3.2 Electrophoresis

Electrophoresis (*Electro* refers to the energy of electricity and *Phoresis*, from the Greek verb *phoros*, means to carry across) is a technique for separating, or resolving, charged molecules (such as amino acids, peptides, proteins, nucleotides, and nucleic acids) in a mixture under the influence of an applied electric field. Charged molecules in an electric field move or migrate, at a speed determined by their charge : mass ratio. According to the laws of electrostatics an ion with charge 'Q' in an electric field of strength 'E' will experience a electric force, $F_{electrical}$

$$F_{electrical} = QE$$

The resulting migration of the charged molecule through the solution is opposed by a frictional force

$$F_{frictional} = Vf$$

Where V is the rate of migration of ion and f is its **frictional coefficient**.

Frictional coefficient depends on the size, shape and viscosity of the solution. In constant electric field, the force on ion balance each other;

$$QE = Vf$$

So that each ion moves with a constant characteristic velocity. An ion's **electrophoretic mobility,** μ is defined as

$$\mu = V/E = Q/f$$

So according to equation, if two molecules have the same mass and shape, the one with the greater net charge will move faster toward an electrode.

Electrophoresis is of two types - *moving boundary electrophoresis* and *zone electrophoresis*. In zone electrophoresis sample is constrained to move in a solid support such as filter paper (paper electrophoresis) or a gel (gel electrophoresis).

GEL

There are two basic types of materials used to make gels: *agarose* and *polyacrylamide*. Agarose is a natural colloid extracted from sea weed. Agarose gels have very large pore size and are used primarily to separate very large molecules with a molecular mass greater than 200 kDa. Agarose is a linear polysaccharide made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose. Agarose is usually used at concentrations between 1% and 3%.

A polyacrylamide gel consists of chains of acrylamide monomers ($CH_2 = CH-CO-NH_2$) cross-linked with *N*, *N*-methylenebisacrylamide units ($CH_2 = CH-CO-NH-CH_2-NH-CO-CH-CH_2$), the latter commonly called *bis*. The pore size of the gel is determined by both the total concentration of monomers (acrylamide + bis) and the ratio of acrylamide to bis. Polymerization of the acrylamide : bis solution is initiated by ammonium persulfate and catalyzed by TEMED (*N, N, N′ N′*-tetramethylethylenediamine).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic separation of proteins is most commonly performed in **polyacrylamide gels**. When a mixture of proteins is applied to a gel and an electric current applied, smaller proteins migrate faster than larger proteins through the gel. The rate of movement is influenced by the gel's pore size and the strength of the electric field. The pores in a highly cross-linked polyacrylamide gel are quite small. Such a gel could resolve small proteins and peptides, but large proteins would not be able to move through it.

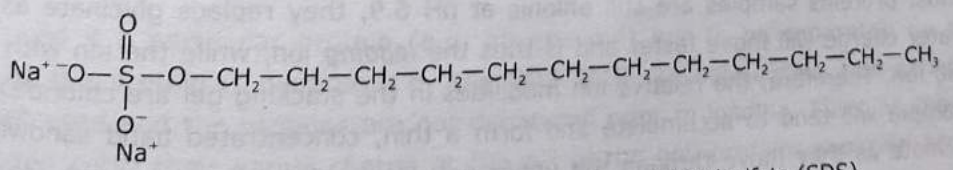


Figure 3.2 : Structure of sodium dodecylsulfate (SDS).

Proteins are exposed to the negatively charged ionic detergent **sodium dodecylsulfate** (SDS) before and during gel electrophoresis. SDS denatures proteins, causing multimeric proteins to dissociate into their subunits, and all polypeptide chains are forced into extended conformations with similar charge:mass ratio. SDS treatment thus eliminates the effect of differences in shape, so that chain length, which reflects mass,

is the sole determinant of the migration rate of proteins in SDS-PAGE. The molecular weight of a protein can be estimated by comparing the distance it migrates through a gel with the distances that proteins of known molecular weight migrate.

SDS-PAGE is rapid, sensitive, and capable of a high degree of resolution. Bands resulting from electrophoretic separation can be located by a variety of techniques. Proteins are often visualized by staining. **Coomassie brilliant blue** is the most widely used dye. **Fluorescamine**, a nonfluorescent molecule is also used as an alternative type of protein stain.

Discontinuous electrophoresis

In *continuous gel system*, separating media consists of a single gel with an uniform pH throughout. In *discontinous gel system*, polyacrylamide gel is divided into three regions called the *sample gel*, *stacking gel*, and *separating gel*. These gels can have different concentrations of the same support media, or may be completely different agents. The sample gel contains the mixture of proteins to be separated and is prepared using low concentration of acrylamide so that pore sizes are large and do not influence the rates of migration of different size proteins. The stacking gel is similar to the sample gel. The proteins in the sample gel will concentrate into a small zone in the stacking gel before entering the separating gel.

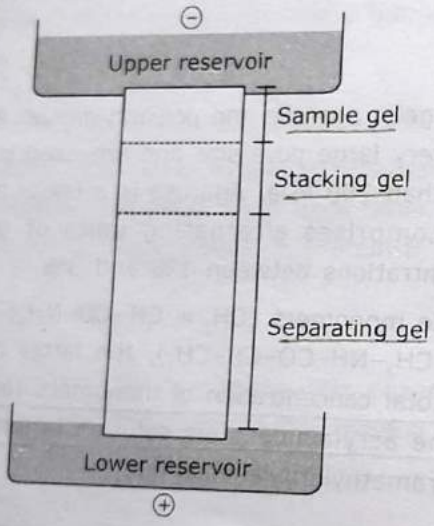


Figure : 3.3
Essential components of discontinuous electrophoresis.

The sample gel, stacking gel, and reservoirs have the same pH. The separating gel differs from the other two regions due to greater concentrations of acrylamide; this results in smaller pore sizes and provides the sieving effect. The sample is usually dissolved in glycine-chloride buffer, pH 8 to 9, before loading on the gel. Glycine exists primarily in two forms at this pH, a zwitterion and an anion. When the voltage is turned on, buffer ions (glycinate and chloride) and protein sample move into the stacking gel, which has a pH of 6.9. Upon entry into the upper gel, the concentration of glycine zwitterion increases and hence no electrophoretic mobility. Since most proteins samples are still anionic at pH 6.9, they replace glycinate as mobile ions. The ion having a greater charge will move faster and is thus the *leading* ion, while the ion with the lesser charge will be the *trailing* ion. Therefore, the relative ion mobilities in the stacking gel are chloride> protein sample> glycinate. The sample will tend to accumulate and form a thin, concentrated band sandwiched between the chloride and glycinate as they move through the upper gel. Now, when the ionic front reaches the lower gel with pH 8 to 9 buffer, the glycinate concentration increases and anionic glycine and chloride carry most of the current. The protein sample molecules, now in a narrow band, encounter both an increase in pH and a decrease in pore size. The increase in pH would, of course, tend to increase electrophoretic mobility, but the smaller pores decrease mobility. The relative rate of movement of anions in the separating gel is chloride> glycinate > protein sample.

The separation of sample components in the resolving gel occurs as described in an earlier section on gel electrophoresis. Each component has a unique charge/mass ratio and a discrete size and shape, which directly influence its mobility.

Two-dimensional gel electrophoresis

Electrophoresis of all cellular proteins through an SDS gel can separate proteins having relatively large difference in molecular weights (e.g., a 41-kDa protein from a 42-kDa protein). To separate proteins of similar mass, another physical characteristic must be exploited.

In two-dimensional electrophoresis, proteins are separated in two sequential steps: first by their charge and then by their mass. In the first step, a cell extract is fully denatured by high concentrations (8 M) of urea and then layered on a glass tube filled with polyacrylamide that is saturated with a solution of **ampholytes**, a mixture of polyanionic and polycationic molecules. When placed in an electric field, the ampholytes will separate and form a continuous gradient based on their net charge. The most highly polyanionic ampholytes will collect at one end of the tube, and the most polycationic ampholytes will collect at the other end. This gradient of ampholytes establishes a pH gradient. Charged proteins will migrate through the gradient until they reach their pI, or isoelectric point, the pH at which the net charge of the protein is zero. This technique, called **isoelectric focusing** (IEF), can resolve proteins that differ by only one charge unit.

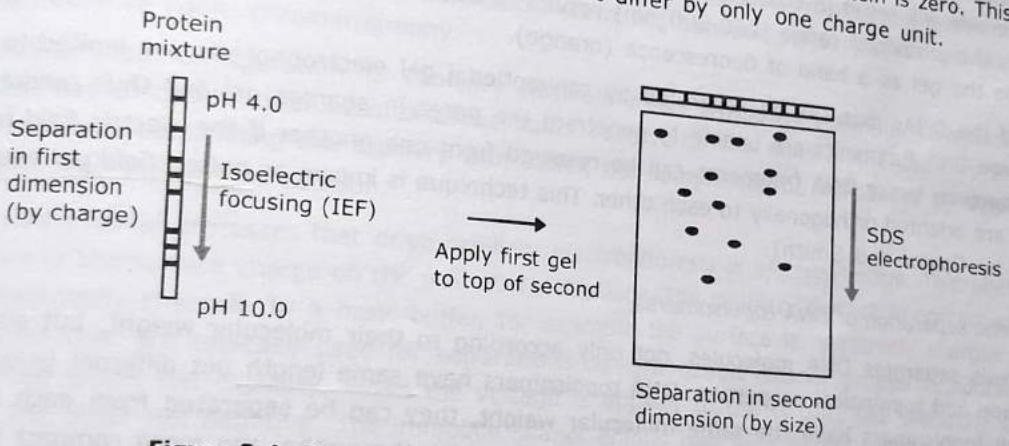


Figure 3.4 : Two-dimensional gel electrophoresis

Proteins that have been separated on an IEF gel can then be separated in a second dimension based on their molecular weights. To accomplish this, the IEF gel is extruded from the tube and placed lengthwise on a second polyacrylamide gel, this time formed as a slab saturated with SDS. When an electric field is imposed, the proteins will migrate from the IEF gel into the SDS slab gel and then separate according to their mass. The sequential resolution of proteins by their charge and mass can achieve excellent separation of cellular proteins.

Native PAGE

SDS-PAGE is not used if a particular protein (e.g. an enzyme) has to be separated on the basis of its biological activity, as the protein is denatured by the SDS-PAGE. In native gels, non-denaturing condition are used. SDS is not used and the proteins are not denatured prior to loading. Since all the proteins in the sample being analyzed carry their native charge at the pH of the gel, proteins separate according to their different electrophoretic mobilities.

Immunoblotting

Separation of a mixture of proteins by electrophoretic techniques usually results in a complex pattern of protein bands or zones. Specific proteins can often be identified using an **immunoblotting** technique (also known as Western blotting). This technique requires an antibody against the test protein. After the initial

separation by electrophoretic technique in a gel, the proteins are transferred (or blotted) from the gel a membrane, usually nitrocellulose. The next step involves treating the membrane with a suitable antibody and allowing the reaction to take place. Excess antibodies are then washed from the membrane and the bound antibody which remains is detected using a second antibody against the first.

Electrophoresis of nucleic acids

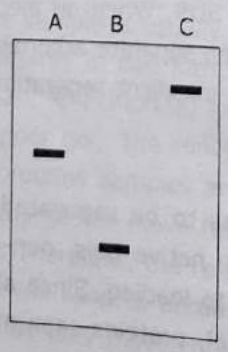
Gel electrophoresis is the process by which scientists can sort pieces of DNA of different size. An agarose or polyacrylamide gel is loaded with the DNA fragments and current is passed through the gel. Since DNA is *negatively* charged, it will migrate towards the positive pole. The DNA will not migrate at the same rate however. Larger pieces of DNA collide with the gel matrix more often and are slowed down, while smaller pieces of DNA move through more quickly. Electrophoretic mobility of DNA in gel not only depends on the size but also on shape.

DNA molecules are invisible to the naked eye, but can be seen in gels by staining them with a solution of planar aromatic cations such as *ethidium bromide, acridine orange* or *proflavin*. Ethidium bromide is a molecule that becomes intercalated between the stacked bases of the DNA molecule. Soaking a DNA containing gel in ethidium bromide will result in concentration of the chemical within the DNA. Illumination of the soaked gel with light in the ultraviolet range (260-300 nm) results in fluorescence of ethidium bromide, and the DNA shows up on the gel as a band of fluorescence (orange).

The sizes of the DNAs that can be separated by conventional gel electrophoresis are limited to ~100 bp. Very large DNA fragments are unable to penetrate the pores in agarose gel and thus cannot readily be resolved. However, larger DNA fragments can be resolved from one another if the electric field is applied in pulses that are oriented orthogonally to each other. This technique is known as *pulsed-field gel electrophoresis* (developed by Cantor and Smith).

Electrophoretic separation of DNA topoisomers

Electrophoresis separates DNA molecules, not only according to their molecular weight, but also according to their shape and topological properties. DNA topoisomers have same length but different linking number. Even though topoisomers have the same molecular weight, they can be separated from each other by electrophoresis. The basis of this separation is that the greater the writhe, the more compact the shape of a covalently closed circular DNA (cccDNA). The more compact the DNA, the more easily it is able to migrate through the gel. Thus a relaxed cccDNA migrates more slowly than a highly supercoiled form of same circular DNA.



Electrophoretic separation of DNA topoisomers. Band on *lane C* represent relaxed circular DNA, *lane B* highly supercoiled and *lane A* less supercoiled cccDNA.

Problem

An enzyme examined by means of gel filtration in aqueous buffer at pH 7.0 had an apparent molecular weight of 160,000. When examined by gel electrophoresis in SDS soultion, a single band of apparent molecular weight 40,000 was formed. Explain these findings.

Solution

The detergent SDS causes the dissociation of quaternary structures and allows the determination of molecular weight of the component subunits. The data suggests that the enzyme comprises four identical subunits of M_r = 40,000, yielding a tetramer of M_r = 160, 000.

Problem

The R_f values of substances A and B are 0.34 and 0.68 when chromatographed on paper using ethanol as a solvent. What is the ratio of the distance moved after two hours?

Solution

B moves twice as far as A at all times.

Capillary electrophoresis

Capillary electrophoresis employ narrow-bore capillaries to perform high efficiency separations of both large and small molecules. These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary. The properties of the separation have characteristics resembling between polyacrylamide gel electrophoresis and high performance liquid chromatography.

The basic instrumental configuration for capillary electrophoresis is relatively simple. It includes a fused-silica capillary, two electrode assemblies, and two buffer reservoirs. The ends of the capillary are placed in the buffer reservoirs. After filling the capillary with buffer, the sample can be introduced by dipping the end of the capillary into the sample solution.

One of the fundamental processes that drive capillary electrophoresis is *electroosmosis*. This phenomenon is a consequence of the surface charge on the wall of the capillary. The capillary surface in contact with a buffer solution is electrically charged. In a basic buffer, for example the surface is negatively charged. The fused silica capillaries that are typically used for separations have ionizable silanol groups in contact with the buffer contained within the capillary. When the voltage is applied to the circuit, one electrode become net positive and the other net negative. The immobile silanol anions pair with mobile buffer cations, forming a double layer along the wall. The remaining buffer cations are attracted to the negative electrode, dragging the bulk buffer solution with them. This is electroosmotic flow.