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ELECTROPHORESIS TECHNIQUES

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ELECTROPHORESIS

Electrophoresis is a separation technique often applied to the analysis of biological or other polymeric samples. It has frequent application to analysis of proteins and DNA fragment mixtures and has been increasingly applied to the analysis of non-biological and non-aqueous samples. The high resolution of electrophoresis has made it an important tool in the advancement of biotechnology. Variations of this methodology are being used for DNA sequencing and fingerprinting, isolating active biological factors associated with diseases such as cystic fibrosis, sickle-cell anemia, myelomas and leukemia, and establishing immunological reactions between individual compounds. Electrophoresis is an effective analytical tool because it does not affect a molecule's structure, and it is highly sensitive to small differences in molecular charge and size.

The term electrophoresis refers to the movement of a particle through a stationary fluid under the influence of an electric field. The study of electrophoresis has included the movement of molecules, colloids, fibers, clay particles, latex spheres, bubbles; anything that forms two phases with the fluid in which the substance is suspended.

The fundamental principle behind electrophoresis is the existence of charge separation between the surface of a particle and the fluid immediately surrounding it. An applied electric field acts on the resulting charge density, causing the particle to migrate and the fluid around the particle to flow.

There are three distinct modes of electrophoresis: zone electrophoresis, isotachophoresis, and isoelectric focusing. These three methods may be used alone or in combination to separate molecules on both an analytical (μL of a mixture separated) and preparative (mL of a mixture separated) scale. Separations in these three modes are based on different physical properties of the particles in the mixture, making at least three different electrophoretic analyses possible on the same solution.

Distinction is also made among electrophoretic techniques in terms of the type of matrix employed for the analysis. Matrices include polymer gels such as agarose and polyacrylamide, paper, capillaries, and flowing buffers. Each matrix is used for different types of mixtures, and each has unique advantages.

There are a variety of techniques for detecting separated sample components including chemical stains, photographic media, and immunochemical reactions, and many instrumental techniques. Each detection technique also gives different information about the identity, quantity, and physical properties of the molecules in the mixture. Detection is often a key part of the electrophoretic analysis, and usually yields basic information about the mixture being studied.

PRINCIPLES

Electrophoresis employs an applied electric field to move charged molecules or particles through a matrix. The electric field exerts a force on the particle's charge or surface potential. This force results in a velocity for the particle that is proportional to the particle's surface potential. Two particles with different velocities will end in different locations after a fixed time in an electric field.

Theory of Electrophoretic Motion

A brief description of the origin of an electric charge at an interface, and the result of applying an external electric field, is given in this section. Considerations for the design of equipment, heating and electrolysis reactions are reviewed as well.

The Electric Double Layer

The interface between two immiscible phases can support an electric potential or charge. The potential arises because some ions preferentially associate, absorb or partition to one phase. Sometimes, one phase has a bound charge, such as the charged amino acid side chains in a protein. There is a microscopic separation of charge that exists in a thin section of the charged particle or surface, and in a thin layer of the fluid phase adjacent to the surface (see Figure 1). These two lay-

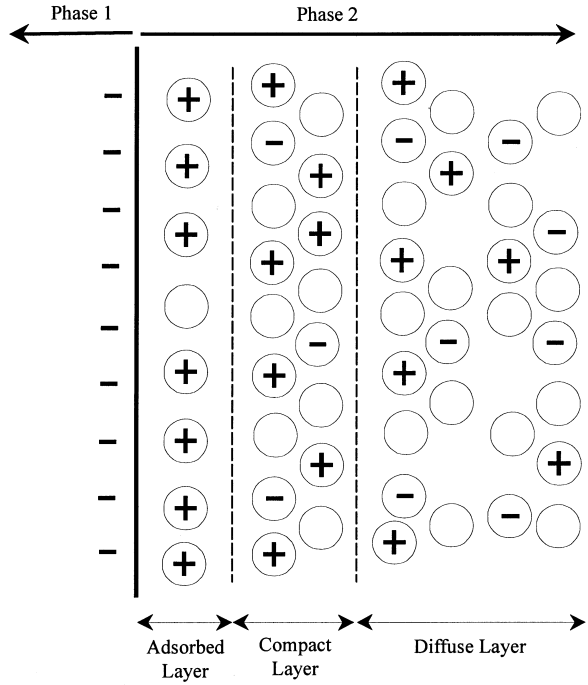

Figure 1. The formation of the electrical double layer at phase interfaces.

ers of charge are called the electric double layer. The charged layer in the fluid phase is called the diffuse layer. The electric potential in the diffuse later extends into the fluid phase, and drops off as a Poisson distribution:

$$\phi = \phi_0 e^{-\kappa x} \tag{1}$$

where ϕ_0 is the potential at the interface, mV; ϕ is the potential x cm from the interface, mV; $1/\kappa$ is the "double layer thickness", a characteristic distance from the interface into the fluid phase, cm; and x is the distance from the interface, cm. The double layer thickness is a function of the concentration and charges of the ions in solution, the temperature, and the permittivity of the solution:

$$\kappa^2 = \left(\frac{F^2 \sum_i c_i z_i}{\varepsilon \varepsilon_0 RT} \right) \tag{2}$$

where ε is the relative permittivity; ε_0 is the permittivity of free space (8.854×10^{-14} C V^{-1} cm^{-1}); z is the valency of each ion (i); c is the concentration of each

ion (i); R is the universal gas constant (8.314 J mol^{-1} K^{-1}); T is the temperature, °K, and F is Faraday's constant (9.65×10^4 C mol^{-1}). The relative permittivity is a measure of the conductance of the pure material relative to a vacuum. Pure water has a relative permittivity of about 80 (unitless).

Not all of the ions in the diffuse layer are necessarily mobile. Sometimes a distinction is made between the location of the true interface, an intermediate interface called the Stern layer, where there are immobilized diffuse-layer ions, and a surface of the shear where the bulk fluid begins to move freely. The electric potential at this surface of shear is called the zeta potential (ζ). The zeta potential is the apparent charge of a particle or surface being acted upon under an electric field.

There are two phenomena associated with the electric double layer that are relevant: electrophoresis, in which a charged particle is moved by an electric field relative to the surrounding fluid, and electroosmosis, in which bulk fluid flow is driven by an electric field along a charged surface.

Electrokinetics

The most basic mathematical description of electrophoresis balances the electrical body force on the charge in the diffuse layer with the viscous forces in the fluid phase that oppose motion. Since the particle velocity is always proportional to the electric field strength,

$$V = \mu E \tag{3}$$

and electrophoretic mobility (μ) can be defined, where E is the electric field strength, and V is the particle velocity V $cm.s^{-1}$; and μ is the electrophoretic mobility. Using the force balance, the electrophoretic mobility is related to the zeta potential as:

$$\mu = \frac{\varepsilon\varepsilon_0\zeta}{\eta} f(\kappa a) \tag{4}$$

where ζ is the zeta potential in mV; η is the fluid viscosity in g cm^{-1} s^{-1}; and a is the particle radius in cm. The function $f(\kappa a)$ is 1 when the particle diameter is much larger than its double layer thickness (high ionic strengths, $\kappa a >> 1$), and is 1.5 for very low ionic strength solutions where the double layer thickness is much greater than the particle diameter ($\kappa a << 1$). The behavior of a particle is complex when the double layer and the particle diameter are close to the same size, which is often the case.

Electroosmotic flow is also related to the zeta potential (at the immobilized surface) and the strength of the electric field. For electroosmosis in a capillary, the

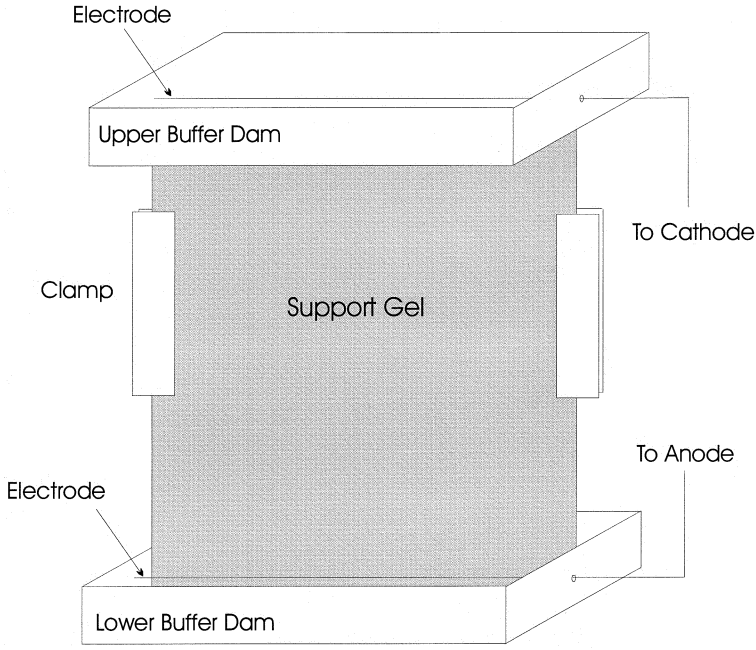

Figure 2. Typical gel electrophoresis apparatus.

flow rate is:

$$Q = \frac{\varepsilon\varepsilon_0\zeta}{\eta}\pi r^2 E \tag{5}$$

where Q is the flow rate in mL s^{-1} and r is the radius of capillary in cm. Electroosmotic flow is generally minimized in polymer networks such as gels (where the apparent viscosity, η, is large or r is very small) but is important in open channels such as capillaries.

A schematic of a typical gel electrophoresis apparatus is shown in Figure 2. The design of analytical gel electrophoresis equipment is influenced by the generation of heat and electrolysis gases, as discussed in the following sections.

Generation of Heat in Electrophoresis Instrumentation

One of the considerations in designing electrophoresis equipment is the dissipation of heat generated by the electric field. The change in temperature in an in-

sulated electrophoretic medium is given by:

$$\Delta T = \frac{W}{C_p M_e} = \frac{EI}{C_p M_e} \tag{6}$$

where W is the power (J s^{-1}), I is the current, A, C_p is the heat capacity of the medium (J K^{-1} g^{-1}) and M_e is the mass of electrophoretic medium (g). The current and the electric field strength are related to the conductivity of the electrophoretic medium by Ohm's law

$$E = \frac{I}{C} \tag{7}$$

where C is the medium conductivity in $(\Omega \cdot cm)^{-1}$. By combining the two expressions, it becomes obvious that heating increases as the square of the current. Electrophoresis is thus most conveniently conducted in highly resistive media (low salt concentrations). The addition of polymer matrices, such as gels, also serves to increase the resistivity of the media by increasing the viscosity.

Heating in electrophoresis causes changes in the viscosity and density of the electrophoretic medium. High temperatures can also damage electrophoretic equipment by warping cooling blocks, melting plastic, or cracking glass plates. When fluids heat unevenly, the hot portion of the fluid tends to rise with respect to cooler portions because of the difference in density. This convection distorts zones and thereby decreases resolution in electrophoretic separations. The ability to remove heat from electrophoretic systems ultimately determines the voltage which can be applied, and the speed and resolution that can be achieved.

Heat is dissipated from electrophoresis equipment by maximizing surface-to-volume ratio. Most electrophoresis is conducted in thin gels (0.5 to 1.5 mm) or in capillaries with channels up to 100 μm in diameter. The narrow cross section also reduces the total current, although the current density (amps/cm^2) remains independent of scale.

Electrolysis Reactions

The electrodes in electrophoresis equipment are typically constructed from platinum wire, and small ions generally carry the current in the electrophoretic medium. At the interface between the electrodes and the electrophoretic medium, electrolysis reactions occur. For aqueous systems, the following reactions are expected:

Cathode $\quad 2\,H_2O + 2\,e^- \rightarrow 2\,OH^- + H_2$

Anode $\quad H_2O \rightarrow 2\,H^+ + \frac{1}{2}\,O_2 + 2\,e^-$

Electrode chambers are usually open to the atmosphere so that electrolysis

gases can vent. The reactions at the electrodes produce acid (anode) and base (cathode), thus requiring electrophoresis systems to be well buffered. Electrophoresis systems sometimes mix the buffers from the individual electrode reservoirs to equalize the pH.

Modes of Electrophoretic Separation

Zone electrophoresis and isoelectric focusing are commonly practiced electrophoretic techniques. Zone electrophoresis separates compounds via differing rates of migration (V in Eq. 3). Isoelectric focusing is used for compounds whose net potentials are titrated through 0 by adjusting pH. Mechanistic details are given later in the text.

Zone Electrophoresis

In zone electrophoresis, samples are applied to an electrophoretic medium, most commonly a gel, an electric field is applied, and after a some length of time or a predetermined power consumption, the electrophoretic medium is inspected for resolution of the sample components. A typical zone electrophoresis result is shown in Figure 3. Each band on the gel represents a highly enriched substance that has been separated from the other bands based upon slightly different charge or size/shape property. As shown, several samples are typically analyzed side-by-side on a gel. Bands that migrate the same distance in different sample mixtures probably have similar mass and charge characteristics. Standards having known mobility or molecular mass are usually run concurrently with samples whose components are to be identified.

Disc Electrophoresis

Resolution in zone electrophoresis depends on getting sample components to migrate in a focused band. Thus techniques are employed to concentrate the sample as it migrates through the gel. The most common technique is referred to as discontinuous pH or "disc electrophoresis". Disc electrophoresis employs a two-gel system, where the pH and other properties of the two gels are different.

A sample is applied to a gel matrix as a solution. The sample solution is typically pipetted into "wells" preformed in the gel. The sample migrates into the first gel under the influence of the electric field. For proteins, this "stacking gel" has a pH near neutral. The protein sample migrates slowly under this condition as the charge on histidines is neutralized. As the proteins slowly migrate the few millimeters into the stacking gel, they concentrate. When the protein sample reaches

the second (separating) gel, the pH is raised to 8 to 9, and the sample proteins migrate more rapidly.

SDS PAGE

The use of sodium dodecyl sulfate (SDS) with polyacrylamide gel electrophoresis (PAGE) was first described in the late 1960's. SDS is an ionic surfactant that solubilizes and denatures proteins. The surfactant binds a protein through hydrophobic interactions with the peptide backbone, and disrupts side-chain interactions that cause proteins to fold and interact with other subunits. Non-reduced proteins bind approximately 0.9 to 1.0 gram(s) of SDS per gram of protein.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) allows separation of molecules according to size or molecular weight. When SDS treated samples migrate into a gel and are electrophoresed, the principal difference between sample components is molecular size or length. Smaller molecules travel through the matrix more quickly than those that are larger. The rate at which molecules migrate through a polyacrylamide gel is inversely linear with the logarithm of their molecular size. Thus, denatured samples can be analyzed alongside denatured standards of known molecular weight to estimate the molecular weight of the analyte.

Other dissociating agents may be used to further break down a protein. Urea is often used to disrupt hydrogen bonds. When urea is the only dissociating agent added (no SDS), a protein's intrinsic charge is not affected and separation based on size and charge may be achieved. If a protein contains internal disulfide bonds, a thiol reagent such as β-mercaptoethanol must be used to reduce the sample and break the disulfide bonds. Proteins having reduced disulfide bonds bind approximately 1.4 g of SDS per gram of protein, compared to about 1 gram per gram for non-reduced. Typically, both reduced and non-reduced samples are run in order to evaluate internal disulfide bond content.

Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis was developed to separate large pieces of DNA in agarose gels. DNA had previously been separated by conventional gel electrophoretic techniques. The resolving power of this technique is inversely proportional to the log of the DNA molecular size. Thus as the molecular weight of the DNA increases, the resolution decreases. In pulsed field electrophoresis, the direction of the field is intermittently changed, either forward or backward or from side to side or some other sequence of directions. This causes DNA chain molecules to reorient in the electric field, and entangles the DNA in the polymer matrix. Molecules that are small relative to the space between polymer strands in

the matrix exhibit no significant difference in their electrophoretic mobility because they can completely reorient in the alternating field without entangling in the matrix. However, the redirection of the electric field causes molecules larger than the entanglement length to travel in a zigzag pattern, putting kinks in the molecule. The longer the molecule, the more kinks it develops, and the slower it travels through the gel. Pulsed field techniques significantly enhance resolution of megabase size strands of DNA.

Isolectric Focusing

Isoelectric focusing is a technique used for protein separation, by driving proteins to a pH where they have no mobility. Resolution depends on the slope of a pH gradient that can be achieved in a gel or capillary.

Ampholytes

An ampholyte is a molecule that can be positively or negatively charged depending on the pH. All amino acids and proteins are ampholytes (or amphoteric). Not only does the sign of the charge of an ampholyte change with pH, but the magnitude of the charge can also vary. The charge on a protein, for example, may vary from $+10$ or more at low pH, to -10 or more at high pH. For a molecule, the pH at which its net charge is zero is called the isoelectric point (pI).

A special class of ampholytes has been synthesized for isoelectric focusing. These ampholytes have an amino end and a carboxyl end that are separated by varying numbers of methylene groups. The further apart the amino and carboxyl groups, the less they affect the ionization of each other; thus a different isoelectric point is established for each molecular species. These ampholytes, which may be added to an electrophoretic medium, migrate according to their charge under the influence of the applied electric field. When they reach a zone where the local pH is the same as that ampholyte's isoelectric point, migration stops. The ampholyte molecules buffer themselves and establish the local pH as they migrate through the gel. As the ampholytes reach an isoelectric pH, they establish a spatially distributed and stable pH gradient in the electrophoretic medium.

Isoelectric Focusing Technique

Isoelectric focusing (IEF) is an electrophoretic technique in which amphoteric samples (such as proteins) are separated according to their isoelectric points along a continuous pH gradient. IEF analyses are carried out in various matrices:

in acrylamide, agarose and capillaries. The agarose or acrylamide gels that are used must be prepared with carrier ampholytes bracketing a specific pH range. After some time, the ampholytes separate and there is a pH gradient that covers the range of all the ampholytes' isoelectric points. Another class of available ampholytes, called Immobilines®, can be added to the matrix, electrophoresed into a gradient, and covalently incorporated into the gel matrix.

Because protein samples are actually ampholytes, when samples are loaded onto the gel and a current is applied, the compounds migrate through the gel until they come to their isoelectric point, where they reach a steady state. This technique measures an intrinsic physicochemical parameter of the protein, the pI, and therefore does not depend on the mode of sample application. In practice, IEF has the highest sample load capacity of any electrophoretic technique.

In slab gels, isoelectric focusing typically requires approximately 3 to 30 hours to complete because sample compounds move increasingly slowly as they approach the pH that corresponds to their isoelectric point. This time can be shortened to approximately 30 minutes in capillaries because of the higher electric field employed. Because the gradient ampholytes and the samples stop where they have no mobility, the resistivity of the system increases dramatically toward the end of the experiment, and the current decreases dramatically. For this reason, isoelectric focusing is usually run in constant voltage mode and has the most stringent requirements for cooling of any electrophoretic technique. A plot of current verses time can be used to determine when the system is properly focused.

ELECTROPHORETIC INSTRUMENTS

Most electrophoresis equipment shares the basic design shown in Figure 2. Electrophoresis equipment typically has two buffer reservoirs, one anodic and one cathodic. The equipment includes some sort of electrophoretic medium connecting the two reservoirs, such as a gel, paper, or capillary, to which a sample is applied. A direct current power supply connects two electrodes suspended in the buffer reservoirs. When an electric potential is applied between the two electrode reservoirs, the sample components migrate through the medium. At completion of an electrophoretic separation, the sample components have been resolved into several zones along the length of the medium, or have been eluted from the medium.

Support Media

The conduction of electric current through an electrophoresis system causes the system temperature to increase if the heat is not dissipated at a rate equal to the rate of its production. Increases in system temperature will increase the elec-

trophoretic mobility of molecules. Additionally, the formation of thermal gradients will result either in convection or a distribution of electrophoretic mobilities for a unique compound, and ultimately in zone spreading. To minimize the influence of convection on the electrophoretic separation, anti-convective support media such as paper, polymer gels or capillaries are commonly employed.

Separation on paper or polymer gels is influenced not only by electrophoretic mobility, but also by sieving of the molecules as they pass through the network of pores and fibers. The finer the weave of this matrix or the larger the gel forming molecule, the slower a molecule travels through it. Therefore, molecular size, as well as charge, can influence the rate of migration in these media.

Paper Electrophoresis

Paper was one of the first matrices[SR1] used for electrophoresis. In paper electrophoresis, the sample is applied directly to a zone on the dry paper, which is then moistened with a buffer solution, and an electric field is applied. Dyes are combined with samples and standards to help visualize the progress of the electrophoresis. The movement of samples on paper is best when the current flow is parallel to the fiber axis in the paper.

Some advantages of paper are that it is readily available, easy to handle, requires no preparation, and new methodologies can be developed rapidly. Besides being easy to obtain, paper does not contain many of the bound charges that can interfere with the separation. The disadvantages of paper electrophoresis are that the porosity of paper cannot be controlled, the technique is not very sensitive, and it is not easily reproducible.

Polyacrylamide Gels

Polyacrylamide gels are synthesized through the polymerization of acrylamide monomer, $CH_2{=}CHCONH_2$, and a bifunctional, cross-linking co-monomer. Typically, the cross-linking co-monomer is N,N'-methylenebisacrylamide (bisacrylamide), $(CH_2CHCONH)_2CH_2$, although other compounds such as ethylenediacrylate (EDA) or N,N'-diallyltartardiamide (DATD) are sometimes used. The sieving properties of the gel are defined by the network of pores established during the polymerization. As the acrylamide concentration of the gel increases, the effective pore size decreases. By convention, a gel is characterized by two parameters, %T and %C, where %T is the mass % of total monomer (acrylamide plus co-monomer in grams per 100 mL), and %C is the proportion by mass of monomer (per 100 g acrylamide) that is the cross-linking agent. Gels typically have %T values between 3 and 30%.

The most commonly used combination of chemicals to produce a polyacrylamide gel is acrylamide, bisacrylamide, buffer, ammonium persulfate, and tetramethylenediamine (TEMED). TEMED and ammonium persulfate are catalysts for the polymerization reaction. The TEMED causes the persulfate to produce free radicals, initiating polymerization. Once the gel is poured into a prepared form (usually between two vertical, parallel glass plates that are sealed on the bottom and sides, see Figure 2), a "comb" can be inserted into the top portion of the gel before polymerization is complete. This comb sets small indentations (wells) into the gel that can be used to apply samples. If the comb is used, samples are typically mixed with a dense liquid such as glycerol to prevent the sample from dispersing into the reservoir buffer. Samples then migrate straight down the gel and appear as columns, or lanes, of separated components at the end of the experiment (see Figure 3).

Polyacrylamide gel electrophoresis is one of the most commonly used electrophoretic methods. Analytical uses of this technique center on protein and nucleic acid characterization, e.g., purity, size, or molecular weight, and composition of a protein. The drawbacks of a polyacrylamide matrix are that acrylamide is a neurotoxin, the reagents must be combined extremely carefully, and the gels are not as pliable as most agarose gels.

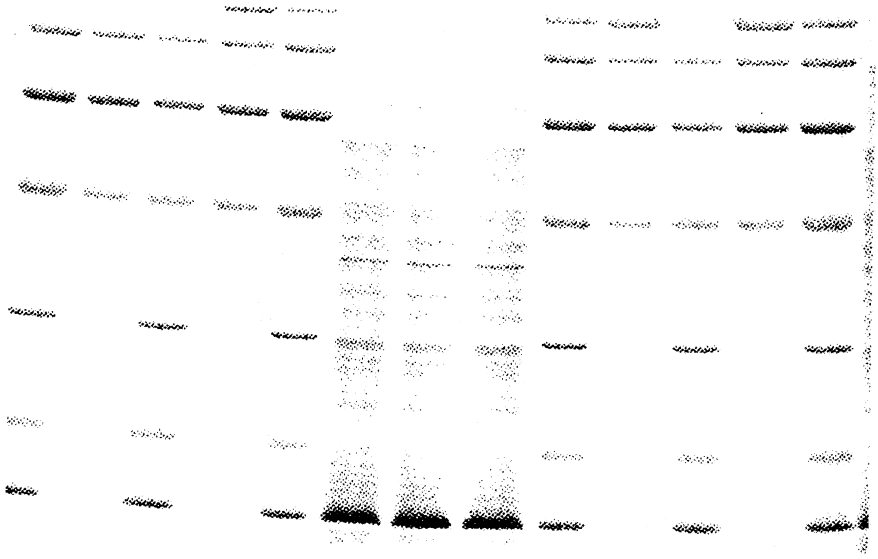

Figure 3. Typical gel electrophoresis result.

Agarose Gels

Agarose is a polymer extracted from red seaweed. When agar is extracted from the seaweed, it is in two components, agaropectin and agarose. The agarose portion is nearly uncharged, making it desirable for use as an electrophoresis matrix. To prepare an agarose gel, a combination of agarose and buffer is heated until the solid is dissolved. The solution is cooled and then poured into a warmed gel casting apparatus and allowed to gel. The advantages of agarose electrophoresis are that it requires no additives or cross-linkers for polymerization, it is not hazardous, low concentration gels are relatively sturdy, and it is inexpensive.

Agarose gels have large pore sizes compared to polyacrylamide gels. Agarose is commonly used for large molecule separation, such as DNA fragments.

Capillary Columns

Capillary electrophoresis is a commercially available technique which is highly automated and bears a strong resemblance to analytical chromatography. Capillaries were first widely used as a support medium for electrophoresis in the early 1980s. The capillaries are typically 20 to 200 μm in diameter, 30 to 100 cm in length, may be filled with buffer or a gel, and are frequently coated on the inside. Capillaries are used because the small cross sectional area minimizes power consumption, while the high surface-to-volume ratio allows efficient dissipation of heat. In practice, electric fields in excess of 300 V cm^{-1} can be utilized without encountering detrimental thermal effects. Capillary electrophoresis separations are rapid (minutes) and produce a chart recording rather than a stained gel for archiving. A typical capillary "electropherogram" is shown in Figure 4.

Samples may be introduced into the capillary via electrophoresis and/or electroosmosis. However, this technique will discriminate against sample components with small electrophoretic velocities and the amount of sample introduced can be affected by the sample matrix. Pressure-based sample introduction techniques are generally preferred because they are more reproducible.

Clearance of sample components from a capillary is a problem that must be considered carefully. Usually, capillary electrophoresis is conducted in a fused-silica capillary that is coated on its inner surface. Capillaries are too expensive to discard after each use, as is done with a standard gel. Therefore, the entire sample has to be cleared from the capillary before the capillary is used for another sample, much like analytical chromatography. Another problem arises when the analytes interact with the walls of the capillary, which leads to broadening of the zone and reduced resolution. This latter problem has slowed the application of capillary electrophoresis to the analysis of macromolecules.

Electroosmotic flow in a capillary makes it possible to simultaneously analyze both cations and anions. The only requirement is that the electroosmotic flow veloc-

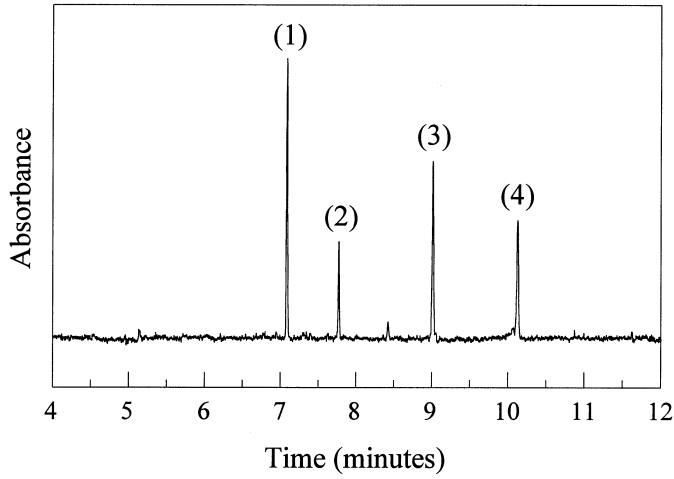

Figure 4. Capillary electrophoresis electropherogram for the separation of a synthetic protein mixture containing (1) α-chymotrypsinogen A (9.5 fmol), (2) ribonuclease A (17.5 fmol), (3) cytochrom c (17.5 fmol), and (4) lysozyme (17.9 fmol). The separation was carried out in 20 mM pH 5.0 sodium acetate buffer with an applied field of -330 V cm^{-1}. The separation capillary was fused silica treated with polyarginine. The capillary dimensions were 50 μm id, 360 μm od, 50 cm from capillary inlet to detector.

Table I. Electrophoresis Matrices Comparison

Matrix	Advantages	Disadvantages
Paper	Contains a low level of bound charge	Porosity not controlled
	Readily available	Low sensitivity
	Easy to handle	Low reproducibility
	Requires no preparation	
Polyacrylamide	Commonly used	Acrylamide is a neurotoxin
	Useful for proteins and nucleic acids	Gels not pliable
Agarose	No additives required	Gels have short shelf life
	Inexpensive	
	Not hazardous	
	Sturdy	
	Used commonly for DNA	
Capillaries	Reusable	Requires sensitive detector
	High resolving power	Relatively expensive equipment
	Permits rapid analyses	
	Consumes a small amount of sample	Longer development cycle
	Easily automated	

ity is greater in magnitude than the electrophoretic velocity of both the positively and negatively charged ions. [SR2]Electroosmosis is the preferred method of generating flow in the capillary because it produces a very flat flow profile, which minimizes zone broadening. In general, electroosmosis is greatest in bare fused silica capillaries when the pH of the buffer is high. Additives to the buffer can be used to enhance or suppress electroosmotic flow by influencing the charges on the walls of the capillary.

The small quantity of material loaded into the capillary requires that high-sensitivity detection be employed with all capillary electrophoresis instruments. Typical sample volumes range from 5 to 50 nL, which is 0.1% to 1% of the volume typically loaded onto polyacrylamide or agarose gels. For trace analysis, a small number of molecules will be present in the capillary after loading. To detect these small amounts of material necessitates very sensitive detectors.

A summary of the advantages and disadvantages of different electrophoresis matrices is given in Table I.

Detection Techniques

Most sample components analyzed with electrophoretic techniques are invisible to the naked eye. Thus, methods have been developed to visualize and quantify separated compounds. These techniques most commonly involve chemically fixing and then staining the compounds in the gel. Other detection techniques can some times provide increased selectivity or sensitivity.

Chemical Staining

The size and type of the compound as well as the electrophoretic matrix dictate and often limit the variety of stains that can be used to help to visualize electrophoretic banding patterns. Molecules can be lost during the staining process, so most staining procedures incorporate a "fixing" step, such as a soak in dilute acetic acid for 1 hr, either before or in conjunction with staining. Once the molecules are fixed, they can be stained without loss of the separated components.

Amido black is a commonly used stain for proteins, but it is not very sensitive. It is often used to visualize components that are readily accessible to dyes. Two of the more sensitive and more frequently used protein stains are Coomassie Brilliant Blue (R250 and G250) and silver stain. The Coomassie stains are approximately five times more sensitive than amido black and are appropriate for both agarose and polyacrylamide gels. The silver stain is approximately 100 times more sensitive than Coomassie, but its use is more complex and often requires more troubleshooting to obtain the desired results.

To quantitate stained sample components, the gel is scanned with a densitometer. These scans can be compared with equivalent scans of quantitative standards to generate a calibration curve.

Fluorescence

Fluorescence detection provides much better detection limits than simple chemical stains. This typically involves the covalent binding of a fluorescent residue to the analyte, but can involve any type of a specific interaction. If the staining process can be performed prior to the electrophoretic analysis, this often allows the electrophoretic process to be followed visually, and minimizes the background signal caused by dye trapping in the electrophoretic matrix.

Fluorescamine is a popular reagent for labeling of proteins. At room temperature and alkaline pH, fluorescamine can react with primary amines on the protein to generate a fluorescent derivative. Fluorescamine may alter the charge to mass ratio of the analytes, and may be used to label samples before electrophoresis, or to stain the gel afterwards, depending on the importance of maintaining charge to mass. The reagent ethidium bromide is commonly used to visualize DNA. The ethidium is incorporated into the structure of DNA. Once incorporated, the fluorescence quantum efficiency of ethidium is dramatically enhanced and the bands can be easily visualized under a UV lamp.

Radioactivity

Another method to visualize and identify separation products on a gel is through radioactivity. If a sample is radioactive, the bands that separate during electrophoresis are subsequently radioactive. After the separation is complete, the electrophoretic matrix (e.g., gel or blotting membrane) may be placed against x-ray film until the radiation makes a mirror image of the banding pattern on the film. When this film is developed, the resulting autoradiograph displays the bands.

Immunoelectrophoretic Techniques

The technique of gel electrophoresis has been successfully combined with immunological techniques to further evaluate molecules. A frequently used method of immunoelectrophoresis is a technique known as “crossed immunoelectrophoresis”. A sample is first run longitudinally through an agarose gel for a predetermined time. Secondly, the gel area where the sample was electrophoresed is typically cut out and placed into a similarly sized area of an antibody-containing gel. As an electrical current is applied to the second gel system, the sample in question electrophoreses through the second gel and forms an antigen-antibody precipitin pattern.

Both agarose and acrylamide gel systems have been used for direct immunofixation. In these gels, samples are electrophoresed and then immunofixed

by either using strips of cellulose acetate soaked in an antibody or the antibody is placed directly over the sample area of the gel.

All of these techniques are most often, but not exclusively, used in the clinical setting in order to diagnose abnormalities or to evaluate inheritance patterns of polymorphic proteins.

On-column/End-column Detection

Capillary electrophoresis is characterized by minute amounts of sample contained in small volumes of liquid. To monitor the zones formed by the analysis requires detection methods that are extremely sensitive and do not give rise to extracolumn band-broadening or dilution. Most detection methods employed with capillary electrophoresis are on-column adaptations of detectors commonly employed with analytical liquid chromatography. By monitoring the zones directly on-column, distortion of the sample zone is minimized while maximizing detection sensitivity.

Absorbance is the most popular detection method employed with capillary electrophoresis. Nearly every commercial capillary electrophoresis instrument comes equipped with an absorbance detector. Relative to equivalent detectors used with analytical liquid chromatography, the concentration limit of detection in capillary electrophoresis can be as much as 3 orders of magnitude lower. For example, where a concentration of 100 ppb may be detectable in a chromatographic detector, 100 ppm may be required for detection by capillary electrophoresis. This results from the short optical pathlength defined by the inner diameter of the capillary and by the poor optical characteristics of the capillary wall. Because of the small volume in the detector path, the mass limits of detection are typically more sensitive than those observed with other separation techniques. In other words, a smaller number of molecules may be detected by capillary electrophoresis, provided the molecules can be concentrated into the volume defined by the width of the detection laser and the cross section of the capillary.

Enhanced detection sensitivity is achieved with on-column fluorescence and electrochemical detectors. With fluorescence detection, the small dimensions of the capillary helps confine the sample to a small volume. Focusing the excitation beam into the small volume increases the irradiance of the fluorophore and the fluorescence signal. Electrochemical detectors are usually formed by inserting a microelectrode into the capillary, or by butting the electrode up against the capillary outlet. The small dimensions of the capillary ensure that the maximum distance the analyte must travel to reach the surface of the electrode is small and therefore the electrochemical conversion efficiency is high.

The coupling of capillary electrophoresis to mass spectrometry is developing rapidly because of the wealth of chemical information which can be obtained.

Most often this coupling is through an on-line electrospray ionization interface. The primary difficulty of using mass spectrometry as the primary detector for capillary electrophoresis is the relative incompatibility of the common electrophoresis buffer salts and solvents with the electrospray interface.

SPECIALIZED TECHNIQUES

Two-Dimensional Electrophoresis

Two-dimensional (2D) electrophoresis is unique, offering an analytical method that is both reproducible and sensitive. It is referred to as 2D because it employs two different methods of electrophoresis (typically IEF and SDS PAGE), in two different directions, to separate the sample components. The combination of the two methods gives better resolution of the compounds than could be achieved with either method alone. For example, each method alone may separate up to 100 components of a sample, whereas together they may separate up to 10,000 components. A 2D analytical technique using IEF and SDS- PAGE to separate total protein results in a gel having bands or spots in an apparently random pattern. Each spot represents a unique component present in the sample. The sample spots are lined up on the gel in order of molecular weight in one direction (typically vertical), and isoelectric point in the other (horizontal). 2D gel electrophoresis is a powerful technique for analyzing very complex samples, such as tissue homogenates, but is not usually applied to purified samples. Only one sample can be analyzed at a time on each gel in 2D gel electrophoresis.

Blotting Techniques

Problems encountered when trying to analyze resolved components of a sample mixture on a gel, with techniques such as direct immunofixation or application of a ligand can be circumvented with the use of blotting techniques followed by staining or autoradiography. It is the inability of some compounds, such as antibodies or ligands, to enter a specific gel matrix that necessitated the development of various blotting techniques. The nucleic acid and protein blotting techniques are useful because they combine electrophoretic analyses and sensitive immunological or hybridization detection tools. These techniques involve the transfer of nucleic acids or proteins, immediately after being separated by electrophoresis, from the gel matrix to another matrix. Typically, the other matrix is nitrocellulose paper, nylon, or other high affinity membrane. The mode of transfer is electrotransfer for proteins and capillary transfer for nucleic acids. On nitro-

Table II. Blotting Techniques Commonly Used with Electrophoresis

Technique	Analyte	Membrane	Probe	Detection
Southern Blot	DNA	Nitrocellulose	Radiolabeled complementary RNA or DNA	Autoradiography
Northern Blot	RNA	Nitrocellulose	Radiolabled complementary RNA or DNA	Autoradiography
Western Blot	Proteins	Nitrocellulose or nylon	antisera	Autoradiography or enzymatic detection

cellulose paper or nylon, nucleic acids and proteins are more accessible than in the original gel matrix. This second matrix is then treated with a ligand to identify a specific component of a sample. Table II summarizes the common electrophoresis blotting techniques.

NOMENCLATURE

a	The particle radius (cm)
c_i	The concentration of ion "i"
C	The medium resistivity ($\Omega.cm^{-1}$)
C_p	Heat capacity ($J.g^{-1}.\Delta\ T^{-1}$)
E	The electric field strength (V/cm)
F	Faraday's constant (9.65×10^4 C mol^{-1})
I	The electric current (A)
M_e	The mass of the medium (g)
Q	Flowrate ($cm^3.s^{-1}$)
r	The radius of a capilary (cm)
R	The universal gas constant (8.314 J mol^{-1} K^{-1})
T (ΔT)	The absolute temperature, or the temperature difference (°K)
V	The velocity of a particle (cm/s)
W	Electrical power (V.A, or $J.s^{-1}$, or Watts)
x	The distance normal to the charged surface (cm)
z_i	The valence of ion "i"
ε	The relative permittivity of the continuous medium (dimensionless)
ε_0	The permittivity of free space (8.854×10^{-14} C V^{-1} cm^{-1})
η	The viscosity of the continuous medium ($g.cm^{-1}.s^{-1}$)

κ	The "double layer thickness" (cm^{-1})
μ	The electrophoretic mobility ($cm^2/V.s$)
π	3.1417
ϕ	The electric potential measured at any given point (mV)
ϕ_0	The electric potential measured at the particle surface (mV)
ζ	The zeta potential, the electric potential measured at the particle's surface of shear (mV)

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