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Capillary electrophoresis and the biopharmaceutical industry: Therapeutic protein analysis and characterization

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ABSTRACT

Capillary electrophoresis (CE) has emerged as a powerful technique for comprehensive physicochemical characterization of monoclonal antibodies (mAbs) as well as other therapeutic modalities. The method provides high resolution separation and high sensitivity characterization for analysis of therapeutic biomolecules. CE based techniques such as sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE, also referred to as CE SDS), capillary zone electrophoresis (CZE) and imaged capillary isoelectric focusing (iCIEF) have been increasingly adopted to assess size heterogeneity, glycosylation heterogeneity and charge heterogeneity in mAbs and related therapeutic modalities. This paper reviews the latest application developments of CE based methods for routine release testing, stability testing and in-depth characterization. In addition, advantages and disadvantages of each of these techniques are critically discussed.

1. Introduction

Monoclonal antibodies (mAbs) and related therapeutic modalities, e.g. highly glycosylated Fc-Fusions, bi- and tri-specific mAbs, mAb-ADCs, mAb fragments, PEGylated proteins and other varieties have entered the market or clinic [1] and represent a rapidly growing class of therapeutics treating numerous diseases such as cancer, autoimmune disorders, and various infectious diseases including the culprit of the recent pandemic. mAbs alone have a revenue potential of USD 300 billion by 2025 [2]. Due to their proteinaceous nature, heterogeneity accumulates throughout bioprocessing, with potentially deleterious variants acquired from mutations during translation, uncontrolled modifications after translation (post-translational modifications, or PTMs), and degradation events occurring during the complex manufacturing process such as fermentation, purification and storage. PTMs of a single protein sequence can lead to large molecular diversity given the number of functional groups subject to various reactions including glycosylation, glycation, phosphorylation, etc. [3]. Protein degradation events are often chemical in nature and can lead to, amongst other things, a reduction in efficacy or increase in aggregation and even immunogenicity, and these events occur at varying rates depending on sequence, formulation, and storage conditions [4]. Thus, stringent analytical methods are necessary to comprehensively identify, characterize and monitor these quality attributes and ultimately to ensure the release of purified and efficacious drug products at the clinic and commercial stages. The latter requires validated testing be performed in a quality control (QC) laboratory with fit-for-purpose instrumentation and use of appropriate reference controls and system suitability tests. The results need to fit within set acceptance limits, or specifications, on the reported level of purity, amount(s) of known impurities, potency, etc., to control

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Abbreviations:

ADC	Antibody Drug Conjugate
BGE	Background Electrolyte
BLA	Biologic License Application
CE	Capillary Electrophoresis
CEX	Cation Exchange Chromatography
CPA	Corrected Peak Area
CZE	Capillary Zone Electrophoresis
DMSO	Dimethyl Sulfoxide
EOF	Electroosmotic Flow
ESI	Electrospray Ionization
FDA	Food and Drug Administration
GU	Glucose Units
HILIC	Hydrophilic Liquid Interaction Chromatography
HMW	High Molecular Weight
iCIEF	Image Capillary Isoelectric Focusing
IEX	Ion Exchange Chromatography
IND	Investigational New Drug Application
LC	Liquid Chromatography
LIF	Laser Induced Fluorescence
LMW	Low Molecular Weight
mAb	Monoclonal Antibody
MS	Mass Spectrometry
NG	Non-Glycosylated
pI	Isoelectric Point
PTMs	Post Translational Modifications
QC	Quality Control
QL	Quantification Limit
RSD	Relative Standard Deviation
SDS-CGE	Sodium Dodecyl Sulphate-Capillary Gel Electrophoresis
SEC	Size Exclusion Chromatography
UV	Ultraviolet

critical quality attributes [5], with Health Authority monitoring and feedback at each stage of the process [6]. Similar requirements apply to the commercialization of biosimilars with the added expectation of establishing comparability to the original product [7].

Capillary electrophoresis (CE) has proven to be an essential separation technique for these purposes because of its fast analysis times with minimal sample consumption, high resolution, general adaptation into the QC environment, and equally importantly, complementarity to chromatographic approaches. The most popular modes in the industry include capillary SDS gel electrophoresis (SDS-CGE), capillary zone electrophoresis (CZE) and capillary isoelectric focusing (regular cIEF and imaging iCIEF modes) [8–13], with some modes readily hyphenated to mass spectrometry (MS), and are routinely utilized to assess size, charge and glycosylation heterogeneity of protein biologics.

2. SDS-CGE and size heterogeneity

Size heterogeneity is a critical aspect of the physicochemical characterization of a biological product [ICH Q6B]. Product related aggregates (dimers, tetramers and even multimers), also known as high molecular weight (HMW) species, and fragments of low molecular weight (LMW) relative to the parent molecule are often considered critical quality attributes requiring close monitoring during manufacturing, release, stability and storage and must meet set acceptance criteria to minimize the risk of negative immune responses and/or reduction in potency [14–16].

SDS-CGE is used for the purpose of evaluating size heterogeneity under denaturing conditions with platform reagents and automated instrumentation available from several commercial manufacturers that can be utilized in the QC space. The performance and usability of some of the more commonly used instruments in the industry have been compared [17]. The method proceeds as follows. Briefly, upon application of an electric field, SDS-protein complexes, ideally of uniform surface charge densities, migrate towards a detector window to produce an electropherogram of peaks representing increasing size over time. The most common (and commercial) approach uses a capillary consisting of an inner surface composed of bare-fused silica and filled with a sieving matrix of dextran cross-linked with borate and other components which serve to alter electroosmotic flow (EOF) [18,19].

Ideally, during electromigration, sample proteins are to be in a uniformly denatured and unfolded state with SDS bound at an optimized ratio with the protein [20]. This may be challenging for proteins that, for example, contain PTMs or highly negatively-charged domains resistant to SDS binding and include proteins with relatively high kinetic stability [21,22]. Method-induced HMW formation can be a consequence of a non-ideal SDS-protein ratio and can be detected by observing unexpectedly large variations in relative % values upon testing across the anticipated concentration range of the assay [12]. Correction may require optimization of the SDS-protein ratio and/or a change of detergent to one that binds protein with higher affinity (Fig. 1A) [13]. Alteration of the monomer/cross-linker ratio has also proven to be useful to increase the resolution of highly glycosylated species [19] as well as increasing the capillary length and optimizing denaturation temperature (Fig. 1B) [23]. In addition, sample preparation requires careful consideration of pH, denaturation time, and concentration of a free-thiol alkylating agent to prevent disulfide scrambling, or disulfide reductant etc., with a primary goal being the prevention of excess method-induced fragmentation [24]. Achieving this goal would predictably improve method robustness and reproducibility and ensures that the observed fragments represent real species present in the sample prior to treatment.

Optimal separation conditions would ensure adequate peak quantification of product and product-related variants of interest, either known or anticipated. The optimization process may require testing of aged samples, specimens from in-process purification steps, and samples forcibly degraded, e.g., by subjecting it to partial reduction, deglycosylation, UV light exposure, high pH, high temperature, and/or peroxide treatment [25]. For example, partial sample reduction assessments are necessary if the upstream fermentation process produces enzymatically-reduced mAb product impurities [26] and peptide N-glycosidase (PNGase) deglycosylation experiments ensure that a protein yielding a broad peak profile is simply a homogeneous protein bound to a highly complex network of N-glycan structures. Representative in-process samples can be useful in the case of antibody-drug conjugates (ADCs) by helping to identify the mAb-drug stoichiometry via comparison of electropherograms of pre- and post-conjugated samples [27,28], or to ensure clearance of monoclonal parental mAb impurities from the target bispecific mAb product [29].

For a typical mAb, SDS-CGE is considered an “LMW method” due to its ability to resolve product-related species smaller than the parent molecule and complements size exclusion chromatography (SEC) when needed to assess the larger HMW species [30]. SDS-CGE is run under denaturing conditions thus can reveal variants not observed by native SEC, for example, a fragmented protein held

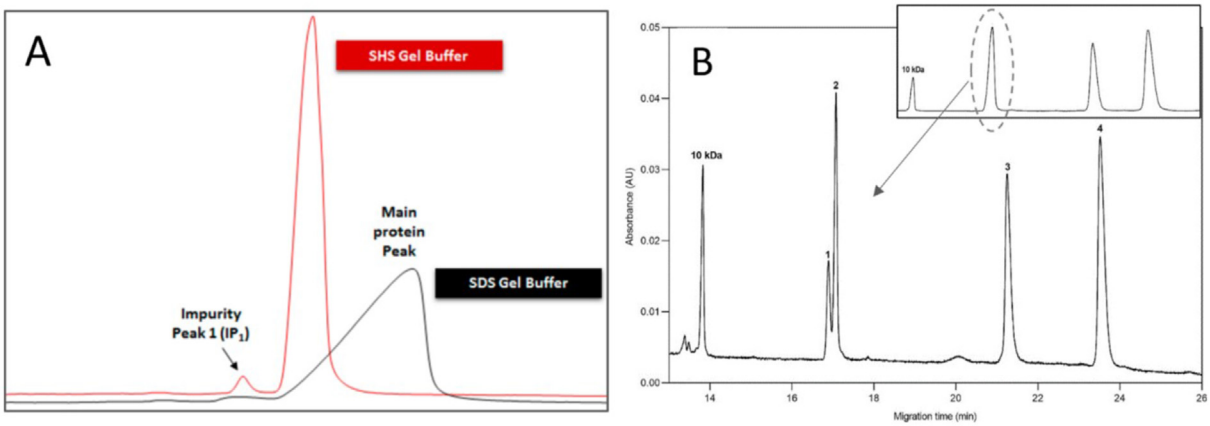

Fig. 1. Improving CGE separation efficiencies when evaluating challenging protein modalities. A) Use of higher affinity detergents, in this case SHS in place of SDS, to improve the resolution of the main peak of an Fc-fusion from a product impurity (with permission from Ref. [13]). B) Optimizing denaturing temperature and increasing the effective capillary length from 10 to 20 cm to resolve two light chains with similar MW of a bi-specific mAb. Inset: 10 cm effective capillary length (modified with permission from Ref. [23]).

together by secondary and/or tertiary interactions [31]. Denaturing and reducing conditions can reveal fragmentation not observed under non-reducing conditions alone, for example, when a CAP256 mAb clip was shown to only be held together by an *intra*-disulfide bond [32]. If an important size variant cannot be resolved from its intact parent molecule due to relatively small molecular weight (MW) differences, dropping the MW via reduction and/or enzymatic cleavage may be sufficient to achieve full resolution. For example, accurate and quantitative tracking of the non-glycosylated (NG) IgG variant, a variant linked to reduced bioactivity and effector function [33], can be achieved by mAb reduction or IdeS cleavage to fully resolve NG-Heavy Chain or NG-Fc/2 from their parent molecules respectively [10].

When sensitivity is a concern, signal can be enhanced through use of CE with laser-induced fluorescence (LIF) detection [34] either using native fluorescence or after proper labeling. SDS-CGE microchip instrumentation using LIF (μSDS-CGE) has been widely implemented in process development laboratories due to its high sensitivity and throughput capabilities, but has had limited success as a QC release method due to reproducibility concerns [35]. Although release testing typically does not require high throughput, bridging this technology into commercial QC laboratories would minimize comparability risks inherent to switching platforms mid-development [36]. To this end, recent vendor-driven μSDS-CGE method improvements, including the addition of an automated standard mAb calibration step helping to optimize dye content within the capillary, have produced reproducibility results comparable to the more established, lower throughput SDS-CGE options, with % area relative standard deviations (RSDs) of ~1% (intra-assay precision) to 4% (inter-assay precision) [17].

With regard to CGE peak variant characterization, online hyphenation of MS to SDS-CGE has not yet been achieved at the commercial stage and requires more indirect means [37,38]. The main bottleneck is MS-incompatibility of sieving matrix components, such as SDS and high concentration co-ions, although great strides have been taken to improve the situation, for example, the use of cyclodextrins in interface solutions to sequester detergents [39].

3. Charge heterogeneity

Many protein variants differ in overall charge relative to the parent molecule and can yield a large range of effects, from complete inactivation to higher relative potency [40], and often cannot be separated by SDS-CGE due to their comparable MWs. The most common charge variant monitoring methods in the biotechnology industry include ion exchange chromatography (IEX), capillary-based isoelectric focusing without (cIEF) or with (iCIEF) capillary-wide imaging capability, and CZE [41]. Several commercial instruments performing CZE and (i)cIEF have been adopted into the QC space [42,43]. IEX will not be discussed here, except to say that it has a valuable place in the industry with well-established MS compatibility [44]. Note, however, that IEX, unlike CZE and cIEF/iCIEF, does not separate analytes based on *overall* charge, but on the charge available for interaction with the solid phase. Thus, a particular proteoform could be separated by CE but not by IEX thereby yielding complementary information as orthogonal techniques. This difference can be important when monitoring the profile of ADCs [45] since the physical characteristics of the conjugated drug could interfere with the IEX solid-phase interaction of the parent mAb. As discussed below and shown in Fig. 2, iCIEF, CZE and its offshoots, such as micellar electrokinetic chromatography (MEKC), are also not expected to yield comparable profiles and together are uniquely informative depending on the specific variant(s) under scrutiny [46]. MEKC and other promising CZE derivatives will not be discussed in detail here in order to focus on the most common methods utilized in the industry today.

CZE migration rates primarily depend on the ratio of the overall charge-to-hydrodynamic radii of the analytes. Typically, the sample is injected as a plug at one end of a capillary filled with a background electrolyte (BGE) buffered at a pH providing the desired mobilities and capable of maintaining a constant field strength. Unlike IEF, isoelectric point (pI) identification is not possible, however, its advantages are sensitivity, MS compatibility and high throughput; the latter particularly when judiciously implementing EOF. These characteristics are ideal for routine quality monitoring tasks such as ensuring batch lot-to-lot conformity. Although EOF can be advantageous in this regard, reproducibility suffers due in large part to the instability of the zeta potential at the capillary inner surface [47]. As with SDS-CGE, these challenges have been largely overcome through use of excipients that virtually eliminate EOF and protein-surface adhesion while using a bare-fused silica capillary. Comparable peak profiles and CPA% within a standard deviation of ± 0.9 were shown across companies [43], at least within the target predicted pI range of 7.4 and 9.5. For this reason, CZE has grown in popularity and is being added to biologics release testing panels [48].

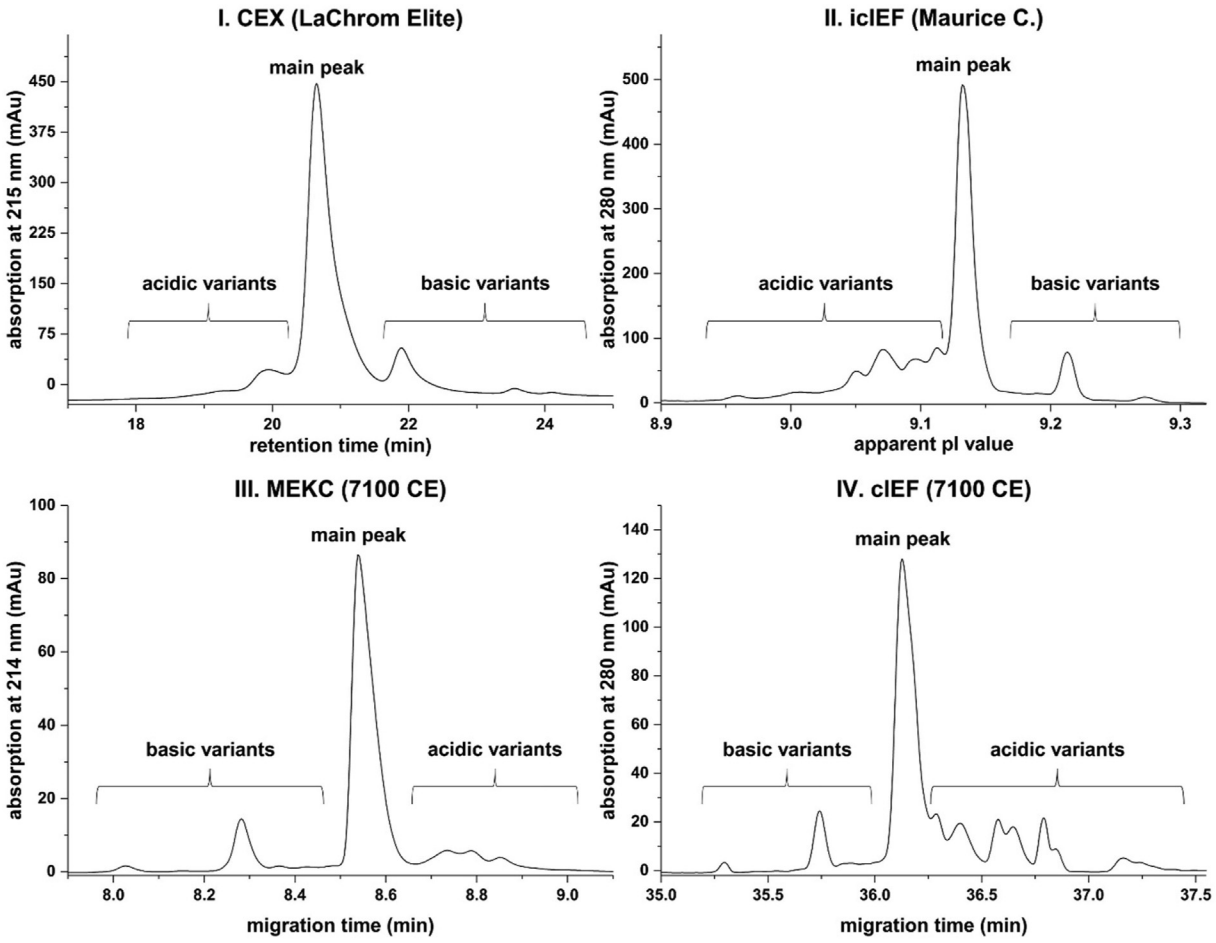

Fig. 2. iCIEF, CZE (MEKC), and IEX (Cation exchange chromatography, CEX) profiles of the NIST mAb standard. These results underscore how methods utilizing the same basic separation principle ("separation by charge") can produce different profiles. The method chosen for monitoring would depend on the method that best resolves the variant(s) of interest (Reprinted with permission from Ref. [46]).

Capillary isoelectric focusing has the unique capability of identifying an effective isoelectric point, or pI, of a protein of interest and of each of its charged variants via migration across a pH gradient to the point of neutrality. Resolving power depends on field strength and the slope of the linear pH gradient established across the medium, with resolution improving with shallower gradients. Theoretically, pIs differing by 0.01 pH units or less can be separated [49], although the requirement is a concentrated mixture of amphoteric compounds, or "ampholytes", collectively covering the pH range of interest with closely-spaced pIs. Each ampholyte must contain functional groups with pKas closely flanking its pI to provide the conductance needed to generate a uniform field strength. Importantly, each protein analyte may require its own solubilizers/stabilizers [50], e.g., DMSO, urea, formamide or one if its derivatives [51], to ensure robust and reproducible performance.

Ampholytic mixtures yielding linear pH gradients of various slopes and ranges are readily available on the vendor market, e.g. Bio-Lyte from Bio-Rad, Pharmalyte from Cytiva, Servalyte from Serva Electrophoresis, and Aeslytes from AES Ltd., and can be mixed and matched to achieve a sufficiently resolved profile. Although Pharmalyte lot-to-lot reproducibility has been demonstrated [52], method development should include lot evaluations to ensure reproducibility for the specific protein of interest, and salts and other additives can impact the linearity of the ampholyte pH gradient [53].

Relative to conventional cIEF, iCIEF has become popular as a monitoring, release and stability test due to its ability to visualize the final, focused variant profile in real time without requiring mobilization of the focused peaks towards a detector window either by chemical or hydrodynamic means. Mobilization may modify the profile [54] or negatively impact resolution due to laminar flow [55], respectively. Recently it has achieved acceptance by the FDA as a characterization and QC method based on its common inclusion in IND and BLA submissions [56] with laboratory support provided by multi-company demonstrations of precision, robustness, etc., all satisfying Q2(R1) ICH guidelines [52,57]. Quantification limits (QLs) of approximately 2–4% of the total protein load were demonstrated when detecting by UV absorbance at 280 nm. This QL lowers several-fold when utilizing the natural fluorescence of tryptophan, allowing an evaluation at lower protein load [58].

With regard to the use of iCIEF as a release and stability test, the reported pI of a typical mAb is simply the peak of highest relative proportion [57]. Product release specifications early in the pipeline may report total relative % acidic and basic species compared to the reported pI. These species may be considered impurities in lieu of characterization evidence identifying particular variant(s) as either confirmed impurities or not, at which point adjustments are made to the specifications [59]. Thus, limits on relative quantities of allowable acidic and/or basic variants are set late in development once the needed characterization data is collected. Due to its

relative simplicity with regard to method preparation and execution, biotechnology companies have utilized iCIEF internally as an identification method for release of clinical material in place of the relatively complex ELISA or peptide mapping methods [57]. However, it has been observed that two mAb therapeutics *within the same product portfolio* can have virtually identical profiles, even after subjecting the product to reducing and/or subunit analysis. This indicates that the risk may be too high to implement iCIEF as a standalone commercial-stage identity method and this risk would increase if testing involved a CRO [60]. Perhaps the entrance of new and complex modalities into the biotherapeutics space, in particular Fc fusions with their accompanying complex charge variant profiles, may lower this risk. However, the tradeoff would be an increased reliance on characterization tools like CE-MS and demand for more innovative cartridge coatings and additives to reduce non-specific interactions and increase sample solubility, stability, proteoform resolution, and method robustness.

4. Glycosylation heterogeneity

N- and O-linked carbohydrates are the most commonly occurring forms of protein glycosylation [61], with some exceptions such as reported in Ref. [62]. In this review we will focus on the analysis of N-glycan since this represents the vast majority of oligosaccharides bound to marketed protein therapeutics.

Glycosylation at the conserved C_H2 Asn297 site of mAbs and Fc fusion proteins can greatly impact the pharmacokinetic (PK) and pharmacodynamic (PD) properties of a protein [63]. Because of the large number of linkage and positional options, glycosylation is highly heterogeneous, resulting in numerous glycosylation patterns [64], and this complexity varies by the host cell and by the manipulation of bioprocessing parameters, such as growth phase, nutrition, oxygen level, pH, temperature, etc. [65]. Taken together, complex glycosylation has the potential to impact patient safety and product efficacy, thus, requires glycan content testing at drug product release to ensure profile consistency [66] and in-depth characterization as discussed below.

CE-based N-glycan analysis can occur at four levels [67–69]; Intact (Level 1), subunit, (Level 2), peptide (Level 3), and released glycan (Level 4). At Level 1, CZE and iCIEF can differentiate glycan-altering modifications such as sialylation, acetylation, sulfation or phosphorylation, due to their impact on the overall charge density of the protein. As discussed earlier, SDS-CGE can readily differentiate between the glycosylated an non-glycosylated product forms [19]. Hyphenation of CE with MS is another excellent way to conduct Level 1 analysis due to the availability of low flow interface operations (nanoliter- or low microliter per minute) in either sheathless or sheathflow setups, which result in excellent separation efficiency and high signal strength due to efficient ionization/low ion suppression at these flow rates [69].

Level 2 analysis, as outlined earlier, can be done using SDS-CGE to evaluate reduced/denatured and/or partially clipped forms using endopeptidases [70] with CZE- or cIEF-MS providing detailed glycoform information [71].

Level 3 requires proteolytic digestion (e.g., trypsin) followed by low nL/min flow rate CE-MS or CE-MS/MS analysis of the resulting glycopeptides [68]. Analysis of glycosylation together with the corresponding glycopeptides provides site specificity information. Furthermore, peptide analytical approaches such as isotope, isobaric and fluorescent labeling for quantification, metabolic incorporation, and mass defect analysis can be simultaneously accomplished using low flow sheathless interfaces with different sheath liquid additives [72]. CE-MS also enables linkage-isomer separation through targeted modification of the isomers resulting in distinguishable mass analytes, e.g., ethyl esterification of sialic acid-containing isomers of the prostate specific antigen [73].

Finally, at Level 4, the attached carbohydrates are either chemically or enzymatically removed from the polypeptide backbone and analyzed at the released glycan level. Liquid phase separation-based carbohydrate analysis, however, requires derivatization of the free glycan structures with a UV or fluorescently active agent, and for CE this tag should also be charged to support proper electromigration [74,75]. Level 4 N-linked carbohydrate analysis starts with specific enzymatic removal of the glycan moiety, in most instances using PNGase F. This endoglycosidase releases most asparagine-linked oligosaccharides except e.g., the alpha 1–3 core fucosylated versions, to mention the most important one. For efficient release, in most instances the polypeptide chains should be unfolded to ensure full access of the glycosidase, typically using mixtures of denaturing and reducing agents such as SDS, dithiothreitol (DTT), iodoacetic acid (IAA), etc. This is especially important for new modalities, such as fusion proteins and multi-specific antibodies, in which cases a denaturing temperature gradient is suggested [76]. For labeling, the most commonly used CE fluorescent agent is the triple negatively charged 8-aminopyrene-1,3,6-trisulfonic acid (APTS), which binds covalently via a classical Schiff-base formation reaction followed by reduction to obtain a stable conjugate. The use of positively charged tags is also possible, but not recommended for sialylated structures as the net charge of the carbohydrate – dye complex can become zero (equal number of negatively charged sialic acids and positive charges on the tag) resulting in no differential electromigration. Other labeling dyes have also emerged utilizing NHS ester-based chemistries rather than the commonly used reductive amination technique [74]. The applied labeling agents have to be added in large excess to the derivatization reaction mixture in order to ensure acceptable reaction speed, and associated parameters such as temperature, derivatization time and catalyst concentration all have to be optimized to improve reaction yields. Due to the large excess of tagging agent, the labeled sample must be purified prior to electrophoresis using either HILIC type micro-columns or magnetic micro-particles [77]. In both instances, the cleanup process is based on binding of the labeled glycans to the applied stationary phase under high organic solvent conditions – typically acetonitrile – and eluted by water. The entire workflow can be fully automated to provide the required robustness of the approach for downstream validation [78].

Detailed structural elucidation of the sugar structures including positional and linkage information is either based on calculated glucose unit (GU) values and an associated database search (www.GlycoStore.com) [79] or by MS identification [80]. For GU calculation, either an oligosaccharide ladder is run prior to or after the sample separation step or a bracketing standard set is co-injected. Alternatively, the ladder can be co-injected with the sample using tags with different excitation/emission wavelengths. In both instances the GU based database search usually provides adequate structural elucidation of the separated glycans [81].

When neither the GU value search nor MS analysis gives unambiguous results, exoglycosidase digestion-based glycan sequencing is necessary to properly identify the sequence of the sugar components along with their positional and linkage information [82]. This can include a type of sequencing workflow utilizing multiple enzymes either consecutively or in an array format to specifically release the carbohydrate building sugar monomers one by one, starting from the non-reducing end of the oligosaccharide structure, as shown in Fig. 3. For most biotherapeutics, sialic acids are the usual capping residues at the non-reducing end, which are readily released by appropriate neuraminidase enzymes with alpha 2–3,4,6 or 8 specificities. Considering the most frequently occurring glycan structures shown in Fig. 3,

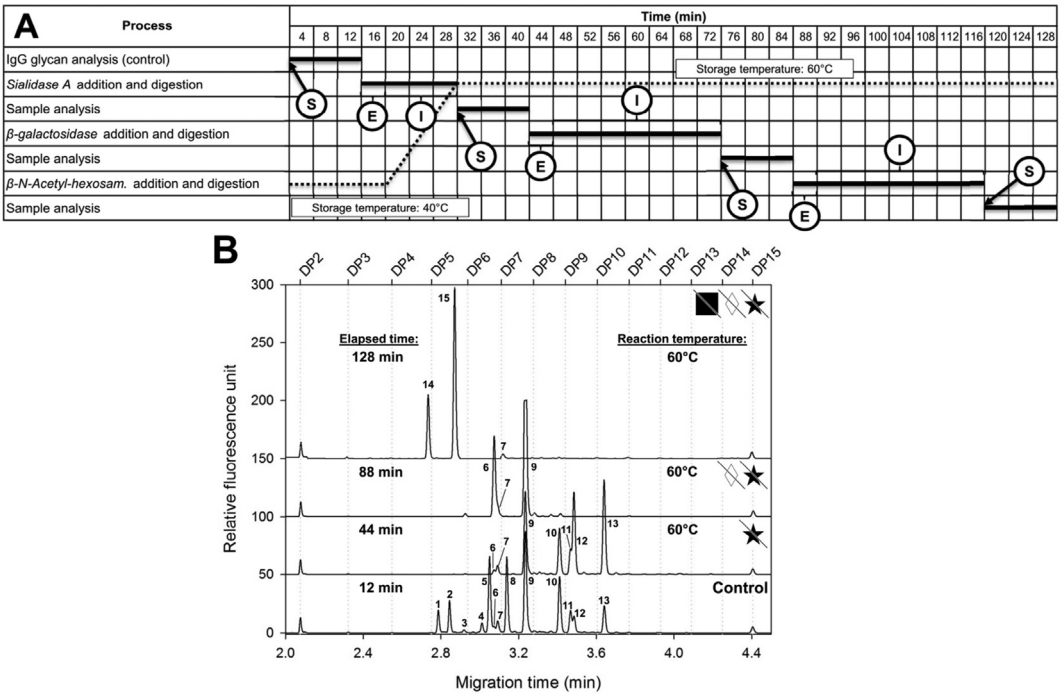

Fig. 3. Fully automated capillary electrophoresis based carbohydrate sequencing of etanercept. Panel A: workflow of the consecutive exoglycosidase digestion steps, Ⓢ sample injection, ① incubation, Ⓔ exoglycosidase enzyme addition. The dotted line depicts the temperature profile during the exoglycosidase digestion steps. Panel B: the resulting CE separation traces after the addition of the exoglycosidases. With permission from Ref. [82].

galactosidases are to be used next to cut β1-3,4 linked Gal residues. This particular treatment is of high importance since it can identify any highly immunogenic alpha 1–3 Gal epitopes [83]. Next, the GlcNAc residues are released, both antennary and bisecting types, using appropriate hexosaminidase enzymes. Finally, the core fucose is removed by fucosidase. Note that in rare cases arm fucosylation is possible, at which case, the fucosidase should be used first because its non-reducing capping position would inhibit further digestion steps. The same thing is applicable for the alpha 1–3 Gal as that is usually at the same non-reducing end position [83]. If necessary for antennary positioning identification, the mannose residues can be released by applying the corresponding 1–3 or 1–6 mannosidases of the partially digested core [83]. The mannosidase enzyme becomes especially important in analyzing hybrid structures and samples containing high mannose glycans. After each digestion step, the target sample is re-analyzed by CE, and from the migration time shifts and peak area changes the type and the number of the removed residues can be readily calculated. Please note that sequencing should start from the non-reductive end usually with the removal of the sialic acids, followed by the galactoses and GlcNAc residues in order to ensure that consecutively used enzymes are not inhibited by non-removed sugars.

5. CE-MS

The various complex mAb-related modalities can yield relatively highly complex charged-isoform profiles relative to a typical mAb. Companies are combining one or more proteins in so-called combination therapies, which further complicate the isoform profile [84]. Consequently, such complexity underscores the importance of MS characterization.

As mentioned earlier, MS-based methodologies are widely used to support characterization and development testing of biopharmaceuticals at various levels of analysis, from intact to peptide map to glycan release, and are becoming increasingly popular even with the potential of GMP implementation [85]. Although LC-MS and LC-MS/MS are the predominant MS-based hyphenated techniques, the role of CE-MS in the biopharmaceutical field has increased and examples have been successfully applied to the analysis of complex glycoproteins, ADCs, bispecifics, and protein mixtures [86–89]. CE-MS can serve as an orthogonal and complementary approach to LC-MS as discussed below and shown in Fig. 4 [90] and its lack of stationary phase poses less risk of nonspecific interaction and injection-to-injection carryover.

Electrospray ionization (ESI) is the most common MS ion source for large molecule analysis including protein charge variants. Its integration with CE requires an interface, the purpose of which is to maintain stable electric contact at the CE outlet electrode and assist in steady spray formation by ESI [72]. Significant amounts of effort have been devoted to CE-ESI interface design and several comprehensive reviews have highlighted the technical innovations in terms of instrumentation and methodology, e.g. Ref. [72]. The application of CE-MS is facilitated by these dedicated designs and their commercialization, for example the sheathless porous tip interface (commercialized as CESI 8000, SCIEX, USA), coaxial sheath-flow interface (G1607B, Agilent Technologies, USA) and nanoflow sheath liquid interface (EMASS-II, CMP Scientific, USA).

The sheathless interface enables the BGE to be electrosprayed directly from the CE capillary without additional dilution, giving rise to enhanced sensitivity and has been used in characterization of complex intact proteins, elucidation of their glycosylated structures and conformational heterogeneities, as well as identification of low-abundance PTMs [86,91]. The method detection range of protein biomarkers could be as low as ng/mL when combined with a pre-concentration process [92]. Although compromised sensitivity may occur in sheath liquid interfaces due to dilution effects by the liquid flow employed to maintain electrical contact,

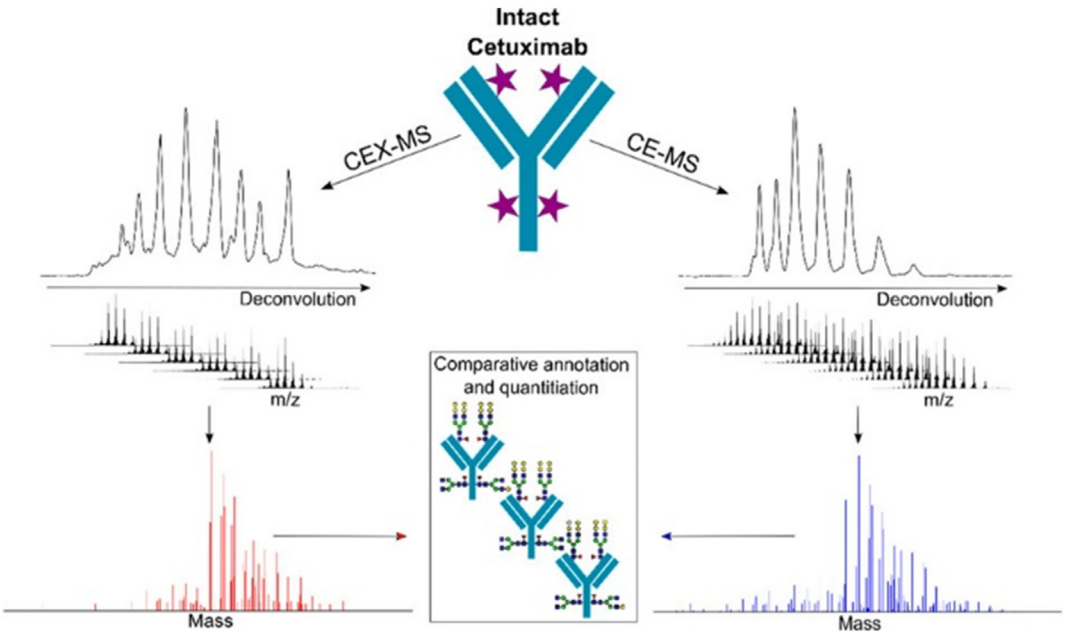

Fig. 4. CEX-MS and CZE-MS comparative characterization of the monoclonal antibody cetuximab. pH gradient-based separation by CEX-MS and CZE-MS were both capable of efficient separation of cetuximab charge variants with eight major peaks baseline resolved, although with different separation selectivity (Reprinted with permission from Ref. [90]. Copyright (2020) American Chemical Society).

introduction of sheath liquid provides BGE component flexibility. Sufficient robustness and accuracy assessments of sheath liquid has been shown by studies characterizing degradation variants of mAbs and their fragments via screening and monitoring of specific modifications [93,94]. By coupling with immunoaffinity capture techniques, sheath liquid CE-MS has also been used to evaluate *in vivo* stability of fusion proteins through monitoring of protein catabolites in serum samples [95].

iCIEF-MS analysis can be achieved by offline fractionation where fractions are first collected, processed with MS-compatible components if needed, and subsequently introduced into MS for characterization. Although more time consuming and labor intensive, offline fractionation is not limited to separation of analytes requiring only MS-compatible components [93] and may be necessary to fully characterize some highly complex proteins at the intact level, for example highly glycosylated Fc-fusions [96]. Moreover, the labor intensive efforts can be alleviated by the availability of preparative systems and high throughput methods [97].

Microchip CE-MS is a trending technology thanks to its fast analysis time, high throughput, high sensitivity and low sample consumption. The narrowing of hydraulic channels enables implementation of higher field strengths which accelerates sample movement and consequently reduces analysis time [72]. Innovative capillary coatings have been applied to improve performance by reducing EOF and protein adsorption [71,98]. Examples of microchip iCIEF-MS and CZE-MS are now commercially available and show great potential for applications such as early development screening and monitoring of specific protein quality attributes [71,99]. The integration of microchip capillary electrophoresis based imaged isoelectric focusing with MS is a promising new combination, allowing in-line peak identification of the separated species [71]. Application of transillumination supporting wafer materials enables direct monitoring of the separation and immobilization steps prior to entrance of the focused molecules to the mass spectrometer for structural elucidation. Recently, the utility of microchip CZE-MS was demonstrated through its generation of a native charge variant profile yielding several low abundant variants not observed by IEX-MS, achieving complementary results to traditional LC-MS with relatively high sensitivity in *minutes* per sample [90]. Note that the platform format of this technology and its pre-mixed buffer kits can reduce method development and optimization timelines [90]. That said, vendors, when possible, should “open” platforms to accommodate molecules not fitting within the typical paradigm by adjusting BGE compositions and/or modifying locked method parameters.

6. Conclusion and future perspectives

The molecular complexity of mAbs and other therapeutic modalities requires a large variety of analytical methods to identify and monitor deleterious modifications and degradation products. CE has emerged as an important component of the overall testing strategy as an orthogonal and complementary technique to LC in the assessment of size, charge, and glycan heterogeneity. CE-MS characterization has gained importance in the field due to its high sensitivity and inherent structural elucidation potential. Robust and reproducible non-hyphenated CGE, CZE, and cIEF/iCIEF methods can be developed for testing at all stages of the manufacturing process, from monitoring of upstream and in-process samples to the release and stability testing of patient-ready products. In particular, the versatility of the above discussed CE-based workflow for rapid analysis of complex glycan structures at different levels, including linkage and positional identification of isomers, is a promising addition to the in-process control toolbox. As the biopharmaceutical industry continues to diversify with increasingly complex modalities (relative to mAbs), in particular bi-and tri-specific mAbs, conjugated proteins with improved activity and/or PK (e.g. PEGylated, fatty acid conjugated, and ADCs), highly N- and O-glycosylated Fc-fusions with high melting temperatures, proteins utilizing specific glycans to manipulate the immune response (e.g., fucosylated versus afucosylated), etc., it will become increasingly important to properly extract the complementary information only these CE methods can provide.

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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.

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