CHAPTER

19 BIOPHARMACEUTICAL APPLICATIONS OF CAPILLARY ELECTROMIGRATION METHODS

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CHAPTER OUTLINE

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19.1 INTRODUCTION: BIOPHARMACEUTICAL PRODUCTS ANALYSIS

Biopharmaceuticals, also referred to as biologics, are designated biomolecules (nucleic acids, peptides, proteins) used for therapeutic purpose. This category of therapeutic products has emerged at an exceptional pace and they are modifying the paradigm in the pharmaceutical industry with several blockbusters currently on the market. They are mainly produced using advanced recombinant technologies, which enable the production of proteins on a large scale while minimizing the variability of the product [1]. Presently, biopharmaceutical products include different types of biomolecules, for instance, hormones such as insulin or growth factors (Epo), interferons, interleukins (IL-2), vaccines, monoclonal antibodies (mAbs) and their associated formats (bispecific antibodies, fusion proteins, antibody-drug conjugates).

Compared to small molecules employed as therapeutic agents, biopharmaceutical products are significantly larger molecules with complex structures. In addition, as well as being similar to biomolecules naturally present in vivo, they exhibit a large diversity of post-translational modifications (PTMs) such as glycosylation. These microheterogeneities may impact the physicochemical and/or pharmacological properties of the product. The regulation agencies have introduced the concept of critical quality attributes (CQA) to designate the PTMs responsible for a change in properties of the biopharmaceutical product [2]. Therefore, each biopharmaceutical product requires a detailed characterization over the different structural levels in order to confirm the production of the desired protein, assess the occurrence of microheterogeneities, and finally ensure consistency of production. As a consequence, an important activity in analytical chemistry research is to address the complexity emerging from the characterization of biopharmaceutical products. A detailed characterization requires a combination of several techniques in order to investigate the different structural features of the biomolecule. The analytical techniques used are typically separation methods such as capillary electrophoresis (CE) or liquid chromatography (HPLC). These allow separation of the main isoform from lowly abundant variants or impurities that provide optimal specificity and sensitivity. Since its introduction in the late 20th century, capillary electromigration techniques have been shown to be particularly suitable for the separation of biomolecules. For these applications, the advantage of CE lies in its excellent separation efficiency and the possibility of using aqueous buffers that preserve the higher-order structure of proteins. Also, therapeutic peptides and proteins are typically charged molecules. In many cases, their modification induces a change in the net charge or hydrodynamic radius due to a conformational change. Therefore, electrokinetic separations using CE-based techniques are well suited for the separation of biopharmaceutical microvariants. CE instrumentation can be coupled to a large number of highly sensitive detection techniques including laser-induced fluorescence (LIF) and mass spectrometry (MS). MS is especially useful for the structural characterization of biopharmaceutical products [3]. The characterization of biologics has been reported using every type of CE separation mode from CE to imaging capillary isoelectric focusing as reported here. The CE-based methods are extensively used in the biopharmaceutical industry. CE can be used for routine analysis, for example, the determination of the glycosylation profile of therapeutic proteins. On the other hand, it also may be used during research and development activities to investigate the presence of amino acid substitution or faint PTMs. This chapter describes the principal applications of CE-based methods for the structural characterization of biopharmaceutical products. Each separation mode is consid-

ered independently and some relevant applications are emphasized with examples reported in the literature.

19.2 CAPILLARY GEL ELECTROPHORESIS

19.2.1 METHOD DESCRIPTION

The principle of capillary gel electrophoresis (CGE) is the separation of macromolecules according to their size. CGE employs the same separation mechanism as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The charge homogeneity of SDS-protein complexes allows separation in a sieving medium under an electrical field based on their hydrodynamic radius. CGE adapts SDS-PAGE to a miniaturized format that reduces the known drawbacks of SDS-PAGE, such as a long separation time and limited reproducibility, limited possibilities to improve separation performance by automation, and simplifying sample treatment and increasing resolution. Shi et al. reported a comparison between SDS-PAGE and CGE for a mAb purity analysis [4]. Advantages of CGE include higher accuracy and resolution of size determination as well as quantitative performance. Miniaturization of SDS-PAGE to a capillary format involved the replacement of the traditional slab gel by soluble polymers employed as a replaceable molecular sieve allowing the separation of the protein directly in the capillary. One major issue of this transfer of methodology concerns the nature of the capillary, which can produce high electroosmotic flow (EOF) depending on the background electrolyte (BGE) composition. In CGE, EOF has to be suppressed to allow the separation based only on differences in the hydrodynamic radius. Several strategies have been described to remove EOF during CGE analysis. Some are based on the use of bare fused-silica capillaries with modifiers such as tris-borate in the BGE to suppress EOF. However, due to a possible adsorption of protein on the inner capillary wall, other solutions based on the chemical modification of the capillary wall by a dynamic or a permanent coating have been described. Due to the need for total EOF suppression, neutral coatings are required. Several home-made solutions such as linear polyacrylamide (LPA) [5], hydroxypropyl cellulose (HPC) [6], polyvinyl alcohol (PVA) [7,8], and some commercial solution [9–13] were developed. Concerning detection modes with CGE, UV, and fluorescence detection are the most common. Using UV detection, common wavelengths are 220 nm and more rarely 200, 214, and 280 nm. LIF offers improved sensitivity. Classically, a laser operating at 488 nm for fluorescence excitation with the resulting emission signal monitored at 520 or 560 nm is used. For glycosylation analysis, CGE methods based on 8-aminopyrene-1,3,6-trisulfonic-acid (APTS) derivatives allow fluorescence detection with the addition of three negative charges, and this appears to be a reference method [10,11]. Indeed, with these modifications, electrophoretic separation can be obtained in less time, with a higher efficiency and sensitivity. APTS is not the only option. Other strategies have been developed to label N-glycan for mAbs analysis as a fluorescent product using 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) [14,15] or 8-aminonaphthalene-1, 3, 6-trisulfonate (ANTS) [10]. All these methods had excellent precision and accuracy. Only differences were observed, particularly with regard to the detection and quantification of minor glycan species [10].

Since the end of the 80s, CGE methods have been developed for protein separations and characterization. In the field of biotherapeutics, CGE is known as a reference method for the size heterogeneity and glycan profiling of mAbs and related products. With the development of commercialized

sieving kits by several companies, CGE has become a high-throughput method in the biopharmaceutical industry.

19.2.2 EXAMPLE OF A PRACTICAL PROTOCOL

19.2.2.1 Size heterogeneity analysis of therapeutic proteins using CGE-LIF

The following experimental protocol is adapted from reference [16].

1. Sample is derivatized with 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA), a fluorescent dye.
2. Free dye is removed by gel filtration (NAP-5 column).
3. For nonreduced proteins, the sample is treated with SDS/40 mM iodoacetamide (IDA) and heated at 70°C for 5 min.
4. For the analysis of reduced proteins, the sample is mixed with SDS to a final concentration of 1% (*v*/*v*) with 10 µL of 1 M dithiothreitol (DTT), and heated at 70°C for 20 min.
5. Preflushing of the capillary with BGE is performed for 10 min at 70 psi.
6. CGE-LIF conditions should be applied as follows:

Bare fused-silica capillary	Detector/total length 21 cm/31 cm; i.d. 50 µm
Background electrolyte	Beckman Coulter sieving gel buffer
CE electrical field	− 15 kV (484 V/cm)
Electrokinetic injection	10 kV, 40 s
LIF detection	Excitation 488 nm, emission 520 nm
Separation time	40 min

19.2.2.2 N-glycosylation profile of therapeutic proteins using CGE-LIF (APTS labeled analysis)

This experimental protocol is adapted from the references [9,10].

1. Protein sample is diluted to approximately 10 mg/mL with deionized water.
2. PNGase F diluted in reaction buffer (50 mM sodium succinate pH 5.5) is added to the sample.
3. The sample is incubated for 15 h at 37°C with mild agitation.
4. Deglycosylated protein is heated and precipitated by centrifugation.
5. Supernatant is dried and reconstituted in an excess solution of 15 µL of acidic APTS (5 mg in 0.5 mL of 15% *v*/*v* glacial acetic acid) and 5 µL of 1 M sodium cyanoborohydride in tetrahydrofuran.
6. The solution is heated at 55°C for 2 h then diluted to a final volume of 250 µL using deionized water.
7. Preliminary flushing of the capillary with BGE is performed for 3 min at 30 psi.
8. CGE-LIF analysis conditions should be applied as follows:

N-CHO-coated capillary	Detector/total length 50 cm/60 cm; i.d. 50 μm
Background electrolyte	N-CHO carbohydrate separation gel buffer
CE electrical field	− 30 kV (500 V/cm)
Injection volume	35 nL (2 psi, 10s)
LIF detection	Excitation 488 nm, emission 520 nm
Separation time	15 min

19.2.3 APPLICATIONS

CGE is widely used for purity analysis of mAbs for lot release and stability studies, and to demonstrate product consistency and shelf life during the production and life cycle of the product. CGE applications are mainly focused on protein size heterogeneity and N-glycan profiling [17]. However, CGE is also used for profiling of ADC positional isomers [18], disulfide scrambled forms during the purification process of Fc-fusion protein [19], and Fc-fusion protein aggregation [12].

19.2.3.1 Size heterogeneity analysis

The size heterogeneity profile of proteins like mAbs is influenced by modifications such as PTMs or fragmentation. A shift of the profile toward acidic forms (deamidation, sialylation, glycation) or basic forms (succinimide formation, C-terminal lysine heterogeneity) [20,21] can be observed and potentially impact the safety and potency of the product [22]. In 1999, Hunt et al. developed a CGE-LIF method using a labeling precolumn as part of the control system for the quality control (QC) release of mAbs [23]. The same group reported the optimization of a generic and quantitative CGE method for QC and stability monitoring of mAbs. The validation of this method under the guidelines of the International Committee on Harmonization (ICH) demonstrates that the assay quantitatively determines the consistency of mAb manufacturing as it relates to size heterogeneity and product purity [24]. Han et al. also proposed an optimal CGE-LIF method for mAb separation based on multiusers experimental practices. Their method was transferred to the QC laboratory as a purity assay for lot release testing of therapeutic antibodies [25]. Several publications using CGE separations reported the efficacy of this approach in pharmaceutical laboratories [4,16,26–29]. To date, the CGE method is considered a reference method for size heterogeneity testing of intact and reduced mAbs. Indeed, reduction of mAbs disulfide bonds using a reducing reagent like tris(2-carboxyethyl)phosphine (TCEP) or DTT allows the separation of light chain (LC) and heavy chain (HC) isoforms in addition to several PTMs modifications. An application to the evaluation of the chemical integrity of mAbs during coupling to microparticle surfaces by tracking reduction fragments was reported [30]. To further improve the performance of CGE, Szekely et al. proposed a generally applicable multicapillary SDS-gel electrophoresis process for the analysis of mAbs for high-throughput QC. They adapted CGE methods for quality analysis with reducing conditions [28]. Gester et al. established a high-throughput method for the determination of antibodies intra- and extracellular LC to HC polypeptide ratio using CGE in reducing conditions. The method demonstrated important information for optimizing the vector design leading to the selection of CHO cell lines with optimized antibody assembly and preferred product quality [31]. Li et al. [32] and Kubota et al. [33] demonstrated the identification of cleaved

fragments of mAbs using orthogonal analytical methods including CGE (Fig. 19.1). They confirmed the complementarity of CGE methods and structural identification methods (including top-down and bottom-up approaches) to identifying the fragment.

Technical progresses in CGE instrumentation to reduce separation times have focused on microchip electrophoresis with sodium dodecyl sulfate (ME-SDS) tools. The intrinsic properties of microchips (separation channel of 14 mm in length and 31 μm in width) make ME-SDS a fast separation approach with separation times (< 40 s). Yagi et al. compared ME-SDS and CGE for the analysis of degradation species from heat-stressed mAbs and favored ME-SDS as an alternative method because of a significant reduction in problems often observed in CGE such as injection failure, occurrence of noise peaks and baseline shift [34]. However, mAbs analyzed by this technique sometimes exhibited different electrophoretic behavior. Cai et al. reported the optimization of ME-SDS for mAbs product quality analysis for three IgG1 and five IgG4 under reducing conditions. The optimized method was further evaluated for specificity, linearity, precision, and limit of quantification, and compared with conventional CE-SDS. While ME-SDS has also been increasingly recognized as an attractive alternative to conventional CE-SDS for protein purity analysis, there are still opportunities for improvement, for example, higher resolution to separate product variants of similar sizes without sacrificing signal response and higher sensitivity to accurately quantify low-expressing proteins [35]. Nonetheless, Smith et al. validated a ME-SDS method for purity analysis of therapeutic mAbs by separation and quantitation of size variants. Once defined through design of experiments studies, the method design space was validated according to ICH Q2 guidelines. The method is appropriate for use as a GMP release and stability assay including accelerated stability/forced degradation studies, and may be used for routine upstream and downstream process support [36].

19.2.3.2 Glycosylation analysis

Biotherapeutics such as mAbs, ADCs, or Fc-fusion proteins are glycosylated proteins whose N-glycans are naturally incorporated in the protein during secretion into the extracellular environment. Extensive glycans characterization in terms of structure and relative abundance is of prime importance. CGE performed for glycosylation analysis of biotherapeutic proteins represents a reference method implemented in the QC laboratories of biopharmaceutical companies. Mainly used as CGE-LIF of APTS labeled N-glycans, some alternatives were described using labeling by other fluorophore groups (see Section 19.2.1). Validation of CGE-LIF (APTS) methodology, in terms of reproducibility of sample preparation and N-glycan profiling, was described in an interlaboratory study performed by several biopharmaceutical companies, analytical contract laboratories, universities, and national authorities in the United States, Asia, and Europe [9]. Migration time, peak area, and peak area percent values were determined for all peaks with > 1% peak areas. Low variability and high reproducibility, regardless of site, was demonstrated. These results allowed standardization and validation of the CGE-LIF (APTS) approach for mAbs glycosylation analysis. CGE was used for a lot-to-lot variability study of major oligosaccharide profiles [13] and to identify all major and most minor glycans in a mAbs produced in NS0 cells using glycan standards [8]. The group of Wuhrer proposed a comparison of separation-based methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles including CGE-LIF (APTS) (Fig. 19.2) [10]. Hydrophilic interaction liquid chromatography of 2-aminobenzamide (2-AB)-labeled glycans was used as a reference method. A therapeutic antibody reference material was analyzed six times on two different days, and the methods were compared for

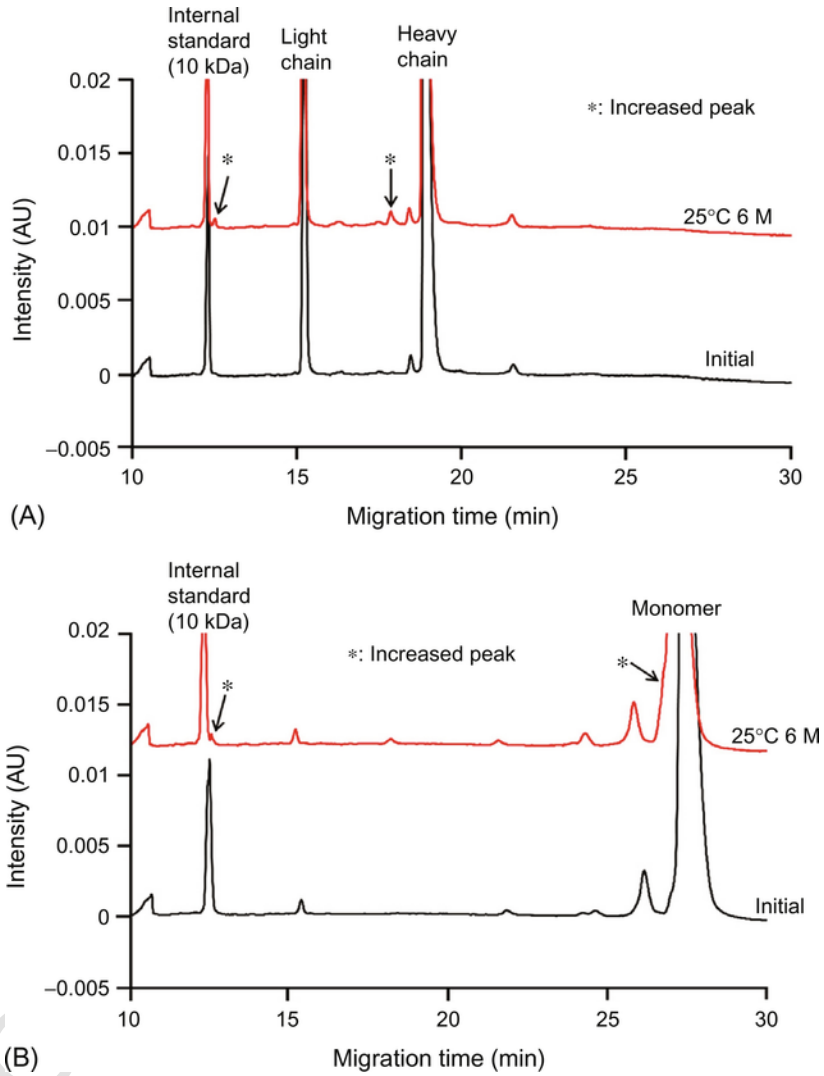

FIG. 19.1

Electropherograms of the mAb-A initial sample (lower trace) and degradation sample (upper trace) obtained by CGE (A) reduced and (B) nonreduced conditions. Analytical conditions: bare capillary, 50 μm i.d. (360 μm o.d.) × 30.2 cm, 20 cm effective; separation voltage, − 15 kV; detection, 220 nm; capillary temperature, 25°C; sample storage temperature, 25°C; injection, − 5 kV for 20 s; running buffer, SDS gel buffer.

- Reprinted from Kubota K, Kobayashi N, Yabuta M, Ohara M, Naito T, Kubo T, et al. Identification and characterization of a thermally cleaved fragment of monoclonal antibody-A detected by sodium dodecyl sulfate-capillary gel electrophoresis. J Pharm Biomed Anal 2017;140:98–104.

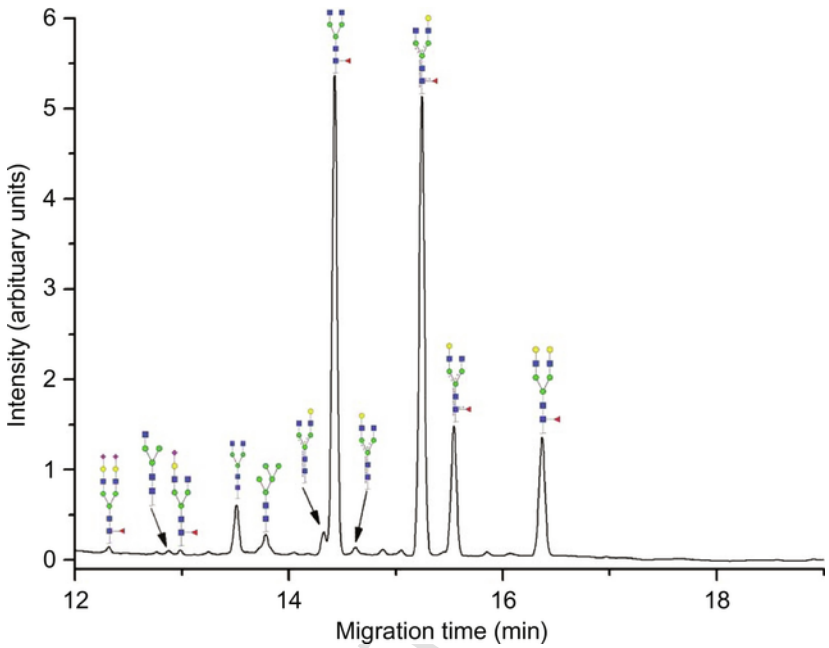

FIG. 19.2

Capillary electrophoresis separation of APTS-labeled N-glycans with laser-induced fluorescence detection.

- Reprinted from Reusch D, Haberger M, Maier B, Maier M, Kloseck R, Zimmermann B, et al. Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles—Part 1: separation-based methods. Mabs 2015;7(1):167–79. Copyright (2015), Taylor and Francis Group.

precision, accuracy, throughput, and other features, with emphasis on the detection of sialic-acid-containing glycans. CGE-LIF (APTS) showed excellent precision and accuracy; some differences were observed, particularly with for the detection and quantification of minor glycan species, such as sialylated glycans [10]. The development of this last method was driven by the need for rapid and automated sample preparation methods for *N*-glycosylation analysis. Szigeti et al. developed a rapid N-glycan release method based on an immobilized recombinant glutathione-*S*-transferase tagged PNGase F enzyme microcolumn [37]. This method resulted in efficient N-glycan removal in 10 min from glycoproteins. The same group developed a triple-internal standard based glycan structural assignment method. Based on the migration times of the co injected standards of maltose, maltotriose, and maltopentadecaose, a data-processing approach was designed and developed to set up a virtual ladder that was used for glucose unit calculation. This approach readily supports high-throughput CE systems by significantly accelerating the processing time for glycan structural assignment [38]. Commercialization of the Fast Glycan Labeling and Analysis Kit by Sciex (Darmstatt, Germany) resulted from the optimization of these last two studies.

19.3 CAPILLARY ISOELECTRIC ELECTROPHORETIC FOCUSING

19.3.1 METHOD DESCRIPTION

Capillary isoelectric electrophoretic focusing (CIEF) provides the highest resolution for the separation of proteins according to their isoelectric point (pI) [39]. CIEF is based on the same mechanism as for IEF slab gel. CIEF methods are mainly developed for the analysis of charge heterogeneity of mAbs and related products [40–45]. An example of a conventional protocol is described in Section 19.3.2. Briefly, a mixture of ampholytes and sample fills the whole capillary. A basic catholyte, generally sodium hydroxide, placed at the cathode end and an acidic anolyte, generally phosphoric acid, is placed at the anode end. Due to the ampholytes properties, a pH gradient is established in the capillary under an electrical field. Proteins are focused along the pH gradient until the region where the local pH and the pI of the protein are equal is reached. At this point, the apparent charge of the protein is zero, thus canceling its electrophoretic mobility. In a second step, sample is forced to migrate toward the detector by electrophoretic or hydrodynamic mobilization. Indeed, in CIEF, the position of the detector at the capillary exit makes the mobilization step mandatory, which may involve negative effects on the migration time and on the resolution due to possible peak broadening. To address these difficulties, whole-column imaging CIEF (iCIEF) was developed to facilitate faster separations with higher resolution, better reproducibility, and reduced sample volume. iCIEF based on the use of a short capillary length (5 cm) with CCD camera detection allows real-time monitoring of the focusing process. No mobilization step is necessary in iCIEF to detect proteins. Moreover, miniaturization of the capillary length permits shorter separation times in routine analyses. During the focalization step in CIEF and iCIEF, EOF must be suppressed and hydrodynamic pressure is prohibited. Similar to EOF suppression strategies described for CGE, neutral capillaries using dynamic or permanent coatings have been used. In CIEF, same coating as described in CGE were used as LPA [42,43,46–48], hydroxypropyl methylcellulose (HPMC) [44,49], and PVA [41,50]. For iCIEF, the use of a fluorocarbon capillary is virtually universal due to the manufacturer's near monopoly (Proteinsimple) of the instrumentation [16,29,40,47,51]. Concerning detection modes coupled with CIEF and iCIEF, UV detection at 280 nm represents almost all applications described to date. This is due to the favorable absorption of proteins at this wavelength and the large cut-off observed at lower wavelengths because of the strong absorbance of the ampholytes. Other detection modes are described to improve the performance of CIEF and iCIEF in terms of sensitivity and structural characterization. A multiplexed iCIEF immunoassay with chemiluminescence detection for mAbs characterization requiring different approaches to immobilization and conjugation allows the detection of product impurities even in the presence of excess host cell protein lysate [40]. For structural information, MS detection coupled with CIEF allowed identification based on pI and m/z ratio [52]. Nonetheless, CIEF-MS coupling is limited by technical issues such as the presence of salts and ampholytes. Presently, CIEF and iCIEF represent a reference method for the characterization of biotherapeutics. Similar to CGE, with the development of commercial kits and instruments, CIEF and iCIEF have become high-throughput methods in the biopharmaceutical industry.

19.3.2 EXAMPLE OF PRACTICAL PROTOCOLS

19.3.2.1 Charge heterogeneity analysis of a therapeutic protein using CIEF-UV

This protocol is inspired by the interlaboratory study described by Salas-Solano et al. [43].

1. Protein sample solution is prepared by adding and mixing the following components:
   - 15 µL of mAb sample into a 0.5-mL microcentrifuge vial
   - 200 µL of the 3 M urea-CIEF gel solution
   - µL or pH 5–8 ampholytes
   - 9.0 µL of cathodic stabilizer
   - 5.0 µL of anodic stabilizer
   - 6.0 µL of the pI marker mix
2. Conditioning of new capillary is performed using the following flushing conditions:
   - 50 min at 50 psi of chemical mobilizer
   - 2 min at 50 psi of deionized water
   - 5 min at 50 psi of CIEF gel
3. Preconditioning of the capillary before application is:
   - 3 min at 50 psi of 4.3 M urea
   - 2 min at 50 psi of deionized water
4. CIEF-UV conditions as follows:

Neutral capillary	Detector/total length 20 cm/30 cm; i.d. 100 µm
Cathodic stabilizer	0.5 M free base arginine
Anodic stabilizer	0.2 M iminodiacetic acid
Anolyte	200 mM phosphoric acid
Catholyte	300 mM sodium hydroxide
Chemical mobilizer	350 mM acetic acid
Voltage focusing	5 min at 25 kV, 28°C, normal polarity
Voltage mobilization	21 min at 30 kV, 28°C, normal polarity
Sample injection	25 psi, 99.9 s
UV detection	280 nm
Analysis time	26 min

19.3.2.2 Charge heterogeneity analysis of therapeutic proteins using iCIEF-UV

This protocol is inspired by the interlaboratory study described by Salas-Solano et al. [46].

1. iCIEF buffer is prepared by mixing the following solutions:
   - 270 µL deionized water
   - 350 µL of 1% methyl-cellulose
   - 36 µL of pH 5–8 ampholytes
   - µL of pI marker 5.8

- 4.5 µL of pI marker 7.6

2. mAb sample solution is prepared by adding and mixing the following reagents:
   - 5 µL of mAb sample at 5.5 mg/mL into a 0.5-mL microcentrifuge vial
   - 70 µL of iCIEF buffer
   - 25 µL of 8 M urea
3. iCIEF-UV conditions:

Fluorocarbon-coated capillary	Length 5 cm, i.d. 100 µm
Anolyte	80 mM phosphoric acid, 0.1% methyl-cellulose
Catholyte	100 mM sodium hydroxide, 0.1% methyl-cellulose
Voltage focusing	1 min at 1500 V, 25°C
Voltage mobilization	4.5 min at 3000 V, 25°C
UV detection	280 nm
Analysis time	8 min

19.3.3 APPLICATIONS

19.3.3.1 Charge heterogeneity analysis

Charge heterogeneity profiles can be influenced by modifications such as PTMs or fragmentation. Therefore, charge heterogeneity analysis is important for the QC testing of protein-based pharmaceuticals. CIEF and iCIEF are fully implemented in QC laboratories and have become reference methods for the characterization of charge heterogeneity in biopharmaceutical companies. Identification of intact and reduced therapeutics and PTMs can be realized using this electrophoretic mode.

Numerous sources cite the efficacy of CIEF and iCIEF as useful tools in the process development of biotherapeutics [3,17,53–59]. To illustrate the power of these methods, Cao et al. optimized the focusing time and temperature to obtain a robust method to analyze trastuzumab biosimilar charge variants profiles [60]. They reported that this method can be applied to evaluate stability, lot consistency, and purity assessment, and concluded that CIEF was a useful tool for the routine analysis of therapeutic mAbs. Suba et al. validated a CIEF method for identification testing of mAb drug products with a pI between 7.0 and 9.0 [42]. The CIEF method provided a good pH gradient for internal calibration ($R^2 > 0.99$) and good resolution between all isoforms ($R = 2$), as well as minimizing the time and complexity for sample preparation. The method is reproducible and is suitable for validation following ICH Q2 and method transfer to any QC laboratory. These authors also found CIEF to be and accurate routine analytical method to confirm protein identity in QC and release tests in the biopharmaceutical industry, especially with the use of commercial standardized kits [42]. CIEF and iCIEF methods are also suitable for the characterization of mAbs-related products. Wu et al. measured the pI of a deglycosylated Fc-protein named conbercept using a CIEF method as a part of a complete structural characterization strategy by different analytical methods [61]. A year earlier, Anderson et al. reported the charge heterogeneity of a heavily sialylated Fc-fusion protein for biosimilar development using iCIEF [62]. Other applications of ADCs charge heterogeneity by CIEF and iCIEF were reported in the last 10 years [45,51,63].

To validate the accuracy of CIEF and iCIEF method as references for biotherapeutic charge variants characterization, Salas-Solano et al. organized two interlaboratory studies based on the same design [43,46]. An international consortium of 12 laboratories from 11 biopharmaceutical companies in North America and Europe was formed to evaluate the precision and robustness of CIEF and iCIEF to determine the charge heterogeneity of mAbs. These interlaboratory studies endorse the application of CIEF and iCIEF methodologies both in process development and QC of biopharmaceutical companies. It was concluded that these methods could also facilitate increased regulatory and industrial acceptance of CE during the development of protein therapeutics [46].

Technical progresses of IEF instruments to further improve performances in terms of separation time resulted in the development of microchip CIEF (mIEF) tools. Kinoshita et al. reported a comparison between mIEF and conventional CIEF methods for the evaluation of mAbs charge heterogeneity (Fig. 19.3) [44]. Despite the decrease by 10-fold in separation time and the excellent correlation of calculated pI values and the relative amounts of each charge variants, these authors admitted the lack of automation as a problem. However, this strategy appears as a promising method and the need for improvements in functionalization will certainly be realized in the future.

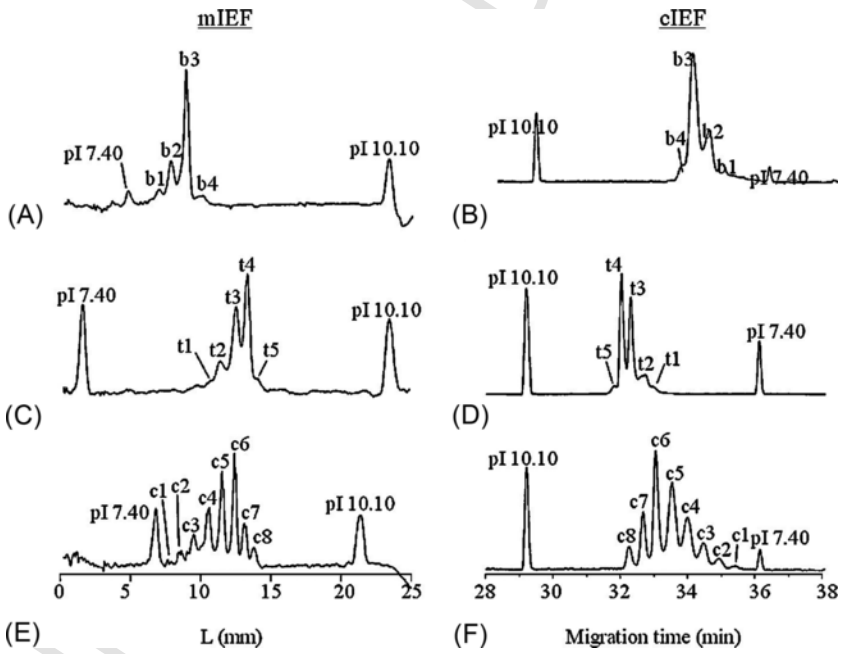

FIG. 19.3

Analysis of charge variants of bevacizumab, trastuzumab, and cetuximab by mIEF (left column) and CIEF (right column). Peaks observed in each sample were labeled with numbers depending on their detection positions. Peak 1 means the most acidic-end peak in the sample, but they were not identical charge variants among the tested samples. Bevacizumab (A and B), trastuzumab (C and D), and cetuximab (E and F).

- Reprinted from Kinoshita M, Nakatsuji Y, Suzuki S, Hayakawa T, Kakehi K. Quality assurance of monoclonal antibody pharmaceuticals based on their charge variants using microchip isoelectric focusing method. J Chromatogr A 2013;1309:76–83. Copyright (2017) Elsevier.

19.4 CAPILLARY ELECTROPHORESIS

19.4.1 METHOD DESCRIPTION

Not considered as a reference method so far, recent improvements in robustness and validation of CE methods are promising for the characterization of biotherapeutics. Separations by CE are based on differences in electrophoretic migration of analytes in a conductive solution in an electrical field following their charge-to-size ratio [64]. CE methods are used mainly for the characterization of charge heterogeneity of mAbs [16,21,65–69]. Examples of classical protocols are described in Section 19.4.2. Briefly, the capillary is rinsed and filled with BGE, the sample introduced by hydrodynamic injection, and the analytes separated by application of an electric field according to their electrophoretic migration. Detection is typically by fixed wavelength absorbance at 200 nm or 214 nm. Unlike CGE or CIEF, CE is more compatible with MS detection, affording higher sensitivity and the possibility of obtaining structural information for biologics. Positively charged proteins impose no or a reverse EOF for mAbs charge heterogeneity analysis. Indeed, due to negatively charged surface silanol groups, coulombic interactions induce protein adsorption phenomena at the inner capillary wall. Peak broadening and a dramatic decrease in resolution are possible consequences. Modification of the inner surface of capillary wall with a dynamic or a permanent coating is the most described strategy to eradicate these phenomena [70–74]. Due to simplicity dynamic coating of the capillary with a solution containing a polymer as a buffer additive, such as HPMC or polybrene, are commonly used for the characterization of mAb charge variants [65–67]. Polymers adsorb on the capillary wall minimizing the adsorption phenomena between mAbs and silanols, but the dynamic process can imply possible release requiring regeneration steps. This strategy is designed for spectrophotometric detection but is generally not compatible with MS detection. As an alternative, modification of the capillary wall by covalent bonding of neutral or charged polymers is a good option. Although modified capillary columns are commercially available, numerous coating procedures are described in the literature [75]. For the analysis of mAbs charge heterogeneity, major trends were observed, for example: (i) for a positively charged coating, EOF and the mAb effective mobility need to be relatively close and (ii) for neutral coatings, EOF has to be near zero to obtain the highest resolution [67]. Moreover, due to other types of interaction, such as hydrophobic interactions, no coating eliminates entirely protein adsorption at the capillary wall. To enhance these phenomena, some publication reported the addition of a surfactant, such as triethylenetetramine (TETA) [21,65,66] or Tween 20 [67], to the BGE. Since miniaturization is a key objective for the future of analytical sciences, microchip zone electrophoresis (MZE) represents the latest development to miniaturize CE separation methods. Improvement of CE performance in terms of separation time for mAbs charge heterogeneity place MZE as a forthcoming approach for the characterization of biopharmaceuticals. Several commercial systems are available with UV, MS, or chemiluminescence detection. As for conventional CE, adsorption phenomena on device surfaces is a concern, and both dynamic and permanent coatings have been developed for the most frequently used microchip materials including glass, poly(methylmehacrylate), poly(dimethylsiloxane), poly(carbonate), and poly(ethylene terephthalate-glycol) [76]. As an MZE-UV approach, dynamic coating with HMPC is most commonly used for mAbs charge heterogeneity analysis using Tween 20 as a BGE additive [49,68,69]. More recently, Ramsey et al. proposed an MZE-MS device for the separation of intact mAbs variants based on a surface coating method utiliz-

ing chemical vapor deposition of an aminopropylsilane layer and covalent modification with polyethylene glycol [77,78].

19.4.2 EXAMPLE OF PRACTICAL PROTOCOL

19.4.2.1 Charge heterogeneity profile of therapeutic proteins using CZE-UV

This protocol is inspired by the intercompany study described by Moritz et al. [21].

1. Protein sample is diluted to a final concentration of 1 mg/mL with purified water.
2. Sample a volume of 80 µL into PCR vials.
3. Preliminary flushing of the capillary with BGE for 10 min.
4. CE-UV conditions as follows:

Bare fused-silica capillary	Detector/total length 40 cm/50 cm; i.d. 50 µm
Background electrolyte	400 mM EACA, 2 mM TETA, 0.05% HPMC
CE electrical field	30 kV (600 V/cm)
Injection volume	10 nL (0.5 psi, 10s)
UV detection	214 nm
Separation time	30 min

EACA, ε-aminocaproic acid; TETA, triethylenetetramine; HPMC, hydroxypropyl methyl cellulose.

19.4.3 APPLICATIONS

CE was investigated for the study of mAbs and related products focused on protein charge heterogeneity [16,21,67,69,79]. Nonetheless, some articles demonstrated the efficacy of CE for the determination of the effective electrophoretic mobilities and charge to characterize Fc1 aggregation, and highlighted the challenges in maintaining long-term stability of biotherapeutics based on human Fc constructs [80], or to enhance treatment of eye diseases such as wet age-related macular degeneration and diabetic retinopathy in the development of iontophoretic drug delivery systems [81]. Feng et al. identified the N-glycan profile with different CE methods including CE, CGE, and MEKC. They demonstrated the low covariance and high orthogonality using multiplexing platforms for the identification of glycans [82]. Jaccoulet et al. also demonstrated the potential of CE for routine and high-throughput applications in a hospital, particularly for drug delivery control in cancer treatment [83].

19.4.3.1 Charge heterogeneity analysis

Charge heterogeneity is considered by regulatory agencies to be a critical product quality attribute due to its potential to impact acidic and basic isoforms on the pharmacokinetics, pharmacodynamics, and stability during long-term storage [84]. High-throughput charge heterogeneity analysis in biopharmaceutical QC laboratories requires reproducible and fast analytical methods. As an alternative to CIEF, CE-based methods were developed for the rapid analysis of mAbs charge variants for the QC of biopharmaceuticals in terms of purity, quantification, and stability testing. Reusch's group can be considered the pioneers of CE separations of intact mAbs charge variants [65,66]. They described

CE-UV methods for the analysis of mAbs based on the optimization of three parameters, (i) BGE pH between 4.5 and 6.0, (ii) ε-aminocaproic acid (EACA) BGE with a concentration between 300 and 600 mM, and (iii) BGE additives with HPMC and TETA to perform dynamic coating of fused-silica capillary columns to assess robustness of the method. This method represents a reference for the analysis of mAbs charge variants. A few articles described mAbs characterization analysis using similar CE-based methods with optimization of only EACA concentration, capillary coating properties, and nature of BGE additives [16,21,67–69,79,85].

Since CE provides an alternative approach to CGE or CIEF strategies for the characterization of intact mAbs, it also can be considered an orthogonal approach to assess the complete physicochemical properties of biotherapeutics. Glover et al. performed a stability study of pertuzumab and trastuzumab admixture in an intravenous infusion bag by several methods including CE, CGE, and iCIEF [16]. They concluded that, using these methods, no observable differences in the pertuzumab/trastuzumab mixtures stored for up to 24 h at either 5°C or 30°C were detected, proving the suitability of CE as a new platform technology for the charge heterogeneity testing of mAbs. Zhao et al. reported their work on charge variants analysis of an avastin biosimilar using several analytical methods including CE and iCIEF (Fig. 19.4). As expected, they concluded that these separation techniques contribute to the biopharmaceutical drug innovation and development [86]. Within the biopharmaceutical industry, to achieve the needs of GMP requirements, CE approaches have to pass method validation requirements according to ICH Q2. Evaluation of CE-UV methodology, in terms of pI range and high-throughput capabilities, was described in an international cross company study with 11 participating laboratories [21]. It was shown that CE can be applied to a broad pI range between 7.4 and 9.5. Correlation coefficients for linearity, precision by repeatability, and accuracy by recovery were 0.99 and around 1% and 100%, respectively. A direct comparison with ion exchange chromatography and iCIEF showed comparable results for percentage of corrected peak area for all three methods, but the highest resolution was obtained by CE for the investigated mAbs [21]. This major article confirms the potential of CE as a reference technology for the charge heterogeneity testing of biotherapeutics in the biopharmaceutical industry. ME-SDS or MIEF, and MZE have continued to develop for high-throughput charge heterogeneity analysis of mAbs [49,68,69]. These methods utilize high-speed microchip electrophoresis separations (from 8- to 90-fold faster) and are well suited for high-throughput charge profiling of antibodies during process and formulation development [49].

19.5 CE HYPHENATED WITH MASS SPECTROMETRY

19.5.1 METHOD DESCRIPTION

The development of electrospray (ESI) ionization and matrix-assisted laser desorption ionization (MALDI) led to the application of MS to biomolecules. As the performance of mass spectrometers improved in term of sensitivity, mass accuracy, and scanning frequencies, MS progressively became a key technique for the analysis of biomolecules, especially as it allows structural information to be obtained. CE quickly appeared as a suitable separation technique for direct hyphenation with MS in order to analyze complex samples [87]. The different modes of separation provided by CE techniques are particularly adapted to biomolecules separation, including, glycans, peptides, and proteins. The example that emphasizes this approach the best is deoxyribonucleic acid sequencing by CGE. CE separations in most cases are utilized for charged analytes in solution, which is favorable for MS

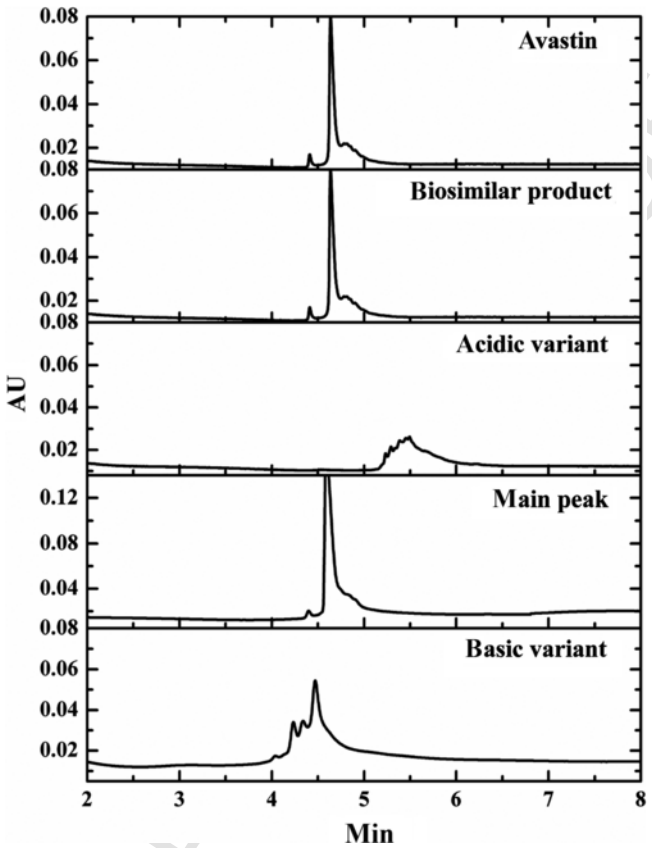

FIG. 19.4

Charge variants of mAbs, biosimilar product, and avastin profile obtained by CZE analysis.

- Reprinted from Zhao YY, Wang N, Liu WH, Tao WJ, Liu LL, Shen ZD. Charge variants of an avastin biosimilar isolation, characterization, in vitro properties and pharmacokinetics in rat. PLOS One 2016;11(3):13. Copyright (2016) Zhao et al.

analysis. Finally, MS delivers an outstanding sensitivity compared to other analysis techniques. The sensitivity provided by MS can counterbalance the limited injection volumes typical of CE.

CE hyphenated with mass spectrometry (CE-MS) hyphenation is usually implemented with ESI ionization mass spectrometers; however, CE-MALDI-MS specific applications are also described in the literature. Regarding the coupling of CE-MS, it requires continuity of the electrical field applied to induce the migration of analytes whereas the capillary outlet is fixed inside the source instead of the outlet vial. Therefore, different designs have been developed to meet the technical requirements and ensure compatibility with ESI and MALDI ionization. CE-ESI-MS interfaces can be categorized based on the method used to maintain the electrical field [88]. The most common CE-MS interface uses a sheath liquid, connected to the outlet electrode and constantly flowing, in order to form a junction with the BGE flowing out from the capillary outlet. The constant introduction of the sheath liq-

uid to the mass spectrometer reduces the ionization yield of analytes and thus the signal response. To address the limitations of sheath-liquid CE-MS interfaces, several research groups have developed CE-MS interfaces that do not rely on a sheath liquid or reducing to the minimum the flow rate of sheath liquid involved during the analysis, some of which are commercially available [89–91]. The formation of an OEF during the separation produces a constant flow of BGE of a few dozens of nL/min that is directed toward the MS source. The characteristics of this flow are favorable for nanoESI. NanoESI has favorable characteristics in terms of ionization yield and provides optimal sensitivity. In addition, capillary inner diameter below 100 nm enables smaller droplets to be produced, which improves the production of ions in the gas phase [92].

With dedicated conditions, CE-MS analysis is described for the characterization of therapeutic proteins over different levels defining the structure of the macromolecule:

- Primary structure: amino acid sequence, PTMs including glycosylations
- Secondary structure: partially digested proteins for glycoforms and isoforms characterization
- Tertiary and quaternary structure: intact protein analysis

CE-MS demonstrated the contribution of the selectivity provided by electrokinetic separation for the comprehensive characterization of therapeutic proteins, especially at the peptide level. Indeed, CE enables peptides to be transferred to the MS regardless of structure or hydrophobicity. In addition, it has demonstrated the possibility to separate peptides with PTMs from the intact counterparts or glycopeptides containing different glycans [93]. These characteristics are particularly important for the development and approval of new therapeutic proteins. The analysis of intact proteins using liquid-chromatography-based methods is limited due to interactions with the stationary phase, especially for therapeutic proteins with high-molecular masses like mAbs. Because CE does not incorporate a stationary phase, it is well suited to the analysis of intact proteins. For biotherapeutic molecules, the objective is to provide relevant characterization while reducing sample pretreatment.

19.5.2 EXAMPLE OF PRACTICAL PROTOCOL

19.5.2.1 Amino acid sequence and N-glycosylation characterization of therapeutic proteins using CE-ESI-MS/MS

This protocol is adapted from [94]. The following protocol is adapted to a quantity of 100 µg of therapeutic protein.

1. Dilute/reconstitute the sample in 50 mM ammonium bicarbonate (pH = 8.0) to a final concentration of 5 µg/µL. Heat the sample at a temperature of 40°C for 20 min, especially if the sample requires reconstitution.
   n.b.: For proteins difficult to digest (e.g., mAbs), perform the previous step using rapigest SF surfactant instead of 50 mM ammonium bicarbonate in order to guarantee optimal digestion efficiency.
2. Using a solution of 100 mM dithiotreitol, add DTT to the sample to a final concentration of 25 mM DTT. Heat the mixture for 5 min at 95°C.
3. Using a solution of 100 mM IDA, add IDA to the mixture to a final concentration of 10 mM of IAD. Leave the sample in the dark for 20 min.
   n.b.: IAD is a photosensitive compound. It should be kept away from light as much as possible.

4. Add 1 μL of trypsin enzyme (MS grade) at a concentration of 0.5 μg/μL. Leave the sample for incubation for 3 h at 37°C.
5. Add another 1 μL of trypsin. Leave for incubation overnight at 37°C.
6. If Rapigest SF was used, add 1 μL of 98% formic acid in order to cleave surfactant, leave sample 2 h at room temperature.
7. Dilute sample to the final concentration with 50 mM ammonium acetate (pH 4.0).
8. CE-ESI-MS conditions as follows:

Bare fused-silica capillary	Length 95 cm; i.d. 30 μm/50 μm
Background electrolyte	10% acetic acid
CE electrical field	− 20 kV (210.5 V/cm)
Injection volume	90 nL (30 μm)
Separation time	45 min

9. Perform data analysis manually or with identification algorithm software.
10. Perform data analysis manually. To ease glycosylated peptide identification, fragments 204.08 (GlcNac) and 366.10 (GlcNac-Man) fragment should be present in the fragmentation of N-glycosylated peptides.

19.5.3 APPLICATIONS

19.5.3.1 Amino acid sequence characterization

Even though most therapeutic proteins are produced by highly accurate recombinant technologies, amino acid sequence characterization is essential for lot release in order to demonstrate the absence of amino acid substitution during the production or conservation processes [95]. CE-MALDI-TOF MS was implemented using an automated fractionation/deposition system and demonstrated the ability to perform peptide mapping of trastuzumab with a sequence coverage of 92%–100% for the HC and LC, respectively [96]. Gennaro et al. demonstrated the complete characterization of mAbs using CE-ESI-MS analysis of a Lys-C digest in combination with HPLC–MS analysis [97]. The approach was based on sheathless CE-ESI-MS/MS. This analysis with trypsin proteolysis demonstrated the consistent characterization of the amino acid sequence with 100% coverage for various mAbs in a single analysis while strictly considering peptides without miscleavage. The datasets generated enabled the characterization of *N*-glycosylations, N-terminal glutamic acid cyclization, asparagine deamidations, methionine oxidations, and aspartic acid isomerizations [93,94]. The same method was used to assess the biosimilarity of trastuzumab and cetuximab to their respective biosimilar candidate. This analytical workflow demonstrated the possibility to achieve a detailed characterization of complex therapeutic proteins with a reduced number of analyses in a robust manner [98]. Said and coworkers adapted a CE-ESI-MS/MS method for the characterization of the primary structure of antibody-drug-conjugates brentuximab vedotin. They demonstrated complete characterization with a single injection and the ability to locate the position of drug molecules on the peptide backbone and to estimate the conjugation level independently for each site [99].

19.5.3.2 Glycosylation characterization

In a regulatory environment for therapeutic proteins, glycosylation is considered one of the major CQA. Indeed, glycosylation represents the most common PTMs and the characterization of glycosylation is complex due to the potential presence of several glycosylation sites on a single protein in addition to the different carbohydrate structures expressed for a designated site. They impact significantly the physicochemical properties of therapeutic proteins (solubility, protein–protein interactions) and may be the cause of immunogenic reactions if not properly controlled. Regulation agencies therefore require for approval a detailed investigation of the product glycoprofile, the demonstration of analytical methods able to provide a robust and confident characterization of this aspect of the protein. The analysis of glycosylations using CE-MS methods is commonly performed on released glycans or at the peptide level whereas it is also emerging at the middle-up and intact protein level. Gennaro and coworkers utilized CE-ESI-MS of glycopeptides to study glycosilation of several mAbs. The glycopeptides were generated through Lys-C proteolysis of the therapeutic protein followed by APTS labeling. Detection was realized in the negative ionization mode and allowed the identification of the major *N*-glycosylation and *O*-glycosylation sites [97]. The same group described a CE-LIF/ESI-MS method using a PVA-coated capillary for the analysis of therapeutic mAbs glycosylation after glycans release and APTS labeling [100]. A CE-ESI-MS method with a basic BGE was used for the analysis of glycans released from therapeutic mAbs as native or APTS-labeled glycans [101]. A similar CE-ESI-MS approach using an acidic BGE was used for the characterization of released glycans [7]. CE-ESI-MS/MS method could be used for characterization of glycosylation together with amino acid sequence information. The characterization based on MS/MS data enabled the position on the peptide backbone of the glycosylation site to be precisely determined and the structure of the glycan moiety was identified from the y/b ions (Fig. 19.5). Finally, the intensities of each glycopeptide could be exploited to estimate the relative abundance of each glycoform [94]. This methodology demonstrated the possibility of using glycoprofiles for therapeutic mAbs to compare biosimilar candidates [98,102].

The characterization of glycosylation using CE-ESI-MS/MS at the peptide level for ADCs suggests the technique may be adaptable for emerging therapeutic proteins. The localization of glycosylation sites of erythropoietin was described using a CE-ESI-MS analysis. In this case, the identification of glycosylation at the peptide level was demonstrated by proteolytic sample digestion [103]. Kammeijer and coworkers described a CE-ESI-MS method with a coaxial flow of nitrogen as a dopant in the ionization process for the characterization of therapeutic mAbs glycopeptides. The results demonstrate an increase in the sensitivity with improvement of the robustness of the glycoprofiles generated [104].

19.5.3.3 Middle-up analysis

Middle-up analysis refers to the characterization of therapeutic proteins after limited proteolysis, which generates peptides usually larger than 10 kDa. Usually proteolytic treatment is realized with dedicated enzymes: pepsin, papain, streptococcal cysteine proteinase, for example. From an MS perspective, the use of a limited digestion is interesting. It allows the complexity of the sample to be reduced in order to focus the analysis on specific parts of the investigated protein, whereas the other sources of heterogeneities can be excluded. As a consequence, it is employed mainly for the analysis

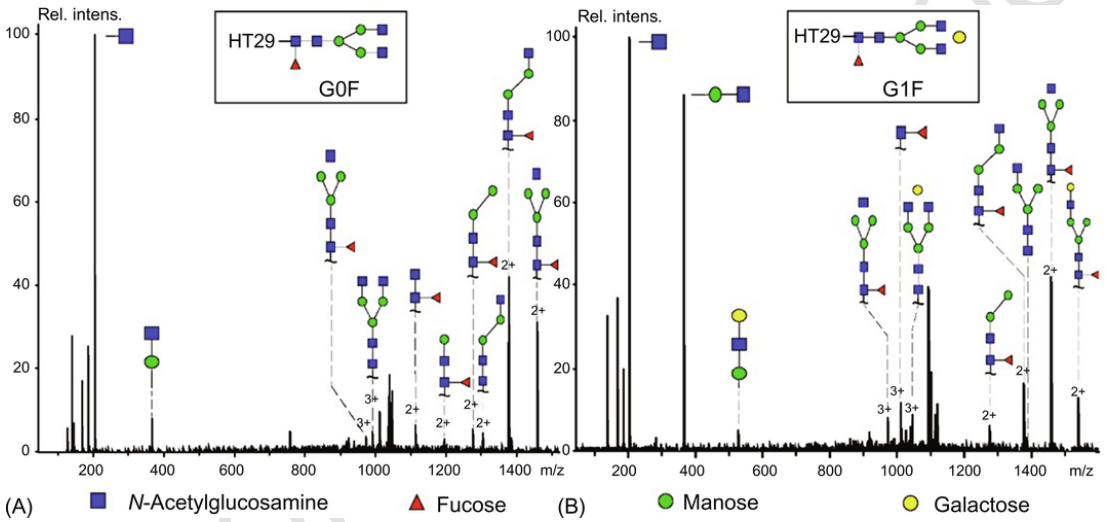

FIG. 19.5

Fragmentation spectra obtained for the CE-ESI-MS/MS analysis of trastuzumab corresponding to the glycopeptide HT29-G0F and HT29-G1F.

- Adapted from Gahoual R, Burr A, Busnel J-M, Kuhn L, Hammann P, Beck A, et al. Rapid and multi-level characterization of trastuzumab using sheathless capillary electrophoresis-tandem mass spectrometry. mAbs 2013;5(3):479–90. Copyright (2013), Taylor and Francis Group.

of highly complex proteins like mAbs, especially because highly specific enzymes for IgG1 proteolysis are available [105].

The peptide fragment generated from limited proteolysis is significantly larger compared with those typically generated by conventional enzymes like trypsin, chemotrypsin, Lys-C, Asp-N, or proteinase-K. One of the major issues for middle-up and intact protein analysis is the adsorption of the protein due to electrostatic interactions with the silanol groups on the capillary surface. This occurs even in acidic conditions. To address this limitation, physically coated or chemically bonded modified capillaries may be necessary. For CE-MS, permanent coatings like HPC and LPA are typically used. Biacchi and coworkers developed an offline CE-MALDI/ESI-MS method for the analysis of different fragments produced from the IdeS digestion of cetuximab. They demonstrated the baseline separation of Fc lysine variants and the independent characterization of the *N*-glycosylation sites present on the protein [106]. This method was also used to investigate the formation of Fc/2 dimers from the IdeS digestion and their conservation in case of analysis in native conditions [107]. The analysis of a mAbs after reduction of disulfide bridges was developed using online CE-ESI-MS. Results showed the separation of the mAbs HC and LC in a mixture of reduced mAbs. CE-ESI-MS was used for the middle-up characterization of therapeutic mAbs after different sample pretreatments. The complete separation of the mixture composed of mAbs HC, LC, and fragments produced by IdeS digestion was demonstrated. In addition, the different lysine variants for the Fc fragments were identified as well. A study of mAbs stability demonstrated the ability of this method to separate Fd fragment carrying a succinimide intermediate on an asparagine residue [108]. The various applications of CE-ESI-MS middle-up characterization demonstrate show CE is suitable for the separation of large protein fragments even in the case of a single modification.

19.5.3.4 Intact protein analysis

In the context of biopharmaceutical products manufacturing, the analysis of intact proteins represents "the ideal analysis." MS-based methods applied on a routine basis are delivering an unprecedented level of characterization, constantly enriched with methodological and technical improvements. However, most of these methods use extensive sample preparation, which risk introducing artificial modifications. The analysis of the intact protein has the advantage to be more representative of the actual sample. An automated CE-MALDI-MS instrument was designed by Biacchi and coworkers for offline analysis. The CE-MALDI-MS method demonstrated the separation and MS analysis of intact charge variants of trastuzumab [96]. CE-ESI-MS using a sheathless interfacing was used to develop a method for the characterization of human erythropoietin (Epo). This method allowed fractionation and subsequent characterization of a large number of Epo glycoforms in a single analysis. In this case, the separation of isoforms prior to their transfer to the MS improved sensitivity and significantly increased the number of identified isoforms [109]. The characterization of intact interferon-β1 was realized using CE-ESI-MS/MS analysis. This methodology allowed the identification of glycoforms and modified variants by MS/MS analysis. The concomitant use of electron transfer dissociation and high-energy collision dissociation fragmentation demonstrated the possibility of performing a comprehensive characterization of isoform diversity and the occurrence of PTMs [110]. The separation and identification of deglycosylated therapeutic mAbs was described using CE-ESI-MS for the identification of low levels of free LC in IgG1 reference material [108]. A CE-nanoESI-MS microchip was used for the analysis of intact infliximab. In this application, the sample did not undergo

any pretreatment. Lysine variants were successfully separated and identified by MS. An IgG1 drug conjugate was characterized using this method. The results showed the ability to discriminate between nonconjugated species and species conjugated with one or two drugs [77]. The same system was used for the characterization of intact ADCs approved as therapeutic treatment. The separation of charge variants emerging from N-terminal glutamic acid cyclization was achieved. In addition, decarboxylation of the sample prior to the CE-nanoESI-MS analysis enabled the characterization of the unconjugated species and ADCs exhibiting different numbers of conjugated drugs. The microchip CE-nanoESI-MS analysis demonstrated the possibility to accurately determine the drug-to-antibody ratio (DAR) [78].

Based on the IgG1 format, ADCs include an additional level of complexity coming from the presence of different numbers of conjugated drugs compared with conventional mAbs. At the moment, they represent the most complex type of sample in the field of therapeutic protein products. To provide the most detailed characterization of this category of therapeutic proteins, Said and coworkers developed an integrated analytical methodology based on ADCs analysis on the peptide, middle-up, and intact level. This integrated workflow is mainly based on sheathless CE-ESI-MS. It allow the characterization of the protein amino acid sequence, to precisely localize the different conjugation sites, and to estimate the relative conjugation levels for each site and obtain the glycoprofile of the protein. Finally, the different species produced from the conjugation reaction and their relative abundance were determined allowing the DAR of the protein to be deduced [99]. Because the ESI ionization yield is significantly lower for intact proteins compared to peptides, the CE-ESI-MS method should profit from the introduction of a more sensitive CE-MS coupling. The characterization of therapeutic proteins on the intact level using CE-MS based analysis still remains a challenge. However, the potential of the applications recently reported is undeniable and further developments are expected in the near future.

19.6 CONCLUSION

The development of biological products still represents a challenge for the analytical sciences. The complexity inherent to this category of therapeutic products and the important number of new products approved each year urge the development of analytical methods capable of delivering structural information for a designated biomolecule. On top of that, the introduction of innovative biopharmaceutical formats like ADCs and the development of biosimilar products are going to sustain this demand in the medium term. The number of research articles regarding the characterization of biotherapeutic proteins has grown rapidly in the last two decades. Among the state-of-the-art techniques used for the analysis of biopharmaceutical products, the intrinsic characteristics and the selectivity provided by CE separation have demonstrated major benefits. CE is a technique adapted to the separation of complex macromolecules and can be applied for the characterization of proteins over different structural levels. Especially CE, CGE, and cIEF have indubitably demonstrated their relevance for the characterization of size and charge variants as well as glycosylation analysis. Their robustness was demonstrated through numerous independent inter laboratory studies and they are currently used on a routine basis in the biopharmaceutical industry for production consistency, purity, and stability studies in a QC environment. More recently, CE-MS has been recognized as a powerful technique for the characterization of primary structure of biological therapeutic products with the possibility to ob-

tain information regarding the amino acid sequence or endogenous modifications including glycosylation. CE-MS facilitates the precisely location of PTMs on the peptide backbone and to obtain the relative abundance level of modifications. Also the development of CE-MS instruments suggests the possibility to use CE hyphenated to MS for the analysis of intact proteins. In the near future, CE and CE-MS methodologies should be expected to satisfy the requirements for the analytical characterization of biopharmaceutical products.

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