Imaged Capillary Isoelectric Focusing (icIEF) and Mass Spectrometry (MS) for In-Depth Characterization of Therapeutic Protein Charge Heterogeneity

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1. Introduction

In-depth characterization of therapeutic proteins is required to understand their charge heterogeneity as it relates to drug safety and efficacy. Imaged capillary isoelectric focusing (icIEF) is critical to this process because of its ability to separate protein charge isomers with a high resolution and reproducibility [1]. In recent years icIEF has experienced rapid progress to keep pace with an industry that aims to accelerate the development of monoclonal antibodies (mAbs) and other emerging biologics:

1. icIEF fractionation and icIEF-MS modes are now available to characterize the post-translational modifications (PTMs) and chemical degradation underlying each protein charge isomer, thus providing a convenient and comprehensive alternative to the resource-intensive ion exchange chromatography (IEX) [1-4].

2. Novel carrier ampholytes and capillary coatings allow for fast and easy method optimization to resolve challenging molecules like antibody-drug conjugates (ADCs), fusion proteins, and bispecific antibodies [5-6].

Altogether these innovations can deliver high quality information to help avoid costly setbacks during development and manufacturing. In this work, we present method development case studies highlighting how these technologies can most effectively be used in an end-to-end icIEF platform (see Fig 1).

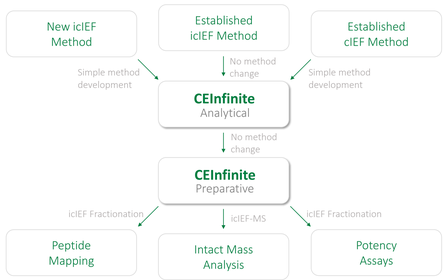


Fig 1. The CEInfinite developed by Advanced Electrophoresis Solutions (AES) is capable of analytical icIEF, icIEF fractionation, and icIEF-MS with seamless method transfer between each mode.

2. Materials and Methods

2.1 CEInfinite Experimental Setup

The experimental setup for CEInfinite Preparative is shown in Fig. 2 with the ability to run icIEF fractionation and icIEF-MS for offline and online MS, respectively. The icIEF-MS mode can easily couple directly to a variety of commercial mass spectrometers as shown in Fig 3.

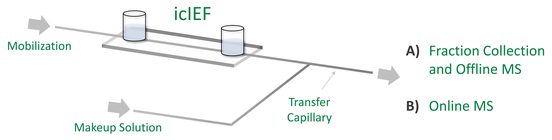


Fig 2. In a two-step process, the icIEF separation takes place before pressure mobilization is initiated and drives the icIEF peaks through a transfer capillary. **A)** icIEF fractionation process where the transfer capillary leads to a switch valve that directs the selected fractions into one of nine collection vials in preparation for offline MS. **B)** Online MS involves direct coupling of the transfer capillary to the users preferred mass spectrometer.

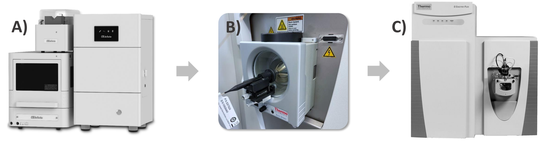


Fig 3. A) The transfer capillary from CEInfinite is coupled to the **B)** Thermo EASY-Spray Ion Source coupling using a nanospray adapter developed by AES. **C)** This interface connects directly to the Thermo Q Exactive Plus mass spectrometer with the entire setup taking approximately 5 min to complete.

2.2 Chromeleon Software Driver

The CEInfinite analytical icIEF and icIEF-MS modes can be directly controlled using the Chromeleon™ 7 Chromatography Data System (CDS). This tool enables the integration of icIEF and MS into one software for comprehensive charge variant analysis that is 21 CFR Part 11 compliance ready.

3. Results and Discussion

3.1 icIEF Platform Method Optimization

The CEInfinite analytical icIEF mode enables simple method transfer from established icIEF platform methods to support the quality control (QC) of charge variants prior to manufacture release. However, additional options for method optimization can be considered for more comprehensive charge variant characterization (See Fig 4).

Carrier Ampholytes. AESlytes® are designed for lot-to-lot consistency and high resolution [5]. The HR AESlyte® series is typically employed for monoclonal antibodies (mAbs) to achieve an icIEF profile comparable to Pharmalyte® (Fig 4A and 4B), while the UH AESlyte® series offers an improved resolution (Fig 4C). The HR and UH series can also be combined to offer better performance in some cases (Fig 4D). These options can provide more in-depth charge information for ADCs, fusion proteins, and other complex modalities that can be, for example, heavily conjugated or glycosylated [5].

CEInfinite Capillary Coatings. Durable hydrophilic coatings in the form of acrylamide derivative (AD) and methylcellulose (MC) provide an alternative to the hydrophobic fluorocarbon (FC) coating [3], thus opening the door to a more diverse set of modalities that can be characterized by icIEF. Moreover, the AD and MC coatings eliminate the need for MC in solution to help mitigate errors that typically arise from increased viscosity and bubble formation.

pI Markers. There are more than 40 pI markers available to narrow down the separation region and maximize the accuracy of the pH calibration. Some of these markers are also fluorescent and carboxypeptidase B (CpB) enzyme resistant for additional versatility.

3.2 icIEF-MS Online for Intact Mass Analysis

The CEInfinite icIEF-MS mode which takes less than 45 min to run enables the identification of several PTMs and chemical degradation (see Fig 5). There are several method development considerations to maximize resolution and sensitivity [1-4].

Carrier Ampholytes. AESlytes® are also designed to reduce the background noise that has traditionally been an issue for icIEF-MS.

CEInfinite Capillary Coatings and Formamide. AD and MC coatings become critical for icIEF-MS because it avoids the MC in solution that interferes with the MS signal. Likewise, formamide can be used to replace urea which poses the same concern as MC.

Mobilization. Pressure mobilization is used for reproducibility while avoiding the need to add a chemical mobilization agent to the sample matrix. The speed of mobilization can be a tradeoff between throughput and resolution.

Makeup Solution. Acetonitrile and formic acid can be added to solution prior to MS for an improved electrospray ionization process. It is also possible to use less denaturing conditions for more sensitive molecules such as ADCs.

Capillary Diameter. The diameter of the separation capillary is increased from 100 µm to 200 µm for improved MS sensitivity, while a 50 µm diameter transfer capillary diameter is used to minimize the resolution loss during pressure mobilization.

MS Instrument and Interface. The setup uses an nanospray ion source and a Thermo Fisher Orbitrap instrument (Fig 3) for high resolution and sensitivity, but it is also possible to connect to other instruments from Waters, Agilent, Bruker, and more.

3.3 icIEF Fractionation for Peptide Mapping

The CEInfinite icIEF fractionation mode enables the collection of 4-20 µg of sample per run with a high purity for each peak (see Fig 6). Up to 30 consecutive runs per day enables the collection of quantities suitable for peptide mapping [2-3], and can be extended further to potency assays.

Carrier Ampholytes. Choose from a wide selection of narrow range carrier ampholytes to maximize the purity of each icIEF peak after fractionation.

Capillary Coatings. Select either an FC, AD, or MC coating depending on the surface properties of the protein to minimize resolution loss during mobilization due to extra-column effects.

Sample Concentration. A high sample concentration between 1-5 mg/mL is typically used to collect larger quantities. It is possible to increase this further depending on the tradeoff between throughput and fraction purity.

Makeup Solution. The make up solution serves to increase the speed of fraction collection. It can consist of water, formulation buffer, or any solution needed to prepare the fractions for further analysis.

Capillary Diameter. The diameter of the separation capillary is increased from 100 µm to 320 µm for increased sample throughput, while a 50 µm diameter transfer capillary diameter is used to minimize the resolution loss during pressure mobilization.

Dynamic Fractionation and Automation. Manually select each fraction interval during the first run, and these settings can be applied to automate subsequent runs.

Reverse Polarity. The purity of the acidic regions can be improved by reversing the polarity and allowing them to elute first.

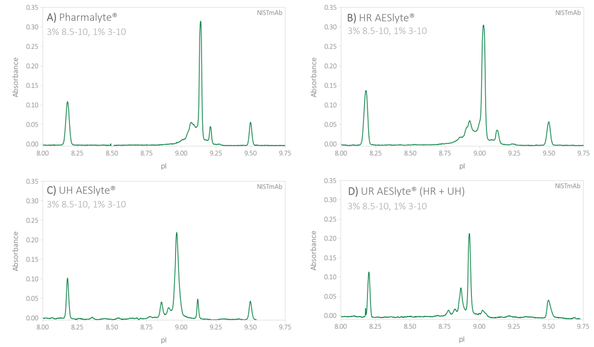


Fig 4. A) and **B)** demonstrate comparable profiles for Pharmalyte® and HR AESlyte® although there is slightly improved resolution for the latter. **C)** UH AESlyte® shows a substantially improved resolution for the acidic region although the baseline is slightly increased. **D)** A combination of HR and UH AESlyte® leverages the improved resolution of UH with the lower baseline noise of HR.

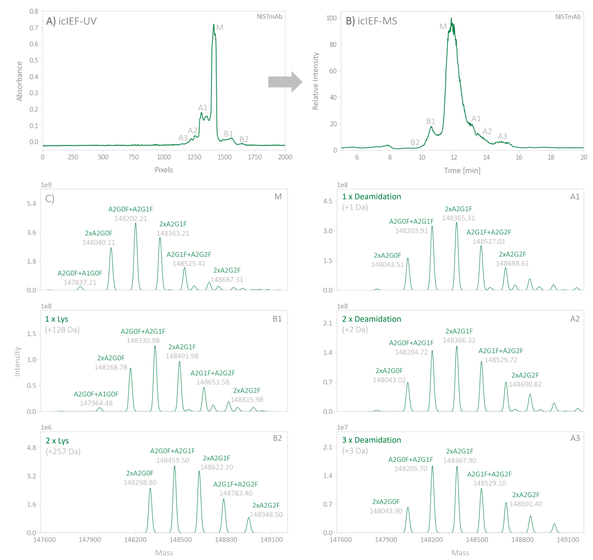


Fig 5. A) The icIEF profile with 2% 8.5-10 UR AESlyte® and 10% formamide which is then mobilized towards the MS instrument to produce a **B)** Total Ion Electropherogram (TIE) that displays the corresponding icIEF peaks [4]. **C)** The deconvoluted MS data demonstrates the identification of chemical degradation and PTMs including glycosylation, lysine residues, and deamidation. The +1 Dalton shift by deamidation is less certain due to the inherent limitations of the MS, but using icIEF as a front-end separation technique helps build confidence in this measurement.

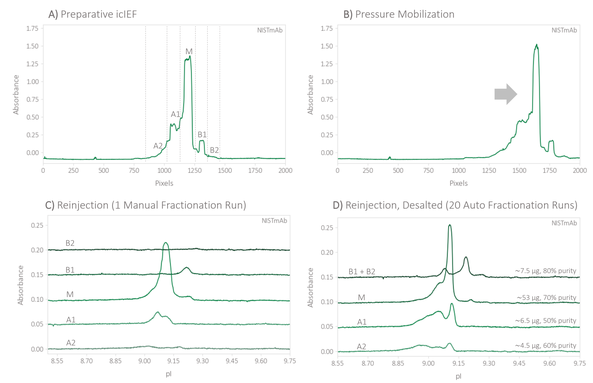
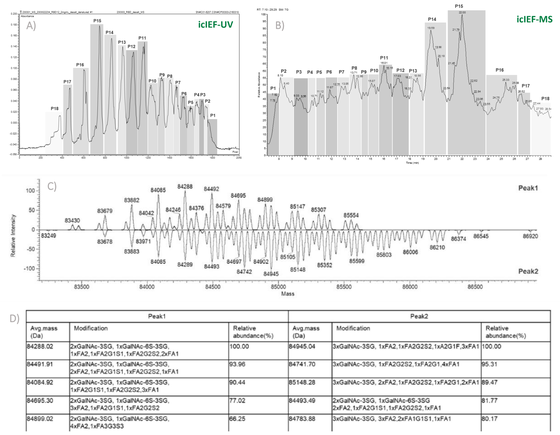


Fig 6. A) The preparative icIEF profile which is comparable to the analytical profile seen in Fig 4. The width of each fraction interval can be manually selected during this initial run. **B)** The pressure mobilization which is capable of maintaining focusing and has outstanding reproducibility. **C)** The reinjection of each fraction after mobilization and collection. **D)** The reinjection of 20 consecutive automated runs in which the fractions were pooled together in their respective collection vials and then desalted.

3.4 Next Generation Biotherapeutics

ADCs, fusion proteins, bispecific antibodies, lipid nanoparticles (LNPs), and adeno-associated viruses (AAVs) all offer potential as the next generation of life-saving medicine. However, these modalities present unique challenges for charge variant characterization by icIEF and IEX due to a number of factors including increased glycosylation, conjugation, and hydrophobicity. Consequently, emerging tools for icIEF method optimization, fractionation, direct MS coupling seem poised to play a more prominent role in biotherapeutic development. Fig 7 illustrates how this suite of end-to-end icIEF technologies can provide in-depth characterization for a highly heterogenous recombinant protein that was developed as a vaccine for SARS-CoV2 [7].



Peak1			Peak2		
Avg.mass (Da)	Modification	Relative abundance(%)	Avg.mass (Da)	Modification	Relative abundance(%)
84288.02	2xGalNAc-3SO, 1xGalNAc-6S-3SO, 1xFA2,1xFA2G1S1,1xFA2G2S2,2xFA1	100.00	84945.04	3xGalNAc-3SO, 1xFA2,1xFA2G2S2,1xA2G1F,3xFA1	100.00
84491.91	2xGalNAc-3SO, 1xGalNAc-6S-3SO, 2xFA2,1xFA2G1S1,1xFA2G2S2,1xFA1	93.96	84741.70	3xGalNAc-3SO, 1xFA2G2S2,1xFA2G1,4xFA1	95.31
84084.92	2xGalNAc-3SO, 1xGalNAc-6S-3SO, 1xFA2G1S1,1xFA2G2S2,3xFA1	90.44	85148.28	3xGalNAc-3SO, 2xFA2,1xFA2G2S2,1xFA2G1,2xFA1	89.47
84695.30	2xGalNAc-3SO, 1xGalNAc-6S-3SO, 3xFA2,1xFA2G1S1,1xFA2G2S2	77.02	84493.49	2xGalNAc-3SO, 1xGalNAc-6S-3SO, 2xFA2,1xFA2G1S1,1xFA2G2S2,1xFA1	81.77
84899.02	2xGalNAc-3SO, 1xGalNAc-6S-3SO, 4xFA2,1xFA3G3S3	66.25	84783.88	3xGalNAc-3SO, 3xFA2,2xFA1G1S1,1xFA1	80.17

Fig 7. A collaborative study demonstrating icIEF characterization of the recombinant protein vaccine which was extremely heterogeneous due to PTMs including N-glycans, O-glycans, deamidation, and oxidation [7]. **A)** The icIEF profile and **B)** the corresponding TIE by MS illustrating at least 18 different charge isomers. **C)** The deconvoluted MS data for the first and second peaks highlighting **D)** extensive glycosylation as shown in the table.

4. Conclusions

There are a variety of new tools available for icIEF method development and characterization, including carrier ampholytes, capillary coatings, icIEF-MS, and icIEF fractionation. These tools have the potential to transform all areas of biotherapeutic development by reducing the cost of existing workflows and creating new opportunities (Fig 8). Furthermore, as companies become more ambitious in their attempt to develop impactful biotherapeutics with higher complexity, these new icIEF technologies can begin to play a more impactful role.

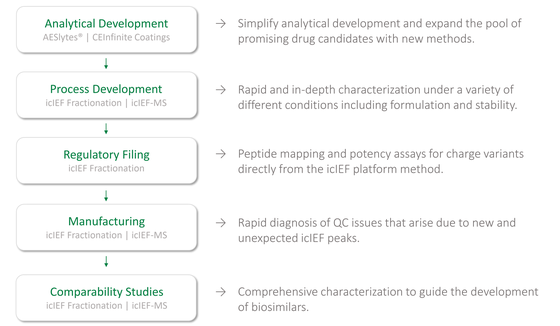


Fig 8. Summary of the opportunities for biotherapeutic development that leverage the new suite of icIEF tools including CEInfinite and AESlyte®.

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