Low level quantification of impurities in bispecific proteins using CE-SDS with LIF detection

Bispecific protein analysis by capillary electrophoresis (CE) using LIF detection

Stephen Lock[1,3], Marcin Moczulski[1] and Pranav Bheamadu[2]
[1] SCIEX, Europe; [2] Immunocore, Abingdon, UK; [3]Horváth Csaba Memorial Laboratory, University of Pannonia, Veszprém, Hungary

A highly sensitive capillary electrophoresis solution composed of commercial kits and prebuilt cartridges, providing an easy-to-install application for impurity profiling of bispecific proteins, is shown in this technical note. The method is flexible and can be easily adapted to other protein targets.

Capillary electrophoresis (CE) has been used in the QC of biopharmaceutical monoclonal antibodies for over 15 years and was designed as a substitute for SDS-PAGE.[1] This methodology traditionally uses UV based detection as derivatization approaches to improve sensitivity, including TAMRA and FQ derivatization, can be lengthy. [2] Recently a new simple LIF derivatization has been used to improve the sensitivity of viral protein analysis and is being utilized in standard monoclonal antibody testing.[3]

Over the last few years, research into the use of more complex protein drugs, such as bispecific fusion proteins, has increased. Bispecific fusion proteins are artificial proteins that have promising applications in the field of cancer immunotherapy. They are usually composed of a targeting domain and an effector function and this, as the name suggests, makes them able to bind to two different antigens.

In this case, the bispecific was immune mobilizing monoclonal T-cell receptors against cancer or ImmTAC.[4] These newer classes of target therapeutics are often of lower concentration and therefore more sensitive approaches are required to detect low level impurities.

In this technical note, a bispecific protein was analyzed by CE with sodium dodecyl sulphate (CE-SDS), using laser induced fluorescence (LIF) detection, commercially available kits and prebuilt cartridges. Figure 1 highlights a typical result from the analysis of a sample demonstrating the method sensitivity.

Key features of CE-SDS with LIF detection of low-level impurities in bispecifics

- High resolution separation for impurity profiling
- Sensitive and reproducible assay with % RSD of 0.11 and 0.12 for migration time and corrected peak areas, respectively.
- A linear response for the bispecific protein with detection of impurities down to 2%.

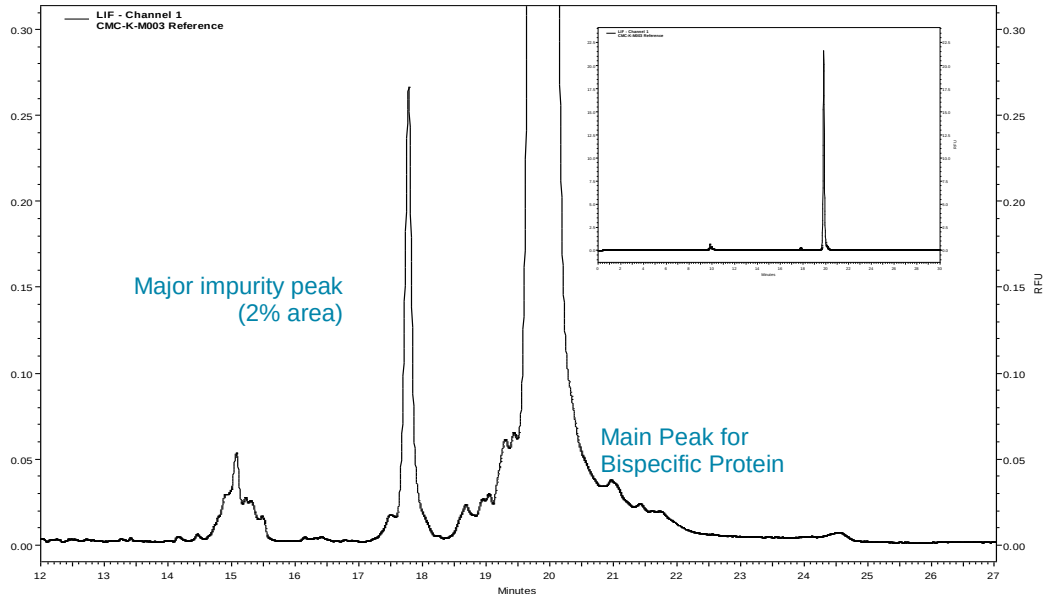

Figure 1. **Electropherograms of injection of 0.2 mg/mL of the bispecific protein.** The baseline has been expanded so that you can see the impurity peaks (most of these peaks were not seen by UV detection). The inlayed electropherogram is the unexpanded full picture.

Methods

Sample preparation: Methanol (200 µL, Sigma p/n 10675112) was added to Chromeo P503 dye (1 mg, Sigma p/n 30693) to produce a stock solution (5.0 mg/mL). The solution was vortexed thoroughly (~1.5 hours) and 5 µL was added to ultrapure water (45 µL) to prepare the labeling solution which was stored at -20°C. A Chromeo P503 dye labeling solution of 0.4 mg/mL was found to be optimal during method development, therefore 12.5 µL of ultrapure (12.5 µL) water was added to a 50 µL aliquot of labeling solution (0.5 mg/mL) before use. An IS spike solution was prepared by mixing internal standard (10 µL, CE-SDS kit supplied by SCIEX, p/n A26487) with 90 µL of SDS-MW sample buffer (90 µL, SCIEX CE-SDS kit, p/n 390953).

The test sample (50 µL, bispecific protein) was mixed with SDS-MW sample buffer (50 µL) and 5 µL of IS spike solution (5 µL). This mixture was incubated to denature the protein (5 minutes, 70°C). Following denaturation, Chromeo P503 dye labeling solution (5 µL, 0.4 mg/mL) was added and the sample derivatized (30 minutes at 70°C). The derivatized solution was cooled to room temperature (10 minutes) and mixed (50 µL) with ultrapure water (400 µL) to prepare a sample for analysis.

Capillary electrophoresis: Separations were performed using a prebuilt cartridge (p/n A55625) with a capillary length of 30.2 cm (effective length 20 cm) on a PA 800 Plus pharmaceutical analysis system. At the start of every sequence, a conditioning run was performed. This involved a 0.1 M NaOH rinse (20 psi, 10 minutes) followed by 0.1 M HCl (20 psi, 5 minutes), ultrapure water (20 psi, 2 minutes) and SDS-MW gel buffer (70 psi, 10 minutes). This was followed by a 15 kV separation step (10 minutes).

Before each injection, the capillary was cleaned with 0.1 M NaOH (70 psi, 3 minutes) and 0.1 M HCl (70 psi, 1 minute) and rinsed with ultrapure water (70 psi, 1 minute) and SDS-MW gel buffer (70 psi, 10 minutes). Ultrapure water was injected (20 psi, 10 seconds) prior to sample injection to act as a “stacking zone”.

The exterior of the capillary was cleaned by dipping in water and a sample was injected (5 kV, 5 seconds). The exterior of the capillary was cleaned again with water and the test sample separated (15 kV, 30 minutes).

At the end of every sequence a shutdown method was run using 0.1 M NaOH (70 psi, 10 minutes), 0.1 M HCl (50 psi, 5 minutes), ultrapure water (50 psi, 2 minutes) and SDS-MW gel buffer rinses (70 psi, 10 minutes) followed by a 10 minute 15 kV conditioning step with SDS-MW gel buffer and a step where the capillary is dipped into ultrapure water.

Results and discussion

During the development of the next generation of biologics, such as bispecific proteins, the concentration of the samples is often lower. In this case, the bispecific concentration of the stock solution was only 0.2 mg/mL which is 5 to 10-fold less than the typical concentration available for traditional monoclonal antibodies [1]. UV detection can struggle to pick-up low-level impurities at these concentrations, which is highlighted in Figure 2 and 3.

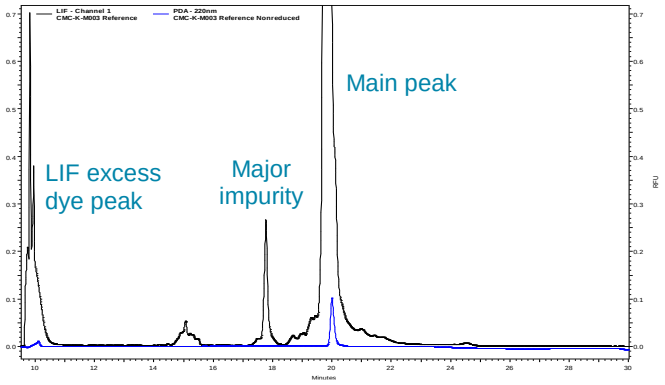

Figure 2. Comparison of UV (blue trace) and LIF (black trace) detection of a 0.20 mg/mL standard. The vertical intensity axis for both UV and LIF was magnified (zoom in) to clearly observe the bispecific peak in the UV trace.

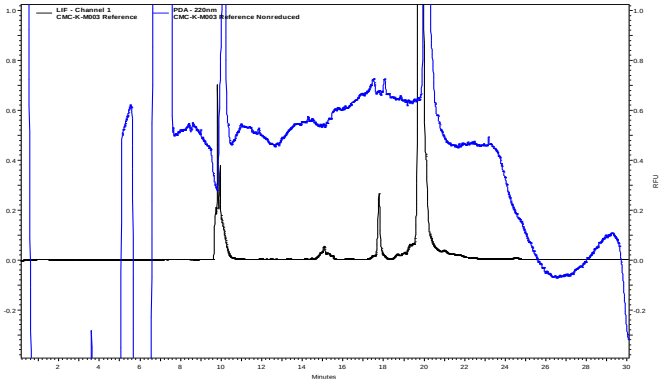

Figure 3. Comparison of UV (blue trace) and LIF (black trace) detection of a 0.20 mg/mL standard. The UV and LIF traces were normalized on the Y-axis in order to review the bispecific impurity peaks.

In Figure 2, the increased sensitivity of LIF was observed and represents a response increase of approximately 100-fold, with a major low molecular impurity peak observed (17.9 minutes). The additional peak at 10 minutes in the LIF trace corresponded to excess dye left in the sample. When the baseline was expanded for the UV trace (Figure 3), the impurity peaks detected in LIF were not observed due to the lower sensitivity of the UV detection.

During development of the LIF assay, several of the derivatization process parameters were evaluated. It was generally found that different concentrations of the stock dye solution didn't significantly alter the protein profile but the 0.4mg/mL concentration generated a higher signal and was therefore used in these evaluation studies. 30 minutes was used as the incubation time for the dye derivatization as this increased the fluorescence signal with no significant fragmentation or aggregation observed. Increasing the final dilution of the sample with ultrapure water provided a very good signal intensity and slightly better resolution of the shoulder peak from the higher molecular weight main peak.

To assess the intra-assay precision for the main peak migration time and % corrected area for the CE-LIF method, the bispecific was injected across a range of concentration levels from 0.04 mg/mL to 0.20 mg/mL, which represents a range of 20% to 100% of the standard protein load of 0.20 mg/mL. Table 1 provides a summary of the main peak migration time (min) and % corrected area for each dilution level tested. The %RSD was very low for both migration time and % corrected main peak area demonstrating that the method is very reproducible.

Sample concentration	Injection	Migration time (min)	% Corrected area
0.20 mg/mL	1	19.76	95.6
0.20 mg/mL	2	19.76	95.5
0.12 mg/mL	1	19.81	95.3
0.12 mg/mL	2	19.81	95.4
0.04 mg/mL	1	19.80	95.4
0.04 mg/mL	2	19.80	95.4
Average:		**19.79**	**95.43**
Std Dev:		**0.02**	**0.10**
%RSD:		**0.12**	**0.11**

Table 1. Comparison of the migration time and corrected peak area from the injections of the bispecific protein at 3 concentrations levels in duplicate.

Linearity of response is important for accurate impurity peak detection in CE-SDS analysis, so calibration standards were prepared over an order of magnitude. Initially these were prepared without the use of the IS spike solution, which contained the 10 kDa internal standard provided with the CE-SDS kit, but the R^2 values obtained were poor in the range of 0.6-0.7. The internal standard was therefore used to normalize the derivatization process and reduce the variability sometimes observed with electro-kinetic injections. The internal standard peak area was then used to normalize the peaks resulting from the bispecific protein. The resulting calibration lines for both the main impurity peak and the major peak are shown in Figures 2 and 3.

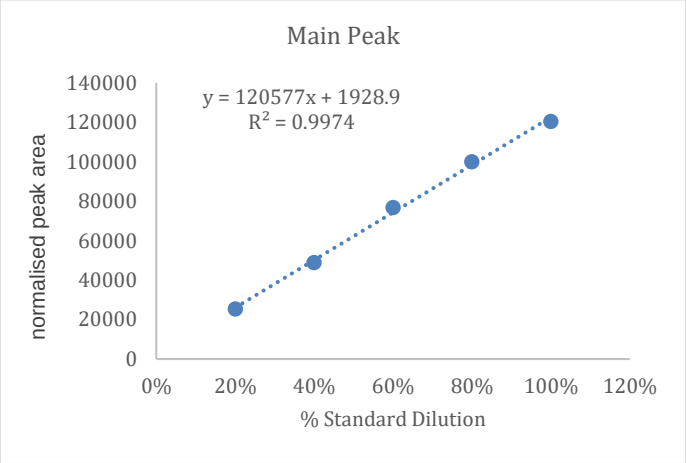

Figure 4. Linear regression plot for corrected peak area of the main peak versus % standard solution of the stock bispecific sample which was at 0.20 mg/mL (100% standard dilution).

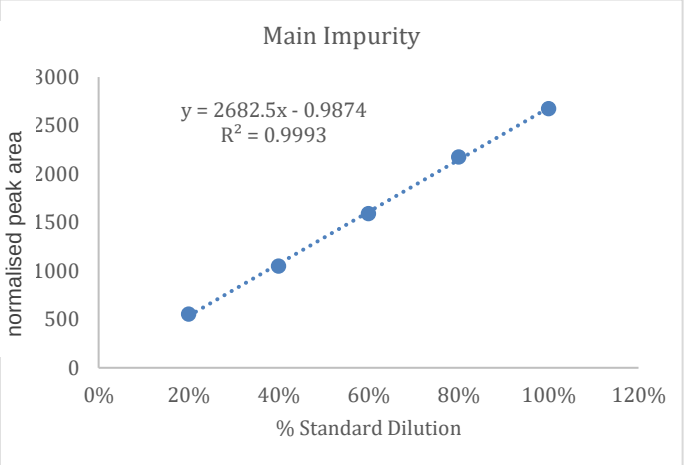

Figure 5. Linear regression plot for corrected peak area of the main impurity peak (2.1% of the area of the main peak) versus the % standard solution of the stock bispecific sample which was at 0.20 mg/mL (100% standard dilution).

When the internal standard was used to normalize the peak responses, the R^2 values obtained were > 0.995. The data used for these calibration lines is shown in Table 2 and it highlights the reproducibility of % area for the main impurity at the different concentration of the samples. The data also shows the variability observed for the internal 10kDa standard which doesn't affect the % peak area for the impurities. This highlights the need for the internal standard to normalize the response observed with different samples.

Sample (mg/mL)	10kDa Peak Area	Main peak area (normalized peak area)	Impurity peak Area (normalized peak area)	% Area main impurity
0.04	31867	37720 (25969)	821 (553)	2.06
0.08	23547	53532 (49907)	1150 (1049)	2.03
0.12	39742	14191 (78094)	2946 (1592)	1.97
0.16	25847	120323 (101965)	2617 (2175)	2.06
0.20	21480	120422 (122668)	2673 (2673)	2.10

Table 2. Peak areas observed for the injection of a diluted standard curve for the bispecific protein.

Conclusions

- The method, using prebuilt cartridges and commercial kits enables an easy setup and can be transferable to other protein targets
- The use of LIF detection improved sensitivity by at least 100-fold and allowed the detection of impurity peaks not visible by UV detection
- The CE-SDS analysis developed produced a robust and reproducible method for bispecific protein impurity profiling
- The use of an internal standard and corrected peak areas provided a linear response for the bispecific protein tested, over the range of 0.04 – 0.2 mg/mL for the main peak and also for an impurity which was only 2% of the total sample peak area—providing an accurate calculation for low level % impurities

References

1. Nunnally B. et al. (2006). A series of collaborations between various pharmaceutical companies and regulatory authorities concerning the analysis of biomolecules using CE *Chromatographia*, **64**, 359-368.
2. Using fluorescent labels to increase the sensitivity of IgG purity and heterogeneity assay on PA 800 Plus. SCIEX technical note, RUO-MKT-02-8609-A.
3. Using laser induced fluorescence to improve CE-SDS analyses. SCIEX technical note, RUO-MKT-02-12769-A.
4. Lowe KL et al. (2019). Novel TCR-based biologics: mobilising T cells to warm 'cold' tumours. Cancer Treat Rev. 2019 Jul;77:35-43. doi: 10.1016/j.ctrv.2019.06.001. Epub 2019 Jun 12. PMID: 31207478.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices