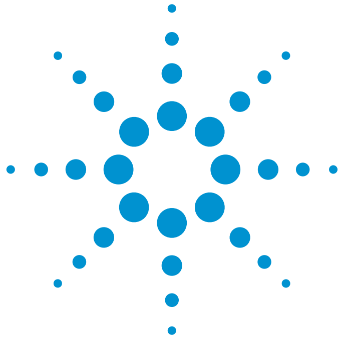

Impurity Detection with a New Light Emitting Diode Induced Fluorescence Detector Coupled to the Agilent 7100 Capillary Electrophoresis System

Application Note

Biologics & Biosimilars, Proteomics & Protein Science

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Abstract

Light emitting diodes could present alternatives to lasers as light sources for fluorescence detectors in applications requiring high sensitivity such as impurity detection in purified monoclonal antibody preparations by capillary gel electrophoresis. This Application Note describes the performance of a detector for diode induced fluorescence from Picometrics coupled to the Agilent 7100 Capillary Electrophoresis System. It shows that a small spiked-in protein impurity in a monoclonal antibody sample derivatized with 3-(2-furoyl)-quinoline-2-carboxaldehyde can be quantified with good accuracy and precision down to a level of 0.05 %. The extrapolated limit of detection was 0.02 %, a value that is close to limits of detection reported for laser-based systems.

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Introduction

Capillary gel electrophoresis (CGE) is an important tool for biopharmaceutical product development and quality control[1]. It is used to monitor product purity, detect minor product or process related impurities, and confirm batch-to-batch consistency of therapeutic proteins, especially monoclonal antibodies (mAb). In addition to UV detection[2], protein derivatization combined with laser-induced fluorescence (LIF) is often used to enhance the sensitivity of the method. Examples of popular derivatization reagents are 5-carboxytetramethylrhodamine succinimidyl ester[3] and 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ)[4,5]. However, it has been shown that light emitting diodes (LEDs) can be used as alternative light sources for fluorescence detection in CGE. A recently published comparative study of LED and laser-based systems showed a very comparable signal-to-noise (S/N) for an antibody sample derivatized with the above-mentioned reagents, provided that a similar excitation wavelength was used in both cases[6]. Potential benefits of LED in comparison to lasers are lower energy consumption, increased baseline stability, and longer lifetime.

This Application Note describes the performance of a detector for LED-induced fluorescence (LEDIF) from Picometrics coupled to the Agilent 7100 Capillary Electrophoresis system for the detection of low-level impurities in an mAb sample. Impurities were simulated by artificially contaminating a purified mAb preparation with small standard proteins. Prior to CGE analysis, samples were derivatized with FQ. Derivatization with this reagent is convenient because it becomes fluorescent only after reaction with a primary amine and, therefore, purification of the derivatized protein is not necessary.

Experimental

Instrumentation

Agilent 7100 Capillary Electrophoresis System (G7100A)

Materials

The mAB used was provided by a biotechnical company. Standard proteins, citrate-phosphate buffer concentrate, N-ethylmaleimide (NEM), and sodium dodecyl sulfate (SDS) were purchased from Sigma Aldrich (St. Louis, MO, USA). Potassium cyanide (KCN) and FQ were purchased from Life Technologies (Paisley, UK). SDS Gel Buffer was purchased from Beckman Coulter (Fullerton, CA, USA). Protein concentrations were measured with the Qubit assay (Life Technologies, Paisley, UK). The Zetalif LED detector equipped with a 480 nm LED and a high pass 510 nm emission filter was obtained from Picometrics (Toulouse, France). All other materials and instrumentation were from Agilent Technologies, Inc. (Waldbronn, Germany).

Sample preparation

A stock solution of 40 mM FQ in methanol was prepared and stored at –20 °C. Immediately before use, an aliquot of this stock solution was diluted with water to 1 mM. NEM solutions were prepared fresh daily. Prior to derivatization, the mAb sample was diluted with citrate-phosphate buffer (90 mM sodium citrate, 77 mM sodium phosphate, pH 6.5) to a protein concentration of 2 mg/mL. For derivatization with FQ, the following reagents were mixed in 0.5-mL microcentrifuge vials:

- 35 µL of diluted mAb
- 7.0 µL of 10 % SDS
- 4.0 µL of 100 mM NEM
- 0.5 µL of 100 mM KCN
- 25 µL of 1 mM FQ

Final concentrations in the reaction mixture were 1 mg/mL mAb, 1 % SDS, 5.6 mM NEM, 0.7 mM KCN, and 0.35 mM FQ. The solution was incubated for 5 minutes at 75 °C and then cooled down on ice. After the addition of 30 µL 10 % SDS, the solution was transferred into 100-µL CE sample vials. For reducing analysis, samples were additionally incubated with 50 mM DTT for 10 minutes at 70 °C.

CE conditions

The LEDIF detector was connected to the CE instrument through the integrated analog-to-digital converter. The range used was 50 RFU/V. The rise time and photomultiplier high voltage were set to 0.5 s and 610 V, respectively. Fifty µm id bare fused silica capillaries with a total length of 33 cm and an effective length 21 cm were used. Once a day, capillaries were conditioned as follows:

1. High-pressure flush at 2 bar with 0.1 N NaOH for 10 minutes, with 0.1 N HCl for 5 minutes, and with water for 2 minutes
2. High-pressure flush at 4 bar with SDS Gel Buffer for 10 minutes
3. Water dip for both electrodes
4. Voltage equilibration at –16.5 V for 10 minutes with 5 minutes ramping.

Prior to every run, capillaries were conditioned as follows:

1. High-pressure flush at 4 bar with 0.1 N NaOH for 3 minutes, with 0.1 N HCl for 1 minute, with water for 1 minute and with SDS Gel Buffer for 10 minutes
2. Water dip of both electrodes

Samples were electrokinetically injected from the outlet vial position by applying 5 kV for 20 seconds and, after a water dip of the outlet electrode, separated by applying 16.5 kV (500 V/cm) for 30 minutes. Two bar pressure was applied to both inlet and outlet home vials during the run. After use, capillaries were conditioned as follows:

1. High-pressure flush at 4 bar with 0.1 N NaOH for 15 minutes

2. High-pressure flush at 3.5 bar with 0.1 N HCl for 5 minutes, and with water for 10 minutes.

Flushes for daily preconditioning and conditioning after use were done in forward direction (that is, pressure was applied to the inlet vial). Prerun flushes were done in backward direction. The capillary temperature was kept at 40 °C. For all reagents, 2-mL glass vials were used. The fill volume was 1.2 mL, except for the water dip vials that contained 1.6 mL water and the waste vials that contained 0.6 mL of water. Three separate waste vials were used for the collection of 0.1 N NaOH, 0.1 N HCl/water, and SDS Gel Buffer, respectively. SDS Gel Buffer containing inlet and outlet home vials were exchanged after every sequence of eight runs.

Results and Discussion

Sensitivity of CGE with LEDIF detection

The analysis of a FQ-derivatized mAb sample by CGE with LEDIF detection is shown Figure 1. Two detector parameters were optimized in pilot experiments. Firstly, the photomultiplier high voltage was adjusted to a value that resulted in main peak maxima in the range of 30–40 RFU. This was done to take advantage of the full measuring range without risking the rise of the signal beyond the 50 RFU limit. Secondly, the baseline noise reduction parameter rise time was varied between 0 and 5 seconds and then set to a value of 0.5 seconds that resulted in an acceptable background noise level without affecting the peak shape of small mAb impurities (data not shown). The peak present in all runs at approximately 7.5 minutes might represent a buffer contaminant[4] (Figure 1). The background noise was calculated according to the ASTM over time intervals of 2 minutes around the migration times of the peaks of interest and was between 2–2.5 mRFU. To estimate the sensitivity of the method, the mAb sample was spiked with four standard proteins at a level of 0.25 % (Figure 1). Under nonreducing conditions, where all standard proteins were clearly resolved from mAb derived peaks, peak heights of approximately 100 mRFU were observed for three out of four protein spikes, corresponding to an S/N of 40 and an extrapolated limit of detection (LOD) of approximately 0.02 % (S/N > 3). This value is close to LODs reported for protein impurities in FQ-derivatized mAb samples obtained with laser-based systems[4,5].

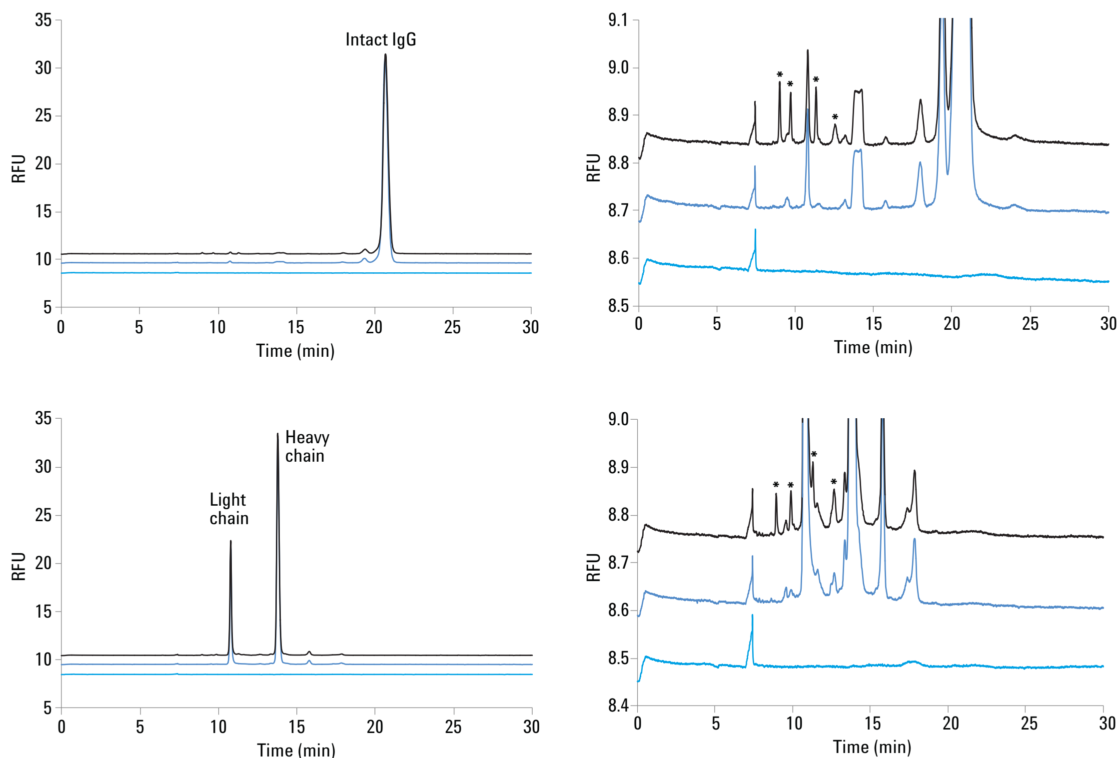

Figure 1. Sensitivity of CGE with LEDIF detection. Samples were analyzed under nonreducing (top) and reducing conditions (bottom). Shown are full-scale electropherograms (left) and an expanded view (right) of the original mAb sample (dark blue), the mAb sample artificially contaminated with standard proteins at a concentration of 0.25 % (blue) and a control sample without protein (light blue). Molecular weights of standard proteins were 12, 18, 29, and 45 kDa, respectively. Standard protein peaks are labeled (*).

Impurity quantification

The performance of the method for low-level impurity quantification was evaluated with mAb samples spiked with a single standard protein at concentrations of 0.05–0.25 % (Figure 2). Samples were derivatized and analyzed on two days using different CE instruments. Peak integration was done automatically with the same set of parameters (Table 1). The maximum deviation of the measured standard protein % area from the target value was 12 %, and the average deviation was 7.1 %. A precision of 10 RSD % or better was observed for standard protein concentrations ≥ 0.1 %. Only for the lowest concentration was the precision 15 RSD %. An S/N of 8 was calculated for the 0.05 % sample, which confirms the above-mentioned LOD estimation.

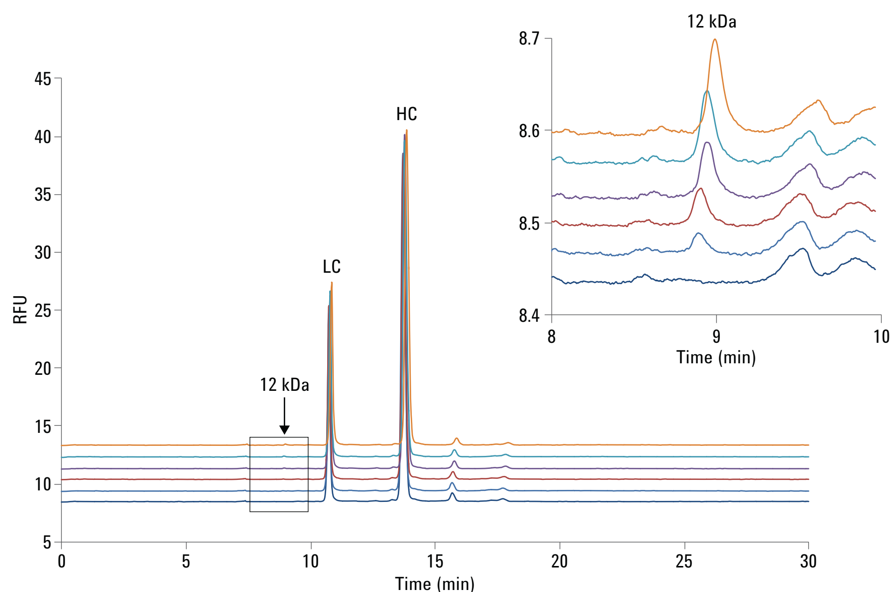

Figure 2. Limit of quantification. A 12 kDa standard protein was added at concentrations between 0.05–0.25 % to the mAb sample and analyzed under reducing conditions. Shown is an overlay of full-scale electropherograms of mAb without (bottom) and with 12 kDa protein added at concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25 % (top) with a zoom on the 12 kDa protein peaks in the inset.

Table 1. Accuracy and precision of low level impurity detection by CGE with LEDIF detection. Samples of mAb with different amounts of added 12 kDa protein (cf. Figure 2) were derivatized and analyzed on two different days with two CE instruments and four runs per instrument (n = 8). Shown is the relative time corrected area (% Area) and the S/N for the 12 kDa protein.

% Area					S/N		
Target	Average	SD	RSD %	%Deviation from target	Average	SD	RSD %
0.050	0.048	0.007	15.0	–4.0	7.9	0.9	12.0
0.100	0.092	0.008	8.9	–8.1	14.5	1.8	12.6
0.150	0.131	0.010	8.0	–12.4	22.7	3.0	13.2
0.200	0.181	0.009	4.8	–9.6	29.7	3.4	11.5
0.250	0.247	0.025	10.2	–1.3	37.0	3.6	9.6

Conclusion

This work shows that the Agilent 7100 CE instrument equipped with the Picometrics LED-based fluorescence detector is well suited for low-level impurity detection by CGE with FQ-derivatized mAb samples. The method allowed the detection of standard proteins spiked into an mAb preparation down to a level of 0.02 %, a value that is close to published LODs obtained with laser-based systems. Good accuracy and precision was observed for the quantification of a single protein spike at concentrations in the range of 0.05–0.25 %. Given the similar performance combined with lower energy consumption, increased stability, and longer lifetime of LEDs, the described approach may present an attractive alternative to CE systems with laser-based fluorescence detectors.

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