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ELECTROPHORESIS

SHORT COMMUNICATION

Evaluation of an icIEF-MS system for comparable charge variant analysis of biotherapeutics with rapid peak identification by mass spectrometry

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Abstract

Protein therapeutics are usually produced in heterogeneous forms during bioproduction and bioprocessing. Heterogeneity results from post-translational modifications that can yield charge variants and require characterization throughout product development and manufacturing. Isoelectric focusing (IEF) with UV detection is one of the most common methods to evaluate protein charge heterogeneity in the biopharmaceutical industry. To identify charge variant peaks, a new imaged microfluidic chip-based isoelectric focusing (icIEF) system coupled directly to mass spectrometry was recently reported. Bridging is required to demonstrate comparability between existing and new technology. As such, here we demonstrate the comparability of the pI value measurement and relative charge species distributions between the icIEF-MS system and the control data from a frequently utilized methodology in the biopharmaceutical industry for several blinded development-phase biopharmaceutical monoclonal antibodies across a wide pI range of 7.3–9.0. Hyphenation of the icIEF system with mass spectrometry enabled direct and detailed structural determination of a test molecule, with masses suggesting acidic and basic shifts are caused by sialic acid additions and the presence of unprocessed lysine residues. In addition, MS analysis further identified several low-abundance glycoforms. The icIEF-MS system provides sample quantification, characterization, and identification of mAb proteoforms without sacrificing icIEF quantification comparability or speed.

KEYWORDS

biotherapeutics, capillary electrophoresis, charge variants, glycosylation, mass spectrometry

Recombinant proteins, such as monoclonal antibodies, may acquire heterogeneities during different stages of the manufacturing process including potential differences in their amino acid sequences and various PTMs [1]. Such heterogeneity includes glycosylation, deamidation, succinimide formation, glycation, C-terminal clipping, cysteinylation, etc. [2, 3]. The variations generated by these modifications can lead to alterations in the efficacy of the therapeutic molecule ranging from inactivation to enhanced potency [4]. Most of these changes lead to a

Abbreviations: DEA, diethylamine; icIEF, imaged capillary isoelectric focusing; IEX, ion exchange chromatography; GlcNAc, *N*-acetylglucosamine.

shift in the pI of the molecules and these charge variants can be measured by imaged capillary IEF (icIEF) [5]. These charge variants, an important class of heterogeneity, represent a critical analytical reportable for the biopharmaceutical industry in release and stability testing as well as during in-process control since these variants can be generated during the bioproduction process. Consequently, fast and comprehensive analytical methods for charge heterogeneity determination are of great interest to the biopharmaceutical industry.

Conventionally used methods for analyzing protein charge variants include IEX and various forms of IEF including preparative modes [6–9]. While the frequently used IEX-based techniques are compatible with MS detection [10], the process is usually time consuming and the separation of analyte molecules is not based on their overall charge but limited to their interaction with the stationary phase [11]. Since CE-based methods, including cIEF, separate species based on an orthogonal principle to stationary-phase interaction, particular forms of biotherapeutic proteins that are not amenable to separation by IEX can be separated by cIEF, an established platform method used in the biopharmaceutical industry [9, 12, 13]. However, cIEF requires additional detection methods for adequate peak assignment and identification of the charge variants, preferably MS. One of the options for such analysis is by fraction collection and subsequent MS characterization [14]. The other option is direct hyphenation, as CE-based separation methods can be interfaced with MS via various sprayer setups such as coaxial or sheath-less interfaces [15]. It is important to note that the nanoliter per minute flow rates in capillary formats support reduced ion suppression and increased performance in direct CE-MS coupling. Unfortunately, lower sensitivity coaxial sprayer setups can induce sensitivity issues via dilution, and higher sensitivity sheathless approaches have historically struggled with easy and direct IEF connection. Attempts to alleviate these and other issues have led to the development of special sprayer designs to reduce sample dilution [16] and 2D setups to decouple the CE and ESI processes [17], as well as cIEF and MS coupling via an electrokinetically pumped sheath-flow nanospray [18]. Imaged cIEF has been coupled to MS through a nanoliter valve or via CZE to alleviate the interference from MS-incompatible icIEF reagent [19]. Connecting liquid-phase charge-variant separation methods to MS offers structural elucidation of sample molecules and is advantageous for the biopharmaceutical industry and regulatory agencies.

The combination of microfluidic chip-based separation devices with MS is promising, offering high-resolution separation performance with immediate structural identification of the separated molecules [20]. Microfluidic designs offer on-chip integration of key functions, including separation, mobilization, and electrospray, fabricated on a single chip, which is not easily achieved with simple capillary-based setups. In addition, the actual separation channel in microfluidic chip devices is significantly shorter than regularly used CE capillaries, resulting in a decrease in the analysis time. Finally, fabrication of the microfluidic chip using materials with properties amenable to transillumination and optical detection allows direct monitoring of the focusing, separation, and mobilization processes, offering unique optimization possibilities, a key advantage of imaged cIEF.

Imaged cIEF directly connected to MS (icIEF-MS) is a recently introduced technology for high resolution separation of therapeutic charge variants, enabling rapid isoelectric point measurement, direct UV-based charge variant quantitation, and mass characterization of charge variants [7]. The method is readily applicable to mAb protein therapeutics and can be extended to new modalities including fusion proteins and antibody drug conjugates. In this article, the analysis of different IgG subclasses covering the pI range of 7.3–9.0 is described. UV quantitation and pI assessment of the icIEF results were shown to be comparable to established methodologies. In addition to direct charge variant characterization by integrated MS of blinded mAbs, the approach also detected low abundance PTMs.

Histidine, sucrose, EDTA, and Polysorbate 80 were from J. T. Baker (Philipsburg, NJ). Formamide, glacial (HPLC grade) acetic acid, and LC/MS-grade formic acid and ACN, were acquired from Fisher Chemical (Fair Lawn, NJ). Diethylamine (DEA) 99.5% (extra pure, redistilled) was from ACROS Organics (Fair Lawn, NJ). The pI markers of 6.14 and 9.50 were from ProteinSimple (San Jose, CA), and the Pharmalytes from Cytiva (Marlborough, MA), Urea, L-Arginine, and iminodiacetic acid were from Sigma–Aldrich (St. Louis, MO).

Pfizer provided blinded mAbs initially denoted mAbs 1–4, and later revealed that mAbs 1 and 2 were the same molecule. Consequently, samples are denoted mAb1: IgG1 subclass (10.0 mg/mL in 20 mM histidine, 85 mg/mL sucrose, pH 5.8); mAb2 (TSmAb1): thermal stressed mAb1 (10.0 mg/mL in 20 mM histidine, 85 mg/mL sucrose, pH 5.8); and mAb3 and mAb4: IgG2 subclasses (10.0 mg/mL in 20 mM Histidine, 85 mg/mL sucrose, 0.05 mg/mL EDTA, and 0.2 mg/mL polysorbate 80, pH 5.5). The thermal stress conditions were 4 weeks of 40°C incubation of 10.0 mg/mL mAb1 in 20 mM histidine and 85 mg/mL sucrose at pH 5.8).

The breadboard Blaze icIEF-MS system (developed by Intabio Inc., Fremont, CA since acquired by SCIEX) was used for all icIEF-MS analyses. The inner surface of the microfluidic chip separation channel was coated to suppress EOF. Separation of the mAb1 and mAb2/TSmAb1 samples utilized 0.01 mg/mL 6.14 and 9.50 pI markers in

4.0% pH 3–10 Pharmalyte with 8 mM arginine as a spacer. The focusing voltage/time program applied for all antibody samples was 1500 V for 1 min, 3000 V for 1 min, and 4500 V for 4.5 min. Analysis of mAbs 3 and 4 was accomplished using 0.0125 mg/mL pH 3.38 and 9.99 pI markers in 4.0% pH 3–10 Pharmalyte with 8 mM arginine and 0.125 mM iminodiacetic acid as spacers. Anolyte and mobilizer concentrations were 1% formic acid and catholye concentration was 1% DEA. Final mAb concentrations were 250 µg/mL in the solutions described above.

All icIEF control experiments were performed on an iCE3 Analyzer (ProteinSimple). The separation cartridge column was a 5 cm long coated fused-silica capillary (100 µm ID, 200 µm OD). The detection system imaged the whole column with a UV absorption detector monitoring at a fixed wavelength of 280 nm. The control mAb solutions analyzed with the iCE3 were prepared with two pI markers (6.14 and 9.50), 4.0% carrier ampholytes (pH 3–10), 0.25% methyl cellulose, 2.0 M urea, and the mAb samples at 0.3 mg/mL concentration. The samples were prefocused at 1.5 kV for 1 min, followed by focusing for 6 min at 3 kV at ambient temperature and the charge profiles were acquired and analyzed.

In both types of IEF analyses, the unstressed peak with the greatest area was defined as the main peak of the monoclonal antibody. Total acidic and basic species were the sum of all peaks with pI values lower and higher than that of the main peak, respectively, expressed as a percentage of the total peak area.

The research breadboard Blaze icIEF-MS system ("icIEF-MS system") was operated through control software programmed to automatically apply pressures to solutions for microfluidic control; adjust voltages for stepped separation and mobilization; acquire full-channel 280 nm absorbance data for monitoring of separation and relative quantitation of peaks; detect currents for maintaining electrospray stability; and trigger synchronized MS analysis. For each injection, the system primed the microfluidic chip channels (anolyte, catholyte, mobilizer, and sample) with the appropriate solution leaving the separation channel of the chip filled with the sample. After taking dark and background UV absorbance images of the channel, focusing began by applying 1.5 kV between the anolyte and catholyte for 1 min, followed by 3 kV for 1 min, and 4.5 kV for 4.5 min. During focusing, compounds migrated to their respective isoelectric points while being monitored by UV imaging every 4 s. After focusing, an electrophoretic voltage offset of 3 kV was applied between the anode and mobilizer to electrophorese the focused proteoform bands into the MS-compatible mobilizer solution flowing at the chip tip. The system continuously monitored the currents at each microfluidic channel and algorithmically adjusted the voltages to maintain the chip tip electrospray voltage at 4 kV for stable electrospray into the mass spectrometer. The microfluidic chip-based integrated icIEF-MS technology included an integrated ESI tip and was coupled to a Q Exactive+ mass spectrometer (Thermo, San Jose, USA) to enable mass identification of the separated charge variants. MS acquisition was performed in a high mass range, positive ion mode with a capillary temperature of 350°C. Collisional energy was set to 50 eV with an S Lens RF level of 150. Data were acquired from 2000–6000 m/z at 35 000 resolution. Data were processed and reports were produced using Protein Metrics Byos® software (Cupertino, CA) for spectral deconvolution and assignment. Analysis of replicate injections was performed with an in-house statistical software package. Data were initially analyzed blind, with receipt and inclusion of expected mass information from FASTA files after initial successful reporting.

icIEF was used for comprehensive charge variant analysis of the nonstressed and thermally stressed versions of an IgG1 molecule as well as for two IgG2 subclass monoclonal antibodies with various charge distributions and a range of pI values spanning 7.3–9.0. The system was also connected to a mass spectrometer for detailed analysis of one of the IgG2 molecules.

Figure 1 delineates the capabilities of the microfluidic chip-based icIEF-MS workflow, with close-up views of the key elements on the analysis. A schematic of microfluidic separation and ionization chip with electrospray outlet, inlets for solvents, and electrodes at (1) anode, (2) cathode, and (3) mobilizer is shown in Figure 1A). The separation channel was filled with the sample solution mixed with the carrier ampholytes and pI markers (B). The focusing step started by applying an electric potential between the anolyte and catholyte ports. The entire time, the detection system imaged the entire separation profile, within the UV detection window shown, generating traces for the charge variant profiles along the pI gradient (C). For mobilization, the electrophoresis circuit was switched to apply voltage between the anolyte and mobilizer ports to drive the focused peaks toward mobilizer solution flowing out of the ESI tip (D) for simultaneous ESI into the downstream mass spectrometer for detection and analysis (E).

An enabling aspect to this icIEF-MS system is the comparability between the icIEF-UV results from this system and the data from the existing capillary-based icIEF-UV system. Although the cIEF-separation reagents were different between the two systems, this work shows the similarity in results, despite being a different separation channel architecture and reagent conditions. To enable icIEF-MS, separation methods with MS-friendly reagents were developed. Instead of phosphoric acid, formic acid – a volatile, MS-friendly acid– was used in the anolyte and mobilizer solution. Instead of sodium hydroxide, DEA was

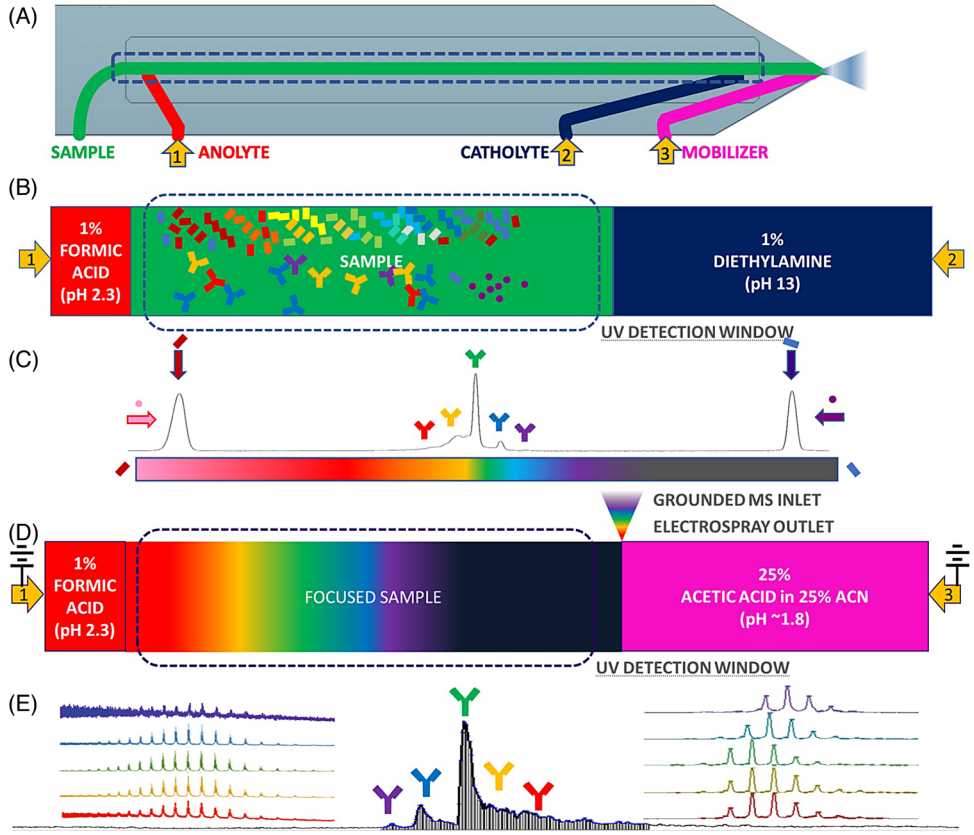

FIGURE 1 The integrated icIEF-MS workflow for the analysis of intact protein therapeutics. (A) Schematic of the prototype microfluidic separation and ionization chip with electrospray outlet, inlets for solvents, and electrodes at (1) anode, (2) cathode, and (3) mobilizer. (B) Schematic of path for separation showing primed anolyte, sample, and catholyte solutions in the channels. (C) Focused sample UV absorbance electropherogram with highlighted pI markers, sample, ampholyte gradient, and stackers. (D) Schematic for path for initiation of mobilization and ESI of the separated sample. (E) Time-resolved base peak intensity plot with inset normalized raw (right) and deconvoluted (left) mass spectra corresponding to highlighted peaks matching the icIEF data collected on the prototype

used in the formulation of the catholyte. In addition, the separation channel in the microfluidic chips was covered by a hydrophilic coating, reducing EOF and eliminating the need for methylcellulose, an MS-incompatible reagent, in the sample solution. Likewise, the urea that is regularly used in conventional IEF systems can be replaced by formamide, a volatile MS-compatible reagent.

To establish comparability in the icIEF separation and quantitation by UV between the icIEF-MS platform and the control icIEF methodology described in the experimental section, the icIEF analysis of the four development-stage monoclonal antibodies (including a thermal exposure-stressed sample) was performed using only the imaged cIEF part of the platform with UV detection. This was important to determine the appropriate focusing parameters to obtain equivalent separation performance.

Figure 2 shows the icIEF-UV traces of the four samples analyzed on the icIEF-MS system, with quantitated results compared to control icIEF data in Table 1. Traces A and B depict the electropherograms of the nonstressed IgG 1 molecule (mAb1) and its thermally stressed form (mAb2/TSmAb1), respectively. For the nonstressed sample, in trace A, five well-separated components can be observed, with peak 3 being the main component (relative area: 50.2%, Table 1), having the pI value of 8.4. Peaks 1 and 2 (combined relative area: 21.4%) are acidic and peaks 4 and 5 (combined relative areas: 28.5%) are basic variants of the main component. Trace B shows the resulting electropherogram with significant changes in the peak profile for the mAb1 sample exposed to thermal stress conditions (TSmAb1). While the relative peak area of the acidic variants only slightly decreased, as shown in Table 1 (21.4 to 16.1%), the main component (peak 3) significantly decreased from 50.2 to 23.6%. Consequently, the total relative peak areas of the basic variants of peaks 4 and 5 increased from 28.5 to 60.9%. Measurements from both systems show comparable UV separation profiles and comparable quantitative results for both unstressed and stressed mAbs.

Analysis of the mAb3 sample (trace C) also shows five separated peaks with two acidic (peaks 1 and 2: 18.5%) and two basic variants (peaks 4 and 5: 13.7%) besides the main

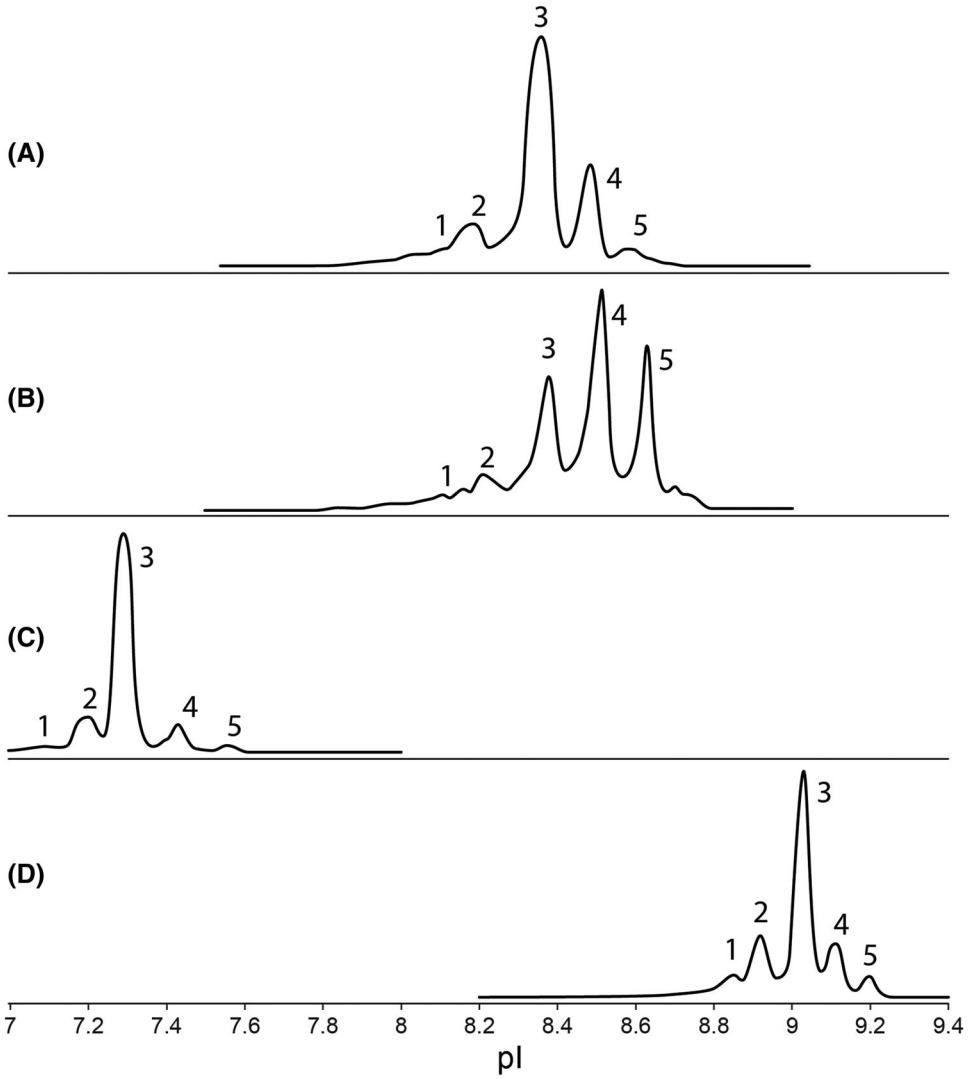

FIGURE 2 Imaged cIEF analysis of four development phase monoclonal antibodies spanning a range of pI values from 7.3 to 9.0 and both subclass IgG1 and IgG2. Traces: (A) mAb1 (IgG1 subclass), (B) mAb2/TSmAb1 (thermal stressed mAb1), (C) mAb3 (IgG2 subclass), and (D) mAb4 (IgG2 subclass)

TABLE 1 Comparison of the apparent pI values and relative peak area % distributions between the icIEF and control system experiments for the four test mAbs across a wide range of pI values, representing two mAb subtypes

		Main peak pI	Total % acidic	Main %	Total % basic
mAb1	**Control**	8.5	17.4	51.5	31.1
	icIEF-MS	8.4	21.4	50.2	28.5
mAb2/TSmA b1	**Control**	8.5	16.0	23.1	60.9
	icIEF-MS	8.5	16.1	23.6	60.9
mAb3	**Control**	7.2	19.8	67.9	12.2
	icIEF-MS	7.3	18.5	67.8	13.7
mAb4	**Control**	8.9	31.0	48.9	20.2
	icIEF-MS	9.0	30.0	47.3	22.6

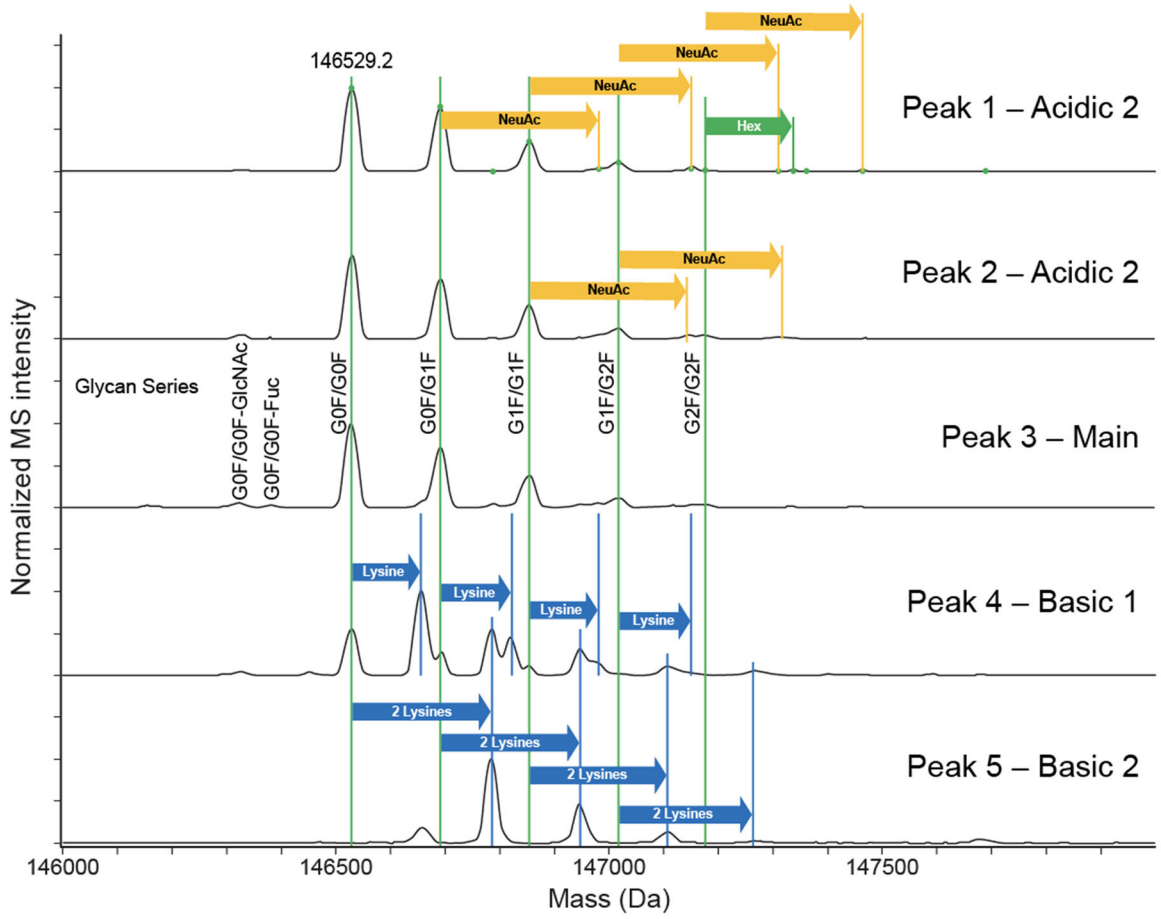

FIGURE 3 Deconvoluted mass spectra for detailed charge variant analysis of mAb 4. The most abundant peak 3 is comprised of a typical glycosylation pattern comprised on combinations of G0F, G1F, and G2F. Low abundance glycans, including G0 and G0F-GlcNAc, are also measured. Proteoforms that comprise peaks 1 and 2 have a lower pI and contain glycans with *N*-acetyl neuraminic acid resulting in an acidic pI shift. A hexose addition was measured in the acidic peak 1, and putatively identified as a glycation, a glucose modification to lysine that also results in an acidic pI shift. Basic peaks (4 and 5) were modified by either one or two unprocessed lysines that result in a basic shift

component with a pI of 7.3 (peak 3: 67.8%). The overall charge of this IgG2 molecule was at the lower pI region of the range investigated. Conversely, the icIEF analysis of mAb4 (trace D), another IgG2 subtype mAb, showed five peaks, all toward the higher pI region of the analyzed pH interval. The main component (peak 3) had a measured pI value of 9.0 and comprised 47.3% relative peak area of the separated components. Peaks 1 and 2 represent the acidic, while peaks 4 and 5 the basic variants with total relative peak areas of 30.0% (peaks 1 and 2) and 22.6% (peaks 4 and 5), respectively.

Table 1 lists main peak pI values and relative peak area % distributions in comparison to the control data, demonstrating the very high comparability of the icIEF-MS platform used in the experiments to one of the frequently utilized methods in the biopharmaceutical industry (Control lines in Table 1).

The icIEF system used in this study was coupled to a high-resolution mass spectrometer, thus, comprehensive structural identification—otherwise not available by using the control cIEF alone—was generated. The downstream deconvoluted MS characterization of the charge variants is shown in Figure 3, which shows the mass assignment details for the charge variants in the mAb4 sample (IgG2 subtype) analyzed with the icIEF-MS system. The middle trace (Main) shows the deconvoluted mass spectrum of the main component with multiple 162 Da mass shifts, corresponding to the increasing galactosylation level in the G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, G2F/G2F (where F indicates the presence of core fucose and the number corresponds to the galactoses on the biantennary glycan) containing species, representing all permutations of these biantennary glycan structures. It is important to note that the high sensitivity of the system used also identified the trace components of the low abundance G0F/G0F-*N*-acetylglucosamine (GlcNAc) and the G0F/G0 afucosylated glycoforms. For the two basic components (peaks 4 and 5 in Figure 2, trace D), the respective nominal mass additions

of 128 (trace Basic 1) and 256 Da (trace Basic 2) shifting all distributions relative to the main galactosylation series peaks suggested the presence of one and two additional lysine residues, respectively, representing proteoforms containing unprocessed lysine. At the acidic side (peaks 1 and 2 in Figure 2, trace D), addition of two and four sialic acids (with mass increments of 291 Da) were identified in the acidic 1 and acidic 2 groups (upper traces), respectively. An interesting feature in the upper trace is a hexose addition to the acidic 2 variant, seen as the 162 Da mass addition to the G2F/G2F peak. The most abundant proteoform of mAb4 in the main peak was G0F/G0F. The measured mass was 1 46 528 Da, revealing mass agreement of within 1 Da between the blinded data acquired on the icIEF-MS system and 1 46 529 Da measured for the control MS experiments accomplished by infusion.

IEF with quantitation, identification, and characterization of the charge variant peaks of several biotherapeutic mAbs was demonstrated by using the icIEF-MS system. First, rapid and high-resolution imaged IEF analysis, including pI measurement and relative peak quantitation of IgG1 and IgG2 subclass mAbs, was demonstrated to be comparable to historical icIEF results from the classic approach used in the biopharmaceutical industry. The IgG1 subclass mAb was analyzed in its nonstressed and thermal-stressed forms, revealing a decomposition profile in agreement with control data. Primary structural elucidation of one of the IgG2 subclass mAbs was also demonstrated, revealing basic and acidic shifts corresponding to unprocessed lysine residues and additions of sialic acids, respectively. In addition to the comprehensive charge variant determination, the icIEF-MS system detected glycation and very low abundance glycoforms including G0 and G0F-GlcNAc. While the results revealed good comparability with other commercially imaged cIEF methods, the online icIEF-MS platform offered additional immediate peak identification by MS, thereby significantly accelerating the elucidation of icIEF peaks.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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