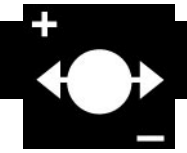
PROTEINS



Capillary Electrophoresis

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Electrophoresis, mostly in the slab gel format, and high performance liquid chromatography (HPLC) are the two techniques commonly employed for protein separation during the past decades. After emerging in the early 1980s, capillary electrophoresis (CE) has been recognized as a new tool for protein analysis and characterization: it combines a number of aspects of both electrophoresis and HPLC. Based on differences in charge-to-size ratio or isoelectric point (pI) of protein macroions, the separations in CE are inherently electrophoretic. However, online detection producing quantitative information in the form of peak area or height, single sample analysis in a serial fashion, and the possibility of performing separation in the presence of flow (generated by electroosmotic current) are features of CE similar to those of HPLC. These features lend CE to easy automation, in contrast to the labour-intensive methods of conventional (gel) electrophoresis. Since heat dissipation by convection is effectively suppressed in capillaries of less than 0.2 mm i.d., the separation can be performed in free solutions without a gel. The high heat dissipation properties of thin fused silica capillaries also enable one to apply field strengths of several hundred volts per centimetre, thus greatly reducing the time of separation.

To date, CE techniques analogous to a number of conventional electrophoretic methods such as zone electrophoresis, isoelectric focusing and sieving (size-dependent) separations have been developed and numerous applications of CE to separation and characterization of proteins have been demonstrated. In the particular mode of CE as capillary zone electrophoresis, short separation times (often a few minutes) combined with relatively large diffusion constants of proteins were expected to provide separation efficiences exceeding a million theoretical plates. However, such potential efficiency has never been practically achieved, and this mostly appears to be due to the interactions of proteins with the capillary walls.

The approaches to reducing protein interactions with the capillary walls and the modes of CE applied to protein analysis are briefly considered below.

Approaches to Reducing Protein–Silica Surface Interactions

The high surface activity of fused silica at neutral pH, combined with the high surface-to-volume ratio of thin capillaries is a major problem in applying CE to protein separation. In general, the protein–silica surface interactions give rise to peak broadening and asymmetry, compromising the resolution. In bare fused silica capillaries, these interactions often result in uncontrolled alterations of the electroosmotic flow (EOF) and irreproducible migration times, low mass recovery of proteins or even their irreversible adsorption with loss of sample. Over the last 10 years, great efforts have been made to develop conditions for protein analysis under which the protein–capillary wall interactions are minimized and the EOF is either suppressed or stabilized.

In the pH range of 3–10, the charge density of the capillary inner surface is known to increase progressively due to the ionization of weakly acidic silanol groups. The charge density on the wall is near zero at pH < 3 (silanol groups become fully protonated) and saturated above pH 10 (silanol groups are fully dissociated). Thus, protein species possessing a pI higher than the pH of the electrophoretic buffer will experience an electrostatic attraction to the negatively charged silanols. Beside silanol groups, fused silica bears a variety of other active sites such as inert siloxane bridges and hydrogen-bonding sites. These active sites can join in the protein immobilization on the inner surface of the capillary, by interacting with the hydrogen-bonding and hydrophobic regions of the protein.

The electrostatic attraction between protein molecules with a net positive charge at a given pH and ionized silica is believed to play a key role in the protein–capillary wall interactions. Thus, operating at extremes of pH (at pH < 3 where silanol ionization is very low or at pH > 11 where proteins carry a net negative charge) appears to be the simplest approach to their minimization. Though such an approach has been demonstrated to be successful in a number of applications, operation at pH extremes in general reduces charge diversity, diminishing the separation selectivity. The pH extremes also tend to

denature proteins and induce formation of multiple conformers. The electrostatic attraction between proteins and the silica surface may be reduced by increasing the ionic strength of electrolyte solutions (100 mmol L^{-1} or greater). However, the high ionic strength limits the applied voltage, consequently decreasing efficiency and increasing the analysis time. Deactivation of silanol groups may also be achieved by derivatizing them with organosilanes but the carbon moieties of organosilanes make the capillary wall highly hydrophobic.

Two approaches appear to be the most successful in rendering CE suitable for routine protein separations: first, incorporation of appropriate additives into the electrolyte solution to mask or compete for either the silanol groups or the basic amino acid residues of the protein which are exposed to the solution; and second, use of capillaries with an inner suface modified by an adsorbed or covalently attached polymeric coating. A large variety of chemicals and modification procedures currently exist which can effectively reduce protein–wall interactions and control the EOF so that a separation efficiency of several hundred thousands of theoretical plates has become practically achievable. Several types of coated capillaries are commercially available, and are described below.

The incorporation of buffer additives permitting successful protein separations in bare fused silica capillaries has the advantage of simplicity. Ideally, the buffer additive should not compromise the selectivity of separation by interacting with the analyte, alter the buffer pH or increase the operating current, and should in general exhibit low UV absorbance. Organic compounds of different kinds have been extensively examined as the buffer additives. Since mostly they interact with the silica surface in a dynamic fashion, the method of modifying the capillary walls by using buffer additives is known as dynamic coating.

A large database of organic compunds and buffer components suitable for improving CE performance in protein separations has been established. This database includes zwitterionic salts (methylglycine and trimethylglycine, trimethylammoniumpropyl and butyl sulfonates), an extensive number of mono-, di- and polyamines, surfactants (ionic and zwitterionic fluorosurfactants as well as nonionic surfactants of the Brij and Tween series) and neutral polymers (cellulose derivatives, dextran, polyvinyl alcohol, polyethylene glycol). However, the effectiveness of dynamic coatings is mostly evaluated with standard mixtures containing a small number of proteins or with variants of a single protein. Therefore, it is not possible to predict how a particular additive will act in conjunction with complex biological samples. Such samples may consist of a broad spectrum of proteins ranging widely in their degree of hydrophobicity, pI values and molecular weight. Despite this limitation, the present database of buffer additives known to improve protein separations may be very useful in developing methods for a targeted component analysis like the purity control of recombinant proteins, food analysis, or electrophoretic analysis of haemoglobins, serum or urine proteins.

Though the main mechanism by which the buffer additives improve protein separation appears to be their interaction with the silica surface, they can play an additional role – binding to the protein. In a number of cases, the additives have been shown to modulate selectivity by enhancing differences in electrophoretic mobility (e.g. some surfactants upon complexation to proteins, or alkyl diamines and their derivatives upon binding to protein glycoforms).

The incorporation of diaminealkanes, polyamines and fluorinated cationic and zwitterionic surfactants in the electrophoretic buffer effectively controls both the magnitude and direction of EOF.

Adsorbed coating differs from dynamic coating by the degree of permanence, but the demarcation line between them is arbitrary. In the case of an adsorbed coating, the coating agent should not be present in the electrophoretic buffer during a run. As a rule, polymeric species are used for adsorbed coating. Permanence can result from the high binding affinity of the coating agent to the silica surface (polymeric amines, polyethylene oxide) and may be enhanced by subsequently cross-linking the adsorbed species into a continuous, permanent film (e.g. polyethyleneimines treated with diepoxide after adsorption to capillary walls). The permanence can also result from the ability of the coating agent to form, upon a particular treatment, polymeric films physically covering the silica surface (cellulose acetate and polyvinyl alcohol films are examples). The polymers may be adsorbed not directly to the silica surface but to a hydrophobic layer formed by moieties of a surfactant covalently attached to the capillary inner walls (hybrid coating). Hybrid coating appears to be the most flexible, since the polymeric layer can easily be removed by rinsing the capillary with an organic solvent and polymeric species of other types may be adsorbed, depending on the separation goal. An example of protein separation in a capillary with the hybrid coating is presented in **Figure 1**.

The covalently attached polymeric coating is usually carried out by grafting polymers to a silica surface derivatized with organosilanes. In the subsequent step, polymer chains may be cross-linked to help stabilize the coating. Several neutral (cellulose

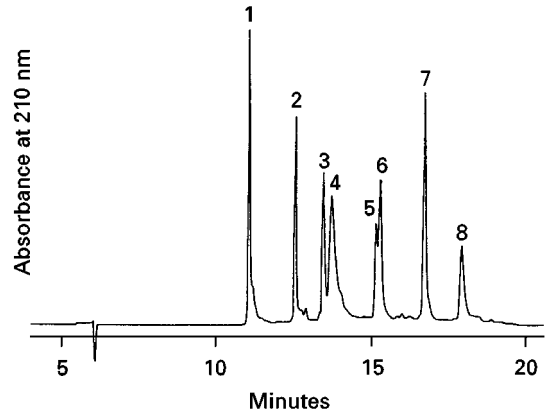

Figure 1 Electropherogram of some acidic and basic proteins in a capillary with hybrid coating (after derivatizing with the organosilane, the capillary was coated with epoxybutane-modified hydroxypropylcellulose). Other conditions: 0.05 mol L^{-1} NaH_2PO_4, pH 3.0; detection at 210 nm, + 21 kV, 30 mA. Capillary total length, 85 cm; effective length, 50 cm, 50 μm i.d. Peak assignment: 1, cytochrome *c*, pI 10.2; 2, lysozyme, pI 11.0; 3, β-lactoglobulin A, pI 5.1; 4, conalbumin, pI 6.0; 5, haemoglobin, pI 5.6; ribonuclease A, pI 9.3; 7, α-chymotrypsinogen A, pI 9.2; 8, trypsin inhibitor, pI 4.2. Reproduced with permission from Yang C and El Rassi Z (1998) Capillary zone electrophoresis of proteins with fused-silica capillaries having polymers and surfactants adsorbed onto surfactant moieties previously covalently bound to the capillary column surface. *Electrophoresis* 19: 2278–84. Copyright: Wiley-VCM.

derivatives, epoxy polymers, dextran) and cationic (polyvinylimidazole, polyethyleneimine derivatives) polymers have been employed for the covalently attached coating. The most popular polymer used for the polymeric coating is polyacrylamide. This coating is mostly performed with polyacrylamide chains polymerized *in situ*.

It should be noted that, despite the variety of materials and procedures used to prepare the coated capillaries, they do not appear to vary markedly in separation properties. The diversity of chemistries underlying the capillary coating is likely to reflect the continuous search for a 'magic' coating and inadequacy of any single approach to provide satisfactory results for all applications. A particular problem arises due to difficulties in optimizing the coating process. The quality of coating appears to depend on the quality of the fused silica surface, which may vary between different sources of capillaries and even between different batches of silica; this requires a corresponding adjustment in capillary pretreatment and coupling chemistries.

Modes of Capillary Electrophoresis Applied to Protein Analysis

Capillary zone electrophoresis (CZE), capillary isoelectric focusing and sieving capillary electrophoresis in polymeric media are the modes of CE most widely used for separating and characterizing proteins. A number of commercial kits for capillary isoelectric focusing and sieving separations are now available. Capillary isotachophoresis has in rare cases been employed for protein analysis. Micellar electrokinetic chromatography has generally exhibited a poor selectivity in separating proteins. This is probably due to the inability of relatively large protein molecules to partition into the detergent micelle.

Capillary Zone Electrophoresis

CZE is the simplest of the CE modes and straightforward to perform (Figure 1 depicts a typical example of a CZE separation). When employing CZE for protein separation, the choice of capillary (uncoated or with the particular type of coating) and buffer additives should be made carefully depending on sample composition. The uncoated capillaries generally require a prior conditioning step. Detection based on either UV adsorption or laser-induced fluorescence (LIF) is most often employed in the CZE of proteins. Depending on the detection mode, a sample pretreatment may be necessary.

The sensitivity of the detection by UV absorbance is limited since both the optical length (= capillary internal diameter) and sample volumes (typically, a few nanolitres) are very small in CZE. Though the sensitivity can be greatly increased by detecting proteins in the wavelength range of 200–220 nm, UV detection still requires a relatively high concentration of analyte in a sample. That is not always the case and a preconcentration of the sample, often of a volume of a few microlitres, is needed. Several online and offline preconcentration techniques can be employed in CZE. The first and simplest approach to online sample preconcentration is zone sharpening by stacking. Proteins dissolved in a buffer with a conductivity lower than that of the run buffer (commonly, the diluted run buffer) become concentrated at the interface between the sample and the run buffer due to a high voltage drop in the sample zone. Preliminary sample desalting is often necessary for this approach and special methods have been developed for desalting (and concentrating) microlitre volumes of protein samples, using small pore polyacrylamide gels.

Isotachophoresis is the other popular technique to concentrate samples. The preconcentration may be performed either online or in a coupled column, and in the presence of salts. The gain in detection limit is 10- to 100-fold and can be increased up to 1000-fold when a hydrodynamic counterflow is employed. Another efficient method of protein preconcentration is selective accumulation of the proteins on

a solid-phase affinity support. This method has been used in both online and offline modes, with several hundred-fold concentration.

After derivatization with a fluorophore, proteins may be detected online by LIF. A number of fluorescent dyes capable of covalently binding to protein molecules (e.g. fluorescein, naphthalenedicarboxaldehyde and fluorescamine) have been used, providing mass detection limits in the attomole range (initial sample concentrations of 10^{-8} to 10^{-10} mol L^{-1}). However, covalent binding of the dyes frequently results in a broadening of protein peaks or even in the formation of multiple peaks due to multiple derivatization.

Capillary Isoelectric Focusing

Like conventional isoelectric focusing (IEF), capillary isoelectric focusing (cIEF) is based on differences in isoelectric points (pIs) of proteins. In cIEF, a stabilizing gel is not required and, due to the high field strength, the focusing process usually takes only 5–15 min. The cIEF can provide resolution of up to 0.01 pH units, comparable with that of the most successful applications of conventional IEF.

Sensitivity of detection based on UV absorbance (at 280 nm) is generally satisfactory for cIEF, due to the concentration of proteins from a relatively large injected volume into a small volume of the focused zone. Capillaries with a hydrolytically stable coating effectively preventing protein adsorption and changes in the EOF are required for successful cIEF separations. As in conventional IEF, protein precipitation due to the high protein concentration at the isoelectric point is a potential problem in cIEF and is addressed in the same way: using strong solubilizing agents, such as urea and nonionic detergents, in the ampholyte mixture.

Size-dependent Separation of Proteins by Capillary Electrophoresis

Although the use of narrow bore capillaries abolishes the need for gel media to suppress convection, another important feature of gels – their capability to provide size-dependent separation of macromolecules – is clearly beneficial for protein analysis. Efforts to adopt gels to the capillary format were made in the early days of CE. However, technical difficulties, such as bubble formation and the fast deterioration of polyacrylamide gels during serial runs, limit the use of gel-filled capillaries. These difficulties have been overcome by using replaceable sieving media such as solutions of entangled polymers. While gels are polymerized *in situ*, polymer solutions are usually prepared by dissolving commercially available polymers in the run buffer and are pumped into the capillary before each run. Solutions of dextran, polyethylene oxide, polyvinyl alcohol and linear polyacrylamide have been demonstrated to be suitable for protein analysis. Though the use of coated capillaries is generally recommended, uncoated capillaries may also be employed if a polymer solution produces sufficient viscosity (typically >100 cP). The main drawback of polymer solutions is that resolution is not as high as that obtained with gel-filled capillaries.

Size-dependent separation by CE of protein–sodium dodecyl sulfate (SDS) complexes provides information similar to that obtained from conventional SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as illustrated in **Figure 2**. The limits of UV detection are comparable to those obtained in SDS-PAGE with Coomassie blue staining, whereas total analysis time for multiple samples is even shorter for CE than that for, e.g. a 16-channel slab gel. Size-based analysis by CE in sieving media under native conditions has been demonstrated for proteins. Usually, such analysis employs constructing a Ferguson plot (the logarithm of protein mobility vs. polymer (gel) concentration). Such construction is extremely time-consuming in traditional PAGE but becomes practical by using CE in replaceable sieving media. The Ferguson plot-based analysis may also be useful in estimating the molecular weight of proteins whose binding with SDS is anomalous (e.g. membrane proteins, glycoproteins, highly basic proteins) and that of aggregates and complexes of proteins.

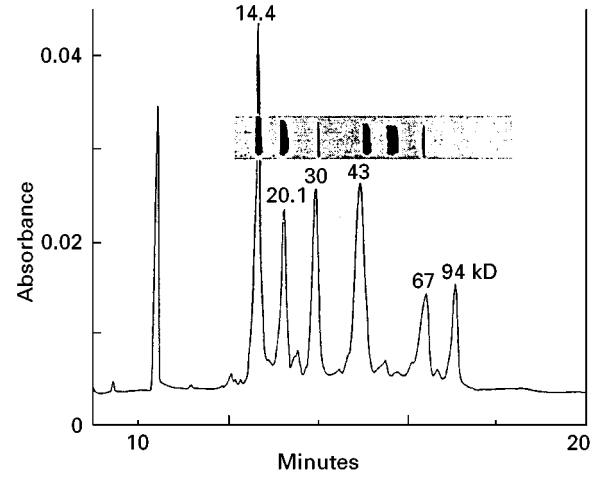

Figure 2 Capillary electrophoresis sieving separation of a standard SDS–protein mixture. Sieving matrix, 3% solution of polyethylene oxide. Inset shows the SDS-PAGE trace of the same mixture. Numbers above the peaks correspond to protein molecular weight. Buffer: 100 mmol L^{-1} Tris-CHES, pH 8.8, 0.1% SDS. Condition: 300 V cm^{-1}, 20°C. Detection at 214 nm. Reproduced with permission from Guttman A (1996) Capillary sodium dodecyl sulfate-gel electrophoresis of proteins. *Electrophoresis* 17: 1333–41. Copyright: Wiley-VCM.

Protein Characterization by Capillary Electrophoresis

In addition to the targeted component analysis and sample profiling, CE has been employed in a number of specific electrophoresis-based approaches aiming at the characterization of protein–ligand interactions, protein functional activity and structure.

Affinity capillary electrophoresis (ACE) has been applied to the study of protein interactions with drugs, carbohydrates, nucleic acids and other proteins. In a typical ACE experiment, the receptor is subjected to electrophoresis in a capillary containing free ligand at different concentrations. The receptor–ligand binding (K_{on}) and dissociation (K_{off}) constants are estimated by Scatchard analysis of shifts in the receptor's mobility in response to ligand concentrations. The analysis of peak broadening has been shown to be useful for estimating K_{on} and K_{off} constants, if the characteristic times of receptor–ligand interactions are comparable with migration time of the analyte. The receptor–ligand complex equilibrium and separation process can be affected by capillary wall effects and/or the use of buffer additives. These limitations must be addressed when developing an ACE method.

The potential of CE in analysing antibody–antigen complexes has been extensively studied over the last few years in order to develop CE-based immunoassays (IA). The CE-based IA offers advantages of high speed of a single analysis, detection of antigen at trace concentrations (10^{-10} mol L^{-1} if the LIF detection and fluorescently derivatized antibodies are employed) and the potential for automation. Despite some successful examples, CE has generally exhibited the inability to perform direct IA due to the lack of separation between bound and free antibodies. Though this drawback has been overcome in the competitive CE-based IA, the sensitivity of the competitive IA does not meet the detection levels required for many important clinical tests.

A CZE-based approach to microassaying enzyme activity has recently been developed. In this 'in-tube' approach, the enzyme and substrate are electrophoretically mixed inside the capillary under conditions where their mobilities differ. Another new application of CZE to protein characterization is studying protein folding/unfolding transitions. Due to recent advances in capillary coating and the inherent ability to perform electrophoresis in short time intervals, CZE appears capable of reliably distinguishing different forms of protein conformations, providing a new instrumental approach to the quantitative analysis of the conformational equilibrium of proteins.

Coupling Capillary Electrophoresis to Other Techniques for Protein Analysis

Several multidimensional separation systems for protein analysis, incorporating CE, have been proposed over the last decade. Two-dimensional (2-D) techniques such as CE-CE (using CE separations with two different carrier systems), HPLC-CZE, size exclusion chromatography (SEC)-CZE, and even a 3-D technique combining SEC-HPLC-CZE have been reported. The multidimensional systems possess tremendous resolving power and may be extremely useful in analysing complex biological samples, but there are drawbacks. Separation times are generally long and can last 2–12 h. Beside the technical difficulties of interfacing different separation systems, the compatibility of mobile-phase and run buffers as well as maintaining the detection sensitivity adequate for trace analysis in the sequential separations are the most significant problems.

By coupling CE with mass spectrometry (MS), the molecular weight of separated proteins can easily be determined. It is also possible, using MS, to detect proteolysis and deamidation, find glycosylation variants, and, with peptide mapping, confirm the primary structure of proteins. Though online interfacing of CE with MS has progressed substantially in recent years, the successful online combination of CE and MS is still a challenging instrumental problem. In addition to the interface design issue, practical considerations concerning the compatibility between the run buffer and the sheath liquid as well as differences in their flow rates at the interface must be addressed when dealing with CE-MS coupling.

Conclusions

After an initial period of fast technical progress, over the last decade CE has been increasingly focused on developing practical methods for protein separation and characterization. To date, due to advances in capillary coating, CE may be viewed as a practical tool for rapid, sensitive and quantitative analysis of minute amounts of protein samples, with great utility in targeted component analysis. For many applications, it can replace HPLC and conventional gel electrophoresis but more often it should be used in conjunction with other separation techniques, providing different selectivity or automated analysis. Since the acceptance of CE in the clinical laboratory for routine protein-based diagnostics depends considerably on meeting adequate throughput, attempts to develop high throughput CE systems are likely to be intensified. With a decrease in the real cost and improvement in sensitivity and resolving power of

MS detection, the increasing use of a combined CE-MS technique can be expected.

To be more widely accepted in the area of biomedical research, CE-based protein separations must demonstrate a number of features that match the success of conventional (gel) electrophoretic systems. Besides profiling complex protein samples, these systems allow for immunological and enzymatic assaying of separated proteins as well as for simultaneous transfer of sample components into another separation dimension without altering the separation in the first one. All of these are achieved with minimal disturbance of zone integrity. Thus, the major efforts will probably be made in developing both multidimensional separation systems involving CE and CE-based separation systems permitting post- or on-column enzymatic and immunological analysis of the separated components of complex biological samples. Incorporating immobilized enzymes or antibodies into CE-MS systems will revolutionize the analysis of protein structure and, especially, glycoprotein analysis.

Further Reading

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Centrifugation

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Introduction

Modern technological developments have made centrifugation one of the most important and widely applied techniques in experimental research. In biological studies centrifugation is used for the extraction and isolation of biological materials and for the measurement of physical properties of macromolecules. Indeed, biological materials have been extracted and isolated for more than a thousand years using centrifugal forces. In the 1920s, Svedberg and other researchers developed motor-driven centrifuges which had an optical system to observe sedimentation of macromolecules during centrifugation, and used these instruments for the measurement of physical properties of macromolecules, especially proteins. The molecular mass of most proteins was determined using these analytical centrifuges until 1970, but they have not been recently used for that purpose because much easier methods for the measurement of molecular mass, such as size exclusion chromatography and sodium dodecyl sulfate (SDS)-gel electrophoresis, have been developed. More recently, centrifugation has become an indispensable tool for the isolation of proteins, nucleic acids and subcellular particles. The use of centrifuges has also been revived for the measurement of physical properties of proteins, especially for the characterization of protein associations and protein–protein interactions. In this section, important points of theory and practice for the separation and isolation of proteins by centrifugation are summarized.

Theoretical Basis of Centrifugation

Although a rigorous understanding of sedimentation theory is not required for the separation and isolation of proteins, a review of some basic principles will be helpful for understanding the establishment of conditions and the interpretation of experimental results obtained. Because of their random thermal motion,