Increased Throughput for the Characterization of Bispecific Antibodies on Subunit Level using SelexION® Differential Mobility Separation (DMS) Technology

Featuring the SCIEX TripleTOF® 6600 LC-MS/MS System with SelexION DMS Technology

Kerstin Pohl[1], Jennifer Schanz[2], Jason Tonillo[2], Denise Mehl[3], Roland Kellner[2], Sean McCarthy[1]

[1]SCIEX, Framingham, MA, USA; [2]Merck Healthcare KGaA, Darmstadt, Germany; [3]SCIEX, Villebon sur Yvette, France

The landscape of biopharmaceuticals is getting more and more diverse nowadays. New modalities are emerging that are enabling new options for the treatment of diseases. Recently the emergence of bispecific antibodies has gained significant attention with greater than 85 bispecific antibodies in clinical studies.[1] As molecular classes expand it is evident that there is a demand for high quality and high throughput analytical approaches to support the development of biotherapeutics. Liquid chromatography-mass spectrometry (LC-MS) instrumentation is frequently used for characterization of protein therapeutics. Subunit analysis by LC-MS enables the analysis of the individual chains in a single method. For bispecific therapeutics, chromatographic separation of protein chains prior to MS analysis is challenging and can complicate data analysis.

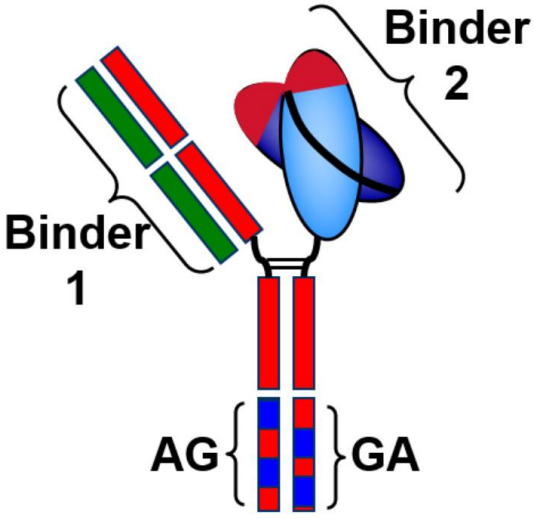

Figure 1. Schematic of a **bispecific antibody.** Heterodimer with two different binder domains and complementary AG and GA sequences in the CH3 domains.

SCIEX TripleTOF 6600 System equipped with SelexION® device.

To address this challenge an orthogonal separation approach using DMS with a SelexION device coupled to the TripleTOF 6600 System offers an additional dimension of separation. Presented here is an example of the separation of protein subunits using differential mobility to enable unambiguous characterization of each chain in a bispecific antibody. The analysis is accomplished in a single injection making data collection and analysis simple and reducing overall time required to complete studies.

Key Features for the Characterization of BioPharmaceuticals

- IonDrive™ Turbo V Ion Source ensures efficient desolvation and ionization particularly well suited for proteins
- SelexION Technology provides additional separation that complements or replaces chromatographic separation
- Increased throughput for subunit analysis for antibodies and new modalities such as bispecific antibodies

Methods

Sample Preparation:

A bispecific antibody sample (Figure 1) was obtained from Merck Healthcare KGaA, Darmstadt, Germany (conc. 10 mg/ml). The protein sample was reduced at room temperature using tris(2-carboxyethyl)phosphine (TCEP) with a final concentration of 50 mM for 30 min. Subsequently, the sample was diluted to a final concentration of 0.1 mg/ml in 10 % Acetonitrile/1 % formic acid (*v*:*v*).

Chromatography:

Table 1. Chromatographic Conditions.

Parameter	Value
Column	*MassPREP desalting column 2.1 mm × 5 mm, 20 µm, 1000 Å*
Mobile Phase A	*Water + 0.1 % formic acid*
Mobile Phase B	*Acetonitrile + 0.1 % formic acid*
Column Temperature	*80 °C*
Injection amount	*10 µl / 1 µg*

Table 2. Gradient Conditions.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]	Flow rate [µL/min]
Initial	*90*	*10*	*500*
0.5	*90*	*10*	*500*
1.5	*10*	*90*	*300*
2.0	*10*	*90*	*500*
2.5	*90*	*10*	*500*
3.0	*90*	*10*	*500*

Mass Spectrometry:

An ExionLC™ system coupled to a SCIEX TripleTOF 6600 System equipped with an IonDrive Turbo V source and SelexION Differential Mobility Separation device was used for data acquisition. A short chromatography of 3 min in total was used, not being able to separate the different protein chains of the bispecific antibody therapeutic (Table 2). Data were acquired using positive ionization in TOF-MS mode with the intact protein mode turned on and detector decrease off. Detailed MS settings are listed in Table 3 and DMS settings in Table 4.

Table 3. MS Parameters.

Parameter	Setting
Scan Mode	*TOF MS positive*
Gas 1	*40 psi*
Gas 2	*40 psi*
Curtain Gas	*30 psi*
Source Temperature	*300 °C*
Ion Spray Voltage	*5500 V*
Time Bins to Sum	*40*
Accumulation Time	*0.5 sec*
Mass Range	*700 - 3,000 m/z*
Declustering Potential	*150 V*
Collision Energy	*10 eV*

Table 4. SelexION Device Parameters for Differential Mobility Separation.

Parameter	Setting
DMS temperature	*150 °C*
Separation Voltage	*3500 V*
Compensation Voltage	*-9 / -3 / 9 V*
Resolution Gas	*off*

Data Processing:

Data was processed using SCIEX OS 1.6.1 software with the BioToolKit and BPV Flex 2.0 software.

Subunit Analysis of a Bispecific Antibody without SelexION Technology

The bispecific sample was generated by Merck Healthcare KGaA, Darmstadt, Germany using the strand-exchange engineered domain (SEED) platform.[2] This strategy results in alternating sequences of Ig**A** and Ig**G** CH3 sequences and favors the formation of heterodimers with complementary AG and GA in the CH3 domains (Figure1).[2] The bispecific protein sample consists of three distinct amino acid chains: One light chain (Figure 1, green chain), one AG chain and one GA chain, forming two different binding domains.

First, the reduced bispecific sample was run without the use of DMS technology, resulting in all protein chains coeluting in one single peak at around 1.3 min (Figure 2 top). Although the short LC method in combination with the source conditions used provide sufficient desolvation and declustering, the corresponding spectrum for the chromatographic peak contains several charge state envelopes derived from the multiple chains of the bispecific sample (Figure 2 middle). The overlapping charge state envelopes can be a challenge during data interpretation, particularly when investigating raw data. In addition, different charge states derived from different features can end up as a complete overlap, impacting the accuracies of reconstructed data in terms of intensities.

It is possible to separate the different chains chromatographically; however, LC methods of greater than 20 min are frequently required, which presents challenges when there is a demand for high throughput.

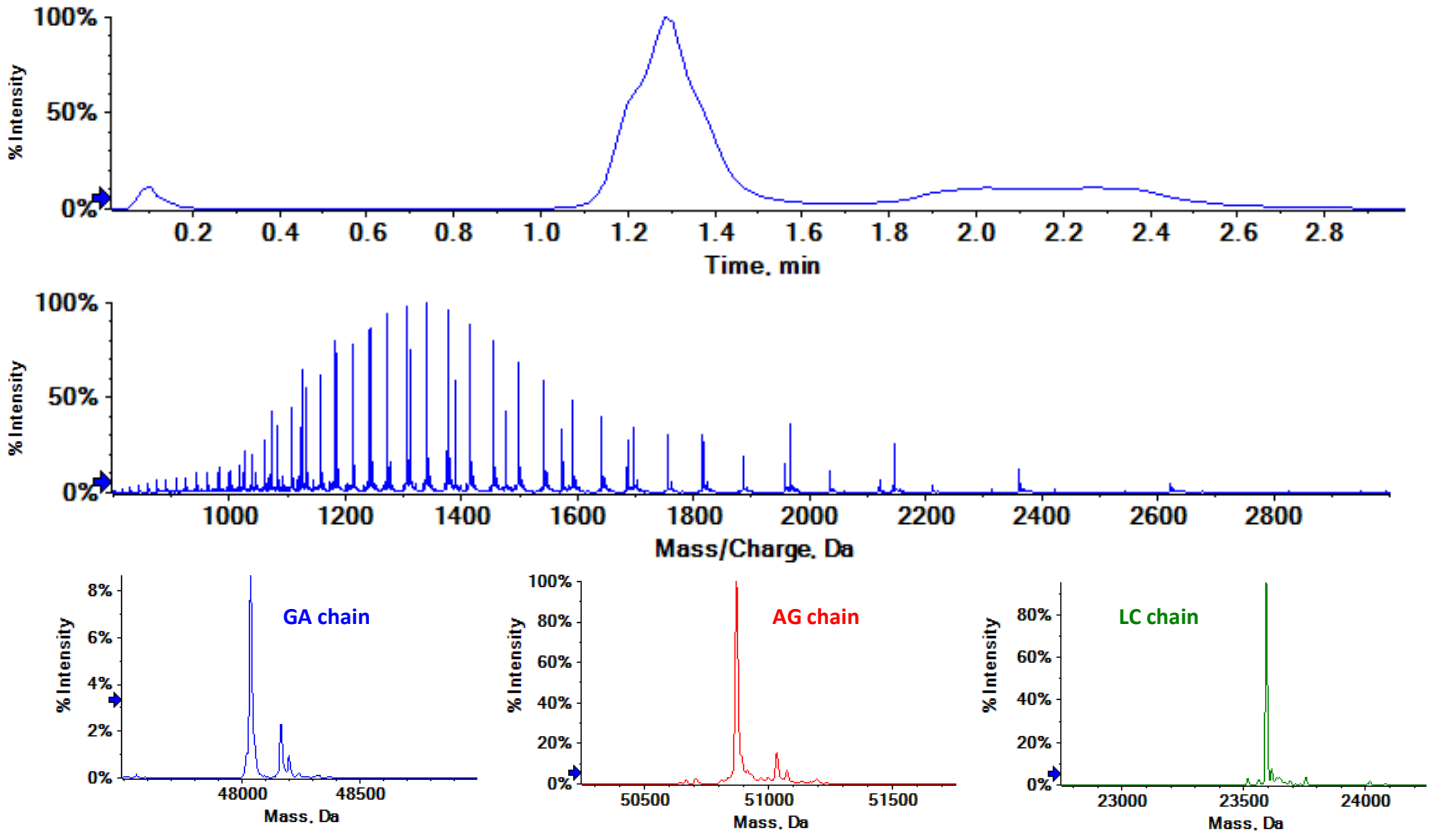

Figure 2: Data on Bispecific Sample Without SelexION Technology. Top: Total ion chromatogram showing no separation of individual chains. All chains are eluting within the major peak. Middle: Average MS spectra from 1.1-1.45 min. Overlapping charge state envelopes of all three chains of the bispecific mAb sample. Bottom: Zoom into the reconstructed mass ranges of the three different subunits.

Subunit Analysis of a Bispecific Antibody with SelexION DMS Technology

As a next step, the DMS cell was installed within a couple of minutes without the need to break vacuum. Previously, the use of SelexION technology has been successfully used to improve the S/N for quantitation of peptides and separating different peptide species as well as improving the data quality in complex antibody-drug-conjugate studies.[3,4,5]

The DMS mobility cell is composed of two parallel flat plates to define the mobility region (Figure 3, bottom). The ions are drawn by the transport gas flow towards the MS. Radio frequency (RF) voltages are applied across the ion transport channel, perpendicular to the direction of the transport gas flow which is referred to as the separation voltage (*SV*). Due to the difference between high and low field ion mobility coefficients, ions will migrate toward the walls and leave the flight path. Their trajectory is corrected by a compensating voltage (*CoV*). The *CoV* is scanned serially to pass ions according to their differential mobility, or set to a fixed value to pass only the ion species with a particular differential mobility.

For this study, three different *CoV*s were scanned serially within a single injection to separate the three different protein chains in

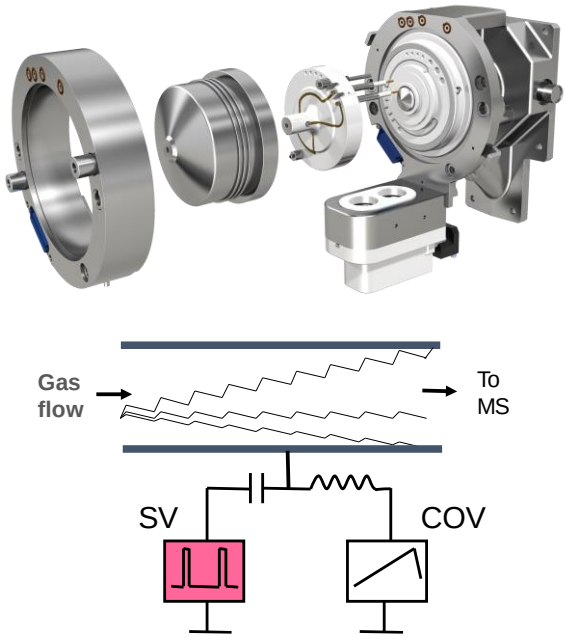

Figure 3. SelexION Device DMS Cell. Top: Individual parts of the SelexION DMS device. Bottom: Schematic function of the DMS cell.

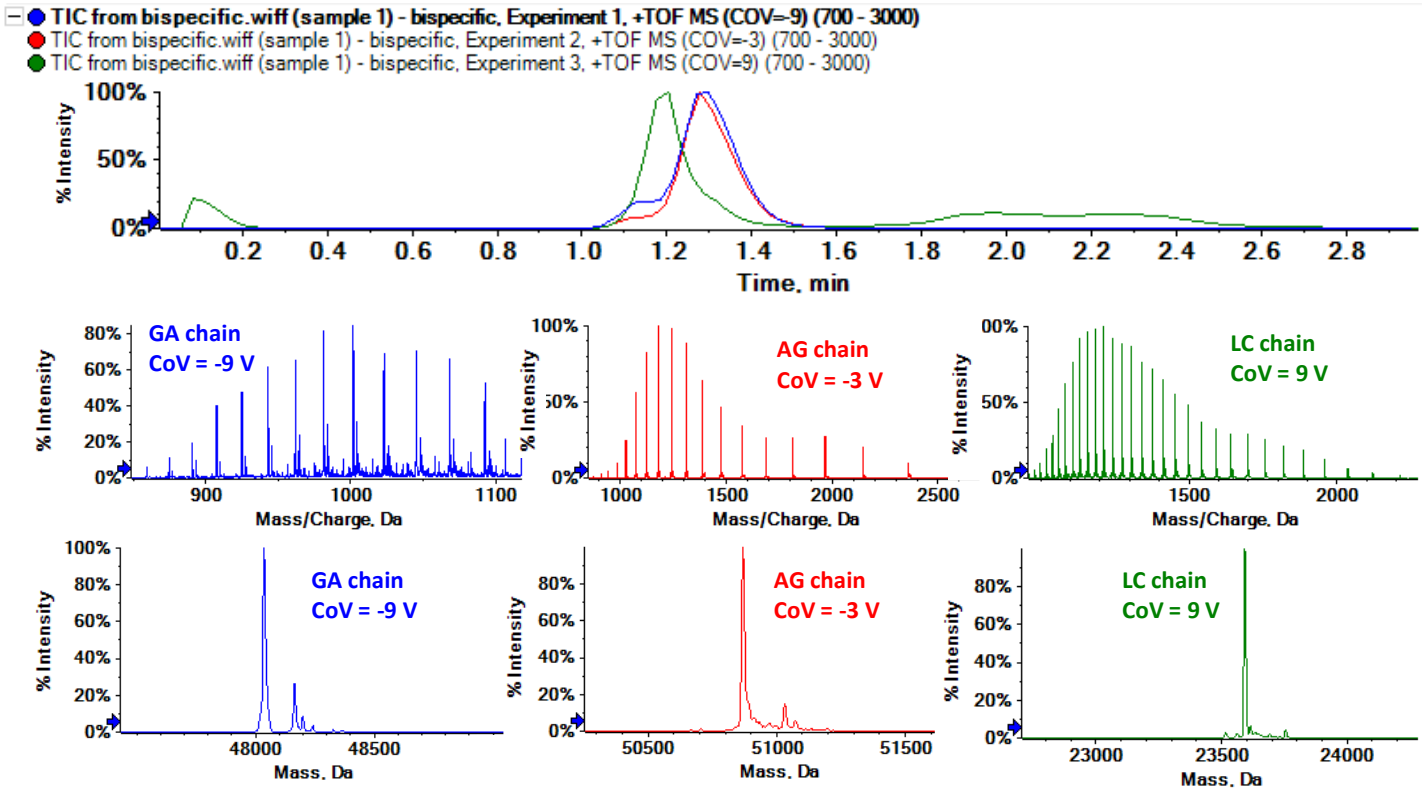

Figure 4: Data on the Bispecific Sample using SelexION Technology. Top: Serially scanned *CoV*s of -9, -3 and 9 V within one single injection. Middle: Averaged MS spectra derived from the three different *CoV*s. Bottom: Reconstructed masses for each *CoV* channel.

ID	Validated	Use	Chain Names	RT	Reconstruction Area	Observed Average MW
Data File: bispecific_inj1.wiff (3 items)						
1	✓	✓ ? ✗	GA chain	1.11	3.84e4	48036.94
2	✓	✓ ? ✗	AG chain	1.30	4.65e5	50869.79
3	✓	✓ ? ✗	Light Chain	1.20	1.80e6	23592.60
Data File: bispecific_inj2.wiff (3 items)						
4	✓	✓ ? ✗	GA chain	1.11	3.40e4	48036.96
5	✓	✓ ? ✗	AG chain	1.30	4.51e5	50869.50
6	✓	✓ ? ✗	Light Chain	1.20	1.71e6	23592.58
Data File: bispecific_inj3.wiff (3 items)						
7	✓	✓ ? ✗	GA chain	1.11	3.50e4	48037.05
8	✓	✓ ? ✗	AG chain	1.30	4.47e5	50869.78
9	✓	✓ ? ✗	Light Chain	1.20	1.70e6	23592.68

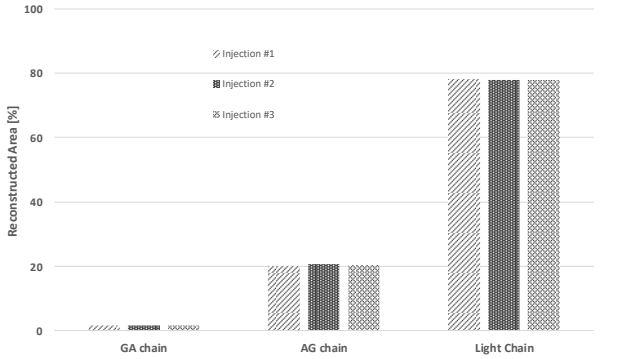

Figure 5. SelexION device data processing for three replicate injections. Top: Reconstructed data matching in BPV Flex. Bottom: Plot of relative percentages of areas for each chain.

the DMS cell. The total ion chromatograms for each *CoV* can be visualized separately and the raw data evaluated (Figure 4, top and middle). In contrast to the overlapping charge state envelopes resulting in complex mixed spectra without using the SelexION device (Figure 2, bottom), the spectra derived from the data using SelexION technology are much cleaner and enable higher confidence in the interpretation of the reconstructed results (Figure 4, bottom).

Data from replicate injections were subsequently processed using BPV Flex software, including the reconstruction of the different chains, matching against predefined target masses without the need for a sequence input and the comparison of the obtained reconstructed areas (Figure 5). As expected, the replicate injections show similar amounts for each chain (Figure 5, bottom). However, during the development of bispecific biotherapeutics, differences could be quickly spotted with this approach enabling a scientist to evaluate different conditions much faster. In addition, interfering background can be separated from the target analytes as previously reported for complex samples such as antibody-drug-conjugates which can reduce the need for sample preparation.[5]

Conclusions

- Reduction of complexity of subunit data using SelexION Differential Mobility Separationtechnology
- Separation of individual chains using the SelexION device without the need for chromatographic separation
- Increased throughput for subunit analysis for antibodies and new modalities such as bispecific antibodies during development

References

1. Labrijn, A.F., Janmaat, M.L., Reichert, J.M. *et al.* Bispecific antibodies: a mechanistic review of the pipeline. Nat Rev Drug Discov 18, 585–608 (2019).
2. Davis, J.H., Aperlo, C., Li, Y. et al. SEEDbodies: fusion proteins based on strand-exchange engineered domain (SEED) CH3 heterodimers in an Fc analogue platform for asymmetric binders or immunofusions and bispecific antibodies. Protein Eng Des Sel 23(4), 195-202 (2010).
3. Using Differential Mobility Spectrometry to Separate and Localize Sites of Post-Translational Modifications on Peptides. SCIEX Technical Note RUO-MKT-02-2750-A.
4. Analysis of non-deglycosylated antibody-drug-conjugates by TripleTOF high resolution quadupole-time-of-flight instrument and effective reconstruction software. SCIEX Technical Note RUO-MKT-02-2814-A.
5. Differential Mobility Separation Mass Spectrometry for Quantitation of Large Peptides in Biological Matrices. SCIEX Technical Note RUO-MKT-02-4792-A.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices