# Unit – 3 Electrophoretic techniques

**(Syllabus: Basic principles of electrophoresis, theory and application of paper, starch gel, agarose, native and denaturing PAGE, isoelectric focusing, capillary, microchip and 2 D electrophoresis).**

## Basic principles of electrophoresis

Electrophoresis is a method used to separate charged particles from one another based on differences in their migration speed.

The movement of particles under spatially uniform electric field in a fluid is called **electrophoresis**.

In 1807, Ferdinand Frederic Reuss observed clay particles dispersed in water to migrate on applying constant electric field for the first time. It is caused by a charged interface present between the particle surface and the surrounding fluid.

The rate of migration of particle depends on the strength of the field, on the net charge size and shape of the molecules and also on the ionic strength, viscosity and temperature of medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species and as a separation technique. It provides the basis for a number of analytical techniques used for separating molecules by size, charge, or binding affinity, example- for the separation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein molecules using an electric field applied to a gel matrix.

Gel matrix used mainly is polyacrylamide and agarose. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

In the course of electrophoresis, two electrodes (typically made of an inert metal, e.g. platinum) are immersed in two separate buffer chambers. The two chambers are not fully isolated from each other. Charged particles can migrate from one chamber to the other (Figure).

By using an electric power supply, electric potential (E) is generated between the two electrodes. Due to the electric potential, electrons move by a wire between the two electrodes. More specifically, electrons move from the anode to the cathode. Hence, the anode will be positively charged, while the cathode will be negatively charged. As mentioned above, the two electrodes are immersed in two buffer chambers. Electrons driven to the cathode will leave the electrode and participate in a reduction reaction with water generating hydrogen gas and hydroxide ions. In the meantime, at the positive anode an oxidation reaction occurs.

Electrons released from water molecules enter the electrode generating oxygen gas and free protons (which immediately form hydroxonium ions with water molecules). The amount of electrons leaving the cathode equals the amount of electrons entering the cathode. As mentioned, the two buffer chambers are interconnected such that charged particles can migrate between the two chambers. These particles are driven by the electric potential between the two electrodes. Negatively charged ions, called anions, move towards the positively charged anode, while positively charged ions, called cations, move towards the positively charged cathode.

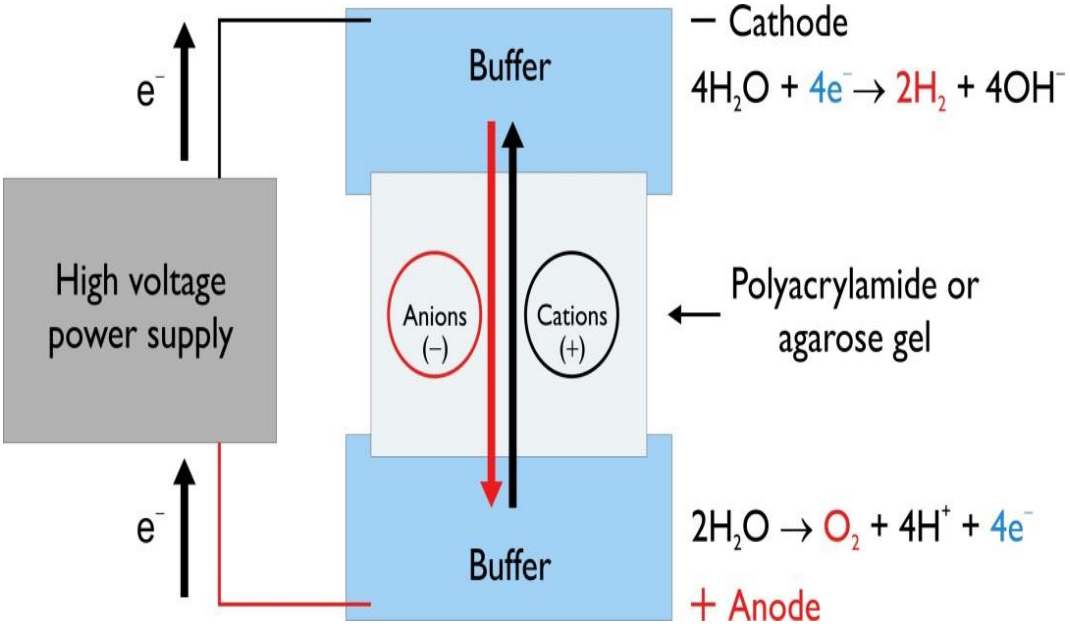


Figure. The principle of electrophoresis. In the course of electrophoresis, two electrodes are immersed in two separate buffer chambers. The two chambers are connected such that charged particles can migrate from one chamber to the other. By using a power supply, electric potential difference is generated between the two electrodes. As a result, electrons flow from one of the

electrodes, the anode, towards the other electrode, the cathode. Electrons from the cathode are taken up by water molecules of the buffer, resulting in a chemical reaction which generates hydrogen gas and hydroxide ions. In the other buffer chamber, water molecules transfer electrons to the anode an in another chemical reaction that generates oxygen gas and protons. (Protons are immediately taken up by water molecules to form hydroxonium ions.) As charged particles can migrate between the two chambers due to the electric potential difference, positive ions (cations) move towards the negatively charged cathode while negatively charged ions (anions) move towards the positively charged anode.

Different ions migrate at different speeds dictated by their sizes and by the number of charges they carry. As a result, different ions can be separated from each other by electrophoresis. It is very important to understand the basic physics describing the dependence of the speed of the ion as a function of the number of charges on the ion, the size of the ion, the magnitude of the applied electric field and the nature of the medium in which the ions migrate. By understanding these basic relationships, the principles of the many different specific electrophoresis methods become comprehensible.

Electrophoresis of positively charged particles (cations) is called **cataphoresis**, while electrophoresis of negatively charged particles (anions) is called **anaphoresis.**

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- **Paper electrophoresis**

**Principle**

A small amount of serum is applied on a Whatmann filter paper No. 1 strip previously soaked in buffer solution, the two ends of the strip dip in two compartments of an electrophoresis tank containing the buffer solution. A direct current is applied to the ends of the paper for a desired period.

During this time different fractions of the protein mixture separate into bands depending upon the amount of charge, the shape and size of the protein particles. After that electric current is disconnected, paper is removed from the tank, dried and stained with a protein stain to demonstrate different protein bands.

The fastest band is albumin, behind it are $\alpha_1$-globulin, $\alpha_2$-globulin, β-globulin and γ-globulin. The stained fractions are eluted in suitable solvent and the eluted colour is measured in a colorimeter.

**Two types of electrophoretic tanks are available:**

a. Vertical and

b. Horizontal.

a. In the vertical type, there are five compartments. The electrodes are present in two outermost compartments, which is connected to adjoining compartments by wicks. By this arrangement the pH change occurring in the electrode compartment is prevented from affecting the adjoining compartments in which the ends of the paper dip.

In the middle, there is a central compartment. The arrangement is shown in Fig. All compartments excepting the central one are filled by buffer.

b. In the horizontal type, paper lies in horizontal position supported by strings. The paper ends dip in two compartments on the two sides of the tank. These compartments are connected by means of wicks to adjoining compartments containing electrodes. Buffer is kept in all compartments to equal levels.

**Procedure:**

Pour sufficient buffer into the four compartments up to the same level, leaving the central compartment empty, cut Whatmann No. 1. paper into strips of 35 cm long and 5 cm wide. Mark the centre of the strip with a pencil and mark the point of application of sample by another line 2.5 cm in length parallel to and 5 cm from the mid-line (for the vertical tank) or 7.5 cm away from the mid-line for the horizontal tank.

Now apply the paper in such a way that the two ends of the paper dip in the two buffer compartments adjoining the electrode compartments. Replace the lid of the tank. In about 2 hours-time the whole paper is soaked with the buffer.

Now apply 10 µ1 of the serum (or plasma) by a micropipette on the line marked for sample application. Replace the paper in its place and cover the tank. At a time, several such strips can be set up for different samples. Now connect one electrode with the cathode and the other with anode of direct current with constant voltage.

The electrode nearer the site of application is connected to the cathode. Switch on the current and allow the electrophoresis to run for 16 to 18 hours. A voltage gradient of 3.2 volts per cm length of the paper, or a current of 1.5 to 2.0 milli amps for each 5 cm wide strip is suitable.

At the end of the desired length of time, first disconnect the instrument from the mains, remove the strip from the tank, and dry it at 100°C to 110°C for 30 minutes.

**Detection & Quantitative assay:**

To identify the unknown electrophorogram, compare the Unknown electog ram with standardelectrogram under standard conditions.Individual compou nds are usually identified by physical properties by the following methods.

**i) Fluorescence:**

a) Staining with "**Ethidium bromide**" and subsequent visualization of the electrophoreticgram under UV light makes DNA & RNA fluoresce and thus facilitates their detection.

b) **Flourescamine** staining is utilized for detecting amino acids, amino acid derivatives, peptides & proteins.

c) DANSYl chloride may be used in place of **fluorescamine.**

**ii) UV absorption:** Proteins, Peptides & nucleic acids absorb in the range of 260 to 280nm, this property these can be detect.

**iii) Staining:**

Pour sufficient stain in a large size glass or enamel tray and put the dried paper strips in it for at least 6 hours (overnight staining is always preferable). After removing from staining tray, wash for 6 minutes each time in two changes of wash solution, then in the fixative solution for a further 6 minutes. Dry the paper in air or in an oven at 100°C – 110°C.

After staining bands corresponding to albumin, $\alpha_1$, $\alpha_2$, $\beta$, and $\gamma$-globulins are noticed. It is possible to guess from the electro- phoretogram if some protein fraction is absent, present in decreased or increased amount, or some extra band such as myeloma protein is present.

However, the relative amount of different fractions can be accurately estimated by scanning the paper under photoelectric scanner or different bands

are cut and eluted in 6 ml of 0.01 N. NaOH for 30 minutes. These eluted coloured solutions are read in a photoelectric colorimeter at 540 mμ.

**Reagents:**

**1. Buffer (pH 8.6, ionic strength 0.083 M):**

Dissolve 3.12 grams of diethyl-barbiturate in water and make up to 1 litre.

**2. Dye solution:**

Add 25 ml of 95 per cent ethanol to 0.1 gram of bromphenol blue and 50 grams of $ZnSO_4$. Mix well, add 5 per cent acetic acid (V/V), mix to dissolve and make up to a litre with further 5 per cent acetic acid.

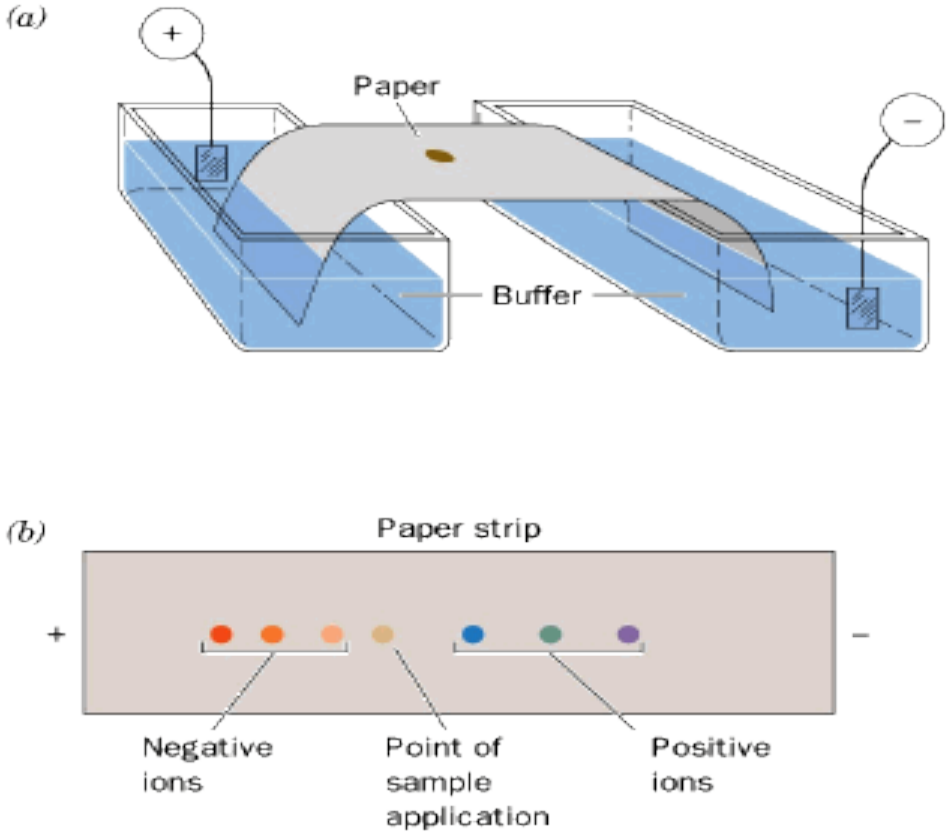
**3. Wash solution:**

Acetic acid (5 per cent, V/ V).

**4. Fixation solution:**

Acetic acid (5 per cent) containing 0.3 per cent of sodium acetate ($CH_3COONa$, $3H_2O$).

| Compound | Dye | Comments |
|---|---|---|
| Proteins | Bromophenol blue in acetic acid<br>DANSYl chloride<br>Fluorescamine | Visual, quantitative<br>Fluorescent, Quantitative<br>Fluorescent, Very sensitive |
| Nucleic acids | Methyl green-pyronine<br>Ethedium bromide | DNA-Blue, RNA-Red, Sensitive<br>Fluorescent, Very sensitive |
| Polysaccharide | Iodine | Visual, Sensitive |
| Lipoprotein | Sudan black in 60% ethanol | Visual, Sensitive |
| Glycoprotein | Alcian blue | Visual, Sensitive |

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❖ **Gel electrophoresis**

**Gel electrophoresis** is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge.

❖ **Starch gel electrophoresis**

Starch gel is one of a wide variety of supporting media that can be used for horizontal zone electrophoresis. Such gels are prepared by heating and cooling a quantity of partially hydrolyzed starch in an appropriate buffer solution. The choice of buffer is somewhat empirical and a wide variety of compositions have been used successfully. Starch gel electrophoresis method

for separation of Hb Fractions at alkaline pH is still valuable under many circumstances. Because of the low price is applicable in laboratories with limited resources.

**Reagents**

1. Acetic Acid,
2. Boric acid,
3. Sodium chloride
4. TRIS
5. Hydrogen peroxide 30% ($H_2O_2$).
6. 3,3'-Dimethoxybenzidine (o-Diansidine) ($C14H16N_2O_2$).
7. Ethylene Diamine Tetra Acetate (Na2EDTA).
8. Sodium Acetate Trihydrate ( $CH_3COONa\text{-}3H_2O$)
9. Methanol, 96%, technical.
10. Saponin, white pure.
11. Starch (potato), hydrolysed Smithies (Stärke nach Smithies, Carl Roth GmbH),
12. ($C_6H_{10}O_5$) Brunschwig.

**Solutions**

1. **Smitthies-buffer (stock)**

   For 2 Liter: 219 g Tris, 11.68 g $Na_2EDTA$, 70 g boric acid, pH = 8.6. Adjust pH with boric acid.

2. **Smitthies Gel buffer:**

   Dilute the Smitthies-buffer 1:20; by adding 12.5 ml of Smitthies-buffer to 250 ml distilled water.

3. **Smitthies running buffer:**

   Dilute the Smitthies-buffer 1:7; by adding 50 ml of Smitthies-buffer to 300 ml distilled water.

4. **Saponin Lysis buffer**

   For 0,5 liter: 2,2 gram TRIS, 0.5 g Boric Acid, 0,136 g $Na_2EDTA$, 1.5 g Saponin, 0.5 g KCN. Adjust to pH 8.6 with Tris or boric acid.

5. **Sodium Acetate, 1 M**

Add 272 g. of Na-acetate ($CH_3COONa\text{-}3H_2O$) in 1700 distilled water. Adjust to pH 4.7 with 80-90 ml Acetic Acid.

6. **Phosphate Buffered Saline**

   Add 7 g NaCl to 1 L of 0.1 M phosphate buffer pH 7,4.

7. **Staining solution**

   Add under fume cupboard a 'tea spoon' of dimethoxybenzidine to 150 ml 96% technical methanol, 50 ml sodium-acetate and 2 ml $H_2O_2$. Mix for 15 minutes. This staining solution can only be used once.

## Preparing a starch-gel

Home made glass plates are needed, provided with thin glued edges 3mm high of the same material. Plates of 16 x 15 cm are suitable for an average of 10-15 samples per run.

1. Purchase a cooling plate to accommodate one or more glass plates.
2. Place the empty tray on the cooling system kept at $5^oC$.
3. Weigh 22.7 g of Starch and transfer it to a flame resistant round bottom flask.
4. Prepare and add the Smitthies gel buffer to the starch.
5. Mix well before heating on a flame, and keep heating while shaking vigorously until bubbles appear.
6. When the boiling point is almost reached connect the flask to a vacuum system and keep the flask for 30 seconds shaking under vacuum until air bubbles are removed.
7. Pour the gel carefully of the glass tray from the center to the corners.
8. Let the gel solidify for 30 – 40 minutes before loading the samples.

## Preparation of the samples

1. Mix the blood sample kept in the refrigerator by inverting the tube a few times
2. Pipet 200 ul of blood into a tube filled with PBS.
3. Centrifuge for 5 minutes at 3000 rpm.
4. Remove the PBS by vacuum suspiration.
5. Add 100 ul of saponin lysis buffer to the pellet.

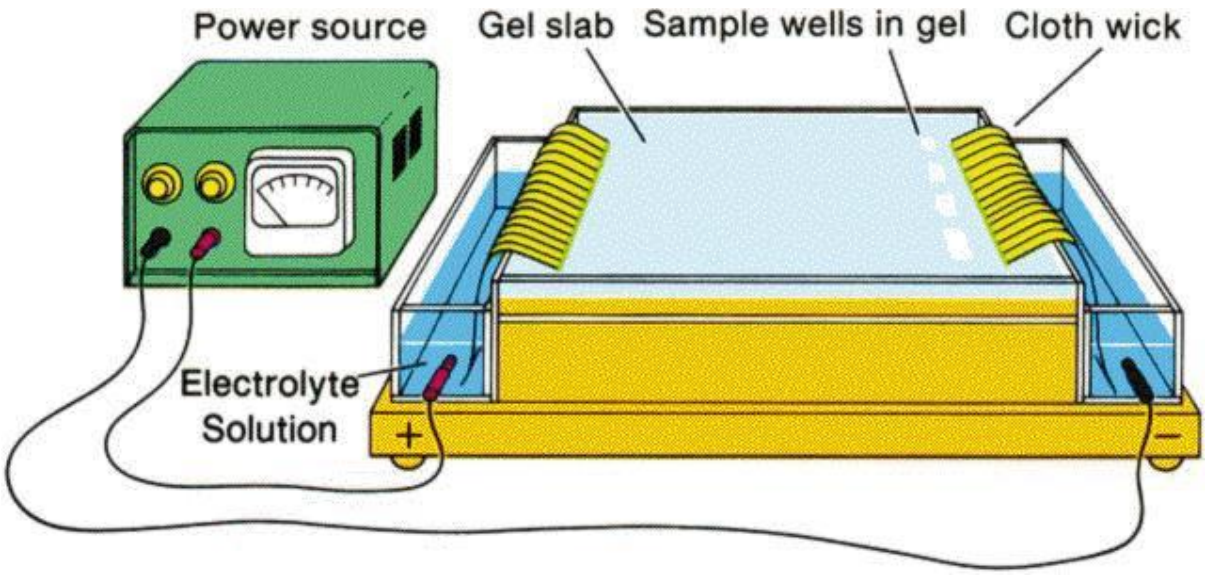
Vortex the tubes several time, until the lysis of the erythrocytes is complete.

## Loading and running of the starch-gel

1. Cut, with a scalpel and a ruler, a straight incision across the width of the gel, 3.5 cm from the edge.
2. Cut for every sample a loading paper of convenient proportions (3MM Whatman).
3. Take a loading paper with a pair of tweezers and immerge it in the tube containing the lysate.
4. Remove the excess of lysate by placing the soaked paper on a sheet of filter paper for a few seconds.
5. Place with the tweezers the loading papers in the incision. Ensure yourself that the loading paper doesn't touch the bottom of the gel tray and doesn't protrude above the gel surface. If necessary adjust the loading paper by cutting with a pair of scissors.
6. Fill the 2 buffer tanks with the running buffer.
7. Apply the shammy bridges (see figure 1) and place a glass cover of the gel plate and on the bridges.
8. Connect the power supply with the electrodes of the buffer tanks. The samples migrate from (-) to (+) pole.
9. Place the safety cover over the electrophoresis equipment and switch on the power supply.
10. Run for 4 – 6 hours at 0.3 kV and 7 mA, until the Hb A-band is 2 cm away from the upper bridge. If case a faster moving band is visible, end the run when this band is about 2 cm away from the bridge.

## Staining of the gel

1. Prepare the staining solution, under a flow cabinet.
2. Switch off the power supply and the cooling system.
3. Remove the loading papers from the gel, using tweezers.
4. Place the cover glass back on the gel, separate the upper part of the gel from mthe main body by cutting the gel with a steel wire leaning on the edges of the plate and remove the upper part of the gel.
5. Free the gel from the edges of the plate using the tip of a Pasteur pipet.
6. Place the plate in a staining tray and add the staining solution (use glows).
7. Remove the staining solution after about 20 minutes (use glows).
8. Rinse once with 200 ml of 50% methanol dry the starch surface with tissue paper and make a picture with the digital camera (use glows).
9. Keep the gel in 200 ml 50% methanol. Stained starch electrophoresis gels can be kept for over a one month and subsequently discarded as chemical waste.

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❖ **Agarose gel electrophoresis**

**Agarose gel electrophoresis** is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and/or size (isoelectric focusing agarose electrophoresis is essentially size independent), and the DNA and RNA fragments by length.[1] Biomolecules are separated by applying an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix.

**Principle**

Electrophoresis is a process which enables the sorting of molecules based on size. Using an electric field, molecules (such as DNA) can be made to move through a gel made of agarose. The electric field consists of a negative charge at one end which pushes the molecules through the gel, and a positive charge at the other end that pulls the molecules through the gel. The molecules being sorted are dispensed into a well in the gel material. The gel is placed in an electrophoresis chamber, which is then connected to a power source. When the electric current is applied, the larger molecules move more slowly through the gel while the smaller molecules move faster. The different sized molecules form distinct bands on the gel.

Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins. Gel electrophoresis can also be used for separation of nanoparticles.

**Requirements**

**1. Buffers and Solutions:**

Agarose solutions.
Ethidium bromide.
Electrophoresis buffer.

2.

**1.** An electrophoresis chamber and power supply. **Gel casting trays,** which are available in a variety of sizes and composed of UV-transparent plastic.

**2. Sample combs,** around which molten agarose is poured to form sample wells in the gel.

**3. Electrophoresis buffer,** usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).

**4. Loading buffer,** which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, Bromophenol blue and xylene cyanol dyes which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.

5. Agarose gel. For this usually 1 to 2 grams of agarose is added to 100ml of electrophoresis buffer.

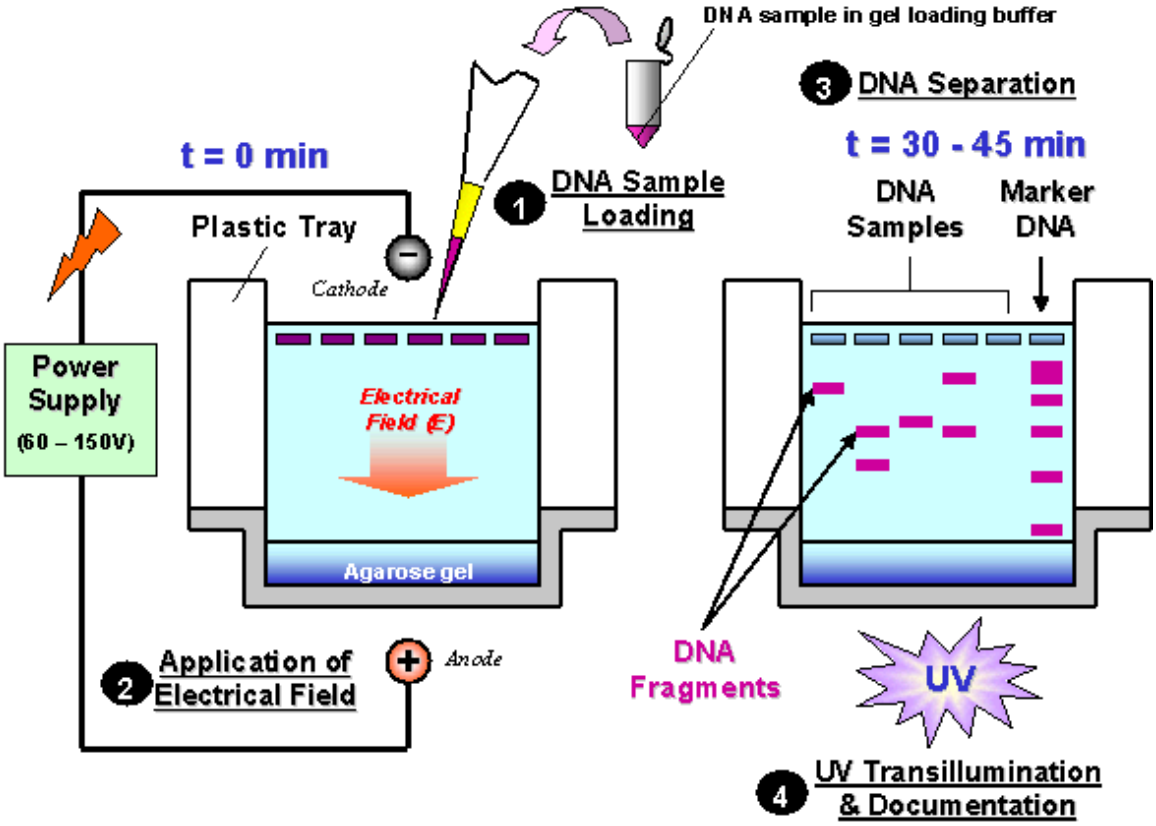
6. **Nucleic Acids and Oligonucleotides:** DNA samples. DNA Ladders.

**7. Ethidium bromide,** a fluorescent dye used for staining nucleic acids.

**8. UV light box / Transilluminator**, which is used to visualize ethidium bromide-stained DNA in gels.

## Procedure

1. To pour a gel, 1 gm agarose powder is mixed with electrophoresis buffer and then heated until completely melted. Most commonly, Ethidium bromide is added to the gel (final concentration 0.5 μg/ml) at this point to facilitate visualization of DNA after electrophoresis.
2. After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.
3. After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer.
4. Samples containing DNA mixed with loading buffer are then pipeted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied (50 V). You can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.
5. The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gel at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively.
6. When adequate migration has occurred, DNA fragments are visualized by staining with Ethidium bromide. This fluorescent dye intercalates between bases
7. of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of Ethidium bromide.
8. To visualize DNA or RNA, the gel is placed on a ultraviolet transilluminator. We can see orange bands of DNA.

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**Polyacrylamide gel electrophoresis (PAGE)**

Polyacrylamide gels can be used for the separation and analysis of proteins and relatively small nucleic acid molecules. For example, when it was first invented, Sanger's DNA sequencing method applied PAGE to separate linear single-stranded DNA molecules based on their length.

The resolution of the PAGE method is so high that, in the size range of about 10-1000 nucleotide units, it is capable of separating DNA molecules that differ in length only by a single monomer unit. In the case of single-stranded DNA, individual molecules are separated solely based on their length. This is due to the fact that, in the case of DNA (or RNA), the number of negative charges is a simple linear function of the number of monomer units (i.e. the length of the molecule).

In other words, the specific charge (number of charges per particle mass) is invariant, i.e. it is the same for all DNA molecules. It is so because each monomer unit has one phosphate moiety that carries the negative charge. When

an appropriate denaturing agent, such as urea, is added to the DNA sample and the gel is heated, the shape of the varying-length linear DNA molecules becomes identical. As a consequence, denatured molecules will be separated exclusively based on their size.

There are several PAGE methods

SDS-PAGE,

isoelectric focusing,

2D PAGE

At a given pH, different proteins carry different amounts of electric charge. Moreover, different proteins have different shapes and sizes, too. Consequently, during electrophoresis, proteins are separated by a complex combination of their charge, shape and size. PAGE separation of proteins provides high resolution. However, as three independent molecular properties simultaneously influence electrophoretic mobility, it will provide limited room for precise interpretation. For example, when two proteins are compared, it remains hidden what makes one of them migrate faster: a larger number of electric charges, a smaller size, or a more spherical shape. Nevertheless, even the simplest PAGE method, which will be referred to as native PAGE, provides many particular advantages.

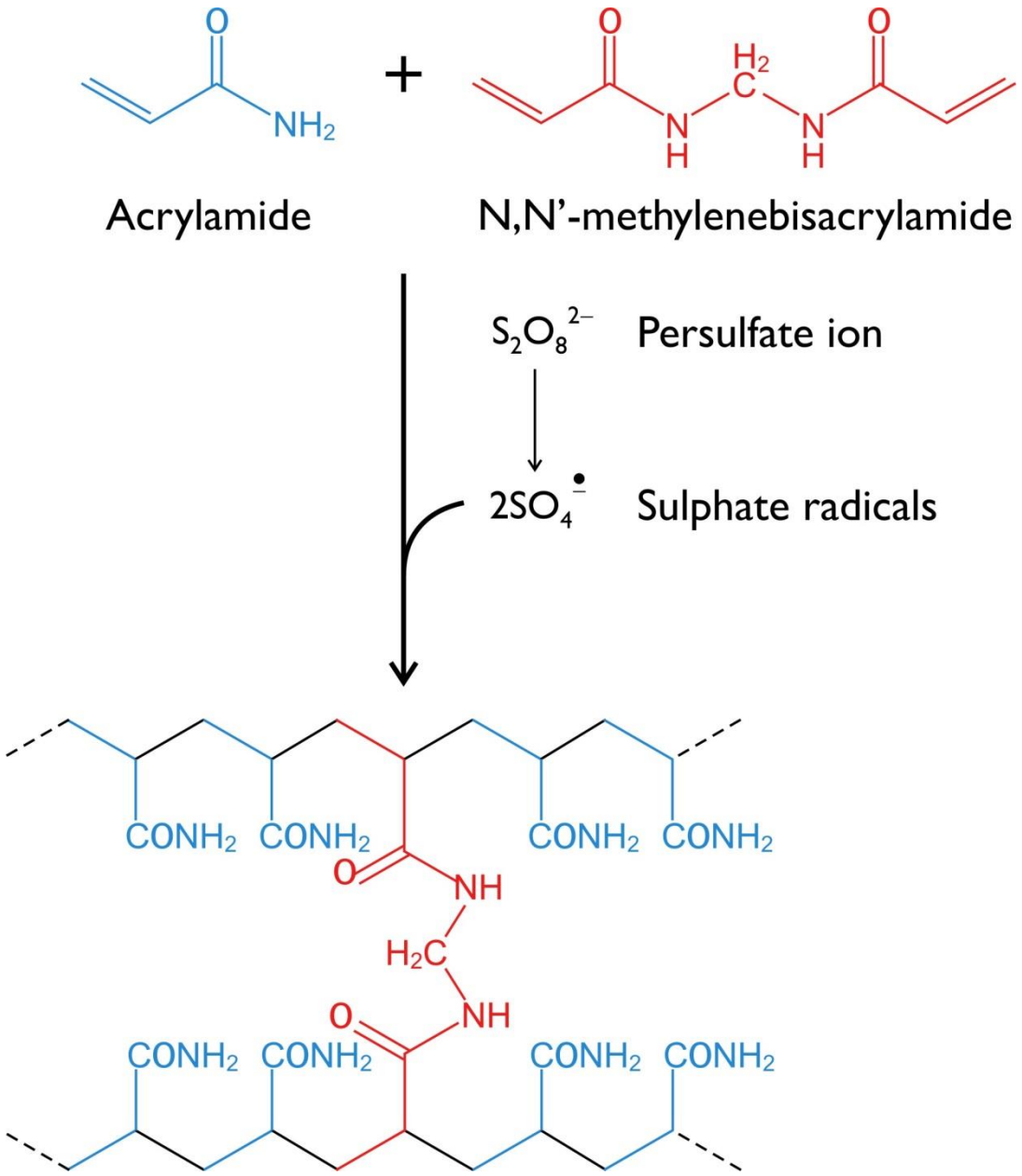


Figure. Molecular structure of the polyacrylamide gel. The three-dimensional molecular network comes into being by a radical polymerisation of acrylamide monomers and cross-linking *N*,*N'*-methylenebisacrylamide components.

Without any modification, polyacrylamide electrophoresis separates macromolecular ions based on a combination of charge, size and shape. Size (and shape) separation is due to the molecular sieving property of the gel. The size range in which molecules can be separated is dictated by the average pore size of the gel. In the case of polyacrylamide gels, this can be controlled through the concentration of the acrylamide monomer and the proportion of the cross-linking *N*,*N'*-methylenebisacrylamide. The acrylamide concentration can be set

in the range of about 4-20 % as this is the range in which the mechanical properties of the gel are appropriate. Below this range the gel will be too soft and it will not keep its shape, while above this range it will be too rigid and prone to break. The polyacrylamide gel possesses all advantageous properties necessary for a good electrophoresis medium, i.e. it is hydrophilic, free of electric charges and chemically stable. A further very important property of the polyacrylamide gel is that it does not participate in any non-specific or specific binding interaction with proteins. Furthermore, the polyacrylamide gel does not interfere with common protein staining reactions.

When electrophoresis is performed under native (non-denaturing) conditions, such as near neutral pH and ambient or lower temperature, many enzymes retain their native conformation and, in turn, their enzymatic activity. This way, many enzymes can be separated and specifically detected in the gel after electrophoretic separation.

There are two types of gels according to their geometry. In early gel electrophoretic applications, gel tubes were used that allowed only a single sample to be run. Gel slabs were later introduced, allowing for many samples to be run at the same time in the same gel in parallel. Gel slabs became much more common than gel tubes. Gel slabs are created by pouring the gel-forming solution between two parallel glass sheets prior to polymerization. Besides its higher throughput, this gel geometry provides another important advantage over gel tubes: samples are loaded side by side on such slabs and are run in the same gel at the same time. This allows for a more reliable comparison of the samples, facilitating the interpretation of experimental results.

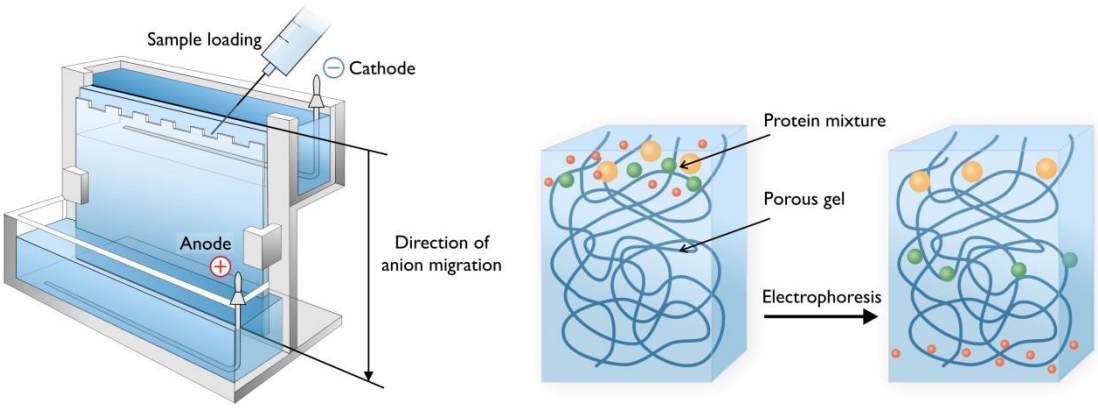


Figure 7.3. Separation of proteins in a polyacrylamide gel. As illustrated in the left panel, several samples can be run in parallel in a slab gel. Ions can move between the two electrodes only through the gel interconnecting the two chambers. The gel acts as a molecular sieve. The larger the molecule, the larger the drag force exerted on it by the gel.

Proper selection of pH and acrylamide concentration is instrumental for successful electrophoresis. For protein electrophoresis, the pH is set usually higher than the pI value of the proteins in the sample. At such a pH, all proteins will be negatively charged and will move towards the anode. The buffer in the medium serves two purposes. One is to set and maintain the proper pH during electrophoresis. The other function of the buffer is to establish the electric current in the medium.

The majority of the electric current is carried by the ions of the buffer. Normally, the protein-ions that are separated by electrophoresis have only a negligible contribution to the current. In other words, proteins have a low ion transport number. However, if the buffer concentration is set too low, the contribution of proteins to carrying the current will increase, and the protein molecules will migrate rapidly. This usually leads to smearing of the bands of migrating proteins. On the other hand, if the buffer concentration is set too high, the mobility of the proteins will be too low. In this case the electrophoresis process would take a very long time. Unnecessary lengthening of the process provides excess time for diffusion, which lowers the resolution of separation.

According to the applied buffer system, gel electrophoretic methods can be classified into two types: continuous and discontinuous.

Continuous methods apply the same buffer in the gel and in the two buffer chambers containing the electrodes. The only advantage of this method lies in its simplicity. More complex discontinuous methods were introduced to provide higher resolution. SDS polyacrylamide gel electrophoresis is usually associated with such a discontinuous system.

The discontinuous system applies two gels of different pore size and three different buffers. One of the gels, the resolving gel, is polymerised at a higher acrylamide concentration. The pore size of this gel is set according to the size range of the proteins to be separated. Another gel, the stacking gel is created on top of the resolving gel. (The gels are mounted in a vertical format.) The stacking gel is polymerised from a more dilute acrylamide solution to provide larger pores. This pore size does not provide a molecular sieving effect.

As mentioned above, there are three buffers: different ones in each of the two gels and a third one, the so-called ‘running buffer’ in the buffer chambers containing the electrodes. In the gel buffers, the anion originates from a strong acid; it is usually chloride ion. Dissociation of strong acids does not depend on the pH: these acids always fully dissociate. Consequently, chloride ion is never protonated in the solution: its ionisation state is independent of the pH. On the other hand, the anion component of the running buffer is the conjugate base of a weak acid. Consequently, the ionisation state of this ion depends on the pH of

the buffer. Glycinate ion is one of the most frequently used compounds for this purpose. The pH in the running buffer is set to 8.3.

The protein sample is layered on the top of the stacking gel. When an electric field is generated by the power supply, the protein ions and the ions of the running buffer enter the stacking gel. The pH in the stacking gel is set to 6.8. This value is only slightly higher than the pI value of glycine (6.5). At this pH, most glycine molecules are in a neutral zwitterionic state, and only a small portion of the molecules carry a net negative charge. As the electric current must be of the same magnitude at any segments of the electric circuit (there is no macroscopic charge separation), the voltage will increase according to Ohm's law. Due to this effect, the migration speed of the proteins will be relatively high and the protein front will reach the chloride front in the stacking gel. The ion concentration in the chloride front is high and, therefore, here the electric resistance and the voltage are low. This slows down the protein front. This effect results in a very sharp protein front, with the protein molecules being crowded right behind the chloride ion front.

The protein sample will thus enter the resolving gel in a sharp band. The pH in the resolving gel is set to about 8.8. At this pH, almost all glycinate molecules are in the anionic state. Thus, the electric mobility of glycinate increases, and the concentrating effect applied by the stacking gel ends in the resolving gel. Different proteins will be separated in the resolving gel according to their charge, size and shape.

In most electrophoretic methods, a tracking dye is mixed in the sample. Usually, this dye is chosen to have a higher electrophoretic mobility than any of the components of interest (proteins or nucleic acids) in the sample. The function of the tracking dye is to visualise the running front and, in turn, the completeness of the run. The most popular tracking dye is bromophenol blue.

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- **Native PAGE gel electrophoresis**

Native PAGE is an electrophoresis method to separate native proteins. The conditions are set such that the migrating proteins are kept in their native state. The buffers provide a non-denaturing, native-like milieu, and the electrophoresis is performed at low temperature in order to dissipate heat.

Many enzymes retain their native conformation and their enzymatic activities while running in the gel. If certain conditions apply, these enzymes can be highly selectively detected within the gel through a specific 'staining' reaction even in the presence of a large excess of 'contaminating' proteins.

After completion of electrophoresis, the gel is soaked in a solution containing the substrate of the enzyme. As the substrate is usually a small molecule, it quickly diffuses into the gel while the large enzyme molecules do not diffuse out. In an optimal case, the natural product of the enzymatic reaction is a coloured and insoluble compound that precipitates inside the gel and marks the exact location of the enzyme. Of course, most enzymes do not have such natural substrates. However, once the molecular mechanism of catalysis is revealed, synthetic substrates can be designed that, on the one hand, mimic natural substrates and, on the other hand, lead to colourful insoluble products.

Native PAGE is also a useful method for checking the uniformity of the isolated protein. Even if the purified protein sample contains only a single type of protein, the sample might not be uniform. Some of the molecules might be unfolded or have undergone chemical modifications. Unfolding changes the overall shape of the molecule, while most chemical modifications change the electric charge of native molecules. These alterations can be detected after traditional staining of the purified sample. If no such side products are present, protein molecules will run in a single sharp band. Otherwise, multiple bands or smearing of the band is expected.

In addition, native PAGE can also be used to detect complex formation between proteins. If two (or more) proteins (or proteins and non-proteinous ligands) form a complex, the complex can be detected as an extra band in the gel. This is because in native-like conditions, many non-covalent (subunit-subunit, receptor-ligand, enzyme-inhibitor) interactions are maintained and the complex migrates apparently as a single molecule.

In the course of native PAGE, it is highly important to pay attention to the relationship of the pI values of the proteins or protein complexes and the pH of the gel buffer, as this will determine where individual proteins will migrate in the gel.

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- **SDS-PAGE electrophoresis**

SDS-PAGE is an electrophoresis method to separate proteins. However, unlike in the case of native PAGE, here the proteins migrate in their denatured state. As it was mentioned in the general introduction to traditional (native) PAGE, the migration velocity of proteins is a function of their size, shape and the number of electric charges they carry. As the velocity is a complex function of these properties, native PAGE cannot be used to estimate the molecular mass of proteins. The traditional native PAGE method is similarly unable to assess

whether a purified protein is composed of a single subunit or multiple subunits. Even a multi-subunit protein may migrate in a single sharp band.

SDS-PAGE was introduced to analyse such cases and to allow the estimation of the molecular mass of single-subunit proteins or those of individual subunits of multi-subunit proteins. SDS-PAGE is the most prevalent PAGE method currently in use.

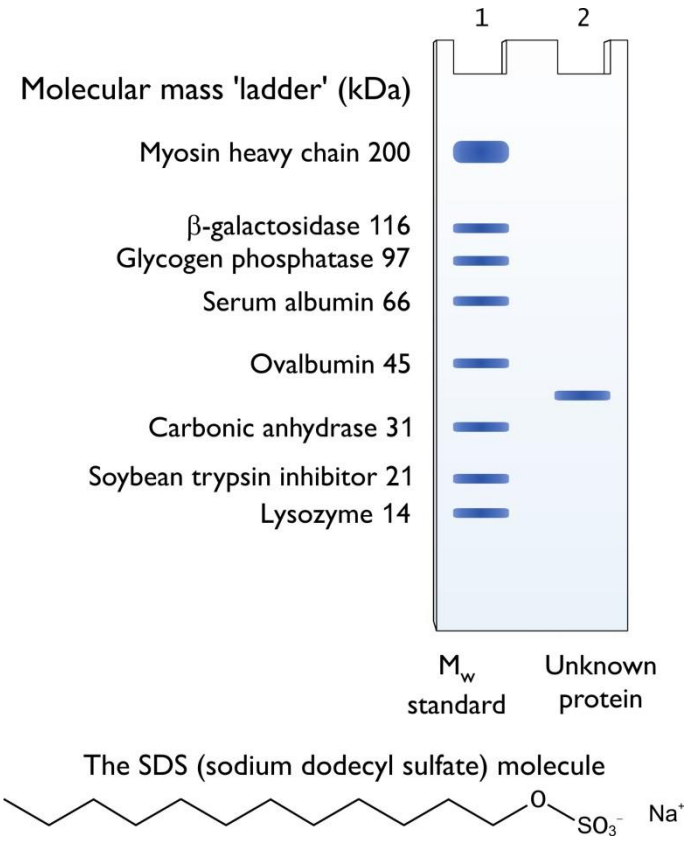


**Figure.** SDS polyacrylamide gel electrophoresis. SDS (sodium dodecyl sulphate) is an anionic detergent that unfolds proteins and provides them with extra negative charges. The amount of the associated SDS molecules—and therefore the number of charges—is proportional to the length of the polypeptide chain.

The SDS gel separates individual polypeptide chains (monomeric proteins and subunits of multimeric proteins) according to their size. The velocity of the proteins is an inverse linear function of the logarithm of their molecular mass. Proteins of known molecular mass can be used to establish a calibration curve (a descending line) along which the unknown molecular mass of other proteins can be estimated.

SDS (sodium dodecyl sulphate) is an anionic detergent. When proteins are treated with SDS at high temperature, radical conformational changes occur. The treatment breaks all native non-covalent intermolecular (inter-subunit) and intramolecular interactions. The subunit structure of multi-subunit proteins disintegrates and the proteins unfold. If the native structure is stabilized by disulfide bridges, reducing agents are also added to open up these connections. SDS molecules bind to unfolded proteins in large excess, providing extra negative charges to the molecules.

The amount of the bound SDS molecules is largely independent of the amino acid sequence of the polypeptide chain and it is roughly a linear function of polypeptide length—i.e. the molecular mass of the protein. Therefore, upon SDS-treatment, the specific charge (the charge-to-mass ratio) of different proteins will become roughly identical. Another result of the treatment is that the shape of the different proteins becomes similar.

The negatively charged SDS molecules repel each other, which lends a (presumably) rod-like shape to the SDS-treated proteins. These factors together result in a situation analogous to the one already discussed in this chapter for the PAGE separation of linear single-stranded (denatured) DNA molecules. Instead of being separated simultaneously by charge, shape and size, SDS-treated proteins—just like denatured linear DNA molecules—will be separated solely based on their size. As size is a linear function of mass, SDS-PAGE ultimately separates proteins based on their molecular mass.

SDS-PAGE is the most popular cost-effective method to estimate the molecular mass of protein subunits with considerable accuracy. The relative mobility (i.e. the running distance of the protein divided by the running distance of the tracking dye) of the SDS-treated protein is in inverse linear proportion to the logarithm of the molecular mass of the protein. By running several proteins of known molecular mass simultaneously alongside the protein of interest, a log molecular mass – relative mobility calibration curve (a descending linear graph) can be created. Based on the calibration curve, the estimated molecular mass of the protein in question can be easily calculated.

SDS-PAGE is a standard method for assessing whether the sample of an isolated protein is homogeneous. Besides that, SDS-PAGE is a robust method for the analysis of large supramolecular complexes such as multi-enzyme complexes or the myofibril, as discussed below. SDS-PAGE separates and denatures individual subunits of these complexes. Thus, all polypeptide chains will migrate separately in the gel. Via various staining procedures, all subunits

can be visualised and the relative amounts of these proteins (subunits) can also be determined. This allows for the identification of each subunit of a complex and provides a good estimate of the stoichiometry of subunits, too.

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❖ **Isoelectric focusing**

In the course of isoelectric focusing, the conditions are set in a way that proteins will be separated exclusively based on their isoelectric point (Figure). The two termini and many side chains of proteins contain dissociable groups (weak acids or bases). The dissociation state of these groups is a function of the pH of the environment.

Isoelectric focusing is based on the pH-dependent dissociation of these groups. Due to this pH-dependent phenomenon, the net electric charge of a protein molecule will be a function of the pH of the medium. If, in a given protein, the number of acidic residues (Asp, Glu) exceeds that of the basic ones (Arg, Lys, His), the protein will have a net negative charge at neutral pH. The isoelectric point (pI) of the protein—i.e. the pH at which the net charge of the protein is zero—will be in the acidic pH range. Such proteins are often denoted as acidic proteins. If the number of basic residues exceeds that of the acidic ones, the protein will be positively charged at neutral pH, and its pI value will be in the basic pH range. These proteins are often called basic proteins.

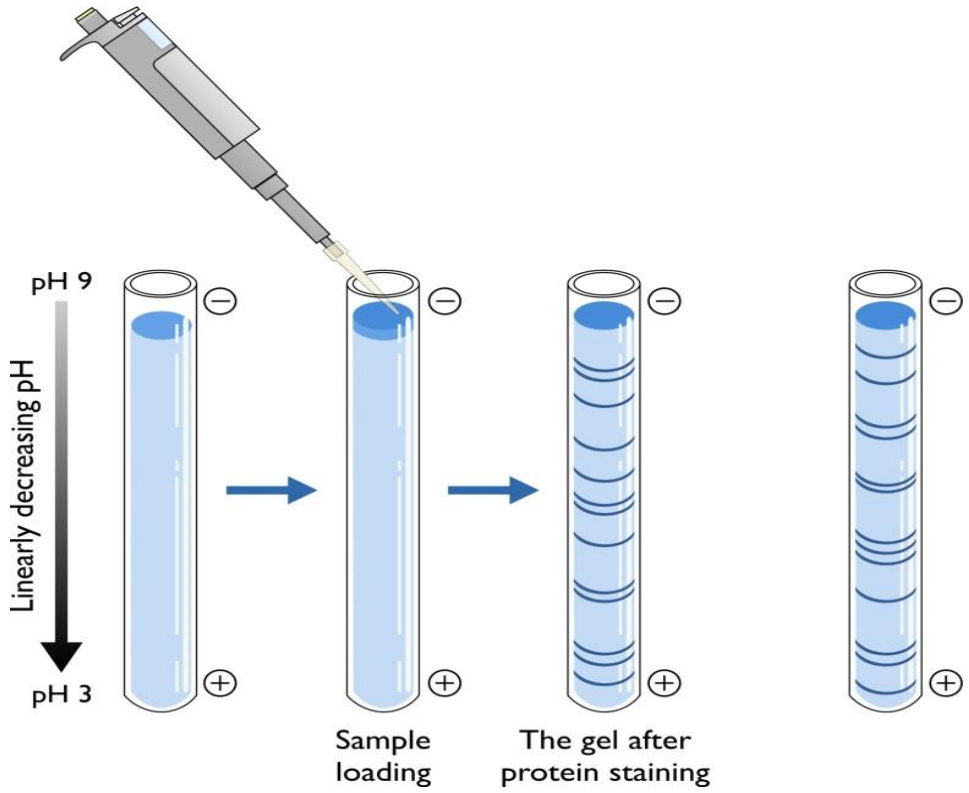

**Figure.** Isoelectric focusing. In the course of isoelectric focusing, a pH gradient is created in the gel (usually made of polyacrylamide, less frequently agarose). Upon electrophoresis, various proteins will accumulate in different narrow regions of the gel where the pH equals their individual pI value. At this pH, the number of positive charges equals that of the negative charges on the protein—the net charge will thus be zero. Consequently, no resultant electric force is exerted on the protein.

Isoelectric focusing is an efficient high-resolution method because the pI values of various proteins are spread across a broad range. If the pH is lower than the pI of the protein, the protein will be positively charged and will move towards the cathode during electrophoresis. If the pH is higher than the pH of the protein, the protein will be negatively charged and will migrate towards the anode. If the pH equals the pI value, the net charge of the protein will be zero and the protein will not migrate in the gel any further.

In the course of isoelectric focusing, proteins are placed in a gel representing a special medium in which the pH gradually decreases by going from the negative cathode towards the positive anode. As the protein migrates, it encounters a gradually changing pH and its net charge will also change accordingly. If it has a net negative charge and therefore moves towards the cathode, it will encounter a gradually decreasing pH, i.e. a more and more acidic environment. Consequently, the protein will take on more and more protons—up to a level where its net charge will be zero. This state is reached when the protein reaches a location where the pH equals its pI value. At this point, the protein will stop moving because no electric force will be exerted on it. If it spontaneously diffused further towards the anode, it would take on more protons, would become positively charged and would turn back to migrate towards the cathode.

Following the same line of thinking, if a positively charged protein moves towards the cathode, it will encounter increasing pH and lose more and more protons. It will migrate to the place where the pH equals its pI value and will thus stop. If it diffused further towards the cathode, it would become negatively charged and would turn back towards the anode. As one can see, by performing electrophoresis in a medium in which the pH decreases from the cathode towards the anode, each protein will “find its place” according to its pI value and will become sharply focused at that location. In addition, it does not matter where exactly the proteins were introduced in the medium between the cathode and the anode.

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## ❖ Capillary electrophoresis (CE)

**Capillary electrophoresis** (**CE**) is a family of electrokinetic separation methods performed in submillimeter diameter capillaries and in micro- and nanofluidic channels.

In CE methods, analytes migrate through electrolyte solutions under the influence of an electric field. Analytes can be separated according to ionic mobility and/or partitioning into an alternate phase via non-covalent interactions. Additionally, analytes may be concentrated or "focused" by means of gradients in conductivity and pH.

### Instrumentation

The instrumentation needed to perform capillary electrophoresis is relatively simple. A basic schematic of a capillary electrophoresis system is shown in *figure*. The system's main components are a sample vial, source and destination vials, a capillary, electrodes, a high voltage power supply, a detector, and a data output and handling device.

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. Sample is introduced into the capillary via capillary action, pressure, siphoning, or electrokinetically, and the capillary is then returned to the source vial. The migration of the analytes is 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. In the most common mode of CE, all ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow.

The analytes separate as they migrate due to their electrophoretic mobility, and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as an integrator or 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.

Capillary electrophoresis was first combined with mass spectrometry by Richard D. Smith and coworkers, and provides extremely high sensitivity for the analysis of very small sample sizes. Despite the very small sample sizes

(typically only a few nanoliters of liquid are introduced into the capillary), high sensitivity and sharp peaks are achieved in part due to injection strategies that result in concentration of analytes into a narrow zone near the inlet of the capillary. This is achieved in either pressure or electrokinetic injections simply by suspending the sample in a buffer of lower conductivity (*e.g.* lower salt concentration) than the running buffer. A process called field-amplified sample stacking (a form of isotachophoresis) results in concentration of analyte in a narrow zone at the boundary between the low-conductivity sample and the higher-conductivity running buffer.

**Detection**

Separation by capillary electrophoresis can be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. In these systems, a section of the capillary itself is used as the detection cell. The use of on-tube detection enables detection of separated analytes with no loss of resolution. In general, capillaries used in capillary electrophoresis are coated with a polymer (frequently polyimide or Teflon) for increased flexibility. The portion of the capillary used for UV detection, however, must be optically transparent. For polyimide-coated capillaries, a segment of the coating is typically burned or scraped off to provide a bare window several millimeters long. This bare section of capillary can break easily, and capillaries with transparent coatings are available to increase the stability of the cell window. The path length of the detection cell in capillary electrophoresis (~ 50 micrometers) is far less than that of a traditional UV cell (~ 1 cm). According to the Beer-Lambert law, the sensitivity of the detector is proportional to the path length of the cell.

Fluorescence detection can also be used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. This mode of detection offers high sensitivity and improved selectivity for these samples, but cannot be utilized for samples that do not fluoresce. Numerous labeling strategies are used to create fluorescent derivatives or conjugates of non-fluorescent molecules, including proteins and DNA.

## Applications

Capillary electrophoresis may be used for the simultaneous determination of the ions $NH_4^{+}$, $Na^{+}$, $K^{+}$, $Mg^{2+}$ and $Ca^{2+}$ in saliva.

One of the main application of CE in forensic science is the development of methods for amplification and detection of DNA fragments using polymerase chain reaction (PCR) which has to lead to rapid and dramatic advances in DNA typing in forensic.

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### ❖ Two-dimensional (2D) electrophoresis / (2D-PAGE)

The various separation methods are all aimed at separating complex systems to individual components. Separation is always based on at least one physicochemical property that shows diversity among the components. The general problem encountered in the case of complex mixtures is that not all components differ significantly from all other components when only one property is considered. Accordingly, separation based on a single property rarely results in single-component fractions. Some components will be efficiently separated from all others, while some other components will remain in the mixture.

The remaining mixtures can be further fractionated by another separation technique that relies on a different physicochemical property. The most effective separation can be achieved if the combined consecutive separation steps rely on absolutely independent physicochemical properties. A good example of this is the very high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) that combines two already discussed electrophoresis methods, isoelectric focusing and SDS-PAGE (Figure).

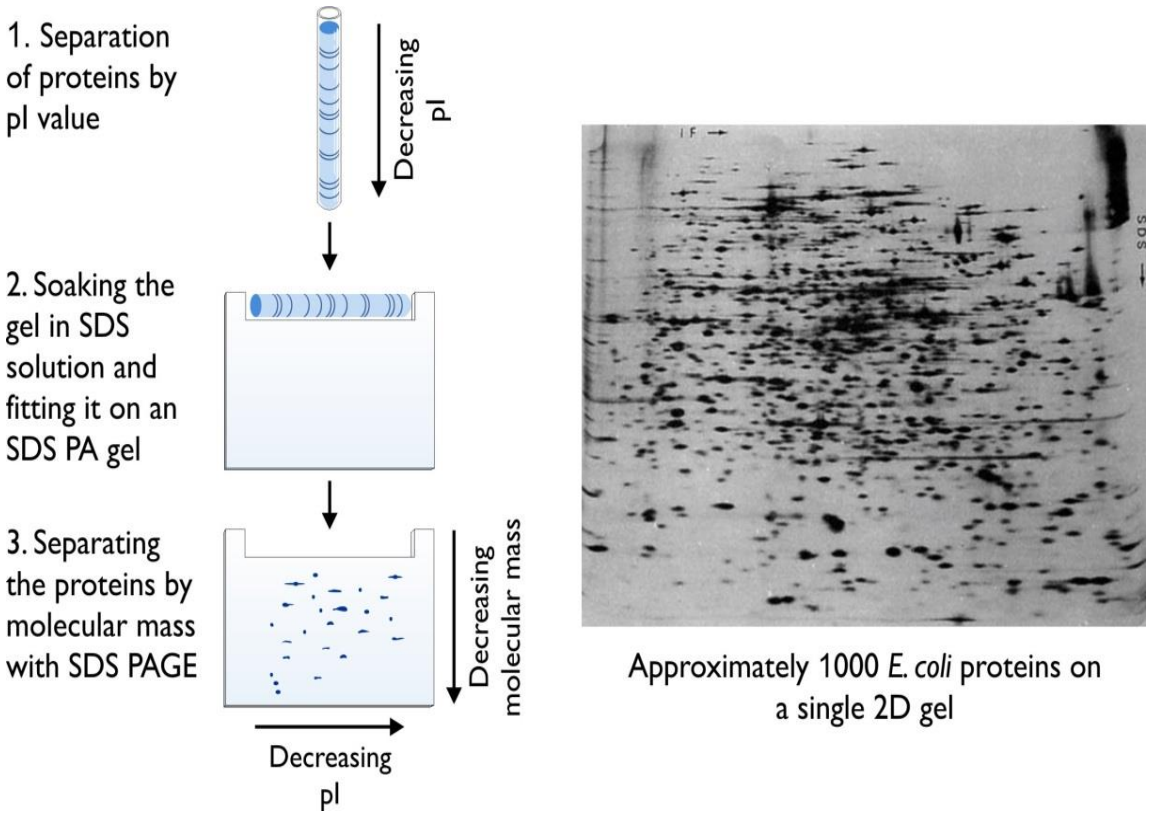


**Figure.** Two-dimensional (2D) electrophoresis. 2D electrophoresis is the combination of isoelectric focusing and SDS-PAGE. Proteins are first separated based on their pI values and then based on their molecular mass. As these properties are completely independent, the combination of the two separation methods provides much higher resolution than either of the two methods alone.

As the first step of 2D gel electrophoresis, isoelectric focusing is performed to separate proteins based on their pI values. Only a single sample is loaded on a gel strip in this step. The sample is separated in one dimension both in a primary and in a figurative sense. In a primary sense because the components are separated along a single line, and in a figurative sense as the separation is based on a single well-defined property, the pI value.

After the first separation step has been completed in the first dimension, the gel strip is soaked in an SDS solution and is fitted tightly to one side of a "classical" SDS polyacrylamide gel. The second separation step is traditional SDS-PAGE, which separates proteins based on their molecular mass. This second step represents a second dimension in both a primary and a figurative sense. The second separation is performed in a second dimension in a direction rectangular to that of the first separation, and the property utilized in the second step (molecular mass) is completely independent of the one utilized in the first step (pI).

If, after the first step, some gel regions contain different proteins that coincidentally have identical pI values, these proteins will be separated from each other in the second step if their molecular mass is different. Note that every aspect discussed for SDS-PAGE also applies to the second separation step of 2D-PAGE. Van der Waals interactions that might have held protein subunits together in the course of isoelectric focusing will break and individual subunits will become separated. If disulfide bridges need to be opened up, some kind of reducing agent needs to be added. Accordingly, in the second separation step, single polypeptide chains will migrate in the gel. If isoelectric focusing collects a multimeric protein at a certain gel location, the second electrophoresis step will dissect it into individual chains. If the multimer contains subunits of different sizes, these subunits will be separated from each other in the second separation step.

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## ❖ Microchip electrophoresis

Microchip capillary electrophoresis emerged in the early 1990s as an intresting and novel approach to the high-speed separation of biological compounds, including DNA and proteins.

### Introduction

Microchip capillary electrophoresis (CE) has appeared over the decades as a result of the marriage of chemical analysis and microfabrication techniques from the integrated circuit world. CE proved to be an excellent match for microchip technologies because it easily manipulates volumes at the nanoliter scale, requires no moving parts, and provides fast, high-resolution separations.

Unlike traditional CE instrumentation, which consists of essentially a single capillary (or an array of capillaries in parallel), many different capillaries and fluidic channels can be patterned on a microfluidic device, providing the potential for high-throughput, massively parallel analysis.

Microchip CE has also garnered. polymerase chain reaction (PCR), enzymatic digestion, and solid phase extraction, can be incorporated into the microchip to provide pre- and postsample processing. This capability is important because it allows a raw sample to be added to the microchip and a final quantitative answer provided.

## Theory and Mechanisms of Action in Microchip CE

### *Injection*

Mixtures of proteins and peptides were separated in glass capillaries. Standard analysis protocols for microchip CE involve injection, separation, and detection. Injection in conventional CE is accomplished by placing one end of the capillary in the sample solution and applying either pressure or voltage for a short period of time to introduce a finite volume of sample into the capillary.

Microfluidic channels cannot be physically manipulated in the same way and, therefore, new injection protocols had to be developed for microchip CE. There are two common forms of injection in microchip CE, gated injection and cross injection. In gated injection, a flow boundary is established between two solutions, the mobile phase and the sample solution, at the intersection of several channels.

No mixing of solutions occurs at this intersection because the interaction time is small, minimizing diffusion and the flow regime is laminar. At a specific point in time, the voltage is turned off to the mobile phase channel and the sample solution is injected into the separation channel. After the specified time length, the voltage to the mobile phase reservoir is re-established and the injection plug is defined. Gated injection is simple but results in the biasing of sample injection toward cations over anions owing to their higher mobility under normal flow conditions.

### *Separation*

Microchip CE separations occur in much the same way as conventional CE separations. It is easier to fabricate a monolithic column with open injectors for microchip CEC than in conventional CEC.

### *Detection*

Several modes of detection have been demonstrated with microchip CE with the most common being laser-induced fluorescence (LIF). LIF has been used for many applications with microchip CE including DNA analysis, protein analysis, and measurement of small molecules.

Mass spectrometry (MS) has been coupled to microchip CE for a variety of applications but most reports have focused on protein sequencing (proteomics). The disadvantages of MS include high-instrument cost, a high level of technical expertise needed for operation, and large instrument size.

Electrochemistry (EC) relies on selective electron transfer reactions at an electrode surface to achieve detection. Selectivity can be achieved by control of

the detection potential. Many more molecules are naturally electrochemically active than are fluorescent, especially when pulsed potential methods are used.

## Microchip CE Applications

### *DNA Separations*

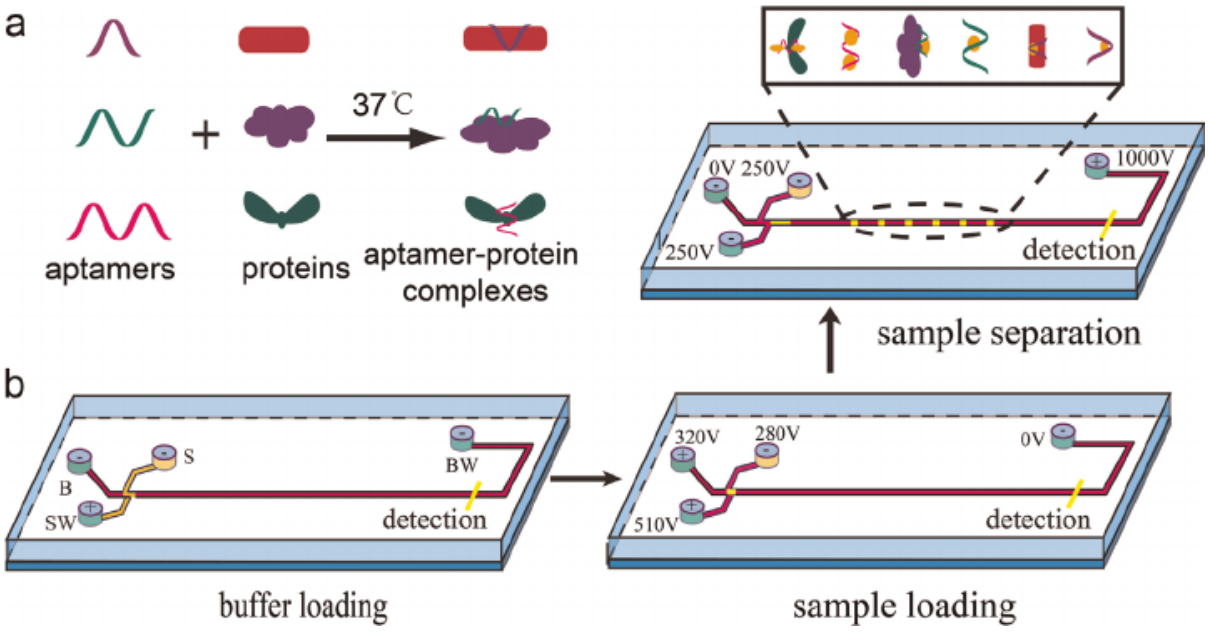
DNA separations are performed in a manner very similar to traditional capillary gel electrophoresis experiments. The same gel matrixes are often used and detection is achieved using LIF. The short columns of microchip CE decrease the separation time relative to conventional CE and should also decrease the resolution. However, the reduced injection volumes used in microchip CE (pL) help maintain resolution. In addition to DNA separation, the PCR has been demonstrated onchip both independently and directly coupled to microchip CE.

### *Protein Analysis*

Direct separation of fluorescently labeled proteins has been demonstrated numerous times for both characterization of new microchip CE devices and for measuring the purity of protein samples. Microchip Capillary electrophoretic immunoassays CEIA allows for the direct measurement of protein analytes from complex samples such as blood and urine, making it a very useful tool clinically. A second useful analysis method being developed focuses on measuring enzyme kinetics and activities.

### *Small Molecule Analysis*

Specific applications range from detection of explosive components to the measurement of clinical analytes in urine and serum.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