Abstract

This Application Note shows the performance of a high resolution capillary isoelectric focusing method on a fluorocarbon coated capillary. A well resolved charge isoform pattern was observed for three commercially available monoclonal antibody samples. Good repeatability was obtained by introducing an additional capillary cleaning step comprising a flush with dilute base after every sixth run. In terms of intermediate precision of measured charge variant isoelectric point and relative abundance, the fluorocarbon coating was similar to other widely used coating types. The coating stability was assessed by monitoring charge variant isoelectric point, peak area-to-height ratio, and resolution over many injections of the same sample on a single capillary. The observed capillary longevity was above 200 runs. Furthermore, the performance of a new detector filter assembly developed for protein detection in capillary isoelectric focusing is described.
Introduction

Capillary isoelectric focusing (cIEF) has proven to be one of the most powerful methods for protein charge heterogeneity analysis, and is among the most important capillary electrophoresis methods employed in biopharmaceutical product development and quality control. A high resolution cIEF method that is used for the analysis of monoclonal antibodies (mAb) employs a capillary with a permanent coating of linear polyacrylamide to suppress protein adsorption and electroendosmotic flow (EOF). However, a remaining technical challenge with this system is capillary longevity. Deterioration of charge isoform resolution with multiple types of proteins, antigen, antibodies, and antibody fragments after a few consecutive injections was reported recently. Presumably, this loss of performance was due to protein adsorption to the capillary surface. As alternatives to linear polyacrylamide, other commercially available permanent coatings were suggested, for example, polyvinylalcohol (PVA) and dimethylpolysiloxane (DB-1). A fluorocarbon (FC) coating could present an alternative. This permanent coating consists of a 0.075-µm thick film of FC polymer. It is chemically inert, stable from pH 2.5 to 10.0, and can withstand short flushes with 0.1 N NaOH for removal of contaminants. In contrast to capillaries coated with linear polyacrylamide, an FC coated capillary can be stored conveniently in a dry state. This Application Note shows the performance of a high resolution cIEF method on a FC coated capillary. The intermediate precision of the method and the capillary longevity were evaluated in detail. A new detector filter assembly designed for protein detection at 280 nm on the 7100 CE instrument was used for all measurements.

Experimental

Materials

IgG1 Kappa from murine myeloma clone number MOPC 21 (mlg1–k), methyl cellulose, urea, L-arginine, iminodiacetic acid, and tris(hydroxymethyl) aminomethane were obtained from Sigma Aldrich (St. Louis, MO, USA), Pharmalyte 5-8 from GE Healthcare Bio-Sciences AB (Uppsala, Sweden), hydrochloric acid and glacial acetic acid from Merck Millipore (Darmstadt, Germany), and phosphoric acid from JT Baker (Austin, TX, USA). IEF markers were from Sigma Aldrich (St. Louis, MO, USA) or Beckman Coulter (Fullerton, CA, USA). The cIEF gel was from Beckman Coulter (p/n 477497). An anti-α1-antitrypsin mouse mAb was obtained from Merck Millipore (Darmstadt, Germany). A rat anti-DYKDDDDK mAb (p/n 200474), and all other materials and instrumentation were from Agilent Technologies (Waldbronn, Germany).

Preparation of 1 % MC

1. Heat 15 mL ultra pure CE water (p/n 5062-8578) to approximately 80 °C.
2. Add 0.4 g methyl cellulose powder (Sigma Aldrich p/n M 0387) slowly while stirring into the hot water; continue stirring for 5 minutes.
3. Remove the dispersion from the heater, add ice-cold water to a final volume of 40 mL, and mix.
4. Cool the solution to –20 °C, and mix every 30 minutes until it is frozen.
5. Store the mixture overnight at 4–8 °C.

Preparation of 0.6 % MC containing 3 M urea

1. Add 1.8 g urea to 6 mL of 1 % MC.
2. Add water to a final volume of approximately 9 mL.
3. Mix until the urea is completely dissolved.
4. Add water to a final volume of 10 mL.
5. Filter the solution.

Preparation of 0.5 % MC

• Dilute 1 % MC with the same volume of water.

Preparation of methyl cellulose (MC) containing solutions

Preparation of 1 % MC

Preparation of 0.6 % MC containing 3 M urea
Sample preparation
Prior to CE analysis, mAb samples were desalted using Microcon YM-30 centrifugal filter devices (Millipore, Bedford, MA, USA) and a buffer containing 20 mM Tris/HCl, pH 8. Protein concentrations were measured with the Qubit assay (Life Technologies, Paisley, UK) and were 2–5 mg/mL after desalting. Sample solutions for cIEF analysis were prepared by adding the following reagents into 0.5-mL microcentrifuge vials.

- 100 µL of 0.6 % MC containing 3 M urea (or cIEF gel containing 3 M urea)
- 3.0 µL of Pharmalyte 5-8
- 4.5 µL of 500 mM L-arginine (cathodic stabilizer)
- 5.0 µL of 200 mM iminodiacetic acid (anodic stabilizer)
- 1.4 µL of IEF-marker mix
- 5 µL of desalted mAb

Final concentrations in the sample solution were 0.5 % MC (or 84 % cIEF gel), 2.5 M urea, 2.5 % Pharmalyte 5-8, 19 mM L-arginine, 8 mM iminodiacetic acid, and 0.08–0.21 mg/mL mAb.

The volumes used for IEF-markers 5.5, 7.2, and 7.6 were 1.0, 0.2, and 0.2 µL, respectively. The mixtures were vortexed for 10 seconds, centrifuged for approximately 1 minute, and transferred into 100-µL CE sample vials. Sample solutions were kept in the autosampler carousel of the CE instrument at approximately 10 °C and analyzed within 24 hours.

CE Conditions
For all CE runs, Agilent 7100 CE instruments equipped with an external waterbath set to 6 °C, a 280 nm high pass detector filter assembly (p/n G7100-68750), and 4 bar external pressure were used. A µSIL-FC capillary (p/n 194-8111) was cut at both ends at a distance of 8.5 cm and 24.5 cm from the detection window, respectively, equipped with a green alignment interface (p/n G7100-60210) and fitted into the Agilent capillary cassette. Once a day, and after cleaning, the capillaries were conditioned as follows: high pressure flush at 3.5 bar with 350 mM acetic acid for 5 minutes, with water for 2 minutes, and with 0.5 % MC (or cIEF gel) for 5 minutes. Prior to every run, the capillaries were conditioned as follows: high pressure flush at 3.5 bar with 4.3 M urea solution for 5 minutes, and with water for 2 minutes. The samples were injected by applying 2 bar high pressure for 100 seconds, followed by a water dip of both inlet and outlet electrode. Focusing was done for 100 seconds, followed by a water dip of both inlet and outlet electrode. Focusing was done for 5 minutes at 25 kV with 200 mM phosphoric acid as anolyte and 300 mM NaOH as catholyte. For chemical mobilization, the outlet vial was exchanged for 350 mM acetic acid, and 30 kV was applied for 30 minutes. After each run, a high pressure flush at 3.5 bar with water was done for 2 minutes. For cleaning, capillaries were flushed at 1 bar with 0.1 M NaOH for 2 minutes, with water for 30 minutes. Prior to storage, the capillaries were flushed at 1 bar with water for 20 minutes, with methanol for 5 minutes, and then dried (5 minutes flush from an empty vial). All flushes were done in forward direction (that is, pressure was applied to the inlet vial). The capillary temperature was kept at 20 °C. The detection wavelength was 280/20 nm, the reference wavelength 550/100 nm, and the response time 1 second. For all reagents, 2-mL glass vials were used. The fill volume was 1.6 mL, except for the waste vials that were empty. All reagent vials were exchanged after six runs.

Data Processing
Apparent isoelectric points were calculated by linear regression analysis of marker pl versus migrations time. Resolution was calculated using the half-width method.
Results and Discussion

A new detector filter assembly for protein analysis

Typically, optical filters that do not transmit the high energy UV light in order to protect sample components from denaturation are employed in diode array detection for cIEF. In a recent application note, a 260 nm band-pass filter that gave optimal results at a detection wavelength of 270 nm was used for this purpose. However, a new 280 nm high-pass filter designed for protein detection in cIEF is now available for the 7100 CE instrument. This filter enables detection at 280 nm, and is supposed to improve the protein detection sensitivity in cIEF relative to the filter used in C. Wenz note due to (i) the increased absorption of proteins and decreased absorption of carrier ampholytes at 280 nm and (ii) the higher available light intensity at this wavelength. Figure 1 shows that a 4–5 fold reduced noise was obtained with the new filter in comparison to the filter used before. All of the following data were obtained with the new filter.

![Figure 1. Comparison of results obtained with the 260 nm band-pass filter used in C. Wenz (red) and the new 280 nm high-pass filter (blue). A sample containing IEF-markers only and a MC-based gel was analyzed by cIEF. Shown are full scale electropherograms and a magnification of the signal recorded in the range of the mAb isoform migration. The average ASTM noise between 17 and 21 minutes was 0.14 ± 0.01 mAU with the filter used in C. Wenz and 0.031 ± 0.002 mAU with the new filter (n = 9).]
**Method performance on FC coated capillaries**

A high resolution cIEF method\(^2,3\) in a slightly modified form and with the mAb sample as described in C. Wenz\(^9\) was tested. Very similar results in terms of migration time and mAb isoform resolution were obtained on the FC coated capillary as compared to the capillary coated with linear polyacrylamide employed in C. Wenz\(^9\) (compare Figure 2A, red, to the Figures in C. Wenz\(^9\)). Next, it was tested to see if the cIEF gel used in\(^2,3\) could be replaced by a low-cost methylcellulose (MC)-based gel. Figure 2A (blue) shows that shorter migration times were obtained with 0.5 % MC relative to the cIEF gel. This might be due to a less efficient suppression of the EOF by MC. However, the decreased migration time difference between the peaks obtained with the MC polymer was balanced by an improvement in peak width. Hence, the resolution of mAb isoform peaks for both gels was within 3 % (Figure 2A). In addition to the mAb used in C. Wenz\(^9\), two other commercially available samples were analyzed using the MC-based polymer: a mouse anti-\(\alpha_1\)-antitrypsin mAb\(^\,10\) and a rat anti-DYKDDDDK mAb. Similar electropherograms with multiple well separated isoform peaks were obtained in both cases (Figures 2B and 2C).

**Figure 2.** CIEF analysis of commercially available mAb samples on fluorocarbon coated capillaries. Electropherograms of samples containing mouse IgG1 and either cIEF gel (red) or 0.5 % MC (blue) are shown in (A). The resolution of main mAb isoform peaks was 2.74 ± 0.05 with the cIEF gel and 2.66 ± 0.09 with 0.5 % MC (n = 5). Samples containing an mouse anti-\(\alpha_1\)-antitrypsin mAb (B) or a rat anti-DYKDDDDK mAb (C) were analyzed in presence of 0.5 % MC. For the rat mAb, electropherograms obtained on a new capillary (red) and after more than 200 injections of the same sample (blue; with peaks labeled from 1 to 8) are shown.
Capillary longevity

The long-term capillary stability was tested by repeated injections of the rat mAb sample on a single capillary. Initially, in long overnight sequences comprising more than 20 runs, a drift of migration times and gradual loss of resolution was observed after several injections (data not shown). However, a capillary cleaning step including a 2-minute flush with 0.1 M NaOH after every sixth sample injection was found to suppress this loss of performance. Presumably adsorbed protein that slowly accumulates at the inner capillary wall is removed by this procedure. A consistent mAb isoform profile was observed after > 200 injections (Figure 2C). Parameters that were reported to be indicative for system performance, namely isoform pI, peak area-to-height ratio and resolution, were evaluated for a set of 204 runs (Figure 3). The experimental pI values for all eight mAb isoforms were stable over the entire set of runs (Figure 3A). The observed slopes of linear trendlines were ≤ 0.01 pI units/100 runs in all cases. The area-to-height ratio and resolution was calculated for main mAb isoform peaks only (Figures 3B and 3C). Among the examined parameters, only the area-to-height ratio of peak 4 showed a slight trend over time.

Figure 3. Capillary longevity. Performance parameters for 204 consecutive injections of the rat mAb on the same capillary are shown: apparent pI values for isoforms 1–8 (A), area-to-height ratios for main isoforms 3 and 4 (B) and resolution of main isoform peak pairs 3–4 and 4–5 (C). Linear trendlines are shown in (B) and (C). For the assignment of isoform peaks 1–8, refer to Figure 2C.
Intermediate precision
The precision of the method including within-laboratory variations such as different day, capillary batch, and instrument, was determined for isoform pl and relative abundance of the rat mAb (Table 1). The intermediate precision for apparent pl was for all isoforms around 0.1 % RSD. For relative peaks area, the precision was < 5 % RSD for all major isoforms. Values > 10 % RSD were only observed for two minor isoforms accounting for less than 2 % total peak area. These results agree well with results obtained with different capillary coatings2,6,7,9.

Table 1. Intermediate precision of high resolution cIEF on FC coated capillaries. Four sets of 12 runs were done on four different days, with three instruments, and two capillary batches (n = 48).

<table>
<thead>
<tr>
<th>Isoform peak</th>
<th>Apparent pl</th>
<th>Peak area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average SD RSD%</td>
<td>Average SD RSD%</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.892</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>6.769</td>
<td>0.004</td>
</tr>
<tr>
<td>3</td>
<td>6.654</td>
<td>0.005</td>
</tr>
<tr>
<td>4</td>
<td>6.552</td>
<td>0.005</td>
</tr>
<tr>
<td>5</td>
<td>6.458</td>
<td>0.006</td>
</tr>
<tr>
<td>6</td>
<td>6.371</td>
<td>0.006</td>
</tr>
<tr>
<td>7</td>
<td>6.293</td>
<td>0.006</td>
</tr>
<tr>
<td>8</td>
<td>6.222</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Conclusion
This Application Note shows the robust and reliable performance of a high resolution cIEF method on FC coated capillaries. The stability of the FC coating allowed the routine use of a cleaning step employing a diluted base that increased the repeatability significantly, presumably due to the suppression of protein adsorption. A high capillary lifespan was observed; more than 200 injections could be done on a single capillary without loss of performance. The intermediate precision of measured mAb isoform isoelectric point and relative abundance was very similar to other widely used capillary coatings. Given their excellent performance, high stability and ease-of-use, the FC coating presents an attractive choice for high resolution mAb isoform analysis by cIEF.
References


8. Care and use information µSIL-FC Capillaries, Agilent Technologies Part No. 830-0601.
