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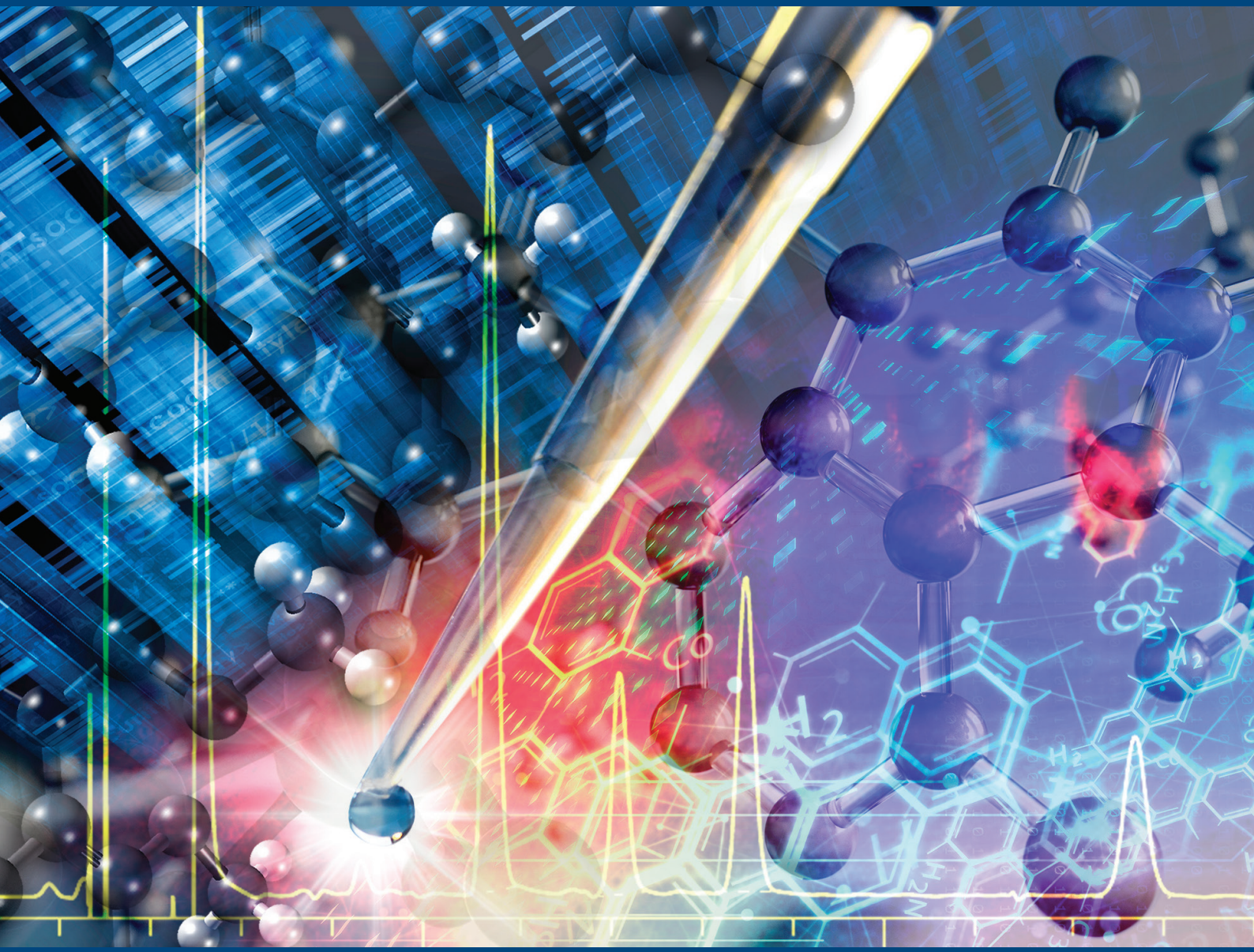

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REVIEW ARTICLE OPEN ACCESS

Recent Developments and Applications of Capillary and Microchip Electrophoresis in Proteomics and Peptidomics (2023–2025)

Sille Štěpánová | Václav Kašička

Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic

Correspondence: Václav Kašička (kasicka@uochb.cas.cz)

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ABSTRACT

This review article presents a broad overview of developments and applications of capillary electrophoresis (CE) and microchip electrophoresis (MCE) in peptidomics and proteomics in the period 2023–2025. It deals with various methodological aspects, such as sample preparation including preseparation and preconcentration, multidimensional separations by on-line or off-line coupled different CE, MCE, and LC techniques, and their hyphenation with MS detection. Innovations in bottom-up and top-down proteomics are reported. Furthermore, application of CE and MCE techniques in various peptidomic and proteomic studies are demonstrated. They embrace monitoring of posttranslational modifications, basic biological and biochemical research, and analysis of clinical and food samples.

1 | Introduction

Peptides and proteins belong to the most important bio(macro)molecules. They regulate many vitally relevant biochemical processes in all living organisms. Proteomics is a large-scale study of proteins. It investigates various protein properties (structure, expression, localization, post-translational modifications [PTMs], interactions etc.) and functions to obtain a wide view of the healthy organisms and of the changes occurring during various diseases, disorders or other special conditions at the molecular level [1].

Bottom-up proteomics (BUP), sometimes also known as shotgun proteomics [2], characterizes proteins through MS and MS/MS analysis of peptides obtained by digestion of proteins with sequence-specific proteases (usually trypsin) and separated by LC or CE methods [3–6]. BUP is a very effective technique in identifying high number of proteins in complex samples. It is the most widely used proteomic approach [7–9]. A different strategy, where the enzymatic cleavage of proteins is avoided, is used for the characterization of intact proteins by MS and MS/MS [10–12] combined with LC and CE methods [6, 13, 14], and is called top-down proteomics (TDP) [11, 12, 15]. The advantage of TDP is the ability to distinguish different proteoforms of the particular proteins. The term proteoform describes different protein forms produced from the same gene but varying in their molecular structures resulting from genetic variations, alternative RNA splicing and PTMs [16, 17]. The information about proteoforms is important for better understanding and description of various molecular processes. Hybrid of BUP and TDP, middle-down

Abbreviations: AcOH, acetic acid; BSA, bovine serum albumin; BUP, bottom-up proteomics; CID, collision-induced dissociation; DDA, data dependent acquisition; DIA, data independent acquisition; EtOH, ethanol; FA, formic acid; FAIMS, high-field asymmetric waveform ion mobility spectrometry; FITC, fluorescein isothiocyanate; FS, fused silica; IMER, immobilized-enzyme reactors; IPA, isopropyl alcohol (propan-2-ol); LPA, linear polyacrylamide; MCE, microchip electrophoresis; MDP, middle-down proteomics; MeOH, methanol; NP, nanoparticle; PEI, polyethyleneimine; PTM, post-translational modification; SL, sheath liquid; TDP, top-down proteomics.

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proteomics (MDP) [18, 19], employs limited proteolysis, thus larger peptide fragments than in BUP are analyzed.

Native proteomics investigates endogenous proteins and protein complexes under near physiological conditions by LC or CE methods coupled with MS and MS/MS detection [20–22]. It allows to characterize protein complexes while preserving their non-covalent inter- and intramolecular interactions [23–25]. In multiple proteomics approach, the proteins are described in all, BUP, TDP, MDP, and native proteomics levels [26].

Besides MS, several other technologies can be used for protein sequencing [27, 28], such as Edman degradation [29], DNA point accumulation in nanoscale topology (DNA-PAINT) [30], recognition tunneling [31], and nanopore sensing [32, 33]. Although MS is the main platform for peptidomic and proteomic analyses [5, 10, 34, 35], coupling of MS analysis with liquid-phase separations is often necessary due to the high complexity of biological samples.

Peptidomics involves identification and quantification of endogenous peptides in biological samples with the aim to study their roles in specific biological or disease processes, and to discover new potential biomarkers and peptide-based therapeutics [36–39]. Both proteomic and peptidomic workflows are composed of sample preparation steps, followed by peptide and protein LC or CE separation, MS/MS analysis, comparison of obtained spectra with the theoretical spectra available in protein databases (database searching), and statistical analysis [7, 8, 12, 40]. To interpret and analyze complex proteomics data, application of machine learning techniques (e.g., support vector machines, random forest, deep learning) is very useful [41]. These methods are utilized for the identification and characterization of proteins, retention/migration time prediction of peptides and proteoforms, discovery of biomarkers of various diseases, or development of predictive models for disease diagnosis and prognosis.

Complex biological samples are frequently pre-fractionated or separated before MS analysis. RPLC coupled to MS is commonly utilized technique in proteomic and peptidomic studies [4, 6, 42]. CE-MS is considered as a complementary and, in some cases, an alternative to LC-MS [3, 14, 42, 43]. Due to its orthogonality, CE-MS can improve the peptide sequence identification. CE methods provide high separation efficiency, and fast analysis, while requiring very small amounts and volumes of samples and buffers, which makes them suitable for various scientific fields [44–47], including biomolecular research [48]. Intrinsic characteristics of CE make it a good choice for the cellular and subcellular peptidomics [36] and proteomics [49].

In peptidomic and proteomic studies using CE methods, coated capillaries are often employed to reduce the adsorption of peptides and proteins on to a capillary inner surface. Various types of capillary surface coatings for protein separations were reviewed in [50, 51]. Alternatively, bare FS capillaries in combination with strongly acidic BGEs are used [52, 53].

Microchip electrophoresis (MCE) and MCE-MS provide further miniaturization for peptidomic and proteomic analyses [54]. Recent developments and applications of microfluidic systems in proteomics, known as proteomics-on-a-chip, were reviewed in [55]. Due to considerable amount of research dealing with the application of CE and MCE methods in proteomics, a recently published review [51] avoided this topic, except a partial overlap in the section “Peptide mapping”. Therefore, herein, we present another review that is devoted to this rapidly progressing and exciting field and that is a complement to reference [51].

The aim of this review is to present new trends and interesting applications of the CE and MCE methods for proteomic and peptidomic analyses in the years 2023–2025. It is a continuation of our previous reviews [56–58] on the same topic. This review focuses on the sample preparation and preconcentration procedures, coupling of 1D and multidimensional CE methods with MS detection, and on the application of CE methods in BUP and TDP. In addition, the usage of CE methods for monitoring of PTMs, as well as their application in biological and biochemical research, in clinical studies and in food analysis are presented.

2 | Sample Preparation

Sample preparation is very important in the peptidomic and proteomic workflows. Sample preparation of BUP and TDP approaches have some differences. Typical sample preparation process in BUP comprises cell lysis/homogenization, protein extraction/precipitation, membrane filtering, reduction, alkylation, enzymatic digestion, desalting, and fractionation [59, 60]. The sample preparation methods for cell surface proteomics have recently been reviewed [61].

For cell samples, lysis buffer and sonication are applied to disrupt the cell membranes and homogenize the sample. For protein extraction, often 50–100 mM buffers with neutral pH (e.g., phosphate buffered saline [PBS], Tris(hydroxymethyl)aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], ammonium bicarbonate, triethanolamine bicarbonate) containing a strong denaturant (e.g., urea, sodium dodecyl sulphate [SDS] or sodium deoxycholate) are used [8]. The detergents are removed by protein precipitation using acetone, trichloroacetic acid, chloroform/methanol (MeOH)/water, or other organic solvents. Then, disulfide bonds in proteins are reduced, for example by dithiothreitol (DTT), tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), or β-mercaptoethanol), and alkylated by iodoacetamide, N-ethyl maleimide, acrylamide, or other reagents. Thereafter, the proteins are site-specifically cleaved into peptide fragments by protease(s). Most widely used protease in BUPs is trypsin [62] but alternative proteases with different specificity, for example, chymotrypsin, Lys-C endoprotease, Lys-N endoprotease, Asp-N endoprotease, Glu-C endoprotease, Arg-C endoprotease, and pepsin, are used as well [63, 64].

In-capillary enzymatic digestion (ICD)-CE-MS was employed for BUP analysis of α-synuclein (α-syn), a protein biomarker of Parkinson disease [65]. Instead of trypsin, endoproteinase GluC was used. This approach involved sequential injection of digestion buffer, endoproteinase GluC (20 µg/mL) in digestion buffer, α-syn (100 µg/mL) in water, a second plug of endoproteinase GluC and digestion buffer, and finally BGE to the BGE-filled capillary, followed by 30 min waiting time. Under these conditions, the RSDs of the peak areas of potentially acetylated and phosphorylated peptides were 7.1% and 3.4%, and the LODs were equal

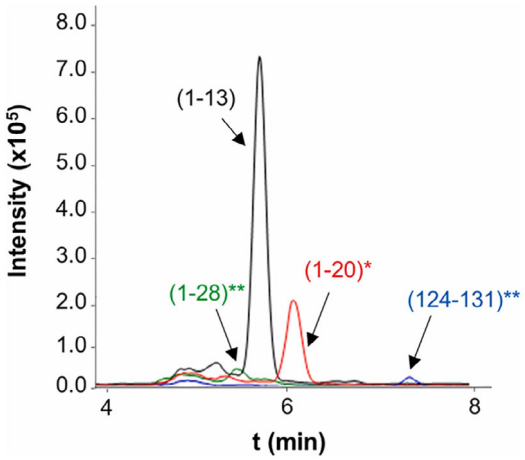

FIGURE 1 | Extracted ion electropherograms (EIEs) of the three α-synuclein acetylated peptides and the potentially phosphorylated peptide obtained under optimized conditions by GluC-in-capillary enzymatic digestion (ICD)-CE-MS for the analysis of endogenous α-synuclein from human red blood cells. Asterisks indicate peptides resulting from missed cleavages, with the number of asterisks denoting the number of cleavage events. Figure reprinted with permission from Reference [65].

to 25 and 15 μg/mL, respectively. During ICD-CE-MS analysis of endogenous α-syn from human blood, 3 acetylated peptides characteristic of the acetylated N-terminal amino group of the most abundant blood α-syn proteoform (peptides (1–13), (1–20), and (1–28)), as well as the potentially phosphorylated peptide (124–131) were detected, see Figure 1.

Classically, during the digestion, enzyme and substrate are mixed in a solution. Immobilized-enzyme reactors (IMERs) have some advantages over this traditional approach, such as prevention of protease autolysis, shorter digestion times, smaller reagent consumption, and possibility for automation and integration with HPLC or CE [66]. The common immobilization methods, various μIMER designs, application in MS proteomics, and challenges of their long-term use are presented in recent review [67].

The main difference in TDP sample preparation strategy compared to BUP is the avoidance of the enzymatic digestion. Recent study investigated the influence of the distinct sample preparation steps on proteoform identification by TDP [68]. Generally, different strategies resulted in complementary identifications that substantially increased proteome coverage. Critical points during sample preparation, such as conditions for cell lysis, protein precipitation/re-solubilization, reduction and alkylation of the proteoforms, and choice of the prefractionation/purification methods together with possible solutions to avoid protein loss and artifacts were presented. Sample preparation strategies for clinical TDP, such as targeted enrichment of individual proteins (non-affinity- and affinity-based), reduction of sample complexity by untargeted approaches (gel-based fractionation, size exclusion chromatography (SEC), solid-phase extraction (SPE), molecular-weight cut-off (MWCO) filter, nanoparticles (NPs) and intact protein clean-up methods (dialysis, SPE, MWCO filters, precipitation, magnetic beds) were reviewed in [69].

For proteomic characterization of PTMs of proteins [70] or low abundance proteins in complex samples, protein enrichment and depletion methods are used [8, 71, 72]. To reduce the sample complexity in TDP, sample fractionation by SEC [73, 74] or SDS-PAGE [75] are applied before the analytical separation. In addition, CZE has been employed as fractionation method before LC-MS. Namely, CZE-UV was used for fraction collection before NanoRPLC-ESI-MS/MS analysis of Hela protein digest [76]. Before CE-MS analysis, peptides and proteins are purified by SPE, to remove salts and denaturants [77, 78]. On-line aptamer affinity (AA)-SPE was applied for purification and preconcentration of intact protein biomarkers from biological fluid and food samples before CZE-MS analysis [79].

A sufficiency of very small amounts of sample makes CE especially appropriate for nanoscale omics, such as single cell analysis [49]. An automated spray-capillary platform, where the spray-capillary device is coupled with the commercial CE autosampler suitable for handling pL and nL samples was utilized for BUP analysis of *Escherichia coli* lysate digest (1 μg/μL in 0.1% formic acid [FA] and 45% ACN, spiked with 10 μM standard peptide angiotensin II) [80]. For sample injection, ESI was used to create a pressure difference between the sample end and the MS end of the capillary (ESI voltage was applied to the MS end of the capillary). CE separation was performed in poly(ethyleneimine) (PEI)-coated capillary, 0.1% FA was used as BGE, and −30 kV as separation voltage. Fifty continuous runs with angiotensin II resulted in RSDs of 14.70% and 0.62% for intensity and migration time, respectively. From *E. coli* lysate digest, 749 unique peptides from 170 protein groups were identified.

Another approach for BUP of single cells used RP-solid-phase microextraction (SPME)-CZE-MS/MS system built in a single capillary [81]. In CZE, the linear polyacrylamide (LPA)-coated FS capillaries were employed. For SPME (~2 mm long section), the C8 beads were packed into the separation capillary with an in-situ synthesized frit. For peptides elution from SPME section, a buffer composed of 50 mM ammonium acetate, pH 6.5, and 30 % (v/v) ACN (~100 nL) was used. This was followed by CE-MS analysis utilizing dynamic pH junction preconcentration and 5% acetic acid (AcOH) as BGE. SPME-CZE-MS allowed efficient peptide analysis from very low amounts of sample. From the only 0.25 ng of the HeLa cell digest (equivalent to the protein mass of one HeLa cell), 0.1 ng was injected and 257 proteins and 523 peptides were identified.

Novel robotic capillary (RoboCap) platform was developed for more automated CE-MS single-cell proteomics [82]. In this device, CE separation capillary was immobilized on an electrically insulated post, while sample and BGE vials were attached onto a miniaturized three-axis translation stage. Actuators controlled by a special software enabled to remotely manipulate with positions of both vials. This system was enclosed into a hermetically sealed chamber, allowing an electropneumatic system to apply a pressurized nitrogen over a controlled time for sample injection. Robocap platform allowed to inject only ~10–250 nL of sample with injection errors <3% RSD and was applied for CE-ESI-MS proteomic analysis of ~20 ng of HeLa proteome digest (from 100 nL of sample on RoboCap). The RoboCap significantly improved the use of sample during the injection compared to the manual injection system. In average, 1800 proteins were identified (~12% RSD, $n = 8$).

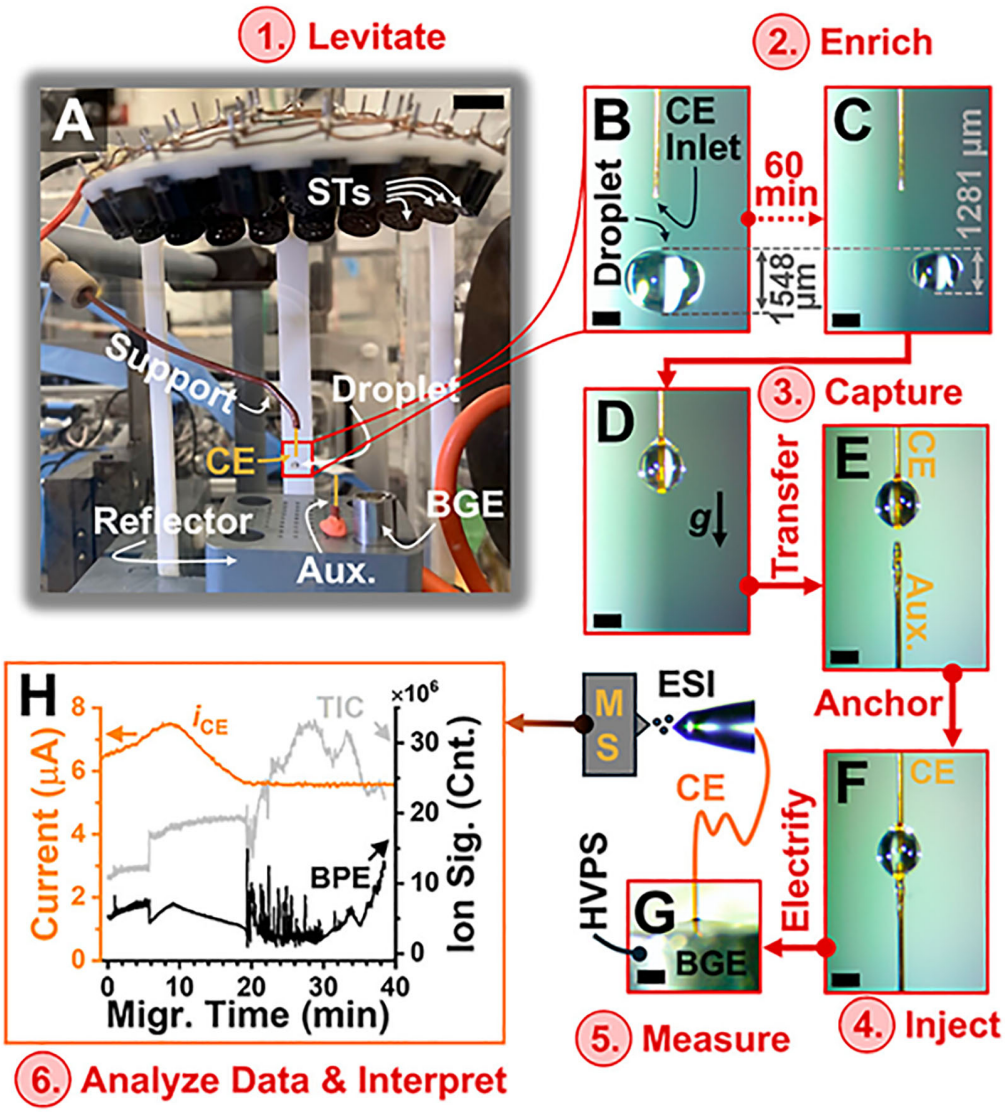

FIGURE 2 | Sequential analytical steps in the levitational CE-MS workflow. (A) Asymmetric acoustic levitator with 32 top-mounted transducers and a reflective bottom surface. (B) Stable levitation of a 1.6 µL HeLa cells digest droplet in 50 mM ammonium bicarbonate. (C) Over 60 min, the droplet reproducibly shrank by ~4.4-fold (RSD <2.8%, $n = 4$). (D) Direct sampling via capillary inlet showed droplet adherence and limited bulk access; electrophoretic current confirmed air ingress as a failure mode. (E,F) Symmetric suspension between two capillaries allowed controlled pressure-driven injection. (G) Control runs analyzed 3 ng digest (~10 cells) from levitated-only droplets. (H) Stable electrophoretic and capillary (i_{CE}) and generated total ion current (TIC) and base-peak electropherogram (BPE) traces supported robust CE-ESI-MS operation. Key: HVPS, high-voltage power supply. Figure reprinted with permission from Reference [83].

To increase sensitivity, acoustic droplet levitation was online integrated with RoboCap CE-ESI-MS [83]. This strategy involved concentration of analytes before CE-MS analysis by evaporation of levitating droplets containing proteome digests (see Figure 2). After 1 h of passive evaporation, ~4.4-fold reduction in sample volume was observed by optical imaging. After this enrichment, ~14 ng of digest instead of ~3.2 ng (without enrichment) was introduced into CE-MS that resulted in identification of ~2169 proteins compared to ~1140 proteins, respectively.

As part of a BUP workflow, NP protein corona-based sample preparation was coupled with CZE-MS/MS analysis, to reduce blood plasma proteome complexity [84]. In this approach, human plasma (diluted to 55%, v/v) was incubated with magnetic NPs (four types) to form a NP protein corona. Unbound proteins were removed by washing steps. This was followed by 1 h on-bead tryptic digestion, with hydrophilic peptides remaining in the aqueous phase. Then, the NPs were shortly incubated with the elution buffer containing 20% ACN. Thereafter, second eluted peptides were combined with the initial aqueous phase and analyzed by CZE-MS/MS. NPs enriched the low-abundance peptides. Application of this workflow for the analysis of healthy and nuclear protein in testis carcinoma mouse serum samples resulted in discovery of some novel potential cancer biomarkers.

Samples commonly analyzed in peptidomics include body fluids, cells, tissues, secretory vesicles, etc. After collection, the biological samples should be properly handled and kept in cold until peptide extraction to avoid their degradation or modification. Depending on the stability of the samples and purpose of

the study, inhibition of proteases may be necessary (e.g., heat denaturing, addition of protease inhibitors, organic solvents, or chaotropic agents). Also, the analysis of low abundance endogenous peptides is often disturbed by the presence of high abundance proteins, sugars, lipids, or salts present in biological samples. Thus, for the clean-up and enrichment of endogenous peptides, different strategies are used, such as organic solvent precipitation (ACN, MeOH, ethanol (EtOH], acetone), centrifugal ultrafiltration, differential solubilization, and SPE (reversed phase, HILIC, affinity-based nanoporous materials) [36, 85]. BUP approach for peptidomics includes also enzymatic hydrolysis or microbial fermentation steps [37].

Important points for plant sample preparation for peptidomics and other omics approaches were discussed in recent review [86]. Various on- and off-line preconcentration strategies can be applied to increase the sensitivity of peptidomic and proteomic analyses by CE [87–89] and MCE [90, 91].

In sample preparation for multi-omics analysis, after homogenization, the raw sample should be equally divided for further sample preparation for individual "omics" or if it is not possible, the sample preparation according to the preferable "omics" method should be carried out [92].

The greenness of sample preparation approaches for proteomic analysis was investigated, showing that the proteomic analyses should focus on miniaturization of analytical devices, utilization of in-line and on-line sample preparation techniques, application of less toxic solvents and reagents, and use of more sustainable materials [93]. In terms of extraction recovery, comparable results were obtained when ACN as elution solvent was replaced with greener EtOH in SPE of low molecular mass proteins spiked in biological fluids [94]. Also, the extraction recovery was not improved by addition of acidic modifiers to elution solvent and by nitrogen evaporation of the eluate. The latter resulted in loss of the proteins compared to simple dilution of the eluate.

3 | Hyphenation of CE and MCE Methods With Mass Spectrometry

3.1 | General

MS is the main tool in peptidomic and proteomic analysis allowing identification and quantification of individual peptides and proteins [8–10, 14, 36]. Due to the complexity of biological samples, MS is frequently combined with separation methods, mostly by LC [4, 6] and to lesser extent with CE [13, 14]. MS-based proteomics/peptidomics is divided into qualitative and quantitative analyses [95, 96]. Qualitative proteomics/peptidomics give information about the presence of proteins and peptides in a biological sample, whereas quantitative proteomics/peptidomics determine the amounts of these proteins and peptides. It is possible to perform absolute or relative quantification. Absolute quantification determines absolute concentration of protein/peptide in a sample. Relative quantification provides information about relative ratio of the amounts of a specific protein/peptide in different samples. Labeling and label-free strategies are distinguished in quantitative proteomics and peptidomics. For label-free quantification that measures the correlation between the abundance of a protein and its signal intensity, spectral counting and signal intensity measurement are used. During labeling-based quantification, the known amount of the labeled (mass-tagged) internal standard is introduced into the sample before the analysis and the abundance of the protein (peptide) is determined from the signal intensity ratio of the unlabeled/labeled protein (peptide) pairs. This internal standard should have sufficiently different mass but the same physicochemical properties as the analyte. This is achieved by stable isotope labeling. Common approaches employed for the labeling-based quantification include metabolic labeling (e.g., stable isotope labeling with amino acids in cell culture [SILAC]), chemical labeling (e.g., tandem mass tags), isobaric tags for relative and absolute quantification (iTRAQ), and enzymatic labeling (^{18}O labeling) [97, 98].

MS-based proteomics is performed either under denaturing or native conditions. The main difference between traditional denaturing and native MS is the composition and pH of the used solutions [99]. Solutions with neutral pH and minimal concentrations of organic acids preserve protein non-covalent interactions. Native protein MS investigates intact proteins and protein complexes while attempting to maintain as much as possible the native structural features of the analytes [100–102].

Native CZE coupled to ultra-high mass range Orbitrap mass spectrometer enabled to detect from *E. coli* cell lysate 72 proteoforms or protein complexes in a mass range of 30–400 kDa during single 1-h run, while consuming only 50 ng of the *E. coli* sample [22]. The mass distribution of detected proteoforms or protein complexes was in good agreement with that from mass photometry measurement.

Approach combining native CZE-MS, in-source collision-induced dissociation (CID) and denatured TDP enabled to quantify 33 proteoforms/complexoforms during the transition of *E. coli* cells from logarithmic (log) to stationary growth phase [103]. In addition, differentially expressed complexoforms were identified, including glutamate decarboxylase beta hexamer (~317 kDa) that showed significantly higher abundance at the stationary phase compared with the log phase agreeing with its biological function.

3.2 | 1D-CE Methods Coupled With ESI-MS and ESI-MS/MS

ESI-MS is a major method in proteomic studies. ESI generates multiply charged intact molecular ions that effectively reduce the mass-to-charge (m/z) ratio of the analyte ions. Thus, the mass of large proteins or peptides can be effectively measured by ESI-MS. To reduce the sample complexity before ESI-MS, it is frequently combined with LC (mostly RPLC) [4] or CE methods [13, 14]. ESI interfaces for coupling with CE, and possibilities for minimizing ion suppression in CE-MS are discussed in [13]. Hyphenation of Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) with CE, LC, and GC methods was overviewed in [104].

CIEF offers high resolution of proteoforms. However, CIEF coupling with MS is problematic as carrier ampholytes suppress ionization and contaminant MS instrument. Sometimes, to

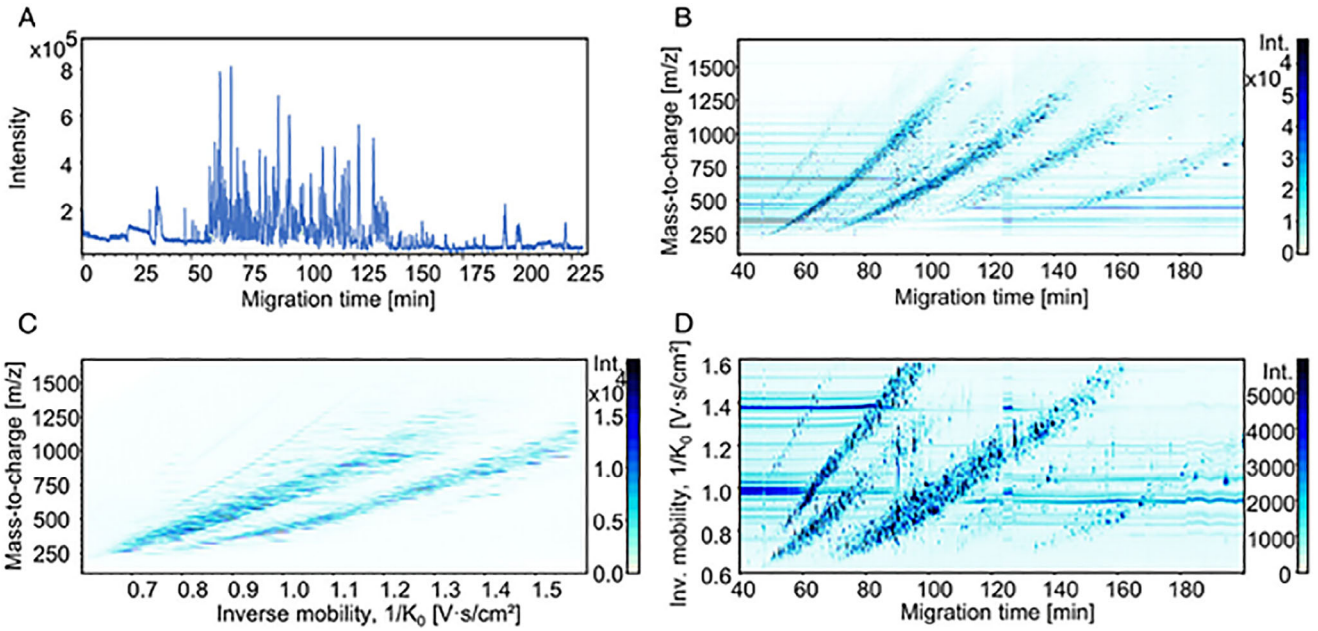

FIGURE 3 | Separating tryptic HeLa cells digest using 1.5-m long poly(ethylene oxide) (PEO) coated fused silica capillary. (A) Base peak electropherogram (BPE; 350–2200 *m/z*). (B) Heatmap of migration time and *m/z* value. (C) Heatmap of gas phase mobility and *m/z* value. (D) Combined heatmap of liquid and gas phase mobility. Figure reprinted with permission from Reference [106].

reduce these effects, instead of commercial ampholytes, amino acid ampholytes are used for establishing pH gradient for CIEF separation. To lower the amount of amino acid ampholytes entering MS detector, automated in-line CIEF-ESI-MS interface was used [105]. The interface was composed of Nafion tubing enclosed within a stainless tube that allowed liquid counter flow for desalting. Ampholytes permeated the membrane and were eliminated as the analytes were mobilized into the MS. The interface was used for the analysis of protein standards as well as tryptic BSA digest. It did not compromise the CIEF linearity of the intact protein separations and its application substantially increased peak area (~90%).

Combining of different methods for peptidomic or proteomic analysis is attractive as it can substantially improve separation of complex samples. Performance of the combined method depends on the orthogonality of single modes. The orthogonality of CZE and ion-mobility MS (IM-MS), a technique that separates ions in the gas phase based on their size, shape, and charge with consecutive mass determination, was investigated by analyzing tryptic digest of HeLa proteins (see Figure 3) [106]. NanoCEasy interface was employed to couple CZE to trapped ion mobility MS (TIMS-MS) instrument. For CZE separations, poly(ethylene oxide) (PEO)-coated FS capillary (50 μm ID; 1.5 m), 1 M FA in 10% IPA, pH 1.9, as BGE, and separation voltage 30 kV were used. CZE and IM-MS were proved to be orthogonal methods providing total peak capacity up to 9500. Solvation effects resulted in different charges and sizes, and thus distinct selectivity of peptide separations in the liquid and gas phases.

A new nanoelectrospray interface for coupling CE with MS using the principle of liquid junction was composed of nanosprayer and nanospray module [107]. Nanosprayer was fabricated using silicone technology, and it incorporated an emitter tip on the front side of the silicon wafer with spraying channel in the middle. The latter extended across the substrate toward a star-shaped liquid-junction structure on the opposite side of the chip. The nanospray module was used to position and operate the nanosprayer in front of MS entrance. CE-nano-ESI/MS was utilized for TDP and BUP analysis of cytochrome c and its tryptic digest.

A special CE-ESI-Hydrogen Deuterium Exchange (HDX)-MS at 0°C was employed for BUP analysis of the denatured and native bovine hemoglobin, and was estimated as promising method for structural proteomics [108].

3.3 | Multidimensional CE and LC-CE Methods Coupled With MS

Multidimensional methods coupling two or more orthogonal CE and LC techniques significantly improve identifications of low abundance species. However, they usually require more analysis time. Many multidimensional separations combining different LC, CE, and/or gel electrophoresis-based separations have been developed and applied for both BUP [76, 109] and TDP analysis of complex samples [110]. SDS-PAGE protein fractionation (passively eluting proteins from polyacrylamide gels as intact species for MS (PEPPI-MS) was combined with CZE-MS/MS to characterize histone proteoforms [111]. SDS-based prefractionation resulted in six fractions (diluted in 50 mM NH_4OAc, pH 9) that were further analyzed by CZE-MS/MS. CZE analysis was performed in LPA-coated FS capillary with 5% (v/v) AcOH, pH 2.4, as BGE, and 30 kV separation voltage. The sheath liquid (SL) was composed of 0.2% (v/v) FA and 10% MeOH (v/v) in water. This combined method enabled to identify more than 200 histone proteoforms.

2D RP nanoLC-CZE-MS platform used for nontargeted TDP of human cell lysate enabled to detect much higher number of proteoforms than 1D nanoLC-MS and 1D CZE-MS methods

[112]. A targeted analysis of Histone H4 using nanoLC-CZE-MS enabled to detect additional proteoforms attributed to certain PTMs (acetylation) by calculating the mobility change of the proteoforms and estimating their mass differences.

The developments of a spatial 3D microchip that integrates IEF, SEC, and RPLC methods were recently reviewed [113]. The chip contained interconnected channel structure with one 1D, sixteen parallel 2D and 256 parallel 3D channels. Altogether this microdevice had more than 4000 microchannels. This 3D approach was predicted to achieve peak capacities >30 000 within a 1 h of analysis time).

4 | Bottom-Up Proteomics

BUP is the main method used for protein identifications in complex mixtures [2, 7–9]. Compared to TDP, it is a more mature method and offers better bioinformatics tools. BUP workflow embraces sample preparation including enzymatic digestion of proteoforms into peptides, followed by their separation by LC or CE methods, MS-based detection and sequencing (identification), and inference into protein groups by bioinformatics approaches. Typically, peptides analyzed in BUP have M_r < 3 kDa. BUP-MS is the main method for proteome analysis [7–9]. RP-LC-MS is commonly employed in BUP applications [4, 6, 9]. To a smaller extent, CZE coupled to ESI-MS has been used [3, 13, 65, 81].

In CZE-MS proteomic studies, data dependent acquisition (DDA) is traditionally used. It limits the number of identified proteins since only the most intense precursor ions are selected for further MS/MS fragmentation analysis. Data independent acquisition (DIA), on the other hand, selects all the precursor ions within a pre-defined *m/z* window for MS/MS fragmentation analyses. Recently, CZE-DIA-MS was compared to the CZE-DDA-MS and showed that the numbers of identified peptides and proteins were 1.8-fold and 2-fold higher than the ones acquired by DDA mode using 12.5 ng HeLa cells digests as a sample [114]. The proteins identified in DIA mode covered almost all the proteins identified in DDA mode. For CZE separation, LPA-coated FS capillary, 1 M AcOH as BGE, and 21.7 kV separation voltage were used.

CE-MS technologies were used also in single-cell proteomics [49]. The throughput of CE-HRMS single cell proteomics of commercial HeLa proteome digest was increased by applying higher separation voltage and thereby shortening the separation window as well as by employing DIA (library-free) instead of DDA [115]. From single HeLa-cell-equivalent (~200 pg) proteome digest within 15 min effective separation using DIA and DDA methods, 1161 and 401 proteins were identified, respectively. Using label-free quantification, ~76% of the DIA-quantified and ~43% of DDA-quantified proteins had coefficient of variation <20%.

The reproducibility and the accuracy of the tandem mass tag-based isobaric multiplexing quantification in CE-ESI-MS and in nanoLC-MS were compared using MS^2 and simultaneous precursor selection (SPS)-MS^3 strategies and validated mouse-yeast two-proteome model [116]. The reproducibility and the accuracy for both CE and nanoLC methods using MS^2 or SPS-MS^3 quantification were the same but about 12-fold sensitivity enhancement was observed in CE.

5 | Top-Down Proteomics

TDP is used for identification and characterization of proteoforms in biological samples [10–12, 15]. TDP workflow incorporates protein extraction from biological samples, separation of proteoforms, measurement of their masses and fragmentation by MS, and interpretation of proteomics data by bioinformatics tools (identification, characterization, quantification).

Commonly, RPLC is combined with MS for TDP analyses [15, 71]. However, alternatively CZE can be used [14]. Studies combining the results of CZE-MS and RPLC-MS methods have shown increased coverage of proteoforms compared to single methods [117, 118]. From CE methods, CZE and CIEF have been combined with ESI-MS for TDP of intact proteoforms.

CIEF-MS/MS TDP enabled to measure different proteoforms of the gene product in the protein corona of NPs and to identify over 70 proteoforms of 16 cancer-related genes [119]. Using high-resolution for both, MS1 and MS2, 80% of the identified proteoforms were smaller than 10 kDa, and 20% in the mass range 11–30 kDa. However, employing low resolution MS1 and high-resolution MS2, 24 proteoforms of ~28 kDa or higher (up to ~66 kDa) were detected.

The improved CIEF-MS/MS method for the TDP of proteoforms in protein corona lasted only 30 min, and RSD of migration time was <4% for 25 runs [120]. Fifty-three genes were identified, and 1–102 proteoforms containing different sequence variations or PTMs per gene were detected. Thirty-three identified genes are potential protein biomarkers of various diseases. So-called "sandwich" injection was used, where 6 cm catholyte plug containing 0.3% NH_4OH was followed by 20 cm mixture of sample (contained about 600 ng of corona proteins) and ampholyte plug containing 0.6% ampholytes (pH 3–10, 5–8, and 8–10.5). Then, 50 cm anolyte plug was injected containing 5% AcOH. Analysis was conducted in LPA-coated FS capillary, and separation voltage was 30 kV.

CZE-MS/MS has also been employed for the plant TDP, namely for characterization of proteoforms of *Arabidopsis thaliana* leaf tissue, using prefractionation with SEC [121]. For CZE, LPA-coated FS capillary, 10% AcOH as BGE, and separation voltage of 30 kV were utilized. For total leaf, 3198, and for chloroplast, 1836 proteoforms were identified, of them 1024, and 363 proteforms having PTMs, respectively. PTMs that affect the charge state (acetylation, phosphorylation) were additionally validated based on calculated theoretical effective mobilities of proteoforms in CZE.

High-field asymmetric waveform ion mobility spectrometry (FAIMS), a gas-phase fractionation strategy providing online filtering of ions based on their differential mobilities in oscillating high and low electric fields was combined with CZE-MS/MS for the TDP of complex proteome (yeast lysate) [122]. Compared to CZE-MS/MS alone, this joined approach improved the identifications of proteoforms ~3-fold (in the mass range 20-45 kDa 6-fold). In addition, the signal-to-noise ratio was improved ~20-fold.

CZE-FAIMS-MS/MS was applied also for TDP of histones [123]. CZE analyses were conducted in LPA-coated FS capillaries using 20 mM NH_4OAc, pH 5.0, as BGE (sample buffer was 50 mM

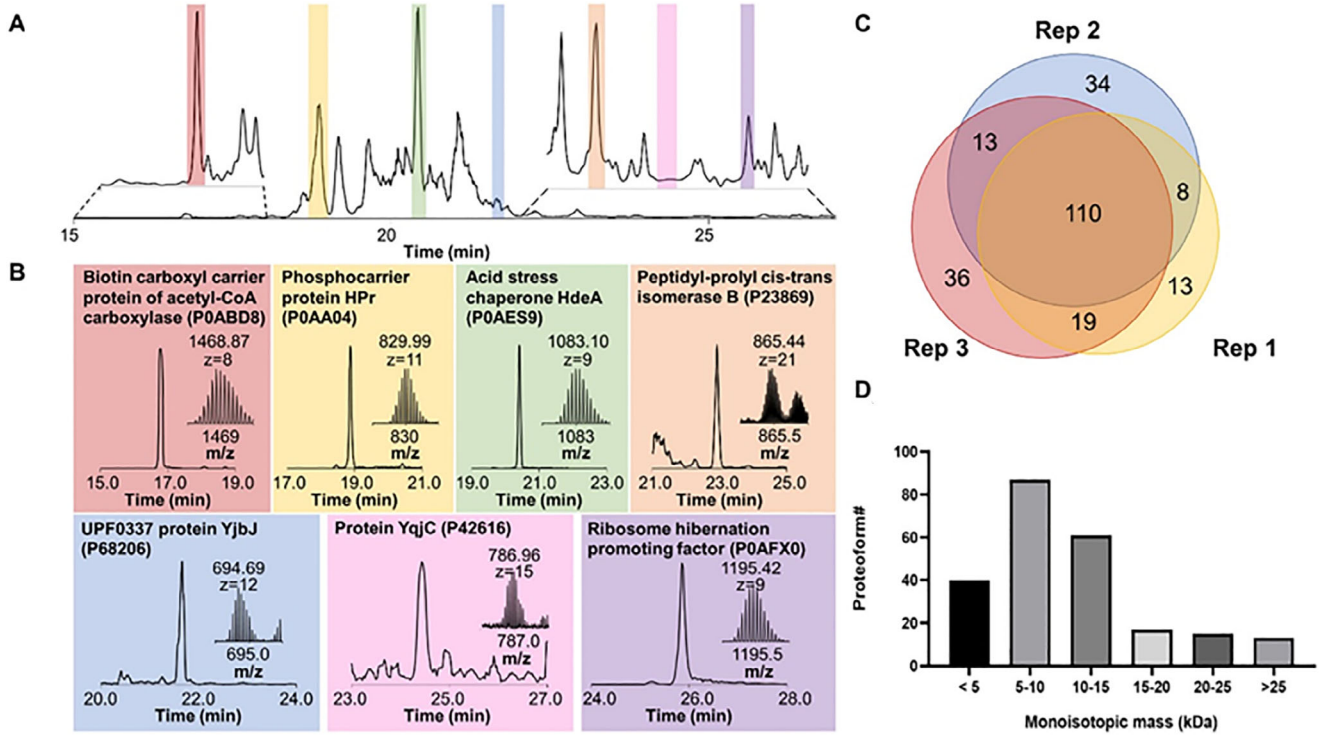

FIGURE 4 | Ultrasensitive top-down analysis of 50 pg of *E. coli* cell lysate using spray-capillary CE–MS (20 μm ID, PEI-coated). (A) BPE (700–1600 *m/z*) of a representative run; colored highlights correspond to (B) arbitrarily selected proteins (extracted ion electropherograms [EIEs] and isotopic peak distributions). (C) Venn diagram of detected proteoforms for triplicate analyses. (D) Mass distribution of detected proteoforms. Figure reprinted with permission from Reference [125].

NH_4OAc, pH 9.0). The developed method enabled to identify 366 (ProSight PD software) and 602 (Top-PIC software) histone proteoforms from commercial calf histone sample. It was 3-fold more than those identified by CZE-MS/MS without FAIMS.

Dynamic pH junction-based CZE-MS/MS identified 263 proteoforms (2–70 kDa) in the protein corona of polystyrene NPs incubated with human plasma using TDP [118, 124]. CZE was performed in LPA coated FS capillary using 5 % (v/v) AcOH, pH 2.4, as BGE (sample buffer, 100 mM ammonium bicarbonate, pH 8.0), and 30 kV separation voltage. SL was composed of 0.2% FA (v/v) and 10 % (v/v) MeOH. Combining this method with CZE-FAIMS-MS/MS and RPLC-MS/MS enabled to identify 883 proteoforms, among them proteoforms of 48 protein biomarkers [118].

CZE-MS/MS was utilized also for the TDP of integral membrane proteins (IMPs, enriched from mouse brain) that are fundamental for vital cellular functions as transporters, receptors, channels, and enzymes [74]. To reduce the sample complexity, SEC was used for fractionation before CZE-MS/MS. By this combined method, 65 proteins and 343 proteoforms from the mouse brain sample were identified, 276 of which were IMPs. With single-shot CZE-MS/MS without SEC fractionation only 21 proteins and 65 proteoforms, 51 of which were IMPs, were identified.

A spray-capillary-CE-MS platform [80] was used for the TDP analysis of 50 pg of *E. coli* lysate [125] resulting in detection of over 200 proteoforms. Use of PEI-coated spray FS capillary instead of bare FS, and spray-capillary with lower ID (20 μm instead of 50 μm) improved the sensitivity of CE-MS analysis of intact protein mixtures (see Figure 4). In addition, by coupling nanodroplet-based sample preparation (to lyse cells and extract intact proteins) with this CE-MS platform (50 μm ID capillary was used), 261 ± 65 and 174 ± 45 proteoforms were detected from less than 50 *HeLa* and OVCAR-8 cells, respectively.

In addition, multisegment sample injection was integrated with the above-mentioned spray-capillary-CE-MS platform [126]. In this approach, sample and spacer (10% AcOH) segments were sequentially introduced. The optimization of the spacer volume (7.5 nL), improved the peak resolution of proteoforms. Up to 17 segments of sample could be injected during single 75 min lasting CE-MS run (occupying ~50% of the capillary) without negatively affecting resolution or reproducibility.

The investigation of the long-term qualitative and quantitative reproducibility of CZE-MS/MS for TDP of complex proteome sample (yeast cell lysate) showed that after ~10 CZE-MS/MS runs, migration times and intensities of proteoforms substantially changed due to their adsorption onto the inner wall of LPA-coated FS capillary [127]. Nevertheless, more than 1000 proteoforms were identified per run across 62 runs utilizing the same LPA-coated FS capillary. Consecutive washing with 0.5% NH_4OH, water and BGE (5% (v/v) AcOH) enabled to effectively remove the adsorbed proteins. After clean-up, at least 23 CZE-MS/MS measurements provided highly reproducible results.

Poly(acrylamide-*co*-(3-acrylamidopropyl)trimethylammonium chloride) (PAMAPTAC)-coated FS capillaries with cationic tunable EOF prepared with some modification according to [128] were used for TDP of yeast cell lysate [129]. PAMAPTAC-

coating provided better separation resolution and efficiency than commonly employed neutral LPA coating. CZE-MS with PAMAPTAC-coated capillaries allowed to detect large proteoforms (≥ 30 kDa) without size-based prefractionation. In addition, in PAMAPTAC-coated capillary, the mobilities of proteoforms were accurately predicted using the semi-empirical model [129].

To simplify the capillary coating procedure, it was further modified. Only one monomer, (3-acrylamidopropyl)trimethylammonium chloride (APTAC), and methanol as the solvent were used. CZE-MS analysis of *E. coli* cell lysate using this capillary allowed to detect large proteoforms, 26–35 kDa [130]. Across 35 CZE-MS runs, the RSD of migration time was <2.1% after migration time corrections, and the RSDs of the intensities of the three large proteoforms were from 20.9% to 32.8%.

Also, cationic sulfobetaine-modified poly(α-L-lysine) (α-PLL) SMIL coatings were utilized to improve separations of intact proteins and proteoforms by CE-MS [131]. The EOF mobility in α-PLL-coated capillaries could be adjusted depending on the number of lysine side chains modified with the zwitterionic sulfobetaine functionality. These coatings with medium EOF velocity were well-suitable for the separation of large proteins and their proteoforms with a low positive net charge such as proteins with high amounts of acidic PTMs (phosphorylation, glycosylation, acetylation).

In microchips designed for TDP, the separation of proteins is usually achieved by CE methods (CZE, CIEF, CEC), LC or field-flow fractionation [132].

A cross-laboratory study embracing 12 research groups around the world applied CZE-MS for TDPs of two model proteoform mixtures, standard mixture of 6 proteins (9-68 kDa) and yeast (*Saccharomyces cerevisiae*) cell lysate [133]. CE and MCE set-ups, different CE-MS interfaces, and various MS platforms were used. By different groups either bare FS, or various coated capillaries (i.e., LPA, PEO, PVA, PEI, PEG [microchip]) were employed. In 11 laboratories, RSDs of migration times of CE-MS measurements were <1% (after internal standard correction). In addition, CE-MS and LC-MS methods were compared within one laboratory using for both techniques the same MS instrument and data acquisition parameters. CE and LC were found to be highly complementary as nearly 50% of unique proteoforms identified by CE-MS were not detected by the LC-MS.

6 | Applications

6.1 | Monitoring of Posttranslational Modifications (PTMs)

PTMs of proteins increase their diversity by altering their structure and biological functions. Abnormal PTMs play an important role in the development of diseases. Thus, investigation and interpretation of PTMs is essential for the prevention, diagnosis, and treatment of diseases [134]. Recently, the strategies for PTM identification and quantification in MS-based proteomics in cardiovascular diseases were briefly overviewed [135]. PTMs are explored using both BUP and TDP approaches and also by peptide mapping of proteins and polypeptides [136, 137].

Proteoforms of G protein-coupled receptors, the largest family of integral human membrane receptors, and important drug targets, were characterized by CZE-MS TDP (BGE: 3% AcOH, 10% sulfolane) and MDP (BGE: 3% AcOH) approaches [138]. Only 40 nL of sample containing ~200 ng of β2-adrenergic receptor was injected. BGE additive sulfolane and selection of precursors at <2000 *m/z* improved fragmentation coverage of CID and higher-energy collisional dissociation (HCD). With CZE-MS/MS, nine proteoforms for human β2-adrenergic receptor A purified from insect cells were identified, and several phosphorylation sites were localized.

CZE-MS/MS and RPLC-MS/MS were combined for BUP of recombinant human *tau-0N3R* protein expressed in *E. coli* cells. They enabled to identify peptides with multiple PTMs [139]. RPLC-MS/MS identified more hydrophobic peptides, while CZE-MS/MS was more successful for hydrophilic peptides with phosphate or succinyl groups. The peptide identifications were further validated by examining the correlation between their predicted and experimental electrophoretic mobilities. CZE and RPLC methods were found to be highly complemental in characterizing tau protein PTMs. Using pseudonative CIEF-MS, three putative tau-0N3R dimers carrying phosphate groups were observed, see Figure 5.

Microchip-based SPE-integrated CZE device coupled with MS was applied for the analysis of 225 phosphorylated peptide standards and phosphorylated peptide-enriched mouse brain tissue [140]. BGE was composed of 1% FA in 50% ACN. For sixty percent of these standards, reliable quantification was possible even at 640 zmol amount. Analysis of the phosphorylated peptide enriched mouse brain tissue showed that phosphorylation sites determined by SPE-CZE-MS and nano-LC-MS were highly complementary.

6.2 | Biological and Biochemical Research

Proteomic studies help to increase understanding of complex biological systems. CE-MS techniques have been successfully applied for the single-cell proteomic analysis at both the peptide and intact protein levels [49, 141]. Below, some examples of the use of CE-MS in single-cell proteomics are presented.

Electrophoresis-correlative data-independent acquisition (Eco-DIA) was developed to increase the sensitivity of CE-MS-based single-cell proteomics [142]. It is based on the characteristic feature of CE-MS method to sort peptide ions into reproducible mass-to-charge (*m/z*) versus migration time trends in the liquid phase, prior to ionization and MS detection (see Figure 6). This approach was combined with DIA while not the entire *m/z* range but narrower *m/z* regions containing a dense distribution of the temporally *m/z*-sorted peptide ions were scanned. From 1 ng of the HeLa proteome digest, Eco-DIA quantified 51% more proteins with <10% coefficient of variation than classical DIA.

Eco combined with ion mobility MS (IMS) was employed for the single cell CE-ESI-MS proteomics to profile single blas-

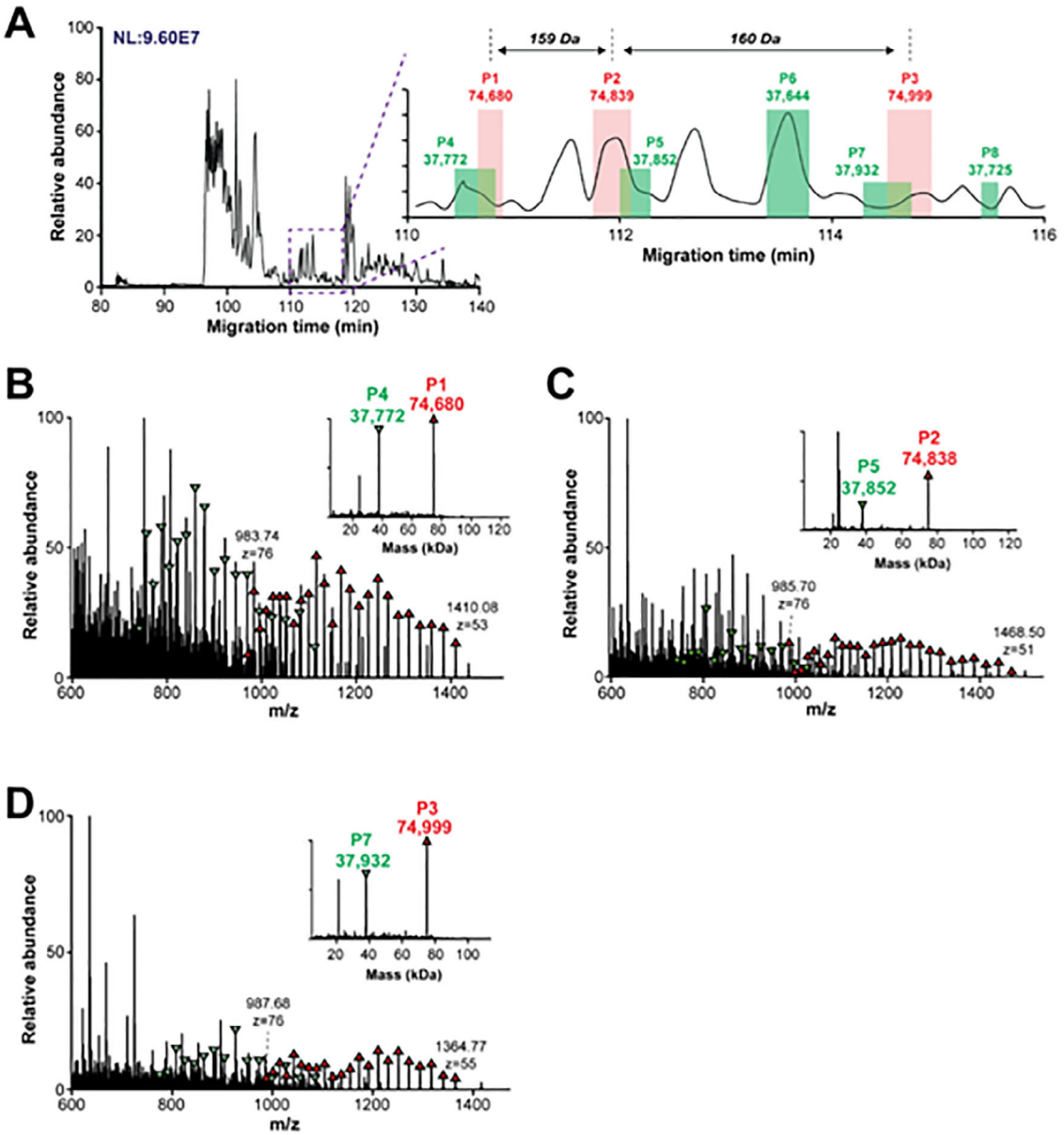

FIGURE 5 | CIEF-MS analysis of human p-tau-0N3R protein under pseudonative conditions. (A) Base peak electropherogram of p-tau-0N3R proteoforms with a zoomed-in base peak electropherogram of the 110–116 min region (inserted figure). The deconvoluted masses of different tau proteoforms are labeled. (B–D) Averaged mass spectra and deconvoluted masses (insets) of p-tau-0N3R proteoforms detected in P1–P3. Figure reprinted with permission from Reference [139].

tomeres from the animal cap, a tissue of pluripotent cells, in the frog *Xenopus laevis* embryo [143]. The method enabled to detect 1157 proteins from <4% of the total proteome content in single cells (~80 μm). Quantitative profiling of different blastomeres ($n = 9$) revealed detectable differences between these cells.

Combination of CE, DDA with Eco ion sorting and artificial intelligence (AI)-assisted spectral deconvolution via CHIMERYS (Real-Time Eco-AI) was developed as a cost-effective approach for the single cell proteomics [144]. In this workflow, custom-built CE coupled to a legacy hybrid quadrupole-orbitrap mass spectrometer (Q Exactive Plus) was used. This method enabled to identify 1799 proteins from a single HeLa cell equivalent (~250 pg) digest within 15 min. Achieved identification rates were comparable with modern MS instruments (Orbitrap Fusion Lumos and Exploris). From *X. laevis* embryonic cells, this novel approach allowed to identify 1524 proteins from ~500 pg of proteome during the 10 min of CE-MS analysis.

CE-LIF method was employed for the preliminary quantification of the copy number of human epidermal growth factor receptor-2 (HER2) membrane proteins in single cells [145]. For this, HeLa cells were labelled by fluorescein isothiocyanate (FITC) affinity bodies specifically targeting HER2 membrane proteins. The calibration curve was obtained by measuring a series of fluorescent microspheres with known number of FITC molecules on the surface. Calibration was used to convert the fluorescence intensity of the cells to the molecule number of HER2. The copy number of HER2 in HeLa cells ranged from 4036 to 1,224,920 ±

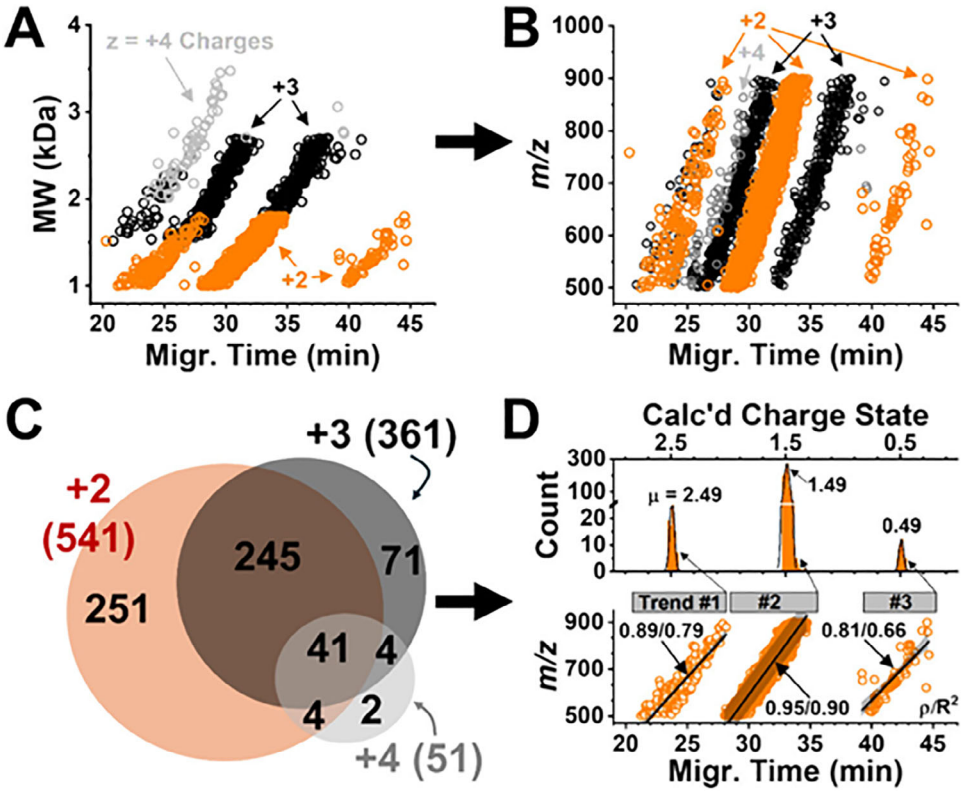

FIGURE 6 | Recognition of "ion sorting" in CE-ESI-MS. (A) Experimentally observed correlation between the measured peptide mass (molecular weight, MW), migration time, and detected charge state (+2 in orange; +3 in black; and +4 in gray) for the HeLa proteome standard. (B) These correlations translated into the domain of the detected mass-to-charge (m/z) versus migration time. (C) Distribution of the detected peptide charge states, revealing most protein identifications originating from precursors carrying +2 charges (total protein identifications in parentheses). (D) Modeling of the observed trend lines. (Top panel) Charge states calculated in the background electrolyte (pH 2.3), revealing charge-driven differentiation of the trend lines. (Bottom panel) Fitting of the experimental m/z–migration time trends with linear regression, revealing a strong correlation and predictability. Key: R^2, linear regression coefficient; ρ, Pearson correlation coefficient. Figure reprinted with permission from Reference [142].

100 (2.5%–97.5%), and the median of copy numbers were 104,438 ± 100 per cell.

CE has been applied in analysis of mass-limited samples in diverse biological studies. For example, CE-ESI with trapped ion mobility spectrometry-time-of-flight mass spectrometry (TIMS-TOF PRO MS) and STochastic Optical Reconstruction Microscopy (STORM) were applied for the characterization of suprachiasmatic nucleus (SCN) proteome before and after eye-opening [146]. CE-MS identified 1894 proteins and quantified 1066 proteins by analyzing ~10 ng of proteome digest corresponding to <~0.5% of the tissue proteome. During quantitative profiling, significant developmental changes were detected for 166 proteins, many of them having important biological functions such as axon guidance and synapse function.

6.3 | Clinical Applications

Proteomic and peptidomic strategies are used to search, determine, and validate clinically relevant biomarkers (and/or their combination) in biological samples with the aim to implement these biomarkers into clinical practice [147], in order to improve early disease detection [148, 149], prognosis of its progression [150–152], prediction of treatment response [153], or monitor the effects of dietary interventions [154]. The use of MS-based approaches (CE-MS, LC-MS, MALDI-MS) in urinary peptidomics and proteomics were recently reviewed [155]. For determination of profile of clinically relevant peptides in urine samples of diabetic nephropathy patients, CE-MS/MS, LC-MS/MS and MALDI-MS methods were compared [156]. Front-end separation improved the accuracy of peptide identifications as besides intact mass, elution/migration times were utilized for identification.

Analysis of urine by CE-MS has been applied in many peptidomic and proteomic studies for diagnosis [157, 158] and prognosis [159, 160] of different diseases.

For example, urinary peptidomics profile for hypertension in young adults was investigated using CE-MS [161]. The BGE in CZE was composed of 1:20:79 (v/v/v) FA, ACN and water, and SL consisted of 0.4% FA and of 30% IPA in HPLC grade water. Migration times and ion signal intensities were normalized based on the reference signal of internal peptide standards. Several urinary peptides (collagen type I and III fragments) potentially associated with hypertension were identified.

In addition, CE-MS was used for the analysis of endogenous urinary peptides of patients with prostate cancer and compared with those of non-cancerous prostatic diseases patients (see Figure 7)

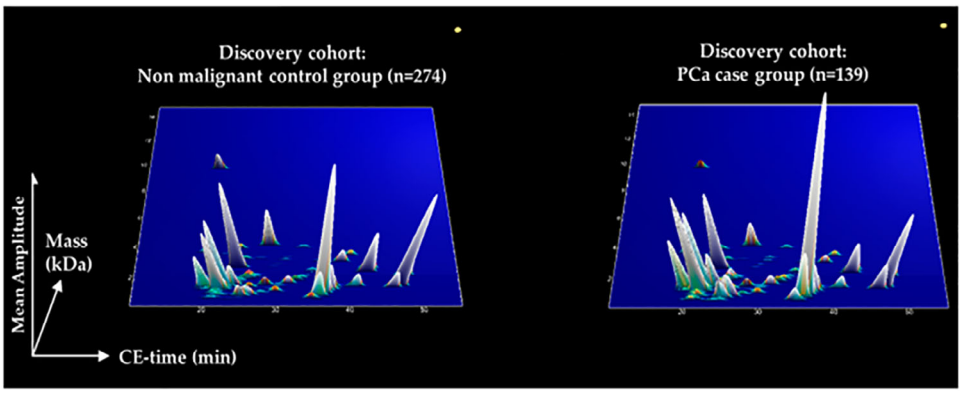

FIGURE 7 | Compiled urinary profiling signatures of prostate cancer (PC) patients ($n = 139$) compared to those with non-prostate cancer etiologies ($n = 274$). In this plot, each peptide is plotted based on the normalized migration time (10–55 min) on the x-axis against the log molecular mass (0.8–15 kDa) on the y-axis. The signal intensity is represented by the peak height, corresponding to the mean values of each peptide. Figure reprinted with permission from Reference [162].

[162]. The developed biomarker model based on machine learning was composed of 181 biomarkers and demonstrated good accuracy in detecting prostate cancer.

Peptidomic and proteomic analysis of urine provide additional tools not only for diagnosis of urogenital diseases but also for other disorders, such as osteoporosis [163, 164], post-acute sequelae of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection [165] or possible clinical complications of preterm infants [166]. To lesser extent, CE-MS has been applied in studies of other body fluids, such as plasma [52], cerebrospinal fluid [167], or saliva [53].

By CZE-MS, 291 plasma samples (from patients with chronic kidney disease and end-stage kidney failure) were analyzed resulting in identification and quantification of 3920 unique plasma peptides [52]. Sequence information was obtained for 661 peptides by CE-MS/MS and by database searching 135 unique proteins were identified. Analysis of pre- and post-dialysis samples allowed to compose a model enabling to classify pre- or post-dialysis samples with high precision.

CZE-MS and CZE-MS/MS were used also for the peptidome analysis of human saliva [53]. A total of 6170 peptides were identified with sequence information obtained for 630 peptides, originating from 82 proteins. Substantial shift in the peptide composition of saliva before and after breakfast was detected.

Quantitative TDP employing SDS-PAGE fractionation followed by CE-MS analysis was used to study histone proteoforms between metastatic (SW620) and nonmetastatic (SW480) colorectal cancer (CRC) cells [75]. CZE-MS analysis was carried out in LPA-coated FS capillary using 5% (v/v) AcOH, pH 2.4, as BGE, and 0.2% (v/v) FA and 10% (v/v) MeOH in water as SL. In SW480 and SW620 cell lines, 230 histone proteoforms were quantified. Between the two CRC cell lines, substantial differences in histone proteoform profile were found. Differentially expressed histone proteoforms, such as acetylated histone H4 proteoforms and histone H2A proteoforms with N-terminal acetylation and phosphorylation were suggested as possible proteoform biomarkers for CRC metastasis.

Quantitative TDP using SEC fractionation before CZE-MS/MS analysis discovered substantial differences between the healthy and Alzheimer disease (AD) brain samples [168]. Over 2200 proteoforms were identified solely in AD or in healthy control samples, and 157 proteoforms identified in both samples showed statistically significant differences in their abundance between two conditions. Broad changes in biological processes in AD brains were revealed by Gene Ontology and Pathway analysis of the genes associated with those proteoforms.

Linear poly(N-(2-hydroxypropyl)-methacrylamide) (LP(HPMA)) coated FS capillaries prepared by surface-confined aqueous reversible addition-fragmentation chain transfer (SCARAFT) polymerization method showed comparable EOF mobility (3.7×10^{-6} $cm^2 \cdot V^{-1} \cdot s^{-1}$) but better stability at physiological pH than LPA coated capillaries [169]. Using LP(HPMA) capillaries, non-small cell lung cancer tissues and corresponding paracancerous tissues were analyzed by CZE-ESI-MS/MS (100 ng) and compared to nanoRPLC-MS (1 μg). Overall more proteins were identified by nanoRPLC-MS but the two methods were complementary. CZE-MS identified more peptides with pI 7–8 than with pI < 4, and more hydrophilic than hydrophobic peptides. Principal component analysis (PCA) of the CE-MS data divided the analyzed samples into tumor tissue clusters and paracancerous tissue clusters. In addition, samples from different patients were further separated into subclusters.

6.4 | Food Analysis

Peptidomics and proteomics are applied in different areas of food analyses [170], to ascertain food quality [171], origin, authentication [172, 173], and adulteration [174], to identify and characterize bioactive peptides [175–177], or to determine protein allergens [178, 179].

CE was used for detection of cow milk adulteration in seven types of milks (buffalo, yak, goat, sheep, camel, horse, and donkey) and milk products (raw and pasteurized milk, and freeze-dried and spray-dried milk powder) [180]. Proteins, such as bovine α_{s1}-

TABLE 1 | Selected proteomic and peptidomic analyses by CE and MCE methods.

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
Arabidopsis thaliana leaf and chloroplast proteomes	~500 ng of protein and ~160 ng of protein	TDP	CZE; MS/MS; prefractionation by off-line SEC	10% (v/v) AcOH, 50 μm × 1 m, LPA, 30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	Over 4700 unique proteoforms across total leaf and chloroplast samples were identified.	[121]
BSA tryptic digest (in mixture with amino acid ampholytes)	0.07 mg/mL	BUP	CIEF-Nafion; ESI-MS	Catholyte: 0.2 M NH_4OH, 10% glycerol, anolyte: 0.1% (v/v) FA, 10% glycerol, 100 μm × 60 cm, LPA, Nafion (5 cm), BFS (18.2 cm), 20 kV, n.p. 0.1 % (v/v) FA, 50% MeOH (v/v)	Peptides with 0.02 pI unit difference were baseline separated.	[105]
BSA	n.p.	BUP	CZE; UV@200 nm, off- line pepsin immobilized enzyme particle (IEP) or in-situ immobilized enzyme microreactor (IMER); LC-MS/MS	0.44% (v/v) FA, pH 2.0, 50 μm × 50 cm, 15 kV, 25°C	Peptide maps showed the similarity of digestion obtained by IEP and IMER indicating a comparable enzyme-to substrate ratio within the capillary	[66]
Brain tissue (human; inferior temporal cortex)	n.p.	TDP	CZE; MS/MS; SEC fractionation	5% (v/v) AcOH, pH 2.4, 50 μm × 1 m, LPA, 20 kV, n.p. 0.2% (v/v) FA, 10% (v/v) MeOH	1232 and 1223 proteoforms were unique to Alzheimer's disease and to healthy control samples, respectively.	[168]
α_{s1}-casein, β-lactoglobulin and β-casein A1	0.8 mg/mL	TDP	CZE; UV@214 nm	10 mM phosphate, pH 2.5, 8 M urea, 8.5 mM HPMC, 50 μm × 60/51.5 cm, 25 kV, 25°C	These proteins were identified as suitable markers for detecting cow milk adulteration in distinct types of milks.	[180]
Cell lysate of human Caucasian colon adenocarcinoma cells	n.p.	TDP	NanoLC-CZE; MS	2 M AcOH, 50 μm × 35 cm, 5-layer PDADMAC-poly-(styrenesulfonate) (PSS) SMIL, −15 kV, n.p. 50% (v/v) IPA, 0.5% FA	~2 and 3–4 times more proteoforms were identified by nanoLC-CZE-MS than by 1D CZE-MS and 1D nano-LC-MS methods, respectively.	[112]

(Continues)

TABLE 1 | (Continued)

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
Cytochrome c from bovine heart	0.1 mg/mL	TDP BUP	CZE; nanoESI-MS	50 mM AcOH, 25 μm × 60 cm, n.p., 25°C 50% (v/v) MeOH in BGE 15 kV	A signal-to-noise ratio of approximately 230:1 with an infusion of 8.3 nM cytochrome c solution was achieved. Six specific fragments were detected.	[107]
Epidermal growth factor receptor-2 membrane proteins	1000 particles/mL (cells or FluoSpheres)	BUP	CZE; LIF	20 mM $Na_2B_4O_7$-93 mM H_3BO_3, pH 8.3, the ID of the FS capillaries was equal to ~1–3 times of the diameter of the cells, 10 kV, n.p.	The copy number of human epidermal growth factor receptor-2 in HeLa cells ranged from 4036 to 1,224,920 ± 100, with a median value of 104,438 ± 100 molecules per cell.	[145]
Escherichia coli cell lysate Intact *HeLa* cells and ovarian cancer cells	50 pg ~45 cells per injection	TDP	CZE; MS	5% AcOH, 20 μm × 85 cm, PEI, −15 kV, n.p. sheathless 50 μm × 80 cm, PEI	233 proteoforms were detected. 261 and 174 intact proteoforms were detected from *HeLa* cells and ovarian cancer cells, respectively, using nanodroplet sample preparation.	[125]
Escherichia coli cell lysate digest (spiked with standard peptide angiotensin II)	1 μg/μL (10 μM Ang II)	BUP	CZE; ESI-MS	0.1% FA, 50 μm × 1 m, PEI, −30 kV, n.p. sheathless	749 unique peptides from 170 protein groups were identified.	[80]
Escherichia coli cell lysate	8–250 pg	TDP	Multisegment spray-capillary CZE; MS	5% AcOH, 20 μm × 85 cm, PEI, −15 kV, n.p. 0.1% FA	Multisegment injection approach allowed to produce six-point calibration curve for *E. coli* lysate proteoforms during single analysis within one hour.	[126]

(Continues)

TABLE 1 | (Continued)

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
Escherichia coli cell lysate	~125 ng	Native proteomics (TDP) Denaturing TDP	Native CZE-MS Denaturing CZE-MS	25 mM NH_4OAc, pH ~7, 50 µm × 90 cm, LPA, 30 kV (68,9 mbar) or 20 kV (130 mbar) for native CZE-MS with in-source CID, n.p. 10 mM NH_4OAc 5% AcOH; 0.2 % FA and 10% MeOH	33 large proteoforms/ complexoforms were quantified during the logarithmic and stationary growth phases of *E. coli*.	[103]
Escherichia coli cell lysate	50 ng	Native proteomics (TDP)	Native CZE-MS/MS	25 mM NH_4OAc, pH ~7, 50 µm × 1 m, LPA, 30 kV, n.p. 10 mM NH_4OAc	Nearly 100 proteoforms or protein complexes in a mass range of 10–400 kDa were detected.	[22]
Escherichia coli cell lysate	n.p.	TDP	CZE; MS	5% AcOH, pH 2.4, 50 µm × 1 m, (3-acrylamidopropyl) trimethylammonium chloride (APTAC), -30 kV, n.p. 0.2% FA, 10% (v/v) MeOH	Repeatability of the migration times <2.1% ($n = 35$; after migration time correction)	[130]
HeLa proteome digest	1 ng	BUP	CZE; Electrophoresis-correlative-MS/MS	1 M FA in 25% (v/v) ACN in water, pH 2.3, 40 µm × 1 m, 23 kV, n.p. 0.5% (v/v) AcOH in 10% (v/v) ACN in water	Compared to classical DIA, electrophoresis-correlative-DIA identified and quantified ~38% more proteins.	[142]
HeLa proteome digest Blastomeres isolated from the animal cap of South African clawed frog *X. laevis* embryo	~200 pg ~80 µm	BUP	CZE; Electrophoresis-correlative-ion mobility-MS	1 M FA, 25% (v/v) ACN, pH 2.3, 40 µm × 1 m, 23 kV, n.p. 10% (v/v) MeOH, 0.5% (v/v) AcOH	~962 proteins were identified. 1175 proteins were detected by analyzing <4% of the total proteome content in single, yolk-laden embryonic stem cells.	[143]
HeLa cells protein digest standard	0.1 ng	BUP	RP-SPME-CZE; MS/MS	5% AcOH, 50 µm × 80 cm, LPA, 30 kV, n.p. 10% MeOH and 0.2% FA	It was possible to inject ~40% of the available peptide material. 257 protein groups and 523 peptides were identified.	[81]

(Continues)

TABLE 1 | (Continued)

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
HeLa cells protein digest standard	1 µg/µL (20 ng)	BUP	Robotic capillary platform nanoCE; ESI-MS	25% (v/v) ACN with 1 M FA in water, pH 1.94, 40 µm × 75 cm, n.p. 10% (v/v) MeOH and 1% FA in water, pH 2.30	The nanoCE platform is able to analyze <100 µL of starting sample (in automation). In average, 1800 proteins were identified.	[82]
HeLa cells protein digest standard	50 ng/µL (~64 nL; ~14 ng after enrichment)	BUP	Acoustic droplet levitator-Robotic capillary platform CE; ESI-MS	10% (v/v) AcOH, pH 2.42, 40 µm × 75 cm, 15 kV, n.p. 1% (v/v) FA in 10% (v/v) MeOH, pH 2.3	60 min-lasting midair evaporative enrichment reduced ~4.4-fold sample volume, which corresponded to ~3.4-fold concentration enrichment.	[83]
HeLa cells proteome digest Single precursor cells in *X. laevis* blastulae	1 ng (~250 pg) 50–75 µm diameter	BUP	CZE; Electrophoresis-correlative-DDA-artificial intelligence-MS/MS	1 M FA in 25% (v/v) ACN, 40 µm × 1 m, 250 V/cm, n.p. 0.5 % AcOH in 10% (v/v) MeOH	2142 (1799) proteins were identified. 1524 proteins were identified; at the single-cell level proteomic differences between dorsal and ventral lineages were detected.	[144]
HeLa cells proteome digest	~200 pg (single HeLa-cell-equivalent)	BUP	CZE; DIA-MS	1 M FA in 25% (v/v) ACN, 40 µm × 1 m, 28 kV, n.p. 10% (v/v) MeOH, 0.5% (v/v) AcOH	1161 proteins within a 15 min effective separation were identified by DIA method (by DDA 401 proteins).	[115]
HeLa cells proteome digest	1 µg/µL (200 ng)	BUP	CZE; TIMS-MS	1 M FA in 10% IPA, pH 1.9, 50 µm × 150 cm, PEO, 30 kV, n.p. n.p.	High orthogonality for CE and gas-phase ion mobility was observed.	[106]
HeLa cell digest	12.5 ng	BUP	CZE; DIA-MS	1 M AcOH, 50 µm × 1 m, LPA, 21.7 kV, n.p. 0.5% (v/v) FA, 10% (v/v) MeOH	A potential cancer biomarker protein, carbohydrate antigen 125, undetected in the DDA mode, was identified in the DIA mode.	[114]
Histone extract from calf thymus (commercial)	2 mg/mL (50 ng loaded)	TDP	CZE-MS/MS CZE-FAIMS-MS/MS	20 mM NH_4OAc, pH 5, 50 µm × 1 m, LPA, 30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	366 and 602 histone proteoforms were identified by ProSightPD and TopPIC Suite software, respectively.	[123]

(Continues)

TABLE 1 | (Continued)

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
Histone protein	1 mg/mL	TDP	CZE; MS-MS; SDS-PAGE fractionation	5% (v/v) AcOH, pH 2.4, 50 μm × 1 m, LPA, 30 kV, n.p. 0.2% (v/v) FA, 10% (v/v) MeOH in water	Significant differences in histone proteoform profile between metastatic (SW620) and nonmetastatic (SW480) colorectal cancer cell lines were observed.	[75]
Histone proteoforms	20 μg	TDP	CZE; MS/MS; PEPPI-MS	5% (v/v) AcOH, pH 2.4, 50 μm × 1 m, LPA, 30 kV, n.p. 0.2% (v/v) FA, 10% MeOH in water	Over 200 histone proteoforms were identified from a commercial calf thymus histone sample.	[111]
Integral membrane proteins from mouse brain	1.4 mg/mL (average protein concentration of SEC fraction)	TDP	CZE; MS/MS; Prefractionation with SEC (4 fractions collected)	30% AcOH and 30% MeOH in water (v/v), 50 μm × 1 m, LPA, 30 kV (pressure: 6.9 mbar for 80 min, 34.5 mbar for 20 min, 137.9 mbar for 10 min), n.p. 0.2% (v/v) FA, 10% (v/v) MeOH	65 proteins and 343 proteoforms, 276 of which were integral membrane proteoforms containing 1–4 transmembrane domains, were identified.	[74]
β-lactoglobulin A and B in foods	LODs were 0.03 and 0.02 μg/mL, respectively	TDP	AA-SPE-CZE; MS	2 M AcOH, pH 2.2, 75 μm × (7.5+52) cm, (microcartridge, 250 μm × 0.7 cm), 20 kV with pressure 50 mbar, 25°C 60:40 (v/v) IPA/water with 0.25% (v/v) FA	RSDs for migration time and peak areas was 0.5% and 2.4%, respectively. LOD for β-lactoglobulin with AA-SPE-CZE-MS was 200 times lower than with CZE-MS.	[178]
Mouse brain tissue enriched by standards of phosphorylated peptides	32 ng down to 50 pg	BUP	SPE-MCE; MS/MS	1% FA, 50% ACN, ~30 μm × 22 cm (channel), ZipChip, 500 V/cm, n.p.	Relative quantification was reproducible when sample loading was scaled down to 1 ng on-chip.	[140]
Mouse neuronal proteome digest and yeast proteome digest (standard)	~40 ng (tandem mass tag-tagged proteome mixture)	BUP	CZE; MS/MS; SPS-MS^3, nanoLC-MS	1% FA in 25% (v/v) ACN, 40 μm × 1 m, ~20 kV, n.p. 10% (v/v) ACN, 0.05% (v/v) AcOH	The reproducibility and accuracy between CE and nanoLC using MS^2 or SPS-MS^3 quantification was indistinguishable.	[116]

(Continues)

TABLE 1 | (Continued)

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
Non-small cell lung cancer tissues and corresponding paracancerous tissues	n.p.	BUP	CZE; ESI-MS/MS; nanoRPLC-MS	1 M AcOH, 50 μm × 1 m, LP(HPMA), 21.7 kV, n.p. 10% (v/v) MeOH, 0.5 % FA	The differentially expressed proteins in lung cancer tissues identified by CZE-MS and nanoRPLC-MS were complementary.	[169]
Plasma	0.5 μg/μL protein	Peptidomics	CZE; MS	250 mM FA in 20% (v/v) ACN, 50 μm × 95 cm, n.p. n.p.	169 plasma peptides with distinct pre- and post-dialysis plasma abundance were identified.	[52]
Protein corona of human plasma and mouse serum NPs	~30 ng	BUP	CZE; MS/MS	5% (v/v) AcOH, 50 μm × 80 cm, LPA, 30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	Different NPs captured different pools of the plasma/serum proteome in the protein coronas.	[84]
Protein corona of polystyrene (PS) coated NPs	2.4 mg/mL (protein concentration), ~240 ng of corona proteins	TDP	CZE; MS/MS	5% (v/v) AcOH, 50 μm × 100 cm, LPA, 30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	~900 proteoforms (2–70 kDa) in the protein corona of PSNPs, including the proteoforms of 48 protein biomarkers were identified.	[118]
Protein corona of polystyrene coated NPs incubated in human plasma	1.5 mg/mL (protein concentration)	TDP	CIEF; MS/MS	Catholyte: 0.3% NH_4OH, ampholyte: mixture of pI 3–10 and 5–8 ampholytes (volume ratio of 1:2), 50 μm × 80 cm, LPA, 30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	Over 70 proteoforms of 16 cancer-related genes were identified.	[119]
Protein corona of polystyrene coated NPs incubated in human plasma	1.5 mg/mL (protein concentration), ~600 ng of corona proteins	TDP	CIEF; MS/MS	Catholyte: 0.3% NH_4OH, ampholyte: mixture of pI 3–10, 5–8, and 8–10.5 ampholytes, anolyte: 5% AcOH, 50 μm × 80 cm, LPA, 30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	53 genes were identified, 33 of them are potential biomarkers of various diseases. 1–102 proteoforms per potential protein biomarker were identified containing various sequence variations or PTMs.	[120]

(Continues)

TABLE 1 | (Continued)

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
Recombinant human *tau-0N3R* protein	digest of p-tau protein, n.p. 0.5 mg/mL protein, 1.5 % ampholyte	BUP TDP	CZE; MS/MS; RPLC-MS CIEF; MS	5% (v/v) AcOH, 50 µm × 1 m, LPA, 30 kV, n.p. 0.2 % FA, 10 % MeOH, pH 2.2 Catholyte: 0.5% NH_4OH, 15% glycerol, anolyte: 0.1% FA, 15% glycerol, ampholyte: pI 8–10.5 and 3–10 at a ratio of 4:1, 20 kV, at 20 min additional pressure: 13.8 mbar	49 phosphorylation sites, 14 acetylation sites, 12 methylation sites, 21 succinylation sites were identified. 11 putative tau-0N3R proteoforms carrying up to nine phosphorylation sites were detected.	[139]
Saliva	n.p.	Peptidomics	CZE; MS/MS	0.94% FA, 20% ACN in water, 50 µm × 90 cm, 25 kV, 35°C, pressure gradient 30% IPA, 0.4% FA in water	For 630 peptides, fragments of 82 proteins, high confidence sequence information was obtained, majority of which were derived from proline-rich proteins. Best time for saliva collection is 1–4 h after breakfast.	[53]
Snake venom	n.p.	TDP	CZE; UV; MS	1 M FA, pH 1.9, 50 µm × 65/56.5 cm, 25 kV, n.p. 50 µm × 85 cm, 25 kV, n.p. IPA:water 1:1, with 0.1% (v/v) FA	More than 250 different neuropeptides (7–10 kDa) were detected in the venoms obtained from snakes of 9 different subspecies.	[182]
Standard proteins spiked into human urine, serum, plasma and saliva	100–64 000 ng/mL	TDP	tITP-CZE; ESI-MS; preconcentration by off-line SPE	500 mM FA, pH 1.96, 5% ACN, (tITP, LE: 200 mM NH_4COOH, pH 4.00, TE: BGE), 50 µm × 85 cm, 20 kV, n.p. 50% MeOH, 0.1% FA (v/v)	The accuracy of the method ranged from −22.9% to +27.4%, and the precision from 0.37% to 22.0%.	[77]
Suprachiasmatic nucleus proteome	~10 ng of protein digest (~40 cells)	BUP	CZE; ESI-MS; TIMS-TOF MS; STORM	n.p, 40 µm × 1 m, ~220 V/cm 50% MeOH in 1% (v/v) FA	1894 proteins were identified and 1066 proteins were quantified.	[146]

(Continues)

TABLE 1 | (Continued)

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
(Recombinant human) α-synuclein expressed in *Escherichia coli*	100 μg/mL	BUP	Immobilized enzyme microreactor (IMER)-CE; MS	50 mM AcOH, 50 mM FA, pH 2.3, 75 μm × 72 (7.5 + 64.5) cm, (IMER, 250 μm × 0.7 cm), 25 kV, 37°C 60:40 (v/v) IPA/water with 0.05% FA	RSDs of migration time and peak area ($n = 5$) of (124–131) α-syn peptide were 7.2% and 3.4%, respectively. LOD was 15 μg/mL.	[35]
Urine	2 μg/μL	Peptidomics	CZE; TOF-MS	79:20:1 (v/v/v) H_2O, ACN and FA, n.p., 25 kV, 25°C; 30% IPA, 0.4 % FA in H_2O	Multiple urinary peptides, especially collagen type I and III fragments were found to change in hypertension.	[161]
Urine	0.8 μg/μL	Peptidomics	CZE; MS; LC-MS/MS	5% (v/v) AcOH, 50 μm × n.p., HPC, 30 kV, n.p. n.p.	To improve the accuracy of MS1 profiling of peptides, their separation before MS is necessary.	[156]
Urine	n.p.	Peptidomics	CZE; MS	1% FA (v/v), 20% (v/v) ACN, pH≈2.0, 50 μm × 90 cm, 25 kV, 35°C 0.1% (v/v) FA and 50% (v/v) IPA	Glycocalyx-mimetic supplementation was found to improve the urinary peptidomic signature associated with heart-failure risk.	[154]
Urine	n.p.	Peptidomics	CZE; MS/MS; LC-MS/MS	0.94% FA (v/v), 20% (v/v) ACN, n.p., 25 kV, n.p. n.p.	The abundance of 195 urine peptides among post-acute sequelae of SARS-CoV-2-patients substantially differed from that of controls.	[165]
Yeast cells lysate	1 mg/mL (protein concentration)	TDP	CZE; MS	5% (v/v) AcOH, pH 2.4, 50 μm × 1 m, LPA, 30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	Over 1000 proteoforms were identified per run across 62 runs.	[127]
Yeast cells lysate	~70 ng	TDP	CZE; MS	5% (v/v) AcOH, pH 2.4, 50 μm × 1 m, PAMAPTAC, −30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	The method allowed the detection of large proteoforms (≥30 kDa) reproducibly without any size-based prefractionation.	[129]

(Continues)

TABLE 1 | (Continued)

Proteomic or peptidomic sample	Sample concentration (amount)	Proteomic/ lpeptidomic approach	CE method; detection; other methods applied	Separation conditions (BGE, fused silica capillary dimensions[a] and coating, separation voltage and temperature) Sheath liquid (SL)	Results	Ref.
Yeast cells lysate	200 ng	TDP	CZE; MS/MS, FAIMS-MS/MS	5% AcOH, pH 2.4, 50 µm × 1 m, LPA, 30 kV, n.p. 0.2% (v/v) FA and 10% (v/v) MeOH	Compared to CZE-MS/MS alone, CZE-FAIMS-MS/MS enhanced the identification of large proteoforms (>30 kDa) and significantly improved the sensitivity of detection.	[122]
Yeast cells protein extract	n.p.	TDP	CZE; MS/MS	2 M AcOH, 50 µm × 180 cm, sulfobetaine-modified poly(α-L-lysine)-based SMIL, -30 kV, n.p. 50% (v/v) IPA, and 0.5% (v/v) FA	The mobility of the EOF depends on the number of modified lysine side chains. 978 proteoforms from 214 proteins were identified.	[131]

Abbreviations: AcOH, acetic acid; CID, collisional induced dissociation; DDA, data dependent acquisition; DIA, data independent acquisition; FA, formic acid; FAIMS, high-field asymmetric waveform ion mobility spectrometry; HPC, hydroxylpropyl cellulose; HPMC, hydroxypropyl methylcellulose; IPA, isopropyl alcohol (propan-2-ol); LE, leading electrolyte; LP(HPMA), linear poly(N-(2-hydroxypropyl)methacrylamide); LPA, linear polyacrylamide; n.p., not presented; NH_4OAc, ammonium acetate; NPs, nanoparticles; PDADAMAC, poly(diallyldimethylammonium chloride); PEI, polyethylenimine; PEPPI-MS, passively eluting proteins from polyacrylamide gels as intact species for MS; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SMIL, successive multiple ionic polymer layer; SPME, solid-phase microextraction; SPS, simultaneous precursor selection; STORM, Stochastic Optical Reconstruction Microscopy; TE, terminating electrolyte; TIMS, trapped ion mobility spectrometry.

[a]ID × total length/effective length (to the detector).

casein, β-lactoglobulin, and β-casein A1 were reliable biomarkers for detecting adulteration with a minimum detection limit of 2%.

MCE was used to detect milk adulteration with cheese whey and compared with SDS-PAGE method [181]. In raw, pasteurized, ultra-high temperature (UHT) and powdered milks, the addition of cheese whey was detected at 1% level, using α-casein and β-casein as markers. Change of intensity of protein bands in the SDS gel was observed only after addition of 5%–20% of cheese whey.

Selected proteomic and peptidomic analyses in all above application fields are presented in Table 1.

7 | Conclusions

As shown in many above examples, CE and MCE are very effective techniques for peptidomic and proteomic analyses. From the successful utilization of CE methods in diverse peptidomic and proteomic studies, it is obvious that CE methods have a good perspective to continuously be employed as complementary or, in some cases, as alternative methods to LC techniques. Due to the inherent property of CE to analyze low volumes (nL scale) of samples, it is especially appropriate for the analysis where the limited amounts of material are available, such as proteomic analysis at the single-cell level. Moreover, new technologies handling very small sample amounts and introducing maximum of the available sample to CE-MS have been developed together with interesting approaches to concentrate these tiny amounts of samples before their injection to CE-MS system (acoustic droplet levitation). To deplete high abundance proteins and concentrate low abundance peptides and proteins in complex biological samples, various prefractionation techniques (e.g., SEC) and different sample preconcentration methods, for example, dynamic pH junction, field-amplified sample stacking, or transient ITP, are combined with CE-MS. In peptidomic and proteomic CE studies, both bare FS and coated FS capillaries are employed to a similar extent. Nevertheless, nonspecific adsorption of proteins and peptides on the inner capillary wall is substantially reduced by the use of effective and stable capillary coatings. Most commonly, LPA coating is used. It eliminates also EOF and thereby enlarges the separation window, improves the resolution of analytes and increases the number of peptide and protein identifications. However, preparation of coated capillaries is laborious and time-consuming, while commercially coated capillaries are expensive. In addition, long-term stability of capillary coatings is problematic and most coatings are less stable at extreme pH values. Therefore, in many clinical studies uncoated capillaries are preferred.

In recent years, there is a growing trend in utilization of CE-MS in TDP. To reduce the complexity of biological samples, often prefractionation by SEC prior to CE-MS is employed in TDP. From CE methods, CZE and CIEF are utilized for the characterization of intact proteoforms. With both techniques high-resolution separations are achieved. However, the sensitivity of CIEF-MS is hampered because of the suppression of the ionization of proteoforms and protein complexes in ESI-MS interface due to the interference of carrier ampholytes. This effect can be decreased by application of amino acid ampholytes or even further minimized by a special CIEF-Nafion-ESI-MS interface designed to decrease in-line the amino acid ampholytes after CIEF and before ESI. TDP studies by CE-MS are performed under denaturing or native conditions depending on the buffers used for CE separation and ESI.

In addition, the native CE-MS is more frequently used for investigation of native proteins and protein complexes. Furthermore, native MS coupled with CE is applied not only for the characterization of low-complexity protein samples but also for the measurements of large proteoforms and protein complexes (up to 400 kDa) in a complex whole cell lysate. Moreover, recent results suggest that during the native CZE-MS, the native-like structural topology of protein complexes is maintained. Besides, native CZE-MS is successfully applied for quantitative native proteomics of complex proteome samples (*E. coli* cell lysate).

In the future, even broader utilization of CE-MS methods in diverse peptidomic and proteomic studies is expected, especially in the single-cell and subcellular peptidomics and proteomics, or clinical peptidomics and proteomics. Wider application of CE-MS in various fields of proteomics and peptidomics will be accompanied by the improvements in sample preparation, capillary coatings, CE-MS instrumentation, and data acquisition and interpretation strategies.

Author Contributions

Sille Štěpánová: conceptualization, writing – original draft preparation, literature searching, writing – editing. **Václav Kašička**: conceptualization, literature searching, review and writing – editing, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data are available from the corresponding author upon reasonable request.

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