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Bioanalytical Applications of Capillary Electrophoresis

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Review Contents

This review focuses on the period from January 2004 to December 2005. Nearly 5000 papers and 500 reviews on capillary electrophoresis are listed in *SciFinder* for this period. Clearly, it is not possible to produce a comprehensive review of this massive literature. As in the last biannual review (*1*), we restrict ourselves to 200 references. Also, as in the last review, we focus on the use of capillary electrophoresis for the analysis of biopolymers: proteins and peptides, oligonucleotides, carbohydrates, and lipids. To this list we add sections on the analysis of single cells and applications in neurochemistry. Even with these restrictions, we have had to select representative publications; much important work was not included.

PROTEINS, PEPTIDES, AND POLYAMINES

Sample Preparation. Chemical analysis can be broken down into the steps of sampling, sample preparation, separation and detection, and data processing. We do not address the issue of sampling in this review; nevertheless, it is vital that any sample taken for analysis is representative of the whole. This issue becomes critical when dealing with single-cell analysis, where enough cells must be analyzed to replicate the characteristics of the larger tissue from which the cells are taken.

Sample Preconcentration. Because of the minute sample volumes and masses used in capillary electrophoresis, concentration detection limits are often relatively poor. However, by using preconcentration techniques, improved concentration limits of detection and separation properties can be realized for biological analytes. Some of the most commonly used preconcentration techniques include chromatographic, immunoaffinity, electric field-enhanced sample injection, large-volume sample stacking with polarity switching, pH junction concentration, micellar electrokinetic capillary chromatography (MECC) sweeping, and isotachophoresis (ITP).

Electric field-enhanced sample injection is used as a preconcentration method and is based on the principle that analytes migrate with greater velocity in higher electric fields. Where the conductivity is lower, the electric field experienced by the analyte is greater and therefore its velocity is greater. By contrast, an analyte's velocity is lower where the solution conductivity is greater. Thus, if the sample matrix has lower conductivity than the separation buffer, then analytes will migrate rapidly to the boundary between the two buffers, at which point their velocities abruptly decrease. The result is analyte concentration at the boundary. Monton et al. demonstrated a greater than 3000-fold signal enhancement using this method with protein analytes (*2*).

ITP is another ionic boundary preconcentration method utilized with proteins. With ITP, large sample volumes can be injected and are subsequently focused in an electric field. By appropriately selecting a leading buffer, which has a velocity greater than the analytes and a trailing buffer, which has a velocity lower than the analytes, the analyte zone width can be narrowed in an applied electric field. This method is frequently employed prior to capillary zone electrophoresis (CZE) separation. However, when compared with other preconcentration methods, ITP requires more careful optimization of buffer characteristics. Stutz et al. described the use of CITP and CZE in their characterization of metal-binding proteins with UV and MS detection. They observed limits of detection on the order of 50−160 fmol (*3*).

In a discontinuous buffer system, where the pHs of the sample and the separation buffer differ, a pH junction develops. Within this junction, analytes are focused into narrow zones according to their isoelectric points (pIs). In recent work, Nesbitt et al. achieved over 1000-fold protein preconcentration using a pH junction (*4*). In a similar study by Monton et al., band narrowing was induced through the use of a dynamic pH junction (*5*). With this technique, peptide detection limits improved by a factor of 124.

Wu and co-workers utilized a cellulose acetate-coated porous joint for protein preconcentration (*6*). A segment of capillary near its inlet was etched, causing the wall to become thin and porous to small buffer ions. An electric field was applied between the

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injection end and this porous joint. In the presence of an electric field, protein analytes migrated to the joint and accumulated there. After concentration, a voltage was then applied across both ends of the capillary to drive separation. Preconcentration factors for this method were on the order of 65−800, depending upon the analyte.

In other work, Wang et al. tailored the electroosmotic flow (EOF) velocity through electric field manipulation to selectively isolate and concentrate a given set of analytes with comparable electrophoretic mobilities (*7*). A greater than 150-fold increase in signal was observed using this technique. Liang et al. demonstrated on-line sample concentration in pressurized capillary electrochromatography (*8*). Because a stationary phase is used in this mode, band broadening is also affected by analyte interactions with the stationary phase. These interactions diminish the band narrowing achieved with field-enhanced stacking. However, Liang and co-workers demonstrated that the use of a more strongly eluting mobile phase could lead to band narrowing. A 100-fold improvement in detection limit for three standard proteins was achieved.

Separations. Last year was the 30th anniversary of O'Farrell's paper describing two-dimensional electrophoresis for protein separations. In that technology, proteins are first separated on an isoelectric focusing gel, typically in a thin strip. The strip is placed on a large plate coated with polyacrylamide in an SDS buffer. Proteins are then separated by molecular weight and visualized by staining, creating a two-dimensional map of protein expression. This technology is extremely powerful, producing spot capacity exceeding 10 000. However, the technology is very cumbersome and has resisted most attempts at automation.

Capillary electrophoresis (CE) provides an attractive alternative to conventional gel electrophoresis for protein analysis. As was demonstrated with astonishing success in the human genome project, capillary electrophoresis is very easy to automate, certainly easer than either conventional slab gel electrophoresis or microfluidic devices. Capillary electrophoresis provides fast and efficient separations and produces very impressive mass detection limits, which is important when small amounts of protein must be studied.

A number of CE modes are available for separation of proteins and polypeptides. These modes include CZE, MECC, capillary sieving electrophoresis (CSE), capillary isoelectric focusing (CIEF), and capillary electrochromatography (CEC).

(a) Capillary Zone Electrophoresis. The simplest mode of separation in capillary electrophoresis is CZE, where analytes are separated based on their size-to-charge ratio. Polyamines and catecholamines in PC-12 tumor cells were analyzed simultaneously using CZE-laser-induced fluorescence detection (LIF) (*9*). Modifying labeling reactions to incorporate a fluorescent porphyrin into myoglobin in place of its nonfluorescent heme group resulted in 50 nM limits of detection in CZE-LIF (*10*).

Zone electrophoresis is particularly useful for the separation of peptides, such as those prepared from tryptic digests. There has been interest in the development and incorporation of solid-phase immobilized reactors for on-line digestion before electrophoresis analysis. Migneault reported the use of glutaraldehyde to immobilize trypsin (*11*). We reported the use of a monolithic column with immobilized trypsin as a reactor to digest proteins before separation by free zone electrophoresis (*12*). Dulay reported enhanced enzymatic activity of immobilized enzyme in a photoployerized sol gel (*13*).

Although free zone electrophoresis is a rapid and efficient separation method for lower molecular weight compounds, it is less useful for proteins. Analyte adsorption onto the capillary wall leads to instabilities in the electroosmotic flow, sample loss, and peak talking. Adjusting the buffer pH to highly acidic or basic values gives the capillary walls and the analyte the same charge, which results in electrostatic repulsion and minimizes adsorption. More often, however, surfactants and wall coatings are used to reduce analyte adsorption.

(b) Micellar Electrokinetic Capillary Chromatography and Submicellar Electrophoresis. Surfactants are used to modify the mobility of proteins and other analytes and to minimize adsorption of compounds to capillary walls. If the concentration of the micelle exceeds the critical micelle concentration (cmc), the micelles can act as a pseudostationary phase, in which case the separation is called micellar electrokinetic capillary chromatography. At surfactant concentrations below the cmc, the surfactant acts as a complexing agent, interacting with both charged and hydrophobic regions of proteins, in which case the separation is called submicellar electrophoresis. Free surfactant molecules are always present in the separation buffer in MECC, and there usually is not a dramatic change in the separation of proteins as the buffer concentration is increased above the cmc.

There is a large literature on the use of MECC and submicellar electrophoresis for medical and biological analysis. As an example, nucleotidase and transferase activities of cytosolic 5′-nucleotidase III have been investigated using MECC methods, in particular (*14*). Using human erythrocyte lysates obtained from a healthy population of volunteers, the study showed excellent reproducibility and reliability, and the assay has been employed to monitor red cell enzyme disorders associated with hemolytic anemia.

Typically, MECC is performed on unmodified or uncoated fused silica. However, an MECC method employing a dynamically coated capillary was used for the separation of the isomerization and hydrolysis product of acyl glucuronides (*15*). Coated as well as uncoated capillaries were investigated using MECC for the analysis of the polydispersed fluorosurfactant FC134, which can be used in CE runs to reduce wall adsorption of proteins (*16*). Fractions were collected after the migration of certain components and analyzed with matrix-assisted laser desorption/ionization (MALDI)-MS to determine how this surfactant interacts with different buffer systems and analytes.

An MECC approach with LIF has been developed for the determination of phosphoamino acids in different hydrolyzed protein samples (*17*). The phosphoamino acids were labeled with 5-(4,6-dichloro-*s*-triazin-2-ylamino)fluorescein, and detection limits of 0.5 nM were reported. LIF and MS detection coupled with MECC have also led to some new and interesting developments in meat authentication (*18*). Bovine spongiform encephalopathy has become a recent crisis for the meat industry, resulting in a loss of consumer confidence. Implementing CE to all-meat derived products has increased reliability and traceability to meat authenticity issues.

(c) Capillary Sieving Electrophoresis. CSE is the capillary version of traditional gel electrophoresis (SDS−PAGE). A polymer

is dissolved in solution to create a molecular sieve, also known as an expendable physical gel. CSE separates analytes by size as they migrate through the polymer matrix under near-zero EOF conditions. To reduce EOF, the capillary is coated with either a static or a dynamic coating. This technique is commonly employed in SDS−gel molecular weight analysis of proteins and in sizing applications in DNA sequencing and genotyping.

Purified cider proteins were tested with CSE using a linear polyacrylamide as the sieving matrix (*19*). Four different sample treatments (ethanol precipitation, dialysis, ultrafiltration, gel filtration) were compared; ultrafiltration proved to be the best sample cleanup method before CSE analysis. These results were confirmed with SDS−PAGE analysis aligning the molecular weight bands with the respective peaks in the electropherograms. Tissue proteins from human squamous cell lung carcinomas (SQCLC) and small cell lung carcinomas (SCLC) were separated in 0.1% hydroxypropylmethyl cellulose linear polymer sieving solutions (*20*). Molecular classifications of SQCLC and SCLC were obtained from comparisons of the CSE patterns.

(d) Capillary Isoelectric Focusing. CIEF separates amphoteric molecules, such as proteins or peptides, by electrophoresis in a pH gradient generated between the cathode and anode. A solute will focus at a point where its net charge is zero. After analyte are focused, the zone is usually mobilized past an end-column detector by either hydraulic pressure or chemical means. Alternatively, whole-column imaging can be employed to simultaneously monitor all analytes during the separation.

Carrier ampholyte-free IEF was demonstrated using a single capillary with an etched porous wall and an inlet end vial with protein sample. A pH step was established at the interface of the fronts of H^+ and OH^- so that proteins could electromigrate to the boundary from the sample vial. Over a 100-fold concentration factor was reported for bovine serum albumin (BSA), lsozyme, and ribonuclease A (*21*).

By coupling CIEF with other proteomic methods, analysis of complex samples can be improved. Crowley has demonstrated the off-line coupling of CIEF with MALDI-MS in the analysis of human blood serum. This technique has allowed for quicker analysis and ease of automation in comparison to traditional 2-DE. The volume of the sample used in the analysis was reported to be ~100 nL, demonstrating the sensitivity of this technique (*22*). CIEF has also been coupled with electrospray ionization (ESI)-MS in the separation of *Escherichia coli* whole-cell lysate (*23*). CIEF-ESI-MS, however, often presents problems with ampholyte interference and signal quenching. The use of reversed-phase liquid chromatography (RPLC) between CIEF and MS has been shown to remove this interference for separation of proteins. Protein detection at the low-femtomole level was demonstrated using CIEF−RPLC−MS (*24*).

CIEF with liquid-core waveguide LIF whole-column imaging detection has been demonstrated for the separation of naturally fluorescent phycobiliproteins (*25*). This system has also been used to study the conformational and chemical microheterogeneity of characterized proteins with identical pI values through denaturing CIEF.

CIEF has also been demonstrated using a sampling capillary as a bypass fixed to the separation capillary, providing an established pH gradient from the sample capillary for sample solutions (*26*). The separation of ampholytic compounds with isoelectric points beyond the pH gradient was demonstrated using this system and termed as pH gradient-driven electrophoresis.

While CIEF separations are usually performed with neutral coated fused-silica capillaries in aqueous anticonvective media, a 30:70 v/v glycerol−water medium has been demonstrated as an alternative medium in stabilizing proteins without imposing increasing viscosity (*27*). Better resolution was achieved with this system than a conventional aqueous CIEF system, separating two model proteins that could not be separated by conventional CIEF.

(e) Capillary Electrochromatography. CEC is a hybrid technique combining electrokinetic pumping and stationary-phase retention capacity of high-performance liquid chromatography. Columns for CEC typically have larger inner diameter than those used for CE methods, which more easily facilitates MS detection schemes. The CEC column can be packed with an alkylsilica stationary phase that may be modified for a specific or selective retention. Under reversed-phase conditions, an uncoated capillary has a relatively high EOF due the charged silanol surface and silica particles. Bulk flow of solvent moves analyte through the stationary phase, producing high separation efficiencies. Frits are commonly used to prevent loss of the packed stationary phase, but often result in bubble formation and decreased EOF. In other cases, the capillary surface or packing material is chemically modified to reduce absorption effects and stabilize EOF. Monoliths and monomers, such as cationic alkylaminosilane, are covalently bound to all or part of the capillary (*28*, *29*). Because the coating is chemically bonded to the capillary, exit frits are not required. Proteins and peptide are separated at neutral pH by RP-CEC using a neutral monolith (*30*). The nonpolar monolith generates a strong EOF and is void of electrostatic interactions with charged analytes. Separation of proteins standards gave a peak capacity of ~100.

The most common packing materials are silica−octadecyl silica (ODS). CEC separations on ODS packed capillaries were able to resolve all 20 phenyl thiohydantoin amino acids in less than 15 min (*31*). Histamine and methylhistamine were derivatized on-line and separated by CEC on and ODS packed capillary (*32*).

CEC is used to monitor levels of caffeine during in vivo microdialysis of the rat brain (*33*). Columns are packed with NAIP silyl silica gel immobilized with organic dyes or aromatic dicarboxylic anhydrides. Buffer pH and additive concentration were optimized to resolve caffeine and its two metabolites in less than 4 min. RSD in component migration was less than 1% for both within-day and day-to-day experiments.

Cationic alkylaminosilyl monomers were used to modify capillaries for CE and CEC of neuropeptides and tryptic digests (*29*). TAC, TeDAC, and ODAC monomers were covalently bound or absorbed to the silanol surface to produce a reversed EOF below pH 9. Dynamic absorption of the monomers was found to provide higher resolution and separation efficiency than static coating procedures. An ODAC-treated capillary was used to separate a tryptic digest of BSA in under 5 min. No interfering peaks from monomer desorption are seen in the MS profile, suggesting these coatings are suitable for on-line CE−MS experiments.

(f) Multidimensional Separations. Despite the exquisite resolving power of CE separation methods, no one-dimensional separation technique to date can resolve proteins found in tissue

and cellular homogenates, which may contain over 10 000 components. Instead, much recent effort has been given to the development of multidimensional separation platforms where electrophoretic and/or chromatographic methods are hyphenated for the high-resolution separation of proteins and peptides. Where two orthogonal separation modes are interfaced, the multidimensional spot capacity is equal to the product of each dimension's peak capacity. Both on-line and off-line separation systems are currently in use, and the general trend is toward more automated, higher resolution, and higher throughput separation systems.

The majority of multidimensional CE applications have utilized a chromatographic separation mode coupled with an electrophoretic separation mode. One reason for this is that the chromatographic and electrophoretic separation mechanisms are almost completely orthogonal, and therefore, high spot capacities can be achieved in multidimensional systems.

Janini et al. described the use of an LC−CE system using RPLC and CZE (*34*). In this study, highly abundant proteins from a serum sample were first removed using methanol precipitation, after which proteins were digested to peptides with trypsin and then separated by RPLC. Fractions were collected off-line and loaded sequentially onto a capillary for CZE separation. Detection of peptides was accomplished using tandem mode mass spectrometry (MS/MS). Using these methods, 130 unique proteins were identified from a serum sample.

Jia and co-workers also described a two-dimensional separation system using LC, CZE, and MECC (*35*, *36*). However, these experiments utilized on-line fraction transfer from the first to the second dimension as opposed to the off-line fraction collection used by Janini. Capillary liquid chromatography with a monolithic silica-octadecyl silica column was used in two separate reports from Jia et al., to investigate the separation of metabolites from *Bacillus subtilis* and *E. coli* cellular homogenates. For these studies, CZE was employed for early-eluting analytes and sweeping MECC was utilized for late-eluting analytes. Two interfacing strategies were used, field-enhanced stacking and a dynamic pH junction. Both of these interfacing systems also have the advantage of concentrating analytes at transfer. UV absorbance was utilized in these studies for metabolite detection.

Zhang and co-workers also described the use of a comprehensive LC−CE system for the separation of trypsin-digested proteins from liver cancer tissues (*37*). For this study, RPLC was used for the first-dimension separation, and CZE was utilized for the second-dimension separation. A valve-free hydrodynamic sampling interface was employed for analyte transfer from the first to the second dimension. The eluent stream from the first dimension was regularly sampled for separation in the second dimension. Fractions were collected for subsequent analysis by MALDI-TOF-TOF-MS. Using these methods, over 300 proteins from the D_{20} tissue were identified.

While a large proportion of the studies done using LC−CE separations employ CZE, other studies have utilized CIEF because of its exceptional resolution and ability to concentrate peptide and protein analytes into narrowly focused zones. In one such study, Wang et al. performed automated and integrated two-dimensional separations using CIEF and nano-RPLC (*38*). Proteome analysis was performed using microdissected glioblastoma tumor samples. Proteins from the tissue sample were digested with trypsin to yield peptides, which were then separated in the first dimension by CIEF. Peptides in the first dimension were loaded into either 14 or 28 trap columns, after which they were separated in the second-dimension nano-RPLC column. Analytes were detected using ESI tandem MS analysis. Using only 10 μg of microdissected tumor tissue (~20 000 cells), Wang and co-workers were able to identify 1820 proteins. This low-level sample consumption is advantageous for clinical applications because small tissue samples are obtained less invasively than larger samples. In another similar report, Wang and co-workers described the use of this instrumentation to study yeast protein lysates (*39*). However, in this study, intact proteins were characterized and 534 proteins were identified.

Zhou and Johnston described a similar separation system (*24*). In this study, standard proteins were first separated by CIEF. The cathodic cell of the CIEF setup was fitted with a microdialysis membrane, which enabled fractions to be collected, washed for ampholyte removal, and transferred to the RPLC column for the second-dimension separation. ESI-MS was used for protein detection. With this instrumentation, seven CIEF fractions could be analyzed by RPLC over a 2-h period, and low-femtomole-level detection limits were demonstrated. While CIEF has exceptional resolving power, for each of the CIEF separation systems described here, the CIEF dimension was divided into 28 or fewer fractions. In doing so, the resolution of the first dimension was diminished.

Other studies employing multidimensional capillary electrophoretic separation of proteins and peptides have utilized CE modes in both dimensions. Liu et al. describe a CIEF-CZE separation system for use with protein samples, in which the two dimensions were coupled using an on-column etched fused-silica porous junction (*40*). Liu and co-workers also reported another study using a CIEF-CSE separation platform (*41*). This system is analogous to the separation mechanisms used in two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS−PAGE), a powerful gel-based separation method used for protein characterization. Proteins excreted from rat lung cancer cells were investigated in this study, and UV absorbance was used for detection.

We have described a two-dimensional CE instrument for the automated and comprehensive separation of 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ)-labeled proteins from cellular samples using LIF detection (*42*, *43*). In this system, proteins were separated in the first dimension according to their molecular weights by CSE in an SDS−pullulan polymer buffer system, and in the second dimension by MECC. The two capillaries were coupled at a simple gap junction, in which the capillaries are aligned in a buffer-filled interface. In one application, we studied the single-cell expression from native MC3T3-E1 osteoprogenitor cells, MC3T3-E1 cells transfected with the human transcription regulator TWIST and MCF-7 breast cancer cells before and after treatment with an apoptosis-inducing treatment (*42*). In another application, we studied protein expression in cellular homogenates of the bacteria *Deinococcus radiodurans* (*43*).

Guzman described the use of immunoaffinity capillary electrophoresis (IACE) in multidimensional CE separations where select analytes were first bound by immobilized antibodies in a small zone at the injection end of a separation capillary (*44*). Analytes were then desorbed through the use of an elution buffer

and separated in the remaining length of the capillary. The IACE system can be hyphenated with other CE or LC modes for multidimensional separation.

Fused-Silica Coatings for CE of Biomolecules. Biomolecules contain charged functional groups that can interact with the negatively charged silanol groups of the capillary wall (*45*). Absorption can lead to sample loss, peak broadening, poor resolution, unstable EOF, and long migration times. To reduce protein absorption and stabilize the EOF, capillaries are commonly coated prior to the electrophoretic separation.

Coatings are typically classified as either static or dynamic. Static coatings are covalent silanol modifications or physical coating of the wall with cross-linked polymers. Preparation of static coatings is time-consuming. Covalently coated capillaries are available commercially but are relatively expensive. Other drawbacks of static coatings include degradation of the coating due to pH sensitivity and batch-to-batch variations in coating efficiencies. As pointed out by Lee over 20 years ago, surface tension tends to produce droplets of derivatizing reagents when applied to the tightly curved surface of narrow inner diameter capillaries (*46*).

A recent report demonstrates improvements to established linear polyacrylamide (LPA) coating methodology by incorporating cross-linked polyacrylamide (CPA) (*47*). Bridged cross-linkers help to cover pockets or gaps left uncoated by the linear polyacrylamide. CZE and CEIF separations of standards proteins on CPA-coated capillaries were reproducible after 80 and 240 runs, respectively. The CPA-coated capillaries showed a significantly higher resistance to alkaline buffers as compared to LPA-coated capillaries. Classic silane chemistry was used to covalently coat capillary walls with poly(dimethylacrylamide) for separations of DNA and proteins by CGE and CZE (*48*).

Dynamic coatings are applied by filling the capillary with high concentrations of a coating reagent. After relatively brief exposure times, excess reagent is rinsed from the capillary, leaving an adsorbed layer of reagent. Dynamic coatings usually suffer from limited stability, but the modified surface can be stripped and replenished as needed. In some cases, the dynamic coating can simply be added to the running buffer at lower concentrations, and the coating is continuously replenished during electrophoresis. A host of proprietary dynamic coating reagents has become available.

A family of commercial dynamic coating reagents is used for a number of applications (*49*, *50*). EOTrol and UltraTrol have been used to separate protein isoforms using CZE. EOTrol coatings are added to the running buffers at low concentrations (0.15% w/v) to replenish the absorbed coating. UltraTrol coatings are applied prior to the separation and are not incorporated in to the buffer, facilitating CE−MS studies.

Another commercial dynamic coating is used to separate a large group of seized drugs by CE using β-cyclodextrins (*51*). The polymeric cationic dynamic coating CElixir was optimized for a high and stable EOF at pH <7. Using short capillaries, rapid and reproducible results were obtained by screening racemic mixtures of phenethylamines and propoxyphene.

A noncovalent bilayer coating was developed for CE at medium pH. Capillary treatment is accomplished in two simple rinses of Polybrene and poly(vinyl sulfonate) solutions (*52*). Integrity of the coatings was measured by repeated injections of terbutaline as an EOF marker. The PB−PVS coating was found to perform as well as other commercially available coatings when standard proteins were separated.

Several reports discuss the use of dynamic coatings for CE−ESI-MS analysis (*53*−*55*). A robust and high EOF is desirable for stable electrospray. A polymer, PolyE-323, is reported to be quickly absorbed to the capillary wall. Capillaries demonstrated a constant anodal EOF at pH 4−8. Reproducible migration (RSD < 1.5%) for four basic proteins by CZE is shown after successive regeneration of the PolyE-323-coated capillary. The PolyE-323 coating's tolerance toward biological matrixes was investigated during CE−ESI-MS of human plasma and CSF. While only major proteins were effectively separated and detected, the polymer coating was stable and did not bleed into the mass spectrometer.

A series of ionene polymers was used as a dynamic capillary coating for analysis of urinary and recombinant human erythropoietin (rhEPO) by CE and CE−ESI-MS (*16*). Synthetic ionenes are polycationic polymers that strongly absorb to the capillary surface. Ionenes of different molecular weight and charge density are compared while separating rhEPO isoforms near pH 5.5. Under optimized conditions, a 6,6 ionene-coated capillary was used to separate and detect rhEPO and uEPO by CE−ESI-MS. However, large sample injections on the capillary are necessary for reliable MS detection.

Double-chained surfactants are used as semipermanent dynamic coatings for CE analysis of cationic and anionic proteins (*56*−*59*). Bilayer structures are generated using didodecylmethylammonium bromide and cetyltrimethylammonium bromide, which aggregate on the capillary surface. As with most dynamic coatings, degradation of the coatings is seen over time. In an effort to improve the stability of these coatings, a solution of phospholipid vesicles was polymerized ex situ (*57*). The oligomerized phospholipid bilayers were then rinsed through the capillary to generate a coating. Capillary performance was monitored while separating mixtures of basic proteins at low ionic strength and neutral pH. RSD in migration times of around 1% run to run and 3% day to day. Stability of the coatings was improved with increased reagent hydrophobicity (chain length), increased buffer pH, and other factors that help to decrease the cmc of the dynamic surfactant (*58*). Coating stability was monitored by measuring the EOF over time and at various buffer pH and ionic strength. Migration times varied less than 2.3% with no loss in efficiency over 60 successive runs without regeneration of the coating (*55*).

An investigation on the influence of pH for the formation and stability of liposome coatings was reported in a series of papers from Riekkola's laboratory (*60*−*63*). Phosphatidylcholine and phosphatidylserine formation of unilaminar vesicles is dependent on the pH of the liposome solution. HEPES was chosen as the solvent for liposome coating. Protonation of phospholipids and HEPES amines increases the electrostatic attachments of phospholipids to the capillary wall. Evidence of liposome desorption was seen in the presence of more basic pH. Addition of positively charged diamines such as 1,2-ethylenediamine and 1,3-diaminopropane was found to increase the liposome coating stability (*64*). Other additives and buffers had negative effects on the coatings and separation efficiencies. The liposome coatings and additives were used to improve resolution of a mixture of five steroids at pH 4.5.

Detection. Detection of proteins and peptides can be implemented by UV absorbance, fluorescence, mass spectrometry, and other methods. In addition, the use of sample concentration was covered in the first page of this review.

(a) UV Absorbance Detection. UV detection of proteins and peptides is usually based on absorbance of the aromatic amino acid residues tryptophan, tyrosine, and phenylalanine. In CZE, UV generally produces micromolar or submicromolar concentration detection limits for proteins and peptides. This limit becomes even worse in SDS-DALT CE due to the use of UV-absorptive polymers. However, UV demonstrates lower concentration detection limits in CIEF due to the high injection volume and on-column concentration resulting from IEF (*48, 53, 65*). A UV detection system utilizes a liquid-core waveguide as a very long path length UV window, resulting in limits of detection to the order of nanomolar (*52*). In addition, CIEF is carried out in a Teflon capillary creating efficient separation and superb detection limits. The LCW is also capable of performing fluorescence detection for pM detection limits.

(b) Fluorescence. Detection of native protein fluorescence depends on the presence of aromatic amino acids and can be subject to interference from the sample matrix (*66*). Improved sensitivity is achieved by fluorescent derivatization of proteins. Fluorescent labeling strategies fall into three categories: covalent and noncovalent labeling with small-molecule dyes and genetic manipulation of an organism to produce autofluorescent proteins. Labels are available with a range of optical characteristics, target specificities, and reaction chemistries. Many of these labels have been restricted in use to the separation of standard proteins; this review will focus mainly on applications involving complex biological samples.

Covalent derivatization of amine groups with a small-molecule dye remains the most common strategy for fluorescent labeling of biomolecules in complex mixtures. The fluorogenic reagent FQ provides zeptomole detection limits for proteins and has been applied to several biological samples, including the analysis of trace proteins in milk (*18*). Unfortunately, multiple labeling products and a change in the charge state of the protein following reaction make quantitation difficult, reduce resolution, and prevent the application of these dyes to IEF separation. To improve separation resolution for the analysis of cancer cell proteins and peptide digests, we reported postcolumn labeling with naphthalene-2,3-dicarboxaldehyde (*12, 67*). The Wolfbeis group developed novel pyrilium-derived amine-reactive dyes that maintain the charge state of the native protein and provide nanomolar detection limits (*65*). The consequences of limited dye solubility in aqueous buffer and the interference of detergents in the labeling reaction remain to be determined for complex mixture analysis.

Noncovalent labels have also been applied to protein separations in CE, though few have been reported for complex samples. Noncovalent labels do not generate multiply labeled products and are relatively simple and inexpensive. Unfortunately, the sensitivity of these labels to detergents limits their application in many proteomic separations. Also, these compounds tend to be highly fluorescent in the presence of surfactants, such as SDS. However, the NanoOrange dye has been successfully applied for a collegenase activity assay, tolerating up to 0.05% SDS (*68*). Specific protein detection can also be carried out using noncovalent immunoassays with fluorescently tagged antibodies; prions in sheep blood samples have been detected at the nanogram per milliliter level using a fluorescein isothiocyanate tagged protein A immunocomplex (*69*). In this case, the instability of immunocomplexes limits the mode of separation to rapid zone separations.

The most specific fluorescent tagging method is genetic manipulation of an organism to generate a fusion between the protein of interest and an autofluorescent protein. Fusions with the green fluorescent protein (GFP) family have been used to monitor cell division (*70*), to assay calcium binding proteins with subattomole limits of detection (*71*), and to monitor phosphorylation of kinase proteins in vivo (*72*). Autofluorescent protein vectors are available with a range of optical characteristics; their specificity and the exquisite sensitivity of fusion protein analysis make this system very useful for targeted protein analysis. The generation of a stable expression system is not possible for all proteins of interest, however, and can have detrimental metabolic consequences.

(c) Refractive Index Detection. Bornhop's group continued its work on the development and application of backscatter interferometry for high-sensitivity refractive index measurement. They reported a dual-column detector that automatically compensates for temperature drift, resulting in detection limits in the 10^{-9} ΔRI range (*73*). This technology was used to monitor molecular interactions without the need for labeling (*74*). The technique has also been used to measure flow velocity by heating a solution upstream from the detector and measuring the time for the refractive index perturbation to reach the refractive index detector (*75*).

(d) Mass Spectrometric Detection. CE and mass spectrometry are most commonly coupled via ESI, with the two main types of CE−MS interfaces being sheathless and sheath flow interfaces. Nilsson et al. analyzed the effects of sheath liquid flow rate, nebulizer gas flow rate, electrospray voltage, and electrospray needle position in a detailed chemometric study (*76*).

Unlike in LC−MS, an electrical connection has to be made at the spray tip for CE operation in CE−MS. With sheath flow CE−MS interfaces, the connection is established via the sheath liquid, whereas it is more difficult to establish at the capillary outlet in sheathless designs. Many different variants of sheathless interfaces have thus been developed. For example, the connection is often made at a junction between the separation capillary and an emitter tip. Viberg et al. devised such a sheathless CE−MS interface in which they coated a tube sleeve with epoxy and graphite and then inserted a nanoESI fused-silica emitter into the sleeve (*77*). The emitter was butted against the separation capillary outlet via a zero dead-volume union. An alligator clip was attached to the tube sleeve, and electrical contact with the CE buffer was made via the graphite coating at the liquid junction inside the union. The coating was stable for more than 80 h, and only the tubing had to be exchanged if necessary, not the emitter tip. The electrical connection for sheathless interfaces can also be made by coating the capillary tip with a conductive material. Trapp and co-workers developed a simple procedure for coating tapered capillary ends first with silver and then with gold (*78*). The capillaries were used for sheathless CE-Hadamard transform-time-of-flight (HT-TOF)-MS analyses of standard peptide mixtures, and the coating was stable for 600 h. Both Viberg and Trapp applied

a makeup pressure to the capillary inlet to obtain a stable electrospray during CE−MS operation because the EOF was not sufficient. Establishing direct electrical contact at the capillary outlet is not necessarily essential, however. For instance, Wu and Chen showed that standard proteins and peptides could be analyzed using a pulled fused-silica capillary whose tip had not been modified to accommodate an applied voltage (*79*). Instead, voltages were only applied at the CE inlet buffer reservoir and at the mass spectrometer inlet.

Recent reports on sheath flow interfaces mainly focus on decreasing the sheath flow rate to diminish sample dilution. Tseng and Chen developed beveled emitter tips that were beveled on one side or on two sides (*80*, *81*). The tip orifices had relatively large, e.g., 75 μm, inner diameters, but because of the beveled shape, lower flow rates could be used than with unbeveled tips, and smaller liquid droplets were produced with the beveled tips, which aided sensitivity. The large i.d.s also alleviated tip clogging compared with smaller i.d., flat-tipped emitters. We have reported a low-flow sheath flow CE−MS interface in which a capillary was inserted into a pulled fused-silica capillary (*82*). The outside of the inserted end of the capillary was coated with gold to enable a stable electrospray. The orifice of the pulled capillary was only 30 μm in diameter, so that sheath flow rates below 1 μL could be used, which allowed improved sensitivities than with a conventional ion spray interface. A similar sheath flow interface was developed that used a tapered stainless steel emitter, which was part of commercial MS instrumentation, into which a CE separation capillary was inserted (*83*). The stainless steel emitter provided robustness, and capillaries could be easily and quickly exchanged within minutes if necessary.

Because sheath flow interfaces are generally more robust than sheathless interfaces, the former are more commonly used in applications of CE−MS to real biological samples. There has been a series of papers that uses sheath flow CE-ESI-TOF-MS to establish polypeptide patterns of urine, serum, or cerebrospinal fluids based on m/z versus migration time (and intensity) in three-dimensional plots (*84*−*87*). Differential polypeptide patterns with more than 1000 polypeptides from cerebrospinal fluid or urine were observed for healthy persons versus persons with, for instance, Alzheimer's disease, schizophrenia (*85*), and various chronic kidney diseases, and different stages of kidney damage could also be discerned (*84*). Selected polypeptides could be identified by off-line CE−MALDI-MS/MS for which the CE effluent was spotted onto MALDI target plates before MALDI-MS/MS analysis. The low signal intensities of some polypeptides made their identification impossible. In a later report, identification of potential biomarkers was achieved using preparative CE to collect fractions for subsequent FT-ICR-MS/MS analysis (*87*).

Simó et al. applied sheath flow CE−MS for the study of phycobiliproteins from *Spirulina platensis* extracts (*88*). Simó et al. also studied the enzymatic cleavage specificities of recombinant and natural bovine pepsin A using sheath flow CE-ESI-ion trap-MS (*55*).

CE−MS has also been applied to study protein posttranslational modifications. Bonneil et al. used a sheath flow interface and a hybrid quadrupole/time-of-flight mass spectrometer to analyze the sequence homology between lectin proteins and to study their glycosylations (*89*). Demelbauer et al. established preliminary carbohydrate structures of different glycoprotein (antithrombin) isoforms using sheath flow CE−ion trap-MS of the whole proteins, and Neusüss et al. used sheath flow CE−TOF-MS to study intact glycosylated recombinant human erythropoietin proteins (*90*, *91*).

Moini and Huang used sheathless CE-ESI-ion trap-MS to detect potential methylation and acetylation modifications of intact proteins from fractions of *E. coli* lysates (*92*). Samples containing as little as ~3.4 ng of ribosomal proteins were analyzed. A sheathless CE−ESI-MS interface was used in a proof-of-principle study by Sassi et al. to detect and discern proteins and peptides that were spiked into human serum samples at concentration ratios ranging from 2 to 20 (*93*). Transient isotachophoresis was used for on-line preconcentration, and the lowest concentration of spiked analyte was 10 nM angiotensin I. A pattern recognition/feature selection algorithm that included using a training data set was able to establish which m/z values of the spiked analytes would yield distinguishing markers of the two groups. The algorithm assigned samples to the two groups with 95% accuracy.

Inductively coupled plasma-MS (ICPMS) has also been employed as a detector for CE separations. Timerbaev and co-workers used sheath flow CZE-ESI-ICPMS to study the binding kinetics of several platinum coordination compounds to human serum albumin (*94*). The platinum complexes are, or have potential as, anticancer metallodrugs, and a better understanding of their interactions with blood proteins might shed light on their efficacy as drugs.

CZE is the simplest form of CE and has therefore been coupled to MS most commonly, but other modes of CE have been used as well. Storms et al. reported sheath flow CIEF-ion trap-MS for analyzing standard and *E. coli* protein digests (*95*, *96*). A low concentration (0.20%) of carrier ampholytes was used to avoid excessive analyte ion suppression and contamination of the MS yet still obtain reliable CIEF results. Protein pI information from the CIEF separation facilitated the protein identification with SEQUEST. Liang et al. used pressurized CEC-ESI-ion trap-MS to analyze tryptic digests of cytochrome *c* and a mixture of peptides (*97*).

An alternative to ESI is MALDI-TOF. MALDI-TOF-MS affords high selectivity, mass accuracy, and sensitivity, and its mass spectra are often more easily interpreted than ESI spectra because most analyte ions become only singly charged in MALDI-MS ionization (*98*). CE has been coupled to MALDI-TOF-MS either on- or off-line. For off-line analysis, the effluent from the CE capillary is commonly spotted onto a MALDI target plate, or it is collected in small vials. One of the advantages of off-line MALDI analysis is that one CE fraction can be interrogated multiple times, whereas in CE-ESI-MS, the whole experiment has to be repeated to obtain information that might have been missed during an earlier run. MALDI also tolerates sample contaminants slightly better than ESI (*99*).

Zhang et al. used a two-dimensional RPLC−CE system in conjunction with MALDI-TOF-TOF-MS/MS (*37*). The CE effluent plus a matrix sheath fluid were spotted onto MALDI target plates by an in-house-built CE−MALDI interface that allowed 20 spots/min to be deposited, whereas a commercial spotting interface had a frequency of 12 spots/min. A total of 388 proteins of a tryptic

digest of a D_{20} mouse liver cancer tissue protein extract were identified.

Off-line CE-MALDI-TOF-MS/MS analysis of standard proteins and proteins in tear fluid was performed by Zuberovic et al. (*98*). CE effluent, along with a sheath fluid, was spotted onto MALDI plates onto which matrix had previously been applied. Proteins were identified based on their molecular weight and based on MALDI-TOF-MS/MS data of their tryptic digests and database searching. Protein digestion was done in individual spots directly on the MALDI target plate.

A report on on-line CE-MALDI-TOF-MS has also been published that used a rotating ball as a deposition surface for the capillary effluent (*100*). The ball was made of stainless steel to provide the cathode for the CE separations and a voltage for MS operation. Three capillaries deposited liquid onto the ball; one was a CE separation capillary from which the peptide sample migrated, the second capillary provided a makeup buffer to aid in maintaining electrical contact between the separation capillary and the ball, and the third provided the MALDI matrix. The ball was pressed against a Teflon gasket that was placed in an opening to the vacuum chamber of the mass spectrometer. Liquid deposition occurred at atmospheric pressure, and the rotation of the ball delivered the quickly dried sample into the vacuum region. The ball was cleaned by a solvent-saturated felt piece that made contact with the ball where the ball rotated out of the vacuum region. Three standard peptides were fully resolved with this on-line CE-MALDI system.

Because samples can be very complex, multidimensional separation systems have been coupled with MS detection, and these have been mentioned in the 2D section of this review. An on-line enzymatic microreactor-CE-MS system has been reported by Kato et al., which enables reduced sample handling and increased analysis speed compared with analyses using bulk solution digestion (*101*). They prepared a methacryloxypropyltrimethoxysilane monolith inside a capillary and then coated the monolith surface with a sol−gel that contained the enzyme pepsin. Only the inlet end of the capillary was modified with the monolith, and the rest of the capillary was used for separation of the digests of lysozyme and insulin. This system was on-line connected to ESI-MS. Pepsin was used because it is active at low pHs. Low-pH buffers, in turn, enhance analyte protonation in the ESI process.

CHEMICAL CYTOMETRY: SINGLE-CELL ANALYSIS BY CAPILLARY ELECTROPHORESIS

With a greater demand for technology to probe deeper into the biological process, instrumentation built to handle small sample volumes is being developed. Capillary electrophoresis, with its exquisite detection limits and resolving power, is a useful technique for single cell-analysis, especially when coupled to LIF detection.

CE-LIF of single cells requires method development for labeling the cell either on-column or in tissue culture. For example, FQ is a fluorogenic reagent used for on-column labeling, while fluorescein isothiocyanate can be injected into cells in tissue culture (*42, 102, 103*). To assist researchers in adopting on-column FQ labeling, a complete methodology has been compiled detailing procedures for labeling and then separating proteins by capillary electrophoresis with special emphasis on single-cell analysis (*103*).

Manipulating single cells is a challenging technique that has inspired the introduction of several sampling techniques. Nanopipettors, levitating, optical tweezers, and laser micropipets were all used to introduce a single cell to the capillary, as were electrokinetic and electroosmotic injections. Injection methods have been reviewed for single-cell CE (*104*−*106*).

CE-LIF with an on-column microreactor was used to study different forms of human interferon-γ (*107*). CE-LIF was also used in the analysis of amino acids in single wheat embryonic protoplasts, where a single derivatized protoplast was lysed on column and amino acids were detected and compared to lysate (*102*). In another study, subcellular compartments of ΔH2-1 cells intact nuclei were probed by on-column treatment with digitonin to separate the plasma membrane-bound farnesylated enhanced green fluorescent protein from the nuclear-targeted protein nuDsRed2 (*108*). Two distinct spectral regions were probed. These data were initially probed using fluorescence microscopy, but CE-LIF appears to be a more sensitive general method for nuclear localization studies (*109*).

Multiple sampling is an advantage of using single-cell analysis. A novel approach to replicate studies has been introduced for the Sf9 cell line (*104*). Micromanipulators isolated individual cells, where they were lysed with a solution containing substrate and then nanopipettors were used to inject only 5 nL of sample, so enzyme activity could be monitored in triplicate or over time by CE-LIF. Enzyme activities were also studied in single 4T1 and NIH3T3 cells to detect catalyzed reactions products farnesyltransferase, endoprotease, and methyltransferase, which are potential therapeutic targets (*110*). Laser scanning confocal microscopy was used to monitor cell uptake of the substrate, and then CE-LIF was performed. Reaction products were compared to the conversion of the substrate in solution and were found to differ. 4T1 cells were also probed in a study noting the differences in GFP expression patterns for sister cells using two-channel chemical cytometry (*70*).

RT-PCR has been used for detection and semiquantitative determination of GFAP mRNA in mouse brain; capillary electrophoresis was much more sensitive and rapid than agarose gel electrophoresis (*111*). RT-PCR typically requires a second round of nested PCR; however, a recent qualitative study with CE-LIF of single MCF-7 cells and their multiplex products from RT-PCR required only one round (*112*). Zabzdyr and Lillard monitored estrogen receptor α- and β-actin with fragment sizes between 318 and 838 bp using a sieving matrix for a size-based separation. CE-LIF combined with SC-RT-PCR was also used to monitor gene expression in tiny plant tissues with detection limits of 0.04 ng/mL for LEAFY (*113*).

Different detection methods have also been utilized. Amperometric detection was used with etched electrodes and capillaries to inject cytoplasmic samples from intact single mammalian cells of the PC12 cell line using electroporation (*114*). Dopamine in rat pheochromocytoma cells was also detected at the 240 μM level using this technique. Amperometric detection at a carbon fiber microdisk bundle electrode with constant potential was used to detect the reaction product of a single fibroblast cell of mouse bone marrow exposed to disodium phosphate as enzyme substrate in the running buffer for 30 min on-column prior to CE (*115*). Nitrate and nitrite concentrations were observed using CZE with

a Crystal-1000 conductivity detector in submicroliter samples of mammalian neuronal tissues and large individual cells of invertebrates (*116*). Detection limits were observed at 8.9 and 3.54 nM for nitrite and nitrate, respectively. Other detection schemes for single-cell CE have been reviewed (*117*).

We have an interest in single-cell analysis for fingerprinting cell line protein expression through 2D CE-LIF. Single MC3T3-E1 osteoprogenitor cells, MC3T3-E1 transfected cells with the human transcription regulator TWIST, and MCF-7 cells before and after induced apoptosis were sampled to create protein expression patterns (*42*). A first dimension of CSE in a pullulan buffer system was performed coupled to a second-dimension separation by MECC. A prerun brought the sample to the interface of the two dimensions. Then, over 100 successive transfers were performed and analyzed in the second dimension over a 3.5-h-long period.

There has also been some interest in the analysis of single cells by the microfluidic community. Wu reported the analysis of amino acids from single Jurkat T cells (*118*).

NEUROSCIENCE

The application of capillary electrophoresis in neurochemistry and neurobiology has continued to evolve in recent years, providing improved molecular, physiological, and pharmacological understanding of complex brain chemistries. The ultimate goal of neuroanalytical chemistry is to provide better models of neurotransmission as well as more accurate chemical, spatial, and temporal information available in the brain. Different modes of CE coupled to various detection techniques have been successfully applied to analyze biogenic amines, amino acids, and neuropeptides from tissue samples, extracellular fluids, and the content of single neuron or neuron-like cells. An excellent review summarizing the application of CE to neuroscience has been published recently (*119*).

Extracellular Fluid Analysis. The nervous system is surrounded by extracellular fluid where many important cellular processes such as chemical communication and nutrient and metabolite transport between nerve and glial cells take place. The qualitative and quantitative characterization of amino acids, biogenic amines, and peptides present in this fluid could be linked to the neuronal functionality. The most commonly used sampling techniques for extracellular fluid analysis are microdialysis, low-flow push−pull perfusion, and direct sampling. In microdialysis-based measurements, the temporal resolution is limited by the mass detection limit of the assay used. Due to the requirement of a large volume of samples, when coupled with high-performance liquid chromatography, microdialysis samples need to be collected for at least 5 min and therefore offer poor temporal resolution. Capillary electrophoresis is able to analyze nanoliters of sample with low limits of detection and allows temporal resolution to be improved to better than 10 s.

The Kennedy group has reported the first on-line microdialysis sampling coupled with CE-LIF to investigate amino acids in freely moving rats (*120*). Fifteen amino acids were separated in 30 s and were identified based on migration times and coinjection of standards using MECC from dialysate samples of rat nucleus accumbuns at basal levels. This method was applied to monitor amino acids during ethanol injection in freely moving rats. Taurine, a neuroactive compound, showed a 2-fold increase over basal levels within 5 min of ethanol injection. In a similar report, Parrot et al. demonstrated monitoring the extracellular concentrations of five neurotransmitters in freely moving rats using microdialysis and CE-LIF (*121*). Using rats with genetic absence epilepsy, they showed that GABA concentration increased concomitantly with the seizures.

A CE-LIF method has been reported for monitoring GABA, glutamate, and L-aspartate in human spinal dialysates obtained during preoperative microdialysis from patients undergoing surgery against chronic, pharmacoresistant pain (*122*). When a 1-min sampling rate was used, an enhancement in GABA level was detected in the microdialysate following an electrical stimulation, which was not detectable when a 5-min sampling rate was used. This observation highlights the importance of high temporal resolution in tracking rapid chemical changes.

Several research groups have investigated the changes in amino acid neurotransmitters in microdialysates as a result of drug delivery. Using in vivo microdialysis sampling, the concentration of vigabatrin, an antiepileptic drug, and amino acid transmitters in microdialysates from the rat striatum were monitored and rapid vigabatrin-induced changes in GABA and glutamate levels were detected (*123*). The effect of the extracellular concentration of ascorbate, a water-soluble vitamin, on glutamate transmission in rats was also studied (*124*). Ascorbate elevated striatal glutamate in a concentration-dependent fashion for both basal level and electrically evoked glutamate response. The Bowser group has reported the first direct sampling of multiple neurotransmitters from perfused, isolated larval salamander retinas using on-line microdialysis and CE-LIF (*125*). They successfully demonstrated hard-to-achieve enantioseparation of D- and L-serine. An increase in the efflux of GABA, glutamate, taurine, and L-serine was observed as a result of pharmacological stimulation with potassium ion. The same group then studied D-serine dynamics in rat brain (*126*) and tiger salamander retinas (*127*). D-Serine uptake by retinas was found to be Na^+ dependent.

One very interesting application of microdialysis sampling coupled on-line to the CE-LIF system was to monitor the intrastriatal neurochemistry of freely moving stereotypic deer mice during episodes of stereotypic and nonstereotypic behavior (*128*). Motor stereotypes are characterized as repetitive, topologically invariant, apparently purposeless behaviors and hypothesized as a consequence of heightened neuronal activity along cortico-basal ganglia-cortical feedback circuits. Indeed, rearing behavior was found to be associated with significant and selective elevation of striatal glutamate and aspartate concentration.

The spatial resolution of microdialysis sampling is poor because of the comparatively large probe size (1−4 mm). Low-flow, push−pull perfusion and direct sampling provide up to 500-fold improvement in spatial resolution over microdialysis. In push−pull perfusion, two concentric or side-by-side tubes are used as sampling probe. Sample is extracted from one tube while artificial cerebrospinal fluid is delivered through the other tube. The Shippy group reported low-flow, push−pull perfusion method for sampling amino acids from rat vitreous humor perfusates (*129*). Seventeen amino acids including D-ser and D-asp were detected using CE-LIF. The same group demonstrated simultaneous detection of NO_2^- and NO_3^- in push−pull perfusion samples from rat striatum (*130*). Complete separation of NO_2^- and NO_3^-

was achieved within 1.5 min using UV detection.

The Ewing group introduced a miniature tissue homogenizer for reproducible homogenization of single *Drosophila* heads in volumes as small as 100 nL (*131*). MECC with amperometric detection was used to separate nine biogenic amines from homogenized samples. Using the same detection system, this group also reported the significantly reduced amount of many biogenic amines and their metabolites present in the *Drosophila* mutant head homogenates when compared to those in the wild-type samples (*132*). In an interesting report, Quan et al. detected D-Ser concentration in eight different sections of rat brain (*133*). Microdialysis coupled to CE-LIF showed that D-Ser was not homogeneously distributed in rat brain. D-Ser concentration was found to be highest in frontal cerebral cortex while midbrain had the lowest concentration.

When fluorescence derivatization is problematic due to slow reaction kinetics, nonquantitative derivatization at low analyte concentration, or possible formation of multiple products, native fluorescence detection could be employed. A CE method for detecting serotonin in rat brain microdialysate using native fluorescence has been reported (*134*). A pH-mediated on-capillary preconcentration of samples produced ~150 times lower LOD than ones achieved with CE-native LIF detection methods published previously.

Analysis of Single Neurons. Single-cell analysis has been increasingly important for neuroscience, where the tissues are highly heterogeneous and information of intercellular communication can lead to the understanding of neuronal function and dysfunction. The Sweedler group used CE-LIF for measuring D-amino acid-containing neuropeptides present in individual neurons from *Aplysia californica* (*135*). Although proteins and peptides present in animal cells are almost exclusively made up of L-amino acids, D-amino acid-containing peptides have been detected in several animals. The same group demonstrated detection of serotonin and serotonin metabolites in the somata of serotonergic metacerebral cells of *Aplysia* (*136*). The serotonin sulfate concentration was found to be at least 10-fold higher during the light portion of a 12:12-h light/dark cycle than during the dark portion. In another report, this group showed that electrokinetic injection in CE can cause sampling bias by inducing electrochemical reactions of easily oxidized species (*137*). Formation of serotonin dimmer was detected when electrokinetic injection was used, but with hydrodynamic injection no dimer was observed.

CE-LIF analysis of major nitric oxide synthase (NOS)-related metabolites using *Lymnaea stagnalis* neurons was reported recently (*138*). The data suggested that the ratio of Arg/Cit would be a better reliable marker of NOS than the absolute concentration of other NOS metabolites.

Subcellular Sample Analysis. In the nervous system, individual neurons use processes for transferring the chemical and electrical signals from one neuron to the next. Knowledge of the distribution pattern of neurotransmitters, the basis of chemical signaling, in a single neuron could be used to develop models for neurotransmitter synthesis, transportation, storage, and release. Although whole-cell analysis provides valuable information about the content and behavior of single cells, the spatial information is lost.

Sweedler reported the quantitative investigation of D-Asp content in the mechanically separated individual subcellular regions of single neurons (*139*). Using CE-LIF, D-Asp was detected in both nucleus and processes of *Aplysia* neurons. D-Asp percentages in processes from different identified neurons differed significantly. Morphologically distinct regions of the same neuron exhibited similar ratios of D-Asp despite differences in the amounts observed.

Ewing used nanometer inner diameter capillaries coupled to amperomatric detection for analysis of subcellular samples from intact single mammalian cells (*140*). Separations of cytoplasmic samples (as little as 8% of the total cell volume) taken from PC12 cells had been achieved, and dopamine was identified and quantified using this technique. In a follow-up article, the same group reported a method for nondestructive sampling from cells (*141*). This technique involves the use of electroporation to insert a microinjector across the cell membrane and provides the small-volume capabilities as well as gentle injection process. A separation of cytoplasmic sample taken from intact PC12 cells produced a few peaks, but they were not identified.

DNA ANALYSIS

DNA analysis by capillary electrophoresis has become a mature field in a remarkably short period of time. The first DNA sequence generated by capillary electrophoresis was submitted to GenBank in 1997; today, capillary electrophoresis has been the workhorse tool used for genomic sequencing, and the sequences of 323 prokaryote and 41 eukaryote genomes are available on public databases. The sequence of a new organisms is now posted online every three days, all generated by capillary electrophoresis.

As expected for a mature field, advances in capillary electrophoresis for DNA sequencing are incremental and tend to focus on coatings and sieving materials. The stability of poly(dimethylacrylamide) and poly(diethylacrylamide) coatings was studied using atomic force microscopy (*142*). These polymer coatings that also function as sieving media were successfully used in CSE of DNA fragments and detection of the single point mutation of C677T in the human methylenetetrahydrofolate reductase gene. An alternative sieving matrix, replaceable cross-linked polyacrylamide (rCPA), has been developed for CSE separation of proteins (*143*). In comparison with the most widely used sieving matrixes, rCPA is claimed to produce increased resolution and speed for protein separations. The main drawback to this particular matrix is its intense absorption in the UV spectra, but this is not a concern in LIF detection schemes. Zhang and Wirth report the migration of single molecules of DNA in a crystalline array of 300-nm silica colloids (*144*).

CSE with LIF detection has been demonstrated for the qualitative analysis of single-cell multiplex products of RT-PCR (*112*). A replaceable hydroxypropylmethylcellulose sieving matrix was used to provide a molecular weight separation of ethidium bromide-bound DNA.

Polymorphisms. There has been a fairly large effort to use capillary electrophoresis for polymorphism studies. Applied Biosystems has commercialized a single-nucleotide polymorphism genotyping system, which is based on the polymerase chain reaction and capillary electrophoresis (*145*). Hitachi has performed high-resolution single-stranded conformation polymor-

phism by capillary electrophoresis for characterization of single-nucleotide polymorphisms (*146*). Thilly and Karger have reported a multiple capillary instrument with fraction collection for DNA mutation discovery during constant denaturant capillary electrophoresis (*147*). Finally, there have been several reports of the use of capillary electrophoresis to scan for mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 (*148*, *149*).

Methylation and Histone Modification. 5-Methylcytosine is the fifth base found in mammalian genomes. It is found in GC islands that form the promoter region of genes, and its presence is usually associated with downregulation of the gene. DNA methylation provides a powerful mechanism for an organism to control gene expression in specific tissues and organs. Changes in methylation status are associated with some cancers, and demethylation chemotherapies are being developed. Capillary electrophoresis is a vital tool in measuring the effects of these agents (*150*). There have been a number of reports of capillary electrophoresis for quantitative analysis of 5-methylcytosine in tumor samples (*151*−*153*).

Similarly, histone acetylation and methylation cause tight binding between DNA and the histone, modulating access to a gene by the translational machinery. Fraga and colleagues have reported the use of capillary electrophoresis (*154*).

BINDING ASSAYS AND APTAMERS

Gomez and colleague published a review of affinity capillary electrophoresis to study receptor−ligand interactions (*155*). As an important example, DNA adducts can be recognized by affinity reagents, and capillary electrophoresis has proven to be an ideal tool for the study of the adducts through mobility shift assays (*156*).

Krylova has reported nonequilibrium capillary electrophoresis of equilibrium mixtures as a general tool to extract binding information from affinity capillary electrophoresis (*157*). They have used this tool to extract thermochemical information on DNA−protein interactions (*158*) and as a general probe for the temperature rise within a capillary during electrophoresis (*159*). This technology was used to demonstrate that the tau protein binds single-stranded DNA with sequence specificity (*160*). Fang and Chen have published a series of papers that consider models of affinity capillary electrophoresis (161−164). Zou and co-workers presented a review of bioanalytical tools to characterize the interaction of small molecules and biomarcormolecules (*164*).

There has been much interest in the use of aptamers as affinity reagents for protein analysis. Huang and co-workers employed the α-thrombin-binding aptamer to monitor the free protein and the protein complexed with an antibody (*165*). Mendonsa and Bowser continued their impressive work in generating aptamers through use of capillary electrophoresis. They generated a highly specific and strong binding aptamer against human IgE in four rounds of selection (*166*). Similarly, they obtained a high-affinity aptamer against HIV-1 reverse transcriptase with dissociation constants of 180 pM after four rounds of selection (*167*). Berezovski reported the similar use of nonequilibrium capillary electrophoresis of equilibrium mixtures to generate aptamers with tuned binding constants (*168*).

Wang reported an aptamer-based exonuclease protection assay (*169*). Bound aptamer was protected from exonuclease I digestion and amplified by PCR. The assay was reported to detect several hundred copies of the target protein.

Whelan reported the use of fluorescence anisotropy with capillary electrophoresis for affinity assays (*170*). In this assay, a low molecular weight fluorescent probe is allowed to bind to its higher molecular weight target. Binding leads to both a mobility shift and an increase in the polarization anisotropy of the fluorescent probe.

In a demonstration of affinity-probe CIEF, fluorescent BODIPY FL GTP analogue (BGTPγS) and G protein were incubated and separated for detection of Ras-like G proteins using LIF (*171*). A 3−10 pH gradient was used in separating excess BGTPγS from BGTPγS-G protein complex by CIEF, and detection was done in whole-column imaging mode. While affinity CE has been used to determine the binding constant of a specific antibody (Ab) to the target antigen (Ag) using specific Abs against BSA and healthy prion protein (PrPc), CIEF was used in the same study to determine the complexity and recognition of various isoforms of PrPc Abs toward their Ag, PrPc (*172*).

CARBOHYDRATES AND LIPIDS

Carbohydrates. Carbohydrate chemistry is much more complex than that of either proteins or nucleic acids and, as a result, has received much less attention. However, they tend to be very well behaved in capillary electrophoresis separations because of their highly polar nature and the ease with which they form complexes with borate.

Most carbohydrates are spectroscopically silent at convenient wavelengths, and most studies employ either derivatization chemistry or mass spectrometry for detection. There are several notable exceptions. For example, Mikus et al. used conductivity detection to characterize heparin (*173*).

Taking advantage of very old chemistry, Herrero-Martinez reported the determination of the amylose/amylopectin ratio of starches based on capillary affinity electrophoresis wherein the starches were complexed with iodine (*174*). They report the use of a bubble cell detector to obtain detection limits of 0.1 mg/mL for the strongly absorbing complex.

In a brute force approach, capillary electrophoresis is used with absorbance detection at short wavelengths. Carchon et al. used absorbance at 214-nm absorbance in the diagnosis of congenital glycosylation disorders (*175*). Absorbance detection at 200 nm was used to monitor purification of the *E. coli* K5 capsular polysaccharide (*176*). Similarly, Lanz and colleagues report the use of capillary electrophoresis with absorbance detection at 200 nm for the high-throughput analysis of carbohydrate-deficient transferring in patient sera (*177*). Na and colleagues employed detection at 220 nm to characterize ricin and its subunits by use of capillary sieving electrophoresis (*178*). Finally, Wu and Birch doped a buffer with copper sulfate and used detection at 254 nm to resolve sucrose, isomaltulose, and glucose under alkaline conditions (pH 11.6) by capillary electrophoresis (*179*).

Fluorescence-Based Carbohydrate Analysis. Fluorescence derivatization of carbohydrates often results in superior detection limits. Beaudoin and colleagues reported an improved fluorescent labeling procedure for characterization of chitin and chitosan oligosaccharides (*180*). Kamoda et al. report the use of 3-aminobenzoic acid to derivatize oligosaccharides released from

antibody pharmaceuticals (*181*). Guttman and colleagues presented a series of reports on the use of capillary array electrophoresis for large-scale analysis of APTS-labeled oligosaccharides (*182*−*184*).

Callewaert and colleagues also used APTS to characterize oligosaccharides, in this case isolated from human serum (*185*). Soares reported the use of APTS and capillary electrophoresis to characterize polymorphisms in lipophosphoglycans in *Leishmania tropica* (*186*), and Le Floch and colleagues used APTS and capillary electrophoresis to monitor the N-glycosylation pattern of erythropoietin produced by CHO cells in batch processes (*187*).

Mass Spectrometry-Based Carbohydrate Analysis. Zamir and Peter-Katalinic review the use of capillary electrophoresis with mass spectrometry detection for glycoscreening in biomedical research (*188*). Sandra and colleagues report the use of capillary isoelectric focusing and nanospray mass spectrometry to characterize glycosylation of cellobiohydrolase I from *Trichoderma reesei* (*189*). Wang and colleagues employed a suite of techniques, including capillary electrophoresis−mass spectrometry, for structural studies of the capsular polysaccharide antigen of *A. salmonicida* (*190*).

Itoh and colleagues employed capillary electrophoresis and mass spectrometry to monitor glycolysis in a long enzymatic pathway (*191*). Demelbauer and colleagues report the use of capillary electrophoresis−electrospray ionization mass spectrometry to characterize glycoforms of plasma-derived antithrobin (*90*). Similar instrumentation was used by Li and colleagues to characterize lipopolysaccharides of *N. meningitides* (*192*). Koller and colleagues used a sheathless electrospray interface to study high-mannose-type olicosaccharides (*193*). Thibault and colleagues used CE−MS to characterize glycoform distribution in lectins (*89*).

Lipid Analysis. Lipids are highly polar compounds, and their analysis by capillary electrophoresis is almost always performed by micellar electrokinetic capillary chromatography. Several papers have appeared that deal with the study of lipoproteins by capillary electrophoresis. Human serum has been profiled for low-density lipoproteins (*194*) and high-density lipoproteins (*195*). Differences in mobilities of low-density lipoproteins in type 2 diabetic patients has been studied (*196*).

Lipopolysaccharides have been characterized from *H. indluenzae* (*197*) and *T. paurometabola* (*198*). Ion mobility spectrometry and capillary electrophoresis were used for the analysis of complex lipopolysaccharides (*199*). Finally, Amadzadeh and colleagues used 10-nonly acridine orange to form a fluorescent complex with cardiolipin, a phospholipid found only in mitochondria, to characterize individual mitochondria sampled directly from muscle tissue (*200*).

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