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Microchip Zone Electrophoresis for High-Throughput Analysis of Monoclonal Antibody Charge Variants

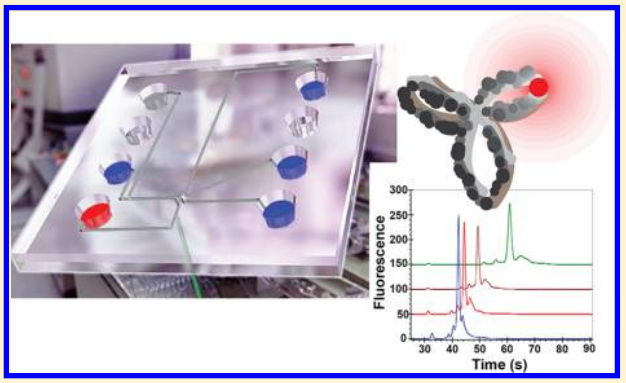
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Supporting Information

ABSTRACT: A high-throughput screening assay on a microfluidic chip was developed for the determination of charge variants of monocolonal antibodies (mAbs) in pI range of 7−10. This method utilizes microchip zone electrophoresis for rapid separation (<90 s) of mAb charge variants that are labeled fluorescently without altering the overall charge. The microfluidic assay achieves between 8- and 90-fold times faster separation time over conventional methods while maintaining comparable resolution and profiles of charge variant distributions. We further characterized the assay with respect to (i) the effect of pH on resolution, (ii) the effect of excipients and buffering agents, (iii) the performance of the assay compared to conventional methods, and (vi) the reproducibility of charge variant profiles. Finally, we explored the utility of the assay with four case studies: (i) monitoring C-terminal lysine modification of a mAb, (ii) quantifying the extent of deamidation of a mAb, (iii) providing charge variant information on which to base clone selection, and (iv) making process parameter-related decisions from a "design of experiment" (DoE) study. The results of these case studies demonstrate the applicability of the microfluidic assay for high-throughput monitoring of mAb quality in process development of biopharmaceuticals.



Monoclonal antibodies (mAbs) are large and complex molecules that comprise an important class of biotherapeutic agents and are currently a major focus of the biopharmaceutical industry.[1] The complexity poses a substantial challenge to their design and production. Compounding this challenge are the numerous processes by which the composition of a mAb, and therefore its quality (i.e., efficacy, safety, and consistency), can be altered inadvertently through post-translational processes. These alterations, which typically occur by enzymatic reactions or spontaneous degradation, must be monitored and then controlled for the consistent manufacture and storage of biotherapeutic mAbs.[2]

Characterization assays for monitoring the quality of mAbs include analytical measurement of the heterogeneity in the size, glycosylation pattern, and net charge of the molecules in a sample. The latter is determined by the pH of the surrounding solution and by the pK_a of the biochemical functional groups that are on the exterior surface of the molecule. Changes to these functional groups that are brought about, for example, by glycosylation, C-terminal lysine processing, deamidation, disulfide bond formation, isomerization, and oxidation,[3] can generate a heterogeneous population of charge variants. Conventional separation techniques for the measurement of this heterogeneity are (1) ion-exchange chromatography (IEX) techniques, such as weak cation-exchange high-pressure liquid chromatography (wCEX-HPLC), in which differently charged variants bond with different strengths to an oppositely charged resin and are then eluted via a salt or pH gradient, (2) capillary zone electrophoresis (CZE), in which differently charged variants migrate with different speeds when subjected to an electric field,[4−7] and (3) capillary isoelectric focusing, including imaged capillaries (icIEF), in which differently charge variants are separated by their isoelectric point (pI) when an electric field is applied in the presence of a pH gradient. Vlasak and Ionescu provide a detailed review of charge-based methods for determining the charge heterogeneity of mAbs.[8]

While the above separation techniques have proved useful in monitoring charge variants, the manner in which they are performed imparts severe limitations to throughput: conventional, IEX, CZE, and icIEF separations require run times on the order of 8−90 min per sample (see Table 1). At these rates, instruments are unable to keep up with the number of samples that "design of experiment" (DoE) studies can generate. These studies, as well as high-throughput process development approaches,[9,10] are vital for a complete understanding of the

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Table 1. Comparison of Analysis Times

n	IEX	icIEF	CZE	MZE
1	30−90 min	8−20 min	8−30 min	1−2 min
96	48−144 h	24−32 h	16−48 h	2−3 h

upstream and downstream process conditions that affect the quality of mAbs and their consistent production.

To address this issue, we developed a CZE separation technique on a microfluidic chip that automates and integrates the assay steps. The assay was developed for a commercial high-throughput microfluidic platform (LabChip GXII) that performs electrophoretic separation and laser-induced fluorescence (LIF) detection. Previously developed high-throughput assays for the platform include screening of mAb purity and fragmentation by microchip CE-SDS[11−13] and determination of relative amounts of N-linked glycans.[14] Han et al.[15] developed a microchip zone electrophoresis assay for the analysis of charge heterogeneity of antibodies on the LabChip GXII. In each of these assays, the conversion of macroscale separations to microfluidic systems results in 10−100-fold reductions in separation times, allowing the analysis of larger DoE studies with more processing variables. The assays also leverages additional benefits of microfluidics: low-microliter-scale consumption of samples and reagents, compatibility with automation, and highly reproducible heat and mass transfer.

We report a new microchip zone electrophoresis (MZE) assay for the determination of charge variants. To determine the capabilities of the assay, we (1) investigate the effect of pH on resolution of charge variants, (2) determine the influence of typical buffer compounds and excipients found in drug substances and products on the assay, (3) compare the quantitation of relative amounts of charge variants by MZE to quantitation by conventional separation techniques, and (4) assess the reproducibility of the quantitation of charge variants by MZE. In addition, we demonstrate the implementation of MZE in four studies of charge heterogeneity: (1) detection of C-terminal lysine modification, (2) quantification of degree of deamidation, (3) clonal selection based on charge variant profiles, and (4) applicability for DoE approaches.

MATERIALS AND METHODS

Materials. HT Protein Charge Variant reagent kits and HT DNA5K/RNA/CZE chips were purchased from PerkinElmer (Waltham, MA, U.S.A.). Zeba Micro Spin desalting columns (7K MWCO, 75 μL microcentrifuge columns) were purchased from Thermo Scientific (cat. 89877, Rockford, IL, U.S.A.). Dimethylacetamide (cat. 271012) and dimethylformamide (cat. 227056) were purchased from Sigma (St. Louis, MO, U.S.A.). icIEF cartridges (cat. 101701), pI markers 6.14 (cat. 102220), and 8.79 (cat. 102230) were obtained from Proteinsimple (Santa Clara, CA, U.S.A.). Pharmalyte 3−10 (cat. 17-0456−01) and Servalyte 7−9 (cat. 42907) ampholyte solutions were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden) and SERVA (Heidelberg, Germany). Ammonium bicarbonate (cat. 09830) was purchased from Sigma, TSKgel G3000SWXL column (7.8 mm × 30 cm) for semipreparative size exclusion chromatography (SEC) fractionation was obtained from Tosoh Bioscience GmbH (cat. 08541, Stuttgart, Germany), and analytical ProPac WCX-10 weak cation-exchange column (4 mm × 250 mm, cat. 054993) was purchased from Thermo Scientific. POROS HS 50 μm bulk strong cation-exchange resin was purchased from Life Technologies Ltd. (Paisley, UK). Rapigest surfactant was purchased from Waters (Milford, MA, U.S.A.), and sequencing grade trypsin was from Promega (Fitchburg, WI, U.S.A.). Carboxypeptidase B was purchased from Worthington Biochemical (Lakewood, NJ, U.S.A.).

Preparation of Samples and Microfluidic Chips for MZE Assay. mAb samples were processed according to the instructions provided by PerkinElmer (both with and without desalting using Zeba columns). Microfluidic chips were also prepared according to instructions. See the Supporting Information for additional details, including a schematic diagram of the microfluidic chip and a description of chip operation, the effects of labeling reaction on the charge distribution of a sample, and the composition of the running buffer.

Studies of Charge Variants. *C-Terminal Lysine Modification.* Carboxypeptidase B (CPB) diluted in 100 mM sodium chloride was incubated with desalted samples containing 5 mg/mL of mAb at ∼1 mol of enzyme to 20 mol of mAb for 2 h or overnight, at 37 °C.

Deamidation. mAb samples were temperature- and pH-stressed to induce deamidation. After buffer exchange into 50 mM ammonium bicarbonate, pH 7.9 (containing protease inhibitors PMSF, benzamidine, aprotinin, leupeptin, pepstatin) using Zeba columns, samples at 13 mg/mL were incubated at 30 °C for 0, 2, 4, 8, 24, 48 h. To exclude charge pattern variations due to aggregate formation,[16,17] samples were run over a semipreparative TSKgel G3000SWXL SEC column (50 mM sodium phosphate, 500 mM NaCl, pH 6.7) at flow rates of 0.5 mL/min. Monomeric peak fractions were concentrated and buffer-exchanged to DI water using Zeba columns.

Clonal Selection. mAb samples derived from two different cell clones were buffer-exchanged to DI water using Zeba columns. Charge variant analysis was performed for each sample using the MZE assay, icIEF, and wCEX-HPLC.

DoE. Robustness of a preparative strong cation-exchange chromatographic step using a POROS HS 50 column (1 cm × 25 cm, 19.6 mL column volume, self-packed) was evaluated by a DoE approach. The process response basic peak groups were quantified in the elution pool. Elution buffer pH and conductivity were examined as input variables. A central composite face-centered (CCF) design with four center points was chosen. For each run the column was equilibrated with five column volumes of 50 mM sodium acetate, pH 5.0, and loaded with 40 mg of mAb H/mL of bed volume (5 min contact time). The column was washed with five column volumes of 50 mM sodium acetate, pH 5.0, before the protein was eluted by 50 mM sodium acetate with varying pH and conductivity (pH 4.85, 5.0 and 5.15, conductivity 8.5, 10.5, and 12.5 mS/cm). Cutting criteria for elution pools were from 0.2 to 1 according to optical density at the column outlet. Elution pools were analyzed by MZE and wCEX-HPLC. Data analysis of the DoE was performed using MODDE v. 9.1 software (MKS Umetrics AB, Umea, Sweden). A quadratic process model was applied. Data were considered statistically significant when $p < 0.05$.

Conventional CZE, icIEF, wCEX-HPLC, and MS Assays. *icIEF.* Analysis of mAbs was performed with an iCE280 Fast IEF analyzer (Proteinsimple). The ampholyte buffer constituents were 0.3 mg/mL mAb, 0.35% hydroxypropyl methylcellulose, 3% sucrose, 1 M urea, pI markers 6.14 and 8.79, 0.4% (w/v) Servalyte 7−9, 10.8 μequiv/mL Pharmalyte 3−10. A prefocusing step of 1500 V was carried out for 1 min, before samples were focused at 3000 V for 8 min.

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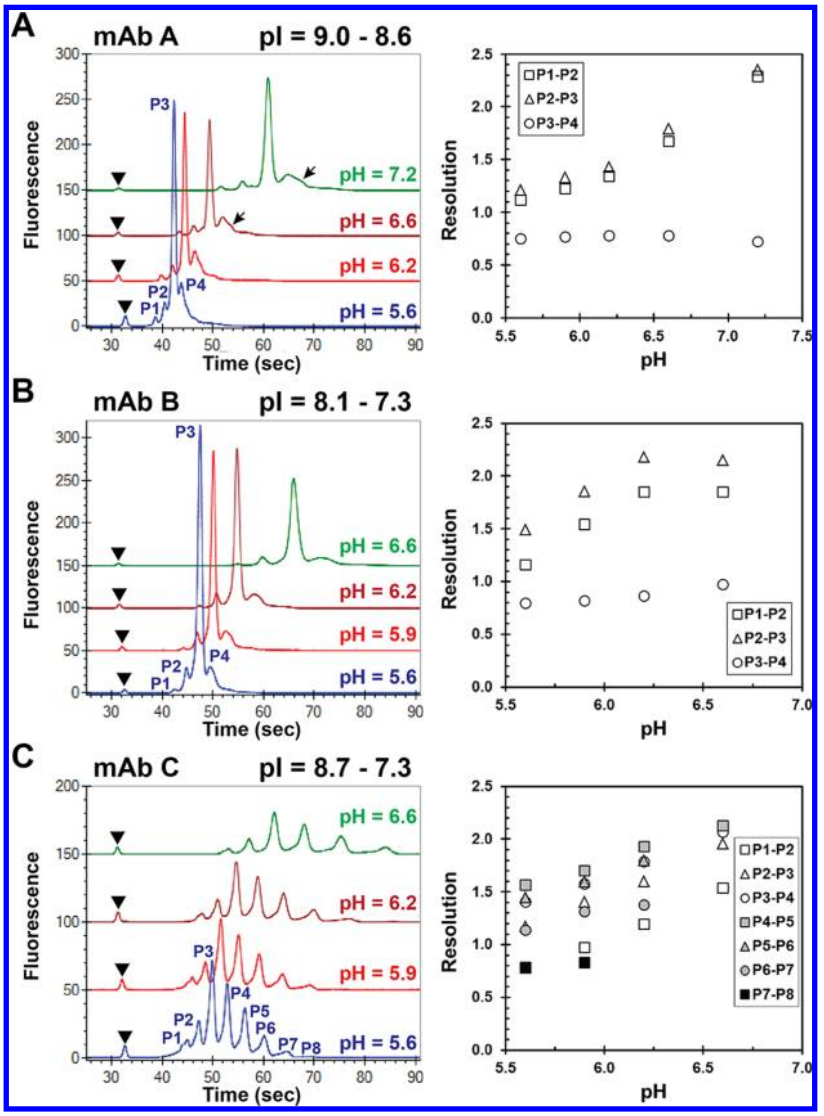


Figure 1. Electropherograms showing the charge variant profile of (A) mAb A, (B) mAb B, and (C) mAb C, at several running buffer pHs. Electropherograms at each pH are offset by 50 relative fluorescence units (RFU). In each profile, peaks P1 and P2 are basic variants, peak P3 is the main variant, and peaks P4 and higher are acidic variants. Inverted black triangles indicate the peak corresponding to free dye. The pI range of all variants, as determined by icIEF, is indicated above each set of electropherograms. Next to each set of electropherograms are plots of resolution vs pH for each pair of adjacent peaks labeled in the electropherogram.

wCEX-HPLC. Analysis of mAbs was performed using ProPac WCX-10 columns at flow rates of 1 mL/min (mAb F, G) or 0.7 mL/min (mAb H). Samples were prepared in buffer A (mAb F, 10 mM sodium phosphate, pH 6.0; mAb G, 10 mM sodium phosphate, pH 7.0; mAb H, 20 mM sodium acetate, pH 5.2) or DI water. Amounts of 20−75 μg of sample were injected and monitored at 280 nm at 25 °C (mAb H) or 40 °C (mAb F, G) column temperature. The following gradients were used: mAb F, 0−90 mM NaCl in 30 min (buffer B, 10 mM sodium phosphate, 500 mM NaCl, pH 6.0); mAb G, 0−95 mM NaCl in 35 min (buffer B, 10 mM sodium phosphate, 500 mM NaCl, pH 7.0); mAb H, 0−300 mM NaCl in 35 min (buffer B, 20 mM sodium acetate, 300 mM NaCl, pH 5.2).

CZE. Analysis of mAbs by conventional CZE was performed using the method described in He et al.[6]

MS Analysis. Preparation of mAb G samples started with 10 kDa diafiltration against 50 mM ammonium bicarbonate buffer followed by incubation with Rapigest surfactant at 50 °C for 30 min. Reduction was performed by addition of 9 mM DTT and incubation for 30 min at 50 °C. After cooling, alkylation was done in the dark at room temperature by addition of 16 mM iodoacetamide. Digestion was performed by incubation with sequencing grade trypsin at a protease/protein ratio of 1:20, at 37 °C for 5 h. Peptides were then treated with 2% formic acid to stop digestion, to cleave Rapigest surfactant, and to acidify peptides for ionization.

Desalted and acidified peptides were separated online by an Acquity I-Class UPLC system (Waters) before analysis by a Synapt G2-S HDMS mass spectrometer plus ESI Z-Spray LockSpray source (both Waters). For each run, 1 μg of total protein (corresponding to 6.7 pmol of digested mAb) was loaded onto a C18 column (Acquity BEHC18, i.d. 2.1 mm, 150 mm, 1.7 μm particle size, 300 Å pore size, Waters) at 98% mobile phase A (0.1% formic acid, deionized water) and 2% mobile phase B (0.1% formic acid, acetonitrile). Peptides were eluted by increasing mobile phase B from 2% to 40% over a period of 55 min at a flow rate of 0.20 mL/min and column temperature of 60 °C. Data were acquired in positive ion

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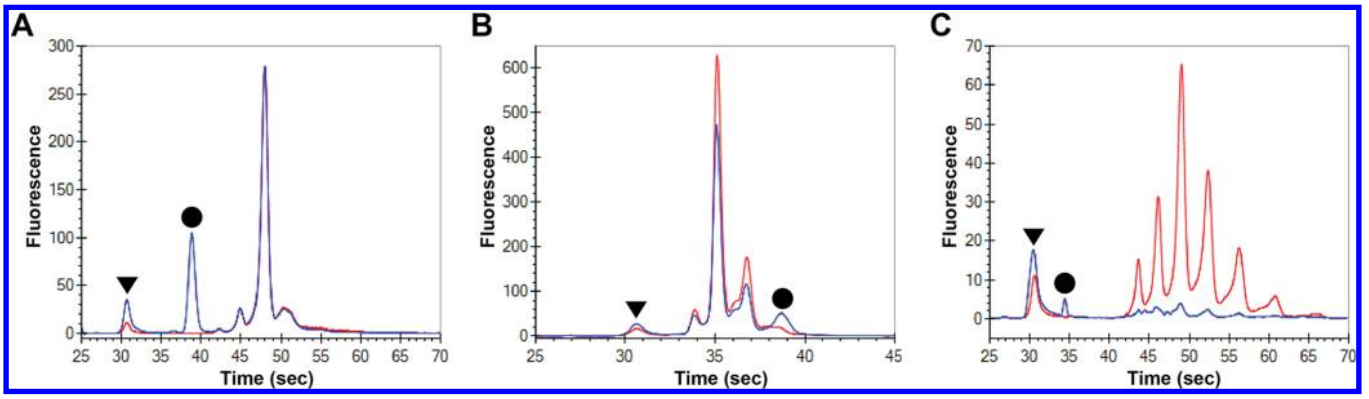


Figure 2. Electropherograms of desalted (red) and nondesalted (blue) samples. Inverted triangles indicate the free dye peak, and circles indicate excipient or buffer compound peaks.

resolution mode with an m/z range from 50 to 2000 and MSe function enabled. Source parameters were set as following: desolvation gas flow 950 L/h, desolvation temperature 450 °C, and source temperature 130 °C. The capillary voltage was set to 2.8 kV. Collision energy in the trap cell was ramped through scans from 20 to 45 V. Data analysis was performed using the software BioPharmaLynx version 1.3.2 (Waters). A sequence coverage rate of at least 92% was achieved for all samples.

Deamidation rates were quantified after processing by BioPharmaLynx software including lock mass correction for obtaining accurate masses, Apex3D detection of peaks, and deconvolution of multiply charged ions by the MaxEnt 3 algorithm (Waters) with aligning accurate masses to a theoretical peptide list of mAb G including investigated modifications ending up in summed intensity counts for matched peptides. Mass accuracy for peptide matching was set to 5 ppm for peptides in MS data track and to 10 ppm for peptide fragments in MSe data track. The intensity counts were taken to perform a relative quantification of deamidated peptides and compared to their nondeamidated counterparts.

RESULTS AND DISCUSSION

Assay Characterization. To characterize the MZE assay, we examined four aspects: (1) the effect of pH on the resolution of charge variants, (2) the effects of buffer compounds or excipients on the assay, (3) the agreement between charge variant profiles obtained by MZE and those obtained by conventional assays, and (4) the reproducibility of charge variant profiles obtained by MZE.

Effect of pH on Resolution. We examined the influence of the running buffer pH over the migration behavior and resolution of charge variants. The electropherograms in Figure 1 show the charge variant profiles of three mAbs at various pHs; the migration speed of each mAb was inversely proportional to the pH of the running buffer. This deceleration was caused by a decrease in the net charge of the molecules, as the pH approached the pIs of the variants,[6] and indicates that transport is predominantly by electrophoretic migration. If transport were instead by bulk electroosmotic flow of the running buffer, then the migration speed would increase at higher pH, since the stronger ζ-potential at higher pH leads to higher electroosmotic flow rates.[18]

Also apparent in Figure 1 is that the separation between peaks generally increased with increasing pH. This increase was likely caused by greater differences between the net charges of the variants, as the pH approached the pI.[6] To quantitatively assess the effect of pH on the separation of peaks, we calculated the resolution (see the Supporting Information); plots of resolution versus pH are shown in Figure 1. Generally, increasing pH improved resolution, particularly for basic charge variants. For acidic variants, the improvement was less than that for basic variants, and was negligible in some cases. More specifically, for mAb A (Figure 1A), the resolution of basic variants improved markedly with increasing pH. The resolution of the acidic variant (P4) from the main variant (P3) remained constant, but an additional acidic variant appeared as a shoulder on P4 at pH 6.6 (indicated by an arrow). For mAb B (Figure 1B), the separation between basic variants increased with increasing pH. However, all peaks were noticeably broader at higher pH. This broadening negated the gain in resolution from the increased separation, as reflected by the plateau in the plot of resolution versus pH. Lastly, for mAb C (Figure 1C), the resolution of all variants increased as the pH was increased to 6.6. At and above this pH, however, the peak heights of the trailing acidic variants diminished below the detection limit, due to broadening.

The broadening of peaks as the pH of the running buffer approaches the pI of the sample was likely a side effect of the decrease in the net charge of the variants. This decrease in charge, and the accompanying reduction of migration speed, may have allowed hydrophobic interactions between the mAb molecules and the microchannel surface to increase the dispersion of samples as they migrated along the separation channel.[19] Thus, while pH ≈ pI theoretically achieves the best separation of charge variants,[6] increased dispersion at this condition established an upper limit to the resolution at pH < pI.

Effects of Excipients and Buffer Compounds. The labeling reaction used in the MZE assay occurs via the conjugation of a dye molecule to a lysine residue on the exposed surface of the mAb. The same reaction may also occur for amino acids and other amine-containing buffer components that are present in a sample. If the net charge of labeled excipients is positive at the pH of the running buffer, then they may appear in an electropherogram. A comparison of the profiles shown in Figure 2, parts A and B, reveals that desalting a sample with a Zeba column removed the peaks of such excipients. Figure 2B also shows that desalting increased the peak height by ~20%. An even greater increase in signal from desalting (~20-fold) is seen in Figure 2C. Three changes to the sample may have led to this increase: (1) a decrease in the conductivity of the sample, resulting in greater field-amplified sample stacking

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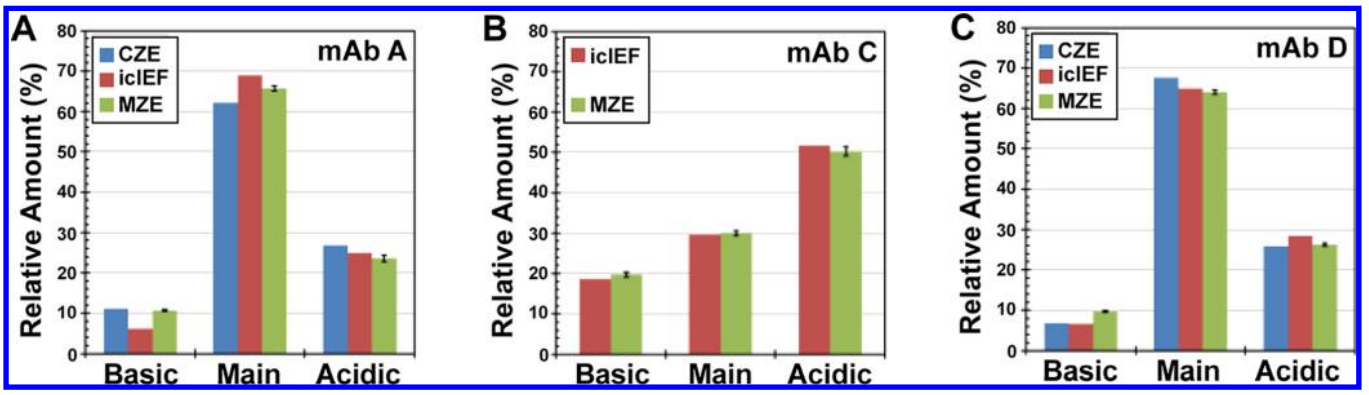


Figure 3. Comparison of relative amounts between methods. Relative amounts obtained by conventional CZE (blue), conventional icIEF (red), and MZE (green) are shown for (A) mAb A, (B) mAb C, and (C) mAb D. Data obtained by MZE are averages with n = 9 (A), n = 8 (B), and n = 20 (C); error bars represent one standard deviation.

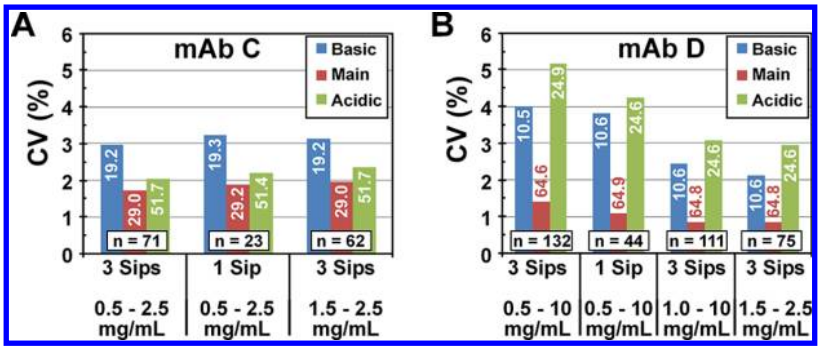


Figure 4. Intermediate assay reproducibility. Panel A shows the coefficient of variance (CV) of relative amount measurements for mAb C, corresponding to peaks P1 + P2 (basic), P3 (main), and P4−P8 (acidic) in Figure 1C, respectively; panel B shows the CV of relative amount measurements for mAb D. n is the number of data points used to calculate each CV, and the value at the top of each bar is the average relative amount (%) for that group of variants.

during the electrokinetic load (see the Supporting Information), (2) removal of a sample buffer that has a higher buffering capacity than the labeling buffer and maintains the pH < 8.0, in which case the labeling efficiency is reduced, and (3) a decrease in the concentration of buffer components that compete with the mAb for dye molecules in the labeling reaction. In summary, the results indicate that desalting is potentially an important step for ensuring the highest signal possible and that no excipients contaminate the profiles of mAb charge variants.

Comparison to Conventional Methods. We determined the equivalence between the charge variant profiles generated by MZE and those generated by conventional CZE and icIEF, for three mAbs. We assessed this equivalence by comparing the percent relative amounts (%RA) of the basic, main, and acidic variant groups. These relative amounts are equal to the percentage of the total area under an electropherogram that an individual variant or group of variants contributes and provide a semiquantitative means of measuring the charge heterogeneity of a mAb sample. The graphs in Figure 3 show that the various analysis methods compared well for all three mAbs tested: the deviations of relative amounts determined between all methods were less than 5% for mAbs A (Figure 3A) and D (Figure 3C), and those determined by MZE and icIEF were less than 3% for mAb C (Figure 3B). Overall, both in this study and in the work of Han et al.,[15] comparisons between charge variant profiles generated by MZE and by CZE indicate that MZE provides information that is equivalent to that of the conventional assay. Comparisons between MZE and icIEF profiles also shows good agreement, though for some molecules we observed better resolution of acidic peaks with icIEF.

Reproducibility of Charge Variant Profiles. To evaluate the intermediate assay reproducibility of data generated by MZE, we calculated the coefficient of variance (CV) of %RA measurements of basic, main, and acidic variant groups for mAbs C and D. We included data from four chips on four instruments that three analysts accumulated over 3 days. A new set of samples at varying concentration (0.5−10 mg/mL) was labeled each day, and each sample was analyzed three times per run, using running buffer at pH 6.2.

For both mAbs, the CVs of all %RA measurements were less than 5.5% (Figure 4). For mAb C, which was only labeled at concentrations of 0.5, 1.5, 2.0, and 2.5 mg/mL, the variability was lower than that of mAb D. This difference is attributable partially to the larger %RA values for the basic and acidic variant groups of mAb C, compared to those of mAb D (see values above bars in Figure 4); the corresponding standard deviations of the %RA of each variant group of both mAbs are similar in magnitude. The higher reproducibility obtained for mAb D may also be attributable to differences in the distribution of peaks and the resolution between peaks. For mAb D, the CV decreases to less than ∼3% when data from samples at 0.5 mg/mL are excluded. Further limitation to concentrations of 1.5−2.5 mg/mL did not significantly reduce the CV, indicating that samples at concentrations above this range do not contribute substantially to variability. MZE achieved the low degree of variability reported here without flushing the microchannel with buffer between samples; unlike

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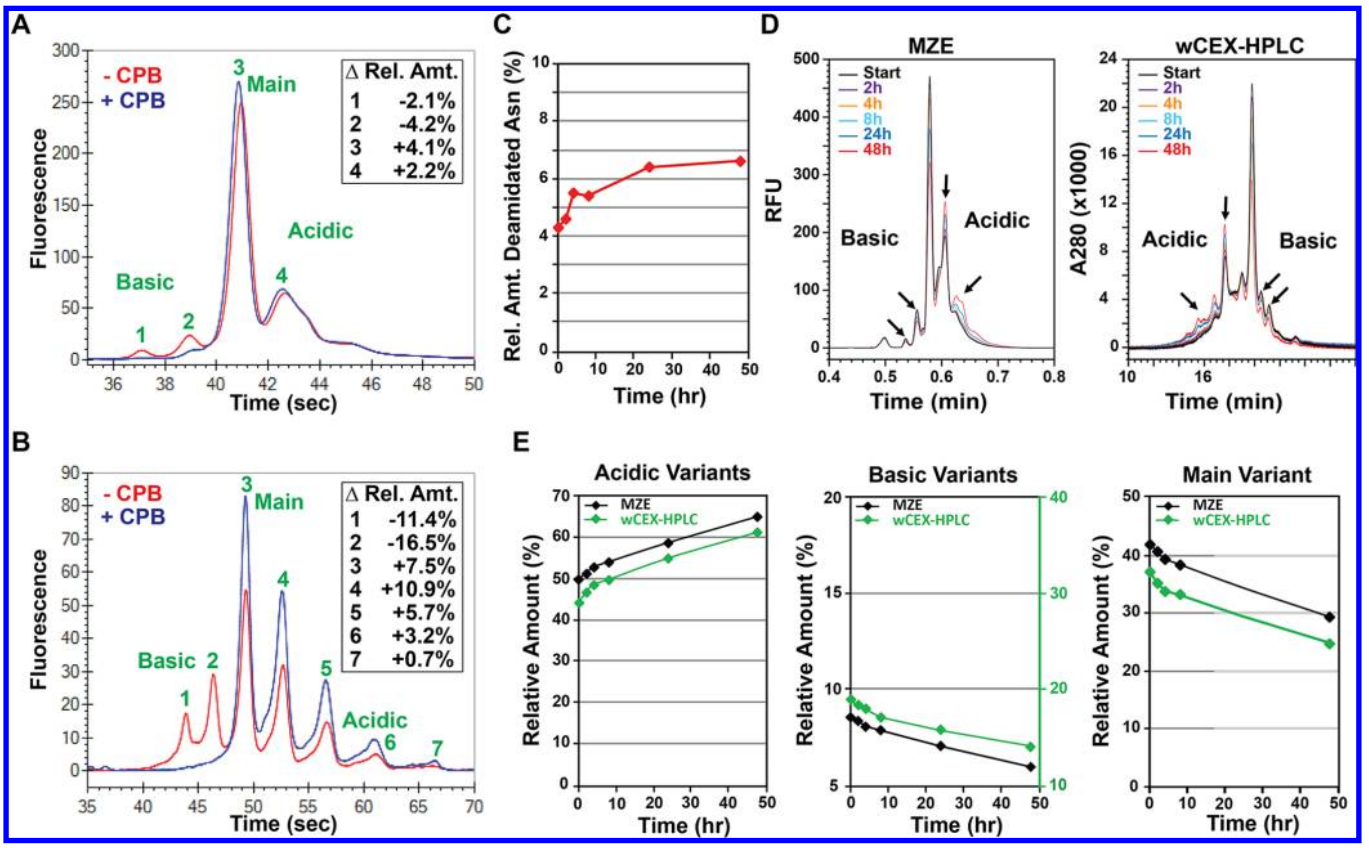


Figure 5. Carboxypeptidase B (A and B) and forced deamidation studies (C–E). Panels A and B show charge variant profiles of mAb A and C, respectively, before (− CPB) and after (+ CPB) digestion. Inset tables list the change in the relative amount of each peak. Panel C shows the extent of mAb G deamidation over time at pH and temperature stress, measured by LC–MS/MS. Panel D shows charge variant profiles measured by MZE and wCEX-HPLC. Plots in panel E are relative amounts of the variant groups of profiles shown in panel D.

Han et al.,[15] we found that it was unnecessary to recondition the surface of the microchannel after the separation of each sample. Chip lifetimes (400 samples) were also unaffected by lack of reconditioning.

Charge Variant Studies. Process development for biotherapeutic mAbs requires comprehensive analysis of the degree of charge heterogeneity of the resulting drug substance and thorough investigation of the cause(s) of this heterogeneity. The wide variety of these causes, and of the process conditions that affect them, can give rise to extensive DoE experiments that include many parameters, thus requiring the analysis of hundreds of samples. The information that is gained from such experiments is essential for ensuring the quality of a drug product and for successfully obtaining its approval by regulatory agencies.[2] At the molecular level, major causes of acidic variants include the attachment of sialic acid or mannose to N-glycans, deamidation, modifications to disulfide bonds, and nonenzymatic glycation; basic variants are mainly caused by incomplete removal of C-terminal lysines, incomplete formation of pyroglutamate at the mAb N-terminus, isomerization of aspartate, succinimide formation, and aggregates.[20] In the following sections, we provide four examples of how MZE can be employed to develop a more complete understanding of the process conditions and clone attributes that affect the charge heterogeneity of a drug substance.

Detection of C-Terminal Lysine Modifications. C-Terminal lysine processing is one of the most common chemical modifications that leads to charge heterogeneity. All human IgGs have a lysine at the C-terminus of their heavy chains that can be removed by the activity of basic carboxypeptidases present in cell culture. Depending on conditions, varying fractions of a population of mAbs in a sample may lose their C-terminal lysines by this mechanism, resulting in charge heterogeneity. C-Terminal lysine variants are not expected to affect efficacy or safety of a mAb;[21] however, it is important to monitor these variants to ensure the consistency of the development and manufacturing process. We determined whether MZE is able to perform this task by analyzing samples that we first treated with carboxypeptidase B (CPB), an exopeptidase that specifically cleaves heavy chain C-terminal lysines. Parts A and B of Figure 5 show the profiles of mAb A and mAb C, before and after this digestion. The basic variant peaks of both molecules (peaks 1 and 2) were significantly diminished after the digestion. This change suggests that the basic variants had one or more C-terminal lysines that the enzyme removed, thereby reducing their net charge. Additionally, the pattern of the main and acidic peaks remained unchanged, suggesting that these peaks did not contain variants with C-terminal lysine residues. In both profiles, the relative amounts of the main and acidic variants increased after digestion, corresponding to the decrease in relative amount of the basic variant group.

These results indicate that MZE is able to detect changes in the charge variant profiles of mAbs that occur as a result of C-terminal lysine modifications. This ability suggests that the assay is able to monitor changes in product consistency that result from these modifications, and to reveal conditions that give rise to such charge heterogeneity, via DoE experiments.

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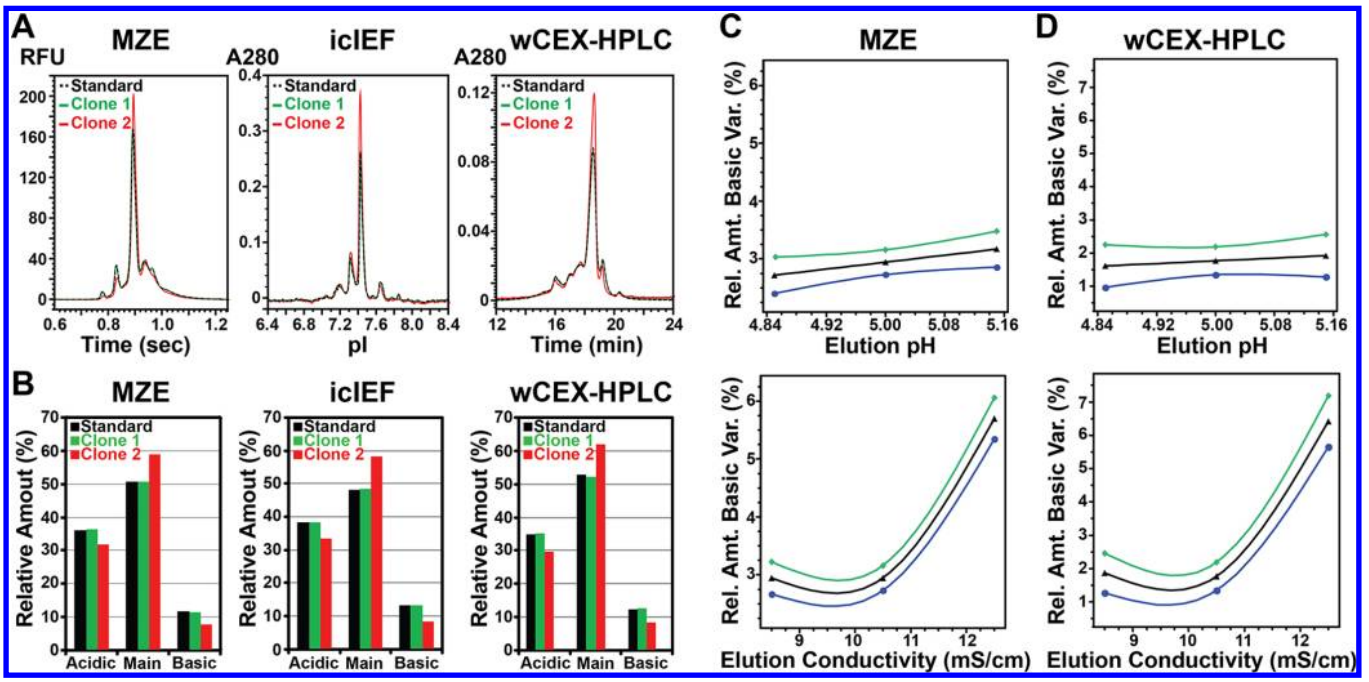


Figure 6. Clonal differences in variant profiles (A and B) and analysis of DoE experiments (C and D). In panels A and B, drug substance from clone 1 (green) and clone 2 (red) is compared with the standard (black), using three charge analysis methods: MZE (left), icIEF (center), and wCEX-HPLC (right). Panel C shows DoE prediction blots using MZE data. Panel D shows DoE prediction blots using wCEX-HPLC data. Upper graphs show the correlation of basic variant amount with pH while keeping conductivity constant at high (green), middle (black), or low (blue) values. Lower graphs show the change in basic variant amount with conductivity, while keeping pH constant at high (green), middle (black), or low (blue) values.

Moreover, the analytical setup consisting of an enzymatic reaction followed by MZE that we demonstrated here with CBP can also enable the detection of other charge heterogeneity-causing modifications, e.g., N-glycosylation with PNGase F treatment or sialic acid with sialidase treatment.

Quantification of Deamidation. A critical quality attribute in process development is deamidation, the nonenzymatic removal of an amide functional group of asparagine (or less frequently of glutamine). This removal results in the formation of aspartate, isoaspartate, a succinimide intermediate, or glutamate.[22] Isoaspartate in particular may destabilize the secondary structure of the protein and can increase the formation of aggregates.[23] Depending on the site of action, deamidation can have functional implications: deamidation of Asn55 in the CDR2 region of mAbs was shown to lead to an 80% decrease in potency.[24] Deamidation is induced by elevated pH, by elevated temperatures, and by buffer constituents such as ammonium ions[22,25] and often results in an increase of acidic isoforms that can be monitored by wCEX-HPLC.[20,26,27] It is essential to follow such charge pattern variations carefully, e.g., during drug substance stability studies. In this section, we evaluate the ability of MZE to detect increases of acidic variants for this purpose and make a direct comparison to wCEX-HPLC results.

We induced deamidation of mAb samples as described in the Material and Methods section. Figure 5C shows an increase in the extent of deamidation of the mAb with time, as determined by LC−MS/MS. The kinetics of deamidation shown in Figure 5C represent deamidation events of all detected asparagine residues within the mAb molecule, as measured after tryptic digestion. The rate of deamidation was especially rapid during the first 4 h; the extent of deamidation increased to over 5% for particular deamidation hot spots and was negligible for other asparagine-containing sites (data not shown).

Charge profiling of the same deamidated samples by MZE and wCEX-HPLC shows an increase in acidic variants and a decrease in basic and main variants (Figure 5D). Moreover, as shown in Figure 5E, data from both methods reveal the same change in deamidation over time, with the fastest increase of acidic species occurring within the first 4 h of stress induction. This result is consistent with the LC−MS/MS data in Figure 5C and suggests that MZE displays similar sensitivity to the current "gold standard", the ProPac WCX-10 column, in monitoring variations in the charge isoform distributions of deamidated samples. MZE may therefore be applied routinely for drug substance stability studies and has the advantage of a 35-fold higher sample throughput.

Clonal Selection. An important task of drug development is the identification of a suitable expression clone. Drug substances from different clones of the same cell line can differ in physicochemical properties and post-translational modifications. Prominent examples include differences in molecular integrity, aggregation affinity, and glycosylation pattern (e.g., fucose content);[27−29] these differences can be readily revealed by charge pattern detection methods.[20] Identification of the clone that creates mAbs with the desired charge pattern can be a key selection step in the early development phase of a drug substance (a "quality by design" (QbD) principle), especially when the particular pattern of a standard must be achieved.

To evaluate the ability of MZE to detect differences in clonal charge patterns, we compared MZE data to those of icIEF and wCEX-HPLC. mAb F samples that were purified from two clones and a corresponding standard were analyzed by each method. Figure 6A shows overlays of charge profiles obtained by MZE (left), icIEF (middle), and wCEX-HPLC (right) of the

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standard material (black traces) and drug substances from both clone 1 (green traces) and 2 (red traces). All three detection methods reveal that while the charge profile of clone 1 matches the profile of the standard closely, some discrepancies are present in the profile of clone 2. All three methods point to the selection of clone 1 for future process development. These findings are further supported by the quantitative analysis of the charge profiles in Figure 6B. Again, all three methods show that the charge isoform distribution of the product from clone 1 matches that of the standard with greater fidelity than does the distribution of the product from clone 2. These results demonstrate that MZE is well-suited to assess clonal similarity to given standards and to subsequently drive decision making during process development.

Analysis of DoE Studies. DoE studies are routinely applied in screening for optimal process conditions or for defining robust ranges of process parameters for a particular purification step. When combined with robotic purification platforms to generate many samples in relatively short times, these studies become powerful tools for understanding the effects of large parameter sets on drug quality. As a consequence of large sample numbers, a need for rapid analytical tests arises. The short analysis time and high-throughput capability of MZE could meet this need, and so we tested its performance in the detection of basic variants in samples derived from a DoE study.

For this study, we characterized the robustness of a strong cation-exchange chromatography step, by varying elution pH and elution conductivity. In 12 separate runs, elution conditions of mAb H were modified in a multivariate manner that followed a CCF design (elution conditions of low, midpoint, and high pH values were systematically combined with low, midpoint, and high elution conductivity). The basic isoform content was considered a critical metric for this purification step, and so we measured this content in all elution fractions by MZE and by wCEX-HPLC (ProPac WCX-10 column) for comparison. We used data from both methods as input values for DoE prediction models to test whether the quality of MZE data is sufficient for this purpose.

Parts C and D of Figure 6 show the prediction blots for the basic isoform content as a function of elution pH (upper graphs) and elution conductivity (lower graphs), as determined by MZE or wCEX-HPLC. Both methods display a minor, linear influence of elution pH and a substantial influence of the elution conductivity that follows a quadratic function. The minimum basic variant content at a conductivity of 9.7 mS/cm was predicted independently by both methods. These results demonstrate that the high quality of MZE data allows the calculation of accurate prediction models for process development decision making via DoE studies. This small-scale DoE study served as proof of principle; the high throughput of MZE facilitates the scaling up of DoE experiments, to cover large numbers of process conditions and parameters.

CONCLUSIONS

We presented a microchip zone electrophoresis assay for the separation of mAb charge variants with separation times of 90 s or less. By leveraging the small scale of microfluidic systems, the assay is able to achieve separations on the order of 8−90 times faster than conventional methods. This improvement in throughput addresses a significant limitation in the execution of DoE studies for determining optimal production or storage conditions for mAb biotherapeutics. To characterize the microfluidic assay, we examined its performance with respect to the effect of pH on resolution and observed the theoretically expected increase in resolution as the pH of the running buffer approached the pI of the antibody. Peak broadening, presumably a result of hydrophobic interactions between the sample and the surface of the microchannel, also occurred under this condition, resulting in a plateau or decrease in resolution as a function of pH. Second, we described the effects of buffer components on the assay; removal of these species can both improve the sensitivity of the assay and remove extraneous peaks from the charge variant profile. Third, we found that relative amounts of charge variants determined by the microfluidic assay compared well to those obtained by conventional methods. The results of this comparison indicate that the microfluidic assay is able to provide improved throughput while maintaining a level of quality to the data that is comparable to that of conventional methods. Finally, we performed a reproducibility study with two antibodies and determined a CV of ∼3% for the relative amounts of all variant peaks at mAb input concentrations between 1 and 10 mg/mL. To demonstrate the usefulness of MZE in process development, we performed four studies of charge heterogeneity that are relevant to the biotherapeutics industry: (1) monitoring C-terminal lysine modification, (2) quantifying the extent of deamidation, (3) determining charge variant information on which to base clone selection, and (4) making process parameter-related decisions from a DoE study. With these studies, we established that the sensitivity of the assay to changes in charge variant profiles was comparable to that of conventional methods and could be used in clone selection and to optimize process conditions.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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