First Internal Examination (2019-20)
B.Sc. (BT) I Year
Bioanalytical Techniques and Instrumentation
SET-A

Time: 1:30 Hours **Max. Marks-30**

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1. Short Answers Questions (1 Marks Each; 1x8= 8 Marks)

i. Define SDS-PAGE?

ANS…A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

ii. What is Electrophoresis?

ANS… Electrophoresis is a method used to separate charged particles from one another based on differences in their migration speed. Electrophoresis (from the Greek meaning "to bear electrons") is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field.

iii. The speed of migration of ions in electric field depends upon what factors?

ANS… The rate of migration (Separation of particles) during electrophoresis will depend on the following factors:

1. The Sample
2. The Electric Field
3. The Medium
4. The Buffer

iv. What does the electrophoresis apparatus consist of?

ANS… The apparatus consists of power pack and electrophoresis unit.

v. If proteins are separated according to their electrophoretic mobility then the type of electrophoresis is?

ANS… In the technique of SDS page, the proteins are separated according to their electrophoretic mobility.

vi. The electrophoretic mobility denoted as μ is mathematically expressed as?

ANS… v/E is the formula for electrophoretic mobility which is the ratio of velocity of the biomolecule in the electric field and it's intensity.

vii. What kind of molecules cannot be separated using electrophoresis technique?

ANS… Electrophoresis cannot be used in separation of uncharged molecules such as lipids.

viii. What is Ethidium Bromide?

ANS… Ethidium bromide is an intercalating agent commonly used as a fluorescent tag (nucleic acid stain) in molecular biology laboratories for techniques such as agarose gel electrophoresis.

2. Medium Answer Questions (4 Marks each 2x4= 8 Marks)

i. What is the role of pH in electrophoresis?

ANS… In solution glycine exists as a zwitterion. A low pH would protonate the -COO- group in Glycine and cause it to move towards the negative electrode. A high pH would deprotonate the -NH3+ group and cause it to move towards the positive electrode. A neutral pH would mean that Glycine was at the isoelectric point and hence, will not move in either direction. Same concept with gel electrophoresis of DNA - DNA contains a sugar-phosphate backbone, of which the phosphate group is negatively charged. If pH is low, the negatively charged phosphate group would be protonated, and thus the DNA wouldn't move towards the correct electrode. The buffer is needed to maintain the pH of the DNA solution at close to neutral level because if it can become acidic through electrolysis.

ii. Write short note on different types of support media used in electrophoresis?

ANS… Various types of support media are used in electrophoresis including gels and membranes. The sample for electrophorectic separation is placed on the gel or membrane. Common gels are **cellulose acetate** (formed by a chemical reaction of **cellulose** with **acetic anhydride**), **agarose**, and **polyacrylamide**. Cellulose requires soaking before sample application and a clearing step for detection of separated solutes or bands. Agarose gel is therefore used more often than cellulose acetate gel for clinical electrophoresis because it does not require these steps. These gels are formed into sheets or slabs, or inserted into columns or tubes. The gel can be positioned horizontally or vertically.

3. Long Answer Questions (7 Marks each 2x7= 14 Marks)

i. Explain the principle of electrophoresis?

ANS… 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.

The mathematical description of the force during electrophoresis is simple. An electric force F_e is exerted on the charged particle. The magnitude of the electric force equals the product of the charge q of the particle and the electric field E generated between the two electrodes:

$$F_e = q \times E \qquad (1)$$

Dimensions of the electric field E are defined either in newton/coulomb or volt/cm units. During electrophoresis, the magnitude of the electric field E is defined in volt/cm units. It can be easily calculated using the value of the voltage (volt) set by the electric power supply and the distance of the two electrodes (cm).

As soon as the electric field is applied and the charged particles are accelerated by the electric force, a drag force (F_d) called friction will also be immediately exerted on the particles by the medium. This force, whose direction is opposite to the direction of particle movement, is proportional to the velocity of the particle. At the typically very low speed of particle migration during electrophoresis, the force F_d is a linear function of the velocity (v) of the particle, as described by Equation 2:

$$F_d = f \times v \qquad (2)$$

The ratio of the force and the velocity is defined as the frictional coefficient (f). The value of f is a function of the size and shape of the particle and the viscosity of the medium. The larger the particle and the more obstructing the medium, the higher the value of f.

When electrophoresis is started, particles accelerate instantaneously to a velocity (v) at which the magnitude of the drag force equals the magnitude of the (opposite) accelerating electric force:

$$q \times E = f \times v \quad (3)$$

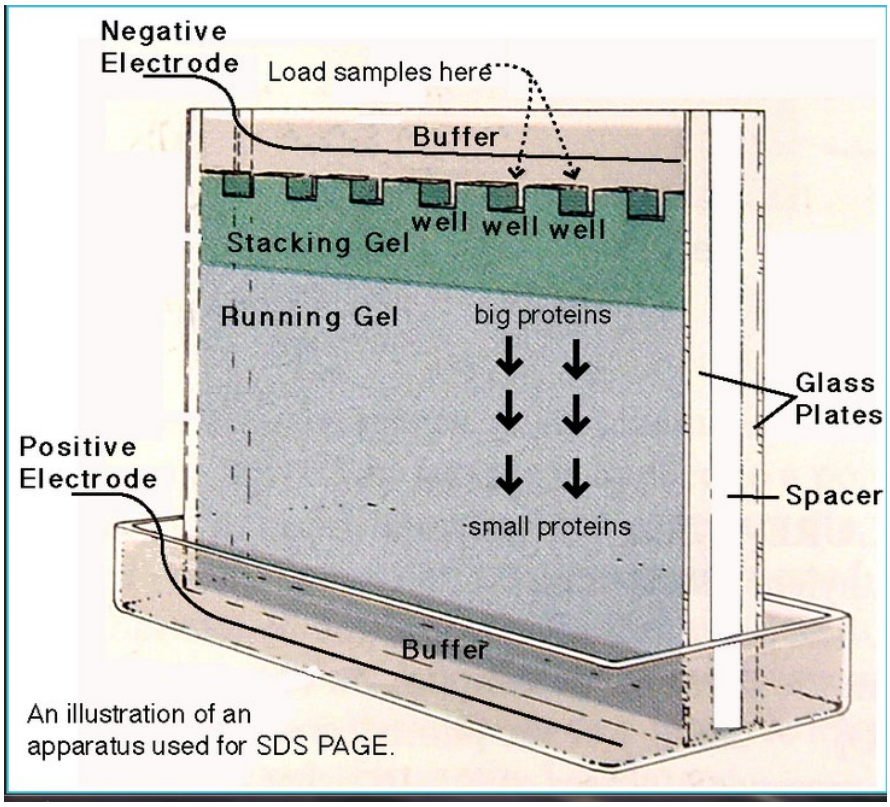
Once the magnitude of the two opposing forces becomes equal, the resultant force becomes zero. Therefore, each particle will move at a constant velocity characteristic of the given particle at the given accelerating potential and medium. A useful parameter, the electrophoretic mobility (μ) of the particle, defines the velocity of the particle in a given medium when one unit of electric field is applied. Electrophoretic mobility is a linear function of the charge of the particle and it is a reciprocal function of the frictional coefficient (which depends on both the size of the particle and the nature of the medium):

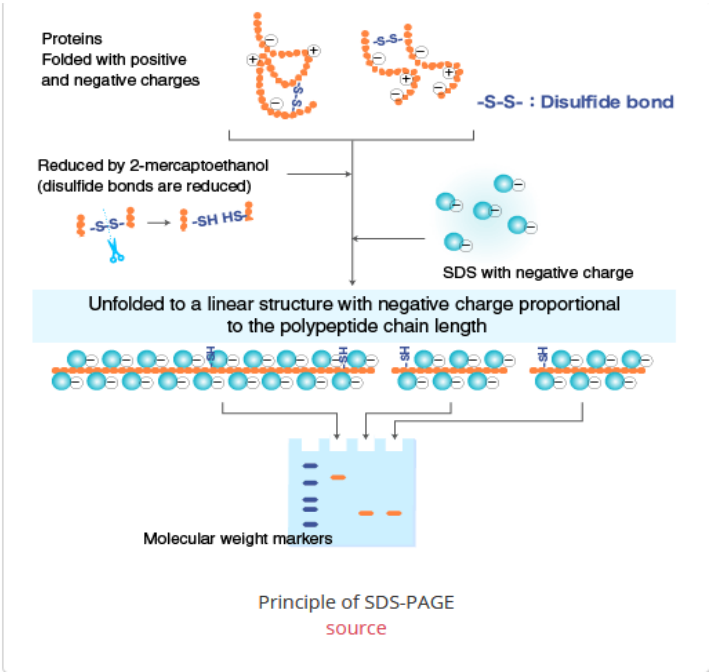
$$\mu = \frac{v}{E} = \frac{q}{f} \quad (4)$$

Particles having different electrophoretic mobility, i.e. those that migrate at different speeds in the same medium and electric field, can be separated by electrophoresis.

ii. Write short note on SDS-PAGE?

ANS… A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sodium dodecyl sulfate (SDS) is an amphipathic detergent. It has an anionic headgroup and a lipophilic tail. It binds non-covalently to proteins, with a stoichiometry of around one SDS molecule per two amino acids. SDS causes proteins to denature and dissassociate from each other (excluding covalent cross-linking). It also confers negative charge. In the presence of SDS, the intrinsic charge of a protein is masked. During SDS PAGE, all proteins migrate toward the anode (the positively charged electrode). Polymerization of the gel occurs by free radical mechanism. TEMED ((N,N,N,N-tetramethylethylenediamine) acts as a catalyst and generate free radicals of Sulfate. These sulfates for free radical generation is provided by ammonium persulfate. SDS-treated proteins have very similar charge-to-mass ratios, and similar shapes. During PAGE, the rate of migration of SDS-treated proteins is effectively determined by molecular weight.





	Stacking Gel	**Resolving Gel**
Polyacrylamide concentration	Low	High
Pore size	Larger	Smaller
pH of Tris-Cl used	6.8	8.8

Purpose	To stack the polypeptides on the interface of stacking gel and resolving gel.	To separate the polypeptides solely on the basis of size.
Electrophoretic mobility	glycine <protein mixture <BPB <Cl.	Protein mixture <glycine <BPB <Cl.

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1. Short Answers Questions (1 Marks Each; 1x8= 8 Marks)

i. Electrophoresis was developed by whom?

ANS… The development of gel electrophoresis began with the pioneering work of **Arne Tiselius**, a Swedish biochemist who had published his first paper on electrophoresis in the paper "A New Apparatus for Electrophoretic Analysis of Colloidal Mixtures" in 1937 and awarded the Noble prize on his work in 1948.

ii. Which technique separates charged particles using electric field?

ANS… Electrophoresis is the **technique** that **separates charged particles using electric field.**

iii. What is loading dye?

ANS… Ethidium bromide is a molecule commonly used to visualize DNA in agarose gel electrophoresis experiments. It **intercalates** between the nitrogenous bases of DNA and **fluoresces** under UV light. Loading buffer is a solution added to an electrophoresis sample to give it color and density.

iv. What is the function of β-mercaptoethanol in SDS-PAGE?

ANS… **BME** is suitable for reducing protein disulfide bonds prior to polyacrylamide **gel** electrophoresis and is usually included in a sample buffer for **SDS-PAGE** at a concentration of 5%. Cleaving intermolecular (between subunits) disulfide bonds allows the subunits of a protein to separate independently on **SDS-PAGE**.

v. What is the use of sodium dodecyl sulfate in SDS-PAGE?

ANS… **SDS** is a detergent that is present in the **SDS-PAGE** sample buffer where, along with a bit of boiling, and a reducing agent (normally DTT or B-ME to break down protein-protein disulphide bonds), it disrupts the tertiary structure of proteins. This brings the folded proteins down to linear molecules.

vi. What is the pH of stacking gel and separation gel (resolving gel) in SDS-PAGE?

ANS… The **stacking gel** is a lower polyacrylamide concentration **gel** that is placed on top of the more concentrated **resolving gel** in a PAGE. It is used to improve the resolution of the electrophoresis due to its concentrating effect on the proteins in the sample, right at the beginning of the focusing **gel**. **pH of** stacking gel and separation gel (resolving gel) in SDS-PAGE is 6.8 & 8.3 respectively.

vii. What is the purpose of adding glycerol in protein sample before SDS-PAGE?

ANS… Glycerol is added so the sample loaded become more viscous and won't diffuse out of the well in the time it takes to load the other samples before the current is turned on.

viii. What is tracking dye in electrophoresis?

ANS… **Bromophenol blue** is often **used** as a tracking dye during agarose or polyacrylamide **gel electrophoresis**. **Bromophenol blue** has a slight negative charge and will migrate the same direction as DNA, allowing the user to monitor the progress of molecules moving through the **gel**. The rate of migration varies with **gel** composition.

2. Medium Answer Questions (4 Marks each 2x4= 8 Marks)

i. Explain electrophoretic mobility?

ANS… Electrophoretic mobility. 1. The rate of migration (usually in cm/s) per unit electric field strength (usually V/cm) of a charged particle in electrophoresis. Symbol μ.

The velocity with which a solute moves in response to the applied electric field is called its electrophoretic velocity, v_{ep}; it is defined as

$$v\text{ep}=\mu\text{ep}E \qquad (1)$$

where μ_{ep} is the solute's electrophoretic mobility, and E is the magnitude of the applied electrical field. A solute's electrophoretic mobility is defined as

$$\mu\text{ep}=q6\pi\eta r \qquad (2)$$

where

- q is the solute's charge,
- η is the buffer viscosity, and
- r is the solute's radius.

Using Equation (1) and Equation (2)
we can make several important conclusions about a solute's electrophoretic velocity. Electrophoretic mobility and, therefore, electrophoretic velocity, increases for more highly charged solutes and for solutes of smaller size. Because q is positive for a cation and negative for an anion, these species migrate in opposite directions. Neutral species, for which q is zero, has an electrophoretic velocity of zero.

ii. Explain horizontal & vertical electrophoresis system?

ANS… **Horizontal Gel Electrophoresis**

In horizontal gel electrophoresis, a gel is cast in a horizontal orientation and submerged in running buffer within the gel box. The gel box is divided into two compartments, with agarose gel separating the two. An anode is located at one end, while a cathode is located at the other. The ionic running buffer allows for a charge gradient to be created when a current is applied. In addition, the buffer serves to cool the gel, which heats up as a charge is applied. The running buffer is often recirculated to prevent a pH gradient from forming.

Vertical Gel Electrophoresis

A vertical gel method is slightly more complex than its horizontal counterpart. A vertical system utilizes a discontinuous buffer system, where the top chamber contains the cathode and the bottom chamber contains the anode. A thin gel (less than 2 mm) is poured between two glass plates and mounted so that the bottom of the gel is submerged in buffer in one chamber and the top is submerged in buffer in another chamber. When current is applied, a small amount of buffer migrates through the gel from the top chamber to the bottom chamber.

Unlike horizontal systems, the buffer can only flow through the gel, which allows for precise control of voltage gradients during separation. When combined with the smaller pore size of the acrylamide gel, greater separation and resolution can be achieved with this system compared to horizontal systems.

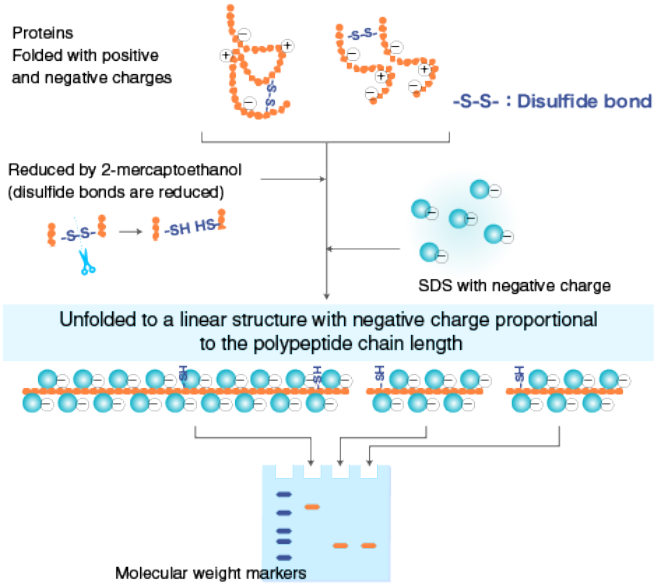
3. Long Answer Questions (7 Marks each 2x7= 14 Marks)

i. What type of electrophoresis can be used for separation of protein sample according to their electrophoretic mobility, Explain?

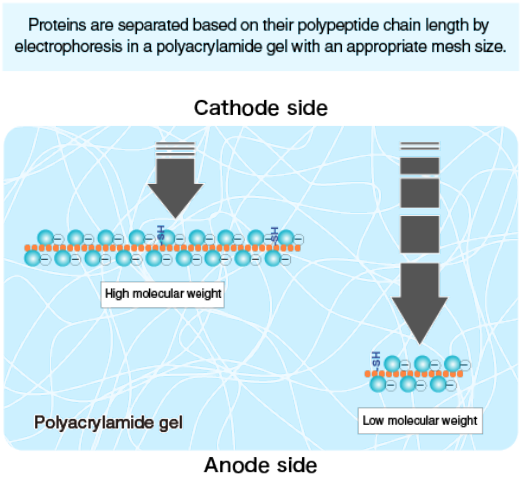
ANS… SDS-PAGE (**sodium dodecyl sulfate-polyacrylamide gel electrophoresis**) is a technique used to separate the proteins according to their masses. Separation of macromolecules under the influence of the charge is called electrophoresis. Polyacrylamide gel electrophoresis (PAGE) is one of the most widely used laboratory methods to separate biological macromolecules such as proteins and nucleic acids. Macromolecules will be differentiated according to their electrophoresis mobility which is a function of the length, conformation and charge of the molecule. In general, macromolecules may be run in their native state or in denatured forms. To separate molecules based on their lengths, samples are run in denaturing conditions. For proteins, sodium dodecyl sulfate (SDS) is used to linearize proteins and to negatively charge the proteins. The binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass. As a result, negatively charged proteins will migrate towards the positive electrode and will be fractionated by approximate size during electrophoresis. This procedure is called SDS-PAGE. Western blotting is the combination of SDS-PAGE and antibody based detection and is a commonly used antibody application to detect proteins from complex biological mixtures. When proteins are separated by electrophoresis through a gel matrix, smaller proteins migrate faster due to less resistance from the gel matrix. Other influences on the rate of migration through the gel matrix include the structure and charge of the proteins.

In SDS-PAGE, the use of sodium dodecyl sulfate (SDS, also known as sodium lauryl sulfate) and polyacrylamide gel largely eliminates the influence of the structure and charge, and proteins are separated solely based on polypeptide chain length.

SDS is a detergent with a strong protein-denaturing effect and binds to the protein backbone at a constant molar ratio. In the presence of SDS and a reducing agent that cleaves disulfide bonds critical for proper folding, proteins unfold into linear chains with negative charge proportional to the polypeptide chain length.



Polymerized acrylamide (polyacrylamide) forms a mesh-like matrix suitable for the separation of proteins of typical size. The strength of the gel allows easy handling. Polyacrylamide gel electrophoresis of SDS-treated proteins allows researchers to separate proteins based on their length in an easy, inexpensive, and relatively accurate manner.



ii. What are the factors affecting the results of electrophoresis separation technique?

ANS… **Factors affecting on Electrophoresis:**

The rate of migration (Separation of particles) during electrophoresis will depend on the following factors:

1. **The Sample**
2. **The Electric Field**

3. The Medium

4. The Buffer

1. The Sample:

Charge/mass ratio of the sample dictates its electrophoretic mobility. The mass consists of not only the size (molecular weight) but also the shape of the molecule.

a) **Charge:** The higher the charge, greater is the electrophoretic mobility. The charge is dependent on pH of the medium.

b) **Size:** The bigger molecules have a small electrophoretic mobility compared to the smaller particles.

c) **c) Shape:** The globular protein will migrate faster than the fibrous protein

2. The Electric Field:

The rate of migration under unit potential gradient is referred to as "Mobility of the ion". An increase in potential gradient increases the rate of migration.

3. The Medium:

The inert medium can exert adsorption & molecular sieving effects on the particle, influencing its rate of migration.

a) Adsorption: It means retention of a component on the surface of supporting medium. The rate and resolution of the electrophoretic separation can be efficiently reduced by adsorption.

b) Molecular sieving: Media such as "Polyacrylamide", "Agar", "Starch" & "Sephadex" have cross-linked structures giving rise to pores within the gel beads.

- **Sephadex,** molecules larger than the pores are excluded from entering the gel beads & these molecules migrate faster.

- **Polyacrylamide, Starch & Agarose** the larger molecules also are made to squeeze through the pores. The smaller molecules pass through the pores easily, but the larger molecules are retarded.

4. The Buffer: The buffer can affect the electrophoretic mobility of the sample in various ways.

A) Composition: Commonly used buffers are "Formate", "Acetate", "Citrate", " Phospahate", "EDTA" "Acetate" "Pyridine", "Tris" (2-amino, 2-hydroxymethyl, 1,3-diol pentane) and "Barbitone" etc. The choice of buffer depends upon the type of sample being electrophoresed.

b) Ionic Strength: "Ionic Strength (I) is a measure of the electrical environment of ions in a sol". When increase ionic strength of the buffer means a larger share of the current being carried by the buffer ions & meager (small quantity) proportion carried by the sample ions.

When decrease ionic strength, a larger share of the current being carried by the sample ions leading to a faster separation.

Note: The ionic strength used is usually between 0.05 to 0.1M.

d) **pH:** The pH determines the degree of ionization of organic compounds; it can also affect the rate of migration of these compounds. When increase pH, increases ionization of organic acids. Decrease in pH, increases ionization of organic bases. **E.g.:** an Ampholyte (Amino acid) - The amino acid has both acidic & basic properties