Capillary Electrophoresis in Quality Control PART II: CE-SDS: Method Development and Robustness

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Abstract

Capillary electrophoresis sodium dodecyl sulfate (CE-SDS), is the modern equivalent of the slabgel sizing technique SDS-PAGE. Common uses of SDS-PAGE include monitoring of manufacturing consistency and apparent molecular weight. Although used frequently, SDS-PAGE is a poor technique for quantitative protein purity due to inherent sample preparation artifacts, migration time and staining variability. With greater reproducibility and online detection capability, CE-SDS has not only been able to overcome some of the apparent drawbacks of SDS-PAGE,[1] but it also matches, and in some instances, surpasses techniques such as high performance liquid chromatography (HPLC) in resolution and reproducibility.

In the biopharmaceutical industry today, CE-SDS is applied at all stages of the pharmaceutical development process, including high-throughput process development,[2] structural isoform analysis,[3] carbohydrate occupancy,[4] and more common molecular size variant analysis for characterization and release.[5] Part II of “Applications of CE in Quality Control” is focused on method development and robustness approaches for CE-SDS. This article has been divided into 3 subsections: I) Method Purpose; II) Key method development aspects; and III) Critical robustness studies.

Method Purpose

As with any analytical methodology, it is critical to define the purpose of the method on the control system prior to initiating development. CE-SDS can serve a variety of purposes during the pharmaceutical development process so the specific requirements should be considered prior to initiating development. For example, a CE-SDS method used for protein titer determination in process optimization has different requirements than CE-SDS for identity or for purity.

The purpose of the method will guide initial method development. In order to develop a CE-SDS method for purity on a Drug Substance/Drug Product control system, you will first need to establish what level of detection and quantitation sensitivity is desired. The desired quantitation sensitivity will determine whether to use UV or Laser Induced Fluorescence (LIF) detection. LIF detection offers the benefit of about a 100-fold increase in sensitivity, yet it also requires additional sample manipulation. In common purity determination, i.e. lot-to-lot purity with respect to size variants, UV detection, which is comparable to Comassie stained SDS-PAGE, is likely to be sufficient. Some organizations, however, choose to use CE-SDS for both protein purity and as a complementary method for detection of potential host cell impurities. In this case, additional sensitivity may be required to detect minute amounts of foreign protein matter that may be present. Finally, a decision needs to be made with respect to sample preparation, for example whether a sample is reduced or nonreduced. For the non-reduced analysis, the native protein is treated with SDS prior to separation to mask the protein native charges. For reduced analysis, the sample is treated with SDS, and either dithiothreitol (DDT) or beta-mercaptoethanol (BME) to reduce the native protein structure. Recombinant monoclonal antibodies (rhuMAb, r-MAb), which represent the majority of currently approved biopharmaceuticals, will be reduced to glycosylated heavy chain (HC), non glycosylated heavy chain (NGHC) and light chain (LC). Both non-reduced and reduced offer advantages on a QC system. Specifically for r-MAb, the reduced form can allow for monitoring of the heavy chain glycosylation occupancy. If product degradation or stability is your primary goal, you may need to consider analyzing the non-reduced samples, which offer a much higher aptitude for detecting an increase in fragmentation. Often, companies will choose to develop both reduced and non-reduced CE-SDS methods for characterization and opt to use CE-SDS reduced for its ability to quantitate the non-glycosylated heavy chain (NGHC) for bulk release, yet apply non-reduced CE-SDS for stability monitoring.

Figure 1 illustrates key aspects for consideration when developing a CE-SDS method.

Purpose of the Assay?	**Identity**	• Detector UV: Sensitivity generally not a key factor • Most important: reproducibility for comparison to a reference material • Resolution must be sufficient to ensure specificity • Reduced versus non-reduced depends on specificity needs
	MW	• Detector UV: Sensitivity generally not a key factor • Critical to select correct MW ladder • If Markers are run separately, reproducibility must be considered • Generally run non-reduced
	Content	• Detector UV: Sensitivity generally not a key factor • Resolution must be sufficient to ensure specificity • Specificity and Accuracy/Reproducibility critical (i.e. corrected peak area) • Sample prep. artifacts (i.e. induced fragmentation) should be minimized
	Purity	• Detector: UV or LIF, depends on required LOQ • Reduced or non-reduced: depends on peaks of interest • Resolution generally critical • Accuracy/Reproducibility critical (i.e. % CPA) • Sample prep. artifacts (i.e. induced fragmentation) should be avoided

Figure 1. Purpose-driven critical method properties for CE-SDS.

Key Method Development Aspects

Protein analysis by CE-SDS relies on separation of SDS-labeled protein variants by a sieving matrix (i.e. polymer) in a constant electric field, with the following critical method parameters: SDS labeling technique, sieving matrix, and electrophoretic conditions.

For the purpose of this article, we will work through the critical steps in the development of a CE-SDS purity method for bulk drug substance. In this example, the method shall be used to monitor lot-to-lot consistency on release and degradation on stability. As previously discussed, this will require development of a reduced method for lot-to-lot consistency and a non-reduced method for increased ability to detect new degradation products. Because fragments are generally not immunogenic, we do not require the detection power of LIF, and thus, will focus on UV development.

Once the detection system has been selected, the next step is to consider the type of sample to be analyzed and the required MW range for separation. It is critical to choose the appropriate separation medium to achieve resolution in the desired range. CE-SDS uses linear or slightly branched polymers such as linear polyacrylamide, polyethylene oxide, polyethylene glycol, dextran, and pullulan as the sieving matrix.[6-7] In comparison to cross-linked polyacrylamide gel matrices, these polymers add great flexibility to CE-SDS since they are water-soluble and replaceable after each CE analysis, resulting in enhanced overall precision and robustness. It should be noted that optimizing gel composition is quite a challenging task, and although an option, one should first consider commercial sources with defined MW separation ranges to ensure reproducible gel separation performance. Commercial gel matrices routinely have adequate separation efficiency ranging from 10kD - 225KD. While these gel formulations are often optimized for antibody separation, they do prove to have adequate resolution for a wide variety of proteins. It is recommended to choose a suitable commercial gel matrix and to first focus on the development of sample preparation

steps. Once this parameter has been optimized, additional gel matrices can be evaluated to fine-tune resolution, if needed.

The most difficult aspect of CE-SDS method development is the reproducibility of the sample preparation protocol. CE-SDS requires the formation of SDS-protein complexes prior to electrophoretic separation. Traditional sample preparation conditions include heat treatment at elevated temperatures (e.g. 90º C for up to 10 min). In the case of non-reduced rMAbs, this could lead to sample preparation artifacts in the form of thermally induced fragmentation attributed to disulfide reduction and exchange reaction.4 It has been reported that high pH conditions during heat treatment also enhanced the fragmentation of SDS-rMAb complexes, and thus affected sample stability.4 These artifacts significantly altered the true representation of the size heterogeneity of a protein and also increased the variability of quantitative CE-SDS methodologies of non-reduced samples. The desired application will determine the required reproducibility of the sample preparation method. If the assay is primarily used to establish protein identity, then some variability in the fragmentation pattern may be acceptable. However, if the application is quantitative in determination of size distribution for purity/stability, then achieving reproducible SDS-labeling with minimal artifact creation is a key aspect of development.

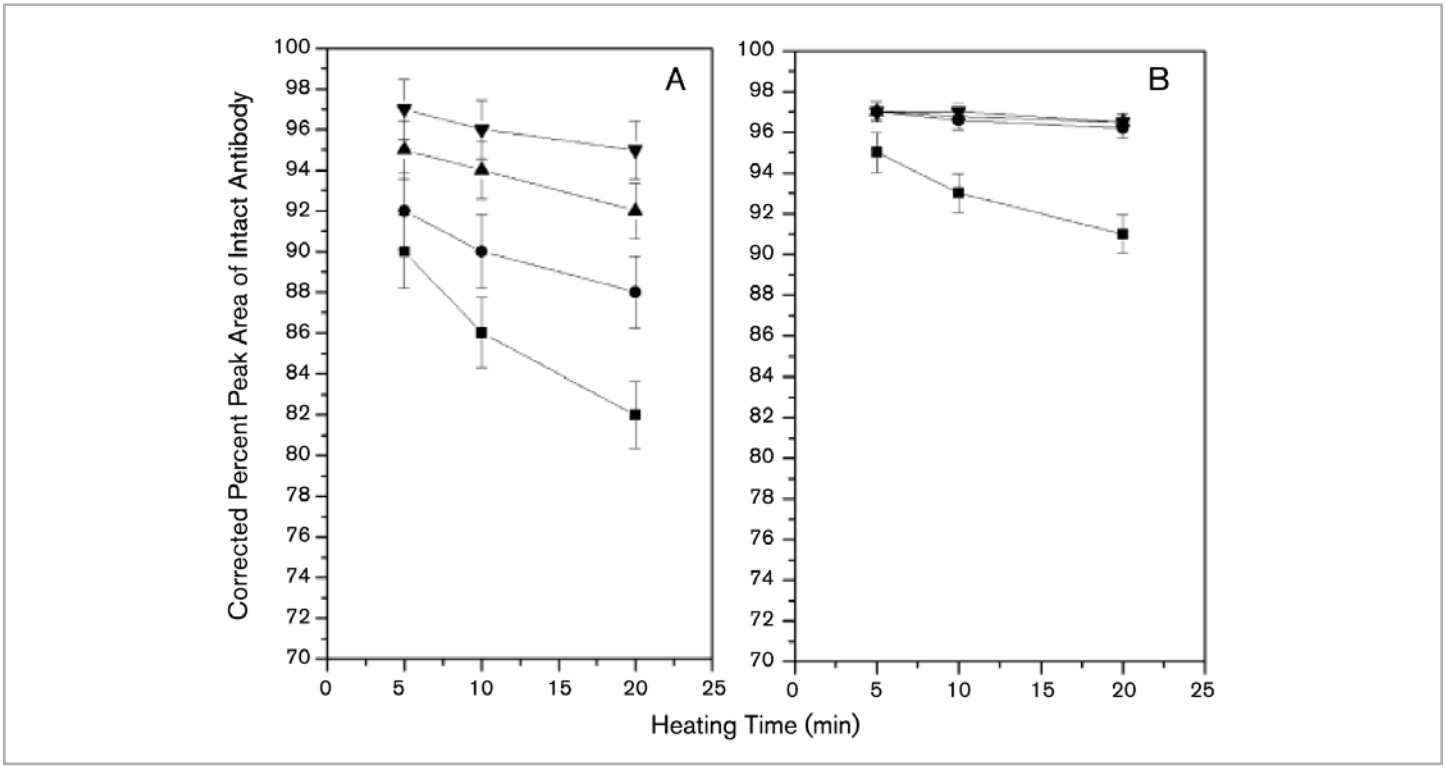

Figure 2. Corrected percent peak area of intact antibody vs. heating time at several incubation temperatures: (▲) 45, (▲) 60, (●) 70, and (■) 90° C. (A) no IAM, (B) IAM. Error bars are shown at the 95% confidence interval (n =3). Electrophoretic conditions were as follows: SCIEX PA 800 Series instrument with LIF detection, effective length 10.2 cm, total length 31.2 cm, 50-µm i.d., 375-µm o.d. uncoated fused silica capillary; both anode and cathode buffers were the SCIEX CE-SDS polymer solution. The samples were injected at a constant electric field of 160 V/cm for 20 seconds and electrophoresed at 480 V/cm (32.5 µA). Reprinted with author's permission from Reference 4.

Antibodies are especially vulnerable to heat-induced fragmentation, as described by Salas, et al. Thermally induced fragmentation of non-reduced rMAbs, can be greatly reduced through systematic optimization of the sample preparation conditions prior to CE-SDS analysis.[4] This includes optimization of sample incubation buffers, incubation time and incubation temperature. Due to the fragile nature of the hinge region present in rMab molecules, subjecting samples to heat treatment with SBS labeling, can lead to an increase in fragmentation.

Figure 2 shows the impact of both time and temperature on the measured rMAb fragmentation levels upon increased exposure to heat. The molecule can be stabilized against heat-induced degradation through the inclusion of an alkylation step. Using 40 mM iodoacetamide (IAM) during heat treatment of the non-reduced rMAb sample, combined with optimization of incubation temperature and time (70° C for 5 minutes, in the presence of SDS), Salas, et al., were able to significantly suppress the extent of thermally-induced fragmentation (Figure 2B, 3).

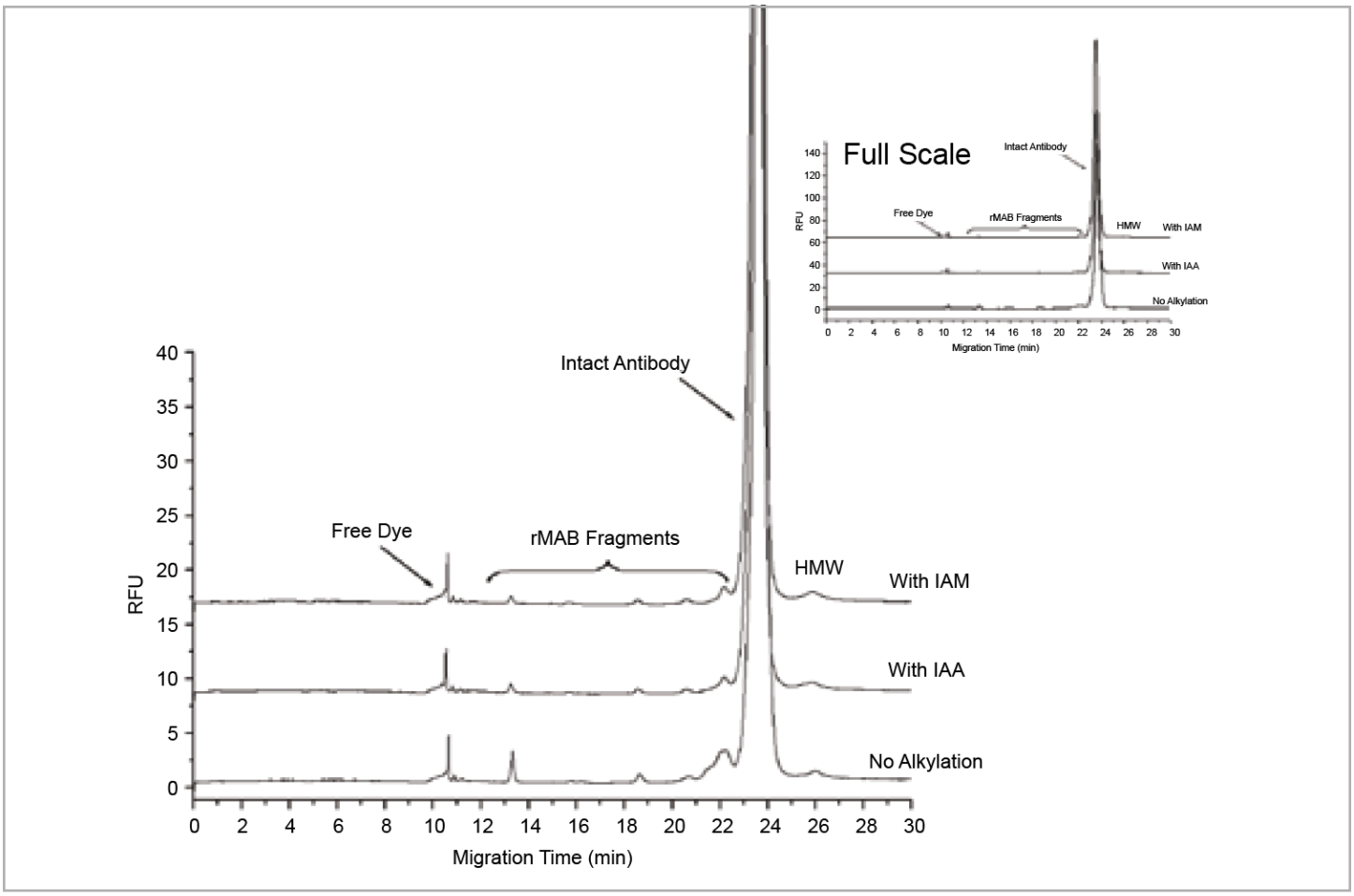

Figure 3. Expanded view of CE-SDS separations of non-reduced labeled-rMAb samples in the presence of different alkylating agents. The inset show the full scale view of the electropherograms. Electrophoretic conditions were as follows: SCIEX PA 800 Series instrument with LIF detection, effective length 10.2 cm, total length 31.2 cm, 50-µm i.d., 375-µm o.d. uncoated fused silica capillary; both anode and cathode buffers were the SCIEX CE-SDS polymer solution. The samples were injected at a constant electric field of 160 V/cm for 20 seconds and electrophoresed at 480 V/cm (32.5 µA). Reprinted with authors permission from Reference 4.

The quantitative studies of their work also demonstrated that utilizing an 85 mM citratephosphate, 1% SDS sample buffers at pH levels of 6.5, further decreased the induced fragmentation of non-reduced rMAb samples and improved sample stability. A significant decrease of the corrected peak areas corresponding to the rMAb fragments was observed for a sample treated with the optimized sample preparation scheme compared to the rMAb control sample. The control sample was prepared by traditional sample preparation conditions that included using a 1% SDS, 100 mM Tris-HCl buffer pH 9.0 as CE-SDS sample buffer and incubated the sample at 90º C for 5 minutes. The corrected percent peak area (%CPA) of the intact antibody increased from 90.0% in the control sample to 98.5 % in the sample containing 40 mM IAM, reconstituted in 86 mM citrate-phophate pH 6.5 and incubated at 70º C for 5 minutes.

The addition of the alkylating agent to reduced rMAb samples showed no benefit, as the sample was fully fragmented into HC, LC and NGHC as part of the reduction step.

The most crucial part of developing a reduced CESDS method will be optimizing the reduction to ensure complete dissociation of existing di-sulfides. Both DDT and BME are adequate reduction agents for concurrent use during the SDS sample treatment. The amount of reducing agent required as well as the time/temperature needed to achieve complete reduction will be dependent on the nature of the protein to be analyzed and should be optimized for each sample. It should be noted that DDT must be handled with proper care as it degrades easily, (i.e. store as a dried powder in a desiccator and prepare fresh reduction solution).

Lastly, electrophoretic analysis parameters need to be established. As the resolution is predominantly defined by the selection of the sieving matrix, key aspects of optimization should focus on sample injection and detection. As discussed earlier, we chose to use UV detection as a technique. As CE uses online detection, the sensitivity of the assay is directly proportional to the diameter of the capillary, thus favoring large bore capillaries. In contrast, when using the same separation matrix, increased capillary dimension will also induce higher currents – creating heat that will negatively affect your sieving matrix and negatively impact resolution and/or robustness. It is a fine balance between robust sample analysis and highest sensitivity. If using a commercially available sieving matrix, it is best to follow the manufacturer's recommendation for capillary dimensions. CE-SDS predominantly uses electrokinetic injection. Pressure injection is not recommended, as the injection process will reduce overall separation length by replacing the sieving matrix with sample solution. Additionally, due to the resistance of the sieving matrix, pressure injections tend to lack reproducibility.

Finally, it should be noted that although concentration of SDS in the SDS sample buffer is not crucial with respect to complex formation, as long as it is in excess of the sample, it can significantly affect your separation. Electrokinetic injections are biased and small, and charged compounds present in the sample will be preferentially injected. Increasing sensitivity can be accomplished by maintaining salt concentration, i.e. SDS concentration in the SDS sample buffer, at a minimal level. Commonly, 1% of SDS is used for CE-SDS reduced and non-reduced labeling of rhuMAbs.

Although not discussed in detail in this review, if fluorescence labeling is desired, several excellent application papers have been published which describe the development and optimization of the rhuMAb/protein labeling prior to SDS treatment.[4,8]

Critical Robustness Studies

Robustness is an integral part of establishing a new method, as it will allow you to define operating ranges for the critical method parameters. Additionally if well planned and performed in a GMP compliant manner, robustness data can be used for future discrepancy resolution and can also serve as supporting documentation for method changes.

If method development was carried out systematically or, even better, using a design of experiment (DOE) approach, one should already have defined the method's main critical parameters. The common contenders here are: incubation time/temperature; reagent expiration dating; electrokinetic injection parameters; sample concentration; sieving matrix lot-to-lot variability; SDS labeling buffer composition, and sample stability. Additionally, one should evaluate alternate instruments, not only by focusing on the CE instrument, but also by taking into account instrumentation used during the sample preparation, such as a water-bath used for SDS incubation. Heating of the samples can vary significantly depending on number of samples and type of heating used. For example, water bath and plate heaters may show significantly different heating profiles for any given sample. To facilitate future method transfers to external laboratories, it is beneficial to study the impact of multiple heating elements in sample preparation. Similarly, it is important to study different sample vials as well. As the method is in use, analysts may need to vary from the specified hardware (e.g. water bath, sample vials, PD 10 buffer exchange column vendors, DDT sources, etc) if the substitute hardware was covered in robustness. The data can be used to supplement a justification and allow for the deviation from the final procedure. To facilitate and speed-up robustness, one can choose to run multivariant DOE studies, which offer multiple benefits over single-variable experiments.

When performing robustness experiments, it is critical to also establish a method's precision at target, as it allows for comparison of method variability when critical parameters are varied. Target is defined as the mid-point, or set-point for a critical parameter. Precision of the method can be measured by evaluating relative migration times and/or % corrected peak area (%CPA) for peaks of interest. It is recommended to analyze at least one target sample at the beginning and at the end of the analysis sequence to confirm daily suitability of the overall system. Additionally, it is recommended to perform a sample preparation repeatability study, using an n=6 at target to evaluate general method precision. This can be done as a separate study, or as part of a larger DOE robustness study, using additional target data points. During development, crude ranges should have been established for all critical parameters, indicating when the method will fail. The goal of robustness is to establish workable ranges for all the critical parameters, i.e. for robustness studies, ranges should at minimum cover the desired final method range, and if possible, cover a larger range to allow for possible reagent/instrument changes as necessary. Finally, the target data points collected during robustness can be used to support quantitative system suitability criteria.

Conclusion

In the end, CE-SDS development and robustness is no different than any other analytical method development and robustness study. You must define the purpose of the method as it will guide you in selecting your development goals, i.e. desired resolution and/or sensitivity. Once the purpose is defined, a systematic method development can be performed. Many excellent application papers with preselected critical method parameters have been published on the development of robust and sensitive CE-SDS separations of proteins and rMAbs, thus facilitating the development process. Once initial critical method parameters have been set, robustness can be initiated to confirm the method's working range to document the impact of minor variability to the method's precision and accuracy.

Post robustness, the next step in the lifecycle of a GMP method will be the process of method validation. In the next article, we will discuss the necessary validation documentation and experiments required for a method used during the release of a commercial drug product.

Reference

1. Hunt, G., Moorhouse, K. G., Chen, A. B., *J. Chromatogr.* A 1996, 744, 295–301.
2. Rustandi R., Washabaugh M. , Wang Y.; *Electrophoresis* 2008, 29, 3612–3620.
3. Guo A., Han M., Martinez T., Ketchem, R. et al.; *Electrophoresis* 2008, 29, 2550–2556.
4. Salas-Solano, O., Tomlinson, B., Du, S., Parker, M. et al.; *Anal. Chem.* 2006, 78, 6583–6594.
5. Good D., Cummins-Bitz S., Fields R., Nunnally B.; Methods in Molecular Biology, vol. 276: *Capillary Electrophoresis of Proteins and Peptides* Edited by: M. A. Strege and A. L. Lagu © Humana Press Inc., Totowa, NJ.
6. Nakatani, M., Shibukawa, A., and Nakagawa, T. (1994). *J. Chromatogr.* A 672, 213–218.
7. Benedek, K., and Guttman, A. (1994). *J. Chromatogr.* A 680, 375–381.
8. Michels D., Brady L., Guo A., Balland A.; *Anal. Chem.* 2007, 79, 5963-5971.

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