12
Trends in CE-MS and Applications

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12.1
Introduction

After the invention of soft ionization techniques, mass spectrometry (MS), once a domain of inorganic chemistry and small molecules, became an important tool for analysis of biomolecules. Especially the protein researchers were waiting for an effective nondestructive ionization for a long time. Although the first promising results were obtained with fast atom bombardment (FAB) and laser desorption ionization (LDI), the revolution came in the 1980s with the development of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) [1].

The use of MS for bioanalytical purposes was further enhanced by the development of interfaces suitable for coupling of mass spectrometry to separation techniques either in online or in offline modes. Such combined techniques not only provide a lot of information about complex samples but also improve detection limits due to reduction of the ion suppression effect, often plaguing the analysis of complex mixtures.

Liquid chromatography (HPLC) and capillary electrophoresis (CE) belong among the most frequently used separation techniques. For online coupling with MS, the majority of applications use capillary zone electrophoretic (CZE) mode since most of other CE modes, including electrokinetic chromatography (EKC), SDS-gel protein electrophoresis or isoelectric focusing (IEF), limit the ESI-MS sensitivity by high content of nonvolatile additives resulting in ion suppression effects and adduct/cluster formation [2–4]. Since the separation mechanisms of CE and HPLC are quite different, the analysis data are complementary and running both can significantly improve the amount of information obtained for complex samples [5–9]. Despite the fact that CE provides instrumental simplicity with high resolution, short analysis time, and very low sample consumption, HPLC is often considered to be a mature technology with better robustness and reproducibility compared to CE.

A number of instruments including mass spectrometry interfaces are available from several well-established manufacturers. Although the first successful

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CE-MS interfacing has already been demonstrated in the late 1980s [10], the CE systems have been mostly operated with UV or fluorescence detectors and the MS coupling was largely a research domain. This is now changing since both the major CE manufacturers (AB Sciex, Framingham, MA, USA – formerly Beckman Coulter – and Agilent Technologies, Santa Clara, CA, USA) have introduced commercial systems for CE-ESI-MS. While most ionic substances can be separated and analyzed by CE, it is expected that the main applications of CE-MS will include especially analyses of intact proteins and protein complexes and other samples difficult to analyze by HPLC.

12.2 Ionization Sources for CE-MS

While electrospray is the universal technology for online coupling of liquid phase separations with mass spectrometry, there are other possibilities for specific applications. Investigation of metalloproteins by CE-ICP-MS [11] may serve as an example here. While inductively coupled plasma (ICP) is incompatible with proteins, metal atoms incorporated in the protein structure can undergo ICP ionization and provide information about the specific protein form and abundance [12,13].

Other variations of ionization methods developed mainly for small organic molecules analysis including, for example, direct analysis in real time (DART) applying metastable helium stream [14] or the flowing atmospheric pressure afterglow (FAPA) working with helium plasma [15,16] may eventually find some use for CE-MS coupling. Lower ion suppression effects compared to electrospray have already been observed to reach reasonable sensitivity even for MEKC-MS analyses [14].

The need for cocrystallyzing the sample with a suitable matrix makes online coupling of MALDI with separations very challenging. Most often discrete fractions from separation are collected and placed on the MALDI target. Alternatively, to minimize the loss of the separation resolution, separated zones exiting the separation column can also be deposited in a continuous trace. Several studies have described the development of in-line [17] or online [18–20] instrumentation for CE-MALDI-MS based mostly on the deposition of continuous trace on a moving surface [21]. However, at present MALDI is practically exclusively used only in the offline mode.

Electrospray ionization is based on the flow of a sample solution through a capillary placed in front of the mass spectrometer sampling orifice. The application of a potential drop (usually 1–5 kV) between the ESI capillary tip and the MS sampling orifice results in charging of the droplet formed at the ESI tip. At certain voltage (depending mainly on the solvent viscosity and the electric field strength at the ESI tip), the droplet deforms into a Taylor cone with a stream of small charged droplets released from its tip. The charged droplets evaporate during the flight to the MS sampling orifice leading to further droplet fission and

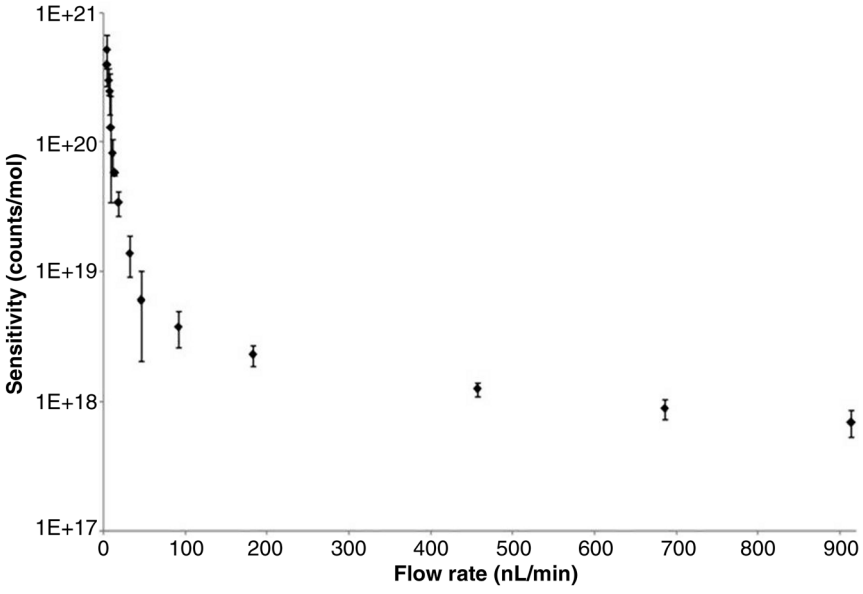

Figure 12.1 Evolution of myoglobin (250 nM) signal sensitivity in logarithmic scale as a function of the infusion flow rate. (Reproduced from Ref. [31].)

ion evaporation. Under optimized conditions, individual ions form before entering the mass spectrometer analyzer. To increase the electric field intensity, the ESI capillary is often sharpened into a tip. The most common procedures of the tip fabrication include grinding [22,23], etching [24], or flame pulling [25,26]. The main aim of fabrication is a highly symmetrical tip; however, beveled tips have also been investigated [27,28]. In contrast to HPLC, where flow rates of 10 µl/min (or more) are common, CE best performs at much lower flows in the sub-µl/min rate. Electrospray run under this condition is often called a nano-(electro)spray. It has been shown experimentally that very low flow rates result in higher ionization efficiency and better sample utilization in terms of mass sensitivity as documented in Figure 12.1 [29–31].

Insulating fine fused silica electrospray tips are most commonly used for nanospray construction. There is a variety of possibilities for the application of the electrospray voltage. In this respect, the interfaces are often divided into two basic groups. In the first arrangement, the CE separation column is inserted into a stainless steel capillary supplied with a conducting liquid, flowing around the outside of the ESI tip. This arrangement, called sheath–flow interface, has been developed first by Smith, Barinaga, and Udseth [32] and allows the use of different buffers for the CE separation and electrospray ionization. The sheath liquid also assures good electric contact for the CE separation current. Often noted disadvantage is the dilution of the CE zones with the sheath liquid. The second design, called sheathless interface, uses the same capillary for the separation and electrospray. Thus a flow inside the separation capillary is needed to

form stable electrospray, and the composition of the separation buffer is selected as a compromise for both the required separation and the electrospray stability. Both approaches have practical strengths and weaknesses as described in several studies [33–37].

12.3 Practical Implementations of ESI-CE-MS Interfaces

12.3.1 Interfaces Implementing Supporting Spray Liquid

12.3.1.1 Sheath–Flow Interfacing

The sheath–flow interfacing is popular for its robustness and commercial availability. In its construction, the sheath liquid (or makeup liquid) establishes the electrical connection for the CE current (Figure 12.2) [38]. Moreover, the chemical composition of the sheath liquid can be optimized with respect to conductivity, pH, viscosity, volatility and surface tension to support sample ionization, droplet formation and solvent evaporation. At higher flow rates (several µl/min), the stability of the interface is often improved by incorporating another coaxial capillary supplying a nebulizer gas. For best results, the sheath liquid and gas flow rates must be carefully optimized [39,40].

Besides creating optimum electrospray conditions, the sheath liquid may also be used for studies of postcolumn reactions of the separated substances, that is, "sheath–flow chemistry". This technique is based on reaction between additive (which is a part of sheath liquid) and analyte eluted from the separation capillary. The reactant is mixed with the analyte within the electrospray plume. Since the reaction time is limited by the time of the droplet flight to the mass orifice, fast reactions can be monitored. Typical examples include hydrogen/deuterium exchange [41,42] or reactions of an antioxidant with a radical giving information about the antioxidant activity [43].

Since its introduction, the sheath–flow interface has been used especially for its robustness in many interesting applications and it is practically the only commonly used interface in industry. The higher robustness resulting from electrospray-friendly (often high percentage of organic solvent) sheath liquid composition helps liquid evaporation and improves analyte ionization efficiency.

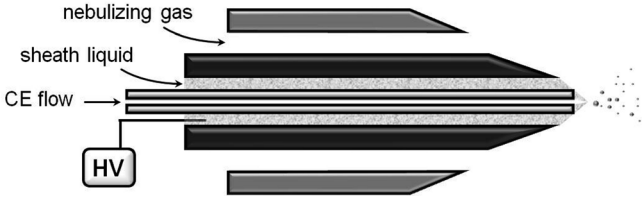

Figure 12.2 Sheath-flow interface for CE-ESI-MS analysis. (Reproduced from Ref. [38].)

However, sheath–flow interfacing has also been criticized for problems inherent to its design.

Since electrospray-MS is considered a concentration-sensitive technique at flow rates above 10 nl/min [44], the most often mentioned disadvantage is the dilution of the separated zones by the sheath liquid. In addition, if not eliminated completely, the sheath liquid impurities can cause ion suppression and higher background noise in the mass spectra leading to poor detection limits. Depending on the flow rate of the sheath liquid, the sample zones exiting the CE separation capillary may be diluted by several orders of magnitude. Thus, it is critical to optimize the sheath liquid/analyte ratio during the experiment as recently documented by Neusüss, Pelzing, and Macht [45] in the example of analysis of protein tryptic digests. With the 75 µm ID separation capillary, the sensitivity of analysis was substantially better at flow rates of 1–2 µl/min compared to the more common 3–10 µl/min. The LOD was further improved by implementing online sample stacking resulting in the sensitivity level approaching that of the sheathless interface.

12.3.1.2 Liquid Junction Interfacing

The main disadvantage of the early sheath–flow interfaces was the relatively large size of the electrospray needle supplying the sheath liquid. Since it had to accommodate the separation capillary, its internal diameter was typically 400–600 µm. While sheath–liquid interfacing operating at flow rates as low as 250 nl/min has been described [46], the typical flow rate, necessary for achieving stable spray, is in the µl/min range. This results in excess dilution of zones occupying nanoliter volumes inside the separation capillary. In addition, a key factor, influencing the ESI ionization, is the electric field strength at the electrospray tip [47], which in turn relates to its diameter. Thus, very narrow pointing tips are preferred for achieving the best ESI sensitivity. At submicroliter per minute flow rates, such an arrangement is commonly termed as nanospray [48]. In CE-MS, interfacing nanospray arrangement can be achieved by mechanical decoupling of the separation and electrospray capillaries using a liquid junction construction [49,50] – see Figure 12.3. Its function is based on a small gap (20–200 µm) between the separation and the electrospray capillaries. This gap is surrounded by spray liquid, providing an electrode for attaching of the electrospray voltage as well as voltage for electrophoretic separation. Both capillaries

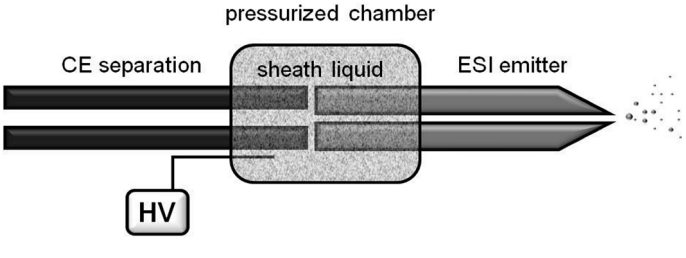

Figure 12.3 Liquid junction interface for CE-MS coupling with nanospray ionization.

can be about the same diameter [51]; however, decreasing the ESI emitter diameter leads to better ionization efficiency [23,52].

The required flow of the spray fluid (typically 10–300 nl/min) can be maintained either by pressurizing the liquid junction reservoir together with the CE capillary inlet (to prevent hydrodynamic flow inside the separation capillary) [23], or by reducing the pressure in the electrospray chamber – subatmospheric interface [50].

While the precise distance between the separation and the electrospray capillaries is not critical, it is recommended to keep the gap dimension between 50 and 200 μm. At the same time, the pressure difference needs to be optimized especially with respect to the diameter and length of the electrospray capillary [53,54]. Once properly optimized, the zone dilution in the liquid junction interface can be 10–100 lower than in the sheath liquid arrangement leading to a better sensitivity. Besides the mechanical arrangement depicted in Figure 12.3, other designs have been described including interface based on partly inserted thin capillary ESI emitter (10 μm ID, 30 μm OD) into the wider separation capillary [55] or the double-junction interface developed for the removal of agents decreasing analyte ionization (e.g., buffer ions or cyclodextrin) [56,57].

In another, liquid junction-based design, "junction-at-the-tip" [58], the electrospray capillary was formed by a beveled stainless steel needle. The instrumentation is based on inserting thin separation capillary (usually OD <150 μm) into a slightly wider emitter with a tapered end. Since the space between the exit of the separation capillary and the electrospray tip is low (on the order of nl), consumption of the sheath liquid and sample dilution is significantly decreased compared to the sheath–flow interface bringing significant improvement of sensitivity. The mixing space is given by dimensions of used capillaries; however, it should be minimized for best results. Both [46,59] or just the electrospray [60] capillaries are tapered. Several arrangements for the ESI-CE high-voltage connection have been described, including metal coating of the CE capillary [60], stainless steel ESI capillary [61], or the platinum electrode immersed into the sheath liquid reservoir [62,63].

Further decrease of the sheath liquid flow rate (100–500 nl/min) was achieved by thinning the emitter tip (2–10 μm) and flow generation by electroosmotic action on the glass surface of the emitter. Thus, neither external pumps nor the nebulizer gas was required yet and a very good repeatability was achieved [64,65] allowing full automation [66] and multiplexing [67] for routine analyses. A general scheme of the "junction-at-the-tip" instrumentation is shown in Figure 12.4.

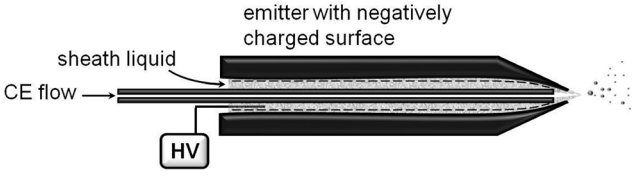

Figure 12.4 Electrokinetically pumped sheath-flow nanoelectrospray interface.

12.3.2 Interfaces without Additional Liquid

Both the sheath–flow and the liquid junction interfacing provides universal means for coupling CE to electrospray ionization, where the separation is decoupled from the ESI process. This means that bare fused silica CE capillaries operating with strong electroosmotic or hydrodynamic flows as well as capillaries coated with neutral polymers preventing the electroosmotic flow during the separation can be used. The liquid flow necessary for the electrospray stability is supplied independently under the flow conditions inside the separation capillary. In addition, the composition of the spray fluid can differ from the separation buffer allowing further optimization of the ESI conditions after the CE separation. On the other hand, especially when not properly optimized, both the sheath liquid and the liquid junction interfaces can cause sample zone dilution leading to decreased sensitivity.

Sheathless CE-MS interfacing works without addition of a spray fluid into the zones exiting the separation capillary. In this case, the fluid flow necessary for obtaining a stable electrospray has to be delivered through the separation capillary, that is, the separation proceeds in a flowing liquid. Either the electroosmotic flow or the pressure-driven flow can be employed. Since both the separation and the ESI conditions have to be optimized at the same time, the range of optimum conditions with respect to the separation/electrospray buffer composition, separation voltage, and flow rate is limited. However, the separated zones exit the CE capillary without being diluted by additional spray fluid.

The absence of the sheath liquid or liquid junction for connection of the separation/spray voltage resulted in the introduction of several designs of electrical connections at the ESI tip. In the first design, a conductive emitter was attached to the exit of the CE capillary [5,26]. Later designs included nonconductive emitters attached to the separation capillary through metallic [68], dialysis [69], or ionophore tubing [70]; however, the connections often resulted in extracolumn peak broadening. This problem was eliminated by using conductive coating at the CE capillary exit terminating it directly by electrospray without any externally connected components. The original metal [71,72] and graphite [73] coatings are being gradually replaced by polymeric ones [74] due to their better operational stability.

In another approach, a thin Pt wire was inserted through a small hole close to the capillary terminus acting as an in-capillary electrode [75]. Nevertheless, this design is not being widely used.

One of the practically promising designs includes the use of a porous segment close to the separation capillary exit as introduced in 2003 by Moini (Figure 12.5) [76]. The porous part was created by etching of the outer capillary by hydrofluoric acid. Once the fused silica thickness approaches few micrometers, it becomes permeable for small ions allowing electrical connection without liquid flow. Porous segment is situated in a metallic sleeve filled with static conductive liquid held at spraying voltage via an external metal electrode [77].

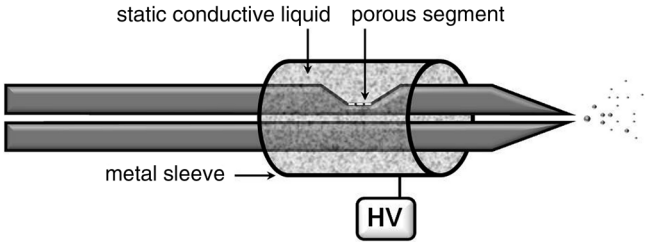

Figure 12.5 Schematic representation of the split-flow interface. (Reproduced from Ref. [76].)

This concept has been commercialized by Beckman Coulter Company and is often referred to as CESI–MS (sheathless capillary electrophoresis–mass spectrometry) [31]. There is a growing number of applications showing the performance of the interface in metabolomics [78,79], proteomics [30,80], or glycomics [81]. Direct comparison of the analysis of intact proteins with sheath–flow and sheathless interface with porous tip [82] showed up to 140-fold better sensitivity of the sheathless design. The difference was attributed mainly to the significant difference in flow rates – 2 µl/min in the sheath–flow versus 140 nl/min in the sheathless interface. In the following study, the picomolar-range detection limit in intact protein glycoforms analysis was reported at the flow rate of only 5 nl/min [80]. This improvement in sensitivity was attributed to increased ionization efficiency in the nanospray as discussed earlier.

In the latest experiments, flow rates in the pl/min range were tested using 2 µm ID capillary for electrophoretic separation [83] using adjustable porous tip as shown in Figure 12.6. This design enabled electrokinetic injection even if positive coating on the capillary wall is used. Positive (or neutral coating) is often used to avoid absorption of proteins and peptides on the capillary wall. To prevent electrolyte loss at the ESI tip during the electrokinetic injection, the porous tip was immersed in a grounded metallic sleeve filled with background electrolyte during sample loading. After injection, the ESI tip was pulled out to perform

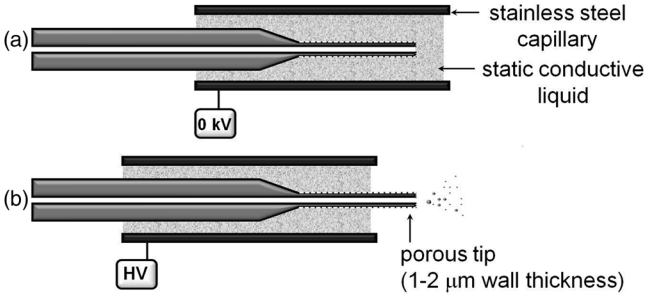

Figure 12.6 Adjustable porous tip. (a) The porous tip in the injection mode, positioned inside the sheath metal. (b) The porous tip in the CE-ESI-MS mode, positioned outside the sheath metal. (Reproduced from Ref. [83].)

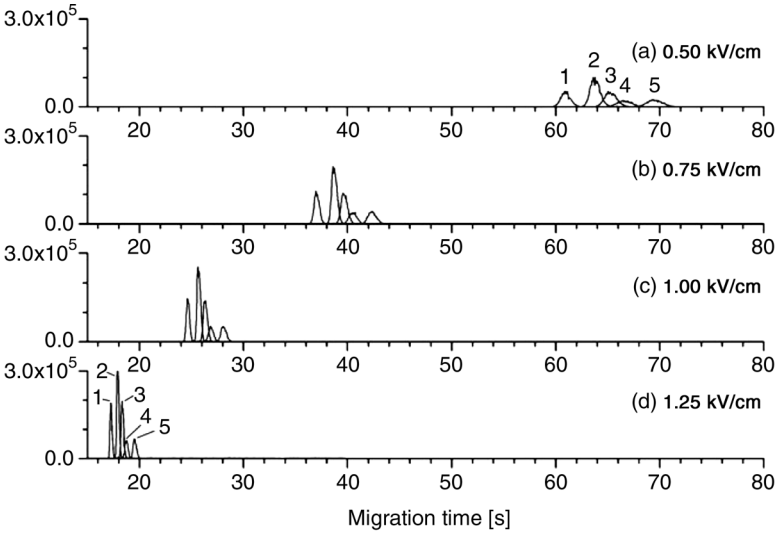

Figure 12.7 Separations at different field strengths. 1, histidine; 2, dopamine; 3, norepinephrine; 4, epinephrine; 5, isoproterenol. (Reproduced with permission from Ref. [85].)

the CE-MS analysis. The process of loading and spraying can be easily automated, and with the high separation electric field strength (>1000 V/cm), it was shown to be very fast and sensitive way of CE-MS analysis.

Capillary electrophoretic separations at high field strength (up to 2 kV/cm) [84] allow very fast analyses, often in less than a minute (Figure 12.7). While such high electric fields typically lead to excessive Joule heating and cooling of the separation capillary becomes vital with common separation capillaries of 75 μm internal diameter, the usage of thin capillaries minimizes this problem and gives more freedom in the background electrolyte selection [83,85]. In addition, narrow capillaries have very low total volume resulting in minimal sample consumption and low flow rate compatible with the nanospray regime [86]. Moreover, if pressure-assisted capillary electrophoresis was used, thin capillaries reduce contribution of the hydrodynamic flow band broadening leading to higher separation efficiency [39,87].

As the diameter of the CE capillary decreases so does the electric current during the separation (e.g., from 15 μA in a 75 μm capillary to 67 nA in a 5 μm capillary). At certain capillary diameter (depending also on the conductivity of the background electrolyte), the electrophoretic current may be as low as the current of the electrospray ionization. Thus, in principle, the ESI current could be used not only for the ionization but also for driving the separation. While the potential drop due to the passage of the electrospray current is small in larger capillaries (e.g., 50 or 75 μm ID) [88,89], it is sufficiently high (especially when using low conductivity background electrolyte) in narrow capillaries (<15 μm ID). Since the resistance of the CE separation capillary is indirectly proportional to

the square of its diameter at certain size, the separation current will be the same as the electrospray current. In such a case, the CE column could directly spray the separated zones at its outlet without the need of any additional power supply [90]. The main advantage of this approach is the instrumental simplicity; however, more work on the optimization of the experimental conditions is still needed.

12.4 Applications

Capillary electrophoresis is at present considered to be a niche technique complementing chromatography; nevertheless, the importance of the CE is increasing with the introduction of the commercial instrumentation. Indeed, CE has been shown to be a very powerful tool for analysis of ionic samples ranging from small molecules and amino acids [91] to protein complexes and lysates of whole cells [92–94] up to extensive clinical studies [95–98]. An important limitation of CE is its lower loading capacity compared to HPLC. While this is often mentioned as a drawback, this may be an advantage in cases when only a limited amount of sample is available for analysis. In addition, several in-capillary preconcentration procedures exist for improving the concentration-based limit of detection in CE [99]. Besides the most common stacking and transient isotachophoresis (tITP) protocols that are used most frequently, solid-phase microextraction (SPE) [37,100,101] or the use of molecularly imprinted polymers [102] was also tested. An example of the online SPE preconcentration setup, practically identical to the setups previously described for use with UV and fluorescence detectors [103], is given in Figure 12.8. Here the SPE microcartidge body is connected to the CE separation [101,104]. Using porous C_{18} particles larger than the separation channel (<50 μm) enables fritless approach leading to lower band broadening [100]. Peptides are eluted from sorbent by suitable organic phase (usually ACN) containing background electrolyte. This setup is typically operated in a reusable mode.

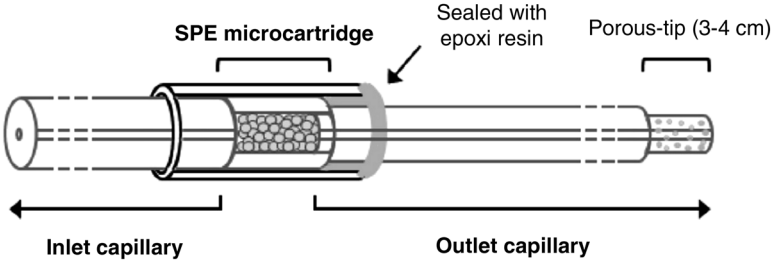

Figure 12.8 Scheme of the SPE microcartridge construction for CE-ESI-MS preconcentration. (Reproduced with permission from Ref. [100].)

12.4.1 Proteins and Peptides

The analysis of proteins and peptides is the main domain of proteomics. While most separations on the peptide level are performed using liquid chromatography, there is a renewed interest in CE-MS instrumentation for proteomic applications [105], especially for the analysis of intact proteins and protein complexes.

Electrospray ionization is characteristic of the formation of multiple charged ions. While peptides, ionized in cationic mode, typically carry only one–three charges (protons), proteins tend to be charged many times more, and the so-called protein envelope is observed in mass spectra [106]. Although the distribution of the signal between several peaks may reduce MS sensitivity, it enables the detection of large proteins with masses much higher than a common mass analyzer could detect as singly charged ions. Moreover, most current mass spectrometers provide better resolution for lower m/z values. For these reasons, there were attempts to influence protein envelope not only by pH but also by additives into the sheath liquid influencing protein ionization. Such so-called supercharging agents [107] include, for example, nitrobenzyl alcohols or sulfolane causing slight denaturation of the protein structure and formation of higher charge states (lower m/z).

While cationic ESI-MS is generally considered to be more sensitive compared to the negative ionization mode, positively charged proteins tend to adsorb onto the wall of the separation capillary. This problem can be (partially) suppressed either by using low pH separation electrolytes (reduction of the ionization of silanol groups on the capillary wall) or by coating the capillary wall with positively charged or neutral hydrophilic coatings. Besides covalently bound coatings [108], dynamic coatings are also frequently used and prepared by simple flushing a cationic polymer solution through the capillary [109–111].

12.4.1.1 Peptides

Peptides represent one of the most important group of analytes being studied by CE-MS. While a broad diversity of samples containing peptides and peptide-like substances include, for example, peptides from soil [112], opiates [100,113], or peptide hormones [114], the main aim of CE-MS peptide analysis is in protein characterization. Since the analysis of intact proteins is challenging due to high molecular mass and a variety of post-translational modifications (PTM), many different protocols for their analysis have been developed. Two main approaches, labeled as the bottom-up [115] or top-down [116] techniques, have been under development since the early 2000s. In the top-down approach, the intact protein is fragmented inside a mass spectrometer and its structure deciphered from the fragmentation information. In the bottom-up approach, the protein is first enzymatically cleaved to peptides (e.g., by trypsin) and the resulting peptide mixture is analyzed by separation MS in the following step. The protein identification is done from measured m/z peptide values by database searching or by using additional MS fragmentation for peptide sequencing. Often two-dimensional

techniques (most often combination of ion exchange and reverse-phase chromatography) are applied for high-resolution separation prior to the MS analysis [117,118]. The use of CE in this field is also evolving as it provides different mode of separation.

An example of the bottom-up approach using CE-MS for the analysis of the *E. coli* lysate [119] is in shown Figure 12.9. The miniaturized electrokinetically driven sheath–flow interface [63] was used with extremely low sample consumption (only 25 nl was loaded). In the first step, all proteins from the *E. coli* lysate

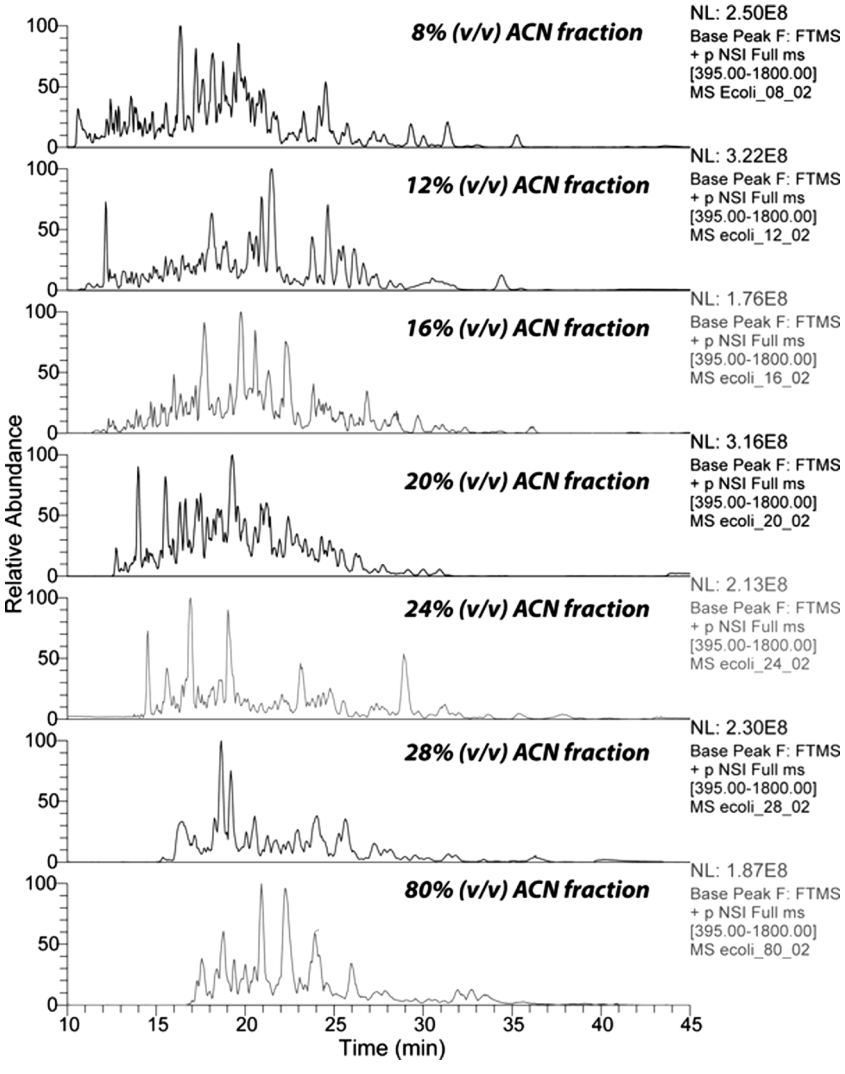

Figure 12.9 Base peak electropherograms of seven fractions of *E. coli* digests analyzed by CE-ESI-MS/MS. (Reproduced with permission from Ref. [119].)

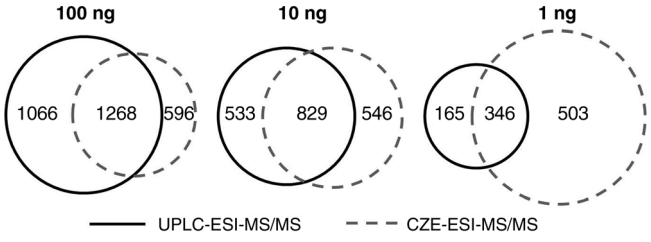

Figure 12.10 Overlap of peptide and protein groups from CE-ESI-MS/MS and UPLC-ESI-MS/MS analysis of 1–100 ng *E. coli* digests. (Reproduced from Ref. [122].)

were digested by trypsin and separated into seven fractions using SPE C_{18} column. The following elution was performed by using a series of acetonitrile solutions. Each fraction underwent CE-MS analysis with MS/MS fragmentation. By searching the MASCOT [120] database, 810 protein groups and nearly 5000 peptides were identified from the 350 min long analysis.

This technique was later modified to improve the speed of analysis by substituting the SPE C_{18} step by a pH-mediated preconcentration, where the sample was injected between two zones of an acidic buffer [121].

As already noted, one advantage of the CE separation is the capability to handle very small sample amounts. This was demonstrated in a study comparing the number of identified proteins in the *E. coli* lysate digest obtained with chromatography and electrophoretic separations – CE-MS and UPLC–MS [122]. While the liquid chromatography-based protocol enabled identification of a higher number of proteins when sufficient amount of sample was available, capillary electrophoresis provided better results with higher reproducibility for limited sample amounts (Figure 12.10).

Combining the powers of chromatography and electrophoresis may bring new quality to the current, mainly chromatography-based, analytical protocols [123–125]. A comprehensive analysis of proteins and peptides in blood serum [126] may serve as an example. Here the high-abundance proteins were first removed by methanol precipitation. The supernatant was then digested by trypsin in the following step and the resulting complex peptide mixture was separated by RPLC into 96 fractions, which were further analyzed with CE-MS. Although the offline collection of fractions may appear time-consuming, the CE provided fast analyses with high resolution, making the analysis time comparable to the common 2D chromatography systems.

12.4.1.2 Post-Translational Modifications of Proteins

Capillary electrophoresis provides very sensitive and selective analysis of intact proteins that is used especially in biopharmaceutics [80,127] and biotechnology [128,129]. Moreover, electrospray provides a very soft ionization enabling analyses of intact protein–protein complexes or complexes of proteins with other species [94,130]. In addition, the ESI-MS is also preferred for investigation

of post-translational modifications (PTM) of intact protein. From approximately 200 types of known PTM, glycosylation and phosphorylation belong to the most frequent ones. Each modification makes identification of the particular protein more complicated. While glycosylation brings in extensive structural variation, phosphorylation often significantly decreases ESI-MS response compared to the nonphosphorylated species.

Glycosylation is known to have crucial influence on protein functionality. Thus, the structural characterization, no matter how challenging it is, is a must in the production of all recombinant proteins used for therapeutic purposes. Besides neutral glycans, many glycosylated proteins also contain sialic acid providing a negative charge at any physiologically relevant pH. Thus, the sialic acid containing glycoproteins are often readily migrating electrophoretically regardless of the ionization of the protein part. This allows estimation of the number of sialic acid residues in the glycoprotein structure, which has been demonstrated for identifications of intact glycoproteins, such as recombinant human erythropoietin [131,132] or intact bovine fetuin [133]. In the example shown in Figure 12.11, there is a mass spectrum of sialylated species bearing negative charge, allowing effective separation from the nonsialylated recombinant human erythropoietin. The online mass spectrometric detection with TOF analyzer provided sufficient accuracy and resolution to identify the glycan composition. The presence of hexoses, fucoses, acetylations, and oxidation could be recognized based on the measured mass shifts, and 44 glycoforms and 135 isoforms were identified. This approach was later further improved by using separation capillaries with positive [131,133] or neutral coatings [80] and mass analyzer with higher resolution [134] and increased ionization efficiency [80].

The second important PTM, phosphorylation, was recently investigated by CE-MS with the use of the sheathless porous interface [30]. As expected at higher ESI flow rates and higher degree of phosphorylation, the observed signal of phosphopeptides was lower. By decreasing the flow rate, the sensitivity improvement was observed (Figure 12.12) with the best results achieved at the lowest used flow rate (15 nl/min) with nearly equimolar response for all analytes. The highest improvement of sensitivity was reached especially for multiphosphorylated peptides. Using tITP-CE-MS strategy in a model sample of trypsin digest of bovine milk, it was demonstrated that significantly better sensitivity can be achieved in comparison to nano-LC–MS, which detected only monophosphorylated peptides.

12.4.2 Glycans

Glycosylation is one of the most frequent and also the most complex PTM observed in proteomics. The protein glycosylation has a number of roles, for example, protein stability, folding, solubility, or protection from enzymatic cleavage. Since glycans are hydrophilic species, they are commonly separated with

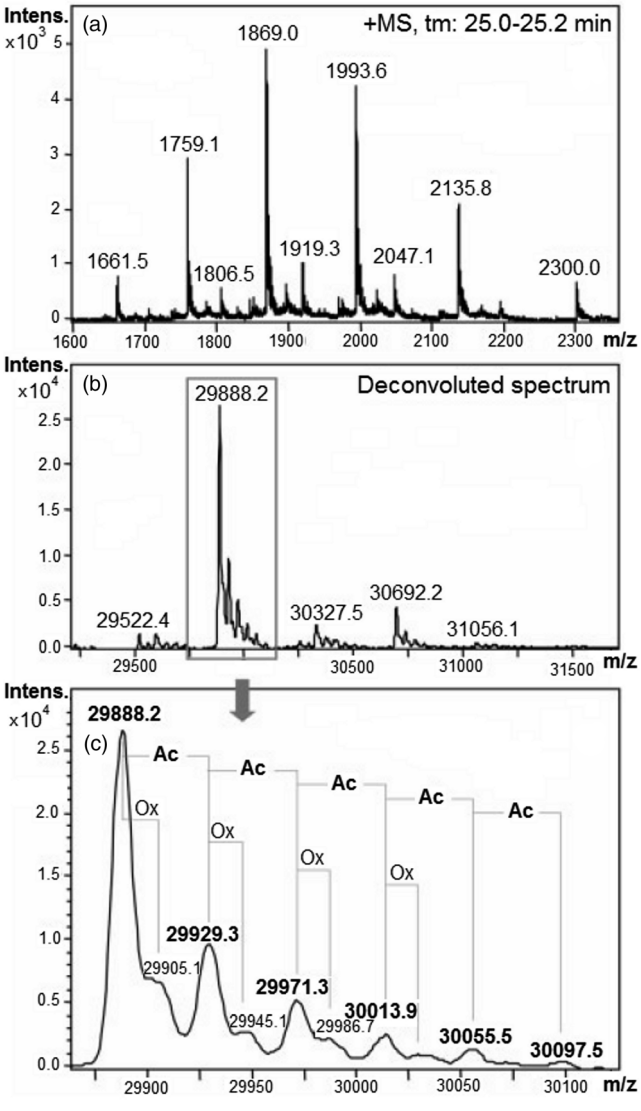

Figure 12.11 Mass spectrum from recombinant human erythropoietin analysis. (a) Mass spectrum from CE separation. (b) Deconvoluted spectrum. (c) Detail of the deconvoluted spectrum (Ac, acetylation; Ox, oxidation). (Reproduced from Ref. [131].)

hydrophilic interaction chromatography (HILIC) or porous graphitized carbon (PGC) LC–MS. Since such analyses may be time-consuming and require larger sample amounts, fast capillary electrophoresis is being investigated for this application as well. Despite the possibility of investigation of glycolysation on intact proteins, as discussed in Section 12.4.1.2, more common procedures include

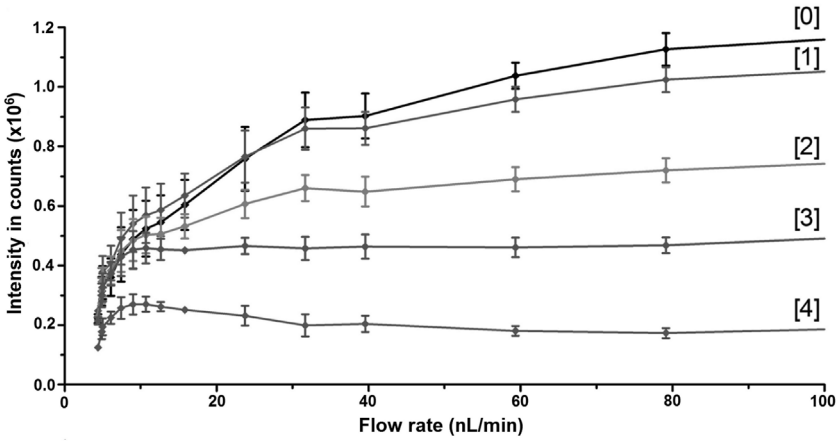

Figure 12.12 Evolution of peak intensity of model phosphopeptides below 100 nl/min: [0] nonphosphorylated [1], monophosphorylated [2], diphosphorylated [3], triphosphorylated, and [4] tetraphosphorylated. (Reproduced from Ref. [30].)

enzymatic release of glycans (e.g., by PNGase F) from proteins in the first step followed by their separation and MS analysis.

Unless the released glycans contain sialic acids in their structure, their native electromigration is possible only under extremely basic conditions (pH > 11), where the hydroxyl groups become ionized [135]. Thus, most of the sample preparation protocols include a labeling step attaching a permanent charge to the glycan molecule. In CE the most popular labeling agent is negatively charged 8-aminopyrene-1,3,6-trisulfonic acid (APTS) providing three negative charges and excellent sensitivity for fluorescence detection with 400–500 nm excitation. Unfortunately, the very sensitive CE with Ar^+ laser (488 nm) induced fluorescence detection can provide only limited information about the glycan structure based usually on different effective size of the glycans. For more detailed information, the CE analysis must be performed either on multiple samples prepared by consecutive digestion with carbohydrate-specific enzymes [136], or using coupling with a MS analyzer of high mass accuracy providing also MS/MS data (usually QTOF). Since APTS agents, sialic acid, and ionized hydroxyl groups have negative charges, the MS analysis has to be performed in negative mode. While positively charged labels might bring higher sensitivity [137], more work will be needed before their practical applications.

Two separation approaches are commonly used for the CE separation of the labeled glycans. In the first approach, a strongly basic background electrolyte with pH ≈ 12 (e.g., aqueous ammonium acetate/ammonia) is used. Under such basic conditions, the strong electroosmotic flow toward MS that carries the negatively charged native glycans (bearing sialic acids, sulfonic acids, or phosphates) as well as APTS-labeled glycans toward the MS interface. The second possibility is the separation in an acidic background electrolyte with pH < 4 (e.g., formic acid). Since the electroosmotic flow is practically negligible at low pH, the

glycans migrate toward the ESI as anions in the order of their electrophoretic mobilities. The repeatability of the acidic and alkaline systems has been compared by Bunz, Rapp, and Neusüss [138]. Under acidic conditions, glycans gave better RSD of migration time and relative peak intensity. On the other hand, in the alkaline system an increased peak height by a factor 3–8 was found. In the same work, CE-MS and CE-LIF analysis was run and was shown that electrospray interfacing does not affect the CE resolution.

As in all fields of bioanalysis, in glycomics also there is a need of improved sensitivity. As shown in previous examples, in ESI-MS the most useful way is decreasing the flow rate of the electrosprayed liquid. For example, the flow-through microvial interface was used for investigation of oligomers containing up to 24 glucose units [139], glycoprofiling of recombinant human erythropoietin [81], and fish serum of Atlantic salmon [140].

12.4.3 Metabolites

The analysis of metabolites (metabolomics, metabonomics) is becoming one of the most important research areas as documented in recent reviews [95,97,141–143]. Many metabolites or sets of metabolites are used as important disease markers in medicine. Metabolic profiling has great potential for diagnosis of changes in organism caused by diseases [144–147], drugs of abuse [52,148,149], alcohol consumption [150], and so on. On the other hand, the plant metabolomes were shown to be influenced by the geographical origin [151] or ripening state [152]. The comprehensive analysis of the metabolites content in biological fluids provides a fingerprint of the current state of an organism. Under specific conditions, some metabolites may increase their abundance or new substances may form as the metabolic pathway changes. While thousands of metabolites are formed in an organism, at present only few of them are useable as disease markers. It is expected that a profile of a set of metabolites will provide better information than a concentration of a single one. Thus, the main challenge is the resolution and identification of as many components in the sample as possible. While identification of a small molecule may appear simpler than in the case of a peptide, it is mostly more complex due to the vast number of chemical species that may have the same mass and elemental composition. Thus, accurate mass information must be supplemented by migration times/ionic state, MS/MS data, and comparison with authentic standards for unequivocal substance identification.

The most important applications relate to medical research. Differential metabolomics study on child diabetes may serve as an example [153]. Collected plasma samples from the patients were analyzed for changes associated with lipidic metabolism, and markers of the differential activity of the gut microflora were identified. At the same time, changes associated with protein and amino acid metabolism were found in urine. Metabolic profiling of urine brilliantly demonstrated the impact of increased ionization efficiency of the sheathless CE-MS system [78]. In this study, the performance of the sheathless interface

with porous emitter was compared with the sheath–flow system in terms of LODs, migration time and peak area repeatability, and response linearity. It was clearly demonstrated that in all selected parameters, the sheathless interface provides better results. For example, 900 molecular features were detected using the sheathless system, while the sheath–flow arrangement could find only 300 distinct compounds (Figure 12.13). In addition, approximately eightfold improvement of sensitivity was observed with the sheathless instrumentation. Similar results have been obtained in another study on metabolic profiling of mouse cerebrospinal fluid [154] or metabolic profiling of zebrafish embryos where the sheath–flow and flow-through microbial-assisted CE-MS was compared [65]. Again, more than threefold increase of sensitivity was demonstrated together with five times improvement of LOD.

Studying metabolic variations between individual organisms is becoming important not only on the level of the whole organism but also on the single cell level. The metabolic variations on the single-cell level have been demonstrated on metabolic investigation of neuron cells of *Aplysia californica* [155]. Over 50 single neuron cells with diameters ranging from 80 to 300 µm were isolated from eight animals. The cells underwent lyses and extraction. A volume of 6 nl from each neuron extract was hydrostatically introduced into the separation capillary. Using a custom-built coaxial sheath–flow ESI source, the

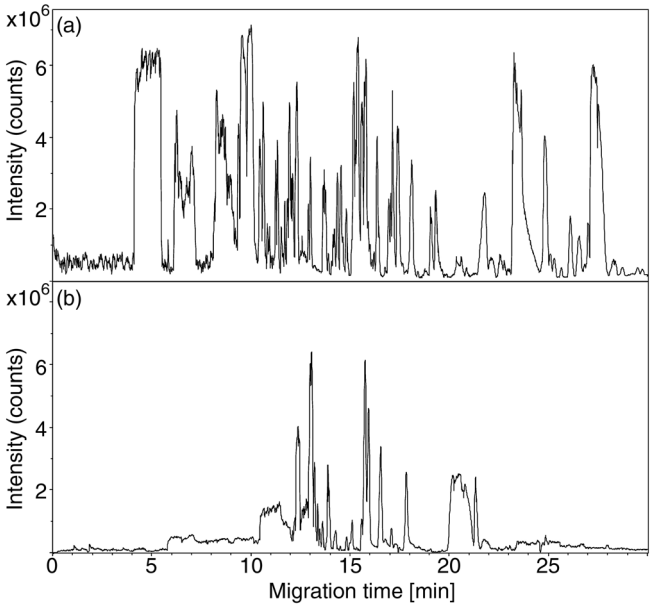

Figure 12.13 Base peak electropherogram (*m/z* 50–450) of human urine obtained with (a) sheathless CE-MS using a porous tip sprayer, (b) CE-MS using a sheath–liquid interface. (Reproduced with permission from Ref. [78].)

MS/MS analysis led to identification of 300 different metabolites from one neuron cell. The obtained data revealed significant differences in metabolite content not only among the cell types but also among neurons of the same type from the same individual.

12.5 Conclusions

Although several interface designs have been proposed in the past two decades, the ideal design, combining sensitivity and robustness, is still waiting for its perfection. Whether the final design will be based on the sheath–flow or sheathless approach is unimportant as long as the performance matches the practical needs. At this moment, both designs have very auspicious representatives. On the side of sheathless interfaces, the most promising results have been achieved by the instrumentation with the porous segment. However, it is quite possible that potential of the sheath–flow arrangement have not been exhausted, yet.

Regardless of the winning setup, the CE-ESI-MS has matured to become a very powerful tool utilized not only in the academic research but also in the industrial environment. The excellent sensitivity and minimal sample consumption of CE has gained an irreplaceable position among analytical separation methods. Moreover, since it is based on a completely different separation mechanism, it represents a very viable complement to most chromatographic techniques.

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