Ultra-High Resolution Separation Comes of Age

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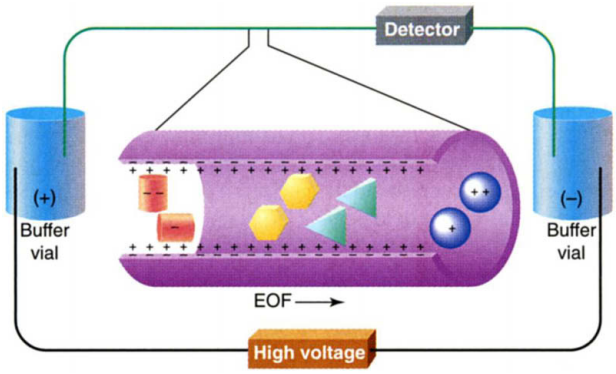
Born in the late 1960s (*1*), popularized in its infancy for its high resolution (*2*), and reaching puberty in the mid 1980s with the introduction of commercial instrumentation (*3*), capillary electrophoresis (CE) has finally entered adulthood as a reliable and widely useful analytical technology.

In 1937, Nobel Prize–winner Arne Tiselius first described electrophoresis, a method for separating charged molecules in an externally applied electric field. With the introduction of agarose and polyacrylamide gels, electrophoresis evolved as the principal means of high-resolution biopolymer (e.g., DNA, protein, and glycan) analysis. Gel electrophoresis, however, remains a qualitative, time-consuming, and labor-intensive analytical method. As we enter the new millennium, we have begun to realize the potential of CE for the automated and rapid analysis of complex mixtures with high resolution and high sensitivity.

The Method

In CE, a sample is separated into its components as it migrates through a capillary under the driving force of an electric field. Separation is typically performed in a long (10 to 100 cm), narrow (10 to 100 μm), electrolyte-filled, fused silica capillary across which a current is applied (see figure, right). The charge on the inner wall of the capillary causes the flow of electrolyte from the anode (where the sample is applied) to the cathode (where the sample is analyzed). The flow of electrolyte through the capillary is called electro-osmotic flow (EOF), and it drives positively charged, neutral, and negatively charged analytes through the capillary to the detector. A second force, electrophoresis, which drives cations toward the cathode and anions toward the anode, enhances EOF for cations and opposes EOF for anions, resulting in their further separation. Adjusting the pH of the electrolyte can control the balance between the EOF and the force associated with electrophoresis. At pH above 5, the silanol groups on the inner wall of the silica capillary are ionized, giving the wall a negative charge and resulting in an EOF. At pH below 5, the capillary wall loses its charge, causing the EOF to disappear and making electrophoresis the dominant force.



CZE separation in a CE system. A open-ended capillary (purple) extends between two buffer reservoirs, across which a high voltage is applied. This voltage causes analytes (red, yellow, green, and blue shapes) to migrate from the site of sample application at the cathode (+) buffer through a detector to the anode (-). The EOF that results from wall-pumping drives the separation of analytes. Electrophoresis speeds the migration of cations and retards the migration of anions through the capillary.

CE is best viewed from front-end (sample preparation) to back-end (data collection and processing) as the sum of its component parts. Analytes, particularly biological samples, often require cleanup and concentration before electrical, capillary, or pressure injection of picoliter to nanoliter volumes into the capillary. Though typically encompassing an aqueous electrolyte, the mobile phase is open to innovations, such as the use of ionic liquids and ion-pairing agents to facilitate separations in nonaqueous media for the analysis of water-sensitive compounds. In most applications, a constant current (or constant voltage) is applied at a high voltage (~20 kV), but current gradients and ultra-high voltages can be applied to very narrow capillaries to avoid decreased resolution due to Joule heating. Separation is based on a variety of principles, from simple capillary zone electrophoresis (CZE) (see figure) to more complex methods. Micellar electrokinetic chromatography (MEKC) (*4*) uses added surfactant to partition the analyte according to phase, offering advantages in separating uncharged or hydrophobic samples. Affinity capillary electrophoresis (ACE) (*5*) uses a soluble analyte-binding partner (either a protein or molecular imprinting agent) in the mobile phase or immobilized on the capillary wall to enhance selectivity. Chiral CE (*6*) uses a chiral selector (such as a cyclodextrin) to separate enantiomers. Capillaries packed with silica have been used in capillary electrokinetic chromatography (CEC) (*7*). The capillaries can be etched to enhance their surface area, coated with polymer (*8*) or filled with gels (*9*). Coated capillaries, for example, can improve resolution in the separation of proteins. Proteins contain both positively and negatively charged functional groups. The positively charged functional groups can interact with the negatively charged silanol groups on the capillary wall, which can result in an undesirable adsorption of the protein and lead to band broadening and poor resolution. By coating the inner wall of the capillary, its negative charge can be reduced and adsorption of the protein analyte prevented. Furthermore, two-dimensional separations [e.g., high-performance liquid chromatography–CE (HPLC-CE)] can greatly enhance separation quality and improve the resolution of complex mixtures, such as those encountered in proteomics.

Newer approaches have relied on ultra-thin plates, etched glass microchips, or plastic microchannels (*10*) promising massively parallel CE for high-throughput applications. Separation of biological molecules within channels on the surface of microchips with the use of microfluidics has attracted substantial attention recently. Microchips offer a number of advantages including rapid analysis, portability, ability to perform multiple analyses, and compatibility with integration allowing development of sophisticated microanalytical systems. In microchip CE, buffer is forced through the channels etched into a glass microchip by EOF, eliminating the need for pumps. Most commercially available instruments rely on ultraviolet (UV) detection of the resolved components, but laser-induced fluo-

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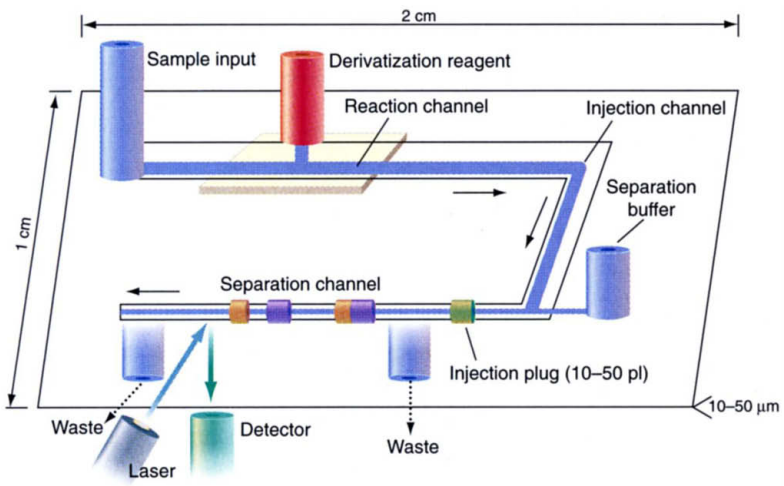

Microchip incorporating a CE separation. Sample is put into the chip (blue chamber) and "derivatized" in a reaction channel to enable LIF detection after fractionation in a separation channel, where the analyte mixture (green) is separated into its component parts (purple and orange).

rescence (LIF) detection is becoming increasingly popular, on the basis of its high (zeptomole) sensitivity (*11*). The high inherent sensitivity of fluorescence detection is enhanced further by confining the sample in the very small volume of a narrow capillary. Derivatization before column, on the column, or after separation (*12*) and indirect detection (using a fluorescent mobile phase additive) are popular ways of enabling LIF detection. Other detectors under intensive development include electrochemical or conductivity (*13*), refractive index, electrospray ionization–mass spectroscopy (ESI-MS) (*14*), nuclear magnetic resonance (NMR), biosensors, chemo- or bioluminescence, and protein or DNA chip array detectors. For example, the high sample concentrations required for NMR analysis are typically found inside the narrow interior of the CE capillary, and deuterium oxide can replace water in the electrolyte without adversely affecting resolution. Electrochemical or conductivity detection is particularly well suited for detection on microchips. Sophisticated data collection and data processing and computer control facilitate the integration of CE with other analytical, chemical, and/or biochemical steps to facilitate development of lab-on-a-chip technologies (*15*).

Applications

The hallmarks of CE are its high resolution, high sensitivity, and rapid, automatable analysis. CE requires only a fraction (1 to 10%) of the amount of sample and time needed by other microanalytical methods, such as capillary HPLC. High-resolution, automated, parallel CE has played an important role in genomics, gene sequencing (*16*), and the diagnosis of genetic disorders (*17*). Proteomics and protein or peptide sequencing (*18*), combining CE separation with ESI-MS detection, hold great promise. A split-flow CE–ESI-MS interface has been introduced in which the electrical connection to the CE capillary outlet is achieved by diverting part of the CE buffer from the capillary through an opening near the capillary outlet. The CE buffer exiting the opening contacts a metal tube sheath that acts as the CE outlet and ESI shared electrode. In cases in which the ESI source uses a metal needle, the voltage contact to the CE buffer is achieved by simply inserting the outlet of the CE capillary into the existing ESI needle, thereby greatly simplifying such an interface. There are no "dead volume" or bubble formation problems associated with this interface. Glycomics, the study of the biological activity of glycan structure at the cellular, tissue, and organism levels, requires carbohydrate sequencing and relies heavily on CE because of the highly polar nature of sugars. The pharmaceutical industry is beginning to accept CE (*19*) because problems with the precision, accuracy, reliability, and robustness of the method have been addressed. The high resolution and enantioselectivity possible with CE have offered irresistible advantages in drug analysis and pharmacokinetic studies. Analysis of combinatorial libraries based on natural products, biotechnology-derived products, and the products of chemical synthesis now routinely rely on CE (*20*). The high sensitivity of CE has led to its widespread use in the food and beverage industries and in agriculture to monitor pesticides and herbicides. In clinical chemistry and forensic toxicology (*21*), CE has been used successfully to detect trace substances in biological samples. CE-based enzyme assays and immunoassays also have a number of advantages over conventional methods. These include rapid and high-resolution separation, automation, high-sensitivity LIF detection, and the ability to simultaneously discriminate between and identify multiple components of similar structure. In biological research, it is now possible to sample single cells (*22*) and organelles or to analyze the concentrations of neurotransmitters at synapses with the use of CE (*23*).

Miniaturization through nanotechnology and the microfabrication of automated capillary arrays on microplates or integrated microchips further the promise of a lab-on-a-chip (*15*) (see figure, left). Such chips, powered by externally applied electric fields, might be implanted in the body to monitor physiological conditions (e.g., ovulation and fertility) and to control pathophysiological states (e.g., diabetes, Parkinson's disease). Chips might also be used unobtrusively, scattered through a subway station or airport, to monitor for bioterrorism agents and explosives. Single molecule detection as well as giant analytical arrays with incredible selectivity could also be possible in the future. The rapid, parallel separation of small amounts of samples on microchips will undoubtedly afford high-throughput analysis of complex, multi-component mixtures. In the analytical community, CE's maturation has generated palpable excitement.

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