Application Note

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Sensitive Native Mass Spectrometry of Macromolecules Using Standard Flow LC/MS

Author

David L. Wong
Agilent Technologies, Inc.

Abstract

Native mass spectrometry can be used for a variety of protein-based applications, such as protein-protein interaction, protein-ligand binding, protein complex structures, protein folding and antibody-drug conjugates. Most of the native MS analyses are using a nano-electrospray approach which faces significant challenges.

This application note describes a robust and sensitive LC/MS method using standard LC flow for the analysis of native protein analysis. The workflow comprised the Agilent 1290 Infinity II LC, the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software.

Introduction

Native mass spectrometry (MS) has emerged as a widely used technique for the characterization of intact proteins and noncovalent protein complexes. Various sizes of protein complex structures (protein-ligand binding or protein-protein interaction) ranging from a few kDa to more than 1 MDa have successfully been analyzed and studied by this technique despite its tremendous analytical challenges.[1-4] Without organic solvent and acid to enhance sample desolvation and ionization, native MS analysis of protein samples at neutral pH conditions tends to have fewer charges per molecule and much lower abundance MS signals at higher *m/z* ranges. In the past decade, the nano-electrospray ionization (nESI) approach has become a crucial method used in native protein analysis. The nESI forms fine charged droplets, which can dramatically increase the sample desolvation and ionization efficiency while preserving the noncovalent protein-protein complexes. However, it has commonly been observed that the neutral aqueous protein samples tend to aggregate easily under the unstable nanoflow rate condition and cause the nanospray emitter to clog. Also, well trained or experienced researchers are needed to produce good-quality MS data using the nESI technique.

In this study, we demonstrate a highly sensitive analytical flow LC/MS methodology for the analysis of native proteins and protein complexes. This workflow uses the AdvanceBio size exclusion chromatography (SEC) column for online sample separation. The 6545XT AdvanceBio LC/Q-TOF, featuring large molecule SWARM autotune and 30,000 *m/z* extended mass range, was used for rapid and reproducible native protein analysis (Figure 1).

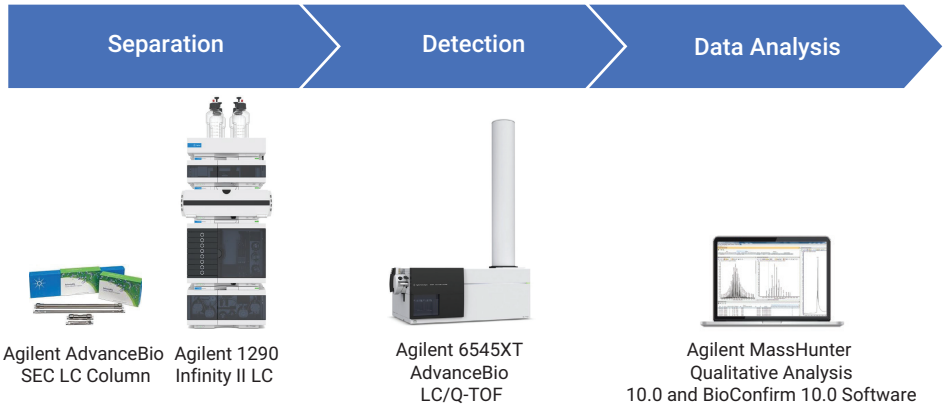

Figure 1. Analytical components of the native protein analysis workflow.

Experimental

Materials and methods

Monoclonal antibody standard, RM 8671, was purchased from the National Institute of Standards and Technology (NIST), often referred to as NIST-mAb. The formulated Herceptin (trastuzumab) was obtained from Genentech (South San Francisco, California, USA). The formulated trastuzumab emtansine (TDM1, ADC) was also from Genentech. All other protein samples and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

To perform native MS analysis, it is crucial to preserve the protein samples at neutral pH and volatile aqueous solutions, such as ammonium acetate or ammonium formate. Therefore, sample desalting and buffer exchange are usually needed prior to the MS analysis. Briefly, protein stock solutions (1 to 10 mg/mL) were desalted and solvent exchanged into 100 mM ammonium acetate using Bio-Rad Bio-Spin P-6 (6,000 MW limit) or P-30 (40,000 MW limit) cartridges. The cartridge was first fully equilibrated with 100 mM ammonium acetate buffer. Protein sample was then pipetted to the top of the column and centrifuged for 5 min at 1000 × g. The protein was then buffer exchanged into the 100 mM ammonium acetate and was ready for MS analysis. This desalting protocol caused minimal sample loss and much less structural alteration of the protein molecule.

Instrumentation

- Agilent 1290 Infinity II LC including:
  - Agilent 1290 Infinity II high-speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent Jet Stream source. Agilent MassHunter Acquisition (B.09.00) workstation software with the large molecule SWARM autotune feature was used.

LC separation was obtained with an Agilent AdvanceBio SEC guard column (4.6 × 30 mm, 200 Å, 1.9 µm).

Tables 1 and 2 list the LC/MS parameters used.

Data processing

All MS data of the native intact mAbs or protein complexes were processed using Agilent MassHunter Qualitative Analysis 10.0 and BioConfirm 10.0 software.

Results and discussion

Method optimization for native protein and protein complex analysis

To overcome the challenges of native protein analysis, some key method developments and optimizations were made:

- The use of offline desalting cartridges (Bio-Rad Bio-Spin P-30) for sample preparation (desalting and buffer exchange) prior to the MS analysis and online SEC column further separated the target protein from background salts, which led to higher MS sensitivity and improved MS data quality.
- The use of a conventional flow rate (0.2 mL/min) of 100 mM ammonium acetate buffer not only eliminated the sample aggregation but also improved LC/MS analytical reproducibility for well-preserved native protein samples.
- The 6545XT AdvanceBio LC/Q-TOF system was equipped with large molecule SWARM autotune for optimizing macromolecular ions transmission, and the extended mass range of up to *m/z* 30,000 for the native protein complex analysis with high sensitivity.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC	
Column	AdvanceBio SEC (200 Å, 4.6 × 30 mm, 1.9 µm) (p/n: PL1580-1201)
Thermostat	4 °C
Solvent (A)	100 mM NH4OAc (pH 7)
Isocratic Elution	0–5 min, 100% A
Column Temperature	Room temperature
Flow Rate	0.2 mL/min
Injection Volume	1–5 µL

Table 2. Native MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF			
Sample Type	Myoglobin	Intact mAbs	Intact Macroprotein Complexes
Source	Agilent Jet Stream	Agilent Jet Stream	Agilent Jet Stream
Dry Gas Temperature	150 °C	365 °C	365 °C
Dry Gas Flow	10 L/min	12 L/min	12 L/min
Nebulizer	30 psig	35 psig	35 psig
Sheath Gas Temperature	150 °C	300 °C	300 °C
Sheath Gas Flow	10 L/min	12 L/min	12 L/min
VCap	5000 V	5500 V	5500 V
Nozzle Voltage	2000 V	2000 V	2000 V
Fragmentor	250 V	300 V	300 V
Skimmer	100 V	220 V	220 V
Quad AMU	*m/z* 500	*m/z* 1000	*m/z* 3000
Mass Range	*m/z* 300–7000	*m/z* 3000–10,000	*m/z* 5000–25,000
Acquisition Rate	1.0 spectrum/s	1.0 spectrum/s	1.0 spectrum/s
Acquisition Mode	Positive, extended (*m/z* 10,000) mass range	Positive, extended (*m/z* 10,000) mass range	Positive, extended (*m/z* 25,000) mass range

Native MS analysis of intact myoglobin (with heme)

Native MS analysis of noncovalent interactions of myoglobin has been well-studied.[4] In myoglobin, heme is noncovalently attached to the globin through hydrogen bonds and hydrophobic interactions. When the heme is attached to the globin, the protein is referred to as holomyoglobin (the native conformation). Monitoring of the charge state distributions of myoglobin ions in mass spectra of ESI-MS has been used in protein folding/unfolding studies.[4] The apomyoglobin (with no heme) with high charge states indicated the disruption of the native heme-protein interaction, which led to a considerable degree of protein unfolding. As shown in Figure 2A, myoglobin was denatured in the organic and acid solvent, and under harsh MS source conditions. The charge envelope of the denatured myoglobin ranged from *m/z* 1,000 (17+) to 3,500 (5+) while the most intense charged ion was 12+. Most of the native holomyoglobin was denatured into apomyoglobin and heme (inset in Figure 2A). Our optimized native MS analysis of myoglobin clearly demonstrated that the native conformation of myoglobin was retained (Figure 2B). Only trace amounts of apomyoglobin and heme could be detected. The charge envelope of holomyoglobin was from 9+ to 5+ and the charge state of 8+ was the most abundant ion. The overall MS signal intensities of the native MS ions were about 1/10 of those in the denatured MS spectrum.

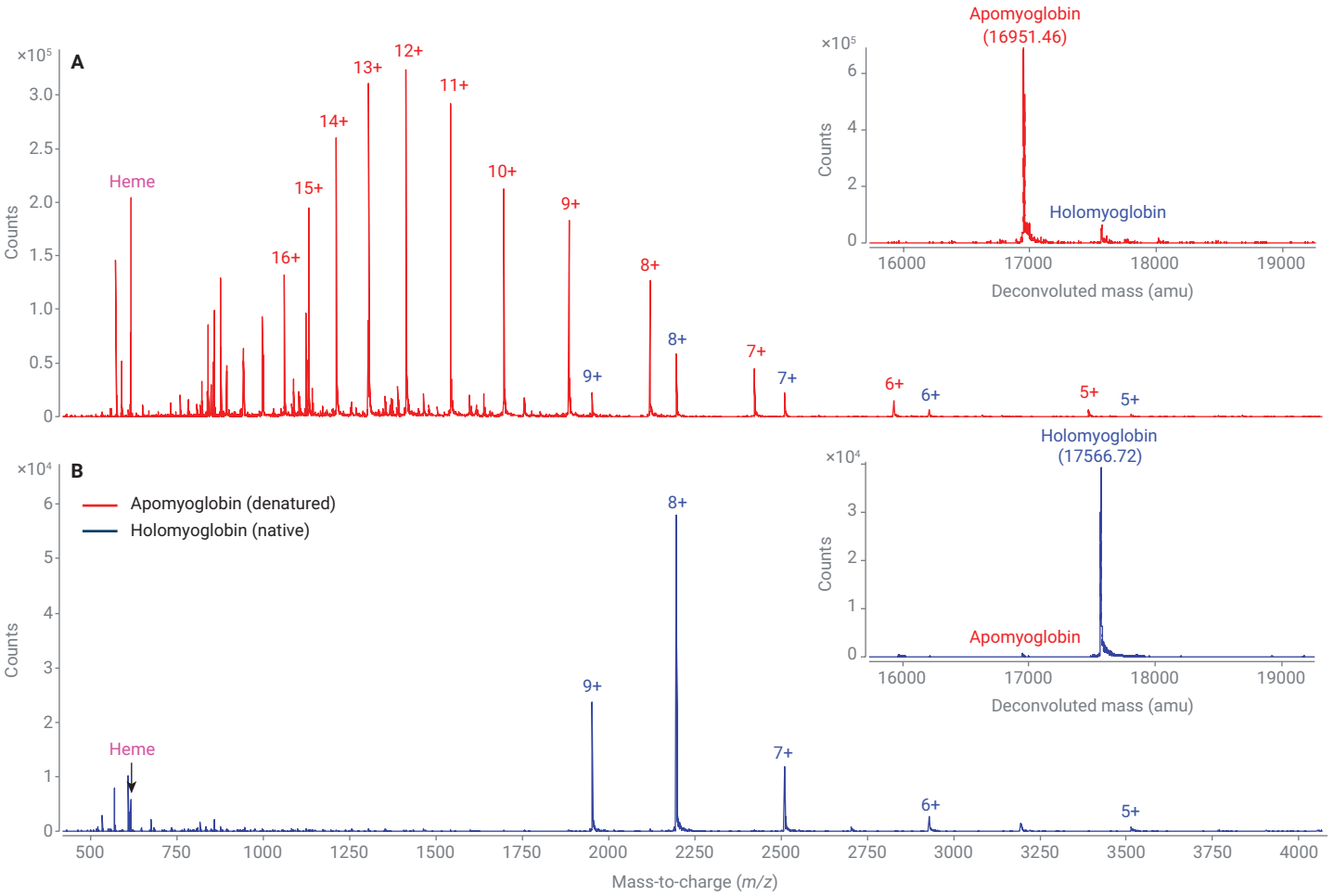

Figure 2. LC/MS analysis of intact myoglobin sample. A) Myoglobin sample was analyzed under denatured LC/MS conditions (previous studies). The heme group was dissociated from the protein complex and the majority of the protein was apomyoglobin (inset figure). B) Native MS analysis of myoglobin. The holomyoglobin (with heme) structure was preserved and only trace amount of heme was detected.

The native MS analysis results confirmed that ionic strength of the SEC column mobile phase also played a key role in maintaining the protein native conformation.[2] Figure 3 demonstrates the native MS analysis of alcohol dehydrogenase (ADH, tetramer) under two mobile phase conditions. Even though both mobile phases were at neutral pH, protein dissociation products (dimer) were observed when ammonium formate was substituted for ammonium acetate in the mobile phase (Figure 3A and 3B). Also, the charge state envelope of the intact native ADH in the 50 mM ammonium formate was shifted to a lower *m/z* range compared to that in the 100 mM ammonium acetate (Figure 3C). The results indicate that use of ammonium formate in the mobile phase increases the number and extent of multiply charged ions (max at 26+ compared to 23+ using ammonium acetate, Figure 3), although the ADH species may still be considered an intact protein tetrameric complex. Therefore, we believe that the 100 mM ammonium acetate solution offered better structural protection to protein complexes during the native MS analysis.

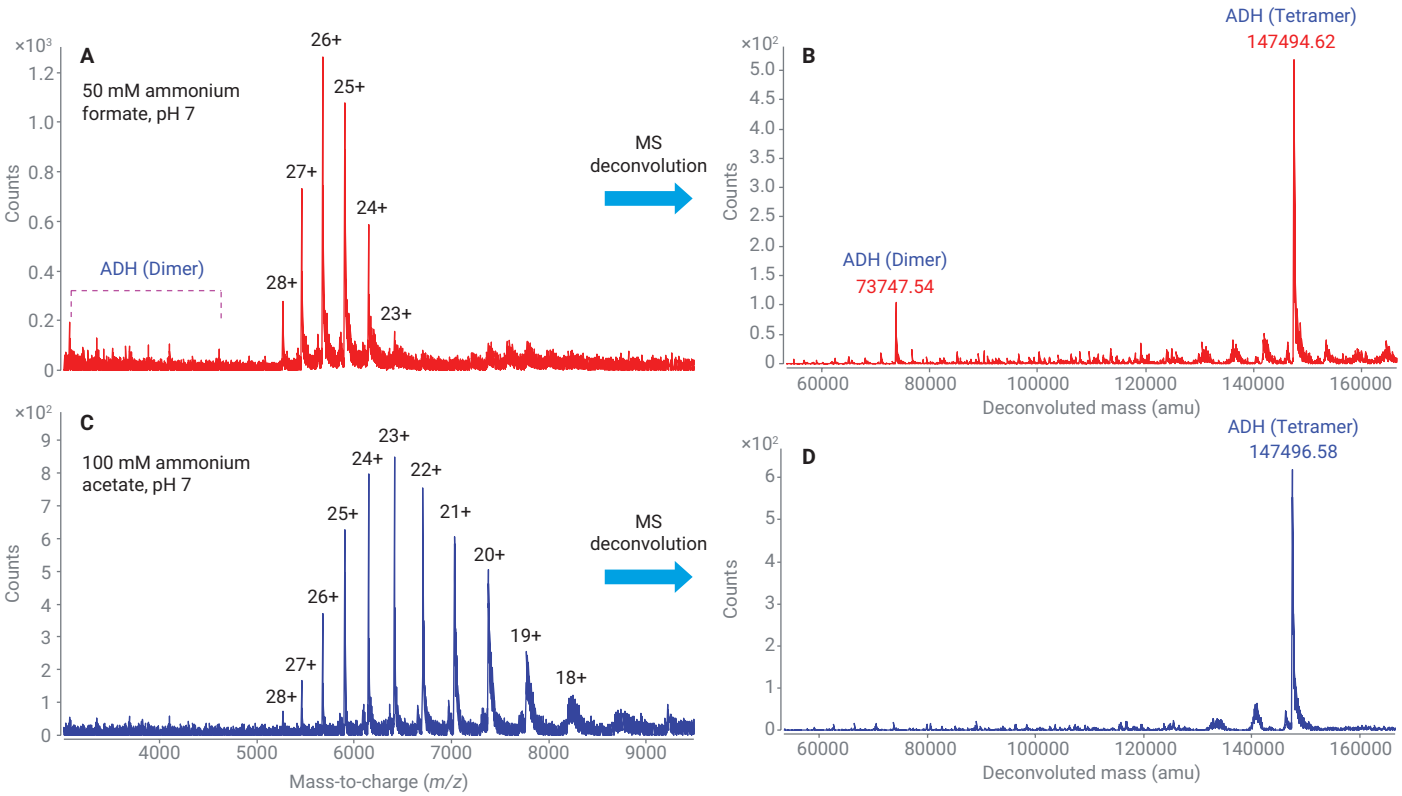

Figure 3. Native alcohol dehydrogenase (ADH, tetramer) analysis under various solvent conditions. A) Native ADH in 50 mM ammonium formate, pH 7. C) Native ADH in 100 mM ammonium acetate, pH 7. B) and D) deconvoluted spectrum of both samples.

Native MS analysis of intact mAbs

Monoclonal antibodies (mAbs) and their derivative products have quickly become an important class of biopharmaceutical molecules with a wide range of therapeutic applications. Native MS analysis of mAbs can provide valuable information, such as: protein folding, mAb aggregation (mAb dimer or trimer), antibody drug conjugates (ADCs), bispecific mAbs, etc.

In this study, we applied the online SEC method for rapid and robust native mAbs MS analysis. Approximately 0.5 to 1.0 µg of mAb was injected onto an AdvanceBio SEC guard column using a 5 min isocratic flow at 0.2 mL/min of 100 mM ammonium acetate solvent. The Q-TOF source conditions were optimized for excellent quality of native MS spectra over the mass range from *m/z* 5,000 to 10,000. Figure 4 demonstrates the LC/MS analysis of intact NIST mAb standard under the denaturing MS conditions (Figure 4A and 4B) as well as the native MS conditions (Figure 4C and 4D). In both conditions, all major glycoforms of the NIST mAb were well resolved (Figure 4A and 4C, inset). The charge state distribution of denatured NIST mAb spanned the mass range of *m/z* 2,000 to 5,000 (30+ to 75+), while the native NIST mAb had a charge envelope in the range of *m/z* 5,000 to 10,000 (15+ to 30+). As shown in the MS deconvoluted spectra (Figure 4B and 4D), low ppm in mass errors were obtained for all major glycoforms. We also achieved very good agreement with the data for the intact NIST mAb analysis under both MS conditions.

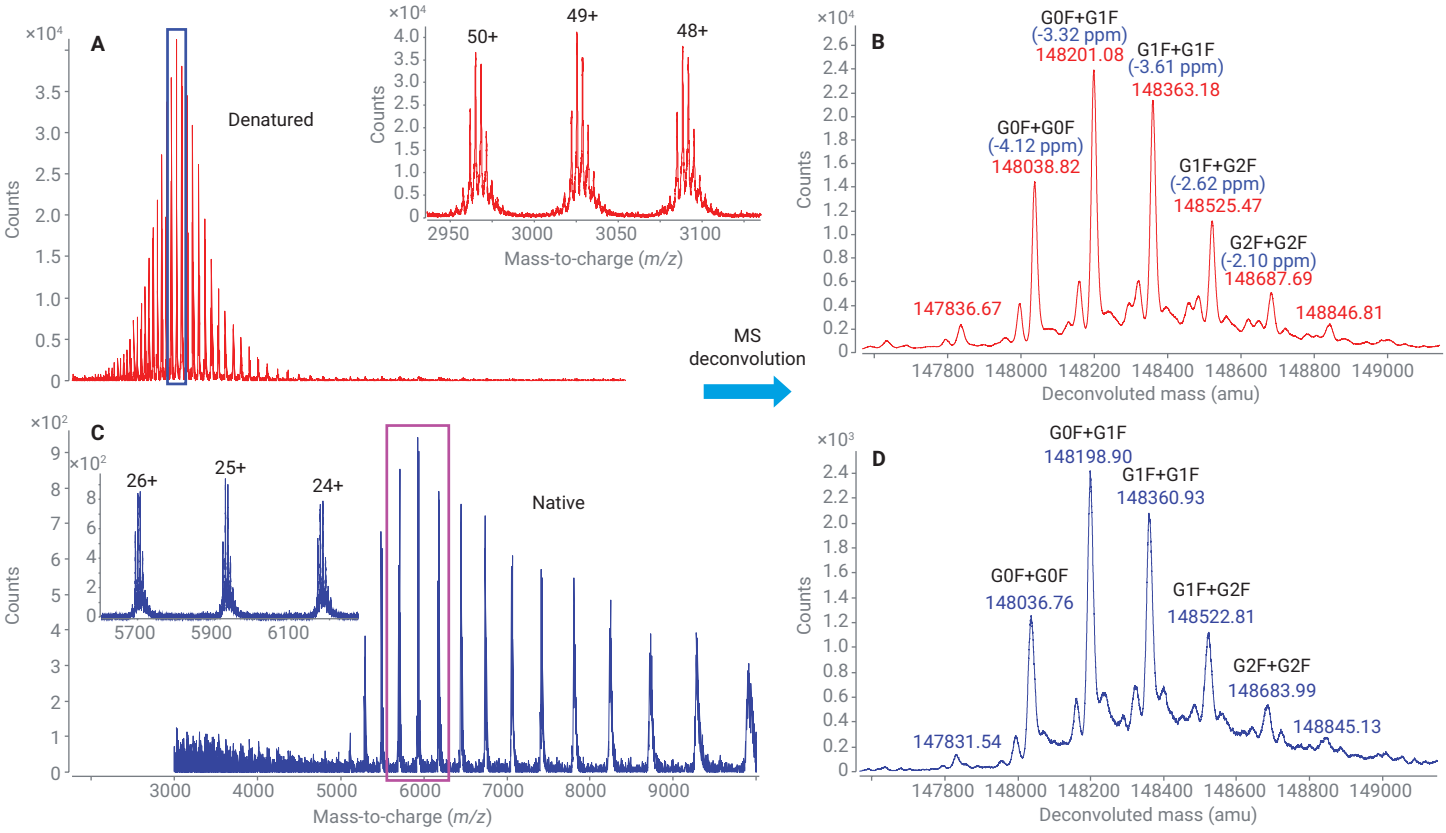

Figure 4. LC/MS analysis of NISTmAb under: A) denaturing MS conditions (acetonitrile and 0.1% formic acid) (previous work, Ref. 5) and C) native MS conditions (in 100 mM ammonium acetate, pH 7). The deconvoluted MS spectra of both samples are shown in B) and D), respectively.

Similarly, native MS analysis of a biotherapeutic drug (trastuzumab, brand name: Herceptin) and its ADC (trastuzumab emtansine, T-DM1) was performed and compared. Figure 5A illustrates the native mass spectrum of intact Herceptin, showing a nicely distributed charge envelope from *m/z* 5,000 to 10,000 with charge states between 15+ and 28+. The most prominent charge state was at 24+ which indicated the intact Herceptin was in its native/folded conformation. High mass accuracies for the major glycoforms were achieved as shown in the inset deconvoluted spectrum.

Native MS analysis enables probing of protein molecules while preserving their native structural conformation. As this method minimizes the interferences from organic solvent and acid in the mobile phase, it is an ideal analytical tool for noncovalent protein complexes or acid labile protein conjugates, such as some ADCs. Figure 5 shows the native raw and deconvoluted (inset) MS spectrum of T-DM1. The average DAR value calculated using the BioConfirm DAR Calculator was 3.5 (Figure 5B, inset), which is consistent with the DAR values of the intact ADC reported by Genentech (the manufacturer).

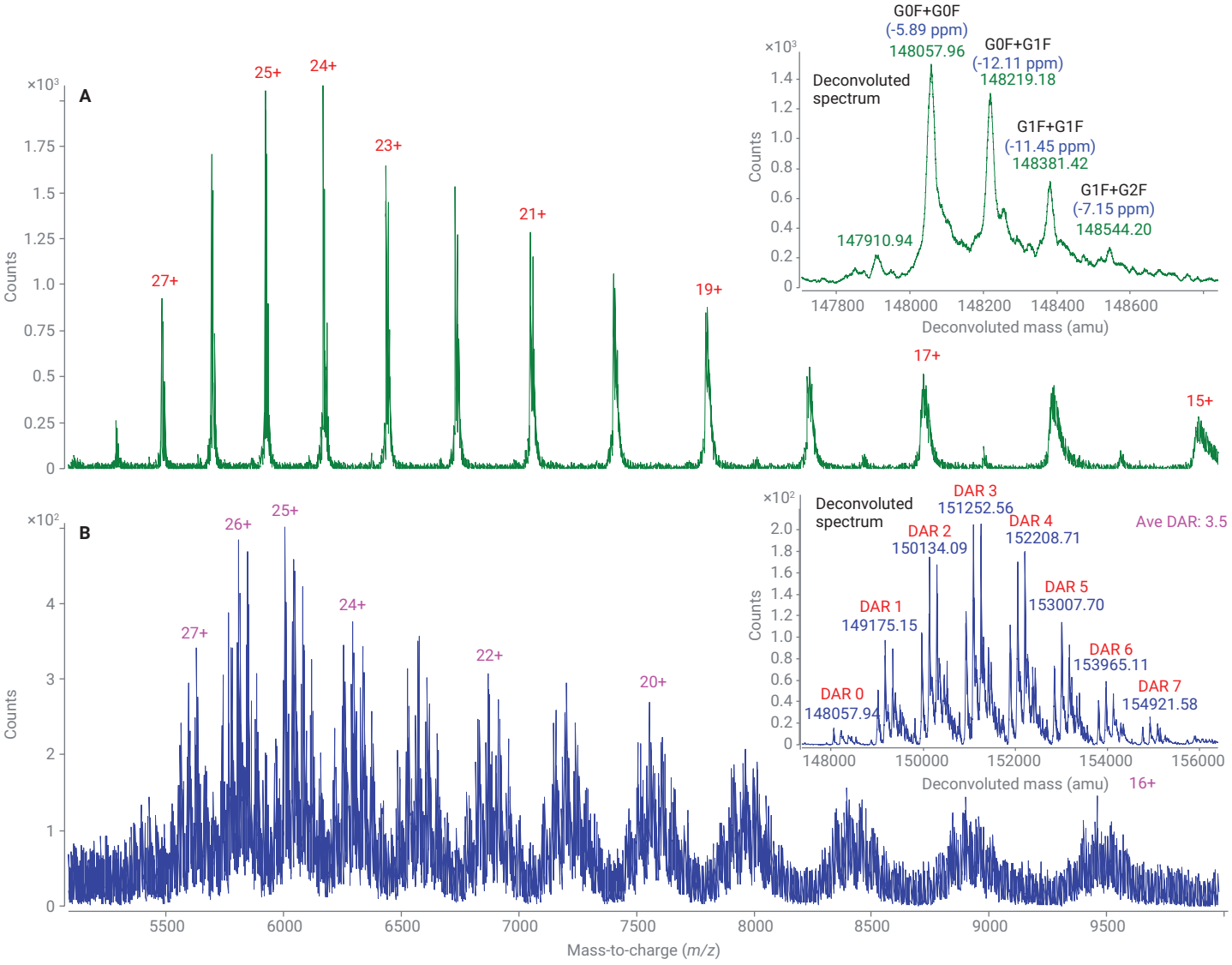

Figure 5. Native LC/MS analysis of mAb and its antibody drug conjugate (ADC): A) Herceptin and B) T-DM1. The deconvoluted MS spectra of both samples are shown in the inset figures.

Native MS analysis of intact protein complexes

The optimized native MS analysis method was also evaluated by three protein complex samples. They were: tetrameric pyruvate kinase (PK, 232 kDa), hexameric glutamate dehydrogenase (GDH, 335 kDa), and tetrameric β-galactosidase (466 kDa). The 6545XT AdvanceBio LC/Q-TOF system offers large molecule SWARM autotune for optimizing macromolecular ions transmission in the extended mass range up to *m/z* 30,000. It is an ideal LC/MS system for native protein complex analysis. Figure 6A shows the native mass spectrum of the tetrameric pyruvate kinase. Two major charge envelopes ranging from *m/z* 6,000 to 10,000 with charge state of 24+ to 37+ were detected. The deconvoluted spectrum revealed that there were two multi-proteoform complexes of PK tetramers in the sample: full-length pyruvate kinase and truncated PK tetramer (three intact subunits plus one PK proteoform with N-terminal cleavage).[3]

The 6545XT system also demonstrated excellent detection sensitivity for protein complexes at higher *m/z* (>*m/z* 10,000). Figure 6B and 6C show the native MS spectrum of GDH and β-galactosidase. Both of their protein charge envelopes were greater than *m/z* 8,000, whereas the most abundant ions were at *m/z* 9,566 (35+) for GDH and *m/z* 10,832 (43+) for β-galactosidase, respectively. The molecular mass of the intact hexameric GDH was determined to be 334,754 and 465,788 Da for the tetrameric β-galactosidase with 1 to 10 µg sample injections.

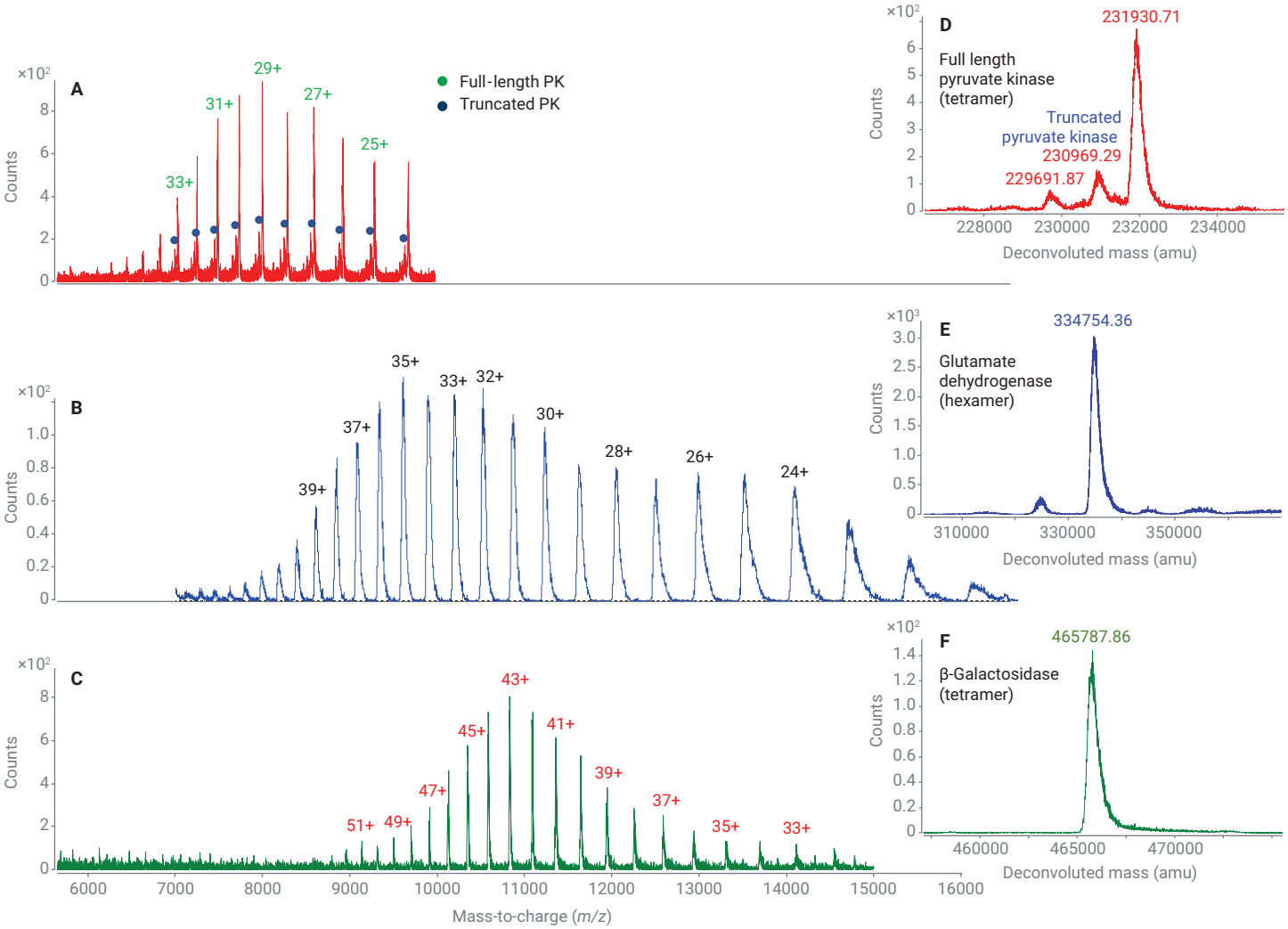

Figure 6. Native LC/MS analysis of various intact protein complexes. A) Pyruvate kinase (PK, tetramer, 232 kDa), B) glutamate dehydrogenase (GDH, hexamer, 335 kDa) and C) β-galactosidase (tetramer, 466 kDa). The deconvoluted spectra are shown in D) to F), respectively. The raw MS spectrum in Figure 6B was smoothed using the mMass open-source MS software tool.

Conclusion

We have developed a highly sensitive and robust LC/MS workflow methodology for native protein analysis. This optimized workflow utilizes the 1290 Infinity II LC with the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF with extended mass range up to *m/z* 30,000, and MassHunter BioConfirm software. The following benefits have been demonstrated by this native MS analysis method:

- Use of an online SEC column at typical analytical LC flow rates eliminates the challenging issues (protein aggregation and instable spraying flow) associated with nanoESI analysis.
- The optimized native MS conditions provide high confidence in ADC characterization, with accurate determined DAR values.
- The large molecule SWARM autotune feature, along with the extended mass range of the 6545XT AdvanceBio LC/Q-TOF, enables the sensitive detection and characterization of native intact macroprotein complexes.

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