Application Note

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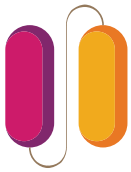
Analysis of Antibody Fragment-Drug Conjugates Using an Agilent AdvanceBio SEC 120 Å 1.9 µm PEEK-Lined Column

Monoclonal antibody



Single-chain Fv (scFv)

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Abstract

Antibody drug conjugates (ADCs) are usually formed by conjugating a drug linker (~2 kDa) to a whole IgG antibody (~150 kDa), achieving a drug-to-antibody ratio (DAR) of up to 8. This resulting antibody is approximately 10% w/w modified and key properties of the antibody largely remain unaltered. Antibody fragments such as single-chain Fvs (V_H and V_L) of an antibody are approximately one-fifth the size of an IgG (~30 kDa), and yet these can be modified to have much higher DARs (~10). In the resulting antibody fragment-drug conjugate (FDC), this represents ~65% w/w modification of the protein. Such drastic modification can lead to extreme changes in properties of the conjugate, creating additional challenges for analysis.

Introduction

The FDCs analyzed in this application note have very low isoelectric points (due to lysine conjugation), precluding the use of formic acid in mobile phases for reverse-phase (RP) chromatography due to rapid conjugate aggregation. In electrospray ionization, formic acid has the dual function of protonating analytes to give charged species, and to help minimize sodium adducts. High DARs can make FDCs prone to aggregation, so in-line desalting methods are preferred to minimize steps between conjugate purification, formulation, and analysis. This application note has developed a mass spectrometry-compatible, native-mode size exclusion (SEC/MS) method for characterization of a challenging FDC with very low pI. The right pore size provides excellent separation of the molecule of interest, and this method provides superior desalting with fewer sodium adducts, making it suitable for both FDC DAR analysis and monitoring of conjugation in the reaction mixture.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Sample preparation

scFv and FDC samples were produced by Antikor.

Instrumentation

For HPLC experiments, an Agilent 1290 Infinity LC was used, comprising:

- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)
- Agilent 1260 Infinity II diode array detector WR (DAD) (G7115A)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Method conditions

Agilent 1290 Infinity LC		
HPLC Conditions	**Reversed-Phase Method**	**Native SEC Method**
Column	Agilent PLRP-S, 2.1 × 50 mm (p/n PL1912-1502)	AdvanceBio SEC 1.9 µm 120 Å, 2.1 × 150 mm PEEK-lined (p/n PL1980-3250PK)
Mobile Phase	A = 20 mM NH_4OAc, pH 7.0 B = MeCN	75 mM NH_4OAc, pH 7.0
Gradient	0 min 10% B 8.4 min 10% B 14.4 min 90% B 20.4 min 90% B 21.6 min 10% B 25.8 min 10% B	
Flow Rate	0.6 mL/min	0.05 mL/min
Column Temperature	70 °C	30 °C
Injection Volume	2.2 µL	5 µL

Agilent 6545XT AdvanceBio LC/Q-TOF		
Parameter	**Reversed-Phase Method**	**Native SEC Method**
Source	Dual Agilent Jet Stream	Dual Agilent Jet Stream
Gas Temperature	325 °C	300 °C
Drying Gas Flow	11 L/min	8 L/min
Nebulizer Gas	40 psi	40 psi
Sheath Gas Temperature	400 °C	300 °C
Sheath Gas Flow	12 L/min	8 L/min
Capillary Voltage	4,500 V	3,000 V
Nozzle Voltage	300 V	2,000 V
Fragmentor	275 V	200 V
Skimmer	65 V	65 V
Oct 1 RF Vpp	750 V	750 V
Mass Range	*m/z* 300 to 3,200	*m/z* 900 to 10,000
MS Scan Rate (Spectra/s)	1	1
Acquisition Mode	Positive, extended dynamic range (2 GHz)	Positive, extended dynamic range (2 GHz)

Data processing

LC/MS data was processed by Agilent MassHunter Qualitative Analysis 10.0 and BioConfirm B.07 software.

Results and discussion

In attempting to desalt our scFvs in-line using RP chromatography in neutral buffer, the scFv peak eluted around 12.7 minutes (Figure 1). Figure 2 shows the mass spectrum of the scFv peak. When deconvoluted, this study observes many sodium adducts (Figure 3), making analysis too complex.

The high source temperatures required with RP analysis can also lead to degradation of linkers, therefore an alternative approach was sought.

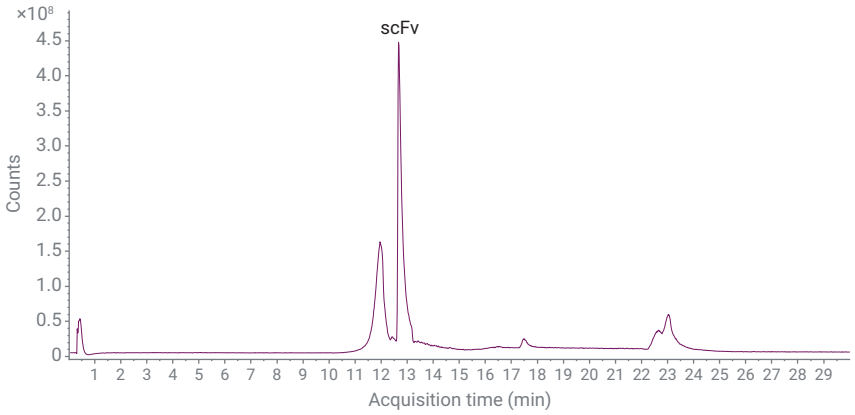

Figure 1. Reversed-phase TIC of scFv.

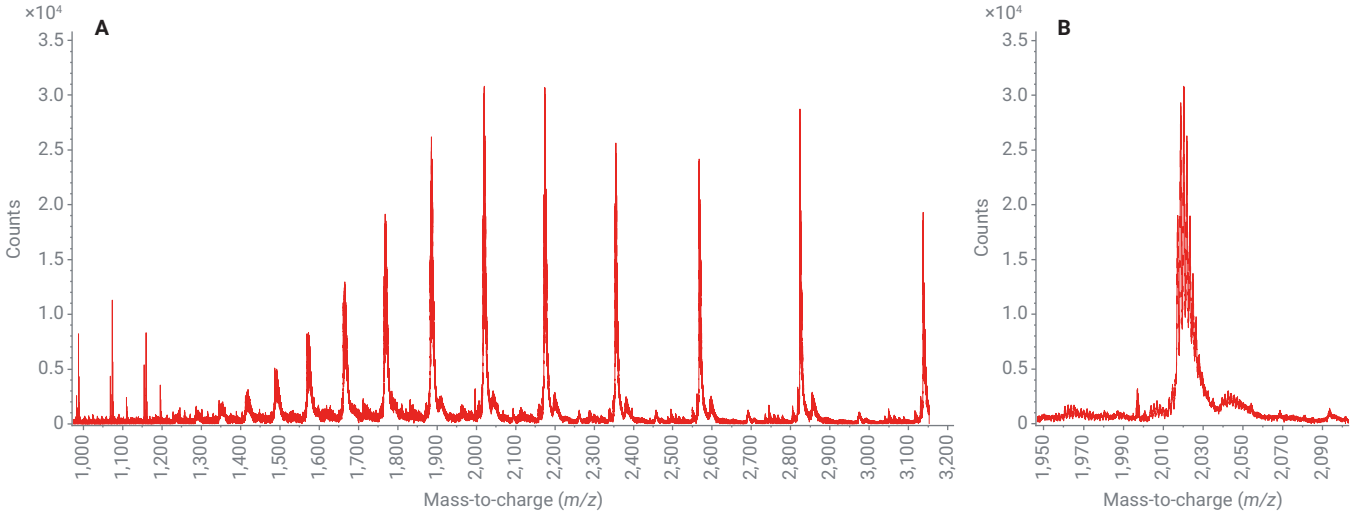

Figure 2. (A) Mass spectrum of scFv peak. (B) Zoomed-in region.

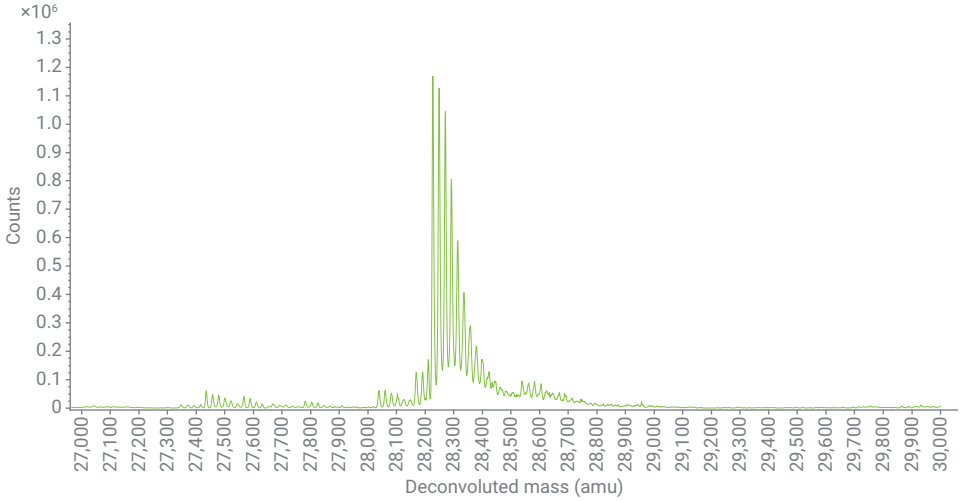

Figure 3. Deconvoluted spectrum showing multiple Na adducts.

SEC can be performed using volatile mobile phase buffers but requires the use of stationary phases with reduced secondary interactions. Peak shape can also be further improved for some molecules by using bio-inert (PEEK-lined) SEC columns, rather than stainless steel.

Also, by using a 2.1 mm id column, the flow rate can be as low as 0.05 mL/min so that lower desolvation gas flows and lower temperatures can be used. Using a lower flow rate, the protein has longer to equilibrate once chromatographically separated from sodium ions, and reduction in sodium adducts results (Figures 4 and 5). Figure 4 also shows that both the scFv and drug-conjugated FDC are well separated from the drug linker (~2 kDa) and salts, so this method could be easily applied for monitoring of the FDC and the payload linker during the reaction. The distribution of individual DAR species can now be determined from the deconvoluted spectrum (Figure 6). Figure 6 also reveals some smaller peaks between the main DAR peaks, which are a result of in-solution peptide fragmentation with loss of amino acids at the C-terminus.

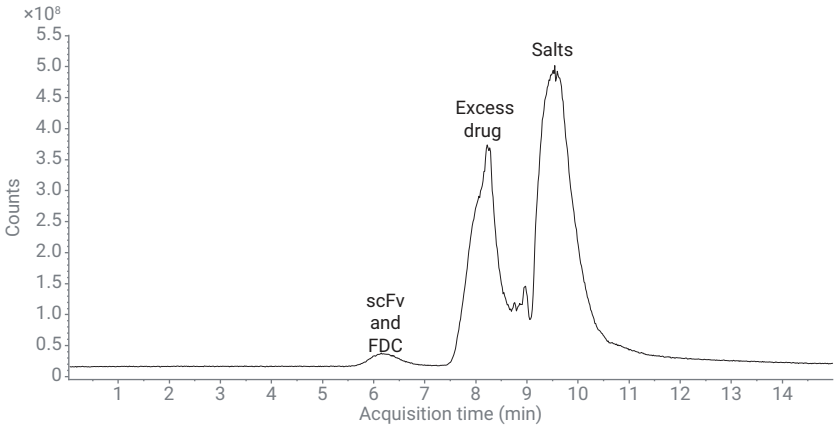

Figure 4. Native-mode SEC TIC of crude reaction mix.

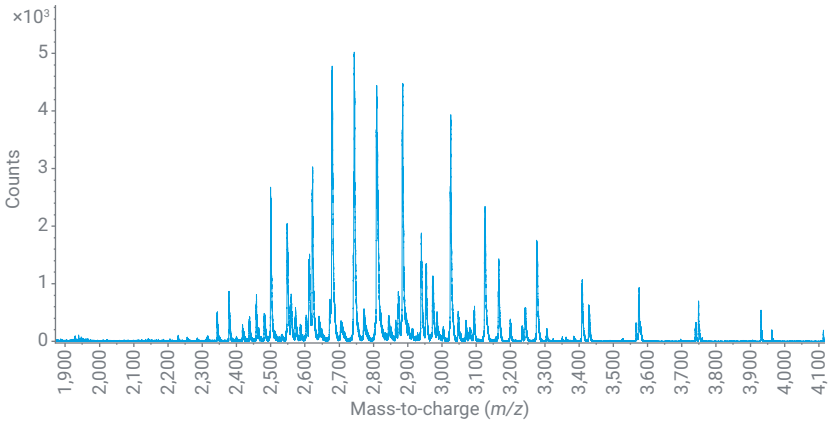

Figure 5. Mass spectrum of scFv and FDC.

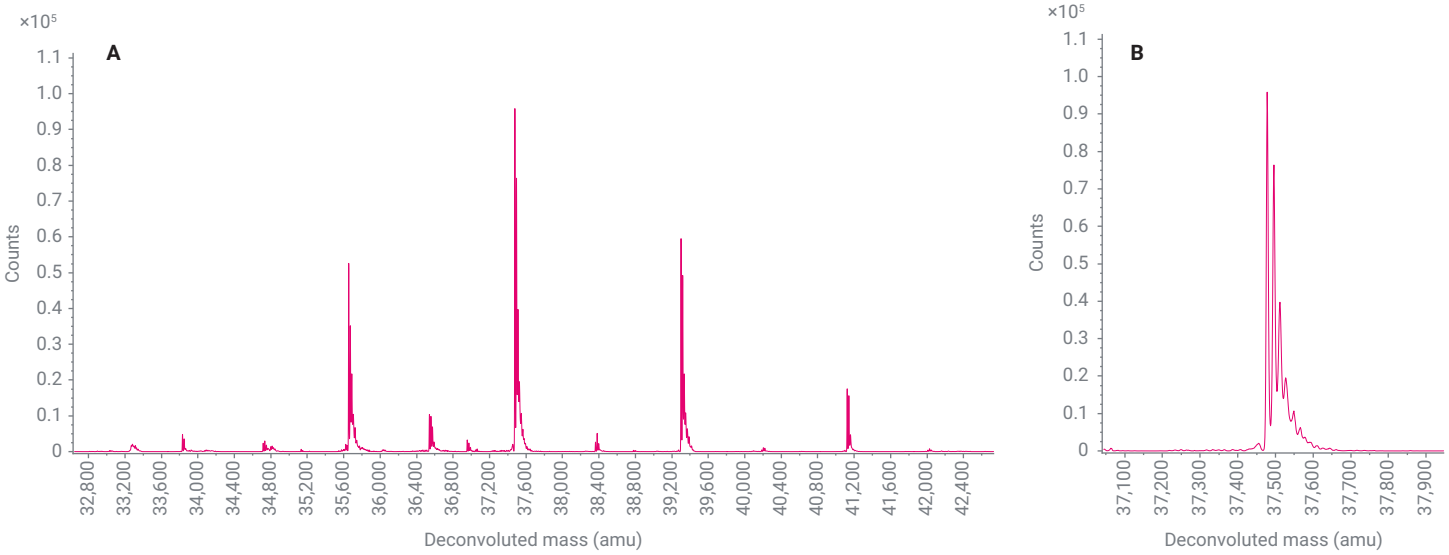

Figure 6. (A) Deconvoluted mass spectrum of scFv and FDC showing DAR species. (B) Zoomed-in region.

Conclusion

In this study, native SEC/MS provides superior desalting with less sodium adducts than a reversed-phase approach, with the neutral pH ensuring the FDC remains stable during the analysis. The PEEK-lined SEC column and hydrophilic, 120 Å-pore-size stationary phase ensures minimal secondary interactions for this type of sample separation conducted using a relatively dilute volatile mobile phase. This new method can be used for FDC DAR analysis and monitoring of conjugation in the reaction mix.

References

1. Richards, D. A. Exploring Alternative Antibody Scaffolds: Antibody Fragments and Antibody Mimics for Targeted Drug Delivery. *Drug Discov. Today Technol.* **2018**, *30*, 35–46.

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