9

Capillary Electrophoresis of Biopharmaceutical Proteins

Roberto Rodriguez-Diaz, Stephen Tuck, Rowena Ng, Fiona Haycock, Tim Wehr, and Mingde Zhu

INTRODUCTION

In the development of new biopharmaceutical molecules, there is a constant need for analytical methods that provide critical information in areas that range from early characterization to routine analysis of approved products. Past experience indicates there are few projects in drug development that can be addressed by "standard" analytical procedures. Even well-established techniques often have to be modified to better suit the analysis of new samples. For this reason, a broad range of techniques is already an integral part of laboratories in the biopharmaceutical industry.

Methods development starts with a relatively high number of techniques to characterize and test samples. The number of protocols is often reduced once the critical parameters and the methods that identify them have been defined. The analyst must evaluate the initial techniques with respect to their purposes. If the goal is to generate research data, the practicality of the method and its limitations are not of primary concern; if the goal is to use the technique as part of a test procedure, it has to be evaluated in terms of its potential to meet full validation. Critical procedures (e.g., release testing) that cannot be validated will bring a project to an expensive halt. For these reasons, this chapter provides basic principles as well as limitations of capillary electrophoresis (CE) as applied to the analysis of real biopharmaceutical molecules.

When a pharmaceutical is in the early stages of drug development (e.g., preclinical phases), the amount of material is limited and little is known about the characteristics of the molecule. Thus, CE is an attractive technique for developmental work due to its low sample consumption. Multiple injections can be made from a few microliters of sample, thus allowing optimization and early qualification of methods.

CE provides analysis based on orthogonal separation principles compared to other techniques as well as high resolving power. Like slab gel electrophoresis, CE is a family of techniques that resolve sample components by differences in intrinsic molecular characteristics such as size, mass, charge, differential interaction, and isoelectric point (pI).

As an analytical chemist whose task is to make measurements, knowing how experimental parameters affect measurements results in increased troubleshooting ability and expertise. For these reasons, basic theoretical principles and their applications will be presented.

Critical Factors for CE Methods Development

When developing a CE method for routine analysis of samples in our laboratory, the first three parameters to be checked are reproducibility, sensitivity, and throughput. There is little use for any analytical method that does not meet these three requirements.

Reproducibility in CE is affected by many parameters, and a detailed description of these factors is beyond the scope of this chapter. Some of the most common problems associated with poor reproducibility include sample composition, sample–capillary wall interactions, and sample instability. Sample composition may affect injection of the analyte and cause fluctuations in the current. Instability of sample components, including excipients, under analysis conditions can hinder the separation and reproducibility of the method. Undesirable sample–capillary wall interactions and sample instability have a profound effect on peak pattern, migration time, and resolution. Peak pattern irreproducibility and loss of resolution are unpredictable and weaken the value of the data obtained. Migration-time variations can be addressed by using internal standards or reference standards. In addition, coinjections of reference and sample materials are useful for identifying peaks. The main approaches to minimizing sample interactions with the column wall are capillary coatings and buffer additives. In some cases it may be necessary to use both. Although there are some logical guidelines to follow in the selection of the capillary and additives, the final choice relies heavily on empirical experimentation.

CE is based on the use of narrow-bore capillaries with internal diameters typically betwen 20 and 150 μm. Because most commercial instruments equipped with ultraviolet/visible (UV-Vis) absorption detectors use a segment of the same capillary as the detection cell, the path length in CE is much less compared to those in HPLC or spectrometry. Therefore, the most commonly used CE detectors

can present problems with sensitivity, especially when detecting impurities at or below the 1% level if the sample is not concentrated enough. Because UV-Vis absorption is so simple and universal, a number of strategies have been developed to extend the sensitivity of CE. One of these approaches is the use of extended path-length cells (e.g., bubble cells or Z-cells). Although the improvement in detection can be significant, alternative capillaries like these should be carefully evaluated because resolution may be compromised, and the available configurations and internal wall modifications are limited. Detectors that employ fluorescence with continuum sources do not greatly improve sensitivity, but fluorescence detection with laser-based sources dramatically increases sensitivity. The advantages and drawbacks of the most common detectors will be addressed later in this chapter.

In CE, a single sample is injected at the inlet of the capillary and multiple samples are analyzed in series. This contrasts with conventional electrophoresis in which multiple samples are run in parallel as lanes on the same gel. When sample throughput is a problem in slab gel electrophoresis, it is easy to order another set of gel boxes and a power supply. Due to the cost, ordering another CE system may not be a viable solution. This limitation in sample throughput is somewhat compensated by the ability to process samples automatically using an autosampler and by the speed of the analysis. CE is highly suitable in applications with a small number of samples requiring short analysis time, e.g., during process monitoring or in a QC laboratory with low sample analysis requirements for that particular application. If high throughput is essential, multiple capillary systems are commercially available.

By evaluating the limitations of CE, one is left to wonder if there are any real applications that can be developed using this technique. The fact is that researchers have found clever ways to overcome the problems described in the preceding text. Their efforts resulted in procedures that provide greater convenience or information hard to obtain with other technologies.

PRINCIPLES AND PRACTICE OF CAPILLARY ELECTROPHORESIS

This section provides a brief discussion of the basic theoretical concepts of CE (including separation mechanisms), a description of CE instrumentation, and some guidelines in selecting conditions for a CE separation. Readers interested in more detailed presentations of CE theory and practice may consult References 1 to 8. Several general reviews of CE have been published,[9–11] as well as specific reviews of protein analysis by CE.[12–16]

CE is frequently compared to two of the mainstream techniques in the protein laboratory: HPLC and gel electrophoresis. The comparison to HPLC focuses on the instrumentation format and modes of detection, whereas gel electrophoresis shares its separation principles with CE. CE exploits the same molecular differences of the sample components to achieve separation as slab gel

electrophoresis. The basis of separations in CE is differential migration of proteins in an applied electric field due to intrinsic characteristics of the sample (e.g., mass-to-charge ratio, pI) or to active participation of the separation matrix (e.g., sieving separations based on size). Despite the similarities, CE also provides capabilities that are not possible with either of the two other techniques.

Gel electrophoresis provides a simple method for separating complex protein mixtures. Because proteins are visualized using stains that may not be linearly incorporated in the gel, the intensity of the stained bands may be poorly correlated with the amount of protein. For this reason, gel electrophoresis is at best a semiquantitative technique capable of generating relative purity results. In CE, separations are commonly performed in free solution, i.e., in the absence of any support such as gel matrices. This allows the replacement of the capillary's content in between analyses and therefore the automation of the process. The use of UV-transparent fused-silica capillaries enables direct on-line optical detection of focused protein zones, eliminating the requirement for sample staining. The detection systems available to CE provide true quantitative capabilities.

Basic Theory of Capillary Electrophoresis

CE is a technique with a very high power of resolution. This is attributed to low diffusion and high plate numbers obtained from the absence of band-broadening factors (e.g., eddy diffusion, equilibrium dynamics, etc.) other than diffusion, which is also minimized by short analysis time.

CE is a family of techniques similar to those found in conventional electrophoresis: zone electrophoresis, displacement electrophoresis, isoelectric focusing (IEF), and sieving separations. Other modes of operation unique to CE include micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC).

As the name implies, CE separates sample components within the lumen of a narrow-bore capillary (20 to 150 μm) filled with a buffered electrolyte. High electric fields (hundreds of volts/centimeters in practice, but sometimes in excess of 1000 V/cm) can be used in CE because the capillary contains a small volume of electrolyte and a relatively large surface area to dissipate the heat generated by the electric current (Joule heat). High-voltage applications result in reduced analysis time and therefore less diffusion.

A schematic representation of a CE system is presented in Figure 9.1. In this diagram, the CE components have obvious counterparts to those found in slab gel electrophoresis. Instead of buffer tanks there are two small buffer reservoirs, and the capillary takes the place of the gel (or more accurately, a gel lane). The capillary is immersed in the electrolyte-filled reservoirs, which also make contact with the electrodes connected to a high-voltage power supply. A new feature to the conventional gel electrophoresis format is the presence of an on-line detection system.

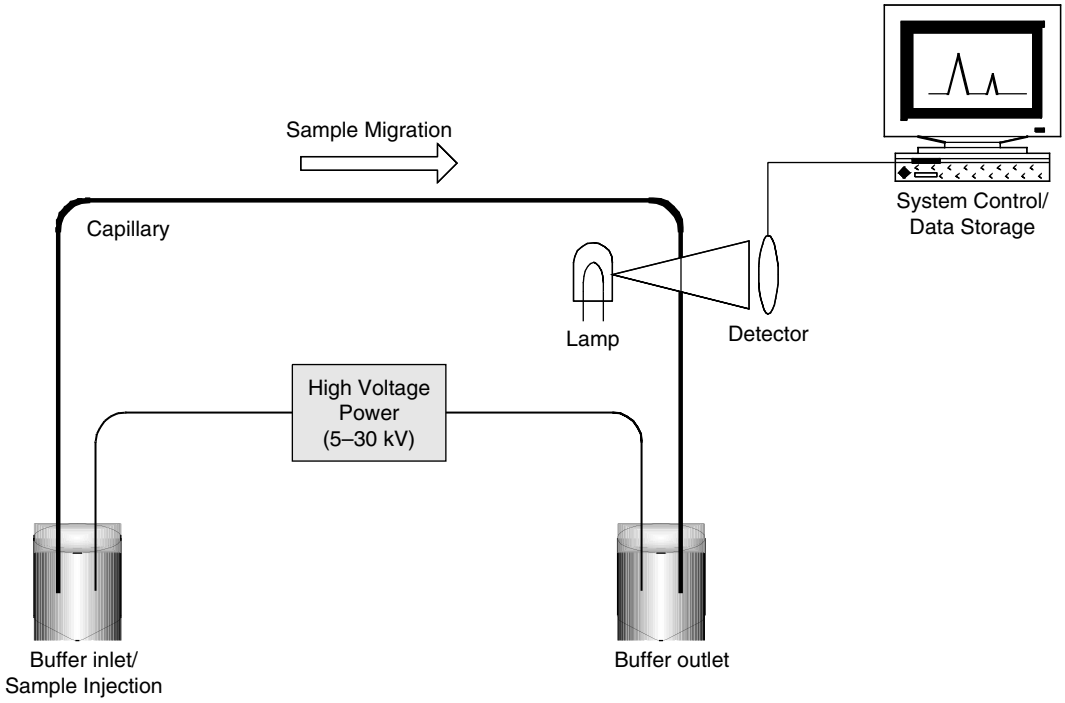

Figure 9.1 Schematic representation of a basic capillary electrophoresis system. The main components include a capillary (commonly contained within a housing that allows for temperature control), a power supply, and a detector. Automation is achieved through the use of computer-controlled setting of solutions and samples, displacement forces (to replace capillary contents and for hydrodynamic injection), and automatic data collection. (Courtesy of Agilent Technologies.)

A typical analysis starts by filling the capillary with fresh electrolyte, then the sample is introduced at one end of the capillary (the inlet), and analytes are separated as they migrate through the capillary toward the outlet end. As separated components migrate through a section at the far end of the capillary, they are sensed by a detector and an electronic signal is sent to a recording device. The data output (peaks on a baseline similar to those obtained with HPLC) can be displayed as an electropherogram and integrated to produce quantitative information in the form of peak area or height.

Separations in CE are based on the different velocities of charged species when they encounter an electric field; thus a key parameter in CE is electrophoretic mobility. Mobility (μ) is the rate of migration of sample components under a given set of conditions:

$$\mu = (L_t \times L_d)/(t_m \times V)$$

where L_t is the total length of the capillary, L_d is the length of capillary to the detection point, t_m is the migration time, and V is the voltage. Mobility is expressed in $cm^2\ V^{-1}\ s^{-1}$.

In the presence of electroosmotic flow (EOF), the mobility of a given molecule is a combination of its own mobility (which is now called *apparent mobility*, μ_{app}) and the mobility of EOF (μ_{EOF}). True mobility is then calculated by subtracting the mobility of EOF from apparent mobility:

$$\mu = \mu_{app} - \mu_{EOF}$$

The mobility of EOF is estimated using the migration time of a neutral marker.

Electroosmosis

When a capillary with electrical charges present on the wall is filled with a buffer solution, an electrical double layer is formed at the inside surface. Because counter-ions from the buffer form a layer close to the capillary surface, this layer is of opposite charge to that of the wall. When an electric field is applied, the fixed layer of charges on the wall is unable to move, but the buffer layer migrates toward the electrode with opposite charge. The result is the bulk movement of the liquid contained within the capillary, referred to as *electroosmosis* or *electroendosmosis* (Figure 9.2). The two most important practical considerations of EOF are its velocity and its direction. The direction of EOF is toward the electrode with the same charge as the capillary wall. In practice, the velocity of EOF (v_{EOF}) is determined using a UV-absorbing neutral molecule as sample. Because EOF is easily affected by many parameters including protein interaction with silica surfaces, an internal marker, usually the same neutral molecule employed to determine EOF, is used as an internal standard. Under these conditions, proteins with net positive charge will migrate faster than the rate of EOF. (EOF velocity is higher than the mobility of most proteins, and thus the polarity of the power

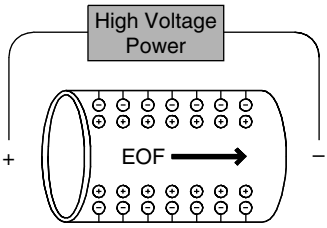

Figure 9.2 Formation of an electrical double layer responsible for electroendosmotic flow in an uncoated fused-silica capillary. The negative charges on the surface of the capillary are "neutralized" by positive charges of cations present in the buffer, which form an electrical layer near the surface of the capillary. When the electric field is applied, the positive charges migrate toward the negative electrode, generating a bulk flow of the solution contained within the column. Electroosmosis exhibits a flat profile, in contrast to hydraulic flow, which is parabolic.

supply is set so that EOF flows toward the detector side of the capillary.) Anionic proteins will migrate toward the cathode at a rate that is the difference between their electrophoretic velocity and ν_{EOF}. A variety of capillary surface treatments and buffer additives developed for reducing protein adsorption and controlling EOF are described in detail in "Minimization of Nonspecific Protein–Wall Interactions."

Capillary Electrophoresis Instrumentation

The core components of a CE instrument are a power supply, a detector, and devices that allow for temperature control of the capillary and sample compartment. A wide variety of commercial CE instruments are available, from simple modular systems to fully integrated automated systems under computer control.

Power Supply

Most systems are equipped with power supplies that can be operated in constant voltage, constant current, and constant power modes. Common limits are 30 kV and 300 μA for constant voltage and constant current, respectively. Software-controlled polarity switching and programmable gradients are conveniences.

Detectors

The main detectors used in CE are briefly described in "Detection of Proteins." The most common detectors include absorbance, fluorescence, and on-line coupling with mass spectrometry (MS).

Capillary Temperature Control

Temperature control of the capillary environment is essential for attaining satisfactory reproducibility. Inadequate temperature control results in variable migration

times. In CE, peak area depends upon the residence time of the component in the detector light path and therefore is dependent upon migration velocity. If migration times vary because of inadequate temperature control, peak area precision will be poor. Control of capillary temperature above or below ambient temperature may be desirable in special applications, such as kinetic studies, on-column enzyme assays, or in the study of protein folding. The effectiveness of capillary thermostating can be determined by variation in current as a function of voltage. According to Ohm's law, this should be a linear relationship, and deviation from linearity in an Ohm's law plot is indicative of poor efficiency in heat dissipation by the capillary temperature control system. For the use of CE as a research tool, programmable temperature gradients are useful for some applications.

Other devices or capabilities may be necessary for the performance of techniques such as electrochromatography (high pressure and pressure on during the run), sieving using polymer solutions (pressure higher than 50 psi), and capillary isoelectric focusing (CIEF) with pressure mobilization (programmable pressure).

Preparative Capillary Electrophoresis

Although the total amount of sample that is loaded into the capillary is extremely small, the use of CE as a "preparative" technique is highly desirable. A preparative technique is one in which samples are collected and then used as product or starting material for other analytical assays. Modern characterization methods require minute amounts of sample to provide such information as amino acid content, sequence, and activity. The use of larger-bore capillaries (e.g., 100 to 200 μm) is recommended for preparative analysis because more sample volume is injected for these capillaries. For short columns with large internal diameters, the use of viscosity-enhancing agents may be necessary to avoid siphoning. In addition, it is important that the additive does not interfere with the technique used for further analysis. Multiple analyses to enrich sample concentration are often necessary.

Capillary Electrophoresis Separation Modes

One of the major advantages of CE as a separation technique is the wide variety of separation modes available. Analytes can be separated on the basis of charge, molecular size or shape, pI, or hydrophobicity. The same CE instrument can be used for zone electrophoresis, IEF, sieving separations, isotachophoresis, and chromatographic techniques such as MEKC and capillary electrokinetic chromatography. This section provides a brief description of each separation mode. Zone electrophoresis, IEF, and sieving are the primary modes used for protein separations, and these will be discussed in detail in the following sections.

Capillary Zone Electrophoresis (CZE)

In CZE, the capillary, inlet reservoir, and outlet reservoir are filled with the same electrolyte solution. This solution is variously termed *background electrolyte*, *analysis buffer,* or *run buffer.* In CZE, the sample is injected at the inlet end of the capillary, and components migrate toward the detection point according to their mass-to-charge ratio by the electrophoretic mobility and separations principles outlined in the preceding text. It is the simplest form of CE and the most widely used, particularly for protein separations. CZE is described in "Capillary Zone Electrophoresis."

Capillary Isoelectric Focusing (CIEF)

IEF is similar in concept to conventional gel IEF; a stable pH gradient is formed in the capillary using carrier ampholytes, and proteins are focused in the gradient at their pIs. The major difference in performing IEF in the capillary format rather than slab gel is the requirement for mobilizing focused protein zones past the detection point. IEF is described in "Capillary Isoelectronic Focusing."

Capillary Sieving Techniques

Sieving techniques are required for separation of species that have no difference in mass-to-charge ratio. This includes native proteins composed of varying numbers of identical subunits, protein aggregates, and sodium dodecylsulfate (SDS)–protein complexes. Sieving systems include cross-linked or linear polymeric gels cast in the capillary or replaceable polymer solutions. Sieving techniques are described in "Sieving Separations."

Capillary Electrochromatography (CEC)

CEC, or capillary electrokinetic chromatography, is a chromatographic technique performed with CE instrumentation. It employs fused silica capillaries packed with 1.5 to 5 μm microparticulate porous silica beads, usually derivatized with a hydrophobic ligand such as C18. Mobile phases are similar to those used for conventional reversed-phase HPLC, e.g., mixtures of aqueous buffers and an organic modifier such as acetonitrile. The silica surface of the derivatized beads has a sufficient density of ionized silanol groups to generate a high electroendosmotic flow when a voltage is applied to the system, thus pumping mobile phase through the column. In contrast to the pressure-driven flow of HPLC, the EOF-driven flow of CEC generates negligible pressure and allows the use of small-diameter beads to improve efficiencies and resolution. In addition, EOF is pluglike rather than laminar in nature, so efficiencies in CEC can be much higher than in HPLC. Like MEKC, CEC is used primarily for small-molecule analysis. Some of the obstacles to using CEC for reversed-phase protein applications include forming reproducible solvent gradients and improving chromatographic supports to maintain stable EOF and increase protein recovery.

Micellar Electrokinetic Chromatography (MEKC)

MEKC is a mode of CE that applies mostly to the analysis of small molecules. The technique is usually performed in uncoated capillaries under alkaline conditions to generate a high electroendosmotic flow. Like CEC, MEKC is a chromatographic technique in which sample components are separated by differential partitioning between two phases: the analysis buffer and a pseudostationary phase usually consisting of detergent micelles. The main differences between MEKC and true chromatography are the presence of the pseudostationary phase and the fact that molecules are transported by an electric field rather than the pressure used in chromatography. The analysis buffer contains a surfactant at a concentration above its critical micelle concentration (CMC). The most widely used MEKC system employs SDS as the surfactant. The sulfate groups of SDS are anionic, so both surfactant monomers and micelles have electrophoretic mobility counter to the direction of EOF. Sample molecules will be distributed between the bulk aqueous phase and the micellar phase depending upon their hydrophobicity (Figure 9.3). Hydrophilic neutral species with no affinity for the micelle will remain in the aqueous phase and reach the detector in the time required for EOF to travel the effective length of the column. Hydrophobic neutral species will spend varying amounts of time in the micellar phase depending on their hydrophobicity, and their migration will therefore be retarded by the anodically moving micelles. Charged species will display more complex interactions because they have the potential for electrophoretic migration and electrostatic interaction with the micelles in addition to hydrophobic partitioning. The selectivity of MEKC can be expanded with the introduction of chiral selectors or chiral surfactants to the system. MEKC is used almost exclusively for small molecules such as drugs and metabolites; it has been used occasionally for peptides.

The use of MEKC for the analysis of proteins has been reported in the literature,[17] but the separation mechanism described in the preceding text is not applicable. SDS micelles range from 3 to 6 nm in diameter, which is too small to accommodate molecules larger than about 5 kDa. However, proteins can bind tenaciously to SDS monomers and micelles via hydrophobic, hydrophilic, and electrostatic interactions. Evidence indicates that SDS–protein complexes consist of protein-enclosed micelles distributed along the protein chain.[18] Perhaps differential binding of SDS at low concentration by different proteins can provide or improve resolution for some protein samples. Under this concept, those proteins that bind more SDS will acquire a higher rate of mobility. One concern would be the reproducibility of the method because variations in detergent concentration (run-to-run, day-to-day) might change the mobility of the high SDS-binding proteins accordingly. Too much SDS (or a long incubation time) can mask the natural charges of the polypeptides, thus giving them a near-equal mass-to-charge ratio. Other detergents can improve performance by increasing sample solubility and by minimizing sample–capillary wall interaction.

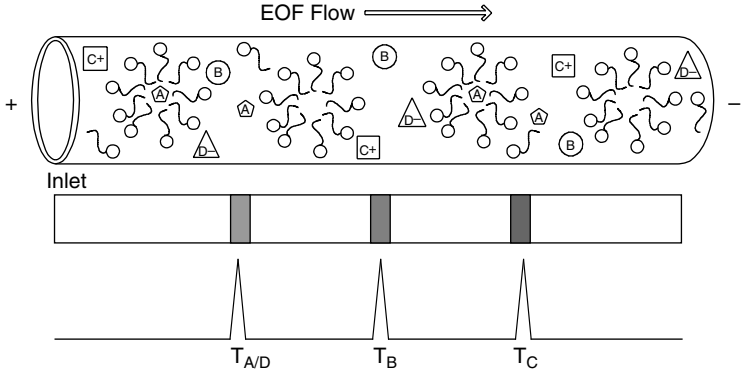

Figure 9.3 An MEKC system (top) includes detergent micelles that possess a hydrophobic core and an aqueous phase represented by the buffer solution. The hydrophobicity of the micelle depends on the chain length of the detergent. The migration profile during analysis of samples depends on charge and degree of interaction with the micelles (bottom). Because EOF is faster than the migration of most analytes, the detector is placed near the negative electrode (cathode) for capillaries with negative wall charge. Micelles of SDS exhibit a strong negative charge and migrate toward the positve electrode (anode). However, the force of EOF carries the negatively charged detergent against its electrophoretic mobility and toward the cathode. In this example, analyte C, with a migration time of T_C, has a positive charge, and therefore its mobility is a combination of electrophoretic mobility toward the cathode and EOF. Analyte B is a neutral molecule interacting loosely with the micelle and thus exhibits a longer migration time (T_B) as it is carried by EOF alone. Analyte A, a neutral molecule that interracts with the hydrophobic core of the SDS micelles, migrates toward the anode but is eventually carried to the cathode by EOF. Thus, T_A is later than T_B. Like the negatively charged SDS, analyte D also migrates toward the anode before being overcome by EOF. Migration of samples that do not interact with the detergent is based solely on electrophoretic mobility and EOF. A high degree of selectivity is achieved because of the combination of electrophoretic mobility and partitioning within the micelles.

Although protein behavior in SDS-containing buffers is qualitatively different from small molecules, applications of MEKC-type conditions have been applied to many protein separations. In applications using uncoated capillaries, protein–wall interactions are eliminated because of the anionic character of SDS–protein complexes. In applications using coated capillaries with no EOF, the high electrophoretic mobility of SDS–protein complexes can decrease analysis time.

DETECTION OF PROTEINS

CE is increasingly applied to analysis of proteins present at trace levels in biological materials or to determination of impurities and degradation products in formulations

of protein therapeutics. In these instances, detection sensitivity becomes a limiting factor in obtaining quantitative information. Several strategies have been pursued to increase detection sensitivity in CE of proteins.

Absorbance Detection

In liquid chromatography, absorbance detection at 280 nm is typically used to monitor the separation of proteins. The detector signal at this wavelength is due to the absorbance of aromatic residues in the polypeptide, e.g., tryptophan, tyrosine, and phenylalanine to a lesser extent. This detection wavelength is sufficiently selective for polypeptides so that nonprotein interferences in the sample are not detected, and the path length of an HPLC absorbance detector (nominally 1 cm) provides sufficient signal for satisfactory sensitivity. However, reduction of the detector path length to 25 to 75 μm in CE cuts the detector signal by a factor of 100 to 400. Therefore, detection at 280 nm rarely provides sufficient signal for satisfactory sensitivity. Instead, detection at 200 nm is typically employed where proteins exhibit 50- to 100-fold greater absorbance.

As in HPLC, absorbance detection is used in the vast majority of CE applications, and all commercial CE systems employ UV or UV-Vis absorbance as the primary mode of detection. All commercial CE absorbance detectors employ on-tube detection in which a section of the capillary itself is used as the detection cell. In accordance with Beer's law, the sensitivity of a concentration-sensitive detector is a direct function of the length of the light path. Therefore, in comparison to an HPLC detector with a 1-cm path length, detector signal strength is reduced 200-fold in a CE system equipped with a 50-μm I.D. capillary. Concentration sensitivity can be improved by employing focusing lenses to collect light at the capillary lumen, by detecting at low wavelengths (where most analytes have greater absorbance), and by using sample-focusing techniques during the injection process. However, even under ideal conditions, the concentration limit of detection (CLOD) is about 10^{-7} *M*.

Several commercial CE systems incorporate absorbance spectra detectors. Absorbance spectra detection enables on-the-fly acquisition of spectra as analytes migrate through the detection point; this information can assist in the identification of peaks based on spectral patterns, in the detection of peak impurities by variation in spectral profiles across a peak, or in the determination of the absorbance maximum of an unknown compound. Two different designs are used to accomplish this type of detection in CE instruments. In photodiode array (PDA) detectors, the capillary is illuminated with full-spectrum source light; the light passing through the capillary is dispersed by grating onto an array of photodiodes that individually sample a narrow spectral range. In fast-scanning detectors, monochromatic light is collected from the source using a movable grating and slit assembly and directed to the capillary; light transmitted by the capillary is detected by a single photodiode. Scanning is accomplished

by rapidly rotating the grating through an angle to "slew" across the desired spectral range.

Fluorescence Detection

The high sensitivity and selectivity of fluorescence detection make this the obvious choice for improving detection of proteins. Three approaches have been used: direct detection of intrinsic protein fluorescence, indirect fluorescence detection, and protein derivatization for fluorescence detection.

Fluorescence detection offers the possibility of high sensitivity and, in the case of complex samples, improved selectivity. However, this mode of detection requires that the analyte exhibit native fluorescence or contain a group to which a fluorophore can be attached by chemical derivatization. Because only tryptophan and tyrosine exhibit significant native fluorescence, fluorescence detection of proteins usually requires derivatization.

When compared to fluorescence detectors for HPLC, the design of a fluorescence detector for CE presents some technical problems. In order to obtain acceptable sensitivity, it is necessary to focus sufficient excitation light on the capillary lumen. This is difficult to achieve with a conventional light source but is easily accomplished using a laser. The most popular source for laser-induced fluorescence (LIF) detection is the argon ion laser, which is stable and relatively inexpensive. The 488-nm argon ion laser line is close to the desired excitation wavelength for several common fluorophores. The CLOD for a laser-based fluorescence detector can be as low as 10^{-12} *M*.

For SDS–protein complexes, the proteins can be derivatized with fluorescent dyes prior to the analysis. Derivatized proteins can be detected in the attomole (amol) level, and because the complex is resolved by sieving, multiple reaction products are detected as peak broadening instead of multiple peaks.[19]

Intrinsic Protein Fluorescence

Compared to absorbance detection, direct detection of proteins rich in aromatic amino acids by the intrinsic fluorescence of tryptophan and tyrosine residues provides enhanced sensitivity without the complexity of pre- or postcolumn derivatization. The optimal excitation wavelengths for these amino acids are in the 270- to 280-nm range.

Indirect Protein Fluorescence

A simple alternative to direct detection of intrinsic protein fluorescence detection is the technique of indirect fluorescence detection proposed by Kuhr and Yeung.[20] In this approach, the analysis buffer contains a fluorescent anion that produces a high background fluorescence signal. Nonfluorescent analyte anions displace the fluorescent species, producing a zone of reduced signal. Sensitivity in indirect fluorescence detection is determined by the dynamic reserve (ratio of signal

intensity to signal fluctuation, S/N) and the displacement ratio. Using a coated capillary and salicylate as the background fluorophore, a detection limit of 100 amol was demonstrated for lysozyme with a stabilized HeCd laser providing excitation at 325 nm and collection of emission at 405.1 nm.

Protein Derivatization for Fluorescence Detection

Some precolumn derivatization procedures described for absorbance detection can also be applied to fluorescence detection. In fact, many of the reagents used for absorbance detection (OPA, NDA, fluorescamine) are also highly fluorescent. Although many analytes contain reactive groups (e.g., amino, carboxyl, hydroxyl), most derivatization chemistries are limited by such disadvantages as slow reaction kinetics, complicated reaction or cleanup conditions, poor yields, interference by matrix components, derivative instability, and interference by reaction side products or unreacted fluorescence agent. In addition, most proteins possess multiple reactive sites, and incomplete derivatization yields a family of products varying in the number of fluorophores. The reactive sites are usually side-chain amino groups, and the derivatized products (which vary in mass and charge) may be resolved into multiple peaks or migrate as a single broad peak. Loss of efficiency and resolution of multiple species has been observed in CZE separation of proteins following precolumn derivatization with fluorescein isothiocyanate[21] and OPA.[22] Bardelmeijer et al.[23] provide an extensive review of fluorescent labeling of proteins.

Mass Spectrometry

With the increasing need to obtain absolute identification of separated components and the gradual price reduction of mass spectrometers, there is a growing demand for direct coupling of CE with MS instruments. On-line coupling of a CE system to a mass spectrometer enables molecular weight and structural information to be obtained for separated components.[24] The most frequent configuration is introduction of the capillary outlet into an electrospray interface (ESI) coupled to the mass spectrometer. In this configuration, the outlet electrode of the CE is eliminated and the MS becomes the ground. Because the volumetric flow out of the capillary is negligible or nil, separated components are usually transported from the capillary to the electrospray using a liquid sheath flow.

Compatibility with MS requires the use of volatile buffer systems such as acetic and formic acids or their ammonium salts for low-pH separations, or ammonium carbonate for high-pH applications. This limits the choice of CE separation modes and selectivity of the CE separation system. The background electrolyte causes discrimination against analyte ions by charge competition in the electrospray, thus reducing overall sensitivity. Using low concentrations of electrolytes in the analysis buffer can minimize this problem. Another factor in reducing sensitivity is the dilution of ions by the sheath flow liquid at the CE–MS interface. This can be minimized by reducing sheath flow rates to 2 to 5 μl/min

and by using sheath liquids composed of low concentrations of electrolyte in organic solvents (e.g., acetic acid in methanol).

Samples collected during CE analysis can be used for off-line mass analysis by matrix-assisted laser desorption ionization (MALDI) and time-of-flight (TOF) MS.

MINIMIZATION OF NONSPECIFIC PROTEIN–WALL INTERACTIONS

Interactions of proteins with the surface of the capillary, especially silica columns, are not different from those encountered when using unmodified chromatographic silica supports. These interactions are often strong or irreversible and have been major obstacles to successfully applying CE to protein separations. Bare silica capillaries contain weakly acidic silanol groups on the surface that ionize rapidly above pH 3. The charge density on the wall increases until the silanol groups are fully dissociated at pH 10. Under these conditions, proteins with basic amino acid residues positioned on the protein surface can participate in electrostatic interactions with ionized silanols. This results in band broadening, tailing, and, in the case of strong interaction, reduced detector response or complete absence of peaks. Such interactions change the state of the capillary wall during an analysis and can alter the magnitude of EOF from run to run, resulting in poor reproducibility. Three strategies have been employed to minimize protein–wall interactions: operation at pH extremes, use of buffer additives, and use of wall-coated capillaries. The simple solution of using "HPLC-like" conditions (e.g., high salt-eluting conditions for ion exchangers) is often not suitable for CE because of negative effects to other aspects of the system (e.g., excessive electric current).

Operation at pH Extremes

The simplest approach to minimizing protein–wall interaction is to use a buffer pH at which interactions do not occur. At acidic pH the silanols on the surface of the capillary are protonated, and the net charge of the proteins is positive. At high pH, the wall is negatively charged, and so are the sample components. Both conditions result in electrostatic repulsion. Problems associated with operation at pH extremes include the potential instability of proteins (denaturation, degradation, and precipitation) and the limited pH range in which to achieve resolution. Additionally, operation at extreme pH does not eliminate all nonspecific interactions.

Use of Buffer Additives

Buffer additives can overcome some of the protein interactions with the capillary wall. Some of these additives are widely used in HPLC to elute proteins off the chromatographic supports. Precautions should be taken when selecting buffer additives, especially in the areas of detector interference and their impact in the conductivity of the buffer. A list of additives used for various CZE applications can be found in Table 9.1.

Table 9.1 Additives Used in CZE

Additive	Application	Reference
Betaine	Basic proteins	154
Cadaverine	Acidic and basic proteins	159
Ethylene glycol (20%)	Acidic and basic proteins, serum proteins	160
Cationic and zwitterionic fluorosurfactants	Acid and basic proteins	155–158, 161
2-(*N*-cyclohexylamino)ethanesulphonic acid (CHES)	Insulins	162
N,*N*-bis(2-hydroxyethyl)-2-aminoethane-sulphonic acid (BES)	Insulins	162
3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid (AMPSO)	Insulins	162
3-(cyclohexylamino)-2-hydroxy-1-propanesulphonic acid (CAPSO)	Insulins	162
N-alkyl-*N*,*N*-dimethylammonio-1-propane sulfonic acid	Basic proteins	163
1,4-diaminobutane	Protein glycoforms	148, 152, 164
Spermine,spermidine	Protein glycoforms	165
Triethylamine	Basic proteins	149, 151, 166
Triethanolamine	Basic proteins	149, 151
Galactosamine	Basic proteins	150
Glucosamine	Basic proteins	150
Trimethylammonium propylsulfonate (TMAPS), trimethylammonium chloride (TMAC)	Monoclonal antibody	167
Trimethylammonium propylsulfonate (TMAPS)	Acidic, neutral, and basic proteins	168
(Trimethyl)ammonium butylsulfonate (TMABS)	Acidic, neutral, and basic proteins	168
2-hydroxyl-3-trimethylammonium propylsulfonate (HTMAPS)	Acidic, neutral, and basic proteins	168
3-(dimethyldodecylammonio)-propanesulfonate	Basic proteins	169
Cetyltrimethylammonium bromide	Acidic and basic proteins	170, 171
Chitosan	Basic proteins	172
Amino acids	Basic proteins	149
Hexamethonium bromide, hexamethonium chloride	Protein glycoforms	153
Decamethonium bromide	Protein glycoforms	153
Polydimethyldiallylammonium chloride	Basic proteins	173, 174
Phytic acid	Acidic and basic proteins	175–177
Ethylenediamine	Basic proteins	148
1,3-diaminopropane	Basic proteins	148
N,*N*-diethylethanolamine	Basic proteins	149
N-ethyldiethylamine	Basic proteins	149
Morpholine, tetraazomacrocycles	Basic proteins	178

Capillary Coatings and Other Surface Modifications

Capillary coatings restrict or eliminate access of sample molecules to the capillary wall. Surface modifications to the capillary wall may be grouped into two categories: covalent and dynamic coatings. Covalent coatings are attached to the wall through a chemical bond, whereas dynamic coatings physically interact with the silica or a first layer deposited over the silica. Depending on the strength of the interaction of the dynamic coatings, it might be necessary to add the wall-interacting compound in the run or conditioning buffer.

The presence of a coating modifies or eliminates EOF. Thus, degradation of the coating through physical loss or chemical changes leads to loss of efficiency and poor reproducibility. There are many coating chemistries described in the literature, which reflect the continuous search for more stable coatings and the inadequacy of any single approach to provide satisfactory results for all applications. Coating chemistries have been extensively reviewed previously.[25–27]

When developing a CE method that requires coatings, the end use of the method itself must first be defined. If the information to be gained is for research, the use of any chemistry is considered to achieve results. On the other hand, if the technique will be used in production or quality control environments, the capillary coating chemistries are limited to those commercially available. An in-house-produced coated capillary or buffer with additives must have a production protocol and quality control release testing. The benefits of having an optimal method must be weighed against the cost, and it is hard to justify all the extra work required if there is a simpler option available.

SAMPLE INJECTION AND PREPARATION

Sample Injection

Sample injection is an event common to all modes of CE except CIEF, and the most relevant aspects of this step are described here. Because the total volume of the capillary is very low, sample injection in CE requires the precise introduction of very small amounts of analyte at the capillary inlet. There are two basic procedures for sample injection: electromigration and hydraulic displacement. All commercial instruments offer electromigration, or electrophoretic, injection and at least one type of displacement injection.

In electrophoretic injection, the capillary inlet is immersed in the sample solution and a voltage is applied for a determined period of time. The amount of sample introduced into the capillary depends on the voltage and the time it was applied. Sample injection is a compromise between detection and resolution, and its parameters are often best determined experimentally. If detection is not a problem, resolution can be greatly improved by maintaining the sample "plug" as narrow as possible. If EOF is present, sample ions will be introduced by a combination of electrophoretic mobility and EOF; under these conditions, this injection mode is generally termed electrokinetic injection.

Electrophoretic injection can be used as a means for zone sharpening or sample concentration if the amount of ions, particularly salt or buffer ions, is lower in the sample than the running buffer. Because sample ions enter the capillary based on mobility, low-mobility ions will be loaded to a lesser extent than high-mobility ions. For this reason, the presence of nonsample ions will reduce injection efficiency, so electrophoretic injection is very sensitive to the presence of salts or buffers in the sample matrix. The disadvantages of electrophoretic injection argue against its use in routine analysis except in cases where displacement injection is not possible, e.g., in capillary gel electrophoresis (CGE) or when sample concentration by stacking is necessary.

Displacement injection is usually the preferred method because analyte ions are present in the sample zone in proportion to their concentration in the bulk sample. In addition, injection efficiency is less sensitive to variations in sample ionic strength. However, it should be noted that the presence of high salt can affect detector response and variations in the sample viscosity due to temperature, or the presence of viscosity-modifying components can affect displacement injection efficiency.

For quantitative purposes, the peak area must be corrected for mobility when using displacement injection. This correction is necessary because peak area is a result of sample response and time. Analytes with lower mobility spend more time in front of the detector, thus generating larger relative area counts. Such correction is not necessary for electrophoretic injections.

Sample Preparation

Sample preparation is often a parameter whose impact on the analysis is overlooked or underestimated. In reality, the composition of the sample matrix is often key to the quality of the data obtained at the end of the analysis. An important consideration for biopharmaceutical molecules is to minimize sample preparation because the impact of sample manipulations must be evaluated during methods validation.

In some cases, sample preparation for CZE requires only the dilution of the sample, mostly to accommodate detection (for signal and linearity of response). However, as was previously mentioned, sample characteristics such as viscosity, buffer composition (pH and excipients), and salt content can especially affect electrophoretic injection and performance.

Sample preconcentration techniques are used with two purposes: (1) to increase concentration in order to achieve detection and (2) to eliminate disturbances of the electrophoretic system during hydraulic or electrokinetic sample introduction when the conductivity of the sample is significantly different from that of the analysis buffer. It is important to keep sample manipulations and modifications to a minimum, and a rule of thumb is to prepare the sample so that its composition is at the same pH as the analysis buffer. It is also advantageous

to reduce the sample buffer composition to a conductivity approximately 10% of the background electrolyte. The two common reasons to modify sample composition, and the common methods used are given in the following subsections.

Preconcentration to Improve Detection

Several strategies have been described for the preconcentration of sample components present at low concentrations. These techniques include zone sharpening,[28,29] on-line packed columns,[30] and transient capillary isotachophoresis (cITP).[31,32] Other standard laboratory techniques are often used, including solid-phase extraction, protein precipitation, ultrafiltration, etc. Two important points to keep in mind when selecting a concentration protocol are the sample requirements of the method and the potential selectivity on relative concentrations of sample components. The latter point applies to purity and concentration analysis.

Preconcentration to Regulate Sample Conductivity

The introduction of a zone of different conductivity into a capillary produces an uneven distribution of the electric field. If the sample zone represents a small portion of the capillary, the electrical current usually "recuperates," presumably by diffusion of charge to the level normally seen in the analysis buffer. However, when the sample zone is of significant length due to overloading, the migration time can be severely affected[33] and, in extreme cases, the current drops to zero. Caution should be taken when using large bore and short columns because they can be easily overloaded. For this reason it is necessary to preconcentrate diluted samples to increase their conductivity, thus improving reproducibility and often resolution. The length of the sample zone that can be tolerated without major adverse effects depends on the difference in conductivity between the sample zone and the analysis buffer. In addition, due to uneven distribution of the electric field along the capillary, fluctuations in current are magnified when using higher voltage.

CAPILLARY ZONE ELECTROPHORESIS

In slab gel electrophoresis, it is necessary to use a medium that prevents convective disturbances during the analysis. CZE is analogous to native electrophoresis performed in low-percentage acrylamide or agarose gels, but in CE, convection is minimized by the low electric current generated and the high heat-dissipation characteristics of the capillary. Thus, separations can be performed in solution. CZE is often referred to as *free zone electrophoresis*, where "free" is used to specify that the buffer is free of stabilizing media.

In native gel electrophoresis and CZE, the sample components are resolved by their differences in electrophoretic mobility or mass-to-charge ratios. Electrophoretic analysis under native conditions in gel electrophoresis is not as widely used as SDS-PAGE. In gels, disadvantages of native analysis are the low field

strength that is used and the usually low charge density of native proteins. Both factors contribute to long analysis time. Native gels often require several hours for completion of the analysis. In the capillary format, high electric fields allow fast analysis time, and the coupling of on-line detection provides fast quantitative results. At the same time, CZE increases the range of application to smaller (e.g., inorganic ions, peptides) and larger sample species such as nano- or microparticles (Figure 9.4).

Manipulation of buffer pH or use of additives can easily vary separation selectivity. In contrast to gel electrophoresis, a single capillary can be used to evaluate the effect of buffer composition or pH on resolution. In CZE the capillary is simply filled with a fresh electrolyte of the chosen composition between analyses.

CZE offers several advantages in comparison to other CE separation modes, including its inherent simplicity: a single buffer is used throughout the capillary and electrode vessels, and sample is introduced as a zone or plug at one end. Capillary preparation often involves just filling the capillary with the separation buffer, although uncoated capillaries generally require prior washing or conditioning steps.

Developing a CZE Method

The development of a CZE separation requires the definition of instrumental and chemical parameters. There have been several attempts to model the behavior of proteins when placed under the influence of an electric field.[34,35] However, difficulties in estimating the charge of the polypeptide as opposed to free amino acids require that instrumental parameters including temperature, injection mode, detection, and power supply settings be determined experimentally. The main chemical parameter that affects CZE is buffer composition (including the type of buffer, additives, pH, etc.). The selection of the appropriate capillary type is intimately linked to the selection of buffer composition. Some parameters (e.g., temperature) need to be defined experimentally, whereas others (e.g., buffer pH) can be defined by the known characteristics of the sample such as solubility, pI, etc. The following subsections provide some general guidelines to consider during the development of a CZE method.

Buffer Selection

Buffer pH defines the net charges of the sample components and therefore the magnitude and direction of their mobilities. Ideally, the run buffer selected for CE should have good buffering capacity in the pH range that provides optimal resolution, be "transparent" to the detector system, and possess low conductivity at a concentration that still allows good pH buffering. Because the sample characteristics dictate the optimal pH range to be used (i.e., the range of maximum mobility difference for the components of interest), no one buffer is suitable for all applications. The pH of the buffer should be at least one pH unit above or

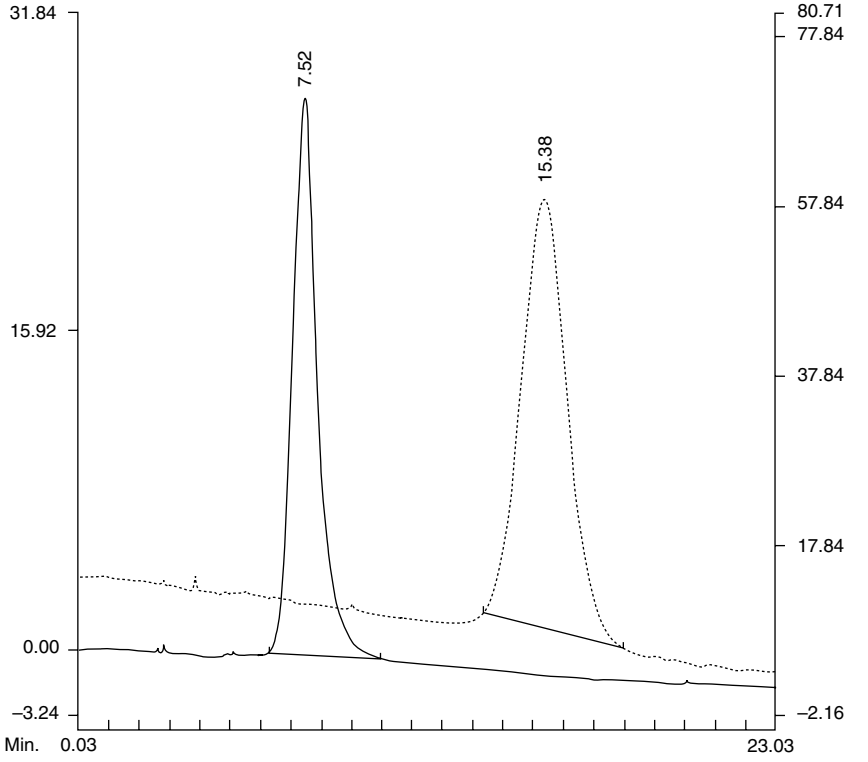

Figure 9.4 Overlay of electropherograms for Hepatitis B surface antigen (HBsAg) and HBsAg chemically linked to immunostimulatory oligonucleotides (ISS) as analyzed by CZE. HBsAg self-assembles into viruslike particles with diameters ranging from 20 to 30 nm. Because of its particle nature, HBsAg is difficult to characterize by analytical techniques such as chromatography (in which the sample particles get stuck in filters and small spaces between packing particles) and gel electrophoresis (in which the sample does not penetrate the pores of the separation matrix). Because CZE can be performed in capillaries of various internal diameters filled with buffer, HBsAg is easily analyzed by this technique. The broken line represents the unmodified particle, whereas the solid trace represents the particle conjugated to oligonucleotide. Notice that the sample conjugated to ISS has an increased mobility (shorter migration time) than the same particle before the attachment of ISS. The increased mobility is due to the high negative charge density of the oligonucleotide compared to its mass, which imparts a higher charge-to-mass ratio to the conjugate. The antigen and conjugate were analyzed using 50 m*M* phosphate buffer, pH 6.5 in a 40-cm × 50-µm coated capillary. The sample was introduced by applying pressure (5 psi) for 1 sec, and the electric field was set to 15 kV with the negative electrode in the injection port. The capillary temperature was maintained at 20°C, and the detection was performed by UV absorption at 200 nm.

below the pI of the protein of interest; at pH values closer to the protein pI, low mobility will result in long analysis times, peak broadening, and increased risk of protein–wall interactions. A variety of buffer systems exist that cumulatively cover a broad pH range appropriate for analyzing many proteins and peptides by CZE.[36] In applications that contain several proteins with a wide range of pIs, three main strategies have been used:

1. The separation may be performed at extremes of pH where all proteins possess net positive charges (e.g., pH 2 to 2.5) or net negative charges (e.g., pH 10 to 11). One concern with this strategy is that extremely high pH may result in protein degradation.
2. The separation may run twice using different polarities.
3. The proteins can be analyzed in the presence of EOF, which will carry all proteins to the detection point regardless of their net charge.

Analysis of polypeptides in the presence of EOF might necessitate the use of additives to decrease interactions between the sample and the capillary wall. This is true even if the analysis is performed at high pH where all polypeptides have a net negative charge. The charges on the surface of the protein are not distributed homogeneously, and positive "patches" can exist that can be attracted to the negative charges of the capillary's silanol groups. Some reagents used to reduce nonspecific interactions are discussed in "Minimization of Nonspecific Protein–Wall Interactions." In some cases, selectivity can be affected by adding molecules known to interact with sample components (e.g., metals or ligands).

Organic solvents (e.g., acetonitrile, methanol, etc.) help solubilize some hydrophobic proteins, and they can affect the Stokes radius, interaction with counterions, and the ionization state of the polypeptide. For more hydrophobic samples, the use of detergents and/or other organic solvents may prove beneficial.

Capillary Selection

Fused silica capillaries are almost universally used in capillary electrophoresis. The inner diameter of fused silica capillaries varies from 20 to 200 μm, and the outer diameter varies from 150 to 360 μm. Selection of the capillary inner diameter is a compromise between resolution, sensitivity, and capacity. Best resolution is achieved by reducing the capillary diameter to maximize heat dissipation. Best sensitivity and sample load capacity are achieved with large internal diameters. A capillary internal diameter of 50 μm is optimal for most applications, but diameters of 75 to 100 μm may be needed for high sensitivity or for micropreparative applications. However, capillary diameters above 75 μm exhibit poor heat dissipation and may require use of low-conductivity buffers and low field strengths to avoid excessive Joule heating.

Selection of capillary length is dictated by the type of capillary used and the required resolution. When using coated capillaries with insignificant EOF, separations can be achieved with relatively short capillaries of 20 to 30 cm effective length

from inlet to detection point. When using uncoated capillaries under conditions in which there is appreciable EOF, longer lengths of 50 cm or greater may be needed to achieve a separation, particularly for basic proteins that are migrating toward the detector under the combined forces of EOF and electrophoresis.

Capillary length is one of the parameters commonly used to improve resolution, but in our experience, more dramatic effects can be accomplished by changing the mobility of the sample components through manipulation of pH or the use of buffer additives. In fact, we usually develop a method in the shortest capillary possible, and only when the separation is adequate do we increase the capillary length for final optimization. This practice saves time in methods development because more data can be collected if the analysis time is short.

High-Voltage Parameters

High-voltage parameters include mode of operation, field strength, and polarity. Most CE systems can be operated in constant voltage or constant current, and some instruments allow the use of constant power. The majority of protein separations reported in the literature have been performed in constant voltage mode. Selection of field strength impacts the analysis time and resolution. Operation at high field strength reduces analysis time but increases band broadening due to thermal effects (which can be minimized by using low-conductivity buffers); operation at low field strength reduces heating but increases analysis time and band broadening due to diffusion. The latter effect is probably small for proteins because of their low diffusion coefficients. In our experience, operation at 400 to 600 V/cm provides optimal separations in terms of speed and resolution. When high voltages >1000 V/cm are used, the areas surrounding the electrodes must be kept clean and well sealed to avoid electric arcing. Selection of high-voltage polarity depends on the sample composition and capillary type. For example, when using uncoated capillaries under neutral to alkaline conditions, positive (inlet side) to negative (detector side) polarity is employed so that EOF will carry all samples toward the detection point.

Temperature

The use of temperature in most CZE applications is to maintain a constant and reproducible environment from one analysis to the next. Several applications also use temperature to improve resolution or provide additional characterization such as elucidation of protein structure.

Applications

A number of CZE applications exist for the separation of proteins and other molecules in purity analysis, structural studies, binding and equilibrium determinations, in-process product analysis, and mobility measurements. The following applications illustrate the use of CZE for both research and routine QC analysis.

Development and Validation of a CE Method for Characterization of Protegrin IB-367

An example of a simple CZE method for peptide analysis and characterization is the one developed for protegrin IB-367.[37] IB-367 is a peptide containing 17 amino acid residues that possess antimicrobial properties, and it is being developed for treatment of oral mucositis associated with aggressive cancer chemotherapy as well as other topical applications. This polycationic product was chemically synthesized using solid-phase and purified by preparative reversed-phase HPLC. IB-367 is rich in cysteine and arginine residues.

Optimized electrophoresis parameters included resolution, reproducibility, and minimal analysis time. The CZE method employs a 100-m*M* phosphate buffer, pH 2.6, and a 50-μm I.D. × 45-cm effective length uncoated capillary. Because the pH of the background electrolyte is 2.6, most silanols should be fully protonated on the capillary wall, thus minimizing undesired sample–capillary wall interactions. The capillary was conditioned with 0.1 *M* NaOH, water, and 0.1 *M* HCl between runs prior to a final water rinse and fill with the run buffer. The capillary was thermostated to 25°C, and detection was performed by UV-absorption at 200 nm. The sample was injected using pressure at 0.5 psi for 5 sec, and the run voltage was set to 20 kV. The voltage was selected by creating an Ohm's plot (voltage vs. electric current) and defining the highest voltage in the linear range of the plot. The linear range of an Ohm's plot defines the range of voltage at which there is still efficient Joule heat dissipation.

Both CE and HPLC methods were capable of resolving the IB-367 peptide from impurities and degradation products. However, the CE method provided better separation and resolution between this polycationic peptide and truncated analogs than HPLC methods. In addition, the CE methods resolved the potential impurities and degradation products from each other, whereas the HPLC methods failed to separate some truncated species.

The CE method was validated in terms of accuracy, precision, linearity, range, limit of detection, limit of quantitation, specificity, system suitability, and robustness. Improved reproducibility of the CZE method was obtained using area normalization to determine the purity and levels of potential impurities and degradation products of IB-367 drug substance. The internal standard compensated mainly for injection variability. Through the use of the internal standard, selected for its close mobility to IB-367, the method achieved reproducibility in relative migration time of 0.13% relative standard deviation (RSD), and relative peak area of 2.75% RSD.

System suitability tests serve to define the level of electrophoretic performance necessary to ensure valid CE assay results. System suitability of the method was evaluated by analyzing the symmetry of the IB-367 peak, theoretical plates of the capillary, and resolution between IB-367 and IB-300, the closest peak to IB-367. The sample concentration of the method was selected at approximately 0.5 mg/ml to assure symmetry below 3.5 and to assume sufficient sensitivity for detecting low

concentrations of impurities. At 0.5 mg/ml, the limit of detection for impurities was 0.1%. The following parameters were summarized from runs with different lots of capillary on a 0.5-mg/ml IB-367 solution:

- Asymmetry <3.5 ($T = W_{5\%}/2f$), where T is the tailing factor, $W_{5\%}$ is peak width at 5% peak height, and f is the width at 5% peak height measured from the leading edge to a vertical line extrapolated from the apex of the peak.
- Theoretical plates >50,000 ($N = 16(t_M/W)^2$, where t_M is the migration time and W the peak width, both in minutes.
- Resolution >2 ($Rs = 2(t_{M2} - t_{M1})/(W_2 + W_1)$, where t_{M1} and t_{M2} are the migration times of the two peaks being measured, and W_1 and W_2 are their peak widths, respectively. Units are all in minutes. Note that the values of the later migrating peak are entered first.

The accuracy of the method was evaluated by assaying six independently prepared solutions of IB-367 against two standard solutions of the same lot as external standards. The mean of 102.3% met the criteria set in validation protocol (97 to 103%).

The intermediate precision (day 2) of the method was evaluated by assaying six independently prepared solutions of IB-367 against two reference standard solutions used as external standards on 2 d. The RSD of 2.1% for the accuracy test (RSD) met the criteria set for repeatability (<4%). The RSDs of 1.9% for the precision test on day 2 and 2.5% for total 12 samples on 2 d met the criteria set for intermediate precision by the validation protocol (<4%).

The linearity and range were determined using solutions of IB-367 at 0.1%, 1%, 10%, 50%, 80%, 100%, and 150% of the specified IB-367 concentration (0.5 mg/ml in water) assayed in duplicate. The method was linear in the 10 to 150% range.

The truncated peptide analogs were used to demonstrate the specificity of the method and to evaluate the limit of quantitation of potential impurities. Potential impurities were spiked into a solution of IB-367 at 0.05%, 0.1%, 0.2%, 0.5%, and 1% to assay the linearity of potential impurities at low concentrations. The method exhibited acceptable linearity for impurities from 0.05 to 1%. The relative response factors of these analogs were assessed to determine area normalization feasibility.

The limit of detection for IB-367 was 0.1% (100% being 0.5 mg/ml) for a peak height at least twice the noise level. The limit of quantitation was determined to be 0.5% by the criteria of signal-to-noise ratio of at least ten. Similar values were obtained for the limits of detection and quantitation of potential impurities and degradation products. The potential degradation products and impurities were synthesized and used to show that they were all resolved from the product peak. The impurities were defined as IB-468 ([des-Arg^1]), IB-469 ([des-Arg^1Gly^2]), and IB-300 ([des-$Arg^1Gly^2Gly^2$]).

The robustness of the method was evaluated by four electrophoretic parameters. The relative migration time of IB-367 and its impurities and the resolution

between the four peptides were monitored by changing the buffer strength, the pH of the analysis buffer, the separation voltage, and the capillary temperature. Because of the relative nature of the results, changes in any of the parameters listed in the preceding text were compensated by the presence of the internal standard, thus rendering the method robust.

Enzyme Assays

Application of CE to determine enzyme activity typically involves off-line incubation of enzyme and substrate with timed injections of the reaction mixture into the capillary to separate and quantitate the low-molecular weight substrate, intermediates, and product. A CE method termed electrophoretically mediated microassay (EMMA) is based on electrophoretic mixing of enzyme and substrate[38–41] under conditions where the mobility of enzyme and product are different. The capillary was prefilled with all of the required components for the assay (buffer, substrate), and the enzyme was introduced at the capillary inlet. Both product and enzyme were transported to the UV-Vis detector, and product was detected at a selective wavelength where the enzyme did not interfere. The relative effective mobility of enzyme and product were adjusted by manipulating the rate of EOF using covalent (e.g., epoxy polymer) or dynamic (e.g., nonionic surfactant adsorbed onto an octadecyl layer) coatings. Highest sensitivity was achieved by operation in zero potential mode; in this mode, enzyme was first injected and mixed with substrate, and then voltage was turned off for a fixed incubation period to accumulate product. In the final step, potential was reapplied to transport product to the detection point. A limitation of the zero potential mode was band broadening caused by diffusion of the product.

Affinity Capillary Electrophoresis

Affinity capillary electrophoresis (ACE), reviewed by Shimura and Kasai,[42] is a method for studying receptor–ligand binding in free solution using CE. The technique depends upon a shift in the electrophoretic mobility of the receptor upon complexation with a charged ligand. Pure receptor preparations or accurate concentration values are not required because only migration times are measured.

In a typical ACE experiment, the receptor is injected into a capillary containing free ligand at a variety of concentrations. Depending upon the kinetics of the on and off processes, incremental shifts in migration times are observed. If association and dissociation have slow kinetics, the sample will be resolved into bound and free components, and binding constants can be calculated from the peak areas at different ligand concentrations in the electrophoresis buffer. In the case of fast kinetics, only one peak will be observed, and affinity is determined by the change in migration time due to variations in time spent in bound and free states during migration through the capillary. The maximum mobility shift will occur as the free receptor becomes fully saturated, and the relationship between mobility shift and ligand concentration can be used to determine the receptor–

ligand association constant. Scatchard analysis of migration time shifts (usually normalized to a neutral EOF marker or a nonbinding reference protein) in response to ligand concentration is used to estimate the ligand–receptor binding or dissociation constant. Mobility corrections using a neutral marker (mesityl oxide) could compensate for variable EOF to yield accurate estimates of binding constants.[43]

Affinity complexation can be detected by an indirect mode termed vacancy affinity capillary electrophoresis, or VACE.[44] The capillary is filled with buffer containing a mixture of receptor and ligand, a plug of buffer is injected at the capillary inlet, and voltage is applied. Differential migration of ligand and receptor through the buffer zone produces zones deficient in ligand and receptor, resulting in the appearance of two negative peaks at steady state. In the VACE experiment, the concentration of ligand or receptor is varied, and the mobility of the two peaks relative to a neutral marker is monitored. The magnitude of the peaks provides information about the degree of complexation, e.g., the receptor-deficient peak reflects the concentration of free receptor and the ligand-deficient peak indicates the level of free ligand. Similarly, the shift in mobility of each peak provides information on the receptor–ligand association constant and the number of receptor binding sites. Using vancomycin and the dipeptide *N*-acetyl-D-alanyl-D-alanine as the receptor–ligand pair, these authors compared the ACE and VACE approaches and found satisfactory agreement of the measured association constants.

ACE has been used to characterize protein–sugar interactions,[45] DNA binding to an anti-DNA monoclonal antibody,[46] antibody–antigen,[47] antisteroidal inflammatory drugs,[48] and prion protein in sheep brain preparations.[49]

In capillary immunoelectrophoresis, antigen–antibody interactions are used to affect resolution.[50] This method can be easily modified to quantitate antigen or antibody, or to study the interaction of the two molecules. To increase the detection sensitivity, one of the polypeptides can be labeled with a fluorescent dye, or the antigen and antibody can be labeled with two different dyes for dual fluorescence. Under these conditions, the free and bound forms may be analyzed simultaneously. If the amount of antibody is not high enough to derivatize, the antibody can be labeled indirectly by using a labeled ligand such as protein A. Capillary immunoelectrophoresis has been used to study interactions between antibody and corresponding antigen or hapten with results comparable to those obtained by ELISA. Concentration of the antigen or antibody must be selected carefully to prevent precipitation, especially when using polyclonal antibodies.

Analysis of Protein Folding

Determination of protein folding is important in various areas of biotechnology and thus is an area of active research for biopharmaceuticals. Crucial parameters such as activity and stability are related to protein folding. Because improper protein folding occurs with high frequency in proteins produced by recombinant technology and proteins tend to denature upon storage, any technique capable of

elucidating tertiary structure or able to monitor its changes will find immediate use in areas such as drug screening, formulation, and QC/QA.

CE has been shown to be a valuable tool for analysis of protein folding. Unlike techniques such as chromatography and gel electrophoresis, CZE is performed in free solution, and migration is a function of the intrinsic properties of the molecule. The ability of CZE to distinguish different folding states of a protein depends upon changes in solvent-accessible charge, and the migration rate thus reflects a cross section of the conformational states. Moreover, peak shape can provide information on the distribution of the protein among folding states.

Rush et al.[51] first described the effect of thermally induced conformational changes on migration behavior of α-lactalbumin. A sigmoidal dependency of the viscosity-corrected mobility on temperature was observed. Transition temperature also agreed closely with that determined by intrinsic fluorescence measurements.

Strege and Lagu[52] used CZE to monitor reformation and interchange of disulfide bonds during reoxidation of reduced trypsinogen. In this study, CE was performed under low-pH conditions to minimize protein–wall interactions for this basic protein. A population of refolding intermediates distributed between native and unfolded trypsinogen was resolved. Resolution was further improved by addition of ethylene glycol and sieving polymers.

These authors also monitored the transition of bovine serum albumin from the native folded state to the unfolded state using CZE in the presence of increasing amounts of urea.[53] The resulting plot of EOF-corrected migration time vs. urea concentration was similar to urea denaturation profiles obtained with other techniques. Using CE in the presence of urea, Kilár and Hjertén[54] detected intermediate unfolding states of transferrin as distinct peaks and were able to resolve unfolding intermediates for each of the five transferrin glycoforms (2, 3, 4, 5, and 6-sialotransferrin). Hilser et al.[55] monitored the migration behavior of lysozyme as a function of capillary temperature and observed a sigmoidal behavior characteristic of a protein-unfolding transition. Calculation by van't Hoff analysis of the transition temperature, entropy, and enthalpy of protein unfolding yielded values in close agreement with those determined by differential scanning calorimetry and confirmed that the temperature-dependent decrease in electrophoretic mobility represented a two-state thermal denaturation. CE has also been used as a confirmatory technique to assess the conformational states of proteins eluted from reversed-phase HPLC columns.[56]

Using CZE, Rochu et al.[57,58] analyzed the thermal denaturation of β-lactoglobulin, which exhibits various oligomeric states depending on the protein concentration, pH, and temperature. A commercial CE instrument was modified by connecting the liquid temperature control to an external water bath providing accurate temperature control up to 95°C. Under various pH conditions, transition temperature (T_m), enthalpy change (ΔH), and entropy change (ΔS) associated with thermal denaturation were determined. The technique is unique in its ability to estimate the heat capacity change (ΔC_p). CZE performed in the presence of EOF was used to determine the stability curves of proteins.

In this application, the authors used an uncoated capillary and a neutral marker to monitor changes in EOF. Because viscosity and electrophoretic mobility change with temperature, the simplest way of determining mobility changes due to unfolding is to estimate the mobility of the unfolding protein to a molecule, in this case the neutral marker, which does not change conformation. Because the data are greatly affected by temperature, the thermostating of the capillary needs to be tightly controlled to within less than 0.1°C.

The van't Hoff plots for thermal denaturation of proteins are linear in the transition region, thus allowing the enthalpy change (ΔH_m) of unfolding at the transition temperature (T_m) to be estimated. Because of the change in free energy in (ΔG) = 0 at T_m (reversible process), the entropy of unfolding (ΔS_m) at the transition midpoint can be calculated from:

$$\Delta S_m = \Delta H_m / T_m$$

For the ΔG of denaturation transition, the temperature (T) data were fitted to the Gibbs–Helmholtz equation:

$$\Delta G_{(T)} = \Delta H_m \, (1 - T/T_m) - \Delta C_p \, [(T - T_m) + T\ln(T/T_m)]$$

where ΔH_m is the enthalpy change at T_m and ΔC_p is the change in heat capacity between the native and denatured state. ΔC_p for the unfolding reaction was calculated using the Kirchoff equation:

$$\Delta C_p = d(\Delta H)/d(T)$$

as the slope of the plot of ΔH_m vs. T_m, measured at different pH values of phosphate buffer.

Glycoproteins

Glycoproteins often exist as multiple glycoforms sharing a common amino acid primary sequence but differing in the number, location, and structure of carbohydrate groups attached to the polypeptide chain. The importance of glycosylation patterns in the biological activity of glycoproteins has generated strong interest in methods for separation of glycoforms, particularly in the case of therapeutic glycoproteins. Variation in the number of sialic acid residues confers charge microheterogeneity to glycoproteins; gel electrophoresis and IEF have been used successfully for their characterization. Therefore, CZE and CIEF are obvious candidates for automated analysis of protein glycoforms. A typical strategy for optimizing CZE separations of glycoforms starts with determination of conditions that yield the best resolution of glycoforms. Enzymatic cleavage (e.g., with neuraminidase) of carbohydrate moieties and the subsequent disappearance of peaks in the electropherogram following enzyme treatment confirm their identity as glycoforms.

Separation of protein glycoforms was first described by Kilár and Hjertén,[59] who used CZE with a Tris-borate + EDTA (pH 8.4) buffer and a coated capillary to separate the di-, tri-, tetra-, penta-, and hexasialo isoforms of iron-free human serum transferrin. Yim[60] used CZE to resolve glycoforms of recombinant tissue plasminogen activator (rtPA), a 60-kDa glycoprotein containing complex N-linked oligosaccharides attached to the polypeptide chain at two (type II) or three (type I) sites. Using an ammonium phosphate buffer at pH 4.6 containing 0.01% reduced Triton X-100 + 0.2 *M* ε-aminocaproic acid (EACA, added to stabilize solubility of the protein) in a linear polyacrylamide-coated capillary, approximately 15 glycoforms were partially resolved. Recently, Thorne et al.[61] at the same institution have expanded this study and found that other ω-amino carboxylic acids were less effective than EACA in achieving glycoform resolution, and the addition of the Tween 80 surfactant was necessary to obtain good recovery.

Purification and Process Monitoring

Because of the speed and high resolution of CZE separations as well as the small sample volumes required to yield information about complex protein samples, CE is increasingly being used to assess protein purity in multistep purification protocols in laboratory, pilot plant, and process scales. Similarly, it is being considered as a candidate for monitoring fermentation.

McNerney et al.[62] described separation of recombinant human growth hormone (rhGH) and its variants from very crude mixtures of *E. coli* cell extracts using CZE in a phosphate-deactivated capillary. The 18-h deactivation procedure included washing the capillary with 0.1 *M* nitric acid and 0.1 *M* sodium hydroxide to remove contaminants prior to conditioning with the run buffer (250 m*M* sodium phosphate + 1% propylene glycol). This method allowed separation of a variety of rhGH variants including deamidated and dideamidated rhGH, desPhe- and desPhePro rhGH, and 2-chain rhGH. In addition, the method could detect changes in fermentation conditions that affected rhGH production. Washing with 3 *M* guanidine HCl + 0.2 *M* sodium phosphate between runs was required to remove adsorbed contaminants. The phosphate-deactivated column provided superior resolution and reproducibility compared to bare fused-silica or PVA-coated capillaries.

Purification of murine antiheparin monoclonal antibody produced in cell culture was monitored by Malsch et al.[63] using a CZE method with a borate or boric acid buffer (pH 9) in an uncoated capillary.

Kundu et al.[64] used MEKC conditions to assess the purity of two recombinant proteins: a cytomegalovirus-CMP-KDO synthetase fusion protein expressed in *E. coli* and a hepatitis C viral protein expressed in CHO cells. Proteins were prepared in a 10-m*M* Tris–1% SDS buffer (pH 8.5) and analyzed in a 10-m*M* borate–100-m*M* SDS buffer (pH 9.5) in uncoated capillaries. The level of impurities, which varied with the method of protein production, agreed within ±5% with results obtained by densitometric scanning of SDS-PAGE gels of the same materials.

Lipoproteins

Serum lipoproteins have been analyzed by isotachophoresis, typically after staining with a lipophilic dye such as Sudan Black.[65,66] Apolipoproteins can be analyzed by CZE as reported by Tadey and Purdy.[67,68] They were able to resolve apoA-I, apoA-II, apoB-100, and apo-B48 from HDL and LDL preparations using uncoated capillaries with a 30-m*M* borate buffer (pH 9) containing SDS. Other detergents were less effective, although either SDS or cetyl trimethylammonium bromide provided good resolution of VLDL apolipoproteins with polyacrylamide-coated capillaries. Lehmann et al.[69] developed a method for direct analysis of apoA-I in serum using an uncoated 50-cm × 50-µm capillary and a proprietary buffer. Dilution of the serum sample in the buffer allowed direct injection and resolution of apoA-I from all other serum proteins. Correlation of apoA-I levels in patient sera correlated well with values from nephelometric determinations. Using a 50-m*M* borate buffer containing 3.5 m*M* SDS and 20% (v/v) acetonitrile, Cruzado et al.[70,71] compared CZE with reversed-phase HPLC for separation of apolipoproteins A-I and A-II. The HPLC method resolved apoA-I and apoA-II into three and two isoforms, respectively, whereas CZE could not. However, the apoA-I and apoA-II isoforms overlapped and could not be resolved by HPLC, thus preventing analysis of the two apolipoproteins in mixtures. Therefore, CZE was the preferred technique for quantitation of apoA-I and apoA-II in HDL. CE values for these two proteins determined in nondelipidated HDL fractions obtained from serum controls by density gradient centrifugation agreed well with immuno-based assay values.

CAPILLARY ISOELECTRIC FOCUSING

Background

Capillary isoelectric focusing (CIEF) combines the high resolving power of conventional gel IEF with the advantages of CE instrumentation. Just as in gel IEF, proteins are separated according to their pI in a pH gradient generally formed by carrier *ampholytes* (amphoteric electrolytes) when an electric potential is applied (Figure 9.5). The use of small-diameter capillaries allows the efficient dissipation of Joule heat and permits the application of high voltage for a rapid focusing of the protein zones. The resolving power of CIEF is usually higher than most protein analysis techniques, including other modes of CE. The introduction of CIEF expanded the use of IEF to include the analysis of peptides, amino acids, and other small organic zwitterions.

As in conventional IEF, the high resolving power of CIEF depends upon the focusing effect of the technique. At steady state, the ampholytes form a stable pH gradient within which proteins become focused at the positions where their net charges are zero, i.e., where pH = pI. Diffusion of a protein toward the anode (positive electrode) will result in the acquisition of positive charge, and the

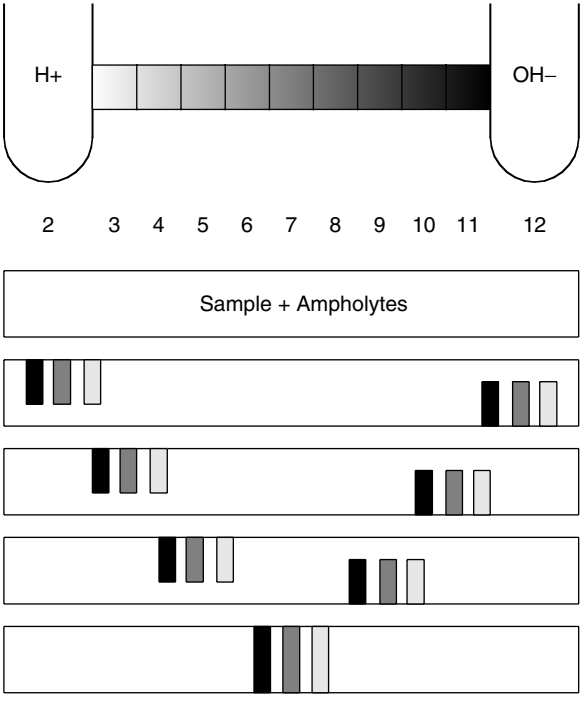

Figure 9.5 Generation of a pH gradient by ampholytes within a capillary flanked by an acid as anodic solution and base as cathodic solution. Ampholyte solutions are composed of high numbers of low-molecular weight amphoteric electrolytes (from which the name is derived) with slightly different pI values. Because ampholytes possess buffering capacity, they maintain a pH value in the specific area occupied by the different molecular species. The sample, which is also amphoteric, focuses in between ampholytes with higher and lower pI. To achieve resolution, there must be at least one ampholyte with a pI intermediate to the two sample components of interest.

molecule will return to the focused zone (attracted by the cathode). Similarly, diffusion toward the cathode (negative electrode) will result in acquisition of negative charge, causing back-migration to the pI zone. As long as the electric field is applied, electrophoretic migration counters the effects of diffusion. Because detection in CIEF is performed on-line, the electric field is maintained throughout the analysis, and resolution is usually very high. CIEF usually produces more complex patterns than conventional IEF because smaller peptides (and even aromatic amino acids) that do not stain well or diffuse out of the gel are also detected. CIEF offers resolution comparable to slab gel IEF but not as high as immobilized pH gradients.[72]

For all CE instruments that use on-line detection at a fixed point along the capillary, CIEF must include a means of transporting the focused zones past the

detection point. This process, commonly referred to as *mobilization*, can occur as an independent stage or be combined with the focusing of the sample–ampholyte matrix components. The forces used to achieve mobilization are various, and in some instances they are applied in combinations of two or more simultaneously.

There are several protocols for performing CIEF that can generally be classified in two groups: two-step CIEF and single-step CIEF. Two-step CIEF is characterized by the performance of focusing and mobilization as two distinct stages. Mobilization can be achieved by ion addition or by applying a hydraulic force such as pressure, vacuum, or gravity. This method requires electroosmosis to be eliminated or reduced to a very low level. In single-step CIEF, focusing occurs while the nascent protein zones are being transported toward the detection point. The forces used to transport the focused zones are the same as those for multiple-step CIEF, but in this case EOF can also be used alone or in combination with any of the other forces.

Sample Preparation and Injection

Sample preparation for CIEF includes selection of the appropriate ampholyte composition, adjustment of sample salt levels, and dilution or concentration of the sample to the proper protein levels required for detection.

Excessive sample ionic strength due to the presence of salts (including buffer) or ionic detergents will interfere with the IEF process, greatly increase focusing times, and cause peak broadening during mobilization. Elevated current due to the presence of salt can increase the risk of precipitation as proteins become concentrated in focused zones. Dilution, dialysis, gel filtration, or ultrafiltration should be used to desalt samples with salt concentration of 50 m*M* or greater. The practical upper limit for ionic strength is 30 to 50 m*M*.

The ampholyte composition should be selected based upon the desired separation range. For separating complex samples containing proteins with widely different pI, or to estimate the pI of an unknown protein, a wide-range ampholyte blend such as pH 3 to 10 is appropriate. The final ampholyte concentration should be between 1 and 2% (w/v). In situations in which enhanced resolution of proteins with similar pI values is desired, the use of narrow-range ampholyte mixtures may be considered. Narrow-range ampholyte mixtures generating gradients spanning 1 to 3 pH units are available from several commercial sources. Improved results have been obtained by adding various ratios (10 to 80%) of narrow-range ampholytes to a "base" of broad-range ampholytes.

In order to detect proteins at the basic end of the gradient during cathodic mobilization, it is necessary that the pH gradient span only the effective length of the capillary, e.g., the distance from the capillary inlet to the detection point. In cases where the total capillary length is much greater than the effective length, many sample components may focus in the "blind" segment distal to the monitor point and be undetected during mobilization. A basic compound such as

N,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) can be used to block the distal section of the capillary. As a rule of thumb, the ratio of TEMED concentration (% v/v) to ampholyte concentration should be approximately equal to the ratio of the "noneffective" (or blind) capillary length to total length.

The final protein concentration in the sample and ampholyte mixture will depend upon sensitivity requirements and the solubility of protein components under focusing conditions. As an approximation, a final concentration of 0.5 mg/ml per protein should provide adequate sensitivity and satisfactory focusing and mobilization performance. However, many proteins may still precipitate during focusing at this starting concentration because the final protein concentration in the focused zone may be as high as 200 mg/ml. Immunoglobulins, membrane proteins, and high-molecular weight or hydrophobic proteins generally have a higher risk of precipitation during CIEF. In such cases, the use of very dilute protein solutions may be required.

Prior to injection, the prepared sample should be centrifuged for 2 to 3 min in a microcentrifuge to remove any particulate material and to degas the solution. This practice is particularly important if the sample contains protein aggregates or other large particles, and when polymers are used to increase the viscosity of sample and ampholyte solution.

Focusing

Although there are several approaches to generating pH gradients, the most widely used to date is with the use of carrier ampholytes. Ampholytes are mixtures of a high number of synthetic chemical species that posses slightly different pIs. Carrier ampholytes are oligoamino acids and oligocarboxylic acids with different pI values.[73] The number of ampholytes per pH unit has been calculated to range between 50 and 1000. Besides having different pI values, carrier ampholytes must be good buffers and conductors at their pI so that they can carry the electric current while also maintaining a steady pH gradient.

During the performance of a CIEF analysis, the capillary is first filled with the sample and ampholyte mixture. The focusing step begins with the immersion of the capillary in the anolyte (dilute phosphoric acid) and catholyte (dilute sodium hydroxide) solutions followed by application of high voltage. Typically, the catholyte solution is 20 to 40 m*M* NaOH, and the anolyte is half the catholyte molarity, e.g., 10 to 20 m*M* phosphoric acid. It is important that the catholyte be prepared fresh because sodium hydroxide solutions will gradually take up carbon dioxide from the atmosphere.

For narrow-bore capillaries (e.g., 50 μm I.D.), field strengths of 300 to 900 V/cm or greater can be used. Our experience indicates that about 600 V/cm is optimal. Upon application of high voltage, the charged ampholytes migrate in the electric field to generate a pH gradient that is defined by the composition of the ampholyte mixture. A pH gradient develops with low-pH components toward the anode (+), and high-pH components toward the cathode (−). At the same time,

protein components in the sample migrate until a steady state is reached, at which point each protein becomes focused in a narrow zone at its pI (Figure 9.5). Focusing is achieved rapidly, typically within a few minutes in short capillaries, and is accompanied by an exponential drop in current. Focusing is usually considered to be complete when the current has dropped to a level approximately 10% of its initial value for samples containing low salt and the rate of change approaches zero. It is generally not advisable to prolong focusing beyond this point because the risk of protein precipitation increases with time.

Two-Step CIEF

Because most commercial CE instruments use on-line detection at a fixed point along the capillary, CIEF must include a means of transporting the focused zones past the detection point. Mobilization has been regarded for the most part as a stage of little importance in the overall performance of the CIEF process, but now it has been shown that mobilization conditions can be manipulated to improve resolution and reproducibility. Three approaches have been used to mobilize focused zones. In *chemical mobilization* (ion addition), changing the chemical composition of the anolyte or catholyte causes a shift in the pH gradient, resulting in electrophoretic migration of focused zones past the detection point.[74,75] In *hydraulic mobilization*, focused zones are transported past the detection point by applying pressure[76,77] or vacuum[78] at one end of the capillary, or by volume height differential of the anolyte and catholyte levels (siphon or gravity).[79] In *electroosmotic mobilization*, focused zones are transported past the detection point by electroosmotic pumping.[80–82] Mobilization by EOF is used only in single-step CIEF.

Chemical Mobilization

At the completion of the focusing step, high voltage is turned off and the anolyte or catholyte is replaced by the mobilization reagent. High voltage is again applied to begin mobilization. As in focusing, field strengths of 300 to 900 V/cm can be used for mobilization, with optimum separations achieved in capillaries with small I.D. using a field strength of about 600 V/cm. The choice of anodic vs. cathodic mobilization and the composition of the mobilizing reagent depend upon the pIs of the protein analytes and the goals of separation. Because the majority of proteins have pIs between 5 and 9, cathodic mobilization (mobilization toward the cathode) is most often used. The most common chemical mobilization method is the addition of a neutral salt such as sodium chloride to the anolyte or catholyte; sodium serves as the nonproton cation in anodic mobilization and chloride functions as the nonhydroxyl anion in cathodic mobilization. A suggested, cathodic mobilization reagent is 80 m*M* NaCl in 40 m*M* NaOH. At the beginning of mobilization, current initially remains at the low value observed at the termination of focusing, but gradually begins to rise as the chloride ions enter the capillary. Later in mobilization, when chloride is present throughout the tube, a rapid rise

in current signals the completion of mobilization. The electrical current at the end of mobilization using NaCl is much higher than the current observed at the beginning of focusing. When using NaCl for mobilization, set the current limit to a value (100 to 150 μA) that will ensure that excessive current heat does not damage the capillary coating.

Use of zwitterions is an alternative approach that provides more effective mobilization of protein zones across a wide pH gradient.[83] For example, cathodic mobilization with a low-pI zwitterion enables efficient mobilization of proteins with pIs ranging from 4.65 to 9.60. The proposed mechanism for zwitterion mobilization couples a pH shift at the proximal end of the tube with a displacement effect at the distal end as the zwitterion forms an expanding zone within the gradient at its pI. Effective zwitterion mobilization depends on the selection of the appropriate mobilization reagent.

Hydraulic Mobilization

Hydraulic mobilization utilizes positive pressure or negative pressure (vacuum) as the force that transports the focused protein zones toward the detection point. During hydraulic mobilization, it is necessary to apply an electric field across the capillary in order to maintain focused protein zones.[78] The main disadvantage of this type of mobilization is the parabolic shape of the hydrodynamic flow profile, which can decrease resolution. For this reason, only weak forces are used.

From an instrument perspective, the simplest hydraulic approach to transport focused zones to the detector is by gravity mobilization.[79] In this technique, focused proteins are transported toward the detection point using a difference in the levels of anolyte and catholyte contained in the reservoirs. The force generated by the liquid-height difference can be manipulated to be extremely small compared with pressure or vacuum. Flow velocity can also be modulated by changing the capillary dimensions or, in the case of large-bore capillaries, with internal diameters greater than 50 μm, by the addition of viscous polymers.

Single-Step CIEF

Single-step or dynamic CIEF is a variation of CIEF in which focusing occurs while sample proteins are simultaneously transported toward the detection point by EOF. Single-step CIEF was first developed as a means to perform CIEF in uncoated capillaries. Uncoated capillaries are inexpensive and have long lifetimes limited only by column plugging. However, sample recovery can be a problem, and capillaries with wall modifications (such as C8) or additives are more commonly used to reduce, but not eliminate, EOF. An advantage of single-step CIEF is simplification of the protein pattern because focusing peaks, generated by proteins migrating past the detection point during the focusing step, are eliminated. However, because the capillary may be only partially filled with sample and ampholyte solution, some resolution and sensitivity are sacrificed.

Approaches to single-step CIEF in the presence of EOF include partial and full capillary injection. In the partial injection method, the sample and ampholyte

mixture is introduced as a plug at the inlet of the capillary prefilled with catholyte. Successful application of partial capillary injection depends upon optimization of the polymer concentration (which modulates EOF), ampholyte concentration, and sample load to minimize protein adsorption and modulate EOF level so that focusing approaches completion before the detection point is reached.

The second single-step CIEF method consisting of full capillary injection was described by Mazzeo and Krull.[80,82] In initial studies using uncoated capillaries, methylcellulose was added to modulate EOF and TEMED was used to block the detector-distal capillary segment. This approach was successful only for neutral and basic proteins due to variations in the rate of EOF during the separation. As the separation progressed, the drop in average pH due to mobilization of the basic segment of the pH gradient into the catholyte resulted in diminished EOF. This, in turn, caused peak broadening and poor resolution for acidic proteins. Improved mobilization of acidic proteins was achieved using commercial C8-coated capillaries in which EOF varied less with pH.[84] However, pH-dependent variation of EOF was still significant enough that plots of pI vs. migration time were not linear over broad pH ranges.[85] Use of multiple internal standards was recommended for accurate pI determination with this method.

Optimal separation of proteins spanning the whole pH range is difficult with EOF-driven CIEF. As for all variations of IEF, salt concentration (higher than 10 m*M*, in this case) greatly diminishes resolution. The concentration of NaOH (catholyte) has a significant effect on migration times of the protein zones, mainly by affecting the rate of electroosmosis. Longer analysis times were observed at higher concentrations of NaOH with an improvement in resolution. On the other hand, higher concentrations of anolyte (phosphoric acid) shortened the analysis time and diminished resolution. Migration was also affected by the concentration of the ampholytes used (1, 2.5, and 5%), with slower mobilization at lower concentrations (1%). The initial length of capillary occupied by the sample affects migration times and resolution, with longer sample zones providing better resolution at the expense of analysis time.

Capillary Selection

To obtain good resolution and reproducibility when performing CIEF with chemical mobilization, it is essential to reduce EOF to a very low level. In the presence of significant levels of EOF, stable focused zones are not maintained. This results in band broadening and, in some instances, multiple peaks caused by incomplete fusion of the nascent protein zones focusing from both capillary ends. Therefore, the use of coated capillaries is necessary for optimal use of chemical mobilization. A viscous polymeric coating is recommended for greatest reduction in EOF. In addition, the use of neutral, hydrophilic coating materials reduces protein–wall interactions.

The low level of EOF in coated capillaries permits separations to be carried out with very short effective capillary lengths. Earlier work using chemical mobilization was performed using capillaries as short as 11 cm with internal

diameters up to 200 μm.[76] More recently, 12- to 25-cm capillaries with internal diameters of 25 or 50 μm have been used.[83] Theoretically, resolution in CIEF should be independent of capillary length because the number of ampholyte species remains constant with only the amount changed. In practice, however, resolution is diminished with very short capillaries, small sample injections, and very dilute sample and ampholytes, particularly in single-step CIEF. Because analysis time increases with capillary length, problems associated with protein precipitation are more severe in longer capillaries.

The length of capillaries used for single-step CIEF is very important, especially when EOF is the driving force. The capillary length must be optimized according to the size of the injection and the velocity of EOF or flow due to hydraulic forces so that the sample will not reach the detection point before it has finished focusing.

Detection

UV-Vis Absorption

Most applications published to date employ on-line detection of mobilized proteins by absorption in the ultraviolet or visible spectrum at a fixed point along the capillary. The strong absorbance of the ampholytes at wavelengths below 240 nm makes detection of proteins in the low-UV region impractical. Therefore, 280 nm is generally used for absorbance detection in CIEF. This results in as much as a 50-fold loss in detection signal relative to the detection at 200 nm, but the high protein concentrations in focused peaks more than compensate for the loss of sensitivity imposed by detection at 280 nm. Because ampholytes may still be detected at 280 nm, care should be exercised when analyzing dilute or low-absorbance proteins.[86] In some instances, proteins possess chromophores that can be detected in the visible range of the spectrum, e.g., hemoglobin and cytochromes.

Concentration Gradient Detection

The use of concentration gradient detectors has been extensively reported.[87–96] The system incorporates a capillary mounted in a holder that aligns the column to a HeNe laser beam. A positioning sensor located at the exit side of the laser beam detects deflections generated by the passage of substances with a refractive index different than that of the background buffer. The main advantage of this detector is its universality in detecting sample components. Although ampholytes may produce signals during the mobilization step, the derivative nature of the detector enables recognition of the sharp bands generated by the protein zones against the background of the broader zones produced by the ampholytes. The detection system can also be built to scan along the capillary, performing detection of focused protein zones without mobilization. Optimization produces fast analysis times (2 min) and detection limits in the 1- to 5-mg/ml range. Use of capillary

arrays greatly improves throughput. An important application for concentration gradient detection involves peptides, many of which do not contain aromatic amino acids required for UV detection at 280 nm. Using this system, peptides produced by the tryptic digestion of bovine and chicken cytochrome C were analyzed.

Laser-Induced Fluorescence (LIF)

LIF is a highly sensitive mode of detection, but lasers that emit at a visible wavelength often require derivatization of the sample prior to analysis. Because chemical modification of the sample molecules can change the pI, often producing multiple peaks, derivatization is not widely used. Instead, this problem is solved by using a laser that emits in the UV range. LIF detection of tagged antibodies directed against the protein of interest can also be used.[97]

CIEF and Mass Spectrometry

The separation power of CIEF often generates a high number of peaks even when relatively pure samples are analyzed. As already discussed, one of the advantages of CIEF is its potential micropreparative capabilities. Capillary IEF allows the collection of fractions that can be further analyzed by other methods. Some of the most widely used characterization tools include MS, peptide mapping, and amino acid analysis.

Foret et al.[98] collected fractions of model proteins and variants of human hemoglobins after fractionation by CIEF, and then analyzed them by matrix-assisted laser desorption–time-of-flight–mass spectrometry (MALDI-TOF-MS). As the authors point out, MS is an orthogonal method to CIEF because it separates according to molecular mass.

Optimizing CIEF Analysis

Resolution in CIEF

As previously mentioned, resolution in CIEF strongly depends on the ampholyte composition. The estimated maximum resolving power of IEF is 0.02 pH units when carrier ampholytes are used to create the pH gradient.[99] The Law of Monotony[99] formulated by Svensson in 1967 states that a natural pH gradient increases continually and monotonically from the anode to the cathode; that the steady state does not allow for reversal of pH at any position along the gradient; and that two ampholytes (in stationary electrolysis) cannot be completely separated from each other unless the system contains a third ampholyte of intermediate pH (or pI). The latter explains why better resolution is obtained when mixing ampholytes from different vendors and production batches; as the number of ampholytes species increases, the chance that one or more ampholytes have intermediate pI relative to those of the sample components also increases.

Additives and Protein Precipitation

A major problem in CIEF is the precipitation of proteins as the focusing step concentrates the sample components. Precipitation may be due possibly to protein denaturation, hydrophobic interactions, and electrostatic interactions resulting from salt removal. Precipitation in CIEF is manifested by current fluctuation and loss, by variations in peak heights or migration patterns, and by spikes in the electropherogram generated as protein aggregate particulates transit to the detection point. This results in poor pattern reproducibility, variable migration times, variable peak areas affecting quantitation, capillary clogging, slow mobilization, and other undesirable effects. Solubilizing agents are commonly used in CIEF to prevent precipitation. Other additives include hydrophilic polymers, usually used for fluid stabilization, to reduce EOF, and to increase viscosity during hydraulic mobilization.

Denaturing CIEF

Poor protein solubility under CIEF conditions has limited the number of applications developed for this technique. Solubilization in some cases may be a problem even before the CIEF analysis is initiated.[100] Some proteins, e.g., membrane proteins, are difficult to analyze in typical electrophoresis buffers. These polypeptides, however, can be rendered soluble in the presence of additives such as SDS. Unfortunately, SDS or similar ionic detergents cannot be used in CIEF because they eliminate the amphoteric properties of proteins. An additive that can be used to increase protein solubility is urea. Unfortunately, hydrophobic interactions and hydrogen bonds play a major role in protein tertiary structure, and because urea disrupts these forces, protein denaturation occurs. When the protein is denatured, all hydrophobic residues are exposed, increasing the possibility of hydrophobic interactions and aggregation. For this reason, detergents are used in combination with urea. Not all detergents are suitable for denaturing CIEF. Some detergents used with success include Tritons (reduced form), Nonidet P-40, CHAPS, octyl glucoside, and lauryl maltoside. Typical concentrations range from 0.1 to 5%.

The use of urea must be approached with caution, because urea solutions often contain ammonium cyanate, the concentration of which increases with temperature and pH. This contaminant can react with the amino group of lysines and the amino terminus of the polypeptide chain, thus leading to artifact peaks. This effect is minimized by the presence of ampholytes, whose primary amines are cyanate scavengers, and by deionizing the urea solution with a mixed-bed resin prior to adding the ampholytes and detergent.

Other CIEF Parameters

Applied Voltage: Theoretically, the best resolution is obtained at high voltages. In practice, variation of the electric field intensity under typical analysis conditions for CIEF (300 to 1000 V/cm) is a parameter of relatively minor

importance in optimizing resolution. Field strength should be kept low enough to avoid excessive Joule heating, particularly at the beginning of focusing. If reduction in analysis time is desired, high field strengths will shorten focusing time but will effect mobilization time only when chemical mobilization or single-step CIEF in the presence of EOF is used.

Capillary Temperature: Protein conformation and solubility are affected by the temperature of the solution. As described above, focused proteins may tend to precipitate during the CIEF process as they become highly concentrated at their pI with low concentration of salt ions. The temperature of the capillary can be manipulated to increase their solubility, but it should not be too high as protein denaturation may occur. Higher temperature is used mainly in combination with other solubilizing agents, and temperature may be used to increase the solubility of additives such as urea rather than the solubility of the proteins.

Because pI, mobility, and viscosity are all affected by temperature, the use of internal standards is recommended. However, the use of synthetic pI standards to estimate pIs should be approached with caution because the pIs of the protein sample and standards may not be affected equally by temperature. Temperature has a direct effect on viscosity and therefore all effects of viscosity on the CIEF process apply as temperature changes.

The Use of Internal Standards: Important characteristics of internal standards include high purity, stability, high absorption at the detection wavelength, nonreactivity with sample components and ampholytes, and availability of species with known pI values spanning the pH range of interest. Protein standards are widely used in slab gel IEF, and they are available from multiple commercial sources. However, they are only available as premixed solutions of a fairly high number of proteins, and they are intended to be applied in a single lane of a slab gel. For CIEF, it is preferable to combine the sample and standard, but the complexity of the resulting pattern often makes identification of the compounds of interest very difficult, if not impossible. Single-protein standards usually lack the necessary purity and appear as several bands that complicate the separation pattern.

A family of substituted aromatic aminophenol compounds has been synthesized,[101] and they possess all of the desirable characteristics of internal standards, including very high UV absorption at 280 nm. They can be introduced into the capillary as a secondary injection that occupies only a small portion of the capillary. By not premixing the sample with the standards, the unused portion of the uncontaminated sample can be recovered. Furthermore, the standards are small molecules that can be removed easily by dialysis (an important factor to consider when collecting fractions). Figure 9.6 displays a CIEF analysis of recombinant human monoclonal antibody rhuMAB against HER2 flanked by synthetic 7.9, 8.4, and 10.1 pI markers. Table 9.2 shows the reproducibility (% RSD) obtained for two hemoglobin variants by analyzing the migration times and using the pI standards to calibrate the protein zones.[102,103] The percent RSD values reported for pI were extremely good (0.061%) as compared with migration times percent

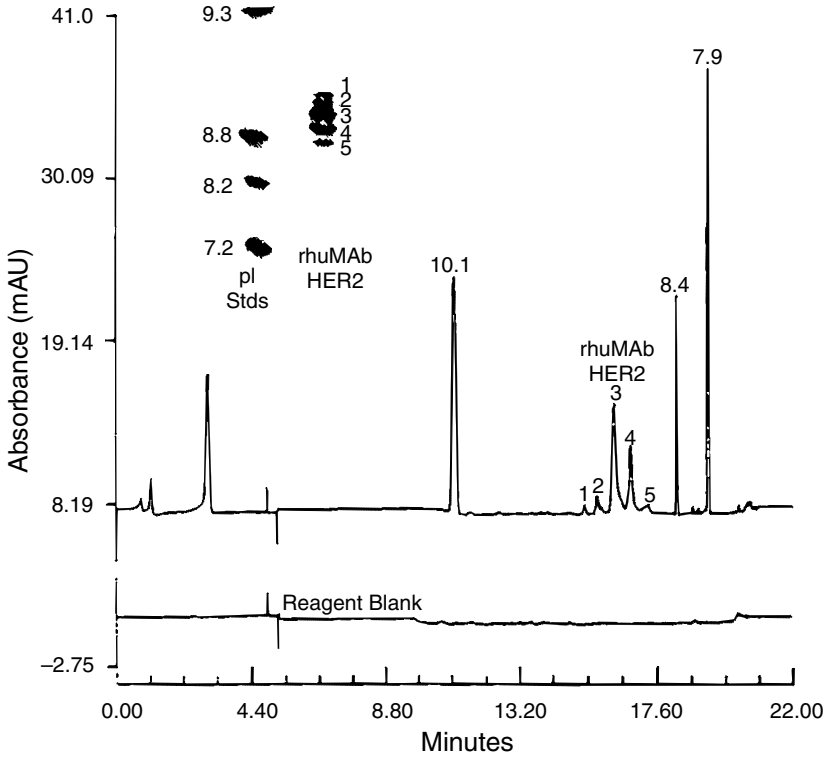

Figure 9.6 Capillary IEF and slab gel IEF of antibodies of rhuMAbHER2. There was good correlation between the number of bands obtained by the two techniques. Notice the excellent resolution of the sample components achieved by CIEF. In this application, the sample was bracketed by synthetic pI markers of known pI that are not only extremely useful in the determination of pI, but can also be used for correction of migration time variations. During methods development, the pI standards can be prepared in ampholytes at a higher concentration (e.g., 10X) and introduced as a second injection after the capillary is first filled with sample prepared in ampholytes. Using this approach, the combination of pI markers can be optimized with a minimal usage of sample (see Reference 112).

RSD (1.1%). Software packages that identify the internal standards, plot their migration time as a standard curve and automatically report the pI of "unknowns," make the CIEF process simpler, more powerful, and reliable.

CIEF as a Micropreparative Technique

Ever since CE was introduced, the desire to collect fractions of pure sample components has existed. The extremely small quantities injected into the capillary in CZE has limited the number of applications in which sufficient amount can

Table 9.2 Migration Time and Isoelectric Point Reproducibility for Human Hemoglobins A and S Analyzed by CIEF

Migration Time Reproducibility			Isoelectric Point Reproducibility		
Run	Hb A	Hb S	Run	Hb A	Hb S
1	19.16	18.77	1	7.39	7.22
2	19.01	18.63	2	7.38	7.23
3	18.88	18.50	3	7.38	7.22
4	18.74	18.36	4	7.38	7.22
5	18.63	18.26	5	7.38	7.22
Average	18.88	18.50	Average	7.38	7.22
Std. Dev.	0.21	0.20	Std. Dev.	0.004	0.004
% RSD	1.11	1.10	% RSD	0.06	0.06

Source: From T. Wehr, R. Rodriguez-Diaz, and M. Zhu, Chromatographic Science Series, Vol. 80 (1999). With permission.

be recovered. CIEF not only utilizes the whole length of the capillary for injection, but protein zones are highly concentrated during the analysis. Thus, CIEF has potential as a micropreparative technique, especially for enzymes, because only very small quantities of these polypeptides are required for enzymatic reactions. It is noteworthy that denaturation may occur when collecting fractions into NaOH (or any other extreme pH solution). Buffered catholyte should be used to avoid protein denaturation.

Applications

A variety of CIEF applications have been published, including the analysis of human transferrin isoforms,[104] recombinant proteins (e.g., human recombinant tissue plasminogen activator,[105] human growth hormone,[106] γ-globulins,[106] and hemoglobin variants.[107,108] As indicated by these reports, CIEF analyses are used to characterize proteins, as well as to determine their purity. It has been suggested that conventional IEF in gels can distinguish conformational states of proteins,[109] although the same has not yet been reported for CIEF. CIEF is a powerful tool for the detection of protein modifications, such as deamidation, deletions, insertions, proteolytic clips, N- or C-terminal modifications, and glycosylation.[73]

Immunoglobulins

Immunoglobulins in the form of monoclonal antibodies are manufactured commercially for therapeutic and diagnostic uses. Major areas of consideration in these applications are quality control and bioactivity. Separation of immunoglobulins has proven to be a challenge even for well-established techniques such as HPLC. Difficulties arise due to the large size of antibodies and their surface properties, which increase their tendency to interact with proteins and matrix.

Monoclonal antibodies have been shown to possess microheterogeneity due to posttranslation modifications such as glycosylation. Gel IEF is used routinely to analyze different batches of antibodies, but this type of analysis presents several drawbacks already discussed. CIEF can be applied to the analysis of antibodies with special consideration to maintaining antibody solubility. The use of additives, short focusing time, low sample concentration, and other precautions mentioned in the subsection titled "Optimizing CIEF Analysis" are a major part of method development to achieve reproducible high-resolution separations. CIEF of monoclonal antibodies with pIs near neutral pH was carried out in single-step mode in the presence of EOF and using protein markers as internal standards.[110]

CIEF was also used to follow the production of recombinant antithrombin III (r-AT III) in cultures of hamster kidney cells.[111] r-AT III inhibits serine proteases such as blood factors (IXa, Xa, and XIa) and thrombin. Interference by the media from which the samples were collected posed some difficulties because some of the media components have similar characteristics to those of the compounds of interest. CIEF was used to determine the pIs of the separated components after sample purification by HPLC. Three major peaks showed pIs of 4.7, 4.75, and 4.85, and three minor peaks had pIs of 5.0, 5.1, and 5.3. These data closely resembled the data already published for serum AT III based on conventional IEF.

The feasibility of using CIEF for analysis of monoclonal antibodies in a quality control environment was demonstrated for recombinant humanized monoclonal antibody HER2 (rhuMAbHER2) by Hunt et al.[112] This protein is present at increased levels in certain breast cancers. Besides primary structure heterogeneity present in the 214-residue light chains and 449- or 450-residue heavy chains, rhuMAbHER2 can exhibit charge differences due to deamidation or C-terminal clipping. Resolution of the five observed components was optimized by mixing Pharmalyte 8–10.5, Bio-Lyte 3–10, and Bio-Lyte 7–9 ampholytes in an 8:1:1 ratio. Figure 9.6 shows a very good correlation of the IEF and the CIEF pattern obtained for rhuMAbHER2. The pIs of the major peaks were determined through the use of internal standards, and the values obtained correlated well with the values obtained from gel IEF. The method was capable of revealing differences due to storage at 5 and 37°C, and the increased acidic peaks observed were consistent with protein deamidation. Intra-assay reproducibility ranged from 0.7 to 0.9% RSD for migration time, 0.8 to 3% RSD for peak area, and from 1 to 3.7% for area percent. Interassay reproducibility for migration time varied from 0.4 to 0.6% RSD, 1.2 to 3.2% for peak area, and 1.1 to 4.2% for area percent. All analyses were performed in the same coated capillary.

Due to the generally low concentration of contaminants, an important parameter for QC labs is the limit of detection. For rhuMAbHER2 this limit was estimated to be 2 ppm. Care should be exercised when analyzing low-concentration samples because ampholytes may show residual absorption even at 280 nm.[86] This problem can be reduced by decreasing the ampholyte concentration to 0.5% w/v.

Glycoform Analysis

A glycoprotein may vary in the location, length, and composition of sugar moieties attached to the polypeptide chain. The saccharide component of these glycoforms may play important roles in cell recognition, protein function, stability, solubility, and immunogenicity. In the development and manufacture of recombinant protein therapeutics, the distribution of glycoforms can therefore determine the efficacy and stability of the product. An understanding of the sugar content of recombinant proteins is particularly important because the glycosylation pattern is defined by an organism other than the end user. Introduction of carbohydrate groups can produce subtle changes in the protein pI, so IEF is a standard method for characterization of glycoforms; CIEF provides an automated quantitative method for glycoform analysis. Applications of glycoform analysis include determination of hemoglobin A1c to monitor diabetes mellitus and determination of elevated transferrin glycosylation as an indicator of alcoholism and pregnancy.

An example of two-step CIEF applied to the analysis of glycoforms is the fractionation of human recombinant tissue plasminogen activator (rtPA).[105] This activator is a protein that degrades blood clots, and its recombinant form is produced for the treatment of myocardial infarction. This 59-kDa glycoprotein possesses three N-glycosylation sites. Type I rtPA is glycosylated at all three sites (residues 117, 184, and 448), whereas type II rtPA is glycosylated at two sites (residues 117 and 448). Although rtPA was purified extensively to yield high purity of the polypeptide, in some instances up to 20 peaks were observed during CIEF. Treatment of rtPA with neuraminidase, an enzyme that removes sialic acid residues, has greatly simplified the pattern and suggests that heterogeneity is due to the variation of sialylation. CIEF performance was suitable for validation of the technique as a routine test.[113]

CIEF analysis of rtPA in the presence of urea was also carried out in an uncoated capillary using pressure mobilization.[114] The final urea concentration used was 4 *M*, and EOF was reduced by adding polymers to the reagents and sample (0.4% hydroxypropylmethyl cellulose produced better results than polyethylene glycol). A one-step CIEF method described by Moorhouse et al.[115] for the analysis of rtPA produced a constant residual EOF in a neutral capillary. The sample was prepared by dilution to 125 to 250 μg of protein per milliliter in 3% ampholytes 3 to 10 and 5 to 8 (1:1) containing 7.5% TEMED and 4 *M* urea. Results obtained by CIEF correlated well with those generated by IEF, and the analysis was completed in less than 10 min.

Protein Concentration and Dynamics of Interaction

It is well known that a very important feature of many biological systems is specific recognition at the molecular level. Antibodies as a group are widely used for molecular recognition, e.g., affinity assays. This feature can be used by labeling an anti-human growth hormone antibody fraction with a fluorescent tag

(tetramethylrhodamine-iodoacetamide) to detect the presence of growth hormone to a level of 0.1 ng/ml. An application of CIEF that exploits the concentration effect of the technique with the advantages of affinity interaction and the detection power of laser-induced fluorescence has been developed.[116]

SIEVING SEPARATIONS

The performance of electrophoresis in narrow-bore capillaries obviated most of the functions of gels in electrophoresis, e.g., elimination of convection through rapid dissipation of Joule heat and reduced diffusion through short analysis time. However, another important feature of gels is their capability to actively participate in the separation process by providing a sieving media that differentially affects the migration velocity of sample components according to molecular size. Macromolecules such as nucleic acids and SDS–protein complexes exhibit no significant mobility differences during free-zone electrophoresis and require the presence of an interactive sieving separation matrix to achieve resolution.

Size-based analysis of SDS–protein complexes in polyacrylamide gels (SDS-PAGE) is the most common type of slab gel electrophoresis for the characterization of polypeptides, and SDS-PAGE is one of the most commonly used methods for the determination of protein molecular masses.[117] The uses for size-based techniques include purity determination, molecular size estimation, and identification of posttranslational modifications.[118,119] Some native protein studies also benefit from size-based separation, e.g., detection of physically interacting oligomers.

Due to the importance and broad spectrum of sieving applications, many groups[120–122] have attempted to adapt gels to the capillary format. Unfortunately, there are several technical difficulties that have limited the use of gel-filled capillaries, including short lifetimes due to bubble formation or contamination after repeated runs. For polypeptides, gel-filled capillaries have poor UV absorbance detection sensitivity because only higher wavelengths can be used. The main advantage of gel-filled capillaries is higher resolution.

An alternative to gel-filled capillaries is the use of polymer solutions.[123] In this chapter, gel-filled capillaries are those containing matrices that are not replaceable. Replaceable matrices are referred to as "polymer solutions." Gels are typically polymerized *in situ*, whereas polymer solutions are pumped into the capillary and are usually replaced between each injection. The use of replaceable gels has been variously termed *entangled polymer CE*, *nongel sieving*, and *dynamic sieving*. The polymer solutions have been referred to as *replaceable gels* and *physical gels*. The advantage of polymer solution is that the whole content of the capillary is replaced in between runs, thus eliminating aggregates that move extremely slowly, do not penetrate the gel, accumulate in the gel matrix, and cause deteriorating results. This practice allows for the analysis of "dirty" samples during process development.

Many polymers have been tested for the analysis of SDS–protein complexes, and the main difference observed is in the resolution (or resolution range) from polymer to polymer and among molecular weight distributions of the same polymer that can be used to improve the resolution. Interestingly, studies on resolution show that it can increase or decrease with increasing temperatures depending on the type of sieving polymer used. Although we occasionally prepare buffers with dissolved polymers for research purposes, commercially available solutions are preferred for routine applications and testing.

Low-melting agarose has been proposed as a sieving medium for CE.[124] This material can be introduced into the capillary by pressure at a temperature above its melting point of 25.6°C and then induced to form a gel by dropping the capillary temperature below the melting point. Following separation, the capillary temperature can be raised and the gel extruded from the capillary by pressure, and then replaced with fresh uncontaminated agarose prior to the next injection. Unfortunately, the pore size of agarose gels is too large to provide sufficient sieving for most proteins.

Analysis of Native Proteins

Native proteins consisting of varying numbers of identical subunits or protein conjugates made up of monomers joined by cross-linking agents may be difficult to resolve by free-zone electrophoresis or CIEF, but they can be easily separated by sizing methods. In some instances, it is desirable to maintain the tertiary and quaternary structures of proteins, which are lost when polypeptides are denatured. In these cases, sieving of native proteins can be performed using either gel-filled capillaries[122,124] or polymer solutions.[125] Examples of size-based analysis of native proteins by CE include bovine serum albumin (BSA), rat liver proteins,[125] and human serum albumin.[124]

Analysis of SDS–Protein Complexes

SDS binds in an approximately stoichiometric fashion to polypeptide chains, with roughly one SDS molecule bound per two amino acid residues[126] or an average of 1.4 g of SDS per gram of protein. Assuming that the contributions of the charged amino acid side chains are low relative to that of the surfactant phosphate groups, the SDS–protein complexes possess the same charge-to-mass ratio independent of polypeptide chain length. SDS complexes of proteins with molecular masses greater than 10 kDa exhibit identical mobility in free solution,[127] although proteins that are not fully complexed with SDS may exhibit variable mobility and may be resolved into multiple species.[128] For smaller proteins, the intrinsic charge of the polypeptide has a more pronounced effect, introducing larger errors when estimating the molecular weight. Most polymer solutions described to date do not provide enough resolution when separating proteins smaller than 10 kDa. In theory, these molecules can be easily resolved by gel-filled capillaries with high-percentage polyacrylamide.

An important advantage of using SDS to denature polypeptides is the solubilizing power of the detergent. This property allows for the study of proteins (e.g., membrane proteins) that easily precipitate under most other conditions.

Size-based analysis by CE provides similar information and comparable limits of detection to analysis by SDS-PAGE with Coomassie blue staining.[120,129] The performance of both electrophoretic techniques for the analysis of polypeptides is far superior to size exclusion chromatography. Figure 9.7 shows the separation of SDS-complexed recombinant protein standards by CE.

Protein separations by CE are often negatively affected by sample–capillary wall interactions and require additives or surface modifications to eliminate undesirable interactions (see "Minimization of Nonspecific Protein–Wall Interactions"). This problem is minimal or nonexistent for SDS–protein complexes. Untreated silica possesses negative charge at pH above 2 to 3, and the SDS–protein complex is also anionic at almost any pH. Electrostatic repulsion between the silica wall and SDS–protein complexes eliminates protein adsorption. Nevertheless, in cases where EOF limits resolution or introduces migration time variations, internally coated capillaries can be used.[130] Capillaries coated with linear polyacrylamide through C-Si bonds were found to be more stable than capillaries coated through siloxane groups.[131–133] Uncoated capillaries have been used with a linear polyacrylamide-sieving matrix that provides sufficient viscosity (>100 cP) to prevent extrusion of the sieving medium from the column by EOF.[117] In some instances, the sieving matrix acts as a surface coating.[134]

Theory

The analysis of SDS–protein complexes is based upon all complexes having the same mobility in free solution and a sieving media providing resolution based on size. Nevertheless, the theory of resolution is important to explore anomalous behavior of sample components, or when the user desires to develop his or her own separation system. Because resolution of sample components is based on molecular size alone, the separation mechanisms include the behavior of a polyionic molecule and the restrictions that the gel imposes on larger analytes. Size-dependent separations can be used to estimate the molecular weight of analytes. Molecular mass can be calculated once the mobility differences of the sample components are cancelled. These mobility variations can be eliminated by two approaches. One is mathematical, and it is performed after mobility measurements in gels of various concentrations (see "Molecular Weight Determination and Ferguson Analysis"). The other involves the binding of a charged ligand that masks the native charge of the protein. The most common practice for the latter approach is the binding of SDS by proteins at a constant ratio, rendering the mass-to-charge relationship close to a constant for most proteins 10 kDa to above 200 kDa. Although the presence of a sieving media suggests size-based separations only, electrophoretic mobilities are affected by differences in shape, size, or net charge.

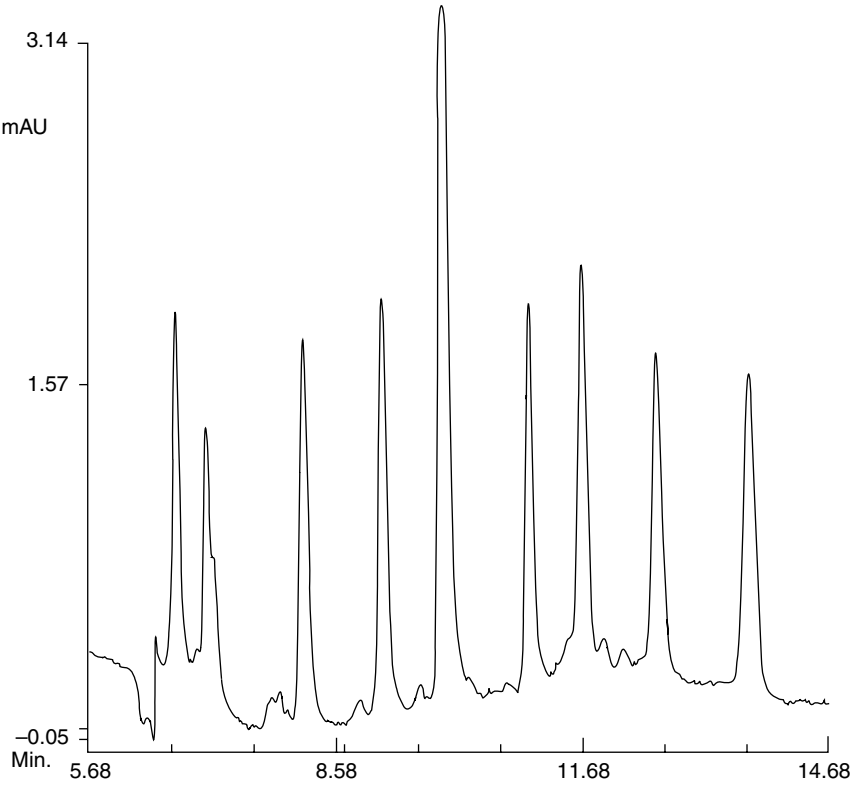

Figure 9.7 Capillary SDS of prestained recombinant protein standards (Bio-Rad Laboratories). The front dye and buffer ions were removed by buffer exchange into CE-SDS sample buffer using a BioSpin 6 (Bio-Rad Laboratories) as suggested by the manufacturer. Due to the high resolving power of CE-SDS as well as detection by UV-absorption, blends of naturally occurring proteins generate a number of peaks that can make peak identity difficult. Recombinant standards, on the other hand, produce sharp peaks and clean electropherograms. Molecular weight of sample components: (1) 10 kDa, (2) 15 kDa, (3) 25 kDa, (4) 37 kDa, (5) 50 kDa (this peak is present at a higher concentration for easy visual reference), (6) 75 kDa, (7) 100 kDa, (8) 150 kDa, and (9) 250 kDa. The proteins were resolved using the Bio-Rad CE-SDS analysis kit and a 50-μm × 30-cm uncoated capillary thermostated at 20°C. The sample was injected by applying 5 psi of pressure for 6 sec, and detection was performed at 220 nm. The polarity was set with the negative electrode at the detector end of the capillary and the electric field as 15 kV constant voltage.

The following explanation of the behavior of analytes during CE-SDS in the presence of sieving media is found in a monograph by Guttman:[135] the electric force (F_e) that a particle experiences when placed in an electric field depends on the net charge (Q) of the particle and on the intensity of the electric field (E):

$$F_e = QE$$

In the presence of a sieving media the motion of the ions is impeded by a frictional force (F_f). The frictional force is dependent on the *translational friction coefficient* (f):

$$F_f = f(dx/dt)$$

where dx and dt are the distance and time increments. The translational friction coefficient is affected by the temperature [$f = C_1 \exp(1/T)$, where C_1 is a constant for a given shape solute]. The effect of temperature is much higher in systems using polymer solutions than high-viscosity gels.

The motion of the charged molecule due to the presence of the electric field can be expressed as:

$$m(d^2x/dt^2) = QE - f(dx/dt) = F_e - F_f$$

When the force from the applied electric field on the charged solute is counterbalanced by the frictional force, the solute will move with a steady-state velocity ($v = dx/dt = QE/f$).

The retardation of the SDS–protein complexes in capillary gel electrophoresis is a function of the separation polymer concentration (P) and the retardation coefficient (K_R):

$$\mu = \mu_0 \exp(-K_R P)$$

where μ is the apparent electrophoretic mobility and μ_0 is the free solution mobility of the analyte.

According to these equations, for a given separation system, the main parameters involved in the separation of SDS–protein complexes are the electric force, the frictional force, and the retardation coefficient. These parameters are in turn affected by the strength of the electric field, molecular charge, analyte shape and size, polymer concentration, and temperature.

Sieving Mechanisms

When the mobility of a molecule is plotted against the gel concentration (Ferguson plots), three regimes are distinguishable. In the Ogston regime the average pore size of the matrix is similar to that of the hydrodynamic radius of the migrating analyte. It is in this region that true sieving occurs, and therefore the retardation coefficient (K_R) is proportional to the molecular mass of the analyte [$\mu \sim \exp(M_r)$]. Under these conditions the logarithm of mobility for a given analyte is a linear function of the molecular mass, and the Ferguson plots are linear. Typically, the plots intersect each other at zero gel concentration.

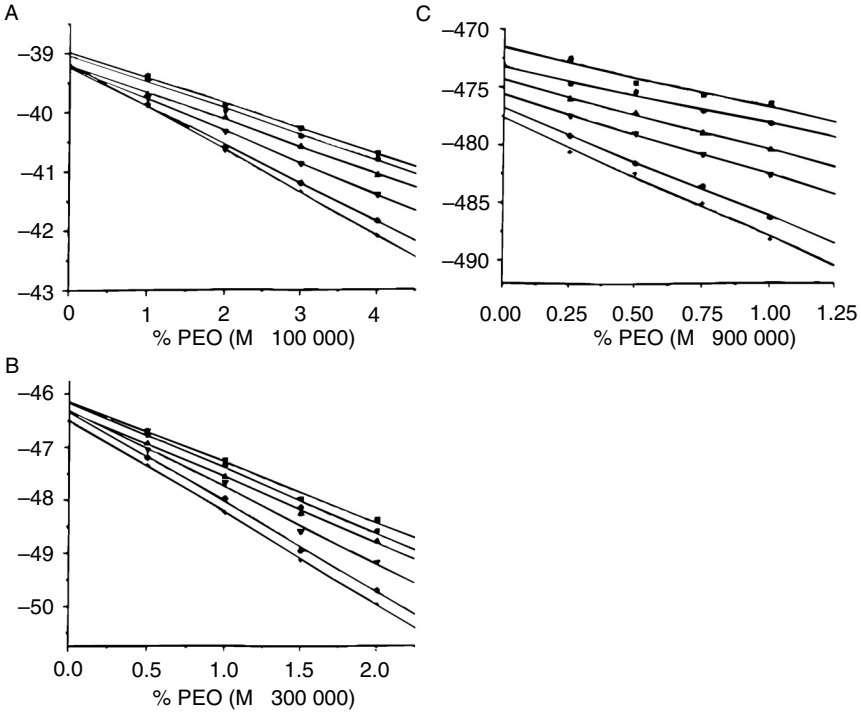

Figure 9.8 Ferguson plots for polyethylene oxide used as sieving polymer. The graphs represent plots of the polymer concentration and the log of electrophoretic mobility for six different samples. Graphs A, B, and C represent polymers with MW of 100, 300, and 900 kDa, respectively (From A. Guttman, *Electrophoresis, 16*: 611 (1995). With permission.)

The Ogston theory assumes that the migrating particles behave as unperturbed spherical objects and that the gel or sieving matrix has similar pore sizes as the analyte. Molecules with large Stokes radii such as the flexible chain of biopolymer molecules (DNA and SDS–protein complexes) can still migrate through pores much smaller than their size would permit. This can be explained by the reptation model (reptation-without-stretching regime), which describes the migration of the polyelectrolyte as a "head first, snakelike" motion through the pores of the sieving media. This model suggests an inverse relationship between the mobility and the molecular mass of the analyte.

When high electric fields are applied, molecular migration is explained by the reptation-with-stretching model. A common way to recognize the Ogston theory, the reptation, or the reptation-with-stretching regimes, is to plot the log solute mobility vs. the log solute's molecular mass curves. Pure reptation is suggested when the values of these slopes are close to −1. Figure 9.8 shows the

plots obtained with the sieving polymer polyethylene oxide (PEO) at various concentration ranges. In graph A, the six plots at 1, 2, 3, and 4% PEO (M_r 100,000) concentrations exhibit consecutively steeper slopes (−0.07, −0.01, −0.014, and −0.018). Similar behavior was observed when the same curves (data not shown) were plotted for the different molecular mass sieving polymers prepared to a 1% polymer concentration. The sieving polymer matrix of 1% M_r 100,000 PEO resulted in the shallowest curvature, with an apparent slope of −0.07. When used in 1% concentration, the larger molecular-mass polyethyne oxides of M_r 300,000 and 900,000 exhibited gradually steeper apparent slopes of −0.11 and −0.15, respectively. The slope values of much lower than 1 in Figure 9.8 implied a reptation-with-stretching mechanism.

Other Parameters Affecting Resolution

Separation of SDS–protein complexes in gel or polymer solutions can be influenced by changing the operating parameters, such as applied electric field strength, temperature, capillary dimensions (diameter and length), and type and concentration of gel or polymer. Because the migration velocity of a given solute is proportional to the applied electric field strength, an increase of the latter should increase the peak efficiency and thus the resolution of the sample components while shortening the analysis time. Temperature is a parameter varied almost exclusively when using polymer solutions because increased temperature is likely to cause bubble formation in gel-filled capillaries. Temperature affects polymer solution analysis in two ways. First, because the viscosity of the solution is usually lower at higher temperatures, an increase in temperature decreases the friction of the migrating SDS–protein complexes. Second, the temperature changes can influence the structure of the dissolved polymer, resulting in differences in pore structure. Experimental data suggest that oriented arrangements (channel-like structures) in concentrated polymer solutions play an important role in the sieving of SDS–proteins complexes. This means that any changes that result in the generation of these organized structures is likely to enhance separation efficiency.

As for CZE, sieving separation of SDS–protein complexes improves at the expense of analysis time when using longer capillaries. According to theoretical formulations, a higher applied electric field results in improved separation efficiency, and the capillary should be short enough to allow a high number of volts per centimeter field to be used. In practice, however, the improvements in separation efficiency are modest or nonexistent. Manipulating the length of the capillary usually produces more tangible effects on resolution than variations on the electric field. Once again, the I.D. of the capillary is a compromise between detection and Joule heat generation and dissipation. A larger sample volume can be loaded into a capillary with a larger I.D. without compromising resolution, and the increased path length of the detection window generates a better signal. For polymer solutions, selection of the capillary I.D. needs to take into consideration the viscosity of the solution. Narrow-bore capillaries may require high

pressure and prolonged purging time in between analyses to replace the capillary contents, unnecessarily increasing total analysis time.

Gel-Filled Capillaries: The first described use of gel-filled capillaries for analysis of SDS-denatured proteins was in 1983 by Hjerten.[122] Since then, most reports employed either of two types of gels: polyacrylamide cross-linked with bis-acrylamide,[121] and linear polyacrylamide. Both gels are polymerized *in situ* because their high viscosities preclude pumping them into the narrow-bore column. The chemical or cross-linked gels have a well-defined pore structure largely determined by the concentrations of the monomer and cross-linker (most commonly acrylamide and *N*,*N*′-methylenebisacrylamide, respectively). Because one of the drawbacks of gel-filled capillaries is poor lifetime, several studies have aimed to increase the useful life of the columns. It was found that a lower degree of cross-linking correlated with longer column lifetime.[136] Thus, linear polyacrylamide gels with no cross-linking the capillaries were introduced. These gels were also more compatible with high electric fields than cross-linked gels.[120] Unfortunately, even gels with zero cross-linking could not be used for more than 20 to 40 runs.

Based on the high resolving power of gels compared to polymer solutions,[120] efforts were made to adapt gels to the capillary format. In CGE one of the main disadvantages of using cross-linked polyacrylamide is the lack of flexibility during the separation and injection process. Sample plugs in the gel-filled capillary may result in bubble formation and poor separation efficiencies. Manufacture and shelf life of gel-filled capillaries are also challenges. Chemical gels are heat sensitive; at slightly higher than room temperature, bubbles can form. Other disadvantages of gel-filled columns include a short lifetime, low reproducibility, and poor detection sensitivity due to high UV absorption of the gel matrix. Protein detection in gels is usually accomplished at 280 nm, but the extinction coefficients of proteins are 20 to 50 times higher at 214 nm. At 214 nm there is also less variability in the intensity of absorbance of proteins.[136] Another drawback of gel-filled capillaries is that the composition of the gel-filled capillary cannot be changed. Coupled with short capillary lifetimes and shelf life, this adds to the cost of the method.

Polymer Solutions: The advantages of using polymer solutions to achieve size-based separations include increased reproducibility (because the capillary's content is replaced at each run), increased capillary lifetime, the possibility of using polymers with low absorption in the 200- to 220-nm range, and ease of storage and handling. Another important advantage of polymer solutions is the possibility of using the less problematic, often more reproducible, pressure injection method of sample injection. Separation parameters are simple to optimize by changing polymer type and concentration, buffer pH, viscosity, and conductivity. As stated above, the main drawback of polymer solutions is that resolution is not as high as that obtained with gel-filled capillaries or SDS-PAGE. Polymer solutions can be applied when the difference in molecular weight between the

product and contaminants or degradation products is at least 5%. SDS-PAGE can be used during methods development for comparative purposes.

Several types of polymers have been shown to be suitable for separation of a broad molecular weight range of polypeptides. Most polymers used to date are noncross-linked linear or branched polymers. Because the polymers are in solution, the structure of their pores is flexible and dynamic. Care should be exercised when selecting polymers and optimizing analysis conditions,[137] because separation parameters such as temperature do not affect all polymers equally.[138–140] Resolution also depends on the type, size, and concentration of the polymer used. Under optimized conditions, polypeptides differing by as little as 4% in molecular mass can be resolved.[134] Resolution achieved using polymer-sieving CE is comparable with that obtained using a 12%T polyacrylamide slab gel.

Early reports on the use of polymer solutions for the analysis of SDS complexes included dextran and polyethylene glycol (PEG).[136] Both of these polymers are practically transparent at 214 nm and thus greatly improve detection over polyacrylamide gels. Migration time (MT) reproducibility is of prime importance in this technique because migration times are used to estimate the molecular size of proteins. The use of a replaceable matrix increases MT reproducibility, and RSD values as low as 0.3% were obtained using dextrans. Similar values were obtained for PEG matrices. Using polymer solutions, the life of the column was also extended to over 300 analyses.

One important consideration when selecting a polymer is the viscosity of the final solution. In most cases, low viscosity is desired for easy replacement of the capillary content in between analyses. However, uncoated capillaries will exhibit higher EOF with lower-viscosity polymers.[139]

Because of the properties of both systems, polymer solutions can be used at any stage of the production process, whereas gel-filled capillaries can be used in late stages when clean samples are available and higher resolution might be required.

Molecular Weight Determination and Ferguson Analysis

Molecular weight (MW) determinations are easily performed when analyzing polypeptides in the presence of SDS and sieving media. The MW is obtained by comparing the mobility of protein standards of known MW and the sample of interest. Plotting the log of the MW against the migration time yields a near-linear relationship. This linearity is observed within a range that depends on the type of polymer used for sieving and on the analysis conditions. The protein standard curve may introduce errors in the estimation of MW if the binding of detergent by the protein is anomalous (e.g., membrane proteins, glycoproteins, or highly basic proteins). Because detergent binding directly affects protein mobility by changing the mass-to-charge ratio, the MW discrepancy originates from differences in free-solution mobilities of the different polypeptides.[141]

A method used to correct such errors is to electrophorese the polypeptide in a series of gels of varying concentration. The size effect is then canceled mathematically by constructing Ferguson plots. In electrophoresis, Ferguson analyses are performed for two main reasons: to optimize resolution by determining the appropriate gel concentration and to estimate the molecular weight of proteins. In CE, Ferguson plots are made by measuring the migration times at different polymer concentrations and constructing a universal calibration curve[142] by plotting the logarithms of the relative migrations as a function of polymer concentration. According to Ferguson, the logarithm of the protein's mobility varies linearly as a function of the gel concentration employed. The slope of this mobility line yields a parameter called the retardation coefficient (K_r), which is proportional to the square of the radius of the protein. Universal standard curves are constructed by plotting the logarithm of known protein MW as a function of the square roots of the retardation coefficients. The slope of the curve represents the retardation coefficient, whereas the intercept at zero polymer concentration corresponds to the free-solution mobility of a protein. Different intercepts at zero polymer concentration is an indication of differences in free-solution mobilities of the SDS–protein complexes.

One important advantage of CE is that the mobility of the protein in free solution can be determined experimentally. If the SDS–protein complexes do not have the same mobility in free solution, a Ferguson plot should be constructed. Proteins with similar molecular radii show the same slope, independently of where they intersect on the concentration axis. Ferguson analysis for traditional SDS-PAGE is time consuming, especially because the analysis is best performed using at least six different gel concentrations. Consequently, this method of analysis was practically abandoned until the use of CE with replaceable polymer networks made the Ferguson analysis more feasible. In CE, Ferguson plots can be generated automatically by using different dilutions of the sieving buffer.[142,143]

Practical Considerations in the Analysis of SDS–Protein Complexes Using Polymer Solutions

Commercial kits for analysis of SDS–protein complexes using entangled-polymer sieving systems are currently available from Bio-Rad Labortories, Beckman Instruments, Sigma, and others. This discussion is based on the authors' experience with the replaceable polymer sieving system from Bio-Rad, which employs a proprietary hydrophilic sieving polymer in 0.4 *M* Tris borate buffer (pH 8.5) containing 0.1% SDS. The chain length and concentration of the sieving polymer were formulated to provide resolution of SDS–protein complexes over a MW range of 14 to 200 kDa. The buffer also contains a low concentration of the polymer modified by charged functional groups. The combination of the high-viscosity sieving polymer and the cationic modified-polymer additive serves to reduce the electroendoosmotic flow in an uncoated capillary to less than 5×10^{-5} cm^2 V^{-1}sec^{-1}. Because proteins

that have been complexed with SDS are strongly anionic, they do not adsorb to the capillary wall under the alkaline run conditions, allowing analyses to be performed in uncoated capillaries in the absence of significant EOF.

Sample preparation for CE-SDS analysis is essentially the same as for SDS-PAGE. Protein samples are diluted 1:1 in a Tris-HCl + SDS (pH 9.2) sample preparation buffer. If the proteins are to be analyzed under reduced conditions, an appropriate reducing agent such as β-mercaptoethanol (final concentration 2.5%) or dithiothreitol (15 m*M*) is added. The Bio-Rad CE-SDS kit employs benzoic acid at a final concentration of 50 μg/ml as the internal standard. Because the marker is a small molecule not subject to sieving, it cannot be used to correct for variations in migration time due to changes such as new polymer or solution batch, temperature, or polymer concentration. For this purpose, it is necessary to find a protein whose migration time differs from that of the main protein and its contaminants or degradation products. In addition, always analyze the sample and internal standard separately to determine peak identities.

After mixing the sample, buffer, and internal standard, the mixture should be heated at 95 to 100°C for 10 to 12 min to complex proteins with SDS. For new proteins, it is advisable to prepare several vials of the same solution and heat them for various lengths of time in order to check for heat degradation artifacts. It is our experience that heating in a water bath is necessary; use of a contact heating block is not always effective and may result in reduced separation efficiency.

The presence of salt in the sample will interfere with the injection process, and the highest sensitivity will be obtained if the sample salt concentration is less than 50 m*M*. Samples containing higher salt or buffer concentrations should be desalted.

Entangled-polymer sieving buffers are quite viscose, and bubbles are frequently trapped in the bottom of the buffer reservoir vials when the analysis buffer is pipetted into them. In this situation, the capillary orifice and high-voltage electrode will not contact the buffer, resulting in erratic current and failed analysis. To prevent this, the buffer vials should be centrifuged for at least 2 min at full speed in a microcentrifuge immediately prior to installing them in the CE instrument.

The Bio-Rad CE-SDS analysis buffer is designed for use with uncoated capillaries, and no prior capillary conditioning is required. However, the capillary should be purged with acid and base wash solutions for the appropriate length of time before replenishing the run buffer prior to each injection. These purge cycles serve to sweep residual buffer and any remaining sample components from the capillary. Fresh run buffer is then introduced. The viscosity of the CE-SDS analysis buffer is approximately 43 cP, and the purge times given by the manufacturer are calculated for the capillary length and instrument purge pressure of 100 psi. If the CE instrument employs lower purge pressures, or different capillary dimensions are used, purge times should be modified appropriately.

Because of the high viscosity of the entangled-polymer solutions, the buffer can be retained on the outer surfaces of the capillary and electrodes after the

replenishment step, resulting in carryover of the buffer into the sample solution during injection. This will reduce injection efficiency and compromise sensitivity. To prevent this, the capillary and electrode surfaces should be immersed in one or two vials of wash solution (water or diluted sample preparation buffer) without application of pressure.

In CZE, electrophoretic or electrokinetic injection is usually not the preferred injection mode because of electrophoretic bias: sample ions of low mobility will migrate more slowly in the injection process and therefore will be at lower relative concentrations in the starting zone. In the case of SDS–protein complexes, all sample components will have approximately the same mass-to-charge ratio because of the constant charge density of SDS on the protein. Therefore, all SDS–protein complexes will be loaded with the same efficiency using electrokinetic injection. The high ionic strength of the analysis buffer (0.4 *M* Tris-borate) provides a stacking effect, thus decreasing the starting zone width and increasing zone concentration. Consequently, electrokinetic injection is the preferred mode for this technique. However, if the sample contains appreciable salt concentration and it is not practical to desalt the sample, pressure injection may be used. The high viscosity of the run buffer requires sufficient injection times to introduce enough material. For example, in the case of a 24-cm × 50-μm capillary, an injection of 12 sec at 5 psi is necessary to inject a 0.3-mm sample zone. If sample salt concentration is greater than 50 m*M*, even pressure injection will not provide satisfactory sensitivity.

The great advantage of entangled-polymer systems is their transparency in the low UV range. However, the absorbance of the buffer and sample components such as Tris and SDS contribute appreciable background signal below 210 nm. Detection at 220 nm reduces background interference without significant loss in protein response. A protocol for detection of proteins using precolumn derivatization and LIF[144] is described in the following subsection, "Analysis of rMAbs."

Operation at a field strength of 625 V/cm provides satisfactory resolution with short run times; typical current is approximately 20 μA using a 24-cm × 50-μm capillary. The capillary should be thermostatted close to ambient temperature (e.g., 20°C) for good reproducibility. It is extremely important not to expose the capillary tips to drying conditions when using entangled-polymer sieving buffers. The sieving polymer will precipitate and plug the capillary.

Repeatability of migration times and peak areas for eight protein standards using electrokinetic injection are presented in Table 9.3. Migration time precision was approximately 0.5% RSD and peak area precision varies by 1 to 3%. Peak area precision using pressure injection was comparable (data not shown). The ability to acquire quantitative information on protein concentration is considered a major advantage of CE compared to SDS-PAGE; in the latter the staining response has only narrow linear ranges, depends on operator technique, and is subject to batch-to-batch variability of the stain. In contrast, protein response using polymer sieving CE with UV detection at 220 nm is linear over three orders

Table 9.3 Migration Time Reproducibility (n = 10) for CE-SDS Using Electrokinetic Injection

Protein	Migration Time, %RSD	Peak Area, %RSD
Lysozyme	0.35	1.51
Trypsin inhibitor	0.40	0.85
Carbonic anhydrase	0.45	1.24
Ovalbumin	0.55	1.57
Serum albumin	0.56	3.30
Phosphorylase B	0.55	3.32
β-Galactosidase	0.59	3.05
Myosin	0.66	1.76

Source: From T. Wehr, R. Rodriguez-Diaz, and M. Zhu, Chromatographic Science Series, Vol. 80 (1999). With permission.

of magnitude and quite reproducible (Table 9.3). In this system, the detection limit for carbonic anhydrase (S/N = 3) is 0.5 μg/ml.

Using the polymer sieving system described in the preceding text, the log of protein MW is correlated with migration time. MW can be determined directly by comparing to migration times of standard proteins. However, small variations in migration times can introduce significant error in the calculated MW value. More reliable estimates may be obtained by normalizing the migration times of protein standards and samples to that of an internal standard.

Applications

Analysis of rMAbs

Recombinant monoclonal antibodies (rMAbs) are therapeutic biomolecules with potential applications covering a broad spectrum of indications. Classical approaches to the analysis of antibodies include extensive use of SDS-PAGE to monitor consistency, purity, and stability of these molecules. Often, detection problems arise if the antibodies are not concentrated enough in final formulations. Coomassie blue staining provides relative quantitative results, but lacks the sensitivity to detect contaminants present in small amounts. Alternatively, silver staining is more labor intensive, requires more skill, and it is nearly impossible to obtain consistent quantitation. With the interest generated by the therapeutic use of rMAbs, a replacement analytical approach for the analysis of size-based rMAb variants is needed. Hunt and Nashabeh[144] built upon the early research of Gump and Monning[145] and Wise et al.[146] to develop a CE method using laser-induced fluorescence to increase the detection limit to values comparable to those obtained with silver staining. The assay was developed and validated according to the guidelines of the International Committee on Harmonization (ICH) for use in routine lot release testing of an rMAb pharmaceutical.

In this assay, rhuMAbs produced in transfected Chinese hamster ovary (CHO) cells were analyzed using a commercially available SDS–protein analysis

kit. The rMAb in solution is derivatized with a neutral fluorophore, e.g., 5-carboxytetramethylrhodamine succinimidyl ester. Perhaps the key to the success of this method is the use of a neutral fluorophore. One of the dangers of using precolumn derivatization of proteins is the high potential for the generation of multiple species, which occurs because the reaction produces a distribution of products. Molecules with a different number of neutral fluorophores will vary slightly in mass, but the resolving power of CE with polymer solutions is not sufficient to separate the components with little variation in Stokes radii into multiple peaks. Thus, only a small increase in peak width is observed. The hydrophobic character of the fluorophore may also bind SDS to maintain a more constant mass-to-charge ratio and limit peak broadening. The increase in mass can also be compensated by the elimination of positive charge, such as an amino group that reacts with the fluorophore.

Recombinant MAb samples (2.5 mg) were buffer exchanged into 800 µl of 0.1 *M* sodium bicarbonate, pH 8.3, using an NAP-5 column. A measure of 10 µl of 5-TAMRA.SE (1.4 mg/ml) dissolved in DMSO was then added to 190 µl of rMAb solution, and the resultant mixture was incubated at 30°C for 2 h. After incubation, 190 µl of the antibody–dye conjugate was loaded into a second NAP-5 column and collected into 700 µl of 0.1 *M* sodium bicarbonate, pH 8.3.

The labeled sample was mixed with SDS-containing sample buffer and incubated at 90°C for 3 min. The samples were incubated with and without reducing agent. The capillary was conditioned by rinsing sequentially with 0.1 *M* NaOH, 0.1 *M* HCl, and running buffer containing a hydrophilic polymer as sieving matrix. The sample was injected electrophoretically, applying 10 kV for 15 sec. The applied voltage during the analysis was 15 kV, and the temperatures of the capillary and sample compartment were maintained at 20°C. For comparative purposes, the same unlabeled samples were analyzed by SDS-PAGE using a 5 to 20% gel. Voltage was applied for 3 h (20 mA), and the proteins were visualized by an Oakley silver stain. Figure 9.9 shows a comparison of CE using UV and LIF, both of which are comparable to SDS-PAGE (not shown).

The authors noted the importance of removing the unreacted fluorophore with a second NAP-5 column because prolonged incubation with the dye resulted in protein aggregation and peak broadening during the analysis. The ratio of dye to protein also must be optimized because high relative amounts of dye increased detectable aggregates. CE-LIF of derivatized rMAb using the labeling conditions described in this study allowed the detection of rMAb at a low nanomolar concentration (9 ng/ml) as determined by a signal-to-noise ratio of 2.5. This level of detection compares well with silver staining and is a 140-fold improvement over detection by UV absorption.

To estimate the recovery of the protein from the capillary, a sample was labeled with ^{125}I to track mass balance. A nonradiolabeled sample was used to show that both samples produced the same CE profile. A baseline value or control was obtained by performing the assay through the injection step, followed by a high-pressure rinse to expel the entire capillary contents into a collection vial. A

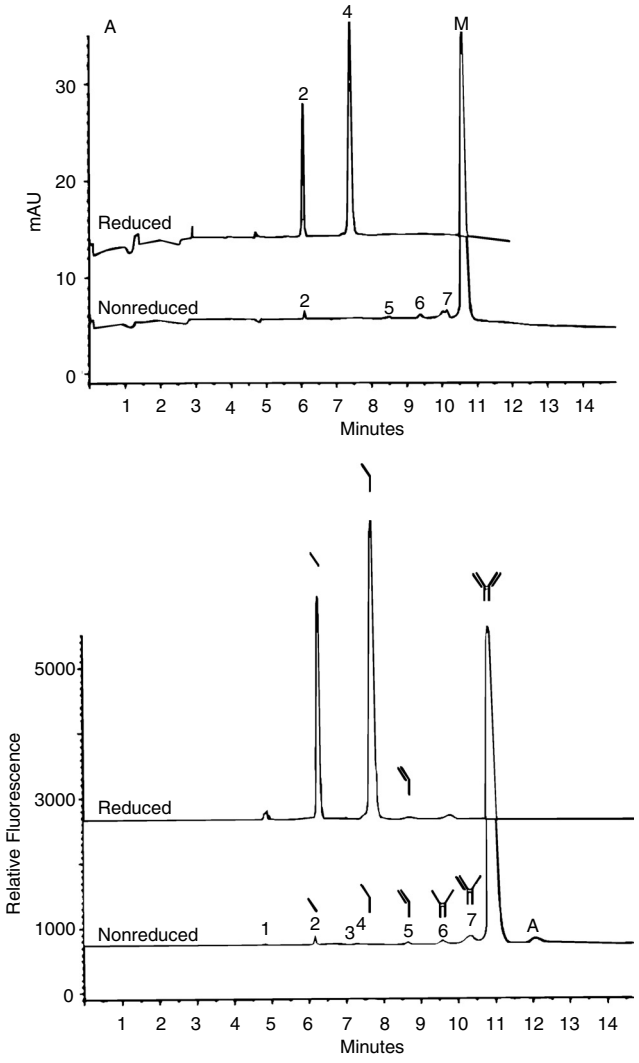

Figure 9.9 Comparison of CE-SDS using UV and LIF detection. A therapeutic recombinant monoclonal antibody (rMAb) was analyzed by CE-SDS with or without a reduction step. The top electropherogram depicts an overlay of reduced and nonreduced samples using detection by UV absorbance at 220 nm. The bottom electropherogram shows the same samples as detected by LIF using a 3.5-mW argon laser (set at 488 nm excitation wavelength, and 520 nm emission wavelength). The samples for CE-SDS with LIF detection were derivatized prior to analysis with 5-TAMRA-SE. The slight increase in the peak width of the monomer (M) for the LIF-detected sample could be the result of heterogeneous derivatization or sample overloading (which can occur when using more sensitive detectors even if the injection is constant). The numbers identifying the peaks correspond to observed bands in SDS-PAGE analysis with silver-stain visualization (see Reference 144).

second analysis was performed under standard protocol conditions. After the last peak had migrated past the detector, the voltage was disconnected, and the capillary content was purged into a collection vial. The counts per minute of both samples were determined using a gamma counter. Comparison of the control and experimental samples resulted in a mean recovery of 95% (n = 3). The authors noted that such recovery studies are often not performed for SDS-PAGE, in which selective loss of material may occur in the stacking gel.

The precision of the assay for nonreduced samples was demonstrated by the evaluation of six independent sample preparations on a single day (repeatability) and the analysis of independent sample preparations on three separate days by two different analysts (intermediate precision). The RSD values for the migration time were 0.9%. The RSD values for peak area percent of the main peak and the minor peaks in the profile were 0.6 and 12.6%, respectively. The higher variability observed with the minor peaks was determined to be primarily related to the sample heating during preparation for the analysis. These results demonstrate that the use of uncoated fused-silica capillaries in combination with a sieving matrix can provide adequate precision and analyte recovery.

During methods development, the authors noticed changes in the peak distribution of the samples. A time–temperature study of sample heating demonstrated that the sample was being degraded at high temperature. Because heating is used to speed up the unfolding of proteins for SDS binding, the best conditions for this rMAb were determined to be 37°C for 8 to 15 min. It is a common practice when performing SDS-PAGE to evaluate the effect of heating on sample profile, especially when working with a new protein. As shown in this application, it is also a useful practice for CE. The main drawback of too little heating temperature or time is the incomplete unfolding of the polypeptide, which results in peak broadening and anomalous migration in CE and in SDS-PAGE.

Recombinant Proteins

SDS-PAGE is widely used in the biopharmaceutical industry to monitor the purification and to estimate the purity of recombinant protein therapeutics. However, SDS-PAGE is a labor-intensive and (at best) semiquantitative technique that is not ideally suited for high-throughput analysis in commercial laboratories. Kundu et al.[147] have evaluated a commercially available entangled-polymer sieving kit for CE of SDS–proteins and compared the results with SDS-PAGE and Coomassie Brilliant Blue (CBB) using a 72-kDa viral-CKS fusion protein. Protein purity was assessed from densitometric scanning of CBB-stained gels and from integration of electropherograms monitored at 220 nm. The authors demonstrated that purity levels determined by the two methods were in good agreement, and that SDS-PAGE quantitation was limited by the nonlinearity of CBB staining at low concentration. Molecular mass estimates obtained using MALDI-TOF MS, CE-SDS, and SDS-PAGE were found to differ from the theoretical molecular mass by 100, 600, and 1400 mass units, respectively.

References

1. P.D. Grossman and J.C. Colburn (Eds.), *Capillary Electrophoresis, Theory and Practice*, Academic Press, San Diego CA, 1992.
2. S.F.Y. Li (Ed.), *Capillary Electrophoresis: Principles, Practice, and Applications*, J. Chromatogr. Library, Vol. 52, Elsevier Science, Amsterdam, 1992.
3. R. Weinberger, *Practical Capillary Electrophoresis*, Academic Press, Boston, 1992.
4. James P. Landers (Ed.), *Handbook of Capillary Electrophoresis*, 2nd ed., CRC Press, Boca Raton, FL, 1996.
5. P. Camilleri (Ed.), *Capillary Electrophoresis: Theory and Practice*, CRC Press, Boca Raton, FL, 1993.
6. N.A. Guzman, *Capillary Electrophoresis Technology*, Marcel Dekker, New York, 1993.
7. J. Vindevogel, *Introduction to Micellar Electrokinetic Chromatography*, Hüthig, Heidelberg, Germany, 1992.
8. S.M. Lunte and D.M. Radzik (Eds.), *Pharmaceutical and Biomedical Applications of Capillary Electrophoresis*, *Progress in Pharmaceuical and Biomedical Analysis*, Vol. 2, Elsevier Science, Oxford, 1996.
9. W.G. Kuhr, *Anal. Chem., 62:* 403R (1990).
10. W.G. Kuhr and C.A. Monnig, *Anal. Chem., 64:* 389R (1992).
11. C.A. Monnig and R.T. Kennedy, *Anal. Chem., 66:* 280R (1994).
12. M.V. Novotny, K.A. Cobb, and J. Liu, *Electrophoresis, 11:* 735 (1990).
13. Z. Deyl and R. Struzinsky, *J. Chromatogr., 569:* 63 (1991).
14. C. Schöneich, S.K. Kwok, G.S. Wilson, S.R. Rabel, J.F. Stobaugh, T.D. Williams, and D.G. Vander Velde, *Anal. Chem., 65:* 67R (1993).
15. C. Schöneich, A.F.R. Hühmer, S.R. Rabel, J.F. Stobaugh, S.D.S. Jois, C.K. Larive, T.J. Siahaan, T.C. Squier, D.J. Bigelow, and T.D. Williams, *Anal. Chem., 67:* 155R (1995).
16. É. Szökö, *Electrophoresis, 18:* 74 (1997).
17. M.A. Strege and A.L. Lagu, *J. Chromatogr. A, 780:* 285 (1997).
18. K. Ibel, R.P. May, K. Kirschner, H. Szadkowski, E. Mascher, and P. Lundahl, *Eur. J. Biochem., 190:* 311 (1990).
19. E.L. Gump and C.A. Monning, *J. Chromatogr. A, 715:* 167 (1995).
20. W.G. Kuhr and E.S. Yeung, *Anal. Chem., 60:* 2642 (1988).
21. D.F. Swaile and M.J. Sepaniak, *J. Liq. Chromatogr., 14:* 869 (1991).
22. B. Nickerson and J.W. Jorgenson, *J. Chromatogr., 480:* 157 (1989).
23. H.A. Bardelmeijer, H. Lingeman, C. de Ruiter, and W.J.M. Underberg, *J. Chromatogr. A, 807:* 3 (1998).
24. J. Cai and J. Henion, *J. Chromatogr., 703:* 667 (1995).
25. T. Wehr, *LC GC, 11:* 14 (1993).
26. K.A. Turner, *LC GC, 9:* 350 (1991).
27. J.R. Mazzeo and I.S. Krull, *Biochromatography, 10:* 638 (1991).
28. R.L. Chien and D.S. Burgi, *J. Chromatogr., 559:* 141 (1991).
29. P. Jandik and W.R. Jones, *J. Chromatogr., 546:* 431 (1991).
30. N.A. Guzman, M.A. Trebilock, and J.P. Advis, *J. Liq. Chromatogr., 14:* 997 (1991).
31. S. Hjertén, K. Elenbring, F. Kilár, J.L. Liao, A.J.C. Chen, C.J. Siebert, and M. Zhu, *J. Chromatogr., 403:* 47 (1987).
32. F. Foret, E. Szoko, and B.L. Karger, *Electrophoresis, 14:* 417 (1993).

33. T. Hirokawa, A. Ohmori, and Y. Kiso, *J. Chromatogr. A, 634:* 101 (1993).
34. V. Dolnik, *Electrophoresis, 18:* 2353 (1997).
35. V. Dolnik, *Electrophoresis, 20:* 3106 (1999).
36. G.M. Janini and H.J. Issaq, *Chormatographia Suppl., 53:* S-18 (2001).
37. J. Chen, J. Fausnaugh-Pollitt, and J. Gu, *J. Chromatogr. A, 853:* 197 (1999).
38. J. Bao and F.E. Regnier, *J. Chromatogr., 608:* 217 (1992).
39. D. Wu and F.E. Regnier, *Anal. Chem., 65:* 2029 (1993).
40. B.J. Harmon, D.H. Patterson, and F.E. Regnier, *Anal. Chem., 65:* 2655 (1993).
41. D.H. Patterson, B.H. Harmon, and F.E. Regnier, *J. Chromatogr. A, 732:* 119 (1996).
42. K. Shimura and K. Kasai, *Anal. Biochem., 251:* 1 (1997).
43. F.A. Gomez, L.Z. Avila, Y.H. Chu, and G.M. Whitesides, *Anal. Chem., 66:* 1785 (1994).
44. M.H.A. Busch, H.F.M. Boelens, J.C. Kraak, and H. Poppe, *J. Chromatogr. A, 775:* 313 (1997).
45. S. Honda, A. Taga, K. Suzuki, S. Suzuki, and K. Kakehi, *J. Chromatogr., 597:* 377 (1992).
46. N.H.H. Heegaard, D.T. Olsen, and K.-L.P. Larsen, *J. Chromatogr. A, 744:* 285 (1996).
47. R.G. Nielsen, E.C. Rickard, P.F. Santa, D.A. Sharknas, and G.S. Sittampalam, *J. Chromatogr., 539:* 177 (1991).
48. P. Sun, A. Hoops, and R.A. Hartwick, *J. Chromatogr. B, 661:* 335 (1994).
49. M.J. Schmerr, K.R. Goodwin, R.C. Cutlip, and A.L. Jenny, *J. Chromatogr. B, 681:* 29 (1996).
50. F.T.A. Chen, *J. Chromatogr. A., 680:* 419 (1994).
51. R.S. Rush, A. Cohen, and B.L. Karger, *Anal. Chem., 63:* 1346 (1991).
52. M.A. Strege and A.L. Lagu, *J. Chromatogr. A., 652:* 179 (1993).
53. M.A. Strege and A.L. Lagu, *Am. Lab., 26:* 48C (1994).
54. F. Kilár and S. Hjertén, *J. Chromatogr., 638:* 269 (1993).
55. V.J. Hilser, G.D. Worosila, and E. Freire, *Anal. Biochem., 208:* 125 (1993).
56. R.T. Bishop, V.E. Turula, and J.A. de Haseth, *Anal. Chem., 68:* 4006 (1996).
57. D. Rochu, G. Ducret, and P. Masson, *J. Chromatogr. A, 838:* 157 (1999).
58. D. Rochu, G. Ducret, F. Ribes, S. Vanin, and P. Masson, *Electrophoresis, 20:* 1586 (1999).
59. F. Kilár and S. Hjertén, *J. Chromatogr., 480:* 351 (1989).
60. K. Yim, *J. Chromatogr., 559:* 401(1991).
61. J.M. Thorne, W.K. Goetzinger, A.B. Chen, K.G. Moorhouse, and B.L. Karger, *J. Chromatogr. A, 744:* 155 (1996).
62. T.M. McNerney, S.K. Watson, J.-H. Sim, and R.L. Bridenbaugh, *J.Chromatogr. A, 744:* 223 (1996).
63. R. Malsch, T. Mrotzek, G. Huhle, and J. Harenberg, *J. Chromatogr. A, 744:* 215 (1996).
64. S. Kundu, C. Fenters, M. Lopez, B. Calfin, M. Winkler, and W.G. Robey, *J. Capillary Electrop., 6:* 301 (1996).
65. D. Josíc, A. Böttcher, and G. Schmitz, *Chromatographia, 30:* 703 (1990).
66. G. Schmitz and C. Möllers, *Electrophoresis,15:* 31 (1994).
67. T. Tadey and W.C. Purdy, *J. Chromatogr., 583:* 111 (1992).
68. T. Tadey and W.C. Purdy, *J. Chromatogr. A, 652:* 131 (1993).
69. R. Lehmann, H. Liebich, G. Grübler, and W. Voelter, *Electrophoresis, 16:* 998 (1995).

70. I.D. Cruzado, A.Z. Hu, and R.D. Macfarlane, *J. Capillary Electrop., 3:* 25 (1996).
71. I.D. Cruzado, S. Song, S.F. Crouse, B.C. O'Brien, and R.D. Macfarlane, *Anal. Biochem., 243:* 100 (1996).
72. T.J. Pritchett, *Electrophoresis, 17:* 1195 (1996).
73. X. Liu, Z. Sosic, and I.S. Krull, *J. Chromatogr. A, 735:* 165 (1996).
74. S. Hjertén and M. Zhu, *J. Chromatogr., 347:* 265 (1985).
75. S. Hjertén, J.-L. Liao, and K. Yao, *J. Chromatogr., 387:* 127 (1987).
76. S. Hjertén and M. Zhu, *J. Chromatogr., 346:* 265 (1985).
77. T.L. Huang, P.C.H. Shieh, and N. Cooke, *Chromatographia, 39:* 543 (1994).
78. S.-M. Chen and J.E. Wiktorowicz, *Anal. Biochem., 206:* 84 (1992).
79. R. Rodriguez and C. Siebert, poster presentation, 6th International Symposium on Capillary Electrophoresis, San Diego, CA, 1994.
80. J.R. Mazzeo and I.S. Krull, *J. Microcol. Sep., 4:* 29 (1992).
81. W. Thormann, J. Caslavska, S. Molteni, and J. Chmelik, *J. Chromatogr., 589:* 321 (1992).
82. J.R. Mazzeo and I.S. Krull, *Anal. Chem., 63:* 2852 (1991).
83. M. Zhu, R. Rodriguez, and T. Wehr, *J. Chromatogr., 559:* 479 (1991).
84. J.R. Mazzeo and I.S. Krull, *J. Chromatogr., 606:* 291 (1992).
85. C. Schwer, *Electrophoresis, 16:* 2121 (1995).
86. A.B. Chen, C.A. Rickel, A. Flanigan, G. Hunt, and K.G. Moorhouse, *J. Chromatogr. A, 744:* 279 (1996).
87. N. Wu, P. Sun, J.H. Aiken, T. Wang, C.W. Huie, and R. Hartwick, *J. Liq. Chromatogr., 16:* 2293 (1993).
88. J. Wu and J. Pawliszyn, *J. Chromatogr., 608:* 121 (1992).
89. J. Wu and J. Pawliszyn, *Electrophoresis, 14:* 469 (1993).
90. J. Wu and J. Pawliszyn, *Electrophoresis, 16:* 670 (1995).
91. J. Wu and J. Pawliszyn, *J. Chromatogr. B, 657:* 327 (1994).
92. L. Vonguyen, J. Wu, and J. Pawliszyn, *J. Chromatogr. B, 657:* 333 (1994).
93. J. Wu and J. Pawliszyn, *Anal. Chem., 66:* 867 (1994).
94. J. Wu and J. Pawliszyn, *Anal. Chem., 64:* 224 (1994).
95. J. Wu and J. Pawliszyn, *Anal. Chem., 64:* 219 (1992).
96. J. Wu and J. Pawliszyn, *J. Liq. Chromatogr., 16:* 3675 (1993).
97. L. Fang, R. Zhang, E.R. Williams, and R.N. Zare, *Anal. Chem., 66:* 33696 (1994).
98. F. Foret, O. Muller, J. Thorne, W. Gotzinger, and B.L. Karger, *J. Chromatogr. A, 716*: 157 (1995).
99. P.G. Righetti, *Isoelectric Focusing: Theory, Methodology, and Applications*, Elsevier Biomedical Press, Amsterdam, 1989.
100. T. Rabilloud, *Electrophoresis, 17:* 813 (1997).
101. K. Slais and Z. Fiedl, *J. Chromatogr. A, 661:* 249 (1994).
102. R. Rodríguez-Díaz, M. Zhu, and T. Wehr, *J. Chromatogr. A, 772:* 145 (1997).
103. R. Rodríguez-Díaz, M. Zhu, V. Levi, and T. Wehr, presented at the 7th Symposium on Capillary Electrophoresis, Würzburg, Germany (1995).
104. F. Kilar and S. Hjerten, *Electrophoresis, 10:* 23 (1989).
105. K.W. Yim, *J. Chromatogr., 559:* 401 (1991).
106. T. Wehr, M. Zhu, R. Rodriguez, D. Burke, and K. Duncan, *Am. Biotech. Lab.,* September 1990.
107. G.L. Klein and C.R. Joliff, in J.P. Landers (Ed.), *Handbook of Capillary Electrophoresis*, CRC Press, Boca Raton, FL, 1993, p. 452.

108. P. Ferranti, A. Malorni, P. Pucci, S. Fanali, A. Nardi, and L. Ossicini, *Anal. Biochem., 194:* 1 (1991).
109. J.W. Drysdale, P.G. Righetti, and H.F. Bunn, *Biochem. Biophys. Acta, 229:* 42 (1971).
110. S. Kundu and C. Fenters, *J. Cap. Elec., 2:* 6 (1995).
111. O-S. Reif and R. Freitag, *J. Chromatogr. A, 680:* 383 (1994).
112. G. Hunt, K.G. Moorhouse, and A.B. Chen, *J. Chromatogr. A, 744:* 295 (1996).
113. J.M. Thorne, W.K. Goetzinger, A.B. Chen, K.G. Moorhouse, and B.L. Karger, *J. Chromatogr. A, 744:* 155 (1996).
114. J. Kubach and R. Grimm, *J. Chromatogr. A, 737:* 281 (1996).
115. K.G. Moorhouse, C.A. Eusebio, G. Hunt, and A.B. Chen, *J. Chromatogr. A, 717:* 61 (1995).
116. K. Shimura and B.L. Karger, *Anal. Chem., 66:* 9 (1994).
117. D. Wu and F. Regnier, *J. Chromatogr., 608:* 349 (1992).
118. K. Tsuji, *J. Chromatogr. A, 652:* 139 (1993).
119. A. Guttman, *Electrophoresis, 16:* 611 (1995).
120. K. Tsuji, *J. Chromatogr. B, 662:* 291 (1994).
121. A.S. Cohen and B.L. Karger, *J. Chromatogr., 397:* 409 (1987).
122. S. Hjerten, *Electrophoresis '83*, H. Hirai (Ed.), Walter de Gruyter, New York, 1994, pp. 71–79.
123. M. Zhu, D.L. Hansen, S. Burd, and F. Gannon, *J. Chromatogr., 480:* 311 (1989).
124. S. Hjerten, T. Srichaiyo, and A. Palm, *Biomed. Chromatogr., 8:* 73 (1994).
125. M. Zhu, V. Levi, and T. Wehr, *Am. Biotech. Lab., 11:* 26 (1993).
126. C. Tanford, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, 2nd ed., John Wiley & Sons, New York, 1980, pp. 159–164.
127. M.R. Karim, S. Shinagawa, and T. Takagi, *Electrophoresis, 14:* 1141 (1994).
128. K. Sasa and K. Taked, *J. Colloid Interface Sci., 147:* 516 (1993).
129. K. Benedek and S. Thiede, *J. Chromatogr. A, 676:* 209 (1994).
130. P.C.H. Shieh, D. Hoang, A. Guttman, and N. Cooke, *J. Chromatogr. A, 676:* 219 (1994).
131. M. Nakatani, A. Shibukawa, and T. Nakagawa, *Biol. Pharm. Bull., 16:* 1185 (1993).
132. M. Nakatani, A. Shibukawa, and T. Nakagawa, *Anal. Sci., 10:* 1 (1994).
133. M. Nakatani, A. Shibukawa, and T. Nakagawa, *J. Chromatogr. A, 672:* 213 (1994).
134. W.E. Werner, D.M. Demorest, J. Stevens, and J.E. Wiktorowicz, *Anal. Biochem., 212:* 253 (1993).
135. A. Guttman, *Electrophoresis, 17:* 1333 (1996).
136. K. Ganzler, K.S. Greve, A.S. Cohen, B.L. Karger, A. Guttman, and N.C. Cooke, *Anal. Chem., 64:* 2665 (1992).
137. A. Guttman, P. Shieh, D. Hoang, J. Horvath, and N. Cooke, *Electrophoresis, 15:* 221 (1994).
138. K. Tsuji, *J. Chromatogr. A, 661:* 257 (1994).
139. E. Simo-Alfonso, M. Conti, C. Gelfi, and P.G. Righetti, *J. Chromatogr. A, 689:* 85 (1995).
140. A. Guttman, J. Horvath, and N. Cooke, *Anal. Chem., 65:* 199 (1993).
141. K. Benedek and S. Thiede, *J. Chromatogr. A, 676:* 209 (1994).
142. W.E. Werner, D.M. Demorest, and J.E. Wiktorowicz, *Electrophoresis, 14:* 759 (1993).
143. A. Guttman, P. Shieh, J. Lindahl, and N. Cooke, *J. Chromatogr. A, 676:* 227 (1994).
144. G. Hunt and W. Nashabeh, *Anal. Chem., 71:* 2390 (1999).

145. E.L. Gump and C.A. Monning, *J. Chromatogr. A, 715:* 167, (1995).
146. E.T. Wise, S. Navjot, and B.L. Hogan, *J. Chromatogr. A, 746:* 109 (1996).
147. S. Kundu, C. Fenters, M. Lopez, A. Varma, J. Brackett, S. Kuemmerle, and J.C. Hunt, *J. Cap. Elec., 4:* 7 (1997).
148. L. Song, Q. Ou, and W. Yu, *J. Liq. Chromatogr., 17:* 1953 (1994).
149. N. Cohen and E. Grushka, *J. Chromatogr. A, 678:* 167 (1994).
150. D. Corradini, A. Rhomberg, and C. Corradini, *J. Chromatogr. A, 661:* 305 (1994).
151. D. Corradini, G. Cannarsa, E. Fabbri, and C. Corradini, *J. Chromatogr. A, 709:* 127 (1995).
152. J.P. Landers, R.P. Oda, B.J. Madden, and T.C. Spelsberg, *Anal. Biochem., 205:* 115 (1992).
153. R.P. Oda, B.J. Madden, T.C. Spelsberg, and J.P. Landers, *J. Chromatogr. A, 680:* 85 (1994).
154. M.M. Bushey and J.W. Jorgenson, *J. Chromatogr., 480:* 301 (1989).
155. A. Emmer, M. Jansson, and J. Roeraade, *J. Chromatogr., 547:* 544 (1991).
156. A. Emmer and J. Roeraade, *J. Liq. Chromatogr., 17:* 3831 (1994).
157. A. Emmer, M. Jansson, and J. Roerrade, *J. High Res. Chromatogr. 14:* 778 (1991).
158. E.L. Hult, A. Emmer, and J. Roeraade, *J. Chromatogr. A, 757:* 255 (1997).
159. V. Rohlicek and Z. Deyl, *J. Chromatogr., 494:* 87 (1989).
160. M.J. Gordon, K.-J. Lee, A.A. Arias, and R.N. Zare, *Anal. Chem., 63:* 69 (1991).
161. W.G.H.M. Muijselaar, C.H.M.M. de Bruijn, and F.M. Everaerts, *J. Chromatogr., 605:* 115 (1992).
162. G. Mandrup, *J. Chromatogr., 604:* 267 (1992).
163. B.Y. Gong and J.W. Ho, *Electrophoresis 18:* 732 (1997).
164. M. Taverna, A. Baillet, D. Biou, M. Schlüter, R. Werner, and D. Ferrier, *Electrophoresis, 13:* 359 (1994).
165. M.E. Legaz and M.M. Pedrosa, *J. Chromatogr. A, 719:* 159 (1996).
166. H.G. Lee and D.M. Desiderio, *J. Chromatogr. B, 691:* 67 (1997).
167. N. Guzman, J. Moschera, K. Iqbal, and A.W. Malick, *J. Chromatogr., 608:* 197 (1992).
168. X.-H. Fang, T. Zhu, and V.-H. Sun, *J. High Res. Chromatogr., 17:* 749 (1994).
169. M.A. Strege and A.L. Lagu, *J. Liquid Chromatogr., 16:* 51 (1993).
170. M.A. Strege and A.L. Lagu, *J. Chromatogr., 630:* 337 (1993).
171. A. Cifuentes, M.A. Rodriguez, and F.G. García-Montelongo, *J. Chromatogr. A, 742:* 257 (1996).
172. Y.J. Yao and S.F.Y. Li., *J. Chromatogr. A, 663:* 97 (1994).
173. N. Cohen and E. Grushka, *J. Cap. Elec., 1:* 112 (1994).
174. M. Morand, D. Blaas, and E. Kenndler, *J. Chromatogr. B, 691:* 192 (1997).
175. G.N. Okafo, H.C. Birrell, M. Greenaway, M. Haran, and P. Camilleri, *Anal. Biochem., 219:* 201 (1994).
176. G.N. Okafo, A. Vinther, T. Kornfelt, and P. Camilleri, *Electrophoresis, 16:* 1917 (1995).
177. J.R. Veraart, Y. Schouten, C. Gooijer, and H. Lingeman, *J. Chromatogr. A, 768:* 307 (1997).
178. A. Cifuentes, J.M. Santos, M. de Frutos, and J.C. Diez-Mesa, *J. Chromatogr. A, 652:* 161 (1993).
179. T. Wehr, R. Rodriguez-Diaz, and M. Zhu, Chromatographic Science Series Vol. 80, Marcel Dekker, 1999.