Characterization of Charge Heterogeneity of Monoclonal Antibodies Using Capillary Electrophoresis (CE) Coupled to Mass Spectrometry (MS)

Featuring the SCIEX TripleTOF® 6600 LC-MS/MS System with microfluidic CE separation via the ZipChip system

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Charge heterogeneity profiling is an important part of the characterization of biotherapeutics during development, production and lot release. Cellular expression and purification processes can lead to a range of modifications including charged glycan structures such as sialic acids, differential lysine clipping at the C-terminus, and deamidation events, to name a few. Monitoring these modifications or product quality attributes (PQA) is often required to ensure products meet expected criteria. Some modifications can have an impact on the pharmacokinetics and binding properties of the molecule as well as immunogenicity. Understanding and controlling these critical quality attributes (CQA) is especially important for developing a safe and efficacious product. Traditional approaches use methods such CE as a standalone technique, whereas more recent approaches are focused on coupling the powerful separation of CE systems with mass spectrometry to understand the nature of charged variants. The feasibility of charge heterogeneity analysis (Figure 1) via the microfluidic ZipChip system in combination with SCIEX high resolution time-of-flight (TOF) technology is demonstrated for different monoclonal antibodies (mAbs).

Key Features of the ZipChip System Coupled to the TripleTOF® 6600 System

- Fast, reproducible and high quality data acquisition with limited optimization of the CE and MS method
- On-chip desalting and removal of detergents means minimal sample prep and avoids carry over issues increasing throughput
- Great separation of different charged isoforms with MS compatible, ready-to-use buffer systems allowing for an additional dimension of information via MS
- Great data quality with generic methods on the TripleTOF® 6600 System

Figure 1. Stacked Electropherograms of Charge Heterogeneity Analysis of NIST mAb, Trastuzumab and Adalimumab.
Methods

Sample Preparation:

In this study, three monoclonal antibodies: the NISTmAb reference material 8671 (10 µg/µL), trastuzumab (20 µg/µL), and adalimumab (50 µg/µL) were used. All three mAb samples were diluted to a concentration of 0.5 µg/µL with water.

Capillary Electrophoresis:

Separation was performed using the ZipChip system (908 Devices Inc.) consisting of an optional autosampler, different options for chips containing a separation channel and a built-in electrospray emitter (Figure 2), and an interface being compatible with SCIEX instrumentation. Here, a charge variant TOF-Kit containing premixed buffers were used. For each sample 1 nL (0.5 ng) was loaded onto the chip with the voltage being set to 500 V/cm. The total analysis time was set to 15 min.

Mass Spectrometry:

Table 1. MS Parameters.

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<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
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<tr>
<td>Scan Mode</td>
<td>TOF MS positive</td>
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<tr>
<td>Gas 1</td>
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<tr>
<td>Gas 2</td>
<td>0 psi</td>
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<tr>
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<td>10 psi</td>
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<td>Time Bins to Sum</td>
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<td>Accumulation Time</td>
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<td>Mass Range</td>
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<tr>
<td>Declustering Potential</td>
<td>160 V</td>
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<tr>
<td>Collision Energy</td>
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Data Processing:

Data were processed using SCIEX OS Software 1.7 with the BioToolKit add on.

Results and Discussion

Working with a ready-to-use buffer system the only CE parameter which needs to be optimized is the voltage being applied to the chip. As a rule of thumb, a higher voltage will shorten the analysis time and vice versa. Here, all samples were analyzed using the exact same conditions: The voltage was set to 500 V/cm allowing for an analysis within 15 min with an MS method set up as indicated in Table 1.

Unlike reversed phase chromatography (RP), which is traditionally used in combination with mass spectrometry, CE separations are based on the size and charge of the molecules. Modifications resulting in a rather small mass shift such as a lysine loss of ~128 Da do not have a high impact on an antibody’s mass, approximately 150,000 Da, or on its hydrophobicity making them difficult to separate using reversed phase approaches. In contrast, such modifications result in measurable changes to the isoelectric point (pI) and therefore the migration time (tm). Until recently, a main challenge for interfacing CE with MS has been the the requirement for using buffers composed of high salt and detergent which are not compatible with MS analysis. The ready-to-go kits from 908 Devices Inc. in combination with generic settings for intact protein analysis on the SCIEX TripleTOF® 6600 system (Table 1) now enable a user to achieve great separation and data quality with a minimal need for optimization.
With the conditions being used, protein species with a higher pI migrate faster than species with a lower pI. This results in basic variants, such as proteoforms containing C-terminal lysines (K), to be detected first followed by acidic species with less positive charge or lower pI.

**NISTmAb**

For the NISTmAb a great separation of several proteoforms was achieved as shown in Figure 3A. The chip and buffer used are suitable for analyzing the molecule without fully denaturating it. Therefore, the protein takes up less charges resulting in higher m/z values (Figure 3B) compared to the denaturating reversed phase liquid chromatography analysis (not shown). In general, native-like analysis can be favorable in many instances as artifacts - modifications being introduced during the analysis - can be reduced or eliminated. Furthermore, less charges on the molecule result in an increased spacing of different features of the molecule on the m/z-axis.

In addition to the separation of several forms, the SCIEX TripleTOF® 6600 System proved its ability to efficiently desolvate and decluster the molecule even under native-like conditions as can be seen by a great raw data quality (Figure 3B). The raw data was subsequently reconstructed and matched in alignment with the literature against the theoretically calculated major protein forms (Figure 4). The antibody with two C-terminal lysine residues (2K), one C-terminal lysine residue (1K), the main species without any C-terminal lysine residue (0K) followed by a group of acidic variants were detected (Figure 3A and Figure 4). Despite the fact that the main form and variants containing C-

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**Figure 3. Analysis of NIST mAb with CE-MS.** A: total ion electropherogram (TIE) of the separation of different mAb proteoforms with 0-2 C-terminal K residues and minor acidic variants. B: Average spectra of TOF-MS raw data from 7.13-7.18 min (main peak; 0K) with zoom into most abundant charge states (insert).

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**Figure 4. Reconstructed Data for NISTmAb.** Top, left: main form (0K); Middle, left: basic variant (1K); Bottom, left: basic variant (2K). B: Top, right: main form (0K); Middle, right: acidic variant with multiple additional proteoforms and slight positive mass shift compared to main form; Bottom, right: acidic variant with slight positive mass shift compared to main form. t_m as indicated.
terminal K can also be detected using reversed phase MS, they cannot be chromatographically separated. Furthermore, the modification of a K modification (Δ 128 Da) is typically not fully resolved on raw data level on a complex molecule like an antibody, limiting the accurate quantitation of such modifications with RP. Therefore the separation power of the chip-based technology combined with the accurate mass data provides an advantage over RP-LC-MS approaches.

The first acidic variant (tₘ = 7.32 min) showed additional heterogeneity (Figure 4, middle left) compared to the main species (tₘ = 7.16 min). These additional peaks might be associated with minor abundant glycoforms. The acidic variants showed a small positive mass shift compared to the main form, which could be the results of modifications which also have an impact on the charge profile such as deamidations. However, for the determination of the exact kind of modification further experiments would be required.

**Trastuzumab**

In order to evaluate the usability for different biotherapeutics, the same chip with the same settings and the same generic MS method, was used for the analysis of the humanized antibody trastuzumab. As mentioned above, basic proteoforms are expected to migrate faster followed by more acidic variants. For the trastuzumab sample, the separation of one C-terminal K, the major form without any C-terminal K, as well as two acidic variants, was achieved (Figure 5 and 6). It is possible that the proteoforms with two terminal K is below the detection limit for this assay. Additional peaks were detected for the basic variant at 7.39 min as well as the acidic variant at 7.82 min, (encircled peaks in Figure 6). As shown in Figure 7, these peaks correlate with the raw data. These peaks are potentially due to low abundant glycoforms, however, this would need to be confirmed using orthogonal approaches.

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**Figure 5. Analysis of Trastuzumab with CE-MS.** A: total ion electropherogram (TIE) of the separation of different mAb proteoforms with 0-1 C-terminal K residues and minor acidic variants. B: average spectra of TOF-MS raw data from 7.56-7.60 min (main peak; 0 K) with zoom into most abundant charge states (insert).
Adalimumab

As a third sample the human antibody adalimumab was evaluated. Also, for this antibody a great separation of different proteoforms could be achieved. The basic variants with one and two C-terminal K were separated from the main from without any C-terminal K (Figure 8 and 9). In addition to the acidic variant with a small positive small mass shift compared to the main form,

Figure 7: Comparison of Raw Data and Reconstructed Data for Trastuzumab Variants. Left hand side: raw data for two different time points. Right hand side: associated reconstructed data. Hypermass feature (red bar) in raw data shown for two isoforms being selected in the reconstructed data (blue highlighted section).

Figure 8. Analysis of Adalimumab with CE-MS. A: total ion electropherogram (TIE) of the separation of different mAb proteoforms with 0-2 C-terminal K residues, an acidic variant and impurity. B: average spectra of TOF-MS raw data from 7.52-7.57 min (main peak; 0 K) with zoom into most abundant charge states (insert).

Figure 9: Reconstructed Data for Adalimumab. Top left: main from (0 K); Middle, left: basic variant (1 K); Bottom left: basic variant (2 K); Top right: main form (0 K); Middle, right: acidic variant with slight positive mass shift compared to main form. Migration times ($t_m$) as indicated.
an impurity peak was observed at 8.29 min in the electropherogram. A molecular weight of approximately 47.3 kDa was determined for this impurity and confirmed by using the hypermass feature, showing a calculation of the charge state based on the reconstructed mass (Figure 10). Since there was no indication of different glycan proteoforms for this impurity, but the size is exceeding the mass for a light chain of the antibody, the mass observed could be a clipping product of the biotherapeutic’s light chain being linked to the heavy chain or a clipping product of two heavy chains being attached to each other. Further investigation is needed in order to confirm this theory.

Conclusions
- The ZipChip system coupled to the TripleTOF® 6600 System was shown to be a turnkey solution providing a great separation power for a variety of antibodies
- Excellent raw data quality from the TripleTOF® 6600 System allowed for assignment of different glycoforms, as well as different basic variants for all antibodies shown being in alignment with published information1, 2
- The additional dimension of high resolution data provides the possibility for detection and characterization of unexpected impurities

References

Figure 10. Data for Impurity for Adalimumab from CE-MS analysis. Top: average spectra of TOF-MS raw data for impurity peak with charge states in red. Bottom: associated reconstructed data for impurity peak.