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Capillary electrophoresis as a sample separation step to mass spectrometry analysis: A primer

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Abbreviations:

CE	capillary electrophoresis	GC	gas chromatography
CIEF	capillary isoelectric focusing	ITP	isotachophoresis
CMT	countercurrent migration technique	LC	liquid chromatography
CZE	capillary zone electrophoresis	LOD	limit of detection
DDA	data-dependent acquisition	LTQ	linear trap quadrupole
EE	electroextracion	mAbs	monoclonal antibodies
EK	electrokinetic	MALDI	matrix-assisted laser desorption ionization
EO	electroosmotic	MS	mass spectrometry
ESI	electrospray ionization	PMT	post-translational modifications
FASI	field amplified sample injection	SPE	solid-phase extraction

Abstract

Capillary electrophoresis (CE) is a separation technique that offers high resolution, great sensitivity, and short processing times. Mass spectrometry (MS) accurately characterizes ionized species based on mass and fragmentation patterns that identify analytes by their mass to charge ratio. Powerful analytical systems are enabled by hyphenating CE with MS. Introducing a sample separated into individual zones minimizes ion suppression and significantly enhances the capacities of MS. This review article is intended as an introduction to scientists and professionals who are already familiar with electrophoresis and are interested in learning about the analytical potential of CE-MS systems. Working principles and basic instrumentation, including the CE-MS interfaces, are included as the robust and sensitive interfaces are crucial to the acceptance of CE-MS systems in the (bio)analytical practice. A discussion of recent reports on CE-MS systems provides an overview of the latest advances, and the conclusions speculate on future developments.

1 Introduction

Electrokinetic (EK) methods refer to the motion of particles and fluid under the effect of an electric field. Electrokinetic separation techniques have proven to be robust platforms for the rapid analysis, separation, and purification of a wide array of species, ranging from small inorganic ions to macromolecules and even multicellular organisms. This capability of being able to work across multiple size scales makes EK methods attractive for clinical analysis and biomedical assessments, as the same technique can separate tiny extracellular vesicles and probe large mammalian cells [1,2].

A large number of EK-based separations have been developed, many of which are relevant in the fields of biomedical and clinical analysis [3,4]. An essential component in all separation systems, including EK devices, is the detection method, whose performance can be significantly improved if the sample is well-separated prior to detection. Moreover, the detection methodology must be appropriate for the type of application. Handling fragile and labile analytes, which is the case in the clinical and biomedical fields, requires specific detection techniques. Mass spectrometry (MS) is a powerful analysis and proven detection method that has been used in a large number of applications [5]. MS has a rich history, as it was first discovered in electromagnetism studies [6], and some of the first applications of MS occurred in the 1920s [7] when it was used for the determination of atomic weights. In the 1940s, MS was used primarily by physicists, but thanks to the work of Alfred Nier in promoting MS to scientists in other fields, MS soon found its first biological applications [8]. The analysis of small molecules with MS was developed in the 1950s by the petrochemical industry. The next significant steps were the combination of MS with gas chromatography (GC) and later liquid chromatography (LC). Important advances, such as the development of soft ionization techniques, have grown even further in the applications of MS [9].

As the applications of MS grew, its coupling to other separation techniques, besides GC and LC, increased. In the late 1980s, the first two reports on MS combined with capillary electrophoresis (CE) employing electrospray ionization (ESI) were published [10,11]. The hyphenation of MS with CE

enables fast and high-resolution separation systems that offer superior sensitivity and selectivity, especially for limited sample amounts [12]. CE-MS quickly became a powerful tool with applications in metabolomics, proteomics, clinical, biomedical, and pharmaceutical fields [13], allowing the identification of thousands of compounds in a single run [14]. Two of the major benefits of CE-MS systems are the enhancement of sensitivity and selectivity that MS provides to CE [15] when compared to traditional rather low resolution UV/vis, detection and the straightforward determination of the mass-to-charge (*m/z*) ratios of the analytes. The applications of CE-MS have grown moderately as CE keeps gradually gaining popularity as an alternative method to LC in pharmaceutical, proteomics, and metabolomics analyses. CE-MS systems are no longer a novelty, however, despite their potential, CE-MS systems have not completely taken off yet. As stated by Vesterberg [16], it takes time, sometimes several decades for new techniques and systems to be fully developed and accepted by the general community. In the future, we anticipate an increase in the utilization of CE-MS systems, as well as their widespread acceptance within the scientific community.

2 Basics of capillary zone electrophoresis and mass spectrometry

2.1 Electrophoresis

Electrophoretic separations rely on differences in electromigration velocity of the analytes. Most CE systems employ fused silica capillaries with a net negative surface charge in the background electrolyte pH range from ~4-12, resulting in bulk electroosmotic (EO) flow from the positive to the negative electrode. **Figure 1a** illustrates the essential components of a CE system: capillary, two buffer reservoirs, two electrodes, a power supply, and a detector [17]. After sample introduction (hydrodynamic or electrokinetic), individual analytes electromigrate through the capillary with velocities proportional to their electrophoretic mobilities and the applied electric field. **Figure 1b** contains a cartoon of electrophoretic separation, depicting the sample before and after it is resolved, as well as a representation of the signal recorded by the detector. Traditionally, CE systems have been coupled with UV/vis detection, which has a much lower resolution than MS. The coupling of CE with MS creates a much more powerful analytical system with higher resolution. /The representation in **Figure 1b** is for positively charged analytes and negatively charged analytes with electrophoretic mobilities (μ_{EP}) that are lower in magnitude than the electroosmotic mobility (μ_{EO}).

2.2 Mass spectrometry

MS is an accurate characterization technique based on mass and fragmentation patterns that is exceptionally powerful when combined with an efficient separation technique [18]. MS identifies analytes by their *m/z* and can be defined as an apparatus that measures the mass of molecules by first converting them into ions [19,20]. Rapid instrumental developments during the past decades allowed MS to become a standard user-friendly technology available in many analytical and clinical laboratories. **Figure 1c** depicts the essential components of an MS system: an ionization source, a mass analyzer, and a detector. For MS analysis, the sample must be first ionized, *i.e.*, part of the sample must

be converted into ions which are then sent to the mass analyzer and subsequently to the detector.

The ionization method in MS is determined by the type of sample being analyzed [21], the four main ionization methods are describe below and listed in **Figure 1c**. ESI, being the softest ionization technique [22], is the preferred method used for online characterization of organic ionic molecules. ESI is very gentle method that permits large bio-macromolecular complexes to be ionized intact [23]. The development of electrospray ionization (ESI) was crucial for the success of CE-MS since it can form multiply charged ions from larger biological molecules, e.g., proteins and peptides. Such ions can be analyzed on standard mass spectrometers with limited m/z range, such as quadrupole and ion trap instruments. Furthermore, the small scale of CE allows the use of interfaces with sub microliter/min flow rates which achieve the best ionization efficiency [23–25]. In ESI, ions are generated by flowing the sample through a small capillary under a high applied voltage, creating an aerosol of electrically charged droplets of analyte and solvent. Biomolecules, such as oligonucleotides, peptides and proteins, which have acid/base functionalities, often have several protonation or deprotonation sites; which leads to multiple peaks in the mass spectra as depicted in **Figure 1d** (left) for the protein cytochrome *c*. One shortcomings of ESI are that ion formation can be hindered by high salt concentrations (above ~1 mM) and that it requires sample to be continuously flowing, which leads to sample waste. Atmospheric-pressure chemical ionization (APCI) employs a corona discharge that produces both positive and negative ions once the analytes reach the discharge region. APCI has the advantages over ESI of being less susceptible to interferences from salts and the capability of ionizing weakly polar analytes [23]. The two other ion sources employed with MS systems are matrix-assisted laser desorption/ionization (MALDI) and inductively coupled plasma (ICP). MALDI employs a matrix that is co-crystalized along with sample by a laser, the sample and the matrix are usually mixed prior to the exposure to the laser, to allow the matrix to evenly coat the sample. In MALDI, ions are formed in a discrete manner as the sample is irradiated with the laser, this allows synchronizing the mass analysis with the ion formation, preventing sample waste and allowing for high sensitivity levels, in the order of sub-femtomoles of sample. Another attractive and unique advantage of MALDI is that the ionization process generally occurs by the addition of a single proton, this means that a single peak is obtained in the mass spectra, as shown in **Figure 1d** (right) for the protein cytochrome *c* [23]. While the coupling of CE with matrix-assisted laser desorption ionization (MALDI) can be demonstrated, it is not routinely used in practice [26,27]. ICP is a hard ionization method where the sample is completely atomized by employing radiofrequency (RF) magnetic fields. ICP is used for the analysis of trace elements, such as metallodrugs assays.

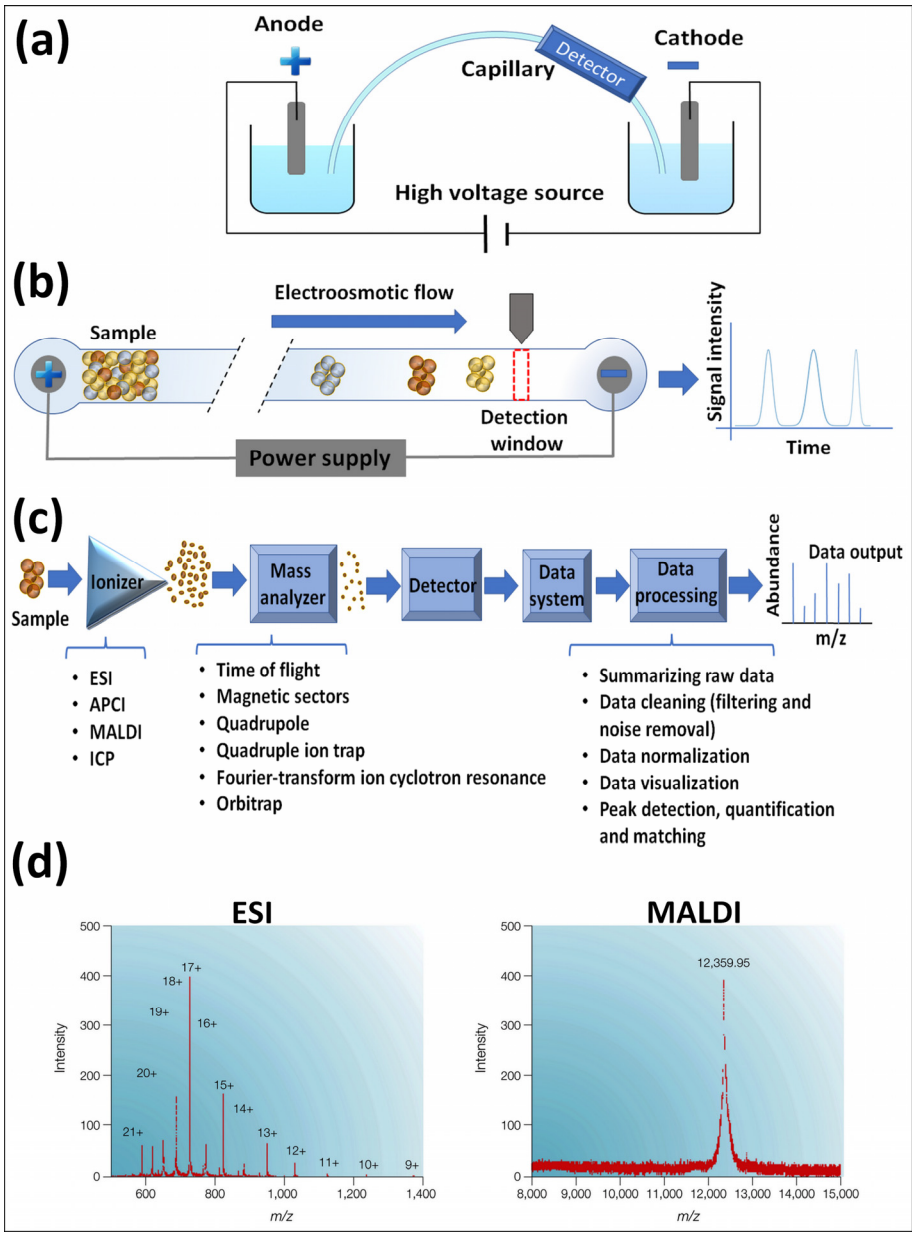

Figure 1. (a) Illustration of a CE system, depicting the basic components. **(b)** Representation of an electrophoretic separation. **(c)** Illustration of the basic components of an MS system, listing some examples of ionization techniques and mass analyzer. **(d)** Comparison of the mass spectra for the protein cytochrome *c*, as obtained with ESI (left) and with MALDI (right). As observed, only one peak is obtained with MALDI. Reprinted with permission from [23], copyright 2003, Nature Publishing Group.

2.3 Data processing in CE-MS systems

Similar to LC-MS, data processing and analysis in CE-MS systems is usually carried out employing software provided by the instrumentation manufacturers and designed for various application fields, i.e., the same software packages are used for LC-MS and CE-MS analysis. However, small migration changes in small molecule analysis in CE can lead to misinterpretation of data. Therefore, the development and the use of software tools for aligning successively recorded electropherograms have

become essential in CE-based metabolomics studies. Gonzalez-Ruiz et al. [28] attempted to solve the migration time shifts with a software tool that utilizes electrophoretic mobility as a more robust characteristic for each compound. The tool ROMANCE (RObust Metabolomic Analysis with Normalized CE) is available under a Creative Common CC BY-NC-ND license and can be downloaded at https://epgl.unige.ch/labs/fanal/romance. This software allows converting the time axis into the effective mobility scale, which only depends on the nature of the chosen background electrolyte (BGE) and the temperature. An updated version [29] is able to solve the quantitative data evaluation issue caused by peak shape distortion in the previous version. Furthermore, Sugimoto et al. [30] developed a stand-alone application tool named JDAMP (Java application for Differential Analysis of Metabolite Profiles), which allows users to identify the metabolites that vary between two groups. Additionally, software tools have been developed for noise reduction, baseline correction, and migration time alignment in metabolomics [31,32], including a very recent open-source tool [33]. For glycosylation analysis, the tools GlycReSoft software (Boston University) [34] and SimGlycan software (Premier Biosoft) [35] have recently been reported.

2.4 Tandem mass spectrometry

A regular practice to obtain structural information about the analyte is combining distinct types of mass analyzing methods, allowing for both the analytes and their fragments to be analyzed. This type of arrangement is known as tandem MS, also known as MS/MS or MS^n, where *n* indicates the number of stages of MS performed in the experiment [36]. In these systems, two (or more) MS are coupled together in series to provide added accuracy to the detection, where the first MS serves as a first-stage *m/z* separator, then ions of specific *m/z* are selected. These selected ions, also called parent ions, undergo an energetic dissociation (e.g., by collisions with a neutral gas, electrons, or radiation). The resulting fragments, referred to as product ions, are then analyzed by the second MS, producing a high accuracy result. Since the fragment ions depend on the structure of the original molecule, its structure can often be deciphered.

3 Interfaces for CE-MS systems

Important efforts have been dedicated to the development of online interfaces for CE. A challenging aspect of interfacing CE with ESI-MS is identifying a balance between robustness, sensitivity, and reproducibility [37], as highly sensitive but fragile interfaces will not enable the development of successful commercial applications. The interfaces for CE-MS systems can be classified and sheath flow and sheathless (no liquid); both types been successfully employed and selecting the type of interface depends on the particular application [38]. A sheathless interface is when the CE capillary is directly connected to the ionization source, without the use of liquid sheath flow. The absence of sheath liquid enables high sensitivity and low detection limits, as there is no liquid diluting the sample. Establishing a stable electrical contact is one of the major challenges in sheathless CE-MS interfaces employing ESI for ionization, as the structure used for electrical contact will serve two functions: function as the terminal electrode for the CE separation and as the emitter electrode for the ESI circuit.

Common practices to provide the required electrical contact are to add a conductive metal coating to the CE capillary, insert a wire into CE capillary, split the flow interfaces where part of the flow comes in contact with an external electrode, use capillaries with porous walls, etc. [38]. **Figure 2a** illustrates one the most common configuration of sheathless interfaces, which features a conductive coating applied directly on the emitter tip; which is simply fashioned at the end of the CE capillary by employing etching, pulling of the capillary of other mechanical means. Other novel configuration of sheathless interfaces have been developed, including the use of a porous tip for the exchange of charge, miniature cracks on the capillary wall and a variety of conductive coatings [25,38,39]. The interface based on the porous capillary junction described by Moini et al. [40] has been commercialized by Sciex [41]. It utilizes a short section of the separation capillary etched from the outside to form a thin fused silica glass membrane permeable for the buffer ions. This part of the capillary, close to its ESI tip, is immersed in the buffer electrolyte reservoir for the connection of the separation and ESI voltages. For its commercial availability and excellent sensitivity this interface became very popular for a wide range of applications [42–45]. It is also worth mentioning, that the simplest sheathless CE-MS experiment can be performed using a piece of a sharpened narrow (15 μm ID or less) fused silica capillary. In this case the CZE current is so low (nA region) that only one high voltage power supply can drive the separation and ESI ionization [46].

Sheath-flow interfaces employ sheath liquid which establishes the required electrical contact between the electrode and the background electrolyte enabling both the CE separation and the ESI process. The sheath liquid also aids the process by mixing with the CE effluent, modifying its composition, making it compatible with the ESI and MS detection. The addition of the sheath liquid also provides a much-needed increase to the flow rate as EO flow produces flow rates that are lower of what is required for ESI. Historically, the first interfaces were developed from LC-MS systems, and since CE could not deliver the necessary flow rate, the sheath liquid solved this problem. A large majority of commercially available CE-MS systems use sheath flow interfaces**. Figure 2b** an illustration of a sheath flow interface where the inner tube is the CE capillary, and the outer tube is for the sheath liquid. Another common sheath-flow configuration is a liquid junction interface shown in **Figure 2c**, where the CE and electrospray capillaries are separated by a gap within the sheath liquid reservoir, enabling the use of capillaries of distinct diameters. Employing liquid junction interfaces allows for independent optimization of the CE separation and the ESI process [37,47]. A number of different mechanical designs for the liquid junction have been developed since its introduction [18]. These include, e.g., the tapered glass or metal capillary tips into which the CE capillary is inserted [48,49], the two-capillary nanoflow interface by Höcker et al. [39] or a microfabricated liquid junction interface with compatible capillaries of different diameters and lengths [37,47]. MS is a popular detection methods employes in microchip electrophoresis, since it offers better resolving capabilities and sensitivity than electrochemical detection, in particular in proteomics analysis. [50]. For more information on both, sheath flow and sheathless interfaces, the reader is referred to an excellent review by Maxwell and Chen [38].

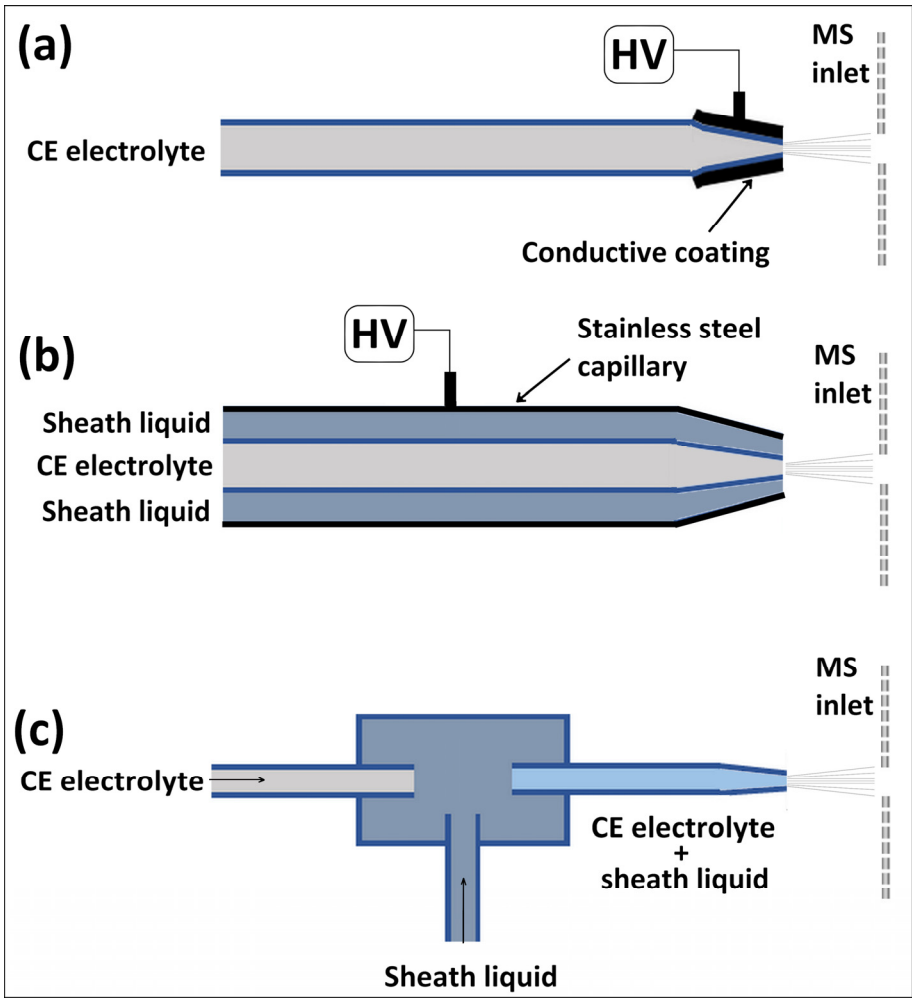

Figure 2. Illustration of CE-Ms interfaces. **(a)** Sheathless (no liquid) interface with a conductive coating applied directly on the emitter tip to provide electrical contact. **(b)** Sheath flow interface where the inner tube carries the CE electrolyte, and the outer tube carries the sheath flow. **(C) S**heath flow liquid junction interface featuring a reservoir for the mixing of the sheath liquid and the CE electrolyte [38].

The first combination of MS with capillary zone electrophoresis (CZE) was reported in 1987 [10]. The following year system was further improved [11]. **Figure 3a-3b** illustrates a schematic of the CZE-MS apparatus and ESI interface. The first CE-MS interfaces reports can be classified as sheathless since no sheath liquid was involved. Instead, they featured a metalized end of the capillary, serving as both the cathode and the ESI needle [10]. The improved system reported in 1988 [11] featured an additional silver layer for improved mechanical strength (**Fig. 3b**). The silver layer provided the electrical contact between the buffer and the cathode with a defined voltage for the ESI in the range of 3 to 5 kV.

The use of metal sheaths at the capillary outlet, while allowing the first demonstration of CE-MS, also had limitations on the composition of the background electrolyte and required very high EO flows to maintain stable electrospray. Thus, there was a need to develop sheath flow interfaces, where employing a flowing sheath liquid provided the required flow rate increase, offering a solution to these

limitations at the cost of reduced sensitivity since the CE effluent was diluted by the sheath liquid [38]. In summary, sheath flow interfaces, with their increased flow rate are the answer in many applications, but this is not a general solution, as some applications may require low flow for improved sensitivity as it was the case of the interface developed by Moini [40].

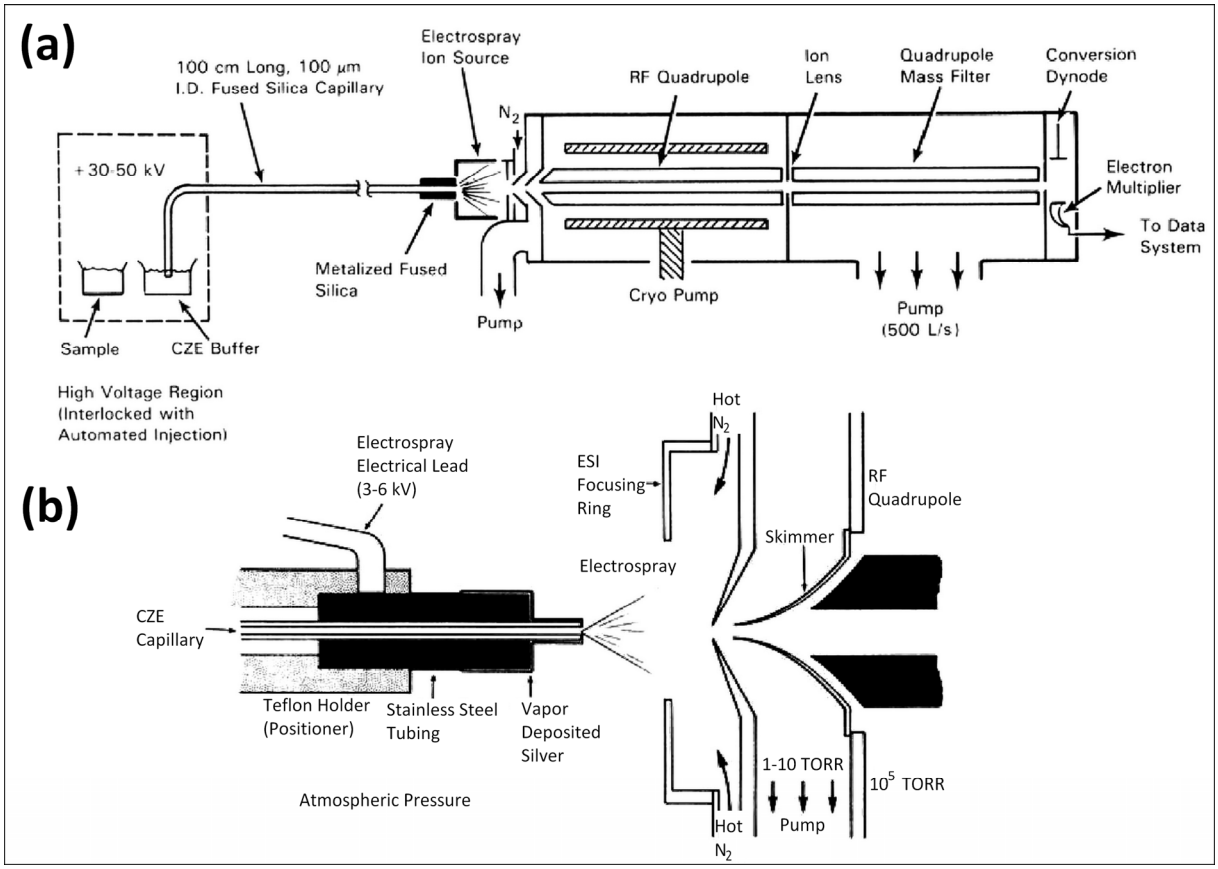

Figure 3. (a) Illustration of the CZE-ESI-MS systems reported by Smith et al. in 1988. **(b)** Detailed schematics of the ESI interface of the system featuring a stainless-steel sheath coated with a layer of silver. Adapted with permission from [11], copyright (1988), American Chemical Society.

4 Applications of CE-MS systems

This section presents some of the most common CE modes including CZE, capillary isoelectric focusing (CIEF) and capillary isotachophoresis (ITP) that have been successfully hyphenated with MS for the analysis of biologically relevant analytes present in trace amounts [25,51]. The main advantage of CE over LC is that CE requires a much smaller sample volume, making CE more appropriate in limited sample applications. Furthermore, as an added benefit of CE over LC is the fact the CE capillaries can be much easily regenerated and restored after contamination than LC columns [52].

4.1 Protein and peptides analysis

The use of MS in proteomics is essential for understanding the mechanisms of disease development and cellular processes. There are three main approaches in MS-based protein analysis: bottom-up

proteomics, top-down proteomics, and native proteomics [53]. Bottom-up proteomics is a peptide-centric approach where proteins are enzymatically cleaved prior to analysis. Top-down proteomics is a gentler approach where proteins are maintained intact; this proteoforms-centric approach is the strategy of choice for the study of proteoforms, which are the different structures of a protein product of a single gene. Characterization of proteoforms by their migration behavior, in terms of their μ_{EP}, has been proposed to strengthen identification (IDs) results [54]. Native proteomics, which characterizes endogenous proteins under physiological conditions, is a protein complex-centric approach. These three distinct approaches allow for multi-level proteomics characterization, where CZE-MS is an alternative method to traditional LC-MS systems [53].

Single-cell analysis is perhaps the area where the combination of CE-MS and proteomics has provided the greatest advancements in the assessment of complex biological samples; CE allows for extremely small sample volumes while MS provides the much-needed high sensitivity detection [12]. This strategic combination that strengthens both the separation and the detection aspects has made possible advances in the area of complex proteome analysis. For example, the Dovichi group reported in 2014 [55] a single shot CZE-MS/MS system that identified 10,000 peptides and 2,100 proteins from the HeLa proteome; this system also identified larger peptides than reversed-phase LC (RPLC). The authors highlighted that the ability to predict CZE migration times [54] strongly increases the confidence in peptide identification.

In 2018, McCool et al. [56] reported the identification of 5,700 proteoforms and 850 proteins from the *Escherichia coli* cell proteome by employing deep top-down proteomics with a CZE-MS/MS system. The cell lysate, first treated with size exclusion chromatography (SEC) and RPLC, produced 20 fractions that were analyzed with CZE-MS/MS. This study was the largest bacterial top-down proteomics report at the time, which included a remarkable increase in the number of proteoforms IDs. These improvements were attributed to 1) the high peak capacity of their SEC-RPLC-CZE separation system, and 2) the high sample loading capacity provided by the dynamic pH junction employed with the CZE separation that allowed focusing the sample. The single CZE-MS/MS analysis of the 20 fractions produced 200-500 proteoforms IDs each, where proteoforms with a mass over 30 kDa were identified, illustrating the potential of this platform for large protein identification. The authors stated that the use of longer capillaries and higher separation voltages during the CZE step could improve performance.

Choi et al. [57] reported another tandem MS strategy for ultrasensitive analysis by developing a HRMS data-dependent acquisition (DDA) system for analyzing protein digest (~500 pg) from mouse neuron cells. The use of HRMS is essential when handling volume-limited samples, such as single-neuron proteomics. The small-scale aspect of CE, which can be considered a disadvantage in other applications, is perfectly suited for volume-limited conditions while providing excellent sensitivity, speed, and simplicity. **Figure 4a** illustrates the iterative DDA procedure and system used for CE-HRMS proteomics of neural mouse cells [57], featuring sheath flow nano-ESI and an orbitrap tandem MS. The objective of this work was to achieve the analysis of a limited sample of 10 neurons. The DDA methodology allowed identifying 428 proteins, of which 415 were also quantified. Effective separation

was achieved with a migration window of 15-30 min in a 90-cm long capillary operated at 23 kV. This report illustrates that CE-HRMS systems are suitable for neuro-proteomics studies with sensitivity close to the single-cell equivalent. Furthermore, this development addresses the technological gap by providing a high sensitivity method able to analyze thousands of distinct molecules, which is ~200-1000 higher than the capacity of nano-LC systems.

Another recent ultra-sensitive CE-MS system for limited cell samples was reported by Johnson et al. [52] employing a bottom-up proteomics approach. They analyzed protein digest samples in the pg range from HeLa cells, demonstrating higher protein and peptide identification than that obtained in similar CE-MS/MS systems. This system was benchmarked against two RP-nano-LC-MS/MS methods (bead packed and monolith LC-MS/MS), employing 8.8-10 ng of protein digest sample. The results illustrated CE and LC are complementary techniques, and both are needed for the complete analysis of cell samples in the low-nanogram range. **Figure 4b(i)** compares the separation and identification results obtained with three distinct methods, where a higher number of proteins and peptides were identified with the two LC systems. However, the sets of proteins and peptide identified by each method are not identical, i.e., each method identified distinct sets of proteins and peptide, with some proteins and peptides in common; but more importantly, each method identified unique proteins and peptides. The system's performance was excellent, with a 60-min migration window in a 150 μm ID, 90-cm long capillary. **Figures 4b(ii-iii)** show the results as Venn diagrams for proteins and peptides, respectively, where is observed the overlap and differences between the three separations methods in terms of the proteins and peptides identified. **Figure 4b(ii)** shows that the CE-MS/MS system identified 139 proteins (4% of the total) that were not identified with the LC-based systems. The results in terms of peptides are more remarkable (**Fig. 4b(iii)**), as CE-MS/MS was able to identify 5,142 (21% of the total) peptides that were not identified by the two LC methods [52]. This study aimed to identify post-translational modifications (PMTs) as they are essential in the understanding of the biological function of cells, for example, phosphorylation, a form of PMT plays a critical role in the regulation of gene expression by signaling transductions pathways. It is challenging to detect PMTs, as the target modified peptides are usually present in low abundance. Two important biological PMTs are O-phosphorylation and protein N-terminal acetylation. CE-MS/MS was far superior to the two LC-based methods in terms of identification of these important PMTs, where 53% of the total identified phosphopeptides and 42% of the total identified N-acetylated peptides were *only* identified with CE-MS/MS. Although in quantitative terms, CE underperformed both LC systems, this work demonstrates that CE is an orthogonal method to LC systems for bottom-up proteomics of limited cell samples. Employing both techniques, CE and LC in a complementary manner, brings unique capabilities which are needed for complete cell proteomic profiling [52].

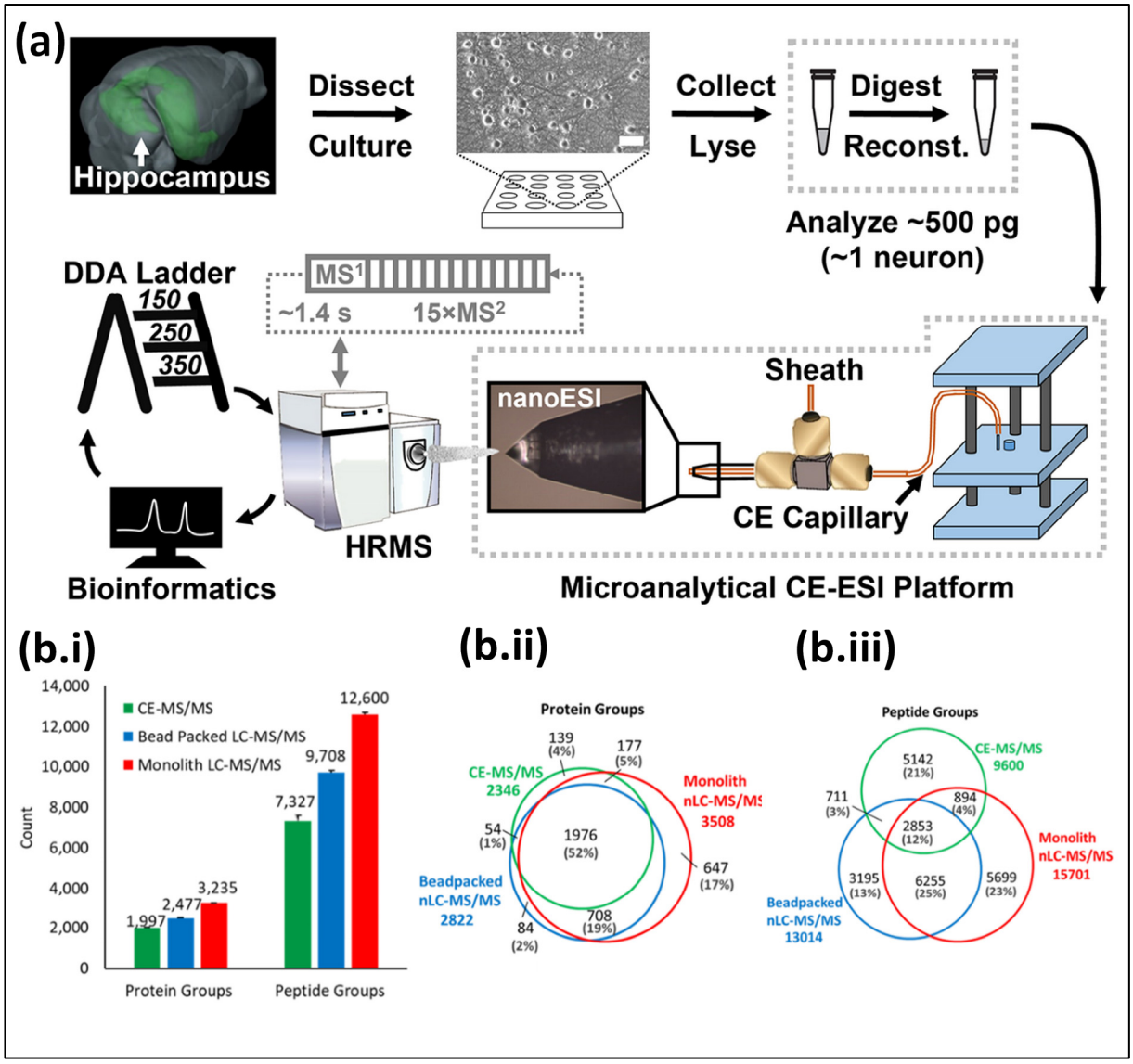

Figure 4. (a) Illustration of the iterative DDA process and system used by Choi et al. for ultrasensitive bottom-up proteomics of mouse neural cells. The scale bar of the culture image is 50 µm. Reprinted with permission from [57], copyright 2021 American Chemical Society. **(b)** Comparison of the separation and identification results of proteins and peptide groups obtained with CE-MS/MS, bead packed LC-MS/MS, and monolith LC-MS/MS. (b.i) Plot of the total count of identified protein and peptide groups, (b.ii)Venn diagram of protein groups, and (b.iii) Venn diagram of peptide groups. A sample of 10 ng of protein digest was used for each one of the two LC-based methods, while 8.8 ng of sample was employed for the CE-based analysis. Reprinted with permission from [52], copyright 2022 American Chemical Society.

4.2 Monoclonal antibody analysis

Monoclonal antibodies (mAbs), are becoming increasingly important in the treatment of many diseases, including cancer and autoimmune disorders. They consist of mixtures of proteoforms where the assessment of PTMs is essential in their differentiation. CE-MS systems are a complementary analytical approach (relying on net charge differences) to LC-MS methods for the comprehensive characterization

of mAbs, as required by drug approval and safety protocols [45]. Belov et al. employed a combination of intact and middle-down methodologies with CZE-MS for the comprehensive analysis of mAbs. Intact analysis allowed determining molecular mass and structural information while middle-down analysis, performed using digestions with ideS protease, allowed detecting and identifying low-abundance PMTs. Some of the PMTs investigated were glycoxidation and deamidation. Middle-down analysis featured the separation by charge employing capillaries with a positive coating that reduced EO flow, operated under reversed polarity, and allowed identification of deamidated species. For the analysis of glycosylated structures, the strategic combination of middle-down (**Fig. 4a**) and intact analysis (**Fig. 4b**) provided comprehensive results. **Figure 5a(i)** shows the deconvoluted mass spectra of ideS-digested mAbs, a ~24 kDa Fc/2 fragment and a ~98 kDa F(ab')$_2$ fragment. The deconvolution, a process to enhance poorly resolved peaks, allowed identifying multiple forms of both types of fragments. **Figure a(ii)** illustrates 16 distinct forms (15 glycoforms and one aglycosylated) for the ~24 kDa fragment Fc/2. The most abundant species was glycoforms G0F with a 14% abundance, with other glycoforms varying abundances between 8-4%. **Figure 5a(iii)** shows one aglycosylated form with an abundance of 40%, and the two distinct glycations forms found for fragment F(ab')2, with abundances of ~7% and ~1%, respectively. The large fragment size hindered the identification of other PMTs, to achieve full proteoforms characterization, it is necessary further decrease the size of the 98.4 kDa F(ab')2 fragment. The results obtained from the analysis of intact mAbs are included in **Figure 5b,** showing three distinct groups of mAb glycans, two glycoforms (6.5 and 81.5% abundance) and one aglycosylated (12% abundance). The degree of glycosylation of mAbs of particular importance as it is linked to mAbs stability, as mAbs with lower degree of glycosylation tend to exhibit higher degradation. This report highlighted the importance of combining middle down and intact approaches for the comprehensive characterization of heterogenous mAbs samples [45]. middle down analysis revealed individual glycosylation states of the fragments, however, since an intact mAb may be composed of asymmetric glycans, an intact analysis is required for the full characterization of glycosylated structures of the mAb.

Haselberg et al. [44] reported a similar CE-MS study on the characterization of PMTs of mAbs by combining intact and middle-up approaches. A low-flow sheathless CE-MS system with a near-zero EO flow (by employing a neutral coating) was used, allowing the successful separation and identification between deamidated isomeric products. Furthermore, middle-up analysis of digested mAbs enabled successful glycoform separation, where non-glycosylated fragments exhibited a higher μ_{EP} than glycosidated ones, probably because of their smaller hydrodynamic volume. The applicability of the proposed CE-MS system was evaluated with digested samples of the mAbs trastuzumab and infliximab, where the migration behavior of the Fc/2 and F(ab)$_2$ fragments was analyzed and related to the pI differences which directly impacted migration time. The results illustrated the presence of low abundant species of glycoforms that have not been reported in similar LC-MS middle-up analysis; further supporting the importance of CE-MS approaches as orthogonal strategies to LC-MS.

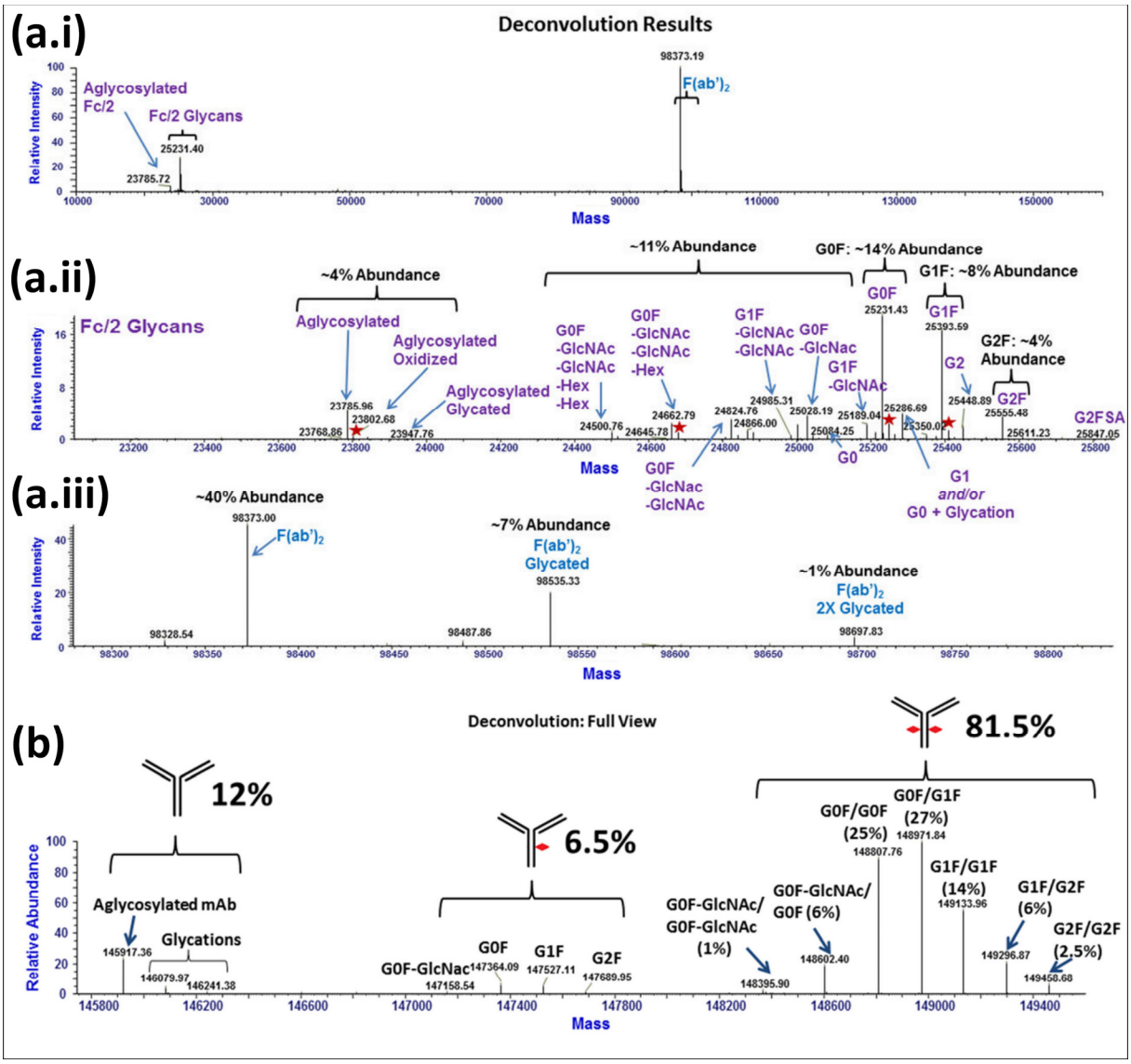

Figure 5. Analysis of mAbs with CZE-MS employing middle-down and intact analysis. **(a)** Analysis of mAbs fragments obtained with digestion with ideS protease. (a.i) Mass spectrum showing the 24 kDa Fc/2 fragment and the ~98 kDa F(ab')2 fragment. (a.ii) Zoomed-in illustration of the mass spectrum region of the 24 kDa Fc/2 fragment, depicting the 16 forms (15 glycosylated and one aglycosylated) with their respective approximate abundances. The red stars represent the several oxidized proteoforms. (a.iii) Zoomed-in illustration of the mass spectrum region of the 98 kDa F(ab')2 fragment, depicting the three forms (two glycosylated and one aglycosylated) with their respective approximate abundances. (b) Mass spectrum of the intact mAbs showing three groups, one aglycosylated form, and two glycosylated forms. The combination of middle-down and intact analysis strategies allowed for complete characterization of mAbs Adapted with permission from [45], copyright 2018 John Wiley and Sons.

Combining separation and analytical strategies is a powerful trend in the comprehensive characterization of mAbs. Shen et al. [58] combined two distinct EK techniques to analyze the antibodies SigmaMab and NISTmAb. They characterized the PTMs of mAbs with CZE, as CZE keeps gaining popularity in quality control of mAbs. In their study, they underscored how low sample loading capacity can represent a major challenge in native CZE separations of large protein molecules, such as

mAbs. Native CZE is the use of native environments for carrying out the separation, avoiding the destruction of the native conformation and noncovalent interactions of the proteins. To handle this limitation, they proposed the use of online CIEF, with a narrow pH range of 6-9, for sample stacking prior to the native CZE separation. The CIEF-focused sample was separated with CZE in 50 μm ID, 70-cm-long capillaries, coated with carbohydrate polymer. The system included ESI and a quadrupole TOF mass spectrometer, which was carefully fine-tuned to handle the native conditions. The results illustrated that CIEF-assisted CZE-MS allowed for high-quality characterization of glycoforms and mAbs variants, which was not achieved with normal native CZE, demonstrating the importance of sample stacking prior to native CZE separations. Another recent study that also employed CIEF was reported by Dai et al. [59] for the characterization of intact mAbs variants; this study aimed to develop a CIEF-MS system that could perform similarly to CIEF-UV systems. They analyzed four commercially available mAbs (bevacizumab, trastuzumab, infliximab, and cetuximab) employing a CIEF-MS system that featured a nanospray shield with a drying gas diverter (replacing the ESI component) followed by TOF mass analyzer. The results showed that CIEF-MS is a suitable approach for the intact analysis of charge variants of mAbs, obtaining similar results to those obtained with CIEF-UV. This work was extended to the characterization of complex mAb charge variants by employing fragments of cetuximab [60]. The studies reviewed above illustrate that CE-MS systems can achieve comprehensive mAbs characterization by strategically combining approaches. CE-MS are still considered newcomers to the clinical laboratory, but their unique capabilities complement LC-MS approaches, making them a powerful analytical tool.

4.3 Drug analysis

An area of drug analysis where hyphenated techniques involving MS are becoming increasingly essential is glycosaminoglycans analysis. These glycans are polydisperse anionic polysaccharides with significant electrical charge and are therefore suitable for CE-based separations. By employing CE, glycans are simply separated by their migration times in an open capillary; there is not a stationary phase that can be clogged or require extra steps to release the analyte after binding to the stationary phase, as needed in some LC modes [61–63]. CE offers higher resolution than LC for glycan analysis, since it acts on both, the shape and the charge of the molecule, differentiating analytes by mass but also isomers and enantiomers [64]. Heparin, an anticoagulant prescription drug, is a pharmacologically important glycosaminoglycan. LC-MS approaches are the current methods of choice for the analysis of glycosaminoglycans; however, there is great potential in CE-based methods for becoming a major player in the analysis of heparins; if proper CE-MS interfaces are developed [65]. The Linhardt group has made major contributions toward the development of CE-MS interfaces for the analysis of glycans; since as they have discussed, that the lack of suitable and reliable interfaces has hindered the acceptance of CE in the clinical laboratory and pharmaceutical industry [61–65]. Recently [62], they achieved the analysis of heparin oligosaccharides with CE-MS, employing negative-ion ESI and a linear trap quadrupole (LTQ) orbitrap mass analyzer. They explained that the disadvantages of employing LC for oligosaccharides separations are an incomplete resolution and incompatibility with ESI (due to LC solvent), or the need for oligosaccharide derivatization. An illustration of their CE-MS system is

included in **Figure 6a**, including a zoomed-in representation of the capillary end, and the forces acting on the analytes. A 50 μm ID, 50-cm-long negatively charged bare silica capillary was employed. The separation was carried under reversed polarity mode since the electrophoretic force (shown as EF in **Fig. 6a**) exerted over the highly charged analytes was dominant over EO; allowing the analytes to migrate down the capillary. The capillary end was capped with a sheath interface that was coated with a protein layer, that served as a spray emitter. The sheath liquid was pumped electrokinetically through EO flow and mixed with the CE effluent resulting in stable electrospray. This novel configuration allowed a successful separation of eight heparin and heparin sulfate disaccharides, in less than 30 min at –30 kV, with good resolution and complete identification of the *m/z* ratios; demonstrating that reversed polarity mode CE-MS is suitable for the analysis of glycosaminoglycans [64]. These results were an improvement over the separation obtained under normal polarity CE-MS [65]. Two of the recent studies [61,64] by this group demonstrated reversed-polarity CZE-MS systems for glycosaminoglycans analysis. Furthermore, the system illustrated in **Figure 6a** was also used for the analysis of Arixtra® and Lovenox®, two commercially available blood thinners. For the former, which is highly charged and challenging to analyze with MS due to loss of sulfo groups; the results illustrated that Arixtra® migrated as an intact molecular ion, allowing for sequence characterization. For Lovenox®, which was analyzed using a bottom-up approach that produced low molecular weight building blocks, the separation was achieved in 20 min identifying 17 distinct building blocks. These reports illustrate that analysis of highly charged glycosaminoglycans is an area where CE has perhaps greater potential than LC; where the development of suitable interfaces, and strategic approaches (use of reversed polarity CE), are essential for the analysis of these important drugs.

4.4 Metabolite analysis

Metabolomics refers to the study of low-molecular weight analytes present in cells, biofluids, and tissues. In single-cell metabolomics, CE-MS systems are gaining importance, as the small-scale of CE combined with the high sensitivity of MS, are excellently suited for characterizing limited cell samples in the nL-μL range. Analysis of metabolites from cells is essential in the assessment of the physiological and health status of organisms [12]. Novel developments in CE-MS systems, as described by Ramautar et al. [66,67] review articles series; have rendered CE-MS as a mature approach for analyzing charged and polar metabolites. Numerous reports in the literature illustrate the metabolomic profiling of a wide array of samples, from body to organ tissue, and single cells [68].

A single-neuron cell metabolomics study was reported by the Sweedler group [69]; as the motivation for this work, the authors explained that handling small analyte amounts is a major challenge in metabolomics, since this limits both, the choice of approaches to employ and the depth of metabolome coverage. Mammalian cells require limits of detection in the atto to zeptomole range to analyze the tiny amounts of metabolites contained in a small cell volume of ~ 500 fL. Two common approaches for optimizing CE-MS protocols are improving the detection limit by modifying the CE-MS interface and minimizing sample dilution during sample preparation. However, as only small sample volumes are injected in CE systems, this work focused on tackling this challenge by performing online sample concentration, ensuring that the limited injected sample contained a sufficient amount of

metabolites. The employed field amplified sample injection (FASI), a sample stacking approach that can produce enrichment up to 1000-fold. **Figure 6b** illustrates a schematic of the FASI CE-ESI-MS system employed for metabolomics of single neuron cells of *A. californica*, a sea slug commonly used as model organism in neuroscience. The bottom inset depicts the FASI process which was essential to improve the LOD of this single-cell CE-ESI-MS analysis. By employing FASI, a sensitivity enhancement from 100 to 300-fold was observed. After FASI, the samples were separated with CE employing 40 μm ID, 80-cm-long fused silica capillaries at 20 kV; all analytes had a migration time <60 min. A sheath flow interface ESI system was employed followed by a high-resolution quadrupole TOF mass analyzer, allowing the detection and identification and detection of 37 metabolites from pleural sensory neuron single-cell analysis. Selected metabolites were quantified, including tyramine and dopamine, two important transmitters and neuromodulators; as well as arginine and citrulline, which are functionally important metabolites. The authors discussed that better analyte fragmentation and the use of a sheathless interface are required to enhance identification confidence; and that FASI may not be suitable for all metabolites of interest. Despite these limitations, the custom system, featuring a sample stacking step, has potential for the analysis of other types of cells, and is a major step forward in the single-cell analysis field.

Another study featuring a sample pre-concentration was published by Oedit et al. [70], they employed an online two-phase electroextraction (EE) step prior to CE-ESI-MS analysis of metabolites, to diminish the number of analytes that fall below the limit of detection (LOD). EE consists of the extraction of the analytes from an organic phase onto an aqueous phase under the effects of an electric field. The online integration of the EE step required fine-tuning of the ESI sprayer tip to avoid chemical corrosion which affects spray performance. The system's performance was characterized by employing nine model metabolites, illustrating LODs enhancements of 50 to 250-fold compared to conventional CE-MS systems. Additional tests with a volume-limited urine sample resulted in the detection of 122 putative metabolites. This report is another example that CE-MS systems that combine several approaches, such as sample stacking, can handle challenging samples in metabolomics analysis.

4.5 Body fluids analysis

The marriage between CE and MS has produced numerus powerful systems that combine the excellent separation resolution of CE with the sensitivity of MS, making CE-MS systems attractive for the analysis of a wide array of samples, including body fluids. Urine samples analysis is a major area for CE-MS systems, as CE enables analysis of negative, neutral, and positive analytes directly in aqueous media, without the need for organic solvents (as required by LC). Urine analysis is highly relevant for diagnostics and clinical studies as it provides a means of assessing the intricate metabolic processes that take place in the human body. For an overview of this area, the reader is referred to an excellent review article by Hrušková et al. [71].

There are numerous reports on the analysis of urine samples employing CE techniques coupled with MS. Pascual-Caro et al. [72] used solid-phase extraction (SPE) followed by CE-MS/MS for the enantiodetermination of amphetamine-type stimulants in urine samples. They focused on evaluating and comparing the six distinct chiral selectors employing the countercurrent migration technique

(CMT), which is when the chosen chiral selector migrates in opposite direction to the EO flow (the choice of the chiral selector is critical in CE-MS systems). Two distinct enantiomers of four stimulants compounds that were present at low concentration levels were successfully determined. Another recent enantiodetermination was reported by Pérez-Alcaraz [73] employing a SPE-CE-MS approach for the analysis of R,S-3,4-methylenedioxypyrovalerone , a major ingredient in illegal synthetic stimulants. They explained that a major challenge in drug abuse detection is that after producing their stimulant effects, these compounds are present and excreted from the body at low concentrations in body fluids, requiring high sensitivity methods for their determination. By employing SPE to enable in-line sample concentration with spiked urine samples, they successfully determined R,S-3,4-methylenedioxypyrovalerone in urine samples with a LOD of only 10 ng/mL and good separation reproducibility. These reports illustrate the effectiveness of CE-MS systems in toxicology, clinical and forensic drug determination analysis in urine samples.

CE techniques have made an impact are the determination of metabolites in body fluids. Piestansky et al. [74] determined serotonin in urine samples employing a clever combination of capillary ITP and CZE followed by MS/MS. Chen et al. [75] reported another study that also employed ITP by developing of a thread-based ITP-desorption-ESI-MS method to analyze model alkaloid compounds in urine, their system included sample clean up, separation, and preconcentration. The thread approach consists of adding a nylon thread, with a knot, to the system over which the ITP migration and separation of the analytes takes place. This novel thread-based- ITP-desorption-ESI-MS approach enabled the analysis of three alkaloid compounds from real urine samples with an 11-fold increase in sensitivity compared to the direct desorption ESI-MS approach, illustrating once more that the CE approaches are an excellent sample preparation step prior to MS analysis. The coupling of CE with MS has also been employed to analyze other body fluids, Marie et al. [76] used CZE-ESI-MS to determine N-glycans at femtomole level blood-derived immunoglobulin G, plasma, and extracellular vesicle isolates. The results showed an increase of 100-fold for some glycans enabling qualitative and quantitative N-glycan profiling; illustrating that CE-MS approaches are suitable for the determination of key biomarkers from complex physiological fluids. All of the studies discussed in **section 4** of this review article illustrate the wide range of applications of CE-MS systems. **Table 1** presents a summary of these applications.

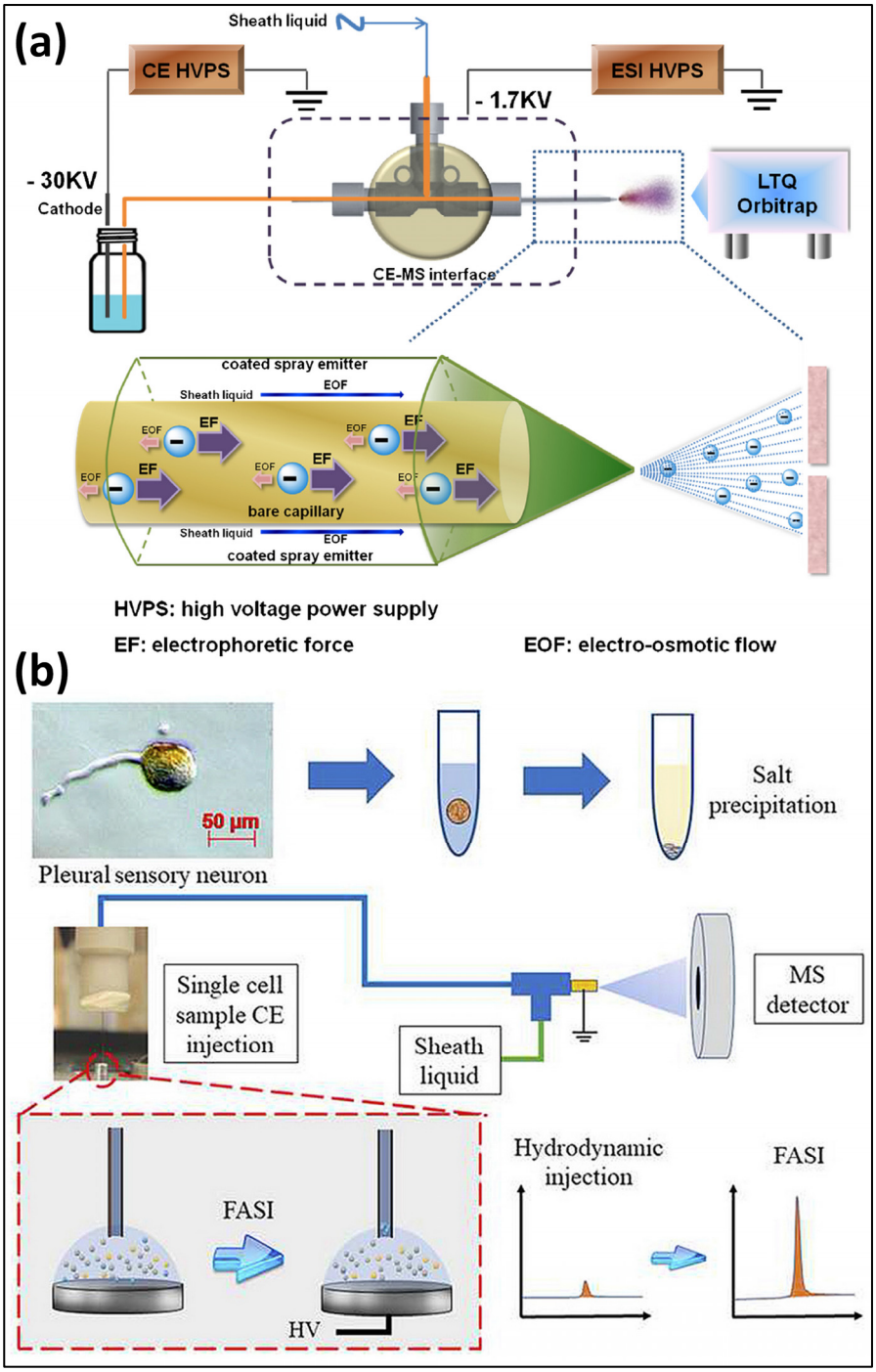

Figure 6. (a) CE-MS system employed by the Linhardt group for the analysis of heparin oligosaccharides employing negative-ion ESI and a linear trap quadrupole (LTQ) orbitrap mass analyzer. In the image EF means electrophoretic force. Reprinted with permission from [62], copyright 2017 Springer Nature. **(b)** Schematic representation of the single-cell analytical process employed by the Sweedler group. The image depicts the pleural sensory neuron cell sample preparation process, the FASI-CE-MS system. The bottom two insets show an illustration of the FASI process and a cartoon comparison between the signal obtained with standard hydrodynamic injection vs. FASI. Reprinted with permission from [69], copyright 2020 Elsevier.

550 **Table 1.** Summary of the applications of CE-MS systems discussed in this article.

Type of system	Target analyte	Description	Ref.
Proteins and peptides			
CZE-MS/MS	Peptides and proteins	Identified 10,000 peptides and 2,100 proteins.	[55]
CZE-MS/MS	Proteoforms and proteins	Identified 5,700 proteoforms and 850 proteins from *E. coli* cell proteome.	[56]
CE-HRMS	Protein digest	Analysis of limited sample of protein digest equivalent to 10 neural mouse cells.	[57]
CE-MS/MS	Peptides and proteins	Analysis of sample of proteins in the low nanogram range, achieved the identification of 130 proteins groups and 5,142 peptide groups.	[52]
Monoclonal antibodies			
CZE-MS	Monoclonal antibodies	Combination of middle down and intact approaches for the comprehensive characterization of PMTs of heterogenous mAbs samples.	[45]
CE-MS	Monoclonal antibodies	Characterization of PMTs of mAbs combining middle down and intact approaches.	[44]
CZE-MS and CIEF-assisted CZE-MS	SigmaMab and NISTmAb monoclonal antibodies	Characterized the PTMs of mAbs employing two distinct EK techniques, CIEF was used for sample stacking prior to CZE-MS.	[58]
CIEF-MS	Monoclonal antibodies	Characterization of intact variants of commercially available mAbs (bevacizumab, trastuzumab, infliximab, and cetuximab).	[59] [60]
Drug analysis			
CE-MS	Heparin oligosaccharides	Analysis of heparin oligosaccharides with negative-ion ESI and orbitrap mass analyzer, achieved the separation of eight heparin and heparin sulfate disaccharides.	[62]
CZE-MS	Glycosaminoglycans	Reversed-polarity CZE-MS systems for the analysis of commercially available glycosaminoglycans (Arixtra® and Lovenox®).	[61,64]
Metabolites			
CE-MS with FASI sample stacking	Single-cell metabolites	Single-neuron cell metabolomics was achieved with FASI. Identified metabolites tyramine, dopamine, arginine and citrulline.	[69]
CE-ESI-MS with prior two-phase electroextraction	Putative metabolites	Employed an extraction method prior to CE-ESI-MS analysis. The system allowed for the detection of 122 putative metabolites.	[70]
Body fluids analysis			
CE-MS/MS with prior solid-phase extraction	Amphetamine-type stimulants in urine	Enantiodetermination of amphetamine-type stimulants in urine samples. Achieved the detection of two distinct enantiomers of four stimulants compounds.	[72]
CE-MS with prior solid-phase extraction	Synthetic stimulants ins piked urine samples	Detection of R,S-3,4-methylenedioxypyrovalerone in spiked urine samples. The use of SPE enabled the detection of low concentration target analytes with LOD of only 10 ng/mL	[73]
ITP-CZE-MS/MS	Serotonin in urine samples	Combined capillary ITP with CZE followed by Ms/MS to detect serotonin in urine samples.	[74]
Thread-based ITP-desorption-ESI-MS	Alkaloid compounds in urine	Integrated system that included sample prep (clean-up, separation and preconcentration) to analyze alkaloid compounds in urine with an 11-fold sensitivity increase.	[75]
CZE-ESI-MS	N-glycans in three distinct samples	Determination of N-glycans at the femtomole level in 3 types of samples: blood-derived immunoglobulin G, plasma, and extracellular vesicle isolates. Achieved up to 100-fold increase in concentration for some glycans enabling further analysis.	[76]

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5 Concluding remarks and future outlook

The present review article is intended as a primer for scientists already familiar with separations sciences like chromatography and electrophoresis that are interested in learning about the capabilities of coupling CE with MS. Traditionally, CE systems employ UV/vis detection, which has a much lower resolution than MS. The objective of the present review article is to underscore the analytical potential of combining CE separations with MS detection. The article describes the history of CE-MS systems, the fundamentals and working principles of both techniques, including a discussion on CE-MS interfaces and a description of applications. A plethora of recent innovations have significantly expanded the applications of CE-MS systems. Although CE-MS systems are not a novelty, these powerful systems are not yet fully accepted nor widely used in the clinical and pharmaceutical laboratory. As it was the case with gel electrophoresis, it can take up to several decades for new techniques to reach their full maturity and be completely accepted [16]. The high separation resolution and short analysis time make CE-MS an attractive analytical technique to be added to the repertoire of any analytical laboratory. CE is orthogonal to LC, and employing both approaches allows a comprehensive and complete characterization of target analytes. CE does offer specific advantages over LC, its small scale, for example, makes CE highly suitable for assessments where sample volume is limited, such as single-cell analysis as discussed in **section 4.1** for proteins analysis and in **section 4.4** for metabolite analysis. Furthermore, CE analysis takes place in open capillaries, thus, there is no stationary phase that could be clogged or need regeneration, and sample preparation is much simpler.

Recent major innovations in the development of robust yet sensitive CE-MS interfaces should enable the growth of more commercial applications, which in the past have been impeded by the lack of reliable interfaces. Furthermore, as miniaturized versions of CE systems are already available, the miniaturization of the mass spectrometer (currently being investigated by NASA [63]), will enable the development of lab on chip CE-MS portable systems We expect an expansion of CE-MS systems in the future, particularly upon the widespread availability of robust commercial interfaces. Researchers and chemists in the areas of clinical chemistry, biotechnology, and pharmaceutical analysis will discover the value of CE analysis as a valuable sample preparation step prior to MS analysis. As scientists become more familiar with CE-MS systems, they will realize the potential of these systems for applications that are simply not possible with LC-MS, which should open the possibility for CE-MS systems to finally become routine techniques in clinical and pharmaceutical laboratories.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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