Application Note

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Comprehensive Approaches to Higher-Order Structure of Intact Proteins by Native Mass Spectrometry

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Abstract

This application note presents different approaches to native mass spectrometry (nMS) using Agilent high resolution mass spectrometers for analysis of intact proteins and noncovalent complexes. This application note covers different analytical approaches including nano electrospray, capillary flow, high flow, and capillary electrophoresis. These approaches also be interfaced with ion mobility for higher order structure studies.

Introduction

MS is emerging as a robust and sensitive technology for the study of protein structure, requiring very little starting material. For intact proteins, mass confirmation and putative post translational modifications are identified. To study protein structure or noncovalent complexes, native MS is used. For native MS, protein is buffer exchanged into a volatile salt in aqueous buffer at neutral pH conditions, usually an ammonium acetate solution, which can be infused with nanoESI into a high-resolution MS, such as a Q-TOF. For a more robust approach, online buffer exchange with size exclusion chromatography (SEC) is used for online desalting using an ammonium acetate solution and introduced into the MS by ESI. This allows the characterization of a protein in its folded state at neutral pH. In a biopharmaceutical environment, native MS helps characterize mAbs, cysteine-linked antibody drug conjugates, or noncovalent complexes. However, challenges arise when sample amounts are limited, especially in pharmacokinetics and drug metabolism (PKDM) studies, where nanoLC and capillary electrophoresis can be advantageous. This application note explores many techniques, including offline and online desalting methods coupled to MS and IM-MS, for high-order structure analysis of intact proteins.

Experimental

Reagents and chemicals

For noncovalent complexes and protein standards; alcohol dehydrogenase (ADH, from Saccharomyces cerevisiae), pyruvate kinase from rabbit (PKM, rabbit muscle), glutamate dehydrogenase (GDH, bovine liver), chaperonin 60 (GroEL, Escherichia coli), and IgG1 (SILu Lite SigmaMAb) were purchased from Sigma-Aldrich. An IgG1 was also expressed in house. Ammonium acetate was purchased from Sigma-Aldrich and diluted to 100 to 200 mM.

Separation

For online desalting using size exclusion chromatography (SEC), a method adapted from Valarie-Douglas *et al.*[1] was developed using 100 to 150 mM ammonium acetate, pH 7. A PolyHYDROXYETHYL A column (1.0 × 50 mm, 5 µm, 051HY0503) was connected via a capillary LC/MS nebulizer (G1385A) to the dual ESI source. For high-throughput workflows, the Agilent AdvanceBio SEC guard column, 4.6 × 30 mm, 1.9 µm, 200 Å (PL1580-1201) was used with an Agilent Jet Stream (AJS) source.

CE/MS

For capillary electrophoresis (CE) using the Agilent 7100 CE system, a PVA capillary was used with the Adapter interface kit (part number G1603A) with the ESI source and the capillary electrophoresis sprayer kit (part number G1607B) for the capillary, sheath gas, and sheath liquid interface to an Agilent 6500 Series accurate-mass quadrupole time-of-flight LC/MS system. The sheath liquid was introduced using an isocratic pump with a 1:100 splitter assembly at a flow rate of 4 µL/min. For denaturing conditions, the background electrolyte (BGE) was 30% acetic acid in water with sheath liquid of 0.1% formic acid, 0.005% (v/v) 2.5 M HP-921 (G1969-85001, reference mass) in 50:50 (v:v) methanol water in water, 1 µL/min flow rate. For native MS conditions, the background electrolyte was 25 mM ammonium acetate with sheath liquid of 100 mM ammonium acetate flowed at 1 µL/min. The CE and isocratic pump were integrated and controlled by Agilent MassHunter Acquisition software for introducing the sample under both native and denaturing MS conditions.

Sample preparation

For offline buffer exchange, Micro Bio-Spin 6 columns were used, and the proteins were buffer exchanged against 100 mM ammonium acetate, pH 7. For the noncovalent complex, GroEL, the protocols by Freeke *et al.*[2] and Campuzano *et al.*[3] were used.

Equipment and software

Online desalting by SEC was performed using an Agilent 1290 Infinity II LC system consisting of:

- Agilent 1290 Infinity II binary pump (G4220B)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)

CE was performed using a 7100 CE interfaced with a 6500 Series MS with:

- Capillary cassette for CE/MS (G1600-60013)
- CE-ESI sprayer (G1607-60002)
- Splitter assembly, CE/MS (G1607-60000)
- Agilent 1260 Infinity II isocratic pump (G7110B)

The UHPLC system was interfaced with the capillary nebulizer (G1385A) to the dual ESI source on a 6500 Series MS or Agilent 6230 TOF LC/MS. MassHunter Acquisition (B.08.01) equipped with the large molecule SWARM autotune was used on the 6545XT AdvanceBio LC/Q-TOF. For data analysis, Agilent MassHunter Qualitative Analysis (version 10.0), BioConfirm (version 10.0, SP1), and Universal Deconvolution of Mass and Ion Mobility Spectra (UniDec)[4] were used.

Methods

Tables 1 and 2 summarize the Agilent 1290 Infinity II LC isocratic conditions for online desalting with SEC with the Agilent 6200 Series MS and 6500 Series MS. Tables 3 and 4 outline the source conditions for use with nanoESI infusion and capillary electrophoresis with the 6200 Series MS and 6500 Series MS. Table 3 describes the nanoLC source conditions used for offline buffer exchange and infusion of larger noncovalent complexes. A recently developed high-mass "SWARM" autotune algorithm was used to optimize the high-mass ion transmission and detection for the Agilent 6545XT AdvanceBio LC/Q-TOF instrument.

Table 1. Agilent 1290 Infinity II LC conditions with the Agilent 6500 Series MS conditions with the capillary LC/MS nebulizer assembly.

LC Conditions: Online SEC	
Column	Poly LC PolyHYDROXYETHYL A, 1.0 × 50 mm, 5 µm, 051HY0503
Column Temperature	Room temperature, not controlled
Injection Volume	1 to 20 µL
Multisampler Temperature	4 °C
Mobile Phase	100 to 200 mM ammonium acetate, pH 7
Flow Rate	0.005 mL/min
Gradient	Isocratic
Stop Time	10 to 30 minutes
MS Parameters: Online SEC with the Capillary Nebulizer (p/n G1385-68700)	
Ion Mode	Dual ESI, positive
Gas Temperature	150 °C
Drying Gas Flow	10 to 13 L/min
Nebulizer Gas	25 psi
Capillary Voltage	4,500V
Fragmentor Voltage	350 to 400V

Table 2. Agilent 1290 Infinity II LC conditions with the Agilent 6500 Series MS conditions with the standard LC/MS nebulizer assembly.

LC Conditions: High-Throughput Online SEC	
Column	Agilent AdvanceBio SEC guard column, 4.6 × 30 mm, 1.9 µm, 200 Å (PL1580-1201)
Column Temperature	Room temperature, not controlled
Injection Volume	1 to 20 µL
Multisampler Temperature	4 °C
Mobile Phase	100 to 200 mM ammonium acetate, pH 7
Flow Rate	0.4 mL/min
Gradient	Isocratic
Stop Time	6 minutes
MS Parameters: Online SEC with the Standard Nebulizer (p/n G1958-67098)	
Ion Mode	Dual Agilent Jet Stream ESI, positive
Gas Temperature	365 °C
Drying Gas Flow	12 L/min
Nebulizer Gas	60 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Fragmentor Voltage	350 to 400 V

Table 3. Agilent 6500 Series MS method parameters with the nanospray source for infusion.

Parameter: Infusion	Setting
Ion Mode	NanoLC Source (G1992A), positive
Gas Temperature	150 °C
Drying Gas Flow	8 L/min
Capillary Voltage	1,800 to 1,950 V
Fragmentor Voltage	350 to 400 V

Table 4. Agilent 6500 Series MS method parameters with the capillary electrophoresis with CE-ESI sprayer.

Parameter: Online SEC	Setting
Ion Mode	Dual ESI, CE-ESI sprayer (p/n G1607-60002), positive
Gas Temperature	300 °C
Drying Gas Flow	6 L/min
Nebulizer Gas	6 psi
Capillary Voltage	5,500 V
Fragmentor Voltage	350 to 400 V

Results and discussion

Optimization of conditions

Native MS of noncovalent complexes requires cool source conditions; otherwise, subunits will readily dissociate in the ion source. Evidence of dissociation involves seeing a charge envelope at a lower *m/z* range. Therefore, gentle and cool source temperatures are needed to maintain the complex in its intact conformation, as well as proper desolvation. Proposed here is the use of the dual ESI source with capillary nebulizer for online buffer exchange with SEC and nanoLC by infusion for offline buffer-exchanged samples. Figure 1 highlights the need for gentler source conditions. The top spectrum shows GDH denaturing into its subunits (*m/z* 3,000 to 6,000) with higher source temperature (350 °C), gas flow rate (10 L/min), and nebulizer pressure (50 psi). Once the source conditions were lowered to optimal conditions, temperature (150 °C), gas flow (11 L/min), and nebulizer pressure (35 psi)—GDH remained in its intact form. Further declustering can be performed by applying additional collision energy in the collision cell and by using a different collision gas, depending on the size of the protein of interest.

For high-throughput online desalting workflows, optimization with the AJS source was used to monitor the behavior of alcohol dehydrogenase (ADH) on the 6545XT AdvanceBio LC/Q-TOF. Figure 2 plots *m/z* 6147 (z = 24+) for ADH monitoring of the response to changes in the sheath gas flow rate and temperature using the standard nebulizer. The microflow nebulizer resulted in a 50% increase in sensitivity.

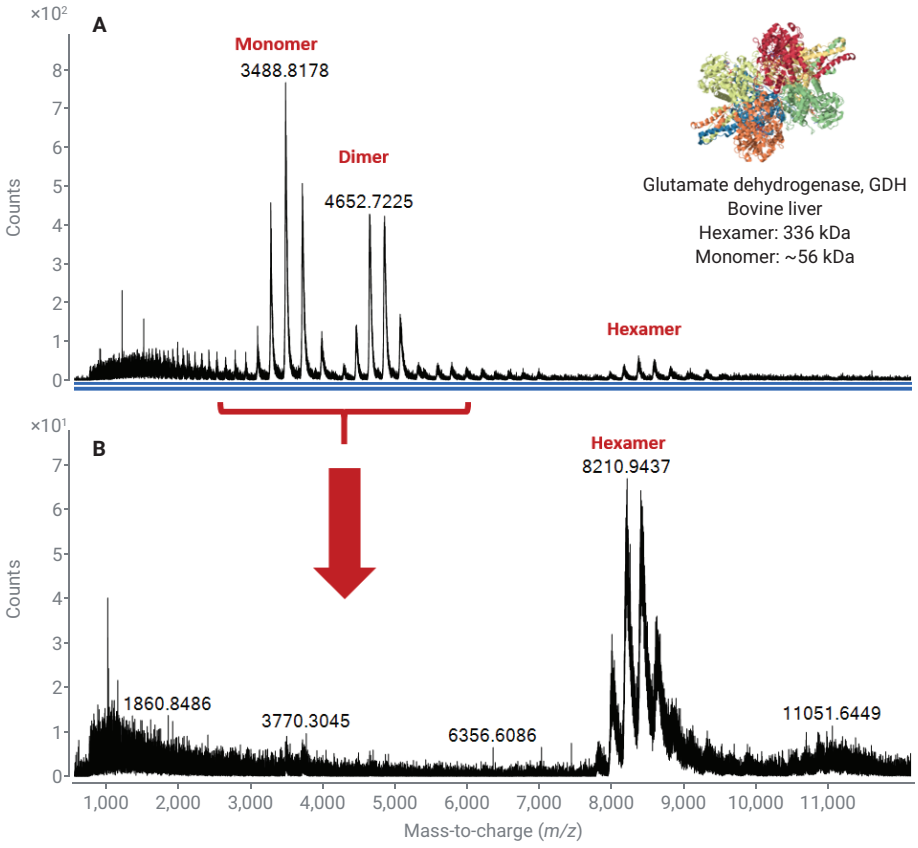

Figure 1. Source optimization using online SEC with the dual ESI source with microflow nebulizer of the homohexameric enzyme, GDH on an Agilent 6530 LC/Q-TOF.

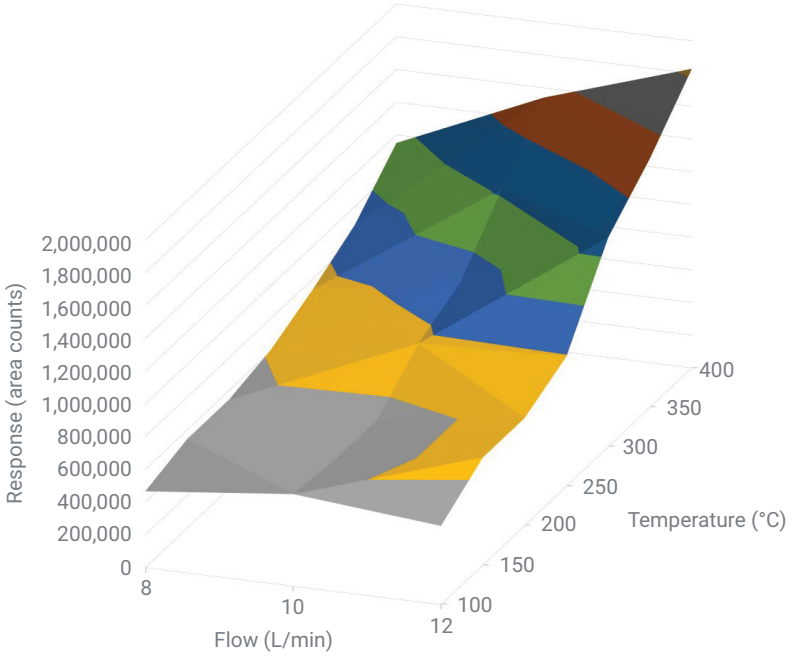

Figure 2. Plot of ADH tetramer (*m/z* 6147 area, 24+ charge state) response to sheath gas parameters temperature and flow.

Extended mass range

The 6200 Series MS and 6520/6530 Q-TOF LC/MS allow MS data acquisition up to *m/z* 20,000, which is needed for native mAbs, and noncovalent complexes up to 350 kDa. For larger noncovalent complexes, the 6545XT can go to *m/z* 30,000 and offers a high-mass SWARM autotune for high *m/z* ion transmission and detection. To highlight the high-mass SWARM autotune and extended mass range capability to *m/z* 30,000, several proteins and noncovalent complexes ranging in molecular weight, from 143 kDa to 801 kDa, were analyzed (Figure 3).

Figure 3 highlights the extended mass range of noncovalent complexes including homo-tetramers (ADH, PKM), homo-hexamer (GDH), and a dual-ringed tetradecamer (GroEL). The mass spectra highlight the optimized source conditions and transmission to keep these noncovalent complexes together. Figure 4 shows GroEL undergoing fragmentation by applying collision energy, using SF_6 as the collision gas. The bottom spectrum shows the intact GroEL and the resulting 13-mer of GroEL after the release of a single subunit.

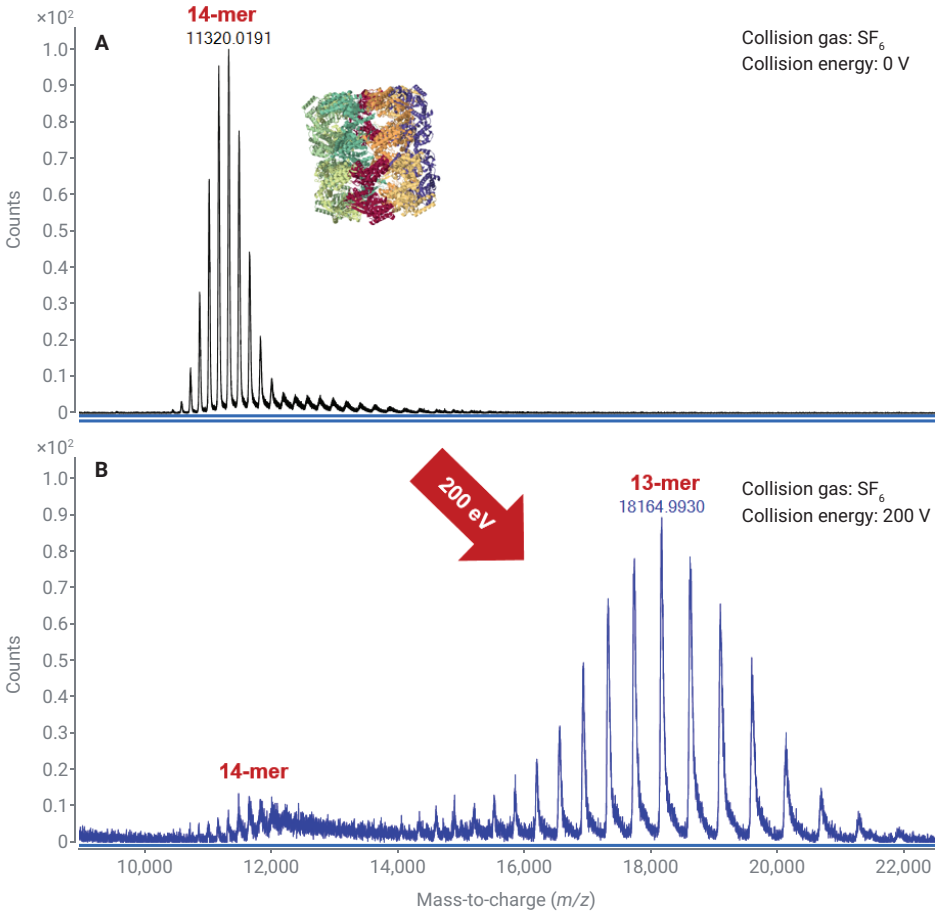

Figure 4. MS/MS on GroEL (A) MS of the intact 14-mer complex (B) MS/MS shows the release of a single subunit, resulting in the 13-mer on the Agilent 6545XT AdvanceBio LC/Q-TOF.

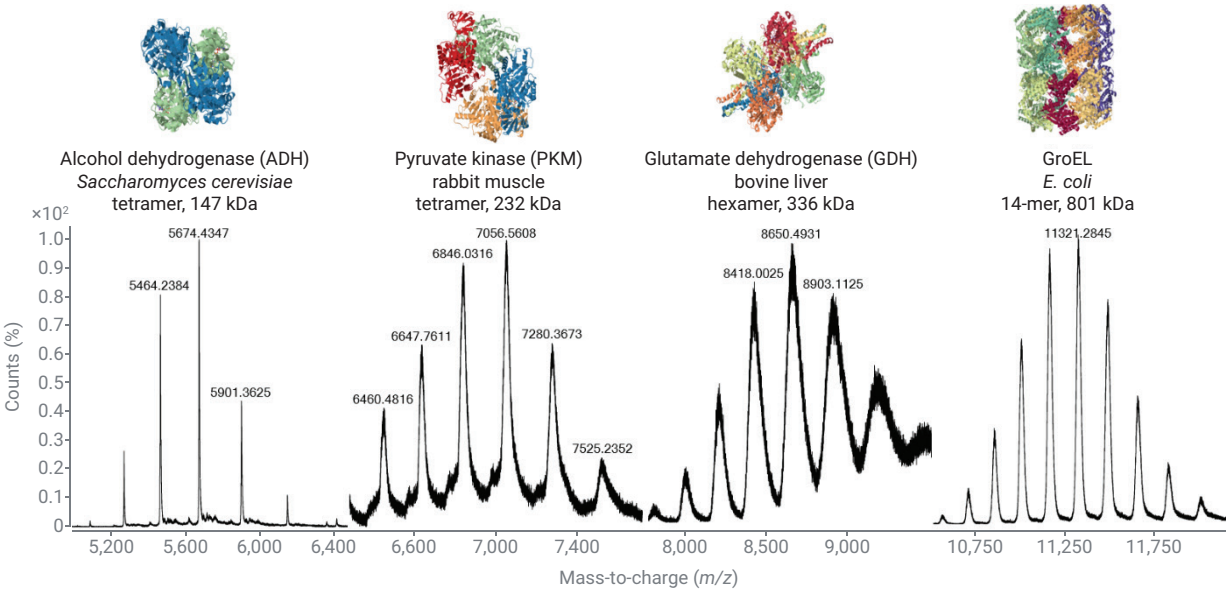

Figure 3. Noncovalent complexes by nanoLC on the Agilent 6545XT AdvanceBio LC/Q-TOF.

Sensitivity

The 6545XT AdvanceBio LC/Q-TOF with the extended mass range offers improved sensitivity for the analysis of intact proteins. Under native MS conditions, the National Institute of Standards and Technology (NIST) monoclonal antibody reference material 8671 was readily observed at 1 µg on column with the online SEC method and ESI source. Figure 5 shows the results of triplicate injections of the NISTmAb, with a blank injection of water, at 1 µg on column (left), 5 µg on column (middle), and 10 µg on column (right).

Native ion mobility mass spectrometry

Native MS applications can be extended to further probe higher-order structure using ion mobility mass spectrometry on the Agilent 6560 ion mobility LC/Q-TOF. Protein conformational changes can be monitored with ion mobility enabled. In Figure 7, ubiquitin under native MS conditions was subjected to collision-induced unfolding in the trap. By varying the trap RF, ubiquitin responds differently. On the left is the abundance map of ubiquitin at the different trap RFs; on the Y-axis is the drift spectrum for each RF voltage for the $[M+7H]^{7+}$ charge state for ubiquitin. When the drift spectra are overlaid from the different trap RF voltages, the lower voltage shows ubiquitin in its native state, and as the trap RF voltages are ramped, ubiquitin unfolds.

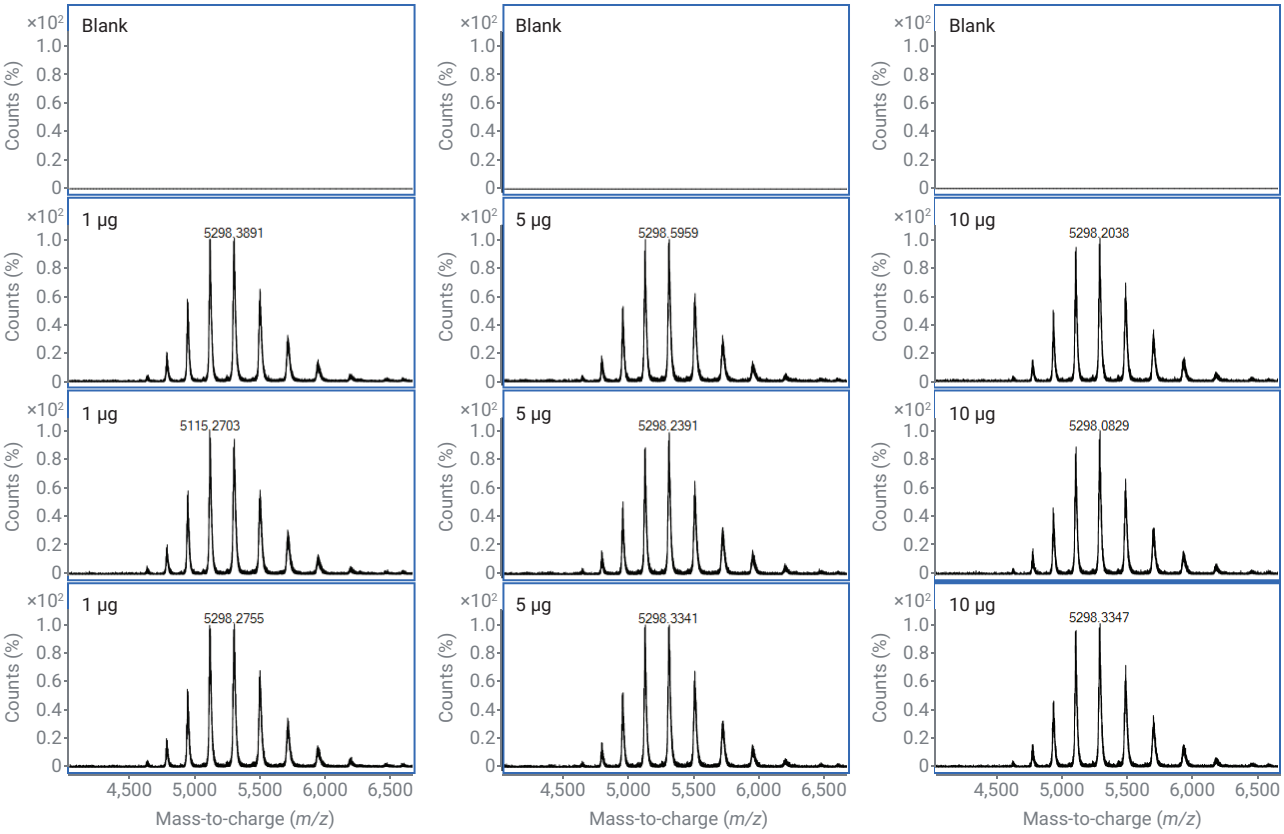

Figure 5. Native MS of triplicate injections of NIST mAb at different amounts on column.

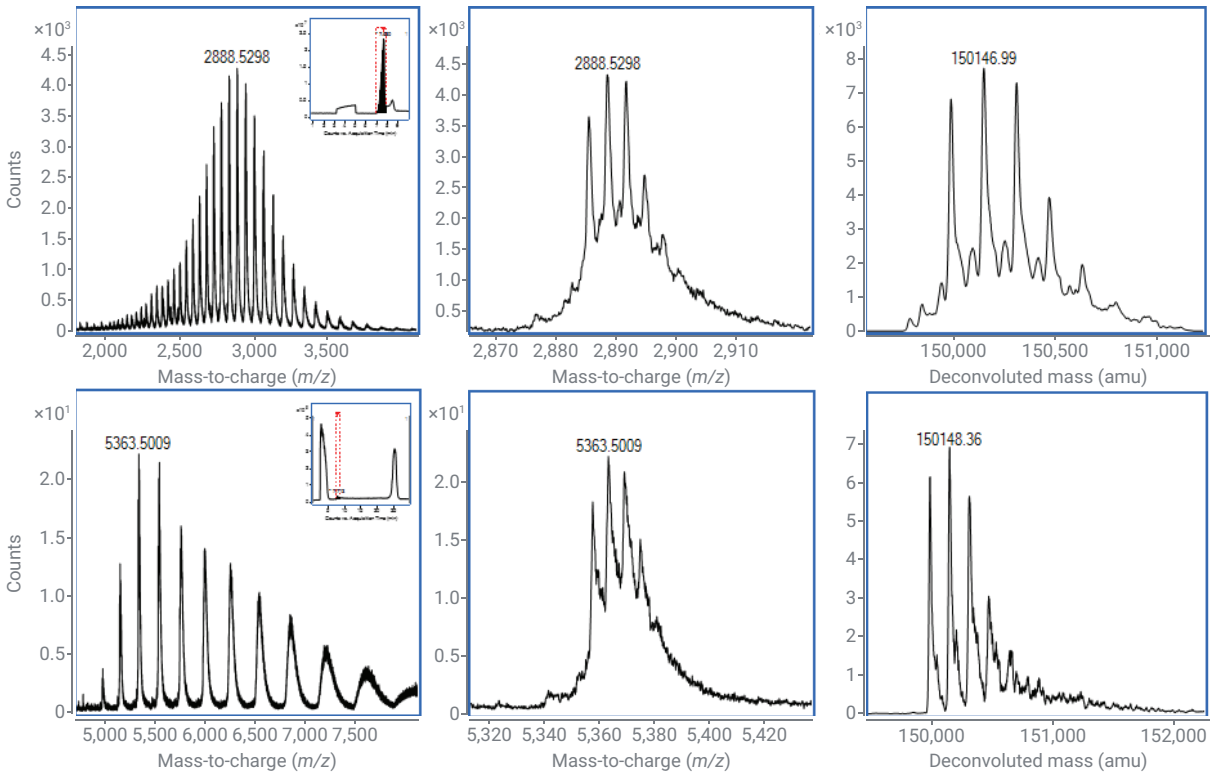

Figure 6. CE/MS of an IgG1 under denaturing MS conditions at low pH (upper) and native MS conditions pH 7 (bottom) using the Agilent 6530 LC/Q-TOF. From left to right, the full charge envelope (left) with the inset of the electropherogram in the upper right, zoomed spectrum of the most abundant charge state (middle) and deconvoluted spectrum (right).

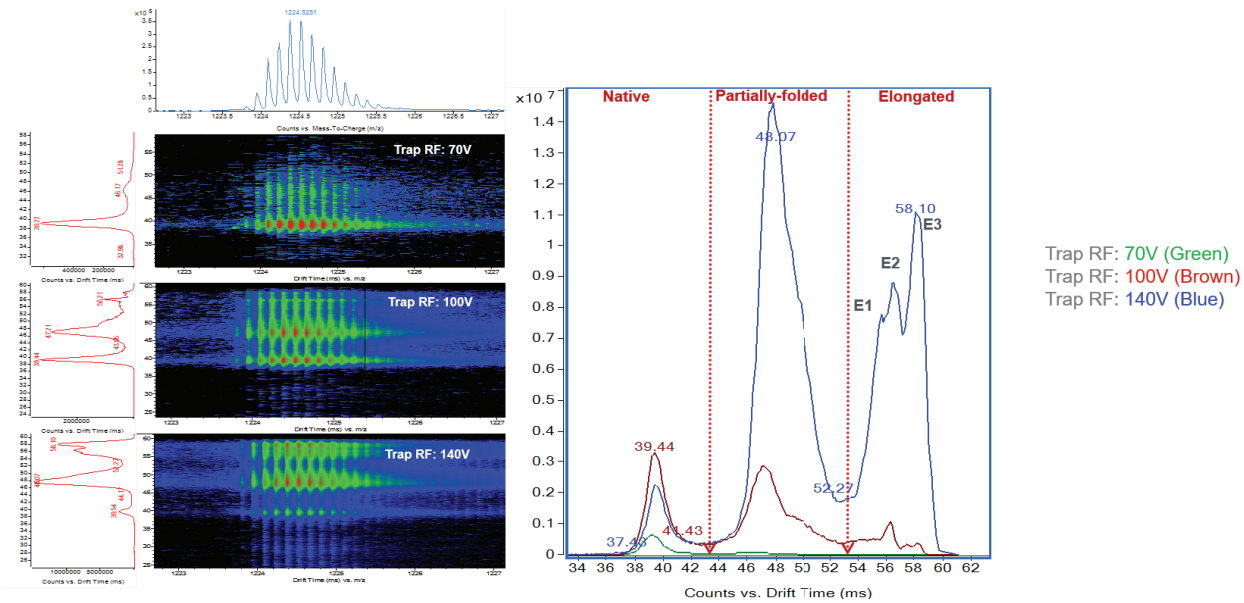

Figure 7. Native MS by online SEC with ESI on the Agilent 6560 ion mobility LC/Q-TOF.

Data analysis

Data analysis can be performed using Agilent BioConfirm or UniDec.[4] UniDec is able to smooth and deconvolute larger protein complexes. Figure 8 shows ADH acquired on the 6545XT deconvoluted to its neutral mass. For the larger complexes such as GroEL, Figure 9 shows the results obtained by UniDec.

Conclusion

Native MS can be readily achieved on the Agilent 6500 Series Q-TOFs. The 6545XT offers extended mass range and excellent sensitivity for intact proteins and noncovalent complexes while maintaining its versatility for small molecules and peptide mapping applications. For higher-order structure analysis, the 6560 ion mobility LC/Q-TOF is suited for protein structure conformation and collision induced unfolding. This application note has explored different approaches for the introduction of protein complexes for native MS, including online SEC with ESI, high-throughput AJS, nanoESI, and CE-ESI with the 6500 Q-TOFs and IM-Q-TOF.

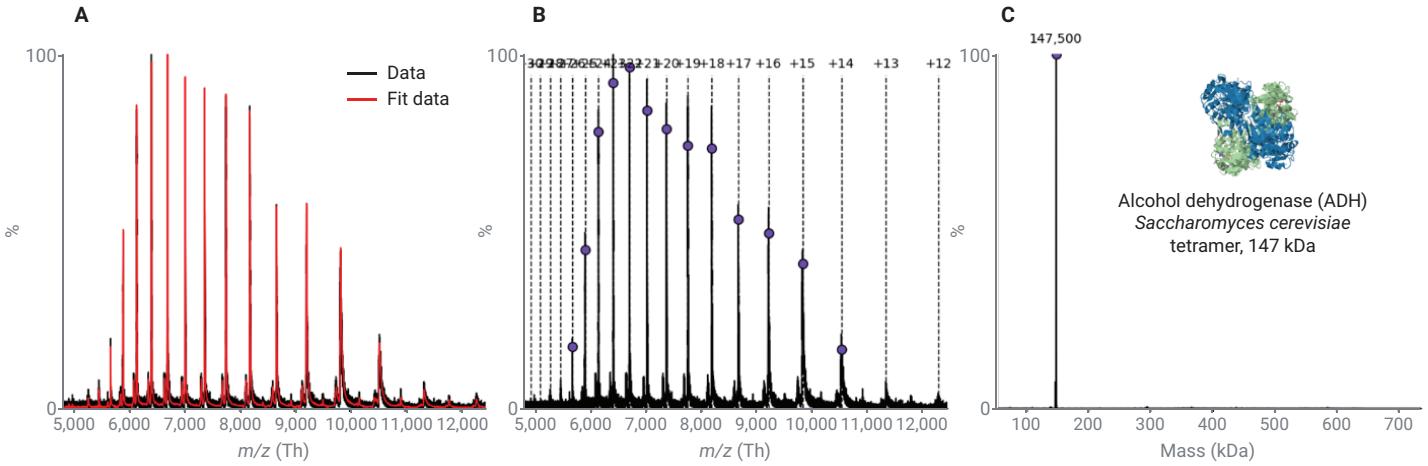

Figure 8. Overlay of the spectra of ADH with (red) and without baseline smoothing and fitting (black) acquired on the Agilent 6545XT AdvanceBio LC/Q-TOF under standard flow LC conditions (A). Charge distribution spectrum (B) and the deconvoluted spectrum of ADH to its neutral mass using UniDec (C).

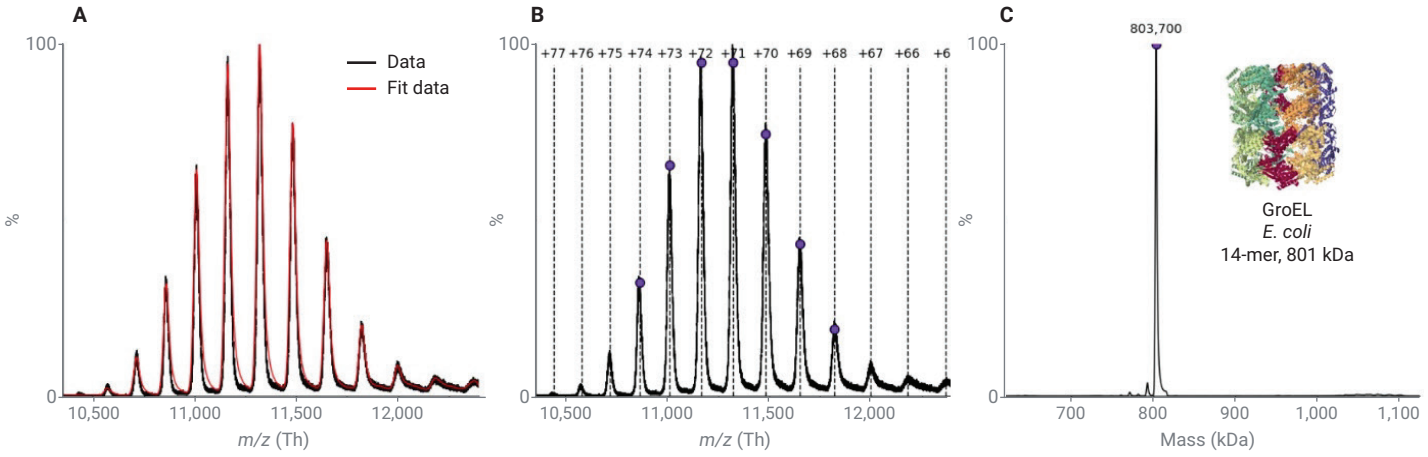

Figure 9. Overlay spectrum of GroEL acquired on the Agilent 6545XT AdvanceBio LC/Q-TOF with smoothing and baseline correction (red) to the unprocessed spectra (black) using UniDec under nanoESI conditions (A), charge state distribution (B), and deconvoluted spectrum (C).

References

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