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Note d'application

Analysis of Proteins by Size-Exclusion Chromatography Coupled with Mass Spectrometry Under Non-Denaturing Conditions

Paula Hong, Stephan M. Koza, Kenneth J. Fountain

Waters Corporation

Abstract

In this application, we describe SEC-MS under non-denaturing conditions. While this application has been evaluated for SE-HPLC, UPLC Technology in combination with sub-2-µm SEC column packing and a time-of-flight mass spectrometer (Xevo G2 Q-Tof) allows for direct analysis with improved resolution and sensitivity. By combining MS conditions with a Xevo G2 Q-Tof, SE-UPLC MS analysis can be used to assist in the identification of unknown components in a biotherapeutic.

Benefits

- Improved resolution and sensitivity with SE-UPLC as compared to traditional SE-HPLC
- Non-denaturing SEC method for MS identification of unknown biotherapeutic components
- Exact molecular weight confirmation of intact bimolecules
- BEH particles provide columns with reduced secondary interactions that allow for mobile phases with reduced salt concentrations

- SEC column with minimal MS column bleed provides improved sensitivity

Introduction

Ultra performance size-exclusion chromatography (SE-UPLC) provides a high throughput, robust method for separation of biomolecules based on size in solution[1]. SE-UPLC is typically performed under non-denaturing conditions, which are intended to preserve the state of self-association of the biomolecule, with a UV detector for quantification. Molecular weight estimates based on this technique require the use of an appropriate set of molecular weight standards for calibration. Other methods capable of providing molecular weight information under non denaturing conditions include on-line multi-angle light scattering (MALS) and off-line analytical ultracentrifugation (AUC), both of which do not rely on molecular weight standards. These low resolution techniques cannot always resolve minor differences in molecular weight due to post-translational modifications or degradation. The combination of SEC using non-denaturing mobile phase and mass spectrometry (MS) provides accurate on-line mass determination for biomolecular species observed by SE-UPLC, however, the form of the noncovalent self-associated species is not provided by this method.

In this application, we describe SEC-MS under non-denaturing conditions. While a similar application has been evaluated for SE-HPLC[2], UPLC Technology in combination with sub-2-µm SEC column packing and a timeof-flight mass spectrometer, Xevo G2 Q-Tof, allows for direct analysis with improved chromatographic resolution and sensitivity. The resulting separations are comparable in retention time to those obtained using typical SEC mobile phases that are not MS compatible. By combining these conditions with a Xevo G2 Q-Tof, SE-UPLC-MS analysis can be used as an effective complementary characterization method to low-resolution, non-denaturing mass determination methods such as MALS or AUC, and low-resolution, denaturing size separations such as capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) to confirm the identification of biomolecular species observed by size-exclusion chromatography.

Experimental

LC Conditions

LC System:	ACQUITY UPLC H-Class Bio System with PDA detector
Flow Cell:	Titanium 5 mm (part number 205000613)
Wavelength:	280 nm
Column:	ACQUITY UPLC BEH200, SEC 1.7 µm, 4.6 x 300 mm (part number 186005226)
Column Temp.:	30 °C
Sample Temp.:	4 °C
Injection Volume:	2 µL
Flow Rate:	0.15 mL/min or 0.2 mL/min
Mobile Phase:	100 mM ammonium formate and 25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8
Additive:	Acetonitrile, 0.8% formic acid, at 0.2 mL/min
External Pump:	Waters 515 HPLC pump
Vials:	LCMS Certified Max Recovery vials (part number 600000755CV)

MS Conditions

MS System:	Xevo G2 QTof
Ionization Mode:	ESI+
Analyser Mode:	Sensitivity
Acquisition Range:	500-5000
Capillary Voltage:	3.00 kV
Cone Voltage:	40.0 V
Source Temp.:	150 °C
Desolvation Temp.:	450 °C
Cone Gas Flow:	0.0 L/Hr
Desolvation Gas Flow:	800.0 L/Hr
Calibration:	NaI 2 µg/µL from 1000-4000 *m/z*

Data Management

MassLynx software

MaxEnt 1 software

Sample Description

The protein standard (obtained from Bio-Rad) containing bovine thyroglobulin (5 mg/mL), bovine γ–globulin (5 mg/mL), chicken ovalbumin (5 mg/mL), horse myoglobin (2.5 µg/µL) and Vitamin B12 (0.5 µg/µL) in deionized water was analyzed. Horse heart myoglobin (Sigma) was prepared at 2 mg/mL in deionized water. A recombinant humanized monoclonal antibody, trastuzumab, was analyzed past expiry undiluted (21 µg/µL).

Results and Discussion

The analysis of proteins by size exclusion chromatography (SEC) is typically performed under non-denaturing conditions which preserve the three dimensional structure and can be correlated with biological activity of the protein. Common mobile phases are 100% aqueous in a physiological pH range (6-8) and typically require non-volatile buffers and salts such as sodium phosphate and sodium chloride[3]. In order to obtain MS characterization of sample fractions separated under these conditions, the most common solution is to desalt the sample prior to analysis; however, this approach can result in sample speciation and can be cumbersome.

Another strategy is to perform SEC under denaturing conditions, so that species are efficiently ionized for detection by MS.[4,5] These methods typically require the use of mobile phases containing acetonitrile, formic acid and trifluoroacetic acid (TFA) for direct coupling of SEC to MS. While TFA does cause ion suppression in MS, it is required to minimize secondary interactions between the column packing material and the biomolecule. This application provides a useful tool for desalting of a sample without the need for column re-equilibration and has been used for the analysis of reduced and alkylated monoclonal antibodies as well as other smaller proteins.[5,6] This method does not typically preserve the self-associated state of the protein. An alternative approach to SEC-MS has been the use of aqueous mobile phases that are MS compatible such as ammonium formate and ammonium acetate at low concentrations (<100 mM). While these mobile phases may not completely preserve the native structures for biomolecules,[3] they have been found to provide MS sensitivity while best preserving protein self-association and size based chromatographic separation.

Method Development

The ACQUITY UPLC BEH200 SEC, 1.7 µm Column was evaluated at varying ammonium formate concentrations (5-200 mM) for resolution and MS sensitivity. Initial screening by UV evaluated the effect of salt concentration on both peak shape and resolution. A protein standard (Bio-Rad Laboratories) was used for the analysis. At low ammonium formate concentrations (<100 mM), secondary interactions result in poor peak shape and increased tailing for most of the proteins compared to phosphate buffers. These interactions can be due to either an “ion-exchange” or “ion exclusion” effect between the free silanols on the packing material and the biomolecules.[7] While peak shape and resolution improved at higher ammonium formate concentrations, ion suppression in the ESI process was also observed with lower intsenity counts. The final mobile-phase conditions were selected to balance resolution and ion suppression. At 100 mM ammonium formate, no tailing significant was observed and the MS signal was adequate for peak identification.

Comparison of the UV chromatograms with 100 mM ammonium formate and PBS (25 mM sodium phosphate, 150 mM sodium chloride, pH 6.8) mobile phases show similar retention and peak shape (Figure 1). For this example, ammonium formate provides an adequate SEC separation. However, not all biomoelcules exhibit the same degree of secondary interactions. In instances in which there are greater secondary interactions, the ammonium formate concentration can be altered to improve peak shape.

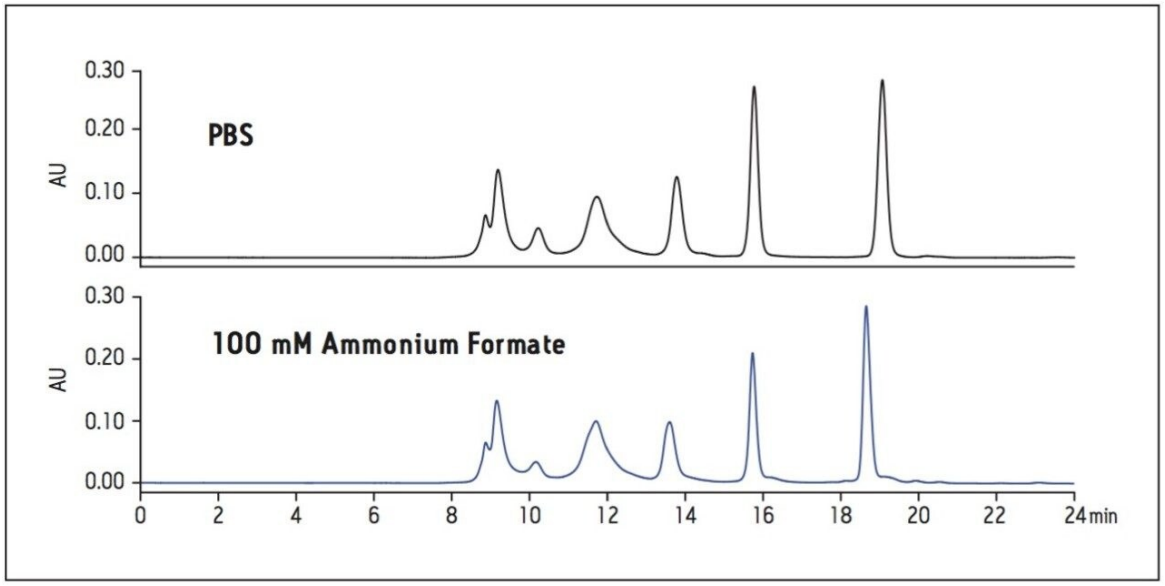

Figure 1. Influence of mobile-phase composition on the SEC separation of a protein standard.

As described above, ammonium formate was selected because of its volatility and MS compatibility. Since the use of non-denaturing mobile phases such as ammonium formate can reduce MS signal by a factor of 10 or greater,8 a denaturing modifier (formic acid in acetonitrile) was added to the eluent post-column. The post-detector tubing and external pump were connected with a tee just prior to the MS inlet valve. Differences in resolution between the UV and TIC were minimal (Figure 2). As expected, there were significant differences in relative peak area ratios of the proteins in the TIC and UV chromatograms due to differential ionization efficiencies of the protein species. In these experiments the ESI-MS TIC was used solely for identification purposes, and the UV traces for quantification, where relevant.

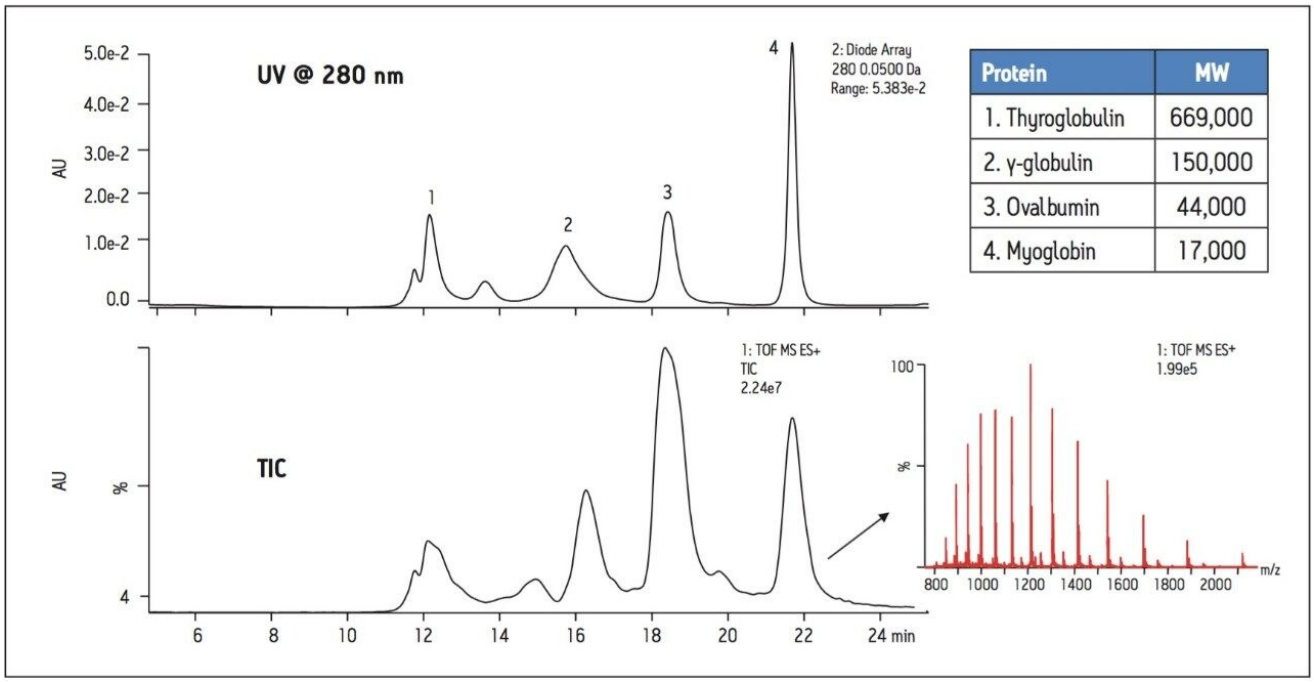

Figure 2. SEC-UV-MS analysis of a protein standard.

Analysis of Myoglobin Aggregates

The ACQUITY UPLC BEH200 SEC, 1.7 µm Column provides adequate resolution and MS sensitivity of the myoglobin size variants, including the monomer (peak 1), dimer (peak 2) and higher order aggregates (peak 3) (Figure 3). The ESI mass spectrum of the myoglobin monomer and dimer show multiple charged ion signals (Figure 4). The spectrum for the monomer reveals multiple-charge states from *m/z* approximately 800 to 2000 corresponding to charge states from $[M+8H]^{+8}$ to $[M+21H]^{+21}$. The deconvoluted spectrum of the monomer mass spectrum confirms the intact mass of myoglobin at 16,951. The MS signal for the dimer is a factor of 10 weaker than that of the monomer. The ESI mass spectrum of the dimer shows multiple charge states from $[M+20H]^{+20}$ to $[M+40H]^{+40}$. The deconvoluted spectrum shows the presence of both myoglobin monomer and the dimer (*m/z* 16,951 and 33,886).

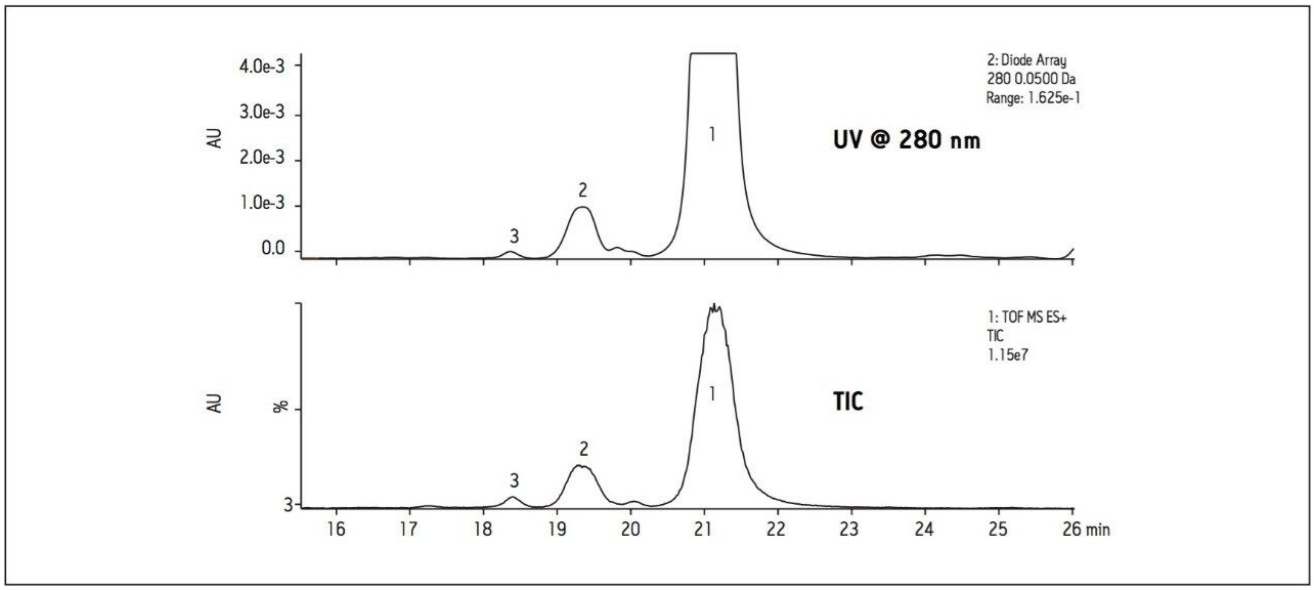

Figure 3. SEC-UV-MS anslysis of myoglobin monomer and aggregates.

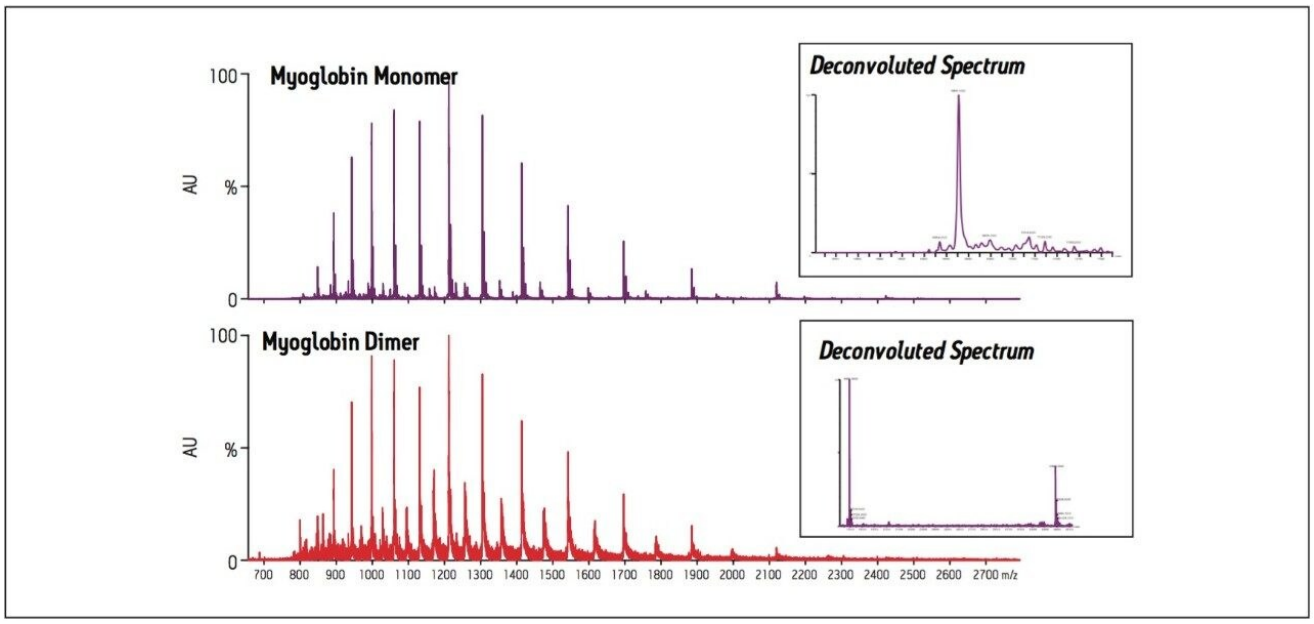

Figure 4. ESI mass spectrum and deconvoluted spectrum (inset) of myoglobin monomer and dimmer.

The simultaneous presence of monomer and dimer in the deconvoluted spectrum may be due to a variety of

factors including dissociation of the non-covalent dimer in source, and/or presence of additional size variants. As described above, an acidic organic modifier is required post-column to provide adequate ionization of the proteins. These sample conditions can cause the proteins to denature, thus disrupting protein-protein interactions including non-covalent interactions.[2] An additional factor may be due to the presence of misfolded forms of myoglobin. While separation of the myoglobin monomer and dimer is achieved, a minor peak is present between the two peaks, possibly due to misfolded proteins or other size variants. These forms may be one factor for the appearance of the monomer mass in the deconvoluted spectrum of the dimer. Nevertheless, the presence of only myoglobin monomer and dimer indicates that the aggregation is primarily related to self-association of myoglobin.

Identification of Unknown Components in a Biotherapeutic

An intact monoclonal antibody biotherapeutic, which was past expiry, was analyzed by SEC (Figure 5) using MS-friendly, non-denaturing conditions. In the UV chromatogram, not only are the mAb aggregate and monomer observed, but a low molecular weight (LMW) peak eluting after the intact mAb is partially resolved as well. In addition to these peaks, the UV chromatogram reveals two other LMW species.

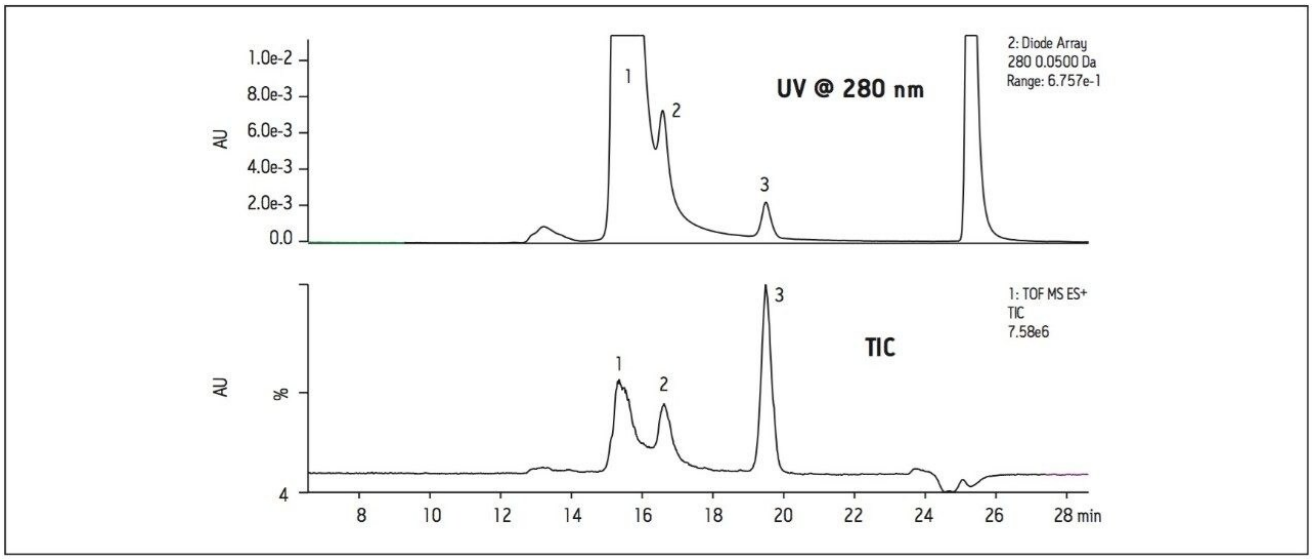

Figure 5. SEC-UV-MS of a recombinant humanized monoclonal antibody.

The ESI-mass spectrum of the monoclonal antibody (1) shows charge-states from $[M+34H]^{+34}$ to $[M+70H]^{+70}$

(Figure 6). The sensitivity of the method is illustrated by the high TIC satellite peaks of the $[M+39H]^{+39}$ and $[M+40H]^{+40}$ charge-states of the monomer . The deconvoluted spectrum of the monomer peak confirms the presence of the major glycosylated forms of the intact antibody with values corresponding to previously published results.9 The exact masses can be assigned to G0F/G0F (148,058 *m/z*), G0F/G1F (148,219 *m/z*) and (G01F)2 or G0F/G2F (142,379 *m/z*).

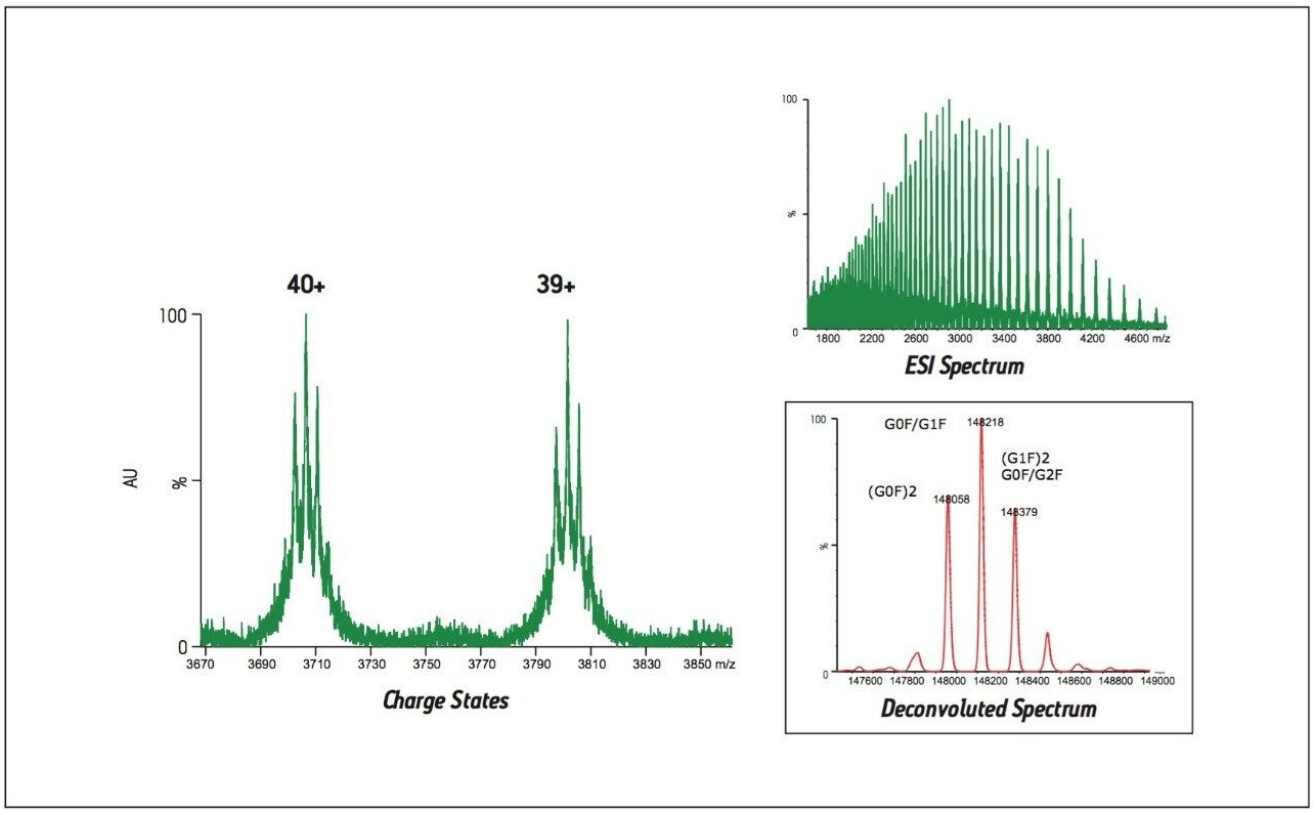

Figure 6. ESI mass spectrum of an intact monoclonal antibody. Deconvoluted spectrum (inset) shows intact mAb as well as gylcosylated forms.

The LMW peak (peak 3) eluting at 19 minutes also provides an adequate MS signal for molecular weight confirmation. Analysis of the ESI spectrum shows the presence of two different charge envelopes from 1100-2400 *m/z* (Figure 7). This is evident in the magnified view in which the satellite peaks for both sets of charge-states are resolved. The deconvoluted spectrum shows multiple peaks (Figure 8 inset), with 47,269 *m/z* (F1) and 47,636 *m/z* (F2) having the highest intensities. These intact masses correspond to the two prominent multiply charged ion states in the ESI mass spectrum: the charge states from $[M+19H]^{+19}$ to $[M+31H]^{+31}$ are shown in the zoomed

spectrum. Based on the sequence of the protein, the main peaks in the deconvoluted spectrum can be assigned to Fab fragments resulting from hydrolytic cleavage of the heavy chain: the mass of F1 (47,270 *m/z*) is consistent with the Fab fragment comprised of the light chain and the heavy chain fragment from the N-terminus to Asp[224] while the mass of F2 (47,637 *m/z*) is consistent with the Fab fragment comprised of the light chain and the heavy chain fragment from the N-terminus to Thr[228].

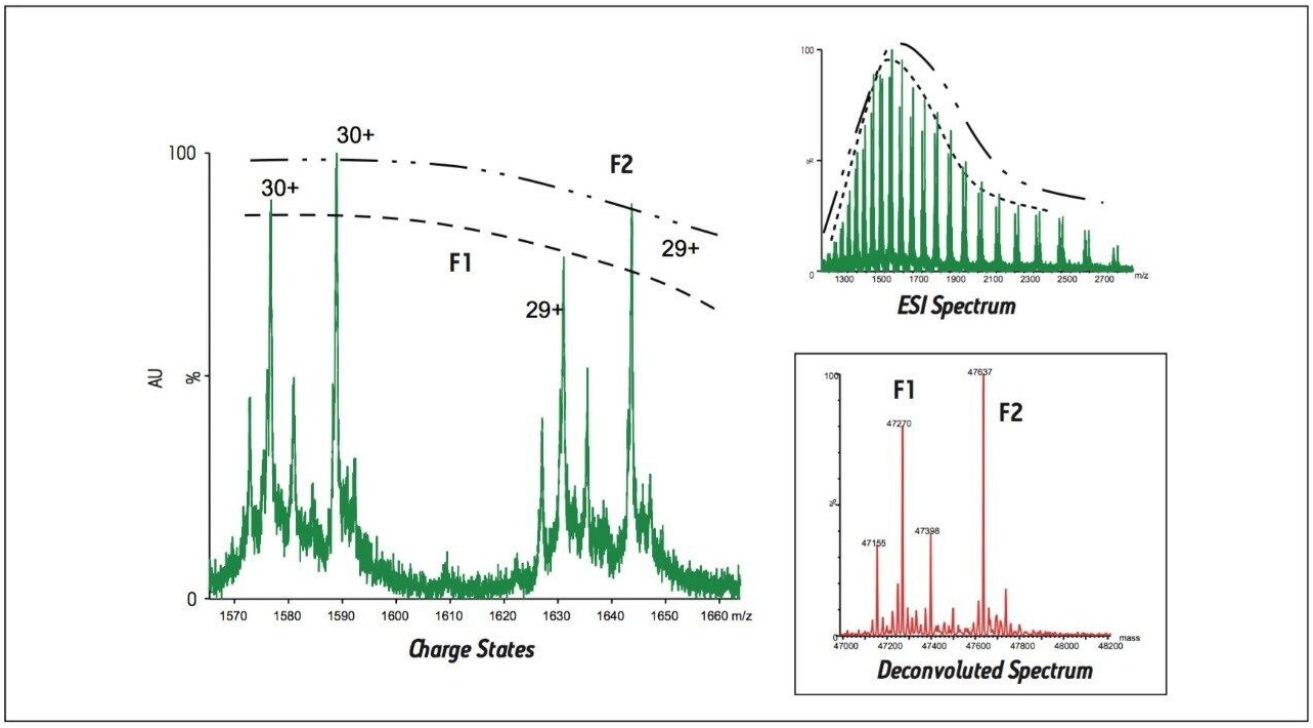

Figure 7. ESI mass spectrum of low molecular weight species (peak 3) in a recombinant humanized monoclonal antibody.

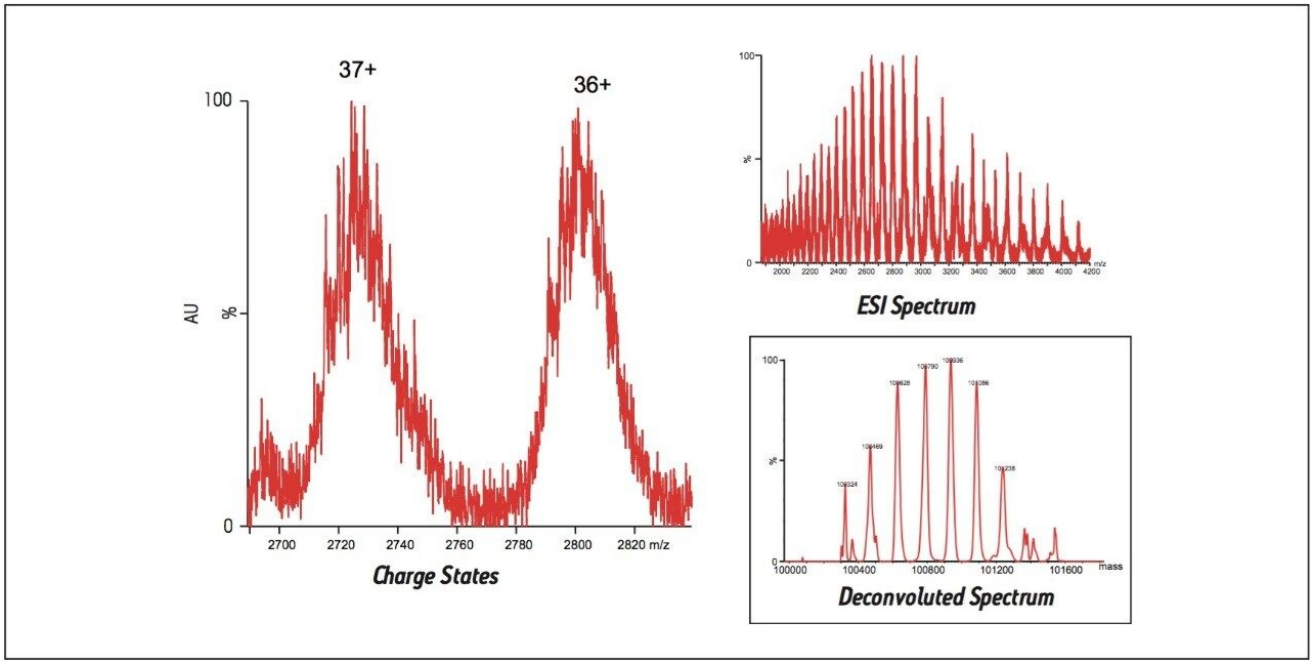

Figure 8. ESI mass spectrum of fragment (peak 2) in a recombinant humanized monoclonal antibody.

A similar analysis can be performed for the partially resolved low molecular weight species (peak 2 in Figure 5). The ESI mass spectrum of the fragment shows charge states from $[M+32H]^{+32}$ to $[M+48H]^{+48}$ at 2200-4000 *m/z* . While the satellite peaks are not well resolved for the lower molecular weight species (Figure 7), the charge states are evident. The deconvoluted spectrum (Figure 8) shows molecular weights consistent with antibodies that have a missing Fab arm with fragments ranging from 100,468 to 101,237 *m/z* (Figure 8 inset). The species observed at 100,468 is consistent with an antibody without one of the Fab arms cleaved at the N-terminal side His229. The confirmation of other, minor fragments that appear to be present is beyond the scope of this application note.

The SEC-MS analysis of the recombinant humanized monoclonal antibody allows for identification of not only the intact monoclonal antibody, but also the lower molecular weight fragments. Deconvolution of the ESI mass spectrum provides intact molecular weight information for the monomer and fragment species.

Conclusion

Size exclusion chromatography under non-denaturing conditions is a standard method for testing biomolecules and their aggregates. MALS and AUC are established detectors but cannot provide exact mass for unknown species with a sufficient accuracy. The presence of an unexpected peak requires further investigation and/ or confirmation of molecular weight, and SE-UPLC MS under aqueous, non-denaturing conditions can provide valuable information that would more rapidly solve an organization's issues with characterization or quality.

While SEC-MS does not typically preserve protein self association, it can assist in identification. The analysis of myoglobin illustrates the utility of an SEC-MS approach by confirming that the HMW forms observed in the myoglobin sample are related to the protein. The SEC-MS analysis of a humanized monoclonal antibody under non-denaturing conditions provides exact masses for LMW antibody fragments. By efficiently combining the ACQUITY UPLC BEH200 SEC, 1.7 µm Column and the benchtop Xevo G2 Q-Tof with an extended *m/z* range, the intact antibody and its associated fragments can be identified, providing a rapid method for exact molecular weight determination of intact biomolecules.

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720004254, March 2012