

Capillary Electrophoresis for the Analysis of Biopolymers

Wes W. C. Quigley and Norman J. Dovichi*

Department of Chemistry, University of Washington, Seattle, Washington 98195-1700

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This review focuses on the period from January 2002 to December 2003. Over 5000 papers and over 600 reviews on capillary electrophoresis are listed in *SciFinder* for this period. Clearly, it is no longer possible to produce a comprehensive review of this massive literature. As in the last biannual review (*1*), this review is restricted 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. Even with this restriction, we have had to select representative publications; much important work was not included.

PROTEINS AND PEPTIDES

Separation. Proteins are directly responsible for cellular structure and function, and analytical methods are required to monitor protein expression and modifications to understand cellular systems. This task is not trivial. Samples can be extremely complex—tissue homogenates may contain tens of thousands of components whose expression levels range over 6 orders of magnitude.

Historically, the most common analytical method for proteomic studies has been one- or two-dimensional gel electrophoresis. In two-dimensional gel electrophoresis, isoelectric focusing is first

used to separate proteins based on their isoelectric point. Proteins are then separated on the basis of their molecular weight, generating an ISO-DALT gel (ISO for isoelectric and DALT for dalton, the unit of molecular mass). The gel is then stained to reveal a two-dimensional pattern of spots corresponding to the proteins present in the original sample. For identification, spot extraction and in-gel digestion, followed by injection of the extracted peptides into a liquid chromatographic mass spectrometer (LC/MS), is used for protein identification. This venerable technique is not without limitations; it requires a large amount of sample, it is laborious and time-consuming, and it is not easily automated for high-throughput operation. Therefore, instrumental designs that are capable of integration, high-throughput, and automation are of great interest.

Capillary electrophoresis (CE) not only provides an attractive alternative to conventional ISO-DALT electrophoresis for protein analysis but also meets the requirements for implementation for high-throughput analysis and automated instrumentation. It provides fast, highly efficient, and automated separation of proteins and peptides. It also requires a minute amount of sample, which can be very important when rare enzymes or antibodies are studied. Moreover, high-throughput analysis can be achieved by use of capillary array electrophoresis instruments. A number of CE modes are available for separation of proteins and polypeptides. These modes include capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), capillary sieving electrophoresis (CSE), capillary isoelectric focusing (CIEF), and capillary electrochromatography (CEC). Table 1 lists some representative literature for different separation CE modes used for protein analysis.

Capillary Zone Electrophoresis. Proteins contain both positively and negatively charged functional groups. Most of the positively charged moieties in proteins are the guanidinium group of arginine residues and NH_2 group of lysine residues. Less positively charged groups include the NH_2 terminal and histidine residues. These cationic residues are responsible for the interaction of proteins with the weakly negative-charged silanol groups on the capillary wall, causing protein adsorption. This interaction between proteins and the capillary is undesirable because it leads to sample loss, peak broadening, poor resolution, and longer migration times.

Generally, modifying the pH to manipulate the separation characteristics and minimize protein interaction with the capillary wall is utilized because it affects both electroosmotic flow (EOF) and protein charge. Common buffers used in CZE include acetate, borate, citrate, phosphate, sulfate, and similar salts. High buffer concentration is useful to minimize protein adsorption and

Table 1. Separation of Proteins by CE^a

mode ^b	sample ^c	capillary	buffer	detection ^d	LOD	ref
CZE	human immunoglobulin G	uncoated	15–100 mM H ₃ PO ₄ , 20 mM borate, various amounts of ACN, PEG, Triton-X, and SDS			3
CZE	model proteins; Lys, Cyt, Chy, Try	coated and uncoated	40 mM acetate, pH 4.5	UV		4
CZE	human serum; globulin isoforms, HAS, transferrin	uncoated	proprietary reagent set	UV		5
CZE	human serum; transferrin isoforms	proprietary dynamic double coating	unreported Tris/borate concn, pH 10	UV		6
CZE	model proteins; RibA, BSA, ADH, Lys	PB dynamic coating	2 mM PB 5mM Tris	LIF	amol	7
CZE	model proteins: Lys, Cyt, RibA, α -Chy, ICA, TI, α -Lal	DLPC	20 mM Tris-HCL, pH 7.4	UV		8
CZE	metallothionein from brain cytol	uncoated	15 mM ammonium nitrate adjusted to pH 7.4 with nitric acid	ICPMS		9
CZE	bovine β -lac					
CZE	mitochondrial proteins		10 mM borate, 20 mM SDS adjusted to pH 9.4 with KOH	LIF		19
CZE	histamine in peritoneal mast cells		25 mM KH ₂ PO ₄ , 20 mM NaOH	ED	0.7 fM	92
CZE	ERK	uncoated	50 mM borate pH 9.3	LIF		125
CZE	HeLa cancer cells	uncoated	10 mM Tris-HCL, 10 mM SDS, pH 8	LIF		113
CZE	phycobiliproteins from Cyanobacteria	uncoated	50 mM borate, 10.0 mM phytic acid pH 8.15	LIF	2–10 fmol	17
CZE	model proteins, BSA digests	uncoated	1 M acetic acid	MS	900 amol	99
MECC	plant seeds	uncoated	50 mM Na H ₂ PO ₄ , 50–125 mM SDS adjusted to pH 7 NaOH	UV		24
MECC	model protein: BSA	uncoated	10 mM borate, 6% SDS, 0, 3.5% methanol	LIF		26
MECC	model protein: BSA	uncoated	10 mM borate, 6% SDS, 3.5% methanol	LIF		27
SDS-DALT	HT29 colon cancer cells	uncoated	100 mM CHES/Tris, 8% pullulan, 0.1% SDS			33
SDS-DALT	model proteins, Lys, TI, β -Cas, Ova, BSA, Con, β -Gal, Cat; saliva and cerebrospinal fluid	uncoated	1–2% PEO, 400mM Tris/Borate	LIF		29
SDS-DALT	model proteins, Lys, TI, CA, Ova, BSA; cider proteins	coated	50 mM Tris/glycine, 0.1% SDS or 50 mM Tris, 35 mM aspartic acid	UV		30
CIEF	tryptophan enantiomers	uncoated	carrier ampholyte, 8 mM β -CD in 25 mM H ₃ PO ₄ titrated to pH 2.13 with LiOH; 80 mM H ₃ PO ₄ catholyte, 100 mM NaOH	UV		126
CIEF	cerebrospinal fluid and model proteins	uncoated	carrier ampholyte, 1% (w/v) HPMC, TEMED and Pharmalyte 3–10 in the ratio of 9:80:3:20 (v/v); anolyte, 10 mM H ₃ PO ₄ catholyte, 20 mM NaOH	UV		35
CIEF	glycated hemoglobin	dimethylpolysiloxane	carrier ampholyte, pH6–8, pH 3–10, 10:1 v/v in 0.40% MC; anolyte, 100 mM H ₃ PO ₄ , 0.40% MC; catholyte, 40 mM NaOH;	UV		36
CIEF	yeast extract	HEC, HPC, hpmc	carrier ampholyte, 2% Pharmalyte, pH 3–10; anolyte, 10 mM H ₃ PO ₄ catholyte, 20 mM NaOH	LIF	fmol	37
CIEF	yeast extract	HPC	carrier ampholyte, 2% Pharmalyte, pH 3–10; anolyte, 100 mM HAC; catholyte, 0.5% NH ₄ OH ₄	MS		38
CIEF	model proteins: R-phy, GFP HSA, BSA	Teflon AF2400 capillary	carrier ampholyte, Pharmalyte, pH 3–10 and pH 4–6.5; anolyte, 100 mM H ₃ PO ₄ catholyte, 100 mM NaOH	UV	1 mM–10 nM	39
CIEF	model proteins: Lys, chy, Myo, CA, β -Lgl A, β -Lgl B	siloxanediol polyacrylamide	carrier ampholyte, 3–10% Pharmalyte; anolyte, 10 mM aspartic acid pH 3; catholyte, 10 mM KAc adjusted to pH 3 with formic acid	LIF	1 pM–1 nM	40
CEC	model proteins: Myo, RibA, α -Chy, Cyt	methacrylate	20 mM phosphate, 0–80% NaCl, pH 6.0	UV		47

^a Chemicals: ACN, acetonitrile; CHES, 2-(*N*-cyclohexylamino)ethane-sulfonic acid; Cy5, sulfoindocyanine succinimidyl ester; DDAB, didodecyltrimethylammonium bromide; DLPC, 1, 2-dilauroyl-*sn*-phosphatidylcholine; FQ, 3-(2-furoyl)quinoline-2-carboxaldehyde; EACA, 6-aminocaproic acid; EB, ethidium bromide; epoxy poly(AG-AA), poly(acrylamide-*co*-allyl β -D-glucopyranoside-*co*-allyl glycidyl ether); HAC, acetic acid; HEC, hydroxyethylcellulose; HPC, hydroxypropylcellulose; HPMC, hydroxypropylmethylcellulose; IDA, iminodiacetic acid; LPA, linear polyacrylamide; MC, methyl cellulose; PA, polyacrylamide; PEG poly(ethylene glycol); PB, pyrenebutanoate; PDA, poly(dimethylacrylamide); PEO, poly(ethylene oxide); PVA, poly(vinyl alcohol); PVP, poly(vinylpyrrolidone); Q-PzI, (*N*-methyl-*N*- ω -iodobutyl)-*N*-methylpiperazine; SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylethylenediamine; TFA, trifluoroacetic acid. ^b CZE, capillary zone electrophoresis; MECC, micellar electrokinetic capillary chromatography; CIEF, capillary isoelectric focusing; SDS–CE, sodium dodecyl sulfate capillary electrophoresis; CEC, capillary electrochromatography. ^c Proteins: ADH, alcohol dehydrogenase; Apr, aprotinin; Chy, chymotrypsinogen; α -Chy, α -chymotrypsinogen A; α -Lal, α -lactalbumin; β -cas, β -casein; β -Gal, β -galactosidase; β -lac, β -lactoglobulin β -Lgl A, β -lactoglobulin A; β -Lgl B, β -lactoglobulin B. BI, bovine insulin; BSA, bovine serum albumin; Cat, catalase; CA, carbonic anhydrase; Con, conalbumin; Cyt, cytochrome *c*; ERK, extracellular signal-regulated protein kinase; GFP, green fluorescent protein; Hem, hemoglobin; HCA, human carbonic anhydrase; HSA, human serum albumin; ICA, insulin chain A; Lec, lentil lectin; Lys, lysozyme; Myo, myoglobin; Ova, ovalbumin; R-phy, (R)-phycoerythrin RibA, ribonuclease A; TI, trypsin inhibitor; Trf, transferrin; Try, trypsinogen. ^d UV, ultraviolet absorbance; LIF, laser-induced fluorescence, ED, electrochemical detection, MS mass spectrometry; ICP, inductively coupled plasma

suppress EOF. However, the conductivity of these buffers is relatively high, resulting in high current, excessive Joule heating,

and the electrolysis of water causing bubble formation during the separation. These phenomena may lead to peak broadening, loss

of resolution, background noise, and other undesirable effects. Other buffers, more commonly used in bioanalysis, are useful for CZE separation of proteins because these buffers usually have low conductivity. Many of these buffers also contain amino groups, which reduce protein adsorption on the capillary wall by saturating sites on the surface that otherwise interact with proteins.

In many cases, capillary coating is essential for CZE separations of proteins. A number of approaches has been proposed to manipulate the inner wall chemistry of silica capillaries including permanent coating with bonded/cross-linked polymers, physical adsorption coating with cationic polymers, and dynamic coating with adsorbed surfactants and other compounds and mixed reagents. The use of coatings is of great interest in reducing protein–capillary wall interactions for proteomic studies in CE, for improved reproducibility as well as long-term stability. Many static coatings only last a few runs, diminish in quality very quickly, and result in exceptionally irreproducible data, leading to increased run times and difficulty in analyte characterization. There are a number of static coatings available that have been used with varying success, including poly(vinyl alcohol) (PVA), poly(dimethylacrylamide) (PDA), and methylcellulose (MC).

A method has been described for quantifying protein adsorption on the capillary wall. In this method, a fluorescently labeled protein is flushed into a capillary, saturating the potential adsorbing sites, followed by desorption and quantifying by using an internal standard (2). That method, and others, has been used to evaluate a number of potential dynamic wall modifiers, including monoamines (triethylamine, triethanolamine, ethylamine), diamines (putrescine, cadaverine, decamethonium bromide, hexamethonium chloride), oligoamines (spermidine, spermine, tetraethylenepentamine), polymers (PVA, PDA, hydroxypropylmethylcellulose, hydroxyethylcellulose, hydroxyethylacrylamide, EOTrol), surfactants (nonionic, zwitterionic), isoelectric buffers (iminodiacetic acid, aspartic acid, quaternarized piperazine), and others (3–12). Oligoamine, zwitterionics, and quaternarized piperazine appear to reduce protein adsorption more than other modifiers. Some coatings claim to universally reduce protein adsorption, while others have specific application. For example, decamethonium bromide and hexamethonium chloride are reported to be effective for glycoprotein separations (4).

Piperazine quaternary diammonium salts show promise for reducing EOF when the alkyl chain terminating with an iodide atom is used (10). Alkyl chains terminating with an iodide atom are able to form a covalent bond with the silica surface at pH 9. The coating is stable, and the authors report that only 1% of the bonds are hydrolyzed per CE run. The coatings appear to strongly modulate and typically invert the EOF. The same piperazine salts without iodide show no such effect (10). One such compound, 1-(4-iodobutyl)-1,4-dimethylpiperazin-1-ium iodide, was shown to effectively coat a capillary for an efficient separation of model proteins.

Peak asymmetry for protein CZE utilizing an “absorbed static coating” epoxy–poly(dimethylacrylamide) was evaluated (13). An investigation of protein and peptide migration behavior due to running buffer composition either with bare fused-silica or polyacrylamide-coated capillaries was performed via CZE. Buffers composed of aliphatic oligoamine triethylenetetramine in combination with a monoprotic or a polyprotic acid and different anions

were tailored to control adsorption of proteins and peptides to the capillary wall over a wide pH range (14). Others have used dextran to coat capillaries for the analysis of basic proteins (15).

Proprietary buffer reagents lead to varying results, but insight to buffer characteristics is an issue (5, 6, 16). One paper describes a multicapillary CE separation of human serum proteins and compares a high-throughput instrument to a standard agarose gel electrophoresis (AGE) system (5). The results are encouraging because of the speed of analysis and ease of use. Low sensitivity, for some analytes, of the multicapillary instrument is the major drawback at this time. The most abundant proteins detected are globulin isoforms, transferrin, albumin, and others, although most components were not baseline resolved. One advantage of this system is the temperature-controlled capillaries allow the dissolution of cryoglobulins that precipitate during the AGE analysis, allowing accurate quantification with the multicapillary instrument. A second paper uses a dynamic double coating to investigate the isoforms of transferrin from human serum (6). This paper resulted in baseline, or near-baseline, resolution by carefully adjusting capillary temperature and running voltage. The results agree quite nicely with a standard method for the determination of carbohydrate-deficient transferrin. Another paper, from a non-peer-reviewed journal, discusses the history of coatings, lists 33 patents on EOF suppressors granted since 1990, reviews EOF suppression theory, and shows some data utilizing dynamic coatings (16).

Another dynamic coating, pyrenebutanoate (PB), was used as a test bed for the separation of some model proteins (7). The authors suggested that PB, a charged molecule, interacts with the silica wall via hydrophobic interaction and hence a PB–protein complex can also interact with the silica wall. However, when the free PB concentration is much greater than the protein content, efficient separations were achieved, while poor efficiency was observed at either lower PB or higher protein concentrations. This observation strongly supports the notion that PB reduces the protein–capillary interaction.

A semipermanent static coating was recently reported based on a double-chained zwitterionic phospholipid, 1, 2-dilauroyl-*sn*-phosphatidylcholine (8). The coating quickly reached equilibrium, and EOF approached zero within 5 min and remained constant for over 35 successive protein separations. Efficiencies for four cationic proteins were reported to be $(0.3–0.56) \times 10^6$ plates, much more efficient than the other methods mentioned above. In addition, variation in the run-to-run migration time was less than 1% RSD, day-to-day migration time was less than 5% RSD, and protein recovery ranged from 64 to 93%. Inspection of tailing in the electropherogram and percent recovery reveals a relationship between peak shape and adsorption. In addition, the paper investigated the efficiency at low, medium, and high pH.

Bare capillaries can be well suited for protein analysis with the proper choice of running buffer. There is no clear line between dynamic coating and proper running buffer in a bare capillary. For instance, a buffer containing small amounts of SDS, ~1 mM, could be categorized as a dynamic coating; in contrast, the system could be considered to be an uncoated capillary with an SDS-containing running buffer. The defining characteristic of dynamic coatings is determined by the equilibrium time of EOF suppression. If that time is very short, the system would be considered

an uncoated capillary, whereas if the time was long, then the system would be considered as a dynamic coating.

CZE with uncoated capillaries was utilized to separate subclasses of immunoglobulins (3). This study was a comprehensive investigation in order to separate the subclasses of immunoglobulin (IgG1, IgG2, IgG3, IgG4) with respect to their minor subclass (the κ and λ chains). Coated and uncoated CZE, CIEF, and MECC were investigated; uncoated CZE with a 50 mM phosphate buffer at a pH of 9.3 produced the best resolution. At this pH, EOF overwhelms the electrophoretic mobility (EM) of IgG, emphasizing the selectivity of the EM of each subclass. The PVA-coated capillary resulted in poor resolution probably due to the similar pI values of the subclasses.

Phycobiliproteins were extracted from cyanobacteria and analyzed with uncoated capillaries in CZE mode (17, 18). After optimization of the method, the major components were separated in under 5 min. In another study, metallothioneins, metal-binding proteins that aid in the detoxification of heavy metals and that exist in several isoforms, were investigated (9). Because of the complicated sample matrix involved with the extraction process, migration times of these analytes vary markedly from sample to sample. An internal standard used for migration time adjustment was used to compare profiles from different samples. Utilizing five time markers, 33–48 separations were analyzed; the average RSD improved from 18 to 3.4% through use of migration time correction.

Subcellular labeling techniques will be a useful approach for simplifying proteomic studies. Mito Tracker Green was used to selectively fluorescently label mitochondrial proteins and analyze via CZE, and laser-induced fluorescence (LIF) was performed (19). This fluorescent label was determined to selectively label a subset of proteins within a mitochondrial-enriched fraction. Genetic variants and degrees of phosphorylation were investigated in caseins using CZE (20). The technique allowed the identification of p - κ -casein, β -casein, γ_2 -casein, and γ_3 -casein. Urea-PAGE and gel IEF were used to verify and complement the CE data. A study describes the application of CZE to investigate interactions between the protein concanavalin A and a variety of carbohydrates (21). A comparison of two fused-silica capillaries of considerably different inner diameter (10 and 75 μm) was made for the separation of five standard proteins; cytochrome *c*, albumin, transferrin, catalase, and chymotrypsinogen A (22). Both capillaries were 27 cm in length, but the smaller capillary produced severe tailing, presumably due to its higher surface-to-volume ratio.

Micellar Electrokinetic Capillary Chromatography. MECC separation is based on the partition of solutes between aqueous and micellar phases. It combines some of the operation principles of micellar liquid chromatography with those of CZE and was originally used for the electrophoretic separation of neutral molecules. The technique can be considered a type of chromatography where the stationary phase is essentially mobile and the mobile phase is electroosmotically pumped. Relatively few applications have been found using MECC for protein separations (23–28). MECC is well suited for separation of proteins, peptides, and other biopolymers that have similar structures or pI (24, 25). Aromatic containing amino acids that are similar in structure are easily separated via MECC (24). This study investigated and

compared MECC to CZE with 27 amino acids. In addition, a dual mode was used to perform MECC and CZE for dual comigration identification, a real-time cross-check for unambiguous identification. Once the method was proven, plant seeds were extracted and analyzed (24). This method could be easily developed for proteomic uses. MECC with LIF has been used to investigate the labeling characteristics, efficiency, and kinetics of sulfoindocyanine succinimidyl ester (26). The flexibility associated with altering the selectivity in MECC provided an easily adaptable method to separate labeled BSA, unreacted fluorescent dye, and an internal standard for a host of kinetic experiments. In a related study, band broadening from multiple labeling of proteins was investigated using MECC (27). The height equivalent-to-plate ratio was affected by running buffer pH, reaction time, and the dye/protein concentration ratio. Bovine serum albumin (BSA) contains 60 amino groups. Incomplete labeling leads to a complex mixture of reaction products. MECC is used to study the specificity of BSA tryptic digests using trypsin conjugated to gold nanoparticles (28). The sensitivity and resolution of the tryptic fragments from BSA is improved with the use of the adsorbed trypsin. Initial results suggest that changes in the conformations and steric effects contribute to the loss of activity and changes in specificity of trypsin adsorbed onto the gold nanoparticles.

Capillary Sieving Electrophoresis. The capillaries used in CSE are often coated to reduce EOF and protein adsorption. In this separation method, proteins are denatured with SDS and then separated based on their size using a replaceable sieving polymer matrix such as linear polyacrylamide, poly(ethylene oxide), poly(ethylene glycol), dextran, or pullulan. This method is the capillary-based version of SDS–PAGE, with advantages of high throughput, ease of automation, and small sample volumes. CSE has been reported for separation of microheterogeneities and isoforms of proteins (29). A segmental filling method is used to separate the proteins of interest, where a plug of SDS is injected onto the capillary prior to sample injection, followed by the poly(ethylene oxide) (PEO) running buffer. The analysis of cider proteins was accomplished using CSE with a coated capillary and compared to a standard SDS–PAGE with UV detection (30). The resulting electropherogram showed 12 peaks. Inspection of UV spectra at the apex of each peak indicates many resolved proteins when compared to SDS–PAGE UV spectra. In addition, the peaks were compared to a molecular mass set of standards and the proteins in the cider appear to have masses ranging from 16 to 110 kDa.

Our group reported a series of papers applying CSE methods for highly sensitive protein analysis for cell homogenates and single cells using 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), a fluorogenic reagent (31–33). The labeled proteins were separated in a replaceable polymer sieving matrix such as PEO or pullulan and then detected using an ultrasensitive sheath-flow laser-induced fluorescence detector. Seven standard proteins were separated on the basis of their size, and their migration times were proportional to the logarithmic of molecular weights of the proteins, $r = 0.986$. This separation method is not affected by the multilabeling problem, where a complex mixture of reaction products results from incomplete labeling of the protein. In CSE, each protein molecule is complexed with many SDS molecules, which helps shield the heterogeneity in the charge-to-size ratio

among the protein population caused by derivatization. In addition, CSE is a size-based separation method; attachment of labels with low molecular mass to a large protein molecule does not dramatically change the size of protein molecule and therefore its migration velocity. Electropherograms are presented for the separation of different fractions of HT29 cancer cells: cytosolic, membrane/organelle, nuclear, and cytoskeletal/nuclear fractions in a 2.5% PEO sieving matrix all with different electropherogram patterns. In that paper, single-cell data for HT29 cancer cells is presented and compared to a cell homogenate (31). Further optimization of single cell protein separations direct another study investigating the variation in protein expression between cells and its relevance to the cell cycle (32). That study revealed that protein content may vary markedly from cell to cell and that the major influence on protein expression is due to the cell cycle. The next paper in the series, as a proof-of-principle experiment, HT29 cancer cells extract was separated by SDS-PAGE, treated with a stain, and a single protein spot was excised from the gel, extracted, and used to spike a cell extract sample (33). That sample was compared to the same extract before the extracted protein was added, and comigration of a single larger peak verified the success of spiking a single protein for CE analysis. The extracted sample was also used to perform LC/MS/MS identification by peptide mapping.

Capillary Isoelectric Focusing. CIEF separates proteins or peptides based on isoelectric point, pI . In CIEF, the sample is normally mixed with ampholytes and the capillary is filled. Next, one end of the capillary is placed in a reservoir containing high-pH solution, the other is placed in a low-pH solution, and an electric field is applied. A pH gradient is quickly formed within the capillary, and proteins or peptides migrate and are focused at the region where the pH is equal to their pI . CIEF can be performed in one step or two steps. One-step CIEF usually employs polymers such as cellulose derivatives to reduce but not eliminate EOF. At this point, either migration past a detection window or whole-column imaging is needed. In two-step CIEF, proteins or peptides are first focused in a coated capillary to eliminate EOF and then mobilized using either chemical or hydraulic mobilization.

CIEF produces the best resolution for proteins and peptides among the CE separation modes, and it is a very powerful tool to resolve protein modifications, to characterize microheterogeneity, and to identify glycoforms (34). CIEF has been proven to be a powerful tool for proteomics studies. A one-step method was used to analyze the proteins contained in cerebrospinal fluid (CSF) (35). A collection of nine standards was used to identify proteins in CSF fractions from numerous patients using comigration techniques. The results for specific protein quantification were used to correctly identify patients with advanced neurological disorders, and the results agreed with results obtained from ELISA. The method is not without challenges, but initial results are promising. CIEF was shown to baseline resolve glycosylated hemoglobins, a task not yet performed using gel or HPLC technology, in just a few minutes (36). The methodology was applied to 31 real samples, and the results yielded excellent correlation with a standard reference method from a local clinical laboratory. Baseline resolution was achieved with pI differences as small as 0.03.

CIEF coupled to capillary reversed-phase liquid chromatography (cRPLC) in a comprehensive two-dimensional system proved to be sensitive and efficient, producing femtomole limits of detection with a peak capacity of $\sim 10\,000$ (37). There are some advantages of a cRPLC-CIEF system: the small amount of sample needed (~ 50 nL), CIEF in a stand-alone method would require a capillary filled with sample. Unfortunately, the system suffered from a serious limitation. Isoelectric focusing is not compatible with known fluorescent labeling technologies. Because of incomplete labeling, each protein is converted to a large number of labeled products, each with its own isoelectric point. The two-dimensional electropherograms in ref 37 show a series of spots with identical LC retention time but different CIEF migration time; presumably, these spots are generated by the heterogeneous mixture of reaction products generated from a single protein. Another paper used a similar separation method for the separation of the tryptic digest of a yeast protein homogenate (but with CIEF in the first dimension and cRPLC in the second dimension), but with mass spectrometry detection (38). This combination of methods facilitates identification of a large number of peptides (1132) with a relatively small amount of sample (~ 1 μg) and appears to be fairly efficient; the peak capacity was over 2500. Last, the identification provided by the MS detection system is a clear advantage over UV and LIF, particularly if large amounts of sample are obtainable.

A CIEF system with a liquid core waveguide (LCW) has also been reported (39). This novel design does not need capillary-coating techniques to suppress EOF due to the relatively inert Teflon capillary that is used for the separation. In addition, like whole-column imaging, the isoelectric focusing step is not changed by moving the focused proteins off column, because the detection method is in situ. The peak capacity was reported to be greater than 100, or ~ 15 per pH unit, a respectable value. A CIEF-like separation mode where the pH is constant and the "gradient" is due different ampholyte composition has also been reported (40). This technique is described as a transient CIEF, part CIEF and part ITP behavior. The unique feature is that the "gradient" can be adjusted to separate specific proteins, although there are no predictive models to explain the separation mode, so the technique is Edisonian in nature. Computer simulation of CIEF process has been of considerable interest because it can be used to predict the separation dynamics, focusing behavior of amphoteric sample components, and pH gradient formation and stability, making this last method a real challenge for simulation studies.

Capillary Electrochromatography. CEC is a hybrid technique of CE and capillary liquid chromatography. Commonly, the CEC column is packed with an alkylsilica stationary phase. Because the silica particles and capillary wall are negatively charged under normal conditions, EOF is generated to pump solvent through the chromatographic bed and to produce high separation efficiency. Initially, CEC separations were mainly performed by using octadecylsilane (ODS) as stationary phase for separation of neutral analytes such as polyaromatic hydrocarbons. In addition, sulfonated poly(styrene-divinylbenzene) (41); poly(2-sulfoethylaspartamide)-silica (42); open tubular silica, porous styrenic sorbents and octadecylsilica (43); pentofluorophenylsilica, trycontylsilica, and octadecylsilica (44); acrylate-based

porous monoliths (45); and wide-pore stationary phases have been recently investigated (46).

Recently, monodisperse poly(glycidyl methacrylate–divinylbenzene) microspheres were functionalized with propanesulfonic acid moieties to attain negatively charged beads with a large pH range. Four model proteins and three cytochrome *c* variants were baseline resolved in isocratic conditions (47). Salt and pH effects on protein separation were also investigated in order to identify the possible modes of separation. It was determined that chromatographic retention and electrophoretic migration were both responsible for separation selectivity. The hydrophilic oligomeric chains are neutral and electrostatic interactions between proteins and sulfonic acid controlled the chromatographic retention at the pH used, near neutral.

The current trend in this technology is the use of monolithic columns, which may be prepared by in situ polymerization (48). One advantage monoliths have over ODS packed columns is the lack of bubble formation that is common with ODS (frit-based) columns. No supporting frits are necessary for monolithic columns because the stationary phase is covalently bound to the inner wall of the capillary. Another advantage is the ease of making the columns, rather than packing a small capillary with an existing stationary phase. Separation of proteins and other neutral and charged species has been demonstrated by using monolithic columns (49, 50). Monolithic phases can be polymer or silica based, for protein separations the polymer-based monolithic phases have shown more promise, but silica-based monoliths are being investigated for protein analysis. The typical charged silica-based monoliths can only produce sufficient EOF above pH 5, limiting separation conditions, and silanol–protein interactions present adsorption issues. Amphiphilic silica, C₁₇-monoliths, and C₁₈-monoliths have been designed for reversed-phased CEC, where octadecyl ligands facilitate sufficient EOF below pH 5 and reduce adsorption issues (49, 50). Another paper describes the separation of proteins using C₄ monolithic phase (51).

Two-Dimensional Separation. There are thousands of proteins in a typical mammalian cell, which can lead to hundreds of thousands peptides, and the concentrations of the proteins may vary by many orders of magnitude. To date, no single chromatographic or electrophoretic separation is capable of analyzing these complex mixtures; therefore, multidimensional separations are inevitably required to resolve those complex mixtures for proteomic studies. Two-dimensional (2-D) separation using two orthogonal separation modes can greatly increase the capacity of the separation and improve resolution. Traditional 2-D gel electrophoresis, the most common approach in proteomics, is a collection of slow and laborious methods. In addition, traditional methods have difficulty in analyzing proteins that are in low abundance, are highly basic or acidic, or have large molecular weight. Since Jorgenson developed 2-D HPLC–CE for separation of proteins and peptides, a number of applications have been demonstrated using either on-line or off-line 2-D HPLC–CE (52).

Work mentioned earlier used CIEF in the first dimension to provide proteome analysis of yeast extracts (38). In that paper, CIEF was able to concentrate the analytes almost 250 times and was able to separate analytes to yield a baseline resolution peak capacity of 160. The second-dimension mode was capillary reversed-phase liquid chromatography coupled to MS/MS, the

combination of methods enables a large number of identifiable peptides with a relatively small amount of sample (~1 μg) and with a peak capacity over 2500. Other methods also use CIEF in the first dimension for proteome analysis, but with other CE modes of separation using dialysis interfaces for the transfer between the first and second dimensions (53, 54). Similarly, a dialysis interface was used to integrate gel electrophoresis to CIEF (55). Another dialysis-based two-dimensional system used valves for the transfer between dimensions, utilizing the dialysis junction more for a pretreatment step (56). All of these methods, and most other similar methods, utilize trypsin, or other digesting agent, to form peptides, further complicating the separation. The digestion of proteins into peptides not only causes the need for more separation power but also necessitates the use of MS detection, complicated software, and database use. In addition, since the proteins are digested, information on phosphorylation and other changes is usually lost.

We have developed a 2-D CE system for automated protein analysis utilizing intact proteins (57). In this system, the FQ-labeled proteins were first separated by submicellar CE at pH 7.5 in the first-dimension capillary. Then the separated fractions were transferred from the first-dimension capillary to the second-dimension capillary, where electrophoresis was performed at pH 11.1 for further separation. Successive transfer followed by a second-dimension separation was repeated and used to generate, in serial fashion, a 2-D electropherogram of intact proteins. This 2-D CE system is computer controlled; there is no operator intervention once the sample has been loaded. Sheath-flow LIF was used as an ultrasensitive detector for this 2-D CE system, enabling zeptomoles of labeled proteins to be detected, demonstrating the exquisite sensitivity of this system.

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 will be covered in this area simply due to the limits of detection interest in this topic.

(1) Sample Preconcentration. Precolumn or on-column concentration can be used to improve the detection sensitivity of proteins and peptides. The techniques used for concentration are usually divided into chromatography based and electrophoretic based. Due to the need for brevity, we will only discuss the electrophoresis-based approaches; a recent review discusses chromatography-based preconcentration (58). Electrophoretic-based approaches for biopolymers include stacking and isotachopheresis (ITP); field-amplified injection is another method, but to date few papers are published utilizing it for proteome analysis (40, 59, 60). Stacking is based on the principle that the velocity of an ion increases with electric field strength. If the conductivity of the sample plug is lower than that of the CZE buffer, the electric field strength in the sample plug will become higher than that in the running buffer. Consequently, the analytes will be focused if the sample plug is positioned between the running buffers. A novel method for concentration of proteins and peptides in a capillary is presented (61). A short section (<1 cm) on the capillary was etched with HF until a porous section was made, the porous area allows electrical conduction but prevents passage of the analyte ions. In addition, the etched area allows the capillary to be isolated into two parts, using three buffer vials to perform CE experiments

in the capillary by applying high voltages independently. Controlling the voltages correctly allowed stacking that resulted in a 50-fold increase in concentration.

Stacking can also be used in the second dimension of the LC–CE system (62). In this configuration, fractions of an HPLC effluent are collected in a 96-well plate and dried, reconstituted, and concentrated on-line using large-volume stacking with polarity switching, allowing analysis of trace proteins in a complicated mixture. Another method uses pH-based stacking, resulting in a robust approach for the detection femtomole amounts using tandem mass spectrometry (63). Another preconcentration device capable of a 40-fold concentration increase was developed for the analysis of proteins by CE (64). The microfluidic device uses an electric field to capture proteins that pass through a flow stream via a balance between hydrodynamic and electric forces. Extremely high peak efficiencies were determined for a CZE analysis of two different kinds of bacteria in bakers yeast (65). Effects due to buffer content and concentration, polymer additive content and concentration, and pH were investigated. The maximum plate count was reported to be 10^9 plates/m.

(2) 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 (35, 40, 62). A novel UV detection system utilizes a LCW (39). This UV detection system uses the LCW as a very long path length UV window, resulting in limits of detection to the order of nanomolars. 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 picomolar detection limits.

(3) Fluorescence. LIF is the most sensitive detection method in chemical analysis. LIF produces a wide dynamic range making it one of the most important tools for biological samples due to simultaneous detection of low-abundant and high-abundant species. In addition, LIF can be used in all CE modes for ultimate flexibility, although multiple labeling limits its utility in CIEF. Fluorescence can be used for kinetic studies (66, 67), enzyme and phosphorylation assays (68, 69), cell constituents (70), and binding assays (71). Tryptophan residues are sometimes used to detect proteins with native fluorescence, but tryptophan is one of the rarest amino acid in many eukaryotic proteomes, and the molar absorptivity and fluorescent quantum yield are relatively low for this amino acid. Generally it is more common to label proteins with fluorescent dyes: Cyanine-5 (27, 72, 73), Alexa Fluor 488 (74), 10-nonyl acridine orange (75), isothiocyanates (73), SYBR Green II (76), hexidium iodide (77), LysoTracker Red (70, 78), MitoTracker Green (19), and others (79). Many dyes used in CE-LIF bind to proteins through noncovalent interactions (79).

We have been using FQ, a covalent dye, for a few years now with good success. FQ reacts with the α -amine of lysine residues, which are quite common; each protein in the yeast proteome contains an average of 35 lysine residues. FQ reagent is a fluorogenic reagent, used in excess, to improve the labeling

efficiency without causing high background. Regrettably, the FQ-labeling reaction does not go to completion under normal circumstances, resulting in a complex mixture of reaction products, which produces complex and broad electrophoresis peaks, particularly in CIEF. This multilabeling problem for FQ-labeled proteins can be eliminated by use of a submicellar buffer containing an anionic surfactant, such as SDS. The surfactant appears to ion pair with the unlabeled lysine residues, generating a complex that mimics the size and charge of the labeled residues. Recently, we have applied FQ to HT29 human colon adenocarcinoma cells (31–33, 57), a single-cell *Caenorhabditis elegans* embryo, human breast cancer cells, and CH27 hybridoma cells (80). The protein patterns produced were found to be species specific, and the multilabeling issues were well addressed via use of SDS. The use of FQ for cell cycle studies revealed that the cell-to-cell variation of protein content, as determined by FQ fluorescence, was due to cell cycle. FQ is efficient enough for single-cell studies. These and other studies consistently reveal efficiencies in the hundreds of thousands of plates with limits of detection at the zeptomole level. The sensitivity of FQ is considerably better than that by noncovalent labeling dyes.

(4) Other Detection Methods. While fluorescence detection provides excellent detection limits, it usually requires the use of derivatization chemistry. In contrast, UV absorbance detection limits are much poorer, particularly for proteins and peptides without aromatic amino acids. A high-sensitivity refractive index detector has been reported for protein separation in capillary electrophoresis based on a complex interaction of a laser beam with the separation chamber (81, 82). Electrochemical detection has increasingly become an important tool for CE analysis, because of its low volume requirements and detection limits (83–94). In addition, a miniaturized surface plasmon resonance (SPR) sensor was developed for a CE-SPR (95). A protein immobilization procedure was used to functionalize a surface for selective protein detection, showing reproducibility of 15% RSD and a detection limit of 2 fmol with a dynamic range of 1000.

(5) Mass Spectrometric Detection. CE–MS has the ability to rapidly separate and detect biological samples present in extremely small amounts. Separation efficiency is very high, and the ability to elucidate molecular structure is invaluable. CIEF, or other concentrating method, and some other separation mode, other CE modes, or liquid chromatography, can separate a wide variety of proteins, protein isoforms, protein heterogeneities, and peptides at very low levels of detection. Liquid-based separations, such as LC and electrophoresis (gel and capillary) coupled to MS, have become important techniques for identification of proteins utilizing peptide mass-fingerprint database searching. However, difficulty in interfacing CE to MS has prevented CE–MS from becoming a widely used analytical technique. Difficulties usually arise due to issues involved in the minute flow rates that are required with efficient small-bore capillaries. Recent advances have resulted in a rapid increase in the use of CE–MS in the analysis of complex biological mixtures. CE–MS has now been successfully applied to the analysis of a wide variety of compounds including amino acids, protein digests, protein mixtures, single cells, oligonucleotides, and various small molecules relevant to the pharmaceutical industry.

Electrospray ionization (ESI) is the most common ionization mode for CE-MS. Common CE-ESI interfaces can be divided into sheath-flow (96–98) and sheathless (99–101) modes. In sheath-flow mode, a sheath liquid is mixed with the running buffer at the distal end of the separation capillary for voltage application and to help facilitate stable spray. A sheath-flow interface is more common than the sheathless type and is typically more reliable, simpler, and easier to implement. While more difficult to implement, the sheathless mode does not dilute the sample at the point of voltage application (102). A CE-ESI-time-of-flight (TOF)-MS was used to investigate protein complex stability (stability of the electrospray) with various amounts of organic modifier added to the sheath flow. Sheath buffers generally contain a weak acid, formic or acetic, and may contain amounts of organic modifier. In one study, increasing the amount of methanol in the sheath flow caused increasing amounts of dissociated protein, forming increasing amounts of apoprotein and prosthetic heme group, and no organic modifier worked best (98). To illustrate the complexity of the dynamics of a sheath buffer, another study determined that a 60% 2-propanol solution gave the best spray stability, for model peptides, and signal characteristics, however, that paper optimized many other parameters as well (97). An on-line sheath-flow system was used to investigate the patterns of peptides and proteins for the establishment of new diagnostic markers (103). Another paper describes a simple pH-mediated stacking method and sheath-flow MS detection to reduce the limit of detection to the tens of femtomole range, very similar to sheathless detection limits.

Sheathless interfaces are better suited for CE due to more compatible flow rates, leading to better ionization efficiencies and sensitivity. For integration of CE to ESI sheathless interfaces, designs that incorporate providing the terminal to the CE separation circuit concomitantly with providing the electric potential to the ESI tip have been accomplished in many ways. A single capillary can be modified near the distal end to accomplish this by inserting a small electrode into a small hole of the separation capillary, coating a small portion of the capillary with a conductive material, or inserting the capillary into a conducting sleeve or a microtee. Another type of interface was created by modifying the distal end of the capillary by etching; a portion of the capillary was fixed to a small support made from a piece of polycarbonate and a small area of the capillary was exposed to HF (99). Once an electrical connection is possible through the already porous tubing wall, the capillary is removed from the HF and rinsed, and a small buffer reservoir is attached to the supporting material and the exposed area of the capillary. A stable sheathless flow was created from this integrated interface, where the CE capillary, an electrical porous junction, and a spray tip were incorporated on a single piece of silica tubing, the separation capillary. Detection limits of 900 and 500 amol are reported (99, 100). In a similar method, a porous junction was made using a dentist's drill to create a well on the capillary and further etching was achieved via HF; the progress was monitored by resistance between two electrodes (104).

There have been various studies coupling CE with ESI-Fourier transform ion cyclotron resonance (ESI-FTICR) MS for proteome analysis. FTICR is advantageous due to high resolution, accuracy, and sensitivity. In addition, due to its nondestructive detection manner, FTICR provides the capability for high-order tandem MS

analyses for structural characterization. A recent CE-FTICR design was used to separate and characterize cerebrospinal fluid proteins (105). A home-built CE was interfaced to a FTICR-MS via a home-built interface. The number of proteins identified in the analysis was over twice that of direct infusion. Direct infusion has issues of ion suppression of the electrospray, space charge effects, and spectral overlapping. All these issues are easily overcome by CE separation with the added benefit of smaller amount of sample is needed for analysis, 40 nL of sample for CE, as opposed to over 2 μ L for direct infusion. Issues regarding data acquisition speed, 1 spectra every 4.5 s, seems to be the greatest negative aspect of this technique. Many peaks were present in only one spectrum, making quantification impossible.

By combining the high-resolution two-dimensional separation of CIEF-CZE, or capillary isotachopheresis (CITP-CZE), with the high-resolution detection of FT-ICR, single CIEF-FT-ICR analyses have revealed over 1000 recognized proteins in the mass range of 10–100 kDa from the cytosolic fraction of *Shewanella oneidensis* (60). The microdialysis system used in sample preparation is believed to be responsible for the lower molecular weight boundary. They also describe and demonstrate an approach that extends the sensitivity, depth, dynamic range, and throughput of proteomic experiments. This approach is based upon the use of peptide "accurate mass tags" (AMTs) produced by global protein enzymatic digestion. The strategy exploits (FTICR-MS) to validate peptide AMTs for a specific organism, tissue, or cell type from potential mass tags identified using a conventional tandem mass spectrometry (MS/MS) method. The result is a method that provides greater confidence in identification, greater sensitivity, less time, and increased throughput.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become an important analytical tool in proteomics due to its accurate mass determination, detection sensitivity, and ease of automation. Although MALDI-TOF-MS can be considered as a method for direct infusion for the analysis of proteins and peptides, direct MALDI-TOF-MS analysis of complex protein or peptide samples can be severely compromised due to ion suppression effects, similar to FTICR. In addition, CE is a microanalysis technique, in which only a few nanoliters is needed for analysis. MALDI-TOF-MS has a mass range of over 300 kDa and is relatively insensitive to the presence of buffering compounds, salts, and surfactants, unlike typical LC-MS. CE and MALDI-TOF-MS provide advantages of high throughput, high resolution, compatible sample flow and volume issues, and minute sample consumption for protein and peptide analysis over conventional analytical methods such as LC methods. CE can be utilized with MALDI-TOF-MS in either on-line or off-line mode.

Off-line CE-MALDI-TOF-MS is normally performed by depositing CE fractions on MALDI probes, followed by MALDI-TOF-MS analysis of these fractions. Generally, signal-to-noise ratio decreases with increasing buffer concentration without degrading mass accuracy. Ionization of organic additives, such as anionic surfactants, nonionic surfactants, and cyclodextrins, is buffer dependent and problematic when the mass of the additive is in the range of the peptide mass. An off-line CEMALDI-TOF-MS examination was performed using BSA as a model analyte (106). An off-line vacuum deposition method was used to continuously

deposit sample eluting from a high-resolution CE system via a serpentine streaked path onto a moving standard stainless steel MALDI target. The target is manipulated via computer-controlled X–Y translation stages, in a vacuum chamber, resulting in a uniform and highly reproducible MALDI signal. In addition, an off-line mode appears to be the only way to implement the current deposition method. The width of the sample streak and the laser spot size are both on the order of 100 μm . The MALDI target moves at the rate of 1 mm/s, resulting in 10 points/s across a typical peak (half-height peak widths are $\sim 3\text{--}5$ s) eluting from the CE separation capillary. The result is a reproducible signal with an excess of data points to define the peak for quantitative purposes.

A comparison of the CE vacuum-deposited trace method and a standard dried droplet method was performed. The shot-to-shot reproducibility for the vacuum-deposited trace method was $\sim 5\%$, 1 order of magnitude better than the standard dried droplet method. In addition, the sequence coverage for the trace method was 50%, versus 30% for the drop method. Furthermore, the target plate can be reanalyzed in TOF/TOF MS mode after an MS analysis is performed, at different segments of the trace relative to peptide elution for better peptide coverage. For example, an unidentified peptide from a BSA digest using CE–MS was resampled in TOF/TOF MS mode, resulting in data that suggested the presence of various residues with an almost complete b and y characterization. The peptide was not observed at all using the dried drop method. It is demonstrated that the off-line coupling of high-resolution CE with MALDI-MS and MALDI-MS/MS allows higher sequence coverage and better sample characterization than the dried droplet method. In addition, due to a lack of time constraints regarding separation timing and MS acquisition speed, the impetus for off-line CE and MS analysis is established. An earlier related paper used the vacuum method for a multicapillary on-line protein analysis with a less complete characterization of CE-MALDI-TOF-MS, but the method appears to be valid (107). A comparison of CE-TOF, LC-TOF, and direct MALDI was performed; between the three methods, the CE-TOF had the highest sequence coverage (108).

CHEMICAL CYTOMETRY: SINGLE-CELL ANALYSIS BY CAPILLARY ELECTROPHORESIS

Single-cell analysis with capillaries has emerged as a growing technique since it was first realized 15 years ago (109). Indeed, for every technological advance there is a greater push to inspect smaller subunits of a biological process. Conventional flow cytometry provides information on a handful of cellular components; chemical cytometry, which is the analysis of cellular contents using analytical methods, allows the potential for analysis of thousands of components per cell. In particular, CE-LIF has the separation power and achievable detection limits for these types of studies. In addition, other detection systems are being investigated.

We have a long-term goal to perform single-cell protein analysis using CE with ultrasensitive sheath-flow LIF detection. Initially, we used submicellar CE to monitor the total proteins expressed in a single HT29 human colon cancer cells. Recently, we reported using CSE for cell cycle-dependent protein fingerprinting of single cells (32). The cellular proteins were denatured with SDS, labeled with FQ on-column, and then separated by using 8% pullulan as

the sieving matrix. This method has provided the first size-based analysis of proteins in a single cell and now is used to investigate cell cycle-dependent protein content (32). We were also able to determine the amount of a specific protein within a single cancer cell (110).

A CE-LIF method has been developed for detection of micrometer- and submicrometer-sized biological vesicles such as individual cell nuclei, which have been labeled with 10-*N*-nonylacridine orange, SYTO-11, Mito-Tracker Green, and hexaiodine oxide (70, 75, 77, 111). The mobility distributions of nuclei isolated from mouse hybridoma cells were successfully measured. The distribution of protein content per mitochondrion and the relative abundance of mitochondrial proteins in density gradient fractions were determined. This method is useful for counting mitochondria and, as a consequence, determining the number of mitochondria per unit volume or estimating mitochondria copy number per cell (77). In a related paper, CE-LIF was utilized to measure the cardiolipin content of individual mitochondria from cultured NS1 cells. Mitochondria were isolated by differential centrifugation and stained with the fluorescent dye 10-*N*-nonylacridine orange. Individual mitochondria were separated and detected by CE with LIF detection; the cardiolipin contents of individual mitochondria were determined and ranged from 1.2 to 920 amol (111).

Single mouse myeloma cells (NS-1) that were treated with doxorubicin, a chemotherapy drug, were injected onto a CE postcolumn LIF instrument. The drug and its metabolites were separated and quantified at the zeptomole level (112). In a similar technique, phosphorothioate antisense drugs were evaluated for interactions with lipids and intracellular proteins in HeLa cells (113). The uptake was measured and cell-to-cell variation determined, $\sim 50\%$ RSD. A technique that could be used to determine gene expression of a single cell has been developed utilizing CE-LIF (114). CE-LIF was utilized to measure the amount of serotonin in single serotonergic matabebral (sea slug) cells (115). Lillard and co-workers have separated protein and RNA from cell lysates and single cells (116, 117). Rat basophilic leukemia cells were loaded with Oregon Green, electrically lysed, and injected onto a capillary for lysing evaluation purposes utilizing CE-LIF (118). There have also been recent advances in wavelength-resolved laser-induced fluorescence detection coupled with CE; see specific review (119).

Other detection methods such as MS and electrochemical detection are also possible for single-cell analysis. Jin and co-workers have published a number of papers for single-cell analysis of enzymes, histamine, amino acids, and glutathione in many types of cells utilizing a carbon fiber disk for electrochemical detection (83, 84, 86, 87, 90, 120). A similar technique is also used to determine dopamine content in single rat pheochromocytoma cells (121). A novel etched electrochemical detector has also been recently reported for single-cell analysis (94). A radionuclide detection technique for CE MALDI-MS for zeptomole detection limits was used to analyze peptides from single neurons (122). A microfabricated device was used to monitor DNA fragmentation associated with apoptosis in cardiomyocytes (124).

DNA ANALYSIS

The history of DNA sequencing technology spans less than three decades. During the lifetime of the authors of this review, that technology has progressed from an era where the determination of a single base would occupy the efforts of a researcher for many years to the current status, wherein the generation of a draft sequence for a mammal requires a few months' effort of a team of a dozen researchers (127).

A cadre of analytical chemists devoted a decade's effort to develop the current generation of sequencing technology, which is based on capillary electrophoretic separation of DNA sequencing fragments. The importance of this technological advance cannot be overstated. This instrumentation is directly responsible for a revolution in the biological sciences. As of early 2004, over 160 microbial genomes are completely sequenced and 44 eukaryote sequences are at various stages of completion. In perhaps the most stunning example, a survey sequence of the dog genome at 1.5X redundancy was reported (127). This project required the effort of a dozen researchers. In contrast, over 200 researchers are listed as coauthors on the paper that reported the sequence of the mouse (128). This astonishing improvement in efficiency is a result of the widespread use of automated capillary electrophoresis sequencing instruments.

Fundamentals of DNA Electrophoresis. The theory of DNA separations by capillary electrophoresis is subtle and difficult. Several reviews have appeared that describe the state of the art in modeling of DNA electrophoresis (129–131), and interesting results have been obtained for the variation of mobility and diffusion coefficients (132, 133).

Slater has addressed an interesting question in DNA separation by capillary sieving electrophoresis (134). In slab-gel electrophoresis, discontinuities and gradients in the polymer composition are used to sharpen bands. In disk electrophoresis, migrating molecules travel from a low-density gel to a higher density region; in doing so, the migrating molecules stack at the interface. This stacking leads to band sharpening. In gradient gel electrophoresis, the gel composition is modified so that the effective viscosity of the polymer increases with distance down the gel. In this case, a wide range of molecular size can be separated, where the gel composition is tailored so that gels have small pores at the bottom, which is optimized for small-molecule separation, and large pores at the top, which is optimized for larger molecules. Slater studied the behavior of molecules in a capillary electrophoresis system with viscosity gradients. Capillary electrophoresis differs in an important respect from slab gel electrophoresis. Capillary electrophoresis employs an end point detector, where all analyte must traverse the length of the capillary to be detected. As a result, all molecules experience the entire composition. In contrast, slab-gel electrophoresis is a snapshot detection, where molecules are detected at a point in time, and different molecules have traversed different portions of the gel. Slater demonstrates that viscosity gradients do not lead to improved resolution in capillary electrophoresis because all molecules experience the entire gradient—there is no improvement in performance in a gradient system.

Instrumentation. Sequencer development appears to have slowed over the past few years. Commercial instruments are approaching maturity, and efforts in instrumentation development have declined compared to the past decade. Those efforts seem

to be focused on the development of high-throughput microfabricated devices (135, 136).

Perhaps the most interesting developments deal with advances in single-molecule detection and analysis of DNA fragments. Yeung's group has reported a high-throughput method to quantify minute amounts of DNA without the need for polymerase chain reaction amplification (137). The same group studied the details of migration of single DNA molecules in the presence of both electrokinetic and pressure-driven flows (138). They observed unexpected radially directed movement of the molecules across the flow gradient and have exploited this phenomenon to separate large DNA fragments in a manner reminiscent of hydrodynamic chromatography (139).

Karger has reported a fraction collector for capillary array electrophoresis, which was applied to the separation and purification of DNA fragments (140, 141).

Both UV absorbance detection and two-color excitation for fluorescence detection has been reported for DNA sequencing by capillary array electrophoresis (142, 143). The former method requires four separation capillaries per sequencing run to determine the sequence. The latter method is reminiscent of Prober's sequencing instrument that was employed in the original DuPont DNA sequencer, albeit with two excitation rather than emission wavelengths (144).

The Simplex algorithm has been used to optimize electrokinetic injection of DNA fragments (145), and a neural network has been used to optimize the separation conditions with hydroxypropyl cellulose (146).

Work continues on the development of near-infrared dyes for DNA sequencing, and conditions have been optimized based on lifetime discrimination (147).

Detection of fluorescence emission intensity in two planes of polarization can be used to estimate the molecular weight of the migrating analyte (148). This polarization detection has been used to detect DNA damage through use of a fluorescently labeled antibody against thymine glycol, which binds to the damaged DNA (149).

Sieving Media. It has become clear that the high-resolution capillary electrophoresis separation of large DNA sequencing fragments requires the use of dilute, high molecular weight polymers, and much work has been devoted to the development of improved polymers for sequencing and mutation detection (150, 151).

Denaturants are important in removing the secondary structure of DNA fragments, eliminating compressions, and ensuring uniform fragment spacing during separation. Urea and dimethyl sulfoxide were used in high-speed sequencing (152), as was operation of capillaries at elevated temperatures (153).

Polyacrylamide is not ideal for DNA separations. It requires the use of rigorous wall coating to eliminate electroosmosis. The material undergoes shear thinning, which is caused by deformation during filling of the capillary; many minutes are required for relaxation of the deformation. Alternative polymers have been developed that combine hydrolytic stability and adsorption to the capillary wall to eliminate electroosmosis (11, 154, 155). Mixed solutions of immiscible polymers have shown interesting properties (156).

Comblike polymers have been reported as another class of low-viscosity, self-coating matrixes for DNA separations (157).

Sample Preparation. Sample preparation looms as an important issue that occupies the attention of large-scale sequencing facilities (158). Conventionally, many preparation reactions are performed by use of a microtiter plate. These plates, while able to process a large number of samples in parallel, require relatively large sample volumes. The expense of the reagents used in these relatively large-volume reactions is a significant factor in large-scale genomic sequencing efforts.

A clever concentrator has been developed based on opposing hydrodynamic and electrophoretic flows (159, 160). Molecules with zero net mobility are concentrated, while low molecular weight ions, which otherwise interfere in electrophoresis, are flushed to waste.

The use of organic solvents for sample preparation has been shown to provide significant advantages in generating high-purity nucleic acids for analysis (161).

Quantitative DNA Analysis. The quantitative determination of DNA can be difficult. Concentrations can span 12 orders of magnitude, which presents a formidable analytical challenge. The polymerase chain reaction is a powerful tool for studying low-concentration oligonucleotides. Unfortunately, the highly nonlinear response of PCR complicates quantitative analysis. TaqMan chemistry has been developed to deal with this wide dynamic range issue; measurement of the amount of product after each cycle is used to determine analyte concentration. However, generation of TaqMan probes can be expensive. A capillary electrophoresis version of this system was demonstrated for the determination of hepatitis B viral titer (162).

Polymorphism Detection. The DNA sequences of any two individuals differ at roughly the part-per-thousand level; those differences account for genetic predisposition for diseases and form the basis for forensic identification and paternity determination. The gold standard for determination of polymorphisms is to sequence the genomic region of interest, and sequencing will be the method of choice as costs plummet. A number of other capillary electrophoresis methods have been developed for both detection of specific mutations and more general mutation screening (163–165). One approach is to employ minisequencing, which consists of a single base extension with a labeled dideoxynucleotide from a primer located at a putative mutations site (166, 167), while another relies on temperature gradient electrophoresis to monitor single-stranded conformation polymorphisms (76).

DNA mutation has many causes, including adduct formation with polycyclic aromatic hydrocarbons. Selected classes of these mutations can be detected if an antibody is available against the adduct. In one example, an antibody against benzo[*a*]pyrene diol epoxide was used to characterize the adduct (168). Similarly, single-stranded binding protein has been used to distinguish single-strand and double-strand DNA in hybridization assays (169).

Aptamers. DNA and RNA oligomers can be used in place of antibodies in the analysis of specific analyte. Binding parameters for these aptamers can be generated using nonequilibrium electrophoresis of equilibrium mixtures (170, 171). The effect of buffer composition and electric field were investigated for detec-

tion of aptamer–ligand complexes by capillary electrophoresis (172).

LIPIDS, FATTY ACIDS, AND CARBOHYDRATES

Lipids and Fatty Acids. The chemical and physical properties of cell membranes are largely dependent on their lipid composition. Lipids are a diverse group of molecules and their analysis, as well as lipidylation analysis, not only provides insight to membrane structure and function but could also contribute to our understanding of the role of lipids in modulating the function of a variety of signaling proteins. Currently, liquid and gas chromatographies are the most commonly used methods for separation of lipids and fatty acids.

There have been a number of reports on the use of capillary electrophoresis to monitor interaction of proteins with lipids. Capillary electrophoresis has been used to characterize apolipoprotein binding with lipids (173). Study of lipid and apolipoprotein binding interactions using vesicle affinity capillary electrophoresis and an NBD-derivatization scheme was used to characterize low-density and high-density lipoproteins (174, 175).

A review has appeared on derivatization chemistry for photolipids, with several examples taken from the capillary electrophoresis literature (176).

A fluorogenic reagent, 10-*N*-nonylacridine orange, has been used to study cardiolipin, an important phospholipid associated with mitochondria (177). This technique was used to study the effect of UV light on translocation of this lipid from the inner to outer mitochondrial membrane. Similarly, the cardiolipin content of individual mitochondria was determined with the same reagent (111).

Lipid-modifying enzymes have also been studied using capillary electrophoresis methods. These studies include use of fluorescently labeled substrates, which undergo enzymatic modification. The substrate and product contain identical labels and may be detected with the same laser-induced fluorescence instrument (178). A particularly interesting example considers the study of the activity of farnesyltransferase, which is the enzyme responsible for the activation of the RAS oncogene (179).

Micellar electrokinetic chromatography was used for the analysis of three classes of aminophospholipids. Each class consists of a set of molecular species, which were resolved with a cyclodextrin-based buffer system (180).

Phospholipids have also been used to create a bilayer coating for separation of proteins by capillary electrophoresis (8). A zwitterionic phospholipid formed a semipermanent wall coating to reduce protein interaction, and the chain length of the lipid was important in generating a useful coating.

Carbohydrates. Most carbohydrates are neutral, and their separation by capillary electrophoresis requires complexation of the carbohydrate with a charged ligand. Rather than using this complexation to effect separation of carbohydrates, capillary electrophoresis can be used to study the complexation phenomenon itself. Capillary electrophoresis was used to determine the binding constants between dextrin oligosaccharides and analgesic drugs (181). Results were similar to those determined by NMR. The interaction of heparin sulfate with growth factors was studied by capillary electrophoresis (182). Similarly, the use of capillary electrophoresis to study protein–carbohydrate interactions was

reviewed (183). Heparin itself was studied by capillary electrophoresis (184).

Most carbohydrates are essentially spectroscopically featureless. Their detection requires derivatization, which has been reviewed by Honda (185). Underivatized carbohydrates can be detected by mass spectrometry (186). Carbohydrates have been derivatized with benzylamine, separated by CE in an ammonium acetate buffer, and detected by off-line MALDI-MS. A much more powerful Fourier transform mass spectrometer was used to characterize complex oligosaccharide mixtures following capillary electrophoresis (187). NMR has been used to characterize monosaccharides in capillary electrophoresis (188).

Although capillary electrophoresis cannot separate neutral compounds, capillary electrochromatography can. Novotny reported the separation of neutral saccharide mixtures using hydrophilic monolithic columns (189). This system was interfaced with a mass spectrometer for identification of low-femtomole amounts of the saccharides.

Finally, a series of papers appeared in *Methods in Molecular Biology* that deal with a number of issues in carbohydrate analysis by capillary electrophoresis, including glycosyl transferase analysis, mass spectrometric analysis of glycoproteins, monitoring protein isoforms, and characterization of glycosaminoglycans (190–193).

ENZYME ANALYSIS

Capillary electrophoresis remains a powerful tool for enzyme analysis. The rapid separation of substrate from product, the minute amount of sample required for analysis, and the identification of unexpected reaction products are all valuable properties of the analytical method. However, manipulation of minute sample volumes is problematic, and Lorieau reported a quantitative nanopipettor for sampling reactions for subsequent capillary electrophoresis analysis (194).

In an elegant application of capillary electrophoresis to enzyme assays, a set of GDP-5-thiosugars was synthesized and used as glycosyl donor substrates for glycotransferases (195). A fluorescently labeled acceptor was synthesized and used as a substrate in the assay. Incorporation of the thiosugar resulted in a mobility shift during capillary electrophoresis/laser-induced fluorescence detection, which provided an extremely facile and sensitive method to monitor the enzymatic reaction. Similarly, Kanie reported a study of glycosidases based on on-column reaction (196). A system for characterization of sialoglycans or sialoglycoproteins was based on capillary electrophoresis that employed *N*-acetylneuraminidase in the running buffer; hydrolysis released *N*-acetylneuraminic acid, which is detected by UV absorbance (197).

A fluorescently labeled gliadin peptide was synthesized and used as a substrate for tissue transglutaminase; deaminated and transaminated products were determined by capillary electrophoresis (198).

Colyer reported a protease assay based on digestion of a labeled protein. Incorporation of a large number of dye molecules resulted in severe quenching. Digestion by a protease generated peptides, which restored the fluorescence (199).

Although capillary electrophoresis produces excellent detection limits and allows the use of a wide range of substrates, conven-

tional enzyme assays performed in multititer plates tend to have higher throughput. He and Yeung reported the use of multiplex capillary electrophoresis with UV absorbance detection for high-throughput screening of kinase inhibitors (200). This system has the potential to generate higher throughput than conventional microtiter plate-based assays.

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Wes Quigley received his B.S. (1993) and M.S. (1995) in chemistry from Western Washington University and his Ph.D. from the University of Washington in 2002, where he is now a postdoctoral fellow.

Norman Dovichi received his B.S. in chemistry and mathematics from Northern Illinois in 1976, and he received his Ph.D. from the Department of Chemistry of the University of Utah in 1980. He spent two years as a postdoctoral fellow with Richard Keller at Los Alamos Scientific Laboratory. His first academic appointment was in the department of chemistry at the University of Wyoming. He moved as an associate professor to the University of Alberta in 1986. He moved to the Department of Chemistry at the University of Washington in 2001, where he holds the endowed professorship of analytical chemistry. He is also an affiliate member of the Institute for Systems Biology in Seattle and holds an honorary professorship in the Chinese Academy of Sciences—Dalian Institute for Chemical Physics.

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