7.1 INTRODUCTION

Gel electrophoresis (GE) is a method that separates macromolecules on the basis of size, electric charge, and other physical properties. The term electrophoresis describes the migration of charged particles under the influence of an electric field. "Electro" refers to electricity and "phoresis," from the Greek word *phoros*, means "to carry across." Thus, GE refers to a technique in which molecules are forced across a span of gel, motivated by an electrical current. The driving force for electrophoresis is the voltage applied to electrodes at either end of the gel. The properties of a molecule determine how rapidly an electric field can move it through a gelatinous medium. The complete GE assembly along with the power pack is shown in Figure 7.1, whereas the various positions in which electrophoresis can be carried out, that is, upright, horizontal, and vertical, are shown in Figures 7.2 and 7.3.

Many important biological macromolecules (e.g., amino acids, peptides, proteins, nucleotides, and nucleic acids) possess ionizable groups and, at any given pH, exist in a solution as electrically charged species, either as cations (+) or anions (–). Depending on the nature of the net charge, the charged particles migrate either to the cathode or to the anode. For example, when an electric field is applied across a gel at a neutral pH, the negatively charged phosphate groups of DNA cause it to migrate toward the anode.

7.1.1 Structure of the Agarose GE Instrument

7.1.1.1 Components of the Agarose GE Instrument

The components of the agarose GE instrument are as follows (Figure 7.4):

Gel casting trays: Gel casting trays are available in a variety of sizes and comprise UV-transparent plastic. The open ends of the tray are closed with tape while the gel is being cast; the tape is removed prior to electrophoresis.

Sample combs: Molten agarose is poured around sample combs to form sample wells in the gel.

Agarose: A natural colloid extracted from seaweed, agarose is a linear polysaccharide (average molecular mass is ~12,000 Da) made up of the basic repeated unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose. Agarose gels have large "pore" sizes and are used primarily to separate large molecules with molecular masses greater than 200 kDa. Table 7.1 gives the recommended agarose gel concentration for resolving linear DNA molecules.

Electrophoresis buffer: The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. Several buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA (pH 8.0) and tris-acetate (TAE), tris-borate (TBE), or tris-phosphate (TPE) at a concentration of approximately 50 mM (pH 7.5–7.8). Electrophoresis buffers are usually prepared as concentrated solutions and stored at room temperature. TBE was originally used at a working strength of 1× for agarose GE.

Marker DNA: For a given voltage and agarose gel and buffer concentrations, migration distance depends on the molecular weight of the starting material. Therefore, marker DNA of a known size should be loaded into slots on both right and left sides of the gel. A marker generally contains a defined number of known DNA segments, which makes it easier to determine the size of the unknown DNAs if any systematic distortion of the gel occurs during electrophoresis.

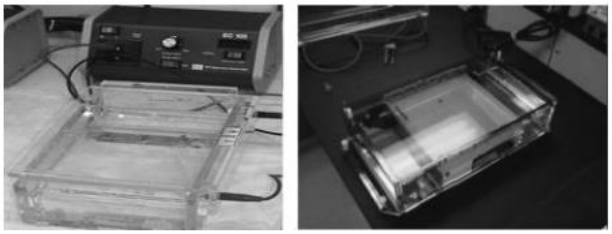


FIGURE 7.1 The GE apparatus.

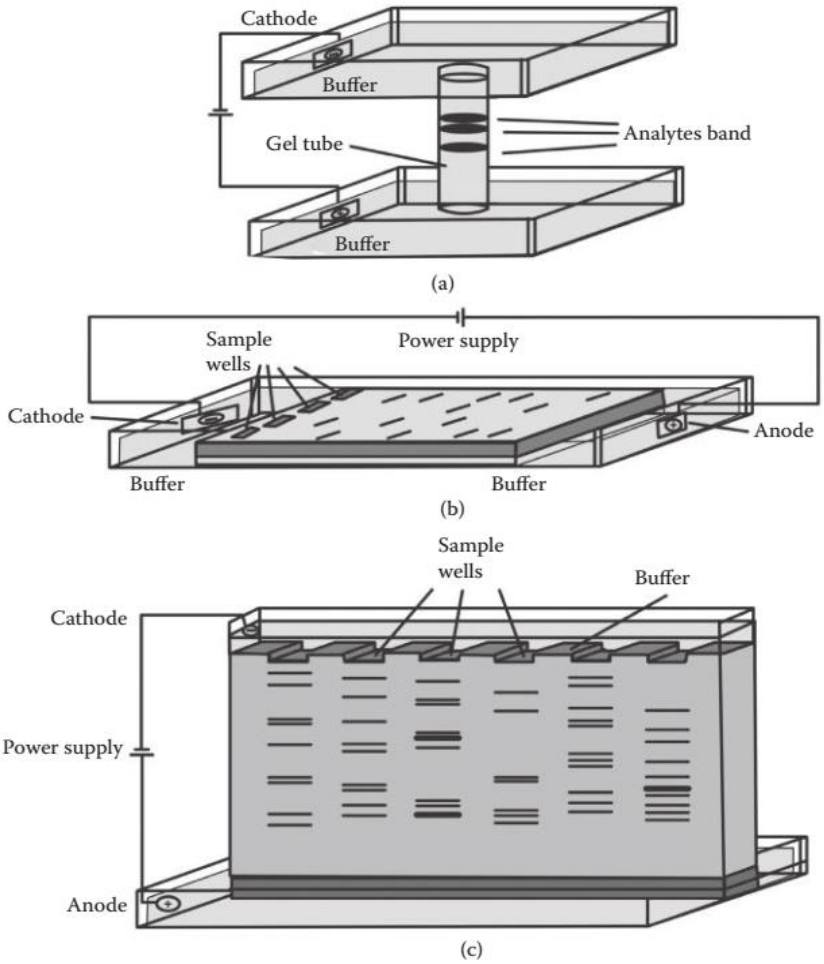


FIGURE 7.2 Schematically, GE can be performed in (a) an upright tube. Alternatively, flat rectangular slab gels can be used, which are positioned (b) horizontally or (c) vertically.

Loading buffer: The DNA samples to be loaded onto the agarose gel are first mixed with the loading buffer, which usually comprises water, sucrose, and a dye (e.g., xylene cyanole, bromophenol blue, bromocresol green). The loading buffer serves three purposes:

1. Increases the density of the sample, ensuring that the DNA drops evenly into the well.
2. Adds color to the sample, thereby simplifying the loading process.
3. Imparts a dye to the sample that moves toward the anode at a predictable rate in an electric field.

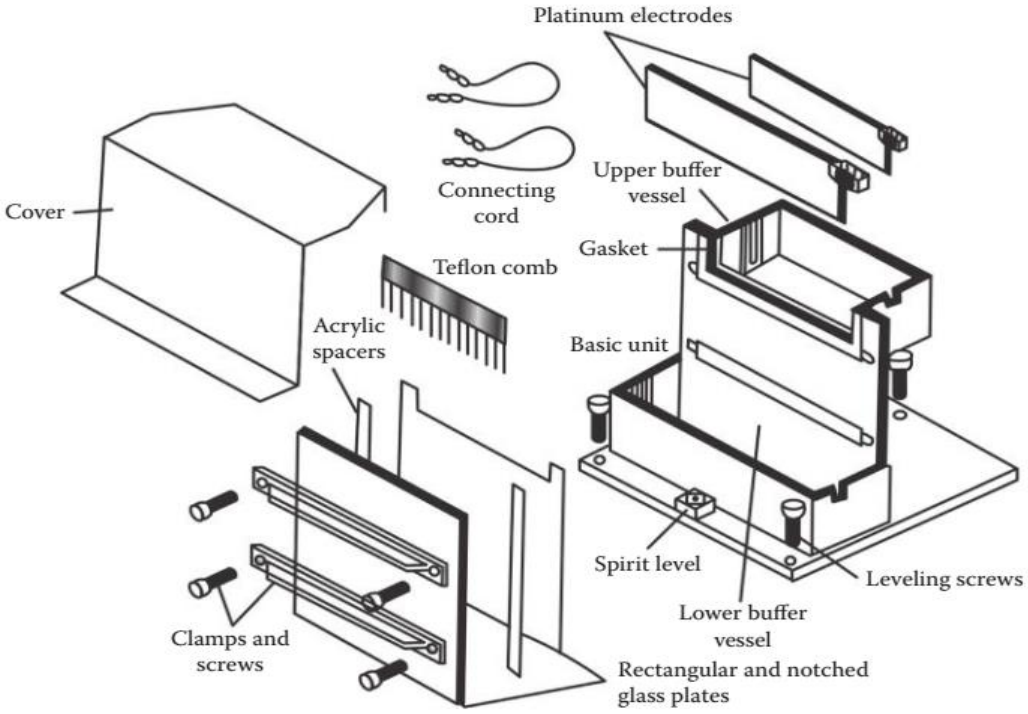


FIGURE 7.3 Vertical slab GE apparatus.

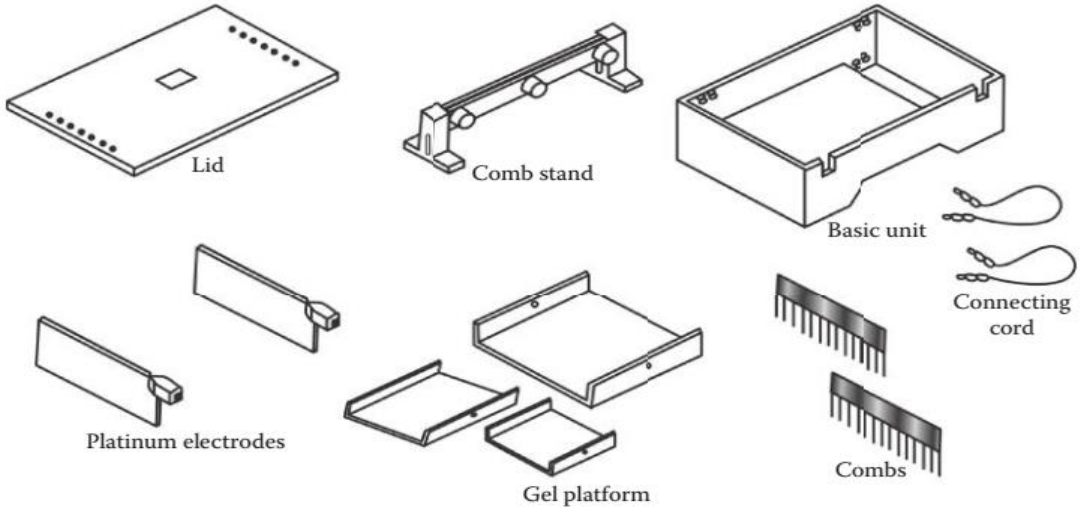


FIGURE 7.4 Parts of a submarine GE apparatus.

Ethidium bromide: Ethidium bromide is a fluorescent dye used for staining nucleic acids. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis; the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. Ethidium bromide is a known mutagen and should be handled as a hazardous chemical; wear gloves while handling it.

Transilluminator: A transilluminator is a UV light box, which is used to visualize ethidium bromide–stained DNA in gels.

TABLE 7.1
Recommended Agarose Gel Concentrations for Resolving Linear DNA Molecules

Agarose (%)	DNA Size Range Base Pair [bp]
0.75	10,000–15,000
1.0	500–10,000
1.25	300–5000
1.5	200–4000
2.0	100–2500
2.5	50–1000

7.2 PRINCIPLES OF GE

GE is a technique used for the separation of nucleic acids and proteins. The separation of macromolecules depends on two variables: (1) charge and (2) mass. When a biological sample, such as DNA, is mixed in a buffer solution and applied to a gel, these two variables act together. The electrical current from one electrode repels the molecules, whereas the other electrode simultaneously attracts the molecules. The frictional force of the gel material acts as a "molecular sieve," separating the molecules by size. The separation principle of electrophoresis is shown diagrammatically in Figure 7.5.

During electrophoresis, macromolecules are forced to move through the pores; their rate of migration through the electric field depends on the following:

- Strength of the field
- Size and shape of the molecules
- Relative hydrophobicity of the samples
- Ionic strength and temperature of the buffer in which the molecules are moving

To completely understand the separation of charged particles in GE, it is important to look at the simple equations relating to electrophoresis. When a voltage is applied across the electrodes, a potential gradient, E, is generated; it can be expressed by the following equation:

$$E = V/d \tag{7.1}$$

where V, measured in volts, is the applied voltage and d is the distance in centimeters between the electrodes.

When the potential gradient, E, is applied, a force, F, on a charged molecule is generated, which is expressed by the following equation:

$$F = Eq \tag{7.2}$$

where q is the charge in coulombs bearing on the molecule. It is this force, measured in newtons, that drives a charged molecule toward an electrode.

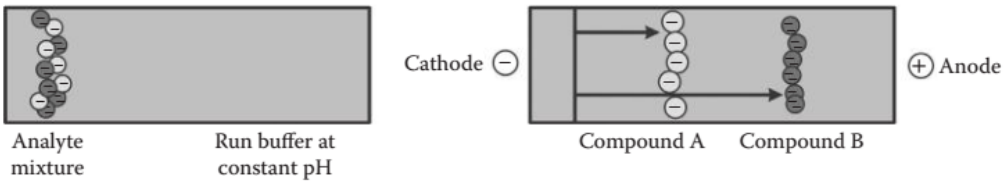


FIGURE 7.5 The separation principle of electrophoresis. Particles with different charges, in this case negative charges, and different sizes migrate toward the electrodes at different velocities in an applied electric field.

7.3 WORKING WITH THE ELECTROPHORESIS APPARATUS

A direct current (DC) power source is connected to the electrophoresis apparatus and an electrical current is applied. Charged molecules in the sample enter the gel through the walls of the wells. Molecules with a net negative charge migrate toward the positive electrode (anode), whereas net positively charged molecules migrate toward the negative electrode (cathode). Within a range, the higher the applied voltage the faster the samples migrate (EDVOTEK Manual). At the end of a run, the separated molecules can be detected in position in the gel by staining or autoradiography and quantified by scanning with a densitometer, and the gel can be dried for permanent storage.

7.3.1 Applications of GE

Major applications of GE are as follows:

- GE is used in forensics, molecular biology, genetics, microbiology, and biochemistry.
- The results can be analyzed quantitatively by visualizing the gel with UV light and a gel imaging device. The image is recorded with a computer-operated camera, and the intensity of the band or spot of interest is measured and compared against standard or markers loaded on the same gel. The measurement and analysis are mostly done with specialized software.
- Depending on the type of analysis being performed, other techniques are often implemented in conjunction with the results of GE, providing a wide range of field-specific applications (Figure 7.6).

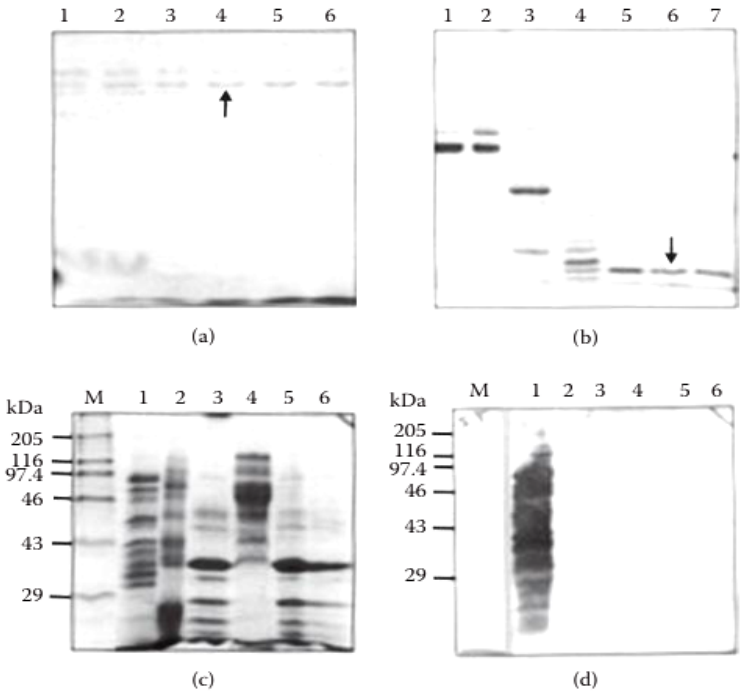


FIGURE 7.6 (See color insert.) Top: Protein profile of different fractions of metabolic antigens of *Aspergillus fumigates* obtained by size-exclusion chromatography on 12% SDS-PAGE stained with Coomassie blue: (a) Arrow shows 70–72 kDa protein doublet bands and (b) arrow shows 18 kDa purified protein. (c) Protein profile of metabolic antigens of *Aspergillus fumigates* precipitated by graded saturation of ammonium sulfate. (d) Identification of immunogenic proteins by Western blotting. Lane M, molecular weight marker; 1-, 2-, 3-, 4-, and 5-proteins precipitated at 20%, 40%, 60%, 80%, and 100% saturation of ammonium sulfate in culture filtrate, respectively.

7.7 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) was developed in the 1980s by James Jorgenson and Krynn Lukas. They separated derivatized amino acids in a tube of 75 μm inner diameter. The technique has variously been referred to as high-performance CE (HPCE), capillary zone electrophoresis (CZE), free solution CE (FSCE), and simply CE. The CE method (Figure 7.14) can be used to separate a wide spectrum of biological molecules including amino acids, peptides, proteins, DNA fragments (e.g., synthetic oligonucleotides), and nucleic acids as well as many small organic molecules such as drugs or even metal molecules.

7.7.1 Principles

CE is based on the same principle as GE. Charged analytes can be separated in an applied electric field based on their mobility. In contrast to GE, however, separations are carried out in a capillary with a small diameter containing a free solution of electrolyte rather than on a slab gel. Moreover, convective flows due to Joule heating occur more easily in a free solution than in a gel. In contrast to GE, electroosmotic flow is often part of the separation process.

7.7.2 Structure of the CE Instrument

The instrumentation required for CE is relatively simple, consisting of vials with samples and buffer, a high-voltage power supply, a capillary enclosed in a thermostatically controlled compartment, an on-column detector, and a data output system as well as a vacuum system for sample injection. The parts of a typical CE instrument are discussed in Sections 7.7.2.1 through 7.7.2.4.

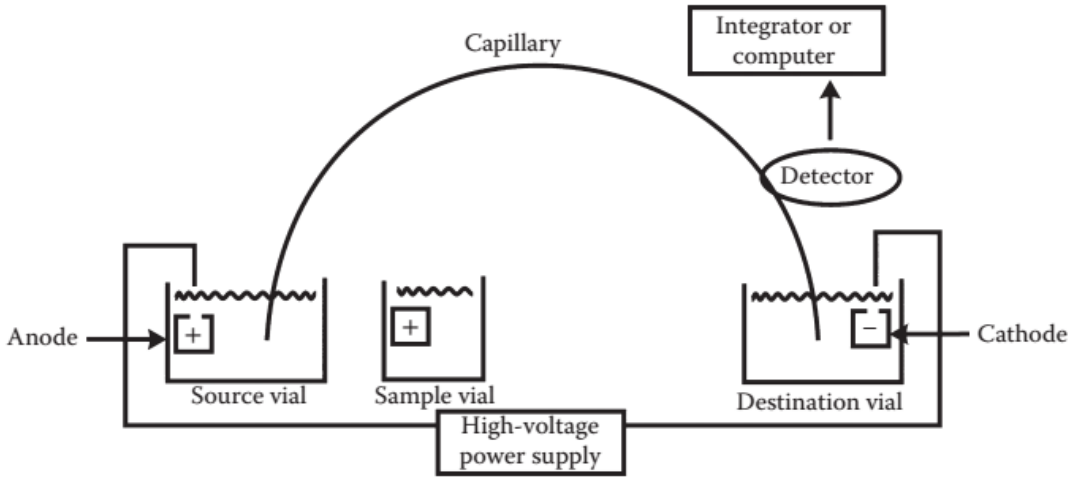


FIGURE 7.14 Schematic of a typical CE instrument.

7.7.2.1 Capillaries

The capillaries used in CE have internal diameters of 20–100 nm and outer diameters of about 400 nm. They are typically between 10 and 100 cm long. The most popular capillary material used is fused silica, that is, amorphous quartz, which is transparent to UV and visible light. These capillaries are externally coated with a polyimide layer of about 10 nm thickness to increase flexibility.

The capillary is usually enclosed in a thermostatically controlled environment for temperature control. This is because the viscosity of the buffer varies with temperature and Joule heating must be dissipated effectively to avoid temperature fluctuations, which can have dramatic effects on the efficiency and reproducibility of CE separations.

7.7.2.2 Buffer

A buffer is also referred to as a carrier electrolyte or background electrolyte. The purpose of a buffer is to maintain the pH as well as the conductivity during an electrophoretic separation. A controlled pH is crucial for maintaining a constant net charge on the biomolecules and thus maintaining their electrophoretic mobility. Buffer concentrations in CE are typically on the order of 10–100 mM.

7.7.2.3 Injection System

The injection system must be capable of reproducibly introducing very small sample volumes into the capillary. Two injection methods are commonly used:

1. In electrokinetic injection, voltages are used to introduce the sample into the capillary. The source ends of the capillary together with the source end of the electrode are placed into the sample solution. A high voltage is applied over the capillary between the sample vial and the destination vial for a given period of time. This causes the sample to move into the capillary according to its apparent mobility.
2. Hydrodynamic injection can be performed in three different ways: (1) In pressure injection, a precisely controlled external pressure is used to force a controlled amount of sample into the capillary. (2) In vacuum injection, a vacuum is applied to the buffer reservoir at the detector end of the capillary for a controlled period of time at a regulated reduced pressure. (3) For gravity flow injection, the sample vial with one end of the capillary is elevated to a certain height above the other end of the capillary for a given period of time. Gravity forces a sample plug into the capillary.

7.7.2.4 Detectors

Detection schemes used for CE include the measurement of UV absorption, fluorescence, and refractive index. Electrochemical signals and conductivity as well as radioactivity from radioisotopes have also been measured. The signals obtained are plotted against the migration time in the form of an electropherogram.

The detection of UV absorption at a chosen wavelength is the most commonly used scheme. Peptides are usually measured at $\lambda = 210$ nm, and proteins and DNA at $\lambda = 260$ nm or $\lambda = 280$ nm. The absorbance is measured directly through a detection window in the capillary, approximately 1 mm long.

7.7.3 Working with the CE Instrument

The source vial, destination vial, and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample and then returned to the source vial. The migration of analytes is then initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the

high-voltage power supply. The output of the detector is sent to a data output and handling device such as an integrator or a computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different retention times in an electropherogram.

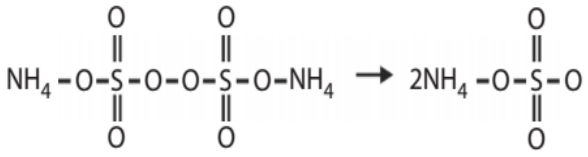
7.7.4 Applications

- CE can be used to quantify DNA. For example, CE analysis of PCR products from Human Immunodeficiency Virus-I [HIV-I] allowed the identification of between 200,000 and 500,000 viral particles per cubic centimeter of serum.
- A range of small molecules, drugs, and metabolites can be measured in physiological solutions such as urine and serum.
- Point mutations in DNA, such as those occurring in a range of human diseases, can be identified by CE.
- Chiral compounds can be resolved using CE. Most of the efforts to this end have been carried out in a free solution using cyclodextrins as chiral selectors.

8.7 Polyacrylamide Gel Electrophoresis

Electrophoresis in polyacrylamide gel is the most widely used technique for analysis and characterization of proteins and nucleic acids. The use of polyacrylamide gels has several advantages: it is chemically inert, gives superior resolution, and is amenable to the preparation of gels with a wide range of pore sizes and that the gels are stable over a wide range of pH, temperature, and ionic strength.

Acrylamide [CH_2–CH–$CONH_2$] is a white, crystalline substance. It is stable inert and nonreactive with sample molecules. Activation of acrylamide by free radicals causes its polymerization into a transparent viscous material. The ammonium persulfate (APS) is the initiator of the polymerization process. The homolytic rupture of O–O bonds produces two rather stable free radicals, each with an unpaired electron at the oxygen atom:



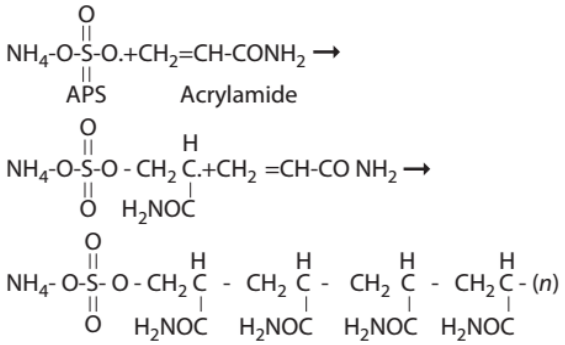


Figure 8.8 Acrylamide chain with *n* number of monomeric molecules.

This radical is added to the double bond of the acrylamide molecule to form another free radical at the carbon atom bearing an unpaired electron. This carbon-centered radical in turn attacks the double bond of another acrylamide monomer giving rise to a new free radical, and the process continues until all the available acrylamide molecules are chained together to form polyacrylamide and two free radicals react to form a covalent bond (Figure 8.8).

The cations have no influence on initiation of polymerization and potassium persulfate may be used equally well. The APS-initiated polymerized viscous acrylamide cannot form a suitable gel unless it is hooked together by a cross-linking agent. This is done by *N*,*N*′-methylene-bisacrylamide [$CH_2(NHCOCH{-}CH_2)_2$] commonly known as bis. One of the vinylic groups of bis is incorporated into the growing linear chain of acrylamide, and another (second) can be built into the other linear polymer chain, thus forming a cross-linkage (Figure 8.9).

Both acrylamide and bis are toxic substances affecting the skin and nervous system; hence, they should be handled with care and skin contact must be avoided. Solutions should be prepared under the hood and rubber gloves should be used. Polymerized substance is almost nontoxic.

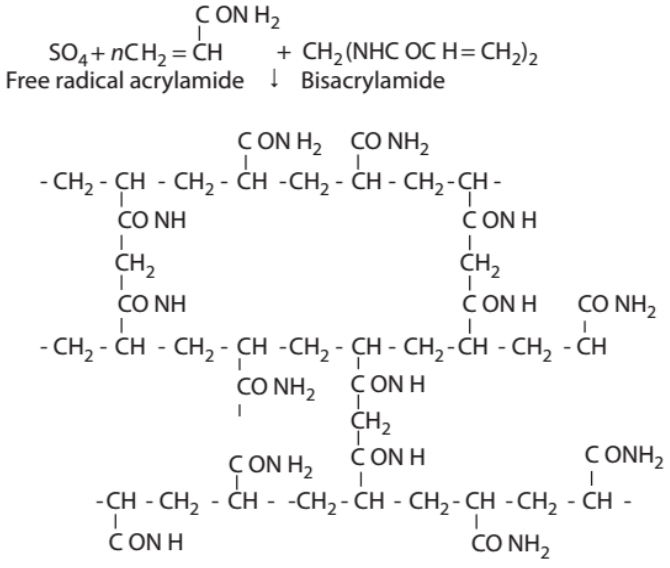


Figure 8.9 Vinyl polymerization.

Riboflavin may also be used as a more potent initiator of polymerization gel mixture in place of APS. Riboflavin is illuminated by a fluorescent lamp (445 nm) for 30–45 min. It is reduced to leucoflavin that is oxidized by dissolved oxygen with the formation of hydrogen peroxide. H_2O_2 decomposes to generate free radicals (HO) necessary for chain polymerization reaction.

The pore size in PAG depends on the concentration of the acrylamide and bisacrylamide and on the ratio of bis- to acrylamide. These two parameters can be critically controlled to get the desired pore size. Gels with high bisacrylamide content are fragile and nontransparent. They easily break away from the glass surface, show cracking during gel drying, and stain intensively. Gels ranging from 3% to 30% acrylamide concentration can be made, and these can be used for separation of molecules of size up to 1×10^6 D. A gel with a low percentage has larger pore size and is suitable for separation of high MW compounds, and a high-percentage gel has smaller pore size and is used for separation of relatively low MW compounds. Gradient gels with linear gradients of increasing acrylamide concentration give better resolution and are quite commonly used. Polyacrylamide gels can also be prepared by using (1) ethylene diacrylate (EDA) [CH_2=CHCOOCH_2=CH_2] and (2) *N*,*N*′-diallyltartardiamine (DATD) [CH_2=$CHCH_2$–NHCO–CHOH–CH_2CH=CH_2].

The gels formed by the previous cross-linking agents are free from the drawbacks as observed with high concentrations of bis. However, these gels can be solubilized. In EDA gels, the ester bonds can be cleaved by treatment with aqueous alkali or piperidine. Similarly, DATD gels are soluble in 2% aqueous periodic acid at room temperature within 20–30 min. EDA and DATD gels are used for some specific applications.

Tetramethyl ethylenediamine (TEMED) [$(CH_3)_2NCH_2CH_2N$)] is a colorless liquid. It is usually added along with APS at a concentration of about 0.4% in gel mixture to serve as a powerful catalyst of the polymerization process because it exists in active free radical form. Dimethylaminopropionitrile (DMPAN) [(CH3)2NCH_2CH_2N)] is a more potent catalyst than TEMED and requires three to four times less concentration.

8.9 SDS Gel Electrophoresis

8.9.1 Principle

Sodium dodecyl sulfate (SDS) (CH_3 $(CH_2)_{10}$ $CH_2OSO_3^-Na^+$) is a detergent that readily binds to proteins. At pH 7, in the presence of 1% w/v SDS and 2-mercaptoethanol, proteins dissociated into their subunits and bind large quantities of the detergent. Under these conditions, most proteins bind about 1.4 g of SDS/g of protein that completely masks the molecules; therefore, the greater the charge, so the electrophoretic mobility of the complex depends on the size (mol. wt) of protein, and a plot of log mol. wt against relative mobility gives a straight line (Figure 8.11). In this experiment, the MW of a protein is determined by comparing its mobility with a series of protein standards. The sieving effect of the polyacrylamide is important in these techniques, and the range of MWs that can be separated on a particular gel depends on the "pore size" of the gel. The amount of cross-linking and hence pore size in a gel can be varied by simply altering the amount of acrylamide to make 5% or 10% gels.

8.15 Isoelectric Focusing

Proteins are ampholytes, that is, they contain both positively and negatively charged groups. All ampholytes have the property that their charge depends on pH; they are positively charged at low pH and negatively charged at high pH. For every ampholyte, there exists a pH at which it is uncharged, and this is called the isoelectric point. At the isoelectric point, the ampholyte will not move in an electric field. If a protein solution is placed in a pH gradient, the molecules will move until they reach a point in the gradient at which they are uncharged; then they will cease to move. With a mixture of different proteins, each type of molecule will come to rest at a point in the pH gradient corresponding to its own isoelectric point. This method of separating proteins according to their isoelectric points in a pH gradient is called isoelectric focusing. The process of the migration of two different proteins with different isoelectric points is shown schematically in Figure 8.17.

The pH gradient is established in an unusual way. If it were established by simply allowing two buffers at different pH to diffuse into one another or by mixing two buffers in the way that is standard for preparing a concentration gradient, the resulting gradient would not be stable in an electric field because the buffer ions would migrate in the field; fractionation of the macromolecules could not occur because

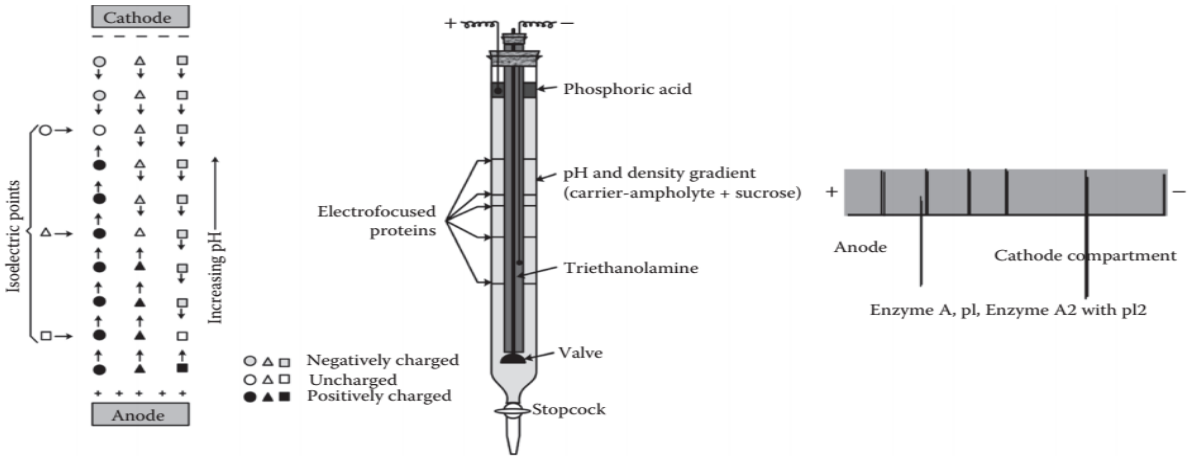
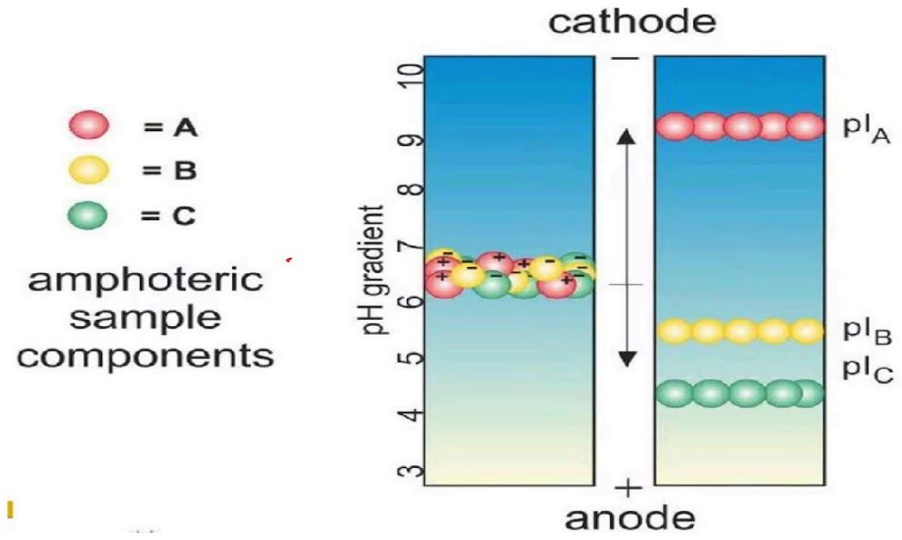


Figure 8.17 Isoelectric focusing.

the macromolecules would migrate much more slowly than the pH gradient is disrupted. The method used to produce a stable pH gradient consists of distributing a mixture of synthetic, low MW (300–600) polyampholytes (multicharged structures) that cover a wide range of isoelectric points (up to 1000 different isoelectric points/interval of 1 pH unit). These polyampholytes are usually mixed polymers of aliphatic amino and either carboxylic or sulfonic acids. They are commercially available as Ampholine, Pharmalyte, and BioLyte. A pH gradient is established by starting with a mixture in distilled water of polyampholytes having isoelectric points covering a range of either 2 or 7 pH units (depending on the resolution required). Before the application of an electric field, the pH throughout the system is constant and is averaged from all the polyampholytes in the solution. When the field is applied, the polyampholytes start to migrate. Because of their own buffering capacities, a pH gradient is gradually established. Soon each particle will come to rest in this self-established gradient at the point corresponding to its own isoelectric point. If the mixture contains proteins of different isoelectric points, they will migrate (but much more slowly) to the positions corresponding to their isoelectric points as long as the concentration and buffering of the protein (which is also a polyampholyte) is not so high that the pH gradient is disrupted.

For purification of a particular type of protein, the pH gradient is formed in a water-cooled glass column containing a cathode tube and a cathode. The tube is filled with a uniform concentration gradient to eliminate convection. The sample material is also contained in the polyampholytes suspension. The cathode tube is filled with a strong base (typically triethanolamine) and the main column is overlaid with phosphoric acid; the anode is in this acid layer. The valve at the bottom of the cathode tube is opened, followed by the application of a few hundred volts between the electrodes. The polyampholyte near the cathode will have a negative charge and will move to the anode. From 1 to 3 days later, the system will be at equilibrium and the proteins distributed throughout the pH gradient according



to their own isoelectric points. The tube is then drained and fractionated through the stopcock at the bottom. The various proteins can be detected by spectrophotometry, enzyme activity, or radioactivity.

A substantial improvement in the resolution of individual molecules is obtained by using as a supporting medium a continuous agarose or polyacrylamide gel rather than a sucrose concentration gradient. When this is done, a protein whose isoelectric points differ by 0.001 pH unit is readily separated. The single requirement of gel is that the pore size must be so large that molecular sieving is unimportant, and the isoelectric point is the single factor that determines the position of the band. This means using 2% polyacrylamide or, even better, 1% agarose. Tubular gels are sometimes used but, because of their greater capacity, horizontal slab gels are preferable. The gel initially contains the polyampholyte. The proteins may be present throughout the gel initially, but they are usually applied in a single zone as in ordinary gel electrophoresis. Following focusing, the bands are visualized by staining with Coomassie brilliant blue and Crocein scarlet. Narrow-range amplification can be obtained by using polyampholytes. This mode of isoelectric focusing is used to test the purity or composition of a sample.

8.16 Agarose Gel Electrophoresis

Agarose gels are more porous and have a larger pore size as compared to polyacrylamide gels and are, therefore, used to fractionate large macromolecules such as nucleic acids that cannot readily penetrate into and move through other types of supporting materials.

Agarose is a linear polymer of D-galactose and 3,6-anhydro-L-galactose. Agarose gels are cast by boiling agarose in the presence of a buffer, then poured into a mold, and allowed to harden to form a matrix. Porosity of gel is determined by the concentration of the agarose. The higher the agarose concentration, the smaller the pore size, and the lower the agarose concentration, the larger the pore size. When an electric field is applied across the gel, DNA molecules that are negatively charged at neutral pH migrate toward oppositely charged electrodes at rates determined by their molecular size and conformation. Since charge/mass ratio in nucleic acids is one, the rate of migration of DNA molecules is inversely proportional to $\log_{10}$ of the MWs, that is, smaller DNA molecules will travel faster as compared to the larger ones. Further, DNA molecules of the same size but with different conformations travel at different rates. The order of migration velocity in the increasing order of various forms of DNA is as follows: supercoiled DNA > linear double-stranded DNA > open circular DNA.

8.16.1 Apparatus

Both vertical and horizontal types of apparatus, for casting of agarose gels, are available. However, horizontal submarine or submerged gels (so named because the gel is immersed in the buffer) are more commonly employed (Figure 8.18).

Horizontal slab gels have advantages over vertical ones, in that low agarose concentration can be used as the entire gel is supported from beneath and also the processes of loading, pouring, and handling of gels are more convenient.

8.19.5 Two-Dimensional Electrophoresis

Isoelectric focusing and chromatography can be combined with SDS-PAGE, to obtain very high-resolution separations. A single sample is first subjected to isoelectric focusing or chromatography. This single-lane gel is then placed horizontally on top of an SDS-polyacrylamide slab. The proteins are thus spread across the top of the polyacrylamide gel according to how far they migrated during isoelectric focusing. They then undergo electrophoresis again in a perpendicular direction (vertical) to yield a 2D pattern of spots. In such a gel, proteins have been separated in the horizontal direction on the basis of isoelectric point and in the vertical direction on the basis of mass. More than a thousand different proteins can be resolved in a single experiment by 2D electrophoresis (Figures 8.24 and 8.25).