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Analysis of Identity, Charge Variants, and Disulfide Isomers of Monoclonal Antibodies with Capillary Zone Electrophoresis in an Uncoated Capillary Column

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A set of related capillary zone electrophoresis (CZE) methods have been developed for the analysis of identity, charge variants, and disulfide isoforms of IgG monoclonal antibodies (mAbs). These methods utilize an uncoated capillary column. The combined use of concentrated zwitterionic (e-amino-caproic acid) buffer and acid flushing was effective in minimizing the adsorption of protein to the inner wall of a bare capillary. Under these conditions, a selective and reproducible separation of multiple IgG1 and IgG2 monoclonal antibodies (mAbs) was obtained with a long capillary column (40 cm effective length), allowing the reliable identification of different mAbs by migration time. A rapid (~10 min) and selective separation of charged variants of IgG mAbs was attained using a short capillary column (10 cm effective length). Finally, the addition of urea in the separation buffer resulted in the separation of disulfide isoforms of IgG2 mAbs by CZE. CZE methods using an uncoated capillary column offer a versatile, generic, and economical approach to the evaluation of identity, charge heterogeneity, and disulfide isoforms of IgG antibodies.

Monoclonal antibodies (mAbs) represent an important class of biotherapeutics in development for many different disease indications.[1,2] Like other biotherapeutics, mAbs are complex molecules and a variety of methods are required to ensure product quality and consistency. Examples of heterogeneities in mAb products include deamidation, glycosylation, oxidation, aggregation, proteolytic cleavage, and disulfide bond isoforms.[3−5]

Capillary electrophoresis (CE), originally developed by Jorgenson and Lucas[6] in the early 1980s, has become a routine tool for the analysis of recombinant protein therapeutics in the biotechnology industry.[7−10] Capillary isoelectric focusing (CIEF),[11,12] imaged capillary isoelectric focusing (ICIEF),[13,14] and capillary zone electrophoresis (CZE)[7,15] have been increasingly accepted as attractive alternatives to slab gel isoelectric focusing (IEF) and ion exchange chromatography (IEC) to assess charge heterogeneity of proteins. CZE separations are based on differences in electrophoretic mobility that are influenced by both the molecular charge and the hydrodynamic radius of the protein under a given set of conditions. The use of CZE as a tool for analysis of identity and charge heterogeneity of therapeutic mAbs was first investigated by Ma and Nashabeh.[7,15] The separation was performed in a permanently coated capillary to minimize protein adsorption to the inner wall of the capillary. The advantages of the CZE method over IEC include the likelihood for one generic platform CZE assay for multiple products offering a rapid analysis time that is applicable for high-throughput process development. Recently, Deng et al.[16] studied the combined use of a concentrated separation buffer and an expensive proprietary dynamic coating in an uncoated capillary to suppress the adsorption of proteins. In this method, recoating of the capillary was required every 10 injections, and the column lifetime was about 100 injections.

Wypych and others recently described disulfide isoforms specifically belonging to the IgG2 subclass of mAbs.[5,17,18] In addition to the classical IgG2-A isoform, two new IgG2-B and IgG2-A/B isoforms were observed. Different analytical methods were

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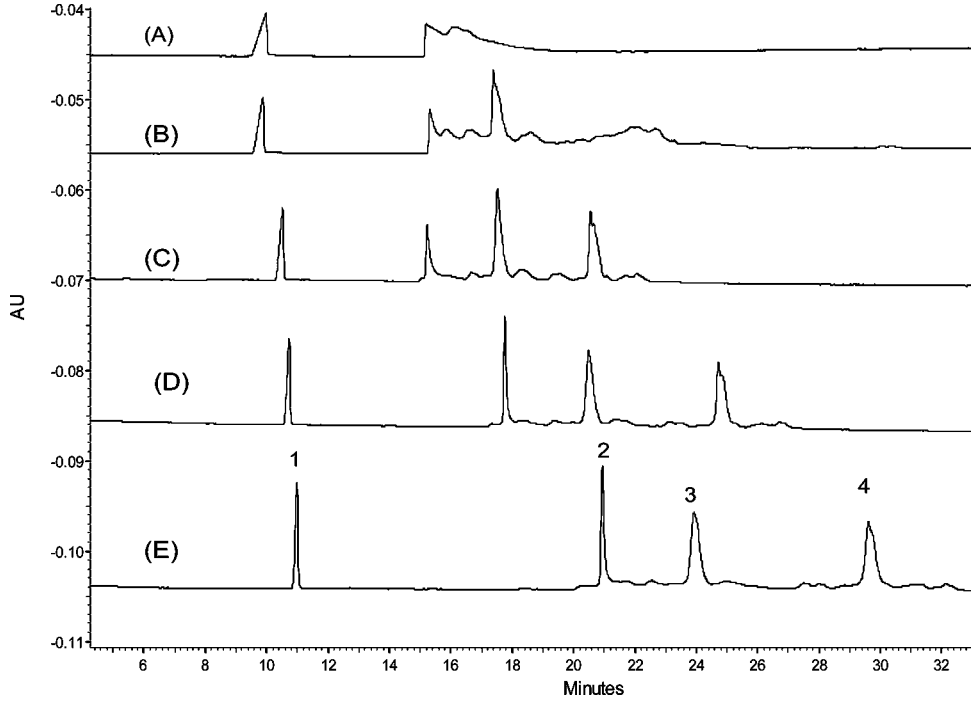


Figure 1. Effect of EACA concentration on the separation of IgG1 and IgG2 mAbs in a bare fused silica capillary. Bare capillary (40 + 10) cm × 50 μm i.d.; capillary temperature 25 °C; separation buffer (A) 50, (B) 100, (C) 200, (D) 400, and (E) 600 mM EACA−acetic acid, 0.1% HPMC, pH 5.4; voltage 24 kV; injection 0.5 psi × 5 s; detection UV 214 nm. Peak identity: peak 1, histidine; peak 2, IgG1 mAb; peaks 3 and 4, IgG2 mAbs.

used to separate the disulfide isoforms in their native or denatured states. Guo et al.[17] demonstrated the application of sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE) as an orthogonal technique to reverse phase liquid chromatography (RPLC) and IEC in the analysis of the disulfide isoforms. A SDS-CGE separation has also been recently optimized by Lacher et al.,[19] and a potential mechanism based on molecular sieving as well as ionic/adsorptive interactions was discussed. However, the commercial gel used for these studies was discontinued. Therefore additional CE based techniques for analysis of disulfide isomers would be desirable.

In this work, a CZE method using an uncoated capillary column was developed for the separation of several mAbs and their charge variants. The combined use of a concentrated zwitterionic buffer and acid flushing was applied to suppress the electroosmotic flow and protein adsorption. The addition of urea to the separation buffer was used to resolve the recently described disulfide isoforms present in IgG2 mAbs.

EXPERIMENTAL SECTION

Chemicals and Materials. E-amino-caproic acid (EACA) was purchased from MP Biomedical (Solon, OH). Acetic acid, hydroxypropyl methyl cellulose (HPMC), urea, cysteine, cystine, isopropyl alcohol (IPA), acetonitrile (ACN), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Saint Louis, MO). HCl (0.1 N) was purchased from Beckman Coulter (Fullerton, CA). PNGase F deglycosylation kits were purchased from Prozyme (Hayward, CA). Bare fused silica capillaries of 50 μm i.d. were obtained from Polymicro Technologies (Phoenix, AZ). μSIL-FC coated capillaries (catalog no. 194-8111) of 50 μm i.d. and Poroshell C8 columns (5 μm, 300 Å, 2.1 mm × 75 mm, part no. 660750-906) were obtained from Agilent Technologies (Santa Clara, CA). IgG1 and IgG2 monoclonal antibodies were produced in-house (Pfizer).

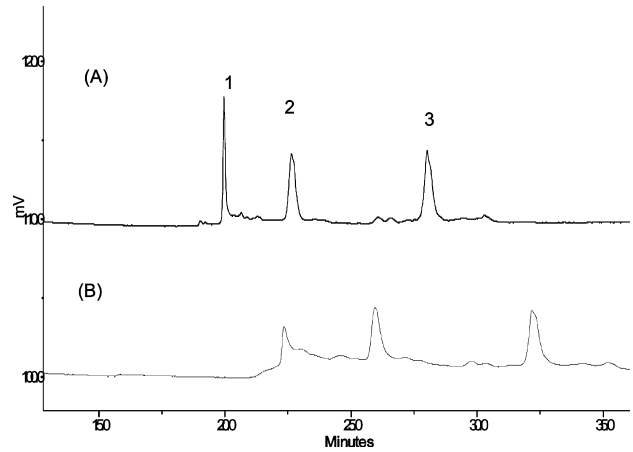


Figure 2. Degraded CZE separation of IgG1 and IgG2 mAbs in an uncoated capillary: (A) 1st injection and (B) 12th injection.

A Beckman Coulter CE-MDQ instrument equipped with a UV detector utilizing 32Karat software and Waters Empower Chromatography data collection system were used for all CE analyses. An Agilent 1200 LC system utilizing a Waters Empower Chromatography data collection system and control was used for all LC analyses unless otherwise noted.

Deglycosylation of IgG2 mAbs. IgG2 mAb was diluted with reaction buffer to 2 mg/mL. PNGase F was added to the diluted sample at an enzyme/substrate ratio of 1:50. The sample was incubated at 37 °C for 15 h and then diluted with water to 1 mg/mL prior to analysis by CZE.

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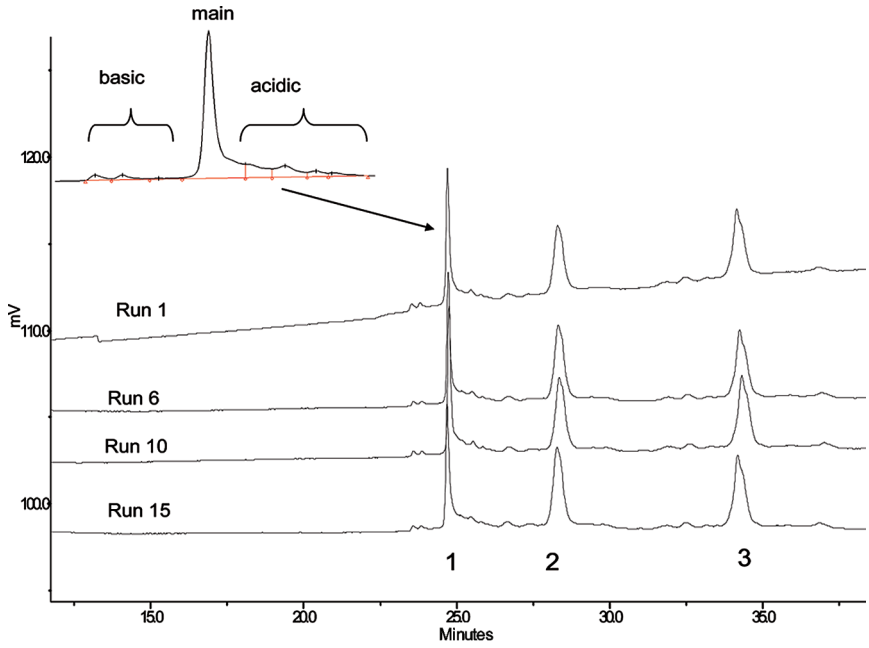


Figure 3. Reproducible CZE separation of IgG1 and IgG2 mAbs on 3 consecutive days.

Redox Treatment of IgG2 mAbs. IgG2 mAb sample (3 mg/mL) was added to a reaction buffer containing 33 mM Tris HCl (pH 8.5), 2 mM cysteine, and 0.2 mM cystine. The sample was put in a sample tray at 15 °C with several reaction times tested prior to analysis by urea-CZE and RPLC.

CZE, Urea-CZE, and RPLC Separation. The optimal CE separation was performed with a bare fused silica capillary (total length of 50 cm, effective length of 40 cm, i.d. of 50 μm) at 25 °C for CZE and 35 °C for urea-CZE. The running buffer for CZE contained 600 mM EACA−acetic acid (pH 5.5), 0.1% HPMC. The running buffer for urea-CZE contained 600 mM EACA−acetic acid (pH 5.5), 0.1% HPMC, 4 M urea. Samples were kept at a temperature of 15 °C and injected at 0.5 psi for 10 s. The separation voltage was 24 kV and detection was by UV at 214 nm. New capillaries were pretreated with 0.1 N HCl at 60 psi for 10 min, separation buffer at 50 psi for 20 min, followed by the application of 24 kV for 30 min. Between injections, the capillary was flushed with 0.1 N HCl at 60 psi for 5 min followed by separation buffer at 50 psi for 10 min. The capillary was filled with 10 mM H_3PO_4, and the two ends of the capillary were immersed in water overnight and for long-term storage.

The CZE running buffer for the μSIL-FC coated capillary (total length of 50 cm, effective length of 40 cm, i.d. of 50 μm) was 40 mM EACA−acetic acid (pH 4.5 and 5.0, 0.2% HPMC). The applied separation voltage was 30 kV, and detection was by UV at 214 nm.

The RPLC method consisted of using an Agilent poroshell C8 column with a gradient from 24% mobile phase (MP) B to 40% MP B in 30 min at a flow rate of 0.5 mL/min. MP A consisted of 2% IPA and 0.1% TFA in H_2O while MP B consisted of 70% IPA, 20% ACN, 10% water, and 0.1% TFA. A column temperature of 80 °C was used with UV detection at 215 nm.

The IEC method used Dionex Propac WCX-10 column (1.0 cm × 25 cm). A column temperature of 25 °C was used with detection at 280 nm. The flow rate was set at 4 mL/min. MP A consisted of 20 mM acetic acid, pH 5.0. MP B consisted of 20 mM acetic acid, 0.5 M NaCl, pH 5.0. The gradient profile: hold 2 min at 22% MP B after injection; 22−32% MP B in 40 min; 32−100% MP B in 5 min.

Table 1. Method Precision: Migration Time (minutes), Percent Total Acidic Peaks, Percent Main Peak, Percent Total Basic Peaks (n = 15)

mAbs	time (min)	RSD %	acidic %	RSD %	main %	RSD %	basic %	RSD %
1	24.6	0.11	22.1	4.2	67.5	1.7	10.7	7.1
2	28.3	0.11	10.6	4.9	81.7	1.8	7.7	7.8
3	34.2	0.21	16.9	3.6	68.5	1.6	14.6	2.6

RESULTS AND DISCUSSION

Separation of IgG mAbs and Their Charge Variants. A separation of three mAbs (one IgG1 and two IgG2 subclasses) with different p*I*s (8.8 for mAb1, 7.8 for mAb2, and 7.7 for mAb3) was investigated using an uncoated capillary (Figure 1). A concentrated zwitterionic buffer was used to suppress protein adsorption to the capillary wall.[20,21] The effect of EACA concentration on the separation is shown in Figure 1. At a low EACA concentration (50 mM), which was similar to the concentration (45 mM) used in the coated capillary,[7] the separation of different mAbs was poor (Figure 1A). Extremely broad peaks suggest adsorption of the proteins to the capillary inner surface. An increase in EACA concentration improved separation efficiency and resolution of the different mAbs. Significantly enhanced separation efficiency and selectivity was obtained when 600 mM EACA was used (Figure 1E). However, in the absence of acid flushing between runs and upon extended storage of the capillary filled with separation buffer, the main peak of each mAb became broad after only about 10 injections (Figure 2). Similar observations were reported by Deng et al.[16] Peak broadening was particularly serious for more basic protein isoforms (e.g., peak 1

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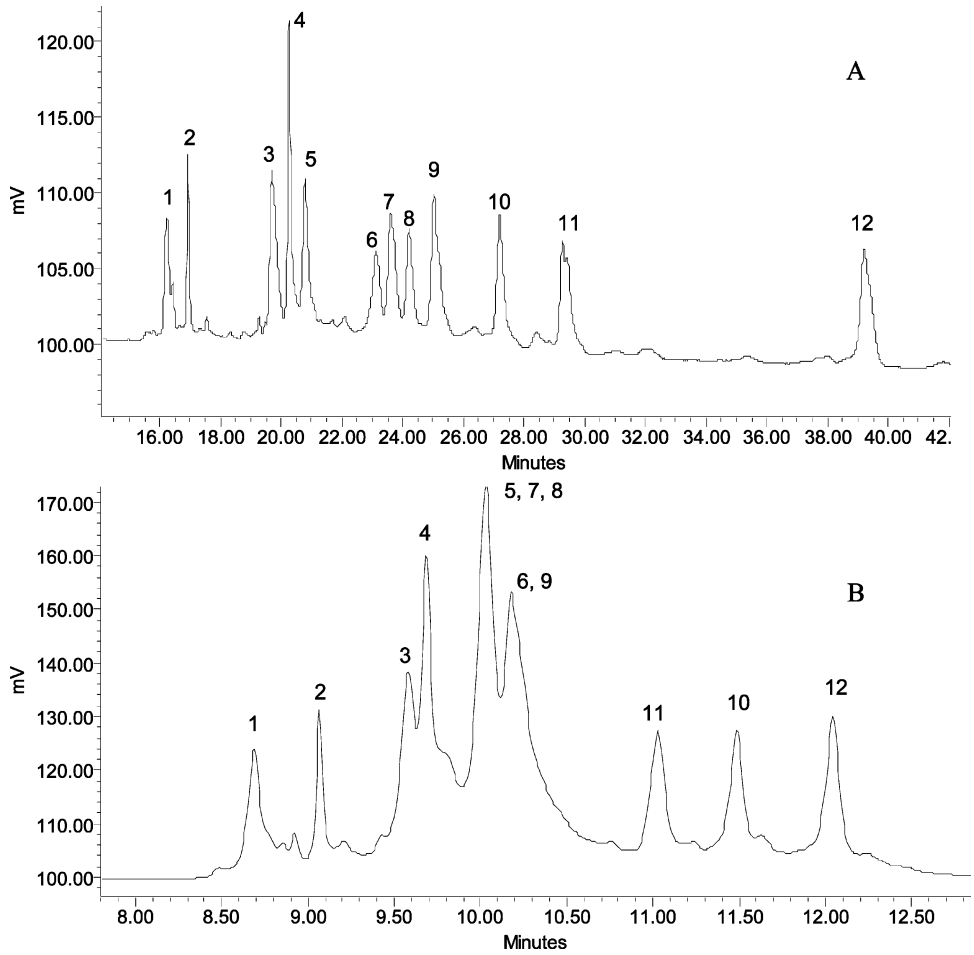


Figure 4. Separation of various IgG1 and IgG2 mAbs with CZE in an uncoated (A) and coated (B) capillary column. Peaks 2 and 4 are IgG1 mAbs; the remaining are IgG2.

in Figure 2B). In order to resolve this issue, the capillary was flushed with 0.1 N HCl between injections and stored in 10 mM H_3PO_4 when not in use. The flushing of a bare capillary with 0.1 N HCl to suppress the electroosmotic flow (EOF) has been previously demonstrated by Yeung and Fung[22] for DNA sequence analysis with CGE performed at pH 8.3 in an uncoated capillary. Nevins and others[23] also used a similar treatment of the capillary to separate topoisomers of plasmid DNA in an uncoated capillary. It was hypothesized that the silanol groups should be fully protonated in a dry fresh fused-silica surface resulting in initially negligible EOF and minimal adsorption of the DNA or proteins to the capillary.[23] However, the ionization of the silanol groups gradually increases due to the pH of the separation matrix (pH 5.5 in this work) and consequently causes the adsorption of protein onto the inner wall of the uncoated capillary over time. Conditioning the capillary in between runs with 0.1 N HCl can remove the adsorbed proteins as well as protonate the residual surface silanol groups, facilitating the adsorption of nonionic hydroxylpropyl methylcellulose (HPMC) to the neutral surface. In addition, concentrated EACA can interact with residual silanol groups that are not covered by HPMC. Good reproducibility of the migration time was demonstrated by two analysts on 3 consecutive days with 5 injections on each day (Figure 3). The migration time % RSDs for three mAbs were in the range of 0.11−0.21% (Table 1). The percentage of total acidic or basic peaks is useful in monitoring mAb charge variants between lots and on stability.[14] The charge variants can be reproducibly quantified (Table 1). The limit of detection (LOD) and limit of quantitation (LOQ) are about 2 and 6 μg/mL, respectively. In addition, good linearity for measurement of charge variants over the expected range of sample concentration (0.3−3.0 mg/mL) was demonstrated for all of the three mAbs. The correlation coefficient (R^2) was in the range of 0.9875−0.9996. It should be noted that the capillary was filled with 10 mM H_3PO_4 for overnight and long-term storage. About 150 runs were performed over 1 month without obvious deterioration of separation performance of the uncoated capillary column.

CZE is one possible approach for determining the identity of various mAbs. In addition to migration time reproducibility, adequate separation selectivity is important for this application. The separation selectivity was evaluated in the separation of 12 different IgG1 and IgG2 mAbs (Figure 4A). Since they are all IgG mAbs, these molecules are of similar molecular weight and share significant sequence homology. Their p*I* values are in the range of 7.0−9.2. The p*I* difference of some mAbs was less than 0.1 pH unit (e.g., peak 1, IgG2; p*I* 9.10; peak 2, IgG1; p*I* 9.10). Reproducible separations with adequate resolution were achieved for all of the 12 mAbs. The % RSDs for the migration time of the main peaks

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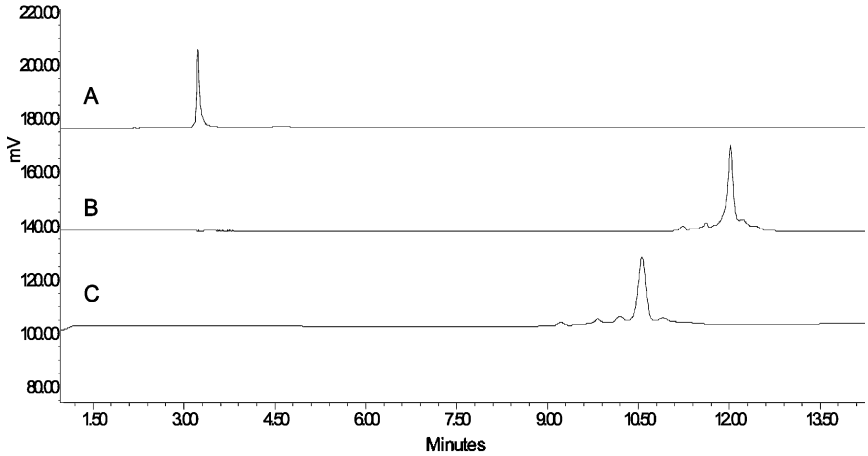


Figure 5. Comparison of charge variants separations of IgG mAbs with CZE in a coated, 10 cm effective length, (A) coated, 40 cm effective length (B), and an uncoated, 10 cm effective length (C) capillary.

for the 12 mAbs in the mixture from six consecutive injections was in the range of 0.1−0.3%. The reproducible and unique migration time of the main species of different mAbs provides the specificity needed for final product identity testing. For comparison, we separated the mAbs with the existing CZE method in a coated capillary (Figure 4B).[7,15] The CZE separation in an uncoated capillary provided better separation selectivity than CZE in a coated capillary. The major reason for the improved selectivity in an uncoated capillary over a coated capillary is due to the use of concentrated EACA buffer (see concentration effect in Figure 1) and higher pH. The separation of IgG mAbs was also tested with diluted EACA buffer (40 mM) and a higher pH (5.5) in a coated capillary. Peak broadening of certain IgG mAbs was observed indicating the adsorption of protein to the inner surface of the coated capillary. It was surprising to find that the combined use of concentrated (600 mM) EACA buffer, diluted HPMC (0.1%), and acid flushing more efficiently eliminated protein adsorption than the combined use of diluted (40 mM) EACA buffer, concentrated HPMC (0.2%), and permanent coating. A similar phenomena (coated capillary vs 0.1 N HCl treated uncoated capillary) in terms of EOF suppression was also observed in Yeung and Fung's work on DNA separation.[22] We also separated the mAbs (Figure S-1 in the Supporting Information) with a recently reported IEC method.[24] The peak capacity provided by CZE in an uncoated capillary is comparable to that by IEC.

Charge variants of mAbs can result from deamidation, C-terminal lysine processing, cysteinylation, and glycation.[3,7,14] High-throughput analysis of charge variants is needed for screening large numbers of in-process and stability samples to support bioprocess and formulation development. For this application, a more rapid separation in a shorter capillary was evaluated. Figure 5B,C compares the separation of charge variants of an IgG mAb using this CZE method in a 10 cm effective length uncoated capillary with a separation achieved with a CZE method in a 40 cm effective length coated capillary. The separation conditions used in the coated capillary were similar to that used in the work by Ma and Nashabeh.[7] CZE at a higher pH (5.5) in a shorter (10 cm effective length) uncoated capillary provided better separation selectivity than that by CZE at lower pH (5.0) in a longer coated capillary (40 cm effective length). Separation in a 10 cm coated capillary (Figure 5A) was very fast at the expense of separation resolution. It was observed that the separation of charge variants in an uncoated capillary could be significantly improved by using a longer uncoated capillary (e.g., 40 cm effective length) at the cost of increased separation time (Figure S-2 in the Supporting Information). As an uncoated capillary is much more economical than the coated capillary, this methodology has the potential to be adapted to a multiplexed CE system[25] to provide both high separation selectivity and high throughput.

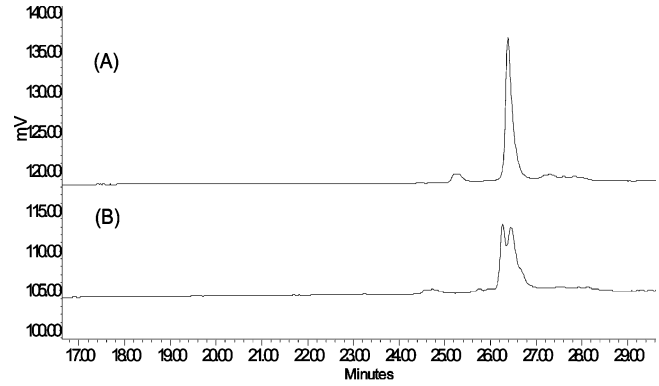


Figure 6. Separation of IgG1 (A) and IgG2 mAb (B) in the presence of urea with CZE. Experimental conditions: electrophoresis buffer, 600 mM EACA−acetic acid, pH 5.5, HPMC 0.1%, urea 2 M. The others parameters are the same as those in Figure 1.

Separation of Disulfide Isoforms. Initially, the CZE separation of IgG1 and IgG2 mAbs was investigated in a separation buffer without urea. The addition of urea to the separation buffer was evaluated to determine if it would improve the separation of the mAbs and their charge variants. While the separation of the different mAbs and their charge variants was not improved by the addition of urea, unexpectedly, the addition of 2 M urea resulted in doublet peaks for the IgG2 mAbs (Figure 6B). Since the IgG1 mAbs retained a single main peak (Figure 6A), the results suggested that the doublet peaks of IgG2 mAbs may result from the presence of IgG2 disulfide isoforms recently described by Wypych et al.[18] Previous manuscripts have described protein unfolding in the presence of urea.[26−28] The conformational stability

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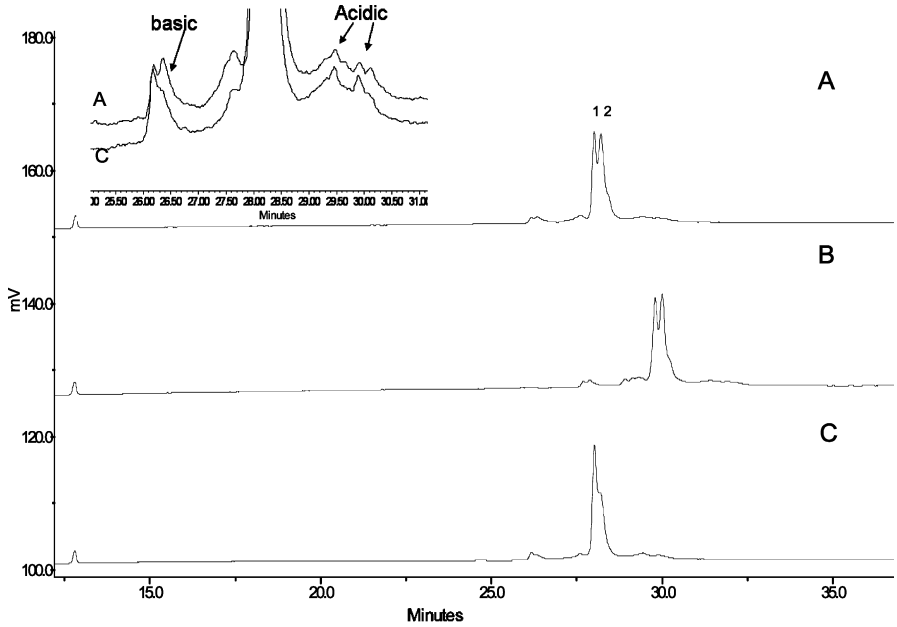


Figure 7. CZE electropherograms of an IgG2 mAb control (A), IgG2 mAb post deglycosylation treatment (B), and IgG2 mAb post redox treatment (C).

of the IgG2 disulfide isoforms was found to be dependent on the arrangements of disulfide bonds.[5,18] Disulfide isoforms may be unfolded to different extents in the separation buffer with urea, resulting in different hydrodynamic sizes and consequently dissimilar electrophoretic mobilities, yielding a separation. This phenomenon may be similar to what was observed by Hjertén et al. in the use of urea-CZE for the separation of folded and unfolded forms of human transferrin as well as for the separation of transferrin isoforms with alternative iron loading.[29]

IgG mAbs have the potential to have numerous post-translational modifications, such as glycosylation, which can change their migration behavior in a CZE separation. To exclude the possibility that the doublet peak of the IgG2 antibody was due to different glycosylation states of the antibody, an IgG2 mAb was treated with PNGase F to remove N-linked glycans prior to the separation by CZE (Figure 7B). The deglycosylation caused an increase in migration time (IgG2 mAb became more acidic after removal of glycans) but did not result in the elimination of the doublet peak. The results suggested that the doublet peak of IgG2 mAb was not glycosylation-related. To provide support for the hypothesis that the doublet peak of IgG2 mAb was disulfide isoform related, the IgG2 mAb was subjected to redox conditions to stimulate isomer exchange. The addition of cysteine and cystine to the sample provided an environment conducive to altering the equilibrium between the isomers.[17−19] Samples were placed in a sample tray at 15 °C and injected at increasing time increments. The significant changes in ratio of the two peaks after redox treatment (Figure 7C) suggested that the doublets resulted from different arrangements of disulfide bonds. It should be noted that the basic and acidic species also showed doublet peaks which changed in the same manner as the doublet of the main peak after redox treatment.

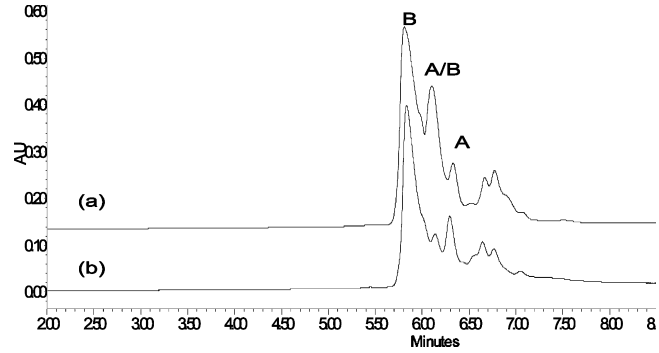


Figure 8. RPLC chromatograms of the first IgG2 mAb control (A) and post redox treatment (B).

Identification of disulfide isomers in the RPLC and IEC separation have been performed with peptide mapping and mass spectrometry (MS).[5,18] Collecting fractions from CE separation for peptide mapping/MS, however, was not feasible due to the extremely small sample volume used in CE. Identification of peaks was initially attempted by analyzing the related RPLC fractions (Figure 8) with CZE. Disulfide isomers were isolated by RPLC (column temperature = 85 °C, high percentage of organic solvent, low pH) yielding fractions that were completely denatured. This resulted in extremely broad peaks (data not shown) in the CZE separation, suggesting the strong adsorption of denatured isoforms on the capillary inner surface. Attempt at identification of peaks by analyzing the related IEC fractions with CZE was also failed due to poor IEC separation of disulfide isomers of this particular IgG2 mAb. Data supporting the identification of doublet peaks was obtained by comparing the variation of peaks in both CZE and RPLC after the IgG2 mAb was subjected to a redox environment. The redox treatment caused a significant decrease in A/B isoform and enrichment of B isoform in RPLC separation (Figure 8). It was conceived that the decreased peak (peak 2 in Figure 7A) in urea-CZE separation should also be the A/B isoform,

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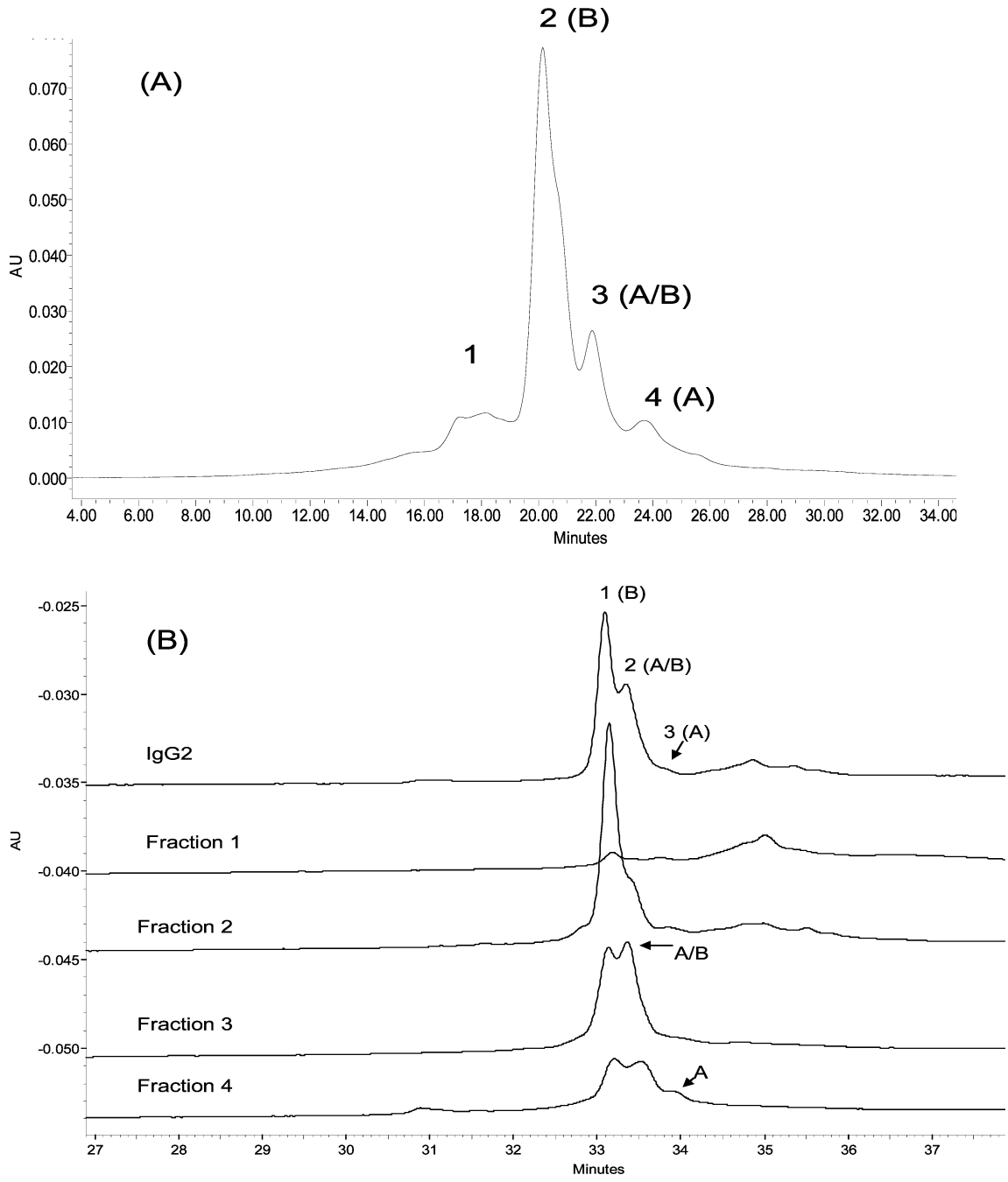


Figure 9. IEC chromatogram of the second IgG2 mAb (A) and CZE electropherograms of the second IgG2 mAb and its IEC fractions (B).

and the increased peak (peak 1 in Figure 7A) should be the B isoform. Assignment of the A isoform was not clear as its change was not significant during exposure to redox conditions. A further experiment was performed using another IgG2 mAb which could be separated by both IEC (Figure 9) and urea-CZE. Fractions were collected from the IEC separation and analyzed by urea CZE. Consistent with the data supporting peak identities for the first IgG2 mAb, the experiment showed that the first peak was the B isoform, the second peak contained the A/B isoform (Figure 9B), and the third small peak was likely the A isoform. The first peak of the doublet peaks for fraction 3 came from carryover of large peak tailing of the B isoform in IEC fractions, and the first and second peaks of the triplet peaks for fraction 4 came from carryover of peak tailing of the B and A/B isoform in IEC fractions. The migration order of the B, A/B, and A isoforms in CZE was in agreement with their conformational stability. It has been shown that the B isoform is more thermally stable than A and A/B isoforms.[5,18] Hence, the B isoform should be more resistant than the A/B and A isoforms to urea denaturation and, thus, would have a smaller hydrodynamic radius and a faster electrophoretic mobility.

Since the separation of the disulfide isoforms was strongly affected by the presence of urea, various levels of urea were added to the separation buffer. As shown in Figure 10, the separation of disulfide isoforms was improved when urea concentration was increased from 0 to 4 M. A further increase of urea concentration from 4 to 6 M, however, caused a decrease in resolution of the doublet peaks. Capillary temperature also influenced the separation of disulfide isoforms as determined by testing several

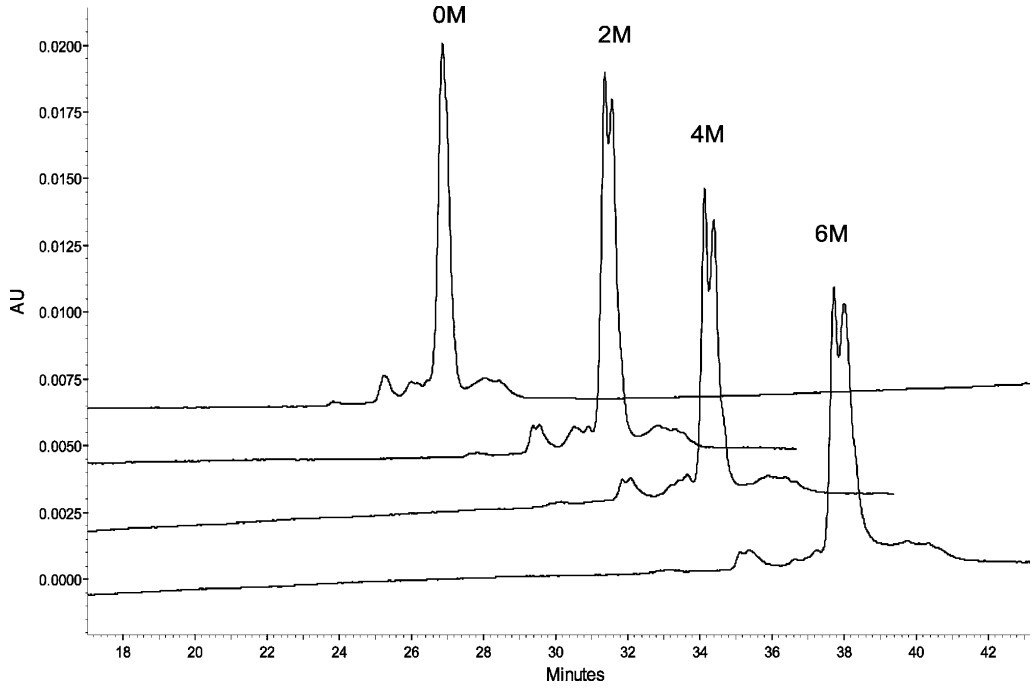


Figure 10. Effect of urea concentration on the separation of disulfide isoforms of the first IgG2 mAb. Urea concentration ranges from 0 to 6 M.

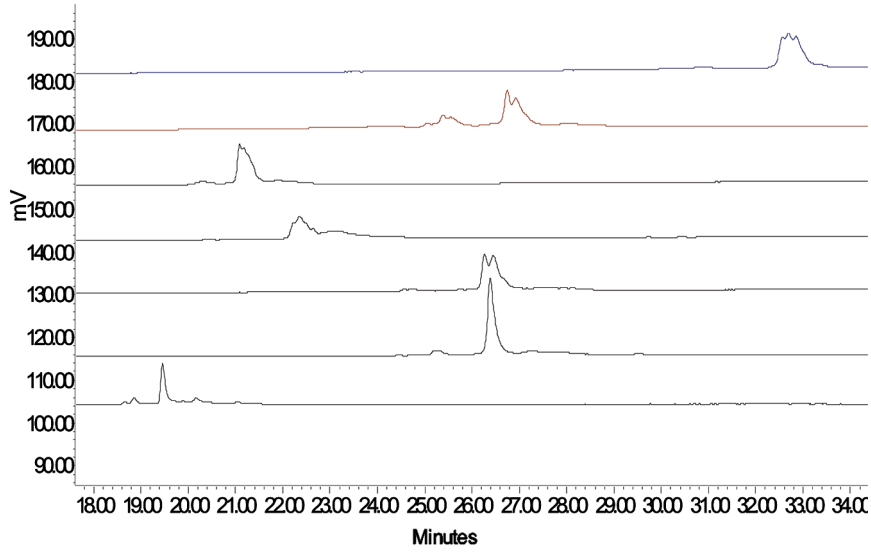


Figure 11. Urea-CZE electropherograms of various IgG1 (lower two) and IgG2 (upper five) mAbs.

temperatures in the range of 17−45 °C. The separation of doublet peaks was enhanced when the temperature was increased from 17 to 35 °C. A further increase of the temperature decreased the resolution of the doublet peak (data not shown). An optimal separation of the doublet peaks was achieved at 35 °C.

Under the conditions optimized for one IgG2 mAb, different IgG1 and IgG2 mAbs were tested (Figure 11). All IgG1 mAbs showed one sharp peak, consistent with the fact IgG1 mAbs are not known to exist as disulfide isoforms.[5,17,18] On the other hand, the IgG2 mAbs showed triplet or doublet peaks, suggesting the presence of disulfide isoforms that could be at least partially resolved by CZE. It was not possible to obtain an equivalent separation for all of the different antibodies analyzed by CZE under identical separation conditions. Experimental conditions may need to be adjusted for optimal separation of disulfide isomers for different IgG2 antibodies. We have found HPLC method (RP and IEC) conditions also often require optimization for different IgG2 molecules; thus, the availability of multiple orthogonal tools for the separation of disulfide isoforms remains important.

In addition to identity, charge variants, and disulfide isomer analysis of IgG antibodies, glycan analysis of IgG antibodies was recently evaluated with CZE using a similar separation buffer in an uncoated capillary column. A rapid, selective and reproducible separation of N-linked glycans was achieved (see Figures S-3−S-5 and Table S-1 in the Supporting Information). The separation was comparable to that of existing methods performed in coated capillaries.[7,30,31]

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CONCLUSIONS

CZE using similar separation buffer in an uncoated capillary provides a versatile, generic, and economical approach to the characterization of IgG mAbs. It can be used to selectively separate a mixture of different IgG mAbs, making it useful as a potential identity test. It also separates charge variants for each IgG mAb and, therefore, may be useful in process and formulation development for mAb therapeutics. The urea-CZE method offers an orthogonal approach to analyze the disulfide isomers of IgG2 mAbs. The migration order of different isoforms in urea-CZE was correlated with their conformational stability. The method also revealed the distribution of disulfide isoforms within basic, main, and acidic species of IgG2 molecules. Preliminary results showed that the CZE method could be readily adapted for glycan analysis of IgG antibodies. The use of uncoated capillaries for these separations offers flexibility for future evaluations of multiplex approaches, which are important for the high-throughput analysis of biotherapeutics in development.

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SUPPORTING INFORMATION AVAILABLE

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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