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Review

Recent developments in CE and CEC of peptides

The article brings a comprehensive survey of recent developments and applications of high-performance capillary electromigration methods, zone electrophoresis, ITP, IEF, affinity electrophoresis, EKC, and electrochromatography, to analysis, preparation, and physicochemical characterization of peptides. New approaches to the theoretical description and experimental verification of electromigration behavior of peptides and to methodology of their separations, such as sample preparation, adsorption suppression, and detection, are presented. Novel developments in individual CE and CEC modes are shown and several types of their applications to peptide analysis are presented: conventional qualitative and quantitative analysis, purity control, determination in biomatrices, monitoring of chemical and enzymatical reactions and physical changes, amino acid and sequence analysis, and peptide mapping of proteins. Some examples of micropreparative peptide separations are given and capabilities of CE and CEC techniques to provide important physicochemical characteristics of peptides are demonstrated.

Keywords:
CE / CEC / Peptides

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1 Introduction

Peptides are an extremely abundant and prominent family of biologically active compounds. They play a significant role in control and regulation of many vitally important processes in all living organisms; their diverse biological functions include operating as hormones, neurotransmitters, immunomodulators, coenzymes, enzyme substrates and inhibitors, receptor ligands, drugs, toxins, and antibiotics. In the current era of proteomics, the comprehensive analysis of proteome, representing now the main approach to understand the molecular bases of biological processes and diseases, and to discover new biomarkers and drug targets, the importance of peptides even increasing. The reason for this fact is that both the structure and function of many proteins are identified *via* their peptide fragments generated by their enzymatic hydrolysis [1–3]. This "bottom-up" or "shotgun" approach is one of the main directions in the proteome research [4]. In addition, for more detailed understanding of both normal and pathological processes of the living cells, a comprehensive investigation of the whole peptide set of a cell (peptidome), peptidomics, a logical sequel to proteomics, has emerged [5–7]. Hence, no wonder that separation, analysis, purification, and characterization of peptides by capillary electromigration methods belong to the most challenging tasks of these high-performance separation techniques.

This article presents a comprehensive survey of the recent advances in CE and CEC of peptides, particularly in the years 2005–2006 with some overlap to the first half of 2007. It is an update of the previous reviews on CE and CEC of peptides [8–11], which cover the period 1997–2004. Fast and vigorous developments of both pure electrophoretic modes, zone electrophoresis (ZE), ITP, IEF, affinity electrophoresis, and mixed, electrokinetic and chromatographic modes, EKC and electrochromatography, in the capillary and microchip format, have gone ahead also in the recent period. Applications of these methods to analysis and characterization of peptides have been further broadened, and CE and

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Abbreviations: μTAS, micrototal analytical systems; **CABCE,** carrier ampholyte-based CE; **CAE,** capillary affinity electrophoresis; **CEKC,** capillary EKC; **CITP,** capillary ITP; **CMEKC,** capillary MEKC; **CSF,** cerebrospinal fluid; **EMMA,** electrophoresis-mediated microanalysis; **FS,** fused-silica; **GnRHs,** gonadotropin-releasing hormones; **GSH,** glutathione; **GSSG,** glutathione-disulfide; **LLE,** liquid–liquid extraction; **MBB,** monobromo-bimane; **MICAE,** multiple-injection CAE; **NBD-F,** 4-fluoro-7-nitro-2,1,3-benzoxadiazole; **NDA,** naphthalene-2,3-dicarboxyaldehyde; **OT-CEC,** open tubular CEC; **SPME,** solid-phase microextraction; **t-ITP,** transient ITP; **ZE,** zone electrophoresis

CEC techniques are nowadays accepted as a recognized counterpart and/or complement to the earlier well-established techniques for peptide analysis and preparation – different modes of HPLC. In addition to the last one of the above reviews [11], the advances of CE and CEC of peptides in recent years are described also in some other reviews dealing with CE and CEC of biomolecules in general [12–14] and with some particular topics of CE and CEC of peptides [15–17], and in the recent special issue of **Electrophoresis** devoted to CE and CEC of amino acids, peptides and proteins [18].

2 Electromigration behavior of peptides and selection of separation conditions

The electromigration behavior of peptides was investigated in terms of a correlation between electrophoretic mobility of peptides and their electric charge, size (expressed by hydrodynamic (Stokes) radius, M_r, or number of amino acid residues), and shape (conformation) of the (poly)peptide chain.

The general form of the equation describing the relation between effective electrophoretic mobility of peptide, m_{ep}, and its effective charge, q, and M_r, is:

$$m_{ep} = \frac{Aq}{M_r^k} \quad (1)$$

where A is a constant, and k, the exponent of M_r, is related to the shape of a peptide molecule [19].

Three basic semiempirical models for peptide mobility differ in the value of exponent k. It approaches 1/3 for the Stokes model in which peptides are modeled as spherical particles with high charge density [19], k is equal to 1/2 for peptides considered as classical polymer (random coil) with a lower charge density [19], and k is close to 2/3 for Offord's model [20] for larger and more rigid structures, the frictional forces for which during the electrophoretic motion are proportional to the surface area of peptide molecules. In addition, some other semiempirical models have been suggested with different specific k values for particular sets of peptides or with logarithmic dependence of effective mobility on charge or on the number of amino acid residues of the (poly)peptide chain [21–23].

The above three basic models were examined by CZE of peptide hormones (oxytocin, eledoisin, bradykinin, Met- and Leu-enkephalins, triptorelin, and buserelin) in a broad pH range, 2–12 [24]. The classical polymer model with $k = 1/2$ resulted in slightly better correlation within the whole investigated pH range. The plots of $q/M_r^{1/2}$ against pH of the BGE were used to predict CE separations of the above hormones and also of the opioid peptides (endomorphin 1, dynorphin A, and enkephalin derivatives) [25] using the accurate acidity constants (pK_a) of the present ionogenic groups (obtained in previous work from the CZE measurement of pH dependence of effective mobilities of these or similar peptides) for calculation of peptide charge. The agreement between the predicted and experimental mobilities was very good and the optimum pH for the separation of the analyzed peptides could be predicted from a limited amount of experimental data. Combination of modeling of peptide mobility and CE-MS was shown to be useful also for a fast and simple characterization of the cleavage specificity of new recombinant enzymes as demonstrated by CE-MS separation of peptide fragments obtained from hydrolysis of cytochrome *c* by recombinant and natural pepsin A [26]. Modeling of peptide mobility using the calculated pK_a values of ionogenic groups [27] helped to elucidate the most probable amino acid sequence of peptide fragments generated by pepsin hydrolysis of cytochrome *c* and in this way to characterize the pepsin cleavage site.

The above three semiempirical models of peptide mobility and logarithmic form of the Eq. (1) introduced by Cross *et al.* [28, 29]

$$\log\left(\frac{m_{ep}}{q}\right) = -k \log M_r \quad (2)$$

have been used to investigate the structure–mobility relationship of gonadotropin-releasing hormones (GnRHs) and their analogs and fragments [30]. None of the models was found to be quite definitively applicable for the whole set of ten GnRHs differing in size (tetrapeptide to decapeptide) and charge (+0.9 to +3.0 elementary charges) which were analyzed in five conventional and isoelectric acidic BGEs, pH 2.18–2.50. Nevertheless, for the dependence of m_{ep} on q/M_r^k, the highest coefficient of correlation, $R = 0.995–0.999$, was obtained for k close to 1/2 for all five acidic BGEs. This indicates that the electrophoretic migration of the set of GnRHs can be best described by the classical polymer model and the most probable structure of GnRHs in the acidic BGEs will be a random coil. The advantage of the application of Eq. (2) is that the value of the exponent k of M_r in the nonlogarithmic form of this relationship can be easily determined as a slope of the logarithmic dependence. Graphs of the model of Cross and the most suitable classical polymer model are shown in Fig. 1.

The quantitative structure–mobility relationship for prediction of peptide mobilities was developed using the Offord's model and artificial neural networks [31, 32]. In this model, the Offord's charge-over-mass term ($q/M_r^{2/3}$) as one descriptor is combined with the corrected steric substituent constant and molar reactivity descriptors to account for the steric effects and bulkiness of the amino acid side chains. The multilinear regression of the data set showed an improved predictive ability of the model over the simple Offord's relationship. Back propagation artificial neural network models resulted in a further significant improvement of predicted mobilities, especially for larger peptides and for peptides with higher charges containing the basic amino acids, His, Lys, and Arg. Linear and nonlinear quantitative structure-property relationship models for prediction of peptide mobilities in CZE were suggested using the linear heuristic method and a nonlinear radial basis function

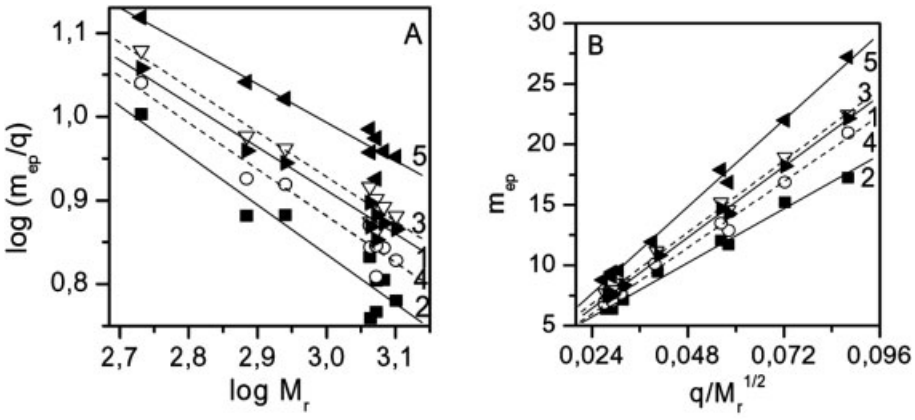

Figure 1. Semiempirical models of the correlation between effective mobility, m_{ep}, of GnRHs and their analogs and fragments, and their charge, q, and M_r. (A) Cross's model; (B) classical polymer model. Lines 1–5 indicate dependencies measured in five acidic BGEs, pH 2.18–2.50. Adapted with permission from [30].

neural network, respectively [33]. The results of the nonlinear model were closer to the experimental data than those of the linear model.

A new approach, bead model for modeling electrophoretic mobility and diffusion of peptides and proteins, is based on an approximate structural model of peptides and it also takes into account the electrohydrodynamic theory [34, 35]. A peptide formed by X amino acids is modeled as $N = 2X$ beads with two beads representing each amino acid in the chain. Peptide conformations are randomly generated and the mobility and diffusion coefficients are computed for at least 100 independent conformations. In general, the mobility is found to be dependent only weakly on peptide conformation, which is in agreement with the small differences of correlation coefficients when different values of exponent k of M_r are used in the above Eq. (1) of the dependence of peptide mobilities on the shape of their molecules. The precedence of this model is that the input parameters are independent of experimental mobilities. Hence, it is hoped that the model will be useful in the prediction of peptide mobility as well as in using peptide mobilities to extract information about peptide structure, conformation, and charge. In the other approach, the NMR measured translational diffusion constants of 12 amino acids are used for modeling of diffusion and electrophoretic mobilities of peptides composed of these amino acids [36].

Piaggio *et al.* [37] have shown that the important characteristics of short oligopeptides, such as effective (net) charge, hydrodynamic spherical radius, and hydration, can be explored through the CZE determined experimental electrophoretic mobilites using the Linderstrøm-Lang electrophoresis model and its perturbed version. The other new approaches include the application of the machine learning techniques, such as back propagation artificial neural network, radial basis function artificial neural network and support vector regression for the prediction of peptide mobility in CE [38], and the use of a novel learning machine, support vector machine, for the development of the nonlinear quantitative structure–mobility relationship model of peptides based on the calculated molecular descriptors representing the structural features of the modeled compounds [39].

A model reflecting the effect of pH, ionic strength, and temperature on electrophoretic behavior for a series of oligopeptides, oligoglycines, oligo-L-alanines, and oligo-L-valines, with a number of residues up to ten, has been developed on the basis of CZE measurement of effective mobilities of these oligopeptides in BGES within a broad pH scale, 1.8–12.0, at two ionic strengths (10 and 100 mM) and at temperatures from 15 to 60°C [40]. Using this broad set of experimental data a semiempirical model was developed allowing to predict pK_a values for any oligopeptide composed of amino acids with neutral lateral chains. The input parameters of this model are only the number of residues and the pK_a of terminal amino acids in their free form. The model can predict the peptide pK_a values at a given ionic strength and temperature and can also be used for selection of the optimum pH for the separation of mixtures of peptides the pK_a of which are known or could be estimated. The proposed relationship allows a significant reduction of the experimental data needed for the development of suitable separation conditions.

The mobilities and charges of the large polypeptides can be approximated by the models applied for modeling charge and mobilities of proteins [41].

Based on the basic electromigration properties of peptides, *i.e.*, their charge, size, conformation, hydrophobicity, binding capabilities, and considering also their other properties, such as solubility, amphoteric character, chemical stability, and biological activity, the experimental conditions of their CE separations are selected, of course taking into account general rules for BGE selection [42, 43]. Different aspects of the selection of the BGE for the analysis of peptides and proteins by CZE, such as concentration and types of the buffering constituents of the BGE with respect to their buffering capacity, mobility and electric conductivity, pH, additives suppressing the adsorption or influencing the EOF and selectivity, organic modifiers, temperature, and Joule's heating effects were thoroughly discussed in the earlier published but still relevant review [44].

3 Sample preparation

3.1 Preconcentration and preseparation

Preconcentration and preseparation (or "sample cleanup") are the necessary operations for sample preparations, when separation power and/or sensitivity of CE and CEC are not sufficient for direct analysis of peptides present at low concentration levels and/or in complex mixtures of different (bio)matrices, such as body fluids, cell lysates, and tissue extracts. The reason for that is that CE techniques generally suffer from relatively low concentration sensitivity due to the minute (nano- to picoliter) applied sample volume and the short optical pathlength (typically 25–75 μm) of the most frequently used UV-absorption detector. The problems associated with sample preparation for peptides and proteins in biological matrices prior to their CE and LC analyses are thoroughly discussed in the recent reviews [45, 46], where the application of various sample-preparation techniques, such as homogenization, centrifugation, precipitation, solvent extraction, liquid–liquid extraction (LLE), solid-phase (micro)extraction (SPE or SPME), affinity (micro)extraction, (micro)dialysis, and ultrafiltration, is described.

The earlier used off-line classical sample cleanup and preconcentration methods, such as SPE, SPME, and LLE, are recently replaced by on-line modes of these techniques. Both preconcentration and preseparation are frequently solved by using a solid-phase packed sorbent at the inlet end of the capillary, a microvariation of SPME cartridge, or more effectively and with smaller disturbance effect for the following CE separation using a microcartridge containing a membrane impregnated with different chromatographic stationary phases. The advances in on-line coupling of SPME and CE were reviewed by the inventor of SPME, Liu and Pawliszyn [47]. One such system, on-column sample enrichment for the high sensitivity CE-MS analysis of peptides was developed by Sandra *et al.* [48]. Peptides or glycopeptides are first retained and concentrated on a short (3–5 mm) C18 RP packed-bed situated in the fused-silica (FS) separation capillary, and they are subsequently liberated for CE separation by injection of a plug of organic solvent (90% v/v ACN or 75% v/v 2-propanol in water). The concentration LODs for standard peptides and for fetuin tryptic peptides were in the high picomolar range with a coaxial sheath-liquid flow CE-MS interface.

The improved cruciform configuration of the analyte concentrator-microreactor device, designed by Guzman and Phillips [49] for use in on-line immunoaffinity CE, enables to specifically trap, enrich, and elute an analyte from any biological fluid or tissue extract without any sample pretreatment except filtration, centrifugation, and/or dilution, allowing the separation and characterization of the target analytes faster, with higher sensitivity and lower cost than existing techniques. Using Fab' fragments of Ig G raised against angiotensin II and neurotensin, these neuropeptides present in low concentration in a complex matrix (urine) were captured by and eluted from the analyte concentrator/microreactor. The following on-line CE analysis confirmed the presence of both peptides in the eluate. With this device 100- to 1000-fold concentration was achieved, permitting quantitation of peptide analytes at the *ca.* 5 ng/mL level using UV-absorption detection.

Recently also monolithic capillary columns have been employed for SPME preconcentration and preseparation various analytes, including peptides [50]. Iron-protoporphyrin-modified monolithic FS capillary columns (150 μm id × 8 mm long) have been prepared for on-line preconcentration of decapaptide hormone, angiotensin I, prior to its CZE analysis [51]. Angiotensin was released from the sorbent by 50 mM sodium phosphate, pH 2.5/ACN, 75/25 v/v, and then analyzed by CZE in 50 mM sodium phosphate, pH 7.0. The monolithic column exhibited a high retention capacity for angiotensin I and showed as much as a 10 000-fold increase of concentration sensitivity achieving values up to 0.5 ng/mL with UV-absorption detection at 214 nm. Similar monolithic FS capillary columns modified with iminodiacetic acid and copper(II) ions were employed for on-line affinity capturing of histidine-containing peptides from the model peptide mixture. His-containing peptides were released from the sorbent by a 5 mM imidazole solution and separated by CZE with UV-absorption detection [52]. The same type of sorbent, monolithic column with Cu(II) iminodiacetic acid functional groups has been developed for preconcentration of low-abundance peptides and proteins from complex biological samples [53]. The practical considerations that permit coupling or integration of sample treatment devices into commercial CE apparatuses are treated in the recent review [54] where the off-line and on-line strategies are critically compared and the integrated in-capillary, in-vial, or in-replenishment-system methodologies for SPME and LLE are described.

In addition to the chromatographic LLE and SPME principles, sample concentration is achieved also by electrophoretic approaches [46]. Electric field-amplified sample injection (FASI) also called field-enhanced sample injection, based on the increased intensity of the electric field in the injected zone of a sample dissolved in water or diluted BGE and possessing higher electric resistance than the surrounding BGE, has been used for high-sensitivity analysis of bioactive peptides by CE with ESI-MS detection [55]. After optimization the injection conditions, such as type and concentration of the acid and organic solvent in the sample solution, length of the water plug, sample injection time, and separation voltage, more than 3000-fold improvement in detection signal was obtained, permitting analysis of bioactive peptides and tryptic protein digests in low nanomolar (fmol/μL) levels. Using the FASI technique, carnosine and its related peptides, anserine, and homocarnosine, could be separated and determined by CE at 10^{-8} mol/L level, with 130- to 160-fold enlargement of sensitivity without loss of separation efficiency as compared to conventional sample injection [56].

The dynamic pH junction technique for peptide concentration [57] is based on the presence of a dynamic pH junction within the capillary, which induces narrowing of the originally broad sample zone due to a sharp reduction in an analyte's migration velocity following a reversal of its electrophoretic direction from the acidic sample zone to the alkaline zone of the BGE, see Fig. 2. The method allowed injection of broad sample zones in relatively high-conductivity matrices, without deteriorating peak shape, resolution, and separation efficiency, which makes the method suitable for analyses of real samples. The size of the original sample zone was reduced almost 40 times and the peak height of analyzed peptides has increased more than 120 times. In the next study, the technique was further improved and about 550- to 1000-fold sensitivity enhancement was achieved in CE-ESI-IT-MS analysis of peptide hormones and tryptic peptides of cytochrome *c*, demonstrating the suitability of this approach for PMF in protein analysis and proteomics [58].

The capturing of a protein or polypeptide in its p*I*, within an applied electric field in the capillary using a pH junction created by discontinuous buffer system is the base of over 1000-fold concentration for microliter volumes of peptide and protein solutions [59]. Further investigation of the discontinuous buffer junctions using pH indicators and their optimization allowed up to 2000-fold preconcentration of myoglobin [60]. A pH-mediated base stacking has been developed for determination of oxidized and reduced forms of glutathione in liver microdialysates [61].

A microfluidic device has been developed for sample cleanup of peptides and proteins *via* their electroimmobilization in a microflow stream [62, 63]. After their capture, the electric field is decreased in a stepwise manner, causing sequential release of the captured peptides according to their electrophoretic mobility. Tryptic peptides were separated and analyzed by MALDI-MS. The concentrating and separation power was sufficient to increase the ionization yield of several peptides originated from the shotgun tryptic digestion of membrane protein extracts, which were not detected in the unprocessed sample.

An extremely high, up to million-fold preconcentration of peptides and proteins has been achieved by a nanofluidic filter based on electrokinetic trapping mechanism [64]. The device, fabricated by photolithography and etching techniques, generates an extended space charge region within a microchannel, in which peptides and proteins are very efficiently collected and trapped. This electrokinetic trapping and collection can proceed for several hours and concentration factors as high as 10^6–10^8 have been demonstrated. Due to its simplicity, performance, and robustness the devices can be integrated in various analytical microsystems, including chip electrophoresis.

Preconcentration and preseparation of bulk components can be achieved also by application of ITP as a preceding step of CZE analysis realized either in column coupling system [65] or as a transient ITP (t-ITP) process which is continuously converted into CZE mode [66–68]. Selective enrichment and ultrasensitive identification of trace peptides in proteome analysis was achieved using t-ITP-CZE coupled with nano-ESI-MS [69]. A new simple and robust ITP system with high concentration of leading ion and EOF suppression can be integrated into microchip CE devices to achieve up to million-fold sample stacking [70, 71].

3.2 Derivatization

Peptides are mostly derivatized with a fluorophore to be detected with (laser-induced) fluorescence (LIF) detection, the sensitivity of which is about two to three orders higher

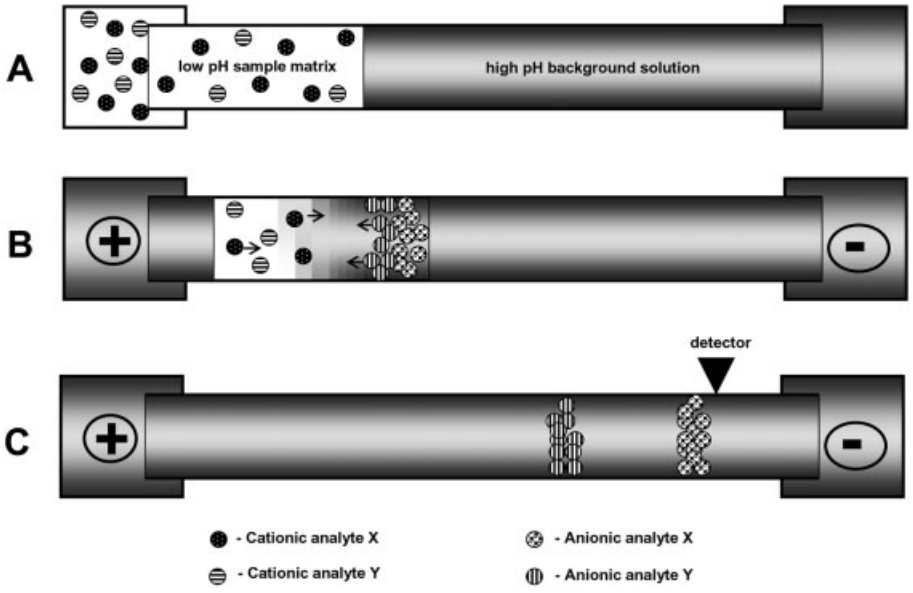

Figure 2. Dynamic pH junction model for acidic peptides and proteins. (A) Long plug of sample in a low-pH matrix is injected into an uncoated capillary filled with a high pH BGS. (B) Steep pH boundary develops at the front end of the plug and sweeps throughout the sample zone during electrophoresis, converting the cationic analyte into anionic, and significantly retarding its migration velocity. (C) Focused peak migrates to the detector. Reprinted with permission from [57].

than that of the most frequently used and no derivatization requiring, UV-absorption detection. The list of selected pre-, on-, and postcolumn derivatization reactions to label peptides and other analytes in the nanomolar and subnanomolar range for CE with LIF detection can be found in the review [72].

The on-line coupling of sequential injection analysis (SIA) and CE *via* an in-line injection microvalve with the port to port volume 51 nL has been developed for on-line derivatization and analysis of peptides and amino acids [73]. The SIA system was used for automated derivatization of amino acids and peptides by dichlorotriazinylaminofluorescein enabling sensitive detection with LOD 30 ng/mL using argon ion LIF detection at excitation/emission wavelengths 488/520 nm.

In addition to more conventional procedures of pre- and postcapillary derivatization, new approach based on in-capillary derivatization, adopted from the concept of capillary electrophoresis-mediated microanalysis (EMMA) [74], has started to be used for peptide and protein labeling. In-capillary derivatization of amino acids and peptide buccaline with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was developed for their subsequent CE analysis with LIF detection (argon ion laser, excitation/emission at 488/520 nm wavelength) [75]. The in-capillary derivatization was achieved *via* zone-passing mode by introducing successive plugs of sample and NBD-F into an fused-silica (FS) capillary previously equilibrated with an alkaline borate buffer. To prevent NBD-F hydrolysis and to achieve a reliable derivatization, NBD-F was prepared daily in absolute ethanol and a plug of absolute ethanol was introduced between the sample and NBD-F reagent plugs. NBD-F/sample molar ratio above 215, sample/NBD-F plug lengths ratio equal to 2 and increased temperature of 45°C for amino acids and 35°C for peptides were found to be optimal for high degree of derivatization. A good linearity between the peak heights and analyte concentrations was achieved allowing quantitative analysis of amino acids and buccaline peptide at the submicromolar level.

In-capillary noncovalent labeling of insulin and a gastrointestinal peptide (Arg-Arg-gastrin) by commercially available dye (Sypro Orange) has been tested for analysis of these biopeptides by CE with LIF detection [76]. The peptide labeling and the fluorescent emission intensity (excitation at 488 nm, emission at 610 nm) were found to be sufficient only in the presence of cationic detergent, tetradecyltrimethylammonium bromide (TTAB), in submicellar concentration in the borate BGE. Although the sensitivity attained was not as high as expected with LIF detection, the advantage of this approach was that a rapid on-line labeling of peptides could be performed without the complications and limitations of multiple labeled species frequently encountered with covalent labeling.

The integrated rapid on-chip sample derivatization employing naphthalene 2,3-dicarboxyaldehyde (NDA) and 2-mercaptoethanol (2-ME) with an easily assembled microdialysis-chip electrophoresis was developed and tested as a prototype of micrototal analytical system for high-temporal resolution monitoring of amino acids and peptides in biological systems [77].

3.3 Micromanipulation

The capability of CE and CEC to analyze extremely small sample volumes (nano- to picoliter scale) put special demands on the techniques and microdevices to manipulate such low-volume samples. Sample micromanipulations are integrated in the so-called micrototal analytical systems (μTAS) [78] where all operations, *i.e.*, sample manipulation, potential preconcentration, clean-up and derivatization, separation, and detection, are performed on the microstructures fabricated on the chips (see Section 7). Different means for sample manipulations in the μTAS, such as sample introduction to the chip, preseparation, derivatization, microdispensing, microinjection (electrokinetic, pressurized, piezoelectric, optical gating, microrotary valves), mixing by static mixers, centrifugal force, capillary force, pneumatic propulsion, and electromanipulation, are discussed in the recent review [79]. These techniques are particularly useful for handling of small sample volumes, such as in single cell analysis [80] and in the analysis of microbiopsy and microdialysis samples [81].

Several integrated systems for protein digestion and CE-based separation have been developed also on the chips, *e.g.*, the chip-based nanovials with immobilized proteolytic enzymes [82, 83]. For *in vivo* monitoring of biomolecules in body fluids and tissues, *e.g.*, neurotransmitters in the brain, microdialysis cells composed of a semipermeable membrane, typically 1–4 mm long and 0.2–0.4 mm in diameter, are fashioned into a probe, which is implanted in the brain or other tissues [84].

4 Suppression of adsorption and control of EOF

Adsorption of peptides, particularly longer polypeptides and peptides with increased number of hydrophobic amino acid residues, represents a serious disturbance factor in their CE analysis performed both in the most frequently used FS capillaries and plastic chips. Thus, suppression of adsorption belongs to the challenging issues in CE of peptides. Various strategies used to suppress the peptide and protein adsorption to the FS capillary or microchip wall have been surveyed in the recent review [85]. In addition to the separations performed in extreme low or high pH and in high ionic strength BGEs, different ways of the FS capillary coatings are employed. Dynamic coatings are based on reversible (dynamic) adsorption of small ions or hydrophilic polymers such as cellulose derivatives or synthetic polysaccharides added into the BGE. Static (permanent) modifications of the inner capillary wall involve the formation of a covalent bond between a suitable derivatized coating agent and the silanol

groups of the silica, giving rise to an immobilized polymeric coating, or the bond may be noncovalent but yet "permanent" resulting from the irreversible physical adsorption of the polymer, *e.g.*, polyvinyl alcohol (PVA) or PEG from the aqueous solution to the capillary wall.

A new adsorptive polyamine coating for CE of basic peptides and proteins in FS capillaries was prepared using polymer poly-LA 313, synthesized from 3,3′-diamino-*n*-methyldipropylamine and 1,4-butanediol diglycidyl ether [86]. The polymeric coating is formed by multisite electrostatic interaction between silanol groups and the positively charged nitrogen atoms, and hydrogen bonding due to the hydroxyl groups of the polymer. The physically adsorbed polymer resulted in stable coating within a broad pH range, 2–10, even in the presence of organic modifiers (ACN, methanol) and complex biological samples (plasma and cerebrospinal fluid (CSF)). The coating procedure was found to be highly reproducible and provided fast (few minutes) and highly efficient separations of basic peptide hormones, *e.g.*, enkephalins, oxytocin, angiotensin, bradykinin, and proteins, such as lysozym, ribonuclease A, α-chymotrypsinogen A, cytochrome *c*, and myoglobin. In addition to suppression of peptide sorption, the polycationic coating reversed the EOF and generated strong anionic EOF, which ensured relatively fast migration of all peptides, negatively charged, neutral and positively charged to anode. Moreover, the coating is well compatible with ESI-MS detection.

Semipermanent noncovalent coatings of the FS capillary by the double-chained surfactants, such as didodecyl- to dioctadecyldimethylammonium bromide and triple-chained tridodecylmethylammonium bromide, which have been successfully applied for CZE separation of proteins in 50 mM ammonium formate BGE, pH 4.5 [87], have the potential to be applied also for separation of peptides.

Using this noncovalent coating concept, bilayers, triple layers, or even multiple layers can be formed by polymers of opposite charge being alternatively adsorbed on each other. Noncovalently bilayer coated FS capillaries, prepared by successive flushing the capillary with cationic polybase (Polybrene) and anionic polyacid, poly(vinyl sulfonate), have been shown to be suitable for separation proteins, such as interferon-α-2b, myoglobin and carbonic anhydrase, and peptide fragments of insulin [88]. With 300 mM Tris-phosphate BGE, pH 7, a high efficiency (150 000–300 000) and favorable repeatability of migration times (RSD <0.8%) were obtained, even at high concentration of HSA in the sample, up to 60 mg/mL. Commercially available dual layers-based dynamic coatings, *e.g.*, CEoFix kits (see www.microsolvtech.com/ceofix.asp), can be also used for CE separations of peptides.

From different strategies tested for noncovalent FS capillary coating in NACE of synthetic polypeptides poly(N_ε-trifluoroacetyl-L-lysine), such as (i) addition of viscous additives, ethyleneglycol, or glycerol, to the BGE, (ii) adsorption of PEG of different M_r *via* hydrogen bonding, (iii) coating with binary mixtures containing ethylene glycol and PEG, and (iv) adsorption of cationic polyelectrolyte (DEAE-dextran) layer, the last one, polycationic coating was found to be the most effective in suppressing the polypeptide adsorption [89].

The problem of peptide and protein adsorption is serious also in plastic microchips. Due to their hydrophobicity, the inner surface strongly interacts with nonpolar analytes or species containing hydrophobic domains, including polypeptides and proteins, resulting in their significant adsorption to the microchannel walls. Cationic quaternary ammonium starch derivatives used as dynamic coating agents were found to suppress the adsorption of fluorescently labeled oligopeptides and amino acids in their CZE separation in poly(methyl methacrylate) (PMMA) microfluidic devices. The effect was valid over the pH range 2.5–8. Good solubility and low viscosity were the other advantages of this dynamic polymeric coating agent.

Effective capillary coating can be achieved also by low molecular mass compounds. Adsorption of cationic diethylentriamine results in masking the silanol groups and other active adsorption sites on the FS capillary wall which are responsible for peptide and protein adsorption to the capillary [90]. Adsorption of barium(II) cations originating from barium tetraborate BGE to ionized silanols and barium silicate precipitation seem to be able to shield the silica surface from separands and to reverse the direction of EOF as shown by CE separations of tryptic digests of peptides [91].

Dynamic or permanent capillary coatings have been used also for control of the EOF, which influences separation efficiency, resolution, and speed of CE analyses of all types of analytes, including peptides and proteins. A high and reproducible anodic or cathodic EOF for CE-MS separation of glycopeptides was achieved by noncovalent coating of the FS capillary with polycationic Polybrene or polycationic Polybrene/polyanionic dextrane sulfate single or multiple layers [92]. The latter type of bilayer coating, produced by subsequently rinsing the FS capillary with a solution of Polybrene and poly(vinyl sulfonate), was shown to be suitable for CE separation of enkephalins and tryptic peptides of cytochrome *c* in acidic 500 mM formic acid-based BGE, pH 2.5, and compatible with ESI-MS detection, causing no ionization suppression or background signals [93]. Dynamic coatings and precoatings with the commercially available agents EOTrol TM and UltraTrol TM have been reported to provide pH and BGE composition independent cathodic or anodic EOF at different flow rates [94].

In addition to chemical ways, EOF can be regulated also by physical tool, external radial electric field applied to the outer low-conductive capillary coating as shown by CE separation of oligoglycines [95].

5 Separations in different CE and CEC modes

5.1 ZE

CZE is the simplest, universal, and most frequently used CE mode applied to separation of peptides. ZE mode is usually

assumed when CE is spoken about without further specification of the separation mode [96]. For that reason only some special new aspects of peptide CZE separations are presented in this section, the others are mentioned also in the other sections.

A new CZE approach, the so-called ion-interaction CZE (II-CZE), has been employed to the separation of 27 synthetic cationic proteomic peptide standards comprising of three groups of nine decapeptides with net charges of +1, +2, and +3 for all nine peptides within a group and with subtle changes of hydrophobicity within each group of nine peptides [97]. This bidimensional CE approach provided an excellent resolution of the peptides with high peak capacity by combining the powerful inherent ZE separation mechanism (different electrophoretic mobilities due to different charge/size ratios) with an hydrophobicity-based mechanism consisting in differences of peptide interactions with BGE constituents, perfluorinated carboxylic acids (trifluoroacetic, pentafluoropropionic, heptafluorobutyric) present in high concentrations (up to 400 mM) and adjusted by LiOH to pH 2.0. Thus, simultaneously with a ZE-based separation of the three differently charged groups of peptides, there is a hydrophobically mediated separation of the peptides within these groups, see Fig. 3. This methology is quite different from other CE modes using complexing agents, such micelles or CDs in EKC.

The principle of II-CZE has been successfully applied also for the separation of peptides differing by a single amino acid substitution in deca- and dodecapeptides with model amino acid sequences [98]. Substitutions differed by a wide range of properties, *e.g.*, alkyl side chains, polar side chains, and charged side chains. The hydrophobic pentafluoropropionic acid anion provided best separation of peptides that differ in hydrophobicity of their hydrophobic side chains but high concentration of the hydrophilic phosphate anion was the best for separation of peptides differing in their polar side chains. Differential hydration/dehydration properties of side chains in the peptide and the hydration/dehydration properties of the hydrophilic/hydrophobic anions as well as the electrostatic attraction between the peptide and the anions in solution play an important role in these solution-based effects.

New types of BGE constituents, "narrow pH cuts" of carrier ampholytes employed for pH gradient formation in IEF, obtained by IEF fractionation in agarose gel or in the Rotofor device, have been investigated in common studies by Peltre's and Righetti's groups [99–101]. They found them promising BGEs for the CZE of proteins and peptides with respect to their low conductivity and good buffering capacity, permitting application of high intensity of electric field and thus achieving short separation times. These properties are provided also by single component isoelectric buffers, such as 100–200 mM iminodiacetic acid, which was used for CZE separation of GnRHs and their analogs and fragments [30].

Extremely fast, about only 4 s separations of JAK2 tyrosine phoshorylated peptide fragment and its complex with

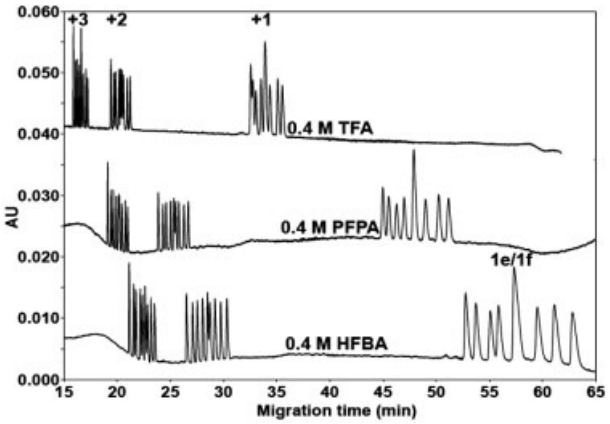

Figure 3. Effect of increasing hydrophobicity of anionic ion-pairing reagents (TFA<PFPA (pentafluoropropionic acid)<HFBA (heptafluorobutyric acid)) on CE separation of peptide standards. Conditions: FS capillary, 60.2 cm (50 cm) × 50 μm id; BGE: 0.4 M aqueous TFA, PFPA, or HFBA, adjusted to pH 2.0 with LiOH. 25 kV, UV-detection at 195 nm. Reprinted with permission from [97].

SH2-Bβ protein were achieved by CZE in 10 μm id, 7 cm/4 cm total/effective length capillary using a very high electric field strength of 2860 V/cm and a BGE composed of 25 mM Tris, 192 mM glycine, pH 8.5 [102].

NACE performed in pure organic solvents (*e.g.*, ACN or methanol) or CZE in hydro-organic solvents, *i.e.*, in the mixtures of organic solvents with water buffers also found their applications in the analysis of peptides and peptide derivatives. NACE with methanol or methanol/ACN mixture (87.5:12.5 v/v) as a solvent of BGEs composed of acetic acid, ammonium acetate, sodium acetate, and tetramethylammonium hydroxide has been used for separation of living (with free *N*-terminal amino group) and dead (with formylated *N*-terminus) water-insoluble synthetic homopolypeptides, poly(N_ε-trifluoroacetyl-L-lysine) [103]. NACE permitted the separation of the oligo- and polymers both according to their size (with the addition of TFA up to a DP of ~50) and according to the nature of the end groups.

Interestingly, NACE has provided better separation selectivities for α-helical polypeptides containing 14–31 amino acids than the aqueous CE under similar conditions [104]. This result was attributed to the significantly increased helical secondary structure of analyzed peptides in methanolic BGEs than in aqueous BGEs as found by circular dichroism method. NACE in different BGEs with electrochemical detection was tested for separation of enkephalin peptides [105]. The best separation was achieved in BGE consisted of 10 mM ammonium acetate in mixed ACN/methanol (3:1 v/v) solvent.

5.2 ITP

The recent applications of ITP both in the capillary [106] and in the microchip [107] formats are more frequently dealing with the determination of low-molecular-mass ions than

with the analysis of peptides and other biopolymers. One of the few exceptions is the application of capillary ITP (CITP) to the determination of the purity of lecirelin, synthetic analog of luteinizing hormone-releasing hormone (LHRH) [108]. In addition to the determination of the purity of this peptide, CITP has been used also for quantitation of anionic counterions of this strongly basic peptide. More frequently, ITP or t-ITP modes are employed as a preconcentration and/or preseparation step [66, 68, 109] prior to CE analysis of peptides and proteins present at low concentrations and/or in complex mixtures, such as, *e.g.*, for selective enrichment and ultrasensitive identification of trace peptides in proteome analysis using t-ITP/ZE mode coupled to nano-ESI-MS detection [69] and in combination with carrier ampholyte-based CE (CABCE) mode [67].

5.3 IEF

CIEF is more suitable for evaluation of microheterogeneity of proteins, glycoproteins, and longer polypeptides than for peptides, since the effective charge of short peptides, similarly as that of amino acids and unlike that of proteins, may approach zero value at rather broad pH zone than at a sharp pH value, p*I*. Different ways of p*I* determination of proteins and peptides by CIEF are presented and critically evaluated in the review [110]. Since the direct measurement of pH inside the capillary is unavailable, the estimation of p*I* values has to be based on the use of different types of p*I* markers, dyes, fluorescently labeled peptides, sets of proteins with known p*I* values.

Denaturing CIEF, *i.e.*, IEF in the presence of amphoteric detergents and/or urea, and CIEF with the whole-column imaging UV-absorption detection and with the liquid-core waveguide LIF whole-column detection have been employed for investigation of protein/polypeptide conformational and chemical microheterogeneity, for characterization of proteins with identical p*I* values and for analysis of proteins and polypeptides of different origins, such as naturally fluorescent phycobiliproteins and noncovalently (NanoOrange) labeled proteins, antibodies, and viruses [111, 112].

Due to its concentrating and focusing effect CIEF is frequently used as the first concentrating step in two- or multidimensional separations of complex mixtures of peptides and proteins. 2-D capillary format was realized by on-line hyphenation of CIEF and nongel sieving CE *via* a hollow-fiber membrane interface [113]. 2-D separation, analogous to the classical slab gel IEF/SDS-PAGE is realized by on-line coupling of CIEF with ESI-MS detection [114], which provides both p*I* and M_r values, similarly as classical 2-DE, however, with much higher precision of M_r determination. This system was applied to determination of peptide and protein concentrations using angiotensin II and human tetrasialo-transferrin as the model analytes [114]. The LOD was 0.22 μM, *i.e.*, about ten times lower than that with UV-detection. Human transferrin could be detected with acceptable accuracy and repeatability at physiological concentration levels (0.5–1.2 mg/mL). A 2-D microcolumn platform consisting of on-line hyphenated CIEF in hydroxypropylcellulose coated capillaries and neutral stearyl-acrylate (C17) monolithic CEC has been used for separation of model mixtures of 15 peptides, 15 proteins, and albumin-depleted human serum [115].

In addition to CIEF, also noncapillary solution IEF systems are worth to be mentioned due to their efficient application in the important areas of proteomics and peptidomics. A six-chamber device with isoelectric membranes as a preseparation step prior to HPLC-MS analysis significantly improved peptide detection and identification in the investigation of insoluble nuclear protein fraction [116]. An efficient fractionation and improved peptide and protein identification of human plasma and of an *Escherichia coli* tryptic digest was attained also by peptide-OFFGEL electrofocusing followed by HPLC-MS analysis [117, 118].

5.4 Affinity electrophoresis

Affinity electrophoresis performed in a capillary format, capillary affinity electrophoresis (CAE), is mostly used as a mild and sensitive tool for the investigation of the (bio)molecular interactions and recognition, and for estimation of binding (association) or dissociation constants of the formed complexes [119, 120]. CAE involves several modes based either on the separation of the interacting species, such as in the Hummel-Dreyer method, the vacancy peak method and frontal analysis, or on the detection of a specific physicochemical property of the complexed ligand or the binding partner. All these methods utilize the differences in the migration velocities of the interacting species. Both theoretical and experimental considerations for these methods and their applications for estimation of binding constants have been outlined in the recent review [119], particular recent applications to peptide interaction studies are presented in Section 8.3.

Methodologically new approach for estimation of binding constants of peptide ligands (*N*-acetyl and *N*-succinyl derivatives of a dipeptide D-Ala-D-Ala) to glycopeptide antibiotic receptor (vancomycin from *Streptomyces orientalis*), flow-through partial-filling CAE (FTPF-CAE), has been developed by Gomez and coworkers [121]. In this technique, the capillary is first partially filled with a zone of ligand followed by a sample plug containing receptor and noninteracting standards. After application of a voltage, the receptor and standards flow into the zone of ligand where a dynamic equilibrium is achieved between receptor and ligand. Due to the continued electrophoretic movement, the receptor and standards migrate through the zone of the ligand plug prior to detection. Evaluation of the change in the relative migration time ratio (RMTR) of the receptor, relative to the noninteracting standards, as a function of the concentration of ligand, yields a value for the binding constant, K_b.

In the technique of multiple-injection CAE (MICAE) [122, 123], separate plugs of sample containing noninteracting standards, peptide I, buffer, and peptide II, are injected

into the capillary and electrophoresed. Peptides I and II migrate through the capillary at similar electrophoretic velocities and remain as distinct zones due to the buffer plug between peptides. The electrophoresis is then carried out in increasing concentrations of receptors, macrocyclic antibiotics, vancomycin, ristocetin, and teicoplanin, in the BGE. Continued electrophoresis results in a shift of migration times of the peptides upon interaction with antibiotic. Analysis of the change in the RMTR of the resultant complexes relative to the noninteracting standards, as a function of the antibiotic concentration gives the K_b. The advantage of MICAE technique is that it is able to measure K_b of interaction of ligands of similar mobilities with the receptor without the need of individual binding experiments for each ligand. Other new modification of MICAE is the partial filling MICAE [124], which has been also applied to investigation of the interactions between macrocyclic antibiotics, vancomycin, teicoplanin, and ristocetin, with fluoren-9-ylmethoxycarbonyl (Fmoc)-derivatized D-Ala-D-Ala terminus peptides.

A special type of CAE is the so called kinetic CE [125], which is defined as CE of species, which interact during electrophoretic movement inside the capillary. It represents a platform for development of homogeneous kinetic affinity methods for quantitative affinity measurements (binding and kinetic constants), affinity purification, and search for molecules with affinity to given ligands.

Affinity electrophoresis was carried out also in a microchip format [126] and it was found suitable for high-throughput screening of biomolecular interactions, capable to provide quantitative data on the strength of the interaction with minimum sample and reagent consumption and within a very short analysis time (8–60 s).

5.5 EKC

The latest developments and applications of CEKC, namely its micellar mode (capillary MEKC, CMEKC) are summarized in the recent reviews [127, 128]. CMEKC with ionogenic detergents, anionic SDS, cationic CTAB, or zwitterionic CHAPS, is suitable for separation of electroneutral peptides, *i.e.*, peptides with blocked or derivatized *N*- and *C*-terminus and other ionogenic groups of peptide chain, and/or for separation of peptides having the same or very similar charge to mass ratio but differing in their hydrophobicity.

A partial filling MEKC (PF-MEKC) with a mixed micelle system composed of a zwitterionic surfactant 3-(*N*,*N*-dimethylhexadecylammonium)propanesulfonate (PAPS) and a nonionic surfactant PEG dodecyl ether (Brij 35) has been applied to peptide mapping of BSA [129]. The highly selective separation of tryptic BSA peptides was achieved in an optimized BGE, composed of 50 mM ammonium formate buffer, pH 2.0, and containing 32 mM PAPS and 0.6% m/v Brij 35. CEKC with two polymeric pseudostationary phases, acrylamide- and siloxane-based polymers, provided very high efficiency and good selectivity for separation of NDA-derivatized amino acids with selectivities different from those observed by SDS micelles but these phases were found to be generally unsuitable for separation of NDA-labeled peptides [130].

5.6 Electrochromatography

Being a hybrid electrokinetic and chromatographic technique, CEC of peptides profits from the high selectivity of numerous stationary phases developed for peptide separation by HPLC and from the low dispersion of electroosmotically driven motion of a mobile phase. Another advantage of CEC is that the composition of its mobile phase is more compatible with on-line coupling with MS detection than that of MEKC.

Recent progress in CEC is described in the reviews [131, 132] and in the special issue devoted to advances in CEC and EKC [133].

CEC separations of peptides have been performed in different separation modes, with RP, cationic or anionic stationary phases. Mixed-mode stationary phase bearing an embedded quaternary ammonium group in a C21 alkyl chain has been used both for CEC and HPLC investigation of the chromatographic behavior of synthetic oligopeptides, eledoisins, [Ile-7]-angiotensin III, Arg-Arg-gastrin, rennin substrate, and epidermal growth factor [134]. In HPLC experiments, the variation of ACN content in the mobile phase indicated that peptides are mainly separated by RP mechanism. The weak or negative retention factors observed as compared to C18 silica stationary phase suggested the involment of electrostatic repulsion in acidic conditions. Comparison of HPLC and CEC separations showed that (i) ion-exclusion is more pronounced in HPLC, and (ii) a high ACN content in the mobile phase induced for some peptides an increase of retention in CEC, pointing out the existence of a retention mechanism other than partitioning mainly involved in a chromatographic process. These facts demonstrate that the electric field has an important effect on peptide retention in CEC and that chromatographic retention in CEC cannot be simply predicted from the retention observed, even under identical experimental conditions, in HPLC.

Performance of mixed-mode, RP (C18)/strong cation exchanger (SCX), sorbents for CEC separation of peptides was tested by thrombin receptor antagonistic peptide and its alanine-scan analogs [135]. By modulating the mobile phase composition, the hydrophobic or ion exchange interactions could be made to dominate the chromatographic retention of peptides in addition to their electrophoretic migration. Using this strategy, a high resolution of six closely related synthetic peptides with very small differences both in their charge and hydrophobicity was achieved, however only within a narrow pH range around 6.5 of the mobile phase.

CEC on silica-based RP (C18) at acidic pH and hydrophobic (C18)/strong-anion-exchange (SAX) mixed mode phases at weakly alkaline pH showed that the former was more suitable than the latter for separation of model mixtures of short peptides (2–4 amino acids) [136].

Simple peptide mixtures are preferably separated under isocratic conditions, where significant changes in the separation are achieved by small changes of the individual parameters, such as ionic strength, organic modifier content, and temperature. On the other hand, complex peptide mixtures, such as protein digests often require gradient elution. The advantage of application of stepwise gradient of buffer concentration in CEC with the mixed-mode stationary phase, 3-(4-sulfo-1,8-naphthalimido)propyl-modified silylsilica, was demonstrated by improved and shortened separation of tryptic digests of cytochrome *c* [137].

Pressurized CEC (pCEC) with the possibility to variate a flow rate and to allow continuous gradient elution, where the mobile phase is driven by both EOF and pressurized flow, facilitating fine tuning in selectivity of neutral and charged species, has been shown to be more efficient for the separation of model oligopeptides compared to the isocratic CEC using 100 μm id capillary column packed with 3 μm size silica particles with C18 RP [138].

Monolithic materials are becoming a well-established stationary phase format for CEC. Both the simplicity of their *in situ* preparation and the large number of readily available chemistries make the monolithic separation columns an attractive alternative to the capillary columns packed with particulate materials. The current state-of-the-art in this rapidly growing area of CEC including its application for peptide separations is summarized in the recent reviews [139, 140].

A novel monolithic column for CEC separation of oligopeptides was prepared using L-phenylalanine as a template and a covalent approach through the formation of Schiff base with orthophthaldialdehyde (OPA). The reaction mixture was introduced into the capillary and after the thermal polymerization the template was extracted with methanolic HCl solution. The capillary column 75(50) cm × 75 μm id with a mobile phase of 40 mM phosphate buffer (pH 7.0)/methanol 5% v/v, applied voltage +15 kV, and detection at 214 nm, could separate several oligopeptides, such as angiotensin I and II, oxytocin, vasopressin, tocinoic acid, β-casomorphin, within 20 min, see Fig. 4. The CEC separation of this set of peptides was meadiated by a combination of chromatographic retention involving hydrophobic, hydrogen bonding, electrostatic interactions as well as Schiff base formation with OPA in the cavity of the templated polymer and also by electrophoretic migration of charged peptides.

A neutral, nonpolar stearyl-acrylate monolithic column providing a relatively strong EOF but being free of electrostatic interactions with charged solutes was developed for the RP-CEC of neutral and charged species including peptides and proteins [141]. Another mixed-mode *n*-alkyl methacrylate-based monolith has been used for separation of therapeutic peptides [142]. While the sulfonic acid (SCX) moiety supported the generation of a stable EOF at both acidic and basic pH, the butyl ligands provided the nonpolar sites for chromatographic resolution.

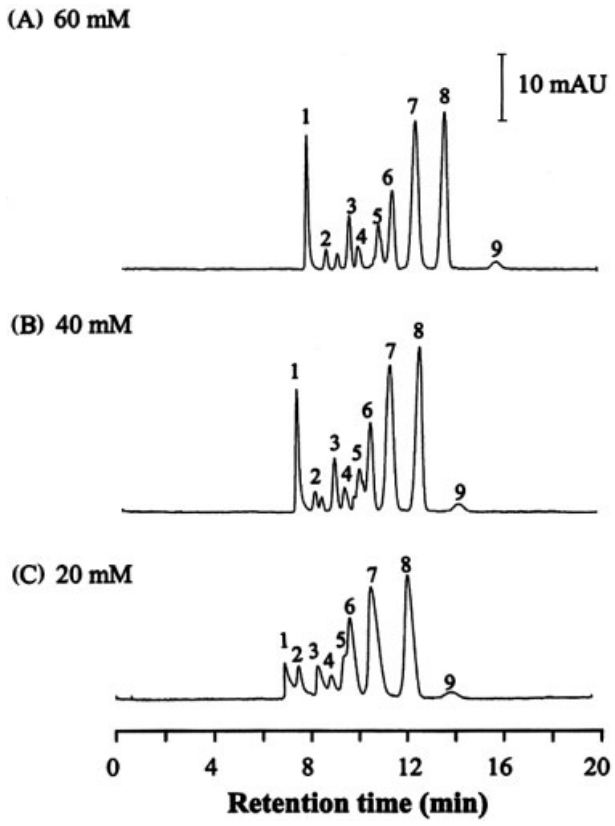

Figure 4. Capillary electrochromatograms of oligopeptides at various ionic strengths of mobile phase. Column: FS capillary monolith with phenylalanine as template, 75 cm (50 cm) × 75 μm; hydrostatic injection (10 cm, 10 s). Mobile phase: phosphate buffer (pH 7.0); voltage +15 kV; UV-detection at 214 nm. Peak identification: 1, tetrapeptide FMRF; 2, oxytocin; 3, angiotensin-ST; 4, vasopressin derivative; 5, angiotensin-I; 6, angiotensin-II; 7, bovine β-casomorphin fragment; 8, tocinoic acid; 9, human β-casomorphin. Reprinted with permission from [255].

Another CEC mode, open tubular CEC (OT-CEC), which is based on the attachment of thin layer stationary phase to the inner capillary wall [143], is also used for separation of peptides and proteins. Novel type of capillary columns for OT-CEC are based on etched chemically modified FS capillaries. Fabrication, properties, and applications of these columns have been recently reviewed by pioneers of OT-CEC, Pesek *et al.* [144]. Etching of the inner capillary wall is performed by heating the capillary at temperature of 300–400°C in the presence of ammonium hydrogen difluoride. Etching process substantially (*ca.* 1000-fold) increases the surface area, which alleviates the relatively low capacity of the bare FS capillary, and creates a surface that is fundamentally different from the bare FS. After the chemical modification of the surface, the bonded organic moiety forms the stationary phase for OT-CEC. Etched FS capillaries chemically modified with *N*-butylphenyl and cholesterol-10-undecanaoate [145] have been used for OT-CEC separation of two sets of peptides, each having structurally

similar amino acid sequences, and the resolving power of the OT-CEC was found to be superior in comparison with gradient RP-HPLC.

OT-CEC in FS capillaries with covalently attached G-quartet-forming DNA oligonucleotides provided better resolution of fibrinogen peptides when G-quartet structure was destabilized by increase of temperature from 25 to 35–40°C [146]. The applications of (metallo)porphyrins-based physically adsorbed or chemically bonded stationary phases for OT-CEC separations of peptides, amino acids, and other analytes have been reviewed by Deyl *et al*. [147].

5.7 Multidimensional separations

In spite of the high separation power of individual CE and CEC modes, for complete separation of complex peptide and protein mixtures present in body fluids, tissue extracts and enzymatic digests of large proteins, apart from super-complex mixtures of proteome or peptidome of different organisms, cells and organelles, a combination of two or more complementary separation principles, such as IEF and SDS-PAGE in the 2-DE [148], is necessary.

The increasingly important role of multidimensional peptide separations in proteomics and peptidomics, *i.e.*, in comprehensive analysis of all or part of protein and peptide content in the cell, organ or biofluid, has been emphasized in recent reviews on this topic [1, 149]. The ultimate goal is to have a rapid separation system that can provide identification and comprehensive monitoring of the changes in concentration, interactions, and structures of proteins and peptides in the proteome and peptidome.

Two- or multidimensional peptide separations are based on utilization of two or more independent physical properties of peptides to separate their mixtures into individual components. When the properties are truly independent, the separation methods can be considered as "orthogonal" and the peak capacity of this multidimensional separation is approximately equal to the product of the individual peak capacities of each dimension.

Different combinations of separation principles are used for 2-D separations of peptides and proteins, such as LC-CE, CE-CE, CE-LC, where LC and CE represent different modes, so that many different combinations are available, such as, *e.g.*, RPLC-CZE, ion-exchange chromatography (IEC)-CZE, SEC-CZE, RPLC-CIEF, affinity LC-CEKC, CZE-CIEF, CZE-CEKC, CEKC-CZE, CITP-CZE, CZE-CGE, CIEF-CGE, CZE-capillary LC (CLC), and CIEF-CLC. When these 2-D systems are on-line connected with MS or MS/MS detection, then really powerful multidimensional systems are obtained capable to identify hundreds up to two thousands of peptides/proteins in complex mixtures such as body fluids, tissue extracts, and cell lysates. For more details see the above mentioned reviews [1, 149], here only few examples will be presented.

A high-speed 2-D CE system with a compact fluorescence detector for high-sensitivity peptide and protein analysis has been developed by Dovichi's group [150]. Fluorescently labeled proteins/polypeptides are separated according to their size by CZE in a replaceable 5% dextran sieving matrix in BGE composed of 100 mM Tris/CHES, 3.5 mM SDS, pH 8.7, in the first dimension. The second-dimensional separation is performed by MEKC in an on-line connected second capillary containing 100 mM Tris/CHES BGE with 15 mM SDS micellar pseudophase. As soon as the first peptide/protein zone approaches the capillary outlet, the fractions from the first capillary are successively transferred to a second capillary, where they are separated according to their hydrophobicity to generate, in serial fashion, a 2-D electrophoregram. The comprehensive 2-D separation involving analysis of 250 fractions lasted 60 min. The system has been applied to 2-D protein/polypeptide fingerprinting of Barrett's esophagus tissues. The same combination of sieving CE and MEKC modes has been used also in the multiplexed 2-D CE system which allows separation of five samples in parallel [151]. Samples are injected into five first-dimensional capillaries, the fractions are transferred across an interface to five second-dimensional capillaries and analytes are detected by LIF in a five-capillary sheath-flow cuvette. The instrument produced LODs at the zeptomole level for 3-(2-furoyl)quinoline-2-carboxyaldehyde-labeled trypsin inhibitor.

Another CE-based 2-D orthogonal separation system, separating proteins/polypeptides according to p*I* by CIEF in the first dimension and according to the M_r in the second dimension by a non-gel sieving CE, employes a hollow-fiber membrane interface for coupling the two capillaries [113]. 2-D microcolumn platform consisting of on-line coupled CIEF in hydroxypropylcellulose-coated FS capillaries and neutral stearyl-acrylate (C17) monolithic CEC has been developed by Zhang and El Rassi [115] and applied to separations of model mixtures of 15 peptides, 15 proteins, and albumin-depleted human serum. The theoretical peak capacity of this orthogonal CIEF-CEC platform was estimated as more than 50 000.

SEC and CE have been on-line coupled *via* SPME and T-split interface, see Fig. 5. This 3-D system was applied to peptide analysis in biological fluids [152]. The SEC column fractionated the sample by M_r; the low-M_r fraction containing peptides is directed to a C18 SPME column, where the peptides are captured and concentrated. The peptides are desorbed from SPME column by 425 nL ACN, and the effluent is introduced into the T-split interface, which hydrodynamically splits (1:40) the flow and in this way it ensures appropriate injection of analytes into the CE system. The system enabled determination of enkephalins in CSF with LODs of about 10 ng/mL and good linearity over 0.25–100 μg/mL range for injection of 20 μL spiked CSF samples.

Since recently there are attempts to integrate multidimensinal separations on chips. An integrated protein/peptide concentration/separation system combining non-native IEF with SDS gel electrophoresis has been constructed on a polymer microfluidic chip with planar dimensions as small as 2 cm × 3 cm [153]. Instead of time-consuming sequential sampling in classical capillary systems, the zones of concentrated and focused proteins are electro-

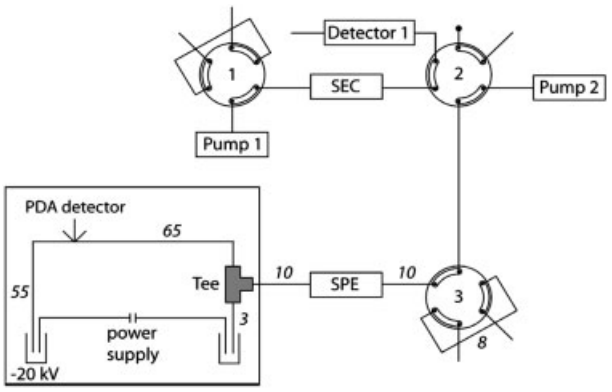

Figure 5. Schematic diagram of the on-line SEC–SPE–CE system with the T-split interface. The CE part of the system is framed. Lengths of FS capillaries (75 μm id) are shown in italics (cm). All other capillaries are made of PEEK (125 μm id). Reprinted with permission from [152].

kinetically transferred into an array of orthogonal microchannels and further resolved by SDS gel electrophoresis in a parallel and high-throughput format. Resolved proteins are monitored by noncovalent, environment-sensitive fluorescent probes such as Sypro Red. A comprehensive 2-D separation is accomplished within 10 min with an overall capacity of about 1700 spots.

Combination of affinity selection of specific peptides, *e.g.,* histidine-containing peptides by immobilized metal affinity chromatography, cysteine-containing peptides by covalent chromatography, glycopeptides by lectin chromatography with subsequent CE or CE-MS separation and detection of these specific peptides represent other examples of off-line multidimensional separations applicable in proteomics studies [154].

MS and MS/MS can be considered as the second and third separation dimensions when used as detection mode in CE or CEC; these set-ups represent a special and numerous class of multidimensional separations, for review see, *e.g.* [17, 155, 156], and Section 6.3. Different aspects of CE-MS analysis of peptides and proteins have been recently thoroughly discussed and reviewed [17, 155]. From the different MS ionization modes, involving atmospheric pressure chemical ionization (APCI), inductively coupled plasma (ICP), FAB, ESI, and MALDI, the last two modes, ESI, and MALDI are most suitable and mostly used for peptides and proteins. Combination of advanced MS technique, Fourier transform ion cyclotron resonance (FT-ICR), with CE or CIEF is suitable for high-throughput analysis of complex peptide/protein mixtures in proteomics [157].

On-line CIEF-ESI-MS was applied to quantitative analysis of peptides and proteins [114]. CIEF-MS using 1% v/v Pharmalyte 3–10 and a sheath liquid containing water/methanol/acetic acid (50/49/1) was capable to resolve angiotensin I and II (differing by ΔpI 0.2) with resolution of 2.3 and to determine human tetrasialo-transferrin in the 0.2 mg/mL concentration.

An extremely powerful 3-D separation system consisting of on-line combination of RP-HPLC, CZE, and ESI-FT-ICR-MS detection has been developed by Bergquist's group [157]. The special on-line interface for coupling LC and CE is a flexible device made of PDMS, which can be used with conventional capillaries, allows coupling to any kind of MS detector and provides high injection repeatability (RSD <3.5 %). The applied FT-ICR-MS detector provides ultrahigh resolution of detected ions, mass accuracy at ppm level, and high sensitivity. Sequence coverage for BSA of 93% showed a high recovery of sample in the different transfer steps through the system. The LOD for peptide identification is in the low picomole range. The system seems to be the most qualified and promising for analysis of complex biological samples, such as those in proteomics and peptidomics.

Another 3-D system, an on-line SPME-CE-MS setup, was developed by Tempels *et al.* [158]. Analytes are preconcentrated using a C18 microcolumn (5 mm $\times$ 0.5 mm) and then introduced into the CE system *via* a valve interface. The CE is connected with ESI-IT-MS using a coaxial sheath-liquid sprayer. The potential of the system was demonstrated by analysis of CSF samples spiked with enkephalins and by separation of tryptic peptides of cytochrome *c*.

Immunoassays combined with CE can be also considered as multidimensional separation systems providing both selectivity and sensitivity, which is competitive with any method currently available for molecular analysis. Immunoassays are mostly coupled with CE in the precapillary off-line mode. In the competitive immunoassays sample antigen is incubated with labeled antigen or antibody and the amount of free or bound antigen or of free or bound antibody is determined by CE. For recent advances of CE-immunoassays see [159].

6 Detection

6.1 UV absorption and interferometry

The relatively strong absorption of a short-wave UV radiation (200–220 nm) by peptide bond allows application of UV-absorption detection at this wavelength range as a universal detection principle for monitoring peptide separations in CE and CEC with LODs in the micromolar range in a *ca.* nanoliter detection cell volume with millisecond time resolution. The earlier developed special detection cell constructions with increased optical path and thus enhanced sensitivity (Z-shaped cell, buble cell, or sleeve cell), and some new designs of the UV-absorption detector for capillary and chip separations are described in the recent reviews on optical detection schemes [160, 161].

Applications of a new type of optical detector, based on the whole-column imaging detection system working alternatively in absorption, refraction index, and fluorescence mode can be found in refs. [111, 112, 162]. The 5 cm section of the FS capillary with removed polyimide coating is illumi-

nated by the light guided from the light source (deuterium lamp, xenon lamp, He-Ne laser, diode laser) by an optical fiber bundle and focused by cylindrical lens to the capillary. The intensity of light after passage across the capillary is measured by a CCD camera, placed in the direction of illuminating light for absorption or refractive index gradient mode, or placed perpendicularly to the direction of the illuminating light for the fluorescence mode. Absorption mode was found to be the most practical due to its quantitative ability and universal characteristics not only for the CIEF of proteins and peptides, for which it was originally developed in order to eliminate the disturbing mobilization step of CIEF required for single point detection after focusing process, but also for CZE separation of these and other analytes allowing to study the dynamics of electroseparation processes.

A new type of UV-absorption detector, utilizing a complementary metal oxide semiconductor (CMOS) active pixel sensor, has been constructed for real time visualization of electrophoretically mediated in-capillary reactions [163]. Imaging of analyte peaks absorbing at 200 nm and migrating over the length of 14 mm in the capillary dimensions enabled measurement of velocities and lengths of reactant and product zones. The system was used for monitoring glutathione oxidation by hydrogen peroxide. CMOS imaging allowed the whole process of reaction, separation, and quantification to be perfomed in nanoliter volumes on-capillary in single run within *ca.* 5 min.

Enhanced sensitivity of peptide detection at ultralow UV region below 200 nm has been utilized, *e.g.*, in CZE determination of zinc-bacitracin in feedstuff when this peptide antibiotic was detected at 192 nm at low μg/mL level [164]. A universal optical detector based on dual capillary backscatter interferometry with density gradient compensation has been constructed for refractive index measurements in nanoliter volume for microchip CE with sensitivity at the femtomole level [165].

6.2 LIF

LIF is the most sensitive detection scheme in CE, CEC, and other microanalytical systems as documented in the recent reviews on advances in optical detection methods [160, 161]. With special designs of detection cells, such as liquid sheath-flow cuvettes and integrated separation and detection devices, LIF detection has a potential to achieve the detection limit of few or even of a single molecule [166]. The latest developments and applications of LIF detector in CE and CEC of peptides are summarized in comprehensive reviews [167, 168].

The disadvantage of the LIF detection of peptides is the necessity of their derivatization by fluorogenic labels. The native fluorescence can be utilized only for detection of peptides containing aromatic amino acid residues of Trp, Tyr, and Phe. However, for their excitation, deep UV-laser systems, such as Nd/YAG laser operating at 266 nm, or multiphoton excitation are necessary. The latter approach has been utilized in a compact LIF detector based on two-photon excitation of the native fluorescence of proteins and peptides containing the above mentioned aromatic amino acids [169]. The fluorescence is excited by a solid-state diode pumped microchip laser operating at 532 nm, with an average power 29.3 mW, a pulse width 500 ps, repetition rate 6.56 kHz, and beam diameter 0.2 mm. Concentration LODs for free amino acids, Phe, Tyr, and Trp, were 62 μM, 2.0 μM, and 470 nM, respectively, in a volume of 3 fL, for BSA the limit was 130 nM.

A multichannel native fluorescence detection system has been constructed for analysis of amino acid and peptide neurotransmitters in single neurons [170]. A hollow-cathode metal vapor HeAg laser emitting at 224 nm is used as excitation source. Fluorescence is collected orthogonally to the excitation optics with an MgF_2-coated, all-reflective microscope objective with a 0.4 numerical aperture and spectrally distributed by a series of dichroic beam-splitters into three wavelength channels: 250–310 nm, 310–400 nm, and >400 nm. A separate photomultiplier tube is used for detection of the fluorescence in each of the three wavelength ranges. With this detector, analytes can be separated and identified not only by their electrophoretic mobilities but also *via* their multichannel signature consisting of the ratios of relative fluorescence intensities in each wavelength channel. The LODs for neurotransmitters are in the low nanomolar (attomole) range.

More frequently, fluorescence detection of peptides in CE and CEC is based on precolumn or postcolumn derivatization of these analytes with a fluorescent label (see Section 3.2). The disadvantage of this approach is that due to the usual presence of multiple derivatization sites in peptide and protein molecules, several derivatives with different electrophoretic mobilities and consequently multiple peaks may be obtained for originally single peptide or protein species.

CIEF with the liquid-core waveguide LIF whole-column detection described in more detail in the above Section 6.1 on UV-absorption detection has been applied for separation and analysis of several different proteins and polypeptides, for microheterogeneity characterization, and p*I* determination of antibodies, glycoproteins, and viruses [111, 112, 162].

A high-brightness blue light-emitting diode (LED) serving as the excitation source at 470 nm and an optical fiber collecting and guiding the emitting fluorescence to photomultiplier tube were used in the combined LED-induced fluorescence and contactless conductivity detection applied for analysis of FITC-labeled amino acids and petides [171]. A home-made instrument with a frequency-doubled Nd/YAG laser operating at 532 nm was used as excitation source for LIF detection of rhodamine B isothiocyanate-derivatized amino acids and peptides (bradykinin, angiotensin, and insulin) [172].

6.3 MS

MS represents an extremely powerful detection principle for CE, CEC, and other separation techniques because of its universality, sensitivity, and selectivity, as evidenced by

impressive recent developments of MS, CE-MS, and CEC-MS methodologies in general [156, 173] and in analysis of peptides and proteins especially [17, 155]. Importance of MS as analytical and structure elucidation method has significantly increased in the last years due to its key role in the proteomics-associated analysis of complex peptide and protein mixtures [174–176]. Consequently, the importance of both on- and off-line coupling (hyphenation) of MS with CE and CEC separations of peptides and proteins, especially in proteomics, peptidomics, and peptide mapping, is also growing, as shown in some reviews [155, 17, 177].

Especially introduction of ESI and MALDI ionization techniques brought tremendous progress in on-line and off-line characterization of electrophoretically separated peptides and proteins by MS [17]. Combination of CE with ESI-MS and MALDI-MS allows not only high-accuracy determination of M_r of CE separated peptides, proteins, and other biomolecules, but also provides important structural data on amino acid sequence, the sites of post-translational modifications, peptide mapping, and noncovalent interactions of peptides and proteins.

ESI is the preferred mode for on-line coupling CE with MS, namely because of its capability to generate the ions with multiple charges so that the ion m/z (mass/charge) values for even very large species as polypeptides and proteins may fall within the limited m/z detection range of most mass spectrometers. A novel, rugged sheathless CE- and CEC-ESI interface, in which an open tubular separation capillary and an electrospray tip are integrated with a Nafion tubing junction, is coupled to MS detector has been developed for CE and CEC separations of amino acids and peptides [178]. A stable electrospray was generated at nanoflow rates by applying a positive electric potential at the Nafion membrane junction. To sustain the stable spray, an EOF to the spray was supported by coating the FS capillary with Lupamin, a high molecular-mass linear positively charged polyvinylamine polymer, which also serves as stationary phase for OT-CEC separation of peptides and proteins.

A simple laboratory-made pressurized liquid junction nanoflow interface for CE-IT-MS was developed and applied to separation and analysis of peptides and tryptic protein digests [179]. In this device, the FS capillary and the spray tip were positioned in the electrode vessel containing the appropriate spray liquid. The electrospray potential was applied on the electrode inside the liquid junction. A stable electrospray was produced at nanoliter *per* minute flow rates generated in the emitter tip by hydrostatic pressure of the spray liquid, see Fig. 6. Another sheathless interface was developed with a graphite-coated ESI tip attached to the separation capillary and has proved to be suitable for CE-ESI-MS separation and characterization of therapeutic peptide hormones [180].

MALDI-MS with TOF mass analyzer (MALDI-TOF-MS) is combined with CE and CEC separations of peptides and proteins more in an off-line mode than with an on-line liquid sample delivery connection [173]. The target plate specifically designed for the CE fraction collection and a semiautomatic fabrication of a prestructured MALDI target coated with silicone has been developed for increased sensitivity in MALDI-TOF-MS analysis of hydrophobic peptides [181]. The small spot size diameter (*ca.* 0.5 mm) and the spot pattern design were compatible with the fraction collection from CE. The matrix, α-cyano-4-hydroxycinnamic acid, was applied on the target plate prior to the CE fractionation, and the fraction deposition was performed directly onto the MALDI target. Using this procedure, nine highly hydrophobic peptides from cyanogen bromide digest of integral membrane protein bacteriorhodpsin were CE-separated, fractionated and off-line detected by MALDI-TOF-MS.

The main advantage of MALDI, soft ionization, and generation of predominantly single charged molecular ions of even polypeptide and protein macromolecules with M_r up to 300 000 is used for the exact determination of M_r with an accuracy of $\pm 0.1\%$ and for identification of peptides and proteins isolated by micropreparative CZE (see Section 8.2). MALDI matrices with high acid concentrations afford enhanced tolerance of CZE buffers to be used for introducing peptides to the mass analyzer.

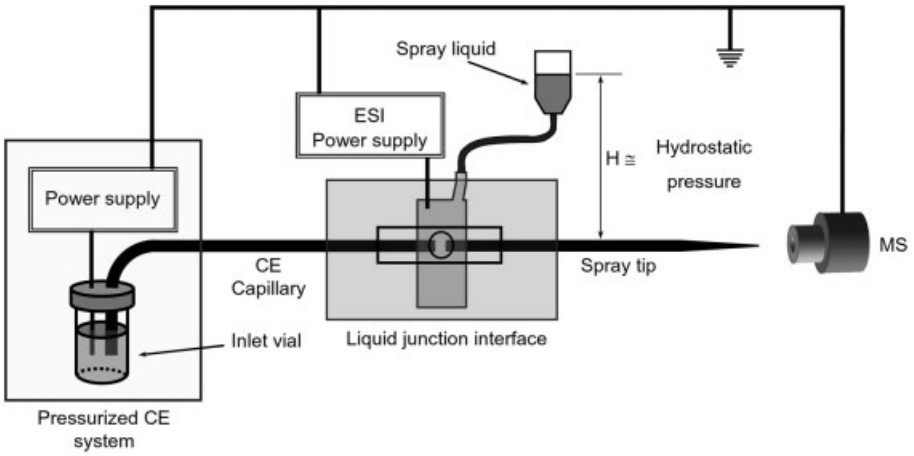

Figure 6. Scheme of the liquid junction interface. Reprinted with permission from [179].

The MS detection enables to detect and characterize also the nonpeptidic parts of peptides and proteins, such as nitration [182] and glycosylation [183]; *e.g.*, sialylated glycopeptides contained in HPLC fractions of tryptic digest of bovine α1-glycoprotein were separated from asialopeptides by CE. CE effluents were deposited directly onto a metallic target and their off-line MALDI-TOF-MS analysis allowed characterization of four glycosylation sites in the glycoprotein. In addititon, MS/MS was used to confirm peptide sequences and glycan content in the glycoforms. MS/MS detection systems are in general applied to determination of amino acid sequence of CE or CEC separated peptides and proteins [184]. A new technique, (CE-MS/MS)(n), in which multiple CE-MS/MS subanalyses (injections followed by analyses) are performed and experimental variables, such as BGE composition and temperature, are manipulated during each CE-MS/MS subanalysis, has been developed in order to maximize amino acid sequence coverage of complex peptide and protein mixture [185].

6.4 Other detection schemes

Other detection schemes, such as electrochemical, contact or contacless electric conductivity, and NMR, are much less suitable and/or were much less applied to peptide detection in CE and CEC in the reviewed period as compared to optical and MS detection. For that reason the reader is referred to the general recent reviews dealing with developments and applications of the former detectors, electrochemical [161, 186, 187], conductivity [188, 189], and NMR [190, 191], where few particular examples of application of these detectors for peptide CE analyses are mentioned. One of them is, *e.g.*, the electrochemical detection of Met-enkephalin, Leu-enkephalin, and [D-Ala-2]-Leu-enkephalin separated by NACE in 10 mM ammonium acetate in ACN/methanol BGE [105]. Using a Pt microdisk electrode with disk diameter 60 μm set to an actual potential +0.65 V (reference electrode Ag/AgCl), LODs in the submicromolar range were observed, *i.e.*, about one order of magnitude lower as compared to UV-detection.

7 Separation in microfluidic devices

CE and CEC separation modes performed on microfabricated microfluidic devices – microchips – represent the platform for a new generation of miniaturized analyzers. In these devices all operations, sample preseparation, preconcentration, mixing, derivatization, separation, and detection, are fully integrated and automated in the above mentioned μTAS or "lab on a chip". The μTAS undergo a period of a rapid and intensive developments, as demonstrated in the recent reviews [79, 132, 192]. They are considered to become the most powerful tools of analytical chemistry in the coming years with a broad application in life sciences, biotechnology, and drug development, particularly in genomic, proteomic, and metabolomic research requiring fast, high-efficient, high-sensitive, and high-throughput separation and characterization of nucleic acids, proteins, peptides, and other biomolecules [193].

An example of a functional model of μTAS is a microchip CE device with on-line microdialysis sampling and on chip-sample derivatization by NDA and 2-ME for LIF detection, which was developed to monitor peptides and amino acids in samples of biological origin, such as microdialysates from brain [77].

CE separations of FITC-derivatized peptide neuromediators, oxytocin, bradykinin, and enkephalins, performed in elastomer (PDMS-PDMS) and hybrid (PDMS-glass) microfluidic devices have shown the perspective of chip CE for analysis of peptides and neuropeptides in small volumes [194]. Using the on-chip electrokinetic sample stacking and LIF detection the fast and effective peptide separations were achieved within tens of seconds at injection volumes of about 100 pL with plate numbers of up 22 000.

Applicability of microchips to affinity electrophoretic separation was demonstrated by investigation of the noncovalent interactions between neurotransmitters and sulfated β-CDs in a commercially available quartz microchip with UV-absorption detection and in a home-made chip station with electrochemical detector [126]. In spite of the fact that microchip CE provided less precise data than classical CAE, in the future, more applications of chip CAE for high-throughput screening and combinatorial chemistry can be expected.

Several types of microchips have been developed for integration of electrophoretic separations of peptides and proteins with ESI-MS detection *via* special, low dead volume and liquid junction connection and for integrated preconcentration and derivatization procedures, micromanipulation and multidimensional separations, see Sections 3 and 5.7, respectively. The microfabricated device has been constructed even for continuous free-flow arrangement of ZE, ITP and IEF [195]. However, due to the limited preparative capacity of theses devices, they are more suitable for continuous monitoring of selected analytes than for really preparative purposes.

8 Applications

8.1 Analysis

8.1.1 Quality control and determination of purity

Peptides are broadly utilized in many fields of biological, biochemical, and biomedical research as well as in biotechnology, pharmaceutical, food, and feed industry, *e.g.*, in the investigation and modeling of the interactions of hormones with receptors, enzymes with substrates and inhibitors, antigens with antibodies, in the mapping of antigenic

determinants (epitopes) of proteins, and in the production of peptide drugs and food and feed additives. This results in enlarged need for CE and CEC application to quality control and purity determination of peptide preparations.

In the majority of the above applications of synthetic, from natural material isolated or biotechnologically prepared peptides, CE and CEC can be employed as sensitive control methods for the determination of their purity, or as a control method of the efficiency of the other, mainly chromatographic separation methods used for their purification. CE and CEC provide rapid and accurate qualitative and quantitative data about the peptide preparations. Further some recent CE and CEC applications to peptide analyses will be given, in addition to those mentioned already in the previous sections on methodology and instrumentation.

CZE has proved to be powerful tool for the quality control/quality assurance of many types of drugs, including peptide and peptidomimetic drugs in the pharmaceutical industry. CZE in acidic BGE, 100 mM phosphoric acid, 50 mM Tris, pH 2.5, with UV-absorption detection at 206 nm was applied to determination of purity degree of lecirelin, synthetic decapeptide analog of LHRH, used as verinary drug for ovulation in livestock, for treatment of ovarian cysts and for improvement of conception rates [108]. Example of CZE analysis of a crude and HPLC-purified preparation of lecirelin is shown on Fig. 7. A macrocyclic glycopeptide antibiotic vancomycin in innovative microparticles and in commercial formulations has been analyzed by fast CZE method using short-end capillary injection (effective length 8.5 cm, total length 18.5 cm) in 12.5 mM phosphate BGE, pH 2.5 [196].

Purity of synthesized glutathione, and its related tetrapetide analogs and their stability and antioxidant activity in different solutions, water, a physiological solution, phosphate buffer, copper(II) sulfate solution, and hydrogen peroxide solution, was checked by MEKC using 25 mM sodium phosphate as BGE and 50 mM SDS as micellar pseudophase [197]. MEKC was found to be superior method for this application over RP-HPLC, since the latter techniques failed in the separation of monomers and dimers of these peptides. Microheterogeneity of peptaibol alamethicin F30, 20-residue peptide isolated from the culture broth of the mold *Trichoderma viride*, was analyzed by NACE-MS using 12.5 mM ammonium formate in methanol, apparent pH 7.4, as BGE, and IT and TOF as mass analyzers [198].

CZE has been applied to purity determination and characterization of synthetic analogs and fragments of [Leu-5]- and [Met-5]-enkephalins and dalargin, bioactive peptides with opiate activity [199]. These oligopeptides (dipeptides to hexapeptides) were analyzed as cations in two acidic BGEs (100 mM H_3PO_4, 50 mM Tris, pH 2.25, and 100 mM iminodiacetic acid, pH 2.30), and some of them as cations and others as anions in alkaline BGE (40 mM Tris, 40 mM Tricine, pH 8.10). Purity degree of these peptides, expressed in three different ways (relative peak height, relative peak area,

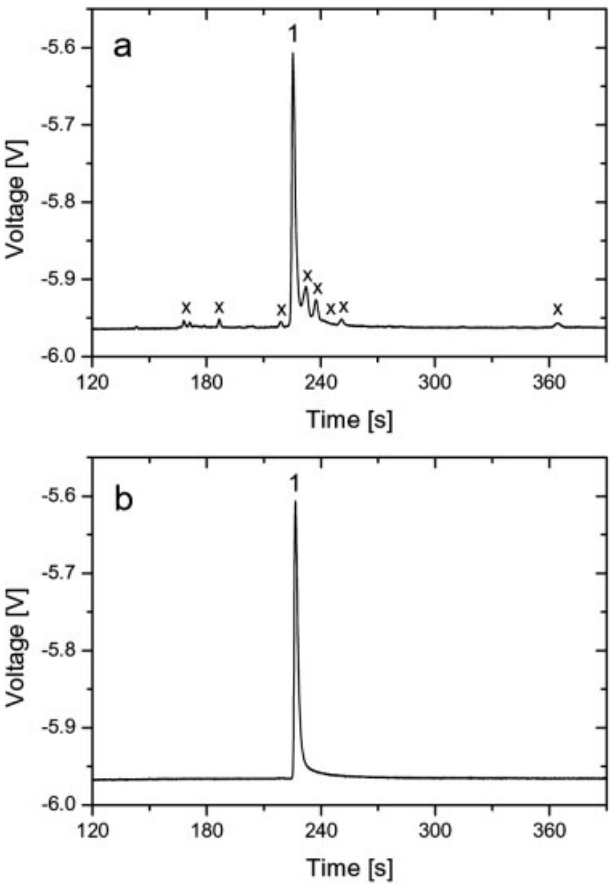

Figure 7. CZE analyses of crude preparation (a) and HPLC-purified preparation (b) of lecirelin, veterinary peptide drug. 1, Lecirelin; x, nonidentified admixtures. Conditions: FS capillary, 300/190 mm, id 50 μm; BGE: 100 mM H_3PO_4, 50 mM Tris, pH 2.25; voltage 10.0 kV; and UV-detection at 206 nm. Reprinted with permission from [108].

and relative corrected peak area), was evaluated, and their effective mobilities at standard temperature 25°C were determined.

Series of opioid peptides, enkephalins, endomorphin I, and dynorphin A were analyzed by CZE with on-line ESI-MS detection in positive ion mode [25] using volatile BGE composed of 50 mM acetic acid, 50 mM formic acid, pH adjusted by ammonium hydroxide to 3.5. The great advantage of MS detection is that from the obtained MS spectra their exact M_r can be determined (see Fig. 8), their quality (identity) can be confirmed or in the case of MS/MS their structure can be elucidated.

For the full characterization of peptide preparations, especially pharmaceuticals and peptides used in biological tests, it is necessary to know also the content of their low-molecular mass ionic admixtures, *e.g.*, fluorides, acetates, and trifluoracetates present as counterions in basic peptides, originating from the synthesis or purification of peptide products. One example of such application is the determi-

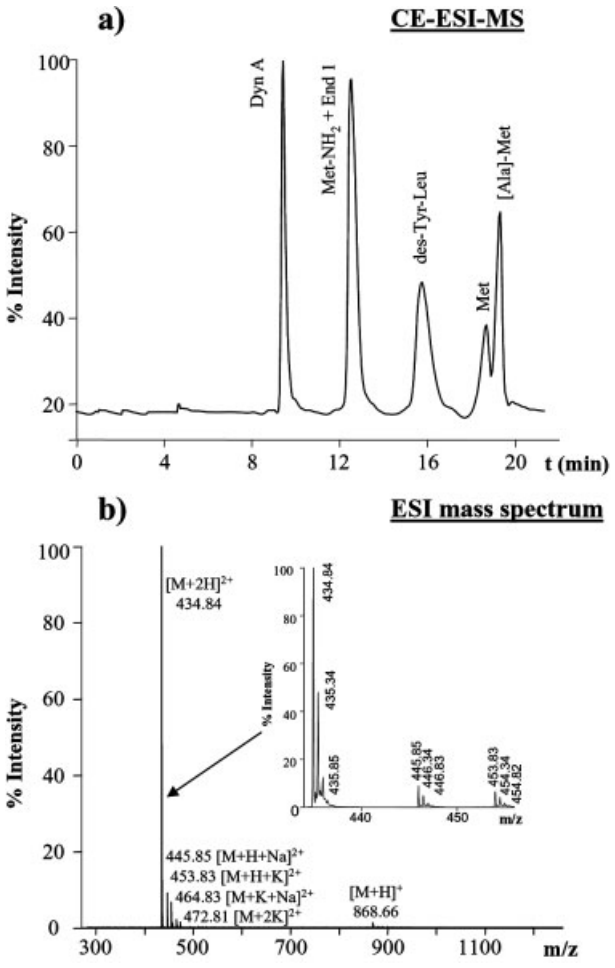

Figure 8. (a) CE-ESI-MS electropherograms of a mixture containing of opioid peptides, using a volatile BGE composed of 50 mM acetic acid, 50 mM formic acid, pH 3.5; (b) ESI mass spectrum obtained for dynorphin A (1–7). Reprinted with permission from [25].

nation of the contaminating anionic counter-ions (acetates, trifluoroacetates, and trifluoromethanesulfonates) of the strongly basic peptide drug, lecirelin, by anionic CITP, using 10 mM HCl/His, pH 6.0 as leading electrolyte, and 10 mM MES, pH 4, as terminating electrolyte [108].

8.1.2 Determination in biomatrices

Thanks to a high sensitivity of some detection schemes, particularly LIF, electrochemical, and MS, frequently enhanced by on-line sample enrichment techniques, CE and CEC methods are applicable also to the analysis of peptides present at low concentration levels in complex biomatrices, such as biological fluids, cell lysates, and tissue extracts.

Several CE methods have been developed for determination of glutathione (GSH) [200], an extremely important biopeptide acting as antioxidant to prevent and limit oxidative damage of proteins in most living cells and participating in the reduction of disulfides and other molecules. Hence, its determination in cells, tissue extracts, and body fluids is of great importance. A new CE method with LIF detection was developed for the rapid separation and sensitive detection of GSH and glutathione-disulfide (GSSG) after derivatization by 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl) [201]. Under the optimized experimental conditions, in 20 mM sodium borate, 4 mM β-CD, pH 9.25 BGE, linear relationships between the peak height and concentrations of the analytes in normal and second-derivative electropherograms were obtained (0.22–45.00 μM). The LODs for GSH and GSSG in normal and second-derivative electropherograms were 0.046 and 0.012 μM and 0.046 and 0.014 μM, respectively. The sensitivity of the method was sufficient for determination of GSH and GSSG in human plasma and tobacco leaves. The other CE-LIF techniques were applied to simultaneous and rapid determination of GSH and reactive oxygen species in individual red blod cells [202, 203], in apoptotic leukemia cells [204], and in probiotic bacteria [205].

Validated CZE methods, using 200 mM or 350 mM sodium borate, BGEs, pH 8, with UV-detection at 200 nm, have been applied to determination of GSH and GSSG in order to monitor oxidative stress and response to antioxidative treatments in an animal model, such as the rat made diabetic by streptozotocin injection [200]. A new CZE method has been developed for determination of GSH and GSSG in microdialysis samples [61]. The analysis was performed in reversed EOF mode using cationic surfactant, 0.5 mM TTAB in 100 mM ammonium chloride BGE, pH adjusted by NaOH to 8.4. A special, on-column preconcentration technique, pH-mediated base stacking, allowed that even medium-sensitive UV-absorption detection at 214 nm was sufficient for detection of GSH and GSSG in liver microdialysates of anesthetized rats with submicromolar detection limits. On the other hand, the content of GSH in must and wines was assayed by CE with LIF detection [206]. Sample preparation involved conjugating of GSH and other potential thiols with monobromo-bimane (MBB) in CHES buffer. The derivatized GSH and other main nonvolatile thiols were separated in 50 mM phosphate BGE and detected by LIF using diode laser with excitation wavelength 410 nm (close to absorption maximum of MBB-GSH adduct, 390 nm) and measuring the emission of thiol MBB-derivatives at 482 nm. The method was used for monitoring the changes in the reduced GSH content in white wines during alcoholic fermentation and barrel aging. The detection limit for GSH was 65 nmol/L, which is much lower than its average concentration in must and wine.

MBB derivatization has been employed also for CZE determination of oligo- and polypeptidic phytochelatins or class III methallothioneins, and their precursors (Cys, γ-Glu-Cys, GSH), in the extracts from marine microalgae [207]. The separation of thiol peptides and phytochelatins was improved when 150 mM phosphate BGE, pH 1.60, was modified with 2.5% v/v methanol. The LOD of GSH was 2.5 μM with UV-detection at 390 nm.

Two chip-based immunoaffinity CE systems have been applied to rapid concentration measurement of inflammatory neuropeptides in tissue fluids of patients with neuropeptide-associated muscle pain [208]. One chip was designed to perform electrokinetic flow immunoassay while the other utilized an immunoaffinity port, containing an array of immobilized antibodies, to capture the analytes of interest. From the two systems the immunoaffinity capture system was found to be superior. Using this system, twelve different inflammation-associated mediators could be determined in approximately 2 min as compared to 30 min when using the flow immunoassay chip. With the expanding array of commercially available antibodies, this chip-based system can be applied to a wide variety of different analytes.

Native and derivatized neuropeptides containing D-amino acids in individual neurons from the marine mollusk, *Aplysia californica*, were characterized by CE with UV-absorption and LIF detection and MALDI-MS [209]. The combination of peptide derivatization by FITC and fluorescamine followed by CE separation with LIF detection proved to be well-suited for single cell analysis due to its ability to analyze peptides in such small sample volumes.

Competitive immunoassay using CE with LIF detection was developed for monitoring of Met-enkephalin [210]. The method is based on competitive reaction between ME and fluorescein-derivatized ME (ME-F) with anti-ME antibody, CE separation of the ME-antibody bound and free ME-F, followed by LIF detection of the fluorescent species. The assay specificity, sensitivity, and accuracy were excellent, allowing determination of ME in the normal and cancer patients plasma on low ng/mL level.

CZE in sodium phosphate BGE, pH 3.1–3.2, with UV-absorption detection has been developed for baseline separation of ten enkephalin-related peptides [211]. The method was used to analyze human CSF samples spiked with these peptides after the samples were pretreated by SPME, and the recovery was found to be in the range 75.7–96.5%. The concentration LODs of these peptides were in the range 0.31–1.94 μg/mL. CMEKC method with 15 mM borate/19 mM phosphate, pH 8.2, BGE containing 20 mM SDS micellar pseudophase and 10% v/v methanol as organic solvent modifier, was elaborated for separation of zinc bacitracin and nystatin and applied for determination of these antibiotics as additives in animal feedstuff [164].

The other applications of CE techniques to peptide analysis in complex matrices include determination of polyglutamyl 5-methyltetrahydrofolate forms in citrus products [212], analysis of microcystins, a group of hepatotoxic heptapeptides produced by various genera of cyanobacteria in contaminated drinking or recreational waters, by CEC with two types of RP monolithic columns (C8 and C12) [213] and analysis of *para*-κ-casein and related peptides as indicators of milk proteolysis and ripening times of ewe's milk cheese [214].

8.1.3 Monitoring of chemical and enzymatical reactions and physical changes

In addition to analysis and characterization of "static" peptide preparations, CE and CEC are capable to monitor also the dynamic changes of peptide samples, such as their chemical and enzymatical reactions and modifications (*e.g.*, oxidation, reduction, deamidation, hydrolysis, racemization), and physical changes – aggregation, denaturation, and folding/unfolding processes.

Hydrolysis, isomerization and enantiomerization of two aspartyl tripeptides, with isomeric amino acid sequences, Gly-Asp-Phe-NH_2 and Phe-Asp-Gly-NH_2, incubated at 80°C in acidic (pH 2) and basic (pH 10) solutions, was in detail studied by validated CE methods [215]. Most of the degradation products, including those arising from isomerization and enantiomerization of the Asp residues were CE separated in 50 mM sodium phosphate BGE, pH 3.0, see Fig. 9. Resolution of comigrating isomers was achieved by addition of CD-based chiral selectors into the BGE. For tripeptide derivatives the assays were linear in the range of 0.015-3.0 mmol/L, for some dipeptides and amino acids the linear range was narrower due to their lower UV-absorption. The LODs were in the range 0.005-0.1 mmol/L. At pH 2, the degradation of peptides proceeded *via C*-terminal deamidation and peptide backbone hydrolysis, whereas isomerization and enatiomerization were observed in combination with deamidation at pH 10.

Cleavage of peptides and proteins by a new method utilizing light-generated radicals from titanium dioxide was monitored by CE in 25 mM acetate BGE, pH 4.5, with UV detection at 214 nm [216]. Reproducible products patterns (electropherograms), consistent with cleavage of peptide bond at proline for angiotensin I, Lys-bradykinin and myoglobin, were obtained, demonstrating that the cleavage procedure is rapid, specific and reproducible.

Monitoring and analytical characterization of degradation of a pseudopeptide composed of esterified tyrosine and lysine linked by urea bonds was carried out by CE hyphenated with IT-TOF-MS analyzer [217]. Several degradation species have been identified and a kinetic analysis of the variation of their concentration with time was obtained.

Several CE applications are dealing with enzymatic conversions of peptides, to study some details of these processes and/or activity of enzymes and kinetics of their acting on peptides and proteins and other compounds using the so called electrophoresis-mediated microanalysis (EMMA) [74, 218]. Two CE modes, CZE and CMEKC, were applied to the screening of combinatorial peptide libraries for their inhibitory activites to botulinum neurotoxin serotype A, a proteolytic enzyme that induces muscle paralysis [219]. The peptide products in this enzyme assay were labeled with 3-(4-carboxy-benzoyl)-2-quinoline-carboxaldehyde (CBQCA) and separated by CZE or MEKC with LIF detection (Ar ion laser, excitation/emission at 488/520 nm). The products were completely separated within 8 min (CZE) or 30 min (MEKC)

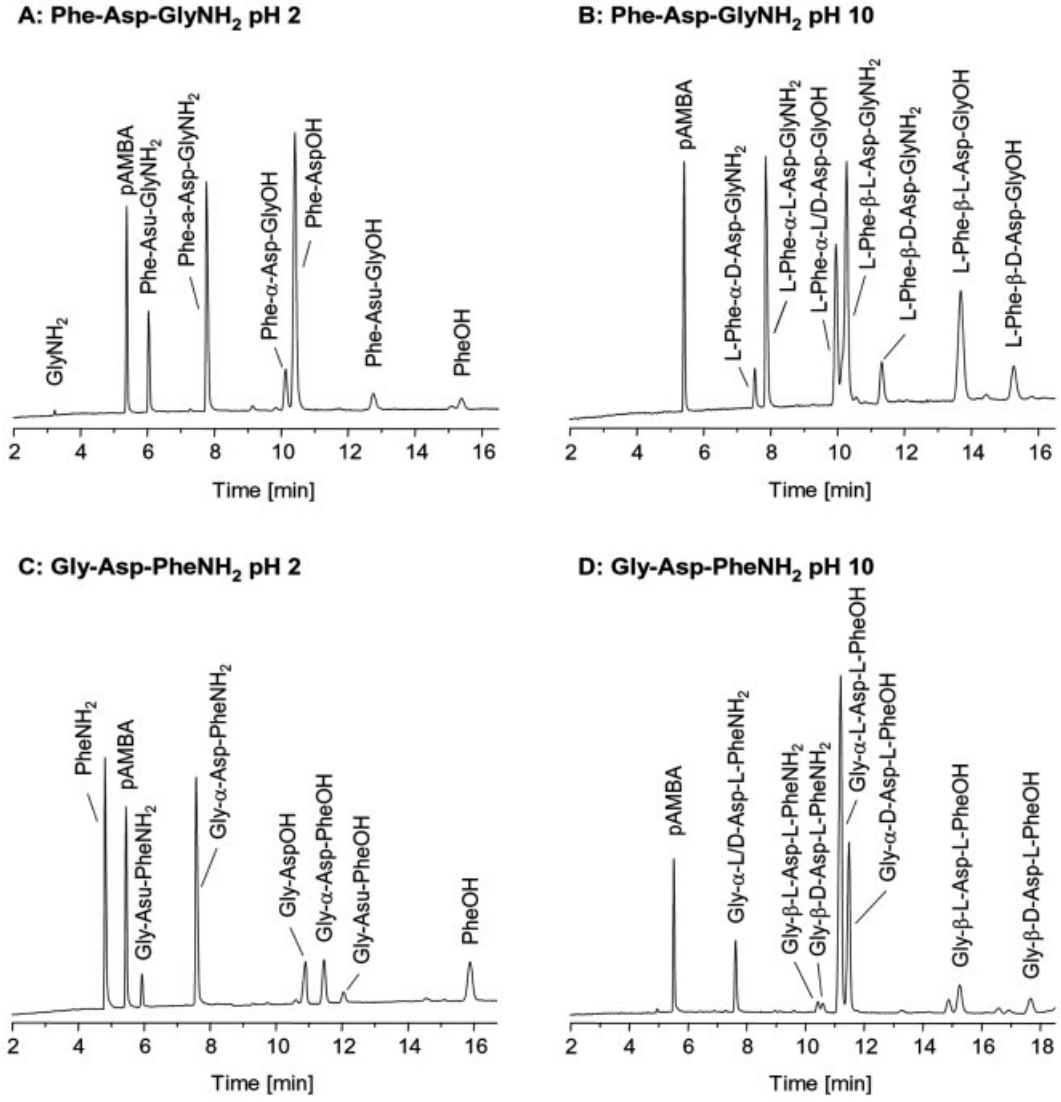

Figure 9. CE separation of isomeric degradation products of aspartyl tripeptides, Phe-Asp-GlyNH$_2$ and Gly-AspPheNH$_2$, incubated for different period in acidic (pH 2) and basic (pH 10) solutions at 80°C. (A) Phe-Asp-GlyNH$_2$, pH 2, 12 h; (B) Phe-Asp-GlyNH$_2$, pH 10, 24 h; (C) Gly-AspPheNH$_2$, pH 2, 36 h; and (D) Gly-AspPheNH$_2$, pH 10, 96 h. Conditions*:* 40/50.2 cm FS capillary, 50 μm id, 50 mM sodium phosphate buffer, pH 3.0, 25 kV. Reprinted with permission from [215].

as compared to their incomplete separation within 1 h by HPLC. A new CZE method has been developed for the monitoring of glutathione *S*-transferase detoxification activity toward styrene oxide [220]. The enzymatic reaction was carried out directly in a thermostatted autosampler vial and the formation of conjugates between GSH and syrene oxide was monitored by sequential MEKC runs. The determinations were performed in 20 mM phosphate/20 mM tetraborate BGE, pH 8.3, with 50 mM SDS micellar pseudophase, with UV-detection at 200 nm.

Monitoring of physical changes is represented by the application of CZE to the studies of folding, unfolding, refolding, and conformational changes of proteins and polypeptides [221].

8.1.4 Amino acid and sequence analysis

CE and CEC techniques are emloyed also in the area of peptide/protein characterization by their amino acid composition and sequence of amino acid analysis. Survey of CE applications for amino acids analysis, including amino acid analysis of complete peptide hydrolysate, can be found in the recent review [222]. Different peptide sequencing platforms consisting of CE separation method coupled on-line or off-line to different MS/MS devices, such as MALDI-TOF-TOF, ESI-IT, ESI-TOF, and FT-ICR, were compared with a conclusion that each of these techniques has its own pros and cons [184].

A computer-aided method has been developed to propose the amino acid composition and sequences of small peptides

(M_r<1000) present in a complex vegetable protein hydrolysate [223]. The method is based on predictive models for peptide hydrophobicity and charge/mass ratio. The former model is related to RP-HPLC-MS analysis and the latter model is related to CE-MS analysis of the protein hydrolysate. The developed program determines the possible amino acid combinations as a function of the mass determination of the data provided by the model. In the hydrolysate of rapeseed proteins by alcalase the amino acid composition and sequence has been determined using the databank sequences of native proteins.

Amino acid sequences of alamethicins F30, 20-residue peptaibol peptides isolated from the culture broth of the mold *T. viride*, forming voltage-dependent ion channels in bilayer lipid membranes and exhibiting antibiotic activities, have been determined by NACE (in 12.5 mM ammonium formate BGE in MeOH) coupled to ESI-IT-MS and ESI-TOF-MS [224]. MS/MS ESI-IT-MS was used for elucidation of the amino acid sequence based on the fragmentation pattern of the selected parent ions.

An improved amino acid sequence coverage of complex peptide and protein mixtures, *e.g.*, tryptic digests of a six-protein mixture with an average M_r around 49 000 and a mixture of *E. coli* ribosomal proteins, was achieved by a new technique, (CE-MS/MS)(n) [185]. In this approach multiple CE-MS/MS subanalyses (injections followed by analyses) are performed and experimental variables, such as BGE composition and temperature, are manipulated during each CE-MS/MS subanalysis, utilizing the advantage of small sample volume (<10 nL) and short analysis time (*ca*. 10 min) of CE. MS/MS was used to confirm amino acid sequences of tryptic peptides of bovine α1-glycoprotein isolated by HPLC and followed by CE-MS/MS analysis [183].

8.1.5 Peptide mapping

High efficiency and high resolving power make CE and CEC very useful methods for peptide mapping, *i.e.*, separation of peptide fragments generated by specific chemical and/or enzymatic cleavage of proteins and polypeptides. Peptide mapping serves as an important tool for protein identification, for sequence determination of internal parts of polypeptide chains, for monitoring of post-translational modification and structure elucidation of proteins. In addition, peptide maps can be obtained also as patterns obtained by separation of peptides present in complex biological fluids, such as serum, plasma, or CSF. Due to the high-complexity of peptide maps, namely of large proteins and biofluids, usually multidimensional separations, 2-DE, 2DE-MS, HPLC-CZE, HPLC-MS, CZE-MS, and HPLC-CZE-MS, are necessary for complete resolution of all peptides present in these mixtures (see Section 5.7).

Monitoring of tryptic digestion of β-lactoglobulin A and B variants in a microscale by CZE allowed developing optimized experimental conditions for specific hydrolysis of these proteins (enzyme/substrate ratio 1/20, incubation in 50 mM ammonium bicarbonate buffer, pH 8, at 37°C for 20 h) [225]. Highly reproducible and highly resolved peptide maps were obtained in BGE consisting of 150 mM formic acid, pH 2.3; they revealed the region where the aberrant peptides of β-lactoglobulin variants may be located.

The highly selective separation of tryptic BSA peptides was achieved by a partial filling MEKC with a mixed micelle system composed of a zwitterionic surfactant, 32 mM 3-(*N*,*N*-dimethylhexadecylammonium)propanesulfonate, and a nonionic surfactant, 0.6% m/v Brij 35, in an optimized BGE, 50 mM ammonium-formate, pH 2.0 [129]. Some therapeutic (glyco)proteins and peptides have been characterized by CE-ESI-IT-MS, CE-UV, and CE-LIF peptide mapping as a complement of RP-HPLC-MS peptide mapping using endoproteinase Lys-C for peptide/protein digestion [226]. Tryptic digests of cytochrome *c* were analyzed by CE-ESI-IT-MS system with simple on-line peptide preconcentration by dynamic pH-junction [58].

In some advanced systems, CE and CEC peptide mapping is on-line connected with previous protein digestion by enzymes (trypsin, pepsin) immobilized in microreactors directly coupled to separation capillary or to ESI-MS [227, 228]. In these arrangements, the advantages of application of immobilized enzymes for peptide mapping, high stability, and high activity of the enzyme and noncontamination of protein peptide maps by enzyme fragments due to the suppressed autolysis, have been confirmed.

8.1.6 Chiral analysis and stereoisomer separation

Chiral compounds applied as drugs, food additives, and agrochemicals represent classes of compounds with high economical and scientific potential. Hence, the separation of enantiomers is of paramount importance. Due to the high efficiency, resolution power, speed, and miniaturization, CE and CEC techniques have become very powerful and frequently used methods for chiral analysis and stereoisomer separation [229] including chiral and stereoselective separations of peptides as demonstrated in the reviews [15, 16]. With respect to the comprehensive survey of peptide stereoseparations in these reviews, only few representative examples of peptide stereoseparations are briefly mentioned in this section.

The enantiomeric and diastereomeric CE separations of peptides were systematically studied in the group of Scriba. Enantiomers, diastereomers, and positional isomers of peptides originated from the degradation of aspartyl tripeptides, Gly-Asp-Phe-NH_2, and Phe-Asp-Gly-NH_2, were separated in acidic phosphate or formate-based BGEs with carboxymethyl-β-CD or sulfated β-CD as chiral selectors [215], see Fig. 9. Separation of the diastereomers of phosphinic pseudopeptides, *i.e.*, peptides with one peptide bond substituted by phosphinic acid moiety, -P(O)OH-CH_2-, derived from the structure *N*-Ac-L-Val-L,D-Alaψ(P(O)OH-CH_2)-L,D-Leu-L-His-NH_2, has been investigated in achiral BGEs within a broad pH range, 1.8–12.0 [230]. The best resolution was achieved in

the acid pH region around the pK_a values of the central phosphinic acid group of the pseudopeptides but successful separation of some diastereomers was achieved also in neutral and alkaline BGEs.

Peptides are not only the subject of CE chiral separations but they are used also as chiral selectors for separation of other classes of enantiomeric compounds. Most important representatives of peptide chiral selectors are macrocyclic glycopeptides, vancomycin, ristocetin, and teicoplanin, used for a broad class of chiral separations both in electromigration and chromatographic techniques [231]. These selectors are mostly used in the countercurrent, partial filling mode, where the solutes reach the detection cell window after the chiral selector has been displaced from the window region, minimizing the background absorbance from the chiral selector and improving sensitivity but they can be also used as immobilized chiral stationary phase in CEC. New peptide chiral selectors are developed using the combinatorial peptide library approach [232].

8.2 Preparative separations

The application of CE and CEC for preparative separations of peptides is much less compared to analytical separations owing to: (i) inherently low preparative capacity of the small id capillary columns, and (ii) more complicated adaptation of analytical capillary setup to preparative one than in chromatographic techniques. The latter problem, caused by the dipping of both capillary ends into the BGE in the electrode vessels through which the electric field is applied over the capillary, has been solved by special modifications of CE systems. These systems and several procedures developed for fraction collections from the capillary have been described in the earlier review [8] and book chapter [233].

Hence, CE and CEC techniques are used for preparative purposes mostly in an indirect way, *via* the analysis of peptide fractions obtained by other methods (preparative chromatography, free-flow electrophoresis) and for evaluation of the efficiency of the separation method used for peptide purification. Direct application is limited to microscale preparation. In commercial devices the autosamplers are used as fraction collectors and the electroelution is sometimes speeded up or completely replaced by hydrodynamic flow introduced at the inlet end of the capillary. Such system has been used, *e.g.*, for micropreparative fractionation of tryptic fragments of β-lactoglobulin A and B [225]. Peptides in collected fractions are later on concentrated by evaporation of the solvent and off-line characterized by (MALDI) MS or by amino acid and sequence analysis, see Section 8.1.4. For the continuous fraction collection in CE it is necessary to use special designs of the capillary outlet for completing the electrical circuit. This is achieved, *e.g.*, by coaxial sheath liquid interface transporting the sample components leaving the exit of the capillary directly on a matrix-coated MALDI target [173, 181].

The problem of limited preparative capacity of CE (usually less than 1 μg *per* run) can be partially solved by increasing the inner diameter of the capillary (compromising the separation efficiency), by repetitive fraction collection or by the use of multicapillary systems. Multiple separations in a single narrow bore capillary and pooling of the fractions with the same mobility are suggested for isolation of peptides in the amounts sufficient for sequence analysis.

Principle solution of the preparative capacity problem is to convert analytical capillary separations to the continous free-flow arrangement on the basis of the earlier developed model of the correlation between capillary and free-flow electrophoresis [233, 234]. Free-flow electrophoresis, particularly the FF-IEF mode has been used for prefractionation of complex peptide/protein mixtures in proteomic studies of cells [235–237] and body fluids, such as human plasma [238] and human saliva [239].

8.3 Physicochemical characterization

CE and CEC are not only high-efficient and high-sensitive analytical techniques but they are also more and more utilized as physicochemical methods, capable to provide important physicochemical characteristics of separated analytes, including peptides and proteins, such as effective and absolute (limiting) electrophoretic mobilities, effective charges, pI, M_r, Stokes radii, pK_a of ionogenic groups, diffusion coefficients, and association (dissociation, binding) constants of peptide complexes [240, 241]. In addition, CE and CEC can be used also for monitoring of the physicochemical processes, *e.g.*, conformation changes during unfolding/folding processes and velocity of chemical reactions and physical changes.

CZE in sieving media can provide data on the size of separated analytes. M_r of polypeptides and proteins can be estimated from CZE of their complexes with SDS (capillary version of SDS-PAGE) [113, 242]. Stokes radii and effective (net) charges of peptides can be assessed from effective mobilities measured by CZE and from the different relations between peptide mobility and their charge-to-size ratio. Due to the approximative character of all models correlating peptide mobility with their charge/size ratio the calculated values of charge and size (M_r) represent also only approximative characteristics. Combination of CZE measurement of electrophoretic mobilities of protein/polypeptide charge ladders and classical electrophoresis theory has been suggested to provide data for estimation of the effective (net) charge and size of larger (poly)peptides and proteins [41]. Net charge, hydrodynamic size, and shape of peptides were explored through CZE measured effective mobilities and have been explored using the Linderstrøm-Lang electrophoresis model and its perturbed version [37].

Another important peptide/protein parameter related to charge, pI, can be determined by CIEF [243, 244], but also in this case one should have in mind that the determined pI value is dependent on the composition of the ampholytes

and other experimental conditions used. Diffusion coefficients of peptides can be estimated from their electrodriven motion in the capillaries and chip channels [245].

Effective and ionic mobilities of phosphinic pseudopeptides, peptide isosteres with one peptide bond substituted by a phosphinic acid moiety – P(O)OH–CH_2–, and pK_a of this moiety and other ionogenic groups (*C*-terminal-, γ-Glu- and β-Asp-carboxyl, *N*-terminal- and ε-Lys amino, His-imidazolyl) have been determined from the precise measurements of the pH dependence of effective mobilities within a broad pH range 1.8–12.0 in BGEs with constant ionic strength (25 mM) [230]. Effective mobilities, corrected to standard temperature 25°C, were subjected to nonlinear regression analysis and the obtained apparent pK_a values were recalculated to thermodynamic pK_a values by extrapolation to zero ionic strength according to extended Debye-Hückel model. Similar measurements of the dependence of effective mobilities on pH within a broad pH range 2–12 provided pK_a values of ionogenic groups in peptide hormones [24] and opioid peptides [25].

In a comprehensive study, the effective mobilities and pK_a values of a series of oligopeptides, oligoglycines, oligo(L-alanines), and oligo(L-valines) with a number of residues up to ten, have been determined by CZE in BGEs within a broad pH scale, 1.8–12.0, at two ionic strengths (10 and 100 mM) and at temperatures from 15 to 60 °C [40]. For each peptide family, the pK_a values were modeled as a function of the number of residues, temperature, and ionic strength. Using this broad set of experimental data a semiempirical model was developed allowing to predict pK_a values for any oligopeptide composed of amino acids with neutral lateral chains. The input parameters of this model are only the number of residues and the pK_a of terminal amino acids in their free form. The model can predict the peptide pK_a values at a given ionic strength and temperature and can also be used for selection of the optimum pH for the separation of mixtures of peptides the pK_a of which are known or could be estimated.

From the CZE measurements of the dependence of effective mobilities of peptides on the charge/size ratio the probable secondary structure of similar peptides was determined, *e.g.*, the random coil structure was predicted for peptide hormones [246], opioid peptides [25], and GnRHs [30]. The electrophoretic mobilities of a series of dipeptides determined by CZE allowed estimation of their transdermal iontophoretic mobility and to evaluate their suitability for iontophoretic delivery [247].

CAE is now widely used for determination of association or dissociation constants of peptide complexes with different types of both low-and high-molecular-mass ligands, for review see [119, 248]. Large series of studies has been performed to estimate receptor-ligand interactions using the system consisting of glycopeptide macrocyclic antibiotics (vancomycin, teicoplanin, ristocetin, and their derivatives) from *S. orientalis* and free or derivatized dipeptides D-Ala-D-Ala [121–124, 249, 250].

New methods, fluorescence anisotropy CE (FACE) and affinity probe CE (APCE) with LIF detection, have been developed for quantitative analysis of peptide-protein interactions, particularly for the interactions between the *Src* homology 2 domain of protein SH2-Bβ and tyrosine-phosphorylated peptide corresponding to the binding sequence of JAK2 [102]. The separation time of 4 s, achieved by high electric field strength of 2860 V/cm in a 7 cm long capillary, was on the same time scale as complex dissociation and allowed determination of both dissociation constant, (K_d = 101 ± 12 nM) and dissociation rate (k_{off} = 0.95 ± 0.02/s corresponding to a half-life 0.73 s). With LOD 100-300 nM both APCE and FACE were found to be suitable for investigation of binding interactions with rapid kinetics in a microscale. The assay was extended to a multipexed system involving the separation of three SH2 domain proteins, Src, SH2-Bβ and Fyn [251].

The interaction between 3C-like proteinase, key target for drug against severe acute respiratory syndrom (SARS) coronavirus, and its octapeptide inhibitor have been studied by CAE [252]. From the mobility shift assay of the inhibitor, *i.e.*, from the changes of its mobility in the BGE with increasing concentrations of the proteinase, the binding constants K_b of 2.44×10^4/M at 20°C and 2.11×10^4/M 37°C were determined. Analysis of the thermodynamic parameters, changes of Gibbs free energy, enthalpy, and entropy, obtained from temperature dependence of K_b, indicated that hydrophobic interactions might play a major role in the binding process, along with electrostatic attractions.

CAE mobility shift assay has been found to be an efficient and sensitive method also for both qualitative and quantitative studies of the interactions between the peptides and oligo- and polynucleotides, it has been employed, *e.g.*, for selection and identification of human GnRH promoter binding peptides using –813/–1081 region of hGnRH promoter and a phage-displayed peptide library [253] and for selection of aptamers with high affinity to neuropeptide Y from random-sequence nucleic acid libraries [254].

9 Concluding remarks and future prospects

As demonstrated in the above sections, CE and CEC possess an extremely high potential for analysis, preparation, and characterization of peptides. Nowadays, these techniques are regarded as accomplished complements and/or counterparts of other high-performance separation methods, particularly HPLC. Applications of CE and CEC methods to analysis, isolation, and characterization of peptides are further broadening; CE and CEC are being utilized not only as highly efficient and highly sensitive analytical techniques, capable to determine femtomole to zeptomole amounts of peptides in nano- to picoliter sample volumes of complex biological matrices, but also as valuable physicochemical methods, which can provide important physicochemical and biochemical

characteristics of peptides, such as their effective mobilities and charges, M_r's, acidity constants of their ionogenic groups, diffusion coefficients, association constants of their complexes, conformation of their molecules, and rate constants of their reactions.

Peptides represent an extremely numerous class of vitally important biomolecules. Many of them and a lot of their functions have been recognized but even more have to be revealed and elucidated. For a more detailed understanding of living processes, a comprehensive investigation of a whole peptide set of a cell, organ, or organism (peptidome), *i.e.*, peptidomics, has to be performed. Also the structure and functions of proteins in proteomic studies are mostly identified *via* their peptide fragments. Hence, undoubtedly, the analysis, purification, and characterization of peptides will belong to the most challenging tasks of CE and CEC methods also in the future. As concerns these techniques themselves, their development will proceed to implementation on microfluidic devices and integration into μTAS, on-line coupled with high-sensitive LIF or MS detection. Multidimensional separation systems based on hyphenation of CE, CEC, LC, and MS techniques will be necessary for analysis of complex peptide and protein mixtures in peptidomics and proteomics.

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