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Research Article

High-resolution separation of monoclonal antibodies mixtures and their charge variants by an alternative and generic CZE method

The determination of mAb critical quality attributes (CQA) is crucial for their successful application in health diseases. A generic CZE method was developed for the high-resolution separation of various mAb charge variants, which are often recognized as important CQA. A dynamic coating of the capillary was obtained with polyethylene oxide (PEO), whereas Bis-Tris allowed the analysis of mAbs under native conditions at pH 7.0. The effect of PEO and Bis-Tris concentrations, as well as the nature of the acidic counter ion on the method performance was systematically studied. The %RSD on migration times was below 5% on three different CE instruments using the optimized method. Additional charge variants (in particular acidic variants) were resolved for 10 out of 17 mAbs compared to a reference CZE approach involving the use of ε-amino-caproic acid (EACA), triethylenetetramine (TETA), and hydroxypropylmethyl cellulose (HPMC). The amount of basic and acidic charge variants of 17 Food and Drug Administration (FDA) approved mAbs covering a wide range of physico-chemical properties, e.g., p*I* between 8.0 and 9.4 and different hydrophobicity, were mainly comprised between 5–15% and 15–30%, respectively. It is noteworthy that applications for the quality control in hospitals as well as for the combination of the immune checkpoint inhibitors nivolumab and ipilimumab were presented.

Keywords:
Capillary-zone electrophoresis / Charge variants / Monoclonal antibodies
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Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

The idea of harnessing the immune system to cancer has recently experienced significant successes with the first approval of T-cell-targeted immunomodulatory mAbs by the Food and Drug Administration (FDA), namely ipilimumab (targeted against CTLA-4) in 2011, nivolumab and pembrolizumab (targeted against PD-1) in 2014, and anti-PD-L1 mAbs such as atezolizumab in 2016 [1]. B-cell targeted therapies have also demonstrated substantial benefits for patient with different types of B-cell lymphomas and autoimmune disorders, with the approval of rituximab (targeted against CD-20) in 1997 by the FDA, and second generation of anti-CD20 mAbs (e.g. ofatumumab and obinituzumab) which were shown to be more effective and better tolerated [2]. Besides immunotherapy, approval rates of antibody-based products have accelerated in 2017 with ten antibodies granted for the first time in a year in either the European Union or United States [3].

According to the International Conference on Harmonization (ICH) guideline Q6B, a set of physico-chemical properties and isoform patterns have to be determined for new biological products to support their marketing applications [4]. Based on the ability of a variant to impact product efficacy, patient safety and its likelihood, high molecular weight aggregates and the isomerization of aspartic acid to iso-aspartic acid

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Abbreviations: CEX, cation exchange chromatography; **CQA**, critical quality attribute; **EACA**, ε-amino-caproic acid; **EMA**, European Medicines Agencies; **FDA**, Food and Drug Administration; **HIC**, Hydrophobic Interaction Chromatography; **HPMC**, hydroxypropylmethyl cellulose; **ICH**, International Conference on Harmonization; **MS**, mass spectrometry; **PEO**, polyethylene oxide; **TETA**, triethylenetetramine

Color Online: See the article online to view Figs. 1–4 in color.

(iso-Asp) were identified as the most important critical quality attribute (CQA), by a product quality attribute risk assessment study [5]. Size exclusion chromatography, gel electrophoresis and analytical ultracentrifugation are the reference methods for the determination of size variants, while IEF, CIEF, IEC and CZE are the main analytical method for the characterization of charge variants.

The most important antibody basic charge variants are iso-Asp modification, C-terminal Lys truncation, aglycosylation, incomplete cyclization of the N-terminal glutamine (Gln) to pyroGlu or methionine oxidation; whereas sialylation, asparagine or glutamine deamidation, glycation, cysteinylation are the most commonly observed acidic variants [5, 6]. Two-dimensional analytical approaches have been proposed both in LC (IEC x RPLC) [7] and CE (CZE x CZE [8], CIEF x CZE [9]) to improve the resolving power of these separation techniques and resolve a higher number of charge variants. In parallel, increasing efforts have been made to directly hyphenate IEC [10], CZE [11–13] and recently iCIEF [14] to mass spectrometry (MS), to quickly identify charge variants at the mAb intact level. However, compromises on the separation performance are often made when directly coupling the LC and CE methods to MS, which explain why CZE-UV methods are still being developed and applied in the biopharmaceutical industry [15, 16].

In CZE, very high peak efficiencies can be obtained as only the longitudinal diffusion contributes to the band broadening. The separation is based on the different charge to hydrodynamic radius of the proteins, resulting in different migration velocities. As shown in a recent review [17], evaluation of charge heterogeneity of mAb products by CZE generally involves a BGE containing around 400 mM ε-amino-caproic acid (EACA), 2 mM of triethylenetetramine (TETA), 0.05% of hydroxypropylmethyl cellulose (HPMC) and the pH is adjusted between 5.5 and 6.0.

The EOF and protein adsorption can be limited with (i) the use of static capillary wall coatings [18] or more generally with (ii) a dynamic coating [15, 16]. Most of the CZE methods listed in the review of Gahoual *et al.* involved the use of HPMC (typical amounts of 0.05%) as dynamic coating [17] while polyethylene oxide (PEO, 0.3%, MW = 600 000 Da) was only selected by Shi *et al.* [19].

This study aimed at developing an alternative CZE method for high-resolution separation of basic mAbs variants. In a first instance, critical CZE method parameters were determined, and then the effect of the buffer and polymer concentrations as well as the separation voltage was systematically studied. The performance of the developed method was compared to a reference CZE method involving the combination of common BGE components (EACA, TETA and HPMC) [16] using 17 FDA- and European Medicine Agencies (EMA)-approved mAbs. The methodology was tested for the separation of mAb mixtures and their charge variants, in particular for a combination therapy approved by the FDA as well as for the quality control in hospitals.

2 Materials and methods

2.1 Chemicals and samples

LC-MS grade water (cat. W/0112/17) was purchased from Fisher Scientific (Reinach, Switzerland). Bis-Tris (cat. B4429), concentrated hydrochloric acid (cat. H1758), EACA (cat. A2504), HPMC (cat. H7509), MOPS (cat. 69947), PEO (cat. 181986, average MW 100 000), propionic acid (cat. 402907), TETA (cat. 90460) were obtained from Sigma-Aldrich (Buchs, Switzerland). LC-MS grade acetic acid glacial (cat. 010741) was purchased from Biosolve (Valkenswaald, The Netherlands). The washing solutions of 0.1 M hydrochloric acid and 0.1 M sodium hydroxide were from SCIEX (Brea, CA, USA).

FDA and EMA-approved adalimumab atezolizumab, belimumab, bevacizumab, cetuximab, denosumab, elotuzumab, ipilimumab, ixekizumab, nivolumab, obinituzumab, ofatumumab, palivizumab, pertuzumab, ramucirumab, rituximab and trastuzumab were obtained as European Union pharmaceutical-grade drug product from their respective manufacturers. Expired bevacizumab (batch #B2009, exp. May 2010,) and rituximab (batch #B2040, exp. January 2009) flasks never opened prior to this study and trastuzumab flask already opened (batch #B1605, exp. September 2014) used during method development were kindly provided by the pharmacy of the Geneva University Hospitals (Geneva, Switzerland). The p*I* values, concentration and formulation components of the mAb products are listed in Supporting Information Table 1. All the mAb products were diluted to 1.0 mg/mL with water to a 100 μL total volume in 200 μL polypropylene vial.

All buffers were prepared by dissolving Bis-Tris in LC-grade water prior to pH adjustment. Polyethylene oxide was added under stirring and the stirring was let for 6 h. The separation gel was stored at room temperature, protected from light and used within 3 days. However, the BGE may be stored for a longer period of time upon further evaluation and in particular for the application of the method in routine analysis. The final BGE contains 200 mM Bis-Tris at pH 7.0 adjusted with acetic acid and 1.0% PEO (current of 33 μA at + 15 kV separation voltage).

2.2 CE-UV instrumentation

CE experiments were performed with three different HP^{3D}CE systems (Agilent, Waldbronn, Germany) equipped with a diode array detector, an autosampler, and a power supply able to deliver up to 30 kV. Separations were performed with a 32.5 cm total length and 24 cm effective length fused silica polyimide coated TSP capillary tubing (cat. TSP050375, 50 μm ID, 363 μm OD) purchased from Polymicro Technologies (Phoenix, AZ USA). New capillaries were pretreated with 0.1 M hydrochloric acid at 1 bar for 20 min and the BGE at 1 bar for 40 min, followed by the application of +15 kV for 10 min with 5 min ramp voltage. Prior to each sample injection, the capillary was rinsed 5 min at 1 bar with 0.1 M

HCl, then 10 min at 1 bar with the BGE. Samples were hydrodynamically injected for 10 s at 30 mbar. Polarity was positive (capillary inlet) to negative (capillary outlet) and the separation voltage was set at +15 kV. The capillary temperature was kept at 25°C, and UV detection was performed at 200 nm with bandwidth of 5 nm and response time of 1.3 s. Better sensitivity was achieved with the less selective 200 nm UV wavelength with the HP^{3D}CE systems compared to the 214 nm one commonly selected with the Beckman Coulter Pharmaceutical Analysis (PA) system equipped with UV detector and 214 nm filter. Same conditions were applied when using the approach from He *et al.* [16], except the separation voltage, which was set at +30 kV. The buffer pH was measured using a SevenMulti S40 pH meter (Mettler Toledo, Greifensee, Switzerland). Instrument control, data acquisition were performed with ChemStation B.04.03-SP2 [105] software (Agilent). Data transferring was achieved by using Excel (Microsoft).

3 Results and discussion

To the best of our knowledge, very few trials have been made with a combination of PEO and Bis-Tris in CE, as only one application could be found for oligonucleotide analysis [20]. Bis-Tris has a very low conductivity and a pKa of 6.5 allowing CZE analysis under non-denaturing conditions for mAbs. A first BGE was prepared with 150 mM Bis-Tris in water at pH 6.5 (adjusted with hydrochloric acid) and addition of 3% (w/v) of PEO (MW 100 000). Encouraging results were obtained with trastuzumab for which several peaks were resolved, but bevacizumab and rituximab were strongly adsorbed (Supporting Information Fig. 1). The potential of such approach for the determination of charge variants was investigated and the method development was performed in two steps (i) evaluation of the critical parameters to optimize, and (ii) fine tuning of the separation conditions.

3.1 Determination and tuning of method critical parameters

The most critical parameters that were identified before achieving suitable CZE performance were (i) the pH of the BGE, (ii) the nature of the acid used to adjust the pH of the Bis-Tris buffer and (iii) the nature of the capillary wash (acidic *vs.* basic). Bis-Tris (pK_a 6.5 at 25°C) has a useful pH range of 5.8 to 7.2; therefore, the pH of the BGE was varied from 6.0 to 7.0 by steps of 0.5 unit. However, acidic pH of 6.0 led PEO to readily flocculate within two days. Such degradation in acidic conditions would modify the solution viscosity, which is likely to affect the repeatability of migration times in CZE. Once the pH was set at 7.0 to limit as much as possible the risk of PEO degradation, preliminary trials to increase the voltage from 10 to 30 kV by steps of 5 kV showed a noticeable increase of current during the CZE analysis from 20 kV, together with an unexpected loss of sensitivity. In addition, random loss of separation performance was observed after five to ten injections. Thus, the voltage was set at 15 kV. The replacement of hydrochloric acid by acetic acid resulted in a current decrease from 35 to 25 μA at 15 kV, better overall performance and above all better stability between injections. Finally, significant improvements on both sensitivity and separation performance were achieved by removing the basic conditioning and preconditioning steps, while solely keeping an acidic wash prior conditioning with the BGE. The unusual acidic wash of the capillary avoid the hydrolysis of the siloxane groups of the untreated silica [21], which significantly improve the method performance with rituximab in particular. Indeed this basic mAb (pI 9.4) was hardly distinguishable from the UV baseline due to probable adsorption to the residual silanolates formed by the basic wash (Supporting Information Fig. 1).

3.2 Evaluation of the BGE components effect on method performance

The effect of PEO and buffer concentrations, the nature of the acidic counter-ion, and the separation voltage were systematically studied using trastuzumab and rituximab. These two mAbs have been widely studied by CZE [17] and can indeed be considered as reference materials for CZE method development. Trastuzumab has a characteristic charge profile with the presence of an intense acidic peak identified as a deamidated variant by Harris *et al.* [22], while rituximab is a strongly basic mAb (pI 9.4) and can therefore be considered as challenging regarding possible electrostatic interactions.

Linear polymers such as PEO or HPMC form a dynamic coating at the surface of the capillary wall in CZE [17] that limits the interactions between the proteins or the BGE cations with the capillary inner surface and drastically reduces the EOF. The effect of PEO on method performance was investigated for rituximab (Fig. 1A) and trastuzumab (Fig. 1B) with concentrations of 0.25, 0.50, 1.0, 2.0 and 3.0%. As expected, the BGE viscosity had a strong effect on the efficacy of the hydrodynamic injection and therefore on peak intensity. Such detrimental effect was more pronounced at PEO concentrations higher than 1% (>50% loss at 3% PEO versus 1% PEO), but less significant for PEO concentrations below 1% (< 25% loss at 1% PEO versus 0.25% PEO). Similar conclusions were drawn on migration times, which decreased from 29.7, 21.3 and 18.9 min for trastuzumab, when the PEO concentration was reduced from 3, 1 and 0.25%, respectively. The BGE viscosity also affected the resolution between protein charge variants. Indeed, lower resolutions were observed for rituximab acidic variants at 0.25 and 0.5% PEO versus 1.0% PEO and higher amounts of the polymer (Fig. 1). In addition, random current drops occurred during separations at 0.25 and 0.5% PEO potentially due to PEO instability at low concentrations under these conditions, which were not observed at 1% PEO. For all these reasons, the better compromise was achieved by reducing the initial PEO concentration from 3.0 to 1.0%.

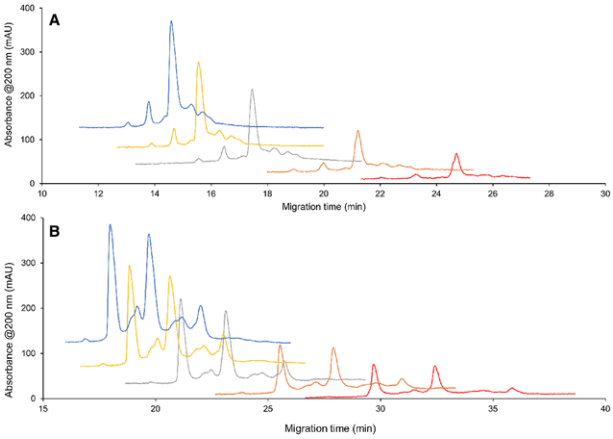

Figure 1. Effects of PEO concentration on the separation of rituximab (A) and trastuzumab (B) charge variants. The polymer concentration was set at 0.25, 0.5, 1.0, 2.0 and 3.0% (blue, yellow, grey, orange and red traces, respectively). The BGE contained 200 mM Bis-Tris, and the pH was adjusted to 7.0 with acetic acid.

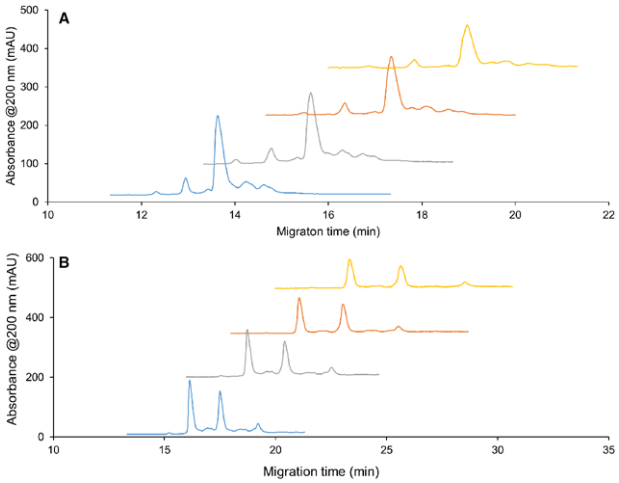

Figure 2. Effects of Bis-Tris concentration on the separation of rituximab (A) and trastuzumab (B) charge variants. The buffer concentration was set at 100, 150, 200 and 250 mM (blue, grey, orange and yellow traces, respectively). The BGE contained 1.0% PEO, and was adjusted to 7.0 with acetic acid.

The effect of pH adjustment on CZE performance was further investigated with propionic acid and MOPS, which have lower conductivity and higher pK_a values of 4.9 and 7.2 compared to acetic acid (pK_a of 4.8). The current of the BGE prepared with 200 mM Bis-Tris and buffered at pH 7.0 decreased from 44, 33, 30 and 20 μA at 15 kV with hydrochloric acid, acetic acid, propionic acid and MOPS, respectively. Slightly lower migration velocities were observed when the acid strength was reduced, probably due to higher BGE ionic strength (Supporting Information Fig. 2). The nature of the acidic counter-ion did not affect the charge variant separations, but the UV baseline was slightly drifted with MOPS and hydrochloric acid (Supporting Information Fig. 2). Acetic acid was finally kept, due to better availability compared to propionic acid.

Bis-Tris concentration was then increased from 100 to 250 mM by steps of 50 mM. Larger migration times were observed for both mAbs when increasing the BGE ionic strength but the peak intensity was also reduced due to peak broadening (Fig. 2A and B). The increase of buffer concentration from 200 mM had a noticeable effect on the number of resolved acidic variants for the most basic antibody (rituximab), but limited effect on trastuzumab. Therefore, the use of high ionic strength is likely to limit the electrostatic interactions between the most basic antibodies and the silanols. For this reason, the final buffer ionic strength was increased from 150 to 200 mM.

Finally, the effect of separation voltage on the CZE performance was evaluated from 10 to 30 kV by steps of 5 kV. The migration times were significantly reduced from 29.0 to 8.3 min for rituximab (Supporting Information Fig. 3A) and from 35.3 to 10.2 min for trastuzumab (Supporting Information Fig.e 3B), when increasing the separation voltage from 10 to 25 kV. Stable UV baseline and similar separations were obtained at 10 and 15 kV, whereas the UV baseline was not stable at high separation voltage (e.g., 20 and 25 kV) and the absence of peak at 30 kV was probably due to the Joule heating effect. The separation voltage was finally set at +15 kV to achieve an effective CZE separation within a suitable run time.

3.3 Comparison of the developed CZE method with a reference method

The performance of our CZE method was assessed on three different CE instruments from the same brand. Method precision was evaluated on both migration times and relative peak areas. Three replicates of each batch of the selected 17 mAbs were successively injected on two instruments (instruments 1 and 2), while each of the three replicates were done on three different days on the third CE instrument (instrument 3). The %RSD on migration times reported in Supporting Information Table 2 were always lower than 5% on each instrument as well as for all instruments together, demonstrated excellent precision (repeatability and intermediate precision). The %RSD of the main peak relative area was below 10% on all instruments, while only 5 and 7 mAbs had %RSD on acidic and basic variants higher than 15% (typically observed for small amounts of variants below 15%) (Table 1). The relative peak areas for the acidic variants, main peak and basic variants were mainly comprised in the ranges 15–30%, 60–75% and 5–15%, respectively (Table 1).

The relative amounts of basic and acidic variants obtained by CZE were plotted against those obtained by CIEF (Supporting Information Fig. 4A and 4B) and cation exchange chromatography (CEX) (Supporting Information Fig. 4C and 4D) from a previous study [23]. Interestingly, 15 mAbs had higher amounts of basic variants with CEX performed in the salt elution mode, whereas 16 mAbs had lower amounts of acidic variants. In CZE, both the charge and the protein

Table 1. Comparison of relative acidic, main and basic variants peak areas (expressed as percentages of the total peak area) measured on three CE instruments

	Instrument 1, $n = 3$			**Instrument 2, $n = 3$**			**Instrument 3, $n = 3$**			**Instruments 1,2 and 3**		
	% acidic peak area	% main peak area	% basic peak area	% acidic peak area	% main peak area	% basic peak area	% acidic peak area	% main peak area	% basic peak area	% acidic peak area	% main peak area	% basic peak area
Adalimumab	15,0 (8)	65,5 (3)	19,4 (15)	15,6 (2)	61,6 (1)	22,7 (2)	15,8 (7)	65,1 (1)	19,0 (7)	15,4 (6)	64,1 (3)	20,4 (12)
Atezolizumab	16,8 (7)	76,6 (2)	6,6 (7)	15,4 (9)	77,7 (2)	6,9 (9)	10,5 (15)	82,0 (2)	7,5 (5)	14,3 (22)	78,8 (4)	7,0 (8)
Belimumab	36,0 (1)	60,6 (1)	3,3 (15)	35,6 (10)	60,7 (7)	3,6 (43)	36,6 (3)	59,0 (2)	4,4 (4)	36,1 (5)	60,1 (4)	3,8 (25)
Bevacizumab	20,9 (14)	70,8 (6)	8,3 (19)	21,4 (18)	69,5 (5)	9,2 (5)	20,6 (8)	70,9 (2)	8,5 (12)	21,0 (12)	70,4 (4)	8,7 (12)
Cetuximab	47,0 (3)	31,5 (7)	21,4 (7)	46,8 (6)	31,9 (7)	21,3 (4)	44,5 (2)	33,0 (2)	22,4 (1)	46,1 (4)	32,1 (5)	21,7 (5)
Denosumab	16,5 (12)	76,0 (4)	7,4 (18)	18,4 (7)	72,1 (2)	9,4 (2)	13,7 (11)	75,6 (3)	10,7 (22)	16,2 (15)	74,6 (4)	9,2 (22)
Elotuzumab	4,6 (15)	53,7 (1)	41,5 (2)	1,7 (44)	54,8 (2)	43,4 (0)	2,7 (64)	52,5 (7)	44,7 (11)	3,0 (53)	53,7 (4)	43,2 (6)
Ipilimumab	27,9 (3)	67,4 (2)	4,0 (39)	34,3 (5)	61,4 (5)	4,7 (29)	32,9 (5)	61,4 (4)	5,7 (14)	31,7 (10)	63,4 (6)	4,8 (28)
Ixekizumab	4,3 (21)	70,1 (1)	25,0 (1)	4,0 (17)	70,2 (1)	25,8 (5)	3,7 (16)	71,7 (2)	24,6 (5)	4,0 (17)	70,7 (2)	25,1 (4)
Nivolumab	17,5 (8)	76,4 (3)	6,1 (16)	15,7 (25)	78,3 (5)	6,0 (6)	14,8 (15)	77,8 (2)	7,4 (7)	16,0 (17)	77,5 (3)	6,5 (14)
Obinutuzumab	11,0 (9)	81,5 (1)	7,5 (5)	16,3 (14)	75,6 (2)	8,1 (11)	11,7 (29)	79,1 (3)	9,2 (7)	13,0 (25)	78,7 (4)	8,2 (11)
Ofatumumab	22,1 (4)	61,8 (4)	16,1 (19)	26,3 (4)	57,8 (3)	15,8 (6)	23,0 (5)	59,7 (3)	17,3 (7)	23,8 (9)	59,8 (4)	16,4 (11)
Palivizumab	20,0 (8)	74,5 (2)	5,6 (3)	22,0 (1)	70,3 (2)	7,7 (15)	20,0 (8)	72,3 (1)	7,8 (28)	20,6 (7)	72,4 (3)	7,0 (23)
Pertuzumab	20,6 (6)	78,4 (2)	1,0 (36)	24,8 (2)	74,2 (0)	1,1 (35)	20,4 (4)	78,8 (1)	0,8 (18)	21,9 (10)	77,1 (3)	1,0 (30)
Ramucirumab	21,1 (6)	68,6 (2)	10,3 (6)	24,3 (3)	64,3 (1)	11,4 (3)	24,0 (9)	62,4 (2)	13,5 (15)	23,1 (9)	65,1 (5)	11,8 (15)
Rituximab	26,9 (6)	70,0 (3)	3,0 (16)	30,4 (5)	66,8 (3)	2,8 (16)	29,3 (2)	66,6 (2)	4,0 (31)	28,9 (7)	67,8 (3)	3,3 (28)
Trastuzumab	29,6 (8)	69,4 (3)	1,0 (26)	33,7 (4)	65,0 (2)	1,3 (28)	32,2 (6)	66,7 (3)	1,1 (14)	31,8 (8)	67,0 (4)	1,1 (24)
Min	4,3	31,5	1,0	1,7	31,9	1,1	2,7	33,0	0,8	3,0	32,1	1,0
Max	47,0	81,5	41,5	46,8	78,3	43,4	44,5	82,0	44,7	46,1	78,8	43,2
Mean	21,0	67,8	11,0	22,8	65,4	11,8	21,0	66,7	12,3	21,6	66,7	11,7
First quartile	16,5	65,5	4,0	15,7	61,4	4,7	13,7	61,4	5,7	15,4	63,4	4,8
Third quartile	26,9	76,0	16,1	30,4	72,1	15,8	29,3	75,6	17,3	28,9	74,6	16,4

Data are shown as mean of $n = 3$ and coefficient of variation (%) in brackets.

hydrodynamic radius affect the migration velocities, whereas only the accessible ionized amino groups interact with the stationary phase in CEX. The increase of basic variants in CEX versus CZE did not correlate to the decrease of acidic variants ($R^2 = 0.17$), therefore the results suggest a better separation of mAb basic variants from the main peak in CEX versus CZE, while the latter would better separate the acidic variants, proving the complementarity of the two modes of separation. Similarly, the charge variants obtained in CIEF were compared to those obtained in CZE. No trend was observed between both techniques regarding the percentage of basic variants ($R^2 = 0.30$). The acidic variants were systematically higher in CIEF, but the generic method might overestimated the amount of acidic variants as discussed in [23]. The choice of the data analysis software and integration parameters can also explain the differences in some cases [24] but unlikely those observed in the present CZE study versus [23] as the same operator manually integrated the peaks.

The developed CZE method was then compared to the well-established CZE approach from He *et al.* [16] involving the use of EACA, TETA and HPMC at pH = 5.7, with 17 representative FDA- and EMA-approved mAbs (Fig. 3 and Supporting Information Fig. 5). The in-house method was found to provide a higher number of resolved variants for several mAbs and in particular for the acidic variants of adalimumab, ipilimumab, nivolumab, obinituzumab, ofatumumab and trastuzumab, but the reference method was still beneficial for the separation of atezolizumab and pertuzumab basic variants. He *et al.* showed that the relative charge difference among charge variants greatly increased when the pH get closer to their model mAb pI [16]. An increase of BGE pH from 5.7 to 7.0 is expected to improve the separation of charge variants for basic mAbs (p$I > 8$) but also increases the risk of protein adsorption to the capillary wall. The successful separations obtained with the developed CZE method for the most basic mAbs, i.e., ipilimumab, palivizumab and rituximab [23], account for a better dynamic coating obtained with 1.0% PEO compared to 0.05% HPMC. Indeed, detrimental effects were observed by He *et al.* at pHs higher than 5.2 with a relatively low basic mAb (p$I = 7.3$) [16]. Similarly, HPMC was replaced by hydroxypropyl cellulose (HPC) to improve the separation of basic antibodies charge variants [15] and a nonionic surfactant (polysorbate 20) to avoid a significant loss of resolution occurring after few injections [24].

Interestingly a narrow peak migrating much earlier than ofatumumab and corresponding to arginine (Supporting Information Table 1, data not shown) was observed with the two CZE approaches, proving their value for the determination of some formulation components. At the end, interesting electrophoretic profiles were obtained with our in-house CZE method for ofatumumab with the presence of a

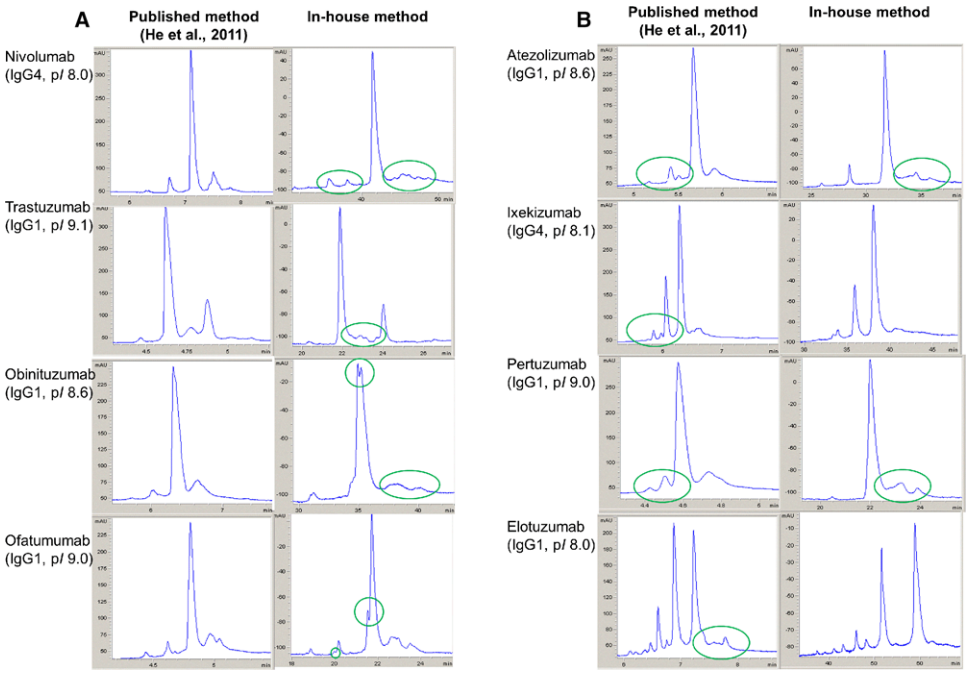

Figure 3. CZE profiles obtained by the final in-house method and according to the methodology of He *et al.* [16], in favourable cases (A) and unfavourable cases (B).

pre-main peak and obinituzumab for which a double peak was observed. Such unusual double peaks would require further investigations for their identification, as they have already been observed with a denaturing CZE method involving the use of high urea concentrations (>1 M), and identified as disulfide scrambling isoforms [25].

At the end, the CZE migration times obtained by both methods were plotted against the experimental mAb p*I* (Supporting Information Fig. 6). The determination coefficients (R^2) were equal to 0.83 with both methodologies, whereas they were equal to 0.94 and 0.93 when discarding three mAbs, namely cetuximab, elotuzumab and obinituzumab. The linear correlations proved the preponderant effect of the charge over the hydrodynamic radius on the migration velocities of selected mAbs in CZE. For the three mAbs that had longer migration times than expected, a lower increase of their net charge is suspected when decreasing the pH in comparison with other mAbs probably due to a lower amount of lysine and arginine residues.

3.4 Application to quality control in hospitals and other perspectives

Bevacizumab, trastuzumab and rituximab are three blockbusters widely used in hospitals and were among the ten molecules in 2017, which generated the highest revenues for the pharmaceutical industry with $ 7.1, 7.4 and 7.8 B worldwide sales [26]. Their compounding before administration to patients is executed in the production units of hospitals. To ensure patients safety, the confirmation of the mAb identity should be performed to prevent potential mistake at the compounding stage. Jaccoulet *et al.* [27] proposed a denaturing CZE method involving the use of a BGE buffered at pH 3.0 for the separation of bevacizumab, cetuximab, trastuzumab and rituximab within 15 min.

In addition to the large separation of the main species of bevacizumab, trastuzumab and rituximab within 45 min, our methodology also successfully separated the charge variants of the three mAbs. Figure 4A shows the separation of bevacizumab, trastuzumab and rituximab and their charge variants from mixtures of valid stock solutions (blue trace) and expired stock solutions provided by the Geneva University Hospitals (orange trace). The amount of basic variants of the expired bevacizumab and rituximab were increased by two-fold and three-fold, respectively, whereas the amount of acidic variants of trastuzumab was almost increased by a factor two (from 32.3 to 53.4%). Such increase of charge variants amounts is likely to indicate some mAb degradation, but it is worth noting that changes in the manufacturing process may also potentially reduce the amount of charge variants [28]. Therefore, the methodology could be applied in hospitals for the quality control (QC) of individual mAbs solutions together with the quantitative analysis of their charge variants. Finally, additional data would be generated from the QC analysis that could be valuable for the evaluation of mAb stability.

Beyond potential application for quality control in hospitals, combination therapies involving multiple mAb products within a single sample increases the analytical challenge. In 2015, the FDA approved the first immune oncology combination of the anti-PD-1 nivolumab and the anti-CTLA-4 ipilimumab, for a certain category of melanoma. The optimization of the formulation included the evaluation of charged heterogeneity by CIEF [29]. Similarly, a mixture of ipilimumab and nivolumab was analysed by our in-house CZE

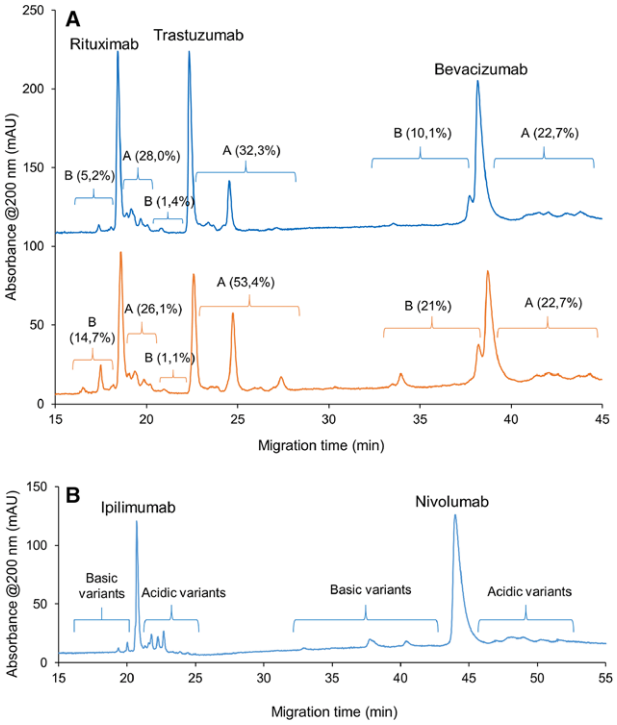

Figure 4. Charge profiles of a mixture comprising valid bevacizumab, trastuzumab and rituximab solutions (blue trace) and expired solutions (orange trace) (A) as well as for a mixture containing valid ipilimumab and nivolumab products by the in-house CZE method. A and B refer to the relative amount of acidic and basic variants, respectively.

method. As shown in Fig. 4B, protein species related to ipilimumab migrated between 19 and 25 min, whereas those related to nivolumab migrated between 32 and 54 min. The separation of several mAbs and their charge variants is likely to gain even more interest in the coming years, due to the growing number of clinical trials involving anti-PD-1/L1 agents in combination with other immune oncology therapies, and more than 1100 combination trials were registered in late 2017 [1]. Furthermore, the potential of the CZE method could be evaluated for the characterization of bispecific antibodies generated by a reduction/oxidation of two parental mAbs and polyclonal antibody products combining a defined number of target-specific antibodies. A limited number of methodologies have been developed for such purpose and they mainly rely on LC-based techniques such as RPLC-MS following reduction and enzymatic digestion of polyclonal antibody product [30] and CEX analysis of intact antibodies [31]. Similarly, CEX and HIC analysis have been performed to separate the two parental mAbs from a bi-specific antibody in order to determine the heterodimerization efficiency [32]. CE-based techniques may gain more popularity for the separation of mAb mixtures, especially when antibodies are strongly adsorbed on the LC column stationary phase as for belimumab and ixekizumab in CEX [23].

4 Concluding remarks

An alternative generic CZE method has been developed using solely a Bis-Tris buffer and PEO dynamic coating. This method was found to be simpler than the reference CZE approach involving EACA, TETA and HPMC. Compared to the original method published earlier, this novel CZE approach allowed separating additional charge variants and in particular more acidic variants, for 10 mAbs out of 17. Conversely, a higher number of basic variants were separated by the reference CZE approach for two mAbs out of 17, demonstrating the complementarity of the two approaches. However, faster analysis time was still achieved using the reference method (5-fold decrease) together with a better sensitivity (2-fold increase). The study also provided the relative amount of basic and acidic charge variants for the 17 EMA- and FDA-approved mAbs, that were mainly comprised between 5 and 15 % and between 15 and 30%, respectively. Potential applications for the quality control in hospital and evaluation of formulations comprising several mAbs were reported, but they are not exhaustive as the high-resolution separation of the mAb charge variants could also be applied to the evaluation of biosimilar products.

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