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Review

Capillary electrochromatography of proteins and peptides

This review summarizes applications of CEC for the analysis of proteins and peptides. This "hybrid" technique is useful for the analysis of a broad spectrum of proteins and peptides and is a complementary approach to liquid chromatographic and capillary electrophoretic analysis. All modes of CEC are described – granular packed columns, monolithic stationary phases as well as open-tubular CEC. Attention is also paid to pressurized CEC and the chip-based platform.

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1 Introduction

CEC is a hybrid technique, utilizing the principles of electromigration techniques (electroosmosis and electrophoresis) and chromatography (distribution between two phases). This technique is currently used for the separation and analysis of a broad spectrum of compounds, both low- and high-molecular, organic and inorganic compounds. There are numerous review articles or books on the principles and application of CEC, and some of the recent ones are worth mentioning [1 – 8].

This review is focused on the analysis of proteins and peptides by CEC. It should be mentioned that the current era of proteomic research needs to develop new and robust methods for the analysis of natural proteomic (and peptidomic) samples. For this reason, analytical chemistry dealing with the analysis of proteins and peptides is an emerging field in modern science. As far as CEC is concerned, there are many review articles dealing with the separation of proteins and/or peptides [9 – 21]. We have to stress that this review is devoted to separation methods, although detection methods, particularly MS methods for the analysis of proteins/peptides, are highly important in proteomic research. These approaches will be often mentioned in the methods described (mainly in pressurized CEC) but readers with a direct interest in these methods are directed to more specialized reviews (*e.g.*, ref. [22]).

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Abbreviations: GPTMS, glycidoxypropyltrimethoxysilane; **VBC,** vinylbenzyl chloride

First, we have to emphasize the separation mechanism and motion of proteins during the separation procedure. Generally speaking, CEC is a combination of electromigration and chromatography. However, proteins and peptides are a heterogeneous group of compounds that differ in their hydrophobicity, charge, molecular mass, and many other properties, and it is impossible to determine only one characteristic that influences their separation efficiency. For uncharged and charged peptides, a synergistic interplay occurs in CEC systems between adsorptive/partitioning events and electrokinetically driven motion. Moreover, at high field strengths, both bulk electrophoretic migration and surface electrodiffusion occur. The separation (and interaction) processes of peptides/proteins during CEC is influenced by various parameters, such as the pH or the level of organic solvent in the mobile phase, which simultaneously influence all other physicochemical aspects of the specific CEC separation. For this reason, the optimization of this (separation) process cannot be realized by single parameter optimization methods but by more sophisticated multiparameter optimization procedures (for details, see the review by Walhagen *et al.* [23]).

In principle, CEC of proteins/peptides can be classified in two ways: according to column format or separation mechanism.

There are three modes of CEC that are distinguished based on their column formats: columns (capillaries) packed with particles, monolithic columns, and open-tubular system (open-tubular electrophoresis, OT-CEC).

The second method of classification is according to separation mode: in principle there are several modes, such as RP, normal phase, ion-exchange, size-exclusion, or affinity-based separations.

In addition, other instrumentation designs that need to be be mentioned are pressurized CEC and the microchip platform.

2 Granular packed columns

The "traditional" approach in CEC is to use packed columns filled with chromatographic media. The methodology of preparation of these columns is outside the scope of this review and we direct readers to more specialized books or reviews (*e.g.*, ref. [24]). In many cases, the materials used for granular packed columns are the same as those used for HPLC or μHPLC. Typically, the materials most often used for protein/peptide separations are RP and ion-exchange materials. It is interesting to note that size-exclusion electrochromatography is used for the separation of synthetic polymers but not frequently for the separation of peptides and proteins [25]. Segmented packed columns or mixed mode stationary phases can also be used.

2.1 RP electrochromatography

RP materials are one of the most popular stationary phases for the separation of proteins/peptides by chromatography, and they are often also used as stationary phases in granular packed columns for CEC. The dominant material is C18-modified silica particles of diameter 3 – 5 μm. It must be stressed that separations by CEC differ from those obtained by HPLC on the same material. An interesting comparison of various tailor-made spherical silica-based stationary phases and the influence of various factors on the separation of model small cyclic peptides was studied by Huber *et al.* [26]. They concluded that care should be taken to select a sorbent which provides sufficient selectivity for the model peptides to guarantee a wide range elution window for the eluates and hence modified beads may prove to be beneficial with respect to detailed requirements.

The choice of the correct stationary phase is a crucial task in RP chromatography similarly as for other methods. Various 3 μm phases were compared (250/335 mm × 100 μm id; Hypersil C8, C18, Hypersil mixed-mode, and Spherisorb C18/SCX columns) for the separation of peptides, where mobile phases composed of ACN-triethylamine-phosphoric acid at pH 3.0 were used [27]. It was concluded that the process of elution of peptides in CEC is mediated by a combination of both electrophoretic migration processes and retention mechanisms, involving hydrophobic as well as silanophilic interactions.

Two stationary phases, Hypersil phenyl and Hypersil C18, were also compared for the separation of four basic proteins [28]. The optimal conditions for the separation on the phenyl stationary phase were 50% ACN, 20% 50 mM Tris, pH 7.5, 30% H_2O as BGE, operating at 20°C and 20 kV high voltage. For the C18 stationary phase, optimal separation conditions were 80% ACN, 20% 30 mM Tris, pH 8.5, again operating at 20°C and 20 kV high voltage. Results show that the phenyl stationary phase is better suited for the separation of basic, hydrophilic peptides.

The separation of structurally related synthetic peptides was studied in the Hypersil C18 column. The migration of these peptides varied in a charge-state-specific manner with the properties of the BGE, such as pH, salt concentration and content of organic modifier, or temperature. Acidic peptides followed similar trends in retention behavior, which was distinctly different from that exhibited by more basic peptides. When the separation forces acting on the peptides were synergistic with the EOF (*i.e.*, positively charged peptides), their retention coefficient decreased with an increase in capillary temperature, whereas when the separation forces worked in opposite directions (*i.e.*, negatively charged peptides), their retention coefficient increased slightly with increase in temperature. When the content of the organic modifier, ACN, was high (*i.e.*, more than 40% v/v) and nonpolar interactions with the C18 sorbent were suppressed, mixtures of both the basic and acidic synthetic peptides could be baseline resolved under isocratic conditions by utilizing the mutual processes of electrophoretic mobility and electrostatic interaction [29].

Gucek *et al.* [30] described the separation of peptides in CEC columns (100 μm id/25 cm long) packed with 3 μm Hypersil C8 or C18 bonded silica particles with an eluent composed of ammonium acetate/ACN. The peptides separated were desmopressin, peptide A, oxytocin, carbetocin, and [Met5]-enkephalin. These columns were successfully coupled to nanoelectrospray MS, where sensitivity in the mid-attomole range was achieved.

2.2 Ion-exchange chromatography

The advantage of ion-exchange electrochromatography is that the surface of the stationary phase is covered with fixed charges which generate EOF. As far as the ion exchanger is concerned, various kinds have been described, including strong cation- and anion-exchangers (SCX, SAX), weak cation exchangers (WCX), and weak anion exchangers (WAX) [31, 32].

Ion-exchange CEC with strong cation-exchange packing (SCX) can be used for the separation of short peptides [31]. The column used was 31 cm (packed length 10 cm × 75 μm id, packed with 5 μm Spherisorb-SCX. Separation was carried out with 60% ACN in 30 mM phosphate (KH_2PO_4) buffer, pH 3.0 under 25 kV. The number of theoretical plates for small peptides varied from 240 000 to 460 000/m and fast separation was achieved for ten peptides in less than 3.5 min.

A strong anion-exchanger can also be used for the CEC of acidic proteins [32]. In this case, a 50 μm id capillary was packed with 5 μm silica beads. As the first step, the stationary phase and capillary wall were silanized and then derivatized with 3-(methacryloylamino)propyltrimethylammonium chloride to form a "tentacular" anion exchanger. It was demonstrated that this tentacular SAX phase can be used for the separation of not only a set of proteins but also protein variants, protein glycoforms (conalbumin and hemoglobin variants), and tryptic digests of proteins. Isocratic elution was carried out with an aqueous phosphate buffer, pH 7.0, containing sodium chloride.

Silica-based tentacular weak cation-exchanger particles were also studied for the separation of proteins [33]. Basic peptides were separated when NaCl was used as the mobile phase modulator.

2.3 Mixed-mode stationary phases

Mixed-mode stationary phases are an interesting alternative, combining two modes of separation (*e.g.*, RP and ion-exchange – C18/SCX). These phases always contain an RP, such as alkyl chains, to interact with the hydrophobic part of the peptides and proteins and a second, charged mode (typically ion-exchange) to maintain a stable EOF. When comparing the phases C18 and SAX/C18, it is obvious that a mixed-mode stationary phase provides more flexibility to alter the separation selectivity of peptides [34].

Columns containing both SCX (sulfonic acid) and *n*-alkyl groups (Hypersil mixed-mode, Spherisorb C18/SCX) were studied for the separation of peptides (linear and cyclic) [27, 35]. It was concluded that these stationary phases can achieve the separation of peptides based on mechanisms involving hydrophobic and ion-exchange interactions as well as electrophoretic migration.

A mixed-mode (C18/SCX) CEC column was also used for the separation of thrombin receptor antagonistic peptides [36]. This group of peptides could only be achieved in a narrow pH range, and the composition of the mobile phase was ACN/100 mM phosphate buffer, pH 6.5/water (32:10:58 v/v).

Sulfonated naphthalimido-modified silyl silica gel has also been described as a mixed-mode stationary phase for the separation of charged analytes [37]. The separation mechanism is a hybrid of electrophoresis and chromatography involving hydrophobic as well as electrostatic interactions. Migration and retention can be altered by changing various mobile phase properties, including buffer pH, buffer concentration, and the concentration of organic solvent. A good separation of short peptides (di- and tripeptides) was achieved using an isocratic elution with a mobile phase consisting of 35 mM phosphate buffer (pH 3.8) and 40% methanol. The same column was also used for the separation of peptides (six peptides and tryptic digests) with a stepwise gradient in buffer concentration [38].

Recently, an embedded ammonium (cationic quaternary amine) in a dodecacyl stationary phase was used for the separation of peptides [39]. EOF was independent of pH over a wide range (2 – 12). Under acidic conditions, the electrokinetic contribution appears to be predominant compared to the chromatographic one. This method was utilized not only for the separation of an artificial mixture of peptides but also for the peptide mapping of β-lactoglobulin and human growth hormone. The mobile phase used was Tris-HCl (50 or 75 mM, pH 3.0 – 2.6)/ACN (60 or 20%). The capillary was packed with end-capped BS-C23 30 nm 5 μm, 31.2 cm (effective length 9.8 cm) × 75 μm. The same embedded stationary phase was used in a comparative study between CEC and HPLC [40]. The critical role of the electric field in the retention mechanisms of peptides on a mixed-mode stationary phase was demonstrated.

2.4 Hydrophilic interaction chromatography

Fu *et al.* [41] developed hydrophilic interaction CEC for the separation of small peptides (dipeptides). The column used was packed with a 5 μm negatively charged strong-cation-exchange stationary phase of PolySULFOETHYL A, 27 cm (packed length, 6.5 cm) × 50 μm id. It was found that the hydrophilic interaction between solutes and stationary phase played a major role in this system; however, the ion-exchange mechanism and electrophoretic mobility also affect the migration. The optimized separation conditions consisted of: mobile phase 80% ACN in 100 mM triethylamine phosphate buffer (pH 2.8) and 15 kV applied voltage; the separation time was only 6 min (Fig. 1).

2.5 Chiral separation

Stationary phases containing teicoplanin aglycone were described as suitable columns for the separation of peptide enantiomers [42 – 44]. These phases were prepared by Schmid's group.

A system utilizing teicoplanin aglycone immobilized onto 3.5 μm silica gel, using a ternary mobile phase of aqueous 0.2% triethylamine acetate, pH 4.1 and organic modifiers (ethanol – ACN) was suitable for the separation of 12 glycyldipetides [42]. Chiral separation of alanyl and leucyl dipeptides and tripeptides with two chiral centers was also possible using a capillary packed with teicoplanin aglycone [43]. A binary mobile phase was used for this separation (in contrast to the ternary system used previously), consisting of 0.2% triethylamine acetate, pH 4.1 and ACN or methanol.

The next approach to chiral separation used particle-loaded monoliths. Teicoplanin aglycone bonded to 3 μm

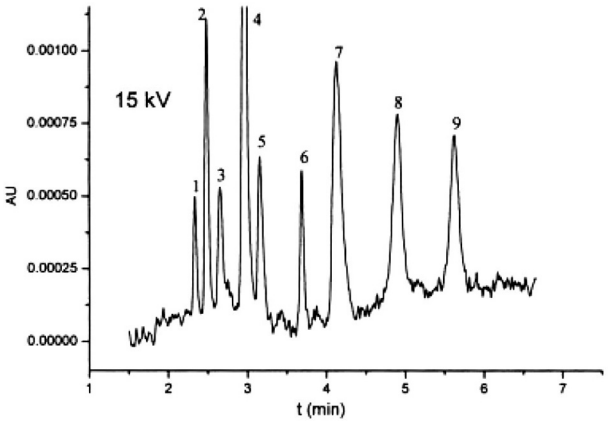

Figure 1. Separation of peptides by hydrophilic interaction CEC. Column packed with PolySULFOETHYL A, 27 cm (packed length, 6.5 cm) × 50 μm id. Mobile phases: ACN concentration 80% v/v in 100 mM triethylamine phosphate buffer (pH 2.8); applied voltage 15 kV. Peptides: (1) Ala-Ile; (2) Gly-Leu; (3) Gly-Phe; (4) Gly-Met; (5) Gly-Val; (6) Gly-Tyr; (7) Gly-Thr; (8) Gly-Ser; (9) Gly-Asp. (Reprinted from ref. [41] with permission. Copyright 2003 Wiley-VCH).

silica particles was used as the chiral selector. The silica particles were suspended in a monomer solution and then drawn into the capillary followed by *in situ* polymerization (a polyacrylamide gel was formed). The optimum conditions were loading 25% of particles and using aqueous triethylamine acetate, pH 4.1, and methanol and ACN as organic modifiers [44]. This system was used for the chiral separation of amino acids, amino acid derivatives, and dipeptides.

3 Monolithic stationary phases

Monoliths are currently popular stationary phases for electrochromatography and are being rapidly developed. There are many reviews, books (*e.g.*, ref. [45, 46]), and a plethora of articles about the preparation and use of monolithic materials in separation science and particularly in electrochromatography.

3.1 Silica-based monoliths

Similar to granular (particle)-based stationary phases, silica-based monoliths are mainly used as stationary phases for RP separations but normal-phase applications have also been described. Because methods for the preparation of silica-based monoliths as well as other monoliths are outside the scope of this review, we direct readers to some detailed overviews, *e.g.*, ref. [47 – 49].

3.1.1 RP separations

A silica-based RP stationary phase in a packed format is a "classical" stationary phase for the separation of proteins and peptides (see above). In principle, the same format of monolith can be used for the same compounds with various separation methods. In the literature, these columns are mainly used for the separation of smaller molecules, charged as well as uncharged compounds. The most comprehensive work on this topic was carried out by the El Rassi group [47, 50].

C18 monoliths can be used for the effective separation of alkylbenzenes or derivatized amino acids. A suitable separation of an artificial mixture of standard proteins was also described using a C18 – NSec monolithic capillary column 20/27 cm × 100 μm id with a hydro-organic mobile phase, 20 mM sodium phosphate monobasic (pH 2.5) at 60% v/v ACN; voltage, 20 kV. Cationic C18-monoliths (C18 – NSec) were prepared by the reaction of (γ-glycidoxypropyl)trimethoxysilane with a silica surface followed by the reaction with octadecylamine [50].

3.1.2 Normal phase

A polar monolithic column with surface-bound cyano functional groups (consisting of a silica-based monolith bonded with 1H-imidazole-4,5-dicarbonitrile) was described for the separation of neutral polar solutes (*e.g.*, mono- and oligosaccharides) and charged polar solutes (*e.g.*, peptides and basic drugs). Multistep-gradient elution enabled the rapid separation of a large number of polar species in a single run [51].

3.2 Polymer-based monoliths

Polymer-based monoliths are very popular stationary phases for CEC. There are many reviews about their preparation and use, for example, see some of the books or reviews mentioned above, or a specialized review, such as in ref. [52]. The neutral surfaces of these monoliths are frequently functionalized. Functionalization is the introduction of charged groups on the surface of monoliths and it enables the generation of EOF. A good method for the preparation of these functionalized monoliths is grafting (*i.e.*, reaction after forming a porous monolith). For example, a technique has been developed for grafting poly(2-acrylamido-2-methyl-1-propanesulfonic acid) or 4,4-dimethyl-2-vinylazlactone [53].

3.2.1 Acrylamide-based columns

Columns based on the polymerization of acrylamide are often used. Of course, the polymers are usually copolymers.

Generally, charged acrylic-based monoliths can be applied in CEC. Alkyl chains on the monolithic stationary phase (since the monomer used can be acrylamide, methylenebisacrylamide, acrylic acid, dodecyl acrylate, poly(oxyethylene)) serve as the retentive ligands while the charged group (typically sulfonic acid) provides the charge necessary to generate EOF. UV-initiated acrylate-

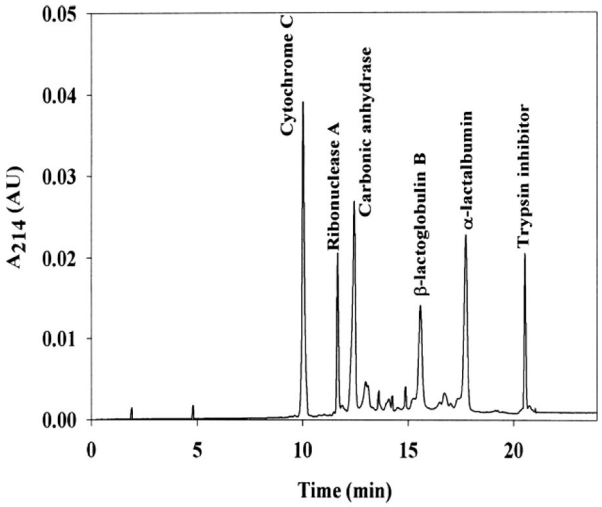

Figure 2. Electrochromatogram of proteins separated by neutral stearyl-acrylate macroporous monolith. Conditions: 40% ACN v/v, 20 mM sodium phosphate monobasic, pH 7.0; capillary 30 cm effective length (37 cm total length) × 100 μm id; voltage, 10 kV. (Reprinted from ref. [57] with permission. Copyright 2005 Wiley-VCH).

based porous polymer monoliths have been described as stationary phases for the capillary- and chip-electrochromatography of cationic, anionic, and neutral amino acids and peptides [54].

Macroporous polyacrylamide/PEG matrixes were used for the effective separation of alkyl phenones but separations of peptides and carbohydrates were also described [55]. In this case, the monomers (acrylamide, bisacrylamide, and acrylic or vinylsulfonic acid), including hydrophobic ligands (C4, C6, or C12) and PEG were polymerized in aqueous formamide (or *N*-methylformamide) solutions inside the capillary.

Cationic C17 monoliths (stearyl-acrylate) are suitable for the elution of various uncharged and charged solutes, including proteins [56]. Monoliths were prepared by the polymerization of pentaerythritol diacrylate monostearate and [2-(acryloyloxy)ethyl]trimethylammonium methyl sulfate monomers. At low pH, the cationic C17 monoliths allowed the separation of proteins with minimal electrostatic interactions between the proteins and cationic sites on the surface of the stationary phase. The utility of cationic C17 monoliths was demonstrated in the rapid and efficient separation of two crude extracts of membrane proteins, namely galactosyl transferase and cytochrome *c* reductase. Short capillary columns (8.5 cm effective length) allowed for rapid and efficient separation of proteins in a matter of seconds.

In principle, the same but neutral (C17) stearyl-acrylate macroporous monolith was prepared for the CEC separation of neutral and charged small species as well as peptides and proteins [57] (Fig. 2). This monolith was prepared by the *in situ* polymerization of pentaerythritol diacrylate monostearate in a ternary porogenic solvent composed of cyclohexanol, ethylene glycol, and water. This neutral monolith did not contain any fixed charges, but the columns exhibited a relatively strong EOF due to the ability of the stearyl-acrylate monolith to adsorb sufficient amounts of electrolyte ions from the mobile phase. This phenomenon allowed the rapid and relatively efficient separations of proteins and peptides at pH 7.0.

A mixed-mode monolithic stationary phase (*N*,*N*-dimethylacrylamide – piperazine diacrylamide-based monolith bearing sulfonic acid groups for EOF generation) was investigated for the separation of positively charged amino acids and peptides. The mobile phase consisted of 40 mM ammonium acetate/800 mM acetic acid in 20% (ACN/methanol, 8:2), and was suitable for the separation of a few peptides when they were eluted before the EOF marker [58].

3.2.2 Polystyrene

After functionalization of the surface, a polystyrene monolith is appropriate for the separation of peptides by CEC [59]. Monolithic stationary phases were prepared by *in situ* copolymerization of divinylbenzene either with styrene or vinylbenzyl chloride (VBC) in the presence of a suitable porogen. Angiotensin-type peptides were separated by CEC using columns packed with a monolithic stationary phase with fixed *n*-octyl chains and quaternary ammonium groups on its surface. Plate heights of about 8 μm were routinely obtained. The mechanism of separation was based on the interplay among EOF, chromatographic retention, and electrophoretic migration of the positively charged peptides.

3.2.3 Polymethacrylates

Methacrylate-based monoliths are very popular and commonly used. Numerous modification reactions have been described and used that enable the modulation of the functionality and chemical properties of monoliths.

A monolithic porous stationary phase prepared by *in situ* copolymerization of VBC and ethylene glycol dimethacrylate was suitable for the separation of synthetic peptides, proteins as well as the tryptic digest of cytochrome *c*. EOF was generated by the positively charged surface by reacting chloromethyl groups (at the surface of the porous monolith) with *N*,*N*-dimethylbutylamine. Typically, the chromatography of a tryptic digest of cytochrome *c* took about 5 min at 55°C and 75 kV/m with hydro-organic mobile phases containing ACN in 50 mM phosphate buffer, pH 2.5 [60].

Monoliths are often prepared with ionizable functional groups attached to the bulk of the stationary phase. These charged surfaces serve to generate EOF. However, it is well known that the charged functional

groups of proteins and peptides can interact with the charged surfaces of the bulk monolithic material and these interactions result in the adsorption of proteins/peptides to the stationary phase. An interesting approach to solve this problem was presented by Li *et al.* [61]. They published a new approach to the design of a monolithic column. This is based on the use of two types of polymers. First, a positively charged polymer forms a layer on the inner wall and generates annular EOF. A second polymer forms a neutral hydrophobic bulk monolithic stationary phase inside the capillary. First, the annular layer was prepared by the pretreatment (silanization) of a fused-silica capillary with 3-glycidoxypropyltrimethoxysilane (GPTMS) and then polyethyleneimine (PEI) was covalently bonded to the GPTMS coating to form an annular positively charged polymer layer for the generation of EOF. Second, a neutral bulk of poly(vinylbenzylchloride-*co*-ethylene dimethacrylate) monolith was prepared by *in situ* copolymerization of VBC and ethylene glycol dimethacrylate in the presence of 1-propanol and formamide as porogens. This monolithic column enables the fast and reproducible separation of peptides in isocratic mode (phosphate buffer 25 mmol/L, pH 2.5, containing 25% ACN) Li *et al.* [61] suggested a dual mechanism of separation that involves a complex interplay between selective chromatographic retention and differential electrophoretic migration.

Szucs and Freitag [62] developed a poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith for the separation of peptides by nano-HPLC or voltage-assisted LC. For the purposes of CEC, the monolith (*i.e.*, for the formation of EOF) was functionalized with *N*-ethylbutylamine.

Another example of the functionalization of a methacrylate-based monolith was presented by Zhang *et al.* [63] for the separation of peptide or protein mixtures. They used a column prepared by *in situ* copolymerization of glycidyl methacrylate, methyl methacrylate, and ethylene glycol dimethacrylate and the surface was functionalized by the reaction with *N*-ethylbutylamine to form fixed tertiary amino functional groups containing ethyl- and butyl-chains. The elution order of proteins was similar to that obtained in RP chromatography. It was proposed that the separation is governed by a dual mechanism that involves a complex interplay between selective chromatographic retention and differential electrophoretic migration [63]. In principle, this monolith can be designated as mixed mode, similar to the use of this designation in the granular packed columns section.

3.2.3.1 Mixed-mode monolith

A mixed-mode *n*-alkyl methacrylate-based monolith was used for the separation of therapeutic peptides [64]. The sulfonic acid (SCX) moiety derived from 2-acrylamido-2-methyl-1-propanesulfonic acid generated a stable EOF and the butyl ligands provided nonpolar sites for the chromatographic resolution. High efficiency separation (5.0×10^5 plates/m) of the peptides was obtained at both low (2.8) and high (9.5) pH values.

Another example of mixed-mode CEC (RP/SCX) is a monolithic capillary column prepared by *in situ* copolymerization of 2-(sulfooxy)ethyl methacrylate and ethylene dimethacrylate in the presence of porogens. Column efficiency was more than 280 000 plates/m. The selectivity of the separation of peptides in this monolithic capillary column could be easily manipulated by varying the mobile phase composition [65].

3.2.3.2 Imprinted monolith

An interesting approach was used by Lin *et al.* [66] when they used a template inside a polymethacrylate-based monolith, *i.e.*, an imprinted column approach was used. L-Phenylalanine served as a template after the formation of a Schiff base with *o*-phthalaldehyde. A mixture of monomers (methacrylic acid, 2-vinylpyridine, ethyleneglycol dimethacrylate, α,α-azobisisobutyronitrile) was thermally polymerized *in situ*. After that, the template was extracted. A capillary column of 75 (50) cm × 75 μm id with a mobile phase of phosphate buffer (pH 7.0, 40 mM)/methanol (5% v/v) could separate angiotensin I, angiotensin II, [Sar1, Thr8] angiotensin, oxytocin, vasopressin, tocinoic acid, β-casomorphin bovine, β-casomorphin human, and FMRF amide within 20 min. It was concluded that the electrochromatographic separation of this set of peptides was mediated by a combination of electrophoretic migration and chromatographic retention involving hydrophobic and electrostatic interaction, hydrogen bonding as well as Schiff base formation with OPA in the cavity of the templated polymer (Fig. 3) [66].

3.2.3.3 Zwitterionic monolith

Zwitterionic stationary phases based on poly(butyl methacrylate-*co*-ethylene dimethacrylate) can be useful for the separation of proteins [67]. Fu *et al.* [68] prepared such zwitterionic monoliths *via* the *in situ* polymerization of butyl methacrylate, ethylene dimethacrylate, methacrylic acid, and 2-(dimethyl amino) ethyl methacrylate. These monoliths have zwitterionic functional groups (tertiary amine and acrylic acid groups); so the ionization of these groups was affected by the pH value of the mobile phase (the strength and direction of EOF is affected). It was demonstrated that the separation order of elution of peptides can be affected by pH.

3.2.3.4 Shielded stationary phases

An interesting new concept in stationary phases is using photografting reactions to polymerize several layers on top of each other. The advantage of this approach is in the ability to generate polymer shells and thus shield

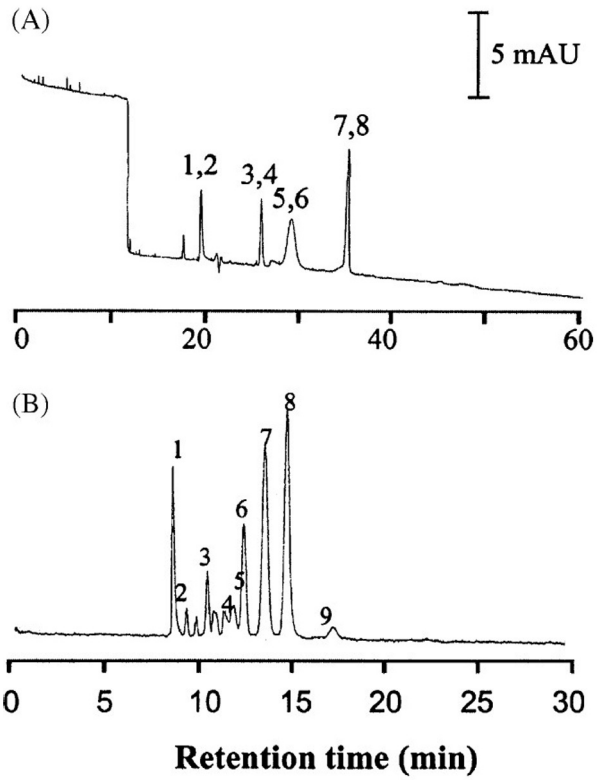

Figure 3. Separation of peptides using template inside polymethacrylate-based monolith – comparison of electrochromatograms of (A) nontemplated column with that of (B) templated column. Column: 75 cm (50 cm to the detector) × 75 μm id; mobile phase: phosphate buffer (pH 7.0, 40 mM) with 5% methanol; applied voltage: +15 kV; detection: 214 nm. Peak identification: (1) FMRF (Phe-Met-Arg-Phe-NH_2), (2) oxytocin, (3) [Sar1, Thr8] angiotensin, (4) vasopressin, (5) angiotensin I, (6) angiotensin II, (7) β-casomorphin bovine, (8) tocinoic acid, (9) β-casomorphin human. (Reprinted from ref. [66] with permission. Copyright 2006 Elsevier).

functional groups in the lower layer from unwanted interactions with the analytes [69]. The capillary was first photografted with a layer of ionizable poly(2-acrylamido-2-methyl-1-propanesulfonic acid) that generates EOF and then this layer was covered with a layer of hydrophobic polymer chains of poly(butyl acrylate). This shielded stationary phase enabled the separation of a variety of highly basic peptides and proteins at neutral pH [69].

3.2.3.5 Affinity electrochromatography

Polymethacrylate-based monoliths were also developed for affinity electrochromatography using immobilized mannan. This design was developed for the separation of mannose-binding proteins by Bedair and El Rassi [70]. The cationic monolith consisted of poly(glycidyl methacrylate-*co*-ethylene dimethacrylate-*co*-[2-(methacryloyloxy)ethyl]trimethyl ammonium chloride). This column allowed a relatively high EOF when mannan was immobilized to the epoxy monolith *via* a positively charged spacer arm, triethylenetetramine. The proteins separated were mannose-binding proteins such as the plant lectins Con and Lens culinaris agglutinin and a mammalian lectin (*e.g.*, rabbit serum mannose-binding protein). A similar neutral monolith (poly(glycidyl methacrylate-*co*-ethylene dimethacrylate)) was only useful for nano-LC (it did not generate EOF).

A monolithic affinity microextractor was used for the analysis of histidine-containing peptides. Monolithic capillary columns retained these peptides and they were subsequently analyzed by CZE. The monolithic column was prepared in a fused-silica capillary (150 μm id) by ionizing radiation-initiated *in situ* polymerization and crosslinking of diethylene glycol dimethacrylate and glycidyl methacrylate, and chemically modified with iminodiacetic acid and copper ions. This microextractor (8 mm long) was coupled online near the inlet of the separation capillary (fused-silica capillary, 75 μm id × 28 cm from the microextractor to the detector). Peptides were eluted from the sorbent by a 5 mM imidazole solution and then separated by CZE [71].

3.3 Other types of monoliths

3.3.1 Incorporation of single-wall carbon nanotubes into the monolith

Li *et al.* [72] introduced an interesting new concept to monolithic capillary columns. They incorporated single-wall carbon nanotubes (SWNT) into a polymeric monolith composed by the polymerization of VBC and ethylene dimethacrylate. This column was used as the stationary phase for HPLC and CEC. The stationary phase was formed inside a fused-silica capillary when the lumen was coated (covalently bonded) with PEI. The annular EOF generated by the capillary coating allowed peptide separation by CEC in the counterdirectional mode. A comparison of peptide separations on the monolith with and without SWNT with annular EOF generation revealed that the incorporation of SWNT into the monolithic stationary phase improved peak efficiency and influenced chromatographic retention.

3.3.2 Ring-opening metathesis polymerization (ROMP)

ROMP [73] is a relatively new method for the preparation of chromatographic materials. Monolithic materials have recently been developed that are usable as the stationary phase for the HPLC separation of various compounds, including proteins and digests of proteins. There is also the possibility of monolith grafting and it is this property that is useful not only for the modification of the chromatographic (HPLC) stationary phase but also

for an application in CEC. There would appear to be a lot of potential for this material in CEC.

The first application in the field of CEC was described by Gatschelhofer *et al.* [74] for the enantioseparation of glycyl-dipeptides. Monoliths were prepared in fused-silica columns with 200 μm id using norborn-2-ene (NBE), 1,4,4a,5,8,8a-hexahydro-1,4,5,8,exo,endo-dimethanonaphthalene (DMN-H6) as monomers, 2-propanol and toluene as porogens, $RuCl_2(PCy_3)_2(CHPh)$ as the initiator and silica-based particles containing the chiral selector. Silica particles bearing the chiral selector were suspended in the polymerization mixture. The chiral selector used was teicoplanin aglycone.

4 Open tubular CEC (OT-CEC)

A relatively recent review about stationary phase design for OT-CEC was published by Guihen and Glennon [21]. However, the presented review is only focused on the methods that can be used for the analysis of peptides and proteins.

OT-CEC is based on the interaction between the analytes and the capillary wall. In the ideal situation, the interaction is strictly chromatographic. This means that the interactions have to be reversible. Probably the first OT-CEC separation was reported by Tsuda *et al.* [75] using an octadecyl-modified 30 μm id capillary.

The inner wall of a fused-silica capillary can be used as a site for the interaction of solutes in electrophoretic measurements (for a review, see, *e.g.*, ref. [76]). By the way, during the first few years of CE, the adsorption of proteins and peptides to the capillary wall (*i.e.*, interaction with silanol groups) was one of the main problems in the analysis of biological samples. To eliminate this adsorption, many various types of coatings of the capillary wall were developed or the peptides/proteins were run under highly acidic or alkaline conditions [77]. Various polymeric coatings are often used. Of these polymeric coatings, we should mention polysaccharide dextran [78], polyacrylamide [79], hydroxylated polyether [80], polyvinylmethylsiloxanediol-polyacrylamide [81], poly(acryloylaminoethoxyethanol) [82], poly (vinyl alcohol) [83], polyarginine [84], cellulose acetate [85], or poly-(ethylene-propylene glycol) [86].

4.1 Chemically bonded ligand phase

A separation based on cation-exchange behavior was described in a capillary with immobilized poly(aspartic acid) [87]. The separation mechanism depends on the relationship of the mobile phase pH to that of protein p*I* and ionic strength. The efficiency of the separation was 10–100-times higher than in HPLC. It was demonstrated

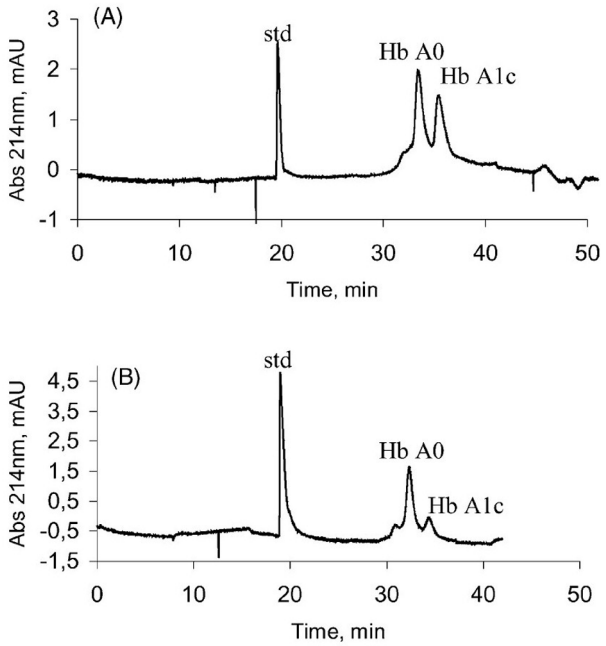

Figure 4. Affinity-based separation for the analysis of glycated hemoglobin (column coated with poly-aminophenylboronic acid) – separation of hemoglobin A_0 and glycated hemoglobin A_{1c}. Running conditions: capillary 37 cm × 50 μm id; 50 mM phosphate buffer at pH 6.0, Tween 0.5%; cathodic direction; 10 kV. Internal standard: glycyl–histidine. (A) Separation obtained for a sample containing a 60:40 ratio of hemoglobin. (B) Separation of a sample containing a ratio 88:12 of A_0/A_{1c}. (Reprinted from ref. [88] with permission. Copyright 2004 Elsevier).

that this isocratic OT-CEC approach for the separation of proteins is equivalent to gradient-elution HPLC.

Affinity-based separation was described for the separation of glycoproteins (accurately glycated proteins) [88]. In this case, poly-aminophenylboronate coatings were used. It should be mentioned that columns with immobilized aminophenylboronic acid are a traditional method for the affinity-based liquid chromatographic separation of glycated hemoglobin. Hemoglobin A_0 and glycated hemoglobin A_{1c} were successfully separated in a coated capillary (37 cm × 50 μm id) using a pH 6.0 running buffer. Because at this pH both glycated and nonglycated proteins strongly adsorb to the ligand, a low concentration of the neutral detergent, Tween-20, was added (0.5%) (Fig. 4) [88].

Dendritic polymers can also serve as a bonded stationary phase [89]. G0, G1, and G2 poly(aryl ether) monodendrons were bonded to the interior of silica capillaries through a triethoxy-(3-isocyanatopropyl)silane linker. The capillary used was 50 cm long and had a 50 μm id The bonded materials tended to reduce the EOF. This method looks to be a promising method for many different compounds (basic proteins, neutral aromatic hydrocarbons). A mixture of three standard proteins (lyso-

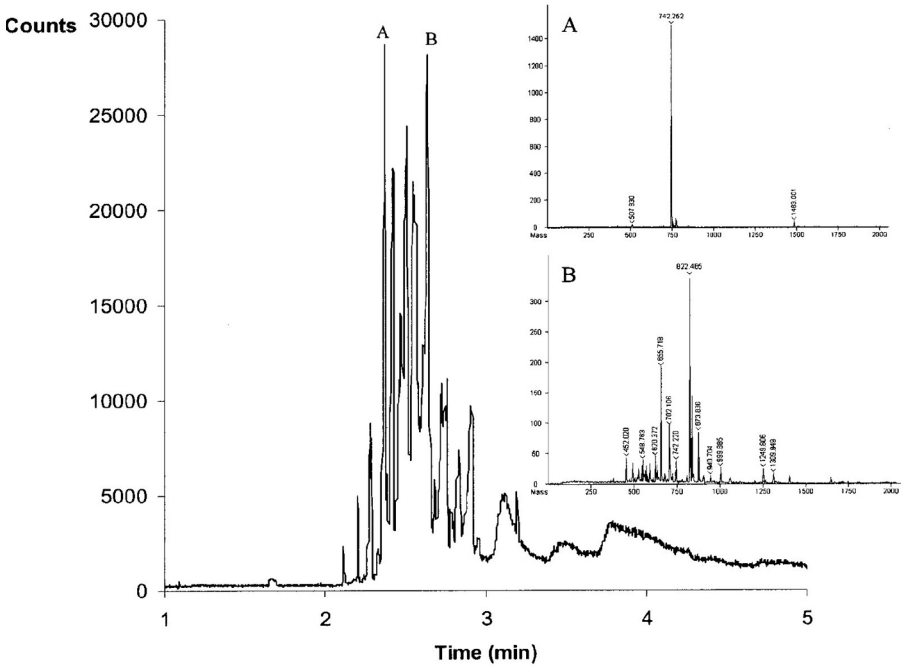

Figure 5. Separation of BSA digest in capillary with inner surface modified with octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride and coupled to ESI-MS (selected ion profile 500–1500 *m/z*). Two insets: (A) single peptide peak and (B) peak including several peptides. Conditions: 40 cm × 50 μm id capillary; mobile phase 5 mM acetic acid with 20% ACN. (Reprinted from ref. [96] with permission. Copyright 2004 Wiley-VCH).

zyme, cytochrome *c*, and ribonuclease A.) was successfully separated in a G1-coated capillary column using a 5 mM MES buffer, pH 6.

Quadruplex (G-quartet) DNA stationary phases prepared by the covalent attachment of the oligonucleotides are other interesting phases for the separation of peptides and proteins. Dick *et al.* [90] demonstrated that various albumins, similar in their structure, can be separated by this type of OT-CEC using 25 mM Tris buffer, pH 7.2 with 1 mM KCl as the BGE. Vo and McGown [91, 92] used a 25 mM potassium phosphate buffer, pH 8.0 with 2 mM KCl for successful separation of a series of dipeptides, including homodipeptides and alanyl dipeptides [91] as well as for the separation of fibrinogen peptides [92]. The resolution of fibrinogen peptides depended on the temperature [92]. This type of coating was also used for the separation of Trp-Arg and Arg-Trp [93], bovine β-lactoglobulin variants A and B [94], or bovine milk proteins, including α-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin [95].

A study was conducted on the applicability of the positively charged alkylaminosilyl monomers as modifiers of the inner surface of fused-silica capillaries for rapid peptide analysis and coupling to ESI-MS. The best choice was to use octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride as a modifier which made it possible to separate and analyze various neuropeptides or BSA tryptic digest (using a 40 cm × 50 μm id capillary and mobile phase 5 mM acetic acid with 20% ACN, Fig. 5) [96].

Peptide diastereomers (series of synthetic 18-residue, amphipathic α-helical monomeric peptide analogs) were separated by OT-CEC using a C8-coated capillary. An increase in temperature from 15 to 25°C significantly improved the peak shape. A further increase in temperature had no effect or even impaired the separation. The addition of 2,2,2-trifluoroethanol or ethanol dramatically improved chiral separation. The best BGE consisted of 100 mM sodium phosphate, pH 3.5 with 25% 2,2,2-trifluoroethanol or ethanol, respectively [97].

4.2 Etched capillaries and their surface modification

The main problem with the OT-CEC technique is the low capacity of the column due to the small area available for bonding a stationary phase. An increase in the available relative surface area can be achieved by reducing the column inner diameter. For example, decrease in the capillary diameter from 75 to 30 μm reduces the volume of the capillary by a factor of 6.25 while the surface of the capillary is only reduced by a factor of 2.5. It means that the ratio of suface-to-volume increases. Another approach is the chemical etching of the inner wall of the capillary. This procedure increases the overall surface area of the capillary by up to 1000-fold [76].

The use of etched octadecyl modified (bonded) capillaries for the separation of peptides/proteins was first described by Pesek and Matyska in 1996 [98]. They compared the separation of peptide and protein mixtures on bare, etched diol, and etched octadecyl capillaries [99]. The authors concluded that diol and octadecyl capillaries have distinct separation capabilities (under identical conditions) and they exhibit significant differences compared to a bare capillary. The same group also demon-

strated the influence of inner diameter, where a smaller diameter (20 μm, compared to 50 μm) exhibits an improvement in the resolution of proteins [100]. They also published that the silanization/hydrosilation reaction sequence is superior to organosilanization for the modification of etched capillaries. In many instances, both the peak symmetry and efficiency in the organosilane column are significantly poorer than on a hydride-based capillary [101]. The effects of the presence of inorganic compounds in the surface matrix on the electromigration behavior of enkephalins were also studied. Capillaries were etched with ammonium bifluoride in the presence of a second inorganic salt ($CuCl_2$, $CrCl_3$, $NaNO_3$, or $(NH_4)_2CO_3$) and the surface was also modified with octadecyl [102]. The influence of immobilized hydrophobic ligands on the etched capillary was also examined. Two immobilized phases were studied (*N*-butylphenyl and cholesterol-10-undecenaoate) were studied and the study was carried out on synthetic peptides [103]. Etched capillaries can also be modified by liquid crystal compounds, cholesterol-10-undeceneoate, and 4-cyano-4′-pentoxybiphenyl. While this coating improves the resolution of smaller molecules, the resolution of proteins is not influenced as much [104].

Etched capillaries can also be coated; this means that the modifier is not chemically bonded. The coating of a capillary with Polybrene (hexadimethrin bromide) is an example of this procedure [105]. This coating results in a better resolution and higher retention for a protein mixture compared to a coated but unetched capillary.

4.3 Sol–gel derived phases

The sol–gel technique can also be used for the preparation of open-tubular columns (for an overview of sol–gel stationary phases in CEC, see the review by Li *et al.* [106]). Zhao *et al.* [107] prepared a C_{18} ester-bonded column for peptide separation. GPTMS (silane agent) was used as the sol–gel precursor to form a thin coating layer on the wall of the fused-silica capillary. The C_{18} groups were introduced into the coating layer *via* an on-column esterification reaction with stearic acid. The usefulness of this column was demonstrated with the separation of seven pentapeptides (separation conditions: capillary 40/48 cm, 25 μm id; mobile phase, ACN/25 mM phosphate buffer, pH 2.5, 25:75 v/v) [107].

4.4 Porous layers

In peptide and protein separations, the term porous layer open-tubular (PLOT) is often used. It describes the situation where a thin porous layer of stationary phase is attached to the inner wall of a fused-silica capillary, irrespective of whether this interaction is based on covalent bonding or other (noncovalent) interactions [15].

An interesting approach utilizes a functionalized rugulose polymeric porous layer grafted to the inner wall of 20 μm id fused-silica capillaries [108]. This polymeric layer was highly crosslinked and prepared by *in situ* polymerization of VBC and divinylbenzene (in the presence of 2-octanol as a porogen). The surface (chloromethyl functions) of the porous polymeric layer was derivatized with alkylamine (*N*,*N*-dimethyldodecylamine) to obtain a positively charged chromatographic surface with fixed C12 alkyl chains. It was demonstrated that a mixture of lysozyme, cytochrome *c*, ribonuclease A, and α-chymotrypsinogen A can be separated by hydro-organic mobile phases containing ACN and phosphate buffer, pH 2.5, and the separation mechanism was a combination of chromatography and electrophoresis [108].

It should be mentioned that a protein-bonded porous-layer open-tubular column can be used for the separation of amino acids [109]. The porous layer was coated onto the capillary inner wall by *in situ* polymerization of 2-hydroxyethyl methacrylate and 2-vinyl-4,4-dimethylazlactone (1-decanol was used as a porogen) after the fused-silica capillary had been silanized with γ-methacryloxypropryltrimethoxysilane. The azlactone functional groups at the surface reacted with BSA to yield a protein-bonded porous-layer open-tubular column. The thickness of the layer was about 1 μm. Three amino acids (histidine, phenylalanine, and tryptophan) were successfully separated using 20 mM phosphate buffer, pH 8.0 [109].

4.5 Physically attached/adsorbed

The surface of the capillary wall can be modified by physically adsorbed or attached layers. Adsorbed layers can be divided into two possibilities: physically (strongly bound stationary phases) and dynamically adsorbed (interaction is weaker). Physically attached layers can involve a variety of chemistries, including various ligands (for review see, *e.g.*, ref. [4, 21, 110, 111]).

Porphyrins can be used as modificators of the capillary wall for OT-CEC (for review see ref. [112]). Two different porphyrin derivatives (free-base or metal form), tetrakis-(phenoxyphenyl)porphyrins ($H_2TPP(m\text{-}OPh)_4$ and $Rh(III)TPP(m\text{-}OPh)_4$), were simply physically adsorbed onto the capillary wall [113]. A set of five aromatic amino acids possessing tripeptides served as the model mixture for separation. The best resolution of peptides was obtained with 100 mmol/L phosphate buffer, pH 2.5, applied voltage 15 kV, using a 43/35.5 cm × 50 mm id capillary coated with $Rh(III)TPP(m\text{-}OPh)_4$ [113]. Four structurally related octapeptides, derivatives of the B23–B30 fragment of the B-chain of human insulin with minor changes in their sequences (the presence of lysine or ornithine in position B-29, the presence or absence of the phenylacetyl protecting group on the amino group of lysine/ornithine or N-terminal amino group of glycine), were

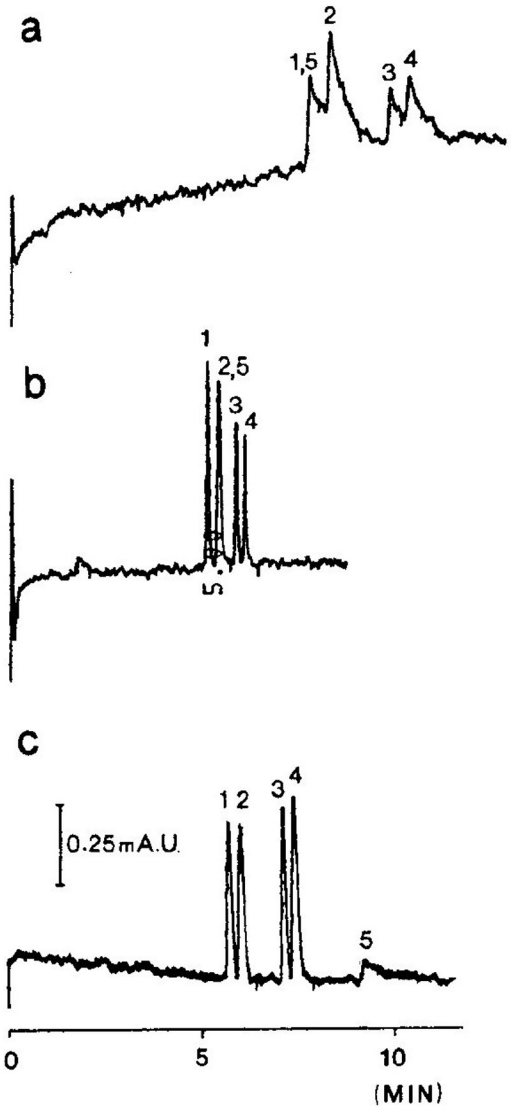

Figure 6. Separation of a mixture of peptides on (a) native fused silica, (b) underivatized titanium dioxide-coated and (c) ODS-modified titanium dioxide-coated capillaries. Conditions: (a) 20 μm id × 60.0 cm (effective length, 39.8 cm), 18 kV; (b) 20 μm id × 59.6 cm (effective length, 39.6 cm), 18 kV; and (c) 10 μm id × 58.5 cm (effective length, 39.0 cm), 20 kV. Mobile phase: (50:50 v/v) ACN/50 mM phosphate buffer (pH 2.5); detection, 200 nm. Peaks: 1, Phe-Leu; 2, Leu-Phe; 3, Leu-enkephalin; 4, Met-enkephalin; and 5, Ile-Ser-bradykinin. (Reprinted from ref. [115] with permission. Copyright 2002 Wiley-VCH).

separated in a capillary with several (metallo) porphyrins adsorbed to its inner surface. The particular porphyrins used were derivatives of tetraphenylporphyrin and complexes of porphyrin derivatives with metal ions (Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Co^{3+}). A decrease in EOF was observed after modification of the capillary. Separations were performed both in alkaline (sodium borate, pH 9.0) and acidic (Tris-phosphate, pH 2.25) BGEs [114].

Titanium dioxide can also be used for coating the inner wall of a fused-silica capillary. Fujimoto [115] treated a capillary wall with the solution of a titanium peroxo complex, followed by heating at an elevated temperature and confirmed by spectroscopic methods that a crystalline form of titanium dioxide had been formed. The capillaries had anodic or cathodic EOF, depending on the pH and composition of the BGE used. The titanium dioxide surface of the capillary could be modified by a silanizing reagent. It was demonstrated that an underivatized titanium dioxide-coated capillary is suitable for the separation of a peptide mixture but chemical (ODS) modification can further improve the separation efficiency. The separation was carried out under acidic conditions – ACN/50 mM phosphate buffer (pH 2.5) 50:50 v/v (Fig. 6) [115]. Hsieh *et al.* [116] studied the suitability of TiO_2 nanoparticle-coated capillaries for the separation of proteins (conalbumin, apotransferrin, ovalbumin, BSA). The surface chemistry of the coated inner wall of the fused-silica was significantly affected by the running buffer. By varying the phosphate buffer pH, cathodic EOF was only detected when TiO_2 nanoparticles existed as a complexed form with the buffer ligand. The selected separation conditions were: phosphate buffer (40 mM, pH 8.0), coated capillary 70 (50) cm × 50 μm id; applied voltage 15 kV. Five peaks of glycoisoforms of ovalbumin were observed under these conditions as well as both acidic and basic proteins in egg white, which were separated in a single run (Fig. 7) [116].

An interesting and useful approach is the utilization of bilayers on the capillary wall. Liposomal bilayers have been used as stationary phases attached to the wall (for review see ref. [117]). The double chained, zwitterionic phospholipid 1,2-dilauroyl-*s*-phosphatidylcholine (DLPC, C12) was used for coating a capillary. This coating allowed the separation of both cationic and anionic proteins over a pH range of 3 – 10 with efficiencies as high as 1.4 million plates/m [118]. 1,2-Dioleyl-3-trimethylammoniumpropane (DOTAP) lipid vesicles were used for the formation of a semipermanent cationic lipid bilayer in a silica capillary. DOTAP coating was stable for the separation of basic proteins (α-chymotrypsinogen A, ribonuclease A, cytochrome *c*, lysozyme), with acidic buffers (40 mM acetate buffer at pH 4) [119]. However, the majority of investigations of these types of coating were carried out using drug-like analytes.

There are some commercially available kits that utilize dynamic coating, such as CEofix™ (Analis S.A., Namur, Belgium) or EOTrol™ (Target Discovery, Palo Alto, CA, USA). For example, CEofix (double dynamic coating of a fused-silica capillary) offers the possibility of analyzing glycated hemoglobin or carbohydrate-deficient transferrin. The technique of bilayer coating for the separation of proteins was studied by Catai *et al.* [120]. Fused-silica capillaries were coated by successively flushing with a

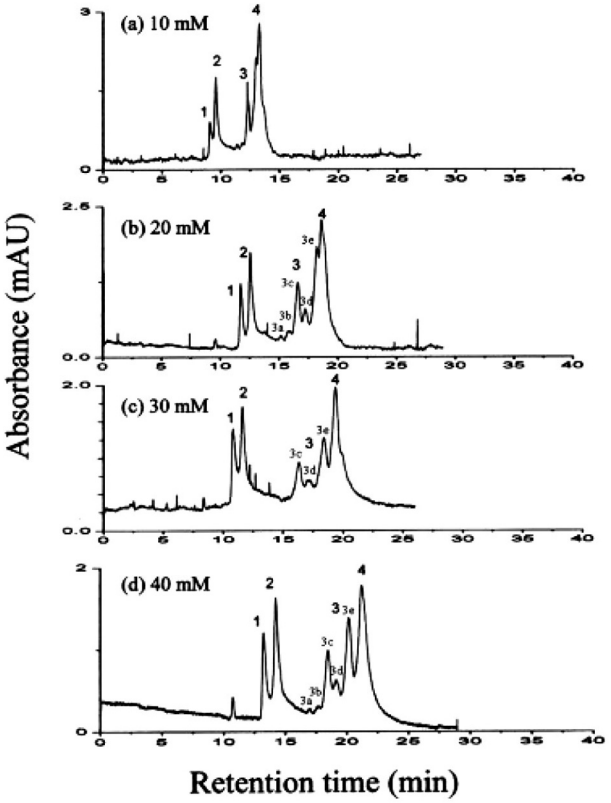

Figure 7. Separation of proteins on TiO_2 nanoparticle-coated capillaries with various concentrations of phosphate buffer. Capillary: 70 (50) cm × 50 μm id; phosphate buffer (pH 8); voltage 15 kV; detection 214 nm. Peak identification: (1) conalbumin; (2) apo-transferrin; (3) ovalbumin (a, b, c, d, and e: glycoisoforms of ovalbumin); (4) BSA. (Reprinted from ref. [116] with permission. Copyright 2006 Wiley-VCH).

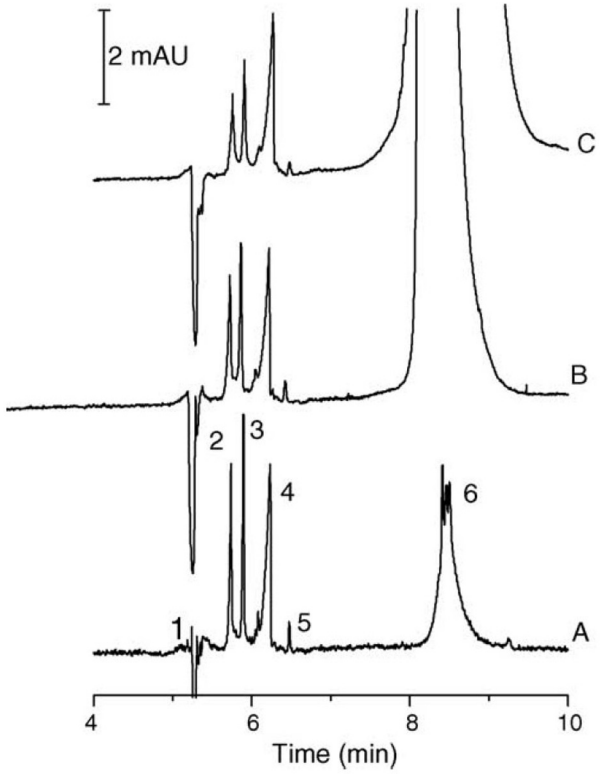

Figure 8. Separation of proteins on capillary with bilayer coating by polybrene-poly(vinyl sulfonate) containing HSA at concentrations of (A) 1 mg/mL, (B) 10 mg/mL, and (C) 60 mg/mL using BGE of 300 mM Tris phosphate (pH 8.5). Peaks: 1, system peak; 2, myoglobin; 3, carbonic anhydrase; 4, interferon-α 2b; 5, impurity from carbonic anhydrase; 6, HSA. (Reprinted from ref. [120] with permission. Copyright 2005 Elsevier).

polybrene (PB) and a poly(vinyl sulfonate) (PVS) solution. The optimum separation performance was obtained for the proteins using high BGE concentrations (300 mM Tris phosphate buffer, pH 7.0). The applicability of bilayer coatings was demonstrated by the separation of proteins such as interferon-α 2b, myoglobin, and carbonic anhydrase, by the analysis of degraded insulin, and by the profiling of the glycoprotein ovalbumin (Fig. 8) [120]. It should be mentioned that during the study of polyelectrolyte multilayer coating stability, it was determined that the 2-bilayer and 20-bilayer of these polyelectrolyte multilayer coatings can be completely removed from the capillary surface after approximately 3.5 and 9.5 h, respectively, of continuous exposure to 1 M NaOH [121].

5 Pressurized CEC (pCEC)

Pressure-assisted CEC (pCEC) or voltage assistance in micro-HPLC (voltage-assisted micro-HPLC) are two names of similar techniques. By definition, the use of the term depends on the origin of dominant flow in the separation process: EOF in the case of pCEC or the flow of eluent in voltage-assisted HPLC [15]. Of course, in many cases it is difficult to determine the dominant mechanism of separation and for this reason, in this review, both methods for the separation of peptides/proteins are presented together in this chapter.

In principle, we can say that pressurized CE is another mode used in the separation of peptides (but not only peptides) when the mobile phase is driven by both EOF and pressurized flow. In this approach, adjustment of the ratio between pressure and voltage also facilitates fine-tuning of the separation selectivity for neutral and charged components. Peptides are positively charged and so the direction of EOF, pressure, and peptide migration are all the same. On the other hand, the application of pressure can also suppress bubble formation during the separation process [122, 123].

The use of pressure in addition to an electric field in CEC was probably first mentioned by Tsuda [124] and

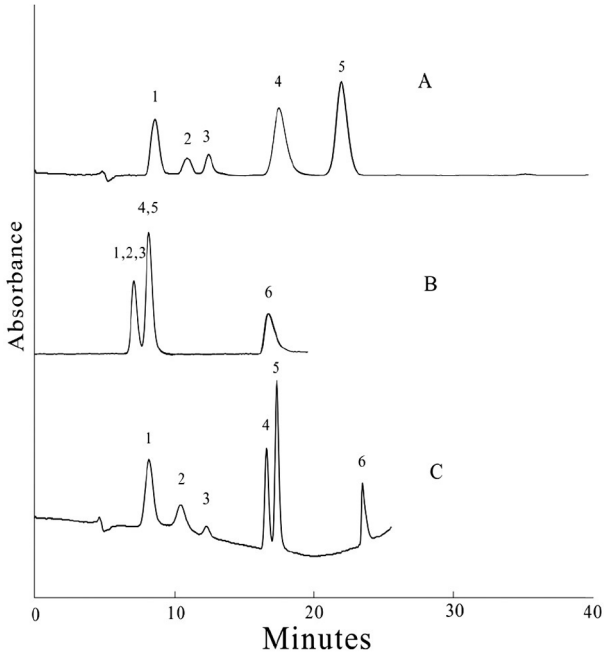

Figure 9. Comparison of isocratic and gradient elution by pressurized CEC using strong cation exchange (SCX) column for the separation of six peptides. Column: 250 mm × 100 μm id packed with 5 μm SCX; flow rate: 0.03 mL/min; voltage: 10 kV; pressure: 6.89 MPa; detection wavelength: 214 nm; peaks: 1 Met-Met; 2 Gly-Leu; 3 Leu-Gly-Gly; 4 Gly-Cys; 5 Gly-Gly-Gly; 6 Gly-Arg-Gly-Asp-Ser-Pro-Lys. A: Isocratic: 75% v/v ACN in 15 mM KH_2PO_4 buffer (pH 2.5); B: Isocratic: 50% v/v ACN in 15 mM KH_2PO_4 (pH 2.5); C: Gradient elution: 80% v/v ACN in 4 min, 80% v/v ACN to 40% v/v ACN in 6 min, buffer: 15 mM KH_2PO_4 (pH 2.5). (Reprinted from ref. [126] with permission. Copyright 2003 Wiley-VCH).

named pressure-assisted electrochromatography [125] or pressurized flow CEC [8].

One of the most interesting applications of pCEC is the gradient elution. Some instruments have been developed in the laboratory for this specific purpose. Descriptions of these instruments are available in papers utilizing this technique. Zhang *et al.* [123] developed a method for the gradient elution of neutral and charged peptides. The column used was a packed capillary (250 mm × 100 μm id) with 3 μm C18. It was demonstrated that the separation of six model peptides by a continuous gradient is more efficient than isocratic separation. The resolution of peptides is affected by the ion-pairing additive (trifluoracetic acid improved separation) and the separation can be fine-tuned by adjusting the electrical field on the column (the electrophoretic mobility of the peptides contributed to selectivity). A change of pressure has only a slight influence on the resolution of peptides [123].

The same authors used gradient pCEC for the separation of peptides using an SCX column [126]. SCX capillary columns (250 mm × 100 μm id and 250 mm × 150 μm id) were packed with 5 μm poly(2-sulfoethylaspartamide)-silica particles. It was reported that the retention mechanism of the peptides in this system is based on a mixed mode of hydrophilic interaction, strong cation exchange, and electrophoresis and it was declared that it is a powerful mode for the separation of hydrophilic peptides with a similar charge-to-mass ratio (Fig. 9).

Another stationary phase usable for the gradient pCEC of peptides is an amide phase [127]. A capillary (id 150 μm) packed with 5 μm particles of TSK gel Amide-80 was used. It was demonstrated that the electrophoretic migration in electrochromatography enhanced the separation of peptides, since the separated peak number of a tryptic digest of BSA was increased from 30 to 40 by the application of +5 kV instead of using pressure-driven capillary LC alone.

Multipurpose columns have also been used (made by the thermally induced *in situ* copolymerization of methacrylic monomers and the surfaces of the monoliths modified by *N*-ethylbutylamine) [128]. These columns were described as multipurpose columns (and the method was named multimode pCEC/pCE) because they exhibited mixed modes of separation mechanisms under different conditions (depending on the pH). For example, at an acidic pH (and high voltage across the column) the monolithic column exhibited a predominantly capillary electrophoretic migration of peptides and at basic pH (and an electric field across the column) it exhibited CEC mechanisms of separation. High efficiencies of peptide separation were reported in various buffers (volatile and nonvolatile, acidic and basic) and in both isocratic and gradient elution.

At present, in the proteomic era, one of the most popular (and useful) techniques for the detection/identification of proteins and peptides is MS. Pressurized CEC is suitable for the direct coupling of pCEC and MS due to the relatively uniform flow of mobile phase inside the capillary. Schmeer *et al.* [129] first published the application of additional pressure to CEC/electrospray-MS for the stabilization of flow (EOF) during electrochromatography. They used a capillary column packed with 1.5-μm RP stationary phase at flow rates of 1 – 2 μL/min. This approach allowed the detection of peptides in picomole quantities.

Wu *et al.* [130] coupled pCEC to an IT storage/reflectron TOF mass spectrometer for the analysis of peptide mixtures and protein digests. Fine-tuning of additional pressure and the electric field allowed the separation of protein digests in a column 6 cm long, when a tryptic digest of bovine cytochrome *c* was separated in 14 min and a tryptic digest of chicken ovalbumin (more than 20 peaks) was resolved within 17 min.

Liang *et al.* [131, 132] coupled ESI-MS using a coaxial sheath liquid interface. The separations of tryptic digests of cytochrome *c* and modified protein as real samples

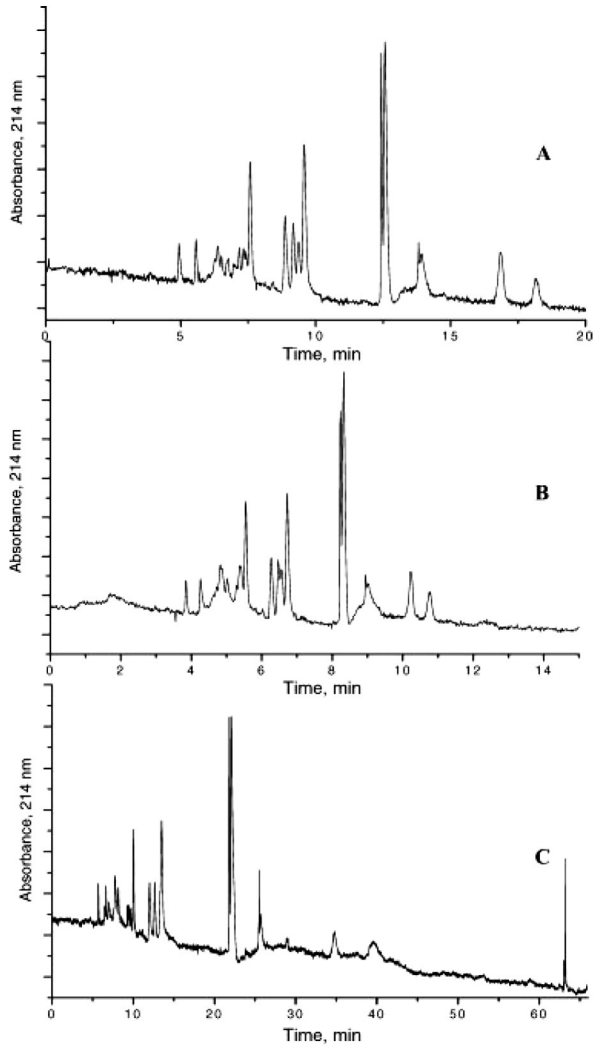

Figure 10. Influence of pressure on the separation of a tryptic digest of cytochrome *c* by multimode pressure-assisted CEC (column 26/44 cm, 50 μm id). Mobile phase: 25 mM Tris-HCl (pH 8.0)/ACN (40:60); separation voltage 20 kV. Applied pressure: (A) atmospheric from both sides; (B) 120 psi (0.83 MPa) on the inlet buffer vial; (C) 120 psi (0.83 MPa) on the outlet buffer vial. (Reprinted from ref. [128] with permission. Copyright 2003 Wiley-VCH).

were studied in a 100 mm id capillary column, 30 cm total length, 20 cm packed with octadecyl silica, where the impact of the pH and concentration of the electrolyte was studied. They also improved the detection sensitivity to online concentration by taking advantage of the field-enhanced sample-stacking effect and chromatographic zone-sharpening effect, and the detection limit was determined to be 10^{-11} mol [132].

The coupling of multimode pCEC/CE (using gradient or isocratic elution) by sheathless electrospray emitter for ESI-MS/MS [128] was also described. This method enabled the analysis of a BSA tryptic digest in less than 5 min yielding a high sequence coverage (73%), demonstrating the potential of the method (Fig. 10).

All previously mentioned methods used EOF as one of the factors for the separation of peptides during the analytical procedure. However, there are also some papers dealing with separation without EOF or with only a limited influence of EOF on the elution from the column.

Wu *et al.* [133] used monolithic column practically without EOF (prepared from the *in situ* copolymerization of lauryl methacrylate and ethylene dimethacrylate to form a C12 hydrophobic stationary phase). Under these conditions, peptides at the acidic buffer were separated on the basis of their differences in electrophoretic mobility and hydrophobic interaction with the stationary phase. It should be stressed that charged compounds elute electrophoretically from such columns, while interacting chromatographically with the neutral hydrophobic stationary phase. The separation was different from CZE. It was demonstrated that some peptide isomers that could not be separated by CZE were separated by this method.

Szucs and Freitag [62] developed the so-called voltage-assisted LC method for the separation of peptides. They compared this method to two other separation modes: CEC and nano-HPLC. With voltage-assisted LC, the charged analytes migrate through a neutral stationary phase driven by electrophoresis while their interaction with the stationary phase provides the basis for a chromatographic separation (it should be mentioned that no pressure was applied). The stationary phases used were poly(glycidyl methacrylate-*co*-ethylene dimethacrylate)-based monoliths and the filled column was 37 cm (effective length 28 cm) × 75 μm id. With CEC, the column was derivatized with ionogenic *N*-ethylbutylamine functional groups. The best separation was observed using voltage-assisted LC. The influence of the mobile phase composition (strength, organic moiety) on the separation was studied, and optimal conditions for the separation by voltage-assisted LC were a 60 mM phosphate buffer at pH 2.5 containing 40% ACN (and the voltage applied was (25 kV). The elution order showed dependencies not only on the charge density but also on the hydrophobicity of the peptides.

6 CEC on a microchip platform

Miniaturization and automation of the whole separation process is nowadays one of the most promising and popular methods in analytical chemistry. Microfabricated devices (microchips) are developing as routine methods for the analysis of proteins, DNA, RNA, cells, etc., using either chromatographic or electromigrative approaches. Analyses are rapid and highly efficient. From this point

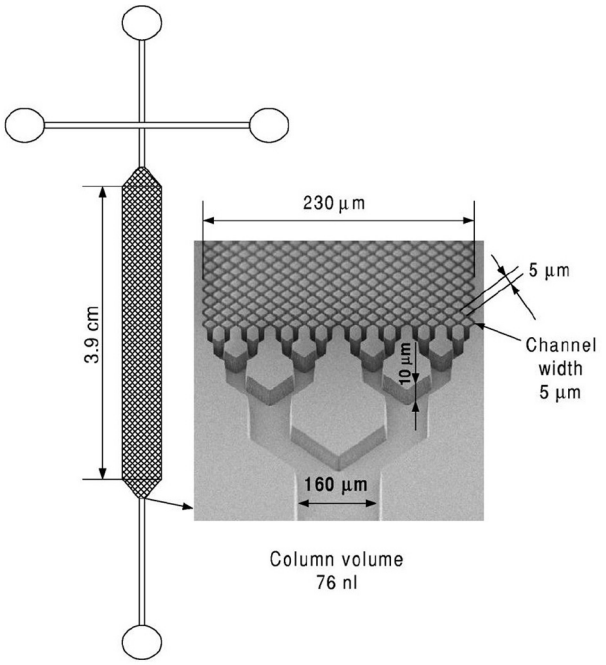

Figure 11. COMOSS (collocate monolith support structures) chip. (Reprinted from ref. [138] with permission. Copyright 2002 Elsevier).

of view, CEC in microfabricated monolith columns are promising techniques [134, 135].

The use of a C18-modified collocate monolith support structures (COMOSS) chip for the separation of a tryptic digest was described [136] and results were comparable to HPLC separations. The usefulness of chips made with poly(dimethyl siloxane) (PDMS) was also demonstrated [137]. Subsequently, a PDMS chip modified by cerium(IV)-catalyzed polymerization was applied [138] (Fig. 11). In this case, vinylsulfonic acid, acrylic acid, 2-acrylamido-2-methylpropanesulfonic acid, 4-styrenesulfonic acid, and stearyl methacrylate were used to modify the PDMS surface by cerium(IV) catalyzed polymerization on microfabricated COMOSS microchips. Modification of the channels by 2-acrylamido-2-methylpropanesulfonic acid and methoxydimethyloctadecylsilane allowed the separation of a protein (BSA) digest with high reproducibility, and an efficiency of about 620 000 plates/m [138].

Chips with a separation channel packed with granular material were also used [139]. The chips were fabricated in PDMS using deep-reactive-ion-etched silicon masters. The separation channel was packed in vacuum with 3-µm octadecylsilanized silica microspheres and the packing was stabilized in the column by a thermal treatment.

Microchips based on a quartz chip with continuous polymer beds were described as a useful platform for the separation of proteins [140]. Acrylate-based porous polymer monoliths were also used as stationary phases for separations in the microchannels of glass chips [54]. In one case, three naphthalene-2,3-dicarboxaldehyde (NDA)-labeled peptides were successfully separated. A similar approach – a microfabricated glass chip containing fluidic channels filled with an acrylate-based porous polymer monolith – was used for the separation of bioactive peptides and amino acids labeled with an NDA dye (followed by LIF detection) [141]. The separation was RP (the polymer was negatively charged lauryl acrylate) and the mobile phase (ACN/25 mM borate, pH 8.2, 3:7 v/v) contained octane sulfonate (10 mM). These separations were fast (six peptides in 45 s) and efficient (up to 600 000 plates/m) [141].

A microfluidic device for CEC can also be used for coupling with MS [142]. The dimensions of one processing line were sufficiently small to enable the integration of 4–8 channel multiplexed structures on a single substrate. A methacrylate-based monolithic material with a positively charged surface was used (to generate EOF). Protein digests were separated to a sensitivity of fmol levels.

One of the most interesting applications is a multidimensional approach. Slentz *et al.* [143] used a chip for a protein proteolysis and the multidimensional electrochromatographic separation of histidine-containing peptide fragments. The first step was trypsin digestion, followed by the copper(II)-immobilized metal affinity chromatography [Cu(II)-IMAC] selection of histidine-containing peptides, and subsequent separation by RP CEC of the selected peptides. Trypsin digestion and affinity chromatography were achieved in particle-based columns with a microfabricated frit, whereas RP separations were executed on a column of collocated monolithic support structures. The applicability of this method was shown by the analysis of FITC-labeled BSA.

Gottschlich *et al.* [144] developed a 2-D separation system on a microfabricated device with combined open-channel electrochromatography as the first dimension and CE as the second dimension. A 25-cm separation channel (OT-CEC) with spiral geometry was chemically modified with octadecylsilane and coupled to a 1.2-cm straight separation channel for CE. The effluent from the first dimension was repetitively injected into the second dimension every few seconds. Fluorescently labeled products from tryptic digests of β-casein were analyzed in 13 min with this system.

However, the interesting development of chip-based analysis is not only associated with the CEC analysis of proteins but also with capillary electrophoretic or liquid chromatographic applications. Le Gac *et al.* [145] developed monoliths for microfluidic devices in proteomics based on the polymerization of lauryl methacrylate and ethylene dimethacrylate (the stationary phases have a C12-functional group). The suitability of the monolithic system for proteomic research was demonstrated by nano-LC/MS of a cytochrome *c* digest. Of course, this sys-

tem was developed for nano-LC but a similar principle could be applied to the CEC approach.

7 Concluding remarks

CEC seems to be a promising and relatively rapidly developing technique for proteomic (and peptidomic) research. CEC combines the advantages and disadvantages of chromatographic and electrophoretic techniques. It is obvious that this technique is not just an alternative, but is a complementary method to both. However many applications of CEC for the analysis of peptides/proteins exist, it is evident that CEC is still a developing technique. Nowadays, the most rapidly developing area is research into new monolithic materials/columns and the multidimensional approach seems to be promising. However fascinating the emerging proteomic research is, a noticeable development of CEC techniques is connected to the analysis of drugs and other lower molecular compounds that are important to industry. However, it is reasonable to suppose that the development on the whole of CEC as well as the CE and HPLC techniques will result in progress in the CEC analysis of proteins/peptides in particular. In the current proteomic era, one can foresee a bright future for this technique.

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