Recent Developments in Capillary Electrophoresis-Mass Spectrometry of Proteins and Peptides

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Many researchers have invested considerable efforts toward improving capillary electrophoresis (CE)-mass spectrometry (MS) systems so they can be applied better to standard analyses. This review highlights the developments in CE-MS of proteins and peptides over the last five years. It includes the developments in interfaces, sample-enrichment techniques, microfabricated devices, and some applications, largely in capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF) and capillary isotachopheresis formats.

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1 Introduction

The last two decades have witnessed the emergence of capillary electrophoresis (CE) as one of the most important tools in analytical science. It provides fast, highly efficient separations with minimum sample volume requirement, which is vital for analytes that are sample-limited. Its combination with mass spectrometry (MS) for detection results in a powerful, high-efficiency, information-rich analytical method, suitable for a wide range of applications, including proteins and peptides. However, its poor concentration sensitivity has precluded its widespread acceptance for routine analyses, especially of biologically-derived analytes, chromatography-based systems still being the methods of choice.

This review highlights selected accomplishments in CE-MS over the last five years that have enhanced its use for protein and peptide analyses (some other recent reviews related to this topic are listed in the Reference section).¹⁻¹⁴ By building on these and earlier efforts, it is anticipated that the popularity of CE-MS will grow, and its advantages will be better appreciated and exploited.

2 Ionization Methods

For interfacing with capillary-based electromigration techniques, the MS ionization modes that have been used

include atmospheric pressure chemical ionization (APCI), inductively coupled plasma (ICP), fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI). For protein and peptide analyses, most CE-MS reports describe combination with ESI¹⁵⁻⁶⁶ (the most common by far), and MALDI.⁶⁷⁻⁸³ Only these two configurations are covered in this review, although, brief descriptions of the other three are given in the succeeding paragraphs.

In ESI, gaseous ions are formed by the application of a strong electric field to a fine spray of their liquid solutions. It is favorable to the generation of multiply charged ions, which enables the analysis of very large molecules even with instruments having a low mass range. By mathematical deconvolution, the multiply charged peaks can be transformed into a singly charged peak for determining the molecular weight of the original species. However, the intricacy of the resulting spectra makes it difficult to analyze highly complex mixtures. The main drawback of ESI, though, is its low tolerance to salts.^{84,85}

In MALDI, the sample cocrystallizes with small organic molecules having strong absorbance in the laser wavelength ("matrix"). A laser beam is directed onto this solid solution, causing vaporization of the matrix material and desorption of the ions. The process is independent of the absorption properties and of the size of the compound to be analyzed; hence, it is compatible with proteins as big as 300 kDa.⁸⁵ MALDI is a more attractive option for heterogeneous samples, and is less susceptible to the effects of impurities.⁸⁴

As its name suggests, in APCI, sample ionization is performed under atmospheric pressure. A common method is by using a

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corona discharge needle, which is held at high voltage, as a source of ionizing electrons that produce ions from nearby gases.⁸⁶ APCI is tolerant of the presence of non-volatile sample components; hence, it could be coupled directly with micellar electrokinetic chromatography (MEKC).^{87,88}

An ICP is a high-temperature source (usually ionized argon, "plasma") sustained with an electromagnetic field. Ionization ensues after the aerosolized sample has penetrated the plasma, and the formed ions (mostly singly charged) are detected by MS.⁸⁵ ICP-MS is widely employed for trace metal analysis. Combined with CE, it has been used to characterize metalloproteins.⁸⁹⁻⁹¹

In FAB, the sample is dissolved in a nonvolatile solvent ("matrix", usually glycerol), placed on a direct insertion probe and bombarded with fast atoms (usually argon or xenon). The high-energy beam of atoms causes desorption of the sample into the gas phase. Compared to ESI and MALDI, it has lower sensitivity, requiring high picomole to low nanomole of the material.^{84,85}

3 Coupling

3-1 ESI-MS

Being compatible with liquid flow, direct coupling of CE with ESI is accomplished more easily. A means to close the CE electrical circuit for analyte separation while simultaneously providing an electrical potential to the spray tip is necessary. The common interfaces can be divided into three categories: sheath-flow^{16,18-20,24,27,32,37,38,40,43,47,50,53-56,60,66} and sheathless^{15,17,25,26,28-30,34,36,44,45,48,49,61} and liquid-junction interfaces.⁹²⁻⁹⁴

In the sheath-flow design, a coaxial sheath liquid, usually a hydroorganic mixture, mixes with the capillary eluent at the outlet end of the capillary. Its flow rate is significantly higher than the CE flow rate; hence, it dilutes the electrophoresis buffer and minimizes the potential incompatibilities for a more stable electrospray.¹ Moreover, it provides another window for optimizing the detection parameters and tuning selectivity. Sanz-Nebot *et al.*,⁵³ in comparing the effect of methanol, 2-propanol and acetonitrile as an organic modifier in the sheath liquid, showed that the highest signal intensities for some peptide hormones were obtained using methanol. A study by Brenner-Weis *et al.*⁵⁴ indicated that increasing the concentration of methanol in the sheath liquid favored the detection of dissociated myoglobin complexes over the intact species, and that even slight changes in the pH of the sheath liquid affected the stability of the complexes. Because of its robustness and ease of implementation, the sheath-flow interface is the most widely used. The disadvantage, however, is that it compromises sensitivity because of dilution at the tip.

The advent of micro- and nanoESI sprayers has prompted the development of sheathless interfaces, which are more compatible with the CE flow rates. Since the sprayer can be positioned closer to the orifice of the mass spectrometer, ionization is improved, and this translates to higher sensitivity.¹ Kelly *et al.*⁹⁵ reported detection limits on the order of 0.1–5 fmol for some standard peptides, a sensitivity approximately ten times better than conventional CE-ESI-MS with sheath-flow configuration. Despite this, sheathless interfaces are not as commonly employed as sheath-flow ones because they generally require special equipment and entail more involved procedures. Several strategies have been developed, and among the most common are coating of the capillary tip with a metal or a polymeric material,^{96,97} the use of an in-capillary electrode,^{98,99}

Fig. 1 Schematic representation of the split-flow CE-ESI-MS interface. Reprinted with permission from Ref. 26. Copyright 2001 American Chemical Society.

and splitting part of the capillary eluent through a small opening at, or near, the capillary outlet for contact with an outer conductive sleeve.¹⁰⁰ Chang and Her¹⁵ carbon-coated the tapered capillary outlet using a soft pencil, while McComb and Perreault¹⁷ used gold. Waterval *et al.*²⁹ compared gold and silverpaint-coated conductive spray tips, and reported an easy-to-prepare T-junction for direct electrode contact, prepared by positioning a gold wire at the gap between the separation capillary and a pulled capillary tip connected by a small transparent rubber. Wetterhall *et al.*³⁴ used a conductive polypropylene/graphite mixture for the production of polymeric nanospray needle emitters. Moini²⁶ drilled a small hole near the capillary outlet through which a small percentage of the capillary eluent exited and contacted a metal tube, acting as the CE outlet/ESI shared electrode (Fig. 1). This was later applied to the analysis of carbonic anhydrase in lysed red blood cells, resulting in detection limits in the low attomole range.³⁶ For better control of the split ratio, the preceding design was modified by chemically etching a small section of the capillary using hydrofluoric acid to render it porous, ion transport through this porous section closing the electrical circuit.⁴⁵ Janini *et al.*⁴⁴ described a rugged interface that integrated the separation capillary, a porous electrical junction, and a spray tip on a single piece of fused-silica capillary. In a subsequent report,⁴⁹ they evaluated capillaries with different internal and external diameters for optimal performance.

In the liquid-junction interface, the CE capillary is separated from the ESI emitter by a small gap ("junction") (10–20 μm) filled with a freely chosen make-up liquid,¹⁰¹ which acts as an electrical conductor. Because of this partial disconnection, CE and ESI conditions can be optimized independently. Its disadvantage, however, is the difficulty in constructing a proper junction.⁴ Only its use in microdevices has been reported recently.⁹²⁻⁹⁴

3-2 MALDI-time-of-flight(TOF)-MS

Though protein digests can be analyzed directly by MALDI-TOF-MS, the performance can be compromised by ion suppression effects; therefore, a separation step beforehand is generally preferred.⁶⁸ Johnson *et al.*⁶⁹ reported on the identification of more peptides from a mixture of tryptic digests by CE-MALDI, compared to direct MALDI analysis.

Except for such case when a liquid matrix is utilized,¹⁰² MALDI uses a solid matrix; hence, CE-MALDI approaches are primarily off-line. In off-line coupling, fractions of the CE eluent are collected and deposited on a MALDI target in the form of spots^{103,104} or as a continuous streak.¹⁰⁵ This approach enables independent optimization of the separation and MS

Fig. 2 Schematic representation of DEC CE-MALDI-MS. Reprinted with permission from Ref. 70. Copyright 2001 WILEY-VCH.

analysis steps. Moreover, since the actual amount of the sample consumed in MALDI is much lower than what is required for analysis, a sample can be reanalyzed further, or recovered, for other experiments.⁶⁹

To minimize sample handling and potential losses, strategies for the on-line coupling of CE with MALDI have also been developed. These include the use of a continuous flow (CF) through a capillary to transport the matrix and the analyte to the tip of the sample probe,¹⁰⁶ a pneumatic nebulizer to spray the solution containing the matrix and the analyte into the mass spectrometer,¹⁰⁷ and a moving target surface that is moved into the mass spectrometer.¹⁰⁸ On the third type, Preisler *et al.*⁶⁸ improved the previous design,¹⁰⁸ in which the liquid sample of the analyte and the matrix was directly deposited onto a quartz wheel in an evacuated source chamber, and transported to a repeller for laser desorption, by depositing the samples on a disposable moving tape, enabling 24 h of continuous operation with detection limits in the low nanomolar concentration range. This approach was extended to a capillary array system.⁷⁶ Ørnsnes *et al.*⁶⁷ constructed an interface based on a rotating ball inlet (ROBIN), in which the sample adhering to the surface of the ball is continuously carried past a polymer gasket into the vacuum chamber. Musyimi *et al.*⁸³ modified this design so that the majority of the ball surface is at atmospheric pressure and easily accessible for sample deposition.

Ojima *et al.*⁷⁰ proposed a method, called droplet electrocoupling (DEC), which is intermediate between the off-line and the on-line modes. In this approach, electro-connection is made by placing the outlet of the separation capillary at a droplet of an electroconductive solution on the MALDI sample plate (Fig. 2).

4 Sample-Enrichment Techniques

Sample loading in CE is typically limited to 1% (a few nL, at most) of the total capillary volume in order to maintain its high efficiency and resolving power. This implies that while the sensitivity in terms of the absolute amount is good, its concentration sensitivity, which is dependent on the amount of the solution injected, is rather poor. For successful analyses of protein and peptide samples at low concentration, it is often necessary to employ a step directed towards enabling large volume injections of the sample followed by a narrowing of the analyte bands within the capillary, or concentrating the components from a relatively large volume of a dilute sample prior to introduction into the separation capillary.

Fig. 3 Evolution of analyte zones in FESI-CE-ESI-MS.

In-capillary sample preconcentration techniques are generally based on electrophoretic stacking. Sample matrices and background solutions (BGSs) are designed to differ in terms of some physicochemical property (*e.g.* conductivity, pH, salt content), which brings about a change in the analyte velocity as it transits the boundary separating these two zones.¹⁰⁹ They are easy to implement because they do not require any modification in existing instruments. Neusüß *et al.*³⁸ reported using a pH-mediated stacking technique, in which the zwitterionic peptides from proteolytic digests were prepared in a basic solution and injected hydrodynamically into a capillary filled with an acidic BGS, the focusing effect being due to a change in their migration velocities following charge reversal. Monton and Terabe⁶⁶ employed field-enhanced sample injection (FESI), in which peptides from a low-conductivity solution were injected electrokinetically and stacked at the head of a capillary filled with a high-conductivity BGS (Fig. 3). Larsson and Mareike Lutz²⁰ and Stutz *et al.*⁶⁰ used transient isotachopheresis (transient ITP), a focusing strategy in which the sample is dissolved in the BGS and is sandwiched between a leading electrolyte and a terminating electrolyte, where its mobility is intermediate.⁴ The preceding four cases used a sheath-flow interface, but the concentration sensitivities obtained approximated those of sheathless designs.

CIEF, a CE mode which separates the analytes on the basis of their isoelectric points (pIs) (hence, particularly compatible with peptides and proteins), is also popular for sensitivity enhancement, since it has a self-sharpening effect on the analyte bands. In CIEF, a pH gradient is generated in the capillary using ampholytes, the high- and low-pH sides of the gradient being the cathode and the anode, respectively. Each component is focused at the position in the pH gradient where it has a net zero charge.^{4,110} The high-resolution capability of CIEF, frequently in conjunction with the high sensitivity, mass resolving power and mass measurement accuracy of an ESI-Fourier transform ion cyclotron resonance (FTICR) mass spectrometer, has been harnessed for characterizing complex protein mixtures, either alone^{16,18,19,31} or in combination with other separation steps.^{23,46,47,58}

In-capillary electrophoretic concentration schemes can provide as much as three orders of magnitude improvement in the signal (*e.g.* using FESI⁶⁶); however, they are limited by the volume of the capillary itself, some length of which has to remain for separation. The optimum volume of a sample that can be loaded is that which will provide the highest sensitivity enhancement without breaking down the resolution and

Fig. 4 Schematic representation of the CE capillary with an on-column mSPE cartridge-sheathless ESI-MS interface. Reprinted with permission from Ref. 49. Copyright 2003 American Chemical Society.

efficiency. In contrast, multiple capillary volumes can be loaded onto preconcentration devices at the inlet end of the CE capillary using a bed of solid-phase packing material, a microvariation of the solid-phase extraction (SPE) cartridge, or a membrane impregnated with a chromatographic stationary phase.¹¹¹ Generally, the analytes are extracted from the sample, which is typically pushed through the system by gas pressure, accumulated on the cartridge, washed with the usual BGS used in the CE separation process, and then eluted with an organic modifier-containing solvent. Significant enhancements in the detection sensitivity could be achieved, with detection limits in the low amol/ μ L reported.¹¹² Additionally, it has the advantage of enabling sample clean-up, which is extremely useful in the analysis of biological samples. The drawback, however, is that it often tends to compromise CE performance, resulting in irregular electroosmotic flow (EOF), broader peaks, and poorer resolution.⁵ Waterval *et al.*²⁹ developed an on-line preconcentrator using commercially available extraction disks containing poly(styrenedivinylbenzene) adsorbent particles in a matrix of inert Teflon, permitting loading of as much as 7.5 capillary volumes of a sample. They applied this later to quantitate low concentrations of gonadorelin and angiotensin II in a plasma.³⁰ Janini *et al.*⁴⁹ assembled a miniaturized, membrane-based solid-phase extractor (mSPE) using a C_{18} -impregnated extraction disk (Fig. 4). In combination with an in-house developed sheathless interface, a mid-attomolar mass limit of detection and a low nM concentration limit for some proteomic samples were achieved.

5 CE-MS in Microfabricated Devices

The use of microfabricated devices (microdevices, chips, microchips) has two distinct advantages: miniaturization and integration;¹¹³ hence, the area of microfabrication has experienced rapid growth in recent years, in response to the demand for high-speed, high-throughput analytical systems capable of handling minute amounts of samples,⁹² especially those of biological importance. Microfabricated devices have been developed for many applications, including chemical separations (CE, HPLC),^{114,115} cell culture and cell handling,^{116,117} clinical diagnoses,^{118,119} immunoassays,^{120,121} DNA separations,^{122,123} and protein analysis.^{124,125}

CE on a microdevice (MCE) enables rapid (within 1 min), and potentially high-throughput analyses, since multiple channels on a chip can be used. Its coupling with nanoESI-MS results in a powerful analytical tool for samples at the nanoscale level. Moreover, the possibility of integrating other processes, like sample clean-up, derivatization and enzyme digestion onto a single chip, makes it an exceedingly attractive format.

Most reports on MCE-ESI-MS have focused on developing designs for facile interfacing of microfabricated devices with MS.^{92-94,113,126-131} Wen *et al.*¹²⁶ demonstrated direct microchannel isoelectric focusing (IEF)-MS using a chip constructed on polycarbonate plates for the characterization of protein mixtures. Zhang *et al.*⁹³ designed a microdevice from glass wafers, which integrated a guiding channel for insertion of the electrospray capillary. No glue was necessary for sealing the electrospray capillary, which made it easy to replace either the microdevice or the electrospray capillary. Sung *et al.*¹³⁰ developed a poly(dimethylsiloxane) (PDMS)-based microchip for protein identification by tandem MS. The microchannel was modified with 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) to generate a stable EOF under acidic condition. Tachibana *et al.*⁹⁴ attached the ESI spray nozzle to the exit of the separation channel of a quartz microchip using a polyether ether ketone (PEEK) screw, thus permitting easy replacement.

There were also a few reports on MCE-MALDI-MS coupling. Liu *et al.*¹³² introduced the concept of rapid open-access channel electrophoresis (ROACHE), where separation is performed in open microchannels cut into glass microchips. In ROACHE, the matrix is added to the buffer solution before separation. When the solvent is evaporated at the end of the separation, the channel yields a MALDI sample complete with a matrix and ready for analysis. Recently, Astorga-Wells *et al.*¹³³ described a microdevice for the desalting, clean-up and enrichment of samples in peptide mapping and protein analysis by MALDI-MS (Fig. 5). It is based on the electrocapture of positively charged analytes in a μ m-diameter fluidic channel for exchanging the salt-containing solution to a solvent suitable for MS, followed by deposition of the concentrated and desalted sample on a MALDI target plate for analysis.

Platforms integrating on-line proteolytic digestion have been developed as well.^{134,135} Aside from their potential to perform sample-handling procedures in minute volumes, they dispense with the long incubation times required for digestions because

Fig. 5 Schematic representation of the microfluidic electrocapture device. Reprinted with permission from Ref. 133. Copyright 2003 American Chemical Society.

Fig. 6 Schematic of the set-up for performing ESI-MS analysis of peptide mixtures from a trypsin membrane reactor. Reprinted with permission from Ref. 137. Copyright 2001 American Chemical Society.

of increased reaction kinetics in low sample volumes.¹³⁵ Wang *et al.*¹³⁶ described a microfluidic device that integrated a protein digestion bed on a monolithic surface using trypsin immobilized on beads. The flow rate at which the substrate solution was pumped through the reactor bed determined the rate and efficiency of digestion. Under the optimum flow rate condition, only 3–6 min at room temperature were required to digest bovine serum albumin (BSA) and cytochrome *c*, with the amino acid sequence coverage ranging from 71% to 92%. Gao *et al.*¹³⁷ constructed a miniaturized membrane reactor by fabricating the microfluidic channels on a PDMS substrate and coupling the microfluidics to a poly(vinylidene fluoride) porous membrane with the adsorbed trypsin (Fig. 6). The extent of digestion could be controlled by the residence time of the substrate inside the membrane, the reaction temperature, and the protein concentration. Additionally, it enabled on-line peptide concentration using transient ITP, preceding CZE-MS analysis.

6 Multi-Dimensional Separations

Generally, the resolving power of one-dimensional separation systems like high-performance liquid chromatography (HPLC), or CE, is adequate for many samples, but not for highly

complex mixtures. This has fueled interest in the development of multi-dimensional separation systems, which can provide a higher total peak capacity (the product of peak capacities in each individual step) and a greater separation space. Ideally, the dimensions should be orthogonal (*i.e.*, independent), and the separation accomplished in the first dimension should be preserved in the next.^{138,139} Combining a reversed-phase chromatographic method with an electrophoretic-based one is particularly attractive because of the complementarity of these techniques; the former resolves the analytes according to their hydrophobic interaction with a stationary phase, while the latter does so according to the analytes' charge-to-size ratios; hence, there is little redundancy in mechanisms.¹⁴⁰

CIEF, as the first dimension, has been coupled to LC-MS. Apart from the extremely high resolving power of such combination, inserting an LC step between CIEF and MS has the advantage of enabling ampholyte removal to minimize interference during the MS analysis. Chen *et al.*⁴⁶ demonstrated that such system has the ability to identify a large number of yeast proteins using a protein loading 2–3 orders of magnitude lower than those employed in other techniques. In this design, an injection loop attached to a microselection valve was placed between the anodic and the cathodic cells to transfer the peptide fractions into the LC, using a C₁₈ trap column for ampholyte

Fig. 7 2-D display of BSA tryptic peptides in one of the CIEF fractions analyzed by transient CITP/CZE-FTICR-MS. The spots are identified as three BSA tryptic peptides with similar pI's, and their spectra are shown in the insets. Reprinted with permission from Ref. 47. Copyright 2003 American Chemical Society.

removal prior to LC. Zhou and Johnston⁵⁸ used a microdialysis membrane to separate the cathodic cell from the separation capillary, which permitted the collection of the fractions and their washing to remove the ampholyte prior to LC-MS analysis.

LC-CE combinations have been investigated as well. An on-line LC-CE approach was developed by Bergström *et al.*⁴⁸ using an interface manufactured in elastomeric PDMS, which allowed the LC and CE eluents to meet with a small contact surface, and ensured that the LC flow did not continuously leak into the CE capillary. LC as the first dimension permits loading of large volumes of the sample, which can be separated into small fractions with minimal overlap between neighboring fractions.⁶¹ This advantage can be better exploited when using the off-line mode, since there will be no constraints on the sample-loading capacity owing to the differences in the dimensions of the LC and CE capillaries. Janini *et al.*⁶¹ reported injecting 200 µL of a tryptic digest of serum proteins into the LC column, separating it into 96 fractions, and later analyzing the fractions by CZE-ESI-MS. Their results showed less than a 15% overlap between adjacent fractions.

Mohan *et al.*⁴⁷ developed an electrokinetic-based platform, which combined CIEF and CITP/CZE-MS using a microdialysis interface. Besides the high resolution provided by CIEF and CZE, the focusing and stacking effects of CIEF and CITP widened the dynamic range and increased the sensitivity of MS detection (Fig. 7).

7 Applications

7.1 Identification of proteins

Proteomics, the identification of the entire complement of cellular proteins, has become one of the major thrusts of biological and medical research in the past decade. MS in combination with high-efficiency separations is acknowledged

as an essential tool for interrogating the proteome. While the traditional two-dimensional polyacrylamide-gel electrophoresis (2-D PAGE) is still widely used, the advantages of liquid-phase separations, often in the capillary format, are increasingly being recognized. Among these are a small sample size requirement, high sensitivity, a wide dynamic range, and better capability for automation.⁹

There are two approaches to protein identification using MS. The more frequently used one is known as the "bottom-up" approach.¹⁴¹ It entails enzymatic digestion of the protein, followed by separation and mass analysis of the resultant peptides. The protein can be identified by database searching using the masses of the peptides and/or the sequence information obtained by tandem MS. Using an on-line combination of CE and an FTICR-MS instrument, Wetterhall *et al.*³⁵ reported the identification of 30 proteins from human cerebrospinal fluid. Zhu *et al.*⁷⁹ utilized CE-ESI-MS and MALDI-MS mapping to obtain high sequence coverage (up to 100% for small proteins and up to 90% for larger ones) of proteins from breast cancer cells. Li *et al.* demonstrated the analytical potential of microdevices for identification of proteins from *Haemophilus influenzae*¹²⁷ and *Neisseria meningitidis*¹²⁹ using both peptide mass-fingerprint database searching and on-line tandem MS. A two-dimensional separation strategy using CIEF-LC, followed by on-line ESI-MS, was employed by Gao *et al.*²³ and Chen *et al.*⁴⁶ for identification of the proteins in *Saccharomyces cerevisiae*. The high efficiency of an LC-CZE-MALDI-MS system was demonstrated by Zhang *et al.*⁸² in the analysis of D₂₀ liver cancer tissue, from which 300 proteins were identified.

The other MS strategy for characterizing proteins, the "top-down" approach, obviates the need for proteolytic digestion. It involves analyzing intact unknowns for accurate mass and amino acid sequence tags.¹⁴¹ Using CIEF and the high mass measurement accuracy of FTICR, Jensen *et al.*,¹⁸ identified 400 – 1000 proteins from cell lysates of *Escherichia coli* and

Deinococcus radiodurans, while Martinović *et al.*³¹ applied it to the analysis of *Escherichia coli* and *Saccharomyces cerevisiae*. Application to subcellular proteomics was shown by Moini and Huang,⁶² who analyzed the ribosomal proteins of *Escherichia coli* by CE-ESI-MS, detecting 55 out of the expected 56. Additionally, they exploited the benefit of having intact molecular weight information for detecting post-translational modifications (PTMs). Since the *E. coli* genome is known, differences between the measured and the calculated average masses of the proteins helped to identify PTMs, the majority in this case being methylation and acetylation.

7.2 Identification of post-translational modifications

PTMs regulate most biological processes. The most common types found in proteins are phosphorylation and glycosylation. Phosphorylation, mainly on serine, threonine and tyrosine residues, is involved in gene expression and protein synthesis which controls cell growth, division or differentiation.¹⁴² Glycosylation, on the other hand, is a major source of protein microheterogeneity.²⁷ The analysis of PTMs is particularly challenging because the modified moieties are often present in low abundance; hence, many approaches are based on the selective enrichment and analysis of peptides harboring specific types of modification.

Cao and Stults²¹ utilized an immobilized metal affinity chromatography (IMAC) column to selectively preconcentrate the phosphopeptides from tryptic digests of α - and β -casein, followed by CE and multi-stage tandem MS for more reliable assignment of phosphorylated residues. Using the known bias of electrokinetic injection for more mobile species, Monton and Terabe¹⁴³ applied a negative voltage to effect discriminate injection of phosphopeptides into the capillary, these being generally more acidic than nonphosphopeptides.

In another application, Nam *et al.*⁷⁷ compared the degree of phosphorylation of three myelin basic protein (MBP) substrates by extracellular signal-regulated kinase (ERK) using CE and MALDI-MS, demonstrating the utility of such combination for characterizing enzyme reactions.

Lai and Her²² developed a method based on CE-ESI-MS for the analysis of glycosylation patterns in the phospholipase A₂ of honeybees, their results indicating no significant difference in the variation and relative abundance of different glycopeptides between young and old bees. Liu *et al.*²⁷ employed the same combination to analyze the high-mannose-type N-glycosylation in ribonuclease B (Rnase B) and in a novel C-type lectin from the venom of *Trimeresurus stejnegeri* (STL), showing the usefulness of the method not only for the characterization of glycosylation sites and glycan structures, but also for the determination of the relative abundance of individual glycoforms. Zamfir and Peter-Katalinic²⁸ reported on the detection of more glycosylated sialylated peptides from complex mixtures using on-line CE-ESI-MS compared to direct ESI-MS analysis.

7.3 Clinical diagnostics

The advantages of CE-MS are also increasingly being exploited in the clinical setting for fast, sensitive diagnoses using biological fluids. Guzman²⁴ applied immunoaffinity CE and MS to the determination of immunoreactive gonadotropin-releasing hormone (GnRH) in serum and urine. In immunoaffinity, a microchamber affinity device, located near the inlet of the separation capillary, contains an immobilized antibody (affinity ligand) to selectively capture and concentrate the analytes (affinity targets). After elution from the microchamber device, the analytes are separated by CE. In this

study, significantly higher amounts of GnRH were found in serum and urine samples from a patient suffering from benign prostatic hyperplasia compared to a normal subject. Using CE-MS, Sanz-Nebot *et al.*⁵³ compared the glycoforms of serum transferrin (Tf) in healthy individuals and Carbohydrate Deficient Glycoprotein Syndrome (CDGS) patients, their results indicating a marked variation in terms of the type and concentration of glycoforms between the two groups. Wittke *et al.*⁵⁶ also used CE-MS to obtain patterns of proteins and peptides present in the urine of healthy individuals ("normal urine polypeptide pattern"). The analysis of urine of patients with kidney disease revealed significant deviations from the normal pattern.

8 Conclusion

Recently, significant advances have been made in the field of CE-MS for protein and peptide analyses. Most research has been directed at improving current designs (*e.g.*, instrument, interfaces, formats) or developing new ones, and exploring the feasibility of combining with other methods (*e.g.* multi-dimensional separation systems) to achieve optimum performance. Many of these have been demonstrated using standard samples, with reports on applications to real samples still limited up to this time. However, with the improvements in sample-handling procedures, the use of sample-enrichment techniques and microdevices, it is expected that full utilization of CE-MS as a bioanalytical tool will be realized in the coming years.

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