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Imaged Capillary Isoelectric Focusing for Analyzing Antibody Drug Conjugates

A new isoelectric focusing method simplifies analysis and characterization of maytansinoid ADCs in ImmunoGen's drug pipeline.

Introduction: A New Option for ADC Analysis

Antibody-drug conjugates (ADCs) are becoming increasingly significant in drug development. An ADC molecule is made of three components: an antibody, a linker, and a cytotoxic payload. The antibody targets the payload to the body tissue or system affected by disease. The linker is designed to be stable in the bloodstream and to control activation when the ADC molecule enters the cell. When building and manufacturing ADCs, an important way to analyze and characterize them is by monitoring charge heterogeneity. Each ADC will have a charge based on the number of C-terminal lysines, N-terminal pyroglutmate cyclization, and other modifications. When using lysine conjugation to attach the payload, each attachment to an epsilon-amino group present in the side chain of lysine residues reduces the pI of the molecule because of the elimination of a positive charge. Monitoring charge heterogeneity is necessary to understand and characterize ADCs and to study the consistency of conjugate manufacturing processes (See **Figure 1**).

ImmunoGen's Maytansinoid ADCs

ImmunoGen, a biopharmaceutical company based in Waltham, MA, has been a pioneer in developing ADC technologies. In collaboration with Roche, they developed an ADC against HER2 positive breast cancer that was approved by the FDA in 2013. The drug combines the recombinant anti-epidermal growth factor receptor 2 (HER2) monoclonal antibody trastuzumab with the maytansinoid cytotoxic agent DM1 through a nonreducible thioether linkage (MCC). Trastuzumab has been used successfully in combination with chemotherapeutics for breast cancer. Maytansine, the parent compound of DM1, was found to effectively kill cancer cells as it targets microtubules causing mitotic arrest. It was never developed as a chemotherapeutic, however, because it was too toxic for non-targeted use. The trastuzumab-DM1 duo combines the anticancer potency of a maytansinoid cancer-killing agent with the targeting specificity of a monoclonal antibody. ImmunoGen is also developing a pipeline of other ADCs using lysine conjugation technologies, specifically by attaching the linker to the epsilon-amino group on the lysine. The charge of the ADC molecule will vary by the number of payload molecules linked to the antibody, or DAR (Drug-Antibody Ratio). Each time a cytotoxic molecule is added to the antibody, one lysine residue is conjugated and the positive charge decreases. Different antibody molecules will have different numbers of the payload covalently linked to them. Antibody conjugation typically results in a tight distribution of compounds with different DAR, plus free payload and naked antibody (1).

Analytical Methods for ADCs

Analytical methods for ADCs cover properties such as target site specificity and binding, payload and linker stability, drug potency, solubility, DAR and heterogeneity. Using UV-vis spectrophotometry, researchers can measure ADC concentrations and DAR. Chromatographic methods can also be used to characterize ADCs, but they are not suitable for charge heterogeneity assessment of ADCs produced with lysine linkage technology due to the high degree of heterogeneity in the sample. Mass spectrometry produces a reliable analysis of ADC mixtures, and is a good complement to other methods. Bioanalytical methods such as an enzyme-linked immunosorbent assay (ELISA) and cell-based assays are also used for ADC analysis.

The development of ADCs typically requires multiple analytic methods from validation of conjugation processes through quality control in manufacturing.

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Charge Heterogeneity

For charge heterogeneity specifically, the most commonly used methods have been ion exchange chromatography, slab gel isoelectric focusing, and traditional capillary isoelectric focusing. These techniques all have drawbacks including poor resolution or lack of ability to directly quantitate the molecules. Slab gel isoelectric focusing is labor intensive and poorly reproducible.

Traditional capillary isoelectric focusing is time-consuming, and due to the nature of the method, it is necessary to mobilize the sample after it is focused, which results in a loss of resolution.

Figure 2 shows a comparison of a monoclonal antibody and an ADC analyzed by cation exchange high performance liquid chromatography (HPLC). The monoclonal antibody in the top graph shows a clean separation, but the various charged species in the ADC sample in the bottom graph do not resolve clearly. Additionally, the cytotoxic payload conjugated to the antibody tends to be hydrophobic, which promotes secondary interactions and poor resolution on HPLC columns. There is some resolution of a single peak, which corresponds to the unconjugated antibody, but in general this method is very ineffective for separating differently charged ADC compounds.

In **Figure 3**, an electropherogram of an ADC sample separated by conventional isoelectric focusing methods shows better separation than the cation exchange method. However, it still does not provide efficient and reproducible resolution. Some resolution is also lost during mobilization step of the sample.

Imaged Capillary Isoelectric Focusing

ProteinSimple's iCE technology solves some of the problems of standard isoelectric focusing and HPLC. **Figure 4** illustrates how sample and carrier ampholytes are injected into the capillary. The carrier ampholytes forms a pH gradient across the capillary, and samples migrate according to their pI values.

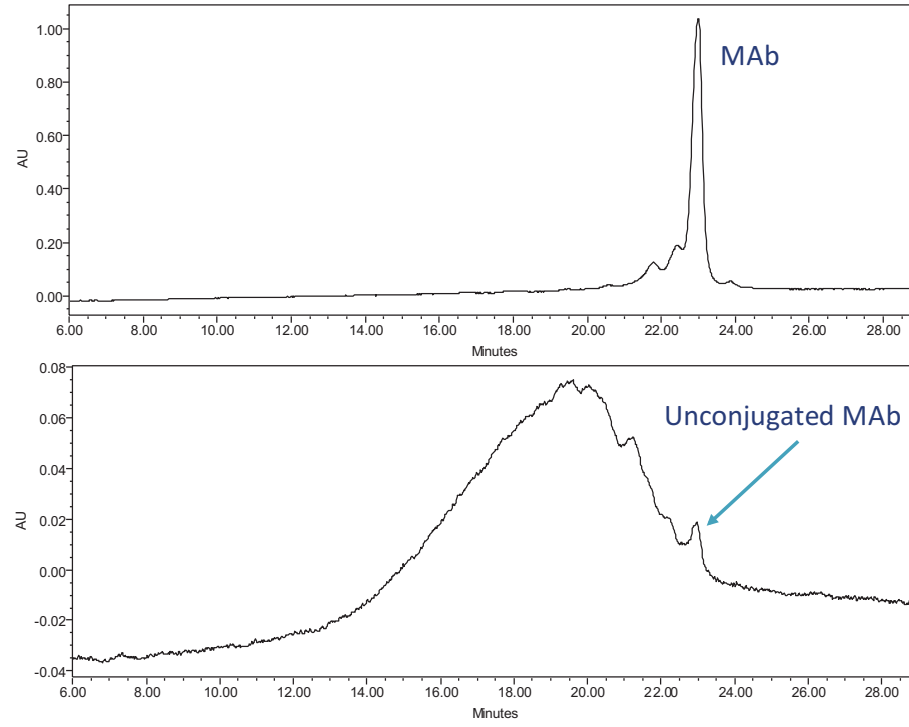
In **Figure 5**, samples separate by charge and detection by absorbance at 280 nm reveals the peaks of individual ADCs. Measurement of absorbance is in real time with a CCD camera every 30 seconds, so that focusing can be observed over time.

Figure 1: Components of an ADC with ImmunoGen's technology

- **Linker**
  - Stable in blood stream
  - Engineered to control activation inside cancer cell
  - Reacts with ε-amino group of lysine residues
- **Payload**
  - Far more potent than standard chemotherapy
- **Antibody (Ab)**
  - Targeting vehicle
  - May or may not have anticancer activity

AN INTEGRATED SYSTEM

Figure 2: ADC Charge Profile by Weak Cation Exchange

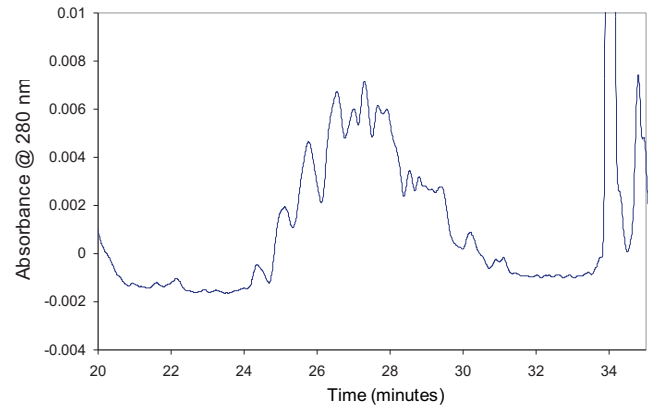


The WCX method has poor resolution.

The next-generation icIEF system, iCE3, includes a CCD camera and has the option to use either a fluorocarbon-coated capillary cartridge or a high-throughput cartridge.

Sample preparation is done by dilution with a loading solution comprised of methylcellulose and carrier ampholytes. The pH range of the carrier ampholytes is determined by the pI of the analyte molecule. Advantages of iCE technology over other methods include higher resolution, reproducibility, and ease of use.

Figure 3: ADC Charge Profile by Conventional cIEF



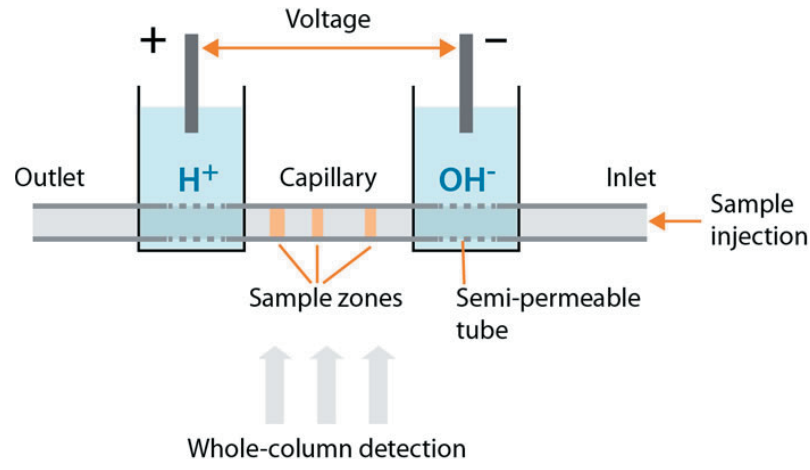
Some resolution was achieved, but development is needed.

Method Development

Method development for analyzing ADCs by icIEF is quite straightforward compared to other methods, as well. Parameters to consider in developing a method are carrier ampholytes, additives, potential interference from buffer components, and focusing time.

Buffer components can improve resolution or interfere with focusing. Adding urea to the buffer helps to keep the various species soluble while voltage is applied, so urea is a good additive to include in the buffer. A concentration between of at least 1 molar and as much as 8 molar may be optimal for solubilizing the compounds. However, urea usually does not correct a problem with solubilization of the antibody. Other options for improving solubility include detergents or sucrose.

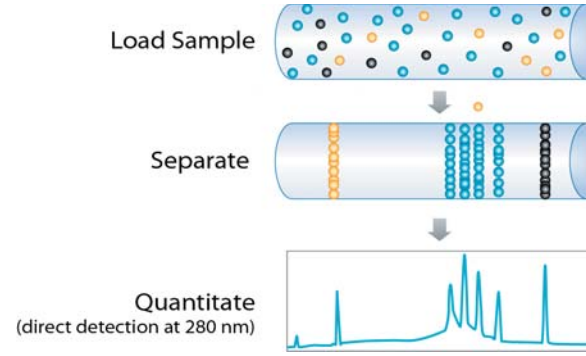
Figure 4: How sample and carrier ampholytes are injected into the capillary



Alternately, a buffer additive such as histidine, which has a pI around 7.5 to 7.6, interferes with the analysis by displacing the charged species of the conjugate. If the starting buffer contains histidine, it will be diluted down to a lower level during sample preparation, and the concentration might be low enough to be undetectable on icIEF, If not, a buffer exchange may be necessary to eliminate interference from histidine.

icIEF can also be used to determine pI and to quantitate unconjugated antibody (uMAB). The level of uMAB in the sample is related to DAR via the payload conjugation process. Ideally, a specific process will yield a predictable uMAB level from one batch to the next. However, in the case of

Figure 5: Samples separate by charge and detection by absorbance



ImmunoGen's maytansinoid ADCs, accurate determination of uMAB concentrations require an external calibration curve because the cytotoxic component of the ADC also has absorbance at 280 nm. The antibody standard used to create the curve should have a very comparable charge heterogeneity to that actually used to make the ADC.

There are some challenges in developing methods for iCE. One is that some ADCs are intrinsically difficult to analyze. The difficulty depends in part on the pI of the antibody or the combination of linker and cytotoxin that is used. Another potential challenge is the presence of buffer components or excipients that interfere with the charge profile of the ADC.

For these reasons, it is recommended that once a method is developed for iCE and used for determination of uMAB concentration, it should be confirmed with orthogonal methods.

Figure 6: Cell line selection (Mab2)

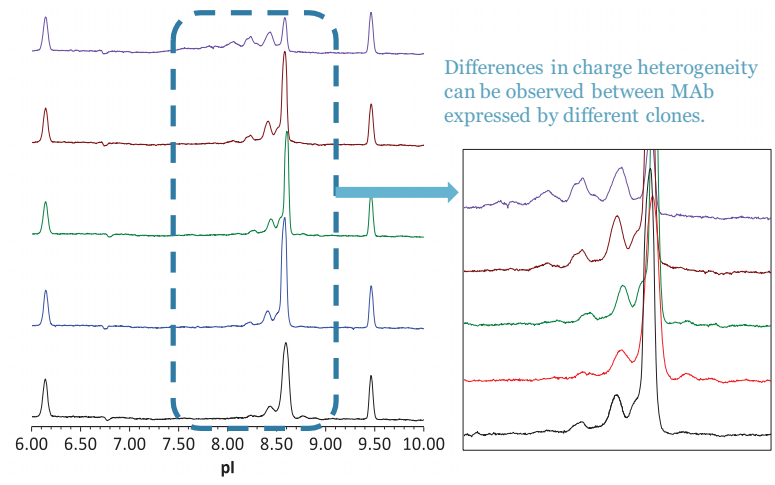
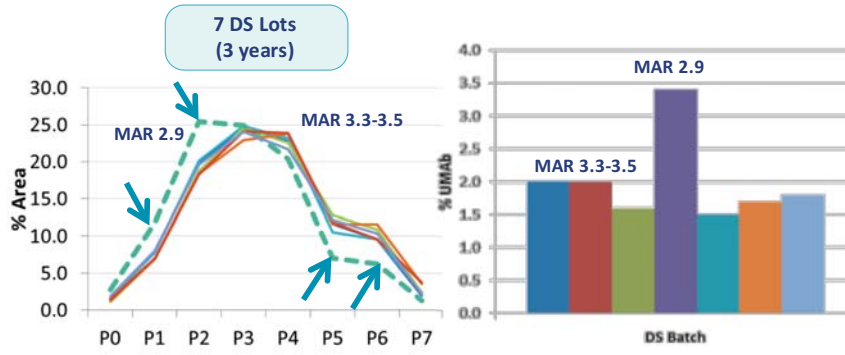


Figure 7: Manufacturing Consistency (ADC3)



- One of the lots had a lower MAR than expected.
- High reproducibility of the method allows for identification of potential outlier processes.

Other applications

An icIEF platform can be put to other uses, including cell line development and process development for antibodies, and process development for ADCs. In a case study of an antibody produced with different subclones of the same cell lines, some subclones produced antibodies with additional acidic species. In **Figure 6**, the icIEF analysis showed that some subclones could be eliminated in favor of those with a desired charge profile. The technology can also be used to compare antibodies from the same cell lines at different generations. Studying cell line stability helps in determining how long a specific cell line can be grown and used for antibody production.

Another case study illustrates the use of icIEF to compare samples in a range of drug-to-antibody ratios. This helps to determine the right amount of linker and drug to use to attain a target drug-to-antibody ratio.

In a case study of manufacturing consistency for an ADC product, five GMP drug substance lots manufactured over a period of two years were compared to each other. The group not only covers processes at different scales, but also different sites of manufacture and changes in conjugation process. In **Figure 7,** the use of icIEF showed that ADC produced was comparable from one batch to another.

Summary

The charge profiles of ADCs from icIEF complement the drug loading profiles that are obtained by mass spectrometry or other methods. Also, icIEF provides an orthogonal method to estimate the level of unconjugated antibody present in ADC samples, assuming a working icIEF method can be developed for that particular ADC.

Lastly, icIEF is a very useful analytic tool for ADC characterization and provides valuable information during process and product development.