Analysis of Monoclonal Antibody Charge Variants by Capillary Zone Electrophoresis

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Charge heterogeneity analysis is important in the characterisation of monoclonal antibodies because it provides important information about product quality and stability. Heterogeneity can be caused by such molecular adaptions as C-terminal lysine modification, deamidation, and post translational modification. One method for separating charge variants is capillary isoelectric focusing (cIEF) which provides information regarding isoelectric point variation for related molecular isoforms. cIEF requires electrophoretic separation in a coated capillary which helps suppress electro-osmotic flow (EOF) and prevents surface protein adsorption but can result in a fairly long cycle time and complex sample preparation.

We demonstrate here that by using a simple separation buffer system and a bare fused silica capillary, it is possible to obtain a highly resolved, reproducible separation of a representative monoclonal antibody in less than 12 minutes. We also demonstrate that while high resolution can be achieved using a short effective length (20cm), the fine structure of the monoclonal antibody used in this study can be revealed by increasing the effective length to 40cm.

Experimental

Capillary Zone Electrophoresis

Preparation of Separation Buffer: 0.05% HPMC , 380 mM EACA, 1.9 mM TETA

Preparation of 400mM EACA, 2mM TETA pH 5.7 Solution

5.25g of EACA (Sigma, cat. no. A-7824) and 30µl of TETA (triethylenetetramine) (Sigma, cat. no. 90460) were dispensed into a 100mL beaker containing 95mL of double distilled and deionised (ddi) water. The contents were allowed to dissolve completely. The pH was adjusted with glacial acetic acid to pH 5.7 + 0.05. The contents were quantitatively transferred to a 100mL volumetric flask and the volume was brought to 100mL with ddi water. The resulting solution was filtered through a 0.2µm filter.

Preparation of 1% HPMC Solution

One gram of HPMC (Sigma, cat. no. H-7509) was dispensed into a large beaker containing 100mL of ddi water. The solid was allowed to dissolve to completion overnight at room temperature.

Preparation of the Separation Buffer

Transfer 8.55mL of the 400mM EACA, 2mM TETA pH 5.7 buffer to a 15mL conical tube and add 450µL of 1% HPMC solution. Mix well. Prepare fresh before each use. The amount prepared is enough for 9 separations.

Capillary Wash Solution

0.1 N HCl solution (Fluka, part no. 94015).

Sample

MAb X is a representative, therapeutic grade monoclonal antibody. A 20mg/mL stock solution of MAb X was diluted to a final concentration of 1mg/mL in ddi water and used in both cIEF and CZE separations.

Capillary

Type: Bare Fused Silica; 50µm i.d. x 360µm o.d.

Effective length: 40cm, total length 50cm for high resolution method and 20cm effective length, total length 30cm for the fast separation method.

CZE of Mab X	Corrected Area			Migration Time		
Run #	Basic	Main	Acidic	Basic	Main	Acidic
Mab X run #1	12899.79	21950.23	4363.77	8.24	8.99	9.81
Mab X run #2	12382.87	21373.5	4155.31	8.13	8.87	9.67
Mab X run #3	12572.88	21441.31	4333.81	8.18	8.91	9.73
Mab X run #4	12483.24	21438.48	4383.71	8.22	8.97	9.78
Mab X run #5	13410.81	22744.52	4581.26	8.15	8.89	9.7
Mab X run #6	12900.86	21703.76	4418.44	8.23	8.98	9.8
Mab X run #7	12906.9	21945.95	4437.96	8.23	8.98	9.8
Mab X run #8	13118.91	22094.07	4569.69	8.23	8.98	9.8
Mab X run #9	13046.59	21908.14	4501.46	8.24	8.99	9.82
Mean:	12858.09	21844.44	4416.16	8.21	8.95	9.77
Std Dev:	328.39	428.21	130.96	0.04	0.05	0.05
%RSD:	2.55	1.96	2.97	0.51	0.53	0.55

Table 1: Raw data for CZE separation of MAb X. Reproducibility of the CZE separation for corrected area and migration time for basic, acidic and main grouped peaks.

Instrument

PA 800 plus Pharmaceutical Analysis System equipped with either a UV or PDA detector was used in these experiments. UV detection was configured with a 214nm filter, with a data rate set at 4Hz to perform the CZE experiment and 280nm filter to perform cIEF. PDA detection was also used in duplicate CZE experiments for later comparison with the UV/vis data. The sample storage and cartridge temperature were kept at 20 and 25°C respectively.

The capillary conditioning method was performed using a series of rinses followed by sample injection and separation as follows: 50 psi rinse of 0.1 N HCl solution for 5 min followed by buffer rinse at 50 psi for 5 min. The sample was injected hydrodynamically at 0.5 psi for 10 seconds. The separation was performed using normal polarity at 30 kV for 30 min.

There are two distinct separation methods for high speed and high resolution analysis. The high speed method used for a 30 cm bare fused silica capillary consists of 50 psi rinse of 0.1 N HCl solution for 5 min followed by buffer rinse at 50 psi for 5 min. The sample is then injected hydrodynamically at 0.5 psi for 10 seconds. The separation was performed using normal polarity at 30 kV. For the molecule used in this study the duration of the separation was only 12 min. The high resolution method used with a 50cm bare fused silica capillary is very similar to the high speed except the buffer rinse step is 10 min long and the separation step is 40 min long. The separation time may vary depending on the pI (protein isoelectic point) of the MAb under test.

The shutdown method consists of a 50 psi rinse with 0.1 N HCl solution for 5 minutes, followed by a wait step where the ends of the capillary are immersed in ddi water and finally a 'lamp off' step.

Results And Discussion

Charge heterogeneity analysis of MAb X was performed using both cIEF and CZE. A typical cIEF separation of MAb X illustrates a complex profile with charge isoforms ranging from pI 6.3 to pI 6.91 (Figure 1). It wasn't clear whether this MAb would separate efficiently using CZE since separation buffer was pH 5.7, potentially leading to solubility issues as discussed by He et al1.

Even though the protein is somewhat acidic and with less than one pH unit difference from the pH of the separation buffer, it was

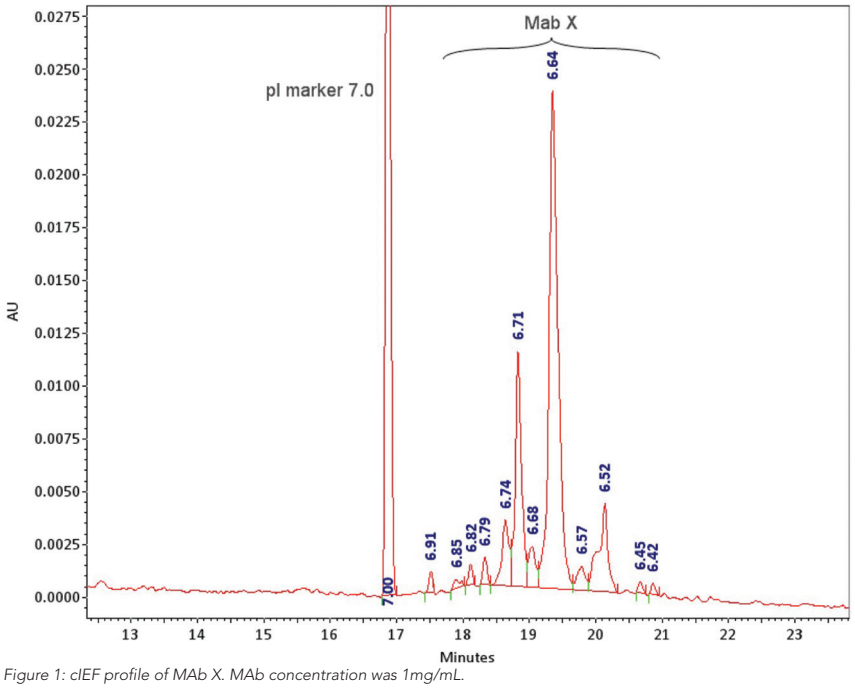

Figure 1: cIEF profile of MAb X. MAb concentration was 1mg/mL.

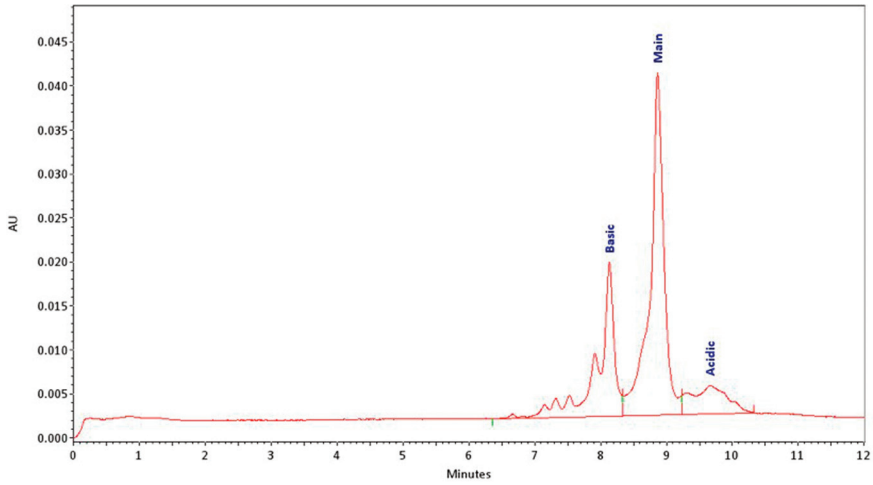

Figure 2: CZE profile and peak integration strategy for MAb X. Peaks were grouped as either Basic, Main or Acidic variants. CZE conditions: MAb X 1mg/mL; Separation buffer: 0.05% HPMC , 380 mM EACA (epsilon amino caproic acid), 1.9 mM TETA; bare fused silica capillary, 30 kV separation voltage.

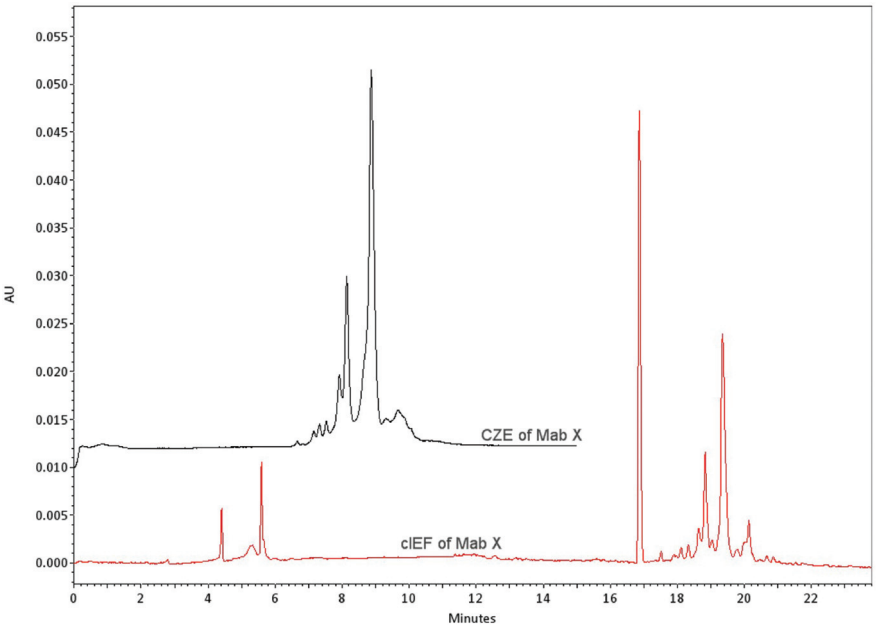

Figure 3: Comparison between cIEF and CZE of MAb X.

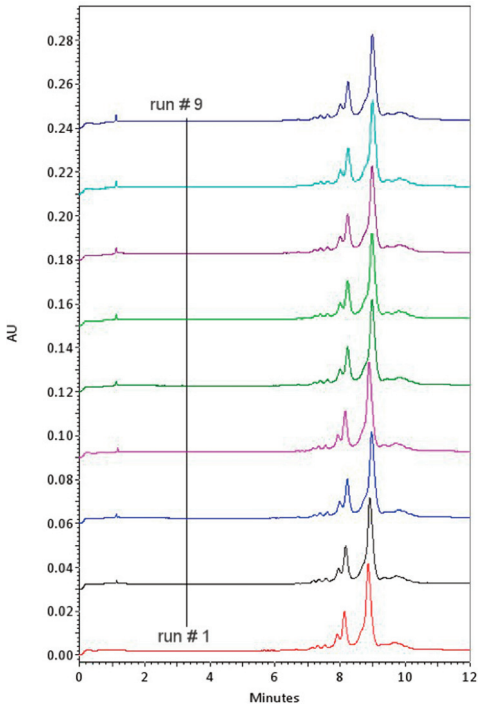

Figure 4: CZE separation of MAb X. Nine consecutive separations of MAb X were performed using UV detection. MAb X concentration was 1mg/mL; separation buffer: 0.05% HPMC , 380 mM EACA, 1.9mM TETA; bare fused silica capillary, 30 kV separation voltage.

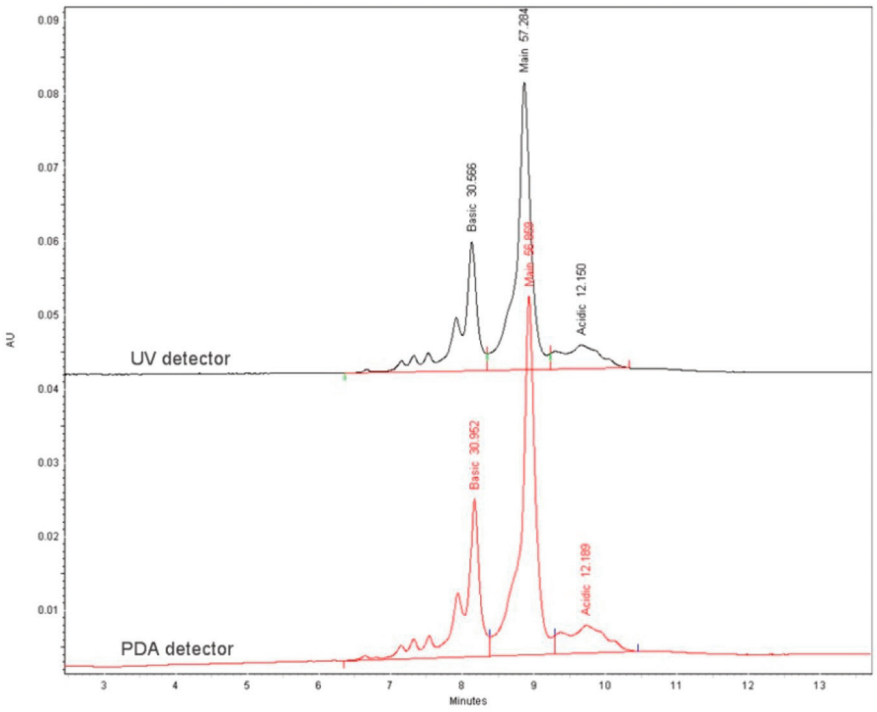

Figure 5: Comparison of UV and PDA in CZE separation of MAb X. CZE was performed using either UV detection (red trace) or PDA detection (black trace). Separation conditions are the same as those described in Figure 1.

possible to obtain a complete high resolution separation in only 12 minutes (Figure 2).

Results illustrate that high resolution separation can be achieved using the CZE methodology (Figure 3). In addition, the resolution in the basic region surpasses that of cIEF. An important attribute of CZE is not only the high resolution of this separation but also the fast separation time. A complete charge heterogeneity profile can be obtained in 12 minutes using CZE compared to 23 minutes required by cIEF.

Given the importance of assay repeatability, a number of CZE separations were performed sequentially to illustrate reproducibility (Figure 4). Data integration on these data was performed so that basic, main, and acidic peak groups could be designated as shown in Figure 2.

This CZE separation resulted in exceptional reproducibility for a number of consecutive runs; better than 3% RSD for corrected peak areas and better than 0.55% RSD for migration time for each of the basic, main and acidic peak groups (Table 1). Corrected area is commonly used in capillary electrophoresis because each peak travels at different velocity past the detection point. Here the corrected area was calculated by dividing the area under the peak by its velocity.

The CZE method also has the flexibility to offer the same performance regardless of the type of detection. Comparison between two CZE separations of MAb X using a photodiode array detector and UV detector illustrates the same resolution can be obtained with both detectors. Additionally, the area % composition is preserved across both detectors (Figure 5).

In cases where resolution is more important than speed of separation, by increasing the effective length of the capillary, it is possible to unveil fine structures that were not obvious in the fast separation method. As shown in figure 6, the separation of MAB X when using a capillary with 40 cm effective length, the pay-off of high resolution is clear even though the total separation time is increased significantly.

The increase in resolution may be a benefit when analysing a degraded form of MAb X,

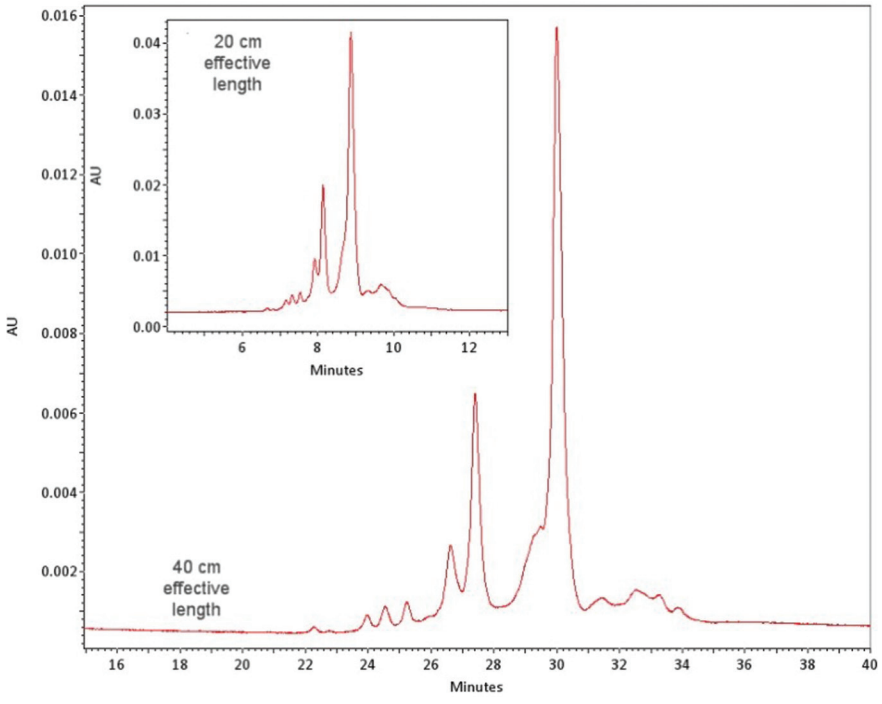

Figure 6: Comparison between 20 and 40cm capillary effective length. CZE separations were performed as described previously using either 40cm capillary (bottom trace) or a 20cm capillary (inset).

exposed to 60°C for a period of 48 hours. However, only by increasing the effective length was it possible to see the fine structure of acidic peaks and basic peaks formed due to temperature instability (Figure 7).

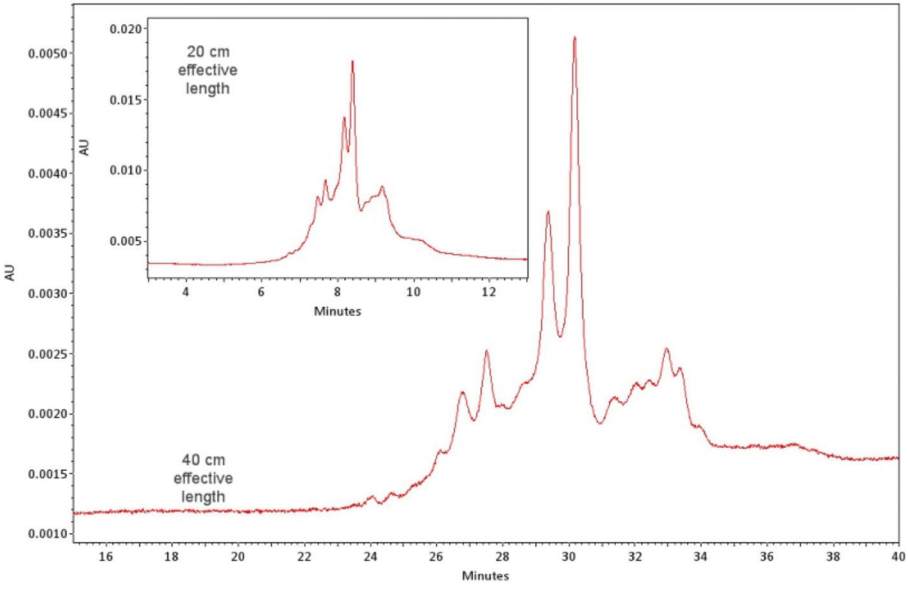

Figure 7: MAb X degraded by exposure to high temperature (60oC) for 5 days. CZE separations were performed as described previously using either 40cm capillary (bottom trace) or a 20cm capillary (inset).

Conclusion

Charge heterogeneity of protein isoforms can easily be determined using CZE and cIEF. While cIEF is commonly used to obtain charge heterogeneity and pI information for heterogeneous isoforms, we illustrate here that CZE is capable of providing faster, highly reproducible separations for a representative monoclonal antibody. This CZE method can be optimised by increasing the effective length of the capillary to increase peak resolution. Quality of this separation was not affected by detection method and reproducibility of this CZE assay is excellent across basic, main, and acidic species. Given the increasing need for sample throughput, separation quality, and repeatability, CZE can be positioned as a faster alternative to cIEF.

References

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2. Ingrid D. Cruzado-Park, Scott Mack and Chitra K. Ratnayake, A Robust cIEF Method: Intermediate Precision for the pH 5-7 Range, PN A-12015

Acknowledgment

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