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Capillary Electrophoresis in Bioanalysis

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For the period January 2006 to December 2007, *Web of Science* reports ~4800 hits on capillary electrophoresis (CE) including 410 reviews. Because of the prominence of CE techniques in bioanalysis, we decided make them the focus of this review and selected 200 hundred papers representing advances in this field. The selected papers cover advances in CE theory, instrumentation, and methodologies that are specific to various analytes of biological origin or relevance. The group of analytes includes nucleic acids, proteins and peptides, carbohydrates, lipids, single cells, and bioparticles. In addition, we have included advances in the use of CE to define functional assays or to investigate biomolecular interactions. The use of microfabricated devices for CE analysis was not included because this is already covered in other review.

Technique Developments

Separation Schemes

Determining the velocity of the electroosmotic flow (EOF) and how it changes during an electrophoretic separation is still an important research topic. A simple method for EOF measurements using so-called thermal marks was reported (1). Here, a tungsten filament caused punctual heating at the capillary wall and caused a perturbation in the electrolyte concentration. A sequence of these "thermal marks" then migrated with the EOF until each mark reached and was detected by a conductivity detector. The feasibility of using thermal marks as internal EOF standards in different separation systems was thereby demonstrated.

Isoelectric focusing separates amphoteric analytes such as proteins or peptides by the differences in their isoelectric points. Most of the reports on capillary isoelectric focusing (cIEF) describe an initial focusing phase after which the focused zones are mobilized and detected. A dynamic cIEF method for protein analysis was reported (2). This technique made it possible to control each protein's position and focused width by moving the pH gradient within the capillary through manipulation of the electric fields. An important advantage of this approach is the capability of collecting focused analytes from the central section, suggesting that there may be great potential for introducing selectively focused proteins to a second separation dimension such as LC or CE.

Micellar electrokinetic capillary chromatography (MEKC) is typically incompatible with electrospray mass spectrometry (ESI-MS) because the nonvolatile surfactants in the micellar phase result in complicated adduct formation and loss of sensitivity during the electrospray process and because the presence of the organic solvent needed for electrospray may cause instability in the micellar phase. These drawbacks were overcome by using synthetic polymeric surfactants that can work as a pseudostationary phase and provide stable electrospray (3). The polymeric surfactant was made by polymerizing three amino acid-derived (L-leucinol, L-isoleucinol, L-valinol) sulfated chiral surfactants. These polymeric

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surfactants showed great compatibility with MS detection as well as enantioselectivity for a broad range of acidic, neutral, and basic analytes.

Ionic liquids are organic salts with a melting point lower than 100 °C. They have been found to be nonvolatile compounds with good solvent properties and good compatibility with the environment. Two reports have been chosen to illustrate the use of ionic liquids in CE. One report describes the potential use of ionic liquids as chiral selectors in the analysis of acidic compounds by MEKC. Two amino acid-derived ionic liquids (leucinol and *N*-methylpyrrolidinol) were synthesized, characterized, and used as chiral selectors in the MEKC analysis of enantiomers of phenoxypropionic acid (4). The enantiomeric separation had a resolution greater than 1.7. In the other report, ionic liquids were also used for reducing adsorption on PDMS channel surfaces (5). Using a buffer that contained a mixture of ionic liquids and the nonionic surfactant Triton X-100, the resolution and efficiency of Cy3-labeled proteins was improved relative to previous reports.

Another recent development has been the use of carbon nanotubes (CNTs) as a pseudostationary phase in electrokinetic chromatographic separations. This application of CNTs stems from their high adsorption capacities and surface areas (6). Single-walled and multiwalled CNTs were coated with surfactants and then used as the chiral selector phase in MEKC (7). Enantiomers of nonephedrine and ephedrine were separated with resolution ranging from 1.35 to 1.45.

The formation of a segmented flow by interspersion of water droplets in an immiscible medium has become a promising tool for the manipulation and analysis of biological materials, particularly single cells and bioanalytes at low concentrations. One major advantage is that analytes can be trapped in femtoliter-size droplets, thereby reducing the diffusion that would be observed in a continuous aqueous environment. One major difficulty has been a lack of detection strategies compatible with the analysis of segmented systems. One approach described the resealing of the immiscible boundaries prior to detection (8). The effects of different wall coatings on the separation, resealing, and detection of fluorescently labeled amino acids trapped in 10-fL droplets were investigated.

Capillary Coatings and Surface Modifications

One of the greatest challenges in CE-based bioanalysis is the interaction of biomolecules with negatively charged, hydrophobic fused-silica capillary surfaces, resulting in poor separation profiles. This problem is particularly severe in the CE analysis of highly basic proteins, which can strongly interact with the capillary surface via electrostatic interactions.

Adsorption effects on the capillary wall surface were studied by combining CE separations and the single-molecule detection of R-phycoerithrin (9). The calculated capacity factors produced using both techniques were in good agreement. This approach appears generally applicable for the investigation of adsorption and separation processes in CE.

New coating procedures that prevent molecular adsorption to the capillary walls during CE separations continue to be attractive due to the simplicity of coating preparation, use, and regeneration. Several relevant coatings procedures that do not rely on covalent modification of the walls, but use surfactants, polymeric materials, or nanoparticles, are included in this review.

One report described the use of the two-tailed gemini cationic surfactant ethylene bis(1-dodecyldimethylammonium) dibromide as a dynamic coating (10). This surfactant reversed the EOF and was better able to control EOF and wall adsorption compared to the commonly used cethyltriethylammonium bromide (CTAB). Efficiencies exceeding 300 000 plates/m

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were achieved for separated proteins over a wide pH range. A third report described the use of another double-chained surfactant containing two C18 chains instead of a C12 chain, to prepare capillary coatings compatible with organic–water systems (11). A tolerance to buffers containing up to 60% of methanol, good migration time reproducibility (0.5% RSD), and high separation efficiencies reaching 600 000 plates/m during analyses of small drugs were achieved.

Another coating procedure utilized bilayer-coated gold nanoparticles with didodecyldimethylammonium bromide as an additive to the separation buffer (12). Modified capillaries had reversed, stable, and EOF at pH <3.5. Following immobilization, the nanoparticles were further modified with 0.05% poly(ethylene oxide) that made feasible the separation of acidic and basic proteins with efficiencies ranging from 7100 to 100 000 plates/m.

Polymeric materials were also used as replaceable coating agents. A cationic polyamine-based polymer, poly-LA 313, proved to be stable in a wide pH range and compatible with ESI-MS (13). The MS compatibility was due to the anodal EOF of the modified capillaries and organic solvent tolerance of the coating. Separations of neuropeptides with a resolution of 1.4 and efficiencies up to 370 000 plates/m were demonstrated. Another report described the synthesis and use of three polyacrylamide–dimethylacrylamide copolymers (P(AM-PDMA)) as a replaceable separation sieving matrix and coating (14). Good efficiency and repeatability of migration times were achieved for four basic globular proteins (RSD ~0.4%). A third report described a highly efficient separation of basic proteins at neutral pH in capillaries coated with poly(diallyldimethylammonium chloride), which show reversed EOF (15). Six proteins were separated with excellent efficiency (0.18–0.6 million plates/m) and had good migration time reproducibility (RSD < 0.4%).

A unique approach for incorporating polymeric coatings was based on entrapping Polybrene on top of the capillary surface with a layer of polymerized silicate (16). A polymerization mixture containing potassium silicate, formamide, and Polybrene was then introduced into the capillary and flushed out after 1 h; this procedure produced a stable coating and reversed EOF. At pH <4.5, the EOF was very stable after 300 runs; at pH >8.5, the EOF drifted 22% after 200 consecutive runs.

New reports on the use of phospholipid bilayers also have appeared recently. One study investigated the effects of vesicle size (25 nm–20 μm), reconstitution buffer type, ionic strength, and Ca^{2+} cations on the use of the lipid 1,2-dimiristoyl-*sn*-glycerophosphocholine (DMPC) as a capillary coating (17). Capillaries prepared from 25–50-nm vesicles produced the best coating separation efficiency (140 000 plates/m) for lysozyme, cytochrome *c*, ribonuclease A, and α-chymotrypsinogen. The coating rate for capillary preparation was enhanced by using HEPES buffer and increasing ionic strength and Ca^{2+} content of the DMPC solution.

In one report, a capillary coating consisting of highly polymerized sorbyl-based polymerizable phospholipid bilayers (bis-SorbPC PLB) was prepared in-capillary using self-assembly and radical polymerization (18). Cross-linking of the PLBs stabilized the coating, prevented desorption from the surface, increased chemical stability, and allowed for the separation of both cationic and anionic proteins. Despite the lower separation resolution achieved, extended stability was demonstrated. The analyses were reproducible even after continuous use of the capillary for 10 h, 10 consecutive days; the capillary stability after 1 year of dry storage was also reported.

Some analytical procedures at the interface of CE and open tube electrochromatography may require coatings with unique molecular properties such as chiral selectivity. A good

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example is the use of covalently immobilized avidin on the capillary surface to produce a stable coating with chiral recognition and MS compatibility (19). Avidin-modified coatings showed good chiral recognition for abscisic acid enantiomers and lasted 50 days with over 100 runs. Another example is the use of capillaries coated with lipid particles to investigate the separation selectivity of steroids (aldosterone, adrostenedione, testoterone, 17-OH progesterone, progesterone) (20). The lipids, in the form of liposomes, were either pure compounds or were isolated from red blood cell ghosts. This coating was suitable for separations in 5:95% v/v methanol/background electrolyte (40 mM HEPES, pH 7.4). After 18 runs, the peaks for each steroid were still resolved, but the migration times had changed dramatically as a result of the stability of the lipid coating. In a third example, oxidized low-density lipoproteins were used to separate steroids (21). This work demonstrated that there are changes in selectivity and retention associated with lipid oxidation that may be relevant to phospholipid oxidation in biological systems.

Processes Prior to CE

(1) Sampling—While in most CE applications, sampling is performed from one vial, either hydro-dynamically or electrokinetically, there are other possibilities, including sampling via microdialysis and directly from solid tissues.

One variation on direct sampling from a vial was reported. A supported liquid membrane was used to assemble a microextraction unit containing the injection end of the capillary and the electrode, thereby permitting application of a voltage within this unit (22). When this voltage was applied, analytes (e.g., nitroimidazoles from a pig liver tissue homogenate) were preconcentrated and successfully cleaned up by the microextraction unit. One disadvantage is that the voltage-assisted extraction and cleanup takes ~20 min per sample.

Sampling control by manipulating the EOF and applying auxiliary pressure bias has been used previously. A new report describes the selective enrichment of multiple phosphorylated peptides by electrokinetic injection in the absence of EOF (23). After sampling, pressure was applied to mobilize the peptides through a polyacrylamide-coated capillary. A related approach, termed pressure-assisted electrokinetic injection, was used for the online preconcentration of small charged molecules (e.g., single nucleotides) (24). Here, pressure is applied to compensate for the EOF-driven flow so that the amount of injected analytes can be larger without causing the added band broadening associated with longer injection plugs.

Photobleached gated sampling of proteins was also used as a sampling technique. Fluorescently labeled proteins were continuously introduced and photobleached on column by an Ar+ laser (351–364 nm). Very short plugs were injected by blocking the gating laser. Analytes were then separated and detected at 488 nm. Separation times of <5 s, extreme efficiencies exceeding 1 000 000 plates/m, and LOD less than 1 nM for selected amino acids and proteins were demonstrated (25).

Another approach used an ultranarrow sampling fracture formed on a quartz capillary held in place with molded PDMS (26). The 25-μm-i.d. capillary was used to sample carbohydrates and then detect them amperometrically. The designed injection fracture reduced sample loss and decreased the contribution of sampling to the final peak width thereby producing efficiencies of 23 900 plates/m.

Procedures for direct sampling from tissues into the CE capillary were reported. In one report, a sample was taken from muscle cross section (27). The sampled region was similar in diameter to that of a muscle fiber; sampling was achieved by bringing into contact the injection end of the capillary and applying negative pressure at the detector end. Similarly, two buffer plugs containing 10-*N*-nonylacridine orange were injected: one before and one

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after the sample. Upon incubation, the reagent plugs and the sample mix by diffusion and the mitochondria in the sample became fluorescently labeled. CE separation and laser-induced fluorescence (LIF) detection then made it possible to detect the mitochondria from a region in a given muscle cross section. A second report describes the sampling of taurine from a resected rat brain. After sampling, the capillary was removed from the brain; then, online preconcentration, labeling, CE separation, and LIF detection took place (28). Different taurine levels in different brain regions were reported.

Microdialysis sampling is commonly used for monitoring dynamic changes in the abundance of small molecules (e.g., dopamine) in the biofluids of organs (e.g., brain) of free moving animals. Recently, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was used as a fluorogenic label for the online, high-speed microdialysis-CE analysis of amino acid neurotransmitters (29). Sixteen amino acids were identified and separated every 20 s with improved detection sensitivity. The method was used to investigate the effect of rat brain stimulation on amino acid neurotransmitter profiles and to investigate correlations between dopamine levels in vivo with the drug-induced behavior of fully moving rats. Another report, using a similar approach, derivatized the dialyzates with NBD-F and defined the separation conditions to separate 16 amino acids, including glutamate, GABA, glycine, taurine, and D-serine in ~20 s; improved limits of detection were also reported (30).

Sampling hemolymph from individual *Drosophila* larvae for amino acid analysis was also reported (31). This clever approach required rupturing of the cuticle of the larvae and suctioning of the hemolymph (50–300 nL) onto a Tygon tube for easy handling. Hemolymph evaporation did not cause any complications as long as the sample was processed within 60 s. The collected sample was derivatized with fluorescamine and analyzed by CE-LIF. This method made it possible to detect 13 amino acids in wild type and in genderbind mutant larvae.

Ultimately, CE sampling procedures need to become compatible with high-throughput approaches. A multiple capillary electrophoresis instrument that simultaneously samples 16 wells has been reported (32). The capillaries in this device were made using printing board technology on laminated material. The utility of the device was tested by separating 15 fragments ranging from 50 to 500 bases; lane-to-lane CV of migration time was 0.38% and a fragment size of 258 ± 15 bases was expected to have a resolution of 0.59.

(2) Electrophoretic Preconcentration—Electrophoretic preconcentration is commonly needed prior to CE analysis in order to improve detection sensitivity. Several reports included in this review used various modes of electrophoretic preconcentration: field-amplified sample stacking, isotachophoresis, and sweeping.

In one report, the fundamental processes behind sweeping and high-salt sample stacking of alkaloids that lead to enrichment in MEKC separations were investigated (33). The effects of different surfactants, sample matrix types and concentrations, conductivity, and the length of the sample plugs on the preconcentration of alkaloids were discussed.

Field-amplified stacking, concentrating analytes at the boundary of low-conductivity and high-conductivity buffers, is the most common approach for fast sample enrichment. For example, field-amplified stacking of peptides in low-nanomolar concentrations produced a 3000-fold enhancement in detection sensitivity in a CE-ESI-MS analysis (34). Another report describes a method for the online concentration of neutral analytes that combines the effects of the field-amplified stacking, reverse migrating micelles, and pressure-driven counterflow (35). Using this approach, some steroids showed up to a 3000-fold concentration factor.

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Isotachophoresis papers are also included in this review. In one paper, transient capillary isotachophoresis/zone electrophoresis was used for the selective enrichment of low-abundance peptides for online proteomic analysis using CE-nano-ESI-MS. This preconcentration approach made it possible to detect low-abundance ovalbumin peptides at concentrations as low as 0.1 nM, in an excess of 500 000 cytochrome *c* peptides (36). Another paper described an isotachophoretic enrichment procedure using gradient elution and moving boundary electrophoresis (37). In this method, enrichment resulted from a counterflow of the leading electrolyte (i.e., low-mobility electrolyte) that continuously pushes analytes, suspended in the terminal electrolyte (i.e., high-mobility electrolyte), away from the capillary; an ionic interface near the capillary inlet then forms. After the sample is concentrated at the boundary, gradient elution is performed by gradually decreasing the counterflow. As the counterflow decreases, plugs of enriched analytes sequentially start entering the capillary. The authors reported a 10 000–130 000-fold improvement in the limits of detection, which makes it feasible to analyze low-picomolar concentrations of DNA, amino acids, and proteins within 2–8 min. A third paper described used pseudotransient isotachophoretic to increase sensitivity of homocysteine detection in plasma samples using commercial CE systems and UV detection (38). Here, acetonitrile was added to the sample containing high concentration of salts (e.g., plasma). After the application of electric field, homocysteine were concentrated by pseudotransient isotachophoretic between the zone of small inorganic cations acted as leading electrolyte and acetonitrile zone acting as pseudoterminator. The LOD of homocysteine labeled with 2-chloro-1-methylquinolinium tetrafluoroborates was 1 μM.

(3) Affinity and Solid-Phase Extraction (SPE) Purification and Enrichment— Affinity purification may decrease sample complexity and preconcentration prior to CE analysis. One report described the use of multifunctional magnetic particles (MFMPs) in the analysis of low-density lipoprotein (LDL) (39). The MFMPs were prepared to serve three purposes: (i) magnetic response, (ii) fluorescent labeling (RITC labeling), and (iii) affinity-based selectivity for the analyte of interest. Sodium dextran sulfate was selected as the affinity ligand for the analysis of LDL. The magnetic particles were trapped in an applied magnetic field within the inlet of the capillary while the sample was injected and concentrated into a narrow band. The electrophoretic mobility of the particles changed as a function of LDL concentration; the LOD was 4.3×10^{-13} mol/L LDL; the electrophoretic mobility response was unaffected by the concentration of high-density lipoprotein.

In a second report, single-walled, carbon nanotubes (c-SWNT), derivatized with carboxylate groups, were used to cleanup carbohydrate samples (40). A plug of 30 mg/L c-SWNT partially fills the capillary prior to the introduction of a juice sample containing multiple carbohydrates containing interferences. This approach allows the complete resolution of glucose, fructose, and maltose from other interferences and makes it possible to quantify the levels of these carbohydrates.

A third report described the use of electrostatic forces between positively charged amino acids (i.e., at low pH) and the negatively charged silica wall of a capillary as a method of preconcentration (41). Etching the inner wall creates a larger surface area for electrostatic interactions. The amino acids are then eluted from the walls of the capillary using a basic buffer and focused into a narrow band; after focusing, the amino acids are electrophoretically separated and detected by a diode array detector.

SPE can be used to remove impurities and preconcentrate the analytes prior to CE analysis. An analysis coupling SPE with CE used a hollow microdialysis fiber as the solid phase (42). Using this design, molecules were concentrated on the fiber and then the fiber was inserted into the CE sample vial through an adapter. After elution from the fiber, macromolecules

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were concentrated (a concentration factor of 1800–2100) in a small-volume macromolecule trap. This further concentrates the macromolecules in the sample vial prior to CE analysis. *β*-Lactoglobulin A and B were used as test analytes. Another report, used liquid-phase microextraction supported on a porous hollow fiber (polypropylene) to extract analytes into an acceptor solution within the fiber. This has been applied in the analysis of hydroxychloroquinone and its metabolites from urine samples (43).

Besides conducting SPE preconcentration and CE as separate steps, new reports describe true in-capillary SPE preconcentrators. By using a temporary frit created within the capillary, octadecyl silica material can be packed into the inlet of the separation capillary (44). This method of preconcentration has been successfully used to concentrate peptides from protein digests prior to CE analysis. It was necessary to maintain a 0.3-MPa pressure throughout the separation to create a stable electrospray for ESI-MS detection. A similar method that uses a frit to trap the solid-phase material was reported for the analysis of 250 pg/mL oxprenolol from urine samples (45). This report stresses ease of use with commercially available CE systems because the in-capillary preconcentrators do not require the attachment of additional columns to the inlet of the separation capillary.

While using in-capillary preconcentration does eliminate the need to attach a concentration column to the inlet of the separation capillary, it then requires that the sample waste be passed through the capillary. This increases the possibility of contaminants adsorption to the walls of the separation capillary, a problem that has been overcome with the use of a hole-opened capillary (46). A small hole, 30 *μ*m, was opened in the capillary wall after the sorbent material of the preconcentrator. When the sample was loaded, the waste was allowed to exit through the hole and bypass the separation capillary. To perform the separation, the electric field was applied between the hole and the outlet of the capillary. A separation of chlorophenols loaded in a solution of CTAB was used to illustrate the elimination of interferents adsorbing to the capillary wall. No EOF suppression was seen when the loading solvent was allowed to pass through the open hole thereby bypassing the separation capillary.

Detection

(1) Fluorescence Detection—Despite the need to label analytes when they do not possess intrinsic fluorescence, this detection scheme is very popular in CE analysis because it is able to detect extremely low concentrations of analytes in very small sample volumes, down to single fluorophore molecules. Single-molecule detection usually uses a high-sensitivity confocal microscope to detect molecules as they pass through narrow channels (i.e., ~500 nm deep and wide) and to reduce external sources of photons. In order to detect single molecules in wider channels, a report described a line-confocal detection geometry to match a 2-*μ*m microchannel width (47). Using this detector, the detection efficiency for single Alexa 488 molecules was found to be 94%, while it was only 34% when using a point-confocal detector. Migration times were also measured using two-beam fluorescent cross-correlation spectroscopy.

Simultaneous monitoring of various spectral regions is desirable to identify analytes that may have different emission spectra and yet comigrate in the CE analysis. A three-channel LIF detection system was reported (48). The system utilized a single excitation laser emitting at 224 nm; using dichroic mirrors, the fluorescence emission was then spectrally separated into three beams. Each of the beams passed through an interference filter that defined the spectral range of interest prior to detection by a respective photomultiplier tube. The system was used for sensitive detection and spectral recognition of neurotransmitters in single neurons with LODs reaching 40 nM.

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Although LIF has been previously used for the CE analysis of fluorescence particles, these detectors did not offer the ability to measure other spectral properties such as scattering. An instrument using an orthogonal scattering detector for CE analysis of individual polystyrene particles was recently reported (49). Using this CE system, particles of different diameters (110–992 nm) were electrophoretically separated; individual scattering intensities were correlated with particle size. Another report described a CE analysis using a detector that measures simultaneously LIF and orthogonal scattering of individual particles (50). Fluorescently labeled latex microspheres (500 and 1000 nm) were used to validate the use of this detector that then was also used to analyze fresh and cryogenically stored mitochondria. In the latter case, changes in scattering patterns were associated with storage.

Light-emitting diodes have become very attractive excitation sources in CE setups. The main advantages are reduced size, easy operation, wide range of available wavelengths including the UV region, low cost, long lifetimes, and miniaturization potential. On the other hand, the LODs obtained using LEDs are generally higher in comparison to those obtained with laser sources, partly because of power losses due to reflections and scattering on the capillary surfaces. In order to increase the power of excitation, an LED source (442 or 480 nm) was coupled to an optical fiber (51). The other end was inserted into the separation capillary, thereby transmitting the excitation light directly to the detection window. The emitted fluorescence was collected by a microscope objective, spatially and spectrally filtered and then detected by a PMT. Detection limits for NDA and FITC-labeled amino acids were 17–23 and 8–12 nM, respectively.

Development of LEDs in the UV region is of particular interest to CE with fluorescence detection, because some derivatizing agents with very good labeling properties such as OPA can be excited only by very expensive UV lasers. Applications of UV LEDs for the fluorescence detection of OPA-labeled amino acids and proteins were reported (52). Detection limits for OPA-labeled glutamic acid and BSA were 10 and 28 nM, respectively. In a separate paper, an integrated LED-induced fluorescence detector utilizing fiber optics for CE was described (53). Despite the reduced dimensions of this detector, its performance (i.e., 5 nM LOD for fluorescein and a dynamic range of 4 orders of magnitude) was comparable to systems equipped with conventional excitation sources.

Most recent CE-LIF developments have utilized laboratory-made LIF systems. An integrated LIF detector for commercial CE instruments was recently reported (54). The detector used a ball lens to focus the laser beam onto the capillary and an ellipsoid mirror glued on the capillary to collect the emitted fluorescence. The whole system could be easily integrated inside a commercial CE cartridge. The detector had LODs of ~1 pM fluorescein and ~50 pg/mL TAMRA-labeled IgG_2.

(2) Mass Spectrometry—The integration of CE with mass spectrometry detection (CE-MS) is a promising approach combining the fast, biologically compatible separations of CE with MS capabilities for obtaining structural information. To find out more about the intensive research in this field, the reader is referred to a specialized review on the subject (55).

The most common ionization method coupled with CE separation has been ESI. The contribution to band broadening by the nebulizer gas, common in many ESI ion sources, has been recently examined (56). It was shown that if the nebulizer gas is on during injection, the injection volume is increased and introduces significant peak broadening in 75- and 50-μm-inner diameter capillaries. A promising solution to this problem was reported in a different paper. A coaxial sheath-flow design of a microspray source allowed the sheath liquid to be electrosprayed without using a sheath nebulization gas because of its small and

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sharp needle geometry (57). In this design, the separation capillary was inserted into a 412-μm-i.d. needle that was to serve as the electrospray source.

A reduction in the sensitivity of CE-MS methods due to the dilution of the analytes migrating out from the capillary by the sheath-flow liquid in electrospray sources has been investigated. An attempt has been made to remedy this problem by using a microsprayer, which reduces the capillary's inner diameter in order to maintain the necessary flow rate and a stable electrospray. A capillary terminated with an in-laboratory shaped microsprayer coated in copper was optimized for the detection of carbohydrates (58). This was shown to be an easily implemented and rugged technique.

While the microsprayer used at the CE–ESI interface addresses the issue of sensitivity, its use causes the capillaries to break or clog more frequently. To avoid this, a pulsed electrospray method has been proposed (59). In this report, the separation capillary was tapered to 30 μm and coated with a conductive rubber. A flow of 500–700 nL was found to be optimum. A duty cycle of 20% and a pulse frequency of 20 Hz were used in the detection of a synthetic drug mixture. Because a very narrow outlet is not necessary with pulsed electrospray, less clogging and breakage of the capillary is observed.

Without the use of sheath flow, electrical contact is maintained by coating the capillary tip with a conductive material. Poor stability and arcing have prevented the use of negative ionization with this type of sheathless interface. To allow the use of negative ionization, a porous junction was utilized to maintain electrical contact (60). This method was used to assess metabolites in *Escherichia coli*, and an improvement over other negative mode techniques was reported because of the sheathless interface.

Another option for coupling the CE separation to MS detection is through a pressurized liquid junction interface. Reports of simple and cost-effective designs for this interface have appeared recently (61,62). The most important consideration is the gap width between the separation and spray capillaries, with a range of 50–200 μm determined to be optimum. Other parameters discussed were interface geometry, separation voltage, and pressure at the liquid junction.

While it is necessary to decrease EOF variations in order to prevent instability in the electrospray process, unfortunately, EOF control and regulation is usually done by modifying fused-silica capillaries walls with coatings that are not necessarily stable at the extreme pHs that may be used in electrospray ionization. A solution to this problem is to use poly(methyl methacrylate) and polyether ether ketone capillaries instead of modified fused-silica capillaries (63). Using these plastic capillaries, common biological anions have been separated and electrosprayed at a high pH (i.e., pH 11).

Precisely controlling the EOF or regulating the pressure-driven flow is necessary when the same CE-MS analysis in fused-silica capillaries involves both anionic and cationic species. Two methods of complete analysis of cationic, anionic, and polyanionic analytes in CE-MS were presented in one report (64). In the first method, the separation was accomplished by using negative voltage polarity and neutrally coated capillaries (poly(methylsiloxane)) and a slight pressure to compensate for residual cathodic EOF. The second method used a dynamic coating of a polycationic polymer, Polybrene. Both methods allowed the separation and detection of all sample anions and almost all cations.

Besides controlling EOF, the possible ionization supression caused by the commonly used nonvolatile CE buffers needs to be addressed when designing CE-MS experiments. Phosphate, for example, is a useful buffer system because of its multiple useful pK_a values. However, it is also known to cause strong ion suppression, forms several background

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clusters, and contaminates the ion optics of the mass spectrometer. In order to continue using these nonvolatile buffers, a method was described in which phosphate ions are prevented from reaching the detector by opposing a stronger EOF to the negative mobility of this anion (65). This method was successfully applied to the CE-MS analysis of a mixture of antihistamines. Another approach using nonvolatile buffer components was designed for use in the analysis of impurities in pharmaceuticals by CE-MS (66). Buffer composition (concentration of TRIS and pH), liquid sheath composition, and nebulizer gas pressure were explored as parameters to optimize the separation and limits of detection for various test compounds. Separation could be successfully performed using a buffer system containing 100 mM TRIS (pH 2.5 adjusted with phosphoric acid).

MEKC and MEEKC are very effective and popular for CE separations of neutral compounds, but these separation modes are not easily compatible with electrospray ionization sources for mass spectrometric analysis. Neutral compounds are not easily ionized, and the addition of common surfactants, such as sodium dodecyl sulfate (SDS), causes a strong ionization suppression effect. An ion source that is not susceptible to ionization suppression by surfactants is the atmospheric pressure photoionization ionization (APPI) source. This source has been successfully coupled to MEEKC (67). Here, it was found that even microemulsions based on nonvolatile buffers and up to 3% SDS showed no negative effects from ionization suppression or source fouling. Another report investigated the mechanisms of ion formation in the APPI source (68). In this report, it was concluded that the ionization of some compounds (e.g., quaternary ammonia compounds and basic amines) proceedes via mechanisms other than photon-induced ionization.

Another strategy to make MEKC techniques compatible with MS detection is to use volatile surfactants. One recently reported MS-friendly surfactant has been ammonium perfluorooctanoate, which can be dissolved in 2-propanol (69). Using *N*-methylcarbamates, it was observed that the detection limits are similar to or lower than previous methods where SDS micelles were used to facilitate the separation.

Polymeric micelles are also an attractive option for the use in MEKC-MS systems. Polymeric micelles provide several advantages when using MS detection: the covalent bonds are difficult to ionize resulting in less background noise, stable micelles allow for the use of a higher organic modifier concentration, and they can be used at any concentration due to the absence of a CMC. Three carbamate-type polymers and three amide-type polymers were compared in the enantioseparation of two benzodiazepines, ((±)-loxazepam and (±)-lorazepam), and one benzoxazocine, ((±)-nefopam) (70). The use of 15 mM poly(sodium *N*-undecanoyl-L-leucinate) along with organic modifiers was successful in providing enantioseparation of the three analytes. Limits of detection of 1.8 μg/mL were achieved, which were 4 times better than UV absorbance detection.

In addition to the developments associated with better coupling of CE with ESI-MS, other efforts have focused on the high-throughput CE-MS. An example of such an effort is the design of an interface that accommodates four sheath-flow inlets that are sampled sequentially (71). This results in a 4-fold increase in overall throughput. This method also avoids the carryover of sample contaminants that can occur when using a single interface and four different capillaries.

Coupling of CE to MALDI-MS has been accomplished in the past, but it is not trivial. Online couplings usually involve significant modification of the MS instrument. A novel offline coupling technique using a closed–open–closed microchannel system has been reported (72). The end of the separation capillary was fed into a microcanal (50 μm deep, 30 mm long) fabricated on a silicon substrate. This was then followed by another closed

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capillary to complete the separation system. The microcanal chip was enclosed in a microclimate chamber to avoid evaporation. The MALDI matrix was added after the separation to the microcanal and sample in a standard MALDI source. External sample transfer to MALDI targets is eliminated by using this method, and no modification to the MALDI-MS instrument is needed.

(3) Other Detection Strategies—Papers referring to other less commonly used detection schemes for CE systems included contactless conductivity, electrochemical, electrogenerated chemiluminescence (ECL), nuclear magnetic resonance (NMR), electrothermal atomic absorption spectrometry (ET-AAS), and Fourier transform. These are discussed below.

A contactless, conductivity-based, absorbance detector for CE was developed (73). When analytes passed through the detector, they absorbed light from a 442-nm HeCd or 488-nm argon ion laser producing a thermal perturbation in the buffer, which changed the buffer conductivity. This change in conductivity was sensed with a contactless conductivity detector.

A microelectrode for end-column amperometric detection was reported (74). The electrode was prepared by depositing a boron-doped diamond thin film on a Pt wire. Good stability, pH-independent background current, low-mass LODs (100–400 amol), and excellent response reproducibility (2–4%) were achieved. The detector was used to identify and quantify 10 different catecholamines and metabolites separated by capillary zone electrophoresis (CZE).

A method for detection of amino acids using ECL was presented (75). Typically, in the presence of tris(2,2′-bipyridine)ruthenium(II), secondary and tertiary amines produce very strong ECL signals but a very weak signal is obtained for primary amines. To overcome lack of sensitivity for primary amines (i.e., arginine, proline, valine and leucine), they were modified with acetaldehyde prior to their CZE analysis; these modified amines produced a strong ECL response when the buffer contained $Ru(bpy)_3^{2+}$ and had LODs ranging from 0.5 to 5 fmol. A separate report described how to analyze mixtures of compounds containg primary, secondary, and tertiary amino groups. This report combined a LED-induced fluorescence and ECL detection (76). Prior to the CE analysis, primary amines were labeled with NDA to make them detectable by fluorescence while the secondary and tertiary amines were reacted with $[Ru(bpy)_3^{2+}]$ to make them detectable by ECL. This dual detection scheme successfully applied to detect amino acids and alkaloids in urine samples and tobacco extracts separated by CZE in borate buffer.

A CE separation was used as the basis of an electrochemical sensor to monitor polymerase chain reaction (PCR) amplification (77). After completion of the PCR, pyrophosphate released during PCR is hydrolyzed to phosphate by pyrophosphatase. Then, the phosphate is separated by CE and detected in a microsensor made of nanoparticles comprising poly-5,2′–5′,2″-terthiophene-3′-carboxylic acid/pyruvate oxidase (PyO). At the microsensor, pyruvate was converted to hydrogen peroxide by PyO in the presence of Pi and oxygen; then, the anodic current produced by hydrogen peroxide was monitored.

The power of NMR lies in its ability to provide structural information, but its Achille's heel is its sensitivity, even when using microcoil NMR. In one report, the limitations in sensitivity were partially overcome by online coupling isotachophoresis cITP to microcoil NMR detection (78). After preconcetration by ITP, microcoil NMR was suitable to detect trace impurities of degradation products in acetaminophen samples.

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A report described the use of a thermospray interface to adapt an E-AAS detector to a CE system (79). The system was used to separate and detect mercury species interacting with DNA; the LOD was 10 nM mercury. The kinetics of binding affinities of Hg(II), MeHg(I), and PhHg(I) species to DNA were also reported.

Improvements in detection capabilities using Fourier transform CE (FTCE) was demonstrated using channels in a PDMS device equipped with LIF detection (80). FTCE not only increases S/N values but also does not require that peaks have complete resolution in the time domain for them to be completely resolved in the frequency domain. In this paper, five parallel channels with channel length increasing according to a square root made it possible to have five simultaneous separations in which analyte peaks migrate out at a frequency given by the differences in length. Signal-to-noise ratio was increased by 2.9 times, and theoretical plate height was improved by a factor of more than 30. A further development in data processing was based on the use of a personal computer sound card (PCSC) to emulate a lock-in amplifier and for data acquisition of an LED-based fluorescence detector (81). High data sampling rates of the PCSC enabled detection of 3.3 and 5.3 fmol of NDA-derivatized tyrosine and alanine, respectively, and wide dynamic range were shown. This was three times better performance than with unmodulated LEDs excitation sources.

The combination of CE with MS techniques produces complex data sets composed of migration times, *m/z* values, and ion intensities. A tool to represent the raw data from a CE-MS analysis of peptides as 2D maps was reported (82). This approach was tested on cytochrome *c* digests, and it was demonstrated that the 2D maps made it simple to inspect large data sets, visually identify differences between the sets, and identify comigrating peptides.

Hyphenation

Integration of several procedures in an automated fashion reduces human error, improves reproducibility, and could lead to higher throughput. When these procedures are orthogonal separation modes, hypenation results in increased peak capacity and in the ability to better characterize the composition of complex samples. The reports described below illustrate the developments in this area.

Combination of online sample cleanup using size exclusion chromatography, preconcentration on a low-volume SPE column, CE separation, and UV detection was demonstrated (83). The system was able to successfully detect enkephalins in cerebrospinal fluids. These peptides were separated and detected down to 100 ng/mL after discarding interfering proteins on the SEC column and enriching on SPE.

Another report illustrated the coupling of sequential injection analysis with CE-LIF using a microvalve interface (84). The valve system made it possible to conduct automated online fluorescent derivatization with subsequent CZE separation of amino acids and peptides. The repeatability of the whole procedure was 3% for migration times and 4.5% for peak areas.

Proteomic research requires separation and identification of proteins based on peptides from proteolytic enzyme digestion. Often these reactions take several hours, and the samples can be very small. A solution to this has been the creation of microreactors within the separation capillary in a CE system. As a proof of principle, a microreactor was incorporated before the separation of peptides via a pressurized liquid junction in a CE-MS analysis (85). Proteins are separated first and then passed through the microreactor in which they are digested by immobilized pepsin; then, the resulting peptides are transferred to a second separation capillary and CE separated and detected by MS. This results in a fully automated analysis of

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a mixture of proteins. Cytochrome *c* and myoglobin were used to test the procedure and were correctly identified.

Multidimensional electrophoresis is an important approach for increasing the separation power in the analysis of complex samples. A method for coupling capillary sieving electrophoresis (CSE) and MEKC-LIF was further improved by using narrow inner diameters capillaries, dynamic coatings, and passive temperature control (86). The improved method was used in the analysis of peptides, proteins, and amino acids of epithelium biopsies taken from Barett's esophagus patients. The analysis time per run was <1 h; the peak capacity was 600; and day-to-day variation in migration times were 1.3 and 0.6% for the CSE and MEKC dimensions, respectively. Another report by the same group demonstrated that the throughput could be increased by using a multicapillary approach that makes it possible to carry out five parallel 2D-CE runs (87).

Another report described a simple 2D-CE method using only single capillary for CZE and MEKC analysis of amino acids (88). In the first dimension, analytes were separated by CZE in borate buffer. Then, a selected portion of the first dimension was transferred back into the capillary and separated in the second dimension by MEKC in borate–SDS buffer. In addition, this approach made it possible to eliminate byproducts of amino acid derivatizations before the MEKC separation. The migration time intraday repeatability was 2%.

Online hyphenation of cIEF with hollow fiber flow field-flow fractionation (HFFlFFF) was used for 2D separation of proteins in urinary samples (89). CIEF-HFFlFF was shown to reduce the sample complexity by removing ampholytes, salts, and the most abundant proteins during the HFFlFF dimension. In addition, no organic solvents and surfactants were present, which made it more compatible with the subsequent proteomic analysis (i.e., tryptic digestion and LC–MS analysis of the resulting peptides). Use of cIEF-HFFlFF and proteomic analysis of the 24 collected fractions resulted in identification of 114 proteins, which is similar to the number of proteins (113) identified when the same sample was analyzed by conventional 2D gel electrophoresis. Furthermore, the new approach of protein fractionation was fully automated and took shorter time than 2D gel electrophoresis.

Applications

Nucleic Acid Analysis

(1) Fundamental Studies—A fundamental study investigated the electrophoretic behavior of linear and branched DNA above and below the critical entanglement concentration (90). Both DNA types had similar mobility below the entanglement concentration, but above the entanglement concentration, the branched DNA has retarded mobility with respect to the linear DNA.

The use of drag tags as a means of performing DNA separations in free solution has been investigated before. A recent report described the design of drag tags based on polypetides and genetically engineered proteins and compare the use of these tags with the theory (91). It was concluded that separations in free solution are feasible if both ends of the DNA are labeled with a drag tag. Another report described the attachment of an *n*-alkylated peptide to the ends of DNA and then separated the species by MEKC (92); micelles interacting with the amphiphilic ends acted as drag tags. A theoretical description of this behavior was presented. PCR products from ~90 to 450 bases were clearly resolved.

Capillary electrophoresis was also used for investigating changes in conformation between the random coil and hairpin structures of GcxxsxGC, where xxxx is GA3, T4, T3C, or A4

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(93). The approach makes it possible to predict the percentages of each hairpin at 20 °C, because the electrophoretic mobility changes as a function of their structural conformation and charge.

Temperature oscillations may also be used to separate homo-duplex and heteroduplex DNA. It was predicted that movement of DNA through temperature zones causes alternate annealing and melting of DNA, ultimately affecting its observed electrophoretic mobility (94). The prediction stated that DNA with and without a point mutation will have a different lag in annealing that would result in different mean velocities. Also, optimal temperature cycles and electric fields were predicted.

Another approach to separate DNA without sieving matrix is based on reducing the dimensions of the separation channel. Feasibility of this concept was demonstrated by conducting separations of fluorescently labeled DNA (10–100 base pair long) in channels with nanometer size dimensions (95). Separations are feasible when the Debye length to channel half-depth ratio is 0.006–02 and the ratio of length of dsDNA to channel half-depth is 0.004–1.7.

(2) Oligonucleotides—Impressive separations of oligonucleotides (i.e., 8–32 bases) were accomplished by using the triblock copolymer $E_{45}B_{14}E_{45}$ (B20–5000), with E, B, and subscript denoting oxyethylene, oxybutylene, and segment lengths, respectively (96). The E block acts as a coating and blocks electroosomotic flow, while the B block becomes hydrophobic when the temperature increases, decreasing the interactions with DNA. The capillaries are filled with the copolymer held at 4 °C at which the copolymer is less viscous; when the capillary is brought to higher temperatures, it becomes a gel.

A reproducible and quantitative method for the analysis of nucleotides was reported using phosphate to mask the silanol groups of silica capillaries before separation using a buffer more compatible with MS detection in order to prevent the interaction of nucleotides with capillary walls (97). Pressure-assisted CE-MS was used to analyze nucleotides, nicotinamide adenine dinucleotides, and CoA compounds simultaneously from biological samples, in this case *E. coli*.

Guanosine gels formed by guanosine 5′-monophosphate were used to separate single-stranded DNA of equal length but different sequence using capillary gel electrokinetic chromatography with LIF detection (98). The approach showed separations superior to CZE, MEKC, or a sieving gel in CGE when analyzing homodimers and homopentamers. Separations of 76-mers are also reported. The principle of separation is based on the strength of the interactions with the G-gel (i.e., T > C > A) and the presence of EOF toward the injection end; thus, oligomers interacting less with the gel are slowed down by EOF and detected last.

A capillary gel electrophoresis method for the analysis of G-quartet-forming nucleotides was designed (99). The 15-, 19-, 23-, and 27-mer oligonucleotides with a sequence of a thrombin-binding aptamer, which results in formation of the G-quartet, were analyzed in this method. Increased migration times observed using capillary gel electrophoresis were attributed to quadruplex formation. The longer oligonucleotides did not show a dependence of migration time on the chain length.

(3) DNA Sequencing and Mutation Analysis—Quasi-interpenetrating networks made of gold nanoparticles and linear PA were used to improve separation of single-stranded DNA, thereby providing a separating matrix that is promising for DNA sequencing (100). The network improves DNA sequencing because of the interactions between the

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nanoparticles and the polymer chains as well as the formation of physical cross-linking points. These networks are less viscous than gels without nanoparticles with similar separation power and offer advantages of high resolution, speediness, high efficiency, reproducibility, adequate shelf life, and facility of automation.

New sieving matrixes for separation of double-stranded DNA were also reported. Molecular imprinted polymers that recognize specific DNA sequences were used to separate double-stranded DNA (101). The feasibility of this approach was investigated with target DNA (Ha-ras gene) and nontarget DNA hosting single-point mutations.

CE was also used to analyze DNA fragments that resulted from photoinduced cleavage in the presence of metallo-intercalators (102). Rh(bpy) (2) (chrysi) (3+) and Rh(bpy) (2) (phzi) (3+) bind to mismatched DNA sites specifically and promote scission next to the mismatch. This is an attractive approach to the analysis of single-nucleotide polymorphisms.

A validated approach for high-throughput detection of DNA variants based on multiple capillary DNA sequencers was reported (103). The samples were prepared by mutiplex-PCR using fluorescently labeled primers and then mixed with reference sequences to produce DNA heteroduplexes. Then, the heteroduplexes were analyzed in the multicapillary DNA sequencer. The approach made it possible to scan variations in the BRCA1, BRCA2, MLH1, MSH2, and MSH6 genes.

(4) Quantification—A capillary electrophoretic analysis of RNA using fluorescently labeled riboprobes was reported (104). These probes, labeled with BODIPY TR, hybridize with target RNA resulting in an RNA–RNA duplex, which is more stable than other DNA–RNA complexes formed when using DNA probes. Separations of the free and complexed probe were done in capillaries filled with hydroxypropylmethylcellulose; detection was based on LIF using excitation from a 488-nm argon ion laser.

Abasic sites are general markers of DNA damage. Detection of abasic DNA, after removal of chemically damaged nucleobases, was accomplished by derivatization with a fluorescent aldehyde-reactive probe, isolation of DNA by ethanolic precipitation, and analysis by CE-LIF (105). It was confirmed that the fluorescence response is proportional to the amount of *N*-7 methylguanines resulting from DNA damage induced by a treatment with methyl methanesulfonate. The detection limit for the method was 1.2 abasic sites/1 million bases, which is better than the alternate ELISA method.

A procedure for quantification of methylation of cytosine in genomic DNA was reported (106). First, a treatment with bisulfite selectively deaminated cytosine (to yield thymine) but not 5-methylcytosine; second, the forward strands are amplified by PCR using a fluorescently labeled primer. Third, the resulting amplicons differ in molecular weight because of the differences in cytosine and thymine, and this factor leads to their separation in a capillary filled with POP 4 polymer. Last, LIF detection was used to quantify the signals resulting from the methylated and unmethylated DNA. The method was applied to the analysis of the hypermethylated region of the fragile-X-FMR1 locus in the human genome.

Detection of a specific DNA sequence was accomplished by affinity capillary electrophoresis (ACE) purification (107). A mixture of sample DNA and nanoparticles covered with target DNA were pressure injected into the capillary. An electric field was applied and the nonhybridized DNA migrated out from the capillary; subsequently, the temperature was raised to 60 °C to melt the captured DNA that then is electrophoretically displaced toward a LIF detector. The system was tested using sequences belonging to the wild type and mutant c-K-ras gene.

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Protein and Peptide Analysis

(1) Theory—New approaches to predict the electrophoretic mobility of peptides and proteins were reported. One report takes into account the size and average electrostatic surface (ζ potential), making it possible to take into account the relaxation effect (108). Low ionic strengths (e.g., ~35 mM monovalent salt) and peptides with absolute electrophoretic mobilities less than 0.2 $cm^2/(kV\ s)$ do not require size or relaxation effect corrections.

Another report considers a deformed sphere model for predicting the electrophoretic mobility of proteins that are globular in solution (e.g., lysozyme) (109). The hydrodynamic radius, the net charge, and the charge quadrupole are important parameters. The authors demonstrate that, when the contribution of the charge quarupole to the electrophoretic mobility is insignificant, the Henry equation can be used.

Another theoretical and experimental report used the unified theory of dynamic chromatography and dynamic electrophoresis to extract kinetic and thermodynamic parameters of conformational equilibria from capillary electrophoretic profiles (110). The conformational equilibrium of *β*2-microglobulin, which is involved in amyloid formation in dialysis-related amyloidosis, was used as a model in this study.

While sodium dodecyl sulfate is an integral part in many protein separation schemes, the dynamics of the interaction of sodium dodecyl sulfate with specific proteins are highly varied. One study reported the use of CE to catalogue the denaturation kinetics of proteins (111); for example, superoxide dismutase, bovine carbonic anhydrase, *β*-lactoglobulin B, *β*-lactalbumin, and ubiquitin showed very different denaturing behavior.

(2) Separations—One report described new poly(*N,N*-dimethylacrylamide) (PDMA) grafted polyacrylamide copolymers that are suitable as a replaceable separation medium in CE analysis of proteins (14). Both size-dependent retardation and supression of adsorption of basic proteins to the capillary walls were adequate for the analysis of native proteins.

Two-dimensional separations based on CSE-MEKC were also used to separately analyze the protein profiles of four subcellular fractions (112). Using this approach, the FQ-labeled proteins in cytosolic, membrane, nuclear, and cytoskeletal fractions from AtT-20 cells produced 231 resolved peaks (231), which is a 4-fold increase with respect to the analysis of the whole-cell homogenate.

New approaches to mapping digests of proteins by CE were also reported. As an example, 77 peaks from a bovine serum albumin (BSA) tryptic digest were reproducibly detected when the separations were done in a mixed micelle system composed of a zwitterionic surfactant, 3-(*N,N*-dimethylhexadecylammonium)propanesulfonate, and a nonionic surfactant, poly(ethylene glycol) dodecyl ether (Brij 35) (113); other separation systems (e.g., CZE or MEKC with SDS as surfactant) produced profiles with fewer number of detected peaks. Another study used a Pluronic F127 gel (e.g., 15% w/v, 20 °C) and phosphate buffer for the separation of small proteins and protein digests prepared with collagenase, trypsin, or CNBr treatments (114). The number of detected peaks improves in relation to separations done in Pluronic F127 or phosphate buffer alone. The separation principle appears to be based on both size and hydrophobicity of the peptides and proteins being separated.

To analyze peptides using capillary electrochromatography (CEC), a new investigation into using open-tubular CEC was shown to be useful over a broad pH range (115). The separation capillary was modified by etching the inner wall and chemically bonding octadecyl or cholesterol functionalities. A mixture of nine peptides was used for testing. By

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modulating the buffer components, such as pH and organic modifier content, the migrations of the peptides were easily modified. High separation efficiencies were seen up to 300 000 theoretical plates.

(3) Labeling and Detection—Labeling strategies for proteins and peptides prior to their analysis by CE were reported. Quantum dots conjugated to BSA and bare quantum dots were successfully separated by CE-LIF (116). Using a similar approach, IgGs against immunoglobulin M were labeled with quantum dots and used as fluorescent labels to selectively detect immunoglobulin M in a sample spiked with 10% human serum (117). After optimization of the CZE separation conditions, it was possible to observe a modest separation of the antibody–antigen complex from the free antibody.

Immunoassay–electrochemical detection of antibody–protein complexes after CE separation of these complexes was reported (118). Prostate specific antigen, carcinoembryonic antigen, or human chorionic gonadotropin were mixed with their respective antibodies; these antibodies are labeled with horseradish peroxidase that produce an electroactive species when they reach the electrochemical detector filled with a horseradish peroxidase substrate. The three antigens mentioned above were detected in the same run.

A fluorescent surfactant (F-16) that has a fluorescein head-group bound to a hexadodecyl alkyl chain was used to enhance the sensitivity of a LIF detection scheme of proteins that are separated in the presence of SDS (119). Proteins labeled with F-12 were size-based separated as cocomplexes of SDS–protein–F-12 in polyacrylamide-filled capillaries; limits of detection were 0.13 ng/mL BSA, dynamic range is 5 orders of magnitude, and fluorescence response is proportional to the square root of the protein concentration.

A report described an approach to selectively analyze glycopeptides separate from their nonglycosylated counterparts using online CE-ESI-MS (120). After the CE separation of glycosylated and nonglycosylated peptides, the eluting peptides were automatically spotted onto a MALDI target and their sequences analyzed by MS and MS/MS. A second study reported the analysis of intact glycoproteins and underivatized glycans by CE-MS (121). The CE separation of intact proteins was performed in capillaries coated with either Polybrene or UltraTrol. After the CE separation of the proteins (e.g., bovine fetuin and bovine R-1-acid glycoprotein), a TOF-MS instrument was used for exact mass determination of glycans while an ion trap MS instrument was used for glycan fragmentation. This analysis provides information about the type and composition of the glycans. The approach does not provide information about the sites at which the glycosylation occurs.

PCR amplification of a labeling aptamer was also the basis of sensitive protein detection (122). This approach was tested with fluorescently labeled aptamer and its binding partner, HIV-1 RTase. After CE separation of the free aptamer and the aptamer–protein complex, the latter was collected and subjected to PCR; as few as 180 molecules of HIV-1 RTase were detected.

Several proteins such as LDL apolipoprotein bind to molecules with thiol groups. When these molecules are not abundant, their detection is challenged by the sensitivity of the detection scheme and the coexistence of more abundant proteins. One report demonstrates by preconcentrating the preparation, optimizing the labeling conditions with 5-iodoacetamidofluorescein, and optimizing the CE separation of the released labeled thiol-containing molecules, that LDL apolipoprotein binds to low-abundance molecules such as glutathione (123).

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An approach to investigate protein profiles in mixtures is by imaging after cIEF. One report describes the optimization of a procedure to monitor the changes in glycosylation of the recombinant human type II interleukin-1 receptor found in Chinese hamster ovary cells (124); UV detection was used in this report.

Another system based on isoelectric focusing of proteins includes photochemical immobilization of the focused proteins to the walls of the capillary. After immobilization of the proteins, they may be detected based on their intrinsic fluorescence properties or on immunolabeling (125). The report concludes that fluorescence detection is better than chemiluminescence detection.

Lipids

Steroids were separated by MEKC in a buffer containing 20 mM sodium tetraborate, 20 mM SDS, and various combinations of organic solvents (e.g., ethanol, acetonitrile, tetrahydrofuran) (126). The abundance and composition of the organic modifier did not seem to affect the interactions between the steroids and the micelles, except for the modifier containing 10% ethanol and 1% tetrahydrofuran. At the optimal separation, which contained 20% ethanol, linear solvation energy relationships suggested that the major parameter affecting the retention and selectivity of the steroids is the total organic solvent percentage, with secondary effect from the hydrogen bond acidity. The optimized separation was also applied to the analysis of steroids in urine.

Another approach useful to separate steroid-related compounds consisted of two sequential micellar systems. This approach was used in the separation of androstenedione, testosterone, epitestosterone, and two synthetic derivatives (fluoxymesterone and methyltestosterone) in 10 min (127). The optimized first pseudostationary phase (close to the capillary entrance) was made of 1.5 mM sodium taurocholate and 29.5 mM SDS in 15.6 mM ammonium acetate (pH 9.5); the optimized second pseudostationary phase had 20 mg/mL sulfated γ-cylcodextrin ODMS, 29.5 mM SDS, and 14.6 mM ammonium acetate (pH 9.5). Urine samples spiked with analyte standards were successfully analyzed.

While MEKC may seem the obvious choice for separation of phosphatidyinositols, one report demonstrated a non-MEKC separation of PiP_2 and PIP_3 at room temperature in less than 15 min (128). The buffer was made of sodium deoxycholate, 1-propanol, the proprietary coating EOTROL LR, and Mg^{2+}; 1-propanol and Mg^{2+} were the key factors controlling selectivity in this separation system.

Another report described efforts toward the separation of phosphatidylcholine and its oxidation products (129). Optimized separation conditions were 10% sodium phosphate buffer (5 mM, pH 7.40), 80% methanol, and 10% acetonitrile. Phosphatidylcholine had one major peak and a small shoulder while oxidized phosphatidylcholine shows several low-mobility peaks.

Due to phospholipids' poor water solubility, typical aqueous CE buffers cannot be used. One solution to this problem is the use of nonaqueous CE (NACE). NACE has been used to analyze phospholipids from the rat peritoneal surface (130). Using MeOH/ACN (40:60 v/v) containing 20 mM ammonium acetate and 0.5% HOAc, a good separation of phospholipids was achieved in 16 min. The low surface tension and volatility of the nonaqueous medium also makes it easily coupled with mass spectrometry detection for collection of structural data.

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Carbohydrates

(1) Separation—Carbohydrate composition of the CH_2 domain of recombinant monoclonal antibodies (e.g., Rituximab) is highly relevant to the antibody's biological function. An approach to profile carbohydrates released initially from Rituximab with PNGase F, labeled with 2-aminobenzoic acid, and then CE separated in a buffer containing borate was reported (131); in some instances, the method also included digestion with mannosidase or sialidase after the initial digestion with PNGase F. The obtained CE profiles were highly reproducible, and the low limits of detection made it possible to detect secondary carbohydrates not usually observed by HPLC.

Electrophoretic separation of neutral carbohydrates requires structural modifications or complexation with a ligand. One reported approach to overcome this limitation was the conversion of mono- and disaccharide to ene–diolates at high pH (e.g., 12.6); these unsaturated products became detectable by UV absorption (e.g., 270 nm) and were electrophoretically separated at high pH (132). Twelve mono- and disaccharides in beverages were successfully analyzed. Another study reported that the use of a glycine–glycine buffer (50 mM, pH 12) improved the resolution of 16 mono- and disaccharides (133); since glycine absorbs at 207 nm, indirect detection of carbohydrates was used.

Sugar–phosphate analysis solely using MS detection is difficult since isomers have the same *m/z* ratio. CE-MS is capable of separating regioisomers and was applied in the analysis of an engineered *E. coli* (134); natural and unnatural sugar phosphates were screened from in vivo galactokinase bioconversions.

(2) Labeling—Some new developments in labeling carbohydrates appeared in the literature. In one report, *p*-nitroanaline was examined as a derivatizing agent for fluorescence detection when excitation occurs at~406-nm with an LED source (135). The derivatization allowed labeling of carbohydrates even at the femtomole level. In addition, an artificial neural network was used to optimize electrolyte conditions by predicting mobilities and response factors for the final separation.

A separate report, described the use of a microwave irradiation (385 W) to accelerate the speed of dansylation of aminosaccharides (136). Dansylated glucosamine, galactosamine, *N*-methylglucamine, *N*-acetylglucosamine, and aminoglucuronic acid were separated in borate buffer and detected by UV absorption.

Lipopolysaccharides (endotoxins) produced by bacteria do not absorb in the UV range, and their derivatization for detection is complicated. One report based lipopolysaccharide detection based on their high affinity for proteins such as hemoglobin that is easily detected by measuring absorbance at 415 nm (137). The lipopolysaccharide–hemoglobin complex and hemoglobin peaks were fully resolved in the reported CE separations.

Chondroitin sulfates are heteropolysaccharides consisting of highly sulfated disaccharide units (i.e., one or more sulfate group per disaccharide unit) low in plasma but of high relevance in biological function. Their low abundance in the plasma makes it necessary derivatize them prior to CE separation. One report described how chondroitin was enzymatic released, labeled with 2-aminoacridone, and separated by CE (138). The separation was faster (12 min) than previous reports and made it possible to detect both sulfated and nonsulfated saccharides from chondroitin sulfate found in human plasma.

Another detection scheme for CE analysis of carbohydrates was based on indirect chemiluminescence quenching (139). 4-Biphenylboronic acid had a dual role in this report because it enhanced the chemiluminescent response of the luminol hydrogen peroxide–

horseradish peroxidase system and served as a selectivity additive in the CE separation buffer.When a sugar–biphenylboric acid complex reached the chemiluminescent detector, the enhancing chemiluminescent effect decreased.

CE-LIF has been applied to the analysis of 9-aminopyrene-1,4,6-trisulfonate (APTS) derivatized monosaccharides and oligosaccharides derived from plant polysaccharides (140). CE-LIF and CE-UV-ESI-MS were used for the identification of APTS–xylooligosaccharides. To the author's knowledge, this is the first time these methods have been used on APTS–xylooligosaccharides.

Metabolites and Small Molecules

Due to the breadth of this subject, we surveyed only three salient areas: metabolites analyzed by CE-MS, chiral analysis of metabolites, and metabolomics.

(1) CE-MS Analysis of Metabolites—Since many metabolites of interest are polar ions, CE is a separation mode of choice in their analysis. New methods have been developed to observe multiple anions of interest including organic acids, sugar phosphates, and nucleotides. One such method used trimethylamine acetate as an electrolyte, positive polarity and pressure to allow the simultaneous analysis of 54 anionic metabolites with relatively high resolution, and the use of mass spectrometry detection (141). No analysis of such varied range of anionic metabolites with a single method had been reported to date.

Improvements in the analysis of low-abundance metabolites using coated capillaries and time-of-flight mass spectrometry were reported. In one study, tryptophan metabolites were monitored in cerebrospinal fluid and separated in a 1-(4-iodobutyl)4-aza-1-azoniabicyclo[2,2,2]octane iodide and monitored by TOF-MS (142). The coating prevented adsorption of components in the matrix, which degrade the separation, and the selection of mass analyzer made it possible to continuously monitor the eluent of the capillary, which is needed for very narrow peaks observed in CE separations.

(2) Chiral Metabolism—Frequently, the metabolic transformation of drugs gives rise to chiral metabolites. Enantiomeric separations capable of monitoring chiral metabolites were reported. Separation of cytalopram (antidepressant) and three chiral metabolites using carboxymethyl-*γ*-cyclodextrin as chiral agent and hydroxypropylmethylcellulose as selectivity agent were reported (143). In this study, the best separation conditions included 0.2% w/v of the chiral agent and 0.05% w/v of the sieving matrix; the method was amply validated and applied to urine samples.

The enantioseparation of risperidone (antipsychotic) and its hydroxylated metabolite was accomplished using a mixture of one neutral and one anionic cyclodextrin (144). Because of the complexity of the system, a chemometric optimization method was used and the sulfated α-cyclodextrin and the hydroxyproylated *β*-cyclodextrin were selected; the metabolic transformation of risperidone in CaCo-2 cells was successfully monitored.

Though CE-MS has been used for targeted metabolite studies, many fewer attempts have been made to collect global metabolite profiles in urine. In those studies that do collect global information, buffers with nonvolatile components were used. One method has been described assessing metabolite profiles in urine using low pH conditions (145). Positive and negative ionization modes were used in order to collect complementary information. While positive ionization showed better reproducibility, negative mode showed metabolites not seen in positive ionization.

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(3) Metabolomics and Metabolic Flux Analysis—CE-MS has also been used for qualitative and quantitative metabolomics. Only one report is described here, but a more comprehensive review of metabolomics analysis using CE-MS appeared elsewhere (146). In the metabolomic study reported here, the levels of 23 sulfur-related metabolites, when yeast is put under cadmium stress, were monitored (147). They observed depletion of glycine and strong accumulation of L-γ-glutamylcysteine, reduction in glutathione levels, and reduction in *O*-acetyl-L-homoserine levels. It is speculated that the availability of glycine may be rate limiting in the production of glutathione.

Advances in using CE-MS for metabolic flux analysis in continuous cultures were reported. One advancement examined glycolysis and the tricarboxylic acid cycle by monitoring C^{13} mass distribution of intracellular metabolites or proteinogenic amino acids (148). If the pool size of the intermediate metabolites is kept small, there is an immediate turnover of metabolites.

Bioparticles

Bioparticles such as cells, organelles, microorganisms, and viruses have negative electrophoretic mobilities at biological pH and can be analyzed by CE. While there are multiple demonstrations of the feasibility of using CE for the analysis of bioparticles, the various factors contributing to their electrophoretic mobilities have not been fully described. In one study, it was confirmed that the electrophoretic mobility of liposomes is affected by the pH gradient across their membranes, which is a common situation in subcellular organelles such as mitochondria and lysosomes (149). Liposomes with inner and outer pHs of 6.8 and 7.4, respectively, showed the most negative electrophoretic mobility; liposomes with higher or lower internal pH displayed less negative electrophoretic mobility. A capacitance effect and the flip-flop theory of phospholipids are possible explanations for the electrophoretic behavior of liposomes with acidic and basic inner pH's, respectively.

Another study described the effects of electric field intensity on liposomal morphology (150). Electric fields between 40 and 330 V/cm were applied to liposomes (200 nm–10 μm), and then the migrating liposomes were quickly frozen and subsequently imaged with SEM. Different morphologies including stretched nanotubules of several micrometers to long-range organized assemblies of liposomes over tens of micrometers were observed.

Individual isolated organelles were analyzed by CE-LIF, which provides both fluorophore content and electrophoretic mobility measurements (151). After endocytosis of a pH ratiometric probe (i.e., dextran labeled with fluorescein and tetramethylrhodamine) by human leukemia cells, a subcellular fraction enriched in acidic organelles was analyzed with a CE system equipped with a dual LIF detector, which made it possible to conduct the ratiometric measurement in acidic organelles. Lower pH values were determined in the acidic organelles of drug-resistant cell lines. Another report described the absolute quantitation of mitochondrial DNA (mtDNA) copy number in individual mitochondrial particles (152). Mitochondria isolated from cells expressing mito-DsRed2 (i.e., mitochondria label) were treated with a DNA intercalator (i.e., PicoGreen) and then analyzed by CE with a dual LIF detector. The codetection of DsRed2 and Picogreen fluorescence was used to identify mitochondria and determine their DNA contents, respectively. Contrary to current theories, detection down to a single mtDNA molecule per mitochondrion suggests that some mitochondria host only one copy of mtDNA.

CE has been used to investigate viral infections. The first event during viral infection is attachment of the viral particle to specific receptors at the host cell membrane. A system for mimicking this process was reported (153). Artificially prepared liposomes with synthetically attached receptor for HRV2 virus were used for modeling the cell membrane.

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Biospecific interaction of HRV2 virus was confirmed by monitoring the change of electrophoretic mobility of the liposome peak. In another study, segregation of viral particles into subviral particles, a process that occurs during viral infection, was mimicked by CE (154). CE separation of subviral particles released from intact human rhinovirus upon treatment with D-dodecylpoly(ethylene glycol ether) was reported; this is a promising approach to investigate viral dynamics.

Fast, selective, and accurate identification of microorganisms is a topic of current relevance. An elegant CE method for examination of bacterial contamination based on bacteria stacking inside a capillary using the spacer method was presented (155). This technique utilized a cationic surfactant buffer to sweep organisms out of the sample zone and a small plug of blocking agent that eliminated cells' electrophoretic mobilities and induced aggregation. Various microbe entities in concentrations down to a single cell were detected using UV–visible or LIF detection in less than 10 min. Another approach described the use of cIEF for separation and detection of microorganisms (156). The buffer contained poly(ethylene glycol)–4-(1-pyrene)butanoate, which simultaneously served as a dynamic capillary coating for reducing microbe–wall interactions and as a fluorescent reporter. Mixed cultures of 11 different microorganisms were focused and separated using this technique. Subsequently, the same group used the same approach to analyze plant pathogens directly from plant tissue suspensions (157).

Single-Cell Analysis

Analysis of a single cell's content is a fundamental approach for understanding cellular functions, to analyze rare cell populations and to investigate the origins of heterogeneity among cells. CE is an excellent tool for single-cell analysis because of its nanoliter sample consumption, high-efficiency separations, and combination with very sensitive detectors. However, technical developments in cell lysis to release and reliably analyze cellular contents are needed. One promising cell lysis approach is the use of pulsed laser to cause cell disruption pulse, while the cell is outside the capillary, followed by immediately injection of the cell contents into the capillary. Using this approach, the effects of laser power and focal position on lysis and sampling efficiency of adherent GFP-HEK 293T cells were investigated (158). Maximum sampling efficiency was found using a 3-ns, 2-μJ laser pulse positioned directly under the cell. Another approach for cell disruption utilized a coaxial buffer exchange system at the capillary inlet that delivered a sheath flow of electrophoretic buffer (cell lysing agent) at the moment of turning on the separation electric field (159). To perform a cell injection and lysis, the capillary tip was lowered until it was ~10 μm above the cell, and the sheath-flow and electric voltage were turned on simultaneously, exposing the cell to both the electrophoretic buffer and the electric field, thereby lysing the cell; other cells were in the same preparation were not affected by the lysis of the selected cell.

Studies reporting cell lysis within the separation capillary also appeared in the literature. A report described a process of mixing the injected cell with lysing surfactant (Triton X-100) using the transverse diffusion of laminar flow profiles (160). Due to the laminar flow in the capillary, nondiffused plugs injected into the capillary by pressure had parabolic profiles tailing along the walls; Thus, a cell injected in a subsequent plugs would be mixed with the surfactant mainly by transverse diffusion rather then longitudinal diffusion; this reduces the amount of surfactant needed to lyse the cell, which also reduces the possibility of artifacts associated with the use of some surfactants with CZE separations.

Previously established single-cell analysis techniques (aka chemical cytometry) were used in several reports. In one of these reports, sphingolipid metabolism in single pituitary tumor cells was investigated (161). The substrate GM1, fluorescently labeled with

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tetramethylrhodamine, was taken and metabolized by a pituitary tumor cells. After incubation, a single cell was injected into separation capillary, lysed upon contact with the surfactant (Triton X-100) in the CE separation buffer (borate, deoxycholate, methyl-β-cyclodextrin), and the released metabolites were separated and detected by LIF. Subzeptomole levels of 10 sphingolipid metabolites were measured. In other report, protein profile was investigated in single mouse embryo (162). By using 2D CSE-MEKC-LIF, ~380 protein spots were resolved, 100 with S/N higher grater than 10. Another reported chemical cytometry approach was based on immunolabeling (163). Anti-IFN-γ monoclonal antibody was introduced into natural killer cells using electroporation. After incubation, single cells were injected into the capillary, lysed, and different forms of IFN-γ were electrophoretically separated. Compared to on-column derivatization, the separation efficiency and resolution increased 4- and 2-fold, respectively.

Besides analyzing fully dissolved single-cell contents, other reports used chemical cytometry to analyze organelles released from single cells. In one report, a procedure for analysis of individual mitochondria released from a 143B osteosarcoma single cell was reported (164). Cells expressing DsRed2 targeted to mitochondria were introduced within the separation capillary where exposure to digitonin and trypsin lead to disruption of the plasma membrane and mitochondrial release; the number of mitochondria, their fluorescence intensities, and electrophoretic properties were reported. Another report utilized the same procedure to analyze doxorubicin, an anticancer agent, sequestered in individual acidic organelles of single CCRF-CEM cells (165).

Affinity and Interactions

(1) Theory—The mass-transfer equation was used to describe the electrophoretic migration of species during ACE regimes (166). In this and other related reports, the authors demonstrate that this equation is suitable for the prediction of ACE experiments using normal affinity capillary electrophoresis, frontal analysis, the Hummel--Dreyer method, and vacancy CE techniques.

In ACE, kinetic properties of affinity interactions are analyzed by injecting one binding analyte as a short plug into the buffers containing binding additive in different concentrations. The resulting affinity binding constants are affected by the sample plug length and by the interactions with capillary walls. A computer simulation model for studying these contributions to the systematic errors in ACE was developed (167). Separation profiles and binding isotherms in the presence and absence of wall adsorption were predicted using this model.

A chemometric approach based on a Box–Behnken design was used to test the relevance of three factors (i.e., injection time, capillary length, and applied voltage) on the optimization of an affinity capillary electrophoresis separation (168). The statistical analysis results were used to create a model, describing surface plots of prediction around a target K_d (e.g., 1.19 μM, which corresponds to a complex of carbonic anhydrase B and 4-carboxybenzesulfonamide). The predictions conditions were successfully tested experimentally.

(2) Enantiomeric Separations—Cyclodextrin continues being a favorite chiral selector in enantiomeric separations. However, recently isolated microbial cyclic β-(1→3),(1→6) glycans are highly promising chiral agents (169). These compounds made it possible to separate, with high resolution, enantiomers of some flavanones such as eriodictyol, homoeriodictyol, hesperetin, naringenin, and isosakuranetin.

A combination of two chiral components in microemulsion electrokinetic chromatography was investigated (170). By using an *S*-dodecoxycarbonylvaline with the cosurfactant *S*-2-

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hexanol, an increased resolution of 3.7 was achieved in the separation of pseudoephedrine enantiomers.

A chiral form of microemulsion electrokinetic chromatography based on the incorporation of chiral oils (*S*)(+)-2-octanol or (*R*)-(−)-2-octanol in the microemulsion droplets was reported (171). Change in the chiral oil reversed the order of enantiomer migration of (±)-norephedrine enantiomers.

A monoclonal antibody against polycyclic aromatic hydrocarbons was used as selector in the electrophoretic separations of (±)-cis- and (±)-trans-benzo[*a*]pyrene tetrols (172). The partial filling technique was used; an antibody plug was introduced first followed by the racemic mixture of tetrols; the separation occurred in 50 mM sodium phosphate, pH 8.8 buffer.

An L-RNA aptamer, which is the mirror image of the D-RNA aptamer was used as a chiral additive to separate L- and D-arginine using the partial filling technique (173). High temperatures (50–60 °C) were needed to resolve the two isomers; at low temperatures, the transfer kinetics of the aptamer impedes suitable separations of the arginine isomers.

(3) DNA Interactions—CE has also been applied to the study of DNA-targeted drugs. One study investigated single-stranded and double-stranded DNAs binding with drugs such as netropsin and distamycin (174). Considerations, such as adding a soluble polymer, were described and assessed. Using the data collected, estimation of K_d values was attempted. Another investigation studied actinomycin D (ActD) interaction with oligonucleotides using microchip electrophoresis with ESI-MS detection (175). It was indicated that ActD/DNA has binding stoichiometry of 1:1 with single-stranded DNA and 1:2 with double-stranded DNA; K_d indicates stronger binding with single-stranded DNA.

Interactions of double-stranded DNA with Hg(II), MeHg(I), EtHg(I), and PhHg(I) were investigated by CE with online electrothermal atomic absorption spectrometric detection (79). The mercury–DNA complexes were clearly separated from the free mercury species; the organomercuric species exhibit the strongest affinity for DNA, which represents more potential damage caused by these species than that caused by Hg(II).

(4) Interactions with Peptides or Proteins—ACE with LIF detection was used to screen β-amyloid aggregation, an important factor in Alzheimer's disease (176). Two different fibrils (precursors and aggregates) were separated in less than 5 min, and the ratio of the fluorescence intensity resulting from their labeling with thioflavine T was used for estimating the degree of aggregation. The effects of Aβ inhibitors (i.e., daunomycin, 3-indolepro-pionic acid, melatonin) on Aβ aggregation was investigated.

A promising approach to screen for various peptide–peptide interactions is multiple injection affinity capillary electrophoresis. In one study, glycopeptide antibiotics vancomycin, teicoplanin, and ristocetin were used as ligand models and a D-Ala-D-Ala terminus peptide was used as a receptor model (177). Five sequential injections were made of the receptor and the mixture electrophoresed simultaneously with buffer containing varying concentrations of the ligands. The data were used in Scatchard plots to estimate binding constants for the ligands. Another approach to simultaneously investigate several protein–protein interactions was based on the use of multiple capillary instruments (178). A labeled protein at a fixed concentration was premixed with different concentrations of the interacting proteins; each mixture was analyzed in separate capillaries. The interactions of (i) labeled conalbumin with succinylated ovalbumin, and (ii) labeled trypsin with anti-insulin monoclonal antibodies were investigated.

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Peptide–protein interactions were also investigated in a CE separation that used a gated-injection system and a fluorescence polarization detector (179). Three proteins with SH2 domains formed complexes with fluorescently labeled phosphopeptides and were detected separately from the unbound peptides; the short capillaries made it possible to conduct a separation in 6 s, allowing for the detection of rapidly dissociating complexes. The approach was used to determine the IC_{50} of various inhibitors.

Whole capillary imaging was also to used to monitor the isoelectric focusing of proteins interacting with DNA (180). Monitoring temporal changes made it possible to identify protein–DNA complexes and DNA separately and monitor the kinetics of dissociation with time, similar to those experiments done by a technique called nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM). This approach appears suitable to monitor the fate of several equilibria when the systems are moved to nonequilibrium.

A recent report suggested selection of aptamers with specific kinetic parameters, which could result in identifying aptamers leading to more suitable separations (181). This principle has been demonstrated by selecting aptamers against the MutS protein. The technique NECEEM was used for this purpose.

Protein–phospholipid interactions were investigated by observing changes in the isoelectric focusing of the proteins as a function of time; whole column imaging detection was used for visualization (182). Protein models included trypsin inhibitor, β-lactoglobulin B, phosphorylase b, and trypsinogen; both phosphatidylcholine and phosphatidylserine were used as model phospholipids; it was observed that interactions are protein and phospholipid specific and may be related to conformational changes in the protein upon lipid binding. Other reports not covered here have demonstrated that parameters that may affect such interactions, such as calcium binding and thermal stability, could also be investigated.

Interactions between organometallic compounds and transport proteins can also be determined with ACE. A report describes the calculation of binding constants of five platinum complexes and human serum albumin (HSA) using normal ACE (183); the calculated binding constants suggest a moderate interaction ($\sim 10^3 - 10^4$ M^{-1}) under simulated physiological conditions (pH 7.4, 100 mM chloride, 37 °C). Another study used the partial filling technique to determine the binding constants between three neutral endogenous steroid hormones or two synthetic analogues with human serum albumin or BSA. Methyltestosterone and testosterone had the strongest binding constants; BSA was a stronger ligand than HSA (184).

Hyphenation of CE to ESI-MS was used in order to evaluate protein/carbohydrate complexes. CE in the frontal analysis format was used to determine characteristics of antithrombin/heparin complexes (185). The mass determination led to the determination of the binding stoichiometry. It was seen that one antithrombin was associated with one heparin pentasaccharide molecule.

Functional Assays

(1) Off-Column Assays—CE methods to monitor and investigate enzymatic activity, metabolism, production of reactive oxygen species, and cellular function were reported.

A CE-LIF assay to measure the interconversion of sphingosine and sphingosine 1-phosphate caused by purified kinase and phosphatase activities was reported (186). Besides determining the K_m and V_{max} for in vitro assays, the fact that the fluorescent substrate is taken up by mammalian cells, made it possible to use this method to monitor the kinase activity in living cells.

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GTPase activity is important in cell signaling and can be used to monitor compounds that modulate G protein-coupled receptors. CE was used to monitor this activity, which results in the transformation of BODIPY FL GTP (substrate) to BODIPY FL GDP (product) (187). The approach was used to determine the EC_{50} of the antagonist yohimbine and UK14,304. Another salient example monitored adenylyl cyclase activity associated with a G-protein coupled receptor (β2 adrenergic receptor) in isolated plasma membranes (188). ATP labeled with BODIPY FL was transformed by this enzyme into BODIPY FL-labeled ADP, cAMP, and AMP. Separation of these products from the substrate made it possible to establish simple assays to determine EC_{50} values when using agonists and antagonists.

A CE assay capable of monitoring glycosphingolipid transformations in cell culture extracts at the yoctomole level was reported (189). The catabolism of the tetramethylrhodamine glycosphingolipid G(M1) produced seven metabolites that were successfully separated and detected at the yoctomole level; at these levels, the reproducibility of the detection method approaches the limits of molecular shot noise.

Enzymatic activities were also monitored by derivatizing the products (and substrates) after the enzymatic reaction. The chiral separation of *o*-phthaldialdehyde derivatized L-serine and D-serine was used to monitor the activity of racemase, an important enzyme in the production of D-serine in the brain (190). The separation system used 2-hydroxypropyl-γ-˜yclodextrin as a chiral agent; the method was used as an enzymatic assay by monitoring the inhibition of racemase by L-erythro-3-hydroxyaspartate.

CE-MS has been used in the discovery of enzymatic activity of previously uncharacterized proteins by analysis of metabolic changes (191). In the study, purified proteins were incubated with a pool of metabolite present in yeast extract. The transformations in this pool of metabolites was used to discover enzymatic activities such as the phosphatase/phosphotransferase activity of two *E. coli* proteins, YbhA and YbiV.

Simultaneous enantiomeric metabolism is important to investigate the chiral selectivity of biological systems. An optimized chiral CE separation was used to characterize the chirality of the metabolic transformation of verapamil into its metabolite norverapamil by cytochrome P450 3A4 isozyme (192); this enzymatic system does not appear to display a dramatic difference in enantioselectivity. This is in contrast with other metabolic transformations (e.g., doxorubicin to doxorubicinol), which appear to favor the formation of one of the two stereoisomers (193).

Functional assays also monitoring the production of reactive oxygen species have been shown. Superoxide produced by mitochondria during respiration may be released to both sides of the mitochondrial inner membrane. The membrane-permeable hydroethidine was combined with isolated respiring mitochondria and reaction with superoxide produced 2-hydroxyethidium; cationic MEKC-LIF was used to separate the superoxide specific product from ethidium, which is not specific (194). This approach also made it possible to monitor the enhanced release of superoxide by antimycin A and menadione. The release of nitric oxide, an important neurotransmitter, was monitored by CE-LIF (195). Because ascorbic acid is an interferent of the reactions of the probe 4,5-diaminofluorescein (DAF-2) with NO, ascorbate oxidase was used to transform ascorbic acid into dehydroascorbic acid; CE separation was needed to separate any interfering product of dehydroascorbic acid with DAF-2 from the DAF-2 triazole, which is the desired product from the NO reaction. This approach made possible the analysis of NO released from single neurons.

(2) On-Column Assays—On-column functional assays included those immobilizing enzymes in the entire or in part of the capillary or unbound enzymes in solution. In one

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assay not using immobilization, alkaline phosphatase activity based on electrophoretic-mediated microanalysis and electrochemical detection was monitored using disodium phenol phosphate as the substrate (196). The electrochemical detector design and better selection of the enzymatic reaction temperature made it possible to detect the activity of yoctomole levels of alkaline phosphatase.

In one assay, the authors electrostatically immobilized glucose oxidase over the entire inner wall of the capillary using mixtures of poly(diallyldimethylammonium chloride) and anionic poly(styrenesulfonate) (197). The latter was needed to maintain an excess of negative charges on walls and the electrosmotic flow in the capillary. Upon injection of a plug of glucose, in the presence of an electric field, glucose oxidase transforms glucose into glucuronic acid and hydrogen peroxide that are then carried toward an amperometric detector in which hydrogen peroxide is detected. The generality of this approach was demonstrated by building a similar reactor for glutamate oxidase.

Other reports immobilized the enzyme just at the entrance of the capillary. For example, an enzymatic reactor was made by electrostatically trapping acetylcholinesterase between two layers of poly(diallyldimethylammonium chloride) that span a short length at the entrance of the capillary (198). Upon injection and incubation of acetylcholine within the reactor length, an electric field is applied to separate the enzymatic products. This system was validated with known inhibitors of acetylcholinesterase and was used to test enzymatic inhibition by a small library of 42 compounds. Another report that also electrostatically trapped an enzyme at the entrance of the capillary used angiotensin-converting enzyme (ACE) (199). In this system, the polycationic electrolyte hexadimethrine bromide was used as immobilization agent. This system was also validated by means of ACE inhibitors added to the substrate during incubation.

A third type of enzymatic reactor places the reactor between two lengths of the capillary. An enzymatic reactor made of a small length of capillary wall, supporting an immobilized enzyme, was combined with a two-pass UV-active pixel detector (200). In this system, the substrates and potential degradation products are monitored prior to reaching the reactor by the detector; upon passage through the reactor, the products of the enzymatic reaction, along with the remaining substrates, continue being electrophoretically separated until they reach the detector again. Comparison of the substrates and products before and after the enzymatic reaction made it possible to identify putative substrates. The system was tested with penicillin G as a substrate and penicillinase as an enzyme.

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