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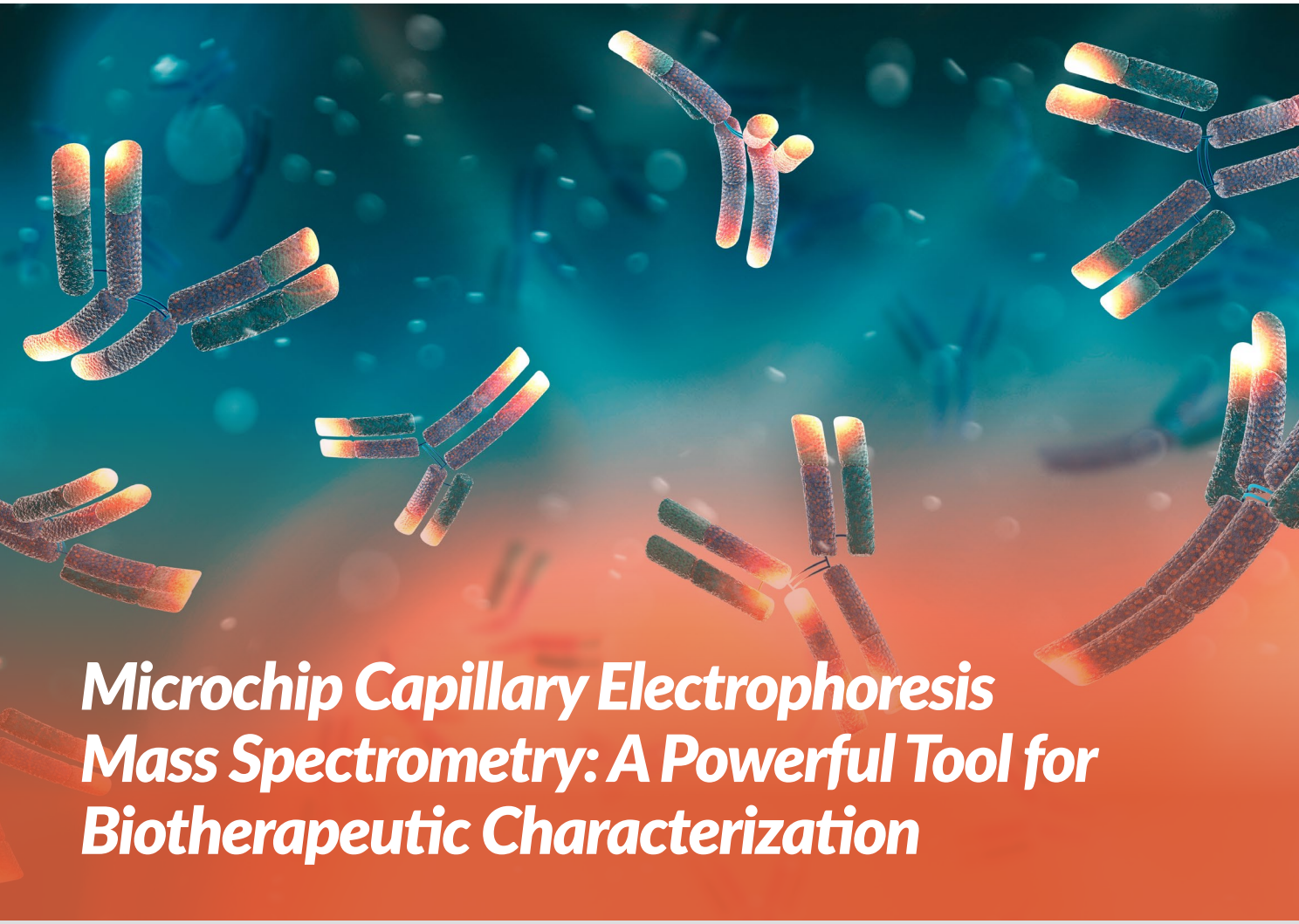

Microchip Capillary Electrophoresis Mass Spectrometry: A Powerful Tool for Biotherapeutic Characterization

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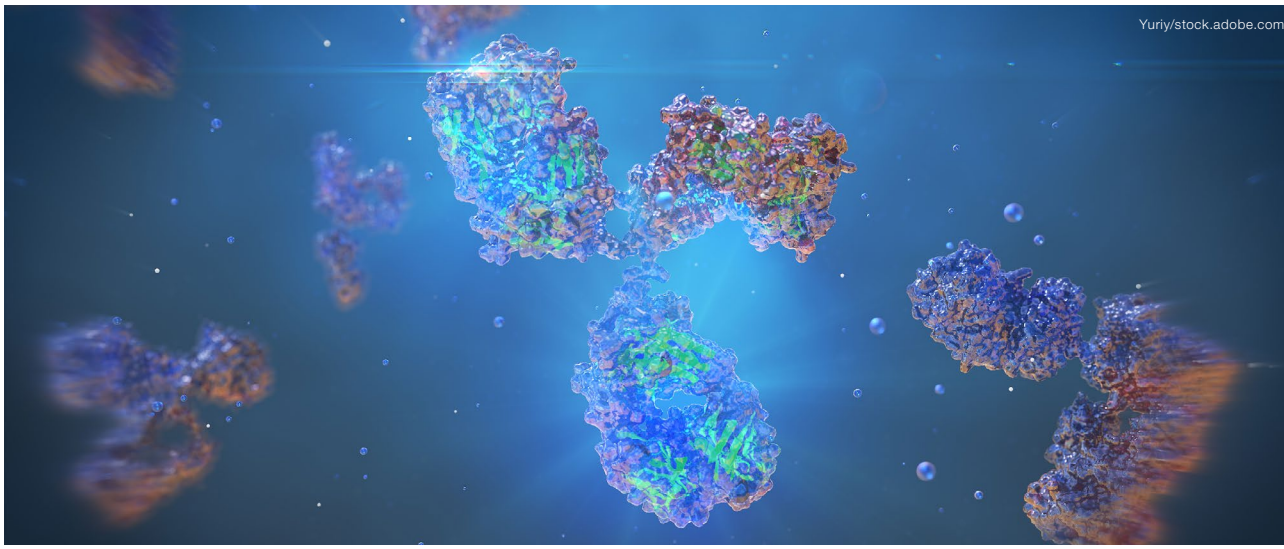

The Critical Role of CE-MS Methods for Biotherapeutic Characterization

ZipChip microchip CE-MS provides an orthogonal, high-throughput solution to conventional LC

Biotherapeutic proteins, such as monoclonal antibodies (mAbs), bi-specifics, fusion proteins, and antibody-drug conjugates, are large, heterogeneous molecules. As many scientists know, the structural heterogeneity of biotherapeutics often make mAbs, in particular, a challenge to characterize in order to ensure their safety, efficacy, and potency during cell culture, purification, and storage.[1]

"Proteins in general, and particularly biotherapeutic proteins, are more complex than small molecule drugs," explains Erin Redman, a senior research scientist at 908 Devices. "So, you need a broad suite of analytical technologies to gather information as you're going through the drug-development process and characterizing the drug product."

"Within the biopharma industry, more traditional optical techniques are being used," she continues. "And each collects little pieces of information that help [drug developers] identify particular characteristics about

the molecule that might cause them to make decisions down the line about how they're manufacturing or packaging—this characterization process can be very complex."

Biotherapeutic proteins can be subject to a variety of post-translational modifications during manufacture, such as lysine truncation, or during the product lifespan, e.g., deamination. Some modifications result in small-to-moderate changes in charge while others can result in a big change in charge. Changes in charge heterogeneity can impact the stability and bioavailability of the drug, which can cause adverse immunological reactions. This is why charge variant analysis plays a critical role in ensuring drug safety.

Charge Variant Analysis

There are three main groups of technologies used for the analysis of charge variants:

- Liquid chromatography is often coupled with mass spectrometry (LC-MS), but it requires lengthy sample preparation, as fractions need to be collected from one of the separation methods. Plus, as Redman points out, "You often struggle in the chromatography world with intact-protein separations and small polar-molecule separations in a way that's also MS-compatible."
- Alternatively, methods using ion-exchange LC coupled with MS and pH gradients offer a full workflow solution and show promising results. However, the dedicated columns required can be time-consuming in terms of method development. Plus, switching between methods can also be time-consuming.
- Traditional methods used for separation include capillary electrophoresis (CE), imaged capillary isoelectric focusing (icIEF), and cation exchange chromatography (CEX). They offer great separation, but some are not compatible with MS. For example, icIEF is a good method for charge heterogeneity and is used for lot release, stability, and characterization; however, it can't identify peaks nor be linked to MS, so it's only good for screening. CE can be coupled to MS, and it can tackle the intact-protein and small polar-molecule separations. "Integrating CE with MS takes advantage of the powerful separations CE can provide along with the in-depth characterization MS provides," Redman says.

So, What is CE?

"CE is a separation technique like chromatography, but it uses a different separation mechanism," Redman explains. "It can give complementary information to chromatography, as it separates molecules based on their net charge as well as conformation in a solution. It can be used for some applications that chromatography

tends to struggle with, such as large intact proteins or small polar molecules. CE, like most techniques, benefits from coupling it to a MS detector to give in-depth information; otherwise, you're limited in the information you're able to obtain from the separation. Charge-variant analysis is a great example.

"A lot of people use CE to do charge variant analysis of mAbs—it gives a nice separation and a great charge variant profile. But identifying what those charge variants are is hard to do if you only have optical detection," she says. "With MS, you're able to get more information about what those variants are and confidently identify them based on their mass, how that mass shifts between the different charge variants, and where that charge variant is present within the separation profile of the molecule."

Microchip CE-MS

CE is not new. Arne Tiselius received a Nobel Prize for his efforts in CE in 1948, and Stellan Hjertén developed the first CE apparatus in the 1960s.[2] James Jorgenson and Krynn Lukacs subsequently published work in the 1980s that ushered in commercial applications.

More recently, CE innovators have developed microchip-based CE platforms that integrate sample injection, electrophoretic separation, and electrospray ionization (ESI) into a mass spectrometer. This eliminates junctions, connectors, and dead volume between individual functions,[3] and as opposed to other liquid separation techniques, microchip CE boasts faster analysis times and greater separation resolution.

In particular, the ZipChip platform from 908 Devices offers a compact, versatile system which is largely plug-and-play with popular mass spectrometers and pre-developed assays. There is little method development required of the chemists, thus switching to ZipChip only takes minutes to accomplish.

ZipChip offers

- Direct ESI of analytes for MS analysis, an integrated ESI emitter in positive mode
- Highly uniform and stable surface modification for high-resolution separations
- A single platform for multi-characterization of proteins
- Minimal sample preparation
- Simple workflows for multiple applications

"When you load a chip into the ZipChip system, the position of the electrospray is fixed and automatically aligned to give a good signal, so customers don't have to position it," Redman explains. "And priming the system is as simple as putting on a background electrolyte and telling the software to prime the system—most everything is push-button operated. We've

From mAbs to Oligos

Synthetic oligos have gained significant interest from the biopharmaceutical industry recently, and several of these candidates have been approved or have progressed to later stages of clinical trials.

As with protein analysis, many labs often consider LC-MS to be the gold standard for the characterization of oligos, especially reversed-phase LC. These methods not only involve the use of ion-pairing agents, but are also demanding in terms of method development, as well as require times for system and column equilibration. It is typical that a dedicated LC-MS system is required for the analysis of oligos to avoid cross contaminations between different assays such as proteins, peptides, or small molecules.

Unlike reversed-phase LC methods, microchip CE-MS uses a MS-friendly background electrolyte that eliminates the use of ion-paring reagents. The analysis times are short (typically less than 5 minutes) with minimum method development with the pre-packed reagent kits.

The method with ZipChip CE-MS, in particular, exhibits linearity over a wide dynamic range, separating phosphorylated, biotinylated, and glycan spacer-containing oligonucleotides from the corresponding unmodified oligonucleotides. Moreover, the method shows excellent peak area and migration time reproducibility.

also put a lot of focus in our R&D labs on method development to make our assay kits a simple way for customers to get good data from the start."

It's as simple as picking a kit based on the application. For example, for charge-variant analysis, one simply needs to prime the system with the background electrolyte included in the kit, prime the chip, and then ready the samples.

"This gives customers good data right out of the box," Redman says. "They don't need to spend a lot of time trying to optimize their separation performance or change conditions to adapt their sample matrix."

Several studies support using microchip CE-MS for mAb characterization including the charge variant profiling of mAbs.[1,4,5,6] In a 2021 study, ZipChip was used to analyze intact mAbs and assess the root cause of increases in acidic and basic variants under stress at high temperatures.[5] The researchers concluded: "The basic variants in the unstressed sample were produced C-terminal amidation, while the acidic variants were produced by deamidation. In stressed samples, change in the acidic and main peaks was caused by deamidation, and changes in the basic peaks were caused by both deamidation and oxidation. These results demonstrate that microfluidic CE-MS is a powerful direct and generic tool for separation and identification of charge heterogeneity of biotherapeutics."

CE-MS vs. LC-MS

One difference between the ZipChip CE-MS and LC-MS is the sample doesn't have to be in a low-organic solution, which alleviates a lot of sample preparation steps.

"With ZipChip, the sample can have high-organic content, so the sample matrix is

more flexible," Redman says. "We use a dilute-and-shoot type of method, and that's it—for most applications, it's a simple sample prep process."

Further, separations using ZipChip are often much faster than chromatography separations. For example, with peptide mapping, runs for a digested antibody take 10 minutes, whereas chromatography separations can take up to an hour and a half.

For protein analysis, one of the simplest things ZipChip does is its intact mass assay: Customers dilute their protein sample with the provided background electrolyte, then run it on ZipChip for a fast intact mass spectrum. And, depending on the protein, customers may also see some separation with it.

"It is extremely tolerant of a wide variety of sample matrices," Redman says. "Proteins come in a variety of different formulations: some are a nicely formulated drug-product candidate; others are purified from cell culture media or a tissue lysate. ZipChip is tolerant of many surfactants, so they don't interfere with the analysis. Therefore, you still get a clean, high-quality mass spectrum out of your protein, even if it has surfactants."

Overall, ZipChip provides a high-throughput solution to traditional LC, combining CE with high-resolution mass data for intact, reduced, subunit, peptide, and metabolite biotherapeutic profiling.

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– **Erin Redman,**
Sr. Research Scientist,
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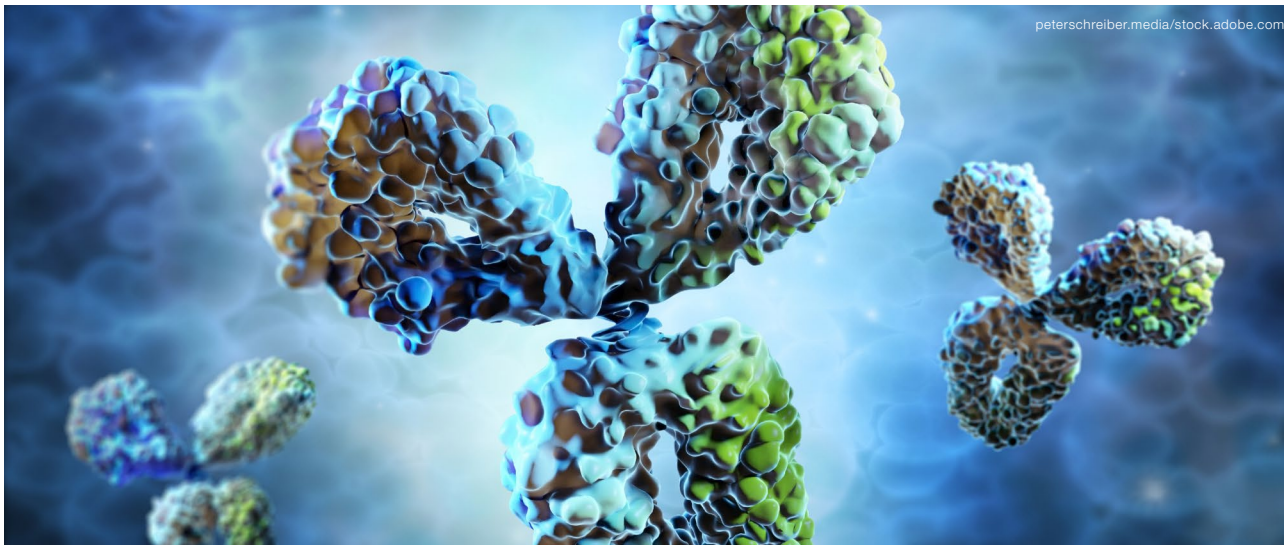


Microchip CE-MS for Monoclonal Antibody Charge Variant Analysis

How charge variant analysis addresses the analytical challenges posed by biotherapy

Biotherapeutics are a rapidly growing class of drugs where the active substance is extracted or produced by a biological source. Examples include monoclonal antibodies (mAbs), growth factors, vaccines, cell-based products, and hormones. The complexity of these biotherapeutics poses a variety of analytical challenges, particularly for traditional analytical techniques. Separations coupled with mass spectrometry can provide a wealth of information for the samples of interest.

Introduction to Charge Variant Analysis

mAbs are protein molecules used to treat many diseases and their complexity far exceeds small molecule drugs. mAbs are composed of four polypeptide chains connected through inter- and intrachain disulfide bonds. Chemical and enzymatic modifications can take place during production, purification, and storage, which creates challenges in monitoring product quality.

During production, for instance, post-translational modifications of mAbs[1] can result in variants that have different charge states or have altered conformations that change the accessible surface charge. These modifications will result in acidic and/or basic variants giving rise to parameters that must be monitored for product efficacy and safety purposes: the charge variant profile.

Charge variant analysis of mAbs comes with several challenges, however. Both chromatographic and electrophoretic methods have been used. For chromatographic approaches, sample preparation steps could include partial enzymatic digestion, reduction of disulfide bonds, and acid hydrolysis depending on the desired result. Typical chromatographic separations used in this context include ion-exchange chromatography (IEX), hydrophobic interaction chromatography (HIC), hydrophilic interaction chromatography (HILIC), and reverse-phase liquid chromatography (RPLC). Meanwhile, electrophoretic approaches include capillary isoelectric focusing (cIEF) and capillary zone electrophoresis (CZE). These methods have advantages and disadvantages, and some are more adaptable for mass spectrometry (MS) detection than others.

Chromatographic Approaches

Liquid chromatography (LC) has been a key to biotherapeutics characterization for decades, though there are challenges to deal with, such as sample preparation requiring numerous steps and risking artefactual sample degradation. Various approaches have been explored to find the most efficient and reliable technique for biopharmaceutical analysis. For instance, IEX is commonly used for charge variant analysis and can have two ramifications.

- First, there could be an increase in the salt concentration in the gradient that weakens the ionic interactions and results in elution of the species of interest.
- Second, a pH gradient could change the protein charge and cause elution with the increasing binding charge. Another challenge for IEX is method development, which requires significant time and resources.[2]

In addition, implementation of MS systems into the workflow can be challenging. Although the chromatography system can be interfaced to a mass spectrometer for identification, the eluent can cause significant interference in the analysis.

Another option among chromatographic techniques is reversed-phase LC (RPLC), which has the distinct advantage of being compatible with MS detection. It is robust and with high separation resolutions, however, solvents

can be harsh for the analytes of interest, for example mAbs, and secondary interactions with the stationary phase can occur.

In general, chromatography techniques commonly rely on optical detection such as UV or fluorescence. They require significant method development to achieve optimum separations without destroying the analytes of interest.

Given these challenges, what other options are available?

Electrophoretic Approaches

Capillary zone electrophoresis (CZE) is widely applied to biopharmaceuticals. In general, all of the electrophoretic techniques are based on applying a high electric field and molecules separating based on differences in charge and size. For instance, cIEF allows separation based on the isoelectric point. With this technique, however, precipitation can occur at net zero charge, which results in clogging or irreproducible results. Again, the detection is optical, so it is not very specific.

CZE is a technique most suited to the separation of mAbs and subsequent analysis by MS systems. Unlike the other electrophoretic methods, CZE takes place in solution and although there are operational constraints, it can readily interface to MS systems for identification of charge variants. Some of the constraints in the classic CZE implementation include possible adsorption onto the capillary wall, low sample loading for proper separation, and a poor detection limit due to the 214 nm detection should a UV detection be used for the analysis.

All the separation approaches described above typically rely on optical detection. This can be ultraviolet or visible absorption, fluorescence, or in some cases, a change in refractive index. These detection schemes are not as specific and any identification will be made based on the retention time or a position in a gel. Another drawback of these optical techniques is that the detector wavelength is typically not characteristic of a species of interest because of the complexity of the mAb. In addition, many separation schemes introduce interfering substances such as salts, detergents, and buffers, which can complicate the analysis or suppress signals of interest.

MS, when used as a detection, offers another form of charge variant identification in addition to their migration or travel in the separation medium based on the mass spectral information. Usually, electrospray ionization is used since it gives a clean mass spectrum of each of the variants of interest. When used in this context, the mass spectrum can differentiate mAbs and both their acidic and basic variants [3]

Microchip CE-MS for Charge Variant Analysis

Using CZE with electrospray ionization provides distinct advantages for the intact mass analysis of charge variants. The technique, in the original implementation, takes place in solution in a capillary so it is

not hindered by extraction from a gel. CZE has many advantages when considered for interfacing to MS for top-down proteomics.[4] It is ideal for separating charge variants, and unlike isoelectric focusing or gel electrophoresis, it is readily interfaced to a mass spectrometer for identification.

The ZipChip microchip CE-MS platform incorporates the advantages of CZE and the soft-ionizing conditions of electrospray ionization into one device that can perform the separation, ionization, and introduction to the mass spectrometer for combined charge variant, intact mass spectrum analysis. This fully integrated system relies on photolithography and wet etching to produce microchannels in a glass substrate where sample introduction, separation, and electrospray ionization all occur.

Some major advantages of the ZipChip mircochip method include:

- Speed of analysis
- Analysis in a wide range of matrices
- Reproducibility
- Minimal sample preparation
- Minimized artifacts due to sample preparation
- Detection of variants with 0.1% abundance in the sample
- Confident identification of variants based on both the migration time and the mass spectrum.

Reproducibility analysis is a major concern when analyzing mAbs. The ZipChip technique is very reproducible as shown in FIGURE 1, especially as compared to alternative techniques. Three replicate analyses of

FIGURE 1: Three replicate analyses of cetuximab with the electropherogram shown on the left and the mass spectral relative intensities are shown on the right indicating very good reproducibility

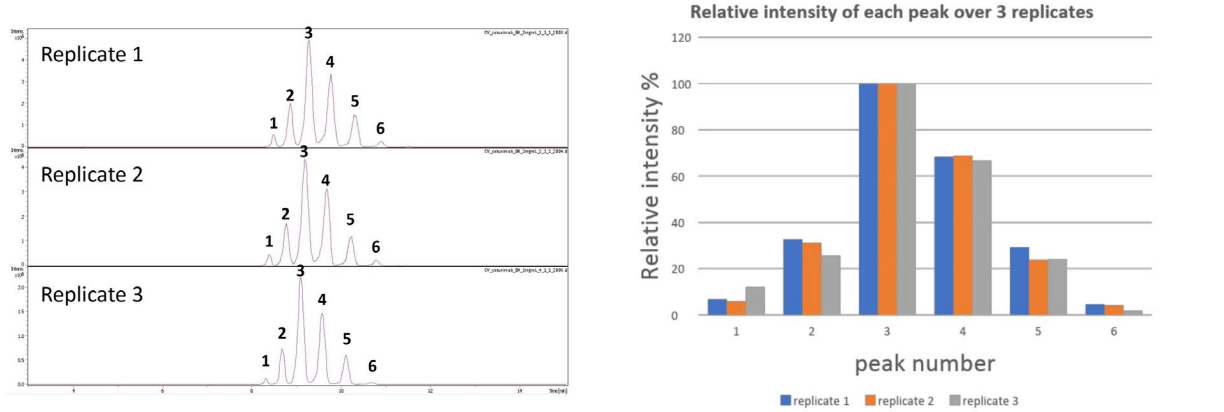


FIGURE 2: Comparison of the NIST mAb standard run in a high throughput chip (top) and a high-resolution chip (bottom)

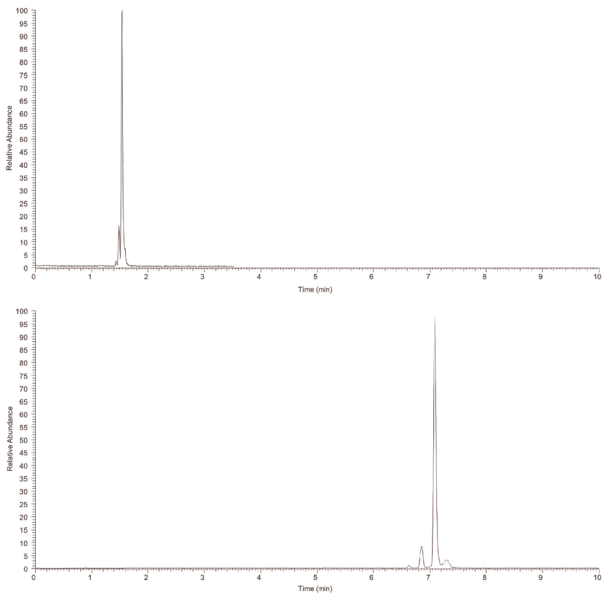
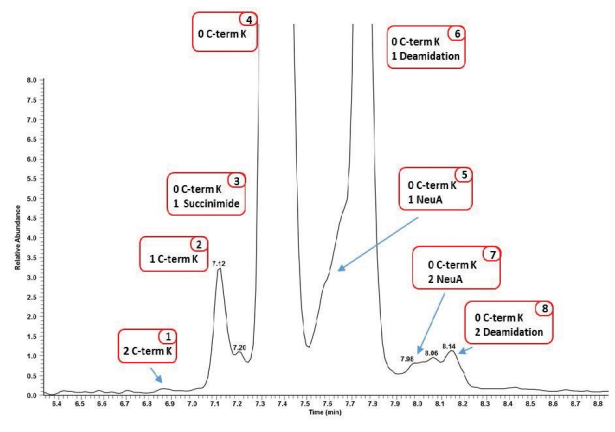


FIGURE 3: Electropherogram from ZipChip CE-ESI-MS analysis of trastuzumb showing both the separation as well as the confident identification from the mass spectrum



cetuximab were conducted using the ZipChip interfaced to a Bruker maXis II mass spectrometer. The electropherograms appear identical and the peaks are very similar when examining the relative intensities—even at low intensities (concentrations).

The sample matrix and sample preparation can have adverse impacts on intact mass analysis. One group that can cause problems is detergents used to stabilize samples. They can wreak havoc on analytical instruments. In the ZipChip experiment, the detergents do not migrate through the channels of the chip, so are not ionized and introduced into the mass spectrometer. Thus, they will not interfere with the final analysis.

Another advantage of the ZipChip workflow for charge variant analysis is the ability to switch from a high throughput charge variant analysis or high-resolution charge variant analysis based simply on the type of chip chosen. FIGURE 2 shows an example of this where the NIST mAb standard is analyzed first using the high throughput chip which yields results in about two minutes and then with a high-resolution chip which better separates the variants for subsequent electrospray ionization (ESI) identification. This flexibility can all be achieved without needing to change the buffer components.

Finally, in FIGURE 3, the confident identification of charge variants by using their intact mass spectra is demonstrated. These charge variants of trastuzumab are identified from their mass spectra

for deep structural characterization of this biotherapeutic.

Conclusion

The characterization of mAbs as well as other biotherapeutics is an important topic when examining product quality attributes during product development, production, and release testing. The traditional methods can suffer from low specificity, analytical interferences, and can be time-consuming. Microchip CE-MS with ZipChip offers major advantages by incorporating the benefits of CZE with on-board electrospray ionization. This results in interference-free, fast, and easy analysis. In addition, this approach can be interfaced to many mass spectrometers from major vendors to provide accurate, interference-free characterization of mAbs.

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Streamlining Therapeutic Protein Characterization with Peptide Mapping

Microfluidic capillary zone electrophoresis for biologic drug characterization

Protein-based biologic drugs are a critical component of the modern pharmacopeia—as of 2019, monoclonal antibody (mAb) drugs alone accounted for roughly 70% of all global pharmaceutical sales.[1] But these powerful drugs are also challenging to manufacture, where even subtle perturbations in cell culture conditions or other stages of the production process can potentially yield a product that delivers subpar performance or poses a potential risk to patient health.

Mass spectrometry (MS) has proven to be a powerful tool for quality control in the production of small-molecule drugs, and many pharmaceutical producers have begun developing MS-based workflows for the characterization of mAbs and other protein-based drugs.

There is a particular excitement around a workflow known as the multi-attribute method (MAM), in which protein targets are digested

into small peptide fragments and then systematically analyzed via MS to identify and map any irregularities or defects present in a given drug batch. By implementing microfluidic capillary zone electrophoresis (CZE) as a preliminary separation step for the MAM process, biologic drug characterization can potentially be performed with unprecedented speed and efficiency.

Quality Control for Protein Production

Anything that alters a protein's structure or chemical composition can profoundly affect its biological function. This can include changes in that protein's posttranslational modification profile, truncation of the polypeptide chain, or chemical alterations to its constituent amino acids that lead to undesirable outcomes such as misfolding, impaired target binding, or aggregation. Manufacturers must therefore define critical quality attributes (CQAs) for every drug product they produce and establish robust monitoring strategies to ensure that every pharmaceutical that reaches patients exhibits all the CQAs associated with a safe and fully functional biomolecule.

Until very recently, this analysis has typically entailed a plethora of specialized techniques for capturing information about specific features of a protein drug. For example, changes in glycosylation can influence a protein's ability to interact with cellular targets or provoke an unexpected host immune response. To characterize these modifications, many labs treat therapeutic proteins with enzymes that shear away glycan groups and then perform hydrophobic interaction liquid chromatography (HILIC) to isolate and analyze those glycans. In parallel, other methods such as cation exchange chromatography (CEX) and reducing capillary electrophoresis (rCE) might be employed to respectively detect abnormalities related to differences in protein charge or truncation of the complete polypeptide sequence.

These experiments can be very informative, but do not provide a complete picture. For example, HILIC analysis alone will not reveal where in the protein an abnormal glycosylation event is taking place, and rCE can indicate that a protein is "clipped" but not the precise site of the truncation.

Thanks to steady advances in MS hardware and software, it has now become feasible to replace many of these steps with a simplified liquid chromatography (LC) – MS workflow for biological quality control. Some groups rely on "top-down" analyses, in which the intact protein (denatured) is characterized. This can provide valuable information about subunit composition and tertiary structure, and it minimizes the potential to introduce artifacts by requiring relatively minimal sample preparation. But as with traditional protein characterization workflows, top-down approaches cannot precisely pinpoint the locations of specific modifications or irregularities within the protein sequence.

Peptide mapping based on so-called "bottom-up" analyses can provide these

critical details. Here, the protein analyte is first subjected to enzymatic digestion, typically with the protease trypsin. The resulting polypeptide fragments are then subjected to reduction and alkylation, and then separated via LC and finally characterized via MS. This process yields a detailed view of the chemical features present throughout the primary structure of the protein, allowing researchers to directly monitor known CQAs while also detecting other abnormalities of potential concern.

A More Productive Pipeline

The MAM peptide mapping workflow was first described as an MS-based quality control solution in 2015 by a team of industry researchers led by Richard Rogers of Just Biotherapeutics.[2] MAM has yet to become a mainstay of biopharmaceutical production processes, but over the past several years we have seen a surge in interest in this approach, including the formation of an independent, industry-wide MAM Consortium focused on developing best practices for deploying this approach in cGMP (current good manufacturing practice) settings.[3]

In a typical MAM process, digested peptides are separated via reversed-phase ultra-high-performance liquid chromatography (UHPLC) followed by MS analysis. The data from this analysis are then subjected to an automated algorithm that identifies a wide range of performance quality attributes (PQAs)—some of which are also CQAs, while others may be non-critical. These can include post-translational modifications, chemical alterations to amino acids, and clipping at specific sites in the polypeptide chain.

The data derived from this initial run are then used as training material, enabling rapid identification of aberrations associated with those PQAs based on LC-MS data collected from subsequent batches of that same protein drug. These subsequent analyses are also used for new peak detection, identifying unusual MS signatures that were overlooked during the initial training experiment, but which may contribute meaningfully to drug safety or efficacy. And critically, each MAM analysis generates an essentially comprehensive protein map with single amino acid resolution, enabling laboratories to accumulate knowledge about defects and aberrations over the entirety of a given protein therapeutic.

Early demonstrations of the MAM process—including the pioneering work by Rogers et al. in 2015–demonstrated that this workflow could deliver comparable analytical performance in terms of discriminating PQAs relative to older methods. For example, MAM analysis can profile glycans as effectively as HILIC-based approaches, while also assigning those glycans to specific sites on a mAb or other protein. Likewise, MAM

can localize residue-specific charge variants, whereas CEX can only generate peaks that vary based on the overall net surface charge of the entire protein.

An Alternative for Accelerated Analysis

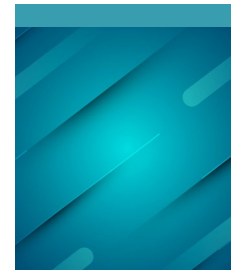
MAM is still a new method, and researchers are continuing to develop new iterations that can further improve its utility and performance. For example, some groups have begun to explore the use of CZE as an alternative to standard UHPLC-based separation procedures.

The ZipChip system from 908 Devices offers a compact microfluidic solution for CZE-based separation of polypeptide fragments. Trypsin digestion products are injected into the device, and then subjected to electrophoretic separation while in solution phase. Since there is no solid gel matrix involved, there is minimal carryover, and no washing steps are required between injections. Furthermore, ZipChip is designed as a monolithic system of channels, with no junctions or connectors that could otherwise add delays to the separation process or reduce the resolution of the final analysis.

This design results in considerable time savings; whereas a conventional UHPLC-based MAM workflow can require more than two hours for chromatographic separation, a ZipChip run typically takes just 10 to 15 minutes. The separation is seamlessly incorporated with an electrospray ionization (ESI) emitter at the corner of the chip, which emits a plume of sample toward the inlet of the MS instrument.

In 2021, a team of Amgen scientists led by Andrew Dykstra demonstrated how a ZipChip-based CZE-MS MAM workflow can help researchers to get a handle on a particularly challenging amino acid modification.[4] Aspartic acid and asparagine residues have a tendency to undergo degradation to another naturally occurring amino acid, isoaspartic acid. This change can be repaired in living cells, but not in purified protein preparations, and drugs containing isoaspartic acid rather than aspartic acid or asparagine are susceptible to reduced therapeutic activity and risk of aggregation.[5] Since isoaspartic acid and the amino acids from which it is derived are isobaric, it can be challenging to discriminate these various residues based on separation with conventional reversed-phase LC. UHPLC-based MAM workflows are suitable for this task but require considerable time to achieve sufficient separation.

Dykstra and colleagues, therefore, set out to assess whether microchip CE-MS might offer a more efficient alternative. They subjected two therapeutic molecules—an IgG1 mAb and a bispecific single-chain protein—to



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Could ZipChip be a solution for MAM bottlenecks?

parallel MAM workflows with UHPLC and CZE, where the proteins were characterized in both an intact state as well as in the aftermath of damaging stressors including heat, pH change, and light exposure. The ZipChip-based analysis was significantly faster, resulting only 10 minutes for data acquisition versus 145 minutes with UHPLC, and achieved excellent detection of stress-induced formation of isoaspartic acid as well as a variety of other potentially problematic amino acid degradation byproducts. Notably, CE-MS also yielded superior sequence coverage for the two proteins than UHPLC-MS, which the authors attributed to more efficient isolation of smaller tryptic peptides.

The pharmaceutical industry is only beginning to explore the potential of MAM as a strategy for biotherapeutic characterization, and more thorough assessment might be called for before CZE can be routinely implemented in these pipelines. But the incremental time- and labor-savings associated with such separation strategies—for example, Dykstra and colleagues estimate that ZipChip could reduce the time required for lead molecule characterization from more than a week to less than a day—create strong incentives to pursue such testing and validation.

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The ZipChip-based analysis was **noticeably faster**, requiring only 10 minutes for data acquisition versus 145 minutes with UHPLC, and also achieved **excellent detection of stress-induced formation of isoaspartic acid** as well as a variety of other potentially problematic amino acid degradation byproducts.