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Top-down proteomic analysis of monoclonal antibodies by capillary zone electrophoresis-mass spectrometry

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ABSTRACT

Capillary zone electrophoresis (CZE) is considered an alternative to advanced chromatographic methods for the analysis of monoclonal antibodies (mAbs) as those are well applicable for the high-resolution separation of intact proteins, proteoforms or even protein complexes. Thus, CZE with mass spectrometry (MS) detection, has the potential to grow into a powerful analytical platform for the extensive investigation of mAbs. The top-down proteomic approach, where the application of CZE-MS might be exceptionally beneficial, provides the determination of both the accurate molecular mass and several microheterogeneities. Although there is a relatively small number of publications about the CZE-MS of mAbs, the pharmaceutical industry has an unambiguous interest on this field and thorough, intensive research has been initiated.

In this review, we surveyed the developments of top-down CZE-MS applied for mAbs. The merits and limitations of the published capillary coatings and running electrolytes used for CZE-MS were discussed. The different aspects of CZE-MS hyphenation, furthermore, the applications of such mAb studies were surveyed, as well.

1. Introduction

A monoclonal antibody (mAb) is a complex protein manufactured for a specific purpose using recombinant DNA technology. MAbs (also their related products like antibody-drug-conjugates) are biotechnology made proteins that mimic the immune system to render adverse antigens harmless. The biopharmaceutical industry is being taken over by mAbs due to their exemplary therapeutic effect in the treatment of several diseases. This excellent capability to combat diseases is achieved via the recombinant biotechnology by which they are manufactured leading to their high specificity. The first monoclonal antibody muromonab-CD3 [1] was licenced in 1986 as an immunosuppressant for patients with organ transplants, and by 2020 > 80 mAbs had been approved [2].

MAbs are large (~150 kDa) and very complex glycoproteins. Due to manufacturing processes and storage (including exposure to light or chemicals), a large number of changes can develop in these proteins: (i) the formation of amino acid sequence variants due to gene mutations, (ii) the appearance of post-translational modifications (PTMs) (e.g., asparagine deamidation, methionine oxidation, sulphation, lipidation, phosphorylation, glycosylation, N-terminal glutamine deamidation, C-terminal lysine clipping, cysteine modifications, isomerization (transpeptidation)), the occurrence of (iii) physical macro-changes (e.g., denaturation, fragmentation, aggregation) or (iv) conformation alterations [3–5]. These modifications can largely increase the heterogeneity and the complexity of mAbs, which may lead not only to instability and a change in their therapeutic potency but also to immune reactions which can cause malfunction or potential toxicity of the drug. From an analytical point of view, their determination, especially the revelation of the induced micro-heterogeneity (i.e., the PTMs) poses a great challenge. The sizes/masses, charges or glycosylation patterns of the modified species often differ only minimally from the original molecules (for instance the deamidation of one asparagine results in only ~ 1 Da difference or a disulfide bridge is responsible for a 2 Da difference in the ~ 150 kDa size proteins). Therefore up-to-date approaches and a large variety of high performance analytical methods are necessary for the investigation of these compounds. At present, the application of separation methods blessed with the highest resolving power (HPLC, CE) in conjunction with high accuracy mass spectrometers is a widely recognized solution for such proteomic studies.

There are two fundamental MS-based approaches for protein characterization applied in proteomics. In the bottom-up proteomic (BUP) workflows the proteins are subjected to proteolytic cleavage, the resulting peptide mixture is then separated and finally analyzed by MS. BUP allows protein identification by the characterization of the relative proteotypic peptides generated by enzymatic digestion prior to MS analysis. Contrarily, in top-down proteomics (TDP), intact proteins or large protein fragments are separated and then exposed to MS analysis (Fig. 1). For MS analysis, the ions are generated mainly by electrospray ionization (ESI), which are then subjected to gas-phase separation, and tandem

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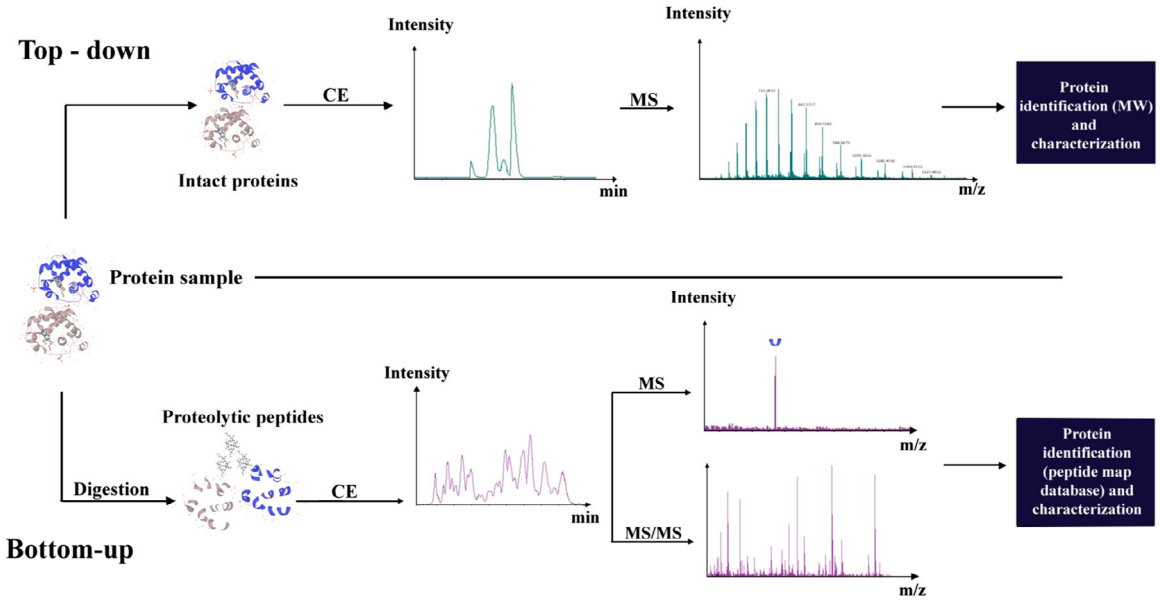

Fig. 1. Fundamental approaches in MS based proteomic analysis.

Nomenclature

ADBAC	benzalkonium chloride
ADCs	antibody-drug conjugates
AFM	atomic force microscopy
APCI	atmospheric pressure chemical ionization
APDIPES	3-(aminopropyl)di-isopropyl-ethoxysilane
APPI	atmospheric pressure photoionization
APS	aminopropylsilane
BFS	bare fused silica
BUP	bottom-up proteomics
CTAB	cetyltrimethylammonium bromide
CVs	charge variants
DAR	drug-to-antibody ratios
Dean	asparagine deamidation
degPs	degradation products of mAb
DS	dextran sulfate
DTT	dithiothreitol
EACA	Ɛ-amino-caproic acid
ECD	electron capture dissociation
ETD	electron transfer dissociation
FC	fluorocarbon
FFE	free flow electrophoresis
FT-ICR	fourier-transform ion cyclotrone resonance
HPC	hydroyxypropyl cellulose
HPMC	hydroyxypropyl methyl cellulose
HRN	high Resolution Native
HC	heavy chain
IAM	iodoacetamide
ISD	in-source decay
isCID	in-source collision induced dissociation
IsoD	aspartic acid isomerization
HHL	heavy-heavy-light chains
LC	ligth chain
LCP	linear carbohydrate polymer
LPA	linear polyacrylamide
M7C4I	1-(4-iodobutyl)4-aza-1-azoniabicyclo[2,2,2] octane iodide
MCE	microfluidic capillary electrophoresis
NHS-PEG450	methyl-terminated polyethylene glycol n-hydroxy succinimide ester
nrCE	non-reducing capillary electrophoresis
PEO	polyethylene oxide
PEI	polyethyleneimine
PEG	polyethylene glycol
PTMs	post translational modifications
PB	polybrene
PVA	polyvinyl alcohol
redFs	reduced forms/parts of mAbs
rCE	reducing capillary electrophoresis
SHS	hexadecyl sulfate
SL	sheath liquid
SMIL	successive multiple ionic polymer layer
SVs	size variants
TDP	top-down proteomics
TETA	triethylenetetramine
HCD	higher-energy collisional dissociation
TCEP	tris (2-carboxyethyl) phosphine hydrochloride;
UVPD	ultraviolet photodissociation.

MS fragmentation in order to obtain the molecular mass and the protein fragmentation pattern.

In the last few decades, the commercial instruments equipped with control, data analysis and bioinformatic pieces of software are well optimized for bottom-up applications [6]. Although the bottom-up approach is the more widely applied strategy due to its high-throughput nature and the easier detectability of smaller compounds (peptides), its utilization might fail to make differences between slightly different proteins or protein isoforms (e.g., proteins formed from the same gene). Generally, using BUP, not all peptides are identified (the sequence coverage does not reach 100%), therefore incomplete sequence information can be gained about the protein of interest. There are several reviews which concluded that some information about PTMs, sequence variants or protein complexes (where protein subunits are attached with non-covalent interactions) – basically the exact depiction of the whole intact protein - can be lost when proteolysis is applied [7–9].

However, TDP allows 100% sequence coverage and is able to provide a bird's eye view of intact proteins because the analysis targets the

given proteoform itself (with PTMs). Another beneficial feature of the TD approach is its simplicity concerning sample preparation compared to the time consuming digestions used in BUP; no dilution or desalting is necessary. During TDP both the accurate molecular mass and several microheterogeneities (PTMs) can be determined because MS measures directly the intact proteoforms and their delta mass values and tandem MS determines the sequence, allowing the identification and localization of PTMs. The main limitation of TDP is that the multiply charged large proteins (M>50 kDa) form very complicated mass spectra, especially when a mixture of proteins is introduced into the MS. Therefore, more sophisticated and expensive MS instruments with wide mass range, high resolution and mass accuracy (eg. FT-ICR, orbitrap) are preferred.

The application of a high performance separation method is crucial for both BUP and TDP. In the case of BUP, the large number of peptides obtained during the digestion need to be separated before entering the ESI-MS. TDP is often applied for a pure protein component without any separation method, but when several proteins are measured, the fractionation of these proteins is practically inevitable. Similarly, for instance, the heterogeneity study of mAbs necessitates the separation of the different variants of the molecule prior to MS analysis. In the TDP analysis of mAbs, chromatographic methods such as reversed-phase liquid chromatography (RP-HPLC) [10], size exclusion chromatography (SEC) [11], ion-exchange chromatography (IEX) [12] or electrophoretic methods like sodium-dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) [13], capillary zone electrophoresis (CZE) [14], capillary gel electrophoresis (CGE) [15] or capillary isoelectric focusing (CIEF) [16,17] can be used. All of these separation methods are amenable for off-line MS hyphenation (i.e. eluted fractions are introduced to the MS); however, for on-line coupling all but SDS-PAGE and CGE are suitable. Although the off-line analysis allows the use of longer time windows for the collection of sample data and the application of usual separation conditions (which are not necessarily MS compatible), the on-line mode is preferred due to the much faster analysis and better economized sample consumption.

Capillary electrophoresis (CE) is well-known for its high resolving power, simple instrumentation, minimal sample consumption and short analysis time. CZE is the simplest CE separation mode based on the differences in the electrophoretic mobilities of the analytes (defined by the size, charge and the shape of the molecule). Moreover, CZE is the most universal separation method which can be applied either for the small monoatomic ions, organic compounds, peptides or even big biomolecules like mAbs. CZE together with CIEF are considered as alternatives to advanced chromatographic methods for the analysis of mAbs as they are well applicable for the high-resolution separation of intact proteins, proteoforms or even protein complexes [18,19]. An additional beneficial feature of CZE for mAb analysis is that the separations can be performed under near-physiological conditions thus the protein conformation, folding or function can be preserved during the analysis. The major limitation of CZE is the poor concentration sensitivity, however, high concentration (but low volume) of mAb pharmaceutical samples are often available for their characterization. The propensity of proteins to strongly adsorb onto the separation capillary wall is another drawback of CZE since it induces peak distortion, poor separation efficiency and low reproducibility of runs.

CZE [20,21], unlike CIEF [22] or other CE techniques, is relatively easy to on-line hyphenate with ESI-MS, thereby providing important information (e.g., molecular mass of the intact protein and its fragmentation pattern) about the separated components. CE cannot be on-line coupled with matrix-assisted laser desorption ionization (MALDI), the other type of MS ion source commonly used for intact protein analysis, since co-crystallization of the protein with special matrix materials on a plate is necessary prior to MS analysis [21]. Since in TDP ESI-MS, the signal of proteins is distributed between clusters of dense multicharged isotopic peaks and several adducts, the sensitivities of CZE-ESI-MS determinations are quite poor which can be remedied principally by analyzing large concentration samples. These very complex precursor spectra necessitate the utilization of (often extremely) high resolution and mass accuracy MS instruments for the accurate peak assignments in mAb analysis. Recently, ongoing research on how to enhance detection sensitivity has resulted in a considerable improvement in interface designs. The most promising and already commercialized design is the porous tip sheathless interface [23].

In the past few decades thousands of papers have been published on the analytical characterization of mAbs, which is recently one of the hottest and most important topics in (pharmaceutical) analytical chemistry. Also, hundreds of papers can be found where a CE method is applied for mAbs. Furthermore, in the last decade over 30 papers appeared which dealt exclusively with CE-MS of mAbs (Fig. 2). It is interesting that although CE-MS and mAb were mentioned together in a relatively large number of documents, the papers about CE-MS analyses of mAbs started to considerably increase only since the last few years. This trend will very likely continue in the future. Numerous reviews have provided an overview of the technical developments of CE-MS [21,24], different proteomic approaches (BUP, TDP) [2,25] and clinical/pharmaceutical applications [26,27] related to mAb analysis (Table 1).

In this review, we focused on the developments of top-down CZE-MS for mAbs. The merits and limitations of the published capillary coatings and running electrolytes used for CZE-MS were compared. The different aspects of coupling CZE with MS, furthermore, the applications of such mAb studies were surveyed, as well.

2.1. Capillary electrophoresis separation techniques for mAb analysis

Capillary electrophoresis includes several techniques like CZE, CGE, CIEF, micellar electrokinetic (MEKC) or capillary electrochromatography (CEC). These separation techniques are based on differences in electrophoretic mobilities, molecular sizes, isoelectric points or chromatographic features of the components. Since CZE, CGE or CIEF are the CE techniques that have been mostly applied for mAbs, only these are discussed in this chapter focusing on the separation conditions used (Table 2). Although in this review we focused on the developments of top-down CZE-MS for mAbs, a short survey of the CGE and CIEF separations of mAbs was found useful for a more comprehensive evaluation of CZE.

2.1.1. Capillary zone electrophoresis

CZE separations are based on the different electrophoretic mobilities of the analytes, which are determined by their charge, size and shape. The larger their charge-to-size ratio and the more spherical the shape of the solutes, the larger their electrophoretic mobility. The charge of the mAbs is determined by their pI value and the pH of the applied background electrolyte (BGE). The shape of mAbs are relatively similar in their intact state (”Y” form), however are quite different upon reduction, denaturation (e.g., with detergents) or aggregation. CZE is predominantly used for separating the charge isoforms of mAbs. The most important CZE parameters include the characteristics of the capillary surface and the BGE (composition, pH, concentration, ionic strength, viscosity, etc.). One of the most significant issues in CZE analysis is the adsorption of biomolecules onto the capillary wall through electrostatic or other intermolecular (e.g., dipole-dipole or H-bond) interactions due to the forces acting between charged or neutral surfaces and the biomolecules of interest. This holds especially true for monoclonal antibodies, since they generally have higher isoelectric point values (7.5–10.5) and thus are attracted more easily to the oppositely charged (deprotonated) silanol groups of BFS capillaries in BGEs with slightly acidic or neutral pH. Such adsorption phenomena cause low efficiency, low recovery, peak broadening and shift in migration time. High separation efficiency can be achieved when the interaction between the mAb and the inner wall of the capillary is eliminated. In order to overcome this problem, several strategies have been applied, including the appropriate choice of pH, the use of different BGE additives or capillary coatings (the capillary coatings applicable for CZE-MS are detailed in a

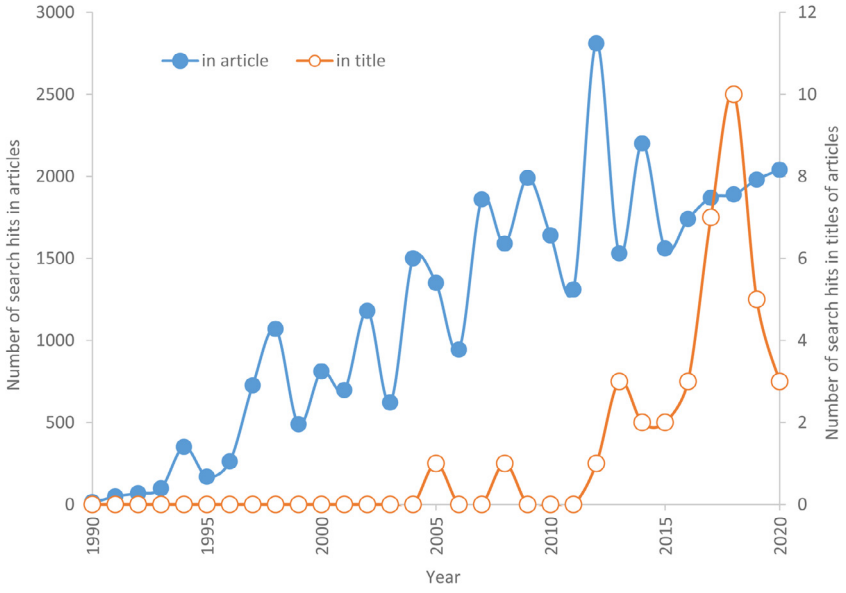

Fig. 2. Number of annual search hits related to ANTIBODIES and CAPILLARY ELECTROPHORESIS MASS SPECTROMETRY (searching with Google Scholar) where the keywords appeared in the titles or in the text anywhere of the articles.

Table 1
Reviews/books including top-down proteomic analysis of mABs by CZE-MS.

Title	Keywords in addition to mAB and CZE-MS	Ref.
Analysis of monoclonal antibodies by capillary electrophoresis: sample preparation, separation and detection	sample preparation, intact, middle-up, bottom-up, proteomics, CIEF, MEKC, CGE, coatings	[2]
Recent advances (2019–2021) of capillary electrophoresis-mass spectrometry for multilevel proteomics	2019–2021, bottom-up, top-down, proteomics, native proteins, SEC-CZE-MS, (SEC)-RPLC-CZE-MS, CIEF-MS, CE-MS interface	[25]
A mini review on capillary isoelectric focusing-mass spectrometry for top-down proteomics	top-down proteomics, CIEF-MS	[55]
Insights from capillary electrophoresis approaches for characterization of monoclonal antibodies and antibody drug conjugates in the period 2016–2018	2016–2018, mAb related products, CE-based methods	[19]
Recent trends of capillary electrophoresis-mass spectrometry in proteomics research	top-down proteomics, bottom-up proteomics, CE-MS interfaces, capillary coating, CIEF, quantitative analysis	[8]
Recent advances in capillary electrophoresis mass spectrometry: Instrumentation, methodology and applications	2016–2018, instrumental developments, two-dimensional separation systems, CGE, CIEF, NACE, MEKC-MS, intact protein and top-down, bottom-up, proteomics, clinical applications	[21]
Recent advances of capillary electrophoresis-mass spectrometry instrumentation and methodology	2013–2017, instrumental developments, CIEF-MS, CGE-ICP-MS, chip-MS, CITP-MS, CEC-MS, NACE-MS, MEKC-MS, MEKC-ICP-MS	[24]
Cutting-edge capillary electrophoresis characterization of monoclonal antibodies and related products	2010–2015, biosimilar, antibody-drug conjugates, Fc-fusion proteins, CGE, CIEF, MZE, MIEF, UV, LIF detection	[26]
Chromatographic, electrophoretic, and mass spectrometric methods for the analytical characterization of protein biopharmaceuticals	RPLC, HIC, SEC, CEX, HILIC, CIEF, ITP-MS, CGE-LIF, LC–CZE–MS, intact protein and middle-up, top-down and middle-down, bottom-up, proteomics	[63]
Developments in interfacing designs for CE–MS: towards enabling tools for proteomics and metabolomics	interfaces, top-down proteomics, bottom-up proteomics, glycomics, metabolomics	[125]
Applications of capillary electrophoresis in characterizing recombinant protein therapeutics	2000–2013, recombinant therapeutic proteins, PTMs, CIEF, iCIEF, SDS-CGE, UV, LIF	[99]
Recent advances in the analysis of therapeutic proteins by capillary and microchip electrophoresis	2012–2013, therapeutic proteins and peptides, biosimilars, coatings, CIEF, microchip electrophoresis	[65]
]Top down proteomics: Facts and perspectives	top-down proteomics, liquid chromatography, electrophoresis, intact proteins, data processing	[7]
CE-MS for the analysis of intact proteins 2010–2012	2010–2012, intact protein, coatings, biopharmaceuticals, intact proteins in biological samples, microreactors, CE-MALDI-MS, CE-ICP-MS	[68]
Capillary electrophoresis–mass spectrometry for the analysis of intact proteins 2007–2010	2007–2010, CIEF-MS, chip-based CE-MS, CE-MALDI-MS, CE-ICP-MS, intact protein, protein-ligand complexes	[67]
Intact protein separation by chromatographic and/or electrophoretic techniques for top-down proteomics	LC–MS, top-down proteomics, off-line MS, CIEF, CEC, SDS-PAGE	[126]

separate chapter). With an uncoated capillary, the simplest way to reduce interactions is if extreme pH values (either very low or very high) are chosen for the BGE. In such cases, the net charge of mAbs and the charge of inner capillary surface have the same sign, hence electrostatic interactions are significantly reduced. Dai et al. analyzed IgG1 mAb and carried out the separation from its host cell impurities in an uncoated capillary using alkaline buffer (pH 9.4) by CZE and MEKC (20 mM borate and 20 mM borate-50 mM SDS, recpectively) [28]. Three of the human IgG subclasses were resolved using an uncoated fused silica capillary and 50 mM phosphate buffer (pH 9.3) [29]. MAb glycoforms were studied using 150 mM borate and 50 mM phosphate buffers (pH 9.4) in an uncoated capillary. Since borate readily forms complexes with carbohydrates, characteristic separation patterns were found in borate buffer, while one single peak was observed using phosphate buffer [30]. Highly acidic or alkaline pH values can cause structural changes in mAbs, which results in low recovery, therefore the use of moderate pH values and capillary coatings are advisable.

The selective and reproducible separation of several mAbs and their charge variants was obtained using zwitterionic Ɛ-amino-caproic acid (EACA) combined with hydroxypropyl methylcellulose (HPMC) in bare fused silica (BFS) and coated capillaries (μSIL-FC, Polymicro Technologies, Phoenix, AZ, USA) [31]. Preconditioning the BFS capillary between

Table. 2
Parameters for the CE-MS analyses of intact mAbs.

CE mode	MAb sample (pretreatment)	Analytes	Detection	Capillary	BGE	Conditioning	Ref.
CE(SDS)-CZE (2D)	NIST mAb (reduction (1 M DTT, 70 °C, 10 min) dilution in SDS-MW)	LC, SVs, redFs, degPs	1st: UV 2nd: ESI-orbitrap-MS	1st: PVA coated, 40 cm, 50 μm ID, 2nd: bare FS, 150 μm ID	1st: SDS-MW, 2nd: 1 M HAc	1st: 0.1 M NaOH, 0.1 M HCl and water at 3 bar for 3 min, SDS-MW, 2nd: water, CTAB, 1 M HAc at 3 bar for 2 min	[96]
(CIEF) assisted CZE	Sigma mAb, NIST mAb (dissolved in water or 10 mM ammonium acetate (pH 6.8)	glyco, aggr.	Q-TOF-MS	LPA and LCP coated, 70 cm, 50 μm ID	25 mM NH_4Ac (pH 6.8), For cIEF, mAB was dissolved with 0.25% Pharmalyte		[62]
CZE	adalimumab, beli-mumab,tocilizumab, infliximab,rituximab, trastuzumab-emtansine in-line and off-line IdeS digestion-TCEP reduction	digPs, redFs	UV MALDI-TOF-MS	fused silica and PEO coated, 60 cm, 30 μm ID,	25, 50, 75, 100 and 150 mM HAc- NH_4Ac (pH 3, 4, 4.5, 5) and 20% mM HAc (pH 2.1)	fused silica: 1 M NaOH, water, and 0.1 M HCl (3 min each), BGE for 5 min, coating: PEO solution for 6 min, water for 5 min, BGE for 5 min	[18]
CZE	adalimumab,natalizumab, nivolumab, palivizumab, infliximab, rituximab, trastuzumab, intact, ideS digestion, IdeS digestion-TCEP reduction	digPs, redFs, glyco, PTMs	Q-TOF-MS	PEI coated, 100 cm, 30 μm ID	3% HAc		[83]
CZE	infliximab reduced and IdeS treated, stressed (4 °C for 6 months, protected from light)	unfolded monomer, dimers digPs, redFs,	Q-TOF-MS, AFM	PB-DS-PB triple layer coating, 60 cm, 50 μm ID	40 mM HAc (pH 3); 40 mM NH_4Ac (pH 4, 5, 6, 7)	BGE at 13.7 psi for 5 min	[14]
CZE	intact, IdeS digestion-TCEP reduction	digPs, redFs, glyco, PTMs	Orbitrap-MS	LPA coated	10% isopropanol-0.2% FA; middle-down: 50% methanol-1% FA	0.1 M HCl at 100 psi for 5 min, water at 100 psi for 5 min, 40 mM NH_4Ac (pH 7.5) at 100 psi for 10 min	[72]
CZE	rastuzumab, rituximab, palivizumab	glyco, CVs	Q-TOF-MS	PEI coated, 100 cm, 30 μm ID	3% acetic acid 30% MeOH	3% acetic acid at 75 psi for 3 min	[82]
CZE	trastuzumab, infliximab, ustekinumab intact, ideS digestion	glyco, CVs, Lys-variants, digPs	Q-TOF-MS	LPA coated, 91 cm, 30 μm ID	intact: 50 mM NH_4Ac (pH 3), IdeS-digested mAb: 10% or 20% acetic acid	0.1 M HCl at 100 psi for 3 min, BGE at 100 psi for 10 min	[73]
CZE	DTT reduction (mAb Hoffmann-La Roche)	HC, LC of reduced mAb	Q-TOF-MS	UltraTrol LN coated, 70 cm, 30 μm ID	10% HAc	BGE	[81]
CZE-CZE (2D)	trastuzumab	CVs	UV QTOF-MS	1st: fused silica 2nd: PVA coated	1st: 380 mM EACA-1.9 mM TETA-0.05% HPMC (pH 5.7) 2nd: 2 M HAc		[75]
CZE	trastuzumab	intact, glyco, dimer	Q Exative Plus MS	LPA coated capillary	20 mM NH_4Ac (pH 8.0)	0.1 M HCl at 100 psi for 5 min, water for 5 min, BGE for 10 min	[66]
CZE	cetuximab, ideS digestion	Fc/2 variants	UV, MALDI-TOF-MS	HPC coated, 60 cm, 50 μm ID	inlet: 200 mM EACA-25 mM NH_4Ac (pH 5.7), outlet: 25 mM NH_4Ac (pH 5.7)		[78]
CZE	reduced SDS-antibody complexes	SDS denatured HC, LC	Q-TOF-MS	UltraTrol™, PVA, PB coated, 55 cm, 50 μm ID	1 M HAc	water for2 min, BGE for 2 min	[53]
CZE-CZE (2D)	trastuzumab	CVs glyco	1st:UV 2nd:Q-TOF-MS	1st: fused-silica, 60 cm, 50 μm ID 2nd: PVA coated 80.0 cm, 50 μm ID	1st: 380 mM EACA-1.9 mM TETA-0.05% HPMC (pH 5.7) 2nd: 2 M HAc	1st: 0.1 M HCl for 5 min, H_2O for 2 min, BGE for 4 min 2nd: BGE at 3 bar for 10 min	[74]
CZE SCX SPE-CE-ESI-MS/MS	CHOK1, CHO K1SV cell line	HCP impurities	LTQ-Orbitrap-MS	LPA coated 85 cm, 50 μm ID	1 M HAc		[127]
CZE	cetuximab, ideS digestion, CP-B treatment	Fc/2 dimers, Fc/2 glyco	UV MALDI-TOF-MS	HPC coated, 82 cm, 75 μm ID	inlet: 200 mM EACA- 25 mM NH_4Ac (pH 5.7), outlet: 25 mM NH_4Ac (pH 5.7)		[79]
CZE	IgG1 manufactured by Amgen, DTT reduction, ideS digestion	HC, LC, declycosylated forms, Fc/2, Fab'2, glyco of Fc/2	TOF-MS	LPA coated, 70 cm, 50 μm ID	5%, 10%, 30% HAc		[71]
nano-ESI	adalimumab	only intact	Q-Exactive-MS	fused silca, 91 cm, 30 μm ID	3% FA		[86]

(*continued on next page*)

Table. 2 (*continued*)

CE mode	MAb sample (pretreatment)	Analytes	Detection	Capillary	BGE	Conditioning	Ref.
CZE	brentuximab-vedotin ADCs, ideS digestion	intact, middle-up, bottom-up, Fc/2, Fab'2, glyco of Fc/2	Q-TOF-MS	fused silica, 100 cm, 30 μm ID	10% HAc	CH_3OH at 75 psi for 10 min, 0.1 M NaOH for 10 min, 0.1 M HCl for 10 min, water for 20 min, 10% HAc for 10 min	[114]
CZE	mouse anti-human IgG4Fc-UNLB, Human/Mouse/RatActivinA Asubunit antibody, Anti-phosphotyrosine antibody, TCEP reduction	HC, LC	LTQ-Orbitrap-MS, LTQ-XL-MS	LPA coated, 50 cm, 50 µm ID	0.1% FA	0.1% FA	[70]
CZE	cetuximab, ideS digestion	CVs, Fc/2, Fab'2, glyco	UV, MALDI-TOF-MS	HPC coated, 82 cm, 75 µm ID	inlet: 200 mM EACA-25 mM NH_4Ac (pH 5.7), outlet: 25 mM NH_4Ac (pH 5.7)	1 M, 0.1 M NaOH at 30 psi for 10 min, water at 30 psi for 10 min	[77]
CZE	commercially marketed humanized mAb	glyco	UV, MALDI-MS	HPC coated, 80 cm, 50 µm ID	400 mM EACA-0.05% HAc (pH 5.7)		[76]
μCE	IgG1 manufactured by HJB Ltd, stressed mAbs at 40 °C for 2 weeks, 4 weeks in the dark, IdeS digestion-DTT reduction	Fc/2, Fab'2, CVs, glyco, Lys-clipping variants	Orbitrap-MS	ZipChip (22 cm channel length)	Native BGE provided in the ZipChip Native Antibodies Kit		[91]
μCE	rituximab, trastuzumab, bevacizumab	CVs (C term Lys-variants, sialyated forms, deaN, pyro-GLu)	Q-Exactive Plus hybrid quadrupole- Orbitrap-MS	ZipChip (22 cm channel length)	BGE provided in Native antibody kit based on NH_4Ac (pH 5.5)	No conditioning step is required and no regeneration step is performed between runs. Chips were only primed before the first use.	[93]
μCE	NIST mAb, intact, PNGase digestion,	Lys-variants, glyco, de-glycosylated mAb, released glycans	Q-Exactive Plus-MS, LIF	ZipChip^{TM} CE (22 cm channel length)	0.2% HAc-10% IPA (pH 3.17)		[89]
μCE	unconjugated IgG-2, ADCs	CVs (pyro-Glu, decarboxylation, glyco), DAR	TOF-MS	CE-ESI device by standard photolithography, NHS-PEG450 coated, APDIPES coated, 23 cm	10% IPA- 0.2% HAc (pH 3.17)		[88]
μCE	infliximab	CVs (C term Lys-variants, glyco	LCT-Premier-TOF-MS	CE-ESI microchip devices by standard photolithography from 0.5 mm thick B270 glass wafers, 23 cm channel, NHS-PEG450 coated, APDIPES coated	10% IPA- 0.2% HAc (pH 3.17)		[87]
CIEF	infliximab, adalimumab	CVs (C-terminal Lys-clipping, deamidation, sialic acid modification)	Q-TOF-MS	PVA coated, 80 cm, 50 µm ID	anolyte: 0.587 M NH_3 in water (pH 11.51)−20% glycerol catholyte: 0.175 M HAc (pH 2.75)- 20% glycerol	water at 15 psi for 5 min, 3 M urea at 15 psi for 10 min	[61]
iCIEF-CZE	trastuzumab	glyco, deamidation	ESI-MS	PVA coated, 50 µm ID	catholyte: 0.1% methylcellulose-100 mM NaOH, anolyte: 80 mM H_3PO_4.	water, 0.5% methylcellulose	[81]
CIEF	cetuximab, IdeS digestion-DTT reduction	CVs, (Fc/2. Fab'2, HC, LC, C-terminal Lys-clipping)	TOF-MS	neutral coated PS1, 75 cm, 50 μm ID	catholyte: 0.2 N NH_4OH-15% glycerol anolyte: 1% FA-15% glycerol		[17]
iCIEF	trastuzumab,	CVs	UV	neutral coated PS1, 75 cm, 50 μm	catholyte: 0.2 N		[16]
CIEF	bevacizumab, infliximab cetuximab,		TOF-MS	ID	NH_4OH-15% glycerol, anolyte: 1% FA- 15% glycerol		
CIEF-CZE	model mAb X, N-glycosidase F-deglycosylation	CVs (deglycosylated)	UV Q-TOF-MS	PVA coated, 60 cm, 50 μm ID	anolyte: 0.1 M H_3PO_4, catholyte: 0.2 M NaOH 0.2 M FA		[59]

Abbreviations: 2D: two dimension separation; LC: ligth chain; SVs: size variants; CVs: charge variants; redFs: reduced forms/parts of mABs; degPs: degradation products of mAB; SDS-MW: SDS-MW gel buffer obtained from AB Sciex (Darmstadt, Germany); aggr.: aggregates; glyco: glyco-proteoforms; digPs: digested products of mAb; TCEP: Tris (2-carboxyethyl) phosphine hydrochloride; PEO: polyethylene oxide; PEI: polyethyleneimine; PTMs: post translational modifications; PB: polybrene; DS: dextransulfate; AFM: atomic force microscopy; μCE: microfluidic capillary electrophoresis; HPC: hydroxypropylcellulose, deaN: asparagine deamidation; isoD: aspartic acid isomerization; ADCs: Antibody-drug conjugates; DAR: drug-to-antibody ratios; APDIPES: 3-(aminopropyl)di-isopropyl-ethoxysilane; NHS-PEG450: methyl-terminated polyethylene glycol n-hydroxy succinimide ester; FFE: free flow electrophoresis; Q-TOF: quadrupole–time of flight

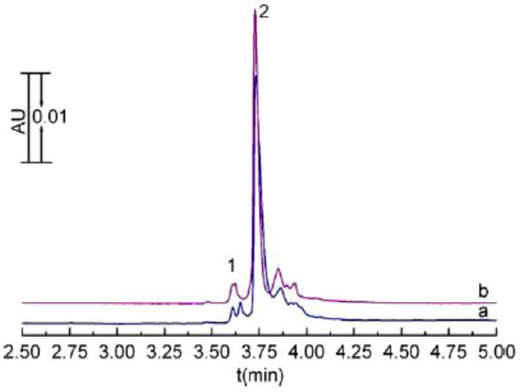

Fig. 3. Charge variant separations conducted using two methods, (a) newly developed method by Shi et al., CZE running buffer: 20 mM acetic–acetate, pH 6.0, 2 mM TETA, 0.3% PEO, (b) reference reported method, CZE running buffer: 400 mM EACA, pH 5.7, 2 mM TETA, 0.05% HPMC, (1) basic peaks, and (2) main peak. Capillary effective length: 20 cm; voltage: +30 kV; injection: 0.5 psi for 10 s; test sample: mAb1 [34].

runs with 0.1 M HCl can remove the adsorbed proteins and protonate the surface silanol groups, facilitating the adsorption of nonionic linear polymer HPMC to the neutral surface forming a dynamic coating. EACA can form an ion pair with proteins reducing their net charge and can also interact with the residual (deprotonated at the pH of BGE) silanol groups that are not covered by HPMC. The application of EACA-HPMC (pH= 5.5) efficiently reduced the interaction between the mAbs and the capillary wall, and a more efficient separation of multiple IgG1 and IgG2 mAbs was achieved compared to a commercially available coated capillary. The addition of triethylenetetramine (TETA) to the separation buffer further improved the separation efficiency. Positively charged TETA interacts with the negatively charged silanolate and reduces peak tailing [32]. Basic and acidic charge variants of a mAb were separated from the main form using BGE of 400 mM EACA–HAc, 0.05% m/v HPMC, 2 mM TETA at pH 5.7. TETA was responsible for reducing the adsorption of basic species of the mAb on the inner wall of the capillary, resulting in a significant improvement of peak shape. The capillary was only treated with HCl (no NaOH), hence the number of residual ionized silanol groups was kept low, which could be titrated quickly by TETA.

An intercompany study involving 11 participating laboratories (Hoffmann-La Roche, Paul-Ehrlich Institut, Boehringer Ingelheim, Novartis, Merck, Pfizer, Seattle Genetics, Amgen, AB Sciex) utilized an identical BGE system (400 mM EACA, 2 mM TETA, 0.05% HPMC at pH 5.7) for the determination of 23 stressed and non-stressed mAbs and their acidic and basic variants [33]. Validation characteristics according to ICH Q2 guideline were demonstrated with 1056 separations performed by these 11 groups. No significant differences were found between the peak profiles of the electropherograms. The coefficients of correlation were above 0.99, precision was 1.4 RSD% (peak area of the main peak) and accuracies were 99.3–101.4%. Accuracy was verified by the comparison of the CZE measurements with IEC and IEF analyses, which showed comparable results with regard to the corrected peak area percent (%CPA) composition for the main peak, the sum of acidic peaks and the sum of basic peaks. The best resolution of the investigated mAbs was obtained by CZE and it was concluded that CZE can be considered an alternative powerful platform for the charge heterogeneity testing of mAbs in the pharmaceutical industry under GMP conditions [33].

Shi et al. developed an alternative CZE method using polyethylene oxide (PEO) dynamic coating [34]. The BGE, containing 20 mM acetate buffer (pH 6.0), 0.3% PEO and 2 mM TETA enhanced peak resolution compared to the EACA-TETA-HPMC system (Fig. 3). This method is useful for the separation of a few (2–6) acidic and basic variants from the main peak. The application of Bis-Tris buffer and PEO dynamic coating agent was compared to the reference CZE approach involving the EACA-TETA-HPMC system for 17 different mAb determinations [35]. The addition of polyamines and zwitterions into the BGE can decrease the adsorption phenomena and/or improve the selectivity of mAbs and their isoforms.

The analysis of mAb isoforms was performed in neutral fluorocarbon (FC)-coated capillaries (J&W Scientific), as well. The recommended BGE for FC-coated capillaries consists of 0.005% FC surfactants. Four isoforms of standard IgG1 were separated using 12.5 mM phosphate - 12.5 mM acetate + 0.005% FC surfactant at pH 7.5 [36]. A similar FC-coated capillary (μSil-FC, Agilent Technologies, Santa Clara, CA, USA) was used for resolving the main isoforms of a commercially available mAb, however, the applied BGE system was based on EACA-TETA-HPMC [37].

Excellent separation of the glycoforms of six commercial mAb pharmaceuticals was achieved using a DB-1 (dimethylpolysiloxane based) capillary (100 μm i.d. J&D Scientific, Palo Alto, CA, USA) and 100 mM Tris−borate-5% polyethylene glycol (PEG70000) buffer (pH 8.3). Borate could improve the selectivity of the glycoform variants by complexation with carbohydrates [38].

Four positively charged (FunCap type A, polybrene-dextran sulfate-polybrene (PB-DS-PB), polyethyleneimine (PEI), UltraTrol HR) and four neutral (FunCap type D, polyvinyl alcohol (PVA), hydroxypropyl cellulose (HPC), UltraTrol LN) static capillary coatings were evaluated in terms of their performance in separating the main isoforms of five pharmaceutical mAbs (infliximab, rituximab, bevacizumab, cetuximab, tocilizumab) [39]. In order to obtain the best possible resolution of the isoforms in the case of a positively charged coating, it is imperative that the pH of the BGE be lower than the pI of the mAb and that there should be no considerable difference in the mobility of EOF and the effective mobility of the mAb. Changing ammonium acetate to EACA (at same pH and ionic strength) remarkably improved the isoform resolution and reduced peak tailing. The concentration of EACA can be higher than that of ammonium acetate, which helps decrease adsorption without producing high Joule heating. The importance of the pH of the BGE was also demonstrated since the resolution of mAb isoforms was driven by their charge differences (Fig. 4) [39].

The separation of mAb charge variants of similar sizes is determined by their charges, which are directly controlled by the pH of the BGE [32,34,40,41]. Additionally, the use of charged additives (such as sodium chloride, ammonium chloride and butanolamine) can result in improved resolution through the slower migration of the mAb isoforms. Organic modifiers (e.g., acetonitrile) can better dissolve the hydrophobic parts of the mAb. The reduction of the hydrophobic interactions between the different charge variants explained the higher resolution in the presence of moderate amounts of acetonitrile. However, using organic solvent in excess causes altered conductivity and thereby a decrease in resolution [41]. The addition of urea to the separation buffer (600 mM EACA-HAc, 0.1% HPMC, 2 M urea, pH 5.5) was used to resolve the disulfide isoforms of IgG2 mAb [31].

2.1.2. *Capillary gel electrophoresis*

CGE is performed in a capillary filled with a sieving matrix as a separation medium, an alternative method to SDS-PAGE for the determination of the size variants of mAbs [31,42–49]. Compared to conventional slab gel electrophoresis, CGE has many advantages such as automated operation, increased separation efficiency, reduced separation time and on-line detection. The first CGE separations were carried out in gel-filled capillaries similarly to conventional SDS-PAGE. There are several difficulties associated with the use of such gels, including the limited lifetime of the capillary, the lack of ease in controlling the polymerization, the formation of air bubbles and their UV absorbance. Several studies showed that size-based CE separations could be obtained with replaceable, UV transparent, low viscosity polymer solutions as molecular sieving matrices [42,43]. Sieving matrices consist of a linear or branched polymer (e.g., dextran, PEG, PEO) and are commercially available as

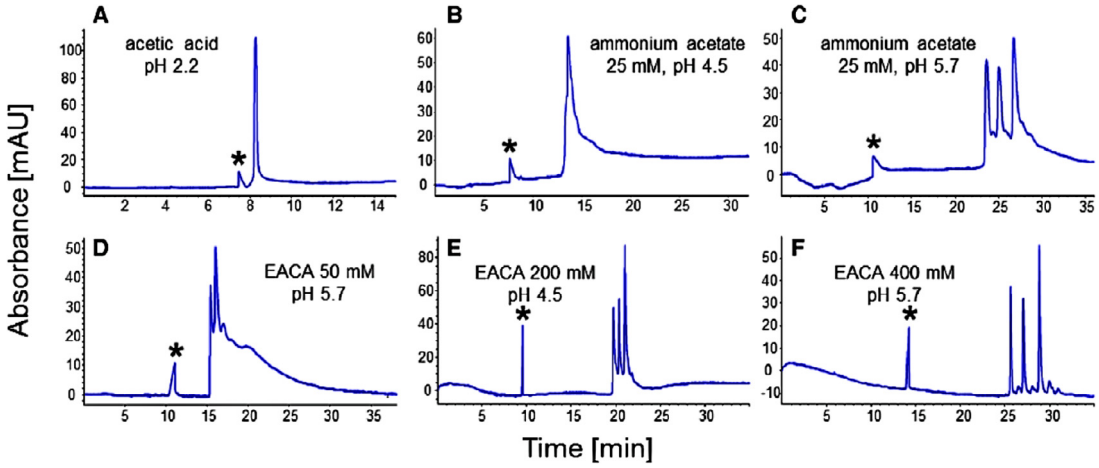

Fig. 4. Comparison of various BGEs for infliximab analysis (0.5 mg/mL). (A) 10% HAc, pH 2.2, (B) 25 mM ammonium acetate, pH 4.5, (C) 25 mM ammonium acetate, pH 5.7, (D) 50 mM EACA, pH 5.7, (E) 200 mM EACA, pH 4.5, and (F) 400 mM EACA, pH 5.7. Capillary: 50 μm id and 64.5 cm long, HPC coating. *control standard: L-histidine [39].

kits. CGE allows the investigation of mAb fragmentation, elongation or truncation, aggregation and N-glycosylation [44–49].

CGE separations are carried out using denaturing conditions with surfactants, mostly SDS under either non-reducing or reducing conditions. Denaturation is performed by the dissolution of the mAb in a sample buffer containing SDS or sodium hexadecyl sulfate (SHS) and its incubation at elevated temperatures for a few minutes. Proteins tend to bind a relatively constant amount of SDS (1.4 g SDS/1 g protein), resulting in SDS-protein complexes with practically identical charge-to-size ratio, thus in a sieving medium, the separation is affected only by the molecular radius of the denatured protein.

The reducing CGE method separates reduced samples. In these cases, sample preparation is completed by adding reducing agent (DTT or mercaptoethanol) to the mAb sample (or SDS sample buffer) in order to break inter-chain disulfide bonds. Reducing CGE is suitable for the identification and quantification of the light chain (LC), heavy-chain (HC) of the mAb of interest as well as the non-glycosylated isoforms [50,51]. Non-reducing CGE is used for the determination of product impurities, low and high molecular mass content (LMWH, HMWH) and covalent aggregates [48,49,51]. The addition of an alkylating reagent (e.g., iodoacetamide (IAM)) during the sample preparation in a nrCE-SDS workflow can minimize the breakage of interchain disulfide bonds (blocking free thiols), thereby prevent the formation of LC, HC and HHL (heavy-heavy-light chains) which would eventually worsen protein purity [48]. In the case of non-reducing conditions, the apparent molecular mass of mAbs is approximately 200 kDa. Such a high value can be attributed to the incomplete unfolding under the non-reducing conditions used and the presence of glycan moieties, which causes a divergence from the usual SDS/protein ratio (1.4/1) (Fig. 5) [43,45]. In the United States Pharmacopeia (USP) and the European Pharmacopoeia (pH.Eur.) both nrCE-SDS and rCE-SDS are being used for the purity and stability determinations of related proteins [49].

The direct hyphenation of CGE to MS is not possible due to incompatibility problems and high ion suppression by the sieving matrix, therefore the modification of the SDS-MW gel buffer is required prior to MS analysis. SDS removal can be performed outside the capillary (off-line) and within the capillary (on-line). Lu et al. deposited mAb-SDS complexes on poly(tetrafluoroethylene) (PTFE) membranes, from which the detergent was eliminated by washing. The mAb remaining on the membrane was then analyzed by MALDI-TOF-MS [52]. Recently, Sanchez-Hernandez developed a method based on the co-injection of a positively charged surfactant (cetyltrimethylammonium bromide (CTAB) or benzalkonium chloride (ADBAC)) and methanol as organic solvent. The successful removal of SDS was shown in neutral coated capillaries as well as in a capillary with a positively charged coating. The usefulness of this in-capillary strategy was demonstrated for antibodies [53]. A similar strategy was developed by Römer et al. using two-dimensional CGE–CZE–MS method for the identification of mAb impurities and fragments. An online SDS removal process was applied in the second dimension, by co-injecting organic solvent (methanol) and a cationic surfactant (CTAB) (Fig. 6) [54].

In conclusion, capillary gel electrophoresis (CGE/CE-SDS) adapts the SDS-PAGE into a capillary format, reducing the drawbacks of the slab gel arrangement. CGE is a commonly applied approach for analyzing the size variants of mAbs in the biopharmaceutical industry. Both non-reducing CE-SDS (nrCE-SDS) and reducing CE-SDS (rCE-SDS) are being utilized for stability and purity testing. Size-based fragments and glycan occupancy are determined with rCE-SDS while nrCE-SDS can reveal size-based fragments, covalently bound aggregates and host cell impurities. However, CGE is not applicable for determining the charge variants as the different charges of the molecules are equalized. Due to the complexity and difficulty of the hyphenation, CGE-MS has not yet become a routine analytical procedure.

2.1.3. *Capillary isoelectric focusing*

CIEF provides the most efficient separation of very similar acidic and basic charge variants, the mechanism of which is based on differences in pI values. In the pharmaceutical industry CIEF has become an important method for the characterization of mAb charge heterogeneity. The separation is carried out in a coated capillary filled with a special sample matrix (ampholyte, mAb sample solution, pI markes, additives). A neutral (e.g., fluorocarbon, PVA) coated capillary is required to eliminate the EOF and to prevent protein adsorption on the capillary wall. The CIEF separation process involves two steps: focusing and mobilization. During the focusing step an applied voltage establishes a pH gradient inside a neutral coated capillary resulting in the separation of mAb charge variants according to their pI. Following the focusing step, the separated zones are transported towards the detector (UV, MS) by means of chemical or pressure mobilization. Due to the high background absorption of the ampholytes at 200–220 nm, detection is carried out at higher wavelengths. Detection at 280 nm may cause the over- or underestimation of charge variants, since charge variants may have different UV absorption at 280 nm.

The composition of the sample matrix determines the attainable separation efficiency. Ampholytes are essential ingredients in the sample matrix as they generate the pH gradient and determine the selectivity and the resolution of separations. (Ampholytes are a mixture of polyamino-polycarboxylic acids of different pI values, with high buffer-

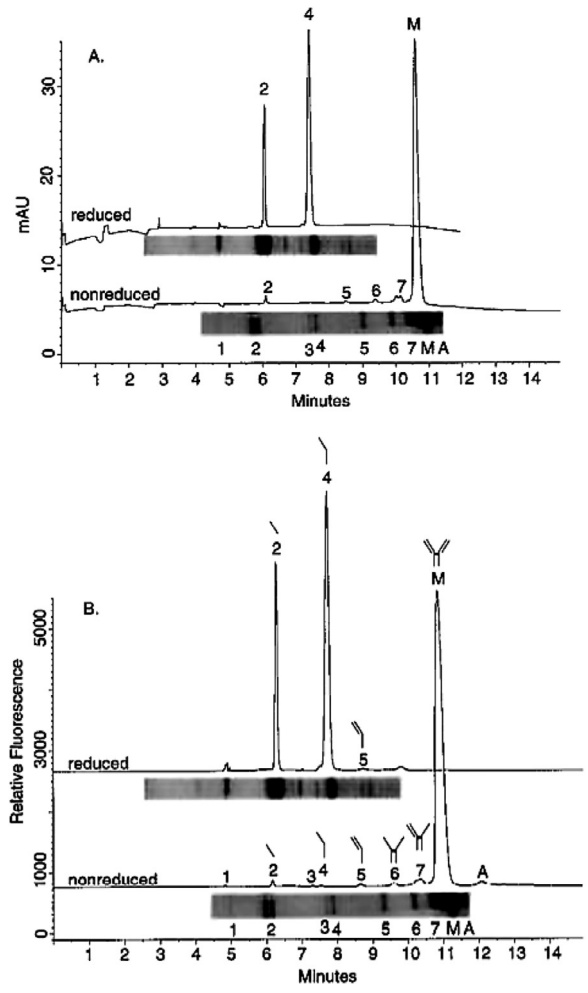

Fig. 5. (A) CE-SDS separations of nonreduced and reduced preparations of a therapeutic rMAb. Insets show silverstained SDS-PAGE traces of the same sample preparations: M, monomer; A, aggregate. Conditions: instrument, Bio-Rad Bio-Focus 3000 capillary electrophoresis system; buffer, Bio-Rad SDS running buffer; capillary, untreated fused silica, 50 μm i.d. and 375 μm o.d.; effective length, 19.4 cm; injection, 15 s at – 417 V/cm; applied electric field, – 625 V/cm; temperature, 20 °C for capillary and sample compartment; detection, UV at 220 nm. (B) CE-SDS separations of nonreduced and reduced preparations of a 5-TAMRA, SE-labeled mAb. All conditions are as in (A), except detection was performed with laser-induced fluorescence using a 3.5-mW argon ion laser, 488 nm excitation, 560 ± 20 nm emission. Insets show silver-stained SDS-PAGE traces of unlabeled sample preparations [44].

ing capacity near their pI.) Cellulose derivatives are used as dynamic coating and the pH gradient is stabilized with viscosity enhancers. As such, glycerol is an alternative additive that is suitable for hyphenation with MS. Other additives such as surfactants or denaturants (e.g., urea, sucrose, formamide, salts) are commonly used to minimize precipitation, aggregation, to achieve a better resolution of charge variants and to ensure reproducibility. The selectivity and duration of the separation are also affected by the BGE composition, the type of catholyte and anolyte [55].

In recent years, imaged CIEF (iCIEF) has been increasingly utilized for monitoring the charge heterogeneity of therapeutic mAbs. The iCIEF tehnique offers improved resolution due to the absence of the mobilization step. The method was introduced in 1992 to avoid possible peak broadening during mobilization [56].

CIEF with MS detection provides an even more in-depth characterization, however, coupling remains problematic due to the high concentrations and non-volatility of ampholytes and additives. The presence of ampholytic components may interfere with sample ionization. The first attempt to on-line hyphenate CIEF and MS for protein analysis was published by Tang et al. [57]. An electrospray ion source with a coaxial sheath flow configuration was used for interfacing. An LPA coated capillary was filled with a solution containing proteins and carrier ampholytes (Pharmalyte 3–10, Pharmacia, Uppsala, Sweden). Focusing was performed with 20 mM phosphoric acid and 20 mM sodium hydroxide as the anolyte and the catholyte, respectively. The focused protein zones were mobilized by replacing the sodium hydroxide catholyte with a solution containing methanol, water and HAc (v/v/v 50: 49:1) at pH 2.6. This solution was also employed as the sheath liquid in the ESI interface [57].

This method was further developed by Zhong et al. [58]. A simple flow-through microvial interfacing strategy for CIEF-MS was demonstrated that combines the advantages of glycerol as an anticonvective medium. Model proteins were mixed with the ampholytes (Fluka pH 3–10), anolyte consisted of 50 mM formic acid (FA) in 30% glycerol, catholyte was 100 mM ammonium hydroxide in 30% glycerol. Modifier solution comprised methanol, water, HAc (v/v/v 50:48:2). The use of glycerol provides increased resolution and reasonable ESI-MS sensitivity [58].

Hühner et al. hyphenated CIEF with CZE-ESI-MS applying a mechanical valve. In general, such 2D CIEF-CZE-MS methods can be divided into two parts. First, a CIEF separation is carried out until the positioning of the analyte in the sample loop of the valve is performed. After switching the valve to the second dimension (CZE-MS), the remaining ESI-interfering components of the CIEF electrolyte are separated from the analytes prior to MS detection. BGE solutions were 0.1 M H_3PO_4 as anolyte, 0.2 M NaOH as a catholyte for the first dimension and 2 M FA for the MS compatible CZE separation. Charge variants from a deglycosylated model antibody were analyzed at the intact level [59].

2D iCIEF-CZE-MS system was applied by Montealegre et al. The focused peaks from the iCIEF system are transferred to a four-port nanoliter valve where peaks of interest are cut and transferred via CZE to ESI-MS. The main charge variants of trastuzumab were characterized. The most intensive acidic variant was attributed to deamidation, while the basic variant was proposed to be either the result of succinimide formation or partial cyclization of N-terminal glutamic acid [60].

A CIEF-MS method was firstly presented by Dai and Zhang [17] for the charge variant analysis of four marketed mAbs (bevacizumab, trastuzumab, infliximab, cetuximab) using an electrokinetically pumped sheath-flow nanospray ion source and TOF-MS with pressure-assisted chemical mobilization. MS-friendly anolyte (1% FA with 15% glycerol) and catholyte (0.2 M NH_4OH with 15% glycerol) were applied. Neutral coating was used for the CIEF-MS separation. The sheath liquid was either 20% HAc with 25% acetonitrile or 0.5% FA with 50% methanol. Protein samples (0.1 – 1.0 mg/mL) were prepared in 1.5% Pharmalyte 3–10 with 5 – 20% glycerol content. The imaged IEF (iCIEF-UV) separations were performed in a fluorocarbon-coated capillary, the anolyte was 80 mM phosphoric acid, the catholyte was 100 mM sodium hydroxide, both in 0.1% methylcellulose. Sample buffer contained 0.35% methylcellulose, 4% Pharmalyte 3–10, 2 M urea and 0.1% pI markers (pI 4.6 and 9.5) [17].

The previously reported CIEF-MS method for intact mAb analysis exhibited excellent resolution of charge variants conforming to those of iCIEF-UV. However, for complex mAbs, CIEF-MS spectra of the intact charge variant peaks may show overlapping, which inhibits successful data processing and interpretation. Charge heterogeneity characterization of intact mAbs represents real analytical challenges, not only for variants with relatively small mass differences but also for heavily glycosylated mAb molecules. Dai implemented a middle-up approach to enhance the capability of the CIEF-MS method for characterizing complex mAb charge variants. The CIEF-MS parameters and BGE solutions were the same as in his previously reported research. In order to reduce sample complexity, Cetuximab was treated by IdeS enzyme and

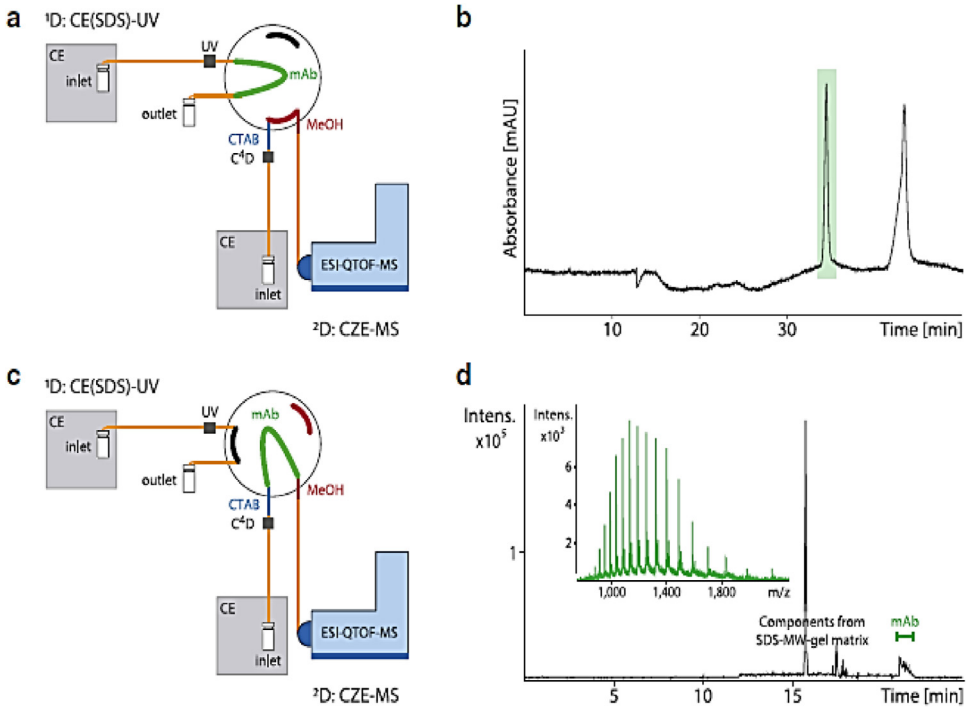

Fig. 6. Setup and workflow of the CE (SDS)-CZE-MS system. **a** Detailed scheme of position A in the nanoliter valve, where the reduced mAb1 is separated in the ^{1}D CE (SDS). The positioning of methanol (red) and cationic surfactant (blue) in the ^{2}D is done simultaneously for the SDS removal. **b** CE (SDS)-UV separation from the reduced mAb1 in the ^{1}D. **c** Detailed scheme of position B where the peak of interest from mAb1 is transferred to the ^{2}D in between the zones of methanol and cationic surfactant. **d** Base peak electropherogram (BPE) showing the ^{2}D CZE-MS separation of the transferred peak from the interfering components of the SDS-MW gel buffer and its raw mass spectrum. Conditions ^{1}D: 50 μm ID fused silica capillary, SDS-MW gel buffer, separation voltage – 15 kV, UV detection 200 nm. Conditions 2D: 50 μm ID PVA-coated capillary, 1 M HAc as separation buffer, separation voltage +10 kV. MS conditions: positive ion mode (4.5 kV), dry gas 4.0 L/min at 170 °C, nebulizer 0.2 bar, sheath liquid 2-propanol:water (50:50, v/v) with 0.2% (v/v) FA with a flow rate of 3.3 μl/min, mass range 700–3500 *m/z*. SDS-removal strategy applied in the ^{2}D: 50 mbar for 12 s methanol plus 50 mbar for 20 s 0.4% (w/v) CTAB in methanol:water (50:50, v/v) [54].

dithiothreitol (DTT). This middle-up approach is a valuable workflow for accurate identification of charge variants for complex therapeutic mAbs [16].

Another group used a catholyte comprising 1.0% v/v HAc (0.175 M, pH 2.75) + 20% v/v glycerol, and an anolyte containing 1.0% w/v ammonia in water (0.587 M, pH 11.51) + 20% v/v glycerol for the charge heterogeneity separation of four mAbs. The CIEF effluent was mixed with a chemical modifier in the flow-through microvial interface to maintain the ESI stability and to decrease ion suppression from the co-eluted carrier ampholytes and glycerol. The CIEF sample mixture was composed of 0.50 mg/mL mAb sample, pI markers (75 μg/mL),1.0% w/v Fluka carrier ampholyte (pH 3–10), 2.50 mM arginine, 2.50 mM iminodiacetic acid and 20% v/v glycerol. The analysis of mAb samples revealed lysine clippings, deamidation and sialic acid modification in oligosaccharide chains [61].

Recently, Shen et al. presented a novel CIEF-assisted CZE-MS platform for the analysis of mAb under native conditions in a linear carbohydrate polymer (LCP)-based neutral coated capillary with much larger sample loading capacity than native CZE-MS alone. First, an online IEF sample stacking process was performed in a narrow pH range to preconcentrate the analytes and increase the loading capacity in native CZE-MS conditions. For CIEF, Sigma mAb was dissolved in 10 mM ammonium acetate (pH 6.8) with 0.25% Pharmalyte 3–10. Before sample injection, 160 nL 50 mM ammonium acetate (pH 9.0) was injected as the catholyte. For the CZE separation, BGE was 25 mM ammonium acetate (pH 6.8). CIEF-assisted CZE-MS achieved high-quality characterization of glyco-proteoforms, charge variants and aggregates of two Sigma mAbs [62].

The hyphenation of CIEF with MS has only recently been published by a few groups. However, such coupling approaches are expected to find extensive application in the future, since there is a great demand in the pharmaceutical industry to facilitate the high level characterization of mAbs.

2.2. Capillary coatings for CZE-MS

The analysis of large biomolecules in BFS capillaries can be accompanied by several difficulties, the most common problem being the adsorption of biomolecules onto the capillary wall [8,63,64]. The strategies generally applied for minimizing these adsorption effects utilizing low-pH BGE solutions or the inclusion of BGE additives [65], however, are known to cause poor efficiency and resolution of the component peaks according to the equation demonstrated by Belov [66]. In cases where the low-pH buffer solution does not provide the desirable results and the application of modifiers shows incompatibility with the CZE-MS interfacing, coating the capillary wall becomes an alternative [67,68]. Capillary coatings act as a shield against large biomolecules and are generally classified as dynamic and static coatings due to their physical adsorption and covalent attachment to the capillary wall, respectively. Some research groups, however, prefer to classify them based on their hydrophobicity (hydrophobic and hydrophilic coatings) or based on their charge (the latter includes charged and neutral coatings) [63]. In Table 3 the coatings used for the top-down analysis of mAbs with CZE-MS were summarized.

Neutral coated capillaries found their wide application in the separation of post-translationally modified mAbs species. Linear polyacrylamide (LPA) coating (a static and neutral coating) excels at minimizing the solute-wall interactions by significantly suppressing the EOF. Components are separated based on their own electrophoretic forces in a slightly acidic buffer condition (between pH 2–6, otherwise polyacrylamide tends to hydrolyze) [69].

LPA coated capillaries have been used for the separation of the light (LC) and heavy chains (HC) of reduced mAbs by Zhao et al. [70] using a CZE-ESI-MS system. The separation of two different reduced mAb mixtures was also achieved by applying identical conditions. The same year Han et al. [71] presented the separation of mAb digests with and without the reduction step in an LPA coated capillary. The digestion was performed by IdeS enzyme (FabRICATOR), which cleaves at a specific site below the hinge region, yielding large F(ab‘)2 (~100 kDa) and Fc/2 (~25 kDa) fragments and upon reduction, F(ab‘)2 fragment was further cleaved into LC (~25 kDa) and Fd (~25 kDa) fragments of the HC. The results suggested that in order to obtain information about the glycosylation forms and other modifications, the incorporation of the reduction step is necessary. Later, Belov et al. [72] performed intact mAb analysis using native CZE-MS conditions in LPA coated capillaries. Thereby the identification of 1X-glycosylated, 2X- glycosylated, agly-

Table 3
The coatings used for CZE-MS analysis of mAbs.

Coating	Surface charge	Bonding/interaction	Coating type	Ref.
LPA	neutral	Covalent	static	70–72
PVA	neutral	Covalent	static	74
LCP	neutral	Covalent	static	62
HPC	neutral	Covalent	static	76–79
PEO	neutral	intermolecular*	dynamic	18
UltraTrol™ LN	neutral	Electrostatic	dynamic	81
PEI	positive	Covalent	static	82
M7C4I	positive	Electrostatic	dynamic	72
PB-DS-PB	positive	Electrostatic	dynamic	68

* Van der Waals or/and hydrogen bonding.

cosylated variants together with the main variant and low abundance dimers was feasible.

A commercial, neutrally coated capillary, ending in a porous tip was employed by Haselberg et al. [73] which is designed for a nanospray sheathless interface (CESI-MS). The coating covers the capillary wall with the hydrophilic polyacrylamide-based agent (Sciex, Neutral OptiMS Cartridge), therefore ensures the extended separation time window and increases sensitivity. The group carried out intact and middle-up analysis of different mAbs. Intact analysis of ustekinumab by low-flow sheathless CE-MS yielded the partial separation of glycosylated and clipped species. However, following the digestion of mAb with IdeS enzyme, a detailed characterization of Fc/2 variants could be achieved. Furthermore, 36 variants were identified in total, including deamidated forms, glycosylated and clipped variants. BGE consisting of 10% HAc was found optimal for the measurements.

The identification of glycosylated variants was reported by Jooss et al. [74] and Schlecht et al. [75] in capillaries utilizing another neutral polymer coating, PVA. The analysis of trastuzumab (glycosylated and deglycosylated) charge variants was carried out, where the acidic variants could most likely be attributed to deamidation. The N-glycosidase F enzyme used for deglycosylation is known to induce additional deamidation and, as a matter of fact, the signal intensities for the acidic variants did increase in the case of deglycosylated mAb charge variant analysis. Authors suggest that the utilization of the proposed heart-cut instrumental setup circumvents the complications arising from using non-MS compatible BGEs. Therefore, this system allowed the use of an EACA based BGE (pH = 5.7) in the first dimension for mAb charge variant separations, whereas the second dimension applied a coated capillary, in which the ESI-interfering components were separated from the mAb variants.

In a recent paper published by Shen et al. [62] a novel neutral coating was developed for the native CZE-MS analysis of mAbs. The in-house made linear carbohydrate polymer (LCP) coating is based on the carbohydrate monomer 3-O-acryloyl-α/β-D-glucopyranose. The coating functions in a similar way to other neutral coatings (as earlier explained for LPA). However, the comparison of the electropherograms obtained with the LCP and LPA coated capillaries suggested that LPA coating still failed to suppress the adsorption of the macromolecules while LCP capillary coating presented a 6-fold increase in mAb peak intensity. The high peak intensity provided by the LCP-coated capillary was associated with the high resistance of carbohydrate-based polymers to non-specific protein adsorption.

Biacchi et al. [76–78] demonstrated the applicability of HPC coating for the separation of mAb variants. Although HPC (and HPMC) is generally used as a BGE additive, several studies show its implementation as a static coating. The coating is attached to the capillary surface by covalent interactions and covers the silanol groups so as to minimize adsorption. Thereby, the middle-up/down characterization of F(ab')2 and Fc/2 charge variants of cetuximab was feasible after fraction collection enrichment was applied to the sample [77,78]. Similarly, François et al. [79] presented the separation and identification of intact (Fc/2) and lysine truncated (Fc/2-K) variants of cetuximab Fc/2 fragment in a HPC coated capillary by using the same fraction collection strategy to off-line couple CZE to MALDI-MS/nanoESI-MS systems.

Dynamic neutral coatings have also been applied by some research groups. PEO is a linear polymer, which can also limit the possible mAb or BGE interactions with the capillary surface. However, in the case of dynamic coatings the effective coverage of the capillary surface and MS compatibility are always under question since a small "bleeding" of the capillary can lead to background noise, suppression of analyte signals and contamination in the ion source [80]. Nevertheless, Dadouch et al. [2] applied PEO coated capillaries for the analysis of intact mAb variants, subunits and domains. They investigated the possibility of integrating the reduction and IdeS digestion steps in-line into the CZE-MS workflow in order to facilitate and accelerate sample preparation for middle-up analysis. Another neutral and commercial dynamic coating, UltraTrol™ LN (Polymicro Technologies AZ, USA) was used by Höcker et al. [81] to compare the performance of nanoflow sheath liquid (SL) interface with the performance of triple-tube SL and sheathless interfaces for CZE-ESI-MS in the separation of reduced mAb subunits.

In the case of positively charged capillary coatings, the surface silanols are covered with positively charged groups, hence the direction of the EOF is reversed towards the anode. The counter directed EOF against the electrostatic forces ensures a better resolving power. Giorgetti et al. [82] used positively charged static PEI coating for the separation and identification of 3 different mAb components at the intact level. Due to the coating and optimized acidic BGE conditions they were able to detect trastuzumab, palivizumab and rituximab isoforms in less than 20 min with CZE-ESI-MS (RSD(t) < 3% for 10 measurements). This method also allowed the detection and identification of 1X-glycosylated and 2X-glucosylated forms. Recently, the same group [83] updated their work by trying the same coating and CZE conditions for the separation of 7 different mAbs not only in their intact forms but also at the middle-up (digestion) and middle-up reduced levels (digestion and reduction). The positively charged coating enabled the separation of acidic variants, main peaks and basic variants of mAbs in this order, in less than 12 min using an acidic BGE. The middle-up strategy allowed the identification of the isoforms of the Fc/2 and F(ab)'2 fragments, while variants containing micro- and macroheterogeneities from N-glycosylation to asparagine deamidation were separated following the reduction of the F(ab)'2 fragments into light chains and Fd parts. Similar experiments were performed by Belov et al. [72], who demonstrated the separation of Fc/2 fragment glycoforms after digestion with IdeS and the baseline separation of the 1X-glycosylated, 2X-glycosylated and aglycosylated forms of the intact species under denaturing conditions without digestion. Separation performance was increased by applying 1-(4-iodobutyl)4-aza-1-azoniabicyclo[2,2,2] octane iodide (M7C4I) as capillary coating, which has a lower reversed EOF mobility compared to the cross-linked PEI (cPEI) coating. The coating has a lower density of positively charged groups hence a lower reversed EOF is generated.

Another positively charged coating is the successive multiple ionic polymer layer (SMIL), which has been widely used for the CZE separa-

tion of smaller biological macromolecules. Previously, Haselberg et al. [68] suggested that a 3-layered coating covers silica surface fully and therefore suppresses the adsorption much better than a single layer of polybrene, due to the increased layer thickness. Additionally, SMIL coatings are known to show compatibility with the MS system causing no ionization suppression of components [84]. The first application of the multiple layer coating with PB-DS-PB for intact therapeutic mAb separation in a CZE-MS setting was presented by Minh et al. [14]. They investigated the denaturing/unfolding and the aggregation of therapeutic mAbs under storage stress. Their findings suggested that IdeS digested stressed samples contain unfolded monomer fragments as well as homodimers of Fab fragments.

In summary, the majority of CZE-MS works mentioned (as can be seen from the Table 2) present the application of static coatings while the use of dynamic and BFS appeared only in a few studies. The reasons could be the wide literature coverage about their efficiency, when applied for the separation of smaller intact proteins as well as the fact that static coated capillaries are typically the capillaries of choice by most pharmaceutical companies. However, recently, Hamidli et al. [85] presented a comparative study of uncoated, static and dynamically coated capillaries for intact protein analysis (differing from 5 kDa to 64 kDa in size). It was demonstrated that BFS capillaries can produce similar precision and efficiency values to coated capillaries, once the ideal separation conditions (e.g., pH 1.7–2.0 for BFS) are employed. Since there are a relatively few studies about the analysis of intact mAbs with CZE-MS with a given capillary coating and several controversial studies exist, there is a necessity for further research in this area.

2.3. Separation media for CZE-MS

The choice of BGE composition is quite limited for MS detection compared to conventional CZE-UV. CZE coupled with ESI-MS requires the use of volatile solutions. The components and additives commonly used in CZE-UV (e.g., phosphate, borate, detergents, polimers) should be avoided, since those would lead to volatility problems, adduct formation or ion suppression in MS. The most frequently utilized BGEs in a CZE-MS setting are HAc, FA and buffers of ammonium acetate and ammonium formate. However, the application of high pH BGEs has not become widespread, since if the pH of the BGE is too close to the pI of the mAb, its solubility is reduced.

The simplest and most often applied approach in CE-MS is the use of one-component acidic solutions as BGE (e.g., FA, HAc). 3% FA was applied for the analysis of intact adalimumab under nanoESI conditions in a fused silica capillary [86]. Reduced mouse IgG4 was analyzed using BGE consisting of 0.1% FA or 5% HAc in an LPA coated capillary [70].

Haselberg et al. performed the CE-MS analysis of intact and IdeS-digested (middle-up approach) mAbs [73]. The use of acidic BGE (10% HAc) for intact mAb separation provided the resolution of clipped species (light chain and light chain-heavy chain fragments) from the native protein. Moreover, glycoforms containing sialic acids were resolved from their non-sialylated counterparts. In the case of IdeS digests, BGEs consisting of either 10% or 20% HAc were used. The F(ab')2 and Fc/2 portions where efficiently resolved for the three mAbs.

CZE-MS separation of seven mAbs (adalimumab, natalizumab, nivolumab, palivizumab, infliximab, rituximab and trastuzumab) at the intact, middle-up and bottom-up levels was conducted with 3 and 10% HAc as BGE (pH 2.1). BFS capillary was used (100 cm, 30 μm i.d.) for bottom-up analysis, while PEI coated (80 cm, 50 μm i.d) capillaries were applied for the intact and middle-up levels. The separation of intact isoforms was obtained in less than 12 min. The MS spectra of intact mAbs proved the presence of major high molecular mass PTMs as N-glycosylations [83].

Using a PEI-coated capillary under acidic conditions, a higher EOF mobility relative to the electrophoretic mobilities of mAb was observed. The EOF mobility was typically two to three times higher than the analyte electrophoretic mobilities. Separation performance could be improved by reducing the EOF. The lowest EOF mobility and highest mAb apparent mobilities was found at BGE composition: 50% methanol + 1% FA. This acidified methanolic BGE was successfully applied for the middle-down analysis of Fc/2 glycoforms. For the intact level CZE-MS, however, the optimal BGE was found to be 10% isopropanol + 0.2% FA [72].

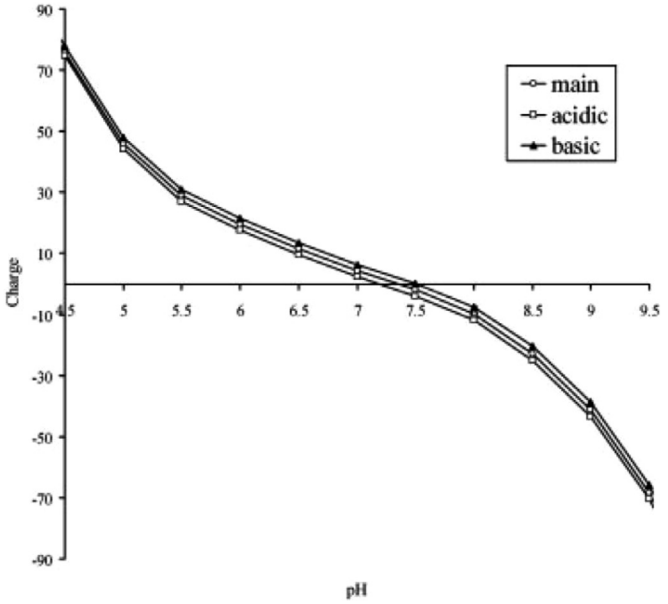

Fig. 7. The change in the calculated charge of basic, acidic and main species with the pH in the range of 4.5–9.5. Protein Calculator was used to calculate the pI and charge at different pH conditions [32].

Trastuzumab, rituximab and palivizumab were analyzed at the intact level applying 3% HAc as BGE in a PEI coated capillary. Sample buffers of different compositions were tested, based on which the optimal conditions were determined as 30% methanol, 1% FA with a 6.7 mM mAb final concentration. For each mAb, glycosylated structures were separated and identified. Deconvoluted mass spectra exhibited the classical glycoform pattern, the three most abundant glycoforms being G0F/G0F, G0F/G1F, and G1F/G1F. Concerning basic and acidic variants, potential aspartic acid isomerization and asparagine deamidation were observed [82].

A BGE of 0.2% HAc-10% IPA (pH 3.17) was applied in a microchip CZE ESI-MS system for the analysis of infliximab, an IgG2 type mAb and PNGase F digested NIST mAb [87–89].

Since charge variants of a given mAb have a similar charge profile in the entire pH range, their proper separation is mainly possible only in a very narrow pH range, where minimal differences arise in their charges. Under strong acidic conditions the differences between charge variants are not obvious. The total charge of charge variants decreases quickly whereas the relative charge differences among charge variants increases significantly when the pH increases from 4, thus the separation of charge variants can be mainly improved between pH 4–7 (Fig. 7) [32].

Different conformational states of infliximab were partly separated using a BGE of near physiological conditions (40 mM ammonium acetate, pH 6.0) in a PB-DS-PB coated capillary. The middle-up CZE-MS analysis of the stressed samples suggested that dimer formation involved mostly Fab-Fab interactions [14].

Six different mAbs were analyzed at the middle-up level using ammonium acetate buffer of differing pH (pH 3, 4, 4.5, 5) and concentrations ranging between 20 and 150 mM in fused silica and PEO coated capillaries. A novel automated methodology for mAb quality control at the middle-up level is described. In-line TCEP reduction of disulfide bonds and simultaneous IdeS digestion-TCEP reduction were successfully integrated inside the capillary. The method was used for MALDI-MS off-line

assays, the developed conditions are compatible with CZE-MS measurements [18].

Two-dimensional (2D, heart-cut) separation techniques have been developed to combine CZE with MS detection. Jooss et al. proposed a CZE-CZE-MS platform, where the 2nd dimension enables the elimination of interfering electrolyte compounds used in the 1st dimension by separating them from the analytes of interest. The two dimensions are interfaced using a mechanical four-port valve. Since the non-ESI compatible components are removed prior to MS analysis, various CE separation modes can be applied as 1st dimension including CZE, CIEF and CE (SDS). The designed 2D approach was utilized for the characterization of intact and deglycosylated trastuzumab. In the 1st dimension a generic EACA–based BGE was used (380 mM EACA, 1.9 mM TETA, and 0.05% HPMC, pH = 5.7). Analyte peaks were transferred from the CZE-UV to the CZE-MS dimension, where the highly ESI-interfering EACA was replaced by 2 M HAc as BGE in a PVA-coated capillary. The co-transferred ESI-interfering EACA was successfully separated from the mAb zones in the 2nd CZE dimension prior to MS detection. Deconvoluted mass spectra revealed the presence of potential deamidation products as acidic variants [74,75].

ZipChip Native Antibody Kit (908 Devices, Boston, MA, USA) with NH_4Ac (pH 5.5) and 4% DMSO as BGE was utilized by Carillo and Sun in microchip-CE-MS. Charge variant profiling of trastuzumab and bevacizumab were carried out, where charge variants derived from deamidation, succinimide formation, sialylation, pyro-Glu-formation, C-term lysine truncation [90,91].

2.4. MS analysis of mAbs

2.4.1. Technical requirements

The difficulties associated with CE-based approaches have already been discussed, however, the MS-linked analysis of mAbs brings an array of additional challenges and potential failures. It is beyond the scope of this review to describe a detailed comparison of ion sources and mass analyzers, herein only some general guidelines are highlighted to provide the reader a better understanding of what aspects to take into consideration when analyzing mAbs with MS.

Depending on the ion source used, the complexity of the mass spectra obtained may differ considerably. Generally, ESI sources are preferred because ESI-MS can be easily on-line coupled with flow systems (HPLC, CE). In ESI, multiply charged ions are generated, whereas using MALDI usually singly or doubly protonated ions are formed. Since resolution deteriorates with increasing molecular mass, MALDI cannot provide accurate mass determination [77]. On the other hand, ESI promotes various adduct formations leading to significant signal heterogeneity, which can also result in deviations from the theoretical mass, although to a much lower extent than with MALDI.

Another important aspect is the efficient transmission of mAb species into the mass spectrometer. MAb formulations often contain high salt concentrations, therefore mAb molecules are most probably present as salt and/or water adducts, which inhibits their sensitive detection. In order to enhance signal intensity, in-source collision induced dissociation (isCID) can be utilized [66,72,82,89,92,93], which assists in the declustering/desolvation of the molecule, without disrupting the non-covalently bound subunits.

The proper choice of mass analyzer is of crucial importance. On the basis of high resolution and high mass accuracy being the fundamental requirements, quadrupole time-of-flight (QTOF), Orbitrap and Fourier-transform ion cyclotrone resonance (FT-ICR) stand a chance in successful mAb analysis. Due to the immensely high purchase price and maintenance cost of FT-ICR instruments, their use is rather limited. Most publications in the CZE-MS literature of mAbs mention the utilization of either QTOF [14,73,74,77,79,81–83] or hybrid quadrupole Orbitrap [72,86,89–96] mass analyzers.

As concerns the true top-down investigation of mAbs, the tandem MS function comes into play. The most common fragmentation techniques include collision induced dissociation (CID), higher-energy collisional dissociation (HCD), electron transfer dissociation (ETD), electron capture dissociation (ECD) and ultraviolet photodissociation (UVPD). One of the main objectives of top-down or middle-down analysis is the sequencing of mAbs or their subunits with respect to the localization of PTMs. With regard to the fulfillment of such endeavours, CID and HCD are the weakest links, mostly because of limited fragmentation coverage [97] and their tendency to fragment the most labile bonds, which can result in the elimination of PTMs [98,99]. In the case of electron-driven fragmentation methods (ECD, ETD), however, PTMs are retained, since the preferential cleavage site is not necessarily the most labile bond, which allows us to reveal the location of PTMs. UVPD utilizes high energy photons and has the potential to enhance fragmentation coverage. Owing to the large size of mAbs and the ”protective” feature of disulfide bonds, the fragmentation of mAb molecules, as a whole, is exceptionally difficult. Therefore, the middle-down approach is preferred, where IdeS digested and/or reduced mAbs are subjected to gas-phase fragmentation [72,96]. Römer et al. performed a comprehensive study on a reduced mAb, exploring the complementary fragmentation pathways of HCD, ETD and UVPD [96].

2.4.2. Intact mass determination

Intact mass analysis can provide detailed insights about protein integrity and domain specific alterations, enabling glycoform characterization, determination of PTMs and disulfide bonds shuffling with high mass accuracy [100,101]. However, the analysis of complete mAbs in their intact state does not always yield the desired high mass accuracy, resolution and sensitivity, since the higher the molecular mass of the component, the more challenging it is to identify and distinguish the small mass changes (deamidation (~1 Da) or disulfide oxidation (~2 Da)) in the molecule. Therefore, middle-up strategies have gained attention, which entail the intact mass determination of larger subunits obtained as a result of specifically cleaving the mAb of interest. Applying simple sample preparation steps (e.g., reduction, IdeS digestion, stressing), it is possible to profile mAb HC and LC subunits [53,70,71], Fc and F(ab) fragments [71,79,83] as well as to identify different PTMs [19,73–76,89] and structural alterations [14,62,66]. So far, there is no method determining these small mass differences in intact mAbs without prior cleavage even by using high resolution mass analyzers [102,103].

The level of difficulty in interpreting intact mAb mass spectra is largely dependent on the chosen ionization method (MALDI vs ESI), as well. In general, ESI is the most frequently utilized technique owing to its ability to impart multiple charges on analytes, facilitating the detection of high molecular mass components in a lower m/z range, compared to MALDI spectra.

Under denaturing conditions (e.g., strongly acidic pH) ESI spectra show the presence of even higher charge states (thus smaller m/z values), resulting in broader charge envelopes. This became the method of choice in the analysis of mAbs to ease the interpretation of mass spectra and mass calculations. However, denaturation also implies that intra- and intermolecular bonds are destroyed and therefore, studying the quaternary, tertiary structures and non-covalent interactions is not possible [101]. For the investigation of higher order structure and protein self-assembly, native MS was introduced, which uses neutral pH to transfer the intact macromolecules into the gas phase, keeping their folded structures [101,102,104]. However, in neutral milieu, mAb molecules acquire a relatively low number of charges and thus, mass spectra display a narrower charge envelope. On the one hand, this increases the signal to noise ratio; on the other hand, the appearance of signals is shifted to a higher m/z range [101,102]. The latter aspect necessitates the use of analyzers with extended upper mass limit and high resolving power (e.g., Orbitrap).

In a recent interlaboratory study, Srzentric et al. [103] investigated the most common mistakes in the mass determination of mAbs. According to their observations these errors originate from i) incomplete reduction of disulfide bonds, ii) misassignment of the monoisotopic mass

and iii) using the average mass instead of the monoisotopic mass for calculations.

2.5. Coupling of CZE with MS

Given the intrinsic complexity of mAbs, direct infusion of such formulations results in complicated, overlapping spectra, inhibiting spectral elucidation. An upfront separation technique is therefore mandatory. Coupled techniques (e.g., CE-MS, LC-MS) play an important role in solving complex analytical problems [105]. Beyond LC-MS approaches, CE-MS has been witnessing an expanding array of applications, which has penetrated into the field of characterizing biopharmaceuticals. This is substantiated by the immense wave of publications related to such workflows [8,21,25,67]. While LC-MS is generally considered to be a go-to method for proteome profiling, there are certain instances where CE proves to be more appropriate due to its separation mechanism based on size-to-charge ratio – often revealing complementary information to those obtained by conventional HPLC approaches. The advantage of CE (regarding the analysis of mAbs) is the ability to separate species differing only slightly in molecular mass, provided that these modifications induce charged state variance. The electrophoretic mobility data acquired from the separation can even promote the correct assignment of mass spectral data, therefore CE, together with MS detection, has the potential to grow into a powerful analytical platform for the determination of mAbs and their microheterogeneities. The choice of interfacing; however, has a remarkable impact on the successful exploitation of what the instrumentation can offer. One of the main considerations is the efficient transfer of the separated analyte from liquid to gas phase. From among the atmospheric ionization techniques, ESI shows exceptional suitability for proteomic studies because of its ability to "make elephants fly" [106]. Moreover, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are better suited for less polar and apolar analytes. There are, however, certain difficulties arising when coupling CE with ESI-MS, namely, the establishment of a closed electrical circuit for CE, the careful choice of volatile BGE, the formation of a stable electrospray (despite the low (or no) liquid flow in the CE capillary) and maintaining harmony between the speed of separation and data acquisition. Ever since its introduction in 1987 by Smith and coworkers [107], CE-ESI-MS has undergone considerable improvements, with the development of various interface designs. In the following sections this progress will be shown concerning on-line and off-line hyphenation, limiting the scope to the platforms used for the determination of mAbs and their related products. Although the present review focuses on top-down analysis, the cited research articles dealing with the analysis of mAbs typically demonstrate the use of multi-level investigation (e.g., native, intact, top-down, middle-down/up and bottom up) in order to perform an exhaustive, in-depth characterization of these therapeutic proteins.

2.5.1. Offline coupling of CZE with MS

The main difficulties associated with CE-MS coupling can be alleviated by resorting to offline approaches. Offline coupling allows a broader sense of flexibility in terms of separation, as we are not restricted by having to find conditions that are MS compatible. By fraction collection, the fractions eluted can be buffer exchanged, if necessary, and infused into the MS. With such infusion experiments, sufficient amount of data points can be collected for each individual protein peak, which might assist in the confident identification and structural elucidation of protein species if a proper mass analyzer is at hand. The utilization of ESI sources prevail in the literature, especially the nanoESI-based implementations. These nanoESI interfaces produce ultra-low flow rates, which promotes ionization efficiency. Moini et al. laid the groundworks for the commercialization of the first robust nanoflow sheathless interface, by using an etched porous tip capillary emitter [23]. Such constructions are actually designed for on-line coupling, therefore their detailed description is given in the corresponding section (2.4.2.). Nevertheless, several research groups have reported on their utilization in an off-line manner, i.e., without a direct connection to a CE separation platform, simply as a means of sample introduction. The performance of such an etched-tip nanoESI interface was investigated by Jarvas et al. with infusion experiments using an intact mAb formulation [86]. Non-desalinated adalimumab was infused at different flow-rates (20 nL/min and 250 nL/min). Results showed no significant difference in spectra quality, despite the large difference in sample consumption. In addition to the better detection sensitivity, ultra-low flow rates are particularly advantageous in cases were limited sample amounts are available only. It is a well known fact, that ESI is rather intolerant to salts and non-volatile buffer components (e.g., EACA, which is a commonly used BGE additive for mAbs isoform separations). Regardless of having the relief of choosing less volatile BGEs in the case of offline couplings, Biacchi et al. [77] developed a CZE-UV/nanoESI-MS platform with fraction collection, where asymmetric CZE conditions were applied at the two ends of the capillary in order to render the collected fractions ESI-compatible. Here, the inlet BGE consisted of a mixture of EACA and ammonium acetate whereas the outlet BGE was chosen to be ammonium acetate. The collected fractions were introduced into the MS via a sheathless nanoESI infusion platform similar to the one mentioned previously. Using this workflow, the authors successfully determined the various glycoforms of cetuximab subunits, furthermore, in another study, Fc/2 dimers were also detected under native conditions [79].

Despite the dominance of ESI interfaces for CE-MS hyphenation, another typical example of the offline coupling is via the MALDI interface. MALDI can be considered a good alternative to ESI, since it is known to have a higher tolerance towards non-volatile BGE constituents. Biacchi et al. [76–78] developed an automated offline CZE-UV/MALDI-MS/MS arrangement for the multi-level analysis of mAbs. The separated protein zones were deposited on the MALDI plate with the assistance of sheath-flow using a home-built spotting device. It was also possible to perform enrichment using this configuration, by pooling the fraction from three consecutive separations. The system was capable of separating intact mAb charge variants; however exact mass determination was not possible. Since MALDI generates mostly singly or doubly ionized protein species, the resolution of the TOF analyzer is insufficient at such high m/z ranges [76]. Enrichment was useful especially for in-source decay (ISD) measurements, since single-run fractionations failed to provide sufficient amount of proteins for fragmentation [78]. The authors also found that the presence of EACA in the BGE compromised the co-crystallization of the sample and the matrix [77].

2.5.2. Online coupling of CZE with MS

The offline coupling approaches described in the previous section circumvent some of the difficulties associated with a true online hyphenation; however, they lack the level of robustness and automation that would be required for a routine and reliable workflow. For establishing the proper circuitry, the previously mentioned ESI is utilized, which can come in two main configurations: sheath flow and sheathless designs. The original setup uses a sheath flow (or make-up flow) which consists of a triple-tube arrangement. The separation capillary is surrounded by a double-walled metal tube in which the sheath liquid (SL) and nebulizing gas are transported. The SL is responsible for (i) creating electrical contact between the two ends of the CE capillary, (ii) stabilizing the electrospray and (iii) enhancing ionization efficiency, especially in cases where MS-incompatible BGE is used for separations.

Although the sheath gas is important for facilitating spray formation from the increased sample volume, the "siphoning-effect" arising at the end of the capillary generates an extra laminar flow, which lowers peak resolution.

The major argument in favor of the coaxial sheath-flow interface is the more flexible choice of BGE, since the effluent is considerably diluted with the make-up flow (mainly consisting of mixtures of aqueous and organic solvents), which reduces potential ion supression phenomena. This very advantage is actually a limitation, as well, because generally

the diluting effect takes its toll on detection sensitivity. Nevertheless, recently, Minh et al. examined what effect increasing SL flow rate (1–20 μL/min) has on the signal intensity of infliximab [14]. Results showed a linear increase in intensity up to 10 μL/min. There might be instances where native mAb analysis is desired, hence the denaturing effect of the organic solvent and acidic additive in the SL needs to be taken into consideration. In the same study Minh et al. also gave account of their findings on mAb unfolding using different SL mixtures. It was found that in contrast to SLs containing acetonitrile, methanol or isopropanol (IPA) did not induce the unfolding of infliximab, preserving its native state. On the other hand, it was hypothesized that higher flow rates (~15 μL/min) might induce unfolding after all, because of the larger amount of IPA and acidic modifier and/or the formation and propagation of an ionic boundary at the capillary outlet. Contrary to the popular and logical belief that coaxial SL shows weaker performance (higher LOD), there are other works demonstrating positive results utilizing such an interface for mAbs analysis [53,74].

Another type of SL interface is the flow-through microvial proposed by Chen and coworkers [108], which, compared to the conventional coaxial setup, allows the application of flow-rates down to 0.1 μL/min, minimizing the unfavorable effects of dilution by the SL. The system utilizes a tapered and beveled emitter tip to increase spray stability. A flow-through microvial is formed at the tip of the emitter, where a modifier solution and the CE effluent meet. The modifier solution is introduced by a second capillary via a tee-union. The platform was successfully exploited for *de novo* monoclonal antibody sequencing [109].

Sensitivity problems commonly associated with the conventional coaxial ESI interface can be overcome by utilizing nanoelectrospray designs instead, which can either be sheath-flow or sheathless configurations. These typically operate at a flow rate of 10–150 nL/min. The Dovichi research group devised a nanoESI interface where the SL is electrokinetically pumped, resulting in a lower diluting effect [110]. The CE capillary is guided into a borosilicate emitter through a cross-piece, to which the SL reservoir is also connected by a tube. Three generations of the interface were introduced: the second generation strived to enhance sensitivity by decreasing the distance between the capillary tip and the emitter orifice. For this purpose the capillary exit was etched that it should better approach the emitter orifice. However, the extremely narrow (2–10 μm) diameter of the orifice often caused clogging. The design was further improved by increasing the diameter (15–35 μm) and reducing the distance from the capillary tip to the emitter orifice. The interface is commercialized under the name EMASS-II and has been used for intact mass [71] a native mAbs [62] studies. The scientific literature is crowded with workflows utilizing an SL containing an organic solvent, which is necessary for providing a favourable environment for ionization. This is why it is quite noteworthy that (for the sake of maintaining native conditions during the ESI process, as well) Shen et al. used an SL containing only ammonium acetate [62].

Most recently, prompted by encountering occasional technical difficulties with the already existing nanoemitter interfaces, Neusüss et al. developed a nanoflow SL interface consisting of two axially movable capillaries – one for separation, the other for delivering the SL [111]. The two capillaries were parallelly threaded through a PEEK tube and cross piece into a borosilicate emitter. Depending on the position of these two capillaries relative to each other, two operating modes were possible: separation and conditioning modes. The SL capillary has a much wider outer diameter and a blunt tip, which does not slide into the tapered glass emitter, therefore, in conditioning mode, when this capillary is positioned closer to the emitter orifice, there is considerable backflow into the separation capillary. The significance of this backflow is two-fold: (i) CE effluents containing ESI-interfering components can be blocked from entering the MS and (ii) the conventional capillary priming with HCl, NaOH or rinsing with coating materials is possible without the need to displace the capillary outlet from the ion source. The proposed interface setup was utilized for the top-down analysis of a reduced mAb in an online two-dimensional CE-MS manner, where the second, CZE dimension was for eliminating the eluting SDS from the first separation dimension [96].

Sheathless approaches generously improve detection sensitivity. In the absence of SL, much creativity has gone into designing ways of creating a closed electrical circuit. Among these the porous tip nanospray sheathless interface is enjoying widespread recognition and is actually commercially available (CESI 8000). Here, the end of the CE capillary is made porous by etching with HF. The capillary is placed into a grounded stainless steel needle containing conductive liquid, making sure the tip protrudes. The movement of ions from the liquid through the pores allows for the electrical contact. The majority of works on mAbs analysis uses such a sheathless interface, which certainly shows what a breakthrough it really was [26,66,72,73,82,83,112–116]. A systematic study comparing the performance of the three most common interfaces (coaxial, nanoflow sheath liquid and sheathless porous-tip) was published by Höcker et al. [81]. The results clearly indicated the positive impact of nanoflow interfaces have on the ionization efficiency of mAbs species.

2.5.3. Chip-based on-line CZE-MS

The growing need for ”cheeper, better, faster” is leading the scientific community to resort to miniaturization (lab-on-a-chip conception). The field of microfluidics has seen an astounding progress over the past couple of decades. In the context of separation sciences the ultimate goal is to develop a user-friendly ”plug and play” microdevice capable of robust, reproducible performance. Downscaling electrophoretic separations to a microchip format seems ideal, given the nearly identical geometric dimensions of the fused silica capillary and microfluidic channels (10–100 μm). Although the fabrication of such microchips as well as their online integration into analytical workflows requires a certain level of craftsmenship, there are several advantages associated with their utilization, e.g., lower reagent and sample consumption, smaller spatial requirement, higher throughput or shorter analysis time.

Microchip CZE (MZE) has long been used for the analysis of mAbs, with on-chip UV [117], LIF [118] or chemiluminescence [119] detection. When coupling MZE to MS (off-chip detection), one faces basically the same challenges as with conventional CZE-MS hyphenation: most importantly the establishment of the electrical circuit and the generation of stable electrospray. The volumetric flow rate during MZE separations matches that of the nanoflow regime. Much effort has been put into forming the nanospray either by direct off-chip spraying or by attaching an external capillary or microfabricated emitter. Out of the proposed techniques, the direct off-chip spraying from the corner of the microchip proved to satisfy nanospray-requirements the most. For a more detailed description of the other implementations, the reader is referred to reference [120].

The pioneering work for a stable MZE-ESI-MS platform was done by the Ramsey group [121] and since then, it has been perfected into a well-established method for the routine analysis of mAbs species [87,88,122]. Microchips were fabricated by means of photolithography and wet-etching in glass substrates. The chip design included a separation channel, injection cross, an electroosmotic (EO) pump and the integrated emitter (Fig. 8). Sample introduction was carried out using the gated injection scheme developed by Jacobson et al. [123]. This method is similar to the standard electrokinetic injection, in the sense that voltage is applied for a plug of sample to be diverted into the separation channel. It relies on the proper switching of voltage profiles of the buffer and analyte reservoirs. The separation channel is a meandering structure, allowing a sufficiently long (23 cm) channel to fit onto the microchip. Channels were asymmetrically tapered at the curvatures in order to minimize zone dispersion [123,124]. Because it was found that a sharp geometric feature is desired for stable and simple spray formation, the outlet of the separation channel was directed towards the corner of the microchip. The EO pump is a key functional element to support the nanospray. The successful incorporation of the proposed chip into the CZE-ESI-MS system culminated in the chip layout actually being used as basis for a commercially available MZE-ESI system (ZipChip^{TM}, 908

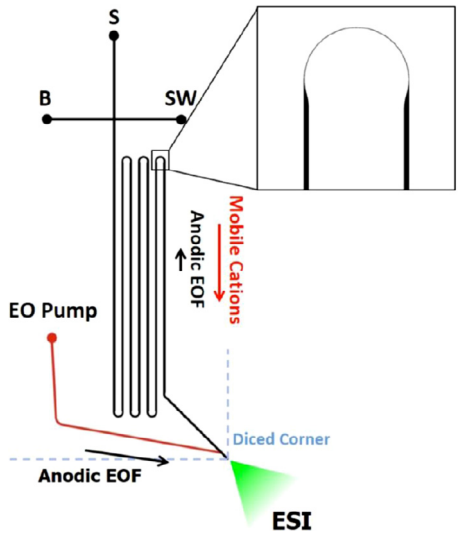

Fig. 8. Schematic drawing for chip-based CE-ESI devices with a 23 cm separation channel, showing an enlarged image of the asymmetric turn tapering. Red channels indicate an APS coating while black channels indicate an APS-PEG_{450} coating. S: sample reservoir; B: background electrolyte reservoir; SW: sample waste reservoir; EO: electroosmotic pump reservoir [87].

Devices Inc., Boston, MA, USA). The operation of the ZipChip platform, therefore is quite similar to the above discussed microchip. The main differenc can be seen in the nature of sample injection, ZipChip utilizes pressure-driven flow, preventing electrophoretic bias. The system comes with the chip itself with the built-in nanoemitter and a housing interface, which can be connected directly to the MS. The manufacturer offers a variety of microchips for specialized fields of application. For the determination of mAbs the High Resolution (HR) [89,91,94,95] and the High Resolution Native (HRN) [90,91] chips can be used (Fig. 8). Analysis times range from 3 to 10 min, which demonstrates the possibility for the rapid screening of mAbs heterogeneities.

2.6. Applications

Ongoing increase of mAb applications in medical treatments increased the need to develop analytical methods for the characterization, quality control and stability testing of preparations [100]. This section summarizes CZE-MS applications that may be useful during the production or preparation of mAbs as well as formulation or stability testing of mAb-containing medicine.

The parameters that can be determined using CZE-MS can be divided into three groups: parameters for characterizing the mAb, parameters for identifying and measuring protein variants and degradation products, and finally, parameters for measuring process related contaminants (Table 4).

Determination of molecular mass is possible by MS but the goal of mAb analysis is rarely limited to molecular mass determination only. More often, the mass characterization of the different variants and PTM products is performed where identification is based on molecular mass determination. Alterations in the primary structure of a protein can occur as a result of changes at the nucleic acid or protein level, which fall into three categories: mutations at the DNA level, misincorporation at the protein level due to mistranslation and miscleavage during the posttranslational processing. Sequence variants are a concern during the production of recombinant human mAbs that are being developed as therapeutics. Detection of sequence variants during clone selection and bioprocess development are important for the biotechnology industry, as well. In order to differentiate between sequence variants and PTM products or to increase the sensitivity and simplify the spectrum, the enzymatic cleavage of glycan chains may be necessary.

Intact mAb analysis better defines the accurate mass and heterogeneity of mAb proteoforms but unfortunately, most of the intact mAb analyses by CZE-MS are performed under denaturing conditions [87] or using reduced and deglycosylated proteins [71], which most likely leads to a loss of information concerning mAb structural changes.

Complete glycosylation profile was identified in an intact mAb using CZE-MS [72]. All three detected populations (aglycosylated, mono- and diglycosylated) were separated using a BGE containing 10% isopropanol and 0,2% FA in a M7C4I coated capillary. Taking a more detailed look into the TIC peaks revealed the presence of seven diglycosylated and four monoglycosylated components in the first and second peaks, respectively. Aglycosylated and glycated mAbs co-migrated in the third peak. Populations were resolved due to differences in the hydrodynamic volume which decrease in the order of the 2X-glycosylated, to the 1X-glycosylated, to the aglycosylated mAb. The ratio of the 2X-glycosylated to the 1X-glycosylated to the aglycosylated mAb species was determined under denaturing and native conditions, which was found similar. In native experiments a polyacrylamide coated capillary, and a rinsing protocol including 0.1 M HCl, water and BGE (40 mM ammonium acetate, pH 7.5) were used. The separation of individual glycosylation states did not occur, however, this approach resulted in the detection of dimers (at less than 1% relative abundance), which actually co-migrated with the main forms of the mAb.

Recently, native CZE-ESI-MS has emerged as a promising tool for the separation and identification of complex proteomes including up to thousands of proteins from only submicrograms of samples [98]. It is important to mention that in native MS, proteins are present in their native form in solution, prior to the ionization event. This requires that the parameters of the solution (such as the pH, ionic strength or the organic solvent content) should be kept under strict control. Avoiding the use of organic solvents during the ESI process results in a sharper charge distribution, which can be advantageous in identifying different proteins. The term "intact protein" is used in the sense that no chemical or enzymatic treatment was performed on the mAb tested during sample preparation.

New solutions have been found to overcome the existing challenges in the native CZE-MS characterization of mAbs, such as inappropriate separation of different mAb variants or low sample loading capacity. An online sample stacking method based on capillary isoelectric focusing (CIEF) in a narrow pH range was developed to expand the loading capacity as well as to improve the CZE separation in the native condition [62]. The authors employed separation capillaries with a carbohydrate-based neutral coating, an electrokinetically pumped sheath flow CE-MS interface, and a high-end QTOF mass spectrometer. Using this method, the separations of different proteoforms of the SigmaMAb and the detection of its various glyco-proteoforms and homodimer were documented.

CZE-native MS reported by Le-Minh et al. [14] was used for the characterization of IgG-type mAbs. Authors used a triple layer coated (PB-DS-PB) capillary to prevent the adsorption of mAbs and applied additional pressure for the analysis of fresh reconstituted and stressed (6 month storage at 4 °C) infliximab samples. CZE allowed for a partial separation of different conformational states, and the MS detection under specific conditions enabled the identification of the native, unfolded monomers as well as the dimers.

A recombinant humanized IgG1κ antibody model (NIST RM8671) containing high abundance of PTMs (C-terminal lysine clipping, N-terminal pyro-glutamination, glycosylation, low abundance of glycation, deamidation, and oxidation) was characterized by CE-MS [89]. A ZipChip^{TM} microfluidic CE device was used for the separation of intact protein forms using a BGE that consists of 0.2% HAc and 10% 2-propanol (pH 3.17). 18 variants were identified amongst proteolytic and glycolytic modifications with a range of relative abundances between 0.1% and 100%. Lysine variants (0, 1, 2), and five to seven glycosylated forms of these variants were identified. In addition, glycation with one or two hexose units was also detected after the enzymatic deglycosylation of the protein.

Table 4
Targeted attributes of mAbs to be determined by CZE-MS.

Quality attribute	Attribute	Capillary coating	Ref. (example)
Characteristics	molecular weight	several	[8]
	primary sequence and amino acid substitution[1]		
	pattern of glycosylation	PEI	[83]
	side specific glycosylation properties	M7C4I	[72]
Product-related impurities (variants/degradants)	aggregation (dimers)	LPA	[72]
		PB-DS-PB	[14]
	fragmentation (proteolytic products)	ZipChip	[89]
	C terminal modification (lysine variants)	PEG	[87]
	oxidation[2]		
	deamidation	LPA	[73]
		2D: BFS + PVA	[74]
	halfmer and Fab arm exchange	LPA	[73]
	N terminal pyroglutamic acid	ZipChip	[93]
		PEI	[83]
Process-related contaminants	host cell proteins (HCPs), residual protein A, host cell DNA (HC-DNA), etc.	LPA	[129]

[1] those are determined by BUP methods.
[2] those are determined by middle-up methods.

Another research using the chip platform was reported by Redman et al., who applied an in-house-built microchip CZE-nanoESI–MS system for the analysis of intact infliximab. In order to prevent analyte adsorption and to reduce the EOF, the channel surface was coated with an aminopropylsilane (APS) base layer followed by its covalent modification with PEG. Minor basic and acidic variants were visible to the left and right of the three major peaks corresponding to the well-separated lysine variants. 0 K, 1 K and 2 K variants were characterized in detail, also revealing the glycosylation structure [87].

A microfluidic chip together with the BGE of the Native Antibody Kit (ammonium acetate at pH 5.5, modified with 4% DMSO) were used for the separation of a mAb type IgG1 manufactured by HJB Ltd [91]. Native-CE-MS spectrum of the unstressed antibody contained the acidic and basic peaks in addition to the main peak. The order of peaks in native CE-MS was reversed relative to cation exchange chromatography (CEX), the acidic peaks migrated after, while the basic peaks appeared before the main peak (Fig. 9). The main peak corresponded to two heavy-chain N-terminal pyroglutamic and C-terminal lysine clipping, and showed two different glycosylation profiles. For basic variants, the molecule of peak B1 and B2 were formed by C-terminal amidation in one and two chains, respectively. For acidic variants A1 and A2 were concluded to be the products of deamidation and different glycan modifications.

Cetuximab charge variants were also succesfully separated by the ZipChip system. The separation yielded eight baseline resolved peaks [93]. Native-like mass spectra were collected for each charge variant peak and more than 200 isoforms were identified. Compounds containing two, one or no C-terminal lysine were identified. The presence of an extremely large number of isoforms is due to the variety of oligosaccharide antennas present at the four glycosylation sites [93].

A detailed CZE-MS study of seven mAbs (adalimumab, infliximab, trastuzumab, nivolumab, palivizumab, natalizumab, rituximab) approved by health authorities was published recently [83]. CZE separations of intact mAbs were performed using a PEI coated capillary, acidic BGE and acidic methanol/water sample buffer. The electropherograms obtained show differences in profiles due to the different heterogeneity and pI values of samples. General profiles contain acidic variants preceding and basic variants after the main peak because of the positive coated capillary and reversed EOF. Analysis of MS data obtained by using a sheathless CZE-ESI-MS coupling resulted in the characterization of several PTMs. Pyroglutamine formation by cyclization of N-terminal Gln-of nivolumab, palivizumab, natalizumab and rituximab was observed, and 1x and 2x glycosylated forms of mAbs were identified in partially separated peaks. Thorough analysis of MS data obtained from separated peaks allowed identification of the fucosylated core and a galactosyl-fucosyl derivative of the core oligosaccharide in monoglycosylated adalimumab. Three other diglycosylated forms were also identified in the peak corresponding to the 2x glycosylated mAb. It was concluded that due to the limitation of mass accuracy, it is impossible to identify the other modifications.

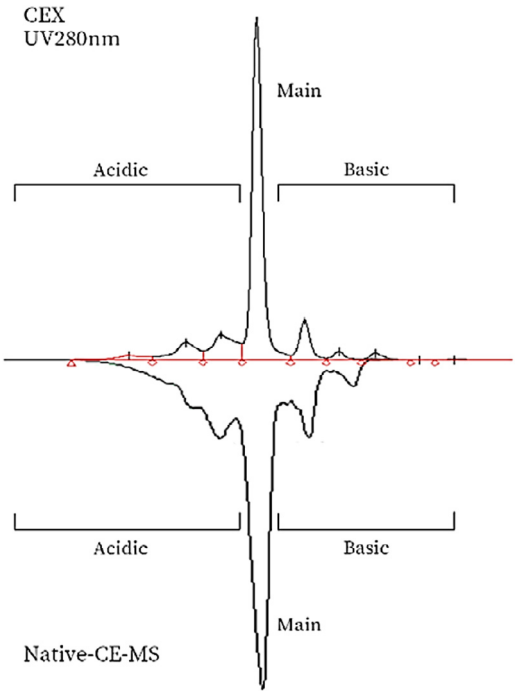

Fig. 9. Comparison of the separation of mAb using CEX (top spectrum) and Native-CE-MS (bottom spectrum) [91].

Ustekinumab and trastuzumab were selected for intact mAb characterization by low-flow sheathless CE-MS [73]. For separation, 10% HAc as BGE was applied in a polyacrylamide-coated capillary. In the case of trastuzumab three peaks appeared in the electropherogram: the main peak contained a mixture of trastuzumab glycoforms (non-sialylated fucosylated diantennary, harboring one glycan species) with loss of a C-terminal lysine, the first minor peak corresponds to a dissociated light chain of trastuzumab while the masses of the second minor peak were the same as those of the main peak. Based on the shift in migration time, this peak was identified as the deamidated forms of trastuzumab, which are less positively charged than the native protein.

A 2D CE-MS system was developed by Jooss et al. [74] for the analysis of intact trastuzumab charge variants. The EACA-based CZE–UV

system, using a fused-silica capillary, was coupled on-line to the CZE–MS system applying a PVA coated capillary and a BGE of 2 M HAc in the second dimension. Charge states ranging from +38 to +62 were observed in the mass spectrum. The CZE–CZE–MS setup was validated regarding migration time, signal intensity, current and flow rate stability. Highly precise mass data were obtained, which enabled the identification and discrimination between single and double deamidated forms of deglycosylated trastuzumab. Authors concluded that the proposed 2D method can become a useful tool for the MS characterization of antibody variants separated in ESI-interfering electrolytes, since it allows the utilization of proven, robust methods routinely used in biopharmaceutical applications (such as the EACA-based CZE separation) in the first dimension.

An example of the process related contaminants is the host cell proteins (HCP) present in recombinant therapeutics. The use of capillary zone electrophoresis (CZE) for the absolute quantification of HCPs in recombinant mAbs has been reported by Zhu et al. [128,129]. Using tryptic digestion as sample preparation without mAb depletion by Protein A affinity chromatography, authors could detect proteins present at an estimated 100 ppm level with respect to the antibody. CZE-MS/MS generated higher peak intensity and more peptide identifications for low-level spiked proteins than those obtained by LC-MS/MS.

Although intact protein analysis still poses challenges for the analyst, its huge advantage lies in the ease of sample handling, which not only simplifies the sample preparation process but can also result in obtaining information that is representative of the entire sample. The data in Table 4 demonstrate that CZE-MS is suitable for the comprehensive characterization of mAbs, including a large variety of potential parameters to be analyzed, however, for certain parameters only a few works have been published or no publication exists at all so far.

3. Conclusions

In the last decade the importance of implementing the TDP approach in mAbs analysis has clearly gotten stronger, and its significance continues to rise due to the intensifying analytical demands of the pharmaceutical industry. TDP allows complete sequence coverage and is able to provide information about the entire intact proteins including the revelation of proteoforms with PTMs. Another beneficial feature of the TD approach is its simplicity concerning sample preparation compared to the time consuming digestions used in BUP. Furthermore, both the accurate molecular mass and several microheterogeneities can be determined when coupled to a separation technique.

CZE is considered an alternative to advanced chromatographic methods for the analysis of mAbs as those are well applicable for the high-resolution separation of intact proteins, proteoforms or even protein complexes. An additional beneficial feature of CZE for mAb analysis is that the separations can be performed under near-physiological conditions thus the protein conformation, folding or function can be preserved during the analysis. Several works were published where CE revealed complementary information to those obtained by conventional HPLC approaches. The advantage of CE is its ability to separate species differing only slightly in molecular mass, provided that these modifications induce charged state variance (e.g., deamidation variants). An intercompany study (based on 1056 separations) involving 11 large pharmaceutical company laboratories proved that validation characteristics according to ICH Q2 guideline can be excellently fulfilled with CZE. The major limitation of CZE is the poor concentration sensitivity, however, high concentration (but low volume) of the pharmaceutical mAb samples for their characterizations can be applied.

Still, there are only a relatively few papers about the CZE-MS analyses of mAbs despite the fact that the ongoing increase of mAb applications in medical treatments is triggering the need to develop analytical methods for the characterization, quality control and stability testing of preparations. However, these methods are often part of the inside information of pharmaceutical companies and are not published. This may be the reason for the relatively small number of publications providing methods for the analysis of real samples (intermediate mixtures, drugs, serum, clinical samples, etc.). Many more publications about mAb analyses with CE-UV are available, however, some electrolyte systems cannot be transferred to MS-coupled systems. Since CZE provides superiority in the determination of charge variants (which are one of the most important components to be targeted in industrial pharmaceutical analytical laboratories), CZE-UV is frequently applied for such tasks, however, we could not find a work with sheath-flow MS detection. Since there are several mAb attributes whose characterization is not widely described, a considerable increase in the number of publications is desired.

Although the TD approach provides an expanding range of answers regarding the quality and composition of mAbs due to an overall development of the proteomics toolbox, further advancements are necessary to explore the full picture. It seems clear that the development of one single method that is applicable for full characterization cannot be expected, but it is certain that the CZE-MS technique is unavoidable/essential in solving analytical tasks for mAbs. The microchip CZE-MS technology using a sheathless arrangement appeared (as a commercial instrument) only a few years ago, but it can be considered as a great promise since it could already demonstrate its applicability in the determination of versatile attributes of mAbs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Cynthia Nagy: Writing – original draft, Writing – review & editing, Investigation. **Melinda Andrási:** Writing – original draft, Investigation, Data curation. **Narmin Hamidli:** Writing – original draft, Investigation, Data curation. **Gyöngyi Gyémánt:** Writing – original draft, Investigation, Data curation. **Attila Gáspár:** Writing – original draft, Writing – review & editing, Conceptualization, Project administration, Funding acquisition.

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