Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 10–15

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Quantitative analysis of a biopharmaceutical protein in cell culture samples using automated capillary electrophoresis (CE) western blot

Dong Xu [1], Kentaro Marchionni [1], Yunli Hu, Wei Zhang, Zoran Sosic *

Biogen Inc., Cambridge, MA, 02142, USA

ARTICLE INFO

Article history:
Received 15 March 2017
Received in revised form 12 June 2017
Accepted 14 June 2017
Available online 15 June 2017

Keywords:
Automated western blot
Capillary electrophoresis
Biopharmaceutical protein titering and characterization

ABSTRACT

An effective control strategy is critical to ensure the safety, purity and potency of biopharmaceuticals. Appropriate analytical tools are needed to realize such goals by providing information on product quality at an early stage to help understanding and control of the manufacturing process. In this work, a fully automated, multi-capillary instrument is utilized for size-based separation and western blot analysis to provide an early readout on product quality in order to enable a more consistent manufacturing process. This approach aims at measuring two important qualities of a biopharmaceutical protein, titer and isoform distribution, in cell culture harvest samples. The acquired data for isoform distribution can then be used to predict the corresponding values of the final drug substance, and potentially provide information for remedy through timely adjustment of the downstream purification process, should the expected values fall out of the accepted range.

1. Introduction

The manufacturing of biopharmaceuticals produces heterogeneous products that are highly dependent on the process. Appropriate control strategy that is defined in industry guidance as a planned set of controls derived from current product and process understanding is critical to ensure process performance and product quality [1]. The Quality by Design (QbD) regulatory initiative outlines that quality is built into the manufacturing process as product testing and control is shifted at early manufacturing steps to ensure final product quality and consistency [2–4]. Clearly, analytical and informatics tools are pivotal in developing and implementing effective and efficient control strategies by providing information on in-process materials and final biopharmaceutical product quality. Traditional analytical methods, such as SDS-PAGE and western blot, are still often applied to monitor the product quality throughout biopharmaceutical development. However, the matrix complexity and low analyte concentration frequently prevent SDS-PAGE to be a practical analytical method for in-process samples. On the other hand, traditional slab gel western blot, often associated with laborious manual operations, does not offer sufficiently accurate and precise quantitation, or desired turnaround time and sample throughput to meet industrial standards and requirements.

Recently, a fully automated, high throughput instrument has been developed for capillary electrophoresis (CE) protein separation and subsequent western blot analysis [5–7]. The method relies on the photochemical capture of resolved proteins onto the inner capillary wall. The immobilized proteins are then subjected to immunoassay in a manner similar to the traditional western blot, except with automatic data acquisition and analysis. This technology has been utilized previously in vaccine development [8–10], analysis of monoclonal

antibody and peptide charge heterogeneity [11,12], biomarker detection [13], characterization of endogenous protein in cell lysates [14,15] and industrial biopharmaceutical protein characterization [16]. Additionally, the applications of CE-based western blot for therapeutic protein and vaccine analyses have been also summarized in a book chapter [17]. In previous work from this group, CE western technology has been used to characterize impurities of a biopharmaceutical Fusion-Fc protein throughout its purification process compared to traditional slab gel western blot and also quantitatively monitor clearance of a selected host cell protein [18].

In the present work, a biopharmaceutical protein in cell culture harvest samples was separated based on size using an automated

Abbreviations: CE, capillary electrophoresis; DS, drug substance; DTT, dithiothreitol; HMWI, high molecular weight isoform; HRP, horseradish peroxidase; RSD, relative standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

* Corresponding author at: Analytical Development, Technical Development, Biogen Inc 125 Broadway, Cambridge, MA 02142, USA.
E-mail address: zoran.sosic@biogen.com (Z. Sosic).

[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.jpba.2017.06.025

CE western system to measure directly its two critical quality attributes. First, a combination of primary antibody and HRP-conjugated secondary antibody was used to measure a total protein expression or titer. Second, use of an HRP-conjugated primary antibody allowed for quantitation of relative distribution of major protein isoforms as an indicator of manufacturing consistency. This approach is fully automated, high-throughput and offers improved data precision measurements compared to the traditional assays, such as ELISA and slab gel western blot.

The relative distribution of protein isoforms as measured by CE western blot in cell culture harvest and purified drug substance samples was correlated with the SDS-PAGE results for drug substance to generate a predictive model for the composition of future drug substance batches. Thus, a novel approach using automated CE western blot has been explored as a potential analytical methodology for advanced process control in the manufacture of biopharmaceutical proteins. Furthermore, the generated data could be potentially used to enable adaptive measures in the downstream purification process to minimize the impact of cell culture variability on the expected product isoform distribution.

2. Materials and methods

2.1. Materials

Biotinylated molecular weight ladder, Streptavidin-HRP, DTT, fluorescent standards, luminol-S, peroxide, sample buffer, stacking matrix, separation matrix, running buffers, and wash buffer were part of the Peggy Sue 66–440 kDa Size Separation Master Kit with Split Buffer (Part# PS-MK24) obtained from ProteinSimple (Santa Clara, CA, USA). The kit also provides capillaries, antibody diluent, sponges, and 384-well sample plates.

Drug substances and the cell culture harvest samples were produced at Biogen. The primary antibody and its HRP-conjugated form were purchased from Enzyme Research Laboratories. The HRP-conjugated secondary antibody, used in the two-step western blot for titer experiments, was purchased from EMD Millipore. All drug substances and harvest samples were thawed from −70 °C prior to use.

2.2. SDS-PAGE under reducing conditions

Freshly prepared β-mercaptoethanol (Bio-Rad) was added to 5 mg/mL drug substance to a final concentration of 0.65 mM. The solution was then mixed with equal volume of 2 × Sample Buffer (Invitrogen), followed by incubation in a boiling water bath for 1 min. The reduced and denatured sample was loaded into a 4–12% Tris-Glycine gradient precast gel (Invitrogen) (25 μL per well), while 10 μL SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was loaded in the adjacent well. The polypeptide components were resolved by electrophoresis at 200 V for 55 min, and subsequently visualized by Coomassie stain (Thermo Fisher). The gel was destained with distilled H_2O, before the image was taken by a scanner (Bio-Rad).

2.3. Automated CE western blot

The experiments were conducted in accordance to the user guide by ProteinSimple [19]. The drug substances and cell culture samples were diluted in deionized H_2O to appropriate concentrations, and mixed at 4:1 ratio with 5 × Master Mix containing SDS, DTT and two fluorescence-labeled protein standards with molecular weights of 57 and 280 kDa (used for normalization of protein migration rates in different capillaries). The samples were incubated at 95 °C for 5 min for protein denaturation and reduction. Treated samples and the ladder (containing five biotinylated proteins with molecular weights of 66, 116, 200, 280 and 440 kDa, and the two fluorescence-labeled standards) were loaded into individual wells of the same row in a 384-well plate. Antibody solutions, blocking solution (antibody diluent), detection reagents (HRP substrates), stacking and separation matrices were loaded into the same 384-well plate. Automated western blot was performed by the Peggy Sue instrument (ProteinSimple) with single-use, silica capillaries (5 cm by length and 100 μm by inner diameter) with a clear Teflon coating to allow light transmission. The inner wall of each capillary contains proprietary coating for photo-activated covalent and non-specific binding of proteins. Capillaries were sequentially filled with separation matrix, stacking matrix and samples using vacuum injection. Proteins were then separated by electrophoresis at 275 V for 50 min in individual sets of 12 capillaries. Subsequently, the resolved proteins were cross-linked to the inner capillary wall through 5 s of exposure to UV light followed by immobilization. The stacking and separation matrices were removed from the capillaries with washing. The capillaries were then incubated with antibody diluent for 23 min to block non-specific binding of antibody. Next, HRP-conjugated primary antibody was introduced to the capillary lumen for 2 h incubation (for isoform distribution determination). Alternatively for titer measurement, the capillaries were incubated with primary antibody for 2 h followed by washing and 2 h incubation with HRP-conjugated secondary antibody. In the first capillary of each 12-capillary set, HRP-conjugated Streptavidin was applied to bind to the Biotin-labeled molecular weight standards. Subsequently, HRP substrates (peroxide and luminol-S) were introduced to generate chemiluminescence responses, which were captured by a built-in CCD camera at 7 exposure periods ranging from 5 to 480 s, in order to accommodate different signal strengths. These signals were automatically converted to electropherograms or lane-view images by Compass (version 2.7.1) (ProteinSimple). In addition, the Compass software also calculates the theoretical chemiluminescent signals at time zero of the HRP reaction by compiling the images taken at all exposure times during the run. Such calculated results, termed "Multi-Image Analysis", are obtained and presented in all figures and tables of this paper.

3. Results and discussion

3.1. Reducing SDS-PAGE analysis of the biopharmaceutical protein

Historically, SDS-PAGE was used to assess purity and composition of the biopharmaceutical protein throughout the purification process and for drug substance analysis as a batch release assay. SDS-PAGE analysis of the biopharmaceutical protein under reducing conditions reveals four major polypeptide components for the purified drug substance as shown in Fig. 1. Among them, the smallest polypeptide is 36 kDa. The two middle-sized polypeptides (90 kDa and 130 kDa, respectively) are the hydrolytic products (resulting from intracellular protease activities) of the largest one, the so-called High Molecular Weight Isoform (HMWI).

3.2. Titer measurement of the biopharmaceutical protein in cell culture harvest

The final production yield of the recombinant biopharmaceutical protein is limited by its expression level (titer) in host cells, which in turn is determined by cell culture conditions and therefore requires monitoring during manufacture. The concentrations of the biopharmaceutical protein in cell culture harvest samples were analyzed by two-antibody CE western blot, which is pre-

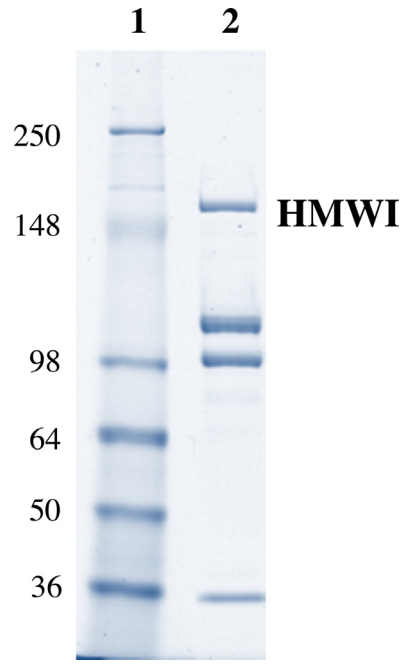

Fig. 1. Reducing SDS-PAGE analysis of biopharmaceutical protein. Lane 1: SeeBlue Plus2 Pre-Stained Standard. Lane 2: drug substance sample resolved to four polypeptides, with the largest one assigned as HMWI.

ferred over one-antibody CE western blot for higher sensitivity and better linearity in quantitation (data not shown). To establish a linear standard curve for quantitation, purified drug substance was diluted to the final concentrations of 0.01, 0.02, 0.05, 0.1 and 0.2 μg/mL before denaturation and reduction. The standards were then resolved by capillary electrophoresis, probed with primary antibody and detected by HRP-conjugated secondary antibody. The results are shown in Fig. 2 with a gel-like image view in Panel A and electropherograms in Panel B. In the figure, the three peaks of 100, 130 and 210 kDa represent the same three large polypeptides observed in SDS-PAGE analysis, respectively (Fig. 1). For titer measurement of cell culture harvest samples, the samples were first diluted 100-fold. This dilution was chosen because it would ensure that the diluted sample signals fall in the middle of the linear range of the calibration curve. The samples were then denatured and reduced, followed by separation through capillary electrophoresis, probing with primary antibody and detection by HRP-conjugated secondary antibody. The CE western blot steps for the standard curve material and cell culture harvest samples were performed in parallel.

Total area of the three peaks is plotted against the concentrations of the biopharmaceutical protein, and a standard curve with an R^2 value of 0.998 and near zero-intercept linear regression fitting was obtained (Fig. 2, Panel C). This relationship is then used to measure the total titer of the biopharmaceutical protein in cell culture harvest samples.

The titer results were compared, per sample, to those obtained by an orthogonal biological activity assay, which is the standard

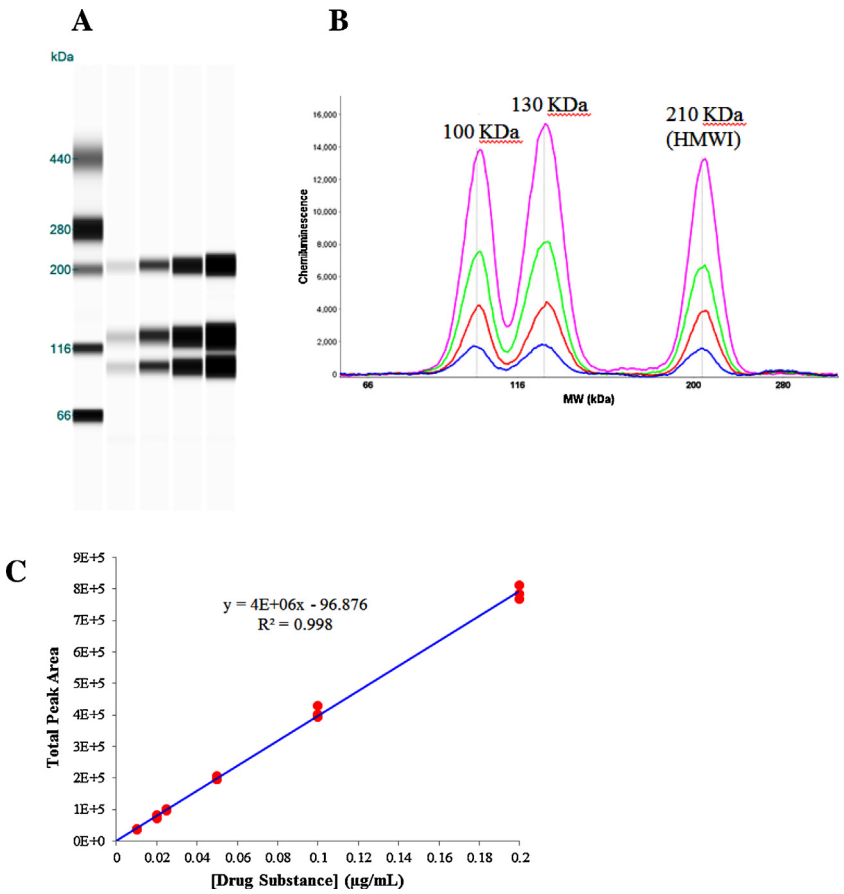

Fig. 2. Two-antibody CE western blot for biopharmaceutical protein titer quantitation. A) Gel-like image view. From left to right: molecular weight ladder, 0.02, 0.05, 0.1 and 0.2 μg/mL drug substance. B) Electropherograms for samples presented in Panel A: 0.02 (blue), 0.05 (red), 0.1 (green) and 0.2 (pink) μg/mL. C) The standard curve is established by plotting the total area of the three peaks against protein concentration standards using linear regression fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
(A) Repeatability for Titer Measurement of a Biopharmaceutical Protein in Cell Culture Harvest Sample. (B) Intermediate Precision of Titer Measurement of a Biopharmaceutical Protein for a Single Cell Culture Harvest Sample on Different Days. (C) Consistency of Titer Measurement using Different Sample Dilution Factors

(A)

N = 9	1	2	3	4	5	6	7	8	9	Average	Stdev	RSD
Titer (μg/mL)	6.0	6.0	6.1	5.9	6.0	6.2	6.0	5.7	6.1	6.0	0.1	2.4%

(B)

	Average (μg/mL)	Stdev (μg/mL)	RSD
Day 1	6.0 (N = 9)	0.1	0.9%
Day 2	5.7 (N = 9)	0.4	7.0%
Day 3	6.7 (N = 9)	0.2	3.6%
Total	6.1 (N = 27)	0.5	8.0%

(C)

Dilution Factor	Titer (μg/mL)	Average (μg/mL)	Stdev (μg/mL)	RSD
50	6.0 (N = 3)	6.0	0.1	0.9%
100	6.0 (N = 3)			
150	5.9 (N = 3)			

and established method used to measure titer in harvest materials for this biopharmaceutical protein. The results from both assays were within acceptable error margins <30% (data not shown). The repeatability of the titer assay was evaluated by replicate analysis (N = 9) of a representative cell culture harvest sample. Shown in Table 1A, are consistent repeatability data, with a relative standard deviation (RSD) of the measurements at 2.4%. The intermediate precision was satisfactory, with the RSD of titer measurements of the same harvest sample across three days below 10% (Table 1B). In addition, lack of impact of sample dilution step on measurement was confirmed by comparing calculated titer for a cell culture harvest sample diluted 50-, 100-, and 150-fold. The values were in agreement with each other, with an RSD of 0.9% (Table 1C).

3.3. Comparison of HMWI in cell culture harvest and drug substance

It has been demonstrated that the isoform distribution of the three largest polypeptides, excluding the smallest polypeptide, is an important product quality that has been monitored to assess manufacturing consistency. SDS-PAGE had been routinely used to obtain the HMWI distribution during drug substance batch release by analyzing the relative ratio of the three largest polypeptides. Since all protein components, including the impurities, are visualized without signal amplification, SDS-PAGE is limited to the analysis of the purified final product formulated at relatively high protein concentration. In this work, CE western has been implemented for the monitoring of HMWI directly in cell culture samples. The biopharmaceutical protein was analyzed by one-antibody CE western blot using an HRP-conjugated primary antibody (Fig. 3). This method was chosen due to its higher precision of individual peak area measurement over the two-antibody system used for titer determination. The ratio of peak area for the largest polypeptide, ~210 kDa, to the total areas of all three peaks, was calculated and reported as the HMWI percentage. Results obtained for HMWI percentage on different assay days demonstrated acceptable day-to-day consistency (Table 2). It is noteworthy that CE western analyses have yielded good experimental precision, with < 10% RSD for all measured values in Tables 1 and 2, and therefore represent a significant improvement over the estimated 30% RSD for traditional slab gel western blot [20].

The HMWI percentages in purified drug substances are higher than the values measured for the cell culture harvests of the same manufactured batches due to its enrichment through the purification process (Fig. 3). HMWI percentages in drug substances measured by CE western (*Y1*) were plotted against those for the

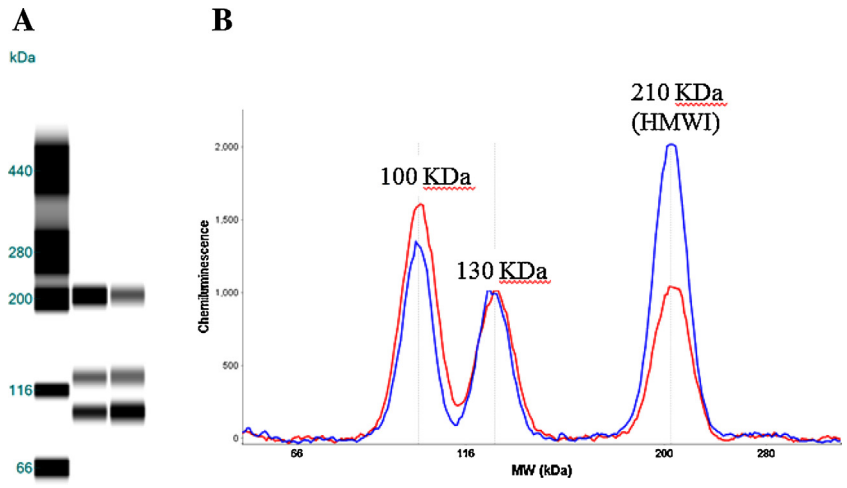

Fig. 3. One-antibody CE western blot for isoform distribution analysis of biopharmaceutical protein. A) Gel-like image view. From left to right: molecular weight ladder, 0.5 μg/mL drug substance, 15 × diluted cell culture harvest. B) Electropherograms for drug substance (blue) and diluted harvest sample (red) from Panel A, including molecular weight peak assignment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
High Molecular Weight Isoform (HMWI) in Drug Substance and Cell Harvest Measured by CE Western on Different Days.

	Drug Substance			Cell Culture Harvest		
	Average (%)	Stdev (%)	RSD	Average (%)	Stdev (%)	RSD
Day 1	43.7 (N = 12)	2.7	6.2%	24.3 (N = 9)	0.9	3.8%
Day 2	44.5 (N = 12)	2.2	5.0%	24.4 (N = 9)	0.9	3.5%
Day 3	44.6 (N = 12)	2.5	5.6%	24.7 (N = 9)	1.0	4.0%
Total	44.3 (N = 36)	2.4	5.5%	24.5 (N = 27)	0.9	3.7%

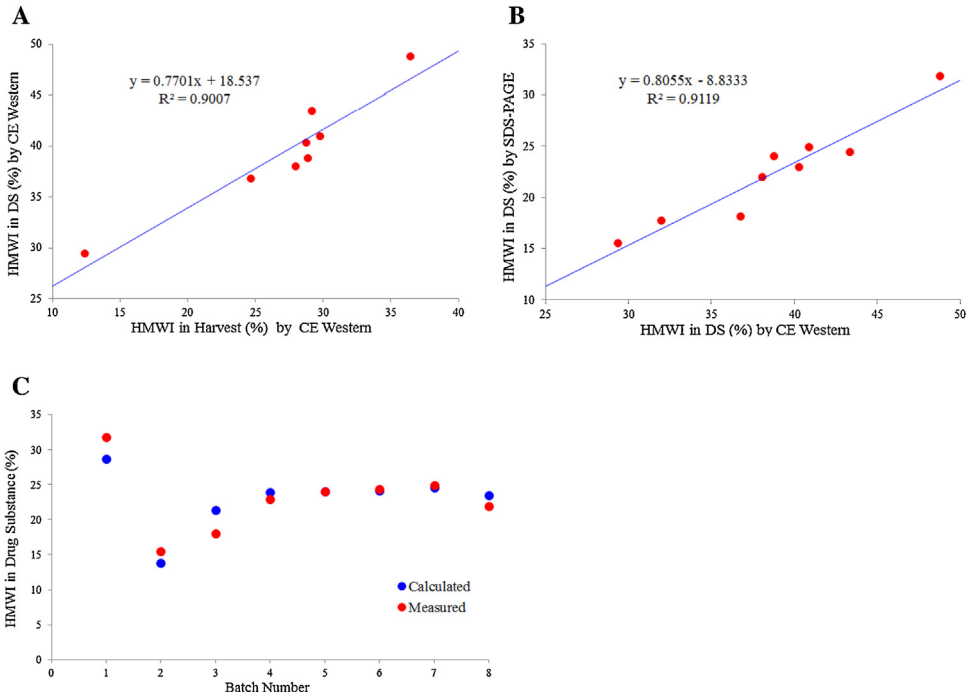

Fig. 4. Prediction of HMWI percentage in drug substance based on the cell culture harvest CE western analysis. A) The plot of HMWI percentages for drug substance and corresponding harvest cell culture samples measured by CE western. B) The HMWI percentages for drug substance samples measured by SDS-PAGE vs CE western. C) Comparison of the predicted HMWI percentages in drug substance samples obtained from CE western analysis of cell culture harvest samples and the fitting equations in Panels A and B (blue) vs the experimental HMWI percentages measured by SDS-PAGE analysis of drug substance samples (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cell harvest samples of the same batches measured by the same method (*X1*) in Fig. 4A. The data are fitted to linear regression, as shown below:

$$Y1 = 0.770 \times X1 + 18.5$$

A positive relationship can also be established between the HMWI percentages in drug substances measured by reducing SDS-PAGE (*Y2*) to the values obtained by CE western for the same materials (*X2*) (Fig. 4B):

$$Y2 = 0.806 \times X2 - 8.83$$

As *Y1* = *X2*, the two equations can be combined as:

$$Y2 = 0.806 \times (0.770 \times X1 + 18.5) - 8.83 \text{ or } Y2 = 0.62 \times X1 + 6.1$$

Therefore, HMWI percentage in the purified drug substance of a production batch measured with SDS-PAGE, (*Y2*), can be predicted using HMWI percentage in cell harvest of the same batch measured by CE western (*X1*). The proposed model is in good agreement with the historical SDS-PAGE data for eight batches, with an RSD $\leq$ 6.6% for%HMWI between measured and expected values. The graphical representation of calculated CE western data and those measured by SDS-PAGE is shown in Fig. 4C.

This indicates that the approach could be successfully applied to predict product isoform distribution of future, manufactured drug substance batches. Recently, use of mass spectrometry based methods in support of advanced process control efforts as part of a control strategy for biopharmaceutical manufacturing has been also reported [21].

4. Concluding remarks

The titer and isoform distribution of a biopharmaceutical protein have been successfully determined by automated CE western blot in cell culture harvest samples. The quantitative measurement of total biopharmaceutical titer in such early stage intermediates is required to provide the level of cellular expression yield prior to its purification process. The relative ratio of HMWI, as an indicator of manufacturing consistency, is another important product quality measured by CE western blot in this work. This approach allows for the assessment isoform distribution of recombinant protein directly after harvesting cell culture materials. The critical information is therefore obtained much earlier than the traditional SDS-PAGE analysis used at batch release. The early readout could be helpful for timely decisions on adaptive downstream measures to minimize potential batch failure due to cell culture variability.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

The authors thank Dr. Shashi Prajapati for the use of the ProteinSimple Peggy Sue CE western Blot instrument, Dr. Canping Jiang and Dr. Kyle Anderson for providing in-process samples and drug substances, and Dr. Kazumi Kobayashi for helpful discussions.

References

[1] International conference on harmonization: guidance for industry Q10 Pharmaceutical Quality System. www.ich.org.
[2] L.X. Yu, G. Amidon, M.A. Khan, S.W. Hoag, J. Polli, G.K. Raju, J. Woodcock, AAPS J. 16 (2014) 771–783.
[3] F. Luciani, S. Galluzzo, A. Gaggioli, N.A. Kruse, P. Venneugues, C.K. Schneider, C. Pini, D. Melchiorri, mAbs 7 (2015) 451–455.
[4] A.S. Rathore, Trends Biotechnol. 27 (2009) 546–553.
[5] R.A. O'Neill, A. Bhamidipati, X. Bi, D. Deb-Basu, L. Cahill, J. Ferrante, E. Gentalen, M. Glazer, J. Gossett, K. Hacker, C. Kirby, J. Knittle, R. Loder, C. Mastoieni, M. MacLaren, T. Mills, U. Nguyen, N. Parker, A. Rice, D. Roach, D. Suich, D. Voehringer, K. Voss, J. Yang, T. Yang, P.B. Vander Horn, Proc. Natl. Acad. Sci U. S. A 103 (2006) 16153–16158.
[6] A.C. Fan, D. Deb-Basu, M.W. Orban, J.R. Gotlib, Y. Natkunam, R. O'Neill, R.A. Padua, L. Xu, D. Taketa, A.E. Shirer, S. Beer, A.X. Yee, D.W. Voehringer, D.W. Felsher, Nat. Med. 15 (2009) 566–571.
[7] A. Harding, Science 339 (2013) 1100–1102.
[8] R.R. Rustandi, J.W. Loughney, M. Hamm, C. Hamm, C. Lancaster, A. Mach, S. Ha, Electrophoresis 33 (2012) 2790–2797.
[9] J.W. Loughney, C. Lancaster, S. Ha, R.R. Rustandi, Anal. Biochem. 461 (2014) 49–56.
[10] M. Hamm, S. Ha, R.R. Rustandi, Anal. Biochem. 478 (2015) 33–39.
[11] D.A. Michels, A.W. Tu, W. McElroy, D. Voehringer, O. Salas-Solano, Anal. Chem. 84 (2012) 5380–5386.
[12] U. Haußmann, O. Jahn, P. Linning, C. Janßen, T. Liepold, E. Portelius, H. Zetterberg, C. Bauer, J. Schuchhardt, H. Knölker, H. Klafki, J. Wiltfang, Anal. Chem. 85 (2013) 8142–8149.
[13] S. Liu, S. Sardi, B. Sonom, D. Zocco, R. McSweeney, A.D. Fraser, A.E. Halleck, H. Li, G.B. Smejkal, S. Munevar, J.G. Jin, T. Kawai, I. Ghiran, J.P. McGrath, M. Whitman, S. Ng, W.P. Kuo, J. Bioanal. Biomed. S3 (2013) 004.
[14] J.Q. Chen, M.R. Heldman, M.A. Herrmann, N. Kedei, W. Woo, P.M. Blumberg, P.K. Goldsmith, Anal. Biochem. 442 (2013) 97–103.
[15] R.R. Rustandi, M. Hamm, J.W. Loughney, S. Ha, Electrophoresis 36 (2015) 2798–2804.
[16] J. Wang, A. Valdez, Y. Chen, J. Pharm. Biomed. Anal. (2016), http://dx.doi.org/10.1016/j.jpba.2016.12.024.
[17] R.R. Rustandi, M. Hamm, C. Lancaster, J.W. Loughney, Capillary electrophoresis of proteins and peptides, in: Tran, Taverna (Eds.), Methods and Protocols Methods in Molecular Biology, vol. 1466, 2016, pp. 197–217, Chapter 16.
[18] D. Xu, S. Mane, Z. Sosic, Electrophoresis 36 (2015) 363–370.
[19] Sue Sally, Sue Peggy, User Guide, Revision 6, ProteinSimple, Inc., 2017.
[20] A. Koller, H. Wätzig, Electrophoresis 26 (2005) 2470–2475.
[21] Y. Lyubarskaya, K. Kobayashi, P. Swann, Pharm. Bioprocess 3 (2015) 313–321.