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Fast separation and analysis of reduced monoclonal antibodies with capillary zone electrophoresis coupled to mass spectrometry

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ABSTRACT

Capillary zone electrophoresis–electrospray ionization–mass spectrometry (CZE–ESI–MS) was used for analysis of reduced antibodies. We first developed a simple protocol to condition commercial linear-polyacrylamide coated capillaries for use in top–down proteomics. We then suspended reduced antibodies in a solution of 35% acetic acid, 50% acetonitrile in water. Heavy and light chains were baseline resolved within 10 min and with 3–30 μg/mL detection limits using a 0.1% aqueous formic acid background electrolyte. Quintuplicate runs of a two-antibody mixture produced relative standard deviations of ~1% in migration time and 10% in peak amplitudes. Resolution was further improved for the two-antibody mixture by using 5% acetic acid as the background electrolyte, highlighting the potential of capillary electrophoresis–mass spectrometry for analysis of antibody mixtures.

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1. Introduction

Capillary zone electrophoresis (CZE) provides both fast and efficiency separations for a wide range of biological molecules [1–3]. CZE has been coupled to mass spectrometers (MS) since the late 1980s [4], and various electrospray ionization (ESI) interfaces have been developed to apply electric field at the distal end of capillary for electrospray [5–10]. Our group developed an electrokinetically pumped sheath-flow nanospray CZE–ESI–MS interface, which transfers the analyte from capillary to a glass emitter filled with sheath liquid for nanospray [11–13]. This nanospray sheath-flow interface provides a stable spray at very low sheath flow rates with neither mechanical pump nor nebulizer gas. The interface has been effective for various bottom–up proteomics experiments [14–18]. With our second generation interface, picogram to femtogram amounts of *E. coli* digests were analyzed [17]. To date, over 10,000 peptides have been identified in a single run with this interface [18].

In addition to bottom–up proteomics, our sheath-flow interface has also been employed to top–down proteomics [19–22]. 56 proteoforms were identified in a single run for the secretome from *Mycobacterium marinum* [20]. A comparison of fragmentation patterns and identification rates of ETD and HCD demonstrated its compatibility to electron transfer dissociation (ETD) [21]. CZE was evaluated for separation of proteins in the 30–80 kDa range [22].

In this manuscript, we extend the application of this CZE–ESI–MS platform to the characterization of intact monoclonal antibodies. Monoclonal antibodies (mAbs) are tetrameric glycoproteins with molecular weights of approximately 150 kDa [23]. They consist of four polypeptide chains: two heavy chains (HCs) (~50 kDa each) and two light chains (LCs) (~25 kDa each) linked by disulfide bonds. The Fc regions on HCs bear highly conserved N-glycosylation sites that contribute to the mAbs heterogeneity. Other modifications such as deamidation and oxidation can also impact the function of mAbs. As a result, quality control is required to ensure mAb efficiency, consistency, and stability. Conventionally, bottom–up liquid chromatography–mass spectrometry (LC–MS) is used to characterize isoforms of a digested mAb. However, this bottom–up approach suffers from incomplete sequence coverage, the introduction of artificial modifications, and time-consuming data analysis [24,25].

To overcome these shortcomings, the intact antibody could be directly analyzed by mass spectrometry [24–27]. However, this top–down analysis requires use of mass spectrometers with extremely high resolving power. Middle–down approaches, on the other hand, analyze subunits of mAbs, including reduced mAbs (HCs and LCs) and large fragments generated by limited proteolysis (Fab and Fc fragments) [28,29]. Middle–down analysis can use mass spectrometers with lower resolution than top–down approaches and introduces fewer artifacts compared to traditional bottom–up approach.

Although liquid chromatography is more often used for mAbs separations, various proteomic studies have investigated CZE–ESI–MS as a promising platform for mAbs characterization, mostly

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through glycan analysis [30,31]. In this manuscript, we report the use of CZE for separation of light and heavy chains of reduced antibodies with top–down analysis.

2. Materials and methods

2.1. Materials and reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. Formic acid (FA) and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Methanol was purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). Water was deionized by a NanoPure system from Thermo Scientific (Marietta, OH). TCEP solution was included in an iTRAQ kit from Ab Sciex. C4 Ziptip was purchased from EMD Millipore (Billerica, MA). Mouse Anti-Human IgG4 Fc-UNLB was purchased from SouthernBiotech. Human/Mouse/Rat Activin A beta A subunit Antibody was purchased from R&D System. Anti-Phosphotyrosine Antibody was purchased from EMD Millipore. Linear polyacrylamide (LPA)-coated fused capillary was purchased from Polymicro Technologies (Phoenix, AZ).

2.2. Sample preparation

A 10 μg aliquot of antibody solution was denatured and reduced by adding twice the volume of 6 M guanidine HCl dissolved in 100 mM NH_4HCO_3 followed by adding 2 μL of 500 mM TCEP solution. The mixture was incubated at 37 °C for 30 min. About half a microliter of solid iodoacetamide (dispensed with a pipette tip) was directly dissolved in the mixture and the mixture was incubated at 37 °C in the dark for 15 min. Finally, the antibody solution was desalted with a C4 Ziptip and eluted with 35% acetic acid and 50% acetonitrile in water.

2.3. CZE–ESI–MS analysis

The CZE system was coupled to an LTQ XL or a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA USA). Electrospray was generated using an electrokinetically pumped sheath flow nanospray emitter [13,17,32]. The borosilicate glass emitter (1.0 mm o.d. × 0.75 mm i.d., 10 cm length) was pulled with a Sutter instrument P-1000 flaming/brown micropipet puller. The emitter orifice inner diameter was 10–12 μm.

Separation was performed using a commercially coated 50 cm long, 30 or 50 μm I.D., 150 μm O.D. LPA-coated fused capillary (Polymicro Technologies, Phoenix AZ USA). Initial separations of antibodies in these commercially-coated capillaries were unsuccessful, presumably due to irreversible adsorption onto active sites within the capillary. To minimize this adsorption, a standard protein mixture was prepared in 0.04% FA, 20% ACN in water by dissolving cytochrome c, myoglobin, carbonic anhydrase, and β-casein with concentrations of 0.1 mg/mL, 0.1 mg/mL, 0.2 mg/mL, and 0.8 mg/mL, respectively. About 20 nL of this standard protein solution was injected into the capillary. After injection, both voltage and pressure were applied to pump the mixture through the capillary. Once a signal was detected by the mass spectrometer, the capillary was flushed with background electrolyte (0.1% FA) for 10 min. This conditioning step was successful in reducing loss to the inner surface and allowed detection of antibody chains.

The background electrolyte was 0.1% (v/v) FA and the electrospray sheath liquid was 10% (v/v) methanol and 0.1% (v/v) FA, unless stated otherwise. The separation voltage was 20 kV. Full MS scans were acquired over the *m*/*z* 600–2000 range in the LTQ-XL mass spectrometer. For LTQ-Orbitrap Velos detection, full MS scans were acquired in the Orbitrap detector over the m/z 600–2000 range at a resolution of 60,000 at *m*/*z* of 400. The c-trap pressure was lowered from 0.5 to a value between 0.1 and 0.2 on the pressure regulator.

3. Results and discussion

3.1. LPA-coated capillary treatment

Fused silica capillary tends to adsorb intact proteins, which causes protein loss during CZE analysis [33]. LPA coating is commonly used to minimize adsorption to the capillary wall. Unfortunately, we have observed both batch-to-batch and within-batch difference in the performance of commercially available LPA-coated capillaries. In some cases, severe protein loss is observed during the first few runs, presumably due to adsorption of proteins onto uncoated regions. However, the signal often recovers for later runs. This observation led to a simple strategy to pre-occupy those uncoated spots with sacrificial proteins before injecting analyte. In this protocol, a plug of relatively high concentration standard proteins is passed through the column using a combination of pressure and electrokinetic flow. The capillary is ready for analysis once the proteins have left the capillary.

We evaluated the performance of the treated capillary by performing electrophoresis with the same standard protein sample that was used to treat the capillary. No signal was observed for the untreated capillary, whereas signal was restored following treatment, Fig. 1. The treatment was also effective after flushing the capillary with 0.1% formic acid.

3.2. High concentration acetic acid aids in solubilizing reduced antibodies

Samples were desalted using a C4 ZipTip before eluting in the injection electrolyte; desalted samples undergo stacking during injection, allowing use of larger injection volumes. We observed no antibody signal after sample desalting with elution in 50% acetonitrile 0.1% formic acid in our initial experiments. We

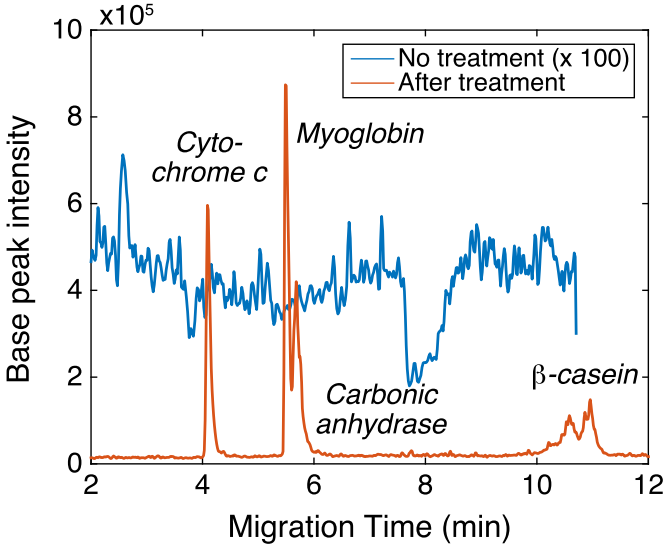


Fig. 1. Base peak electropherograms of a four-standard protein mixture (cytochrome c – 0.1 mg/mL, myoglobin – 0.1 mg/mL, carbonic anhydrase – 0.2 mg/mL and β-casein – 0.8 mg/mL in 0.04% FA, 20% ACN in water). Blue trace: before capillary treatment; this trace was multiplied by 100 before plotting. Orange trace: after capillary treatment. Data were treated with a Lowess filter with five-point span before they were plotted. LTQ-XL mass spectrometer was used for detection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

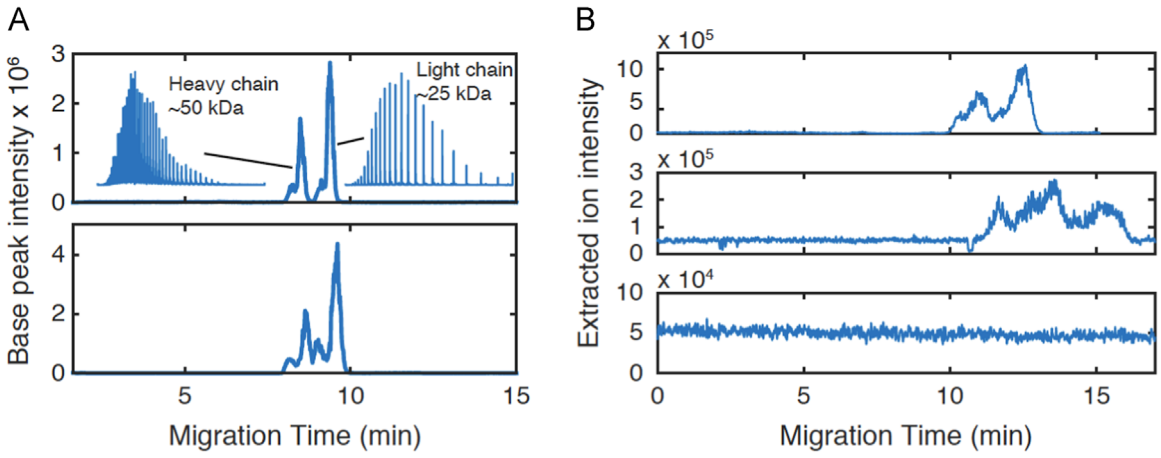


Fig. 2. Base and extracted ion electropherograms of reduced mAbs. (A) Duplicate base peak electropherogram of CZE–ESI–MS analysis of a reduced mAb (mouse anti-human IgG4 Fc-UNLB), including two isoforms of heavy chain and light chain. Mass spectra correspond to elution profiles of the designated polypeptide chains. (B) Extracted ion electropherograms of reduced mouse anti-human IgG4 Fc-UNLB dissolved in 0.01%OG and 50% ACN in water. Top, middle, bottom electropherograms correspond to 10 min, 1 h and 1.5 h after desalting of sample. LTQ-XL mass spectrometer was used for detection.

suspected that reduced antibodies tend to precipitate during eluting in 50% acetonitrile 0.1% formic acid. High concentration acetic acid (70% (v/v)) has been employed to improve the solubility of secretome from *M. marinum* in top–down CZE analysis [20]. However, inefficient ionization was observed during ESI process with the use of an acetic acid solution. In this experiment, we decreased the acetic acid concentration to 35% (v/v) with no observable decrease in ionization efficiency. 35% acetic acid and 50% acetonitrile (ACN) was used to elute the reduced antibody from a C4 ziptip column to help retain the denatured antibody chains in solution. The resulting desalted antibody sample can be kept in solution after desalting for months, and be directly injected for CZE–MS analysis. Duplicate runs were performed for mouse anti-human IgG4 Fc-UNLB, which is a mouse IgG1 antibody specific to the Fc region of human IgG4 antibody (Fig. 2A). Heavy and light chains were baseline separated within 10 min. Two major isoforms for both heavy chain and light chain were partially separated with peak width of about 30 s. Migration profile and retention times for duplicate runs were similar. The second run was performed 2 h after the first run. Since both acetic acid and ACN are volatile, there was a decrease in sample volume after 2 h. As a result, the peak intensity in the second run was about twice as high as in the first run, which matched the increase in sample concentration estimated by observing the volume of sample solution. Table S1 lists the migration times, peak intensities, and their relative standard deviations for each peak.

We also investigated the use of the mass spectrometry-friendly surfactants 0.01% octyl β-D-glucopyranoside (OG) and 50% ACN to elute the reduced antibody from the C4 ZipTip column. As shown in Fig. 2B, CZE–ESI–MS runs were performed 10 min, one hour, and 1.5 hour after desalting. There were two peaks (heavy chain and light chain) observed in the extracted ion electropherogram with reasonable intensities for the first run. After one-hour storage at 4 °C, the signal intensity reduced significantly, and no signal was observed after 1.5 h. Other surfactants (CHAPS and digitonin) were tested, but no antibody signal was detected after 2 h of storage. With the above results, we concluded that the presence of high concentrations of acetic acid in the eluting solution is important for enhancing reduced antibody solubilization.

A standard protocol of reduced antibody sample preparation for CZE–MS analysis was developed, and sample preparation requires 1 h, including reduction, alkylation and desalting. The desalted reduced antibody was eluted in 35% acetic acid and 50% ACN in water, which is compatible with CZE–MS analysis. It is worth mention that this reduced antibody sample can be kept for months with no observed sample loss.

3.3. *Evaluating the performance of CZE–ESI–MS for separation and detection of reduced antibodies*

We next investigated the performance of the CZE–ESI–MS system with three monoclonal antibodies. Mouse anti-human IgG4 Fc-UNLB (antibody A), human/mouse/rat activin A beta A subunit antibody (antibody B), and anti-phosphotyrosine antibody (antibody C) were reduced, alkylated, desalted, and analyzed by our CZE–ESI–MS system. All antibodies were prepared at a starting concentration of 1 mg/mL. The presence of 50% ACN in the sample resulted in stacking, which allowed an increase in the sample volume that can be injected. Approximately 70 nL (~7% of total capillary volume) of each sample was injected into the capillary. Fig. 3 presents the electropherograms for each antibody. The light chains and heavy chains for both antibody A and B were baseline separated. Moreover, two isoforms were partially separated and observed for each polypeptide chain. Figs. S1–S3 show the

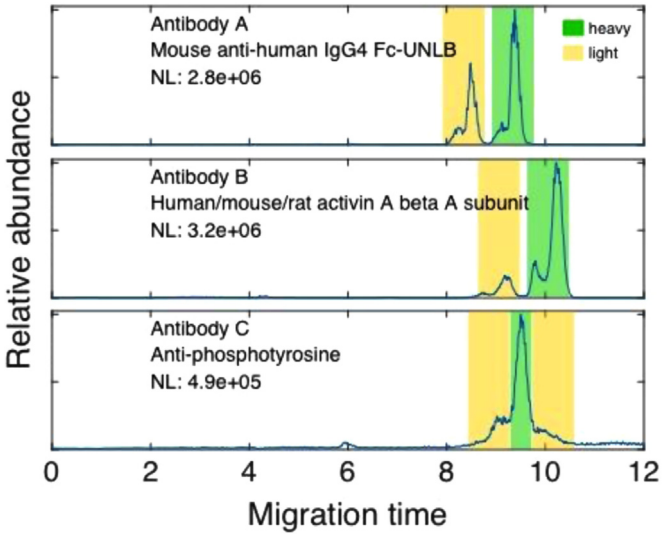


Fig. 3. Base peak electropherograms of CZE–ESI–MS analysis of reduced antibody A, B and C. Data treated with a Lowess filter with five point span before plotting. 50 cm long, 50 μm I.D. capillary was used for separation. About 70 nL sample was injected for each analysis. An LTQ-XL mass spectrometer was used for detection.

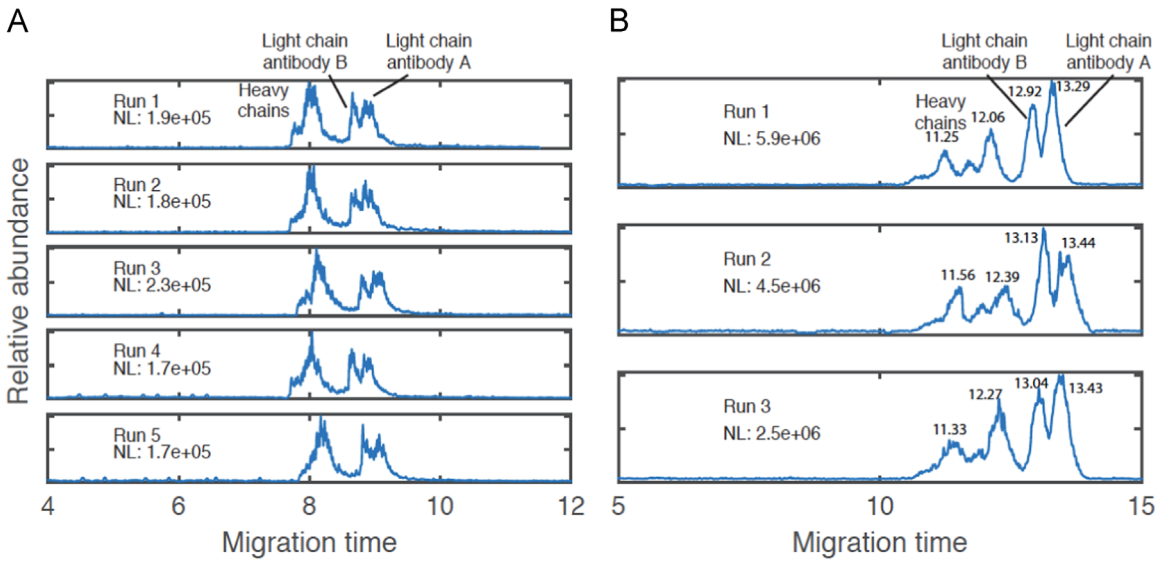


Fig. 4. Base peak electropherograms for a two-antibody mixture (mouse anti-human IgG4 Fc-UNLB and human/mouse/rat activin A beta A subunit antibody), including migration times for each heavy chain and light chain. Major peaks were assigned in the 1st run. (A) Quintuplicate CZE–ESI–MS analysis of a two-antibody mixture. 50 cm long, 30 μm I.D. capillary was used for separation. About 18 nL liquid aliquot was injected for each analysis. Voltages: 21.3 kV/1.3 kV. LTQ-Orbitrap Velos mass spectrometer was used for detection with Orbitrap as detector. (B) Triplicate CZE–ESI–MS analysis of a two-antibody mixture. 50 cm long, 30 μm I.D. capillary was used for separation. About 30 nL liquid aliquot was injected for each analysis. Sheath liquid: 0.5% FA, 10% methanol. Voltages: 23.7 kV/1.2 kV. LTQ-XL mass spectrometer was used for detection. NL is the normalization level.

deconvoluted masses for HCs and LCs. However, the signal intensities for HC of antibody C and some minor isoforms were too low to calculate their masses. There was only ~1 Da difference between LC isoforms. This difference could be the result of deamidation or incomplete reduction of intrachain disulfide bond, which introduce only 1~2 Da mass shift but introduces a difference in charge. The average separation window was about 3 min and the average peak widths (full width at baseline) for each light chain and heavy chain peaks were about 30 s. Plate counts were about 15,000. The light chain and heavy chain peaks overlapped for antibody C, which was caused by an extremely wide heavy chain peak. Based on the spectra complexity (Fig. S3), one possible explanation for the wide migration time range is that the heavy chain for this antibody is highly modified.

The noise in the baseline was estimated as the standard deviation of the region between 2 and 4 min. The signal-to-noise ratio for the peaks ranged from 100 to 1000, corresponding to extrapolated detection limits of ~3–30 μg/mL (~2–20 fmol) of antibody. These data were acquired using an LTQ-XL mass spectrometer; use of a higher sensitivity mass spectrometer will improve the detection limits.

A two-antibody mixture was prepared by mixing 10 μg of antibody A and 2 μg of antibody B in 10 μL of a 35% acetic acid and 50% ACN solution. The mixture was analyzed by the system in quintuplicate runs, Fig. 4A. Heavy chains and light chains were partially resolved within 10 min. The light chains were assigned by calculating their masses, while the signal intensities were not strong enough to calculate the masses of the heavy chains. No flushing was performed between runs, but the protein signals ($\sim 4 \times 10^5$) were about 100-fold higher than the background signals ($\sim 5 \times 10^3$), which was measured at 0–2 min. Sample carryover was negligible in this experiment. The migration times for the polypeptide chains were highly reproducible with relative standard deviations of about 1%. The signal intensities were reasonable consistent with a relative standard deviation of about 13%.

We improved separation efficiency by use of a 5% acetic acid background electrolyte. We have previously demonstrated that 5% acetic acid effectively increases the width of separation window by minimizing electroosmotic flow and increasing viscosity [20]. Fig. 4B shows triplicate runs of a two-antibody mixture. Using 5% acetic acid as background electrolyte generated a separation window of 4 min, which was twice the width produced by 0.1% FA. The analysis was finished within 14 min. Both heavy chains and light chains were separated with improved resolution compared with 0.1% FA.

4. Concluding remarks

We describe the first study of the separation and analysis of reduced monoclonal antibodies on a CZE–ESI–MS system. The system provides fast separation, good reproducibility, and reasonable sensitivity, which shows the potential for CE–MS serving as a quick diagnostic tool for mAbs quality control. Furthermore, we have demonstrated the separation and analysis of a two-antibody mixture, highlighting the possibilities for CZE–MS to characterizing mAb mixture and even polyclonal antibodies. However, the resolution for antibody isoforms was limited in this experiment. A higher resolution mass spectrometer can yield more information on the molecular weight of separated antibody isoforms. Also, employing a pre-fractionation method before CZE–MS analysis would greatly improve the separation resolution for more complex antibody sample, which will be addressed in future work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.11.020.

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