ISSN 1615-9306 · JSSCCJ 40 (1) 1–394 (2017) · Vol. 40 · No. 1 · January 2017 · D 10609

JSS

JOURNAL OF SEPARATION SCIENCE

1 | 17

VOLUME 40

Methods
Chromatography · Electroseparation

Applications
Biomedicine · Foods · Environment

www.jss-journal.com

WILEY-VCH

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Received August 15, 2016
Revised October 1, 2016
Accepted October 1, 2016

Review

Capillary electrochromatography of proteins and peptides (2006–2015)

This review summarizes the development and the applications of capillary electrochromatographic techniques pertinent to the analysis of proteins and peptides, which took place over the last 10 years (2006–mid-2016). This "hybrid" technique is useful for the analysis of a broad spectrum of proteins and peptides and is an approach that is complementary to the liquid chromatographic and the capillary electrophoretic analysis. All modes of capillary electrochromatography are described here, which includes particle-packed columns, monolithic stationary phases, and open-tubular capillary electrochromatography.

Keywords: Capillary electrochromatography / Peptides / Proteins
DOI 10.1002/jssc.201600908

1 Introduction

Although this review is a continuation of our 10-year-old review on this topic [1], it was necessitated by the fact that, not surprisingly, during the past 10 years a number of new articles describing new approaches as well as applications have arisen.

CEC can be defined as a hybrid technique that utilize the principles of electromigration techniques (electroosmosis and electrophoresis) and chromatography (distribution between two phases). Nowadays, this technique is frequently used for the separation and analysis of a broad spectrum of organic and inorganic compounds of both low and high molecular mass and many reviews and studies on this technique are available [2–9].

Notably, this review is focused on the analysis of proteins and peptides by CEC, because the current era of proteomic research required the development of new and robust methods for the analysis of natural proteomic (and peptidomic) samples. It should be also mentioned that the capillary electromigration method consists of many individual methods that are in use, such as CZE, CIEF, ACE, and CEC, and in general this topic has been covered by many reviews [10–16]. The situation is similar with regard to the field of CEC—there are many reviews available that deals with the separation of proteins and/or peptides using this method [12, 17–32].

It should be highlighted that this review is devoted to methods that deal with separation, although methods for the detection, particularly MS for the analysis of proteins/peptides are highly important in proteomic research. Therefore, such approaches will be often mentioned in the description of methods of separation, but readers with a specific interest in the methods related to detection could read more specialized reviews (e.g. [30, 33, 34]).

As it was mentioned above, if we are to talk about separation techniques, we should first look into the mechanism of separation and the motion of analytes during the separation procedure, as CEC is a combination of electromigration technique and chromatography. Proteins and peptides are a heterogeneous group of compounds that differ in their hydrophobicity, charge, molecular mass, and many other properties; and therefore, it is impossible to determine only one parameter that influences their separation efficiency. For peptides, a synergistic interplay occurs in CEC systems between the adsorptive/partitioning events and electrokinetically driven motion. Moreover, at high electric field strengths, both bulk electrophoretic migration and surface electrodiffusion occur. The separation (and interaction) of peptides/proteins during CEC is also influenced by various other parameters, such as 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 optimization based on a single parameter but requires more sophisticated multiparametric optimization procedures (for details, see the reviews by Walhagen et al. [35] and Kleparnik [36]) [1].

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Abbreviations: AG-NSP, affinity-gradient nano-stationary phase; **CLC**, capillary liquid chromatography; **CMC**, carboxymethylchitosan; **CSP**, chiral stationary phase; **DMMSA**, *N*,*N*-dimethyl-*N*-methacryloxyethyl *N*-(3-sulfopropyl) ammonium betaine; **GFP**, green fluorescent protein; **GNP**, gold nanoparticle; **LDL**, low-density lipoprotein; **MIP**, molecular imprinted polymer; **NAPM**, 2-naphthyl methacrylate; **NMM**, naphthyl methacrylate monolith; **ODA**, octadecyl acrylate; **ODM**, octadecyl acrylate monolith; **OPA**, *o*-phthalaldehyde; **OT-CEC**, open tubular capillary electrochromatography; **PAH**, polyaromatic hydrocarbon; **PEM**, polyelectrolyte multilayer; **PC**, photonic crystal; **pCEC**, pressurized capillary electrochromatography; **PETA**, pentaerythritol triacrylate; **PLOT**, porous layer open-tubular; **ROMP**, ring-opening metathesis polymerization; **SMC**, segmented monolithic column

The CEC methods for the analysis of proteins/peptides can be classified in two ways: according to the column format or the mechanism of separation [1]. Based on the formats of the columns, three modes of CEC could be distinguished: the columns (capillaries) packed with particles, monolithic columns, and the open-tubular system (open-tubular electrochromatography, OT-CEC); whereas according to the mode of separation, it can be classified into several modes in principle, but of particular importance are reversed-phase, normal phase, ion-exchange, size-exclusion, or affinity-based separation. In addition to these classificatory schemes, a third possibility is to classify CEC based on instrumentation design, such as pressurized CEC and the microchip platform.

2 Packed (granular) columns

The use of packed columns is the "old" and "traditional" approach in CEC. In this case, the capillary is filled by chromatographic media that is in many cases the same as those used for HPLC, or μHPLC. Typically, the materials most often used for protein/peptide separation are reversed-phase and ion-exchange materials. Segmented packed columns or mixed mode stationary phases can also be used.

In the packed capillaries, the main problem is the packing of capillaries with the separation material as well as the fabrication of the frit [8]. There are five methods in use, which allows packing of a homogeneous and tight bed in a capillary with acceptable speed, namely (i) pressure packing of slurries, (ii) electrokinetic packing, (iii) packing with supercritical CO_2, (iv) packing by centripetal forces and (v) packing by gravity.

Frits are porous plugs (mainly) at both the ends of a packed column to hold the particulate packing materials in place, which may affect the separation efficiency by encouraging bubble formation or by disturbing the flow profile of the mobile phase at the interface between the packed section and frit. The most common frit is silica-based sintered frit. However, other types of frits have also been described, such as: monolithic frit, sol–gel frit, single-particle frit, external taper, internal taper, and magnetically immobilized frit [8].

2.1 Reversed-phase electrochromatography

Li at al. [37] described the new and rapid method for the preparation of CEC columns. This procedure uses CEC mobile phase solution as the packing solvent and it enables column preparation throughput to 1 column/h, including all the fritting, packing, and conditioning steps. This C18 column type was verified by the separation of mixture of standard peptides with a good reproducibility (Fig. 1).

The comparison of nano-LC and CEC was performed by Fanali et al. [38] and there were comparable separations of peptides (cytochrome c tryptic digest) by nano-LC and CEC when peptide mixtures were analyzed by coupling with IT-ESI-MS through a liquid junction interface. Peptides were separated utilizing C18 silica-based stationary phases of different properties and origin, and silica derivatized with cyano groups. The best results were obtained by nano-LC employing the C18 stationary phase (detection of 20 peptides, 88% coverage). The use of CEC revealed a higher separation efficiency and shorter time for analysis. However, the numbers of separated peptides were lower than those observed in nano-LC. In CEC, the use of capillaries packed with cyanosilica particles offered better results; however, it was less satisfactory than those observed in the miniaturized LC technique. Therefore, it was concluded that these two techniques can be considered complementary, offering different information related to the retention times of the studied peptides [38].

Reversed-phase pressurized CEC (pCEC) was used for the analysis of peptides by an on-line 2D system where the first dimension (separation) utilized CIEF. The system

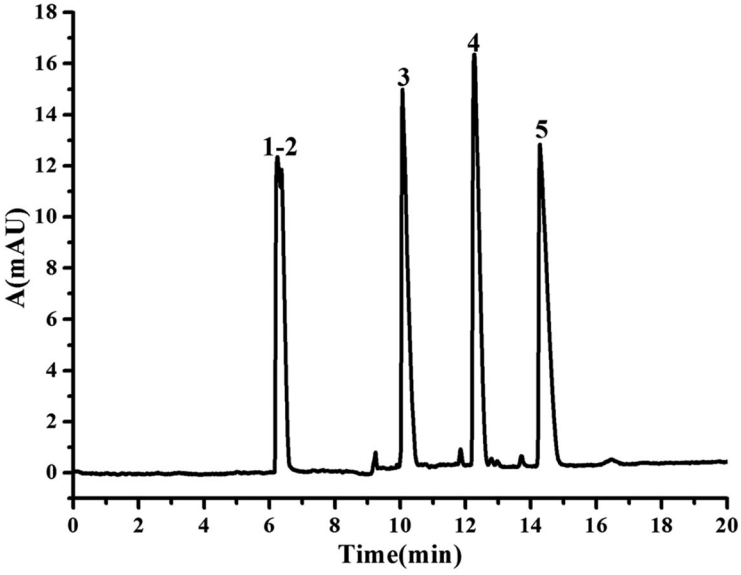

Figure 1. CEC of standard peptides on capillary column packed with Waters Spherisorb SCX/C6 3 μm, 240 mm × 100 μm id; mobile phase, acetonitrile/phosphate (50 mM, pH 2.5), 40:60 v/v; separation voltage, 10 kV; electrokinetic injection: 10 kV/10 s; UV detection: 214 nm. Analytes: (1) Gly-Tyr, (2) Val-Tyr-Val, (3) Met-enkephalin, (4) Leu-enkephalin (5) angiotensin II. Reprinted with permission from [37].

combines CIEF with pCEC using a micro-injection valve as the interface. Sample fractions, which were focused and separated in the first-dimension CIEF based on the differences in their p*I*s, were electrically mobilized and further analyzed at the second-dimension pCEC. Effectiveness of separation with this 2D system was demonstrated by the analysis of tryptic digest of BSA and human red blood cell lysate [39].

RP pCEC was used for 2D separation with SCX LC (LC) [40]. In this case, the combination of SCX-LC–RP-pCEC was used for separation of complex samples. At the first dimension was micro strong cation-exchange LC with capillary column (10 cm × 300 μm id packed with 2.5 μm SCX stationary phase). The second dimension was reversed-phase pCEC with capillary column packed with 3 μm C18 stationary phase (effective length 30 cm × 150 μm id. This 2D system was an offline system equipped with a collector after the first-dimensional column. Samples were fractionated by the first-dimension SCX-LC with a linear solvent gradient and then injected into the second-dimension RP-pCEC for further separation. Separation effectiveness of this 2D system was demonstrated by the analysis of traditional Chinese medicine *Cortex Phellodendri*, BSA tryptic digest, and real serum tryptic digest (Fig. 2).

The analyte zone sharpening of peptides at pCEC was studied on the capillary column (150 μm id, 150 mm packed length) packed with ODS particles (5 μm) [41]. Method dynamic pH junction was used. When the pH of the mobile phase was altered from basic to acidic in a step gradient, the analyte peptides were focused at the basic/acidic interface with the application of voltage. The effect of both pH and pressurized flow velocity on the zone sharpening was studied. The peak height of angiotensin II, [Asn(1), Val(5)]-angiotensin II, and angiotensin III were enhanced 12, 10, and 12 times, respectively. Selective peak zone sharpening for angiotensin II was also observed.

2.2 Ion-exchange chromatography

CEC with a SCX stationary phase (propylsulfonic acid-modified 3 μm diameter silica particles with 10 nm intraparticle porosity) has been applied for the investigation of electric field dependent CEC retention of four counterionic peptides [Met-5]enkephalin, oxytocin, [Arg-8] vasopressin, and luteinizing hormone releasing hormone. The retention behavior of peptides in the studied system depended on the charge-selectivity of the particles of the stationary phase, the applied voltage and the net charge of the peptides [42].

CEC with a SCX stationary phase packed into the fused-silica capillary of 75 μm id has been also applied for the separation of a mixture of insect oostatic peptides. The effect of organic modifier (acetonitrile), ionic strength, pH of the mobile phase, separation voltage, and temperature on the resolution of separation of peptides was investigated. Baseline separation of nine, proline-rich insect oostatic peptides, ranging from dipeptide to decapeptide, was achieved by the use of a mobile phase composed of 100 mM sodium phosphate buffer (pH 2.3)/water/acetonitrile (10:20:70 v/v/v) and a separation voltage of 30 kV at 20°C. Analysis time was reduced to less than 10 min using the short-side mode where the stationary phase was packed only for 7 cm [43].

Capillary hybrid monolithic column with sulfonate strong cation exchanger was prepared for analysis of

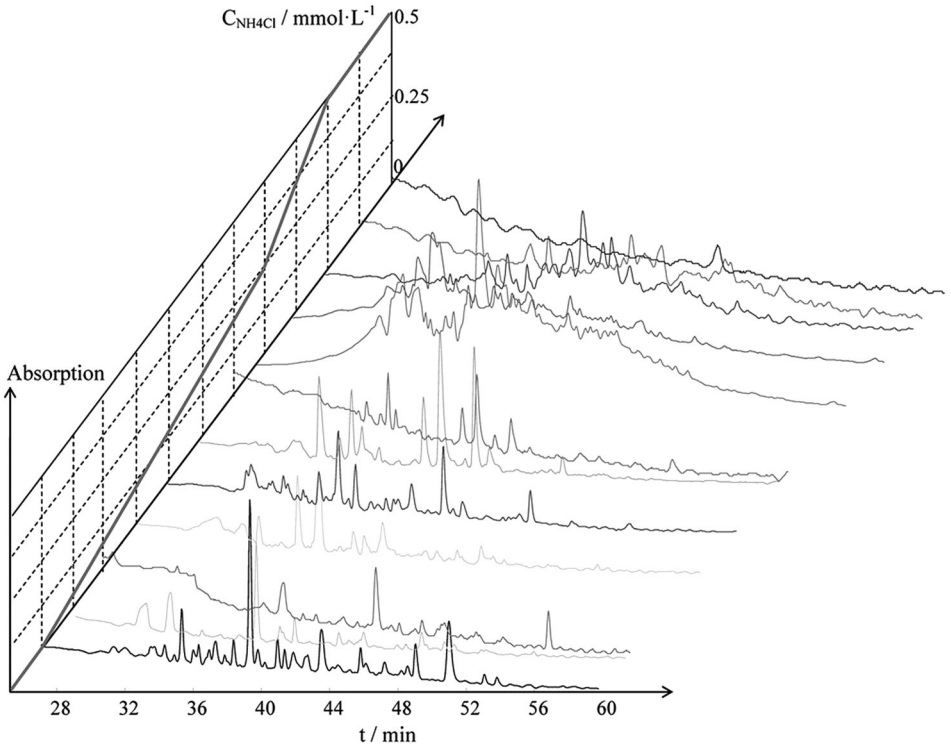

Figure 2. 2D SCX LC/RP-pCEC electrochromatograms for digest of BSA. I: Column: 10 cm × 300 μm id capillary packed with 2.5-μm SCX; mobile phase: (A) TFA/H_2O/acetonitrile (0.1:95:5, v/v/v); (B) TFA/0.5 mol/L NH_4Cl/acetonitrile (0.1:95:5, v/v/v); gradient: 0–5 min, 0% B; 5–40 min, 0–50% B; 40–50 min, 50–100% B; flow rate: 3 μL/min; sample: BSA tryptic digests. II: Column: 55 cm (effective length of 30 cm) × 150 μm id capillary packed with 3-μm C18; mobile phase: (A) H_2O/acetonitrile/trifluoroacetic acid (95:5:0.1, v/v/v); (B) H_2O/acetonitrile/trifluoroacetic acid (5:95:0.1, v/v/v); gradient: 0–5 min, 0–10% B; 5–35 min, 10–50% B; 35–45 min, 50–100% B; flow rate: 400 nL/min; applied voltage: 3 kV; detection: UV at 230 nm. Reprinted with permission from [40].

proteins by "one-pot" strategy [44]. This SCX hybrid monolithic column was prepared using solution of acetic acid, poly(ethylene glycol) (Mn = 10 000), urea, tetramethoxysilane, vinyltrimethoxysilane, and 3-sulfopropyl methacrylate potassium salt with azobisisobutyronitrile. The capillary was incubated at 45 and 60°C for 12 h, for condensation and polymerization, respectively. The developed column had approximately seven times the permeability (in water) and approximately three times the sample loading capacity (tested by dipeptide Gly-Tyr) compared to a particulate SCX column packed with commercial available material. It was successfully used also as trap column for analysis of phosphopeptides. The sulfonate SCX hybrid monolithic column was used also as enzyme reactor for online protein digestion. Comparing to particulate SCX packed column, the identified peptides number increased 40% and the protein coverage increased 10%. This might be ascribed to the high porous structure and relative high surface area that elevated the digestion efficiency.

CEC with packed bead bed (2 mm long) in the microchip channel (18 × 30–50 μm, depth × width) was used for the fast separation of peptides and proteins. In SCX mode, three oligopeptides, Lys2, Lys3, and Lys4, were separated within 40 s with a separation efficiency up to 400 000 theoretical plates/m and in the size-exclusion mode, FITC-derivatized IgG, and FITC-insulin were baseline resolved in less than 10 s (with efficiencies up to 139 000 plates/m) [45].

The effect of ionic capacity on dynamic adsorption behavior of protein in ion-exchange electrochromatography was studied by Yuan and Sun [46]. They studied four anion-exchangers of different ion-exchange capacities (21–129 μmol/mL) prepared by coupling diethylaminoethyl to Sepharose 6FF. The static adsorption capacity of BSA increased in the ionic capacity range, but the effective pore diffusion coefficient of the protein decreased with increasing the ionic capacity due to the hindrance effect of the bound protein molecules at the pore entrance. The dynamic binding capacity of protein in ion-exchange chromatography decreased with increasing the ionic capacity. By applying an electric field of 30 mA, the dynamic-binding capacity in ion-exchange electrochromatography packed with ion-exchangers of high ionic capacities (53 and 129 μmol/mL) increased significantly (over 30 and 100%, respectively). This was because the high surface charge density led to high EOF that enhanced intraparticle mass transfer. The dynamic-binding capacity in ion-exchange electrochromatography packed with ion-exchangers of low ionic capacities (21 and 35 μmol/mL) increased only slightly (ca. 10%) under the same condition. The results indicated the minor effect of electrophoretic mobility on the intraparticle mass transfer. It was concluded that it is beneficial to use ion-exchangers of high ionic capacity for high-capacity purification of proteins by ion-exchange electrochromatography.

2.3 Mixed-mode stationary phases

Chromatographic behavior of peptides on a mixed-mode stationary phase (embedded quaternary ammonium group in a C21 alkyl chain) with an embedded charged group was monitored by CEC and HPLC [47]. In the HPLC mode, the peptides were mainly separated by reversed-phase mechanism and a comparison between HPLC and CEC indicated that (i) ion-exclusion phenomenon was more pronounced in HPLC and (ii) in CEC; higher percentage of acetonitrile in the mobile phase induced an increase in the retention for some peptides. This study pointed out the existence of retention mechanisms involved in the chromatographic process other than partitioning. 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 [47].

2.4 Other types

A special CEC column was prepared by one-step packing of antivoltage photonic crystals (PCs) into microfluidic channels [48] and these packed PCs enabled ultrafast separation of amino acids along a 2.5 mm PC in 4 s, and peptides along a 10 mm PC in 12 s. The separation was highly efficient and reproducible, with a 300 nm plate height and 0.24–0.35% RSD of migration time. This one-step approach is extendable to other gel-forming particles and allows stable analytes.

There was described the usability of silica colloidal crystals for separation of proteins [49]. These crystals were formed from 330 nm nonporous silica spheres inside of 75 μm id fused-silica capillaries. Separation of three proteins was studied, ribonuclease A, cytochrome C, and lysozyme, each covalently labeled with fluorophor. They were well separated over a distance of 1 cm by isocratic electromigration, using 40:60 acetonitrile/water with 0.1% formic acid (voltage was between 400 and 1400 V/cm). The plate height for lysozyme was below 50 nm at almost all of the migration velocities, and it approached 10 nm at the highest velocity.

2.5 Chiral separation

Enantiomers and diastereomers of di- and tripeptides, amino acids, and α-hydroxy acids were separated by ligand-exchange packed CEC using silica-based chiral stationary phases (CSP) [50]. Two phases were tested–the first one contained L-4-hydroxyproline chemically bonded by a spacer to 3 μm silica material, and the second one was *N*-decyl-L-4-hydroxyproline dynamically coated on a reversed-phase packed capillary. The chemically bonded phase provided better resolution of amino acids and dipeptides, but the dynamically coated CSP was much more suitable for the separation of α-hydroxy acids.

3 Monolithic stationary phases

Monoliths are currently very popular in use as stationary phases not only for CEC but also for HPLC. Historically,

Kubin et al. [51] first published the preparation of continuous polymer matrix gel for chromatography (size-exclusion chromatography of proteins using poly(2-hydroxyethyl methacrylate)) in 1967. There are many reviews about monolithic stationary phases [5, 6, 30, 52], books [53] as well as a plethora of articles about their preparation and use in the separation science, not only in electrochromatography.

It is interesting to reflect upon the monoliths for CEC and HPLC coupled to MS [30]. Monolithic CEC–MS techniques have been more focused on the synthesis of highly specialized and selective separation phase materials for fast and efficient separation of specific types of analyte. However, monolithic CLC–MS is more widely used and is often employed, for instance, in the analysis of oligonucleotides, metabolites, and peptides and proteins in proteomic studies. The poly(styrene-divinylbenzene)-based and silica-based monolithic capillaries are currently in use in proteomic analyses when other laboratory-synthesized monoliths still wait for their wider utilization in routine analyses [30].

3.1 Polymer-based monoliths

3.1.1 Acrylate-based monoliths

3.1.1.1 Reversed-phase separation

Karenga and El Rassi introduced a series of monoliths for reversed-phase CEC [54–58].

A neutral octadecyl monolith was prepared by in situ polymerization of octadecyl acrylate as the monomer and trimethylolpropanetrimethacrylate as the cross-linker in a ternary porogenic solvent, containing cyclohexanol, ethylene glycol, and water. This monolithic column exhibited cathodal EOF over a wide range of pH and acetonitrile concentration in the mobile phase, despite the fact that it was devoid of any fixed charges. The EOF was probably due to the adsorption of ions from the mobile phase onto the surface of the monolith, which modulated solute retention and affected the separation selectivity. The wide applications of this neutral monolithic column demonstrated that while the separation of the neutral solutes were based on RP retention mechanism, the charged solutes were separated on the basis of their electrophoretic mobility and hydrophobic interaction with the C18 ligands of the stationary phase. For example, the separation of peptides were achieved quite rapidly with a separation efficiency of nearly 200 000 plates/m and this efficiency was exploited in tryptic peptide mapping of standard proteins, e.g. lysozyme and cytochrome C, by isocratic elution [54].

Another approach involving reversed-phase CEC was attempted by developing a neutral hydroxylated octadecyl monolith [56]. It was prepared by in situ polymerization of octadecyl acrylate and pentaerythritol triacrylate in a ternary porogenic solvent. Pentaerythritol triacrylate possesses a functional hydroxyl group, which donate a hydrophilic group to the monolith. This column exhibited cathodal EOF over a wide range of pH and it was probably due to the presence of polar OH groups on its surface, which would favor stronger adsorption of ions from the mobile phase. As a typical result, the neutral hydroxylated octadecyl monolith was able to separate proteins quite rapidly and with a yield of 200 000 plates/m [56].

A neutral naphthyl methacrylate-phenylene diacrylate-based monolith was applied for RP-CEC of various neutral and charged solute probes by hydrophobic and π interactions. This column was prepared by in situ polymerization of naphthyl methacrylate as the functional monomer and 1,4-phenylene diacrylate as the cross-linker in a ternary porogenic solvent containing cyclohexanol, dodecanol, and water. Despite the fact that it was devoid of any fixed charges, this column also exhibited cathodal EOF that was stronger than its counterpart naphthyl methacrylate monolith, made from the in situ polymerization of naphthyl methacrylate and trimethylolpropane trimethacrylate. The adsorption of mobile phase ions together with the additional π interactions, offered by the aromatic rings of the monolithic matrix, modulated the solute retention, and selectivity of separation. The applicability of this was demonstrated by the separation of a wide range of small and large solutes including peptides, tryptic peptide maps, and proteins [55].

Reversed-phase CEC was also used for the analysis of microcystins with two types of reversed-phase CEC columns containing inorganic (sol–gel with octyldimethylchlorsilane) or organic polymer (acrylamide monomer, piperazine diacrylamide as a cross-linker, and laurylacrylate for RP character) monoliths. Although the columns differed not only in monolith quality (inorganic versus organic), but also in the length of the aliphatic moiety (C8 versus C12), similar results were achieved [59].

Microcystins can be also analyzed by polymethacrylate-based monolithic column using pressurized CEC method. Three types of microcystins (MC-LR, MC-YR, and MC-RR) can be analyzed in less than 6 min with the following conditions: 5 mM borate buffer with a pH of 9.6, 10% acetonitrile as the mobile phases in isocratic elution, a voltage of +13 kV and a supplementary pressure of 7.5 MPa. The method was successfully applied to separate microcystins from other compounds in spiked tap water after SPE when observed lower LODs for MC-LR, MC-YR, and MC-RR were 0.10, 0.13, and 0.16 μg /L, respectively [60].

Another type of column for the RP-CEC of neutral and charged species, including peptides and proteins, can be neutral and nonpolar stearyl-acrylate monolithic column. It was developed to provide a relatively strong EOF but being free of electrostatic interactions with charged solutes [61].

Various neutral monolithic capillary columns with different *n*-alkyl chain lengths were studied by Puangpila et al. [62]. These columns were prepared by copolymerization of the functional monomers C8-methacrylate, C12-acrylate, or C16-methacrylate with the cross-linking monomer pentaerythritol triacrylate to yield monoliths with surface bound C8, C12, and C16 chains. They prepared two series (A and B) of columns. In the case of A columns series, the composition of the functional monomers and cross-linker was adjusted to yield comparable chromatographic retention regardless of the alkyl

chain length. In the B columns series, the composition of the functional monomers and cross-linker was kept constant yielding chromatographic retention, which increased as expected in the order of increasing the *n*-alkyl chain length. The C16-monolith of the A series yielded the highest separation efficiency for small compounds but A columns series were not sufficient for the separation of proteins. The C8-monolith of the B series provided the best separation efficiency for proteins and the C16-monolith of the A series was suitable for tryptic peptide mapping.

3.1.1.2 Ion-exchange monolith

A polyacrylate-based monolithic column ((poly(hexyl acrylate-co-1,4-butanediol diacrylate-co-[2-(acryloyloxy)ethyl]trimethyl ammonium chloride) monolithic column)) bearing cationic functionalities was prepared [63] by photopolymerization of a mixture of hexyl acrylate, butanediol diacrylate, 2-(acryloyloxy) ethyltrimethyl ammonium chloride (monomers), azobisisobutyronitrile (photoinitiator), acetonitrile, phosphate buffer, and ethanol (porogens). The performance of the column was evaluated by means of the separations of alkylbenzenes, substituted anilines, basic drugs, peptides, and a protein digest. The separations of peptides were affected both by their interaction with the stationary phase and their own electrophoretic mobility. Excellent separations, with separation efficiencies up to 160 000 plates/m, were achieved for both a mixture of ten well-defined peptides and a tryptic digest of cytochrome c [63].

3.1.1.3 Mixed mode monolith

Karenga and El Rassi developed mixed ligand monoliths for CEC dedicated for the separation of a wide range of solutes differing in both polarity and size [57]. The compositions of these monolithic columns are based on combining different proportions of octadecyl acrylate (ODA) and 2-naphthyl methacrylate (NAPM) monomers in the presence of trimethylolpropane trimethacrylate cross-linker and a ternary porogenic solvent made up of cyclohexanol, ethylene glycol, and water. Not surprisingly, the magnitude of the EOF changes with the composition of the monolith. As the percent of the monomer ODA in the polymerization mixture was increased, the EOF increased to a maximum at 50 mol% ODA and then leveled off at 75 and 100 mol% ODA. While ODA provided solely nonpolar interactions, NAPM exhibited both nonpolar and π interactions with certain solutes. Applicability of these columns were demonstrated by the separation of various compounds such as polycyclic aromatic hydrocarbons, alkyl phenyl ketones, nitroalkanes, alkylbenzenes, toluene derivatives, peptides, and proteins as well as by peptide mapping of the tryptic digest of lysozyme [57].

The mixed ligand approaches were developed continuously to segmented monolithic columns (SMCs) [58]. In this case, the monolithic capillaries were made of two adjoining segments each filled with a different monolith: one with a naphthyl methacrylate monolith (NMM) to provide hydrophobic and π-interactions, while the other with an octadecyl acrylate monolith (ODM) to provide solely hydrophobic interaction. The ODM segment not only provided hydrophobic interactions, but did also function as the EOF accelerator segment. The SMCs allowed the separation of a wide range of compounds such as benzene and toluene derivatives, polycyclic aromatic hydrocarbons but also charged peptides and proteins. Model mixture of peptides and proteins was successfully separated by SMC consisting of 5 cm NMM segment and 15 cm ODM segment (20 cm effective capillary length, 27 cm total length $\times$ 100 μm id; mobile phase, 50% v/v acetonitrile, 10 mM sodium phosphate monobasic, pH 7.0; running voltage, 12 kV) [58] (Fig. 3).

Hydrophilic and SAX interactions can also be used as mixed mode monolithic stationary phase. This phase is based on in situ copolymerization of pentaerythritol triacrylate (PETA), *N*,*N*-dimethyl-*N*-methacryloxyethyl *N*-(3-sulfopropyl) ammonium betaine (DMMSA), and a selected quaternary amine acrylic monomer [64]. The zwitterionic functionalities of DMMSA and hydroxy groups of PETA on the surface of the monolithic stationary phase function as the hydrophilic interaction sites, and the quaternary amine acrylic monomer controls the magnitude of the EOF and provides the SAX sites. After optimization (three different quaternary amine acrylic monomers were used) the monolith from [2-(acryloyloxy)ethyl]trimethylammonium methylsulfate was chosen as the quaternary amine acrylic monomer. The optimized monolith showed good separation performance for a range of polar analytes including nucleotides, nucleic acid bases and nucleosides, phenols, estrogens, and small peptides [64].

The combination of SCX (sulfonic acid) moiety with butyl ligands (nonpolar sites; reversed-phase mode) were also presented as mixed-mode monolith [65]. The monolith was *n*-alkyl methacrylate-based and it was demonstrated that this butylmethacrylate/SCX monolith was suitable for the analysis of therapeutic peptides containing basic centers, for example arginine, at moderately high pH 9.5 [65].

3.1.1.4 Monolith with gold nanoparticles

Monolithic capillaries can also be modified by gold nanoparticles and this type of column modification was described as useful for the affinity separation, by selective capturing of cysteine-containing peptides (resulting in the reduction of the complexity of peptide mixtures), in bottom-up proteomic analysis. In this case, the column was prepared from a poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith through the reaction of some of its epoxide moieties with cysteamine to create a monolith rich in surface thiol groups. In situ reduction of chloroauric acid was then used within the column to form gold nanoparticles attached to the surface of the pores of the monolith. The cysteine-containing peptides from an analyte were retained in the column due to their high affinity for gold and subsequently the release of the retained peptides was achieved with an excess of 2-mercaptoethanol. Another interesting approach is

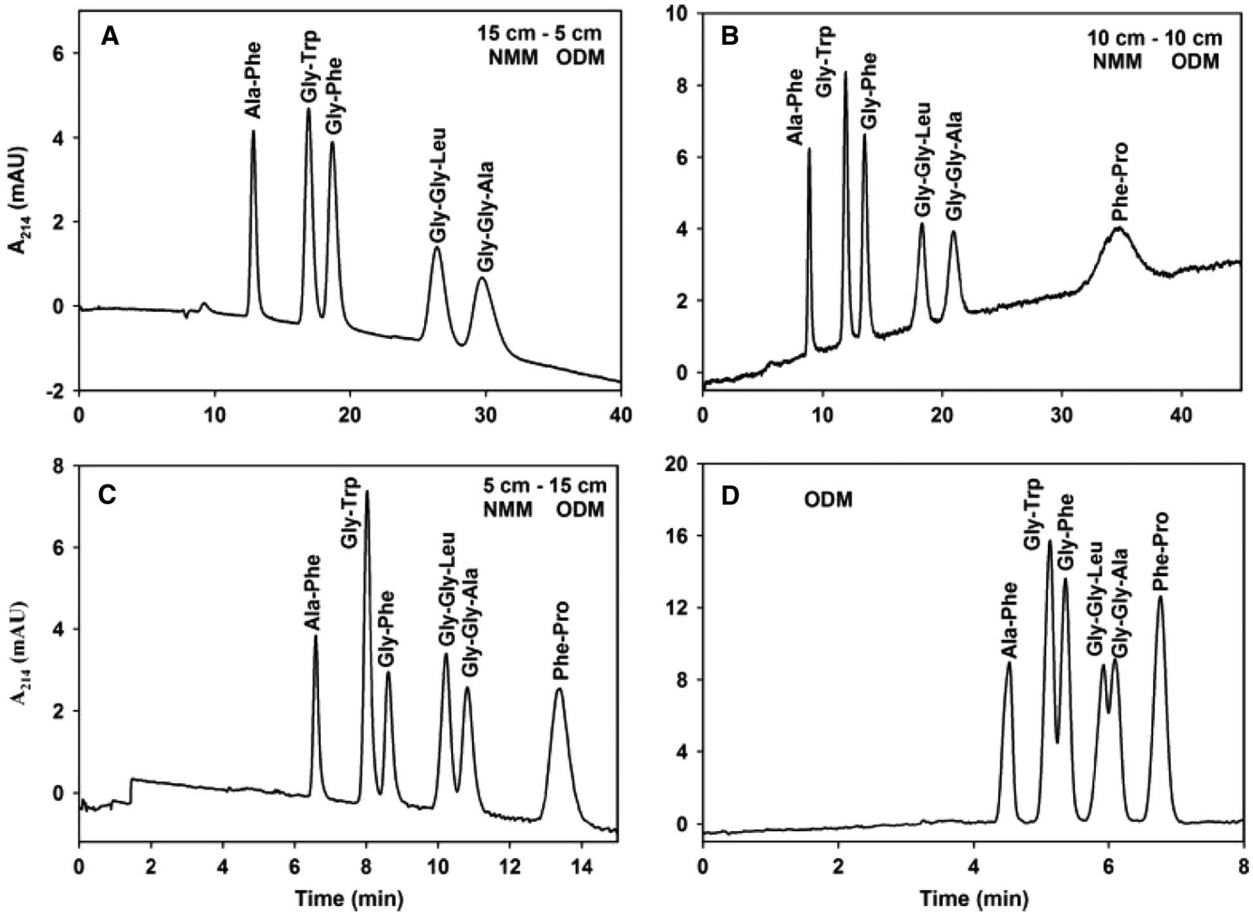

Figure 3. Electrochromatograms for some standard peptides on SMC filled with different fractional length ODM/NMM. Capillary column, 20 cm effective length, 27 cm total length × 100 μm id; mobile phase, 50% v/v acetonitrile, 5 mM sodium phosphate monobasic, pH 6.0; running voltage, 12 kV; electrokinetic injection for 3 s at 10 kV. Reprinted with permission from [58].

to use the gold-modified monolith in tandem with a packed C18 capillary column achieved by a two-step process. The first step involves the retention of cysteine-containing peptides in monolith with RP separation of all other peptides, while the retained peptides are released from the monolith and separated in the second step [66].

The use of porous polymer monolithic capillary columns modified with gold nanoparticles, coated with exchangeable functionalities, has also been described. This approach allowed easy switching of separation modes by a simple ligand exchange process. The columns were prepared from a poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith through the reaction of its epoxide moieties with cysteamine to create a monolith that is rich in surface thiol groups. Gold nanoparticles prepared by in situ reduction of chloroauric acid within the column become attached to the surface of the pores of the monolith. Another method was based on pumping a solution of colloidal gold nanoparticles through the thiol-modified column. Functionalization of the surface of the bound gold nanoparticles was carried out using low molecular weight thiol-containing surface ligands. The dynamic nature of the bonds between gold and these surface ligands enabled the replacement of one surface ligand with another by a simple solution exchange process. The applicability of the columns with exchangeable chemistries was demonstrated on the separation of peptides by CEC as well as nano-HPLC separation of proteins in both RP and ion exchange modes [67].

3.1.1.5 Affinity electrochromatography

The affinity approach is widely used in the separation science, so this principle is also applied in capillary electrochromatographic separation of proteins/peptides. One of the "traditional" use of affinity approach in proteomic (more exactly glycoproteomic) analysis could be exemplified by the method of immobilized lectins using polymethacrylate monoliths [68]. This column can be used for affinity CEC as well as affinity nano-LC with either a single column or columns coupled in series. Both positive and neutral monoliths based on poly(glycidyl methacrylate-co-ethylene dimethacrylate) were used. The positive monoliths (i.e. monoliths with cationic sites) offered relatively high permeability in nano-LC but lacked predictable EOF magnitude and direction, while

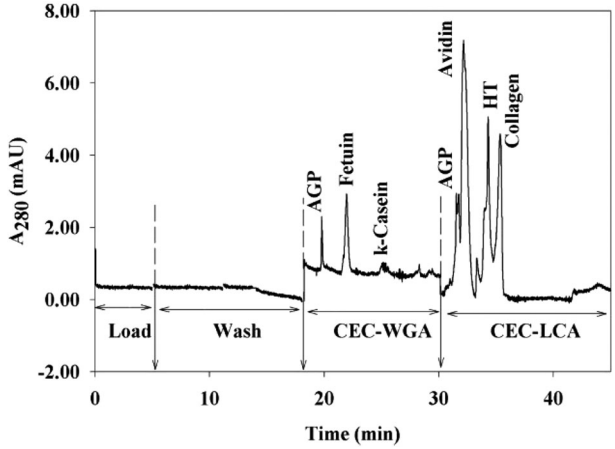

Figure 4. Electrochromatogram of AGP, HT, collagen, κ-casein, avidin, and fetuin, obtained on coupled lectin columns in the order WGA → LCA where the lectins were immobilized on a neutral monolith. Column dimension: 25 cm effective length, 33.5 cm total length × 100 μm id composed of two segments connected butt to butt with a zero-dead-volume Teflon tubing, where the WGA monolith occupies the first segment (12.5 cm) and the LCA monolith the second segment and has an effective length of 12.5 cm and an open portion of 8.5 cm; binding mobile phase: 10 mM DETA, pH 6.0, containing 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$; washing mobile phase: 2.5 mM DETA, pH 6.0 introduced at 5 min; eluting mobile phase: 0.1 M Me-α-D-Man and 0.1 M GlcNAc in 2.5 mM phosphate, pH 3.0 introduced at 18.0 and 30.0 min, respectively, as indicated by the arrows; ΔP, 1.0 MPa for both running mobile phase and sample injection; running voltage, −20 kV for eluting mobile phases; sample injection, 6 s. Reprinted with permission from [68].

neutral monoliths provided a good compromise between reasonable permeability in nano-LC and predictable EOF in CEC. Lectin affinity CEC provided the possibility to simultaneously capture and separate different glycoproteins based on the differences in their charge-to-mass ratios, whereas lectin affinity nano-LC permitted the enrichment of classes of different glycoproteins having similar N-glycans recognized by the immobilized lectin [68] (Fig. 4).

The affinity CEC can also be performed with monolithic capillaries modified by gold nanoparticles (see above section). This approach can selectively capture cysteine-containing peptides, resulting in reducing the complexity of peptide mixtures, in bottom-up proteomic analysis (for details see Section *3.1.1.4*) [66].

The use of affinity principle has also been described in the context of 2D separation (see Section 3.2), where it was applied, e.g. to affinity capture histidine-containing peptides [69].

3.1.1.6 Chiral separation

Acrylamide-based monoliths also can be used for the separation of peptide enantiomers (for a review about chiral separations see e.g. [25, 26]).

The usefulness of hydrophobic monolithic column for this type of separation was amply demonstrated with a neutral hydrophobic monolith that was prepared by radical in situ copolymerization of lauryl methacrylate and ethylene dimethacrylate [70]. Di- and tripeptides composed only of L-configured amino acids migrated faster than peptides containing D-amino acids. A mixture of isomeric Asp tripeptides that could not be completely resolved by either CZE or HPLC as well as the 24 mer peptides tetracosactide and 16[D-Lys]-tetracosactide could also be separated by this approach [70].

Another possibility is to use a weak cation-exchange monolith as stationary phase for this chiral separation in which the monolith was prepared by in situ polymerization of acrylamide, methylenebisacrylamide, and 4-acrylamidobutyric acid in a decanol-dimethylsulfoxide mixture as porogen [71]. The columns were evaluated by the separation of diastereomers and α/β isomers of aspartyl peptides. Analyte separation was done at mobile phases consisting of acidic phosphate buffer and acetonitrile. A comparison between the weak-cation exchange monolith with the reversed-phase monolith and a strong cation-exchange monolith showed different order of elution for some of the peptide diastereomers, so it was concluded that interactions with the stationary phase contribute to the CEC separations are different [71].

3.1.1.7 Imprinted monolith

A selective recognition of oxytocin based on the epitope approach, can be obtained by CEC when tetrapeptide YPLG (Tyr-Pro-Leu-Gly) served as the template. In this instance, the capillary was prepared by a mixture containing functional monomer (methacrylic acid), cross-linking reagent (ethylene glycol dimethacrylate), imprinted molecule, and radical initiator (2,2′-azobis(2-isobutyronitrile)) dissolved in a binary porogen (acetonitrile and isooctane) and polymerized. The imprinted monolith column had a good recognition capacity (the imprinting factors for YPLG and oxytocin were 4.499 and 4.013, respectively) and high column efficiency (theoretical plates for YPLG and oxytocin were 22 995 and 16 952 plates/m, respectively). Furthermore, a mixture of oxytocin and other proteins were analyzed using this monolithic CEC column and oxytocin was eluted much more slowly than other large biomolecules, which demonstrated the ability of such an imprinted monolith toward high selective recognition for oxytocin with PLG (Pro-Leu-Gly) as the epitope [72].

3.1.1.8 Other types of acrylic monoliths

An interesting approach was made for the separation of oligopeptides using monolithic column consisting of L-phenylalanine as template and a covalent formation of Schiff base with *o*-phthalaldehyde (OPA). The mixtures of OPA, allylmercaptan, L-phenylalanine, triethylamine, methacrylic acid, 2-vinylpyridine, ethyleneglycol dimethacrylate, α,α-azobisisobutyronitrile, and propan-1-ol were thermally polymerized in the capillary and then eventually the template was extracted. Phosphate buffer (pH 7.0, 40 mM)/methanol (5%, v/v) served as the mobile phase. This column successfully

separated angiotensin I, angiotensin II, [Sar1, Thr8] angiotensin, oxytocin, vasopressin, tocinoic acid, β-casomorphin bovine, β-casomorphin human, and FMRF amide within 20 min [73].

3.1.2 Other types of monoliths

Monolithic capillary columns prepared by ring-opening metathesis polymerization (ROMP) have been used for the separation of peptide in voltage-assisted capillary LC (CLC). To demonstrate their potential for peptide separation, ROMP-derived monoliths with RP properties were prepared. The procedure for monoliths preparation was adapted from ROMP monoliths optimized for CLC. ROMP monoliths were synthesized within the confines of 200 μm id fused-silica capillaries with a length of 37 cm. After optimizing the chromatographic conditions, the separation performance was tested using: a well-defined set of artificial peptides, two peptide mixtures resulting from a tryptic digest of BSA as well as a collagenase digest of collagen. ROMP monoliths showed comparable performance to other monolithic separation media in voltage-assisted CLC published so far. Therefore, we conclude that by optimizing the composition of the ROMP monoliths as well as by using controlled functionality of the manner it works, ROMP monoliths bear a great potential in CLC and CEC [74].

An organic-inorganic hybrid monolithic column based on 1-vinyl-3-dodecylimidazolium bromide was prepared in a single step by combining radical copolymerization with a nonhydrolytic sol–gel process. The column was applied to separate alkylbenzenes, anilines, and proteins, respectively. This organic–inorganic hybrid polymeric ionic liquid monolithic column exhibited high column efficiency for protein separation when the applicability of it was demonstrated on the separation of egg white sample [75].

Silica sol–gel monoliths can be used for affinity analysis of glycoproteins and glycopeptides when photopolymerized these monoliths are functionalized with boronic acid ligands. These functionalized monoliths were used for separations of proteins and peptides in polydimethylsiloxane microfluidic devices. Usability of method was demonstrated on separation of conalbumin (glycoprotein) and a tryptic digest of the glycoprotein horseradish peroxidase [76].

3.2 Other applications of monoliths (on-line and 2D separation)

The use of monoliths in the field of electromigration techniques, for purposes other than that are strictly devoted to electrochromatography, have also been described and, the most important type of this application is on-line SPE (i.e. monolith that was synthesized in a fused-silica capillary).

In this regard, the technique described by Zhang et al. [77] for the identification of peptide during bottom-up analysis of complex proteomes can serve as an example. They synthesized a strong cation-exchange monolith in a fused-silica capillary with sulfonate-silica hybrid and used it for SPE with online pH gradient elution during CZE–MS/MS proteomic analysis. Tryptic digests were applied onto the strong cation-exchange monolith and fractions were eluted using a series of buffers having lower concentrations but higher pH values than the BGE, i.e. 50 mM formic acid. This combination of elution and BGEs results in both sample stacking and formation of a dynamic pH junction. Application of this method was demonstrated by the *E. coli* tryptic peptides where a series of five pH bumps enabled the identification of 799 protein groups and 3381 peptides from 50 ng of the digest in 2.5 h analysis.

Monolithic RP-silica-based sol–gel concentrator for in-line SPE has been used for CE-MS analysis of Met-enkephalin in cerebrospinal fluid, deproteinized by perchloric acid [78]. After optimization of the operational parameters for SPE, a 40-fold concentrated sample was achieved for 3200 nL loading volume and was detectable at a limit of 1 ng/mL (5 nM).

Another application of on-line preconcentration, by the use of the coupling of monolithic microcolumn with CZE, was described for the analysis of the decapeptide angiotensin I [79]. In this case, iron protoporphyrin-modified monolithic support was prepared by in situ polymerization initiated with ionizing radiation and the cross-linking of diethylene glycol dimethacrylate and glycidyl methacrylate, when the chemical modification was done by iron protoporphyrin IX (Fe-ProP). This monolith (8 mm long) was coupled on-line to the inlet of the separation capillary (75 μm id × 10 cm from the inlet to the micro-column and 27 cm from the micro-column to the detector). Angiotensin I was released from the sorbent by a 50 mM sodium phosphate, pH 2.5/acetonitrile, 75:25 v/v solution, and then analyzed by CZE and reportedly achieved 10 000-fold improvement in concentration sensitivity [79].

A similar monolithic fused-silica capillary column, modified with iminodiacetic acid and copper(II) ions was also employed in another study for on-line affinity capturing of histidine-containing peptides from a model peptide mixture. His-containing peptides were released from the sorbent by 5 mM imidazole solution and separated by CZE with UV-absorption detection [69]. The same type of sorbent, monolithic column with Cu(II) iminodiacetic acid functional groups has been developed for preconcentrating low-abundance peptides and proteins from complex biological samples [80].

2D platform for proteomics was described by Zhang and El Rassi [81] that consisted of on-line coupled CIEF and CEC (RP-CEC). The solvent of the CIEF mode was a weak eluent for the reversed-phase CEC mode, thus allowing the transfer of focused fractions from CIEF to CEC without the induction of band broadening and instead resulted in zone sharpening. The RP-CEC column was based on neutral C17 monolith. The separation was demonstrated on a model mixture of 15 peptides, 15 proteins, and albumin-depleted human serum [81].

For protein analysis, an affinity-gradient nano-stationary phase (AG-NSP), which uses nanofluidic CEC conjugated with MALDI-TOF-MS was also described. A hydrophobicity gradient in AG-NSP was produced photochemically by grafting 4-azidoaniline hydrochloride on vertically arrayed multiwall carbon nanotubes through gray-level exposure to UV light. The reversed-phase gradient stationary phase in AG-NSP was tailored according to the properties of the mobile phase gradient in CEC. The operation of the system could also be automated using a single buffer solution without the need for multiple solvents for elution. This method enabled the separation of proteins and peptides on the chip. The system was tested on the separation of three proteins: cytochrome c, lysozyme, and BSA, and also on the digested IgG fragments. The proposed system provided resolution of 1000 Da for the proteins included in this study and the separation of digested IgG fragments up to a concentration as low as 1.2 pmol/μL [82].

4 Open tubular CEC

Open tubular CEC (OT-CEC) is based on the interaction between analytes and the capillary wall and the most comprehensive review on open tubular CEC was written by Cheong et al. in 2013 [2] followed by Tarongoy et al. [7].

In the ideal situation, the abovementioned interaction is strictly chromatographic. In other words, the column in open tubular chromatography is a capillary or channel where the inner surface is coated with a stationary phase, which serves by chromatographic separation mechanisms (partition, ion exchange, molecular, or chiral recognition) and the mobile phase is electroosmotically driven through the column, as opposed to pressure driven flow in open tubular LC. Probably the first OT-CEC separation was reported by Tsuda et al. [83] using an octadecyl-modified capillary of 30 μm id. Some historical overview can be found in the summarizing table of [29] and a good overview on this technique before 2001 was written by Pesek and Matyska [84].

It should be emphasized that during the first years of CE, the interaction of proteins with the inner wall of a fused-silica capillary, i.e. the adsorption of proteins and peptides to the capillary wall, was one of the main problems in the analysis of biological samples. To eliminate this adsorption, various types of coating were tried on the capillary wall or the peptides/proteins were run under highly acidic or alkaline conditions [85]. Among these various polymeric coatings that are often used, we should mention polysaccharide dextran [86], polyacrylamide [87], hydroxylated polyether [88], polyvinylmethylsiloxanediol-polyacrylamide [89], poly(acryloylaminoethoxyethanol) [90], poly (vinyl alcohol) [91], polyarginine [92], cellulose acetate [93], or poly(ethylene-propylene glycol) [94].

Nowadays, we can see the availability of various types of capillaries for open tubular CEC, chemically bonded, etched capillaries, sol–gel, porous layers, and physical coating.

4.1 Etched capillaries

The main problem with OT-CEC technique is the low capacity of the column due to the small area available for the bonding of a stationary phase. For this reason, the decreasing of the capillary bore can be of help (as the surface-volume ratio of the capillary increases with the decreasing of the capillary inner diameter). Another plausible approach is the chemical etching of the inner wall of capillaries, which can increase the overall surface area of the capillary by up to 1000-fold (for review see [84, 95]).

The etching of capillaries appears to be interesting and usable, but surprisingly, not yet frequently used, probably owing to the complicated procedure involved in its preparation. Proteins can be analyzed by etched capillaries modified with alkyl chains such as C5, for e.g. used in the analysis of carbonic anhydrase, which resulted in excellent peak shape with high separation efficiency (N>1 000 000 plates/m) [96].

Another use of etched capillaries, for the analysis of peptides, were described for chemically (*n*-octadecyl- and cholesterol-) modified capillaries interfaced to an ESI mass spectrometer through a sheath liquid configuration. The stationary phases were fabricated by etching the inner wall of the fused-silica capillary and then chemically modifying the new surface through a silanization/hydrosilation reaction. By this approach, the leaching of the immobilized stationary phase and subsequent contamination of the electrospray ion source was largely avoided (in comparison to other modifications of the method). Significant retention of peptide was recorded even at very low pH with both types of stationary phases, under the conditions in which the electrophoretic migration dominated the separation process [97].

4.2 Chemically bonded phases

Chemically bonded phases are more stable in comparison to physically adsorbed phases as well as have longer lifetimes.

4.2.1 Brush ligands

The oldest type of bonded phase is the stationary phase of brush type ligand (typically C18) and this type of modification has not been so extensively developed in the recent years. In principle, it seems that this modification has low sample loading capacity and the main purpose for using it was to prevent protein adsorption on the capillary wall.

Hydrophilic triamine-ligand bonding for the prevention of adsorption of proteins on the wall (and having anodic EOF at pH below 5.3) has been described [98]. Modification was made by 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane. Separation of four basic proteins (trypsin, ribonuclease A, lysozyme, and cytochrome c) was successfully achieved with 10 mmol/L phosphate buffer at pH 3.5 with column efficiencies in the range between 87 000 and 110 000 plates/m. Ionic compounds were also successfully separated (aromatic acids, nucleosides). This modification

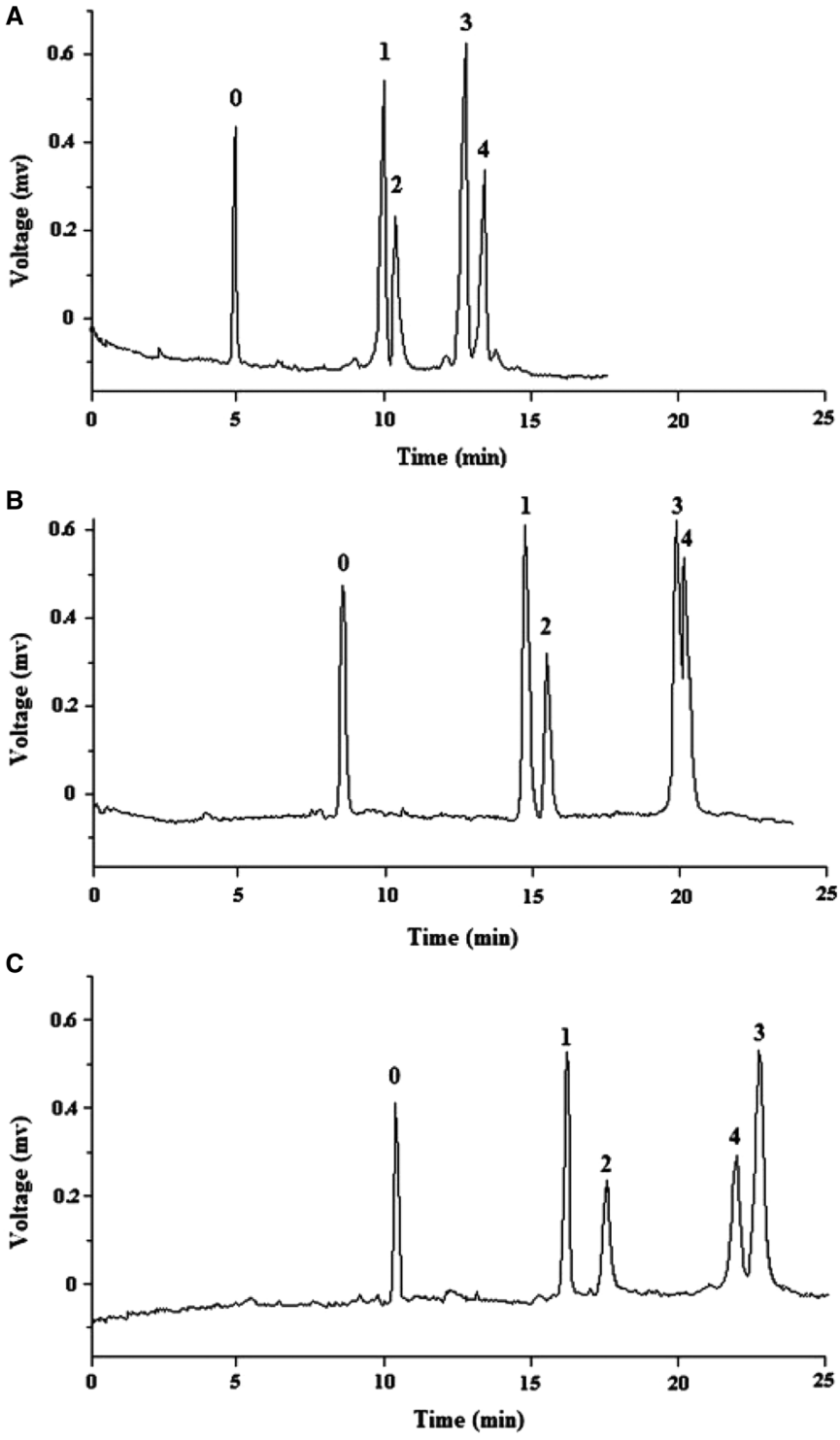

Figure 5. Separation of basic proteins using chemical triamine-bonded OT column: effect of buffer pH on the separation of basic proteins (0, neutral EOF marker (DMSO); 1, trypsin; 2, ribonuclease A; 3, lysozyme; 4, cytochrome c.). Conditions: AEPTS (3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxysilane), 30% v/v; capillary column, 55 cm total length (30 cm to the detector) × 50 μm id; running buffer, 10 mmol/L phosphate, pH values: (A) pH 3.5, (B) pH 4.0, (C) pH 4.5; applied voltage, 18.0 kV; gravity injection, 10 cm, 5 s; detection wavelength, 214 nm. Reprinted with permission from [98].

can be useful for the analysis of basic proteins and anionic analytes (Fig. 5).

4.2.2 Polysaccharides

As was mentioned previously, cellulose derivatives (polysaccharide dextran [86] and cellulose acetate [93]) can be used as a material for coating of the capillary to protect adsorption of proteins on the its wall.

In this context, a column, covalently modified with hydrophilic polysaccharide carboxymethylchitosan (CMC), was developed for the separations of basic proteins and opium alkaloids [99]. The procedure consisted of silanization of capillary wall by 3-aminopropyltrimethoxysilane and combining

glutaraldehyde with amino-silylated silica surface and CMC. This modification exhibited tolerance and chemical stability against 0.1 mol/L HCl, 0.1 mol/L NaOH, and some organic solvents. With anodic EOF mode (pH < 4.3), the separations of basic proteins (trypsin, ribonuclease A, lysozyme, and cytochrome C) were successfully achieved with column efficiencies ranging between 97 000 and 182 000 plates/m, and the undesired adsorptions of basic proteins on the inter-wall of capillary could be avoided. With cathodic EOF mode (pH > 4.3), four opium alkaloids were also baseline separated in phosphate buffer (50 mmol/L, pH 6.0) with column efficiencies ranging between 92 000 and 132 000 plates/m. Therefore, this modification can also be used for the analysis of basic proteins and alkaline analytes.

Nano-chitosan-coated capillary column was prepared by ionic cross-linking technology and coated onto the inner wall of capillary by electrostatic adsorption [100]. EOF was neutralized and reversed at pH < 4.7. It was demonstrated that nano-chitosan coating played the role of hydrophilic stationary phase and the possibility of its use in protein separation was examined on three basic proteins (lysosomes, cytochrome C, and ribonuclease A) [100].

Group of Riekkola [101] developed covalently bounded heparin coatings for 3-aminopropyltriethoxysilane-modified silica capillaries and SiO_2 chips for study of the interactions of heparin with selected peptide fragments of apolipoproteins apoB-100 and apoE. They carried out CEC and quartz crystal microbalance studies. Interactions and affinities were expressed in CEC as retention factors and reduced mobilities. Both studied techniques showed heparin interactions to be stronger with apoB-100 peptide than with apoE peptide fragment, and they confirmed that the sulfate groups in heparin play an especially important role in interactions with apoB-100 peptide fragments.

4.2.3 Tentacles

A stationary phase with tentacle-type polymer chains is an important possibility for OT-CEC. At first the capillary has to be silanized, then in situ polymerized, and finally complexed with metal. The tentacle-type metal-chelating capillary with high ligand capacity has been proven useful for the separation of amino acids and purine derivatives. The capacity of the tentacle-type polymer stationary phase with immobilized copper(II) was nearly 900 times higher than that of the monolayer one [102].

The functionalized (phenylalanine) tentacle-type polymer-coated capillary column was used also for protein separation [103]. The tentacle-type stationary phase was prepared on silanized fused-silica capillaries by glycidyl methacrylate graft polymerization and subsequent functionalization with phenylalanine. Due to the amphoteric functional groups of the phenylalanine bonded to the column, it can perform under both cathodic and anodic EOF by varying the pH of the mobile phase. Model proteins (ribonuclease A, myoglobin, transferrin, and insulin) were baseline separated under cathodic EOF with a mobile phase having a pH of 8.8. Three basic proteins (RNase A, cytochrome c, and lysozyme) were resolved under anodic EOF with an acidic running buffer (pH 2.5). The efficiency of the column for proteins was in the range between 13 000 and 182 000 plates/m [103].

Branched polyethyleneimine-bonded tentacle-type polymer stationary phase was also used for the separation of peptides and proteins [104]. In this case the capillary enabled anodic EOF within a wide pH range of 2.5–7.5. Due to the existence of amine groups on the surface of tentacle-type polymer stationary phase, the silanol effect that occurs between the positively charged biomolecules and the silanols of the capillary column was greatly suppressed. Seven enkephalin-related peptides were well resolved with high efficiencies (144 000–189 000 and 97 000–170 000 plates/m).

4.2.4 Nanoparticles

Nanoparticles are relatively novel material for stationary phases in chromatography and electrochromatography. There are reviews regarding the use of nanoparticles in capillary and microchip electrochromatography [105–107] and regarding the analysis and applications of nanoparticles in CE [108]. Till now, the nanoparticle appears as a promising material but not yet in frequent use. Only a limited number of studies have dealt with the use of nanoparticles, wherein they used gold nanoparticles (GNPs) for the analysis of proteins and peptides.

OT-CEC with bare GNPs-based stationary phase was described for the separation of tryptic peptide fragments of native and glycated proteins, BSA, and human transferrin. The GNPs-based stationary phase was prepared by immobilization of bare GNPs, freshly reduced from tetrachloroaurate(III) ions by citrate reduction, on the sol–gel pretreated inner wall of the fused-silica capillary. The best separation of the above tryptic peptides were achieved with a BGE composed of aqueous 100 mmol/L sodium phosphate buffer (pH 2.5) at a separation voltage of 10 kV, for a capillary having a length of 47 cm and id of 50 μm, thermostatted at 25°C [109] (Fig. 6).

Immobilized bare GNPs, in the sol–gel-pretreated fused-silica, were also used for the separation of hydrophobic polyaromatic hydrocarbons (PAHs) as well as hydrophilic cationic antimicrobial peptides. A model mixture of four PAHs, naphthalene, fluorene, phenanthrene, and anthracene, was resolved by OT-CEC in the GNP-modified fused-silica capillaries, using the hydro-organic BGE composed of 20 mmol/L sodium phosphate buffer (pH 7), modified with acetonitrile at 8:2 v/v ratio. On the other hand, three synthetic analogs of an antimicrobial peptide mastoparan PDD-B, namely, basic tetradecapeptides INWKKLGKKILGAL-NH2, INSLKLGKKILGAL-NH2, and NWLRLGRRILGAL-NH2, were separated in aqueous acidic BGEs (pH 2.1–3.1) composed of weak acids (formic and acetic) or amphoteric amino or imino acids (aspartic or iminodiacetic). The later utilized the advantage of a slow reversed (anodic) EOF and slightly positive charge of the

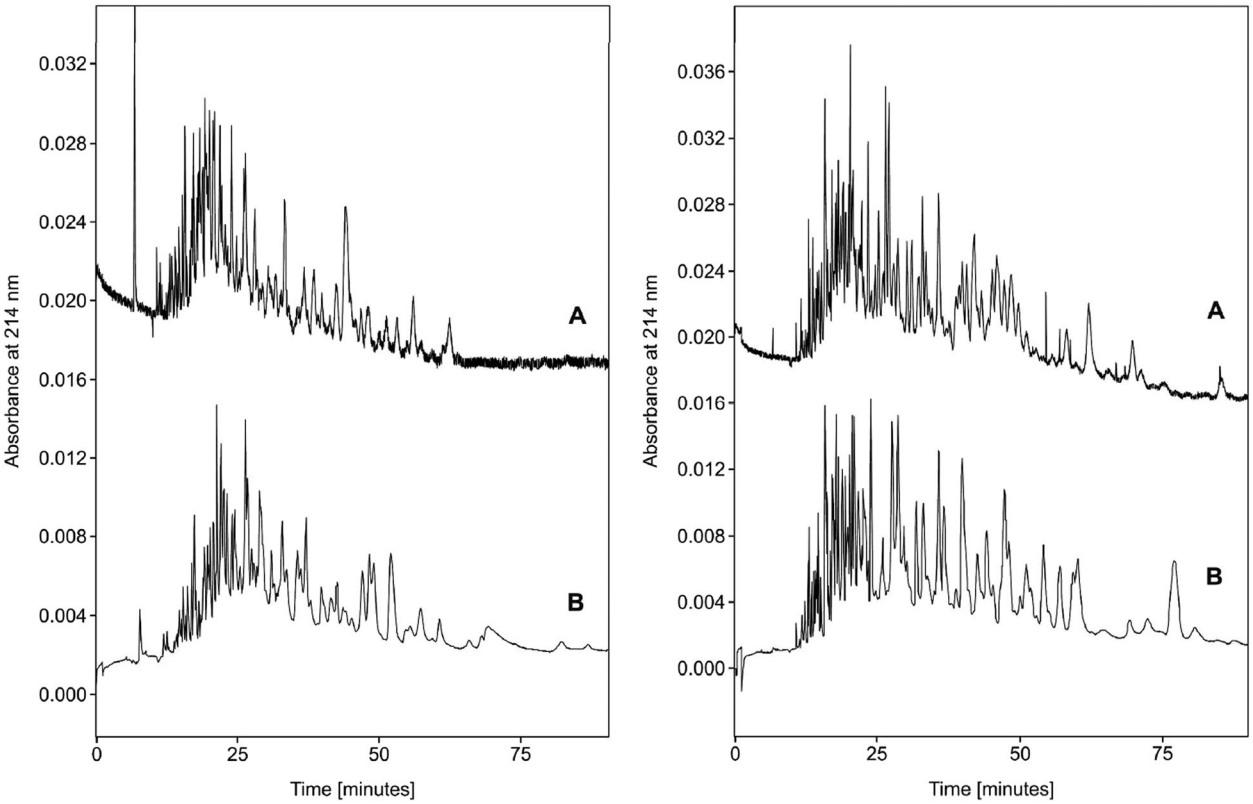

Figure 6. (A = left figure) Separation of tryptic peptides of native BSA by CZE in bare fused-silica capillary (A) and by OT-CEC in GNPs coated fused-silica capillary (B). In both cases, the BGE was 100 mmol/L sodium phosphate, pH 2.5. Injection time 10 s, 3.45 kPa injection pressure. (B = right figure) Separation of tryptic peptides of native human transferrin (HTF) by CZE in bare fused-silica capillary (A) and by OT-CEC in GNPs coated fused-silica capillary (B). Reprinted with permission from [109].

GNP-modified FS capillary, which suppressed the adsorption of cationic peptides on the inner capillary wall and thus improved their resolution [110].

Another method for preparation of gold nanoparticle-coated capillaries used modification of capillaries by 3-amino propyl-triethoxy silane prior treatment with suspension of gold nanoparticles. The method showed a very good stability when it was reused about 900 times. The usability of this approach was demonstrated on the separation of bioactive synthetic peptides (bradykinin, angiotensin I, luteinizing hormone-releasing hormone, oxytocin, and methionine-enkephalin) as well as tryptic peptide fragments of HSA [111].

Carboxyl-modified magnetic nanoparticles (Fe_3O_4-COOH) were constructed on the surface of positively charged poly(diallydimethylammonium chloride) modified capillaries through an approach involving electrostatic self-assembly. The separation of tested analytes (amino acids, dipeptides, and proteins) was primarily based on electrophoretic mechanism in combination with chromatographic mechanism. The nanoparticle coating improved the resolution of separation of these analytes due to their large surface area. With regard to the analysis of proteins, successful separation was demonstrated on three variants of BSA, two variants of β-lactoglobulin and nine glycoisoforms of ovalbumin as well as acidic proteins in egg white [112].

Another approach regarding nanoparticle, involves the use of silica nanoparticles [113]. A simple coating procedure was employed that consisted of a multilayer-by-multilayer process to modify the inner surface of bare fused-silica capillaries with silica nanoparticles. The silica nanoparticles were adsorbed onto the capillary wall by a strong electrostatic interaction between functional amino groups and silica particles. The thickness of the coating was adjustable and ranged between 130 and 600 nm. The method was evaluated with aromatic hydrocarbons but was found to be applicable also for the separate of egg white proteins, when both acidic and basic proteins as well as four glycoisoforms could be separated in a single run [113].

Cationic and anionic lipid-based liquid crystalline nanoparticles can be used also for the separation of proteins by CEC. The anionic nanoparticles were composed of lipids (soy phosphatidylcholine, glycerol dioleate, and Polysorbate 80), oleic acid, and ethanol added to water. In the case of the cationic nanoparticles, oleic acid was excluded and instead 1.6% w/w of dihexadecyldimethylammonium bromide was included. Anionic nanoparticles was used for the separation green fluorescent protein (GFP) and GFP N212Y, while the cationic nanoparticles can separate GFP samples from *Escherichia coli* with high efficiency (800 000 plates/m). A physical attached double-layer coating of cationic and

anionic nanoparticles combined with anionic lipid nanoparticles can be used for the separation of three single amino-acid-substituted GFP variants [114].

The nanoparticle approach can be used also in hydrophobic interaction CEC. In this case, lipid-based liquid crystalline nanoparticles were used as pseudostationary phase [115]. Use of LIF enabled detection at high nanoparticle concentrations. Green fluorescent protein and its mutants harboring single or double amino acid substitutions with the same charge were separated in the described system but not in conventional CE. Separation was achieved by increasing the salt concentration to promote hydrophobic interactions by shielding of the repulsive electrostatic interactions.

The technique of layer-by-layer assembly using electrostatic interaction was used for ionic adsorption of negatively charged graphene oxide nanosheets onto the capillary wall, premodified with positively charged poly(diallydimethylammonium chloride) [116]. The stationary phase displayed characteristic reversed-phase behavior. This column was used for the analysis of egg white proteins and both basic and acidic proteins were separated in a single run [116].

4.2.5 G-quartet DNA stationary phase

An interesting approach is coating of capillaries with G-quartet-forming DNA [117]. G-quartet structures are formed through $\pi-\pi$ stacking of square planar arrays of guanines at guanine-rich DNA sequences. G-quartet structures may be formed intramolecularly (by a single strand of DNA) or intermolecularly (between multiple strands). They are stabilized by certain cations, particularly K^+. Two oligonucleotides of this type were investigated as stationary phases for OT-CEC. Oligonucleotides were covalently attached to the inner surface of the capillary (25 μm id, total length 47 cm, effective length 40 cm) using a heterobifunctional cross-linking agent sulfosuccinimidyl-4-N-maleimidomethyl)cyclohexane-1-carboxylate. The migration of fibrinogen peptides was studied. At 25°C, the peptides eluted in the same order in O-TCEC using a two-plane G-quartet DNA stationary phase as in CZE. At higher run temperatures (35–40°C) baseline resolution of the coeluting peptides could be achieved in the OT-CEC. A stationary phase formed by a scrambled-sequence oligonucleotide that does not form a G-quartet did not provide any resolution of the two coeluting peptides, even at the higher temperatures. The effects of destabilization were explored through variation of the cations (sodium or potassium) used in attachment of the G-quartet oligonucleotide to the capillary surface and in the mobile-phase buffer. Resolution was lower when a more stable, four-plane G-quartet stationary phase was used. The increase in peptide resolution upon destabilization of the G-quartet structure could prove to be an important factor in the application of G-quartet DNA stationary phases for nonaffinity-based separation of native proteins and peptides.

4.3 Sol–gel

The sol–gel technique can be used for the preparation of open-tubular columns (for review see e.g. [118]). In this approach, sol–gel creates organic-inorganic hybrid stationary phases. Various ligands can be chemically immobilized on the inner surface of the capillary by a single-step sol–gel procedure and this technique produces a stable, porous, and thick stationary phase coating. It also enhances the interaction of solute to the stationary phase, exhibits higher sample capacity, and ability to control the EOF (by using charged stationary phases).

Examples of the use of sol–gel in combination with other approaches were demonstrated with the immobilization of GNPs in the sol–gel-pretreated fused-silica, which could be used for the separation of peptides [110].

4.4 Porous layers

The term "porous layer open-tubular" (PLOT) describes a situation wherein a thin porous layer of stationary phase remains attached to the inner wall of fused-silica capillary (this layer can be bonded covalently as well as by other interactions) [22]. In principle, it is a type of monolithic column—the monolith being present only on the surface of the capillary. It should be mentioned that there is another mode of "monolithic open tubular columns" with molecularly imprinted polymers (OTMIP-CEC). However, this approach is mainly used for low-molecular-weight compounds such as drugs, and chiral separations etc. (e.g. [119]).

The possibility of using PLOT technique for CIEF of protein was demonstrated in 2010 by Yang et al. [120]. They immobilized pH gradients by the polymerization of several solutions (contained acrylamide, *N,N′*-methylenebisacrylamide, allylamine, and acrylic acid). These polymers formed a covalently attached hydrophilic layer in the capillary and when these five polymers were orderly lined up in a capillary, an immobilized pH gradient was established.

Porous monolithic layer formed with butyl methacrylate and ethylene glycol dimethacrylate in the presence of porogen (propan-1-ol) was used for the separation of proteins (BSA and cytochrome c). The OTCEC column, prepared as above, showed improved performance compared to the capillary dynamically coated with polyvinylpyrrolidone [121].

A styrene-methacrylic acid-*N*-phenylacrylamide copolymer layer was immobilized on the inner surface of silica capillary (50 μm internal diameter, 500 mm total length, 416 mm effective length) [122]. This capillary was used for the separation of tryptic digest of cytochrome C (as well as the mixture of five synthetic peptides). The sample was well eluted and separated into many peaks when influence of pH and water content in the mobile phase was studied.

Only one article regarding separations of proteins by molecular imprinted polymers (MIPs) can be found till now [123]. It was described that this MIPs was formed by 4-aminostyrene, ethylene glycol dimethacrylate, and azobisisobutyronitrile dissolved in 9:1 v/v acetonitrile/2-propanol,

using PEG (MW 10000) as template molecule and was successfully used for a test mixture of proteins.

4.5 Physical coating

The surface of the capillary wall can be modified by physically adsorbed or attached layers. Adsorbed layers can be divided into two basic categories: physically adsorbed (strongly bound stationary phases) and dynamically adsorbed (interaction is weaker). Physically attached layers can involve a variety of chemistries and can include various ligands (for review see e.g. [124]).

Porphyrins can be used as modifiers of the capillary wall (for older studies on this topic see review [125]) for separation of peptides when tetrakis (phenoxyphenyl)porphyrins (H2TPP(m-OPh)4 and Rh(III)TPP(m-OPh)4) were described as free-base; as well as metal form [126]. Several (metallo)porphyrins, particularly porphyrin derivative tetraphenylporphyrin and complexes of porphyrin derivatives with metal ions (Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Co^{3+}) were successfully used for the separation of structurally related peptides (octapeptides) [127]. Another approach was described by Yone et al. [128] where capillaries modified with different porphyrins (deuteroporphyrin, complexes of deuteroporphyrin with metal ions Fe(III), Cu(II), Zn(II), Ni(II), and Cu(II)-meso-tetra (carboxyphenyl) porphyrin) were used. These porphyrins effectively separated biologically active peptides at the mobile phase, which was composed of 25 mM potassium phosphate (pH 4.0) 5% v/v acetonitrile and 10 mM hydroquinone. Authors concluded that the differences in the metal core of porphyrin and the spatial conformation of attached porphyrin changes the way the analyte interact with the stationary phase [128].

Another interesting approach regarding physical coating is the use of zwitterionic micellar poly (amino acid)-poly-ε-sodium-undecanoyl lysinate. The thickness of the polymer coating ranged between 0.9 and 2.4 nm. This zwitterionic coating allows protein separations in either normal or reverse polarity mode. The utility of this column was demonstrated by excellent separation of a protein mixture composed of four basic proteins and six acidic proteins [129].

Polyelectrolyte multilayer (PEM) coatings can enhance protein separations [130]. There were studies that used four cationic polymers (poly-L-lysine, poly-L-ornithine, poly-L-lysine-serine, and poly-L-glutamic acid-lysine) and three anionic molecular micelles, *Viz*. sodium poly(*N*-undecanoyl-L-leucyl-alaninate) (poly-L-SULA), sodium poly(*N*-undecanoyl-L-leucyl-valinate) (poly-L-SULV), and sodium poly(undecylenic sulfate) (poly-SUS) for the coating of capillaries. Separations were examined with four basic proteins (α-chymotrypsinogen A, lysozyme, ribonuclease A, and cytochrome c) (Fig. 7). Poly-L-glutamic acid-lysine showed the highest resolution and longest migration time. The use of molecular micelles to form PEM coatings resulted in better separations than single cationic coatings. Chiral poly-L-SULA and poly-L-SULV resulted in higher protein resolutions as compared to the achiral, poly-SUS. Furthermore, the use of poly-L-SULV reversed the elution order of lysozyme and cytochrome c when compared to poly-L-SULA and poly-SUS [130].

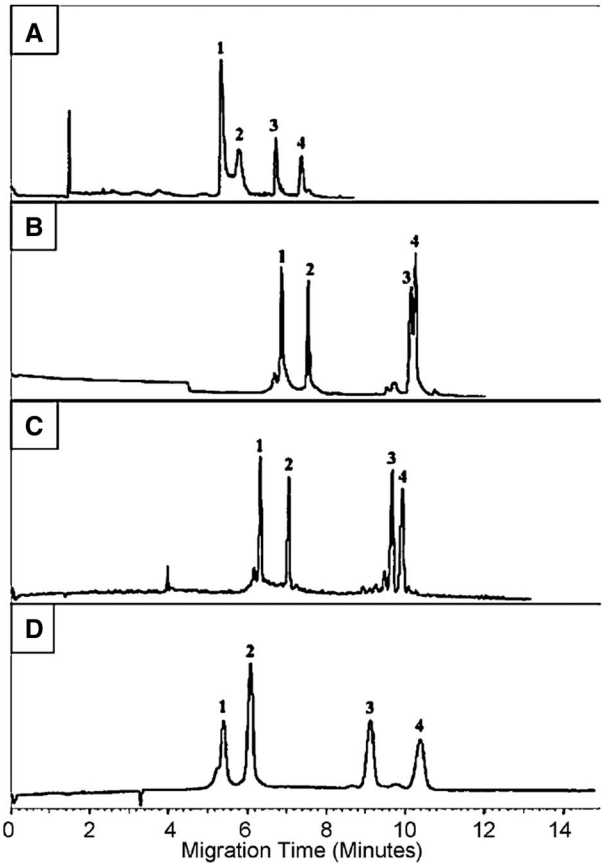

Figure 7. Separation of basic proteins using an uncoated capillary and PEM-coated capillaries. Conditions: coating: (A) uncoated capillary (normal polarity); (B) 0.5 bilayers (reverse polarity); (C) 1.5 bilayers (reverse polarity); (D) 2.5 bilayers (reverse polarity). Cationic polymer: 0.03% w/v poly-L-ornithine; anionic polymer: 0.5% w/v poly-L-SULA. Analytes: 1, α-chymotrypsinogen A; 2, ribonuclease A; 3, lysozyme; 4, cytochrome c; buffer, 20 mM phosphate, pH 4; capillary length, 37 cm total (30 cm effective length); capillary id, 50 μm; voltage, 15 kV; injection, 5 kV for 5 s. poly-L-SULA: poly(*N*-undecanoyl-L-leucyl-alaninate). Reproduced with permission from [130].

Another interesting method involved the use of noncovalent coating by polydopamine, resulting from the self-polymerization of dopamine under alkaline conditions. This polymer acquires a strong adhesive property when the thickness of the polydopamine coating was 106 nm. In this case, ammonium persulfate was used as the source of oxygen to induce and facilitate the polymerization of dopamine to form polydopamine. The EOF of polydopamine-coated open tubular column could be manipulated by varying the pH of the background solutions, owing to the existence of amine and phenolic hydroxyl groups on the polydopamine coating. The applicability of this coating for protein analysis was

demonstrated on real samples (chicken egg white and pure milk) [131].

Interactions of collagen and low-density lipoprotein(s) (LDL) were studied on capillaries coated by collagen. The stable collagen and collagen–decorin coatings (using collagen type I or III) enabled study of the interactions between collagen and selected peptide fragments of apolipoprotein B-100 [132]. Interactions of positive, neutral, and negative peptide fragments of apolipoprotein B-100 were elucidated when selected positive peptide contains the sequence involved in the interaction of LDL with LDL receptor and proteoglycans. The inclusion of decorin in the coatings confirmed the importance of glycosaminoglycans in mediating the interactions between collagens and apolipoprotein B-100 peptide fragments. The similar approach was used in the study of kinetics of glycation reaction on fused-silica collagen I-coated capillary [133]. Results highlighted fact that collagens I and III take part in the retention of LDL particles in the intima through binding with a specific positive site on apolipoprotein B-100.

Cationic lipid vesicles can be also used as coating precursors in CEC [134]. In this case, 1,2-dioleyl-3-trymethylammoniumpropane lipid vesicles were employed as coating precursors to obtain a semipermanent cationic lipid bilayer in silica capillary. This coating was relatively fast and simple. Separation of proteins (α-chymotrypsinogen A, ribonuclease A, cytochrome C, lysozyme) were done using acetate buffer at acidic conditions. Separation was reproducible and reliable and coating was stable under acidic buffers.

5 Concluding remarks

CEC, after 10 years of development, still appears to be a promising method for proteomic (and peptidomic) research and is a continuously developing technique mainly in the front of monolithic stationary phases and capillaries suitable for separation of peptides according to various properties of proteins/peptides. However, similar to HPLC technique, CEC technique has the biggest possibilities in the field of protein and peptide analysis in conjunction with MS. CEC combines the advantages and disadvantages of chromatographic and electrophoretic techniques and therefore it is obvious that this technique is a complementary to both of these methods. However, as very many applications of CEC for the analysis of peptides/proteins already exist, it is evident that new CEC techniques could be developed further. The most promising approaches in this regard include the development of new monolithic materials/columns, capillaries for open-tubular CEC and multidimensional methods/approaches. In the current proteomic era, one can therefore still expect the development of this technique and its applications.

This work was supported by the Czech Science Foundation (Grant No. 15–01948S), and support for long-term conceptual development of research organization RVO: 67985823.

Author declares that has no financial/commercial conflicts of interest.

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