Application Note

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Mass Spectrometric Characterization of Antibody-siRNA Conjugates using the Agilent 6545XT AdvanceBio LC/Q-TOF

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Abstract

Small interfering RNAs (siRNAs) are a promising type of RNA-based therapeutic drugs that demonstrate effectiveness on gene silencing through RNA interference (RNAi). However, due to the chemical nature of siRNA, a vehicle is often required to deliver siRNA into target cells, and a monoclonal antibody (mAb) is typically employed as the delivery vehicle. The characterization of covalently linked mAb-siRNA conjugates is important in drug development and manufacturing processes. Using traditional reversed-phase LC/MS methods presents an analytical challenge due to the unstable nature of the mAb-siRNA conjugates. This application note describes a novel LC/MS method for the characterization of mAb-siRNA conjugates under their native conditions. An Agilent 1290 Infinity II LC, AdvanceBio SEC column, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software were all used in this workflow.

Introduction

With the discovery of RNA interference (RNAi) mechanism and the development of RNAi technology for silencing the target gene expression, small interfering RNAs (siRNAs) have demonstrated their effectiveness as a new class of therapeutic drugs.[1,2] However, one of the main challenges in the siRNA-based drugs development is the low efficiency of siRNA delivery to target cells. There are various proposed approaches to solving this problem, but the use of covalently linked siRNAs to biogenic molecules (human monoclonal antibodies) seems to be a viable solution. These biogenic molecules are specific to target cells to deliver siRNA loads, with minimized human immune reactions.

mAb-oligonucleotide conjugates have been used successfully for targeted delivery of oligonucleotide to specific types of cells and tissues.[3,4] In antibody-siRNA conjugates, the chemically stabilized siRNAs are covalently attached to the partially reduced antibody via its free thiol groups. When controlling the number of siRNA per antibody, antibody components of the conjugates still retain their normal functions and activities. Using this new delivery technology, the oligonucleotide payloads are now enabled efficient targeted treatment.

Biomedical researchers are improving the bioavailability, targeting of delivery, and biological activity (efficacy) of mAb-siRNA conjugates as therapeutic drugs. But there is also an urgent need to have accurate and more reliable analytical methods to characterize novel biomolecules, such as conjugates, that may exhibit different physical and chemical properties from siRNA and mAb.

In this study, an LC/MS-based analytical method for characterization of native intact mAb-siRNA conjugates was developed. This workflow features various AdvanceBio columns for sample separation, and the 6545XT AdvanceBio LC/Q-TOF system with the large molecule SWARM autotune feature and extended mass range of up to 30,000 *m/z* for sample analysis (Figure 1).

Experimental

Materials and methods

Unconjugated mAb, double strand siRNA (control sample), and anion exchange column-purified mAb-siRNA conjugates (DAR1, deglycosylated forms) were provided by an Agilent collaborator.

Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibody-siRNA conjugates sample preparation

The mAb was partially reduced with a commonly used reducing agent. The reduced mAb was then reacted with SMCC linker and siRNA (activated siRNA). The unreacted free thiol groups (–SH) of the mAb were modified with a chemical reagent. The reaction mixture was further purified by anion exchange column. Unreacted antibody elutes were separated, along with the solvent front and late eluting DAR1, DAR2, and unreacted siRNA. The purified DAR1 was then used for mass spectrometry analysis under denaturing and native conditions.

To perform native MS analysis, preserving the protein samples in pH-neutral and volatile aqueous solutions, such as ammonium acetate[5] is crucial. Therefore, sample desalting and buffer exchange are usually needed before the MS analysis. Briefly, the mAb-siRNA conjugates stock solutions (1 to 10 mg/mL) were desalted and solvent exchanged into 100 mM ammonium acetate using a Bio-Rad Bio-Spin P-30 (40,000 mol wt limit) cartridge. The cartridge was first fully equilibrated with 100 mM ammonium acetate buffer. A DAR1 sample was then pipetted to the top of the column and centrifuged for 5 minutes at 1,000 × g. The conjugate was then buffer exchanged into the 100 mM ammonium acetate and was ready for MS analysis.

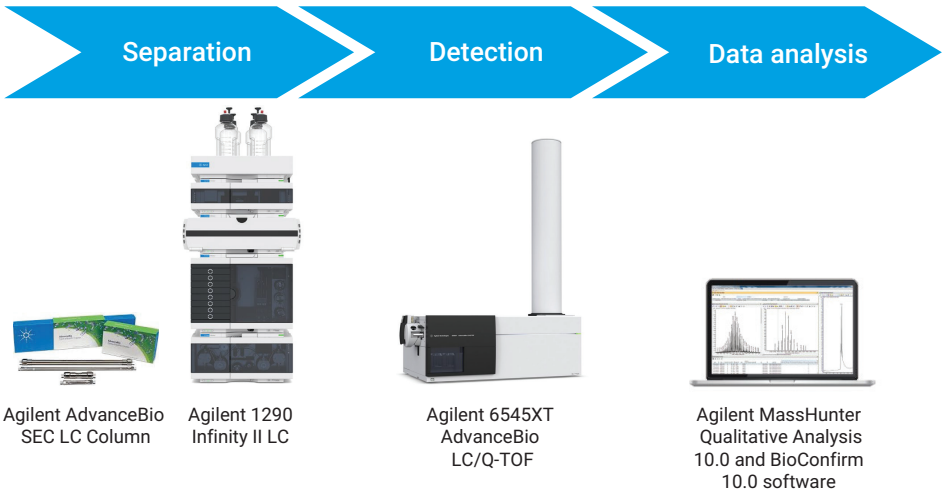

Figure 1. Analytical components of the native protein LC/MS analysis workflow.

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/QTOF

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 LC/MS data acquisition (version 10.1) workstation software was used with the large molecule SWARM autotune feature.

LC/MS analysis of the intact mAb (control) and intact deglycosylated mAb-siRNA conjugate (DAR1) under denaturing conditions was carried out using the traditional reversed-phase HPLC method.[6] An Agilent PLRP-S column (2.1 × 50 mm, 1000 Å, 5 µm, p/n PL1912-1502) was used with acetonitrile as an organic solvent and 0.1% formic acid.

Sample separations under the native LC/MS conditions were obtained with an Agilent AdvanceBio SEC LC column (4.6 × 30 mm) and a 5-minute isocratic run using 100 mM ammonium acetate solvent for the intact mAb (control sample). An AdvanceBio SEC LC column (4.6 × 300 mm, 200 Å, 1.9 µm, p/n PL1580-5201) was used in a 12 minutes isocratic run for the intact deglycosylated mAb-siRNA conjugate.

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

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System			
	Intact mAb and mAb-siRNA	**Native Intact mAb**	**Native Intact mAb-siRNA**
Column	Agilent PLRP-S (1000 Å, 2.1 × 50 mm, 5 µm) (p/n PL1912-1502)	AdvanceBio SEC (200 Å, 4.6 × 30 mm, 1.9 µm) (p/n PL1580-1201)	AdvanceBio SEC (200 Å, 4.6 × 300 mm, 1.9 µm) (p/n PL1580-5201)
Thermostat	4 °C	4 °C	4 °C
Solvent A	0.1% Formic acid in DI water	100 mM NH_4OAc (pH 7)	100 mM NH_4OAc (pH 7)
Solvent B	0.1% Formic acid in 100% acetonitrile		
Gradient	0 to 1 min, 0 to 20% B 1 to 7 min, 20 to 70% B	Isocratic: 0 to 5 min, 100% A	Isocratic: 0 to 12 min, 100% A
Column Temperature	60 °C	Room temperature	Room temperature
Flow Rate	0.5 mL/min	0.2 mL/min	0.3 mL/min
Injection Volume	1 to 5 µL	1 to 5 µL	1 to 5 µL

Table 2. Native MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System		
MS Condition	**Intact mAb-siRNA**	**Native Intact mAb-siRNA**
Source	Agilent Jet Stream	Agilent Jet Stream
Dry Gas Temperature	350 °C	365 °C
Dry Gas Flow	12 L/min	12 L/min
Nebulizer	60 psig	35 psig
Sheath Gas Temperature	400 °C	300 °C
Sheath Gas Flow	11 L/min	12 L/min
VCap	5,500 V	5,500 V
Nozzle Voltage	2,000 V	2,000 V
Fragmentor	380 V	300 V
Skimmer	140 V	220 V
Quad AMU	500 *m/z*	3,000 *m/z*
Mass Range	300 to 8,000 *m/z*	3,000 to 10,000 *m/z*
Acquisition Rate	1 spectrum/sec	1 spectrum/sec
Acquisition Mode	Positive, extended (10,000 *m/z*) mass range	Positive, extended (10,000 *m/z*) mass range

Data processing

All MS data files of the native and intact mAbs or mAb-siRNA conjugates were processed using Agilent MassHunter qualitative analysis 10.0 and BioConfirm 10.0 software.

Results and discussion

Antibody-siRNA conjugates

mAb-siRNA conjugates were synthesized by site-specific conjugation between the free thiol residues of a human IgG antibody and the strand of the siRNA with a linker that can react with the free thiol group. The ratio of siRNA-to-antibody (DAR) is usually 1 or 2 (Figure 2). Synthesis involved the partial reduction reaction using a commonly used reducing agent. The partial reduction did not alter the antibody structure and retained its biological function as a delivery vehicle. After the conjugation reaction, the free thiol was then blocked with a modifier to prevent any further reactions.

HPLC separation of DAR1 on ion exchange column

After the conjugation reaction, all nonconjugated molecules (solvents, excess siRNA, and linker) should be removed. Various techniques exist for the purification of desired mAb-siRNA conjugates from synthetic mixture to remove reactants and by-products. These techniques include gel filtration/size exclusion chromatography, protein A or protein G affinity chromatography for antibodies, and ion-exchange chromatography.

Ion-exchange chromatography is an excellent technique for separating charged molecules and is commonly used to separate and purify oligonucleotides. In this study, mAb-siRNA conjugate target (DAR1) was purified using anion exchange column to remove other impurities.

Various LC/MS methods for mAb-siRNA conjugate characterization

LC/MS analysis of intact mAb and mAb-siRNA conjugates under denaturing conditions

To investigate and characterize the intact mAb and mAb-siRNA conjugates (glycosylated and deglycosylated), the reversed-phase HPLC approach to analyze the samples under denaturing conditions was used first. A PLRP-S column (2.1 × 50 mm, 1000 Å) was used with a 5-minute gradient for intact mAb and an 8-minute gradient for the conjugates at a flow rate of 0.5 mL/min.

Figure 3 shows the LC/MS analysis of intact unconjugated and deglycosylated mAb under denaturing conditions. The charge state distribution of denatured

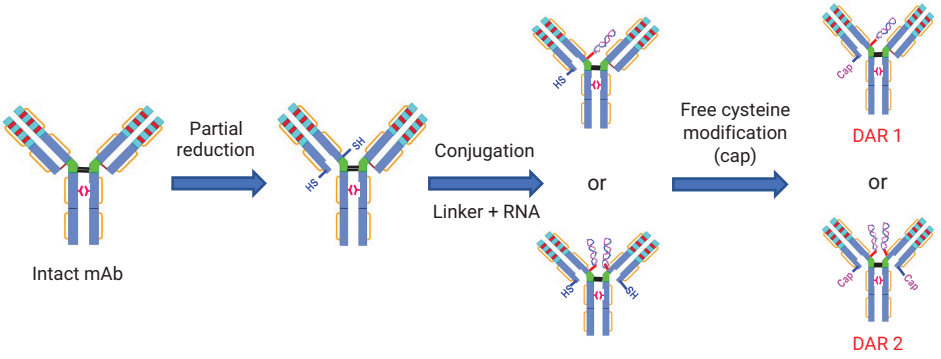

Figure 2. General scheme of antibody-siRNA (mAb-siRNA) conjugate chemical synthesis.

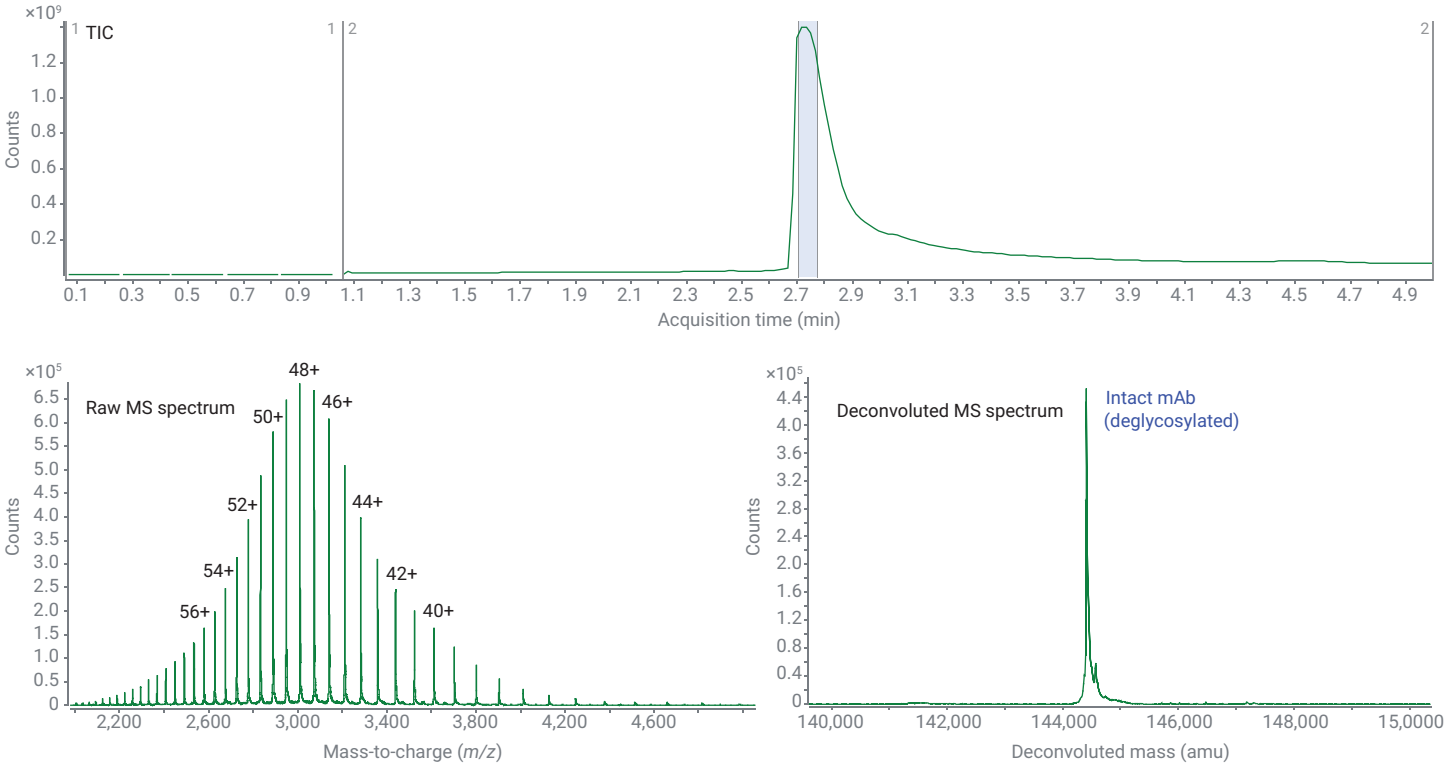

Figure 3. LC/MS analysis of intact deglycosylated mAb under denaturing MS conditions (acetonitrile and 0.1% formic acid).

mAb spanned in the typical mass range of *m/z* 2,000 to 5,000 (30+ to 75+). Only one major protein was detected, and this was the fully deglycosylated antibody sample.

Figure 4 illustrates the LC/MS profile of the intact deglycosylated mAb-siRNA conjugate with a DAR value of 1. The extracted ion chromatogram (EIC, Figure 4B) also shows that there are several charge state distributions over the mass range *m/z* 1,000 to 4,000, indicating that many biomolecules existed in the sample.

Multiple species were expected to be present in this sample, so MS data deconvolution and mass matching to the protein or oligonucleotide sequences were performed on each of the four HPLC peaks over retention time period of 3.4 to 4.3 minutes (Figure 4C).

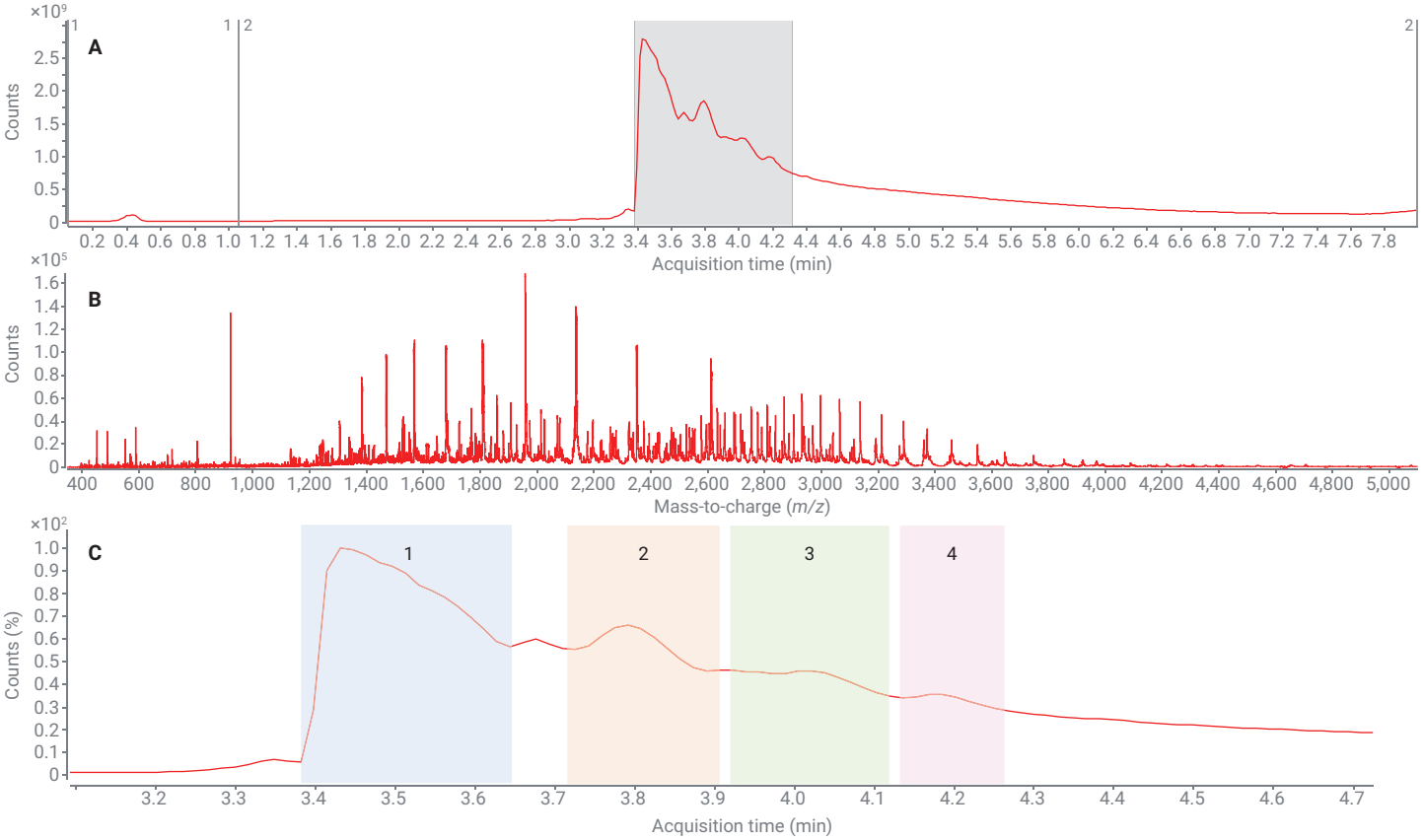

Figure 4. A) MS TIC of an intact deglycosylated mAb-siRNA conjugate on the reversed-phase (PLRP-S, 2.1 × 50 mm, 1000 Å) column. B) Extracted ion chromatogram (EIC) of the chromatographic separated peaks over retention time of 3.4 to 4.3 minutes. C) Zoomed-in chromatogram of the highlighted peaks (in gray box). The MS data from each of the HPLC peaks (1 to 4) were deconvoluted and analyzed. Results are shown in Figure 5.

To obtain as much detailed information as possible, the MS deconvoluted mass range was set from 20 to 160 kDa for the raw MS data of all four LC peaks. This broad mass range should cover small proteins such as the mAb light chain, all the way up to mAb-siRNA conjugate. After careful calculation, many degraded/reduced antibody or antibody-oligo conjugate forms were identified and labeled, as shown in Figure 5 (peaks 1 to 4). These structural assignments are solely based on the mass matching and hypothesis of the conjugation reaction. Further investigation is needed to identify the actual conjugation sites of these molecules.

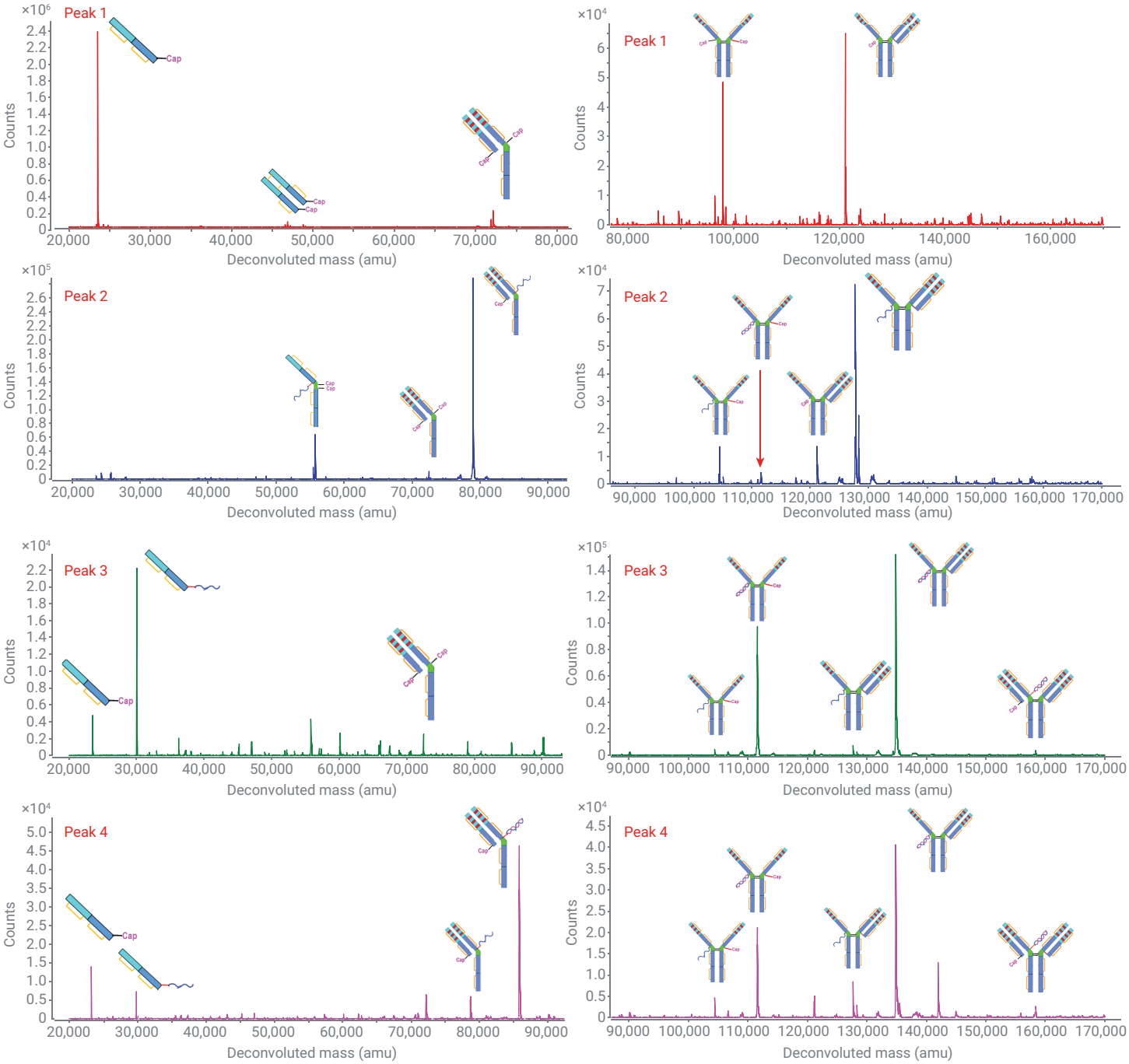

Figure 5. MS deconvoluted spectrum of deglycosylated mAb-siRNA sample (DAR1) (HPLC peak 1-4). LC/MS analysis was performed under denaturing MS conditions.

The results show that many dissociated molecules from mAb-siRNA conjugates were observed in all four LC peaks. They are: mAb light chain (with Cap or RNA), mAb heavy chain (with Cap or RNA), half of mAb-siRNA conjugates, conjugates without 1 or 2 LCs, and more. These results indicate that most of the conjugates were dissociated under the denaturing LC/MS condition. As the conjugation reaction likely occurred at the disulfide bonds between LC and HC, it broke the strong disulfide bond linkages and turned them into weak electrostatic interaction. It is, therefore, difficult to detect the intact conjugates under traditional LC/MS conditions using organic and acidic solvents.

Native LC/MS analysis of intact mAb and its mAb-siRNA conjugates

Native mass spectrometry has emerged as a widely used technique for characterizing intact proteins and noncovalent protein complexes. Native MS analysis enables probing of protein molecules while preserving their native structural conformation. This method minimizes the interferences from organic solvent and acid in the mobile phase, making it an ideal analytical tool for protein complexes with reduced stability or acid labile protein conjugates, such as some mAb-RNA conjugates.

Figure 6 demonstrates the LC/MS profile of intact unconjugated and deglycosylated mAb control under native conditions. Approximately 5 µg of mAb was injected onto an AdvanceBio SEC LC column using a 5-minute isocratic flow at 0.2 mL/min of 100 mM ammonium acetate solvent. The charge envelope of the native mAb was in the mass range of *m/z* 5,000 to 10,000 (15+ to 29+). The Q-TOF source conditions were optimized, and excellent quality native MS spectra with low ppm (<5 ppm) in mass errors were obtained (data not shown).

The 6545XT system demonstrated excellent detection sensitivity for the mAb-siRNA conjugates under the native LC/MS conditions. Approximately 5 µg of the conjugate samples were injected onto an AdvanceBio SEC long column (300 mm) using a 12-minute isocratic flow at 0.3 mL/min. A longer chromatographic run method was developed to optimize the sample separation and maximize the sensitivity of detection.

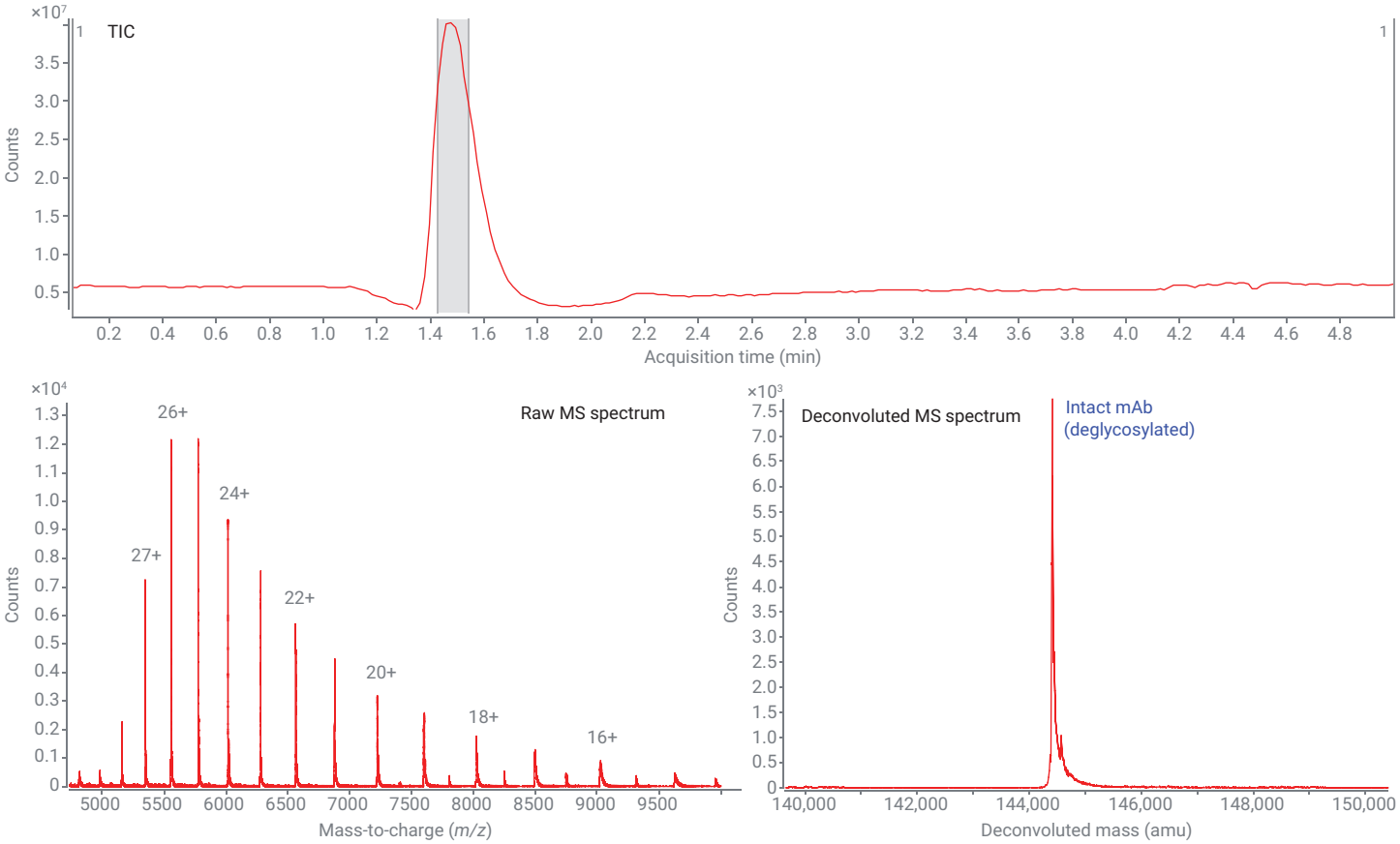

Figure 6. Native LC/MS analysis of intact deglycosylated mAb (in 100 mM ammonium acetate, pH 7).

Figures 7 and 8 illustrate the native SEC column separated LC/MS analysis of an intact deglycosylated mAb-siRNA conjugate (DAR1) sample. Two major LC peaks with MS charge envelopes ranging from *m/z* 5,500 to 10,000 were detected. The deconvoluted spectrum revealed that there were two major forms of mAb-siRNA conjugate in each LC peak. Peak 1 contained DAR1 with one cap and DAR1 with three caps (see inset figures in 7C for structural illustration). Peak 2 included intact mAb + two caps (most abundant) and the tailing of DAR1 with one cap from peak 1 (Figure 8C, inset).

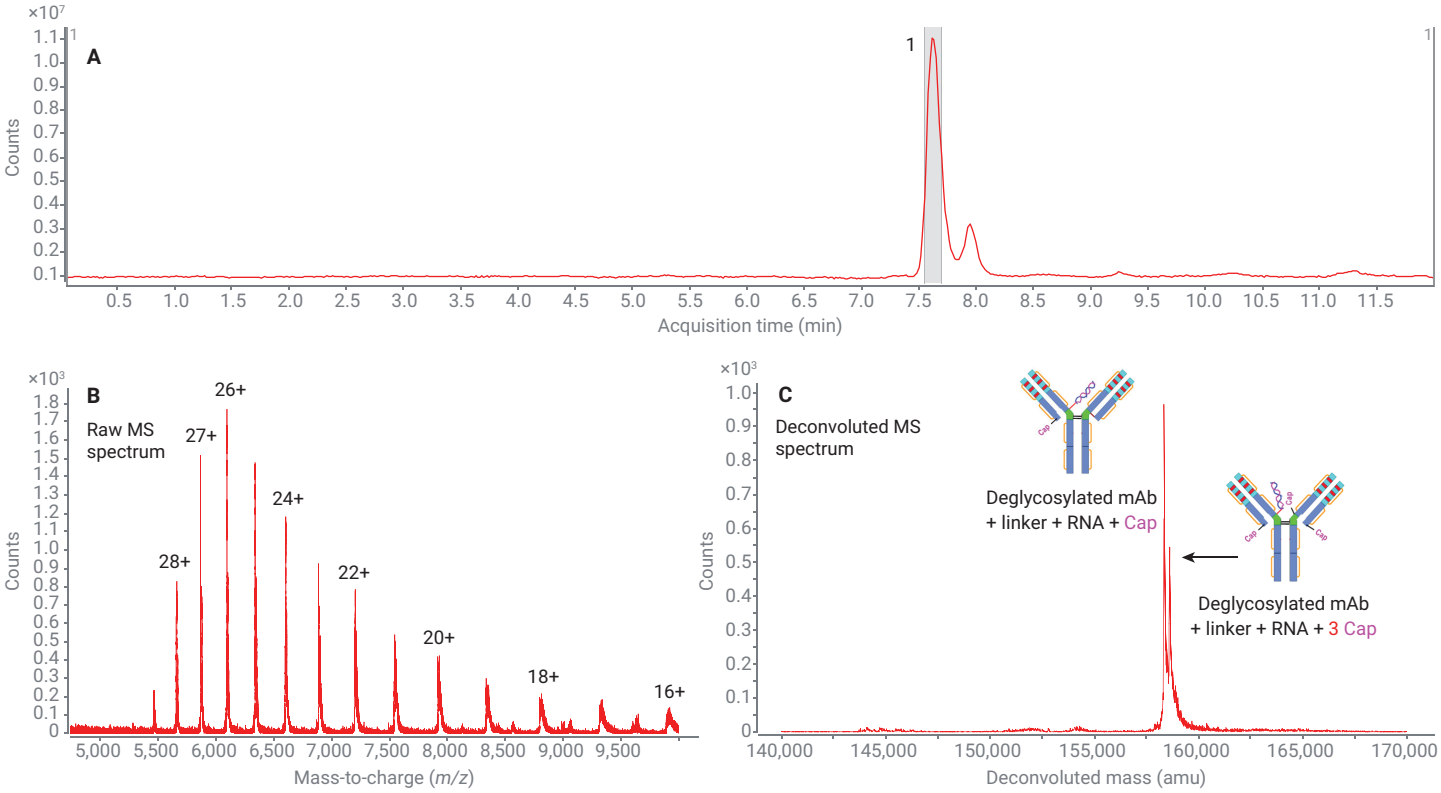

Figure 7. Native SEC LC/MS analysis of mAb-siRNA conjugate (DAR1): (A) HPLC chromatogram of SEC column separated DAR1. (B) Raw MS spectrum of intact DAR1 (peak 1). (C) The deconvoluted MS spectra of intact DAR1 (peak 1), indicating two forms of DAR1 were detected.

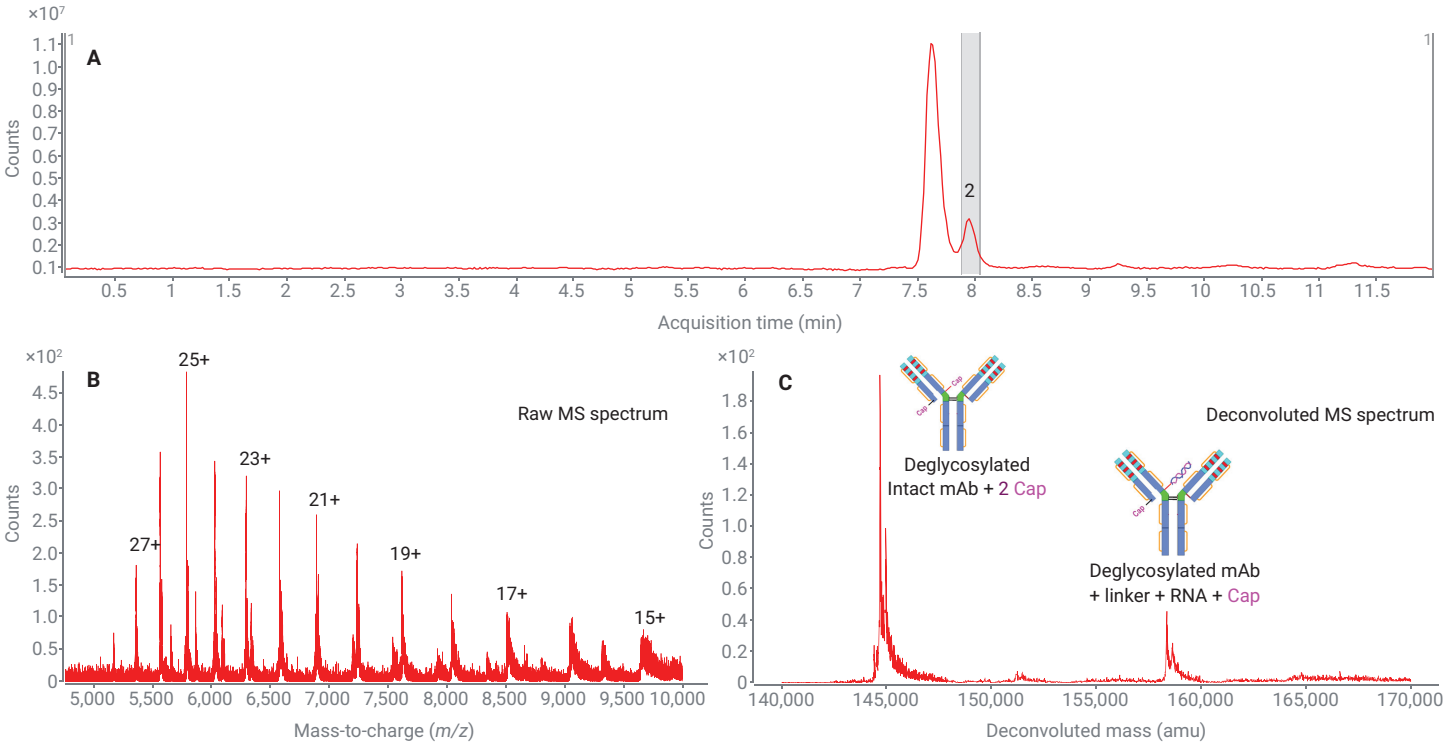

Figure 8. Native SEC LC/MS analysis of mAb-siRNA conjugate (DAR1): (A) HPLC chromatogram of SEC column separated DAR1. (B) Raw MS spectrum of intact DAR1 (peak 2). (C) The deconvoluted MS spectra of intact DAR1 (peak 2), indicating mAb + two caps was the most abundance molecule in peak 2.

Conclusion

A novel LC/MS method was developed for the characterization of mAb-siRNA conjugates under their native conditions. This optimized workflow uses 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. This native MS analysis method not only can provide accurate mass information for mAb-siRNA conjugate structural assignments, but also enable chromatographic separation and relative quantitative analysis on various types of mAb-siRNA conjugates.

References

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Printed in the USA, June 24, 2020
5994-2155EN

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