An Executive Summary

Discover More with Rapid Biotherapeutics Characterization Using CE-ESI MS

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How can the ZipChip™ separations platform achieve improved resolution and sensitivity for greater characterization of biotherapeutic molecules, providing process control and antibody product quality monitoring?

Introduction

Biotherapeutics continue to be one of the fastest growing segments in the pharmaceutical industry and have become a multibillion dollar market. Biological therapies are significantly more complex than traditional small molecule drugs. Thus, analysts need very powerful tools to help characterize the products and to determine their potency, efficacy, and safety. Traditional techniques—including isoelectric focusing (IEF) with ultraviolet (UV) or fluorescent light and infusion mass spectrometry (infusion MS)—cannot provide both peak ID and separation. This necessitates the use of many different techniques to characterize key quality attributes of these large therapeutic proteins. This paper explores the potential of an emerging technique (the ZipChip separations platform from 908 Devices), for improved characterization of biologics.

Novel Microfluidic Platform

The ZipChip platform is a front-end separation system for commercial mass spectrometers that performs capillary zone electrophoresis (CE) separation with integrated electrospray ionization (ESI). The heart of the technology is the chip itself, which contains the actual glass microchip (see **Figure 1**). The injection cross is used to perform rapid injections of the sample directly into to the separation channel. The separation channel is the serpentine channel that traverses nearly the full length of the microfluidic device. This is analogous to the fused silica capillary used in traditional capillary electrophoresis. The separation channel terminates at the fully integrated electrospray ionization emitter at the corner of the device. With this approach, the electrospray is performed directly off the corner of the device without introducing any dead volume. The ZipChip microfluidic technology takes advantage of the inherent speed and efficiency of capillary zone electrophoresis separations and enables nanoflow ESI for sensitive analysis without complex sample prep. A separation of the 20 natural amino acids is completed in less than two minutes (without labeling) at an ESI flow rate of about 150 nL/min, with excellent sensitivity. Using the ZipChip, one can analyze a range of molecules, from large complex molecules (e.g., intact antibodies) to small molecule metabolites.

Extensive work has been performed at 908 Devices using the NIST monoclonal antibody (mAb), a commercially available product from the National Institute of Standards and Technology. This reference material provides researchers access to a molecule that mimics a normal antibody-based therapy from cell culture through purification. The method for intact antibody separations uses gentle solvents that reduces denaturing of the molecules, providing a single method for charge heterogeneity, mass information, and glycoform characterization. The charge states

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obtained, although not fully native, yield less charging on the molecule than denaturing conditions, providing the ability to take advantage of some benefits of native charge states while maintaining the high signal intensity seen with denaturing conditions. The intact analysis of the NIST mAb yields near baseline separation of C-terminal lysine variants as well as glycoform resolution within the charge states in less than four minutes. The data is standard ESI data. Commercial software such as Thermo Scientific™ BioPharma Finder™ software can be used with the methods to process and automatically identify variants.

In addition, the mAb can be reduced to the light chain (LC) and heavy (HC) chain. The polypeptide chains are readily separated using ZipChip. Even with denaturing conditions, high-quality MS data is obtained. Upon deconvolution, modifications can be characterized, as shown in **Figure 2**. The light chain is fairly homogeneous except for a small amount of glycation. Different HC variants can be detected in the separation and deconvoluted spectra, including both C-terminal lysine HC variants and glycoforms.

Figure 1: Anatomy of a ZipChip.

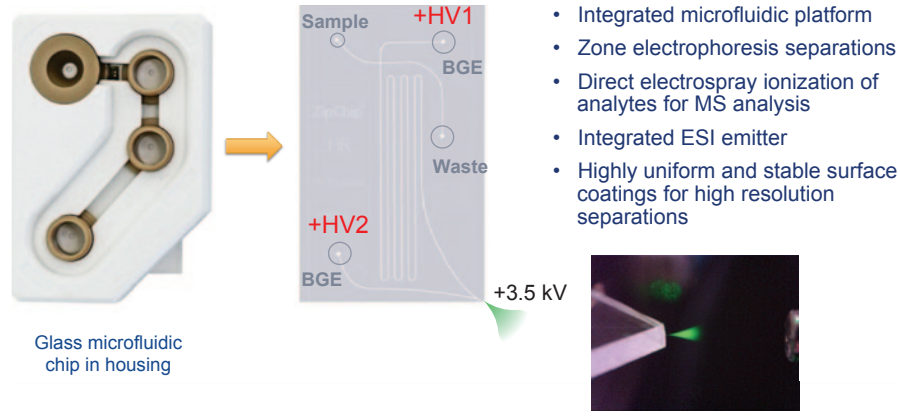
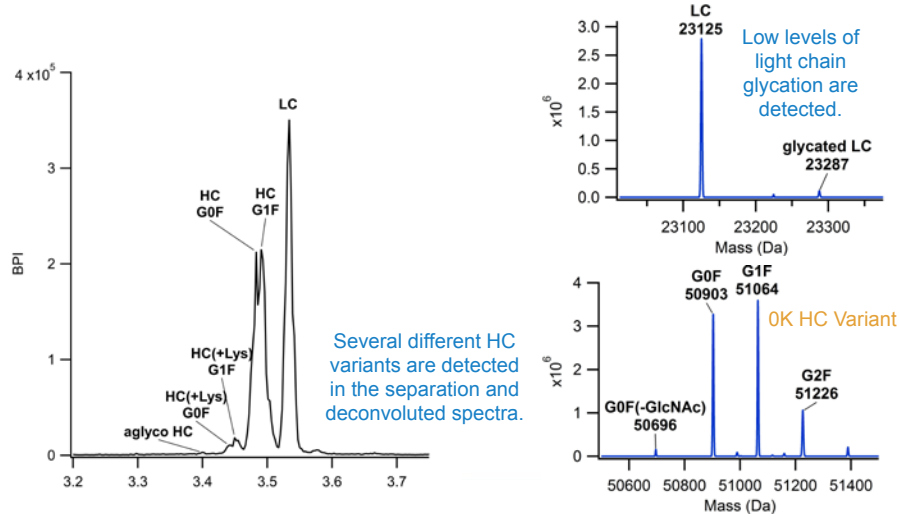


Figure 2: Identification of variants.



The antibody can also be treated with an enzyme to cleave in the hinge region and further reduce it to obtain fragments of about 25 kD in mass. This provides additional site-specific information about modifications to the antibody. The Fc, Fd, and LC fragments of the mAb are easily resolved using ZipChip. Additionally, the smaller Fc fragments of the heavy chain can also be partially separated based on differences in glycan structures.

Finally, the antibody can be fully digested with an enzyme such as trypsin and the ZipChip system is used to perform a rapid peptide map. Collaborators at Thermo Fisher Scientific™ did a trypsin digestion on the NIST mAb and analyzed it using the ZipChip system. The entire separation was completed in about 10 minutes and 98% sequence coverage was obtained just on MS2 identifications alone.

This platform is capable of analyzing small molecules to large intact proteins. In addition, one powerful advantage of the ZipChip system is its ability to perform very rapid fingerprinting of antibodies to assess key degradation points and other critical hotspots for biotherapeutic manufacturing.

Antibody In-Process Monitoring

Scientists at Biogen have been exploring the ZipChip platform for both monitoring and controlling the process as well as the product. The manufacturing process for biotherapeutics involves growing cells in production bioreactors that have final volumes of anywhere from several hundred liters to several thousand liters. The product is harvested and purified using either a two- or three-column system. The product is then further purified, polished, and concentrated to create the final drug product. This is a long, arduous, and expensive process. If the final product fails the quality control testing, the entire process has been a waste. An analysis of the biological

process reveals that the bioreactor is the most strategic step; most of the variability occurs in the bioreactor.

Thus, there has been a shift to try to do more in-process monitoring and testing, to catch any deviations while corrective actions can save the batch or to terminate the batch before going through the extensive purification process. Common methods use pH probes, temperature, dissolved gases probes, and even some Raman spectroscopy. However, there is a limit to the number of probes you can have in a bioreactor, as well as to the specificity and sensitivity of these indirect measurements.

Although the product is a mAb, quite a few post-translational modifications can occur in the bioreactor that result in a fairly heterogeneous product no matter how tightly we control the process. The bioreactor is a very dynamic system; continuous additions of media cause ongoing changes in the sample matrix, metabolite formation is in flux, and product concentration increases, requiring a wide dynamic range in the test methods. The ideal in-process test would be rapid, require limited sample preparation, and report a wide range of product quality attributes.

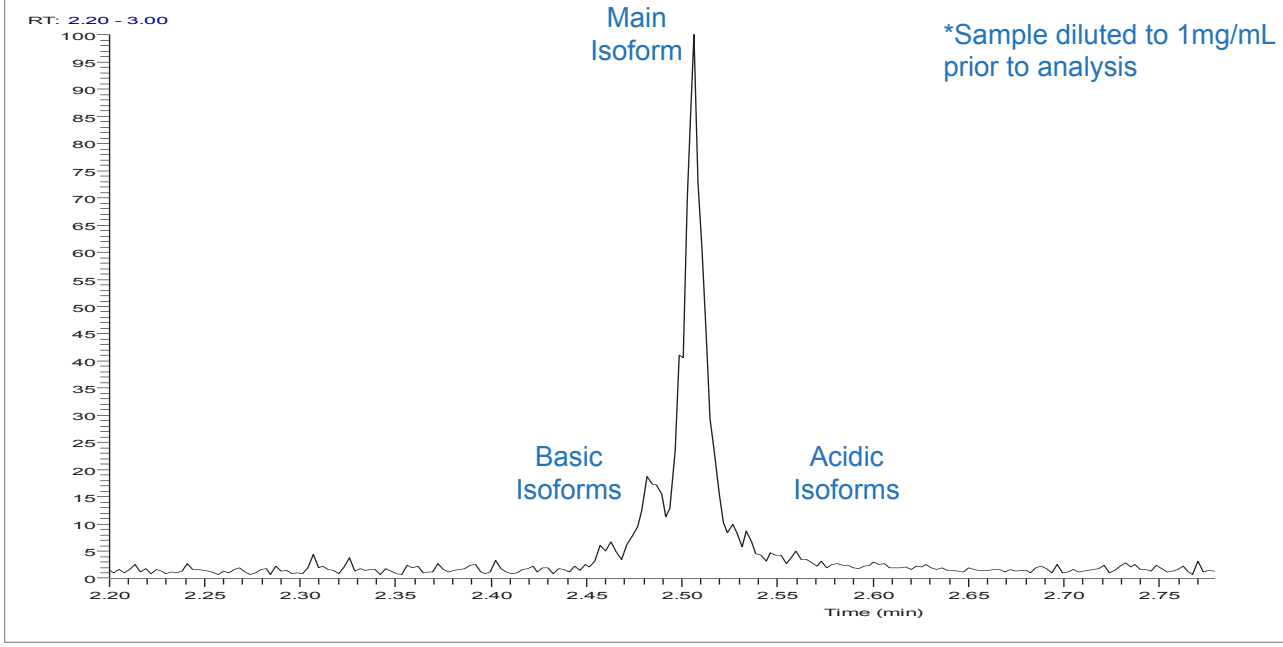
The ZipChip platform offers several advantages for in-process bioreactor testing. As it is a capillary electrophoresis (CE) technique, rather than a liquid chromatography (LC) technique, it doesn't have a stationary phase to foul, greatly simplifying sample prep. Also, CE is innately suited to biotherapeutic work because modifications to the product almost always cause a change in either the charge and/or the size of the molecule, which is the basis for CE migration. In fact, the microfluidic system of the ZipChip permits high electric fields to yield incredibly fast and highly efficient separations. Mass spectrometry is the ideal detector for biotherapeutics because it eliminates the need for fluorescent tags and the dependence on UV absorbance. While UV absorbance may be fine for intact antibodies, it may not be suitable for other molecules that play a role in the bioreactor, such as amino acids. Finally, coupling the MS data with the electrophoretic separation makes it possible to identify both expected post-translational modifications as well as new post-translational modifications that may occur.

Applications for the ZipChip Platform in Biotherapeutic Process Characterization

The initial exploratory work was performed using the NIST mAb. Additional work was then performed with IgG taken directly from a harvest. The ZipChip platform is ideal for in-process testing because one simply dilutes the sample and runs it on the chip. Charge-based separation methods including iCIEF (Imaged Capillary Isoelectric Focusing) are currently accepted for product release. However, the iCIEF is not compatible with mass spectrometry, making it very difficult to identify the peaks.

The ZipChip platform can generate a very similar profile with the benefit of identification by MS. The charge variant separation shows the basic isoforms eluting first, followed by the major isoform and then the acidic isoforms in lower relative abundance as shown in **Figure 3**. This data was run using the 22-cm HR chip from 908 Devices and collected on a Thermo Scientific™ Exactive™ Plus Orbitrap Mass Spectrometer. There is an approximately 17 Da shift between the most abundant glycoforms for these three peaks. The corresponding change in the electrophoretic mobility allows analysts to identify these

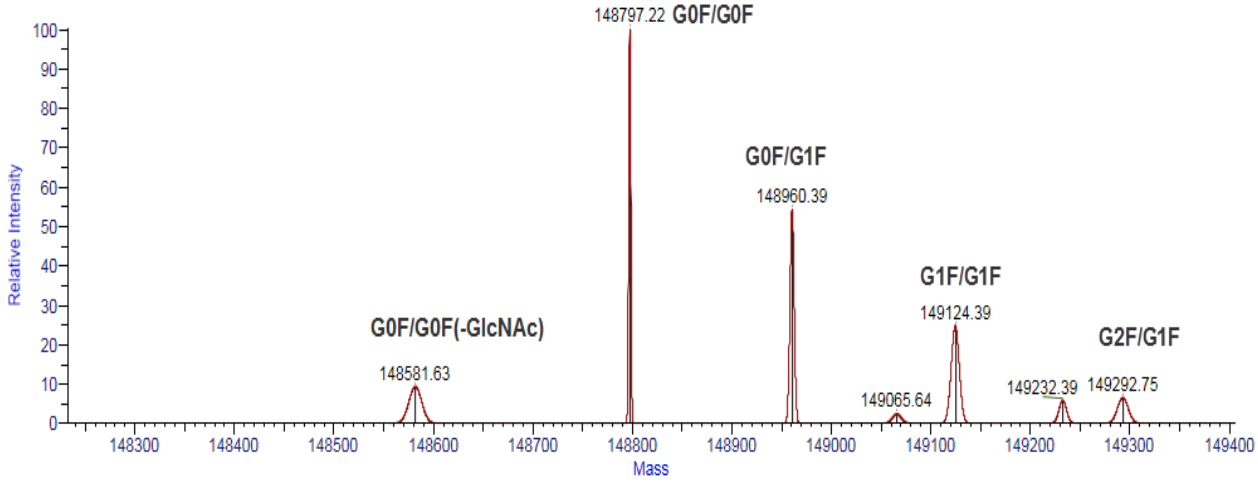
Figure 3: Charge variant separation.



peaks as the cyclization of the N-terminal Q. One can monitor these attributes by looking at the main peak of the intact antibody. Analysts can also look at the deconvoluted spectra where they see the distribution of glycoforms is in good agreement with standard LCMS data **(Figure 4)**.

Identification of the acidic variants became more difficult because the complex multiple variations can comigrate, causing spectral overlap and ion suppression of the low abundance modifications. However, if the antibody is broken down into subunits, the detection and mass accuracy improve.

Figure 4: Glycoform identification.



- Deconvoluted spectra from main isoform peak
- Distribution of glycoforms are comparable to LC-MS results

Figure 5: Glycoform separation in Fc domain.

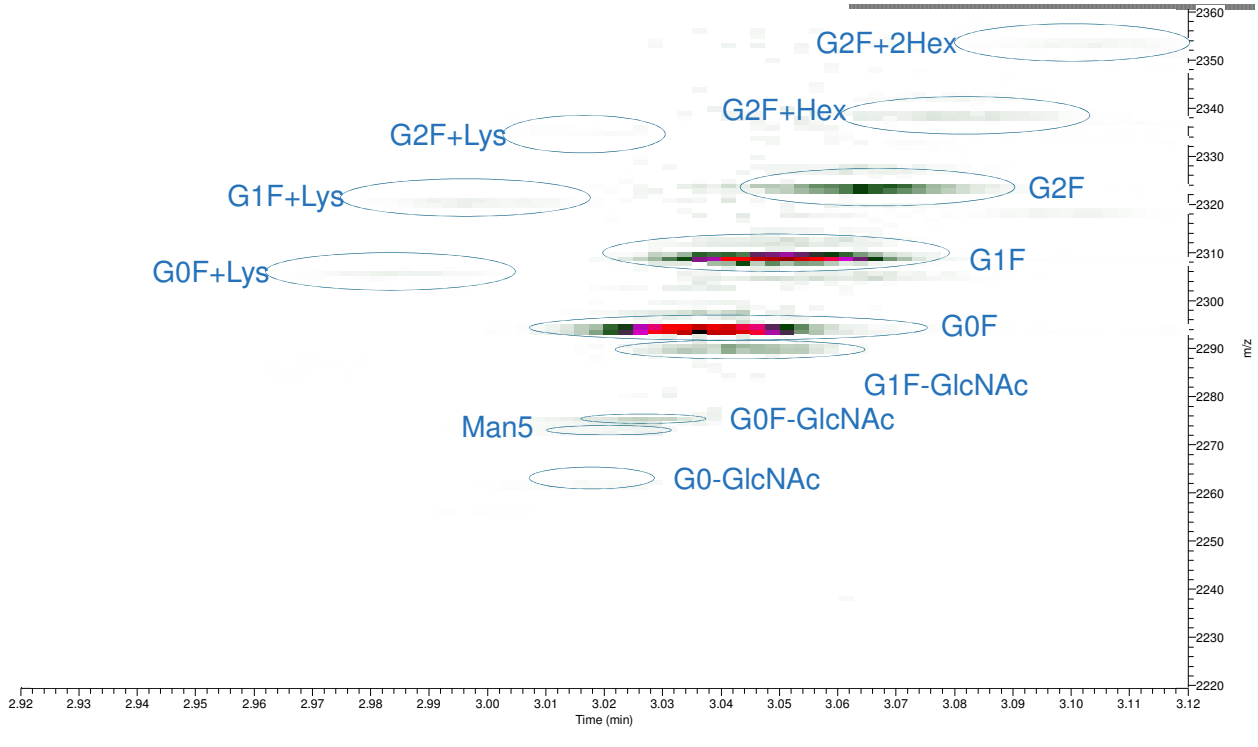
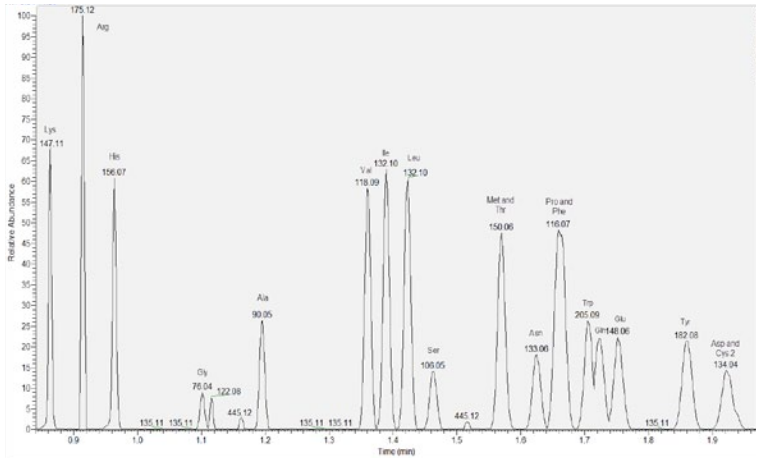


Figure 6: Spent media analysis.



Zipchip separation is excellent at detecting amino acids, which are the primary components of the feed stock for the bioreactor as well as the spent media

As an added benefit, the separation also improves as the overall complexity decreases with the reduction of size of the protein. Hence, researchers explored the use of an IdeS enzyme (i.e., FabRICATOR® enzyme) to cleave the antibody below the hinge and then reduce it. In Biogen's case, the N-glycan profile on the single-chain region is something the team was very interested in controlling as a product quality attribute. After reduction and digestion, researchers simply diluted the sample to 0.5 mg/mL. There was no further sample clean up and desalting is done by the chip. The fragment peaks were easily separated. Moreover, by breaking the antibody down into subunits, one can see peaks for the deamidation of both the LC and Fc regions more readily than in the intact antibody. Further elucidation was performed using a 2D heat map, as shown in **Figure 5**. Using the ZipChip to analyze the subunits allowed the team to detect some low-abundance glycoforms earlier in the process to ensure that the process is within specifications.

Researchers found deamidation identification is enhanced with CE separation as compared to LCMS separations, while the oxidation of the subunits is not as well separated by CE. As a final exploration, they used the highest resolution of the Thermo Scientific™ Q Exactive™ Plus Orbitrap Mass Spectrometer to obtain isotopic resolution of the subunits. In this case, the sampling rate was so low as to prohibit quantitation, perhaps 5 spectra per peak. Nonetheless, the power of isotopic resolution is another advantage of subunit analysis over intact antibody analysis.

Finally, the team investigated the utility of the ZipChip platform for monitoring spent media and feed media, with the thought that while it is a cost saving to discard a batch earlier in the process than after harvest, it would be even more beneficial to determine how to save a batch. Deviations in the feed media or the spent media are easier to detect immediately than are the changes in the product quality they eventually cause. In addition, monitoring of the consumption of nutrients provides a window into cell behavior and can help to optimize and balance feed strategies to yield the highest cell viability.

Figure 6 shows an example of a trace amino acid separation for the spent media. In this case, the team used the shorter HS chip from 908 Devices. Still, analysts were able resolve most of the amino acids in under two minutes. This sample is a good representative control standard for the process. For investigative purposes, the group spiked a sample to simulate a contamination or error and easily detected the fault. In fact, the group is developing a profile from inoculation to harvest, showing the much lower abundance of most amino acids at harvest, with the addition of a few new peaks that represent metabolites.

Conclusions

In summary, rapid characterization of the mAb-based drug product and monitoring of nutrients and metabolites in spent media can be carried out with the ZipChip platform combined with the power of MS detection. Faults can be detected in the media before they impact the final product, saving time and money. The multiple levels of mAb characterization using ZipChip provide important insights into the state of the drug product, including elucidation of low-abundance glycoforms and charge heterogeneity. The intact antibody method brings additional value to the lab as it simplifies the lab workflow, requiring only one simple dilution for sample prep resulting in rapid glycoform and charge heterogeneity characterization, providing both separation and peak ID.

As mentioned, the trend in the industry is to detect failure sooner, to apply corrective action or to end the batch to cut losses. The ZipChip system enables this goal by providing rapid intact antibody testing, subunit detail, and media profiling throughout the process. This system has proven to be an effective and efficient tool for in process characterization and monitoring of biotherapeutics.