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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.

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

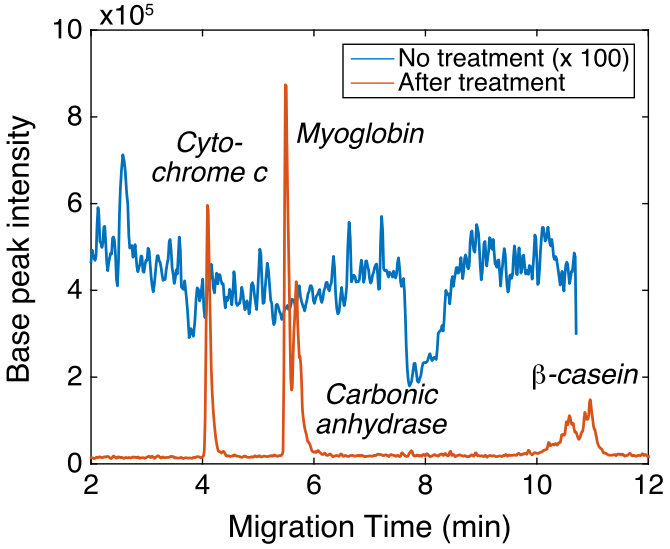

Fig. 1. Base peak electropherograms of a four-standard protein mixture (cytochrome c – 0.1 mg/mL, myoglobin – 0.1 mg/mL, carbonic anhydrase – 0.2 mg/mL and β-casein – 0.8 mg/mL in 0.04% FA, 20% ACN in water). Blue trace: before capillary treatment; this trace was multiplied by 100 before plotting. Orange 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.)