Win the race to FDA approval with iCE™ and MFI™

Robust and fast analytical techniques are critical for achieving commercial success of therapeutic biologics and achieving it first. Speed up the development process with iCE and MFI from ProteinSimple. These highly sensitive technologies will streamline each step in the biologics characterization and manufacturing process so that you can win the race to FDA approval.

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**About Biologics**

Drug development is recognized as one of the most financially risky endeavors in all of science and a major challenge for the biopharmaceutical industry. Failures account for 75% of the total research and development costs and, for biologics, the path to market winds through basic research, discovery, preclinical development tests, clinical trials and finally FDA approval. 10-15 years and millions of dollars later, you have a chance to generate a drug that gains commercial approval void of recalls and lawsuits.1,3

The success of the drug development process is directly proportional to the utilization of accurate, sensitive and fast analytical methods for identification, quantitation, purity and other analyses of the drug substance and the drug product that you don’t get with many technologies. iCE and MFI from ProteinSimple streamline measurement and quantification of the complex interactions between a drug product and the manufacturing process. Whether it’s the infrastructure, the vial or syringe, the cap, or the excipients and stabilizers that are in solution, it is important to understand how these affect the stability and activity of the product in order to prevent enhanced immunogenicity, loss of biologic activity, as well as hyperactivity. All of which constitute a risk for the patients and the company as a whole.

This e-book will take you on the journey through product development and provide specific examples of how iCE and MFI have been used in the race to FDA approval.

**References**


Biologics Characterization

iCE delivers the fastest separation with the best resolution

Taking the extensive pipeline of biologics to market requires fast and robust analytical techniques to monitor product quality throughout development and manufacturing. Isoelectric focusing (IEF) separates proteins and their isoforms based on their charge differences (pI values) in a pH gradient under an electric field. In the past, analysis of charge variants analysis was done using slab-gel IEF methods or ion-exchange liquid chromatography (IEC) methods. Gel-IEF is a time- and labor-intensive qualitative technique, which limits its application for quality control purposes and IEC methods have long method development timelines and do not have sufficient resolution for many therapeutic biologics.

Capillary Isoelectric Focusing (cIEF) offers automation, high separation efficiency and resolution. Traditional cIEF methods involve a two-step process; focusing followed by mobilization for detection, which distorts the pH gradient, causes band spreading or peak splitting, reduces resolution and reproducibility.

iCE systems for identity and charge heterogeneity analysis include the gold-standard iCE3 and the advanced CE platforms, Maurice C. and Maurice. These systems use the innovative whole-column imaging technology (icIEF) delivering the highest resolution and sensitivity, without the need for a mobilization step. The robustness of the platform and the short separation time enables platform method development within a day, making iCE the perfect platform for monitoring the charge heterogeneity analysis of biologics throughout development and post-marketing manufacturing.3,4

- Click here to learn more about iCE3
- Click here to learn more about Maurice

References


When choosing the right clone and conditions, iCE has got you covered

Monitoring sialic acid distribution profiles of the glycoproteins by cIEF is one of the most stringent requirements in developing and marketing these biological therapeutics. Anderson and her colleagues at Merck’s bioprocess and bioanalytical research group studied specific examples of these biopharmaceutical glycoprotein products such as mAbs, erythropoietin (EPO), and recombinant Fc-fusion proteins, using iCE to show the ability of this platform to analyze the charge profiles of this wide variety of proteins. The data presented provide a “taste” of what iCE can do to support the development of biopharmaceutical glycoprotein products. Using the iCE platform, they analyzed icIEF’s efficiency in product identity, product stability, and product characterization for antigen binding and strain selection for heavily sialylated glycoproteins for biosimilar development. We will highlight a few data sets but if you would like to view the data for all of these molecules, feel free to check out the full paper.

Drug development regulations require product identity verification for a site that produces multiple different products, which should be highly specific and based on unique aspects its particular molecular structure. Traditionally, peptide mapping with RP HPLC after trypsin digest is used, but this technique requires two days or longer. Using the iCE3 system, Anderson and her team easily analyzed several different mAbs and other therapeutic glycoproteins to generate their unique fingerprint charge profiles. These electropherograms are shown in Figure 1. Another biologic that they evaluated was an Fc-Fusion protein, which is a sialylated glycoprotein therapeutic currently being used for joint inflammation pain. Its sialic acid distribution makes this particular glycoprotein very complex in terms of charge heterogeneity, making the iCE platform an excellent technology to use for its analysis. During the development of this biosimilar glycoprotein product, the icIEF profiles of two of the candidate strains were compared with the innovator molecule using icIEF. The sialic acid distribution profile as determined by iCE3 is shown in Figure 2.


• Click here to see how iCE works
Making method development easy for a difficult enzyme
cIEF can be run under denaturing conditions by using urea to expose any buried residues that may contribute to the overall charge of the protein. However, urea did not achieve full denaturation of L-asparaginase, a homotetrameric enzyme, from Erwinia chrysanthemi (ErA). ErA is a biopharmaceutical product marketed as Erwinase, which is used to treat Acute Lymphoblastic Leukemia (ALL). Post-translational modifications (PTMs) such as deamidation can reduce activity or have other deleterious effects, making it important to monitor and control PTMs in proteins like ErA. David Gervais and his colleague at the Microbiology Services of Public Health England evaluated various conditions under which all ErA protein residues could participate in the denaturing charge-based cIEF method such that each charge variant species would result in a separated cIEF peak. 8M urea was not sufficient for cIEF analyses of ErA, so they tested to see if Alkylureas such as N-ethylurea increased denaturation of ErA during cIEF. They screened mixtures of 8 M urea with N-methylurea, N-ethylurea and N-butylurea and determined that 8M urea with added 2 M N-ethylurea completely denatured ErA such that the electropherogram profile changed to a profile with one predominant at pI 7.4 (Figure 3). The ability of the iCE platform to handle a wide variety of denaturants/additives, short separation time and reproducible data enabled the development of protein acidic species quantitation of this medically important therapeutic.

- Click here to view this paper
- Click here to view iCE systems technical documents

![Figure 3: Effect of N-ethylurea on cIEF profiles of ErA.](image)
All analyses contained 8M urea as the denaturant, with additional added N-ethylurea.


Biologics Characterization
Scientists find optimal conditions for iCE analyses

Size or charge heterogeneity is an inherent property of monoclonal antibodies (mAbs), which necessitates the development of robust techniques and methods to fully characterize therapeutic mAbs. Xiaoping He and his colleagues at Pfizer and Genentech developed an analytical method using the iCE technology for charge variant analysis and developed it further into a platform method for many mAbs in the development pipeline. They evaluated various parameters that affect the charge profiles, including concentration of sample protein, salt, methyl cellulose and additives, concentration of carrier ampholytes and the pH range. Data from each parameter was analyzed and optimal conditions were defined based on the study results. Since they covered so many parameters, we will go over the data on just a few. Feel free to check out the full paper to learn more!

First, they looked at icIEF separation profiles of proteins at different working concentrations and found that similar charge profiles were obtained for mAb-1 at all three protein concentration levels tested, whereas mAb-2 appeared to be very sensitive to the concentration of the protein (Figure 4). As a result, an optimal protein working concentration for most mAbs was found to be 0.3 mg/mL. Experiments were then conducted to determine the optimal concentrations for single salt or combination of salts. He and his colleagues found that salt concentrations of less than 5 mM sodium chloride and 0.5 mM sodium acetate produced the best results (Figure 5). In order to develop a widely applicable iCE method, they also looked at ampholytes or ampholyte combinations that would provide the best separation for charge variants of various mAbs. Results demonstrated that broad ampholyte pH 3-10 at 4% was optimal for a platform iCE method for analysis of a wide range of mAbs (Figure 6).

To quote the scientists: “When optimized composition of the ampholytes is maintained, reliable results and excellent reproducibility are assured for each experiment.”

• Click here to view a list of iCE publications

Figure 4: icIEF electropherograms of varying concentrations of mAb-1 (A) and mAb-2 (B).

Figure 5: Effects of salt concentrations on mAB-1 separation.

Figure 6: Effects of ampholyte concentration on baseline stability.

iCE has proven robustness for process development and regulatory submissions of therapeutic antibodies

Oscar Salas-Solano and 16 of his colleagues formed an international team including 12 laboratories from 11 independent biopharmaceutical companies in the United States and Europe to evaluate the precision and robustness of iCIEF technology for the charge heterogeneity analysis of mAbs. They performed extensive statistical evaluation of the data to determine laboratory consistencies and outlying information. Described here are the final precision results for the iCIEF charge heterogeneity analysis of the representative rMAb. To see more, check out the full paper. The inter-laboratory variability of the mean values for the percent peak areas of the rMAb isoforms from each of the 12 laboratories was tested using the Mandel’s h statistic and the Grubs test. After rejection of the within and between laboratory outliers, the remaining data were used for calculating the final precision results. Table 1 shows the final precision results for the iCIEF charge heterogeneity analysis of the representative rMAb samples across different laboratories, different analysts, different lots of ampholytes and multiple instruments. The high inter-laboratory precision with RSD values of less than 0.8% for apparent pI and less than 11% for percent peak area support the use of iCE for therapeutic antibodies in the process development and quality control of biopharma organizations.

<table>
<thead>
<tr>
<th>Ampholyte lot A1</th>
<th>Mean</th>
<th>SD</th>
<th>Percent RSD</th>
<th>Mean</th>
<th>SD</th>
<th>Percent RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>2.08</td>
<td>0.36</td>
<td>17</td>
<td>2.20</td>
<td>0.18</td>
<td>8.2</td>
</tr>
<tr>
<td>Peak 2</td>
<td>9.88</td>
<td>0.30</td>
<td>3.1</td>
<td>9.61</td>
<td>0.30</td>
<td>3.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>23.0</td>
<td>0.73</td>
<td>3.2</td>
<td>23.1</td>
<td>0.70</td>
<td>3.0</td>
</tr>
<tr>
<td>Peak 4</td>
<td>31.1</td>
<td>0.45</td>
<td>1.5</td>
<td>31.1</td>
<td>0.45</td>
<td>1.5</td>
</tr>
<tr>
<td>Peak 5</td>
<td>23.9</td>
<td>0.75</td>
<td>3.1</td>
<td>23.9</td>
<td>0.72</td>
<td>3.1</td>
</tr>
<tr>
<td>Peak 6</td>
<td>8.74</td>
<td>0.55</td>
<td>6.3</td>
<td>8.85</td>
<td>0.45</td>
<td>5.3</td>
</tr>
<tr>
<td>Peak 7</td>
<td>1.25</td>
<td>0.22</td>
<td>18</td>
<td>1.30</td>
<td>0.14</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 1: Precision results for the relative distribution of the rMAb-charged variants.

**Size. Count. Morphology. MFI.**

Micro-Flow Imaging (MFI) offers several advantages over traditional techniques in the analysis of sub-visible particles in protein formulations. Its imaging-based analysis offers direct particle detection giving you morphological information, as well as size and count. This allows MFI to provide novel and unique insights into particle characterization and quantification with just a single test. These capabilities give MFI systems the ability to discriminate between protein aggregates and the other contaminants that are commonly found in heterogeneous biopharmaceutical samples. This insight, combined with a sensitive detection method and a class leading 85% sampling rate make MFI an ideal tool for biopharmaceutical development. In this section of the e-book we highlight the areas where MFI provides valuable data in the quest for higher quality products.

- Click here to learn more about MFI
- Click here to learn more about MFI Software
- Click here to view an MFI webinar

**References**


MFI reveals what SEC and LO don’t
Shannon Southall and her colleagues at GSK needed to understand how their formulations protected their product against stresses such as freeze-thaw and shaking. They evaluated different particle analysis techniques including Size Exclusion Chromatography (SEC) and Light Obscuration (LO). They found that MFI typically required a smaller volume, and the data suggested that it was far more sensitive for detecting proteinaceous particles compared to LO.

Using MFI, they were able to capture images during the sample run, which allowed further analysis of particles based on morphological parameters. Data from several samples underwent significant particle formation according to MFI, while SEC and LO counts remained comparable to the controls.

Both shake and freeze-thaw experiments resulted in sub-visible particle formation, especially in the <10 µm range. However, the data achieved by SEC and LO did not always indicate as significant a change in particle count as did MFI (Tables 2 and 3).

Thus, MFI appeared to be a more sensitive method to assess particle formation as compared to LO and SEC most likely due to the tendency of proteinaceous aggregates to be both translucent and amorphous.

• Click here to view this paper
• Click here to checkout the MFI technical library

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Total Particle Count by MFI</th>
<th>% Soluble Aggregate by SEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Shake</td>
</tr>
<tr>
<td>4</td>
<td>1927</td>
<td>9704</td>
</tr>
<tr>
<td>6</td>
<td>18632</td>
<td>22699200</td>
</tr>
<tr>
<td>14</td>
<td>776</td>
<td>2980</td>
</tr>
</tbody>
</table>

**Table 2: Shake analysis.** Particles appear to be stable upon shaking when monitored by SEC, but MFI finds that shaking drastically increases particle count.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Total Particle Count by MFI</th>
<th>Count &gt;10 um by LO</th>
<th>% Soluble Aggregate by SEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Freeze-Thaw</td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>4278</td>
<td>14368</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>8149</td>
<td>85646</td>
<td>156</td>
</tr>
<tr>
<td>9</td>
<td>30296</td>
<td>92762</td>
<td>25</td>
</tr>
<tr>
<td>19</td>
<td>16125</td>
<td>38664</td>
<td>188</td>
</tr>
</tbody>
</table>

**Table 3: Freeze-Thaw analysis.** Data from LO and SEC suggest that the formulations are stable against freeze-thaw stress, while MFI shows drastic changes in particle counts.

Southall, S. Particle analysis as a formulation development tool. Am Pharm Rev article (2011).
Testing m-cresol:Polysorbate formulations with MFI

Polysorbate 20 (PS20) and polysorbate 80 (PS80) are fatty acid esters of polyoxyethylene sorbitan that are used as non-ionic surfactants in biotherapeutics to prevent surface adsorption and stabilize proteins against aggregation caused by agitation and shear. This benefit is complicated by an undesirable reaction with m-cresol, a commonly used phenolic preservative.

The incompatibility of m-cresol and non-ionic surfactants is a well-established fact in the pharmaceutical industry. However, Shuai Shi and his colleagues at Merck, had reason to believe that these incompatibilities were concentration dependent. They decided to see if reducing the concentrations of PS20 and PS80 could avoid complex formation with m-cresol while simultaneously protecting the proteins as needed.

Their agitation study was aimed at determining whether these trace levels of polysorbate in the peptide solution could still stabilize the peptide against agitation induced aggregation and fibrillation. Peptide formulations were prepared with 2.8 mg/mL m-cresol, then spiked with PS20 and PS80 at 20 ppm and 50 ppm respectively. These solutions were then subject to reciprocal shaking at 300 rpm for 3 days.

The particle analysis provided by MFI showed that even at low levels polysorbates can confer their protective property to proteins. Particle concentration was by far the highest for the formulation without polysorbate (Figure 7) and, even at low levels, PS20 and PS80 were able to prevent agitation induced aggregation.

At 50 ppm, PS80 was slightly incompatible with m-cresol, which may suggest that sub visible particles may have formed when mixing 50 ppm PS80 with 2.8 mg/mL m-cresol.

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- Click here to download a brochure

Development of mAbs and antibody-drug conjugates for therapeutic and diagnostic applications requires detailed knowledge of the physicochemical degradation for these protein pharmaceuticals. However, the mechanisms of oxidative protein degradation, aggregation and fragmentation are not well known.

Oliver Mozziconacci and his colleagues at the University of Kansas identified a hydrolysis reaction, catalyzed by Fe II, between Met252 and Ile253 in IgG-1 (Figure 8). They wanted to measure the effect of this hydrolysis on the conformational stability and aggregation propensity of the antibody using the concentration of sub-visible particles measured by MFI as a metric.

To evaluate the extent of aggregation, Mozziconacci and his colleagues used a combination of UV visible absorbance spectroscopy, SEC, and MFI (Table 4). They found an increase in total sub-visible particle concentration of the oxidized IgG-1 sample at 10 mg/mL relative to the unoxidized control. This observation by MFI was able to give them insight into how an extraneous contaminant could catalyze aggregation in a biopharmaceutical product.

- Click here to view this paper
- Click here to view a webinar: MFI as a Stability Indication Method
- Click here to view a list of MFI publications

### Table 4: Effect of oxidation on particle concentration measured by UV-vis, SEC and MFI

<table>
<thead>
<tr>
<th>mABI concn (mg/mL)</th>
<th>Sample</th>
<th>UV-vis (280 nm)</th>
<th>SEC (total aggregates)</th>
<th>MFI TPC (per mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Control 24 h oxidation</td>
<td>0.82 ± 0.002</td>
<td>0.8</td>
<td>457 ± 52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.76 ± 0.003</td>
<td>7.1</td>
<td>7362 ± 285</td>
</tr>
<tr>
<td>100</td>
<td>Control 24 h oxidation</td>
<td>0.82 ± 0.001</td>
<td>1.1</td>
<td>702 ± 22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.79 ± 0.005</td>
<td>3.8</td>
<td>2537 ± 585</td>
</tr>
</tbody>
</table>

Adapted with permission from. Mozziconacci, O. et al. Site-Specific Hydrolysis Reaction C-Terminal of Methionine in Met-His during Metal-Catalyzed Oxidation of IgG-1. Mol. Pharm. 13, 1317–1328 (2016). Copyright 2016 American Chemical Society
MFI reveals what SEC and LO don’t

Formulations for biopharmaceuticals are often exhaustively studied, but lyophilization and reconstitution of those proteins are sometimes neglected. Janice Davis and her team prepared eight lyophilized formulations of an IgG1 monoclonal antibody, each containing increasing levels of sucrose. They also added sorbitol at a level of 5% w/w relative to sucrose in three of the samples. The samples were stored up to 4 weeks at 40 °C and, upon reconstitution, the levels of sub-visible particles were measured using MFI. Davis and her colleagues found that addition of sucrose decreased the number of sub-visible particles and the addition of sorbitol decreased the number of sub-visible particles even more. As seen in Figure 9, the initial sub-visible particle load for freeze-dried LB-060 decreased dramatically as sucrose levels increased. Inclusion of a small amount of sorbitol reduces the initial sub-visible particle load even further despite the fact that the sucrose:protein weight ratio is relatively high. Thanks to MFI, this group also learned that small amounts of sorbitol can further improve the storage stability of a lyophilized antibody formulation.

- Click here to view this paper
- Click here to view a webinar: Process Improvement for Research Use Only Protein Products with MFI
- Click here to learn how MFI complements LO

![Figure 9: Effect of sucrose and sorbitol concentration on particle formation. Addition of sucrose and sorbitol decreases the number of sub-visible particles.](image)

Better insight into your final product.

Aggregates and particulates in liquid protein formulations can have a significant effect on product quality and patient safety. In the past, only particulate analysis in a range > 10 µm was required (USP <788>). However, in many cases, particles below 10 µm can diminish a product’s therapeutic effect, and are more likely to instigate an immunogenic response in patients.  

How are these particles introduced into the sample in the first place?

Protein aggregates can form due to many different types of stress including, pH, heat, freeze-thaw and mechanical stress. In addition, the manufacturing process is filled with opportunities for particle formation. Surfaces in tubing and piston pumps can shed micro and nanoparticles, siliconized surfaces introduce silicone oil microdroplets, and glass syringes can be riddled with tungsten nanoparticles.

With all of this potential for contamination and particle aggregation, it is of upmost importance to establish a robust testing regime that can reliably detect and characterize particles.

In the past, light obscuration has been the “gold standard” particle assessment. However, its scope is limited (Figure 10). Sensitivity and classification are important aspects of particle analysis and light obscuration is limited in these areas. It is susceptible to missing translucent protein aggregates and it only provides information on particle size and count > 10 µm. MFI, on the other hand, gives you all the information you need to differentiate particle populations, as well as a higher degree of sensitivity and accuracy. See more with MFI and be sure that your product is ready for the market.

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References


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