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1 **Charge variant analysis of monoclonal antibodies by CZE-**
2 **MS using a successive multiple ionic-polymer layer (SMIL)**
3 **coating based on diethylaminoethyl-dextran**

4
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19

20 **Keywords:**

21 capillary coatings, capillary electrophoresis, cationic polyelectrolyte multilayers, intact

22 antibody separation, mass spectrometry

23

24

1 **Abbreviations**

2	CEX	cation exchange chromatography
3	DEAED	diethylaminoethyl-dextran
4	PDADMAC	poly(diallyldimethylammonium chloride)
5	PEO	polyethylene oxide
6	PSS	poly(sodium styrene sulfonate)
7	SA	Sialic acid
8	SMIL	successive multiple ionic-polymer layer
9	UPW	ultrapure water

10

11

1 **Abstract**

2 The characterization of the impurities of pharmaceutical monoclonal antibodies is
3 crucial for their function and safety. CZE is one of the most efficient tools to separate
4 charge variants of mAbs, however, peak characterization remains difficult, since the
5 hereby used BGEs are not compatible with ESI-MS. Here, a method that allows the
6 separation of intact mAb charge variants is presented using CZE-ESI-MS, combining
7 a cationic capillary coating and an acidic BGE. Therefore, a successive multiple
8 ionic-polymer layer (SMIL) coating was developed based on diethylaminoethyl-
9 dextran (DEAED) - poly(sodium styrene sulfonate) (PSS). This coating leads to a
10 relatively low reversed EOF with an absolute mobility slightly higher than that of
11 antibodies, enabling the separation of variants with slightly different mobilities. The
12 potential of the coating is demonstrated using USP mAb003, where it was possible
13 to separate C-terminal lysine variants from the main form, as well as several acidic
14 variants and monoglycosylated mAb forms. The presented CZE-MS method can be
15 applied to separate charge variants of a range of other antibodies such as infliximab,
16 NISTmAB, adalimumab, and trastuzumab, demonstrating the general applicability for
17 the separation of proteoforms of mAbs.

18

1 Introduction

2 Monoclonal antibodies (mAbs) are important tools for the treatment of various
3 diseases. To ensure their safety and optimize the production process, it is important
4 to monitor the purity of the product and perform a detailed characterization of the
5 variants. Commonly found variations in antibodies are differences in glycosylation
6 along with missing lysine clipping, deamidation, and fragmentation of the antibody
7 [1]. For analysis, the intact antibody is often enzymatically fragmented into peptides,
8 glycans, or other subunits [2]. Although these methods allow for high sensitivity and
9 good separation, some information about the structure and overarching composition
10 of the intact antibody is lost. In addition, artifacts might be introduced. This makes
11 the characterization of intact antibodies a desirable tool for obtaining the utmost
12 information on microheterogeneity. However, MS does not allow to differentiate
13 modifications of mAbs with small mass differences, such as deamidation (+1 Da) or
14 disulfide bridge reduction (+2 Da), due to overlapping isotopic patterns. Therefore,
15 separation prior to MS characterization is mandatory.

16 To separate charge variants of the intact antibody, three different separation
17 methods are commonly applied: cation exchange chromatography (CEX) [3], CIEF
18 [4], and CZE [5]. However, coupling to MS is often challenging due to the use of non-
19 volatile salts, ampholytes, or additives. To allow coupling of the mentioned
20 separation methods with MS it is possible to use fraction collection in CEX with
21 subsequent LC-MS analysis [6]. When the volumes of collected sample are low,
22 such as in CIEF or CZE, two-dimensional approaches are possible, where interfering
23 substances from the charge variant separation method are removed in the second
24 separation dimension before subsequent MS characterization [7–9]. Recent
25 advances have allowed the direct coupling of CEX [10, 11] and CIEF [12–14] with

1 MS. Acidic and basic charge variants like deamidation, sialic acid variants and,
2 lysine truncations can be separated, and detected subsequently by MS. However,
3 there exists hardly any generic method and both CEX and CIEF have to be adapted
4 to the protein since they rely on the pI of the mAbs [10]. Furthermore, in the case of
5 CIEF, high intense ampholytes may interfere with ESI-MS [12].

6 The most commonly applied CZE separation of mAbs is based on a background
7 electrolyte (BGE) containing ϵ -amino-caproic acid (EACA), hydroxypropyl methyl
8 cellulose, and triethylenetetramine [5]. This universally applicable method shows
9 high separation efficiency and robustness; however, it cannot be directly coupled to
10 MS. Therefore, numerous other approaches were tried to achieve a similar
11 separation using alternative MS-compatible BGEs and coatings. When performing
12 CZE-MS applying a neutral coating (Neutral OptiMS) and an acidic BGE, Haselberg
13 et al. achieved partial separation of the two lysine variants from the main form of
14 ustekinumab [15]. PEI coated capillaries combined with an acidic BGE allowed the
15 separation of some acidic mAb variants with masses that differed in the range of 3-
16 10 Da from the main variant, although no basic variants were separated for intact
17 adalimumab [16].

18 When using microchip electrophoresis (ME)-MS, it was possible to separate the
19 main form and the two lysine variants from each other when using an acidic BGE
20 [17–19]. When using a native BGE, the separation could be further increased [20],
21 resulting in a comparable separation performance using this zone electrophoretic
22 approach compared to CEX [11, 21] or CIEF [22]. However, all CZE-MS or ME-MS
23 approaches do not reach the same resolution as shown for the EACA-based CZE-
24 UV approach. Thus, there is still a strong need for improvement regarding separation
25 selectivity, efficiency, and simplicity of application.

1 The aim of this work is to develop a generic CZE-MS for high-resolution charge
2 variant analysis. As a key point to increase separation under acidic conditions a new
3 cationic capillary coating is introduced with an EOF strength similar to the mAb
4 analyte mobility. For the diethylaminoethyl-dextran (DEAED) - poly(sodium styrene
5 sulfonate) (PSS) coating, the best separation was achieved and optimized regarding
6 resolution, evaluating major parameters, such as BGE and separation voltage. The
7 optimized CZE-ESI-MS method is evaluated regarding the characterization of
8 various proteoforms of USP mAb003. Several other mAbs are analyzed to
9 demonstrate the general applicability of the CZE-MS method with the new coating.

10
11

1 **2 Materials and Methods**

2 **2.1 Materials**

3 The USP mAb003 (lot: F12980) was purchased from Sigma-Aldrich (Steinheim,
4 Germany). Infliximab (charge: 8KMKA90603), adalimumab (charge: 1825216), and
5 trastuzumab (charge: N1050H05) were purchased from Evidentic (Berlin, Germany).
6 NISTmAb (Reference Material 8671, lot: 14HB-D-002) was purchased from the
7 National Institute of Standard and Technology (NIST, Gaithersburg, MD, USA).
8 For capillary coating, DEAE-dextran 500 (DEAED) (Mw = 500000 g/mol, Batch
9 21046) was purchased from TdBLabs (Uppsala, Sweden). Polyethylene oxide (PEO)
10 (Mw 1000000 g/mol) was purchased from Alfa Aesar (Kandel, Germany).
11 Poly(diallyldimethylammonium chloride) (PDADMAC) (Mw 400000-500000 g/mol),
12 PSS (Mw 70000 g/mol), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
13 (HEPES), hydrofluoric acid (40% (v/v)), sodium hydroxide, and hydrochloric acid
14 (37%) were purchased from Sigma-Aldrich (Steinheim, Germany). Isopropyl alcohol
15 (IPA), ACN (all LC-MS grade), formic acid ($\geq 98\%$, FA), and acetic acid (HAc) were
16 obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). All solutions were
17 prepared with ultrapure water (UPW, 18 M Ω *cm at 25 °C, SG Ultra Clear UV from
18 Siemens Water Technologies, USA).

19

20 **2.2 Capillary electrophoresis**

21 CE-UV was performed with a CE 7100A system from Agilent Technologies
22 (Waldbronn, Germany). Bare fused silica capillaries with 50/100 μm inner diameter
23 and 360/240 μm outer diameter (separation capillary/sheath liquid capillary) were
24 obtained from Polymicro Technologies (Phoenix, AZ, USA). Separation capillaries
25 had a length of 40 cm (UV detection) or 60 cm (UV and MS detection).

1 Successive multiple ionic-polymer layer (SMIL) capillaries were coated according to
2 the protocol of Dhellemmes et al. [23]. Briefly, the capillaries were flushed with 1 M
3 NaOH for 10 min, followed by flushing the capillary with UPW for 5 min and 20 mM
4 aqueous HEPES solution (pH 7.4) for 10 min. To create the SMIL coating, the
5 capillary was alternately flushed with polycation and polyanion solution (3 g/L in
6 20 mM HEPES solution at pH 7.4) for 7 min with intermediate rinsing with HEPES
7 solution for 3 min until a total number of five layers was reached. After a waiting step
8 of 5 min, the capillary is flushed with UPW for 3 min followed by a flushing step with
9 BGE for 10 min.

10 PEO capillaries were coated based on a published protocol [24]. Briefly, a PEO stock
11 solution was prepared by dissolving 50 mg PEO in 22.5 mL of UPW at 95 °C. The
12 PEO coating solution was prepared daily by mixing 10% 0.1 M HCl and 90% PEO
13 stock solution. Before the first daily measurement, the capillaries were flushed with
14 1 M NaOH, UPW, and 1 M HCl each for 5 min, PEO coating solution (2 mg/mL in
15 0.01 M HCl) for 10 min, followed by flushing with UPW and BGE for 5 min. During
16 CE-MS measurements, when applying the PEO coating, it was necessary to apply
17 2 mbar of pressure to stabilize the current.

18 All coating steps were performed at 2 bar when using 60 cm capillaries and
19 950 mbar when using 40 cm capillaries. During the measurement sequences, the
20 BGE was exchanged at least every two measurements. Before each measurement,
21 the capillary was flushed for 3 min with BGE. For comparison of the coatings, a
22 voltage of +10 kV was applied for the PEO, and -10 kV was applied for the SMIL
23 coatings. All antibodies were injected with 40 mbar, 5 s using a 1 mg/mL antibody
24 solution in BGE. The SMIL capillaries were stored overnight in BGE. Data

1 processing for CE-UV data was performed using CEval 0.6h9 [25] (available at:
2 <https://echmet.natur.cuni.cz/>).

3

4 **2.3 Mass spectrometry**

5 The CE was coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo
6 Fisher Scientific, San Jose, USA) using the *nanoCEasy* interface [26]. For MS
7 detection, the tip of the CZE capillary was etched with hydrofluoric acid to an outer
8 diameter of roughly 80-100 μm . Emitters with a 30 μm tip were obtained from
9 BioMedical Instruments (Zöllnitz, Germany). IPA:UPW (50:50) + 0.5% FA was used
10 as the sheath liquid. The spray voltage for the nanoCEasy interface was set to
11 2000 V for all MS measurements. All data were acquired in positive mode using a
12 mass range of m/z 700-4000 and a resolution of 30000. Normalized Automatic Gain
13 control Target was set to 100%, maximum injection time to 75 ms with 5 micro
14 scans. The radio frequency was set to 100% and the fragmentation voltage to 80 V.
15 MS data was analyzed using Freestyle 1.8 (Thermo Fisher Scientific) and Intact
16 Mass 3.4 (Protein Metrics, Cupertino, CA, USA). If not otherwise detailed,
17 electropherograms obtained from CE-MS measurements are smoothed by applying
18 Gaussian 5.

19

20

21

1 **3 Results and Discussion**

2 **3.1 Capillary coatings**

3 Capillary coatings are a crucial factor in the CZE separation of proteins by (i)
4 reducing protein-wall interactions and (ii) determining the EOF, thereby influencing
5 the resolution and speed of the separation. Resolution in CZE can be expressed as
6 [27]:

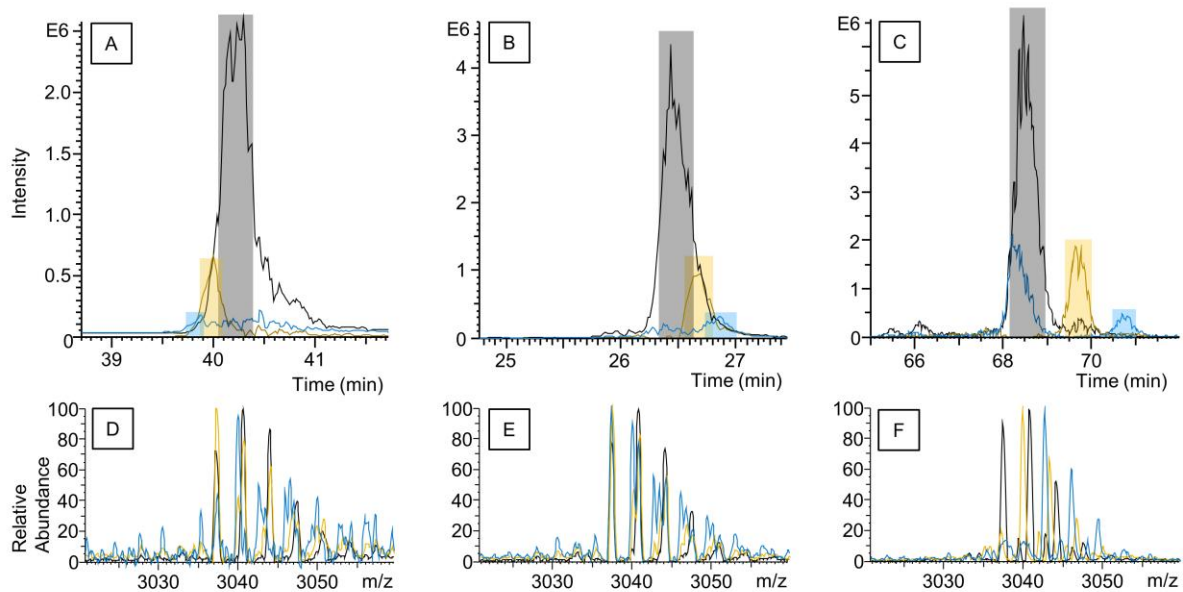
$$7 \quad R = 0.25 \frac{\Delta\mu_e}{|\mu_{EOF} + \bar{\mu}_e|} \sqrt{N} \quad (1)$$

8 where $\Delta\mu_e$ is the effective mobility difference between two analytes, μ_{EOF} is the
9 mobility of the EOF, $\bar{\mu}_e$ is the mean effective mobility of the two analytes and N is the
10 number of theoretical plates. The resolution increases when μ_{EOF} and $\bar{\mu}_e$ are
11 counter-directed and their sum is minimal. In theory, neutral coatings should be
12 preferred over a cationic coating for low mobility analytes, i.e. as soon as the
13 absolute mobility of the reversed EOF provided by the cationic coating is more than
14 a factor of two larger than the absolute mobility of the proteins. It has been
15 demonstrated that proteins with small mobilities can be better separated by neutral
16 coatings while proteins with high mobilities can be better separated by cationic
17 coatings [28]. However, a cationic coating with reduced EOF should lead to better
18 resolution even for low-mobility analytes.

19 For the antibodies described here, we measured mobilities of about 17-18 TU
20 (Tiselius unit, $1 \text{ TU} = 10^{-9} \frac{m^2}{Vs}$) for a 4 M acetic acid BGE. This means that an ideal
21 cationic coating is supposed to be in the range of 20-30 TU for it to perform
22 significantly better than a neutral coating. Here, we introduce a cationic SMIL coating
23 based on DEAED that leads to EOF in this range. This coating is compared to a
24 neutral coating, as well as a previously used SMIL coating with a higher EOF.

1 Because the dextran groups of DEAED are only partially modified with
2 diethylaminoethyl groups, the DEAED coating is expected to have a lower charge
3 density compared to the PDADMAC-PSS coating and, thus, a lower EOF. Indeed,
4 the absolute value of EOF mobility for the 5-layer DEAED-PSS coating was
5 measured to be 24.0 TU, and, thus in the desired range for mAb separation. The
6 reference 5-layer PDADMAC-PSS coating resulted in a higher absolute value of
7 EOF mobility of 35.2 TU. The EOF is highly dependent on the BGE composition and
8 the here given values were calculated for a 4 M HAc (voltage of -10 kV ($n=3$), which
9 turned out to be the best BGE (see 3.2)). The different coatings were tested using
10 USP mAb003 as the model mAb for charge variant separation. This model mAb
11 contains two basic variants with one (1 K) and two (2 K) additional lysine,
12 respectively. Incomplete removal of C-terminal lysine is a common basic variant
13 observed in mAbs [29]. Due to their characteristic mass shift of +128 Da, these
14 variants are easily detectable with MS even when no prior separation is possible,
15 making these variants an ideal tool for studying charge variant separation with
16 subsequent MS analysis. The mobilities of these three USP mAb003 variants are
17 determined to be 16.99 TU (Main), 17.12 TU (1 K), and 17.22 TU (2 K) for the 4 M
18 HAc and the DEAED-PSS system. Applying [equation 1](#), the best resolution should
19 be achieved for the DEAED-PSS coating, while the PEO coating and PDADMAC-
20 PSS coatings should have a similar but lower resolution. This is demonstrated by
21 measurements as illustrated in Figure 1, showing the separation of charge variants
22 of USP mAb003 for the three different coatings.

23



1

2 Figure 1: Separation of the USP mAb003 at A) +10 kV for the neutral PEO coating,
 3 B) -10 kV for the cationic PDADMAC-PSS coating, and C) -10 kV for the cationic
 4 DEAED-PSS coating with EIE (of the five most intense charge states, ± 50 ppm,
 5 Gaussian smoothing 5) of G0F/G0F + 0 K (black), G0F/G0F + 1 K (yellow) and
 6 G0F/G0F + 2 K (blue) applying a BGE of 4 M HAc, a 60 cm capillary and
 7 hydrodynamic injection of 1 mg/mL mAb in BGE (other parameters as described in
 8 material and methods). Marked time slices are integrated to gain the mean mass
 9 spectra shown in D-F for the respective separation figure above.

10

11 For the PEO coating (Figure 1A), analytes with high ion mobility arrive first, while for
 12 the SMIL coatings (Figure 1B and Figure 1C), analytes with high ion mobilities arrive
 13 last. For both PDADMAC-PSS and PEO, it is possible to see a partial separation of
 14 the lysine forms, but the separation is not sufficient to integrate 'clean' mass spectra
 15 (Figure 1D and 1E). In contrast, the DEAED-PSS coating (Figure 1C) with the lower
 16 EOF enables a baseline separation of the lysine variants enabling 'clean' mass
 17 spectra, i.e. that do not include the main variant (Figure 1F). Especially in Figure 1C

1 for the mAb variant with two lysines, the importance of separation becomes apparent
2 as a variant with a similar mass to the two lysine variant was detected below the
3 main peak. This will be discussed in more details later. The measured correlations
4 match the expected theoretical results. However, the high resolution of DEAED-PSS
5 coating goes along with a longer separation time (75 min) compared to both
6 PDADMAC-PSS coating (30 min) and PEO (45 min – numbers for 4 M HAc as BGE,
7 10 kV, and a 60 cm long capillary).

8

9 **3.2 Background electrolyte**

10 In order to find the most suitable acidic BGE for the separation of antibody charge
11 variants on the DEAED-PSS coating different concentrations of formic acid and
12 acetic acid were used (see Supplement Information SI1). In general, a better
13 separation could be achieved when using HAc instead of FA, which is in agreement
14 with previous reports [28, 30]. Increasing the acetic acid concentration from 0.5 M
15 HAc up to 4 M HAc leads to increasing resolution. When further increasing the acetic
16 acid concentration to 8 M HAc the resolution decreases again, which might not be
17 expected based on the decreasing EOF and antibody mobility (see Supplement
18 Information SI2). One explanation could be solvation effects, since 4 M and 8 M HAc
19 correspond to 23% and 46% HAc solutions, respectively, with lower auto dissociation
20 (lower current) for the 8 M HAc.

21 The addition of small amounts (10%) of IPA or MeOH to the BGE is possible while
22 only marginally decreasing the resolution. If the same amount of ACN is added to the
23 BGE, the coating becomes irreversibly damaged with increasing migration times for
24 each subsequent run.

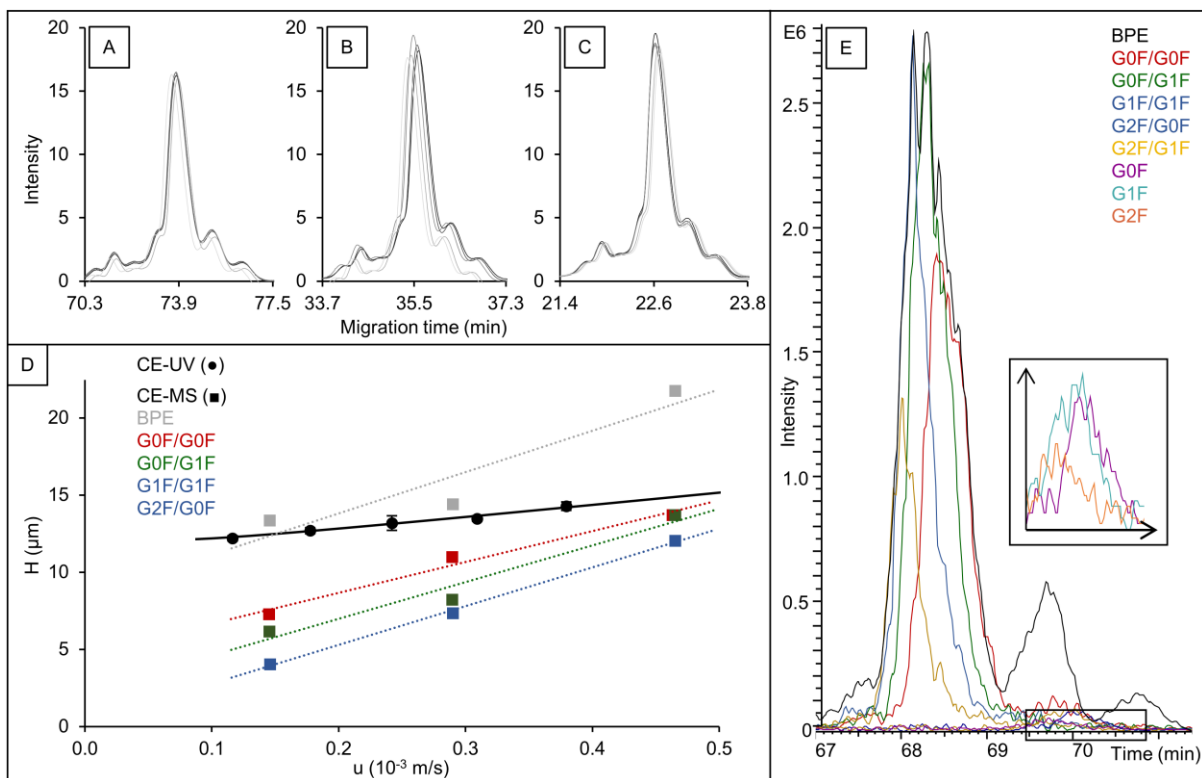
1 Due to the EOF, the separation in cationic (SMIL) systems is less influenced by the
 2 SL in the outlet vial compared to neutral systems. Only cations with a high mobility
 3 can migrate into the capillary from the interface side, therefore here, the nature of the
 4 anion is not critical. Indeed, when exchanging the outlet vial for SL during CE-UV
 5 measurements, no effect on the separation efficiency could be observed (data not
 6 shown). Formic acid was chosen as SL due to its high ionization efficiency.

7

8 3.3 Repeatability and separation voltage

9 To determine the repeatability of the DEAED-PSS coating the same mAb sample
 10 was measured 5 times at five different voltages using CE-UV. In Figures 2A, B, and
 11 C the overlays for -10 kV, -20 kV, and -30 kV are shown.

12



13

14 Figure 2: Influence of the applied separation voltage on CE-UV separation.

15 Electrophoretic separation of the USP mAb003 in 4 M HAc at different voltages (A-C)

1 (overlaid, $n=5$) with a hydrodynamic injection of 1 mg/mL mAb in BGE (other
2 parameters as in Figure 1) A) -10 kV, B) -20 kV, C) -30 kV, D) H vs. u curve for UV-
3 data (experimental points at voltage -10 kV, -15 kV, -20 kV, -25 kV, and -30 kV, $n=5$
4 with a model curve based on equation 2 fitted through the five points) and MS data
5 (experimental points at voltage -10 kV, -20 kV, and -30 kV, $n=2$ with a linear trendline
6 fitted through the three points) (BPE and three EIEs), and E) EIEs of the different
7 glycoforms constructed using the signal for their most intense charge state (± 0.3 Da,
8 Gaussian 7)

9

10 In CE-UV relative standard deviations for the migration times between 0.1% and
11 0.3% were achieved when the BGE was exchanged after every second
12 measurement. For the different voltages, migration times ranging between 25 min for
13 -30 kV and 80 min for -10 kV were measured. When comparing the separation at -
14 10 kV and -30 kV, a slightly better separation can be achieved for the lower voltages
15 with a small shoulder being resolved in front of the main mAb for -10 kV. This is in
16 agreement with recent studies where lower voltages resulted in higher separation
17 efficiencies for small proteins [31]. Similarly, lower H values are reached for lower
18 voltages (10 kV – 12.2 μm , 30 kV – 14.3 μm) as seen in Figure 2D. Plate height H
19 was plotted against solute velocity u using previously described protocols [23, 32,
20 33]. To model the experimental points the following equation was used [33]:

$$21 \quad H = A + \frac{B}{u} + pu \quad (2)$$

22 where A is the constant term (here: $A = 10.97 \mu\text{m} \pm 0.14 \mu\text{m}$), $\frac{B}{u}$ is the term for the
23 axial diffusion (here: $B = 2D = 2 \times 1.81 \times 10^{-11} \text{ m}^2/\text{s}$, D was determined by Taylor
24 dispersion analysis) and pu is the mass transfer term (here: $p (10^{-3} \text{ s}) = 8.25 \pm 0.37$).
25 When comparing the H values for -10 kV for the separation of the USP mAb003 with

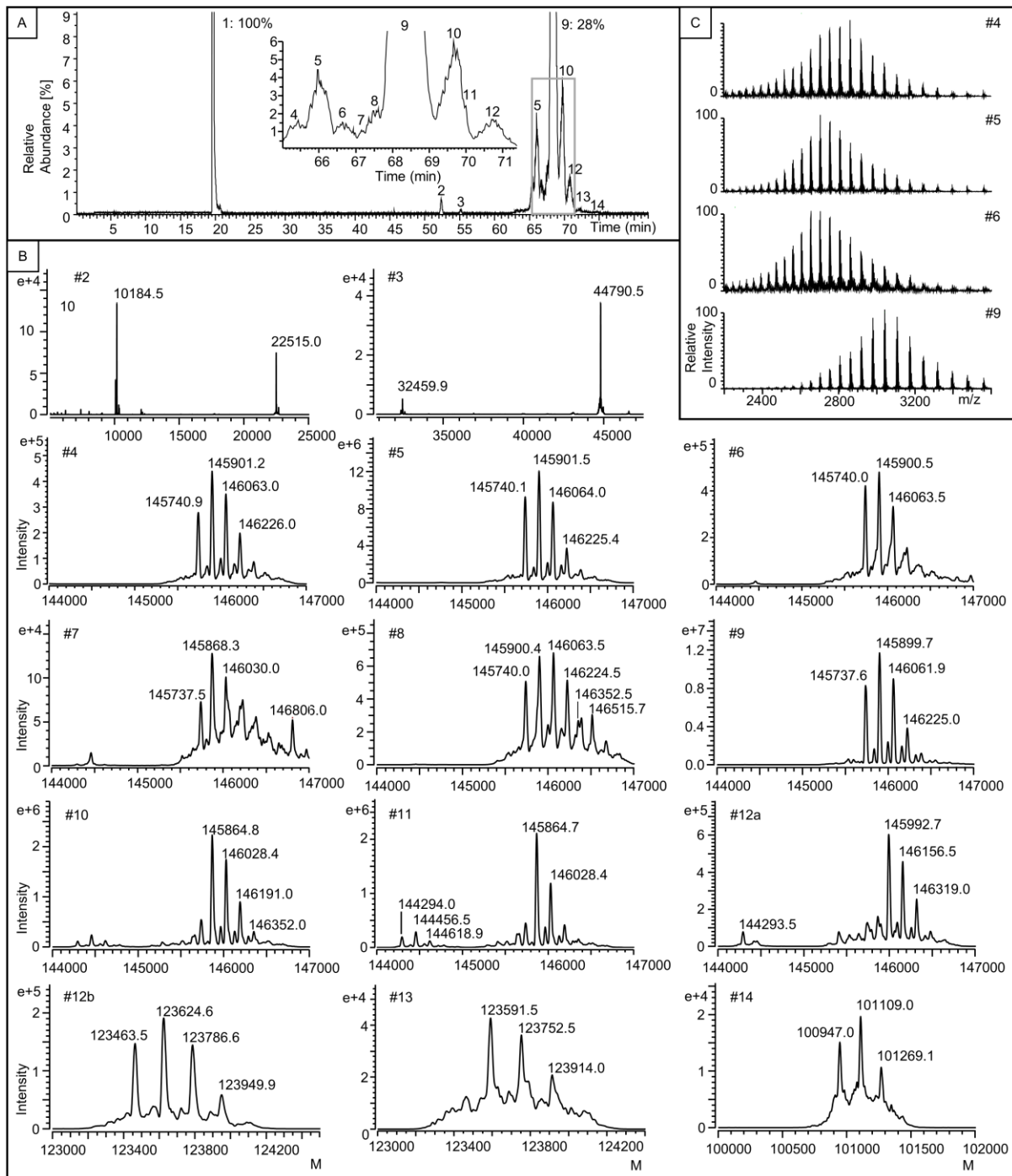
1 a 4 M HAc BGE of the DEAED-PSS coating ($H = 12.2 \mu\text{m}$) to the PDADMAC-PSS
2 coating ($H = 12.3 \mu\text{m}$, data not shown) similar values can be achieved meaning that
3 both coatings have separation efficiencies in a similar range. In Figure 2D, in
4 addition to the CE-UV data, the corresponding CE-MS values are shown for the
5 same capillary length, coating, and BGE. For the BPE, the achieved H values are
6 similar to the UV data, while when the H values for the EIEs of the three most
7 abundant glycoforms are plotted, the H value decreases from $13.4 \mu\text{m}$ (BPE, -10 kV)
8 to $4\text{-}7 \mu\text{m}$ (different glycoforms, -10 kV). In Figure 2E, the EIEs of the glycoforms of
9 the main mAb form are shown, clearly demonstrating narrow and partially separated
10 peaks. This partial separation of heterogeneous samples is not detectable in CE-UV
11 implying there is peak broadening rather than heterogeneity which is disproven by
12 the EIEs of CE-MS.

13 The value of the slope p is controlled by analyte adsorption and charge
14 inhomogeneity induced electroosmotic fluctuations [33]. As seen in Figure 2D the
15 slope increases when using MS detection compared to UV detection, presumably
16 influenced by additional peak broadening induced by the CE-MS interface. However,
17 even the CE-UV slope for the mAb separation is higher ($8.25 \times 10^{-3} \text{ s}$) than what can
18 be typically observed for smaller proteins on a PDADMAC coating ($1.92 \times 10^{-3} \text{ s}$)
19 [33]. The discrepancy between the slopes can be, at least partially, explained by
20 differences in protein diffusion coefficient, and could be also explained by an
21 increase of protein adsorption for larger molecules like the mAb or caused by
22 difference in coating qualities between the PDADMAC-PSS and DEAED-PSS
23 coating.

24

25 **3.4 Characterization of charge variants for USP mAb**

1 To show the separation potential of the developed method, an in-depth
2 characterization of USP mAb003 was performed. In Figure 3 an overview of the
3 separation and the corresponding mass spectra are shown for one data set.
4 Furthermore, all detected species as the mean of five measurements are
5 summarized in Supplement Information SI3.
6



2

3 Figure 3: Overview of the separation and mass spectra for the USP mAb003 at -
 4 10 kV separation voltage, 4 M HAc BGE for the DEAE-D-PSS coating (other
 5 parameters as in Figure 1), including A) the BPE, B) deconvoluted mass spectra of
 6 the peaks annotated in the BPE (intensity (counts) vs mass (Da)), and C) charge
 7 envelope of selected species (relative intensity (%) vs m/z)

1
2 Most analytes can be found between 65 min and 72 min, with the main peak
3 between 67.8 min and 68.8 min (#9). The four most abundant glycoforms of the main
4 form of USP mAb003 are 145737.8 Da \pm 1.1 Da (G0F/G0F), 145899.2 Da \pm 0.4 Da
5 (G0F/G1F), 146062.1 Da \pm 1.0 Da (G1F/G1F or G0F/G2F) and 146225.1 Da \pm
6 0.5 Da (G1F/G2F) (numbers given as mean, cp. Supplement Information S13). A
7 slight separation of these non-charged glycoforms is observed as shown in Figure
8 2E. Similarly, the three monoglycosylated mAbs arrive later (#11) and are baseline
9 separated from the main form. The ability of CZE-MS to separate monoglycosylated
10 mAbs has been shown previously [34].

11 The lysine variants described above are the main components of peak 10 (+1 K,
12 +128 Da) and peak 12 (+2 K, + 256 Da) (Figure 3B). Here, the glycosylation pattern
13 of the lysine variants is different from that of the main form. For the main variant,
14 G0F/G1F is the most abundant variant (#9), while for both lysine variants G0F/G0F
15 is more abundant (#10 and #12). While the reason for this difference remains
16 unknown, this observation underlines the usefulness of analyzing the mAb at the
17 intact level, since such an effect cannot be detected at the peptide level.

18 As part of the main peak (#9), an unknown variant with a mass difference of about
19 +99 Da can be observed. Adding 162 Da (+1 Gal) to +99 Da results in 261 Da which
20 differs only 5 Da from the mass of 256 Da (+2 K), a mass difference that cannot be
21 distinguished by the MS at intact mAb level. Thus, the separation of lysine variants is
22 necessary to detect these proteoforms.

23 Variants before the main peak should have fewer positive charges and are generally
24 considered 'acidic variants'. However, since CZE separates according to differences
25 in charge-to-size, size effects might occur. In the mass spectra of #4-6, a shift of the

1 charge envelope to smaller m/z ratios is observed. While the main peak (#9)
2 generally has the most intense signal at $z=48$, the 'acidic variant' (#5) has the most
3 intense signal at $z=52$. Using the three most abundant glycoforms, a mass difference
4 of $+2.1 \text{ Da} \pm 0.9 \text{ Da}$ ($n=5$) can be calculated between the main form and the most
5 intense 'acidic form' (#5). This shift in mass, the higher charge density, and the shift
6 in the direction of larger analytes may be explained by one open disulfide bridge.
7 Unpaired cysteines in IgG1 mAbs have been identified by RPLC-MS before, where a
8 similar shift to higher charges in MS was observed for the variant with unpaired
9 cysteines compared to the main variant [35]. Similar observations, i.e. CE-MS
10 characterization of variants that showed both a shift to lower mobilities and higher
11 charge state in the ESI-MS, have been made previously, however, no reliable
12 deconvolution could be obtained in this case [34]. Deamidation also causes a slight
13 mass shift (+1 Da) and would appear as an acidic variant, however, is not expected
14 to cause a shift of the charge envelope toward 6 additional charges. The three acidic
15 variants #4, #5, and #6 cannot be further distinguished by their mass as all three
16 show a similar shift around +2 Da. All charge envelopes are shifted to a higher
17 number of charges, which is slightly more pronounced for peak #5 and #6 compared
18 to #4 (Figure 3C). Therefore, it is likely that different disulfide bridges are reduced in
19 peaks #4, #5, and #6. In mass spectrum #7, even a variant containing both +1 K and
20 one reduced disulfide bridge can be found, migrating close to the sialic acid (SA)
21 variant (#8). Again, it would not be possible to distinguish between these two
22 variants by mass alone, since the mass of 2 Gal (+324 Da) + 1 SA (+291 Da) is
23 similar to the mass of 3 Gal (+486) + 1 Lys (+128 Da). Again, a possible annotation
24 was done based on differences in their charge envelope. In addition to intact
25 variants, some fragments were found. In peaks 2 and 3 small fragments with masses

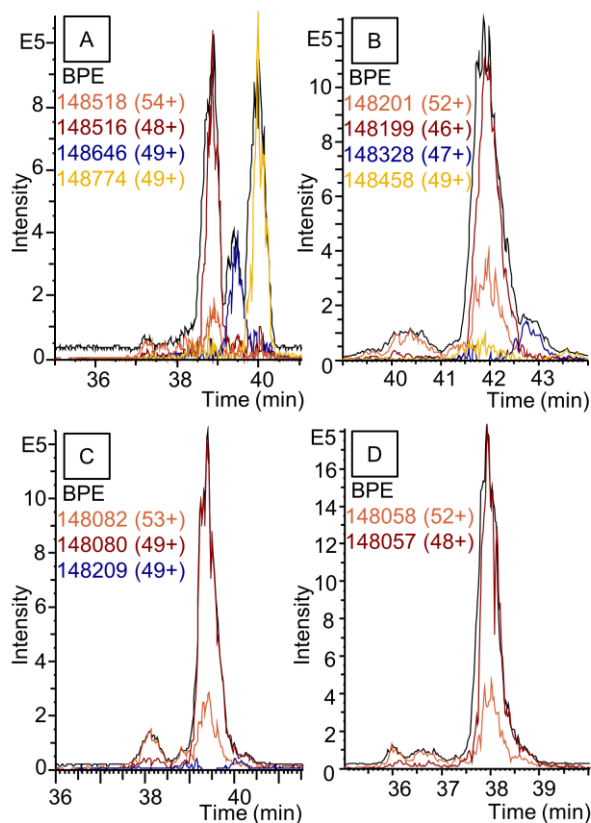
1 between 10 kDa and 50 kDa are present. Behind the main peak migrate, some low-
2 abundance species in the 123 kDa range (#12b and #13) and the 101 kDa range
3 (#14). Here, masses were found that follow the typical difference in glycosylation
4 (+162 Da). Furthermore, the respective lysine variant can be detected (#13;
5 123590.6 Da with a mass difference of about +128 Da compared to #12b;
6 123461.9 Da). Thus, these 'basic' variants can be attributed to fragments including
7 the HC.

8 Overall, about 52 proteoforms are detected with the CZE-MS approach presented
9 here, mainly due to the achieved separation of charge and size variants. The
10 combination of accurate mass, charge distribution, and separation allows a tentative
11 attribution to certain charge and size variants. For further confirmation either a 2D
12 approach using peptide analysis of previously separated intact charge variants [36]
13 or a top-down approach of the intact mAb could be performed [37].

14

15 **3.5 Application of the method to other mAbs**

16 To show the applicability of the method, further mAbs including infliximab, NISTmAb,
17 adalimumab, and trastuzumab were measured. Here, the separation voltage of -
18 20 kV as a compromise between separation efficiency and separation time was
19 chosen. The achieved separation can be found in Figure 4 while a table including the
20 detected variants is in the Supplement Information (SI4-SI7).



1

2 Figure 4: Separation of variants of A) infliximab, B) NISTmAb, C) adalimumab, and
 3 D) trastuzumab at -20 kV using the DEAED-PSS coating with the 4 M HAc BGE
 4 (other parameters as in Figure 1), showing the BPE (black) and the EIE (± 0.3 Da) of
 5 the partial reduced main variant (orange), main variant (red), 1 K variant (blue), 2 K
 6 variant (yellow). Glycoforms G0F/G0F (NIST G0F/G1F) were used for the given
 7 charge state along with the respect deconvoluted mass.

8

9 Lysine variants are detected for three mAbs, agreeing with the literature, where
 10 lysine variants have been described for infliximab [13,17], adalimumab [38] and the
 11 NISTmAb [19, 39]. Using the DEAED-based coating, the lysine variants could be
 12 separated from the main variant in all cases. For trastuzumab (Figure 5D), another
 13 (low-abundance) basic variant is detected with a mass difference of 13.6 Da from the

1 main variant (using the three most intense glycoforms, $n=3$). Again, this mass
2 difference is too small to be resolved by MS, therefore, separation is essential.
3 Similar to the USP mAb003, for all four mAbs some partial separation for the
4 glycovariants could be observed, with G0F/G0F being the most abundant form for
5 adalimumab, trastuzumab and infliximab and G0F/G1F being the most abundant
6 form for the NISTmAb. In the case of the NISTmAb, the glycosylation is especially
7 complex, and after G2F/G2F another form with a mass difference of +162 Da is
8 observed. This could be an additional glycation agreeing with the observation of
9 Chen et al. who observed a mass of mAb+Hex after the removal of the N-glycans by
10 PNGaseF for the NIST mAb [19], although separation might be expected in the case
11 of some glycation due to the removal of a basic amino acid function.
12 Monoglycosylated variants can be annotated for three of the four mAbs (not for
13 infliximab), all of which have higher migration times. Furthermore, for all four mAbs a
14 separation of at least one 'acidic' variant can be achieved. Similarly, to the 'acidic'
15 variants found for the USP mAb003, those forms have a mass of approximately 2 Da
16 higher and are shifted to higher charge states in the ESI, again, most likely attributed
17 to partly reduced mAb variants. For trastuzumab, two separated 'acidic' peaks were
18 detected that both have the characteristics of partially reduced mAbs, indicating
19 positional isomers. Due to the high abundance of the two lysine variants, it is
20 possible to identify partially reduced forms of the lysine variants for infliximab.

21

22 **3.6 Comparison with other CZE-MS approaches**

23 A clear improvement could be reached when applying the DEAED-PSS coating for
24 CZE-MS in comparison with other coatings such as the two discussed in this paper
25 (PEO and PDADMAC-PSS) or for the commercially available neutral coating

1 (Neutral OptiMS) where the lysine variants could be only separated partially from
2 each other and the main variant [15]. The separation of lysine variants achieved here
3 for the DEAED-PSS coating is similar in quality to the one published for ME-MS
4 when using an acidic BGE for infliximab [17], however slightly less efficient than the
5 one published for NIST mAb [19]. The CZE-MS approach demonstrated here,
6 however, can be performed with a standard CZE system by using a simple coating
7 protocol, which is flexible in regards to the length and inner diameter of the capillary.
8 Further improvement of charge variant separation should be expected when shifting
9 the separation from an acidic system to a higher pH value. As previously
10 investigated for the EACA system, a pH close to the pI value of the mAbs allows for
11 a better separation due to a higher difference in the mobility of the charge variants
12 [5]. Similarly, native separation enables improved charge variant separation, as
13 shown for ME-MS [20]. However, applying an acidic BGE, the detected m/z range is
14 typically between m/z 2000 and 4000, facilitating mass spectrometry on various
15 instruments. Furthermore, an acidic BGE does not add cations like ammonium which
16 can lead to undesired adduct formations. Additionally, no separation of size variants
17 such as monoglycosylated antibodies is reported for native BGEs to our best
18 knowledge; indeed, it was reported that monoglycosylated antibodies comigrate with
19 the main forms of the mAb for a native separation applying a neutral coating [40].

20

21 **4 Conclusions**

22 In this work, a new SMIL coating is introduced for the separation of intact mAb
23 variants by CZE-MS under acidic conditions. Due to the denaturing conditions, it is
24 more difficult to separate charge variants, however, it allows the application of
25 standard MS. The cationic properties of the DEAED-PSS coating allow for an

1 efficient reduction of protein adsorption leading to high separation efficiencies similar
2 to other coatings like the PDADMAC-PSS coating. Importantly, the low EOF, being
3 only slightly higher than the mobility of the mAb, allows for the separation of closely
4 related mAb variants. Charge variants like lysine and sialic acid variants are
5 separated. Moreover, the approach enables the separation of size variants like the
6 partial separation of the different glycoforms, the complete separation of the
7 monoglycosylated mAbs as well as the separation of variants differing in the
8 reduction state of one disulfide bridge. The method shows similar performance for
9 various mAbs including the two model mAbs USP mAb003 and NISTmAb as well as
10 infliximab, trastuzumab, and adalimumab. Overall, the here presented CZE-MS
11 method using the new 5-layer DEAED-PSS SMIL coating is an excellent tool for the
12 characterization of mAb variants on the intact level, supporting the development of
13 new protein-based pharmaceuticals.

14

15

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20 **Conflict of interest**

21 The authors have declared no conflict of interest.

22

23 **Data Availability Statement**

1 The data that support the findings of this study are available from the corresponding
2 author upon reasonable request.

3

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