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Review

Applications of capillary electrophoresis in characterizing recombinant protein therapeutics

The use of recombinant protein for therapeutic applications has increased significantly in the last three decades. The heterogeneity of these proteins, often caused by the complex biosynthesis pathways and the subsequent PTMs, poses a challenge for drug characterization to ensure its safety, quality, integrity, and efficacy. CE, with its simple instrumentation, superior separation efficiency, small sample consumption, and short analysis time, is a well-suited analytical tool for therapeutic protein characterization. Different separation modes, including CIEF, SDS-CGE, CZE, and CE-MS, provide complementary information of the proteins. The CE applications for recombinant therapeutic proteins from the year 2000 to June 2013 are reviewed and technical concerns are discussed in this article.

Keywords:
CE-MS / CIEF / CZE / Recombinant therapeutic proteins / SDS-CGE
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1 Introduction

In 1982, recombinant human insulin was approved by the FDA and became the first commercially available recombinant protein therapeutic [1]. In the 31 years since then there has been a remarkable increase in the therapeutic applications of recombinant proteins. Currently, more than 200 protein-based drugs are commercially available and 58 biopharmaceuticals gained approval from January 2006 to June 2010 [2]. Pharmacologically, therapeutic proteins can be categorized in one of five ways based on their function. They can be proteins that augment existing pathways, provide novel function or activity, interfere with a molecule or organism, deliver other proteins or compounds, or act as replacements for deficient or abnormal proteins [3]. Complementing traditional small-molecule drugs, therapeutic proteins hold a great promise for human therapy to treat hematological and solid tumors, autoimmune, inflammatory, infectious, and cardiovascular diseases [4].

Protein therapeutics produced by recombinant technology are almost inevitably heterogeneous due to the biotechnological mode of manufacturing. In addition, potential chemical and physical changes may be introduced from the raw materials, the host cells, and the manufacturing and storage processes [5]. The chemical changes are covalent changes, for example, amino acid sequence variants are often produced in the biosynthesis. Sequence variants may arise from genomic mutations, for example, nucleotide starvation during the clone selection, or amino acid misincorporation during protein translation. Protein PTM is another type of chemical change, which often includes glycosylation, phosphorylation, oxidation, sulphation, lipidation, disulphide bond formation, deamidation, fragmentation, hydrolysis, and isomerization (transpeptidation) [6, 7]. Other types of postproduction covalent modifications can also occur [8]. Different from chemical changes, physical changes are noncovalent, such as aggregation and denaturation.

This inherent heterogeneity can alter the protein activity and/or influence the immunogenicity. The chemical and physical changes may lead to small perturbations in the protein higher order structure, which may cause the drug to lose its efficacy or to malfunction, although sometimes the consequences in terms of side effect are fairly benign [6]. To ensure the safety, quality, integrity, and efficacy of a therapeutic protein, which is usually of extreme structural complexity, it is critical to characterize and monitor the product-related variants throughout the discovery and quality control processes. A wide range of analytical techniques have been applied [5, 9].

CE is an effective technique to characterize proteins. It is beneficial with respect to its simple instrumentation, superior separation efficiency, small sample consumption, and short analysis time. The versatility of CE is attributed to the different separation modes available on the same hardware, and the feasibility of coupling it to various detection methods, such as LIF and MS. The major drawback of CE analysis

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Abbreviations: iCIEF, whole-column imaging CIEF; **G-CSF**, granulocyte colony-stimulating factor; **rhEPO**, recombinant human erythropoietin

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compared to LC is the limited concentration sensitivity. However, in the context of therapeutic protein characterization, high-concentration samples are often easily available. Thus, as an orthogonal and complementary analytical technology, CE has proven its effectiveness to characterize therapeutic proteins and the FDA has fully accepted CE in process development, characterization, and QC release [10].

Another main drawback concerning the analysis of proteins by CE is adsorption to the bare fused silica capillary wall, which could result in irreproducible migration times between runs and additional band broadening [11]. Thus, in recombinant therapeutic protein analysis, capillaries with inner wall modifications are often used to eliminate the sample–wall interaction and many efforts have been devoted to capillary conditioning between runs. By applying the aforementioned methodology, the protein adsorption issue can be mitigated.

In this review article, the application of CE to the analysis of recombinant protein therapeutics from the year 2000 to 2013 is summarized. The reported studies are categorized according to separation mode, including CIEF, SDS-CGE, CZE, and CE-MS. The principles are briefly reviewed and technical concerns are discussed. There have been adequate reviews on other characterization methods for biotechnology-derived therapeutic proteins [6, 12]. Also, CE or CE-MS for protein analysis has been reviewed by other authors [13–16].

2 CIEF

2.1 Principle

CIEF separates molecules based on their p*I*s in a capillary format. The experimental setup usually includes a basic catholyte at the cathode and an acidic anolyte at the anode. A mixture of the protein to be analyzed and carrier ampholytes fills up the majority of the separation capillary. A protein becomes positively charged when its p*I* value is higher than that of the local pH, and negatively charged in the opposite situation. When an electric field is applied to the capillary, the protein will migrate toward the oppositely charged electrode. As the protein migrates along the pH gradient, the charge on the protein will decrease to zero when it reaches the region where the local pH is the same as its own p*I*. At the p*I*, the electrophoretic mobility of the particle should be zero, so that proteins, which fill up the whole capillary at the beginning, are focused into sharp bands according to their p*I*s in a pH gradient along the capillary [17].

2.2 Applications

CIEF has become an often-used separation technique for protein charge variants, which could arise from PTMs, such as deamidation, C-terminal lysine processing, cysteinylation, and glycation [7]. The characterization of charge heterogeneity may also lead to the discovery of sequence variants [18]. CIEF can also be used to identify impurities from the therapeutic proteins. The recent applications of CIEF in protein therapeutic analysis are summarized in Table 1. The types of capillary, electrolytes, carrier ampholytes, and sacrificing agents used in the analyses are included in Table 1. Also provided in the table is the detection mode for the CIEF experiments.

2.3 Technical concerns

2.3.1 Imaging and two-step CIEF

The focused protein bands can be detected in one of two ways: whole-column imaging, or single-point detection with mobilization, also known as two-step CIEF. In this review, concurrent pressure and EOF mobilization during two-step CIEF is not discussed; however, relevant information can be found in other review articles [19]. Whole-column imaging CIEF (iCIEF) enables real-time monitoring of the focusing process. The most popular commercial instrument iCE 280 [20] features a 5 cm long capillary, so the focusing can be accomplished in a short time. The short capillary length also means that a strong electric field can be achieved by applying a relatively low separation voltage. The trade-off here is the resolution: the two-step CIEF method with much longer capillaries offers better resolution. Coupling novel detection methods, such as LIF or MS, is more straightforward with the two-step CIEF.

Both iCIEF and two-step CIEF requires near zero bulk flow to ensure the good focusing, as required by the principles. To achieve the near zero bulk flow, no hydrodynamic pressure should exist during focusing and EOF needs to be substantially eliminated. To decrease EOF, capillaries with neutral inner surface modifications are often used. The capillary types are summarized in Table 1.

2.3.2 Protein solubility enhancement

During a CIEF process, when the current drops to its minimum, the proteins are focused into bands in accordance to their p*I*. At this time, they are neither positively nor negatively charged; they are also at relatively high concentration, which could cause the proteins to precipitate. Protein precipitation could lead to partial clogging of the capillary, chemical mobilization failure and/or irreproducibility of the analysis. To enhance protein solubility, urea, at various concentrations, is usually added to the sample mixture [20–28].

2.3.3 Sacrificing agent

The anodic and cathodic drifts caused by the bidirectional ITP could induce a temporal pH gradient instability in IEF and could lead to sample loss [29]. The use of concentrated anolyte and catholyte could reduce the rate at which extreme p*I*

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Table 1. Protein therapeutics analysis by CIEF in 2000–2012

Sample	Mode	Capillary	Ampholytes	Catholyte	Anolyte	Sacrifizer	Reference
IgG1 mAbs	iCIEF	iCE280	Pharmalyte				Yin et al. [55]
mAb1	iCIEF	iCE280	Pharmalyte 3–10 spiked with 8–10.5	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Anderson et al. [56]
All other mAbs			Biolyte 3–10 spiked with 8–10				
EPO			Pharmalyte 4–6.5, 5–8, and 8–10.5 1:1:1				
Fc-fusion proteins			Servalyt 3–10				
Fc-fusion sialidase A treated samples			Pharmalyte 3–10				
In-house manufactured IgG1 mAbs	iCIEF, immuno-assay	Proprietary photoreactive layer coated	Pharmalytes 5–8 and 8–10.5 3:7	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC	2.5 mM arginine	Michels et al. [21]
Anti-α1-antitrypsin mouse mAb	iCIEF	iCE280	pH 5–8 GE healthcare ampholytes	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Salas-Solano et al. [22]
Ch14.18	iCIEF	iCE280	Pharmalyte 3–10 spiked with 8–10.5	0.1 M NaOH	0.08 M H_3PO_4		Soman et al. [57]
In-house manufactured mAbs	iCIEF	iCE280	Pharmalyte 3–10 spiked with 8–10.5				Lehermayr et al. [58]
In-house manufactured mAbs	Two-step	PVA-coated	Pharmalyte 3–10 spiked with 8–10.5	0.3 M NaOH	0.2 M H_3PO_4	50 mM arginine	Lin et al. [23]
In-house manufactured mAbs	iCIEF	iCE280	Pharmalyte 3–10 spiked with 8–10.5				Ren et al. [18]
In-house manufactured IgG1 mAbs	Two-step	Neutral coated	Pharmalytes 5–8	0.3 M NaOH	0.2 M H_3PO_4	0.5 M arginine and 0.2 M IDA	Salas-Solano et al. [24]
In-house manufactured mAbs	iCIEF	iCE280	pH 3–10 spiked with 6.7–7.7	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Zhang et al. [59]
Deglycosylated gemtuzumab ozogamicin	Two-step	Neutral coated or dimethylpoly-siloxane-coated	Pharmalyte 3–10	0.3 M NaOH	0.2 M H_3PO_4	0.5 M arginine and 0.2 M IDA	Maeda et al. [25]
In-house manufactured IgG2 mAbs	Two-step		Pharmalyte 3—10				Meert et al. [60]
Three proprietary mAbs	Two-step	Neutral coated	Pharmalyte 3–10	0.3 M NaOH	0.2 M H_3PO_4	1.7 mM IDA and 40 mM arginine	Mack et al. [26]
mAbs provided by Pfizer or AmerisourceBergen	iCIEF	iCE280	Ampholine 3–10	NaOH	0.08 M H_3PO_4 in 0.1% MC		He et al. [61]
THIOMABs	iCIEF	iCE280	Pharmalyte 3–10 spiked with 8–10.5	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Chen et al. [62]
IgG1 mAb digested by Carboxypeptidase B	iCIEF	iCE280	Pharmalyte 3–10 spiked with 8–10.5	0.4 M NaOH in 0.1% MC	0.1 M H_3PO_4 in 0.1% MC		Apostol et al. [63]
In-house manufactured mAbs	Two-step	Neutral coated					Dick et al. [64]
rhEPO	iCIEF	iCE280	Fluka Ampholytes 3–10	0.1 M NaOH in 1% w/v PVP	0.1 M H_3PO_4 in 1% w/v PVP		Dou et al. [27]
Hirudin, erythropoietin, and bevacizumab	iCIEF	MCE-2010 linear polyacrylamide coated, 50 μm $\times$ 110 μm $\times$ 25 mm	BioChemika 3–10 spiked with Pharmalyte 2.5–5	0.02 M NaOH	0.01 M H_3PO_4		Vlckova et al. [65]
In-house manufactured recombinant proteins	iCIEF	iCE280	Pharmalyte 3–10 spiked with 8–10.5 or 5–8	0.1 M NaOH	0.08 M H_3PO_4		Sosic et al. [28]
In-house manufactured mAbs	iCIEF	iCE280	Pharmalytes	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Quan et al [66]
A glycosylated, a nonglycosylated and a PEGylated protein model	iCIEF	iCE280	Pharmalyte 3–10 spiked with 8–10.5	0.1 M NaOH	0.08 M H_3PO_4		Li et al. [20]
MU-B3	iCIEF	iCE280	Pharmalyte 3–10 and 5–8 1:3	0.1 M NaOH	0.1 M H_3PO_4		Janini et al. [67]

iCE 280, 50 mm long 100 μm id; fluorocarbon coated.
MC, methylcellulose; IDA, iminodiacetic acid.

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carrier ampholytes and sample lose, but is unable to completely eliminate the situation. Adding sacrificial ampholytes to the sample mixture can sufficiently save the interested part of focused zones. Mack et al. [26] carried out a systematic study on the prevention of the loss of carrier ampholytes and sample components during focusing. Arginine (p*I* 10.7) and iminodiacetic acid (p*I* 2.2) were chosen to be the ideal sacrificing agents (also called blockers or spacers) and the concentrations of these compounds are optimized. Many analyses on therapeutic proteins have incorporated sacrificial compounds [21, 23–25] and arginine and iminodiacetic acid do not have to be present at the same time.

Adding sacrificial ampholytes in two-step CIEF can also ensure the observation of analyte zones during the mobilization step by acting as a spacer to confine the analytes in the front of the detection window, so that it only migrates through the detection window after the mobilization process is initiated [26]. Without the sacrificing agents, peaks that are artifacts of analytes band migrating back and forth around the detection window can be observed, causing confusions when the results are interpreted.

2.3.4 Detection

Almost all current CIEF analyses use UV absorption detection at 280 nm. In iCE 280, the proteins are focused and the whole 5-cm column is imaged by a CCD camera. In the two-step CIEF experiments, the protein train passes the detection window sequentially, and the detection aperture size has an influence on the resolution [26].

UV detection is sufficient when the protein concentration is adequate. Two ways can help to push the detection limit lower. Michels et al. [21] applied a multiplex-based immunoassay to iCIEF for the charge heterogeneity characterization of the in-house manufactured mAbs (Fig. 1). After focusing, the IgG mAbs were immobilized to the capillary wall by UV light and probed using a horseradish peroxidase conjugated secondary anti-IgG. The bound anti-IgG, after washing, was detected by chemiluminescence with a peroxidase reactive substrate. This immunoassay had a LOD of 2 ng/mL for the charge variants.

Another way to increase the sensitivity is to couple CIEF with MS [19]. The hyphenation between CIEF and MS provides the separation based on p*I* and mass-to-charge ratio, which could be equivalent to a simplified version of 2DE. Moreover, with MS/MS, protein structural information can be provided without further separation procedure. Zhu et al. [30] analyzed host cell proteins for a mAb via CIEF-MS. With the in-house made electrospray interface, the LOD can be 70 nM for a myoglobin peptide.

To further exploit the superior resolution of charge variants as well as to reach a more thorough characterization of each charge variants, coupling CIEF with MS is the method to watch. The p*I* based and mass-to-charge-ration based separation can be a promising automated alternative to the traditional 2D gel for the protein therapeutics characterization.

2.3.5 Capillary wall conditioning

Arguably CIEF is the analytical technique with the highest resolution, reproducibility has always been a concern. Even using commercialized protocols, consecutive runs can cause significant capillary degradation. The degradation is often resulted from unstable capillary wall coating, protein adsorption, and carrier ampholytes dynamic interaction with the capillary wall. Much effort has been made for removing the absorbed proteins, including rinsing with hydrochloric acid and urea solutions [31]. Recent articles used urea to rinse between runs to maintain performance [23–26]. Bonn et al. [31] implemented a revival solution wash in combination with a PVA-coated capillary and increased number of reproducible runs from 4–5 injections to 100+ injections per capillary.

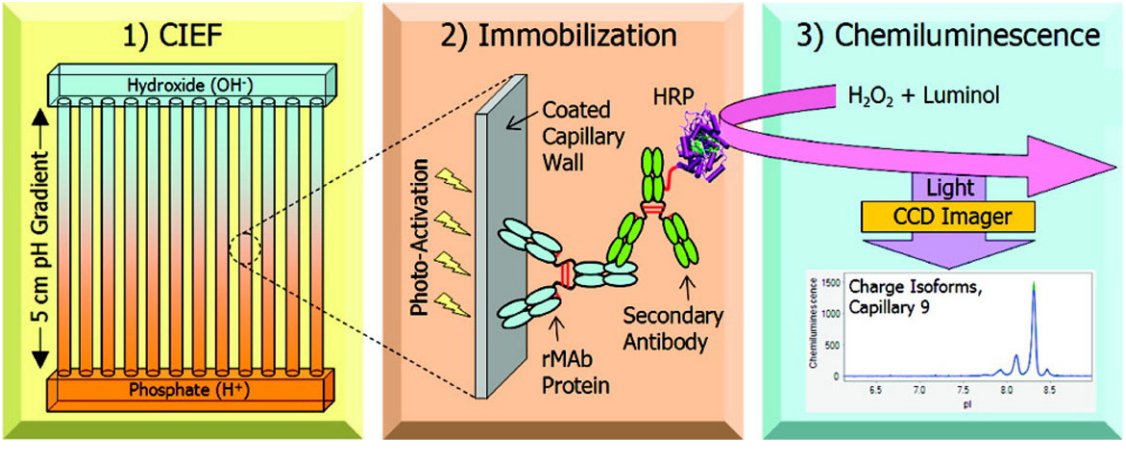


Figure 1. Charge heterogeneity of mAb by multiplexed iCIEF immunoassay with chemiluminescence detection (reprinted with permission from [21]. Copyright (2012) American Chemical Society).

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3 SDS-CGE

3.1 Principles

For most proteins, when SDS binds to the polypeptide chain, the negative charges imparted are in proportion to the protein's molecular mass. At high SDS concentrations (significantly above the CMC), approximately 1.4 g of SDS per gram of protein is coated. The surface charge is dependent on bound SDS, not the native charge of the protein. In a sieving medium, a size or hydrodynamic radius based electrophoretic separation can be achieved, since all the SDS–protein complexes have a similar mass-to-charge ratio. The electrophoretic mobility is in accordance with the logarithm of the protein molecular mass so that the apparent molecular weight can be derived using a calibration curve constructed by a series of molecular weight standards. SDS-PAGE has been used for size-based protein separations traditionally. Transferring SDS-PAGE to a capillary format eliminates drawbacks of conventional gel electrophoresis, including the labor-intensive gel preparation and the irreproducibility associated with CBB or silver staining/destaining detection. SDS-CGE offers on-column UV or fluorescence detection, automation, better resolution and reproducibility, accurate quantification, and more precise molecular weight determination.

3.2 Applications

SDS-CGE is mainly used to determine protein size heterogeneity, assess low-level product fragmentation and aggregation, and detect impurities from host cell proteins. Applying a reducing reagent like 2-mercaptoethanol in sample preparation can reduce disulfide bonds in mAbs, and the heavy chain glycosylation and post-translational proteolytic processing can also be determined. Another scheme is to analyze the nonreduced proteins by including an alkylating reagent like iodoacetamide to prevent the disulfide rearrangement. Recent applications of SDS-CGE on therapeutic proteins are listed in Table 2. The information in Table 2 includes the separation mode, the pretreatment of protein, the separation condition, buffer used in the analysis and the detection methods.

3.3 Technical concerns

3.3.1 Capillary

EOF is eliminated when running in SDS-CGE mode to ensure the separation based solely on the hydrodynamic radius. The reported SDS-CGE analyses from the year 2000 to date have been performed using bare fused silica capillary without modifications except for the report published by Cooper et al. [32], which used a hydroxypropyl cellulose coated capillary for the intrinsic charge ladder test. In the Beckman Coulter SDS buffer, the high-concentration Tris-borate suppresses the EOF. Also, in some cases, Bio-Rad buffer is used and the small percentage of triethanolamine dextran forms a dynamic coating to eliminate the EOF [33, 34].

In 2012, Rustandi et al. evaluated a CE-SDS based Western blot system (Simon™) and the capillary was bare fused silica modified with a proprietary UV-activated covalently linked coating [35]. In this case, the proteins were separated based on size and attached to the capillary wall by UV light. After incubating and washing, the proteins were detected with chemiluminescence by secondary antibodies. This automatic western blotting approach makes CE more applicable for protein characterizations.

3.3.2 Injection

Almost all SDS-CGE analyses on therapeutic proteins were carried out with electrokinetic injection. Theoretically, the sample zone from electrokinetic injection has a plug profile instead of the parabolic profile, which is created by hydrodynamic injection, and thus less band broadening would be observed using electrokinetic injection. Also, the stacking advantages can be used for the separation of proteins that have low diffusion coefficients. In addition, the capillary is filled with a viscous gel medium in SDS-CGE, hydrodynamic injection may not be practical [36].

3.3.3 Detection

The separated proteins are detected by fluorescence or UV and the most common wavelengths used in UV detection are 200 and 220 nm. To enhance the sensitivity, Zhang implemented a bubble cell to increase the light path length [37]. Another way to increase the sensitivity is to utilize LIF detection. Salas-Solano et al. used 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA.SE) to label the in-house manufactured proteins and the detection limit was comparable to that typically obtained with SDS-PAGE silver staining (10 ng/mL) [38]. However, the removal of excess dye was necessary to terminate the reaction and avoid the comigration between dyes and earlier eluting peaks. Michels et al. developed an improved sample preparation scheme for mAbs analysis using the fluorogenic reagent 3-(2-furoyl)-quinoline-2-carboxaldehyde [39]. This dye only fluoresces upon binding to proteins so no dye removal step is required. The optimized scheme also minimized sample preparation artifacts in the form of fragmentation, disulfide reshuffling, covalent interactions, or high molecular weight aggregation, while the detection sensitivity was comparable to that of the 5-TAMRA.SE derivatization method. The subsequent articles concerning SDS-CGE analysis with LIF detection for therapeutic proteins were predicated on this methodology.

3.3.4 Capillary conditioning

If capillary regeneration is mentioned in an article, the methodology described is often quite generic. The rinsing

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Table 2. Protein therapeutics analysis by CE-SDS in 2000–2012

Sample	Mode	Protein treatment	Separation	Buffer	Detection	Reference
IgG1 mAbs	Reduced	Labeled with FQ dye	−15 kV		LIF 488 nm excitation, 600 ± 15 nm emission	Yin et al. [55]
Carbonic anhydrase I, myoglobin, ovalbumin, and BSA			50 μm × 33 (24.5) cm; −16.5 kV	Beckman Coulter SDS-MW gel buffer	UV 220 nm	Cianciulli et al. [68]
Anti-α_1-antitrypsin			50 μm × 50 (45) cm; +20 kV	Tris and borate buffer with various SDS concentrations	UV 200 nm	Cooper et al. [32]
In-house produced IgG1 and IgG4 mAbs		Buffer exchanged and labeled with FQ dye	50 μm × 31.2 (20) cm; −15 kV	Beckman Coulter SDS-MW gel buffer	LIF 488 nm excitation, 600 ± 20 nm emission	Michels et al. [69]
RTA conjugated mAbs	Reduced	Conjugated to RTA after SPDP modification	50 μm × 24 (19.5) cm; −15 kV	Bio-Rad CE-SDS run buffer	UV 220 nm	Na et al. [33]
In-house produced vaccines	Reduced CE-SDS Western blotting		100 μm × 5 cm; 250 V	Protein simple separation matrix and stacking matrix	Proteins immobilized by UV light, probed by a secondary antibody, and detected by chemiluminescence	Rustandi et al. [35]
Bovine serum IgG, and mouse serum IgG	Nonreduced and reduced	Labeled with flurofore Chromeo P503	QIAxcel multicapillary electrophoresis instrument; 6 kV	60 mm Tris–borate containing 0.1 % SDS (ph 8.45)	LED induced fluorescent detection	Szekrenyes et al. [70]
In-house produced mAbs	Reduced and nonreduced	Lys-C and trypsin digestion for nonreduced	50 μm × 31.2 (20) cm; −15 kV	Beckman Coulter SDS-MW gel buffer	UV 220 nm	Hapuarachchi et al. [71]
In-house produced mAbs	Reduced and nonreduced	Buffer exchanged and labeled with FQ dye	50 μm × 31.2 (20) cm; −15 kV	Beckman Coulter sieving gel	LIF 488 nm excitation, 600 nm emission	Kaschak et al. [72]
In-house produced mAbs	Reduced		50 μm × 31.2 (20) cm; −15 kV	Beckman Coulter sieving gel	UV 220 nm	Kotia et al. [73]
In-house produced IgG2 mAbs	Reduced and nonreduced	Lys-C digested	50 μm × 30.2 (20.2) cm	Beckman Coulter sieving gel	UV 220 nm	Lacher et al. [40]
In-house produced mAbs	Reduced and nonreduced		50 μm × 31.2 (20) cm; −15 kV	Beckman Coulter sieving gel	UV 220 nm	Zhang et al. [74]
In-house produced mAbs	Reduced and nonreduced		50 μm × 33 (24.5) cm; −15 kV	Bio-Rad CE-SDS run buffer	UV 220 nm	Guo et al. [34]
				Proprietary borate buffer with additives (ph 9.9) (Sebia)	UV 200 nm	McCudden et al. [75]
In-house produced mAbs	Reduced and nonreduced		50 μm × 31.2 (20) cm; −15 kV	Beckman Coulter sieving gel	UV 220 nm	Rustandi et al. [76]
In-house produced mAbs	Reduced	Labeled with FQ dye	50 μm × 31.2 (20) cm; −15 kV	Beckman Coulter sieving gel	LIF 488 nm excitation, 600 ± 15 nm emission	Michels et al. [39]
In-house produced mAbs	Reduced and nonreduced	Buffer exchanged using NAP-5 and labeled by 5-TAMRA.SE	50 μm × 31.2(20) cm; −480 V/cm	CE-SDS polymer solution	LIF 488 nm excitation, 560 ± m emission	Salas-Solano et al. [77]
In-house produced mAbs	Reduced		50 μm id × 33 cm, bubble factor 3 (extended light path); 25 kV	10% SDS, at a mass ratio of 1:1.7 (protein/SDS)	UV 220 nm	Zhang et al. [37]
In-house produced IgG1	Reduced		50 μm × 38.5 (30) cm; 390 V/cm	Properitery buffer solution	UV 220 nm	Tous et al. [78]

FQ dye, 3-(2-furoyl)-quinoline-2-carboxaldehyde; RTA, ricin toxin A chain; SPDP, *N*-Succinimidyl 3-(2-pyridyldithio)-propionate; 5-TAMRA.SE, 5-carboxytetramethylrhodamine succinimidyl ester.

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sequence includes a short rinse by 0.1 M NaOH, 0.1 M HCl, and water, and then a long rinse (10–15 min) by the running buffer. The base and acid rinses eliminate protein adsorption and the longer rinse with the running buffer allows the pre-equilibrium between the capillary inner wall and the buffer.

3.3.5 Comparison with other techniques

The SDS-CGE method has become a primary method for size variants analysis; while there are other orthogonal analytical techniques for isomer characterization, including reverse-phase HPLC, ion exchange chromatography, and peptide mapping. Lacher et al. reported an optimized SDS-CGE method for IgG2 disulfide isomer separation and compared the method with sorption chromatography [40]. Even with optimized chromatography conditions, it was not possible to achieve baseline separation of the disulfide bond isomers. The CE method used was always able to provide better resolution. A fraction of the isomers that was collected from the LC was run using CE, and showed split peaks.

4 CE-MS

4.1 Principle and applications

Coupling MS to CE significantly increased the potential applications of the CE technique by using an information-rich detection method, providing information about the identity and structure of analytes. MS as a sole analytical technique or as a hyphenated technique with separation methods has been extensively used in the field of characterization of protein therapeutics [8, 15, 16, 41]. Two main types of characterization are performed when analyzing therapeutic proteins by CE-MS. One is to determine the primary structures. Proteins are usually treated by enzymes to release the peptides and the peptides are separated and detected. The obtained mass-to-charge ratios are searched in the databases and the proteins in a mixture can be identified or the amino acid sequence can be elucidated. CE-MS excels at low-mass peptides and is proven to have better sequence coverage compared to LC-MS [42, 43]. Another goal for characterization is to study the intact proteins for additional information. The purity of a therapeutic protein can be determined by CE-MS via the separation of impurity constituents. The isoforms of a protein, such as glycoforms, could also be characterized by CE-MS [44–46].

Note that the coupling between CE and MS is not as straightforward as that of LC-MS. However, the feasibility has been demonstrated by the development of improved interfaces over the last 26 years. Review articles about CE-MS interfaces have been published by many authors [47]. CE-MS can also incorporate other detection methods in the same system to provide additional information. Gennaro et al. demonstrated a CE-LIF-MS system for the analysis of the glycans released from mAbs and obtained both qualitative (structural) and quantitative data in one run [48].

Recent CE-MS applications are listed in Table 3. The information includes the separation mode, the sample pretreatment, and the MS analyzer, capillary, BGE, and interface, sheath flow (if any) used in the analysis.

4.2 Technical concerns

4.2.1 Sequence coverage

Peptide mapping is crucial for the characterization of therapeutic proteins. A protein is usually digested by enzymes like trypsin first, and followed by MALDI-TOF MS or LC-MS analysis to elucidate the primary structure. MALDI-TOF is fast but prone to matrix effect and ion suppression. Reverse phase LC-MS provides high sequence coverage (>95%) but is poor at detecting the low-mass peptides: the retention is insufficient because of the weaker interaction between the stationary phase and the small or hydrophilic peptides when the high-salt-content digestion buffer is present. Because the separation mechanism of CE is charge-based, it tolerates salt ions in the sample and is a useful tool for the analysis of small hydrophilic molecules. Therefore, analyzing "void" peptides that are often lost in the void peak of the chromatograms in LC-MS, by CE-MS is an attractive complement for peptide mapping [43]. Whitmore et al. reported a 100% sequence coverage using CE-MS for the analysis of the in-house manufactured mAbs, while the LC-MS method covered 49 out of 61 predicted peptides, missing the short peptides. The smallest eight peptides were seen only with CE-MS [42].

4.2.2 CE-MS interface

The CE-MS analysis of therapeutic proteins has been carried out mainly using triple-tube interfaces. Coupling to MS limits the range of available BGE for CE analysis. In a triple-tube interface, the make-up flow can modify the chemical properties of the eluent and provide a broader range of BGE choices. The sheath gas also promotes ionization. However, the make-up flow introduces significant dilution to the analytes, which decreases the sensitivity. The sheathless interface has been used in the research community for better sensitivity. However, only one paper reported applying the sheathless interface for peptide mapping of therapeutic proteins [42] and one other for protein isoform characterization [44].

When choosing between sheath-flow and sheathless CE-MS interface, another concern is if it is still possible to utilize neutrally coated capillaries in the analysis. With the high amount of heterogeneities in biotechnology-derived recombinant proteins, neutrally coated capillaries can facilitate the separation by decreasing the sample–wall interaction and EOF bulk flow. When coupling to MS, the neutrally coated capillaries pose a challenge to the flow rate match, since there is no significant EOF. The sheath liquid can make up the flow rate by introducing additional flow but also lead to sample dilutions. An approach to solve this dilution problem is to use

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Table 3. Protein therapeutics analysis by CE-MS in 2000–2012

Sample	Mode	Capillary	BGE	Interface	Sheath flow	MS	Sample pretreatment	Reference
rhEPO	CZE-MS	Beckman neutral bilayer coated	2.0 M CH_3COOH	Beckman Coulter sheathless	None	TOF	Desalted	Haselberg et al. [44]
Interferon-β			50 mM CH_3COOH					
In-house produced mAbs	CZE-MS	PVA-coated or bare fused silica	10% CH_3COOH	Agilent sheath-liquid	0.1% CH_3COOH	TOF	Tryptic digestion	Whitmore and Gennaro [42]
		Bare fused silica	10% CH_3COOH	Beckman Coulter sheathless	None	TOF		
Host cell proteins of a recombinant mAb	CIEF-MS	Linear polyacrylamide-coated	0.1% HCOOH; 0.3% NH_4OH; Pharmalyte 3–10; mobilized by sheath flow	In-house manufactured sheath-liquid	0.05% HCOOH in 50% methanol	Orbitrap	Rhigg depleted, magnetic microsphere-based immobilized trypsin used for digestion	Zhu et al. [30]
In-house synthesized deamidated peptides	CZE-MS	PVA-coated	100 mM or 20 mM CH_3COOH	New Objective metal-coated nanospray		IT		Gennaro and Salas-Solano [79]
rhEPO	CZE-MS	Polybrene or UltraTol dynamic Pre-Coat LN dynamically coated	1 M CH_3COOH in 20% methanol	Agilent sheath-liquid	1% CH_3COOH in 50% 2-propanol	TOF	Desalted and preconcentrated	Balaguer [45]
rhEPO peptide	CZE-MS	Bare fused silica	100 mM HCOOH	Agilent sheath-liquid	0.5% HCOOH in 50% 2-propanol	IT	Desalted, tryptic digestion	
In-house produced mAbs	CZE-MS	PVA-coated	25 mM HCOOH	ThermoFinnigan sheath-liquid	Methanol/water/BGE (80:15:5, v/v/v)	IT	Endoproteinase Lys-C digested	Gennaro et al. [43]
rhEPO	CZE-MS	Polybrene-coated	1 M CH_3COOH in 20% methanol	Agilent sheath-liquid	2-Propanol:1% CH_3COOH in water (1:1)	TOF		Neusüß et al. [46]
Peptide hormones of therapeutical interest	CZE-MS	Bare fused silica	50 mM CH_3COOH and 50 mM HCOOH	Agilent sheath-liquid	2-Propanol-water/0.05% HCOOH (60:40 v/v)	TOF		Sanz-Nebot et al. [80]

LN, low normal.

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Table 4. Protein therapeutics analysis by CZE in 2000–2012

Sample	Capillary	BGE	Running condition	Sample pretreatment	Detection	Run-to-run conditioning	Reference
Rituximab, trastuzumab, and ranibizumab	Polyacrylamide-based hydrophilic	200 mM EACA-CH_3COOH, 30 mM CH_3COOLi, and 0.05% w/v HPMC, pH 4.8	50 μm × 40.2 (30.2) cm; 15 kV		UV 214 nm	0.1 N HCl, H_2O, BGE	Espinosa-de la Garza et al. [81]
Infliximab and bevacizumab		150 mM EACA-CH_3COOH, 20 mM CH_3COOLi, and 0.05% w/v HPMC, pH 5.5.					
Tryptic digest of a mAb	APTES coated	50% CH_3CN, 0.1% HCOOH	Microchip CE channel 91 mm		TOF MS		Mellors et al. [82]
rhEPO	UltraTrol Dynamic pre-coat LN dynamic coated	2.0 M CH_3COOH	UV 3 kV, 75 μm × 70 (60) cm; Flu 17.5 kV, 75 μm × 74 (49) cm (Flu)	Ultracentrifugal desalting	UV 200 nm; Flu 280–295 nm excitation, 335 nm emission	1 M NaOH, H_2O, UltraTrol dynamic precoat LN, BGE	de Kort et al. [49]
Interferon α-2 variant forms	Polybrene coated	50 mM HCOOH in 20% CH_3OH	50 μm × 90 (80); −30 kV	Buffer exchanged	UV 200 nm	H_2O, BGE	Mark et al. [83]
C-terminal Lys variant of TNF-α targeted monoclonal IgG1 and papain-induced degradation of Her-2 targeted monoclonal IgG1	Bare fused silica	20 mM CH_3COOH (pH 6.0), 0.3% PEO and 2 mM triethylenetetramine (TETA)	50 μm × 30.2 (20) cm; 30 kV		UV 214 nm	0.1 M HCl, BGE	Shi et al. [84]
rhEPO	Bare fused silica	10 mM CH_3COONa, 7 M urea, 10 mM Tricine, 3.9 mM putrescine, and 100 mM NaCl at pH 5.50	50 μm × 60 (50) cm; 15 kV	Immunomagnetic beads-based extraction	LIF 266 nm excitation, 340 nm emission	0.1 M NaOH, BGE	Wang et al. [50]
5 Commercialized mAbs	Fused-silica sipper chip	0.2% HPMC and 0.1% polysorbate 20 in 50 mM CH_3COONa pH 6	5 μm × 12.5 mm; 2000 V	Derivatized by Cy5 *N*-hydroxysuccinimide ester	UV 214 nm	Not required	Han et al. [85]
In-house produced mAbs	Bare fused silica	400 mM EACA–CH_3COOH, 0.05% w/v HPMC, 2 mM TETA, pH 5.7	40 μm × 30 (10) cm; 30 kV		UV 214 nm	0.1 M HCl, BGE	He et al. [52]
Bevacizumab and ranibizumab	Bare fused silica	PBS of 0.016 M ionic strength (pH 7.4)	50 or 75 μm × 30 (20) cm; 10 kV or 6 kV		UV 212 and 254 nm	0.1 M NaOH, H_2O, BGE	Li et al. [86]
Recombinant human glycosylated interleukin-7	Diaminobutane dynamically coated	25 mM $Na_2B_4O_7$ at pH 10, 12 mM diaminobutane	50 μm × 47 (40) cm; 20 kV		UV 214 nm	H_2O, 0.1 M NaOH, H_2O, BGE	Alahmad et al. [87]
Granulocyte colony-stimulating factor	Bare fused silica	100 mM phosphate buffer (pH 2.5)	75 μm × 32 (22) cm; 10 kV		UV 214 nm	0.1 M NaOH, H_2O, BGE	Lee and Na [53]
In-house produced mAbs	Bare fused silica	600 mM EACA–CH_3COOH (pH 5.5), 0.1% HPMC	50 μm × 50 (40) cm; 24 kV	Deglycosylated	UV 214 nm	0.1 N HCl, BGE	He et al. [88]
		600 mM EACA–CH_3COOH (pH 5.5), 0.1% HPMC, 4 M urea		Redox by cysteine			
Recombinant human interleukin-7	Bare fused silica	25 mM citrate/TEOA at pH 2.6	75 μm × 57 (50) cm; 15 kV		UV 214 nm	H_2O, 50 mM SDS, H_2O, BGE	Alahmad et al. [89]
Tocilizumab	Carboxylated	25 mM phosphate (pH 5.8) and 0.1% SDS	50 μm × 50 (40) or 40 (30) cm; 20 or 28 kV	PNGase F digested; reduced	UV 214 nm	1M NaCl, BGE	Taga et al. [90]

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Table 4. Continued

Sample	Capillary	BGE	Running condition	Sample pretreatment	Detection	Run-to-run conditioning	Reference
PEGylated human parathyroid hormones	Polyacrylamide-coated	100 mM phosphate buffer (pH 2.5)	50 μm × 24 (19.5) cm; 10 kV	Lys-C digested	UV 200 nm and 280 nm	H_2O, BGE	Na and Lee [91]
Nanoparticle–protein conjugates	Bare fused silica	100 mM $Na_2B_4O_7$	75 μm ×65 (48) cm; 30 kV	Derivatized with naphthalene-2,3-dicarboxaldehyde	LIF 442 nm excitation, 488 nm emission		Wang et al. [92]
rhEPO	Bare fused silica	0.01 M Tricine, 0.01 M NaCl, 0.01 M CH_3COONa, 7 M urea, and 3.9 mM putrescine at pH 5.50	50 μm × 107 (100) cm; 30 kV	Low-molecular-mass excipients eliminated using Microcon-10 cartridges	UV 214 nm	H_2O, 0.1 M NaOH, H_2O, BGE	Lopez-Soto-Yarritu et al. [51]
		80 mM morpholine, 10 mM boric acid, 8 mM NaCl, 8 mM CH_3COONa, 5.6 M urea					
	PEI coated	350 mM CH_3COOH	50 μm × 87 (80) cm; −12 kV			0.1 M NaOH, H_2O, 5% PEI, H_2O, BGE	

LN, low normal; PEI, polyethylene imine.

reduced-flow interface [47]. Regarding sheathless interfaces, recently there is a research article by Haselberg et al. [44] demonstrating the feasibility of analyzing protein glycoforms with low-flow sheathless CE-MS, which makes the future of CE-MS with neutrally coated capillaries more promising.

4.2.3 BGE

BGEs composed of formic acid and/or acetic acid are commonly used for CE-MS analysis of therapeutic proteins. The small organic acid molecules are volatile and therefore are ESI-friendly. Also, the intact protein analytes or the peptides generated from enzymatic digestions are positive ESI amenable and so acidic BGEs facilitate the ionization.

4.2.4 Capillary conditioning

The CE-MS analysis for therapeutic proteins so far has been carried out with acidic BGEs for which the adsorption to the bare fused silica capillary inner surface is minimal. With neutrally coated capillaries, the interaction between proteins/peptides and capillary inner surfaces is also suppressed by the coatings. Buffer flushing for a short period of time between runs is thus usually sufficient for capillary reconditioning between runs.

5 CZE

5.1 Principles and applications

CZE is still the most often used mode of CE due to its simplicity. The separation is based on the different electrophoretic mobilities, which result from the different ratios of charge and hydrodynamic radius of the analytes when they are present in an electric field.

In the manufacturing of therapeutic proteins, different PTMs can be incorporated. CZE as a charge-based separation tool is usually used to confirm the identity of a therapeutic protein, detect the impurities, and characterize the charge heterogeneity. The applications of CZE are summarized in Table 4.

5.2 Technical concerns

5.2.1 Detection

The CZE applications so far (CZE-MS not included) rely mostly on UV detection at 200 or 214 nm, as shown in Table 4. To achieve better sensitivity, deep UV-LIF can be utilized for recombinant human erythropoietin (rhEPO) analysis [49, 50]. The excitation wavelengths are below 300 nm and the emission is around 340 nm, based on the native fluorescence of rhEPO molecules.

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5.2.2 Capillary conditioning

Different types of the capillary wall modifications (bare fused silica, neutrally coated, and positively coated) can be used for CZE applications for therapeutic proteins. For the analysis of rhEPO, which is quite acidic, the rinsing sequence always consists of 0.1 M NaOH rinse followed by water and BGE [49–51]. For products like mAbs, the conditioning process is more likely to include an additional HCl rinse step prior to NaOH wash and/or SDS wash. More details are listed in Table 4.

5.2.3 CZE versus other electrophoretic separations

Since CZE has mostly been applied in charge variant monitoring for therapeutic proteins, and another commonly used assay for the charge variant is CIEF, the efficiency comparison between the two techniques is an interesting topic. Moreover, the current mainstream charge-based separation method for proteins is CIEF, so that the general applicability of a CZE method can be demonstrated by a head-to-head comparison with CIEF assay for the same sample. He et al. [52] reported a comparison between the iCIEF and CZE analysis for the charge variants of five mAbs. The separation profiles were similar and iCIEF showed higher resolution for the acidic analytes while CZE displayed better separation for the basic ones. This is only one example of the head-to-head comparison between the two techniques, and more study is required to further compare the two techniques.

The CZE method was also compared with size based separation assays. Lee et al. [53] carried out a study on the PEG-modified granulocyte colony-stimulating factor (G-CSF). Size exclusion chromatography showed low resolution for separating mono- and di-PEG-G-CSFs. SDS-CGE provided higher resolution but required a longer analysis. CZE could separate both PEG-5K and PEG-20K conjugated G-CSFs successfully within 20 min. In this study, CZE showed better efficiency for PEGylation reaction monitoring and purity test for PEG-G-CSF. Again, more case studies are needed to support these findings.

5.2.4 Future considerations

The reported CZE analyeses have been very successful in resolving the charge variants. However, given the large heterogeneity of the recombinant protein therapeutics, methods fully utilizing the superior resolution of CE are still required. One possible approach is to apply neutrally coated capillaries in the analysis [54]. Thus, the protein adsorption to the capillary wall can be largely eliminated, mitigating the band broadening. Also, when there is no EOF present, the separation without the bulk flow is solely based on the electrophoretic mobilities, therefore the resolution can be improved.

6 Concluding remarks

The use of recombinant protein for human therapeutics demands effective characterizations of the drugs to ensure safety, quality, integrity, and efficacy. Different CE separation modes have been used in the drug discovery and design, development, and quality control phases. CIEF, SDS-CGE, and CZE have been utilized routinely in the biopharmaceutical industry. CE-MS adds another dimension of separation and provides more information about the targeted protein from a single run. The versatility of CE has proven its effectiveness as a promising technique for therapeutic protein characterization. With more research ongoing in the field of therapeutic proteins, CE is playing an increasingly important role.

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