RECENT TRENDS OF CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY IN PROTEOMICS RESEARCH

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Progress in proteomics research has led to a demand for powerful analytical tools with high separation efficiency and sensitivity for confident identification and quantification of proteins, posttranslational modifications, and protein complexes expressed in cells and tissues. This demand has significantly increased interest in capillary electrophoresis-mass spectrometry (CE-MS) in the past few years. This review provides highlights of recent advances in CE-MS for proteomics research, including a short introduction to top-down mass spectrometry and native mass spectrometry (native MS), as well as a detailed overview of CE methods. Both the potential and limitations of these methods for the analysis of proteins and peptides in synthetic and biological samples and the challenges of CE methods are discussed, along with perspectives about the future direction of CE-MS. @ 2019 Wiley Periodicals, Inc. Mass Spec Rev 00:1–16, 2019.

Keywords: *capillary electrophoresis-mass spectrometry; electrospray ionization; native mass spectrometry; proteomics; top-down mass spectrometry*

I. INTRODUCTION

Proteomics is a constantly progressing field that endeavors to comprehensively interrogate biological processes by employing a systematic analysis of the proteins expressed in cells and tissues. Mass spectrometry (MS)-based methods continue to be the most widely used methods for high-throughput proteomics research (Yates et al., 2009; Nilsson et al., 2010; Yates, 2013; Zhang et al., 2013), and liquid chromatography-mass spectrometry (LC-MS) is the prevailing platform. Confident identification and quantification of proteins, posttranslational modifications (PTMs), and peptides in biological samples rely on rigorous separation of peptides prior to introduction into the mass spectrometer (Fonslow & Yates, 2009; Zhang et al., 2013; Aebersold & Mann, 2016). Capillary electrophoresis is a mature separation technology that is effectively coupled with mass spectrometry (CE-MS) and is well-suited for this purpose (Mischak et al., 2009; Belczacka et al., 2018). Recent advances in CE-MS platforms have attracted considerable interest from the proteomics community as an alternative to LC-MS (Bush et al., 2016; Tycova et al., 2017; Stolz et al., 2019). CE offers advantages over LC, including efficient and fast separations paired with low sample consumption. In addition, solvent consumption (aqueous or organic) in CE-MS is very low, making it environmentally advantageous (Tycova et al., 2017). The analytical limitations and advantages of CE and LC and some of their contrasting characteristics are presented in Table 1.

The most common CE-MS technique used in the proteomics field is capillary zone electrophoresis (CZE) coupled with electrospray ionization (ESI). Capillary isoelectric focusing (cIEF) (Kahle et al., 2019) is another useful, but less commonly used CE technique.

In proteomics research, proteins are often identified using a well-established bottom-up approach. This strategy is based on indirect identification of proteins through digestion (enzymatic or chemical) to provide peptides that are further analyzed by either LC-MS or CE-MS (Heemskerk et al., 2016; Toby et al., 2016). Proteins can also be investigated using specific or limited digestion to produce large peptides via middle-down strategies (Cristobal et al., 2017), or with top-down mass spectrometry (TDMS) analysis of intact proteins. TDMS is an emerging approach that provides detailed structural information on intact proteins, including characterization of PTMs (Chen et al., 2018a). PTM patterns can provide insight into potentially significant biological processes (Toby et al., 2016; Aebersold et al., 2018; Chen et al., 2018a) but can often be lost after the protein digestion (Toby et al., 2016; Biacchi et al., 2017) used in bottom-up approaches. Since CE separations can be performed in aqueous buffers, protein complexes can be separated in their native structures, preserving non-covalent interactions within a protein and between subunits of protein assemblies, prior to MS detection (Leney and Heck, 2017; Li et al., 2018; Shen et al., 2018). Antibodies and their related glycosylated forms are often analyzed using CE-MS via TDMS (Han et al., 2016) and CE-MS has been used to determine the purity and the stability of proteins in drug discovery (Jooss et al., 2017).

A growing body of research has demonstrated the efficiency of CE-MS and its successful application to proteomics. Numerous reviews have provided an overview of the technical developments of CE-MS (Jiang et al., 2017; Tycova et al., 2017; Stolz et al., 2019) and its application for peptides, (Heemskerk et al., 2016; Sun et al., 2016; Jansson, 2018; Zhang et al., 2018b) intact protein analysis, (Haselberg et al., 2007, 2013b) and biomarker discovery (Mischak et al., 2009; Pontillo et al., 2015). In this review, we focus on applications of CE-MS in proteomics research that have been reported since 2008. Parameters for maximal selectivity and sensitivity of a CE-MS platform are discussed, as well as a brief introduction to TDMS and native MS.

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TABLE 1. The analytical limitations and advantages of capillary electrophoresis (CE) and liquid chromatography (LC)

CE—advantages	CE—limitations	LC—advantages	LC—limitations
Automation	Sample loading	Automation	High organic solvent consumption
Sample size (nL)	Sensible flow rate (buffer, capillary wall)	Sample size (μL)	–
Sensitivity (fmol-nM)	–	Sensitivity (μM)	–
Speed (sec–min)	–	Speed (min–hr)	–
High resolution	–	Fast gradient	–
Aqueous solvent	–	Good reproducibility	–
Low flow	–	Stable flow	–

II. TOP-DOWN MASS SPECTROMETRY AND NATIVE MASS SPECTROMETRY

The proteomics field has been largely dominated by bottom-up-based techniques, where proteins are digested prior to MS analysis (Zhang et al., 2013). This approach is well-known for its high-throughput performance; however, critical information about PTMs and subunit protein assemblies (via non-covalent interactions) is generally lost after proteolysis (Catherman et al., 2014; Toby et al., 2016). Consequently, peptide-based methods may limit our understanding of the intricate and nuanced control of diverse biological activities by PTMs (Toby et al., 2016). It is commonly accepted that PTMs have significant effects on the fates and functions of cellular proteins (Aebersold et al., 2018). Thus, characterization of PTMs is of key importance for understanding cell signaling processes related to clinical disorders (Chen et al., 2018a). Top-down mass spectrometry (TDMS) is an emerging proteomics approach that interrogates proteins as intact entities without prior digestion. Top-down analysis can provide unique insights into a protein's structure by providing precise molecular weight measurements, the identification of multiple PTMs (Fonslow and Yates, 2009; Haselberg et al., 2013bb; Chen et al., 2018a) and in-depth proteoform characterization (different forms of a single protein generated from a gene) (Smith and Kelleher, 2013). In addition, as demonstrated by Gomes and co-workers (Chen et al., 2018b; Gomes et al., 2019), TDMS is well-suited for identification and characterization of branched systems consisting of two proteins. Recent improvements in mass spectrometry instrumentation and bioinformatics have enabled TDMS to be used to characterize and quantify hundreds to thousands of proteoforms in a single analysis (Chen et al., 2018b). TDMS is now being used in a wide range of applications, including the production of therapeutic proteins, such as antibody-drug conjugates (Chen et al., 2018b). Most TDMS studies are carried out under denaturing conditions, leading to the disruption of the biologically relevant non-covalent interactions, such as protein–protein and protein–ligand complexes (Belov et al., 2017).

While the use of appropriate terminologies to precisely describe the different types of TDMS experiments is still debated, Lermyte et al. (2019) have proposed a standard lexicon for TDMS, and we have adopted their recommendations for this review. The schematic of a TDMS workflow is shown in Figure 1.

Most proteins are assembled into protein complexes to achieve the functional activity, but it is still unclear how these macromolecular complexes are formed. Native MS provides a proteomics tool that enables identification and characterization of these biomolecular structures and advances the understanding of their biological pathways at the molecular level (Belov et al., 2017; Leney and Heck, 2017; Li et al., 2018). Native MS can be used to interrogate the structure of intact proteins in their near-native state, with an emphasis on preserving non-covalent protein–protein and protein–ligand complexes (Leney and Heck, 2017). The success of native MS approaches relies on ESI of proteins in a volatile aqueous buffer that preserves native quaternary and tertiary states in the gas phase (Leney and Heck, 2017; Li et al., 2018). Native MS provides information about ligand binding, dynamics, stoichiometry of individual subunits, stability, topology, and assembly structural properties (Belov et al., 2017). Recently, the Kelleher group reported a standard operating procedure for the native MS (Schachner et al., 2019). This valuable tutorial was designed to guide non- and experienced scientists to obtain

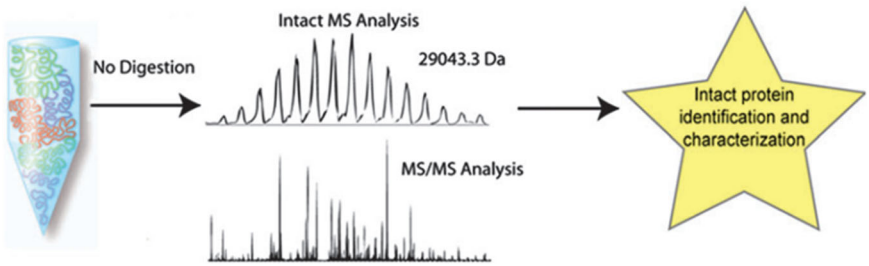


FIGURE 1. Schematic of a top-down mass spectrometry (TDMS) workflow. Reproduced from Kellie et al. (2010), with permission from The Royal Society of Chemistry. [Color figure can be viewed at wileyonlinelibrary.com]

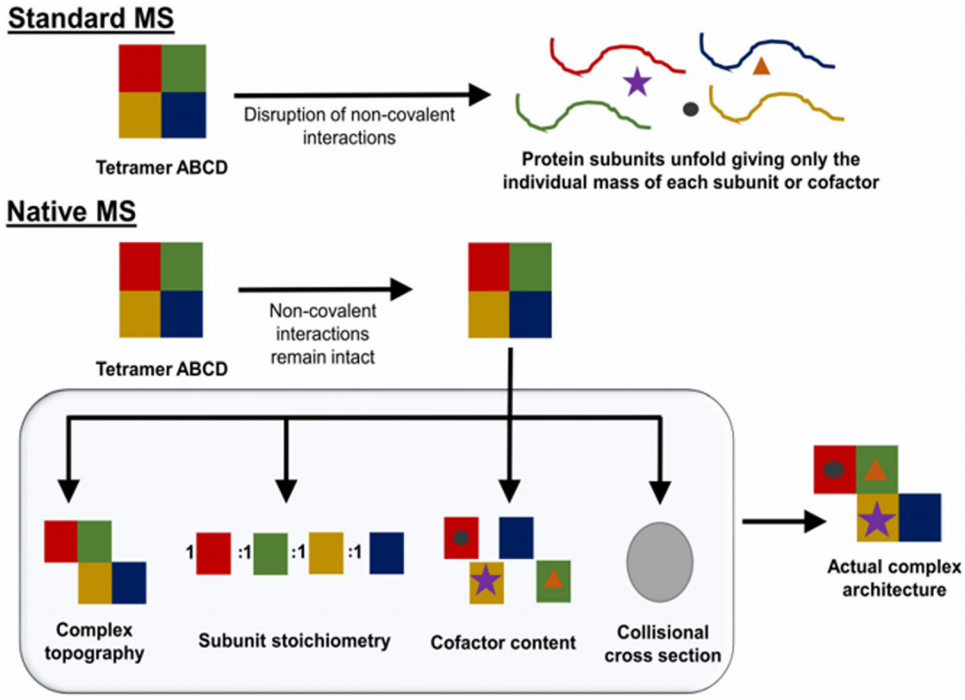


FIGURE 2. Schematic of the information obtained from native mass spectrometry (MS) experiment. Reprinted from Tokmina-Lukaszewska et al. (2018), with permission. [Color figure can be viewed at wileyonlinelibrary.com]

reproducible and high-quality native MS data for proteins ranging from 30 to 300 kDa. Figure 2 illustrates a schematic of the information that can be obtained from a native MS experiment.

III. CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

A. Interface

Since the introduction of CE-MS for analysis of macromolecules, a few soft ionization techniques have been evaluated for CE-MS platforms, but as ESI is the most commonly used technique, we will limit our review to CE-MS applications utilizing ESI. Interfacing CE with ESI requires transferring a voltage to liquid to create the electrospray, which has been done with sheath–liquid or sheathless interfaces. Several thorough reviews have chronicled the considerable progress made in interfacing CE with ESI-MS (Krenkova & Foret, 2012; Ramautar et al., 2012; Tycova et al., 2017; Stolz et al., 2019).

The sheath–liquid interface has proven to be versatile and robust and is currently the more commonly used configuration in CE-MS for proteomics research. This interface uses a coaxial sheath–liquid to produce electrical contact between the CE and ESI source. Conversely, the sheathless configuration is based on the direct application of voltage to the background electrolyte (BGE). The Dovichi group has made significant advances in sheath–liquid interface designs, including the electrokinetically, pumped low sheath–flow nanospray interface. This sheath–liquid prototype is commercially known as EMASS-II ion source. It has no mechanical flow and a closed electrical circuit that is driven exclusively by electrokinetic flow (Wojcik et al., 2010; Peuchen et al., 2017). Its application has been successfully demonstrated for both bottom-up (Zhu et al., 2013; Schiavone et al., 2015; Sun et al., 2015; Yang et al., 2018; Zhang et al., 2018b) and top-down analysis (Zhao et al., 2016b; McCool et al., 2018a,b; Qu et al., 2018). A potential limitation of the sheath–liquid interface is that the high flow rate of the sheath–liquid dilutes the analyte, thereby causing a potential loss in sensitivity (Fonslow and Yates, 2009; Heemskerk et al., 2016). Also, suction effects that are caused by both the sheath–liquid and nebulizing gas can be detrimental for separation efficiency (Mokaddem et al., 2009). However, this interface adds flexibility to the system since the spray conditions can be optimized (e.g., organic content) and thus, the ionization process is less dependent on the BGE composition. Although the sheath–liquid usually contains organic solvents that can adversely affect native protein conformations, Shen et al. (2018) successfully applied an aqueous solution of 25 mM ammonium acetate as a sheath–liquid for the analysis of protein complexes from *Escherichia coli* (Shen et al., 2018).

In contrast, the sheathless interface can significantly enhance signal/noise due to decreased analyte dilution and background noise, as the BGE is the only liquid in the system (Fonslow and Yates, 2009; Yin et al., 2016). In this configuration, the main challenge is maintaining CE flow while generating a closed electrical circuit between the CE separation and the ESI source (Fonslow and Yates, 2009). This has been accomplished in two different ways. The first method includes an ESI tip coated with a highly conductive metal emitter (gold) or detachable porous emitter to transfer the high voltage to the liquid (Yin et al., 2016; Hocker et al., 2018). A second strategy includes a porous region close to the ESI tip for electrical connection through an electrolyte (Fonslow and Yates, 2009). The porous-tip sheathless interface was introduced by the Moini group and is the most popular sheathless prototype in proteomics research (Hocker et al., 2018). The sheathless interface has been widely used in proteomics

research to provide ultrasensitive analysis (Medina-Casanellas et al., 2014; Kammeijer et al., 2016; Snyder et al., 2017), although it is limited by potential blocking of the pores. In a recent paper, Belov et al. (2017) reported a successful application of an etched porous-tip sheathless interface for analysis of complex-forming protein standards, a monoclonal antibody, and a ribosomal proteome of the *E. coli* in native conditions. This interface enabled characterization of proteins without dilution prior to nano-ESI flow rates (10–20 nL/min). The ultra-low flow rates enabled higher sensitivity from this interface than from sheath–liquid designs. Previously, the Yates group demonstrated the power of a porous-tip sheathless interface for highly sensitive top-down analysis (Han et al., 2014a,b). In two studies, the porous-tip interface provided the sensitivity needed for front-end separation and top-down analysis. Nguyen et al. (2016) achieved high sensitivity analysis of tryptic peptides and proteins at low nanoliter flow rates using a sheathless interface that is based on a sub-micrometer fracture located directly in the capillary. In this study, a detection limit of 0.045 pmol/μL was achieved for a model peptide (leucine enkephalin) with gentle ionization, which suggests its suitability to probe proteins in their tertiary and quaternary states. A detailed experimental examination of a sheathless transient capillary isotachophoresis (cITP)/CZE-MS interface was provided by Guo et al. (2016). In this design, the electric contact between the BGE and ESI source enabled the use of a conductive liquid that is in contact with the metal-coated surface of the ESI emitter. The metal-coating on the ESI emitter was found to be highly sensitive and robust for approximately 100 analyses of a mixture of nine peptide standards. The most common types of sheath–liquid and sheathless interfaces are presented in Figure 3.

B. Separation

CE separations rely on the electrophoretic mobility of the analytes in an electric field. While many CE-based techniques have been reported for the analysis of proteins and peptides, CZE is the most commonly used in proteomics research (Zhu et al., 2012; Lamalle et al., 2016; Zhang et al., 2017; Stolz et al., 2019). In CZE separation is based on the analyte migration (according to size/charge) in different zones at different velocities (Zhang et al., 2019). The ionic strength and pH of the BGE drive the solute's electrophoretic mobility. In CZE, band broadening is only caused by longitudinal diffusion. This is particularly attractive for protein analysis because they have small diffusion coefficients (Haselberg et al., 2007, 2013b). Notable examples of CZE-MS include the intact mass analysis of biopharmaceutical proteins (Bush et al., 2016) and monoclonal antibodies (Han et al., 2016; Zhao et al., 2016a) where complete baseline separation is often achieved. Figure 4 shows a CZE separation for top-down proteome analysis of *E. coli* that resulted in high peak capacity (~280, calculated using the average peak width at 50% peak height for the *E. coli* proteome sample) (Lubeckyj et al., 2017).

Zhao et al. (2015) demonstrated the value of combining CZE separations with electron dissociation techniques for top-down analysis. In this study, a baseline separation was first

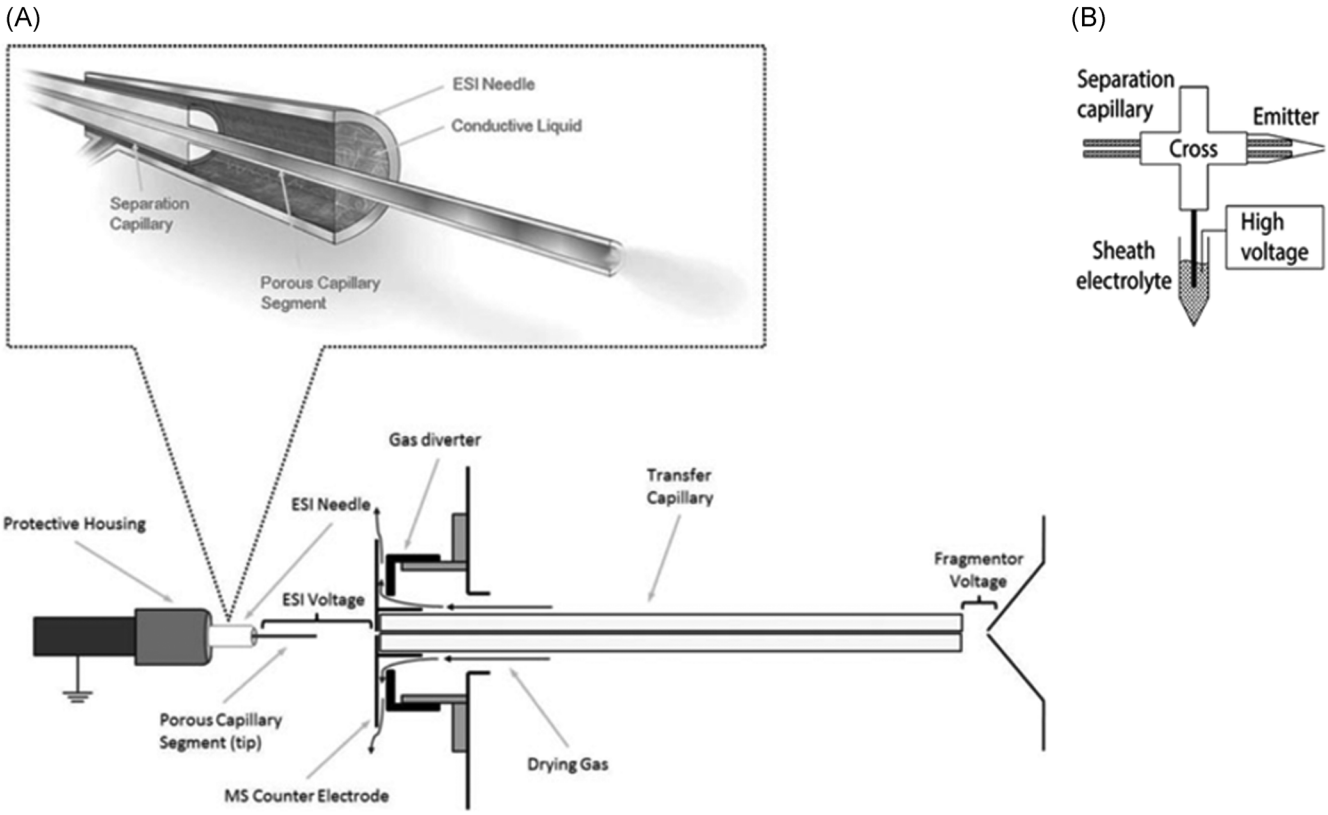


FIGURE 3. Schematic representations of (**a**) porous electrospray ionization (ESI) tip sheathless interface and (**b**) electrokinetically pumped sheath–flow nanospray. Reprinted from Bonvin et al. (2012) and Sun et al. (2015), respectively, with permission

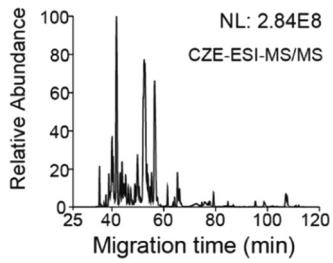


FIGURE 4. Base peak electropherogram of capillary zone electrophoresis (CZE) separation for top-down analysis in *Escherichia coli* proteome. Reprinted from Lubeckyj et al. (2017), with permission

obtained for a mixture of four standard proteins and then the CZE strategy was extended to the analysis of a biologically relevant protein mixture derived from the *Mycobacterium marinum* secretome. More recently, McCool et al. (2019) reported a CZE separation in conjunction with activated ion electron transfer dissociation to interrogate the proteome of *E. coli* via TDMS where 328 proteins and 3,028 proteoforms were identified using 1% spectrum level and 5% proteoform-level false discovery rates. In another study, the precision and robustness of CZE separations were confirmed for the analysis of monoclonal antibody Fc/2 charge variants using top-down and middle-down strategies (Biacchi et al., 2017). CZE has also proven to be a valuable tool for protein identification via bottom-up proteomics where ultrasensitive and fast analysis can be obtained (Sun et al., 2013; Zhu et al., 2016c; Zhang and Dovichi, 2018). Using a bottom-up approach, Zhang et al. (2018a) identified 27,000 peptides and 4,400 proteins with CZE. Despite the high number of identifications, some limitations were observed, including marginal identifications during the first 20 min of the separation, and a dramatic reduction in identifications after 85 min. Additional optimization could improve the methodology.

There have been several theoretical models reported to predict the electrophoretic mobility of a peptide. Since it has been generally accepted that residue shape and size have little effect on the electrophoretic mobility of a peptide, these models are based on the use of BGE pH, side group pKa, N-terminal amine, and C-terminal acid to determine the charge of the peptide. The Dovichi group reported a sequence-specific model to predict peptide electrophoretic mobility via CZE separations (Krokhin et al., 2017). The authors attributed their success to the selection of major sequence-specific features that alter the peptide charge in acidic conditions. Acidic residues (Asp and Glu) next to the N-terminus were observed to most influence the deviation of experimental mobility values from values predicted via classical models. The accuracy of this model suggests *a priori* selection of separation conditions for targeted analysis. More recently, Chen et al. (2019) reported a model to predict the electrophoretic mobility of phosphopeptides via CZE separation. According to this modeling data, phosphorylation reduces the charge of a peptide, thereby its electrophoretic mobility.

CE-MS has been used to study PTMs such as glycosylation. Glycosylation is an important and common PTM in biological systems, but its detailed characterization is challenging because of its heterogeneity in localization and forms (Mechref & Novotny, 2009; Mechref, 2011; Jayo et al., 2014; Lu et al., 2018). CZE separation has frequently been used to analyze glycosylated peptides and glycans (Konermann, 2017; Snyder et al., 2017; Giorgetti et al., 2018; Qu et al., 2018) and CZE separation via TDMS was used for glycosylation characterization (Yang et al., 2017). Posttranslational phosphorylation plays critical roles in biological systems (Mou et al., 2013) and it can also influence the electrophoretic mobility of peptides. Because phosphate groups have two pK_as (acid and neutral) they can add one or two negative charges to the protein or peptide depending on the pH of the BGE. Faserl et al. (2018) demonstrated the value of CZE for the separation of 70 synthetic peptides that were post-translationally modified including phosphorylation, acetylation, methylation, and nitration. These authors observed that multi-phosphorylated peptides were poorly retained on the stationary phase of a reversed-phase LC-MS method, resulting in a decreased number of peptide identification relative to the CE-MS analysis. Ludwig et al. (2015) reported a CZE-MS approach that was able to identify over 2,300 phosphorylated peptides in a single analysis, thus illustrating the advantages of CZE separation over LC for PTM analysis. Many studies have suggested that CZE separations are more capable of identifying hydrophilic peptides than LC (Wang et al., 2012; Zhu et al., 2013). This may be due to the LC gradient, which increases the proportion of the organic modifier throughout the analysis to detect peptides with different hydrophobicity, whereas CE conditions are basically continuous. Since there is a significant difference in size/charge between phosphorylated and unmodified peptides, CZE can achieve outstanding resolution.

Despite the high separation efficiency of CZE for proteins and peptides, separation in only one dimension is not enough to effectively reduce the complexity of proteomic samples prior to MS detection. Multidimensional separation can enhance peak capacity and lead to better separation of proteins or peptides before being introduced into the mass spectrometer. Better separation leads to enhanced ionization efficiency and reduced ion suppression. Qu et al. (2018) reported an LC-CZE strategy for the characterization of site-specific glycan heterogeneity where the use of CZE for analysis of reversed-phase LC glycopeptide fractions reduced the complexity of the sample and enabled glycan structural elucidation. The enhanced sensitivity of CZE in the second dimension is an added benefit for the analysis. Recently, Yang et al. (2018) reported the use of a reversed-phase LC-CZE approach for the identification of proteins in MCF7 proteome via bottom-up proteomics. The authors evaluated C18 ZipTip and nano reversed-phase LC fractionations prior to CZE-MS analysis. Analysis using nano reversed-phase LC combined CZE-MS resulted in the identification of 7,500 proteins and 60,000 peptides. These results demonstrated an increase in sensitivity over analysis after separation using C18 ZipTip only and highlighted the advantages of using an LC-CZE-MS bottom-up approach. Jooss et al. (2019) demonstrated the value of on-line coupling of nano reversed-phase LC and CZE for analysis of intact proteins. In this study, a mechanical valve was used to couple LC with CE which was used to separate a protein mixture (RNAse B, cytochrome *c*, lysozyme *c*, and myoglobin).

McCool et al. (2018b) demonstrated the value of orthogonal multidimensional separation for a large-scale top-down study that identified 850 proteins and 5700 proteoforms from *E. coli*. In this study, size exclusion chromatography

(SEC) and reversed-phase LC were used to reduce the sample complexity prior to CZE-MS analysis. This platform resulted in a peak capacity of ~4,000 for the separation of intact proteins. High peak capacity and resolving power improved the data acquisition, which resulted in an enhanced representation of the proteins in the sample.

Jooss et al. (2017) demonstrated the power of CZE-CZE-MS for the characterization of intact monoclonal antibody charge variants. In another report, Jooss et al. (2018) showed, for the first time, a successful application of CZE in conjunction with drift tube ion mobility-mass spectrometry (DTIM-MS) for the separation and characterization of native and APTS-labeled N-glycans released from different protein sources. This platform seems to have the potential to resolve isomeric forms of glycans. LC and CZE can also independently generate complementary identification of peptides, proteins, and proteoforms, thus expanding proteomic coverage. For instance, Li et al. (2012) reported a bottom-up strategy that combines the data from CZE-MS and reversed-phase LC-MS. This combination resulted in an improved number of identified unique peptides where hydrophilic and basic peptides with low molecular weight were predominantly identified via CZE-MS.

Further advances in CE-MS technologies can enable the identification of many more cellular proteins and add to our understanding of protein interactions with other cellular species. ESI, in conjunction with high-resolution mass spectrometers, enables the identification of protein non-covalent interactions with other proteins or small ligands in protein complexes. The analysis of proteins in complexes by CE-MS is poorly explored, with only a few publications in recent years. An early report for analysis of protein complexes via CZE-MS was described by Belov et al. (2017). More recently, Shen et al. (2018) demonstrated the use of SEC-CZE for analysis of protein complexes and proteoforms under near-native conditions, resulting in the identification of 144 proteins, 672 proteoforms, and 23 protein complexes from the *E. coli* proteome.

Another common CE separation technique used in proteomics research is cIEF. Although it offers high-resolution separations, cIEF has yet to gain widespread use in the proteomics field. In cIEF separation is based on the isoeletric point of the analyte, which is mixed with an ampholyte before being placed in the capillary. A pH gradient in the capillary is initiated by the ampholyte in conjunction with an anolyte and a catholyte. The analytes then migrate through the capillary until they reach their isoelectric points. A limitation of this technique that the catholyte is sprayed into the MS during the ESI process, which can affect the background signal and cause ion suppression. Also, it is not compatible with a sheathless interface.

cIEF-MS with a sheath–liquid interface has been used for the analysis of monoclonal antibodies, with Dai et al. (2018) reporting the separation of intact monoclonal antibody. Resolving intact charge variant peaks is a noted challenge, but using this approach, nine charge variants were resolved. In another report, Dai and Zhang (2018) used cIEF with a middle-down strategy to reduce sample complexity to achieve characterization of the charge heterogeneity for the same monoclonal antibody. cIEF has been also reported for the analysis of other intact proteins (Zhu et al., 2017).

C. Background Electrolyte

BGE selection is critical to ensure successful CE separation and MS sensitivity. In addition to volatility, an effective BGE varies one (±) pH unit around the pK_a value. In CE-MS applications, the type of interface should also be taken into consideration while choosing a BGE. BGE selection is most critical for a sheathless interface because the BGE is the only liquid present during the ESI process and acidic pH is recommended to facilitate protein or peptide ionization efficiency. Conversely, sheath–liquid interface is more flexible and an acidic pH can be obtained from the sheath–liquid reservoir, so the BGE pH range can vary between 1 and 8 units. Compatibility between the BGE and sheath–liquid is of key importance to avoid moving boundaries in the separation medium that can adversely affect the separation efficiency. Table 2 shows the most commonly used BGEs for CE-MS applications in proteomics research (Gimenez et al., 2011; Sarg et al., 2013; Zhao et al., 2014; Boley et al., 2017; Yang et al., 2018; Zhang et al., 2018a).

The electrophoretic mobility of peptides is influenced by the BGE pH. For instance, in a pH lower than ~3.5, migration is predominantly influenced by the basic residues. Acidic pH is especially useful in peptide and protein analysis using uncoated fused silica capillaries. At low pH the free silanols in the capillary wall are protonated and the separation of proteins and peptides is enhanced due to a reduction of the electroosmotic flow (EOF) and reduction of electrostatic interactions. A strategy for large-scale top-down analysis using lysate from *E. coli* was developed by Lubeckyj et al. (2017). In this approach, the sheath–liquid was composed of 0.2% formic acid and 10% methanol in water. The authors compared different

TABLE 2. The most commonly used chemicals for background electrolyte preparation in proteomics research.

Chemicals	pK_a	Buffer range	Formula	pH adjustment (acid/base)
Acetic acid	4.80	–	CH_3COOH	–
Formic acid	3.80	–	HCOOH	–
Ammonium acetate pKa 1	4.76	3.76–5.76	CH_3COONH_4	CH_3COOH or NH_4OH
Ammonium acetate pKa 2	9.20	8.20–10.20	CH_3COONH_4	CH_3COOH or NH_4OH
Ammonium bicarbonate pKa 1	6.35	5.35–7.35	NH_4HCO_3	HCOOH or NH_4OH
Ammonium bicarbonate pKa 2	9.20	8.20–10.20	NH_4HCO_3	HCOOH or NH_4OH
Ammonium bicarbonate pKa 3	10.30	9.30–11.30	NH_4HCO_3	HCOOH or NH_4OH
Ammonium formate pKa 1	3.80	2.80–4.80	NH_4COOH	HCOOH or NH_4OH
Ammonium formate pKa 2	9.20	8.20–10.20	NH_4COOH	HCOOH or NH_4OH
Ammonium hydroxide	9.20	–	NH_4OH	–

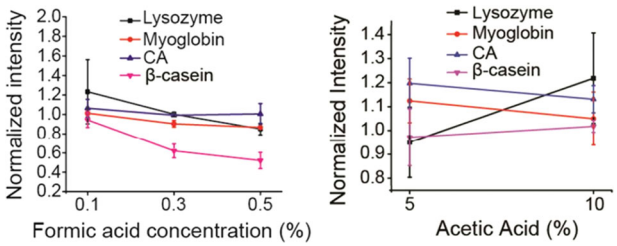


FIGURE 5. Intensity of the proteins from the capillary zone electrophoresis-mass spectrometry method with different background electrolytes and concentrations. Reprinted from Lubeckyj et al. (2017), with permission. [Color figure can be viewed at wileyonlinelibrary.com]

BGE compositions (acetic acid [5–10%]) in water and formic acid ([0.1–0.5%] in water). A BGE composed of 0.1% formic acid (pH 2.8) resulted in peak intensities equivalent to the acetic acid BGEs, as illustrated in the Figure 5.

Nevertheless, acetic acid BGEs (pH ~2.4 and ~2.2) resulted in a wider separation window. The use of acetic acid also increased the migration time of the proteins, which might be attributed to the lower pH of acetic acid that resulted in an increase in hydrodynamics in protein radii, exacerbation of protein unfolding, and reduction of EOF.

Interestingly, addition of organic solvents (e.g., propan-2-ol) to an acidic BGE enhances the separation efficiency and MS detection, as demonstrated by Yates group in a top-down analysis of *Pyrococcus furiosus* proteins using a sheathless interface (Han et al., 2014a). The addition of organic solvent to the BGE reduces the EOF, which results in improved resolution.

Dada et al. (2017) evaluated a BGE composed of aqueous–aprotic dipolar solvent (*N*,*N*-dimethylacetamide [DMA] and *N*,*N*-dimethylformamide [DMF]) for peptide mapping. The use of this BGE dramatically enhanced the separation efficiency over conventional BGEs composed of acetic acid diluted in water. The improvement in separation efficiency might be attributed to the influence of DMA on the electrophoretic mobility of the peptides and EOF.

Ammonia salts (acetate and bicarbonate) have been used in CZE native separations. As can be observed in Table 2, these salts (acetate, bicarbonate, and formate) have more than one pK_a, which allow their use in more than one pH range. Ammonia salts can achieve a pH compatible with physiological conditions (pH 6.8–7.4), which is of key importance to probe structures of cellular proteins in complexes. In native ESI-MS, protein complexes are transferred from solution to the gas phase and environmentally induced changes in pH can perturb their tertiary and quaternary conformations while in solution. Ammonium acetate can achieve a pH between 6.8 and 7.4 when dissolved in water and despite a low buffering capacity (ammonium acetate buffers in the pH ranges of 3.8–5.8 and 8.2–10.20, see Table 2), it is often used in native MS. Since ammonium acetate does not constitute a buffer system that can effectively maintain macromolecular complexes in solution at physiological pH conditions (Kebarle & Verkerk, 2009; Konermann, 2017), it suggests that an ammonium acetate solution in the pH range of 6.8–7.4 becomes acidic during the ESI process. Ammonium bicarbonate has been evaluated as an alternative to ammonium acetate for native MS applications due to its ideal pH stabilization at physiological conditions. Hedges et al. (2013) investigated the behavior of ammonium bicarbonate in protein ESI-MS using holomyoglobin. This protein was chosen as a model due to its rapid unfolding in solvent environmentally induced changes. As illustrated in Figure 6, high charge states (as in denaturing conditions) were formed in native conditions and their signal intensities increased proportionally to the increase in buffer ionic strength.

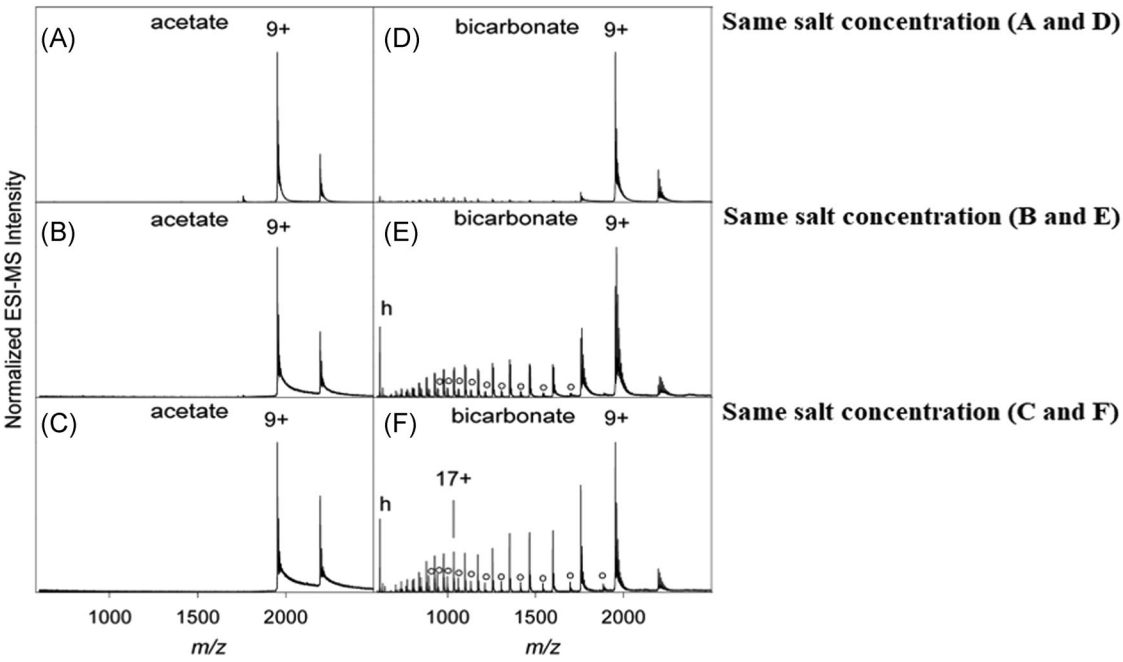


FIGURE 6. Electrospray ionization (ESI) mass spectra of myoglobin in aqueous solution at pH 7. The salt concentrations (ammonium acetate and ammonium bicarbonate) increase from (**a**) to (**c**) and from (**d**) to (**f**). Reprinted from Hedges et al. (2013), with permission

This phenomenon might be attributed to the destabilization effects that are induced by the anion bicarbonate, exacerbating the propensity of the protein to unfold during ESI process.

Interestingly, Bertoletti et al. (2016) reported a CE-ESI-MS sheathless interface strategy for the separation and characterization of the intact protein folding conformers of β2-microglobulin using ammonium bicarbonate. Using this set up, Bertoletti et al. were able to preserve protein structure in native conditions and they did not observe the high charge states that are promoted by ammonium bicarbonate, which might be attributed to the lower molar concentration of ammonium bicarbonate in the BGE. Although a BGE with high ionic strength is useful to avoid band broadening, high ionic strength can increase the diffusion of the sample zone and lead to high current and temperature in the separation capillary. The compositional and site-specific assessment of multiple peptide-deamidation was investigated by Dominguez-Vega et al. (2017). In this approach, a mixture of 150 mM ammonium formate (pH 6.0)-propan-2-ol -acetonitrile was used as a BGE. The addition of 40% of acetonitrile-propan-2-ol (87.5:12.5) to 60% 150 mM ammonium formate (pH 6.0) significantly improved the separation of deamidated and deacetylated TRI-1144 species and positional isomers. Acetonitrile might account for the fast separation and propan-2-ol may provide better resolution of the degradation products.

D. Capillary

Theoretically, since proteins have low diffusion coefficients, they should produce very high separation efficiency in CE. However, hydrophobic and/or ionic interactions between protein and capillary wall surfaces can lead to increased protein retention in the capillary, or to a lesser extent, peak tailing. Coated capillaries can be used to limit protein adsorption on the capillary wall. Although non-permanent coatings such as UltraTrol LN can be used, permanent coatings are preferred, particularly for high-throughput analysis and when interfacing CE with a mass spectrometer. A capillary coating can be achieved using polyacrylamide, aryl pentafluoryl groups, polysaccharides, or polyamines. Examples of coatings are described in Table 3.

EOF suppression or reversal can result in higher separation efficiency, and the EOF can be regulated by the capillary coating. Neutral coating with polyacrylamide is widely used in proteomics research due to its ability to eliminate EOF and maintain effective CE separations. The electropherograms in Figure 7 illustrate a separation of proteins using uncoated and neutral (LPA) coated capillaries.

Belov et al. (2017) investigated both bare fused silica and neutral coated capillaries under native conditions. The bare fused silica capillary produced a marginal separation efficiency for intact proteins and protein complexes as a result of protein adsorption to the capillary wall, whereas the neutral coated capillary generated high separation efficiency by preventing protein adsorption. Haselberg et al. (2018) reported almost zero EOF with the use of a neutral capillary for the analysis of intact proteins. In another report, a neutral coating was successfully used for analysis of conformers and dimers of antithrombin (Marie et al., 2016). Acidic N-linked glycans were also successfully analyzed using a neutral coated capillary (Partyka et al., 2018).

TABLE 3. Most commonly used coatings in proteomics research.

Coating	Charge
Linear polyacrylamide (LPA)	Neutral
Fluorocarbon (FC)	Neutral
Polyvinyl alcohol (PVA)	Neutral
Polyethylenimine (PEI)	Cation
Polybrene-dextran sulfate-polybrene (PB-DS-PB)	Cation
Bovine serum albumin (BSA)	Cation
Poly(arginine) (PA)	Cation

The Dovichi group has described a method using polyacrylamide to produce a neutral coated capillary (Zhu et al., 2016a) which was evaluated for the analysis of four standard proteins (cytochrome *c*, myoglobin, β-lactoglobulin, and carbonic anhydrase), intact antibody, and tryptic digested peptides. These studies showed that high reproducibility with a relative standard deviation for migration time in separation window lower than 1% was achieved.

A capillary with a polyethylenimine (PEI) positively charged coating has also been used for both protein and peptide separations. This coating generally results in high separation efficiency with high number of plates due to its ability to create a positively charged surface on the capillary and reverse the EOF direction. The use of positively charged coatings requires the application of negative voltage on the inlet (reversed polarity); as a result, proteins and peptides will migrate in the opposite direction of the EOF. A potential limitation of this coating is the generation of a very high flow rate in the separation capillary, which becomes more problematic as BGE pH is reduced. Han et al. (2014a, 2014b) demonstrated the high separation efficiency of PEI coated capillaries for the analysis of protein complexes and *P. furiosus* proteins via TDMS.

Other cationic polymers such as PA and polybrene have also been investigated for CE-MS applications (Berardet et al., 2018). The Kelleher group demonstrated the use of a PA coated capillary for the separation of intact proteins with molecular masses ranging from 30 to 80 kDa. In this study, 30 proteins were identified from *Pseudomonas aeruginosa* PA01 whole cell

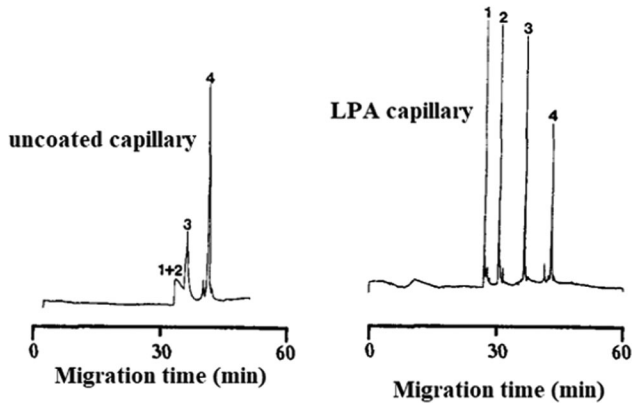


FIGURE 7. Elution profiles of (1) cytochrome *c*, (2) lysozyme, (3) ribonuclease A, and (4) chymotrypsinogen A on uncoated and linear polyacrylamide (LPA) coated capillaries at the same electrophoretic conditions. Reprinted from Zhao et al. (1993), with permission

lysate (Li et al., 2014). A procedure for the production of polybrene coating has been reported by Dominguez-Vega et al. (2017). The capillary was evaluated for the analysis of compositional and site-specific assessment of multiple peptide-deamidation where fast and reproducible separations were achieved. A method to maximize the elimination of SDS interference in antibody separations was reported by Sanchez-Hernandez et al. (2017). In this approach, effective removal of SDS was demonstrated in both neutral and positively charged coated capillaries. A summary of recent CZE-ESI-MS-based methods in proteomics research is shown in Table 4.

E. Preconcentration

The loading capacity of CE is very limited, which can adversely affect the sensitivity of the method and reduce peptide and protein identifications. Many preconcentration techniques to enhance injection volumes in CE-MS applications have been described, based on either electrophoretic (stacking) or chromatographic (solid-phase extraction [SPE]) principles. Recently, a dynamic pH junction has been used in top-down and bottom-up proteomics. This stacking technique relies on the preparation of samples in a basic buffer and injection (with pressure) into the separation capillary. Under application of an electric field, negatively charged proteins or peptides migrate toward the proximal tip of the capillary and are neutralized by an acidic BGE. A focused plug is formed and target analytes become positively charged, migrating toward the ESI source (Sun et al., 2016; Zhu et al., 2016b).

McCool et al. (2018a) described a protocol for top-down analysis of complex samples using a dynamic pH junction where a microliter-scale sample-loading volume was achieved. Zhu et al. (2014) described the use of dynamic pH junction in bottom-up proteomics (Zhu et al., 2014). In this study, a pH junction injection of 130 nL of BSA resulted in the identification of 40 peptides and produced 70% coverage. A 400 nL pH junction injection of an *E. coli* sample enabled the identification of 527 peptides and 179 proteins. The authors found that reproducibility of the migration times between the pH junction and normal injection was good. The pH junction was further extended to three intact standard proteins where the peak intensity increased more than 10-fold compared to conventional injection.

Transient isotachophoresis (tITP) is a less common stacking approach for preconcentration of peptides and proteins in proteomics research. In this strategy, the sample is mixed with two electrolytes, one with very high mobility (leading) and one with very low mobility (terminating). These electrolytes generate a highly conductive zone when the separation voltage is applied. Proteins or peptides are then concentrated into zones between the leading and terminating electrolytes. tITP requires an optimization of both electrolyte concentrations and loading volume to enable efficient stacking of the target analytes (Wang et al., 2012; Zhu et al., 2012; Heemskerk et al., 2013; Wang et al., 2013; Heemskerk et al., 2016; Zhang et al., 2018b).

Sample preconcentration using a chromatographic principle such as SPE was used by Wang et al. where large sample volumes can be trapped on reversed-phase media and eluted using an elution buffer prior to CE separation (Tong et al., 1999). This technology has the advantage that it can be automated and the multistep elution CE can be used as a semi-two-dimensional separation method. Wang et al. (2012) used reversed-phase SPE in conjunction with tITP for sample concentration of a *P. furiosus* tryptic digest, which resulted in low background noise and high sensitivity. The Dovichi group synthetized a sulfonate–silica hybrid strong cation-exchange (SCX) monolith at the inlet of the separation capillary and used it for on-line SPE sample preconcentration in conjunction with a dynamic pH junction for the analysis of tryptic digest peptides (Zhang et al., 2015a). This approach enabled injections at μL levels with a separation efficiency of over 25,000 theoretical plates. In another example, Zhang et al. (2015b) synthetized an SCX monolith in a fused silica capillary and coupled it to a neutral coated capillary. This was used for on-line SPE preconcentration in conjunction with dynamic pH separation for *E. coli* samples via bottom-up proteomics and the results were compared to conventional LC-MS treatment of the same sample. There was no significant difference in the number of peptides and proteins identified by CZE-MS and LC-MS methods. The same group further eliminated the zero dead volume union by preparing SCX and the neutral coated capillary sequentially in a single fused silica capillary, which resulted in an enhanced protein and peptide identifications when compared to previous reports (Zhang et al., 2017).

F. Quantitative Analysis

In proteomics research, quantitative information is critical to gain insights into clinical disorders. Quantification via CE-MS is particularly valuable for clinical studies where samples are often obtained in limited quantities. Several bottom-up strategies are available for protein quantification, including stable isotope labeling by amino acids in cell culture (SILAC), isobaric tag for relative and absolute quantitation (iTRAQ), label-free, and isotope-coded affinity tags (ICAT). Faserl et al. (2015) reported a SILAC approach that resulted in quantification of 1,371 phosphopeptides by CE-MS, without using any enrichment strategy. Varadi et al. (2016) described a two-plex method for quantitative analysis of N-glycans via CE-MS. The CE-MS method was found to be linear and precise. It was further applied for the analysis of a therapeutic protein where the authors observed differences in N-glycan structures from two different lots. The low sample consumption of CE enabled them to perform quantitative studies of the same sample via LC-MS. In terms of quantitative performance, CE-MS was found to be similar to LC-MS. However, the LC-MS platform failed to resolve N-glycan isomers that were resolved via CE-MS. In another report, Mittermayr et al. (2017) described a protocol for robust and reproducible quantification of N-glycans labeled using $^{12/13}C_6$ aniline or 2-aminobenzoic acid by CE-MS. Lombard-Banek et al. (2016) reported a CE-MS approach for the quantification of proteins in single embryonic cells via bottom-up proteomics. In this study, the quantification of ~150 non-redundant protein groups revealed significant translational cell heterogeneity along multiple axes of the embryo at a very early stage of development. More recently, the same research group reported the use of CE-MS in conjunction with label-free single-cell proteomics for quantification ~750 protein groups in 16-cell *Xenopus laevis* (frog) embryo with about 5 ng of protein digest (Lombard-Banek et al., 2019).

Guo et al. (2016) reported quantification of a peptide mixture by CE-MS. In this approach, selected reaction monitoring was used to target the analytes. The method was highly reproducible and robust and provided reliable quantification of a peptide

TABLE 4. Summary of recent capillary zone electrophoresis-electrospray ionization-mass spectrometry (CZE-ESI-MS)-based methods in proteomics research.

Sample	BGE	Capillary	Separation	Interface	Peptide	Protein	Proteoform	Protein complex	MS-based method	References
Escherichia coli	5–10% acetic acid	LPA	CZE	Sheath–liquid	–	200	600	–	Top-down	Lubeckyj et al. (2017)
E. coli	10% acetic acid	LPA	SEC-LC-CZE	Sheath–liquid	–	850	5,700	–	Top-down	McCool et al. (2018b)
Pyrococcus furiosus	0.1% acetic acid-20% IPA	PEI	CZE	Sheathless	–	134	291	–	Top-down	Han et al. (2014a)
E. coli	40 mM ammonium acetate, pH 7.5	LPA	CZE	Sheathless	–	42	144	8	Native MS	Belov et al. (2017)
E. coli	50 mM ammonium acetate, pH 6.9	LPA	SEC-CZE	Sheath–liquid	–	144	672	23	Native MS	Shen et al. (2018)
Yeast	10 mM ammonium acetate (pH 7	Uncoated	CZE	Sheath–liquid	–	49	–	–	Bottom-up	Wojcik et al. (2012)
MCF7 proteome	5% acetic acid	LPA	LC-CZE	Sheath–liquid	60,000	7,500	–	–	Bottom-up	Yang et al. (2018)
Mouse brain proteome	5% acetic acid	LPA	SCX-LC-CZE	Sheath–liquid	65,000	8,200	–	–	Bottom-up	Chen et al. (2018c)
Xenopus laevis	5% acetic acid	LPA	LC-CZE	Sheath–liquid	22,000	4,100	–	–	Bottom-up	Yan et al. (2016)
Monoclonal antibody charge variants	2 M acetic acid	PVA	CZE-CZE	Sheath–liquid	–	–	–	–	Intact analysis	Jooss et al. (2017)
Therapeutic proteins	15–20% DMA or DMF in 20% acetic acid or 2% formic acid	LPA	CZE	Sheath–liquid	–	–	–	–	Peptide mapping	Dada et al. (2017)
Therapeutic antibodies	10% acetic acid	PVA	CZE	Sheath–liquid	–	–	–	–	Peptide mapping	Whitmore and Gennaro (2012)
Monoclonal antibody	40 mM Ammonium acetate, pH 7.5 (native MS) 50% methanol, 1% formic acid (middle-down)	LPA	CZE	Sheathless	–	–	–	–	Native MS, middle-down	Belov et al. (2018)
Monoclonal antibodies	0.1% formic acid	LPA	CZE	Sheath–liquid	–	–	–	–	Top-down	Zhao et al. (2016a)
Monoclonal antibodies	5-30% acetic acid	LPA	CZE	Sheath–liquid	–	–	–	–	Intact	Han

TABLE 4. Continued

Sample	BGE	Capillary	Separation	Interface	Peptide	Protein	Proteoform	Protein complex	MS-based method	References
									analysis	et al. (2016)
Trastuzumab	10% acetic acid	Uncoated	CZE	Sheathless	–	–	–	–	Bottom-up	Gahoual et al. (2013)
Biopharmaceutical proteins	3% acetic acid	PEI	CZE	Sheathless	–	–	–	–	Intact analysis and top-down	Bush et al. (2016)
β2-microglobulin	(50 mM ammonium bicarbonate, pH 7.4	Uncoated	CZE	Sheathless	–	–	–	–	Intact analysis	Bertoletti et al. (2016)
Mixture (9 peptides)	0.1 M acetic acid	Uncoated	CZE	Sheathless	–	–	–	–	Intact analysis	Guo et al. (2016)

LC, Liquid chromatography; LPA, linear polyacrylamide; PEI, polyethylenimine; PVA, polyvinyl alcohol; SCX, strong cation-exchange; SEC, size exclusion chromatography.

mixture with a coefficient of variation (CV) between 0.2 and 1.8% for the migration time. Wang et al. described the use of cITP/CZE for quantification of targeted peptides of a tryptic digest BSA. They observed a quantitation limit of 10 pM with a CV lower than 22% (Wang et al., 2013). Sun et al. (2014) described a quantitative parallel reaction monitoring of peptide abundance where the relative standard deviation (RSD) of the migration time was lower than 4% and the RSD of the fragment ion was approximately 20%. Zhong et al. (2015) reported a quantitative analysis of glycans labeled with multiplex parbonyl-reactive tandem mass tags.

Top-down analysis provides quantitative information on diverse proteoforms that are often missed in bottom-up approaches. In quantification using CE-MS via TDMS, the intensities of the peaks corresponding to unmodified and modified proteins can be compared to obtain the abundance of a proteoform. Bush et al. (2016) characterized and quantitated individual molecular proteoforms of interferon-β1. Haselberg et al. (2013ba) used CE-MS for analysis of intact glycoforms. Quantitative information was based on the relative intensities of the detected glycoforms where glycoform concentrations were estimated to range between 0.35 and 630 nM. In a TDMS study, Lubeckyj et al. (2019) demonstrated a label-free strategy for the quantification of thousands of proteoforms from zebrafish brain using CZE-MS. This quantitative approach revealed that the abundance of proteoforms differs significantly in different regions of the zebrafish brain.

IV. CONCLUSIONS AND FUTURE DIRECTIONS

In the past few years advances in MS technologies, along with the need for separation techniques with high speed and resolving power, have renewed interest in the use of CE-MS for proteomics research. Improvements in interfaces have enhanced the robustness and reproducibility of CE-MS methods, resulting in highly confident identification and characterization of peptides, cellular proteins, PTMs, and protein complexes. These improvements have also enhanced sensitivity, enabling the application of CE to complex and low concentrated samples. Despite susceptibility to more technical difficulties and issues than LC-MS, with improvements in instrumentation CE-MS has, in some applications, outperformed conventional LC-based methods (e.g., separation of intact proteins and protein complexes). Highly sensitive ionization efficiency can be achieved using both sheathless and sheath–liquid interfaces at a lower flow rate (in the separation capillary) than LC, which reduces background noise and increases MS detectability. We suggest the use of sheathless interface for TDMS and native MS due to its unparallel sensitivity. The state-of-the-art single shot CZE-ESI-MS, along with neutrally coated capillaries (~1 m), have substantially enhanced the peak capacity of CE-MS methods in proteomics research. No single dimension, however, can provide a complete reduction of sample complexity. Rather, the utilization of multidimensional separations is the most effective strategy for improving peak capacity and reducing sample complexity. Sample loading is the major drawback of CE, which can be addressed using simple protocols, as demonstrated in top-down and bottom-up applications (Wang et al., 2012; McCool et al., 2018b). The use of CE-MS for quantitative proteomics, TDMS, and native MS is exceedingly low, with

only a few publications over the past few years. Cross-linking and MS have been successfully used to interrogate protein structures; however, cross-linking reports by CE-MS are scarce. The Dovichi group is leading the technological advances to realize the hope that CE-MS can be as routinely used in proteomics research as LC-MS.

Given that CE is well-suited for native separations, applications in native MS will expand to interrogate protein dynamics and conformation. In addition to a detailed proteoform characterization, TDMS can provide structural information of proteins covalently attached to other proteins while native MS provides detailed information on protein subunit arrangements via non-covalent interactions. These individual features suggest that TDMS and native MS can be complementary at several levels and can be merged to provide comprehensive protein structural information. The combination of these MS-based methods will yield fruitful benefits for both structural biology and proteomics research.

The future technological developments in CE-MS applications will continue, influenced in part by the proteomics field. For instance, microchip CE-MS analysis of a bovine hemoglobin pepsin digest has demonstrated high peak capacity and speed when compared to a conventional LC separation of the same sample (Black et al., 2015). The use of microchip CE-MS has also been demonstrated for the separation of intact monoclonal antibody variants where the fast analysis was achieved (~4 min) (Redman et al., 2015). Another example is capillary electrochromatography that has been successfully applied for the separation of peptides (Ali & Cheong, 2015, 2016). Although further development of these CE-based systems is still required, it is clear they are promising for separation of proteins and peptides and will be a step forward in proteomics research.

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ABBREVIATIONS

BGE	background electrolyte
BSA	bovine serum albumin
CE-MS	capillary electrophoresis-mass spectrometry
cIEF	capillary isoelectric focusing
cITP	transient capillary isotachophoresis
CV	coefficient of variation
CZE	capillary zone electrophoresis
DMA	*N*,*N*-dimethylacetamide
DMF	*N*,*N*-dimethylformamide
DTIM-MS	drift tube ion mobility-mass spectrometry
ESI	electrospray ionization
EOF	electroosmotic flow
FC	fluorocarbon
ID	identification
LC-MS	liquid chromatography-mass spectrometry
LPA	linear polyacrylamide
MS	mass spectrometry
native MS	native mass spectrometry
PA	poly(arginine)
PB-DS-PB	polybrene-dextran sulfate-polybrene
PEI	polyethylenimine
PTMs	posttranslational modifications
PVA	polyvinyl alcohol
RSD	relative standard deviation
SCX	strong cation-exchange
SEC	size exclusion chromatography
SPE	solid-phase extraction
TDMS	top-down mass spectrometry
tITP	transient isotachophoresis

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