Question 1:

1. (a) Justify the following: (i) Glass cuvettes are not used to measure absorbance of DNA solutions.

- Glass absorbs ultraviolet (UV) light, and DNA solutions absorb light primarily in the UV range (around 260 nm). Using glass cuvettes would interfere with accurate absorbance measurements. Quartz cuvettes are used instead, as they are transparent to UV light.

(ii) A denser particle will have a higher sedimentation coefficient.

- The sedimentation coefficient (s) is a measure of a particle's sedimentation rate in a centrifugal field. It is directly proportional to the mass and density of the particle. Denser particles experience a greater centrifugal force for a given size, leading to a higher sedimentation rate and thus a higher sedimentation coefficient.

(iii) All fluorescence compounds have cyclic structures.

- While many fluorescent compounds, especially biological ones like those found in proteins (e.g., tryptophan, tyrosine, phenylalanine) or synthetic dyes, contain cyclic structures, it is not an absolute requirement for fluorescence. The presence of delocalized electrons, often found in conjugated systems within cyclic structures, contributes to fluorescence by allowing for efficient absorption and emission of light. However, some non-cyclic compounds with extensive conjugated systems can also exhibit fluorescence.

(iv) Why can molecular weight of proteins not be estimated by Native-gel electrophoresis but can be calculated by SDS-PAGE?

- Native-gel electrophoresis separates proteins based on their charge, size, and shape in their native, folded state. The migration distance is influenced by all these factors, making it difficult to directly estimate molecular weight.

- SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) denatures proteins and coats them with negatively

charged SDS molecules. This gives all proteins a similar charge-to-mass ratio, and they assume a roughly rod-like shape. Therefore, in SDS-PAGE, proteins primarily separate based on their molecular weight, with smaller proteins migrating faster through the gel. This allows for the estimation of molecular weight by comparing their migration distance to a ladder of known molecular weight standards.

(v) A spacer arm is interposed between ligand and the matrix in affinity chromatography.

- A spacer arm is used in affinity chromatography to physically separate the ligand from the solid support (matrix). This is crucial because it reduces steric hindrance, allowing the target molecule to access and bind effectively to the immobilized ligand. Without a spacer arm, the proximity of the matrix might hinder the interaction between the ligand and the target molecule, leading to inefficient binding.

(vi) Electrophoresis cannot be used for separation of lipids.

- Electrophoresis separates molecules based on their charge-to-mass ratio in an electric field. Lipids are generally non-polar and lack a significant net charge in aqueous solutions, which is essential for migration in an electric field. While some lipids can be complexed with charged molecules (e.g., lipoproteins), electrophoresis is not a primary or efficient method for separating a broad range of lipids directly. Other techniques like chromatography (e.g., thin-layer chromatography, gas chromatography) are more suitable for lipid separation.

(b) The absorbance of a 12×10^{-5} M solution at a wavelength of 280 nm is 0.5. The pathlength of the cuvette is 1 cm. Calculate the molar extinction coefficient.

- Beer-Lambert Law: $A = \varepsilon cl$
- Where:

- A = Absorbance = 0.5
- ε = Molar extinction coefficient (L mol^{-1} cm^{-1})
- c = Concentration = 12 × 10^{-5} M
- l = Pathlength = 1 cm

- Rearranging the formula to find ε:
  - ε = A / (c × l)
  - ε = 0.5 / (12 × 10^{-5} M × 1 cm)
  - ε = 0.5 / 0.00012 L cm
  - ε = 4166.67 L mol^{-1} cm^{-1}

Question 2: 2. (a) Define the following: (i) Void volume

- Void volume (V_0) in gel filtration chromatography refers to the volume of the mobile phase outside the stationary phase beads. It represents the space available to molecules that are too large to enter the pores of the gel matrix and therefore elute first.

(ii) Partition coefficient

- In chromatography, the partition coefficient (K or K_D) describes the equilibrium distribution of a solute between two immiscible phases. Specifically, in gel filtration chromatography, it represents the fraction of the stationary phase volume that is accessible to a given solute.

(iii) Exclusion limit

- The exclusion limit in gel filtration chromatography is the molecular weight at which a solute is completely excluded from the pores of the stationary phase beads. Molecules above this molecular weight will elute in the void volume.

(iv) Chromophore

- A chromophore is the part of a molecule that is responsible for its color by absorbing specific wavelengths of visible light. It typically consists of a conjugated system of double bonds or aromatic rings.

(v) Molar extinction coefficient

- The molar extinction coefficient (ε) is a measure of how strongly a chemical species absorbs light at a given wavelength. It is a constant for a particular substance at a specific wavelength and is expressed in units of L mol^{-1} cm^{-1}. It relates absorbance (A) to concentration (c) and pathlength (l) via the Beer-Lambert Law ($A = \varepsilon cl$).

(vi) Stoke's shift

- Stoke's shift is the difference in wavelength between the maximum of the excitation (absorption) spectrum and the maximum of the emission (fluorescence or phosphorescence) spectrum for a given fluorophore. This shift occurs because some energy is lost as heat during vibrational relaxation within the excited state before emission of a photon.

(vii) Retention factor

- The retention factor (R_f) in planar chromatography (e.g., paper or thin-layer chromatography) is the ratio of the distance traveled by the solute (spot) to the distance traveled by the solvent front, both measured from the origin. It is a characteristic value for a given compound under specific chromatographic conditions.

(b) Derive Beer-Lambert's Law and discuss its limitations.

- Derivation of Beer-Lambert's Law:
    - Consider a monochromatic beam of light passing through an absorbing solution of thickness 'dl' and concentration 'c'.
    - Let I be the intensity of the incident light and dI be the decrease in intensity as it passes through the small thickness dl.

- The decrease in light intensity (dI) is proportional to the incident light intensity (I), the concentration of the absorbing substance (c), and the thickness of the solution (dl).
- So, $-dI \propto Icdl$
- $-dI = kIcdl$ (where k is a proportionality constant)
- Rearranging, $dI/I = -kcdl$
- Integrating from initial intensity I_0 (at $l = 0$) to final intensity I (at pathlength l):
  - $\int_{I_0}^{I} d\,I/I = \int_0^l -kcdl$
  - $[lnI]_{I_0}^{I} = -kc[l]_0^l$
  - $lnI - lnI_0 = -kcl$
  - $ln(I/I_0) = -kcl$
- Converting from natural logarithm to base 10 logarithm (by multiplying by 2.303):
  - $2.303log_{10}(I/I_0) = -kcl$
  - $log_{10}(I_0/I) = (k/2.303)cl$
- We define absorbance (A) as $log_{10}(I_0/I)$.
- We define the molar extinction coefficient (ε) as $k/2.303$.
- Therefore, $A = \varepsilon cl$ (Beer-Lambert's Law).

- Limitations of Beer-Lambert's Law:
  - Non-monochromatic light: The law strictly applies to monochromatic light. Using polychromatic light can lead to deviations.

- High concentrations: At very high concentrations, solute molecules can interact with each other, leading to changes in their ability to absorb light, or aggregation may occur, causing deviations from linearity.
- Chemical reactions: If the absorbing substance undergoes chemical reactions (e.g., dissociation, association, or complex formation) with the solvent or other components in the solution, its effective concentration changes, leading to deviations.
- Scattering: The presence of particulate matter or turbidity in the solution can cause light scattering, which is measured as part of the absorbance, leading to falsely high readings.
- Fluorescence or phosphorescence: If the sample fluoresces or phosphoresces, the emitted light can contribute to the measured transmitted light, leading to deviations.
- Temperature and pH changes: The molar extinction coefficient can be affected by temperature and pH, especially for molecules sensitive to these conditions.

(c) Describe the different types of rotors used for centrifugation.

- There are three main types of rotors used in centrifugation:
  - Fixed-Angle Rotors:
    - In a fixed-angle rotor, the tubes are held at a constant, fixed angle (typically between 20° and 45°) relative to the axis of rotation.
    - When the rotor spins, particles move radially outwards and pellet against the outer wall of the tube, sliding down to form a compact pellet at the bottom corner.
    - Advantages: Efficient for pelleting large volumes and rapid sedimentation of particles. They achieve high g-

forces and are commonly used for differential centrifugation.

- Disadvantages: Pellets are often smeared along the side of the tube, making precise collection of small pellets difficult. Not ideal for gradient centrifugation where precise layering is needed.

- Swinging-Bucket Rotors:
  - In a swinging-bucket rotor, the tubes are placed in buckets that are suspended from the rotor body. As the rotor accelerates, the buckets swing out to a horizontal position (90° to the axis of rotation).
  - This results in particles moving directly outwards along the radius of the rotor, forming a compact, flat pellet at the bottom of the tube.
  - Advantages: Ideal for gradient centrifugation (rate zonal or isopycnic) because the sample can be layered on top of the gradient, and the horizontal orientation prevents mixing during acceleration. They produce well-formed, compact pellets that are easy to resuspend.
  - Disadvantages: Generally achieve lower g-forces compared to fixed-angle rotors for the same speed due to a larger average path length of sedimentation. Slower run times for pelleting.
- Vertical Rotors:
  - In a vertical rotor, the tubes are held vertically, parallel to the axis of rotation.
  - During centrifugation, the centrifugal force acts horizontally, forcing particles to sediment across the width of the tube.

- Advantages: Extremely short sedimentation path lengths, leading to very rapid separations, particularly useful for equilibrium (isopycnic) centrifugation where particles reach their buoyant density quickly.
- Disadvantages: Pellets form along the sides of the tubes, similar to fixed-angle rotors, which can be challenging for recovery. Not suitable for layering samples on gradients in the same way as swinging-bucket rotors. Requires careful handling to prevent mixing during deceleration.

Question 3: 3. Mention the role of the following in SDS-PAGE:

(a) SDS in the sample buffer

- Sodium Dodecyl Sulfate (SDS) is an anionic detergent that denatures proteins by breaking non-covalent interactions (e.g., hydrogen bonds, hydrophobic interactions) and coating them with a uniform negative charge. This gives all proteins a similar charge-to-mass ratio, ensuring that separation is primarily based on molecular weight.

(b) β-mercaptoethanol

- β-mercaptoethanol is a reducing agent that breaks disulfide bonds (S-S) within or between protein subunits. This ensures complete denaturation of proteins, allowing them to fully unfold and be coated by SDS, and preventing aggregation. Dithiothreitol (DTT) is another common reducing agent used for this purpose.

(c) TEMED

- TEMED (N,N,N',N'-tetramethylethylenediamine) acts as a catalyst in the polymerization of acrylamide and bis-acrylamide to form the polyacrylamide gel. It catalyzes the formation of free radicals from ammonium persulfate, which then initiate the polymerization process.

(d) Ammonium persulphate

- Ammonium persulfate (APS) is a source of free radicals that initiate the polymerization of acrylamide and bis-acrylamide. These free radicals facilitate the formation of the polyacrylamide gel matrix.

(e) Coomassie Brilliant Blue

- Coomassie Brilliant Blue is a dye used to stain proteins after electrophoresis. It binds non-specifically to proteins, allowing them to be visualized as blue bands on the gel, thus enabling the assessment of protein separation and estimation of molecular weights.

(f) Separating gel

- The separating gel (or resolving gel) is the lower part of the polyacrylamide gel and has a higher concentration of acrylamide, resulting in smaller pore sizes. This gel is where the primary separation of proteins by molecular weight occurs, as proteins migrate through its pores based on their size.

(g) Bromophenol blue

- Bromophenol blue is a negatively charged tracking dye added to the sample buffer. It does not bind to proteins but migrates ahead of the proteins, serving as a visual marker for the progress of electrophoresis and indicating when the run should be stopped before the proteins migrate off the gel.

(h) Glycerol

- Glycerol is added to the sample buffer to increase the density of the sample. This allows the sample to sink to the bottom of the sample wells in the gel, preventing it from floating out when the electrophoresis buffer is added.

(i) Glycine

- Glycine is a component of the running buffer (tank buffer). It acts as a trailing ion in the stacking gel, creating a sharp voltage front that compresses the proteins into a narrow band before they enter the

separating gel. This "stacking effect" leads to sharper protein bands in the separating gel.

(j) Tris buffer

- Tris (Tris(hydroxymethyl)aminomethane) buffer is used in both the stacking and separating gels, as well as in the running buffer. Its primary role is to maintain a stable pH environment during electrophoresis. Different pH values in the stacking and separating gels, maintained by Tris buffer, are crucial for the stacking effect of proteins.

Question 4: 4. Differentiate between the following pairs:

(a) Cation and anion exchangers

- Cation Exchangers:
    - Contain negatively charged functional groups (e.g., sulfonate, carboxylate) covalently attached to the stationary phase matrix.
    - Bind positively charged molecules (cations) from the sample.
    - Separation occurs based on the strength of the positive charge of the molecules; more positively charged molecules bind more strongly.
    - Examples: CM-cellulose (carboxymethyl-cellulose), SP-Sephadex (sulfopropyl-Sephadex).
- Anion Exchangers:
    - Contain positively charged functional groups (e.g., diethylaminoethyl (DEAE), quaternary ammonium) covalently attached to the stationary phase matrix.
    - Bind negatively charged molecules (anions) from the sample.

- Separation occurs based on the strength of the negative charge of the molecules; more negatively charged molecules bind more strongly.
- Examples: DEAE-cellulose, QAE-Sephadex (quaternary aminoethyl-Sephadex).

(b) Fixed angle and swinging bucket rotors

- Fixed-Angle Rotors:
  - Tubes are held at a constant, fixed angle relative to the axis of rotation (e.g., 20-45 degrees).
  - Pellet forms against the outer wall and slides down to the bottom corner of the tube.
  - Best suited for differential centrifugation (pelleting particles) and achieve higher g-forces.
  - Pellets can be smeared, making recovery of small, clean pellets challenging.
- Swinging-Bucket Rotors:
  - Tubes are held in buckets that swing out to a horizontal position (90 degrees to the axis of rotation) during centrifugation.
  - Pellet forms at the true bottom of the tube as a compact, flat disk.
  - Ideal for density gradient centrifugation (rate zonal and isopycnic) due to the horizontal orientation, which prevents mixing of gradients.
  - Generally achieve lower g-forces for a given speed and have longer run times for pelleting.

(c) Paper and thin layer chromatography

- Paper Chromatography:

- Stationary phase: A strip or sheet of specialized paper (cellulose).
- Separation mechanism: Primarily partition chromatography, where solutes partition between the stationary aqueous phase held by the cellulose fibers and the mobile organic solvent phase.
- Development: Often performed in a tank with the solvent moving up (ascending) or down (descending) the paper.
- Detection: Spots are visualized after drying, often by spraying with specific reagents.

- Thin Layer Chromatography (TLC):
  - Stationary phase: A thin layer of adsorbent material (e.g., silica gel, alumina, cellulose) coated on a rigid support (glass plate, plastic sheet, aluminum foil).
  - Separation mechanism: Primarily adsorption chromatography, where solutes adsorb to the surface of the stationary phase, but also partition effects.
  - Development: Performed in a sealed chamber with the solvent moving up the plate by capillary action.
  - Detection: Spots can be visualized using UV light (if compounds absorb UV), spraying with reagents, or by charring. TLC is generally faster and offers better resolution than paper chromatography.

(d) Extrinsic and intrinsic fluorophores

- Extrinsic Fluorophores:
  - These are fluorescent molecules that are not naturally part of the biomolecule of interest but are externally added or attached.

- They are used to label non-fluorescent molecules (e.g., proteins, DNA) to make them detectable by fluorescence.
- Examples: Fluorescein, Rhodamine, DAPI, Ethidium bromide.

- Intrinsic Fluorophores:
  - These are fluorescent molecules that are naturally present within the biomolecule itself.
  - For proteins, the aromatic amino acids tryptophan, tyrosine, and phenylalanine are intrinsic fluorophores (with tryptophan being the most significant contributor to protein fluorescence).
  - For nucleic acids, the bases (adenine, guanine, cytosine, thymine/uracil) have intrinsic fluorescence, though it is generally weak in native structures.
  - Their fluorescence properties can provide information about the conformation and environment of the biomolecule.

(e) Isopycnic and rate zonal gradient centrifugation

- Isopycnic (Equilibrium) Gradient Centrifugation:
  - Principle: Particles are separated based on their buoyant density. The centrifuge tube contains a pre-formed or self-forming density gradient (e.g., CsCl, sucrose).
  - Separation: Particles migrate until they reach a position in the gradient where their buoyant density matches the density of the surrounding medium (their isopycnic point). They then cease to move further.
  - Resolution: Provides excellent resolution for separating particles with small density differences.
  - Application: Often used for separating DNA (e.g., in Meselson-Stahl experiment), plasmids, viruses, and organelles.

- Rate Zonal (Velocity) Gradient Centrifugation:
    - Principle: Particles are separated based on their sedimentation rate, which is influenced by their size, shape, and density. A sample is layered on top of a pre-formed, shallow density gradient (e.g., sucrose).
    - Separation: Particles sediment through the gradient at different rates, forming distinct zones or bands. The run is stopped before particles reach the bottom of the tube.
    - Resolution: Provides good resolution for separating particles of different sizes or shapes, even if they have similar densities.
    - Application: Used for separating macromolecules, ribosomes, organelles, and protein complexes.

Question 5: 5. (a) Describe the principle and applications of affinity chromatography.

- Principle of Affinity Chromatography:
    - Affinity chromatography is a highly specific separation technique based on the reversible and specific binding interaction between a biomolecule (the target molecule) and a specific ligand.
    - A ligand, which has a specific binding affinity for the target molecule, is covalently attached to an inert, insoluble solid support (matrix). A spacer arm is often used to ensure proper access of the target molecule to the ligand.
    - When a sample containing the target molecule is applied to the column, the target molecule specifically binds to the immobilized ligand, while other non-binding components pass through the column (are not retained).

- After washing away the unbound material, the bound target molecule is eluted by altering the binding conditions. This can be achieved by:
    - Changing the pH or ionic strength to weaken the ligand-target molecule interaction.
    - Adding a competing ligand (a soluble form of the ligand or a molecule that competes for the binding site) to displace the bound target molecule.
    - Using a denaturing agent if the binding is very strong, although this might affect the target molecule's activity.
- The purified target molecule is then collected.

- Applications of Affinity Chromatography:
    - Protein purification: Widely used for purifying recombinant proteins (e.g., using His-tag, GST-tag), antibodies (e.g., Protein A/G affinity), enzymes, and receptors.
    - Nucleic acid purification: Purification of mRNA using oligo(dT) cellulose (ligand for poly-A tail).
    - Removal of specific contaminants: For instance, removing unwanted proteins from a mixture.
    - Isolation of specific cell types: Using antibodies as ligands to capture specific cells.
    - Drug discovery and development: Identifying molecules that bind to a specific target.
    - Studying biomolecular interactions: Analyzing binding kinetics and affinity constants between a ligand and its target.

(b) Explain the working of a double beam spectrophotometer with the help of a diagram.

- *Please note: As per your instructions, I cannot provide a schematic diagram. I will describe the working principle in detail.*
- Working of a Double Beam Spectrophotometer:
    - A double beam spectrophotometer is designed to compensate for changes in light source intensity, variations in detector response, and absorbance by the cuvette or solvent. It achieves this by splitting the light beam into two paths: a sample beam and a reference beam.
    - i. Light Source: A stable light source (e.g., deuterium lamp for UV, tungsten-halogen lamp for visible range) emits polychromatic light.
    - ii. Monochromator: The polychromatic light passes through a monochromator (typically a grating or prism), which disperses the light and selects a specific wavelength (monochromatic light) to pass through to the next stage.
    - iii. Beam Splitter: The monochromatic light beam is then split into two beams of equal intensity. This can be done by a rotating chopper mirror that alternately directs the light through the sample and reference paths, or by a fixed beam splitter.
    - iv. Sample and Reference Paths:
        - One beam, the "sample beam," passes through the sample cuvette containing the solution of interest.

- The other beam, the "reference beam," passes through a reference cuvette containing only the solvent or blank solution. This accounts for any absorbance by the solvent or the cuvette itself.

v. Detector: Both the sample beam and the reference beam then alternately (or simultaneously, depending on design) reach a single photodetector (e.g., photomultiplier tube, photodiode array).

vi. Electronic Processing: The detector measures the intensity of light passing through both the sample (I) and the reference (I_0). An electronic circuit then compares these two intensities.

vii. Data Output: The ratio of I to I_0 (I/I_0) is used to calculate the transmittance, and the negative logarithm of transmittance ($log_{10}(I_0/I)$) is calculated to give the absorbance (A).

- The continuous comparison of sample and reference signals ensures high accuracy and stability, as any fluctuations in the light source or detector sensitivity are cancelled out. This allows for direct measurement of the absorbance due to the solute in the sample.

(c) Write the principle of ion-exchange chromatography and write two applications of this technique.

- Principle of Ion-Exchange Chromatography:
  - Ion-exchange chromatography (IEC) separates molecules based on their net charge. The stationary phase consists of an

insoluble matrix (e.g., cellulose, agarose, polystyrene) to which charged functional groups are covalently attached. These functional groups are either positively charged (anion exchanger) or negatively charged (cation exchanger).

- When a sample is applied to the column, molecules with a charge opposite to that of the stationary phase will bind to the resin. Molecules with the same charge as the stationary phase or those that are uncharged will pass through the column without binding.
- Bound molecules are then eluted by changing the ionic strength or pH of the mobile phase.
    - Elution by ionic strength: Increasing the concentration of counter-ions (e.g., NaCl) in the elution buffer competes with the bound molecules for binding sites on the resin. Stronger binding molecules require higher salt concentrations for elution.
    - Elution by pH: Changing the pH alters the net charge of the sample molecules and/or the functional groups on the resin, thereby reducing or eliminating the electrostatic attraction and allowing the bound molecules to be released.
- Molecules are eluted in order of increasing strength of interaction with the stationary phase.

- Two Applications of Ion-Exchange Chromatography:
    -
        ii. Purification of proteins and enzymes: Widely used to purify proteins based on their isoelectric point (pI) and overall charge. For example, separating different isoforms of an enzyme or purifying specific antibodies.
    -

iii. Separation of amino acids, nucleotides, and nucleic acids: Due to their distinct charge profiles at different pH values, ion-exchange chromatography is highly effective for separating these biomolecules. It's used in amino acid analysis and the purification of various DNA and RNA species.

Question 6: 6. (a) What are the properties of good matrix used in chromatography? Write two examples of matrices used in gel filtration chromatography.

- Properties of a good matrix used in chromatography:
    - Inertness: The matrix should be chemically inert and not interact (adsorb or react) with the sample components, ensuring that separation is based solely on the intended mechanism (e.g., size exclusion, charge).
    - Mechanical Stability: It should be mechanically robust to withstand the pressure of high flow rates in chromatography columns without compressing or deforming, especially in high-performance liquid chromatography (HPLC).
    - Rigidity/Porosity: For techniques like gel filtration, the matrix must have well-defined and uniform pore sizes that are stable under varying conditions. For other techniques, it needs a suitable surface area for binding.
    - High Surface Area: For adsorption-based techniques, a high surface area is desirable to provide sufficient binding sites.
    - Chemical Stability: The matrix should be stable over a wide range of pH, temperature, and in the presence of various solvents and denaturants typically used during chromatography, including cleaning solutions.
    - Availability of Reactive Groups: For techniques like affinity chromatography or ion-exchange chromatography, the matrix

needs suitable functional groups that can be easily modified or derivatized to attach ligands or charged groups.

- Non-specific Binding: Low non-specific binding of sample components is crucial to ensure high recovery and purity of the target molecule.
- Cost-effectiveness: The matrix should be reasonably priced, especially for large-scale applications.

- Two examples of matrices used in gel filtration chromatography:
  - iii. Sephadex: A cross-linked dextran polymer. It comes in various pore sizes (e.g., G-25, G-50, G-200), where the number indicates the approximate water regain value (related to pore size).
  - iv. Sepharose: An agarose-based matrix. It is known for its high porosity and is suitable for separating very large molecules like proteins, nucleic acids, and even viruses. Sepharose also comes in different pore sizes.

(b) Elaborate the principle of isoelectric focusing technique.

- Principle of Isoelectric Focusing (IEF):
  - Isoelectric focusing is an electrophoretic technique used to separate proteins (or other amphoteric molecules) based on their isoelectric point (pI). The pI is the specific pH at which a molecule carries no net electrical charge.
  - The technique employs a pH gradient that is stable and continuous across an electrophoresis gel (e.g., polyacrylamide gel). This pH gradient is typically established using a mixture of ampholytes, which are small, amphoteric molecules with

different pIs, that migrate and arrange themselves in the electric field to form a stable pH gradient.

- When an electric field is applied across the gel, proteins in the sample begin to migrate.
- A protein with a net positive charge will migrate towards the cathode (negative electrode). As it moves through the pH gradient towards higher pH values, its net positive charge will decrease.
- Conversely, a protein with a net negative charge will migrate towards the anode (positive electrode). As it moves through the pH gradient towards lower pH values, its net negative charge will decrease.
- Each protein will continue to migrate until it reaches the point in the pH gradient where its net charge is zero, i.e., where the pH of the gel matches its isoelectric point (pI). At this point, the protein stops migrating and becomes "focused" into a sharp band.
- Since each protein has a unique pI, they will focus at different positions along the pH gradient, resulting in their separation.
- IEF is typically the first dimension in two-dimensional gel electrophoresis (2D-PAGE), providing excellent resolution based on charge before separation by size in the second dimension (SDS-PAGE).

(c) Explain the principle of agarose electrophoresis and cite two examples of its applications.

- Principle of Agarose Electrophoresis:
  - Agarose gel electrophoresis is a commonly used technique for the separation and analysis of nucleic acids (DNA and RNA) primarily based on their size (molecular weight).

- The principle relies on the fact that nucleic acids are negatively charged due to their phosphate backbone. When placed in an electric field, they will migrate towards the positively charged anode.
- The separation occurs within a porous gel matrix made of agarose, a linear polysaccharide derived from seaweed. The gel acts as a molecular sieve.
- Smaller nucleic acid molecules can move more easily through the pores of the agarose gel, and thus migrate faster. Larger molecules experience more resistance and migrate more slowly.
- The rate of migration is inversely proportional to the logarithm of the molecular weight of the nucleic acid.
- The concentration of agarose in the gel determines the pore size; higher concentrations create smaller pores, which are better for separating smaller fragments, while lower concentrations create larger pores, suitable for larger fragments.
- After electrophoresis, the separated DNA or RNA fragments are visualized by staining with fluorescent dyes (e.g., Ethidium Bromide, GelRed) that intercalate into the nucleic acid and fluoresce under UV light.

- Two examples of its applications:
  - iv. DNA fragment separation and size determination: Used to separate DNA fragments generated by restriction enzyme digestion, PCR amplification, or cloning experiments. By running a DNA ladder (fragments of known sizes) alongside the samples, the size of unknown DNA fragments can be estimated.

- v. Plasmid DNA analysis and purification: Used to check the integrity and supercoiling status of plasmid DNA, and to separate different forms of plasmid DNA (e.g., supercoiled, relaxed, linear) for purification or further downstream applications.