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Capillary Electrophoresis for the Analysis of Biopolymers

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Review Contents

Analytical Chemistry has changed its policy on its biannual reviews. Rather than providing an encyclopedic listing of papers in a specific topic, authors are asked to focus their reviews more tightly and to list no more than 200 references. There were over 2500 papers and over 200 review articles published on capillary electrophoresis over the past two years. In keeping with the spirit of *Analytical Chemistry*'s policy, we have chosen to focus our review on applications of capillary electrophoresis to chemical biology. In particular, we are interested in the use of this very important analytical tool for the study of four classes of biopolymers: nucleic acids, proteins, lipids, and carbohydrates. While this review formally covers 1998 and 1999, we have taken the liberty of including a few, late-breaking references from this year.

Since the last biannual review (*1*), capillary electrophoresis with laser-induced fluorescence detection has become a dramatically successful analytical tool with widespread application in the biological sciences. Large-scale capillary array electrophoresis instruments have been marketed by PE Biosystems (the model 3700 Genetic Analyzer) and by Molecular Dynamics (MegaBASE). These instruments operate 96 capillaries simultaneously and automatically. In most applications, each instrument generates 10 runs and 1000 electropherograms per day. Over 1000 of the PE Biosystems instruments and a similar number of instruments from Molecular Dynamics were shipped in 1999, providing an astonishing analytical capability: millions of samples are analyzed daily by capillary electrophoresis.

The primary application of capillary electrophoresis has been in DNA analysis, and most interest is in large-scale DNA sequencing. Considering that over 500 different fragments are identified in a typical sequencing run, roughly a *billion* components are separated every day by capillary electrophoresis, which arguably makes this technology the most important analytical tool in use today.

Although genomic research is the most important application of capillary electrophoresis today, we believe that proteomic research will come to dominate the field over the next decade. In many respects, proteomic research today is at a similar stage as genomic research was a decade ago. Semiautomated technology is just becoming available for some parts of proteomic research, but manually intensive slab-gel electrophoresis is the primary tool for protein separations. We believe that fully automated capillary electrophoresis instruments will displace cumbersome slab-gel electrophoresis for protein analysis. We also believe that there will be an explosion of research effort in proteomics once the appropriate instrumentation is widely available.

Lipid and carbohydrate analyses are in their infancy. It has been four decades since researchers began to realize that complex oligosaccharides carry messages essential for the control of many cellular functions. It is now apparent that the oligosaccharide chains of glycoproteins and glycolipids can no longer be ignored. Similarly, it is now realized that lipoproteins are important membrane-bound signal transduction molecules; the lipid portion of the molecule is vital to the function of the protein. However, significant challenges to the analytical community are provided by the extreme difficulty in obtaining large amounts of these compounds, the heterogeneity in their formation, and the complexity in their structure. Capillary electrophoresis will be a powerful tool in lipid and carbohydrate analysis.

NUCLEIC ACID ANALYSIS BY CAPILLARY ELECTROPHORESIS

DNA is the information storage medium of all self-replicating organisms and many viruses. This information is stored in the organism's genome. This genetic information is transcribed into messenger RNA (mRNA), which is transported from the nucleus to ribosomes, which translate the mRNA into protein. These proteins are further modified by addition of lipids, carbohydrates, phosphates, and other groups.

In this section of the review, we first consider the use of capillary electrophoresis for DNA analysis. Sequencing is the primary tool used to characterize DNA. We also consider the analysis of specific genetic markers that are highly variable (polymorphic) between individuals.

We next consider postreplication modifications of DNA. These modifications are analogous to posttranslational modifications of proteins. The only known biological modification in multicellular organisms is cytosine methylation; analysis of this modification

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is in its infancy. Other modifications of DNA can occur, such as DNA adducts, which are not of biological origin and are a form of DNA damage.

At the end of this section, we briefly consider the use of capillary electrophoresis for RNA analysis. Messenger RNA levels provide information on protein expression. However, hybridization arrays are a more powerful method for mRNA analysis, and capillary electrophoresis may not prove particularly important in gene translation studies.

DNA Sequencing. The fundamental tool for DNA analysis is sequencing, which is the determination of the order in which nucleotides are found along the linear DNA polymer. Sanger's dideoxynucleotide method is the technology of choice for DNA sequencing. In this method, a DNA polymerase enzyme is used to synthesize a set of oligonucleotides that all start at a common primer sequence, are complementary to the target sequence, and are terminated by incorporation of dideoxynucleotides. The terminal nucleotide is identified by incorporation of one of four fluorescent labels. This sequencing ladder is separated on the basis of size by means of electrophoresis. The spectral property of the fluorescent label is identified for each fragment as it migrates through a laser-induced fluorescence detector. The sequence of the target DNA is determined from the order in which the fragments migrate from the electrophoresis instrument. Until recently, electrophoresis was performed on slab gels. Although semiautomated instruments were available for slab gels, slab gels nevertheless require many manual manipulations in their preparation and operation.

About a decade ago, analytical chemists began the development of capillary electrophoresis for DNA sequencing. It was realized that the highly flexible capillary systems, when combined with high-sensitivity laser-induced fluorescence detectors, were ideally suited for the automated analysis of DNA sequencing ladders. Two major problems were faced: the development of high-sensitivity laser-induced fluorescence detectors for capillary arrays and the development of replaceable separation media so that the instrument could be fully automated.

These problems have been solved and large-scale capillary array DNA sequencers have been commercialized by Molecular Dynamics and PE Biosystems. These instruments are designed to sample DNA held in microtiter plates and to operate unattended with 2 h required for each separation.

1. Instrumentation and Methods. The first capillary array DNA sequencer was reported in 1990 by Zagusky and McCormick at DuPont (*2*). This system scanned a laser beam across an array of capillaries, recording spectral information from each capillary to identify the migrating DNA fragment. More sophisticated versions of this scanning system were developed by the Mathies group and are employed in the Molecular Dynamics instrument (*3*). A very large-scale instrument has been proposed by that group based on a rotating optical system to interrogate over 1000 capillaries (*4*). Sequencing read lengths of over 500 bases were obtained from a majority of the capillaries.

Rather than using a scanning system, several groups have developed imaging instruments that allow the simultaneous monitoring of the separation from each capillary in the array. These imaging instruments eliminate the dead time between successive illumination of a capillary in a scanning system. We reported a set of instruments based on a linear sheath-flow cuvette (*5*−*7*). In these instruments, the capillary array is inserted into a rectangular cuvette that holds the capillaries like the teeth of a comb. Buffer is pumped between the capillaries, drawing sample from each capillary as a thin stream. A single laser beam skims beneath the capillaries in the sheath fluid. Since the laser beam only traverses the buffer and highly dilute DNA streams, the low-power beam can excite fluorescence from all of the samples simultaneously. This design has been incorporated into the PE Biosystems instrument. In a modification of this design, Yeung's group used an array of square capillaries for separation of the DNA sequencing fragments; side illumination of the array allows simultaneous excitation of the samples without the need for a scanning detector (*8*).

Several groups have investigated the use of fluorescent lifetime, rather than fluorescent spectra, to distinguish the set of four dyes used in the Sanger sequencing reaction. McGown reported the use of phase fluorometry to resolve the fluorescence lifetime of a set of dye-labeled primers (*9*). They observed that the lifetime for one set of dyes is independent of the nature of the sieving matrix (*10*). Soper reported a set of near-IR dyes for DNA sequencing. These dyes relied on heavy-atom modification to produce similar excitation and emission spectra but with lifetimes ranging from 889 to 735 ps (*11*). Sauer and colleagues at the Univerität Heidelberg reported the use of diode laser-excited time-resolved fluorescence detection in capillary electrophoresis DNA sequencing. They used rhodamine, oxazine, and cyanine dyes that have similar excitation and emission spectra. However, the lifetime of the dyes varied from 3.7 to 1.6 ns, which was sufficient to allow discrimination of the dyes and the determination of sequence (*12*).

The size-to-charge ratio and the free solution mobility of DNA depend weakly on the size of the fragment. Therefore, DNA electrophoresis is almost always performed in the presence of a sieving matrix that induces a size-dependent retardation on the DNA mobility. However, the synthesis of high-performance and low-viscosity sieving matrixes is not simple, and the engineering required to pump these matrixes into the capillary adds significant expense to commercial instrumentation. End-labeled free-solution electrophoresis (ELFSE) is one approach to the elimination of the sieving matrix in DNA separations (*13*). In this separation method, a large and weakly charged molecule is used to label the DNA fragment. The mobility of the labeled DNA fragment is dominated by the size of the label and the charge of the DNA fragment. Because the charge of the DNA fragment increases linearly with the number of nucleotides, the size-to-charge ratio, and hence the mobility, of the labeled DNA fragment is inversely proportional to the size of the DNA fragment.

There has been a fair amount of interest in DNA analysis in microchip-based systems. The vast majority of these analyses have been performed with DNA hybridization arrays (*14*). However, those systems do not rely on electrophoresis; they will not be considered further in this review. Instead, we consider electrokinetically pumped chip-based systems.

An important step in genotyping, which is the identification of polymorphisms in DNA, is the polymerase chain reaction. Manz's group reported an elegant chip-based PCR method, wherein the DNA fragments pass in a serpentine pattern through hot and cool regions, undergoing denaturation, hybridization to primers, and

amplification (*15*). Chips have been used for the analysis of PCR and sequencing products (*16*−*22*). The issue of sample cleanup on-chip has also received attention (*23*). To date, the resolution of chip-based DNA separations has lagged that produced by capillaries, primarily because of the short separation path available in most chips. To obtain high-resolution separations, injection and detection volumes must be reduced to the smallest possible values (*24*). Over 500 bases of sequence have been obtained for real-world sequencing applications in microchip instruments (*25*).

2. Sieving Media. The commercial DNA sequencers require the use of a relatively low-viscosity separation medium that can be replaced quickly and under modest pressure. The best results to date have been obtained with a high molecular mass polyacrylamide (*26*). Karger has demonstrated spectacular DNA separations with the high-molecular-weight medium; over 1000 bases of sequence were determined in 2-h period. While the static viscosity of this polymer is very high, the material appears to undergo a dramatic decrease in viscosity under high shear, such as when pumped into a capillary. As a result, relatively modest pressure is required to fill the capillary with the high-molecular-weight material.

Commercial instruments often use dimethylacrylamide (*27*). Dimethylacrylamide appears to be easier to synthesize, is hydrolytically more stable, and is made from less toxic monomers than polyacrylamide.

There have been quite a few papers over the past few years that consider the use of other polymers for DNA separations. The hydrophobic, end-capped polymer *n*-dodecane−poly(ethylene oxide)−*n*-dodecane is interesting because it forms a micellar network at concentrations greater than the critical value (*28*). Other polymers, including pluronic copolymer liquid crystals (*29*), manitol (*30*), poly(*N*-isopropylacrylamide)-*g*-poly(ethylene oxide) (*31*), poly(vinylpyrrolidone) (*32*), poly(acrylamide)−poly(β-D-glucopyranoside) (*33*), poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) (*34*), and poly(ethylene oxide) (*35*), have been used with some success.

Extremely dilute polymer buffers have been used for the separation of very large DNA fragments (*36*). These separations are extraordinarily rapid and can be used for the study of linear and supercoiled DNA.

Low-conductivity isoelectric buffers have been used for nondenaturing separations of double-stranded DNA fragments (*37*, *38*). The low conductivity of these buffers minimizes Joule heating, allowing use of high electric fields for rapid separations. Unfortunately, the onset of biased reptation with orientation results in degradation of separation performance for longer fragments. Interestingly, the cationic form of the buffer appears to ion pair with phosphate groups of the sugar−phosphodiester backbone of the DNA. This ion pairing lowers the effective charge of the DNA and helps stiffen the backbone, which results in greater susceptibility of the DNA to biased reptation with orientation, which degrades resolution for longer fragments. As a result, most applications of the isoelectric buffers appear to be for relatively short fragments produced in genotyping experiments.

3. Sample Preparation. Sample purity is more important in capillary electrophoresis than in slab-gel electrophoresis. Salts, proteins, and other impurities can be present in DNA sequencing samples. These components can cause ionic depletion in the capillary tip due to differences in transference number between the separation medium and the running buffer. In slab gels, current can flow in the space between sample lanes, ensuring the sample experiences a uniform electric field, irrespective of sample impurities. In capillary electrophoresis, there are no current paths that do not pass through the sample, so that these impurities can significantly degrade the separation of DNA sequencing fragments.

Conventionally, DNA sequencing samples are treated to an ethanol precipitation step to remove proteins and some salts. However, this procedure involves centrifugation, which can be difficult to automate in an on-line system. Instead, Karger described sample cleanup based on spin-column technology (*39*, *40*), which still requires centrifugation. Swerdlow pioneered the development of automated on-line sample cleanup (*41*). The use of a simple sample stacking procedure proved particularly useful in the elimination of sample impurities. Yeung reported an automated sample preparation system that processes eight samples simultaneously based on size-exclusion chromatography (*42*, *43*). The system has also been used for genotyping applications (*44*).

4. DNA Sequencing Applications. The first de novo DNA sequence data generated by capillary electrophoresis was 554 bases submitted to GenBank in early 1997 for a gene coding for heat-shock protein from a set of *Staphylococcus* species (*45*). In the past year, well over a billion bases of DNA sequence generated by capillary electrophoresis was submitted to GenBank. Capillary electrophoresis is now the tool of choice for large-scale DNA sequencing efforts.

The highest profile applications are in large-scale DNA sequencing efforts, including the radioresistant bacterium *Deinococcus radiodurans* (*46*), chromosome 2 of *Arabidopsis thaliana* (*47*), and the complete sequence of *Drosophila melanogaster* (*48*). The latter case is best documented; over 1.4 billion bases of raw sequence were generated over a four-month period using 300 capillary array DNA sequencers (PE Biosystems model 3700) to determine the 120 million-base euchromatic portion of the *Drosophila* genome. The accuracy of the assembled sequence was 99.99% in nonrepetitive regions. In the 2.5% of the region comprising the most highly repetitive sequences, the accuracy was 99.5%. This sequencing effort is the largest qualitative analytical project to date, which has determined the primary structure of a group of molecules with a combined molecular mass of 8×10^{10} g/mol.

These large-scale sequencing efforts would have required decades of work using classic electrophoretic technology. In contrast, the combination of whole-genome shot-gun sequencing with capillary electrophoresis required four months to complete the sequence of *Drosophila*. Roughly nine months of work with capillary array electrophoresis will be required to determine the sequence of the human genome, and this effort will be essentially complete by the time that this article is in print.

5. Genotyping and Mutation Detection. DNA sequencing is the ultimate genotyping tool, since it detects all polymorphisms (*49*). However, the sequencing reaction can be cumbersome. Instead, PCR amplification and capillary array electrophoresis instruments are well suited to large-scale genotyping applications, where specific DNA markers are determined (*50*). These markers are short tandem repeats that vary in length between individuals, single-nucleotide polymorphisms that consist of single-base dif-

ferences, and other polymorphisms. If enough polymorphisms are available, then individuals can be identified from forensic samples (*51*). If specific polymorphisms are found to correlate with a disease, then the gene associated with the disease may be found in a neighboring region of the genome. Applications have been found in psychiatry and clinical chemistry (*52*, *53*). Last, variations in viral genetic information may prove valuable in guiding therapy and tracing the origin of infectious outbreaks (*49*).

A mutation is a difference in DNA sequence between an individual and the consensus or wild-type sequence that causes a phenotypic difference. Mutation detection differs from genotyping because the mutation is usually unknown, whereas genotyping is based on well-characterized polymorphisms.

Single-strand conformation polymorphism (SSCP) is the most common capillary electrophoresis-based DNA mutation detection method. In SSCP, a single-stranded DNA fragment is analyzed under nondenaturing conditions. Interstrand hydrogen bonds cause mobility shifts that are characteristic of the internal sequence. If a target and wild-type sequence have different mobilities, then the target likely contains a mutation (*54*). The effects of temperature and pH have been considered in optimizing the sensitivity of the separation to mutations (*55*).

There are other techniques to isolate mutations. The mismatched region in the sequence between the wild-type and target sequence can be chemically cleaved; separation of the resulting fragments can identify the location of a mutation or polymorphism (*56*).

If the mutation is known, then PCR amplification and capillary electrophoresis analysis can be used to detect the mutation. This procedure can be multiplexed so that several mutations can be detected in a single capillary separation (*57*, *58*).

6. Antisense Drug Analysis. There has been a fair amount of interest in the use of antisense therapeutic agents. These compounds are oligomers that have unnatural backbones and contain a sequence of bases that is complementary to that of the gene or mRNA of interest. If the antisense drug binds strongly to the genetic material, transcription of the gene is halted. Similarly, if the nucleotide binds strongly to the mRNA, translation is halted.

Because of their strong binding to DNA or RNA, antisense drugs are not easily assayed by the polymerase chain reaction. Instead, the compounds must be analyzed without amplification, which can be a significant challenge in biological matrixes. Isotachophoresis has proven to be a useful tool in preconcentration of antisense drugs, producing more than a 2 order of magnitude increase in concentration (*59*−*61*).

7. Viral Infection Detection. The detection of low-level virus titers is an important clinical tool to guide therapy. The combination of the polymerase chain reaction and capillary electrophoresis is an obvious tool for the study of these viruses. HIV has received the most attention (*62*, *63*). There has been some interest in hepatitis B and hepatitis C (*49*, *64*). Some chip-based separations have also been performed on hepatitis C (*65*). However, viral titer quantitation by capillary electrophoresis remains in its infancy, and much work remains to be done.

RNA and Gene Expression. The genome contains the information necessary to describe all proteins within an organism. However, only a small portion of the genome is expressed under any particular condition. Expressed genes are transcribed to mRNA, which is translated to protein. mRNA itself is difficult to analyze directly. However, some viruses contain enzymes that allow the reverse transcription of mRNA to complementary DNA (cDNA). The reverse transcriptase enzyme is commonly available and used to form cDNA for analysis by powerful polymerase chain reaction technologies.

Capillary electrophoresis has been used to quantitate mRNA, which is vital to understand the relative expression of different genes (*66*). Rather than working with fresh samples, archived paraffin-embedded tissues have been successfully studied by reverse transcriptase PCR and capillary electrophoresis (*67*). This development facilitates longitudinal and historical studies. Prostate tissues have been studied for chemotherapy resistance genes (*68*).

mRNA analysis has been successfully performed on single oocytes by capillary electrophoresis (*69*). This result is quite exciting. When combined with single-cell protein analysis, mRNA levels can be compared with proteins expressed in single cells. This comparison will allow deep understanding of the relative roles of transcription and translation on protein expression during development.

In addition to its information-carrying role, RNA can also act as a catalyst; these RNAs are known as ribozymes. Capillary electrophoresis has been used to monitor metabolism and reaction of the ribozymes (*70*, *71*).

Postreplication Modification. DNA is conventionally viewed as consisting of the four deoxynucleotides: adenine, cytosine, guanine, and thymidine. However, most mammals, and many other eukaryotes, contain a fifth nucleotide, methylcytosine. This nucleotide is not produced directly during DNA replication. Instead, enzymes modify cytosine after replication. The pattern of methylation in the genome is constant in a particular tissue but varies between tissues. It is very tempting to conclude that methylation patterns help define gene expression and the protein content of a tissue. However, there is surprisingly little known about DNA methylation patterns, primarily because of the very primitive analytical tools available for its study. The most common and powerful method in studying methylation patterns is based on a deamination reaction followed by polymerase chain reaction amplification and DNA sequencing. We reported that this technology suffers from systematic bias, we prescribed an appropriate modification for the standard protocol to correct for this bias, and we demonstrated the use of capillary electrophoresis to monitor the methylation status of DNA (*72*).

Unlike cytosine methylation, which is under biological control, a suite of DNA adducts is formed following exposure to environmental contaminants. Jankowiak and co-workers reported the combination of capillary electrophoresis with fluorescence line-narrowing spectroscopy performed at cryogenic temperatures (*73*, *74*). They identified depurinating DNA adducts in the urine of coal smoke-exposed humans. In a very impressive study, zeptomoles of thymine glycols were detected in γ-irradiated lung cancer cells by a capillary electrophoresis-based immunoassay (*75*). It was shown that the repair mechanism for this DNA adduct was induced by low exposures to γ-radiation.

PROTEINS AND PEPTIDES

Separation. Proteins are key participants in all biological regulatory pathways. The change of cellular phenotype during

development or in response to environmental changes occurs by regulating the expression of genes, which produces differential expression of proteins. Therefore, the protein content varies considerably from cell to cell within an organism, even though each cell contains the same genome.

Peptides are generally shorter than proteins. Peptides have a number of biological functions. They act as neurotransmitters, hormones, immunomodulators, regulators of enzyme activity, toxins, and antibiotics. Due to the important role of proteins and peptides, sensitive and informative methods for measuring their concentration are of growing demand in biology and medicine.

Because of their structural similarity, the general principles of the separation of proteins and peptides are similar. Capillary zone electrophoresis (CZE) is the simplest mode and is usually the first choice. Due to a relative simplicity of peptide structure, peptide mixtures are usually well separated using CZE. In rare cases of very homologous peptides, a method that is orthogonal to CZE may be employed (*76*).

In contrast, the separation of protein mixtures using CZE is often unsatisfactory. A number of other separation modes can be used instead, including micellar electrokinetic chromatography, submicellar capillary electrophoresis, SDS capillary gel electrophoresis, and capillary isoelectric focusing. Table 1 lists representative papers describing method development for the separation of proteins using different modes.

1. Capillary Zone Electrophoresis. CZE is the most attractive mode to separate proteins and peptides because of its simplicity. However, analyte adsorption on the capillary wall plagues protein separations. This adsorption results in sample loss, peak tailing, and unstable electroosmotic flow. Electrostatic interaction between the capillary wall and the proteins contributes the most to such adsorption, although there are reports of strong interaction between positively charged capillary surface and positively charged proteins (*98*). Several ways to reduce such interaction have been used. First, for bare fused-silica capillaries, the buffer pH may be chosen so that both the capillary walls and the proteins have the same charge to introduce repulsion between the walls and the proteins. Therefore, pH $\leq$3, where the capillary wall and most proteins are cationic, and pH $\geq$9, where the capillary wall and most proteins are anionic, are logical choices for protein separation by CZE in uncoated fused-silica capillaries. Phosphate buffer at pH $\leq$3 and borate buffer at pH >9 are the most popular separation media. Unfortunately, these extreme pH conditions do not provide a universal solution to protein adsorption. The proteins with p$I \leq 3$ and p$I \geq 9$ will still interact with the walls at pH $\sim$3 and pH $\sim$9, respectively, while more extreme pH may denature the proteins.

There is another disadvantage associated with the classical sodium phosphate buffer. High buffer concentration is required for high-quality separation. The presence of sodium, which is a high-mobility ion, at high concentration results in high currents that can produce unacceptable Joule heating and peak broadening. Replacing sodium with low-mobility counterions has improved the separation quality; a complex mixture containing $\sim$20 wheat proteins has been successfully analyzed using glycine instead of sodium as a low-mobility counterion (*86*). Another way to decrease the buffer conductivity is using isoelectric buffers at pH close to their pI values so that the net charge of the buffer electrolytes is close to zero. Asparagine (p$I = 2.77$) and diacetic acid (p$I = 2.33$) have been used in isoelectric buffers to separate peptides and proteins (*80*, *94*, *117*). Electric fields of up to 1 kV/cm may be applied resulting in fast and effective separation. Two globulin chains differing by a single neutral amino acid have been baseline separated in an isoelectric buffer in the presence of 3% Tween 20 (*117*).

Additives to classical phosphate and borate buffers can reduce protein interaction with capillary walls. Classic additives are zwitterions such as O-PEA (*85*), surfactants such as CTAB (*98*), and amines such as spermine (*118*). Spermine reduces protein−wall interaction by 90% when present at the concentration of only 1 mM (*118*). A number of other additives, namely, cyclodextrins (*89*), dendrimers (*93*), and some charged polymers (*97*), have been recently reported to improve protein separation by CZE.

Another approach to reduce the protein−wall interaction is to coat the inner walls of the capillary. There are several ways to classify capillary coatings. We define static wall coatings as covalent silanol modifications or physical coating of the wall with cross-linked polymers. Static coatings can considerably improve the efficiency of protein separation.

A new static coating with polystyrene nanoparticles has been recently suggested (*98*). The coating is a $\sim$100-nm-thick monolayer. The nanoparticle coating was stable when treated with phosphate buffers from pH 1.5 to 11.5, 1 mM CTAB, saturated NaCl, and tertahydrofuran. However, phosphate buffer of pH 12.4 resulted in 20% decrease of EOF velocity, indicating the loss of positively charged particles at this very basic pH. Separation efficiencies greater than 10^6 theoretical plates were achieved while separating nine model proteins.

Dynamic wall coatings are usually produced by introducing either a neutral polymer (neutral coating) or a polyelectrolyte (charged coating) to the separation buffer. A dynamic coating is not as efficient as a static coating in protecting the proteins from interacting with silanol groups of capillary walls. However, dynamic coatings are much more flexible because the coating can be changed relatively easily without replacing the capillary. A hybrid "covalent−dynamic" coating combining the advantages of both coatings has recently been demonstrated to be useful for protein separation (*90*). A first layer of cationic surfactant is covalently bound to the capillary wall while the second layer is a dynamic coating. This hybrid approach should allow efficient reduction of protein−wall interaction brought by the first layer along with flexibility of coating replacement provided by the second layer.

Despite its simplicity, CZE can have a relatively high resolving power in protein separations. A few applications dealing with complex biological/physiological samples have been developed. We reported resolution of 30 proteins from a single cancer cell using pentasulfate as a submicellar buffer additive (*77*). Another example is the separation of four major and a number of minor proteins of cerebrospinal fluid using a zwitterionic additive, O-PEA (*85*). In this application, CZE was demonstrated to be superior to a classic agarose gel. About 50 proteins in krill lysates were separated by CZE using anionic and cationic fluorosurfactant additives in the separation buffer (*99*). Separation and quantitation of plasma protein is an important tool in diagnosing many disorders in humans and CZE is becoming a routine tool for

Table 1. Separation of Proteins by CE[a]

mode[b]	proteins[c]	capillary	buffer	detection	LOD	ref
CZE	proteins from single cells	uncoated	50 mM phosphate + 11 mM pentasulfate pH 6.8	LIF, covalent labeling with FQ		77
CZE	model proteins, cell lysate	uncoated	2.5 mM Borax and 5 mM SDS pH 9 50 mM phosphate, 11 mM SPS pH 6.8	LIF, covalent labeling with FQ	0.3 pM	78
CZE	model protein: Ova	uncoated	2.5 mM borate + 5 mM SDS pH 9.5	LIF, covalent labeling with FQ	0.1 nM, 0.2 fmol	79
CZE	model proteins: heme-free globin chains	uncoated	50 mM IDA + 6 M urea and 0.5% HEC pH 3.2	UV absorbance		80
CZE	model protein: HSA	uncoated	25 mM borate pH 8.9	LIF, noncovalent labeling with indocyanine green	1.4 nM	81
CZE	whey proteins	methyl deactivated	(i) 50 mM borate pH 9.3; (ii) 120 mM borate pH 9.2	UV absorbance		82
CZE	model proteins: α-Lal, β-Lgl A, β-Lgl B, BSA	uncoated	20 mM CHES + 10 mM KCl pH 10.2	LIF, noncovalent labeling with 1,8-ANS, bis-ANS, and 2,6-TNS	0.2−4 μM	83
CZE	monoclonal IgG + impurities	uncoated	20 mM borate pH 9.3	UV absorbance		84
CZE	proteins of cerebrospinal fluid	uncoated	129 mM borate + 0.5% PEG (MW 8 kDa) + 75 mM O-PEA pH 9.25	UV absorbance		85
CZE	wheat proteins	uncoated	100 mM phosphate/glycine (Na-free) + 20% CAN + 0.05% HPMC pH 2.5	UV absorbance		86
CZE	model proteins: α-Lal, β-Lgl B, CA, HAT, TI	(i) ethylene glycol-coated; (ii) methyl deactivated	50 mM borate pH 9.5	LIF, covalent postcolumn in sheath-flow labeling with OPA	0.7−10 nM; 2−20 amol	87
CZE	proteins from aqueous humor	polybrene-coated	5% acetic acid + 2 mM ammonium acetate	UV; ESI-MS		88
CZE	model proteins: α-Chy, Cyt, Lys, Rnase,	uncoated	50 mM phosphate pH 2.5 + 20 mM CMBCD	UV absorbance		89
CZE	model proteins: α-Chy, β-Lgl A, β-Lgl B, Con, Cyt, Hem, Lys, Rnase, TI	coated, two layers: first CSM, second EBHPC	50 mM NaH_2PO_4 pH 3.0	UV Absorbance		90
CZE	model proteins: α-Chy BSA, Con, Ova	acrilamide-coated	5 mM HEPES/5 mM SDS pH 8.0 + 7.5% w/v dextran (2 MDa)	LIF, covalent labeling with FQ and FX	<0.1 μM	91
CZE	trancated growth factor	Beckman eCap amine-coated	Beckman eCap 50 mM Tris-HCl pH 8.0	UV absorbance		92
CZE	meat proteins	uncoated	20 mM phosphate + 0.1% g/mL anionic dendrimer	UV absorbance		93
CZE	model proteins: heme-free globin chains	uncoated	50 mM IDA + 7 M urea and 0.5% HEC pH 3.2 + 2% Tween 20 in some cases	UV absorbance		94
CZE	model proteins: β-Lgl A, Cyt, Lys, Myo, Rnase	aminopropylsilane-coated	10 mM acetic acid pH 3.4	UV absorbance ESI-MS	0.6 fmol	95
CZE	model proteins: CA, HSA, Lys, Myo	Polybrene-coated	5% acetic acid + 2% ammonium acetate	UV absorbance		96
CZE	food proteins	uncoated	(i) 5 mM phosphate + 0.01% PDDACl +10 mM sodium octanesulfonate pH 3.7; (ii) 25 mM phosphate + 0.05% DSA pH 7.2	UV absorbance		97
CZE	model proteins: α-Chy, Cyt, α-Lal, β-Lgl A, β-Lgl B, Lys, Myo, Ova, Rnase, TI	polystyrene−ethylenediamine diol-coated	40 mM phosphate pH 3.1	UV absorbance		98
CZE	protein extract from krill	uncoated	50 mM phosphate + 149 μg/mL FC128 + 1 μg/mL FC134 pH 5.5	UV absorbance		99
CZE	proteins of rat airway surface fluid	uncoated	(i) 100 mM borate pH 9.1; (ii) 100 mM phosphate + 0.5 mM spermine pH 2.5	UV absorbance	6 μM	100
CZE	model proteins: Cyt, Myo, BI	uncoated	1% acetic acid	ESI-TOF-MS		101
CZE	human plasma proteins	uncoated	borate	UV absorbance		102
CZE	human plasma proteins	uncoated	borate	UV absorbance		103

Table 1. (Continued)[a]

mode[b]	proteins[c]	capillary	buffer	detection	LOD	ref
ITP−CZE	model proteins: Cyt, Lys A, Lys B, Rnase, rhIL-3, rhIL-6	75 μm × 50 cm, coated neutrally	20 mM triethylamine/ acetate pH 4.2	UV absorbance	25−50 nM	104
CIEF	model proteins: Cyt, Myo, Per	uncoated	Pharmalyte 3−10 ampholyte + HPMC	chemiluminescence	6 nM	105
CIEF	complexes of *scr* SH2 domain with six peptides.	PVA-coated	2% carrier ampholyte (Ampholine 3.5−10)	UV absorbance, ESI-ITMS		106
CIEF	phosphorylated forms of Ova	linear PA coated	0.2% carrier ampholyte (Pharmalyte 4−6.5)	UV absorbance, ESI-MS		107
CIEF	six isoforms of monoclonal antibody	PA- or fluorocarbon-coated	4% carrier ampholyte + 4 M urea	UV absorbance, whole capillary imaging		108
CIEF	scrapie prion protein	neutrally coated	carrier ampholyte 3−10	UV absorbance		109
SDS−CGE	model proteins: α-Lal, β-Gal, BSA, Cal, Ova, Pep	(i) uncoated; (ii) e-CAP-coated; (iii) uncoated Borofloat glass chip	SDS-14−200 gel buffer (Beckman)	LIF, covalent labeling with FMAL and FNHS		110
SDS−CGE	human plasma proteins	non-cross-linked PA coated	0.05 M Tris + 0.05 M tricine + 0.1% SDS + 4% linear PA pH 8.3	UV absorbance		111
SDS−CGE	model proteins: BSA, CA, Con, Ova	uncoated with very viscous 8% linear polyacrylamide	0.1 M Tris + 0.25 M borate + 0.05% SDS + 8% linear PA pH 8.1	LIF, noncovalent labeling with Cypro Red	1.5 nM	112
SDS−CGE	β-trace protein from cerebrospinal fluid	eCAP kit (Beckman)	eCAP kit (Beckman)	UV absorbance		113
SDS−CGE	monoclonal antibody + impurities	uncoated	Bio-Rad SDS running bufer	LIF, covalent labeling with TMR−NHS		114
SDS−CGE	α-amylase and glucoamylase from sake rice koji	uncoated	Bio-Rad SDS running bufer	UV absorbance		115
SDS−CGE	lysates from different bacteria	uncoated	Bio-Rad SDS running bufer	UV absorbance		116

[a]Chemicals: ACN, acetonitrile; 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CMBCD, carboxymethylated β-cyclodextrin; CSM, cationic surfactant moieties; DSA, dextran sulfate; EBHPC, epoxybutane-modified hydroxypropylcellulose (MW 400 000); FC 128, anionic fluorosurfactant from 3M Co. (St. Paul, MN); FC 134, cationic fluorosurfactant from 3M Co.; FMAL, fluorescein-5-maleimide; FNHS, 6-carboxyfluorescein succinimidyl ester; FQ, 3-(2-furoyl)quinoline-2carboxaldehyde; FX, 6-(fluorescein-5-carboxamido)hexanoic acid succimidyl ester; HEC, hydroxyethylcellulose; HPMC, hydroxypropylmethylcellulose; IDA, iminodiacetic acid; NGS, nongel sieving; OPA, *o*-phthaldialdehyde-2-mercaptoethanol; O-PEA, O-phosphorylethylethanolamine; PA, polyacrylamide; PDDACl, polydiallyldimethylammonium chloride; PEG, poly(ethylene glycol); PVA, poly(vinyl alcohol); TMR-NHS, 5-carboxytetramethylrhodamine succinimidyl ester; 2,6-TNS, 2-(*p*-toluidino)naphthalene-6-sulfonic acid. [b] CZE, capillary zone electrophoresis; ITP, isotachophoresis; CIEF, capillary isoelectric focusing; SDS−CGE, sodium dodecyl sulfate capillary gel electrophoresis. [c] Proteins: α-Chy, α-chymotrypsinogen A (p*I* 9.2, MW 25 000); α-Lal, α-lactalbumin (p*I* 4.8, MW 14 200); β-Gal, β-galactosidase (p*I* 4.6, MW 116 000); β-Lgl A, β-lactoglobulin A (p*I* 5.1, MW 36 700); β-Lgl B, β-lactoglobulin B (p*I* 5.3, MW 36 600), BI, bovine insulin (p*I*, MW 57 300); BSA, bovine serum albumin (p*I* 4.7, MW 69 000); Cal, calmodulin (p*I* 4.1, MW 9000); CA, carbonic anhydrase (p*I* 6.0, MW 29 000); Con, conalbumin, (p*I* 6.0, MW 78 000); Cyt, cytochrome *c* (p*I* 10.2, MW 12 400); HAT, human apotransferrin (p*I* 5.2, 6.1, MW 76 500); Hem, hemoglobin (p*I* 6.8, MW 64 500); HSA, human serum albumin (p*I* 4.7, MW 36 700); Lys A, lysozyme A (p*I* 11.1, MW 14 300); Lys B, lysozyme B (p*I* 11.0, MW 14 300); Myo, myoglobin (p*I* 6.8, 7.2, MW 17 500); Ova, ovalbumin (p*I* 4.7, MW 45 000); Pep, pepsinogen (p*I* 4.2, MW 39 000); Per, peroxidase (p*I* 3.5, MW 44 000); rhIL-3, recombinant human interleukin 3 (p*I* 7.0, MW 15 100); rhIL-6, recombinant human interleukin 6 (p*I* 6.3, MW 21 000); Rnase, ribonuclease A (p*I* 9.3, MW 13 700); TI, trypsin inhibitor (p*I* 4.6, MW 20 100); Try, trypsinogen (p*I* 9.3, MW 24 000).

clinical application (*102*, *103*). In the analysis of human plasma proteins, quantitation by CZE shows good correlation with conventional gel techniques and is superior in precision, speed, and automation (*102*).

Attempts have begun to predict the mobility of amines from their fundamental properties (*119*). These efforts may be amply rewarded if peptide mobilities can be predicted. Electrophoretic mobilities of peptides have been studied to compare them with mobilities obtained from mathematical models. The mobilities of 58 peptides ranging 20-fold in mass and 10-fold in their charge were experimentally determined (*120*). It has been shown that Offord's model, $\mu \sim z/M^{2/3}$, fits the data best. Other work using 21 peptides demonstrated that the electrophoretic mobilities of peptides were described by the Offord model; the best agreement was found at acidic pH (2.5) in both pure aqueous and aqueous/2,2,2-trifluoroethanol mixture-based buffers (*121*, *122*). When pH increased to 4.5, the coefficient in the Offord equation decreased from 2/3 to 0.58.

2. Other Separation Modes. CZE does not always provide satisfactory separation of proteins. In such cases, other separation modes can be of use. Capillary isoelectric focusing resolves proteins based on their isoelectric points in a pH gradient created by a mixture of ampholytes. Although the presence of ampholytes reduces protein interaction with capillary walls (*106*), coated capillaries are often used in capillary isoelectric focusing to eliminate electroosmotic flow that can interfere with the separation. Capillary isoelectric focusing was applied to the analysis of scrapie prion protein, which is related to transmissible spongiform encephalopathies or the mad cow disease (*109*). In addition to traditional UV absorbance detection, capillary isoelectric focusing has been successfully combined with chemiluminescence (*105*) and ESI-MS detection (*106*, *107*); see the Detection section.

Chiral peptides were resolved by first derivatizing them with a chiral fluorescent reagent. The diasteromeric products were separated by use of nonionic micellar electrokinetic chromatography (*123*).

SDS−polyacrylamide gel electrophoresis (SDS−PAGE) is a very powerful and common tool in protein analysis. SDS binds the proteins proportionally to their molecular weight (1.4 g of SDS/g of protein). The charge of the complexes is mainly determined by the negative charges of the SDS molecules so that the mass-to-charge ratios and electrophoretic mobilities are similar for all proteins. The SDS−protein complexes are separated according to their sizes by electrophoresis through a polyacrylamide gel. Capillary versions of SDS−PAGE have been developed. Although most capillary versions are based on nongel sieving matrixes, such as linear polyacrylamide, they are usually referred to as SDS−capillary gel electrophoresis for convenience. SDS−capillary gel electrophoresis with 4% linear polyacrylamide was used to separate 6 major and ∼50 minor protein components of human plasma within 1 h (*111*).

Recently SDS−capillary gel electrophoresis of proteins was adapted for microfabricated channels (*110*). The dimensions of the channels (40-μm depth, 100-μm width at the top, 20-μm width at the bottom, and 50-mm length) were chosen to facilitate easy injection of a relatively viscous gel. Six model proteins were separated in the microfabricated channels within 3 min.

Capillary electrochromatography has begun to see application in the separation of proteins and proteins. Early work used sintered frits to retain the chromatographic packing that was used as the stationary phase. The high-activity surface of the sintered frit tended to strongly bind to proteins and peptides, leading to poor separation performance. More recently, capillary electrochromatography has been used for protein separations in fritless columns. Open-tubular electrokinetic chromatography relies on the presence of a thin stationary phase at the capillary inner wall; 20-μm-i.d. columns are commonly used for the separation. Etched and chemically modified columns have been used for the separation of lysozymes and cytochrome *c*'s (*124*). A mixture of lysozyme, cytochrome *c*, ribonuclease A, and α-chymotrypsinogen A was separated isocratically (*125*).

A single separation technique seldom resolves more than ∼30 components in a protein mixture. Jorgenson recently reviewed the use of two-dimensional separations that provide very high resolving power (*126*). In these separations, analyte is slowly eluted from the first dimensional separation capillary, typically based on liquid chromatography, and sequentially injected into a fast second dimension separation, typically based on capillary electrophoresis. If the two separation methods are based on unrelated mechanisms, then a complex, two-dimensional separation map can be generated from complex samples. For example, capillary electrokinetic chromatography and capillary electrophoresis have been combined for analysis of glycoprotein factor associated with cancer cachexia (*127*).

Detection. The amount of sample that can be loaded on a capillary column is limited. The performance of separation is the best if the volume of a sample loaded does not exceed 0.1−0.2% of the total volume of a column; a typical sample volume is a few nanoliters. Because of the small injection volume, capillary electrophoresis inevitably has relatively poor concentration limit of detection, which is particularly problematic in the analysis of minute amounts of biopolymers.

1. Preconcentration To Improve Concentration Detection Limits. Dilute samples must be preconcentrated before analysis. There are two main preconcentration techniques. The first is based on chromatographic principles, such as solid-phase extraction, and the other is based on electrophoretic principles, such as stacking and isotachophoresis. Both preconcentration techniques have been applied to the analysis of diluted samples of proteins and peptides, although solid-phase extraction based on reversed-phase chromatography has been employed the most often (*128*−*133*).

Solid-phase extraction was also implemented using membranes impregnated with silica-based C_2 HPLC phase, which has proven useful for the preconcentration of proteins and peptides (*88*, *96*, *134*). Although solid-phase preconcentration improves the concentration limits of detection by up to a factor of 100, it can introduce into the analysis a bias associated with different selectivity of the solid phase for different proteins; some proteins were lost from the sample during solid-phase preconcentration procedures (*88*). A robust solid-phase preconcentrator has been recently developed based on quartz wool and porous beads (*135*). The preconcentrator improved the concentration limit of detection by a factor of 100, which is typical for a reversed-phase-based extraction. In contrast to reversed-phase-based cartridges, it did not generate high back pressure, did not require frits to hold the packing in place, was tolerant to a wide range of buffer pH (*1*−*14*), and was stable for months.

Transient isotachophoresis is another preconcentration technique that has become relatively common for protein analysis. This method is performed before CZE separation on the same capillary (*104*). In this experiment, a large amount of a low-concentration sample is preconcentrated by isotachophoresis, the trailing electrolyte is removed by hydrodynamic counterflow, and finally the proteins are separated by CZE. The concentration limit of detection improves by a factor of ∼100. One of the requirements for this approach is the optimization of the isotachophoresis focusing time to prevent the loss of unfocused proteins (usually those with low electrophoretic mobilities) when the trailing electrolyte is removed. This requirement may be challenging if the electrophoretic mobilities of the proteins differ significantly. It has been shown that up to 72% of the capillary volume could be filled with the peptide sample without degrading the quality of separation (*136*). A disadvantage of such an approach was that anions and cations must be separated in different capillaries: cations in uncoated capillaries; anions in coated capillaries. In another example, transient isotachophoresis was used to concentrate neuropeptides in a physiological sample (*137*). The capillary was filled with up to 140 nL (∼10% of the total capillary volume) of low-concentration sample from monkey brains. It was also shown that the concentration limit of detection can be further improved if solid-phase extraction is combined with transient isotachophoresis for sequential cleanup and preconcentration (*137*).

2. UV Absorption Detection. Due to its simplicity and despite its relatively poor sensitivity, UV absorbance remains the most popular detection approach for the proteins and peptides in capillary electrophoresis. Proteins have modest absorbance near

~250−300 nm because of the presence of aromatic amino acids (tryptophan, phenylalanine, and tyrosine). Generally, the concentration limits of UV absorbance detection is not much better than ~0.1 μM for proteins and 1 μM for peptides. Therefore, for physiological samples containing low-concentration proteins, sample preconcentration is required (*88*, *96*, *104*, *138*).

UV detection based on whole-capillary imaging was used for capillary isoelectric focusing (*108*). This detection approach allows the exclusion of the mobilization step, which usually takes more time than isoelectric focusing. Salts present in the sample can distort the resulting pattern. A simple two-point correction algorithm can be used to correct for this distortion (*139*). The use of whole-column imaging was recently reviewed (*140*). A commercial system is now available from Convergent Bioscience.

Similarly, UV absorbance detection was performed by use of an linear imaging detector. By stepping the image at a rate proportional to the migration time of analyte, absorbance measurements were averaged along a portion of the detector. Detection limits were improved by roughly the square root of the number of pixels in the detector (*141*).

3. Luminescence. Laser-induced fluorescence produces low limits of detection and wide dynamic range, which minimizes the need for sample preconcentration. Native fluorescence of proteins is primarily associated with emission from tryptophan between 300 and 400 nm. A low-power krypton−fluoride laser was used for native fluorescence detection of proteins in body fluids; detection limits were in the 1−100 nM for tryptophan, proteins, indole-based compounds, and catecholamine urinary metabolites (*142*). Native fluorescence has also been used to monitor ~30 components from single neurons. Wavelength-resolved emission detection allowed identification of aromatic monoamines, aromatic amino acids and peptides containing them, flavins, adenosine and guanosine nucleotide analogues, and other fluorescent compounds (*143*). Despite its convenience, excitation of native fluorescence requires the use of UV lasers, which tend to be expensive and temperamental to operate. Unfortunately, physiological fluids usually contain many components that fluoresce in the same spectral region, which increases the background fluorescence. Native fluorescence of proteins and peptides is not often used as a detection tool in CE.

Proteins can be fluorescently labeled using appropriate reagents for excitation by inexpensive gas or diode lasers that operate in the visible or near-infrared portions of the spectrum. Fluorescent dyes tend to not be desirable because they tend to produce higher backgrounds from unreacted dye and associated impurities. Fluorogenic reagents are more attractive because they do not fluoresce until undergoing the labeling reaction.

Although spectacular detection limits can be obtained for labeled proteins, these detection limits must be interpreted with care. In some cases, a protein is labeled at high concentration and then diluted for analysis. While useful in the production of substrate for some enzymatic experiments (*132*), these dilution experiments usually are useful only in characterizing an instrument, rather than a labeling technique. It is much more useful to determine the detection limit for the labeling reaction itself—the fluorescent labeling of highly dilute proteins can be challenging.

Precolumn or on-column labeling of proteins presents one potential problem. Most labeling reagents react with primary amines, including both ϵ-amines of lysine residues and unblocked N-terminal amino acid. Lysine is a relatively common amino acid, and most proteins contain several lysine residues. Unless the labeling reaction goes to completion, there is a mixture of reaction products generated by the labeling reaction. These multiply labeled proteins can generate a large number of peaks, which complicates electrophoretic analysis of protein mixtures.

The fluorescent labeling of subnanomolar concentrations of ovalbumin was achieved using LIF and precolumn labeling of ovalbumin with a fluorogenic label, FQ (*79*). Two methods were demonstrated to eliminate the effect of multiple labeling. First, the use of a submicellar concentration of SDS in a capillary zone electrophoretic analysis collapsed the multiple labeling envelope into a single sharp peak; nearly 200 000 theoretical plates were obtained for labeled ovalbumin. Second, SDS−PAGE also eliminated the effects of multiple labeling. It was also shown that superior detection limits are obtained if the labeling reaction is performed at an elevated temperature (*78*). It has been demonstrated that sample and standard proteins can be labeled with two different fluorogenic labels, separated simultaneously on the same column, and detected in two optical channels for high-accuracy protein size determination (*91*).

Postcolumn labeling also eliminates the effect of multiple labeling. Sensitivity in the nanomolar range has been demonstrated using the fluorogenic reagent OPA in the sheath fluid used in a sheath-flow cuvette (*87*, *144*).

A new near-infrared fluorescent dye (NN382) was used to improve the detection limit for peptides (*76*), which produced mass limits of detection of 0.1−0.3 amol for four model peptides. The linear dynamic range was 200. Unfortunately, the derivatization reaction is very long, 16 h at room temperature. Also, the labeling efficiency varies highly between different terminal amino acids. Surprisingly, there was no evidence for labeling of lysine residues, so there were no multiple labeling peaks.

Noncovalent labeling is performed with dyes that interact with proteins either by hydrogen bonding or through hydrophobic interaction. Such dyes usually undergo 10−100 times enhancement in their fluorescent yield upon binding to proteins (*83*). Nanomolar detection limits were reported with the noncovalent fluorescent label iodocyanine green (*81*); however, a relatively wide peak of unreacted dye was not baseline separated from the protein peak. A noncovalent reagent was used to fluorescently label proteins separated by SDS−CGE, but only when the concentration of SDS was below the critical micellar concentration (*112*). However, the protein must be labeled before the sample is injected into the capillary. If the label was present in the running buffer for on-column labeling, the label interacted with the SDS, producing an unacceptably large background signal.

Chemiluminescence detection has been combined with capillary isoelectric focusing of heme proteins (*105*). The capillary output was connected to a reaction chamber containing luminol and hydrogen peroxide. Upon exiting the capillary, heme proteins catalyzed oxidation of luminol resulting in a burst of chemiluminescence. The concentration detection limit for cytochrome *c* was 6 nM. Chemiluminescence caused by nonenzymatic oxidation of the substrate was probably the major contributor to a relatively high background luminescence. Chemiluminescence is a highly

selective detector because only a few proteins catalyze the oxidation of chemiluminescent substrates.

4. Mass Spectrometry. Mass spectrometry is becoming a very powerful tool in the detection of proteins separated by capillary electrophoresis. Electrospray ionization (ESI) is a soft ionization mode that provides high sensitivity and that can be coupled relatively easily with capillary electrophoresis for on-line detection. However, ESI requires the use of volatile electrophoresis buffers to avoid contamination of the ionization chamber of the mass spectrometer. This requirement restricts the choice of buffer to components such as acetic acid, formic acid, ammonium acetate, and ammonium formate. Because of the limited range of buffer additives, capillary electrophoresis separations with mass spectrometric detection tend to be of poorer resolution than observed for optical detection. However, the high information content of mass spectrometry compensates for this mediocre separation performance. As examples, the protein component of human aqueous humor was analyzed using solid-phase preconcentration and a sheath-flow CZE-ESI interface (*88*). Similarly, mixtures of model proteins have been analyzed using different types of sheathless interfaces (*95*, *101*).

There has been much interest in the interface of capillary isoelectric focusing and mass spectrometry. This combination is attractive as a replacement for conventional two-dimensional electrophoresis based on isoelectric focusing and SDS−PAGE. The mass spectrometer provides molecular weight information with much higher accuracy than is possible in SDS−PAGE. Karger used capillary isoelectric focusing and mass spectrometry to separate and analyze the affinity complexes formed between the *src* SH domain protein and a number of peptides (*106*). This approach was also used to analyze the mono- and diphosphorylated forms of ovalbumin (*107*). These forms were successfully separated by isoelectric focusing and their identities were confirmed directly by a difference of 80 Da obtained from the mass spectra. Additional glycosylation microheterogeneity was distinguished by ESI-MS within each monophosphoovalbumin and diphosphoovalbumin. The presence of ampholytes did not degrade the detection of the proteins because the m/z values for ampholytes are much lower than those for the proteins. Also, the ampholytes are focused according to their p*I* so that only one or two ampholyte components entered the mass spectrometer with each protein. Clearly, much exciting work will be performed by combining isoelectric focusing with mass spectrometry.

In vivo metabolites of the opioid neuropeptide E were preconcentrated using solid-phase extraction, separated by capillary electrophoresis, and on-line transferred to a matrix, which was analyzed by MALDI-MS off line (*130*). The combination of solid-phase extraction with on-column transfer and MALDI-MS allows the simultaneous optimization of each step, the use of different solid-phase media for extraction, automation of the system, and the use of nonvolatile buffers. However, MALDI imaging required 2.5 h, which slows the analysis dramatically. The overall concentration limit of detection was 10 pM for neurotensin.

In many biological applications, proteins should not only be separated but also identified. One such application is proteomics. The proteome is the protein analogue of the genome: it is the complete set of proteins expressed in a cell, tissue, or organism at certain time. The proteome is a function of a cell's physiological status. Identifying the proteins expressed in response to certain stimuli is a classical biological problem. It is solved by combining the techniques of protein separation and identification. Classically the proteins are separated using slab-gel electrophoresis. If a considerable amount of protein is present then the Edman degradation reaction can be used to determine the protein sequence. However, the Edman degradation reaction is slow, requires a relatively large amount of protein, and fails if the N-terminal amine is blocked by a posttranslational modification. While a useful tool, Edman degradation is seldom used for protein identification.

Instead, mass spectrometry, particularly when coupled with capillary electrophoresis, is a very powerful tool for protein identification. The analysis involves a number of analytical techniques and steps. First, proteins originating from a homogenized tissue sample or from a cell culture are separated using gel electrophoresis. Second, a single spot, presumably containing a single protein, is excised from the gel and a proteolytic enzyme is used to digest the protein into a set of peptides. Third, the peptides in the digest are separated using CZE; incomplete separation is often acceptable for tandem MS/MS. Fourth, the separated peptides are analyzed using ESI-MS/MS to gather information not only on their molecular weight but also on their sequence. This analysis starts with soft ionization of the peptides. The first mass analyzer is used to determine the masses of the peptides and to select an ion corresponding to a single peptide. Collision-induced dissociation is used to break the peptide into short fragments. The masses of these fragments are determined using the second mass analyzer. Fifth, a database search is used to reconstruct the protein based on the structures of the peptides that constitute the protein. Using this technology, a 75-protein complex from yeast ribosome was analyzed and more than 80% of the proteins in this complex were identified (*129*). Capillary electrokinetic chromatography has been coupled with a time-of-flight mass spectrometer for analysis of tryptic digests (*145*).

One of the problems of tandem mass spectrometry detection in capillary electrophoresis is the relatively slow scan speed of the mass spectrometer. The MS/MS analytical cycle includes four steps: peptide mass analysis, peptide ion selection, collision-induced dissociation, and data acquisition for the selected peptide(s) ions. The cycle takes 1−3 s. Under normal CE separation, a large number of peptides may migrate from the capillary into the MS analyzer during this cycle time. However, the mass spectrometer is limited to the analysis of very few and most intense ions. Therefore, the minor components are lost if a large number of different peptides are present. The problem has been recently solved by introducing feedback from the MS to the electrophoresis power supply (*128*). If there were no ions injected into mass spectrometer, then the capillary electrophoresis instrument was operated at a cruising voltage and the mass spectrometer was run in the MS mode. If ions were detected by the mass analyzer, then the electrophoresis power supply was switched to a low voltage and the spectrometer switched to the MS/MS mode. The much slower migration of peptides at low electrophoretic voltage facilitated mass spectral analysis of several comigrating components. The ability to analyze minor peptides in a mixture was demonstrated by determination of in vivo phosphorylation sites of a large protein, endothelial nitric oxide synthase (MW 133 000).

Capillary electrophoresis/mass spectrometric identification of proteins has several advantages compared with Edman sequencing. First, it allows the identification of the proteins present in femtomole amounts. Second, it has much higher throughput; the CZE-MS/MS step takes less than 1 h. The major disadvantage of such an approach is the cost of instrumentation, which makes it inaccessible to the majority of biological and biochemical laboratories. Although MS/MS is usually sufficient for protein identification, it is not always necessary. Using model proteins, Cao and Moini showed that a combination of ESI-MS analysis of the intact proteins, CE/ESI-MS analysis of the tryptic digests of the proteins using high mass accuracy, and high-resolution TOFMS with in-source fragmentation of the digest peptides could characterize and identify the proteins (*146*). Complete separation of the peptide by CZE was essential for such an analysis. Such separation may be a challenge for large proteins.

Interfacing CE with ESI-MS continues to be a serious technical challenge. Traditionally a sheath-flow interface is used (*88*). Sheath fluid helps to maintain stable CE current and stable ESI. However, the sheath flow also dilutes the sample and introduces a large amount of the sheath fluid, which must be considered as a contaminant. There have been many reports on sheathless interfaces. The major challenge of sheathless designs is to provide stable electrical contact for CE separation and stable flow rate for stable ionization. Four different interface designs have been evaluated for separating the tryptic digest of the myosin I heavy chain kinase (*135*). The best performance came from a sheathless interface in which a palladium electrode was placed within the capillary. Disadvantages of such an interface included flow disturbance introduced by the electrode and oxidation of peptides, perhaps due to catalysis on the palladium electrode. A similar interface was evaluated for separating model proteins (*95*). No decrease in the quality of CE separation was observed. A mass detection limit of 0.6 fmol was achieved with a 30-μm-i.d. capillary. Modifications of a sheathless liquid−metal junction interface were used for peptide ionization, which produced a subfemtomole mass detection limit and protein identification through database search using the MS/MS data (*129*, *134*). Another sheathless design was used for the analysis of model proteins; the tip of the capillary was etched and coated with gold (*101*). Such capillaries enabled stable CE separation and ionization. However, the lifetime of such capillaries was 2−8 h, and manufacturing them might be a time-consuming and expensive procedure.

Protein analysis using CZE with MS detection can be applied to abundant proteins in single cells. Two globulin chains (α and β) have been analyzed this way in a single erythrocyte (*147*). A single intact cell was injected into a separation capillary and lysed; the proteins were separated by CZE and detected using ESI-TOFMS. Identifiable MS signals corresponding to the two electropherogram peaks of the two chains were detected. It should be noted that hemoglobin is the major protein in erythrocytes ($\sim$0.45 fmol). The sensitivity of contemporary MS is not enough to monitor less abundant proteins at the single-cell level.

5. Chip-Based Protein Separations. On-chip separation of tryptic digest of proteins has been developed using ESI-MS detection (*148*). Either a disposable nanoelectrospray emitter or a fused-silica capillary was inserted into a low-dead volume connection in the chip. The distal end of the capillary was inserted in a "classical sheath-flow ESI-MS interface". Using disposable nanoelectrospray emitters allowed a 2-min separation of peptides in a 4-cm channel, albeit with a separation efficiency of only 500−3500 theoretical plates. The selectivity and specificity of MS compensated for the relatively low efficiency. A protein digest from 150 fmol of protein was used to gather the amino acid sequence information (MS/MS) sufficient for searching a database. When gold-coated disposable nanoelectrospray emitters were employed, the concentration detection limit improved by 1 order of magnitude ranging from 3.2 to 40 nM for different peptides (*149*). Chips were also used for competitive immunoassay of serum theophylline. Detection limits of 0.3 mg/L were reported (*150*, *151*). Capillary electrochromatography has also been performed on-chip for the separation of peptides (*152*).

Posttranslational Modifications. Posttranslational modifications of proteins such as phosphorylation, glycosylation, and lipidylation play an important role in modulating biological functions of proteins. Phosphorylation participates in most signal transduction pathways. Glycosylation plays an important role in cell recognition and immunoresponse. The role of lypidylation is poorly understood although there a number of examples when it takes a part in signal transducation, with fanresylation of the Ras oncogene being particularly well studied. Other modifications include γ-carboxylation of glutamic acid, which has been studied in recombinant and natural proteins (*153*, *154*). Analytical tools for identifying these modifications are of great importance for biological application.

1. Glycoproteins. Glycoprotein analysis requires both protein and glycan identification. Separation of glycoproteins is achieved using the approaches described above. For example, 13 glycoforms were separated from each of three types of ovalbumin (*155*). The best separation of these forms was achieved in high ionic strength borate buffer in the presence of a putrescine additive (100 mM borate, 1.8 mM putrescine pH 8.6) using uncoated capillaries.

The glycomoieties can be identified in a number of ways. Coupling of CZE separation and tandem MS/MS may be useful not only for identifying the amino acid sequence of glycoproteins but also for determining the structure of glycans. CZE-tandem MS/MS has been used to elucidate the structure of N-linked glycans and amino acid sequences of lectin (*156*). In this application, reversed-phase based solid-phase extraction allowed the analysis at 30 nM concentration.

Useful information about the glycoprotein structure can be obtained by combining CZE with MALDI-MS (*157*). Glycosylation of a model peptide was studied. The peptide (16 amino acids) structure allowed for up to six GalNAc molecules to be added to a single peptide molecule. Six glycoforms were well separated by CZE using 2 M formic acid buffer containing 2.5% (v/v) poly(vinyl alcohol), MW 15 000. The level of glycosylation was monitored using MALDI-MS. Similar model studies were carried out with ESI-MS as well (*158*).

The analysis of the complete composition of saccharides occurring in glycoproteins has been performed by separating the hydrolyzed sugars using CZE (*159*). First, the combination of mild and strong hydrolysis was applied to release the saccharide moieties from glycoproteins and to hydrolyze them into monosaccharides. Second, a set of derivatization reactions, including

enzymatic reactions, was used to fluorescently label the monosaccharides with 8-aminopyrene-1,3,6-trisulfonate. Third, the labeled monosaccharides were separated by CZE using a high-concentration borate buffer, detected using LIF, and identified by comigration with spiked standards. This method could determine not only neutral sugars but also sialic acids. A similar approach was applied to the identification of sialic acids and neutral sugars occurring in glycoproteins (*160*). It has been demonstrated that the presence of the protein or neutral sugars in the reaction mixture did not influence the derivatization of sialic acids allowing for their accurate quantitation.

2. Lipoproteins. The analysis of lipoproteins is mainly used to screen blood plasma for different classes of lipoproteins. ApoB-containing lipoproteins (low-density lipoproteins) are blamed for plaque formation in blood vessels while the ApoA-I containing lipoproteins (high-density lipoproteins) are considered to be protective against the development of atheroma. There are data suggesting that the subfractions of lipoprotein classes may be as important as the classes.

Lipoproteins are very hydrophobic due to the lipid moieties. Therefore, surfactants are used as buffer additives to improve solubilization. High-density lipoproteins have been analyzed using either CZE in neutraly coated capillaries or SDS−capillary gel electrophoresis (*161*). Two forms of the protein, ApoA-I and ApoA-II, were separated by both techniques and their concentrations were in good agreement with those obtained by immunoassay. Oxidation of low-density lipoproteins is believed to be a key event in the development of atherosclerosis. CZE has been used to monitor the change in electrophoretic mobility of lipoproteins undergoing Cu^{2+}-catalyzed lipid peroxidation (*162*). Electrophoretic mobility correlated with the level of peroxidation.

Capillary isotachophoresis is useful for lipoprotein separation. Capillary isotachophoresis has been used to separate 14 subclasses of total blood lipoproteins and to determine the cholesterol and triglyceride content in those subfractions (*163*). Cholesterol- and triglyceride-specific staining reactions were used to monitor them with light absorbance. In another work, low-density lipoproteins were separated in four subfractions using capillary isotachophoresis. Prestaining with a lipophilic dye was used to detect the subfractions (*164*).

3. Phosphopeptides. Phosphopeptides with the same amino acid sequence but with different residues modified with phosphate have been separated by CZE with UV absorption detection (*165*). The best separation was achieved in poly(vinyl alcohol)-coated capillaries using either formic acid or phosphate buffer. This technique will be of great importance in the study of the activity and specificity of kinases and phosphates.

Affinity Capillary Electrophoresis. Affinity capillary electrophoresis exploits the differences in electrophoretic mobilities between a free analyte and an analyte−ligand complex to quantitate the analyte or determine the parameters of the interaction (*166*, *167*). The basic principle involves the measurement of an altered electrophoretic mobility of the complexed species as compared with the free ligand. Affinity capillary electrophoresis can be used to monitor protein−ligand interactions. The major requirement is that the separation does not destroy the complexes.

Affinity capillary electrophoresis can be used to measure the dissociation constants, K_d, for protein−ligand complexes. The K_d values have been measured for the complexation of an 18-mer diphosphopeptide with an antibody (*168*). The peptide had two epitopes that were recognized by the antibody. The average K_d was determined in a large excess of the antigen. K_d values of the complexes were independent of the electric field, confirming that electrophoresis was a nondestructive tool in measuring those constants. It is possible to determine the K_d for multivalent interactions as well. The values of two dissociation constants characterizing binding zinc ions with two zinc-binding sites on a NCp7 protein have been measured by affinity capillary electrophoresis (*169*). It has been shown that binding the first zinc reduces the binding ability for the second zinc by a factor of 2.

Receptors may be immobilized on a capillary wall (*170*). The mobility of the immobilized receptor is zero so that the mobility shifts for the complex are expected to be large. This technique is best suited for measuring low-to-intermediate affinity interactions ($K_d \sim$ mM−μM). If it is used for higher affinities, then the retardation times may be impracticably long. This technique has been used to study relatively weak interactions between heparin and two peptides different only in the stereochemistry of a single amino acid (*170*). The dissociation constants of heparin complexes with the peptides are within micromolar range. They can be barely resolved if heparin is a component of the buffer. However, when heparin is immobilized, the migration time of the peptides doubles, revealing different retardation for the two peptides.

Affinity capillary electrophoresis has also been performed using isoelectric focusing. The analysis of actinavidin by capillary isoelectric focusing revealed 14 peaks associated with different protein conformations existing at native conditions (*171*). When a biotinylated oligonucleotide ligand was added, only 4 major components remained present while 10 other peaks disappeared, which confirmed that protein's conformational heterogeneity is reduced by interaction with the ligand.

Protein−peptide interaction was studied by a mobility shift technique (*172*). Similarly, peroxidase−anti-peroxidase immune complexes were characterized by capillary zone electrophoresis (*173*).

Protein−drug interaction (kedarcidin chromophore and apoprotein) was studied using affinity capillary electrophoresis (*174*). The K_d values were measured in the presence of acetonitrile, which is typically used to improve the solubility of the drug. It has been shown that K_d increases with increasing concentration of acetonitrile, emphasizing that the solvent composition should be taken into account when the equilibrium parameters are being determined.

Affinity capillary electrophoresis may be used for performing immunoassays. In a competitive immunoassay, a known amount of labeled antigen is mixed with the sample; this mixture is treated with an antibody. Two peaks are observed in the electropherogram corresponding to the free labeled antigen and the antibody-bound labeled antigen. The unlabeled antigen in the sample displaces the labeled antigen from the antibody. The ratio of the free and antibody-bound antigen is related to the amount of unlabeled antigen in the sample. A competitive immunoassay has been used to monitor insulin secretion from single islets of Langerhans (*175*). This assay used fluorescently labeled insulin as the labeled antigen and a monoclonal anti-insulin antibody. Single islets were probed after exposure to glucose and tolbutamine.

In noncompetitive immunoassays, the antibody is labeled. The ratio of free and complexed antibody is related to the amount of antigen present in the sample. Unfortunately, there are several problems with noncompetitive immunoassays in capillary electrophoresis. The antigen must be sufficiently large to induce a change in the mobility of the complex; noncompetitive immunoassays are unlikely to be successful for haptens and small antigens. Labeling of the antibody often results in a heterogeneous reaction product that generates several electrophoretic peaks. Last, labeling the antibody may change its specificity toward the antigen. To bypass these problems, antibodies can be replaced with aptamers, which are selective-binding oligonucleotides usually identified from combinatorial libraries. Aptamers can be homogeneously labeled. They are much smaller than antibodies so that their mobility shift will be much higher upon binding with an antigen than that for a large antibody. Aptamers have been used as ligands in affinity capillary electrophoresis for determining two proteins, IgE and thrombin (*176*). Labeling the aptamers increased the dissociation constant of a protein−aptamer complex by roughly 1 order of magnitude. However, this influence did not preclude the use of the labeled aptamer as an immunoassay ligand. Detection limits were 50 pM for IgE and 40 nM for thrombin. This dramatic difference in detection limit results because the thrombin aptamer was a weaker binder than the IgE aptamer. The aptamer approach is limited by their availability. Aptamers are synthetic products requiring a multistep procedure of synthesis and selection.

The formation of a complex between two molecules, one of which is fluorescent, may be detected by measuring the fluorescence polarization ratio. The technique is based on rotational depolarization of fluorescence excited with a polarized light source. Small fluorophores rotate in solution quickly; therefore, they change their orientation during the lifetime of the excited state and their fluorescence is depolarized. Rotational diffusion decreases when a complex forms between the fluorescent molecule and another molecule. Therefore, fluorescence of the complex will be more polarized than that of a free ligand. Measuring the fluorescence polarization can be used to determine the binding constants of the complexes. This technique has been used to study peptide−protein, protein−protein, and DNA−protein interactions (*177*). This detection approach works the best if the size of the complex is much larger than the size of a free fluorophore. It appears well suited to competitive immunoassay and aptamer-based affinity assay described earlier.

Affinity capillary electrophoresis was also coupled with MS detection to determine the affinity of a number of peptides with the model receptor vancomycin (*178*). The K_d values for 19 peptides have been determined in a single experiment. The peptides were separated while interacting with the receptor present at different concentrations in the running buffer. The migration shifts of the 19 peptides were measured and the identities of the peptides were determined with mass spectrometry. Such an approach can be useful for searching the combinatorial libraries for the peptides with the highest affinities.

Enzymatic Activity. A number of assays have been developed to monitor enzymatic modification of proteins or peptides. In these mobility shift assays, a fluorescent protein or peptide is treated with the enzyme. Modification of the substrate results in a mobility shift so that two electrophoretic peaks are observed, corresponding to the unmodified substrate and modified product. The ratio of the areas of the two peaks is used to monitor the enzymatic reaction.

1. Kinase Activity. Kinases are enzymes that add phosphate groups to proteins. They are vital regulatory proteins for a number of cellular functions. Addition of a phosphate by a kinase, or its subsequent removal by a phosphatase, turns on or shuts off regulatory pathways.

To measure kinase activity using CZE-LIF, a fluorescently labeled peptide substrate was synthesized. A kinase was then incubated with the substrate and the phosphorylated substrate was separated from the unphosphorylated starting material (*179*). Classically, this assay would be performed with an unlabeled protein and a radioactive phosphate; mobility shifts would be detected by gel electrophoresis and autoradiography. Clearly, the capillary electrophoresis laser-induced fluorescence method is much faster and avoids the use of radioactive material and visual interpretation of the autoradiogram.

Kinase activity has been recently measured at the single-cell level. Rapid cytoplasmic sampling technique was combined with capillary electrophoresis to monitor the activation of protein kinase C (PKC) in a small region of a relatively large Xenopus oocyte (*180*). A fluorescent PKC substrate was phosphorylated by intracellular PKC upon external stimuli of an oocyte. The product was then separated from the substrate by CZE. More recently, a similar technique was applied to monitor a number of kinase activities (PKC, protein kinase A, calcium-calmodulin activated kinase II, and cdc2 protein kinase) in a relatively small mammalian cell (*181*). This assay strategy should be applicable to the analysis of a broad range of intracellular enzymes in single cells.

2. Glycosyltransferases. This class of enzymes is responsible for the formation of complex carbohydrate structures on proteins. The monosaccharides are added one by one to the protein by highly specific glycosyltransferases. Glycosyltransferase activities have been monitored with CE using UV detection (*182*). The kinetic parameters of the enzymatic reaction, such as K_m and V_{max}, have been determined.

We recently reported the determination of oligosaccharide biosynthesis and biodegradation in a single cancer cell (*183*). A fluorescently labeled disaccharide was introduced into the cell. The substrate was targeted by a set of biosynthetic and biodegradation enzymes to form a number of products, including a monosaccharide, a trisaccharide, and a tetrasaccharide. The phase of each cell in the cell cycle was determined by treating the cells with a vital intercalating dye. This dye produced a fluorescent signal that was proportional to the amount of DNA within the cell. Cells in the G1 phase were diploid and cells in the G2/M phases were tetraploid; an epi-illumination fluorescent microscope was used to monitor the DNA fluorescence and to monitor the cell cycle. Once the phase of the cell in the cell cycle was determined, the cell was injected into a capillary and lysed; the products were separated electrophoretically and detected by laser-induced fluorescence. Each product generated a distinct peak, and an entire enzymatic cascade was monitored in a single electropherogram from a single cell.

3. Proteases. Proteases are another important class of metabolic enzymes. They serve two important roles. Many

proteases cleave a functional protein from a full-length, freshly translated precursor. This function is vital to the function of retroviruses, and the inhibition of this protease activity is an important therapeutic target. Other proteases are less specific and act to degrade proteins during digestion and in apoptosis and necrosis.

Trypsin activity has been measured using a synthetic peptide substrate (*184*). The substrate was fluorescently labeled. Cleavage processed the substrate into two products, one of which possessed a fluorescent label. The substrate was designed in a way that a fluorescently labeled part of the substrate had a p*I* value different from the substrate, which allowed facile separation of the substrate from the product using capillary isoelectric focusing.

A method for determining cathepsin D activity has been developed (*185*). Cathepsin D is an aspartyl protease, and its activity is associated with a number of physiological and pathological conditions including cancer. Classical analysis relies on immunoassay, which measures the concentration of the enzyme. However, immunoassays do not necessarily discriminate between active and inactive forms of an enzyme. The method to determine the enzyme activity is based on using a fluorescently labeled hemoglobin as a substrate. The fluorescently labeled product formed upon cleavage can be separated from the substrate using micellar electrokinetic chromatography with laser-induced fluorescence detection.

We reported a very sensitive protease analysis using solid-phase extraction as a cleanup step for a substrate and a product (*132*). A substrate is a peptide labeled with biotin and a fluorescent label. Biotin is used to purify this double-labeled substrate from the impurities on solid-phase-immobilized streptavidin. After proteolytic cleavage, fluorescently labeled product does not contain a biotin group. A second solid-phase extraction was used to remove unreacted substrate and thus dramatically reduce the fluorescence background level. Using such a technique allowed detection of fluorescent product generated in the presence of as little as 4.6×10^{-13} M of chymotripsin or 5 amol of enzyme in the 10-μL sample volume.

4. Single-Molecule Enzymology. Capillary electrophoresis has been used to monitor the activity of single enzyme molecules (*186*). These enzymes transform a very weakly fluorescent substrate into a highly fluorescent product. A very dilute solution of enzyme was mixed with high concentration substrate, was injected into a capillary, and was allowed to incubate. Each enzyme molecule created a pool of fluorescent product. Electrophoresis was used to sweep the product through a high-sensitivity laser-induced fluorescence detector, where each pool of product generated a peak. Electrophoresis separated the small pools of highly fluorescent product from the large excess of weakly fluorescent substrate.

LIPIDS, FATTY ACIDS, AND CARBOHYDRATES

The analyses of lipids, fatty acids, and carbohydrates by capillary electrophoresis can be considered to be in their infancy, particularly when compared to those of DNA and proteins.

Lipids and Fatty Acids. Because of the hydrophobicity of lipids and fatty acids, they are sparingly soluble in aqueous solutions. Solubility is typically improved by adding surfactants, and micellar electrokinetic chromatography is a natural choice for lipid analysis. Micellar electrokinetic chromatography has been used to separate isomeric epoxy fatty acids in a borate buffer containing 10 mM SDS and 10% acetonitrile (*187*). Separation quality under these conditions critically depended on temperature. Separation efficiency improved considerably at lower temperature. Decreasing temperature changed the partition coefficients of the analytes between the micelles and the bulk solution. Micellar electrokinetic chromatography has been also used to separate the oxidation products of fatty acids (*188*). It was found that an increase of the SDS concentration decreased the quality of separation.

Poor solubility of the lipids and fatty acids in aqueous solutions stimulated the attempts to conduct their separation in nonaqueous environment. Although the separation of hydrophobic analytes may improve while carried out in organic solvents, their detection may become a problem. Many organic solvents exhibit strong UV absorbance that interferes with the detection of the analytes. In this case, indirect detection may be employed. Indirect fluorescence detection with sodium fluorescein has been used to monitor free fatty acids (C_6−C_{24}) separated by nonaqueous CZE (*189*). Although, the detection limit of 10 μM was better than that with indirect UV detection, it is much poorer that obtained with direct LIF of fluorescently labeled compounds (*190*). One of the drawbacks of indirect fluorescence detection in nonaqueous solutions is that the fluorescence of most common water-soluble dyes, such as sodium fluorescein, is dramatically reduced in nonaqueous solvents.

Phospholipids are the major constituencies of the cell membranes and are found in many pharmaceutical, cosmetic, and food products. The conventional methods for the analysis of phospholipids, such as HPLC and TLC, often fail to satisfactory separate them. Attempts to separate phospholipids with CE have been made (*190, 191*). Methyl-β-cyclodextrin was used as an additive in micellar electrokinetic chromatography of fluorescently labeled phospholipids, allowing observation of the microheterogeneity within phospholipid classes (*190*). The combination of laser-induced fluorescence with the use of a fluorogenic reagent produced concentration detection limit of ∼1 nM.

Carbohydrates. Carbohydrate analysis is complicated by the diversity of carbohydrate structures. In terms of ionization ability, carbohydrates may be divided into two classes, weakly ionizable carbohydrates (neutral sugars) and acidic carbohydrates (sialic acids). Weakly ionizable carbohydrates are neutral at mild pH. Therefore, their separation is usually based on their complexation with borate, which forms negatively charged complexes. The presence of surfactants in a borate buffer may enhance the selectivity of their separation. Weakly ionizable carbohydrates can be deprotonated at extremely basic conditions; their separation at very high pH values is possible without the need for complexation agents. Sialic acids are negatively charged at neutral pH values and can be separated similarly to other ionizable species.

Carbohydrates do not strongly absorb light at wavelengths longer than 200 nm and conventional absorbance measurements are not useful in their detection. In contrast, indirect UV detection is often applied. Other techniques, such as electrochemical, fluorescence, refractive index, or MS detection, can also be used.

Derivatization of carbohydrates usually relies on reductive amination. Derivatization with an ionized species can be also used to introduce a charge on a neutral sugar molecule.

Laser-induced fluorescence provides the best concentration limit of detection. Oligosaccharides from glycoprotein have been profiled by CZE and laser-induced fluorescence detection (*192*). The N-linked oligosaccharides were enzymatically released from the protein and fluorescently labeled with 8-aminonaphthalene-1,3,6-trisulfonate by reductive amination. The labeled oligosaccharides, including sialilated ones, were efficiently separated in acetate buffer. Similarly, N-linked oligosaccharides were enzymatically freed from an IgG antibody, derivatized with a fluorescent reagent, and separated by capillary electrophoresis; multiple enzymatic steps were used to determine specific carbohydrate linkages (*193*).

While LIF detection is characterized by good limit of detection, mass spectrometry provides structural information. The combination of these two detection techniques has been used for carbohydrate analysis. Dextran has been recently analyzed using CZE-ESI-ion trap MS (*194*). The polymer was hydrolyzed and the fragments were derivatized with 8-aminonaphthalene-1,3,6-trisulfonate by reductive amination. The derivatized mixture of oligosaccharides was separated in a volatile acetic acid-based buffer and detected with mass spectrometry.

Information on the structure of glycosaminoglycans, components of heparan sulfate proteoglycans, has been acquired by combining capillary electrophoresis separation of oligosaccharide fragments, obtained by enzymatic digestion of the glycosaminoglycans, with MALDI-MS (*195*).

The effect of surfactants and cyclodextrins on the capillary electrophoresis separation of fluorescently labeled neutral and charged sugars has been studied (*196*). Complex mixtures of oligosaccharides originating from two types of glycoproteins have been used to evaluate the separation techniques. It was shown that SDS-based micellar electrokinetic chromatography separates the neutral oligosaccharides but not the charged sialilated carbohydrates. The latter could be separated if SDS was replaced with anionic cyclodextrins. However, neutral oligosaccharides are better resolved in the SDS-based separation.

Structurally similar carbohydrates have been separated using CGE in the capillaries with immobilized antibodies that had affinity to the carbohydrates (*197*). Because the affinity was relatively low, the retention time in a 27-cm capillary did not exceed 10 min. Nevertheless, the system separated two oligosaccharides with slightly different structures.

High-molecular-weight poly(neuraminic acid) was separated in a sieving polymer. Polymers containing over 100 monosaccharide residues were separated (*198*).

Affinity studies have been performed based on carbohydrates (*199*). Amylodextrin oligomers were used to model carbohydrate−drug interaction. Fluorescently labeled oligosaccharides were used in the experiment. Interaction of an oligosaccharide with the drug produced a mobility shift in this affinity capillary electrophoresis experiment. Similarly, drug enatiomer interaction with glycoproteins were also studied (*200*).

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