Consortium for Educational Communication

Module on

Electrophoresis

By
Naseer Ahmad Bhat
Research scholar
Deptt. Food Science & Technology
Cell no. 9906754380
University of Kashmir

Text

PRINCIPLE

An electrophoretic separation is performed by injecting a small band of the sample into an aqueous buffer solution contained in a narrow tube, a flat porous support medium such as paper or a semi-solid gel. The high voltage is applied across the length of the buffer by means of a pair of electrodes located at its each end. This field causes ions of the sample to migrate towards the opposite charged electrodes. The rate of migration of a given species depends on its charge and its size. Separations are then based on differences in charge-to-size ratios for the various analytes in a sample. The larger this ratio, the faster an ion migrates in the electric field.

Consider a situation where a spherical molecule of net charge ***q*** is placed in an electric field. The force ***F*** which will act upon this particle will depend upon: (i) the net charge density of the molecule, and (ii) the strength of the field in which it is placed. The above relation may be mathematically described as:

Where ΔE/d is the field strength applied, (ΔE is the potential difference applied between the two electrodes, *d* being the distance between them. Since the particle has been suspended in a solution, the friction occurring between the accelerating molecule and the solution is to be considered, in order to arrive at a valid relationship of electrophoretic migration. The extent of friction as defined by stoke's equation, will depend upon: (i) the size and shape of the molecule, and (ii) on the viscosity of the medium through which the molecule will migrate. Thus,

Where ***F*** is the friction exerted on the spherical molecule, ***r*** is the radius of the molecule;

η is the viscosity of the solution, and v is the velocity at which the molecule is migrating. The frictional force will oppose the accelerating force generated by the electric field (Fig. 1). Equating the force of acceleration with Stoke's equation, we get

Rearranging the above relation, we get

It will thus be seen that the velocity (v) of the molecule is proportional to (i) the field strength (ΔE/d), and (ii) charge (*q*) on the molecule but is inversely proportional to (i) the particle size (*r*), and (ii) viscosity of the solution (η) .

TYPES OF ELECTROPHORESIS

Electrophoresis can be divided into two main techniques: *free electrophoresis* or electrophoresis without stabilizing media and *zone electrophoresis* or electrophoresis in stabilizing media.

(A) **Free electrophoresis:** Free electrophoresis has two main techniques: *micro-electrophoresis* and *moving boundary electrophoresis.* Both the techniques have now become obsolete but have a best historical significance. They are therefore discussed below very briefly.

1. **Microelectrophoresis:-** This electrophoretic technique involves the observation of motion of small particles in an electric field with a microscope (such as R. B. Cs, neutrophils, bacteria etc.). The suspension is contained in a closed system composed of a thin-walled section for optical observations and of suitable electrode compartments.

2. **Moving boundary electrophoresis:-** In this method, the electrophoresis is carried out in a solution, without a supporting media. The sample is dissolved, the buffer and molecules move to their respective counter charge electrodes. Moving

boundary electrophoresis is carried out in a U shape tube with platinum electrodes attached to the end of both arms (Figure 2). At the respective ends, the tube has a refractometer to measure the change in refractive index of the buffer due to presence of molecule during electrophoresis. Sample is loaded in the middle of the U tube and then the apparatus is connected to the external power supply. Charged molecules move to the opposite electrode during which they pass through the refractometer and a change in the refractive index of the solution is measured. As the desirable molecule passes, sample can be taken out from the apparatus along with the buffer.

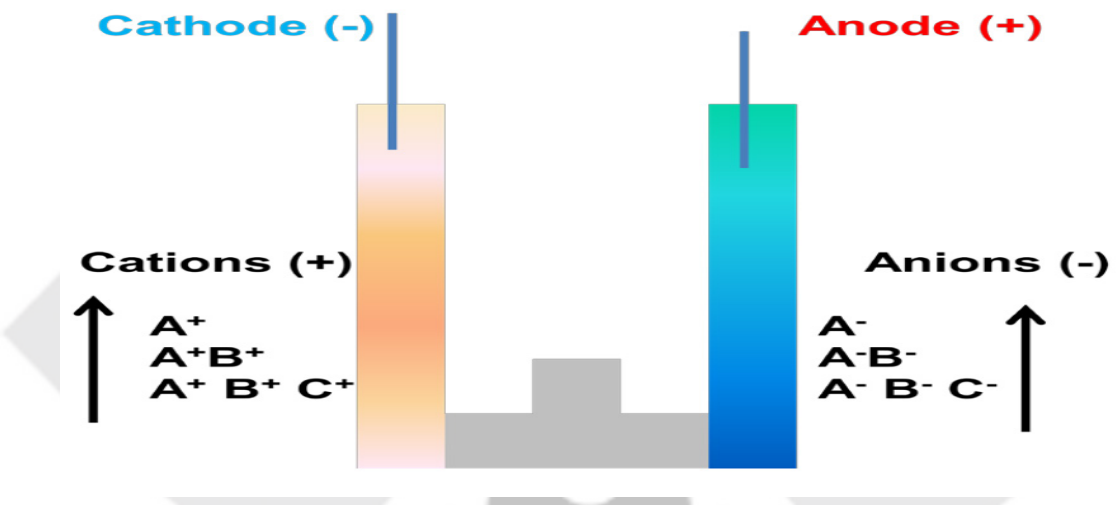

Figure 2: Movement of the charged particle in a moving boundary electrophoresis.

(B) Zone electrophoresis: In this method, an inert polymeric supporting media is used between the electrodes to separate and analyze the sample. The supporting media used in zone electrophoresis are absorbent paper, gel of starch, agar and polyacrylamide. The presence of supporting media minimizes mixing of the sample and makes the analysis and purification of the molecule from the gel much easier than the moving boundary electrophoresis. The gel electrophoresis is the best example of zone electrophoresis.

GENERAL TECHNIQUES OF ZONE ELECTROPHORESIS

I. **Paper Electrophoresis:** Paper electrophoresis is a commonly used electropho-

retic method for analysis and resolution of small molecules. This method is not used to resolve macromolecules (e.g., proteins) because the adsorption and surface tension associated with paper electrophoresis usually alter or denature the macromolecules, causing poor resolution. Two different methods are used routinely to apply samples to the electrophoretic paper. In the dry application procedure, a sample of solutes dissolved in distilled water, or a volatile buffer, is applied as a small spot or a thin strip of line drawn with a pencil called "origin line" on the paper. Appropriate standards of known compounds are applied at other locations on the origin line. If electrophoretic migration towards both poles of the system is anticipated, the origin line should be in the center of the paper. If migration in only one direction is anticipated, the origin line should be near one end of the paper. After the solvent containing the samples has evaporated, the paper is dampened with the electrophoresis buffer, either by uniform spraying or by dipping and blotting the ends of the paper so that wetting of the paper from both ends meets at the origin line simultaneously. In the wet application procedure, samples dissolved as concentrated solutions in distilled water are applied to paper predampened with electrophoresis buffer. The dry application procedure has the advantage of allowing small initial sample spots and better resolution of similarly mobile compounds. However, this method is awkward because the dipping or spraying requires considerable skill to avoid spreading of the applied samples. The wet application procedure is simpler to perform, but usually yields larger spots and poorer resolution because of sample diffusion. After the sample is applied to the moistened piece of paper, the paper is placed in the electrophoresis chamber so that both ends are in contact with reservoirs of the electrophoresis buffer at the electrodes (Fig. 3). If the origin line is not in the center of the paper, the paper must be positioned to allow maximum migration towards the correct electrode. After the chamber is covered or closed to protect against electric shock, an electric field is applied to the system. Application of the electric field and the resultant resistance to current flow in the buffered paper generates heat. This is

the greatest source of difficulty with paper electrophoresis. Heat dries the paper, which in turn leads to more resistance to current flow, which causes greater resistance, and so forth. Even if paper drying is prevented, heating will change the current flow and resistance properties of the system, which will distort the migration of molecules. Because of these difficulties, modern paper electrophoresis systems have been designed to compensate for potential heating problems. Most low voltage systems are portable and can be operated in cold rooms or refrigerated chambers. In contrast, most high-voltage systems either employ a cooled flat-bed system to dissipate the heat or operate in a cooled bath of inert and nonpolar solvent (e.g., Varsol, a petroleum distillate). This solvent absorbs the heat generated by the system without mixing with the water, buffers, or samples on the paper (Fig. 3). After electrophoretic resolution of the samples on the paper for the time and voltage required for optimal separation, the current is turned off, the paper is removed and dried, and the presence and location of the molecules of interest are determined.

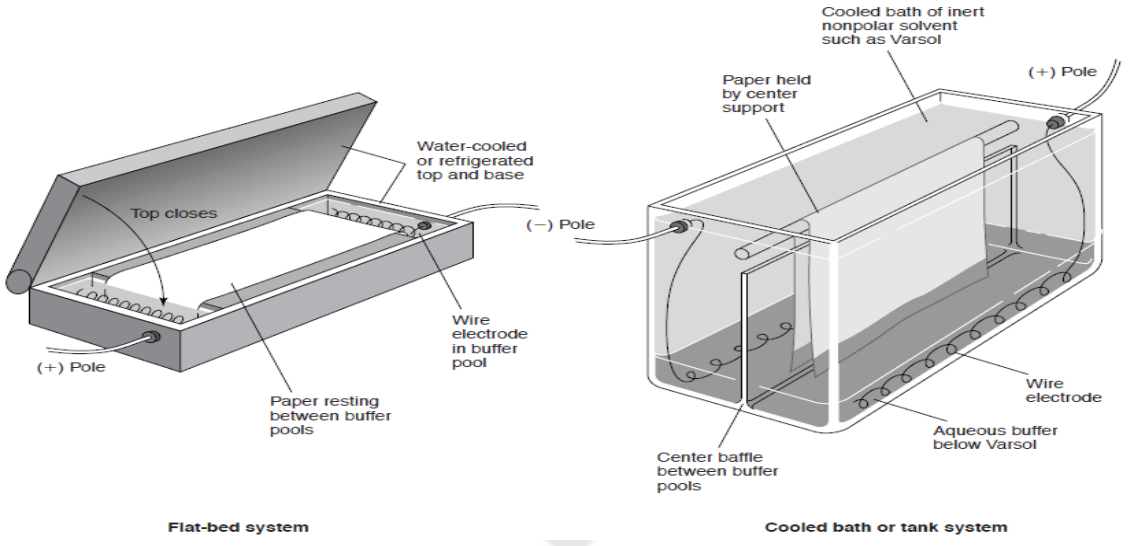

Figure 3. Two paper electrophoresis systems

II. Cellulose acetate electrophoresis:

Cellulose acetate as a medium for electrophoresis was introduced by Kohn in 1958. It was developed from bacteriological cellulose acetate membrane

filters and is commercially available as high purity cellulose acetate strips, which are thin and have a uniform micropore structure.

Although paper electrophoresis is still the choice for routine fast diagnostic analysis, the resolution of a given protein might suffer because of substantial adsorption on paper. This disadvantage of paper is completely taken care of if cellulose acetate strips are used instead of paper. Additional advantages of cellulose acetate are (i) it is chemically pure; it does not contain lignins, hemicelluloses or nitrogen, (ii) cellulose strips are translucent and this makes them suitable for direct photoelectric scanning for separated bands of components, (iii) because of the very low content of glucose these strips are suitable for electrophoresis of polysaccharides; upon staining by schiff's reagent, background staining is negligible. Cellulose acetate is not hydrophilic and thus holds very little buffer. This property is conducive for a better resolution in a shorter time. This lower buffer capacity of the strips, however,is responsible for greater heat production and the electrophoresis has to be carefully performed. Cellulose acetate is not suitable for preparative electrophoresis.

The buffers used for cellulose acetate electrophoresis are essentially the same as used for paper electrophoresis (Table 1). Solvents rendering the strips transparent are preferred rather than staining agents. Such solvents are glacial acetic acid, cotton seed oil, decalin, liquid paraffin etc. The transparency of the strip helps in direct photoelectric quantitative determination of the concentration of separated components without elution being involved.

Gel electrophoresis:

In all the types of electrophoresis discussed so far (free electrophoresis, paper electrophoresis and cellulose acetate electrophoresis) charge on the molecule was the major determinant for its electrophoretic mobility and ultimate separation from the rest of the molecules. The gels, however, are porous and the size of pores relative to that of

the molecule determines whether the molecule will enter the pore and be retarded or will bypass it. The separation thus not only depends on the charge of the molecule but also on its size. Needless to say that resolution of a sample is sharper and better in a gel than in any other type of medium.

In gel electrophoresis, molecules are separated in aqueous buffers supported within a polymeric gel matrix. Gel electrophoretic systems have several distinct advantages. First, they can accommodate larger samples than most paper electrophoretic systems, and so can be used for preparative scale electrophoresis of macromolecules. Second, the character of the gel matrix can be altered at will to fit a particular application. This is possible because the gel enhances the friction that governs the electrophoretic mobility. Low concentrations of matrix material or a low degree of cross-linking of the monomers in polymerized gel systems allow them to be used largely as a stabilizing or anti-convection device with relatively low frictional resistance to the migration of macromolecules. Alternatively, higher concentrations of matrix material or a higher degree of cross-linking of monomers are used to generate greater friction, which results in molecular sieving. Molecular sieving is a situation in which viscosity and pore size largely define electrophoretic mobility and migration of solutes. As a result, the migration of macromolecules in the system will be substantially determined by molecular weight. Many gel-like agents are used in electrophoretic systems. Agarose (a polygalactose polymer) gels have proven quite successful, particularly when applied to very large macromolecules such as nucleic acids, lipoproteins, and others. Polyacrylamide gels are among the most useful and most versatile in gel electrophoretic separations because they readily resolve a wide array of proteins and nucleic acids (Tables 2 and 3 shows how to prepare SDS-PAGE (sodium dodecyl sulphate- polyacrylamide gel electrophoresis) gels and polyacrylamide gels for low molecular weight nucleic acid samples).

Polyacrylamide gels are formed as a result of polymerization of acrylamide (monomer) and *N*,*N*′-methylene-bis-acrylamide (cross-linker) (Fig. 4). The acrylamide monomer and cross-linker are stable even when mixed in solution, but polymerize readily in the presence of a free-radical generating system. Biochemists use either chemical or

photochemical free-radical sources to induce the polymerization process.

Table 2: Polyacrylamide Gels with Various Percent Acrylamide Monomer for Use with SDS-PAGE.

	% Acrylamide in Resolving Gel				
Component (ml)	**7.5**	**10**	**12**	**15**	**20**
Distilled water	9.6	7.9	6.6	4.6	2.7
30% acrylamide solution	5.0	6.7	8.0	10.0	11.9
1.5 M Tris chloride (pH 8.8)	5.0	5.0	5.0	5.0	5.0
10% (w/v) SDS	0.2	0.2	0.2	0.2	0.2
TEMED	0.008	0.008	0.008	0.008	0.008
10% (w/v) ammonium persulfate	0.2	0.2	0.2	0.2	0.2

Prepare the ammonium persulfate fresh and add at last to induce the polymerization process (polymerization of the resolving gel will take approximately 30 min).

Table 3: Preparation of 15% Polyacrylamide gel for use in separating nucleic acids smaller than 500 bases in length.

Component	**Volume or Mass**
Urea	15 g
40% acrylamide solution	11.3 ml
10 × TBE buffer	3.0 ml
Distilled water	4.45 ml
TEMED	30 ml
10% (wt/vol) ammonium persulfate	200 ml

TBE: Tris/Borate/EDTA

In the chemical method (the most commonly used method), the free radical initiator, ammonium persulfate (APS), is added along with the *N*,*N*,*N*′-tetramethylethylenediamine (TEMED) catalyst. These two components, in the presence of the monomer, cross-linker, and appropriate buffer, generate the free radicals needed to induce polymerization. In the photochemical method (less widely used), ammonium persulfate is replaced

by a photosensitive compound (e.g., riboflavin) that will generate free radicals when irradiated with UV light. Many modifications can be made to produce a gel that will be useful for a particular application. If larger pores are required, you could decrease the amount of monomer and/or cross-linker in the polymerization solution. If smaller pores are required, one may increase the concentration of monomer and/or cross-linker. The pore size required for a particular electrophoretic separation will depend on the difference in size of the compounds that are to be resolved.

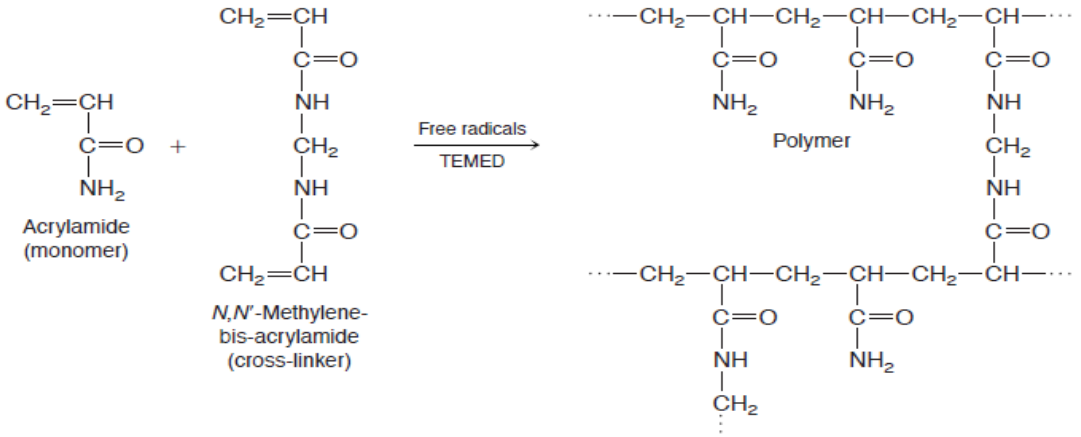

Figure 4: Formation of polyacrylamide gels

For instance, for resolving two small proteins of 8,000 Da and 6,000 Da, a small-pore-size gel is required (15–20% acrylamide). This same percent acrylamide gel would not permit the resolution of two larger proteins of say 150,000 Da and 130,000 Da. A larger pore-size gel would be required for this application (7.5–10% acrylamide). A list of the effective separation range of proteins on acrylamide gels made with various percent acrylamide is shown in Table 4. The method used to produce polyacrylamide gel must be followed exactly each time, since reproducible electrophoretic separations require uniform gel-forming conditions. There are many electrophoretic procedures and applications for polyacrylamide gels. Most of these employ the polyacrylamide gel in

some sort of "slab" format. One of the most commonly used of these procedures is discussed below.

Table 4: The effective separation range of polyacrylamide gels of various percent acrylamide monomers for use with SDS-PAGE.

% Acrylamide in resolving gel	Effective Separation range (Da)
7.5	45,000–200,000
10	20,000–200,000
12	14,000–70,000
15	5,000–70,000
20	5,000–45,000

Sodium Dodecyl Sulfate–Polyacrylamide gel electrophoresis (SDS-PAGE):

One of the most common means of analyzing proteins by electrophoresis is by using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. SDS is a detergent which denatures proteins by binding to the hydrophobic regions and essentially coating the linear protein sequence with a set of SDS molecules. The SDS is negatively charged and thus becomes the dominant charge of the complex. The number of SDS molecules that bind is simply proportional to the size of the protein. Therefore the charge to mass ratio should not change with size. In solution (water), in principle all different sized proteins covered with SDS would run at about the same mobility. However, the proteins are not run through water. Instead they are run through an inert polymer, polyacrylamide. The density and pore size of this polymer can be varied by variation in concentration of monomer and of cross-linking agent. Thus, the size of molecules that can pass through the matrix can be varied. This determines in what molecular weight range the gel will have the highest resolving power.

Sodium dodecyl sulfate gel electrophoretic systems are used to determine the number and size of protein chains or protein subunit chains in a protein preparation. Initially, the protein preparation is treated with an excess of soluble

thiol (usually 2-mercaptoethanol) and SDS. Under these conditions, the thiol reduces all disulfide bonds (–S–S–) present within and/or between peptide units, while the SDS (an ionic or denaturing detergent) binds to all regions of the proteins and disrupts most non-covalent intermolecular and intramolecular protein interactions. These two components result in total denaturation of the proteins in the sample, yielding unfolded, highly anionic (negatively charged) polypeptide chains (Fig. 5). The anionic polypeptide chains are then resolved electrophoretically within a polyacrylamide gel saturated with SDS and the appropriate current carrying buffer. The excess SDS is included in the gel to maintain the denatured state of the proteins during the electrophoretic separation. The SDS coated polypeptides (carrying approximately one SDS molecule per two amino acids) creates a situation in which the charge-to-mass ratio of all of the proteins in the sample is approximately the same.

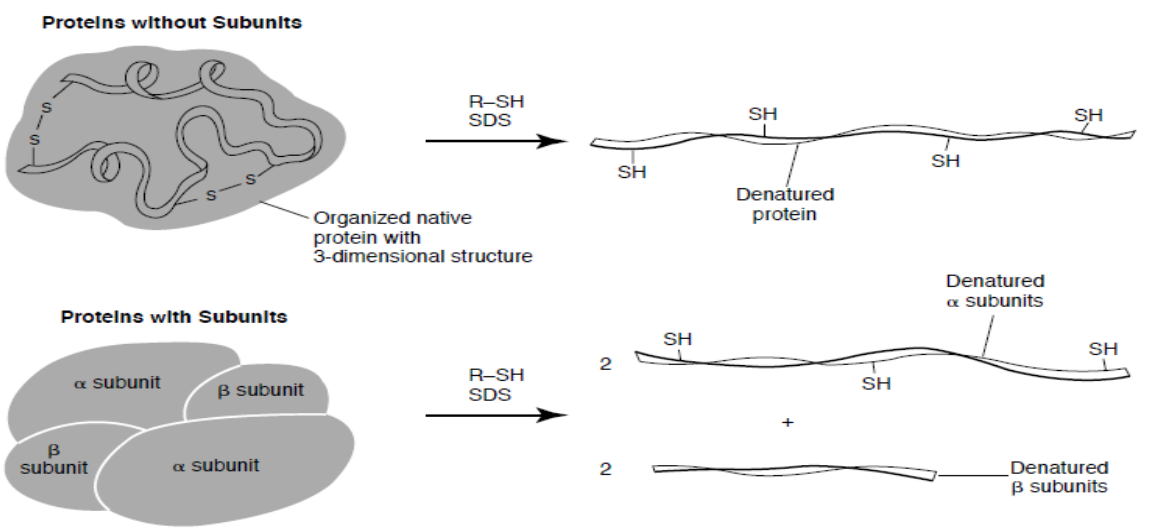

Figure 5: Disruption of proteins with excess thiol and SDS.

At this point, the intrinsic charge on the individual polypeptide chains (i.e., in the absence of SDS) is insignificant as compared with the negative charge imposed on them by the presence of the SDS. The friction experienced by the molecules as they migrate through the polyacrylamide matrix is now the major

factor influencing differences in their mobility. In addition, the friction experienced by the molecules during the separation is governed by the pore size of the polyacrylamide matrix (larger polypeptides will experience greater friction when passing through a gel of defined pore size and will migrate more slowly) than smaller polypeptides (that will migrate more rapidly). In summary, SDS-PAGE allows the separation of proteins on the basis of size. The principles underlying the most commonly used form of SDS-PAGE are best illustrated by a description of the step-by-step progress of a protein migrating in the gel. As shown in Figure 6 and Table 2.

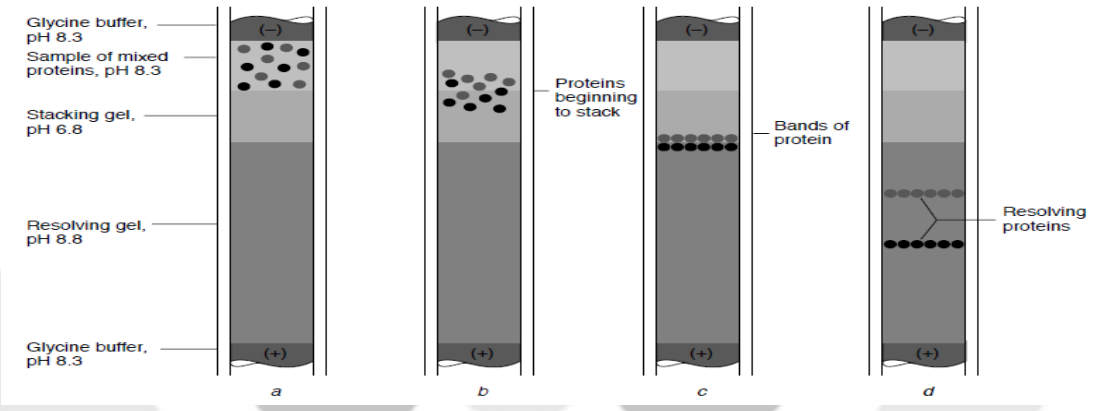

Figure 6: Migration of proteins through stacking and resolving gels during SDS-PAGE.

SDS-PAGE employs two buffer/polyacrylamide gel compositions in a single slab. These are referred to as the "stacking gel" and the "running (or resolving) gel." Protein samples are first introduced into wells, cast within the stacking gel after they are mixed with a viscous sample buffer (30% glycerol) containing SDS and thiols. After the samples have been loaded, voltage is applied to the system (current is carried through the gel, ~3–4 V/cm^2) between two separated pools of glycine buffer, pH 8.3 (Table 5). Thus, greater charge increases mobility, whereas greater size leads to greater friction and decreased mobility. Glycine carries an average charge of about 0.1 per molecule at pH 8.3 (running buffer) and almost no net negative charge at pH 6.8 (stacking gel pH). In contrast, the SDS-coated

proteins in the system carry a high negative charge that is essentially independent of the pH of the system. At low pH in the stacking gel, glycine anions lose negative charge and display decreased mobility in the system. In contrast, the chloride ions contained in the stacking gel migrate ahead of the proteins because of their small size (low friction) and more negative charge. Since the negatively charged proteins in the system have a larger frictional factor, they migrate into the stacking gel at a rate that is slower than that of the chloride ions, but faster than that of the virtually uncharged glycine anions. The resultant scale of anion mobilities in the low percentage acrylamide stacking gel (Cl^->proteins$^-$ >glycine$^-$) causes the proteins to accumulate a head of the advancing glycine front (Fig. 6*b*) and eventually stack into concentrated, narrow bands at the interface between the stacking gel and the running gel (Fig. 6*c*). Meanwhile, the chloride ions in the stacking gel readily migrate into the running gel. As the anions enter the running gel, the high concentration of pH 8.8 buffer and acrylamide (decreased pore size) in the gel triggers two events. First, the high pH buffer imparts a greater negative charge to the glycine anions, whose migration was retarded in the pH 6.8 buffer in the stacking gel. Because of the small size and increased anionic character, the glycine ions quickly overtake the proteins as they migrate through the system.

Second, the reduced pore size of the gel imparts a significant frictional component to the mobility of each individual protein present in the sample. The equal charge-to-mass ratio imparted on the proteins by the SDS present in the system now dictates that all of the proteins in the system will migrate through the gel on the basis of size. As shown in Figure 7, the relative mobility of each of the anionic polypeptide chains is a function of the logarithm of its molecular weight. If a set of polypeptides of known molecular weight are included with the sample during the electrophoretic separation, their relative mobilities may be determined and plotted as log of molecular weight versus relative mobility. The standard curve produced from this analysis of the polypeptides of known molecular weights can then be used, along with the relative mobilities of the unknown polypeptides in

the sample, to estimate their molecular weight (Fig. 7).

Table 6: Preparation of Buffers Used in Agarose Gel Electrophoresis of Nucleic Acids and SDS-PAGE

Tris/Borate/EDTA (TBE) Buffer (0.5× working solution)
Dissolve 5.4 g of Tris base and 2.75 g of boric acid in 700 ml of distilled water. Add 2 ml of 0.5 M EDTA, pH 8.0. Bring the final volume of the solution to 1 liter with distilled water.
Tris/Acetate/EDTA (TAE) Buffer (1× working solution)
Dissolve 4.84 g of Tris base in 700 ml of distilled water. Add 1.14 ml of glacial acetic acid and 2 ml of 0.5 M EDTA, pH 8.0. Bring the final volume of the solution to 1 liter with distilled water.
Electrophoresis Buffer for SDS-PAGE (1× working solution)
Dissolve 3.02 g of Tris base and 18.8 g of glycine in 700 ml of water. Add 10 ml of 10% (wt/vol) SDS and adjust the pH of the solution to 8.3 with HCl. Bring the final volume of the solution to 1 liter with distilled water.

In order to visualize the proteins that have been separated by SDS-PAGE, two methods are most often used. The choice between them depends largely on the sensitivity of detection that is required for your application. The first method involves saturating the gel with a solution of

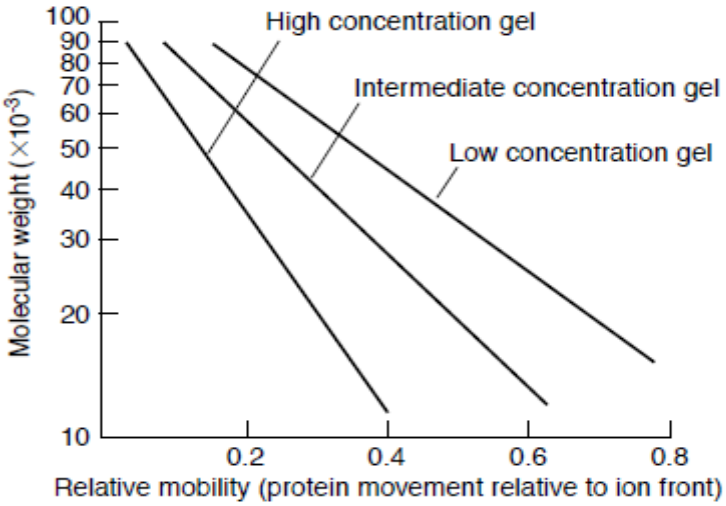

Figure 7: Relation between molecular weight and relative mobility of proteins on SDS gels.

acetic acid, methanol, and water containing *Coomassie Brilliant Blue R-250* dye. As the methanol and acetic acid in the solution work to "fix" the proteins within the gel matrix, *Coomassie Brilliant Blue* binds to the proteins in the gel. The interaction between the *Coomassie* dye and proteins has been shown to be primarily through arginine residues, although weak interactions with tryptophan, tyrosine, phenylalanine, histidine, and lysine are also involved. After the gel is "destained" with the same aqueous acetic acid/methanol solution without the dye (to remove the dye from portions of the gel that do not contain protein), the proteins on the gel are visible as dark blue bands on the polyacrylamide gel. When conducted properly, this method of detection is sufficiently sensitive to detect a protein band containing as little as 0.1 to 0.5 µg of a polypeptide. An alternative staining method, silver staining, is sensitive to about 10 µg of protein contained in a single band on the acrylamide gel. Silver staining begins by saturating the gel with a solution of silver nitrate. Next, a reducing agent is added to cause the reduction of Ag" ions to metallic silver (Ag), which precipitate on the proteins in the gel and cause the appearance of protein bands that are black in color. Silver staining is technically more difficult, and therefore is used only when extreme sensitivity is required.

SPECAILISED ELECTROPHORETIC TECHNIQUES

a) Isoelectric Focusing

Isoelectric focusing is an electrophoretic technique that separates macromolecules on the basis of their isoelectric points (pI, pH values at which they carry no net charge). As with SDS-PAGE, this process can be carried out in a "slab" format. A pH gradient is established in the polyacrylamide gel with the aid of ampholytes, which are small (~5000 Da) polymers containing random distributions of weakly acidic and weakly basic functional groups (e.g., carboxyls, imidazoles, amines, etc.). A polyacrylamide gel containing these ampholytes is connected to an electrophoretic apparatus that contains dilute acidic solution (H^+) in the anode chamber and a dilute basic (OH^-) solution in the cathode chamber (Fig. 8).

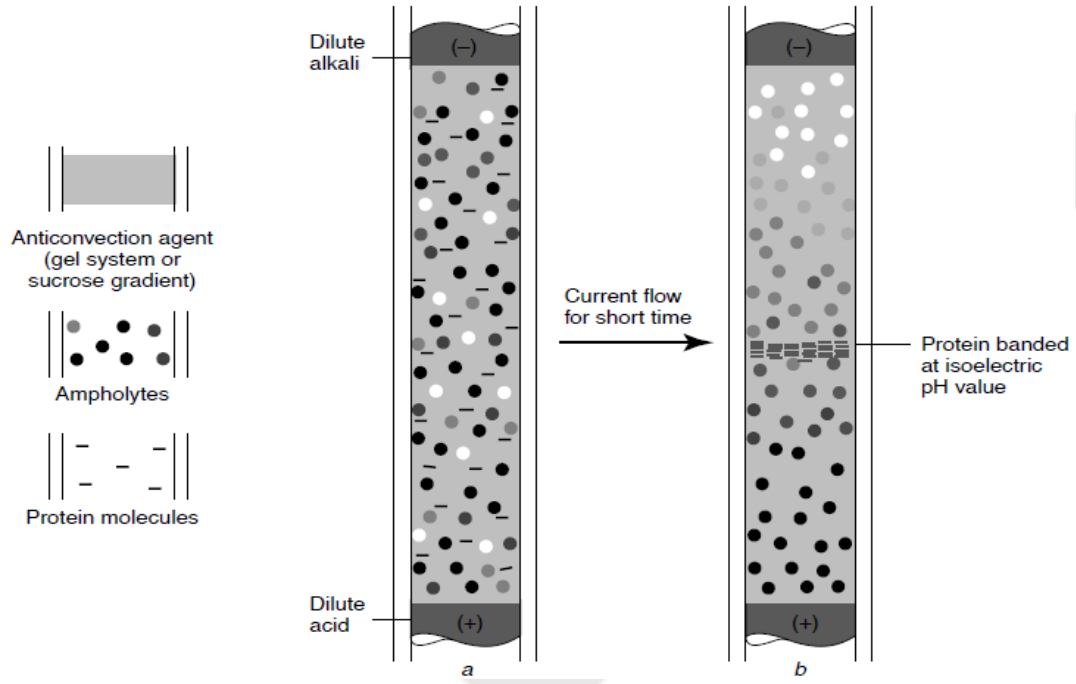

Figure 8: Steps of an isoelectric focusing procedure.

When voltage is applied to the system, the current flow will be largely due to migration of the charged ampholyte species present in the gel. The ampholytes migrate towards either pole in a manner consistent with their charge distributions; ampholytes with lower isoelectric points (e.g., those that contain more carboxyl groups and have a net

negative charge) migrate towards the anode, while ampholytes with higher isoelectric points (e.g., those that contain more amine groups and have a net positive charge) migrate towards the cathode. Eventually, each ampholyte in the system will reach a position in the gel that has a pH equal to its isoelectric point. When this occurs, these ampholytes carry no net charge and will no longer migrate in the gel. The net effect is that, after a sufficient period of electrophoresis, the population of ampholytes will act as local "buffers" to establish a stable pH gradient in the polyacrylamide gel. This pH gradient forms the basis for the separation of macromolecules that are present in the system (or are subsequently introduced into the system). Proteins present in the system act like ampholytes in that they migrate as a function of their net charge. As the ampholytes establish a pH gradient in the gel, the proteins in the system also migrate towards their respective poles until they reach a pH in the gel at which they too carry no net negative charge (the pI of the protein). At this point, the attractive force applied to the protein by the anode and cathode is equal, and the protein no longer migrates in the system. Thus, the different proteins in the sample are focused to particular portions of the gel where the pH is equal to their pI values.

The electrophoretic separation is complete when the ampholytes and proteins reach a pH value in the gel equal to their pI and no longer migrate. Once the proteins and ampholytes no longer migrate, the current (I) in the system decreases dramatically. Because isoelectric focusing is performed in the presence of fairly low concentrations of ampholytes (2–4%), high voltage potential (150 V/cm^2) can be applied to the system without the generation of excess amounts of heat caused from high currents. Since this method is designed to separate proteins on the basis of isoelectric point, one must take care not to impose a significant "friction factor" to the macromolecules being separated. In other words, the pore size of the gel should be large enough that all of the macromolecules in the system can migrate freely to their appropriate isoelectric points. If the pore size of the matrix limits the rate of migration of the proteins with higher molecular weights, the separation may turn out to be influenced more, or as much as, by size as by isoelectric point. If polyacrylamide gels are used for this purpose, the

concentration of acrylamide in the gel should not exceed 8%. If desired, isoelectric focusing can be performed using protocols that employ other "slab" matrix materials, such as agarose, or in a liquid matrix format that uses a sucrose density gradient as the anti-convection agent. The ampholytes are commercially available in a wide array of pH ranges that may be required for separation of a molecule of interest (e.g., pH 3–5, pH 2–10, pH 7–9, etc.).

In the past, isoelectric focusing has been performed in the "slab" format and employed largely as an analytical technique. More recently, systems have been developed that allow isoelectric focusing to be carried out in a preparative (large-scale, non-denaturing) format. Although the principles of the separation are the same as those described above, preparative isoelectric focusing is carried out using a liquid anti-convection agent rather than a gel slab.

b) Agarose Gel Electrophoresis:

Agarose gel electrophoresis is the principal technique used to determine the size of high-molecular-weight nucleic acids (DNA and RNA). Agarose is a long polymer of galactose and 3, 6-anhydrogalactose linked via □ (1□4) glycosidic bonds. This material is readily isolated from seaweed. Agarose polymers may contain up to 100 monomeric units, with an average molecular weight of around 10,000 Da. Agarose gels are made by dissolving the white agarose powder in an aqueous buffer containing EDTA and either Tris-acetate or Tris-borate as the buffering species (TAE or TBE buffer, respectively; Table 6). When the sample is heated to just below boiling point, the agarose powder dissolves in the buffer to form a clear solution. As the solution slowly cools to room temperature, hydrogen bonding within and between the polygalactose units in the solution causes the formation of a rigid gel with a relatively uniform pore size. The induction of this

polymerization event involves no chemical reaction, unlike the polymerization process described above for polyacrylamide gels. The pore size of the gel in polyacrylamide gels can be controlled by the percentage of the agarose dissolved in the solution. A high percent agarose gel (say, 3% wt/wt) will have a smaller pore size than a lower (0.8% wt/wt) agarose gel. The percent of agarose to be cast in the gel will be determined by the size of the various molecules to be resolved during electrophoresis; the smaller the molecular weight of the molecules to be resolved, the higher percent agarose (smaller pore size) the gel should contain. A list of the effective separation ranges with agarose gels of various percent agarose is shown in Table 7. Once the solution is heated to dissolve the agarose, the solution is cooled momentarily and poured into a slab mold fitted at one end with a Teflon or plastic comb. After the solution polymerizes, the comb is removed to create wells into which the desired DNA or RNA samples will be applied. The gel is then transferred to an electrophoresis chamber and is completely covered with the same TAE or TBE buffer that was used to cast the gel. The nucleic acid sample is then mixed with a viscous buffer (30% glycerol) containing one or more tracking dyes that are used to monitor the progress of the electrophoresis. *Bromophenol* blue dye will migrate at the same rate as a DNA molecule of about 500 base pairs, while *xylene cyanole* dye will migrate at the same rate as a DNA molecule of about 4000 base pairs. The nucleic acid samples are loaded into the wells of the gel, along with a sample of DNA fragments of known molecular weight (number of base pairs) in one of the wells. These standards will be used following the electrophoretic separation to aid in the determination of the size of the nucleic acid samples present in the unknown sample. Remember that the DNA is negatively charged and therefore, the cathode (negative electrode) should be connected to the side of the apparatus nearest the wells in the gel, while the anode (positive electrode) is connected to the opposite side of the apparatus. A voltage of about 4 to 6 V/cm is applied to the system (~50–70 mA of current), and the electrophoresis is continued until the bromophenol blue dye front reaches

the end of the gel.

Agarose gel electrophoresis, unlike SDS-PAGE, does not employ the use of a stacking gel. Since the nucleic acids in the sample have a much greater frictional component in the gel than they do in the buffer contained in the wells, the nucleic acids focus very quickly at the buffer–gel interface before entering the matrix.

Table 7: The effective separation range of Agarose Gels of various compositions for separation of nucleic acids

% Agrose (wt/vol)	Effective separation range (base pairs)
0.8	700-9000
1.0	500-7000
1.2	400-5000
1.5	200-3000
2.0	100-300

To visualize the nucleic acids following the electrophoretic separation, *ethidium bromide*, a fluorescent dye that has the ability to intercalate between the stacked bases of nucleic acid duplexes (i.e., the double helix of DNA) is used. After electrophoresis, the gel is placed in a solution of TBE or TAE buffer containing ethidium bromide, which diffuses into the gel and associates with the nucleic acids. Following a short destaining period in buffer without ethidium bromide (to remove it from the areas on the gel that do not contain nucleic acids), the gel is briefly exposed to ultraviolet (UV) light (256–300 nm), revealing the nucleic acid fragments as orange or pink fluorescent bands in the gel. The pattern of fluorescent bands is recorded by photographing the gel in a dark chamber while the gel is exposed to UV light from below. A filter is included to screen out UV light, so that most of the light that exposes the film is from the fluorescence of the nucleic acid bands that contain tightly bound ethidium bromide. The method of determining the molecular weight of an unknown nucleic acid sample is exactly the same as that described for the determination of protein molecular weight in SDS-PAGE. The log molecular weight of the nucleic acid samples of known sizes is plotted against

their relative mobilities to produce a standard curve. From the relative mobility of an unknown nucleic acid fragment on the same gel, the molecular weight and number of base pairs that the fragment contains can be readily determined. (A single base has an average molecular weight of approximately 320 Da, while a single base pair has an approximate average molecular weight of 640 Da).

c) Capillary Electrophoresis

Capillary electrophoresis (CE), can be used to separate ionic species by their charge and frictional forces and hydrodynamic radius. In traditional electrophoresis, electrically charged analytes move in a conductive liquid medium under the influence of an electric field (Fig. 10). Introduced in the 1960s, the technique of capillary electrophoresis (CE) was designed to separate species based on their size to charge ratio in the interior of a small capillary filled with an electrolyte.

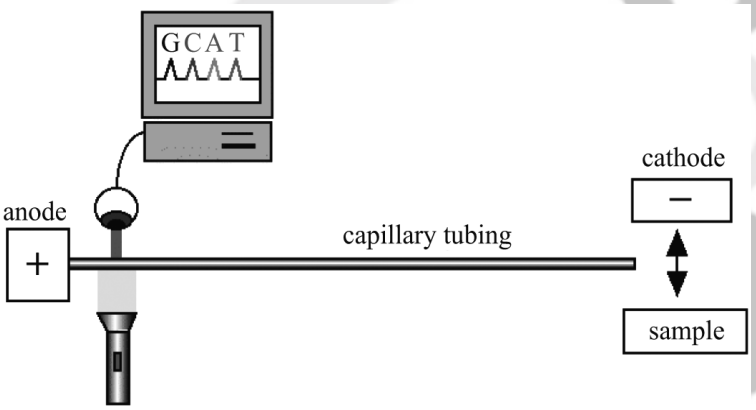

Fig. 10: Capillary Electrophoresis

Capillary electrophoresis offers the advantages of extremely high resolution, speed, and high sensitivity for the analysis of extremely small samples, but is obviously not useful as a preparative method. It is especially useful in the separation of DNA molecules that differ in size by as little as only a single nucleotide. Because of its high resolution, capillary electrophoresis is the basis of separation of polynucleotides in some of the newer designs of DNA sequences. Capillary electrophoresis can also be adapted to the

separation of uncharged molecules by including charged micelles of a detergent (such as SDS) in the aqueous electrophoresis medium.

d) Pulsed field gel electrophoresis

It is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction. While in general small fragments can find their way through the gel matrix more easily than large DNA fragments, a threshold length exists above 30–50 kb where all large fragments will run at the same rate, and appear in a gel as a single large diffuse band. However, with periodic changing of field direction, the various lengths of DNA react to the change at differing rates. That is, larger pieces of DNA will be slower to realign their charge when field direction is changed, while smaller pieces will be quicker. Over the course of time with the consistent changing of directions, each band will begin to separate more and more even at very large lengths. Thus separation of very large DNA pieces using PFGE is made possible.

Applications

- Electrophoresis plays a vital role in the separation of nucleic acids and proteins in the field of genomics and proteomics.
- Electrophoresis such as capillary electrophoresis can be used to analyze inorganic ions (Na, K, Ca and Mg) in foods like, pretzels, breadcrumbs, peanut butter, parsley and Parmesan cheese (Morawski *et al.,* 1993). It can also be used to analyze drinking water for the presence of nitrate and nitrite (Janini *et al.,* 1994) and chloride, sulfate, nitrate and fluoride (Roman0 & Krol, 1993; Rhemrev-Boom, 1994).
- It is used to separate □-, β- K-casein fractions from milk after treatment with different enzymes.
- It is used to characterize the globulin which is a salt soluble protein fraction of

cotton seed meals. It is also used to identify phytates in proteins

- Electrophoresis is used in forensics, molecular biology, genetics, microbiology and biochemistry. The assay can be used for qualitative as well as quantitative analysis.
- The separation of neutral molecules can be conducted efficiently by using this technique.