Research Article

Improved CE(SDS)-CZE-MS method utilizing an 8-port nanoliter valve

Capillary sieving electrophoresis utilizing SDS (CE(SDS)) is one of the most applied methods for the analysis of antibody (mAb) size heterogeneity in the biopharmaceutical industry. Inadequate peak identification of observed protein fragments is still a major issue. In a recent publication, we introduced an electrophoretic 2D system, enabling online mass spectrometric detection of generic CE(SDS) separated peaks and identification of several mAb fragments. However, an improvement regarding system stability and handling of the approach was desired. Here, we introduce a novel 8-port valve in conjunction with an optimized decomplexation strategy. The valve contains four sample loops with increased distances between the separation dimensions. Thus, successively coinjection of solvent and cationic surfactant without any additional detector in the second dimension is enabled, simplifying the decomplexation strategy. Removal efficiency was optimized by testing different volumes of solvents as presample and cationic surfactant as postsample zone. 2D measurements of the light and heavy chain of the reduced NIST mAb with the 8-port valve and the optimized decomplexation strategy demonstrates the increased robustness of the system. The presented novel set-up is a step toward routine application of CE(SDS)-CZE-MS for impurity characterization of proteins in the biopharmaceutical field.

Keywords:
Capillary electrophoresis / CE(SDS) / Mass spectrometry / Monoclonal antibody / Two-dimensional

1 Introduction

Capillary sieving electrophoresis utilizing SDS (CE(SDS)) with ultraviolet (UV) or LIF detection is the most efficient and, thus, standardized method for size heterogeneity analysis of biotherapeutics like antibodies (mAbs) [1]. The identification of observed protein fragments is of outstanding importance to assure drug efficacy and safety. However, direct characterization of peaks in CE(SDS) is not possible since neither fraction collection of the small volumes nor direct ESI-MS is possible because of strong ionization suppression of proteins by SDS [2] and other separation gel components. There are several ways to cope with the challenges of hyphenating MS interfering electrophoretic separation methods summarized by Schlecht et al. [3]. In order to maintain the original separation method, two-dimensional heart-cut approaches can be applied, like presented in several set-up and applications [3,4]. None of these approaches was utilized for the CE(SDS) application so far. Hence, we recently introduced a 2D capillary electrophoretic separation system utilizing a 4-port nanoliter valve [5] for online mass spectrometric detection of CE(SDS)-separated peaks [6]. The generic CE(SDS) separation dimension, as first dimension (1D), is hyphenated via the 4-port nanoliter valve with a MS-compatible CZE method as second dimension (2D), for separating interfering substances from the peak of interest prior to introduction into the MS. One crucial part of this CE(SDS)-CZE-MS approach is the decomplexation of the SDS-protein sample, that is transferred via sample loop from the 1D to the 2D, a presample zone of solvent (methanol) and a postsample zone of CTAB is utilized for decomplexation in the 2D. Therefore, the zones were hydrodynamically injected via the second CZE dimension and positioned with the help of C4D. In this way, several mAb fragments and soybean proteins separated by CE(SDS) were characterized by MS [6]. However, robustness was limited due to frequent current leakage and breakdown, most probably caused by insufficient

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Abbreviations: 1D, first dimension; 2D, second dimension; CE(SDS), capillary sieving electrophoresis sodium dodecyl sulfate; EIE, extracted ion electropherogram; FAc, formic acid; HAc, acetic acid; HC, heavy chain; HCl, hydrochloric acid; LC, light chain; mAb, monoclonal antibody; MW, molecular weight; NaOH, sodium hydroxide; NIST, National Institute of Standard and Technologies; UV, ultraviolet

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Color online: See article online to view Figs. 1–4 in color.
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distance between the separation dimensions and inaccurate positioning of the decomplexation zones in the 1D.

Here, we present a novel 8-port nanoliter valve with increased distances between separation dimensions and additional loops, allowing successive transfer of the SDS-decomplexation zones into the 2D. The decomplexation strategy is adapted to the new design and optimized regarding measurement stability and signal intensity. The performance of the new set-up is demonstrated by the CE(SDS)-CZE-MS analysis of the light chain (LC) and heavy chain (HC) of the reduced mAb of the National Institute of Standard and Technologies (NIST) and compared to previous results obtained with the 4-port valve.

2 Materials and methods

2.1 Chemicals and samples

Methanol (HPLC-MS grade), acetic acid (HAc), and formic acid (FAc) were obtained from Carl Roth GmbH und Co. KG (Karlsruhe, Germany). Ethanol (HPLC grade) was purchased by Fisher Scientific (Schwerte, Germany). Sodium hydroxide (NaOH), hydrochloric acid (HCl), and 1-butanol were obtained from Merck (Darmstadt, Germany). DTT, SDS (10% in water), CTAB, glutaraldehyde solution (50% in water), PVA (≥99%, average molecular weight (MW) 89,000–98,000), and Tris were purchased from Sigma Aldrich (Steinheim, Germany). All solutions were prepared using ultrapure water (18 MΩ cm at 25°C). SG Ultra Clear UV from Siemens Water Technologies, USA. ESI Tuning mix solution was acquired from Agilent Technologies (Waldbronn, Germany). SDS-MW gel buffer was obtained from Sciex (AB Sciex, Darmstadt, Germany). Sample solution of mAb 1 was provided by F. Hoffmann-La Roche (Basel, Switzerland) and NIST mAb was obtained from the NIST. Reduction of mAbs was carried out by adding 0.5 M DTT and heating for 10 min at 70°C in a Thermomixer (Eppendorf, Wesseling-Berzdorf, Germany), followed by centrifugation at 14,100 g during 90 s (Eppendorf). For decomplexation optimization by injection via valve, mAb 1 was diluted in SDS-MW gel buffer to obtain a final concentration of 1 mg/mL. For the 2D analysis, NIST mAb was diluted in SDS solution with a final concentration of 1 mg/mL.

2.2 Technical set-up of 2D system with an 8-port valve

An 8-port-valve was designed consisting of eight symmetrically positioned ports on the stator and four symmetrically positioned sample loops (4 × 20 nL) on the rotor. The stator is made of polyether ether ketone (PEEK) and the rotor of Valcon E (polyaryletherketone (PAEK)/PTFE-based composite) as described before [6]. The valve was custom made by VICI AG International (Schenkon, Switzerland; part number: CSM-4354-CU1), equipped with 360 μm fittings and a multiposition electric microactuator. With this microactuator, the rotor can be switched into each of the four desired positions.

The 1D (position 1) and the 2D (position 3) are placed on the opposite sides and, thus, the loops for the pre- (position 2) and postsample zones (position 4) are chosen to be contiguous to the separation dimensions (Fig. 1). For the 1D and both zone dimensions, fused silica capillaries (Polymer Technologies, Phoenix, AZ, USA) and for the 2D, PVA coated capillaries, prepared in-house as described before [8], all with 50 μm id, are connected to the valve. The inlet position of an Agilent HP110 CE instrument (Agilent Technologies) is utilized for applying voltage and pressure in the 1D and the outlet position for pressure application in the presample zone dimension. The grounding of the 1D dimension is assured by placing a platinum electrode inside the outlet vial with the electrode being connected to an external HV-power supply. Instrument control was performed with the ChemStation software. The separation in the 1D is tracked via an ECD2600 EX UV-VIS detector (ECOM spol. s r.o., Prague, Czech Republic) at a wavelength of 214 nm. A detection window is positioned 4.3 cm in front of the valve on the first capillary of the 1D. The inlet of a second Agilent CE instrument (HP110CE) is utilized for flushing and applying voltage in the 2D and the outlet for the pressure application in the postsample zone dimension. To ensure safety of the user and integrity of the instruments, the valve itself is additionally grounded. An orthogonal electrospray interface (ESI, model G1607A from Agilent Technologies) is used for coupling of CE and MS. For MS detection, a Compact Q-TOF (Bruker Daltonics, Bremen, Germany) is employed. MS control and data analysis are carried out using Data Analysis software (Bruker). ESI is performed in positive ion mode (4.5 kV). The flow rate of dry gas is set at 4.0 L/min and a temperature of 170°C with a nebulizer gas pressure of 0.2 bar. As sheath liquid, a mixture of methanol: water (50:50, v/v) with 0.5% v/v FAc is used, delivered with a flow rate of 4 μL/min. Spectra are acquired in a mass range of 700 to 3500 m/z. External calibration of the MS is performed at the beginning of every day.

2.3 Preconditioning of CE(SDS)-CZE-MS system

In the 1D, capillaries were conditioned prior to use by flushing 0.1 M NaOH, 0.1 M HCl, and water at 3 bar for 3 min. For method development by injection via valve, capillaries were filled with mAb 1 in SDS-MW gel buffer at 3 bar and 2 bar for 10 min each. Meanwhile, the 1D was flushed with mAb 1 in gel, the presample zone dimension was flushed with water, the postsample zone dimension with CTAB solved in ethanol: water: butanol (2:2:1) for 10 min at 2 bar. The capillaries of the 2D were flushed at 2 bar for 5 min with water and 25 min with 1 M HAc.

For the 2D analysis, capillaries of the 1D were flushed after the conditioning step with SDS-MW gel buffer at 3 bar and 2 bar for 30 min each, followed by applying −15 kV for
5 min. Other capillaries were kept filled with air until the separation in the \textsuperscript{1}D was completed. After the analysis in the \textsuperscript{1}D was stopped, both zone dimensions and the \textsuperscript{2}D were flushed with water, CTAB, and 1 M HAc at 3 bar for 2 min, respectively.

2.4 SDS-removal strategy optimization and validation

The transfer of the presample zone into the \textsuperscript{2}D was performed by switching the loop filled with water to position 3 and applying pressure for several seconds. For transferring the post-sample zone into the \textsuperscript{2}D, the loop filled with 0.4\% w/v CTAB solved in ethanol: water: butanol (2:2:1) was switched to position 3 and negative pressure was applied for several seconds. After the pre- and postsample zone were transferred, the sample loop filled mAb 1 sample diluted in SDS-MW gel buffer was positioned in between these zones into the \textsuperscript{2}D by switching the valve to position 3 without further pressure application utilizing the valve as injector (no separation over the \textsuperscript{1}D). After successful positioning of the decomplexation zones and mAb 1 sample, the capillaries of the \textsuperscript{1}D and of the zone dimensions were flushed with air for 2 min at 5 bar, followed by setting the separation voltage in the \textsuperscript{2}D to +10 kV. After each analysis, capillaries were flushed with air for 5 min at 5 bar at position 3 and 1 to prevent possible clogging. For zone length optimization, different injection times for the pre- and post-sample zone and each combination were surveyed. Additionally, a comparison to the 4-port valve decomplexation strategy was done by utilizing methanol as presample zone and CTAB (solved in methanol: water, 1:1) as postsample zone with the 8-port-valve.

2.5 Analysis of mAb with CE(SDS)-CZE-MS system

NIST mAb was injected electrokinetically at $-15$ kV for 60 s in the \textsuperscript{1}D and separated by applying $-15$ kV. As soon as the CE(SDS) peak maxima was detected, the migration time and the effective length of the capillary were used to calculate the migration velocity. With the migration velocity and the known distance from the UV detector to the middle of the sample loop in the valve, the stop time for the CE(SDS) peak to arrive in the sample loop was determined. As soon as the stop time was reached, the separation voltage was set to 0 kV and the zone dimensions and \textsuperscript{2}D were flushed as described before. After transferring the decomplexation zones and the CE(SDS) separated protein fragment into the \textsuperscript{2}D, capillaries of the zone dimensions and \textsuperscript{1}D were flushed with air at 5 bar for 2 min before applying voltage in the \textsuperscript{2}D. The analysis were performed for the NIST LC ($n = 3$) and the HC ($n = 3$). After each measurement, capillaries were flushed with air at 5 bar for 5 min at the end (position 3) and starting position (position 1) to prevent possible clogging.

3 Results and discussion

3.1 Set-up and principle

With the here presented 8-port valve, each separation and zone dimension has its own connection and loop. They can be transferred into the \textsuperscript{2}D separately, by filling the loops, switching the valve, and applying pressure in the \textsuperscript{2}D. This allows the precise placement of defined volumes relative to the SDS-protein sample. Another major advantage of the 8-port valve design is the enlargement of the distances between...
the loops and, thus, between the dimensions. In the commercially available 4-port valve, the distance between the 1D and 2D is around 1 mm. Now, the distances between neighboring dimensions are about 3 mm and by utilizing the opposite positions for the 1D and 2D, the distance is 5 mm. Thus, even if SDS gel is allocated between rotor and stator as a result of flushing and switching the valve several times, current leakage is less observed than before. Especially, when all other loops are kept filled with air during voltage application in the 1D or 2D. The breakthrough voltage was tested by filling the 2D with 1 M HAc and applying voltage (5, 10, 15, 20, 25, 30 kV) for 10 min each (data not shown). Stable current was observed up to 25 kV, at 30 kV a break down and damage of the power supply was observed. For this reason, additional grounding of the valve was implemented to assure integrity of the instruments and safety for the user. With this set-up, separation voltages up to 25 kV can be utilized with the 8-port valve. Nevertheless, the voltage during the CE(SDS) separation utilized as 1D was kept at −15 kV as commonly used in routine analysis. Due to the low surface tension of SDS-containing solutions, they have a high tendency to allocate between rotor and stator. The bigger distances between loops support that current leakage is less frequently observed. However, the separation voltage in the 2D was kept at 10 kV to lower the risk of current breakdown. With this operating method, the analysis stability is still improved compared to the 4-port valve set-up. For other approaches without SDS, higher separation voltages in the 1D and 2D might be feasible with the here presented 8-port valve to speed up the analysis and increase separation efficiency.

3.2 SDS-removal strategy optimization and validation

Different then in our work presented before, decomplexation zones are now transferred separately and do not need to be hydrodynamically injected via the 2D. This assures a more precise decomplexation zone positioning in relation to the SDS-protein sample without the necessity of a C1D detector. Due to known problems with the formation of bubbles of SDS-containing solutions upon application of pressure, sample transfer of the SDS-protein complex from the sample loop into the 2D was aspirated without pressure application. For this reason, the pre- and postsample zones were positioned prior to sample transfer. Applying the previous SDS-removal strategy performed with the 4-port valve [6, 7], with methanol as presample zone and CTAB solution in methanol: water 1:1 as postsample zone, the current was instable or even broke down completely. To understand these current instabilities, offline solubility experiments were performed. When mixing equal amounts of methanol, SDS-MW gel, and CTAB (0.4% solved in methanol: water, 1:1) precipitation was observed. Various combinations and ratios of water/1-alcohol mixtures were tested in a similar way. By substituting methanol with water and solving CTAB in a mixture of ethanol: water: butanol (2:2:1), hardly any precipitation was observed. For this reason, water was used as presample zone and CTAB solved in ethanol: water: butanol (2:2:1) as postsample zone for the SDS-removal strategy with the 8-port valve. For zone length optimization, different injection times for water (30, 35, and 40 s) and for 0.4% w/v CTAB (20, 25, and 30 s) solved in ethanol: water: butanol (2:2:1) and each combination (n = 3) were surveyed. Interestingly, utilizing water and CTAB solved in ethanol: water: butanol (2:2:1), no current instabilities were observed and MS signals of the decomplexed mAb were obtained for all 27 measurements. Beside measurement stability and robustness, the decomplexation strategy with the highest removal efficiency was determined. In addition to the quality of the resulting mass spectrum (absence of noise and adducts), both peak intensity and area can be considered for the evaluation of the removal efficiency. Therefore, both signal intensities and areas of the extracted ion electropherograms (EIE) peaks, resulting from the sum of the eight most abundant m/z for the LC or HC of mAb 1, were evaluated. Optimal decomplexation conditions resulting in the highest peak area were found both for LC and HC in utilizing 35 s water as presample and 25 s CTAB as postsample zone (Fig. 2).

Standard deviations of the obtained signal areas for every decomplexation strategy (n = 3) were determined, ranging from 7 to 24% (mean value = 16%) for the LC and 4 to 24% (mean value = 18%) for the HC. The optimum regarding peak intensity of the LC was found for 40 s water in combination with 25 s CTAB and for the HC 35 s water and 25 s CTAB as pre- and postsample zone. The standard deviation for peak intensities ranged between 4 and 26% (mean value = 14%) for the LC and 1 to 23% (mean value = 8%) for the HC.

For a direct comparison to the decomplexation strategy used with the 4-port valve, methanol and CTAB (solved in methanol: water, 1:1) were applied as pre- and postsample zone with the 8-port valve, too. Here, only one zone length combination, namely 30 s of methanol and 20 s of CTAB (solved in methanol: water, 1:1), was tested, due to observed current instabilities as described before. Otherwise, the same set-up was utilized as performed for the decomplexation optimization by injecting reduced mAb 1 in SDS-MW gel buffer via valve. For the same transfer time into the 2D, obtained peak intensities with water and CTAB (solved in ethanol: water: butanol, 2:2:1) were higher for the LC (25%) and for the HC (20%) compared to the peak intensities obtained with methanol and CTAB (solved in methanol: water, 1:1). Important to note here is that utilizing methanol and CTAB (in methanol: water, 1:1) for decomplexation, a higher number of runs, here five, was necessary for obtaining three runs with stable current in the 2D and MS signals, demonstrating poor robustness and stability. The resulted peak areas obtained with methanol and CTAB (in methanol: water, 1:1) were 24% for the LC and 18% for the HC higher, then the peak areas obtained with water and CTAB (in ethanol: water: butanol, 2:2:1) at the same transfer time into the 2D. This is related to the poor peak shape that was obtained with methanol and CTAB (in methanol: water, 1:1). Hence, higher standard deviations in between successful measurements (n = 3) for the area were observed compared to the new decomplexation strategy.
for the LC, here 20% then 8%, and for the HC, here 39% then 15%. All in all, the experiments by injection via valve with the 8-port valve demonstrate that the optimized decomplexation strategy results in a higher method robustness and stability compared to the methanol decomplexation strategy used before. Additionally, better peak shapes and intensities were obtained for the same transfer times into the 2D.

3.3 Analysis of mAb with CE(SDS)-CZE-MS system

To evaluate the overall performance in a two-dimensional approach, CE(SDS)-CZE-MS analysis were performed with the presented 8-port-valve and optimized decomplexation in terms of peak intensity and area. The obtained results with the optimized decomplexation strategy regarding peak intensity were compared to partly published CE(SDS)-CZE-MS measurements of the NIST mAb LC and HC (c = 1 mg/mL) performed with the 4-port valve and its corresponding methanol decomplexation strategy [6]. The comparison of the runs with both CE(SDS)-CZE-MS systems is demonstrated by the EIEs (sum of the eight most abundant m/z) with the mean intensity (n = 3) for LC (Fig. 3A.1) and HC (Fig. 3B.1).

For the EIEs of the LC (Fig. 3A.1), it can be seen that the 4-port valve approach (dark gray, peak 1) resulted in a higher peak intensity (factor 1.5) but lower peak area (26%) compared to the 8-port valve approach (dark blue, peak 2) and their related decomplexation strategy. Comparing the averaged mass spectra (Fig. 3A.2), almost the same intensity for both approaches was found. Regarding the deconvoluted masses (Fig. 3B.3), factor 3.9 was observed compared to the 4-port valve approach (light grey, peak 1). Additionally, the mass spectra quality was improved as demonstrated by less adducts and artefacts (Fig. 3B.3).

Comparing the averaged EIE peak areas (Fig. 3A.3, n = 3) of the LC (dark blue) and HC (cyan) obtained with the 8-port valve approach to the 4-port valve approach (LC, dark grey and HC, light grey), an increase of a factor of 2.8 for the LC and a factor of 2.6 for the HC could be obtained.

Optimized decomplexation with the 8-port valve resulted in an averaged peak intensity (Fig. 4B) increase of a factor of 1.6 for the HC (cyan) compared to the 4-port valve approach (light grey). For the LC, our 8-port valve approach (dark blue) resulted in a peak intensity of 70% compared to peak intensities obtained with the 4-port valve approach (dark grey). Even if lower peak intensities were observed in average for the LC with the 8-port valve approach, standard deviation was improved from 24 to 12%.

Another interesting point is the comparison of the success rate of both CE(SDS)-CZE-MS set-up. Here, the analysis was stated as successful, if the current in both dimensions was stable and standard deviation was less than 25% regarding MS signal intensity. Comparing the number of performed 2D runs to obtain three successful measurements each for the LC and HC analysis, 14 measurements with the 4-port valve set-up were necessary. This results in a success rate of 43% (6 of 14 runs). With the 8-port valve set-up, nine successful runs were obtained by performing 12 measurements. This leads to a success rate of 75% with the 8-port valve and optimized decomplexation, demonstrating an almost doubled success rate and, thus, an increased measurement stability.

4 Concluding remarks

We presented a new designed 8-port valve for the improved set-up and handling of our previously described CE(SDS)-CZE-MS system. Decomplexation zones are now positioned...
successively via separate zone dimensions. This allows precise positioning for the addition of decomplexation agents before and after the transferred CE(SDS) peak. It turned out that the use of water as presample and CTAB (in ethanol: water: butanol, 2:2:1) as postsample zone resulted in most stable conditions. Best removal efficiency, represented by the highest peak areas, was found by utilizing 35 s at 50 mbar of water as presample zone in combination with 25 s at ~50 mbar of CTAB as postsample zone for both LC and HC. Compared to the previous decomplexation strategy (methanol and CTAB in methanol: water, 1:1), the here developed approach reached better signal intensities both for the LC (25%) and HC (20%) by injection via valve. CE(SDS)-CZE-MS measurements of the NIST mAb LC and HC with the 8-port valve and optimized decomplexation strategy were performed and compared to results obtained with the 4-port valve approach. Even if lower signal intensities of the LC EIEs were observed, it was possible to preserve about the same signal intensity for the mass spectra and for the deconvoluted mass (81%) at same signal quality. An increase of the intensity regarding the HC EIEs (factor 1.5), mass spectra (factor 3.3), and deconvoluted mass (factor 3.9) was observed compared to the 4-port valve approach. Additional, the mass spectra quality for the HC was improved demonstrated by masses showing less adducts and artefacts. Especially, an increase of current stability in the 2D could be achieved. Comparing the 2D measurement for the analysis of the NIST LC and HC performed with the 8-port valve to the 4-port valve, an almost doubled total measurement stability was obtained. Thus, the measurements with the 8-port valve and optimized decomplexation strategy are a significant step toward a routine application of this new technology. The here presented 8-port valve can be utilized for

Figure 3. Comparison of CE(SDS)-CZE-MS measurements of the reduced NIST mAb LC (A.1-A.3) and HC (B.1-B.3) with the 4- and 8-port valve and their related decomplexation strategy. LC and HC (1 mg/mL) were analyzed (each n = 3) with both approaches and the representative EIEs (sum of the eight most abundant m/z) and MS signals are presented. The EIEs obtained in the second CZE-MS dimension of the LC is shown in A.1, where peak 1 (dark gray) was obtained with the 4-port valve approach and peak 2 (dark blue) with the 8-port valve approach. The averaged mass spectra for both LC peaks can be seen in A.2 and the related deconvoluted mass in A.3. The EIEs obtained in the second CZE-MS dimension of the HC is shown in B.1, where peak 1 (light gray) was obtained with the 4-port valve approach and peak 2 (cyan) with the 8-port valve approach. The averaged mass spectra for both HC peaks can be seen in B.2 and the related deconvoluted masses in B.3. For both approaches, a 20 nL sample loop was utilized to transfer the peak of interest from the 1D to the 2D.

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Figure 4. Mean value of the peak areas (A) and heights (B) resulting from the EIEs (sum of the eight most abundant m/z) obtained for reduced NIST mAb LC and HC performing 2D measurements with the 8-port valve (LC, dark blue and HC, cyan) and 4-port valve approach (LC, dark grey and HC, light grey). For both approaches, a 20 nL sample loop was utilized to transfer the peak of interest (here LC or HC) from the 1D to the 2D. Error bars represent standard deviation (n = 3).

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