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Capillary Isoelectric Focusing-Mass Spectrometry Method for the Separation and Online Characterization of Intact Monoclonal Antibody Charge Variants

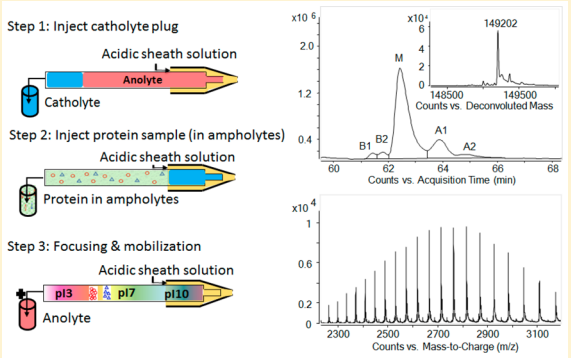
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Supporting Information

ABSTRACT: We report a new online capillary isoelectric focusing-mass spectrometry (CIEF-MS) method for monoclonal antibody (mAb) charge variant analysis using an electrokinetically pumped sheath-flow nanospray ion source and a time-of-flight MS with pressure-assisted chemical mobilization. To develop a successful, reliable CIEF-MS method for mAb, we have selected and optimized many critical, interrelating reagents and parameters that include (1) MS-friendly anolyte and catholyte; (2) a glycerol enhanced sample mixture that reduced non-CIEF electrophoretic mobility and band broadening; (3) ampholyte selected for balancing resolution and MS sensitivity; (4) sheath liquid composition optimized for efficient focusing, mobilization, and electrospray ionization; (5) judiciously selected CIEF running parameters including injection amount, field strength, and applied pressure. The fundamental premise of CIEF was well maintained as verified by the linear correlation (R^2 = 0.99) between p*I* values and migration time using a mixture of p*I* markers. In addition, the charge variant profiles of trastuzumab, bevacizumab, infliximab, and cetuximab, obtained using this CIEF-MS method, were corroborated by imaged CIEF-UV (iCIEF-UV) analyses. The relative standard deviations (RSD) of absolute migration time of p*I* markers were all less than 5% (n = 4). Triplicate analyses of bevacizumab showed RSD less than 1% for relative migration time to an internal standard and RSD of 7% for absolute MS peak area. Moreover, the antibody charge variants were characterized using the online intact MS data. To the best of our knowledge, this is the first time that direct online MS detection and characterization were achieved for mAb charge variants resolved by CIEF as indicated by a well-established linear pH gradient and correlated CIEF-UV charge variant profiles.



Use of recombinant mAbs as therapeutics is rapidly growing throughout the worldwide biopharma industry.[1] Unlike synthesized chemical drugs, biologics are cell-originated and are subject to post-translational modifications (PTMs) such as glycosylation, C-terminal lysine truncation, deamidation, disulfide-scrambling, glycation, and methionine or tryptophan oxidation.[2,3] As these PTMs usually lead to changes in protein isoelectric point (p*I*), mAb drug substances are known to exhibit charge heterogeneity, represented by multiple charge variants with different p*I*s.[2] Comprehensive characterization of mAb charge variants is crucial, as the variants may affect the *in vitro* and *in vivo* properties related to its activity, safety, developability, and quality.[4−8] In addition, charge variant characterization is also important for developing biosimilar mAbs.[9,10]

Charge-based separation techniques such as ion-exchange chromatography,[11,12] capillary zone electrophoresis (CZE),[13,14] and CIEF[15,16] are commonly used for charge variant analysis. CIEF, or later developed imaged CIEF (iCIEF),[17−19] has become an important technique for charge variant analysis and been applied routinely, mainly using optical detection methods. On the other hand, although MS has been well established as a powerful technique for detection, characterization and identification of therapeutic mAbs,[20] effective online hyphenation of CIEF to electrospray ionization MS (ESI-MS) remains to be an unmet challenge.[21,22] The lack of online MS identification has limited CIEF's usefulness in characterizing charge variant profiles of therapeutic proteins. Combining the high-resolution capability of CIEF and the unparalleled characterization power of MS would yield a highly desirable hyphenated analytical technique for deciphering mAb charge heterogeneity.

The online coupling of CIEF separation and ESI-MS detection has been technically challenging for a number of reasons.[22] First, the coupling of capillary electrophoresis (CE) and MS has been difficult due to interference between CE electrical circuits and ESI.[23] Traditional sheath liquid-based CE-MS coupling devices use sheath liquid flow delivered by a syringe pump at several orders of magnitude higher (e.g., triple tube design uses a few microliters per minute of sheath liquid

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flow[24]) than the analyte flow out of the capillary. Severe analyte dilution by sheath liquid diminishes sensitivity that is needed for MS detection of minor charge variant species, which are typically at the abundance level of 1−10% of the main species.[2] Second, the two-step CIEF process, i.e., focusing followed by mobilization, can hinder effective automation for CIEF-MS hyphenation. For traditional coaxial sheath liquid CIEF-MS hyphenation, the solution at the cathodic end needs to be manually switched from a basic catholyte at focusing to an acidic solution at mobilization to provide a stable electrospray.[25] Mokaddem et al. developed an automated protocol using one fixed sheath liquid in which the terminal end of the CIEF capillary was injected with a plug of basic catholyte and the anodic end was filled with the sample ampholyte mixture. Pressure mobilization was applied after focusing for MS detection.[26,27] A number of other strategies have been reported and reviewed for semionline or online CIEF-MS coupling.[22,28]

Moreover, key reagents used in traditional CIEF-UV analysis, such as carrier ampholyte and additives of methylcellulose and urea, are not ESI-MS compatible. In traditional CIEF, neutral high molecular weight polymer additives, such as methylcellulose, are used to modify the capillary inner surface in order to reduce the impeding effect of electroosmotic flow (EOF), to increase viscosity thus reducing band broadening during the mobilization process, and to mitigate protein adsorption on the capillary wall.[29] Because no such additive can be used due to MS incompatibility, diffusion of the focused protein zones becomes problematic, resulting in peak broadening with poor resolution in CIEF-MS analysis. In addition, ampholyte can only be used at a much lower concentration in CIEF-MS due to ion suppression compared to CIEF-UV.[25] Consequently, the CIEF-MS separation has low efficiency and the pH gradient is more easily disturbed during mobilization. Moreover, because of the physical configuration of most conventional CE-MS instrumentation, a longer capillary with a longer mobilization process is used compared to CIEF-UV, thus the above detrimental effects are more severe. Another important potential adverse factor is that proteins are prone to aggregate once being focused into narrow zones at their p*I* values. Without using additives (e.g., urea, methylcellulose) to help maintain protein solubility and reduce wall interactions in CIEF-MS, capillaries are susceptible to degradation due to deposition of protein aggregates on the capillary inner surface, causing poor recovery and irreproducible analysis.[30,31]

Instead of methylcellulose and urea, Varenne and co-workers reported the use of glycerol as an anticonvective medium in CIEF-MS.[26,27,32] By increasing viscosity with glycerol, efficient focusing was observed, leading to thinner and more concentrated bands of myoglobin and other model proteins. In total, 30−40% glycerol was found to be the optimum concentration for their CIEF-MS analysis. All acidic analytes in the study, however, showed significant migration time shifts and p*I* perturbation. This could be caused by the disturbing effects from residual EOF as bare fused-silica capillaries were used and from band diffusion induced by the high pressure (50 mbar) used during mobilization. These disturbing effects were more severe for acidic analytes as they traveled a longer path prior to detection. In addition, Mendes et al. studied the effect of glycerol on ESI signal suppression.[33] They demonstrated that 1% glycerol could completely suppress the signals of myoglobin and ovalbumin, possibly because of the strong hydrogen bonding interactions between glycerol molecules and the polar sites of the side chain of the amino residues from the proteins. In the past several years, use of glycerol for CIEF-MS analysis has been only sporadically reported in the literature,[22,28] despite its potential for being a protein solubilizing and stabilizing reagent and an anticonvective CIEF medium.

Several CE-MS techniques have been used for mAb charge variant analysis. Microfluidic CE-MS devices have recently been reported for the separation of intact mAb charge variants with online ESI-MS detection.[34] In addition, a sheathless CE-MS interface has been introduced for sensitive MS detection and has been applied for mAb charge variant analysis.[35] A mechanical valve based two-dimensional CE-MS strategy has been developed to circumvent the MS interference from the non/low volatile CE reagents.[36] Using this concept, both ampholyte interference-free CIEF-CZE-MS[37] and two-dimensional CZE-CZE-MS[38] have been implemented for mAb charge variant analysis. Although these recent reports hold promise, they rely on either specialized devices or customized parts and multiple CE instruments.

An electrokinetically pumped sheath-flow nanospray CE-MS interface has been developed by the Dovichi group.[39−41] Its low sheath flow (20−50 nL/min) significantly reduces analyte dilution at MS detection. Using this interface and an injection concept similar to the one reported by Mokaddem,[26,27] Zhu et al. reported automated CIEF-MS of small model proteins by effective chemical mobilization without applying pressure.[42] Compared with pressure mobilization, chemical mobilization normally has the advantage of minimum band broadening by overcoming the hydrodynamic parabolic flow profile.[43,44] They demonstrated that once a plug of ammonium hydroxide-based catholyte was injected into the capillary, the acid-based sheath liquid in the electrospray emitter gradually titrated the catholyte, resulting in a unique sequence of simultaneous isoelectric focusing of the sample and titration of the catholyte, followed by chemical mobilization once the titration is complete.[42]

In the present work, we sought to develop a reliable, high-resolution CIEF-MS method tailored for mAb charge variant analysis using a common capillary-based CE and a time-of-flight (TOF) MS system. In order to address the above-mentioned technical challenges of traditional CE-MS interfaces, we adopted a commercial EMASS-II CE-MS ion source that is based on the electrokinetically pumped sheath liquid technology.[39−41]

Separation of mAb charge variants is more challenging than separation of small model proteins, such as those reported by Zhu,[42] because much higher resolution is required for mAb charge variants (with p*I* differences as small as 0.1) than for small proteins and also because high MS sensitivity is needed for minor charge variant species. In addition, the ionization of mAbs can be different and more complex than that of small proteins. To develop a reliable CIEF-MS method for challenging mAb charge variant analyses, many parameters and their interrelating effects need to be investigated and optimized. These include selection and optimization of ampholyte, composition of sheath liquid, injection plug length of catholyte and sample, and determination of CIEF running parameters. Moreover, non-CIEF mechanisms, such as CZE and band diffusion of the analytes must be effectively minimized in order to maintain the CIEF resolution during chemical mobilization prior to MS detection.

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EXPERIMENTAL SECTION

Reagents. Cytochrome C, myoglobin, carbonic anhydrase, Pharmalyte 3-10 (GE Healthcare), glycerol, urea, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). LC−MS grade reagents, including water, acetic acid, formic acid, ammonium hydroxide, acetonitrile, and methanol were also obtained from Sigma-Aldrich. p*I* markers (4.1, 5.5, 7.0, and 9.5) were obtained from Sciex (Framingham, MA). Infliximab, trastuzumab, bevacizumab, and cetuximab were purchased from Komtur Pharmaceuticals (Edgewater, NJ).

Online CIEF-MS. The CIEF separations were performed on an Agilent 7100 CE system (Agilent Technologies, Santa Clara, CA). An Agilent 6224 TOF mass spectrometer was coupled with the Agilent 7100 CE using an EMASS-II CE-MS ion source (CMP Scientific Corp., Brooklyn, NY). Electrospray emitters used for the online CIEF-MS analysis were manufactured by CMP Scientific Corp (1.0 mm o.d., 0.75 mm i.d., 20−30 μm tip size). Neutral coating PS1 capillaries purchased from CMP were used for CIEF-MS separation. Capillaries 75 cm long with 360 μm o.d. and 50 μm i.d. were selected to optimize analysis time, resolution, and MS sensitivity. Ammonium hydroxide aqueous solution (0.2 N NH_4OH with 15% glycerol) was used as catholyte. The anolyte was 1% formic acid with 15% glycerol. Unless stated otherwise, the sheath liquid was either 20% acetic acid with 25% acetonitrile or 0.5% formic acid with 50% methanol. Protein samples (0.1−1.0 mg/mL) were prepared in 1.5% Pharmalyte 3-10 with 5−20% glycerol. Myoglobin at 0.02 mg/mL was added to the mAb samples as an internal standard. The catholyte solution was injected under 950 mbar for 10 s, which was followed by sample injection under 950 mbar for 72−75 s. After catholyte and sample injection, a voltage was applied under normal polarity with a field strength of 250 V/cm. A small pressure of 10 mbar was applied on the capillary inlet (anode) end to assist mobilization. The schematic flow of the CIEF-MS experiment was shown in Supplemental Figure S-1. The electrospray ionization voltage was set at 2.0 to 2.4 kV using the external high voltage power supply that came with the EMASS-II ion source. The distance from the emitter tip to the mass spectrometer was adjusted to be between 2 to 4 mm with the help of a microscope camera.

The regular ESI ion source on the 6224 TOF was modified to accommodate nanospray by replacing the ESI spray shield with a nanospray shield and a drying gas diverter. The TOF capillary voltage was set at 0 V. The drying gas was set at 6 L/min and 350 °C. The fragmentor voltage was set at 125 V for p*I* markers and small proteins and 400 V for mAbs. The m/z range was set at 500−3200 for mAb and 250−3200 for p*I* markers and small proteins. The acquisition rate was one spectrum per second.

Capillary Cleaning Procedure. The PS1 capillary was rinsed at 950 mbar at the end of day for cleaning, in the order of water (5 min), 4.5 M urea (5 min), water (5 min), followed by anolyte buffer rinsing (10 min).

Protein Desalting Procedure. The salt-free protein standards bought from Sigma were used without desalting. The mAb samples were desalted using EMD Millipore (Billerica, MA) Amicon Ultra-30 centrifugal filter units, and buffer exchanged to 5 mM ammonium acetate (pH 6.5).

Data Analysis. The MS data acquisition and analysis were performed using Agilent Mass Hunter software. The CE method setup and operation were performed using Agilent ChemStation software.

Imaged CIEF-UV. The iCIEF-UV separations were performed on an iCE3 (ProteinSimple, San Jose, CA) equipped with an Alcott 720 autosampler. Fluorocarbon-coated capillary cartridges (5 cm × 100 μm i.d.) were used. The anolyte was 80 mM phosphoric acid and the catholyte was 100 mM sodium hydroxide, both in 0.1% methylcellulose. Sample buffer contained 0.35% methylcellulose, 4% Pharmalyte 3-10, 2 M urea, and 0.1% p*I* markers (p*I* 4.6 and 9.5). The final protein concentration in the sample buffer was 0.2 mg/mL. Focusing was conducted at 1.5 kV for 1 min, followed by 3.0 kV for 8 min. UV detection was set at 280 nm. The reagents and capillaries used for iCIEF-UV analyses were obtained from ProteinSimple.

RESULTS AND DISCUSSION

The aim of this work was to develop a reliable and automated CIEF-MS method in order to enable online MS characterization of mAb charge variants that are resolvable in the more established CIEF-UV analyses. For this purpose, extensive studies of interrelating effects of many critical reagents and parameters were conducted. For example, glycerol was identified as an appropriate additive, while sheath liquid composition was investigated as it dictated whether glycerol could be used. The amount of catholyte (determined by the injection plug) and specific sheath liquid titrating the catholyte, along with ampholyte composition and glycerol content determined the focusing time, and impacted mobilization and ionization efficiency. Capillary field strength and pressure were critical to prevent peak migration order inversion by CZE effect, and to minimize band broadening by unwanted laminar flow. Several important method development considerations were evaluated, and the performance of the resulting original CIEF-MS method for the charge variant analyses of four mAbs was determined.

Electrokinetically Pumped Sheath Liquid Nanospray CE-MS on a Time-of-Flight Mass Spectrometer for CIEF-MS. We interfaced an Agilent 7100 CE with a 6224 TOF MS using the EMASS-II ion source that was built based on electrokinetically pumped sheath liquid nanospray CE-MS technology. With the regular ESI source spray shield on the 6224 TOF MS, we discovered that the high volume of drying gas blowing directly toward the electrospray emitter on the EMASS-II ion source prevented efficient introduction of analytes into the MS. In order to avoid this confronting drying gas flow, we adopted a single bore inline spray shield with a radial gas diverter. The drying gas that came out of the MS was diverted radially, thus allowing the nanospray formed on the EMASS-II to be effectively delivered to the MS.

Ampholyte Selection and Concentration. After testing a number of commercially available ampholytes, we selected Pharmalyte 3-10 to minimize interference with intact mAb MS and to compromise between ion suppression and CIEF resolving efficiency. The same ampholyte was used in our iCIEF-UV analysis which allowed direct comparison between iCIEF-UV and CIEF-MS profiles.

While the components of ampholytes are usually from 400 to 1500 Da,[45] mAbs and their charge variants exhibited MS charge envelopes typically at the ranges of m/z 2000−4000. Thus, although MS interference from ampholytes might be troublesome for small proteins, cosprayed ampholytes and mAb exist

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in distinct regions of ampholytes and proteins (Figure S-2). This greatly simplified mass spectral deconvolution of mAbs.

Because ion suppression by ampholytes inevitably compromises the ionization efficiency of proteins, the amount of ampholytes in the sample buffer is critical for successful CIEF-MS analysis. When more than 5% (v/v) ampholyte was used in the sample buffer, no protein MS signal was observed (data not shown), indicating strong ion suppression by this high concentration of ampholyte. On the other hand, less than 1% ampholyte showed poorly resolved protein variants due to insufficient isoelectric focusing power (data not shown). For optimal MS sensitivity and CIEF resolution, we used 1.5% (v/v) ampholyte in the sample buffer.

Use of Glycerol Additive. With a sample medium containing only a low concentration of ampholyte, we observed a narrow p*I* window and insufficient resolutions of mAb charge variants. In addition, CZE instead of CIEF-based separations were found, i.e., a small protein with a lower p*I* value was detected before a large mAb with a higher p*I* (data now shown).

Although glycerol was only reported in CIEF-MS analysis of small proteins for a few applications, we studied the proper amount of glycerol in combination with other CIEF experimental conditions. By adding 15−20% glycerol to the catholyte, samples, and anolyte solutions, we attained a stable, linear pH gradient along the migration path and managed to send the focused mAb charge variants to the ESI-MS with little perturbation in resolution. As shown in the following section, using acetonitrile, instead of methanol, with the properly selected acid in the sheath liquid, we were able to obtain excellent ESI-MS spectra of mAbs in the glycerol medium. To minimize unwanted CZE, and to find a balance between pressure-induced band broadening and reasonable analysis time for the viscous glycerol enhanced CIEF medium, we employed a pressure-assisted chemical mobilization using a moderate field strength (250 V/cm) with a small constant pressure (10 mbar). As glycerol stabilized proteins and reduced their adsorption to the inner capillary wall, it helped us to achieve good reproducibility. In addition, we observed lower current with glycerol in the capillary during focusing, which significantly reduced Joule heating, leading to sharper peaks for higher resolution and MS sensitivity.

Sheath Liquid Selection for mAbs. Sheath liquid must be optimized to ensure a well-controlled, gradual catholyte titration for effective focusing and stable chemical mobilization and to effectively ionize the mAb for MS detection. During method development, we found that formic acid and methanol-based sheath liquid (0.1−0.5% formic acid, 10−50% methanol) worked well for peptides and small proteins (Figure S-3a). However, almost no mAb MS signal was observed using methanol as the organic modifier in the sheath solution (Figure S-3c). This may be due to that, for large proteins, a stronger organic solvent than methanol is needed to break the hydrogen bonding interactions between glycerol and protein in order to realize effective MS ionization. By using acetonitrile in the sheath solution, we obtained excellent mAb spectra without significant interference from ampholyte and glycerol. In addition, we found that acetonitrile and acetic acid based sheath liquid (10−30% acetic acid, 25−50% acetonitrile) generated a 10−20 fold increase in MS signal for mAb samples compared to acetonitrile−formic acid based sheath solution (Figure S-4). This might be because that acetic acid is a better solubilizer for mAb samples compared to formic acid.[46]

Capillary Coating Stability and Urea Rinse. A major technical challenge for a robust CIEF-MS analysis is the capillary coating stability. As proteins migrate to form focused zones inside the capillary, they tend to aggregate and precipitate on the capillary inner wall. After a number of sample injections, the capillary coating can be compromised by the adsorption of proteins, resulting in aberrant capillary current during isoelectric focusing and deteriorated CIEF separation with poor reproducibility.

In CIEF-UV experiments, a urea rinsing procedure has been reported to be effective in cleaning the capillary inner wall after CIEF experiments.[30] Since urea is not compatible with ESI-MS, we implemented a urea cleaning procedure by removing the electrospray emitter and moving the capillary outlet away from the MS entrance. Implementing this urea rinsing procedure at the end of a day has led to good reproducibility and extended the lifetime of coated capillaries.

Throughout this study, we used PS1 capillaries. A few tested neutral coating capillaries from other sources seemed to have instability issues with ammonium hydroxide which was injected as catholyte (data not shown). As most commercially available capillaries have proprietary coating chemistry, looking into their intrinsic properties and comparing them is not possible.

Linearity of Established pH Gradient. An important performance aspect of a CIEF-MS method is its reproducibility and applicability for various types of molecules. For this CIEF-MS method development, we coinjected four peptide p*I* markers (p*I* 4.1, 5.5, 7, and 9.5) with protein standards (cytochrome C, myoglobin, and carbonic anhydrase) to examine reliability and linearity of the pH gradient established by the method. Figure 1 shows the separation of four p*I* markers. Linear regression between the p*I* values and corresponding migration time yielded a correlation coefficient of 0.99 (Figure S-5). This excellent linearity clearly indicates that the pH gradient was effectively established inside the capillary and well retained during mobilization for the CIEF-MS analysis. In addition, the RSD of absolute migration times of all four p*I* markers were less than 5% (n = 4, Table S-1).

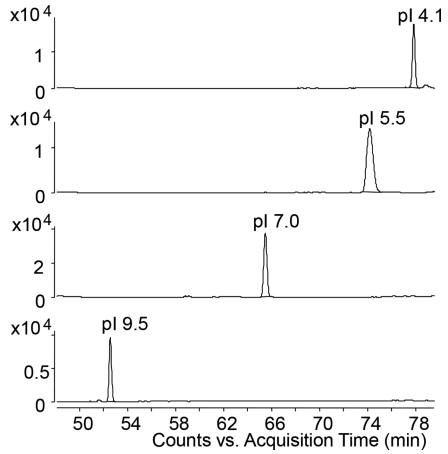


Figure 1. CIEF-MS separation of four p*I* markers. The extracted ion electropherograms of p*I* 4.1 (m/z 591.25), p*I* 5.5 (m/z 471.19), p*I* 7.0 (m/z 627.29), and p*I* 9.5 (m/z 950.47) are displayed from top to bottom.

CIEF-MS Intact Charge Variant Characterization of mAbs. The optimized CIEF-MS method was then applied to analyze four marketed mAbs: bevacizumab, trastuzumab,

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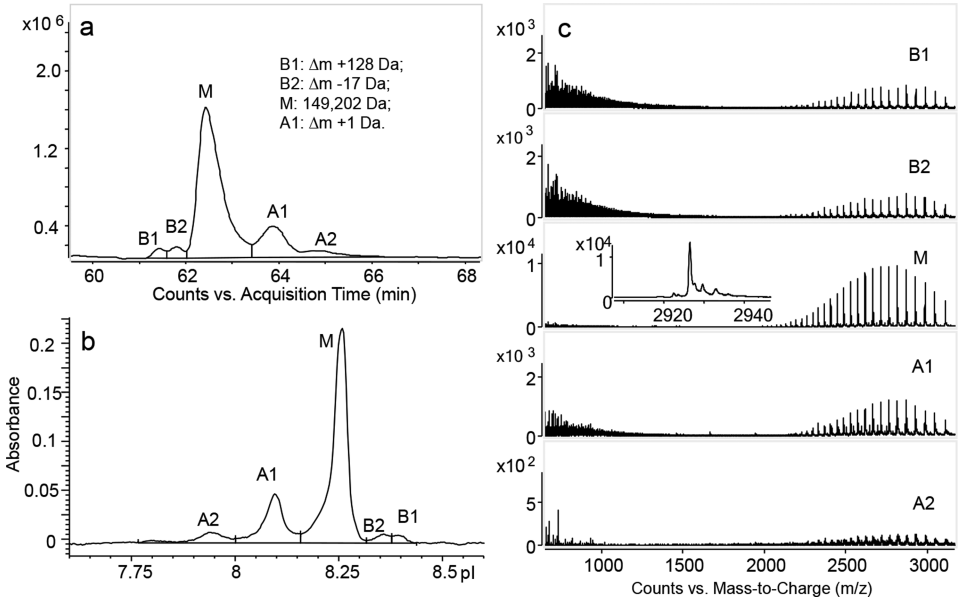


Figure 2. Bevacizumab CIEF-MS analysis in comparison with iCIEF-UV: (a) CIEF-MS extracted ion electropherogram (m/z 2960−3200 Da) showing basic variants B1 and B2, main peak M, and acidic variants A1 and A2; (b) iCIEF-UV electropherogram; (c) CIEF-MS mass spectra of major variants. Mass of each variant shown in part a was based on the most abundant deconvoluted peak. The insert in part c is the expanded view of the mass spectrum of the main peak.

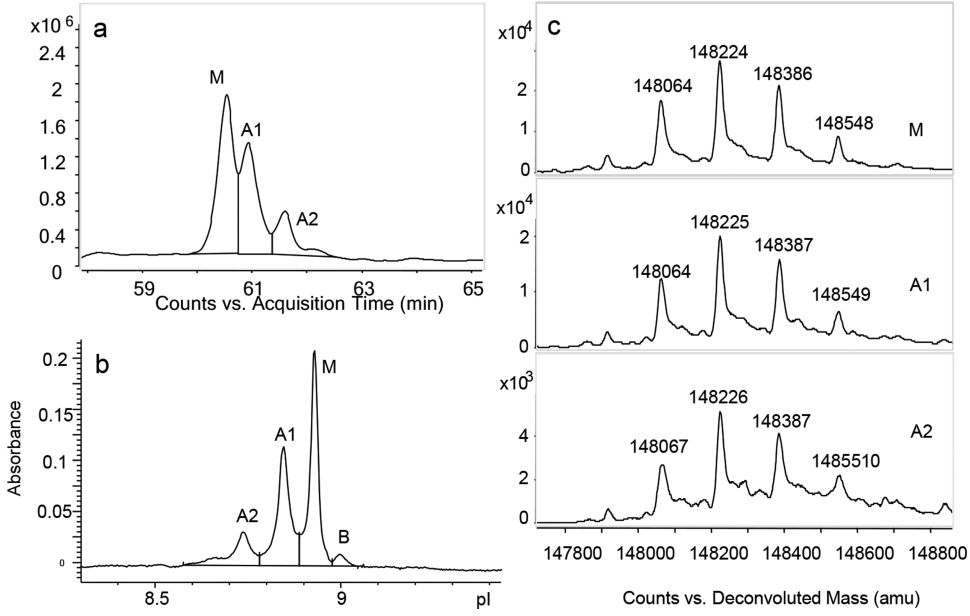


Figure 3. Trastuzumab CIEF-MS analysis in comparison with iCIEF-UV. (a) CIEF-MS extracted ion electropherogram (m/z 2960−3200 Da): main peak M, acidic variants A1 and A2; (b) iCIEF-UV electropherogram, additional basic variant B was observed; and (c) CIEF-MS deconvoluted mass spectra of main peak and acidic variants.

infliximab, and cetuximab. The charge variant profiles revealed by the CIEF-MS analysis were highly consistent with those in iCIEF-UV experiments as shown in Figures 2, 3, 4, and 5. iCIEF-UV achieves high resolution by eliminating the mobilization step using whole capillary imaging detection at the completion of focusing. Good correlations between the CIEF-MS results and the iCIEF-UV profiles (especially in the case of cetuximab) demonstrated unprecedented resolution of mAb charge variants using online CIEF-MS. In addition to resolution, our method also showed good repeatability. As shown in Figure 6, triplicate runs of bevacizumab had RSDs less than 1% on relative migration time (using myoglobin as an internal standard), 2.3% on absolute migration time, and 7% on extracted ion electropherogram peak area.

Figure 2 shows the bevacizumab CIEF-MS and iCIEF-UV results. The two profiles showed similar relative peak areas of the acidic (A1, A2) and basic (B1, B2) variants compared to that of the main species M (acidic variants−main peak−basic variants = 23%:72%:5% for CIEF-MS and 27%:68%:5% for CIEF-UV). Good MS spectra were obtained for the variants, which enabled reliable deconvolution for intact mass. Identifying the exact modifications of mAb at the intact level is challenging and often requires MS instruments with high resolution or MS/MS capability. Some mAb modifications,

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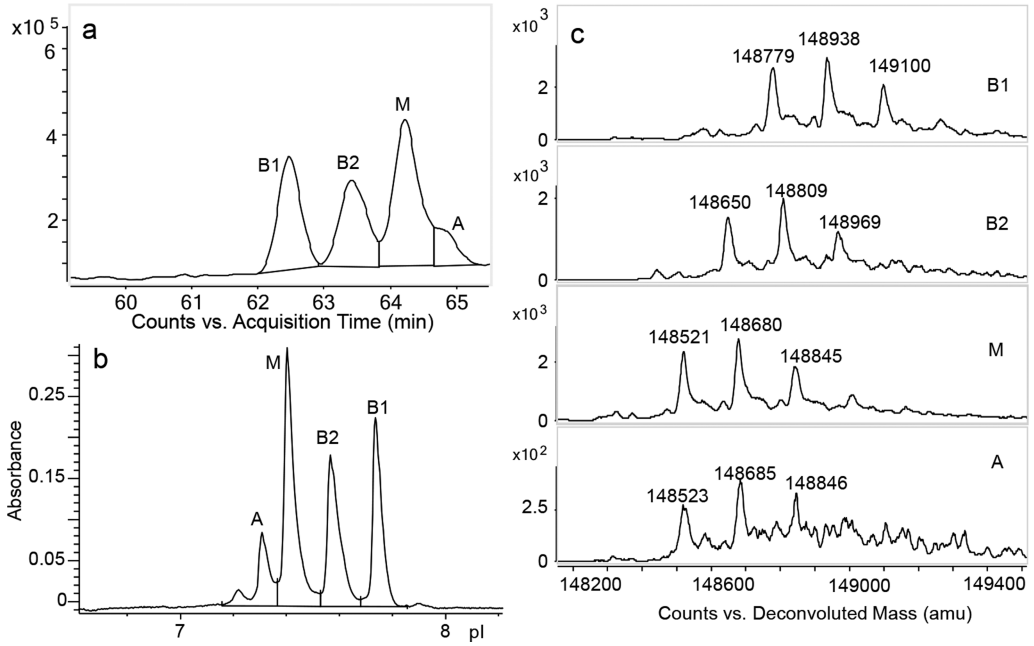


Figure 4. Infliximab CIEF-MS analysis in comparison with iCIEF-UV: (a) CIEF-MS extracted ion electropherogram (m/z 2960−3200 Da): basic variants B1 and B2, main peak M, and acidic variant A; (b) iCIEF-UV electropherogram; and (c) CIEF-MS deconvoluted mass spectra.

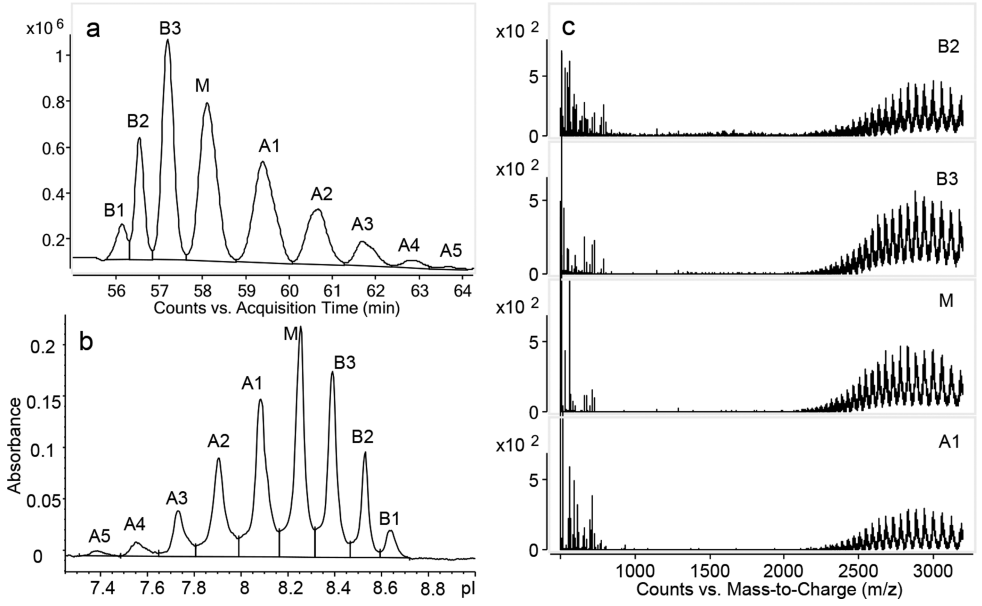


Figure 5. Cetuximab CIEF-MS analysis in comparison with iCIEF-UV. (a) CIEF-MS extracted ion electropherogram (m/z 2960−3200 Da): three basic variants B1−B3, main peak M, and five acidic variants A1−A5; (b) iCIEF-UV electropherogram; and (c) CIEF-MS mass spectra of B2, B3, M, and A1.

such as deamidation, have very small mass differences from the main species. In addition, multiple modifications may yield masses that are not resolved by MS. Nevertheless, the intact MS from CIEF-MS can provide initial insight of charge variant modifications associated with known mass differences.[2,47] According to the intact mass deconvoluted from the mass-to-charge spectra (Figure 2c), main peak M showed a mass of 149 202 Da; basic peak B1 matched the common C-terminal lysine variant with a mass difference from the main species (Δm) of 128 Da (+1K); basic peak B2 had a Δm of −17 Da, which could be due to cyclization of N-terminal glutamic acid. Acid peak A1 had a Δm of +1 Da. Although limited resolution of MS instrumentation could add uncertainty on such a small mass difference, the nature of the acidic variant and the small mass difference reasonably suggested that A1 could be a deamidation species. No reliable intact mass information was derived for peak A2 because of the weak signal. This could be due to highly glycosylated species underlying the low abundance peak A2.

The analysis of trastuzumab showed quite different charge variant distribution from that of bevacizumab (Figure 3). Although there was a minor basic peak in iCIEF-UV (Figure 3b), it was not detected in the CIEF-MS analysis (Figure 3a) likely due to its low abundance. In addition, in iCIEF-UV

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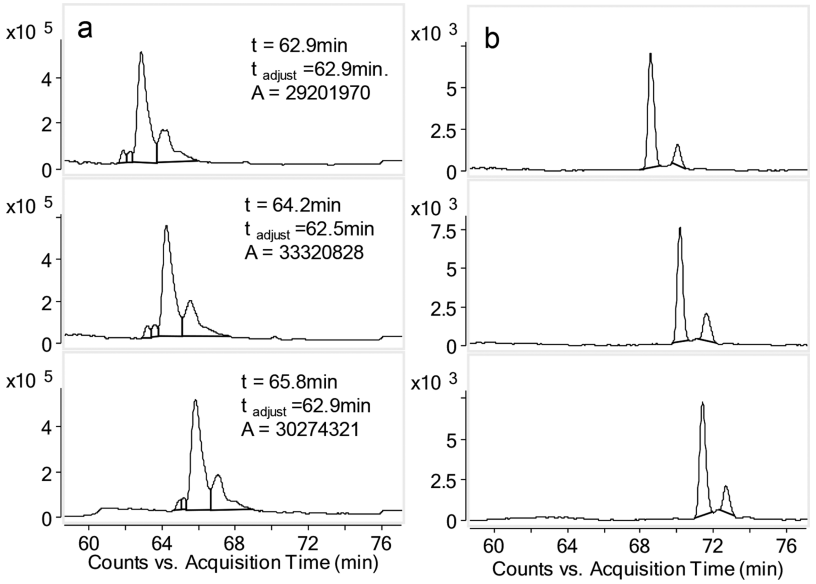


Figure 6. Triplicate CIEF-MS runs of bevacizumab with an internal standard of myoglobin: (a) extracted ion electropherogram (m/z 2960−3200 Da) of bevacizumab; (b) extracted ion electropherogram (m/z 1542 Da) of two variants of myoglobin. t, absolute migration time; t_{adjust}, migration time normalized by myoglobin; A, extracted ion electropherogram peak area.

analysis, the two acidic peaks were better resolved than those in CIEF-MS analysis. The MS spectrum of each variant clearly showed four major glycoforms. The deconvoluted intact mass of the two acidic species were consistent with deamidation species (for the most abundant glycosylation form of each variant, M = 148 224 Da, Δm = +1 Da for A1, and Δm = +2 Da for A2), which agreed with the results obtained by 2D CZE-CZE-MS.[38]

The characteristic three-peak charge variants of infliximab[34,48,49] were well separated by our CIEF-MS (Figure 4a) method. Deconvoluted mass confirmed that the two basic species were the C-terminal lysine variants.[34,48,49] The mass of basic peak B1 matched to +2K (Δm + 258 Da). The basic peak B2 was the +1K (Δm + 129 Da) variant. In addition, an acidic variant that was consistent with iCIEF (Figure 4b) was also detected. Deconvoluted mass revealed intact MS at Δm of +5 Da. Although the nature of the acidic variant modification was not immediately obvious, the small Δm indicated the possibility of deamidation species. Again, the high-quality MS spectra enabled glycoform characterization of each variant.

Cetuximab is a human/murine chimeric IgG-1 mAb[50] that has a large number of microheterogeneities. Its heterogeneity is mainly due to the complex glycosylation (with multiple sialylated glycans) resulting from two glycosylation sites on the Fab and Fc of each heavy chain and incomplete lysine clipping of the heavy chain C-terminus.[51] The high heterogeneity adds difficulty in separating variants of cetuximab at the intact level.[51,52] Our CIEF-MS analysis of cetuximab revealed eight well separated charge variants (Figure 5a). The profile was confirmed by our iCIEF-UV analysis (Figure 5b) and was consistent with that reported in the literature.[53] However, as shown in Figure 5c, the intact mass spectra were very complex for these variants due to the multiple glycoforms. The mass spectrum obtained in an LC−MS analysis (data not shown) of the same sample also showed a complex mass spectrum. Enzymatic treatments might reduce the sample heterogeneity[54] and offer better MS data interpretation. Despite the glycosylation complexity, the high resolution of our CIEF-MS method was clearly demonstrated.

CONCLUSION

We developed a new, automated CIEF-MS method that separated mAb charge variants correlating well with iCIEF-UV profiles and enabled online TOF-MS detection and characterization of the individual mAb charge variants. The method was built upon electrokinetically pumped sheath liquid nanospray CE-MS technology by implementing the EMASS-II ion source and by replacing an ESI spray shield with a nanospray shield with radial gas diverter on the Agilent TOF-MS. In order to attain high CIEF resolution and to enable sensitive MS detection of mAb charge variants, a number of critical method parameters were studied and optimized. The nature and composition of sheath liquid had a dramatic impact on effective focusing, mobilization, and ionization efficiency. We found that the sheath liquid with acetic acid and acetonitrile yielded high MS sensitivity and robust mobilization in particular with the use of glycerol. Combining with sheath liquid, pressure, and field strength optimizations, we found the use of 15−20% glycerol in the catholyte, samples, and anolyte solutions to be critical in maintaining a sufficient separation window and high resolution for the focused mAb charge variants during pressure-assisted chemical mobilization. We also determined that 1.5% (v/v) Pharmalyte 3-10 in the sample buffer was optimal for CIEF resolution and MS sensitivity. A urea-based capillary rinsing procedure was found to be beneficial to ensure repeatability of the CIEF-MS method and to extend the life of coated capillaries.

The performance of this new CIEF-MS method was demonstrated. The linear correlation between pI values and migration times showed a R^2 of 0.99 using a mixture of pI markers. In addition, the charge variant profiles of trastuzumab, bevacizumab, infliximab, and cetuximab, obtained using this CIEF-MS method, correlated well with those obtained by iCIEF-UV. Moreover, charge variants of these mAbs were characterized using the online intact MS data, and they were consistent with those reported in the literature. Reproducibility of the CIEF-MS method was excellent. The RSDs of absolute migration time of four pI markers were less than 5% (n = 4).

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Triplicate analyses of bevacizumab showed less than 1% RSD for relative migration time to an internal standard and 7% RSD for absolute MS peak area.

Although identification of exact mAb modifications at the intact level is challenging, often requiring multiple complementary techniques, the intact mass from online CIEF-MS offers MS resolution in addition to the charge variant separations. The CIEF-TOF-MS results presented here demonstrated the potential applications of the CIEF-MS method for direct charge variant characterization of therapeutic mAb drugs. This new, original method can expand our capability in analyzing and understanding charge heterogeneity for the discovery and development of mAb therapeutic proteins, especially through combination with more advanced MS for high resolution, enzymatic cleavage treatment for reduced sample complexity, and cross-validation via complementary techniques such as peptide mapping.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04608.

Additional data for CIEF-MS method development (PDF)

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Notes

The authors declare no competing financial interest.

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