored using volatile buffer such as 20 mM ammonium formate for SEC separation and directly coupling the SEC column to the Exactive Plus EMR instrument. The figure 2 shows the SEC-MS analysis of a MAb, with figure 2a showing the extracted ion chromatogram of m/z at 5483.08-5483.31 and figure 2b showing the charge envelope of +24 to +29 in the m/z range of 5100-6200. Normally under acidic condition, the charge envelope of MAb is in the m/z range of 2000-4000. Since the 20 mM ammonium formate eluent has near neutral pH (at 6.3), the charge envelope of MAb shifts to higher mass range. The detection of such high m/z charge envelope (m/z above 6000) is made possible with the extended mass range of the Orbitrap instrument. Figure 2c shows the deconvoluted mass spectra of the MAb, with a main peak at m/z 148033 and adjacent peaks at m/z 148198, and 148359, corresponding to different glycoforms with 1 and 2 additional hexoses. Adjacent peak at m/z 148163, is 130 amu above the main peak, corresponding to a lysine variant.

Besides ammonium formate, ammonium acetate buffer was also evaluated. However, when using 20 mM ammonium acetate (pH at 6.8) as eluent, the MAb peak has tailing.

MAb average mass can be measured by SEC-MS under denaturing condition as long as the MAb structure remains intact. Eluent such as 20% acetonitrile, 0.1% formic acid, and 0.05% TFA, can be used for such analysis. When comparing the charge distribution of MAb under acidic and near neutral conditions, MAb is carrying more charge in acidic condition with charge envelope in the range of +33 to +60 (data not shown)

Figure 2: SEC-MS analysis of MAb under non-denaturing condition using 20 mM NH4Fc. (a) extracted ion chromatogram of MAb, (b) mass spectrum of MAb, (c) deconvoluted spectrum of MAb.

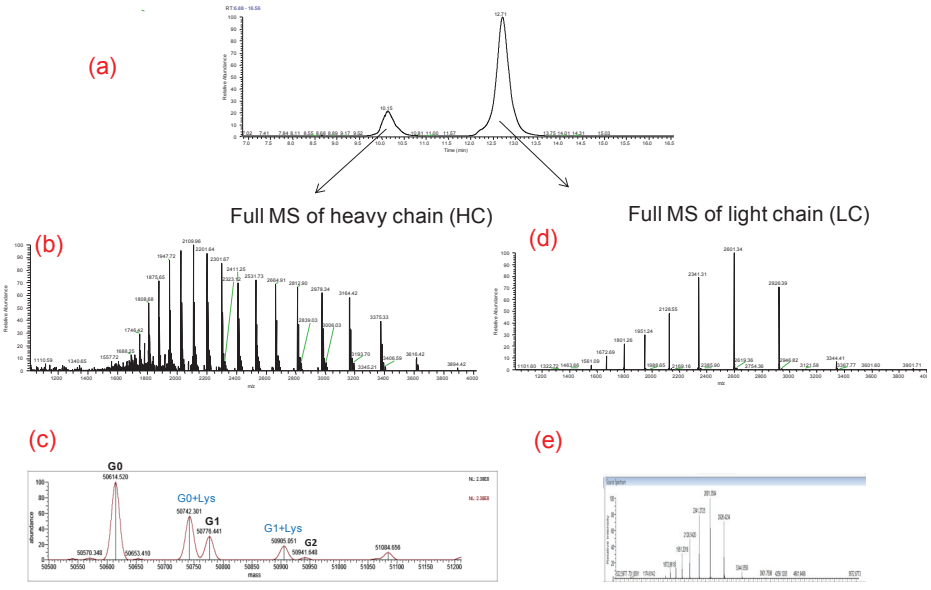


Analysis of MAb fragments by denaturing SEC-MS

Comprehensive analysis of the MAb post translational modifications, such as deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine oxidation, and glycosylation, requires complete digestion of the MAbs and sequencing of all the peptides. However, "peptide mapping" is time consuming. A simpler and faster way to analyze the MAb variants and locate the modifications is to measure the mass of heavy chain and light chain, or Fab and Fc fragments. Heavy chain and light chain are generated by the reduction of MAb. Fab and Fac fragments are generated by papain digestion. For example, the glycan modification is located in the Fc region of the heavy chain, glycan variants can be detected in the heavy chain and Fc fragment mass profiles, while light chain and Fab fragment mass profiles should only show a single polypeptide chain.

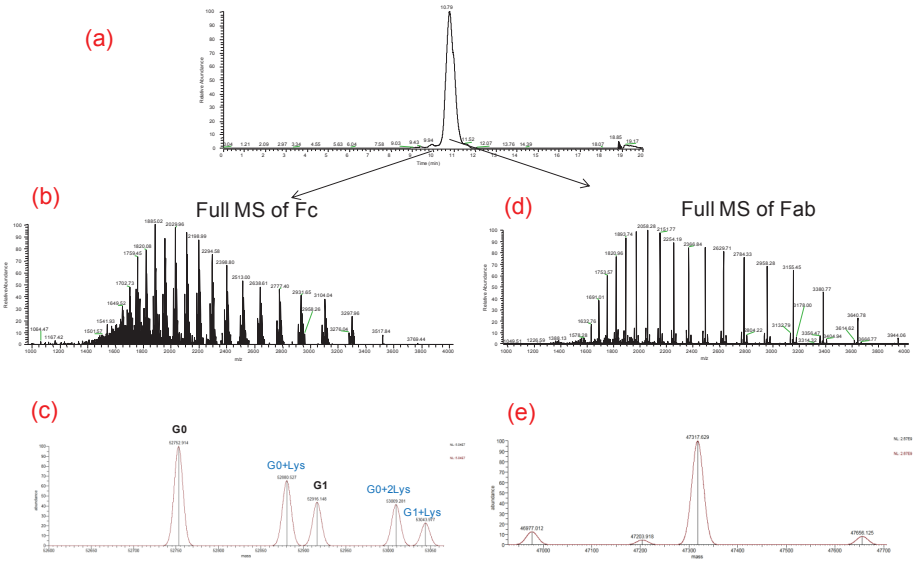
Figure 3 shows the SEC-MS analysis of HC and LC of a MAb using 20% acetonitrile, 0.1% formic acid, and 0.05% TFA. Figure 3a shows the extracted ion chromatogram of HC with m/z at 3163.70-3164.89 and LC with m/z at 2600.78-2601.88. Using this denaturing eluent system, MAb HC elutes at about 10.15 min and MAb LC elutes at about 12.71 min. Different MAbs have been tested and their HC and LC have similar retention time. Therefore, denaturing SEC can be used as a platform method for the separation of HC and LC of MAbs. Figure 3b shows the charge envelope of MAb HC in the m/z range of 1900-3600 and Figure 3c shows the deconvoluted mass spectra of the MAb HC, with a main peak at m/z 50614.5 and adjacent peaks at m/z 50742.3, and 50776.4, corresponding to a lysine variant and a different glycoform with 1 additional hexose. Lysine variant is located at the C-terminal of the HC. Figure 3d shows the charge envelope of MAb LC in the m/z range of 1500-3500 and Figure 3e shows the deconvoluted mass spectra of the MAb LC, with a single peak at m/z 23403.7. The MAb light chain is not glycosylated and does not have C-terminal lysine variants. The intact mass of MAb is determined at m/z 148029 using the equation 2x(HC+LC)-8. The calculated mass is in good agreement with the measured mass at m/z 148035.

Figure 3: SEC-MS analysis of MAb heavy chain and light chain under denaturing condition using 20 acetonitrile, 0.1% formic acid and 0.05% trifluoroacetic acid. (a) extracted ion chromatogram of heavy chain (HC) and light chain (LC), (b) mass spectrum of heavy chain (HC), (c) deconvoluted spectrum of heavy chain (HC), (d) mass spectrum of light chain (LC), (e) deconvoluted spectrum of light chain (LC).



Using the same chromatographic method, Fc and Fab fragments are eluted on the SEC column at 9.94 and 10.79 min (Figure 4a), although the separation is not as good as the HC and LC due to the fact that Fab and Fc fragments are very similar in size. Figure 4b shows the charge envelope of Fc in the m/z range of 1500-3500 and Figure 4c shows the deconvoluted mass spectra of the Fc, with a main peak at m/z 52752.9 and adjacent peaks at m/z 52880.5, and 52916.1, corresponding to a lysine variant and a different glycoform with 1 additional hexose. Figure 4d shows the charge envelope of Fab in the m/z range of 1600-3700 and Figure 4e shows the deconvoluted mass spectra of the Fab, with a single peak at m/z 47317.6. The intact mass of MAb is determined at m/z 147387 using the equation 2xFab+Fc. The calculated mass is 700+ Da away from the measured mass at m/z 148035, indicating additional fragment generated from the papain digestion.

Figure 4: SEC-MS analysis of MAb Fc and Fab under denaturing condition using 20 acetonitrile, 0.1% formic acid and 0.05% trifluoroacetic acid. (a) extracted ion chromatogram of Fc and Fab, (b) mass spectrum of Fc, (c) deconvoluted spectrum of Fc, (d) mass spectrum of Fab, (e) deconvoluted spectrum of Fab.



Conclusions

- MAb intact mass can be measured by SEC-MS under non-denaturing condition using near neutral pH eluent, such as 20 mM ammonium formate.
- Exactive Plus EMR mass spectrometer enables the accurate detection of MAb at m/z 350- 20,000.
- MAbPac SEC-1 column successfully separates the HC and LC, and partially separates Fab, and Fc fragments using denaturing eluent such as 20% acetonitrile, 0.1% formic acid, and 0.05% trifluoroacetic acid.

References

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