We All Have Baggage: A Deep Dive into the Characterization of a Complex cIEF Electropherogram from an Antibody-Drug Conjugate Using a Novel Integrated icIEF-UV/MS System

Overcome the challenges characterizing complex molecules with a fully integrated icIEF-UV/MS workflow

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OVERVIEW

Charge heterogeneity is present in most biopharmaceutical protein products. During the manufacturing process, charge heterogeneity of the protein therapeutics can occur due to enzymatic cleavage and chemical post-translational modifications (PTM). Many of these PTMs, including deamidation, C-terminal lysine truncation, glycation, and sialylation, change protein charge and, thus, isoelectric point (pI). For therapeutics like ADCs, not only the antibody contributes to the heterogeneity but also the linker and payload, which add even more complexity to the charge variant profiles. Characterizing the charge heterogeneity of ADCs is essential for critical quality attribute (CQA) assessment to ensure drug safety, efficacy, and potency.

Capillary isoelectric focusing (cIEF) is a well-adopted technique for monitoring protein charge heterogeneity. However, direct peak identification from electropherograms often requires extensive lab work, including orthogonal LC method development, fractionation, offline mass spectrometry (MS) characterization, and multiple cIEF re-analysis.

Here we discuss the evaluation of the Intabio ZT system -a microfluidic chip-based integrated imaged capillary isoelectric focusing (icIEF)-UV/MS system to identify and monitor the charge variant distribution on an ADC in near real-time.

CHARGE HETEROGENEITY'S CRITICAL ROLE

The evolution of drug pipelines to more complex molecules requires the development of analytical methods to ensure they are fit for purpose. For example, in the past decade, AstraZeneca's pipeline has evolved past monoclonal antibodies (mAbs) to include more complex molecules such as bispecific mAbs, T-cell engagers, and ADCs.

ADCs have become an important class of biotherapeutics to treat a wide variety of diseases due to their high specificity, efficacy, and flexibility. From an analytical perspective, ADCs are generally considered more complex than conventional

monoclonal antibodies (mAbs) as they consist of both a mAb and an attached small molecule known as the payload. This complexity can increase charge heterogeneity making characterization at the intact level more challenging. In addition, manufacturing process changes can result in additional PTMs or alter drug-to-antibody ratios (DAR) that impact product quality. Therefore, continual charge heterogeneity assessment plays a critical role in ADC product development.

Imaged capillary isoelectric focusing (icIEF) is a favored assay for analyzing charge heterogeneity because it can provide quantitative information. It can also be used as an identity method, since - like fingerprints, no two molecules look exactly alike.

An example of a relatively simple icIEF profile is one obtained for NISTmAb, which features a central main peak (**FIGURE 1**). Peaks shown on the left-hand side of the separation profile represent acidic protein isoforms, while peaks on the right are basic isoforms. But not all mAbs are created equally, and conjugation of a mAb can elicit a greater degree of complexity.

For this ADC molecule, the changes due to the conjugation are evident, showing an explosion of peaks. (**FIGURE 2**). This complexity makes it difficult to identify the main peak and underscores the need for robust analytical methods to understand the charge distribution in these complex biopharmaceuticals and to be able to identify those peaks.

TACKLING THE ANALYSIS OF A CHALLENGING ADC

During the development of a favored ADC molecule (ADC-1), the AstraZeneca team identified an unexpectedly complex icIEF profile. Compounding the issue, the team soon discovered the autosampler stability was impaired. It appeared the molecule's charge profile was changing as the sample sat on the autosampler. (**FIGURE 3**) This instability led to the conclusion that their current icIEF method was no longer fit for purpose.

To keep the development project rolling, the team switched to the more time-consuming peptide mapping method to analyze GMP release and stability samples as a temporary solution while concurrently working to resolve the issue with the icIEF method.

FIGURE 1: icIEF profiles are like fingerprints.

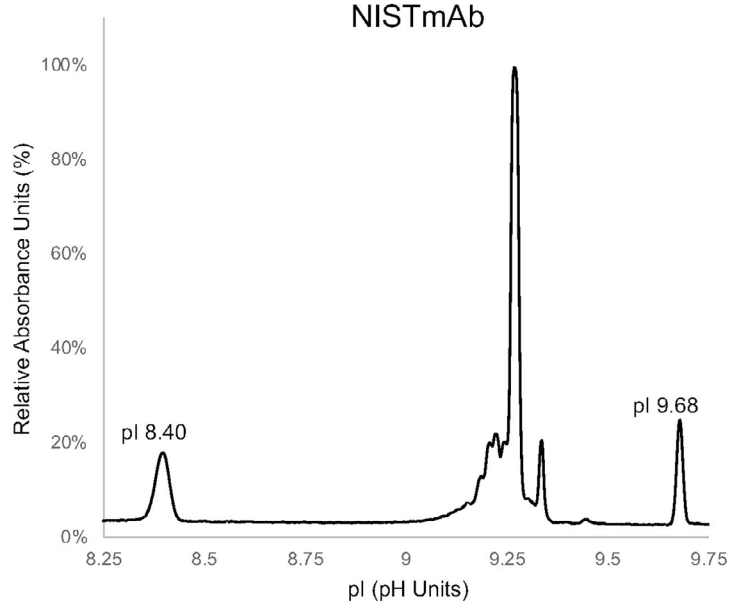


FIGURE 2: An unexpected event observation following conjugation.

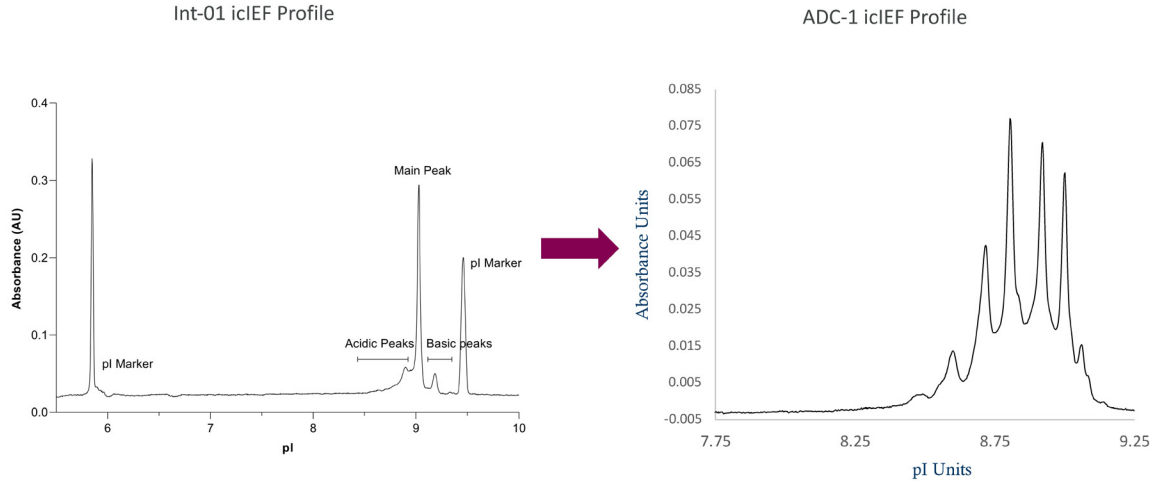
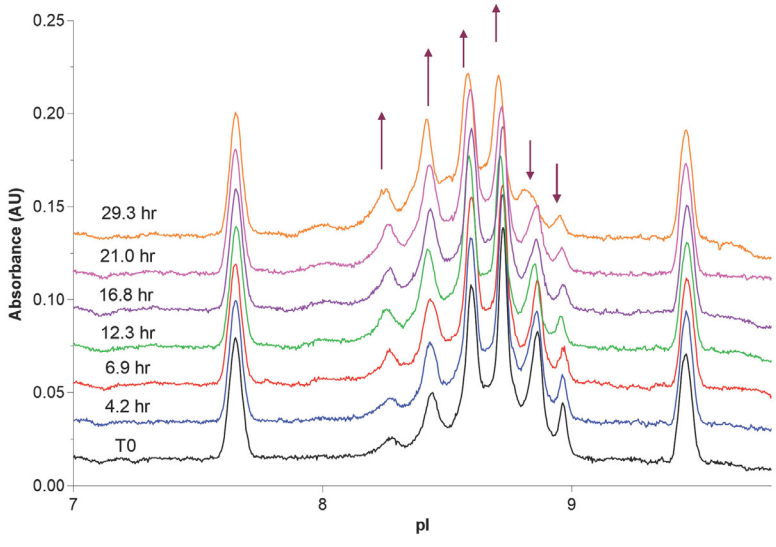


FIGURE 3: Putting salt on the wound: autosampler stability is impaired for ADC-1.



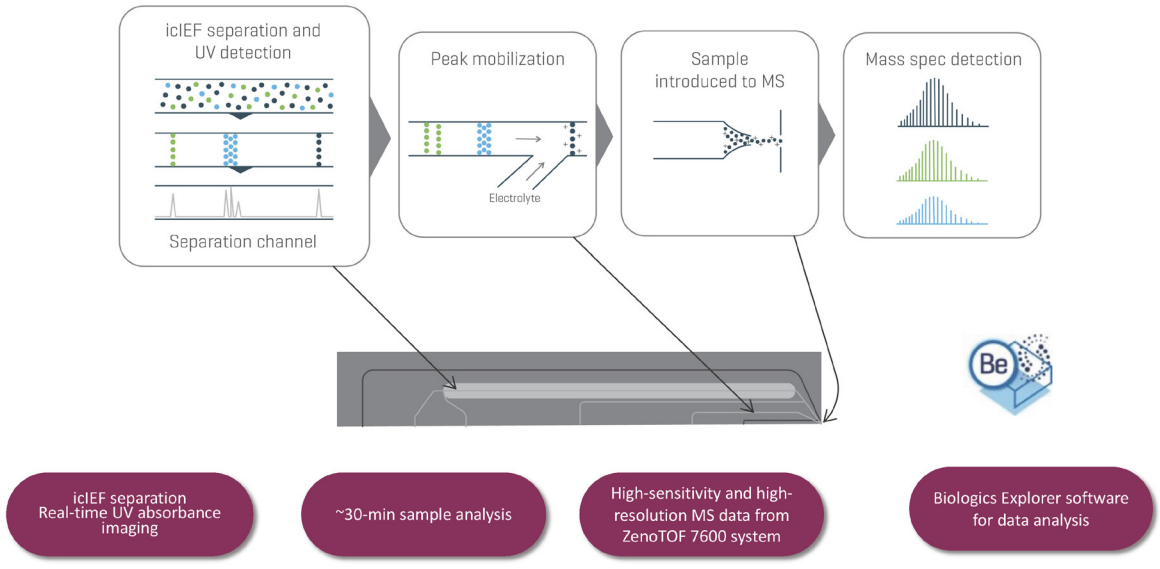
The slide shows icIEF traces in different colors representing the same sample over 30 hours while being held at 4 degrees Celsius on the autosampler. Over time, there is a reduction in the intensity of the basic peaks (on the right) and a simultaneous increase in the acidic peaks (on the left). This instability poses challenges for method qualification because it indicates the sample changes over time on the autosampler.

INNOVATIONS IN icIEF ANALYSIS AND PEAK IDENTIFICATION

One such solution to be investigated was the use of the Intabio ZT system from SCIEX (**FIGURE 4**), which couples microfluidic chip-based icIEF separation, UV detection, and on-chip electrospray ionization for high-resolution mass spectrometry detection on the ZenoTOF 7600 system. It allows for fully integrated icIEF-UV/MS characterization, accomplished via a unique microfluidic chip, where sample solutions are injected, voltage is applied, and proteins focus in real-time.

Then, upon mobilization, the separated proteoforms are electrochemically mobilized to the mass spectrometer for detection. This real-time approach to icIEF separation and mass spectrometry identification significantly reduces analysis time compared to traditional sample fractionation and re-injection

FIGURE 4: Intabio ZT system introduction: microfluidic chip-based integrated icIEF-UV/MS technology.



methods. A process that previously took weeks to perform, at best, though sometimes months and years, can now be completed in hours.

UNTANGLING THE CHARGE PROFILE OF AN ADC

Using the Intabio ZT system, the team attempted to solve the characterization problems with ADC-1, and to develop a method that would identify the root cause of the instability and shift in icIEF profile.

This real-time approach to icIEF separation and mass spectrometry identification significantly reduces analysis time compared to traditional sample fractionation and re-injection methods.

They compared the icIEF-UV/MS profile of a mAb intermediate and the conjugated ADC. The mAb intermediate analysis showed a well-defined main peak, while the ADC exhibited a comparatively small main peak and additional acidic peaks (**FIGURE 5**).

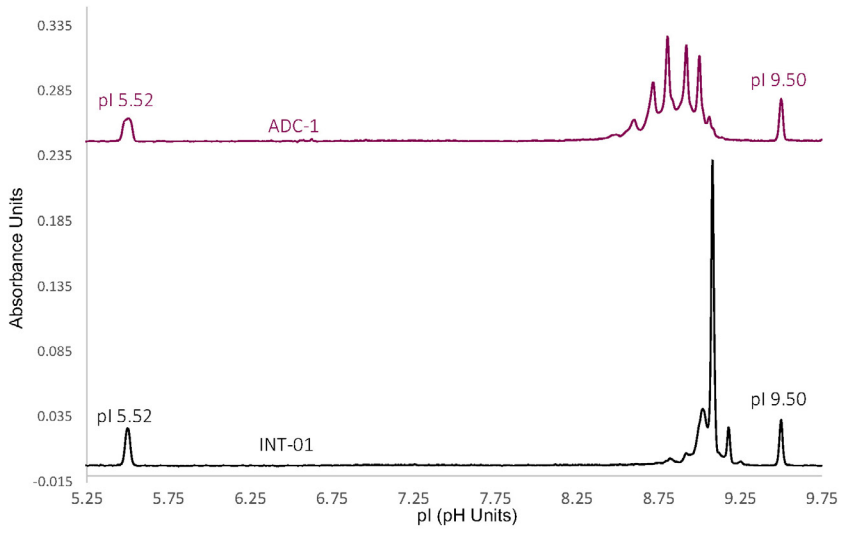
The charge heterogeneity profile of the mAb intermediate included mass shifts that correlated with common modifications, including C-terminal amidation, glycation, as well as the addition of sialic acid.

Importantly, deamidation events were readily identified. Deamidation results in a one Dalton shift which can be challenging to identify in intact mass analysis due to method variability. It is often debated whether mass spec is sensitive enough to actually detect a 1 Dalton shift, or whether the shift is just a sign of method variability. However, coupling icIEF with MS allows for the observation of pI shifts, which enables confirmation of a deamidation event. Through the comparison of shifts between acidic peaks, it was possible to correlate the

FIGURE 5: INT-01 and ADC-1 icIEF-UV profile and focusing conditions.

Platform Method

- 3% Pharmalyte 8 to 10.5
- 3% Pharmalyte 5 to 8
- 15 mM Arginine
- 400-1000 µg/mL Protein
- pI estimated with pI 5.52 and 9.50 peptide markers
- Focusing time 6.5 Min
  - 1500 V 1 Min
  - 3000V 1 Min
  - 4500V 4.5 Min
- Mobilization time 10 Min
  - Mobilization 3000V
  - ESI Tip 5500V



number of deamidation events by comparing changes in pI values. Ultimately, the analysis showed the mAb intermediate to exhibit normal antibody quality attributes.

INTABIO ZT SYSTEM DIGS DEEPER

A comparison of the ADC-1-UV charge profile correlated with its MS charge profile.

Analysis with the Intabio ZT system enabled the team to isolate and assess the molecule with the isolation and examination of both the free light chain (**FIGURE 6**) and the heavy-heavy-light subunit of the ADC (**FIGURE 7**). In both chains, the analysis revealed mass isoform shifts of approximately 18 or 36 Dalton shifts, indicating the possibility of carboxylic acid formation due to hydrolysis of either the succinimide ring or the lactone ring present in the payload.

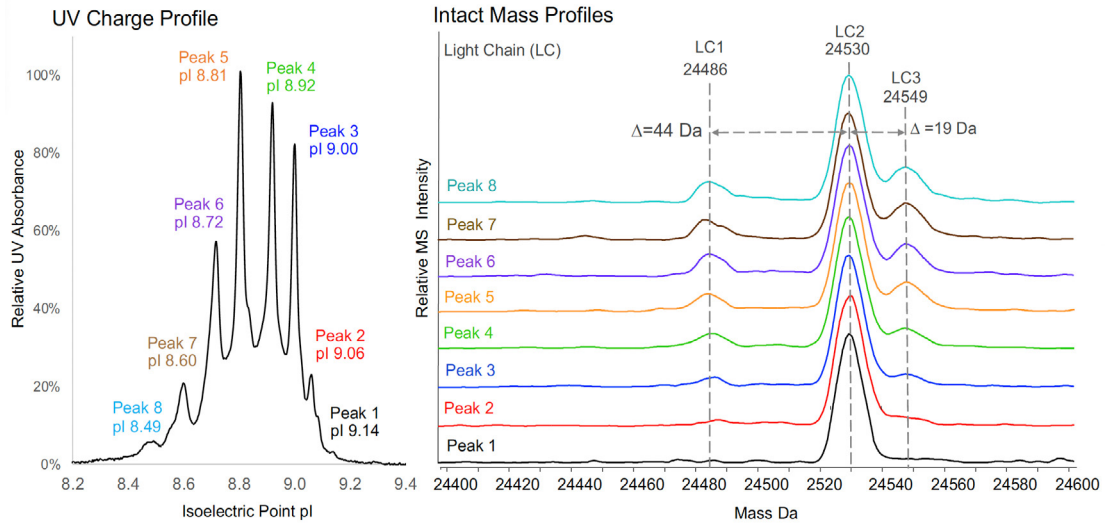
REASSESSING icIEF AS A QC METHOD

The question now is how to best apply the knowledge from this research to re-establish icIEF as a quality control method for charge heterogeneity analysis.

Traditional QC specifications for icIEF are based on numerical limits derived from the main, acidic, and basic peaks. However, these peaks can fluctuate due to ring opening events on the payload during analysis, making traditional specifications inappropriate for this molecule. Additionally, internal data collected by AstraZeneca suggests that the ring opening events on the payload are not critical quality attributes, meaning they do not significantly impact the safety, efficacy, potency, or immunogenicity of the molecule.

Analysis with the Intabio ZT system enabled the team to isolate and assess the molecule with the isolation and examination of both the free light chain and the heavy-heavy-light subunit of the ADC.

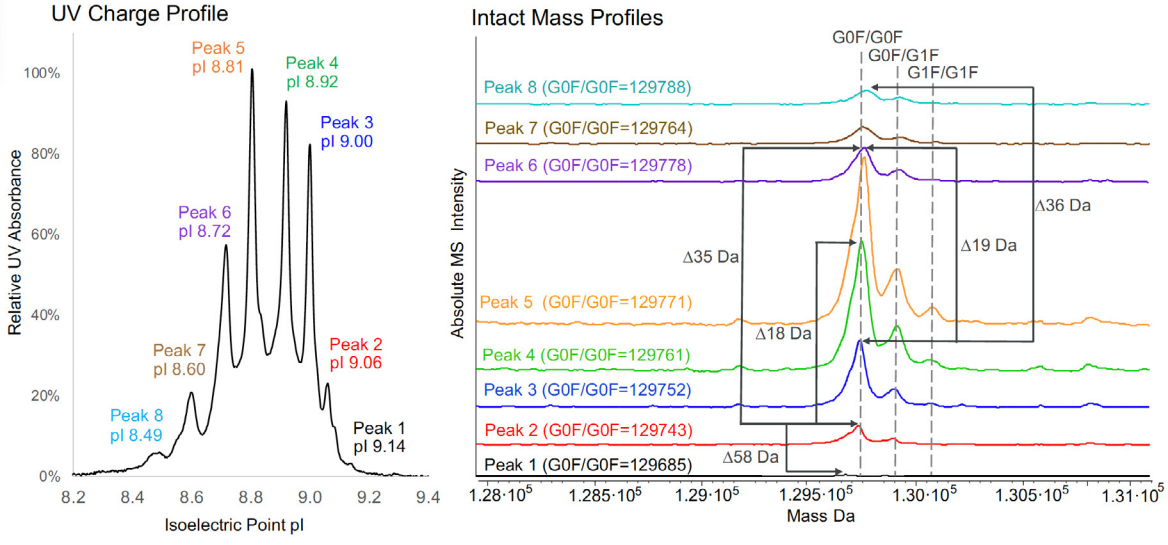
FIGURE 6: ADC-1 light chain charge/mass isoforms show shifts related to ring opening events on the payload.



The +19 Da (we suppose it is actually +18 Da) is associated with lactone ring opening of TOPO. There is a trend that acidic peaks have increased level of LC3 (+18 Da), which matches the hypothesis of higher-level ring opening in acidic cIEF peaks.

The LC1 (-44 Da of LC2 main species), however, is not confirmed by other LC-MS assays. And so far, we don't have an explanation for it. It's interesting to see the trend of increased LC1 in acidic peaks, so it may be related to payload ring opening behavior as well.

FIGURE 7: ADC-1 heavy-heavy-light charge/mass isoforms show shifts related to ring opening events on the payload.



This knowledge acquired through the work described here is being used to shape new specifications for this ADC that align with its safety and efficacy profile. Perhaps a more appropriate specification on which to base charge heterogeneity analysis would be total area counts, rather than individual peak intensity. The new approach could focus on stress material, particularly the region of the icIEF profile where there is significant growth in thermal stress material (from 7.8 pI to 8.5 pI). Additionally, consideration should be given to setting a numerical limit on the total area of the specified region of the icIEF profile.

The team's work to establish the numerical limits is ongoing and includes collecting data and engaging with various experts at conferences, as well as having discussions with regulators.

CONCLUSION

The Intabio ZT system enabled researchers to dig deeper into a complex ADC, and coupled with sensitive MS, could provide more detailed resolution and untangle what was driving the molecule's complex charge heterogeneity profile.

Analysis of both a mAb intermediate and the ADC showed correlation between the UV of older icIEF platforms and the Intabio ZT system. Furthermore, the team observed that the icIEF-UV/MS process with Intabio ZT system does not introduce artifacts into the analysis, strengthening confidence that the mass spec data represents the original sample.

The profile of the ADC captured through the analysis was greatly influenced by ring-opening events that resulted from the hydrolysis of ring structures, which contributed to the charge heterogeneity. Data suggests the ring opening events do not significantly impact the safety, efficacy, pharmacokinetics, potency, or immunogenicity of the molecule.

The team's research illuminates the need to reevaluate and redefine specifications for the charge heterogeneity of its ADC using icIEF-UV/MS to ensure its safety and efficacy for patients. These specifications are often determined by numerical limits associated with the main, basic, and acidic peaks of the molecule shown in an electropherogram. However, on this ADC the analysis by icIEF-UV/MS shows peak variations resulting from the ring openings on the payload, rendering typical specifications unsuitable. The team is developing a strategy to establish a new set of specifications tailored specifically for this molecule, taking its unique characteristics and the non-critical impact of the ring-opening events into account.