1. Electrophoresis

- The term electrophoresis describes the migration of a charged particles under the influence of an electric field.
- Various essential biological molecules, such as amino acids, peptides, proteins, nucleic acids, nucleotides, have ionizable group, which at given pH exist in a solution as electrically charged species either as cation (+ve) and anion (-ve) are separated by electrophoresis
- Under the influence of electric field these charged particles will migrates either to cathode or anode depending on the nature of their net charge

1.1 Principle of electrophoresis:

- When a potential difference is applied, the molecules with different overall charge will begin to separate owing to their different electrophoretic mobility. Even the molecules with similar charge will begins to separate if they have different molecular sizes, since they will experience different frictional forces. Therefore, some form of electrophoresis rely almost totally on the different charges on the molecules for separation while some other form exploits difference in size (molecular size) of molecules.
- Electrophoresis is regarded as incomplete form of electrolysis because the electric field is removed before the molecules in the samples reaches the electrode but the molecules will have been already separated according to their electrophoretic mobilities.
- The separated samples are then located by staining with an appropriate dye or by autoradiography, if the sample is radiolabeled.

Electrophoresis uses an electric field applied across a gel matrix to separate large molecules such as DNA, RNA, and proteins by charge and size. Samples are loaded into the wells of a gel matrix that can separate molecules by size and an electrical field is applied across the gel. This field causes negatively charged molecules to move towards the positive electrode. The gel matrix, itself, acts as a sieve, through which the smallest molecules pass rapidly, while longer molecules are slower-moving.

For DNA and RNA, sorting molecules by size in this way is trivial, because of the uniform negative charge on the phosphate backbone. For proteins, which vary in their charges, a clever trick must be employed to make them mimic nucleic acids - see polyacrylamide gel electrophoresis (PAGE) below. Different kinds of gels have different pore sizes. Like sieves with finer or coarser meshes, some gels do a better job of separating smaller molecules while others work better for larger ones. Gel electrophoresis may be used as a preparative technique (that is, when purifying proteins or nucleic acids), but most often it is used as an analytical tool.

1.2 Factor affecting electrophoresis:

i. Nature of charge:

- Under the influence of an electric field these charged particles will migrate either to cathode or anode depending on the nature of their net charge.

ii. Voltage:

- When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient (E), which is the applied voltage (v) divided by the distance "d" between the two electrodes i.e. p.d. (E) = V/d.
- When this potential gradient 'E' is applied, the force as the molecule bearing a charge of 'q' coulombs is 'E_q' Newtons.
- It is this force that drives the molecule towards the electrodes.

iii. Frictional force:

- There is also a frictional force that retards the movement of this charged molecule.
- This frictional force is the measure of the hydrodynamic size of the molecule, the shape of the molecule, the pore size of the medium in which the electrophoresis is taking place and the viscosity of the buffer.
- The velocity 'v' of the charged molecule is an electric field is therefore given by the equation. $U=E_q/f$, where 'f'= frictional coefficient

iv. Electrophoretic mobility:

- More commonly a term electrophoretic mobility ( ) of an ion is used, which is the ratio of the velocity of the ion and the field strength. i.e. =U/E.
- When a p.d. is applied, the molecule with different overall charges will begin to separate owing to their different electrophoretic mobility.
- Even the molecule with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces.

V. current:

- Ohm's law: V/I=R
- It therefore appears that it is possible to accelerate an electrophoretic separation by increasing the applied voltage, which ultimately results in corresponding increase in the current flowing.
- The distance migrated by the ions will be proportional to both current and time.

vi. Heat:

- One of the major problems for most forms of electrophoresis, that is the generation of heat.
- During electrophoresis, the power (W) generated in one supporting medium is given by $W= I^2R$
- Most of the power generated is dissipated as heat.
- The following effects are seen on heating of the electrophoretic medium has:
    - An increased rate of diffusion of sample and buffer ions which leads to the broadening of the separated samples.
    - Formation of convention currents, which leads to mixing of separated samples.
    - Thermal instability of samples that are sensitive to heat.
    - A decrease of buffer viscosity and hence reduction in the resistance of the medium.

- If a constant voltage is applied, the current increases during electrophoresis owing to the decrease in resistance and this rise in current increases the heat output still further.
- For these reasons, often a stabilized power supply is used, which provides constant power and thus eliminates fluctuations in heating.
- Constant heat generation is however a problem. For which the electrophoresis is run at very low power (low current) to overcome any heating problems, but this can lead to poor separation as a result of the increased amount of diffusion due to long separation time.
- Compromise condition have to be found out with reasonable power settings, to give acceptable separation time and an appropriate cooling system, to remove liberated heat. While such system works fairly well, the effect of heating are not always totally eliminated.

Vii. Elecrtroendosmosis:

- The phenomenon of electroendosmosis (aka- electro-osmotic flow) is a final factor that can affect electrophoretic separation.
- This phenomenon is due to the presence of charged groups on the surface of the support medium.
- For instance, paper has some carboxyl group present, agarose contains sulfate groups depending on the purity grade and the surface of glass walls used in capillary electrophoresis contains silanol (Si-OH) groups.
- These groups, at appropriate pH, will ionize, generating charged sites.
- It is these charges that generate electroendosmosis.
- In case of capillary electrophoresis, the ionized sianol groups creates an electrical double layer, or a region of charge separation, at the capillary wall/electrolytic interface.
- When voltage is applied cations in the electrolyte near the capillary walls migrate towards the cathode, pulling electrolyte solution with them.
- This creates a net electroosmotic flow towards cathode.

1.3 Types of support media used in electrophoresis:

- The earliest supports used were filter paper or cellulose acetate strips, wetted in electrophoresis buffer. Nowadays these media are not is use.
- Nowadays either an agarose gel or polyacrylamide gels are used.
- **Agarose gel:**
  - Agarose- a linear polysaccharide (M.W. 12000 Da) made up of the basic repeat unit of agarobiose (which comprises alternating units of galactose and 3,6-anhydrogalactose.
  - It is one of the components of agar, that is a mixture of polysaccharides from seaweeds.
  - It is used at a concentration between 1% and 3%.
  - Agarose gel is formed by suspending dry agarose in aqueous buffer and then boiling the mixture till it becomes clear solution, which is then poured and allowed to cool at room temperature to form rigid gel.
  - The gelling properties is attributed to inter and intramolecular H-bonding within and between long agarose chains.
  - The pore size of the gel is controlled by the initial concentration of agarose, large pore size corresponds to low concentration and vice versa.
  - Although free from charges, substitution of the alternating sugar residues with carboxyl, methoxyl, pyruvate, and sulfate groups occur to varying degrees which can result in electroendosmosis during electrophoresis.
  - Agarose is therefore sold in different purity grades, based on the sulfate concentration- the lower the sulfate concentration, the higher the purity.
  - These gels are used for the electrophoresis of both proteins and nucleic acids.
  - For proteins, the pore size of a 1% agarose gel is large relative to the sizes of proteins.
  - Therefore, used in techniques such as immune-electrophoresis or flat-bed isoelectric focusing, where proteins are required to move unhindered in the gel matrix according to their native charge.

- Such large pure gels are also used to separate much larger molecules such as RNA and DNA, because the pore sizes are still large enough for RNA and DNA molecule to pass through the gel.
- An advantage of using agarose in the availability of low melting point agarose (62-65^{o}C).
- This gel can be reliquefied by heating to 65^{o}C and thus, for example DNA samples separated can be cut out of the gel, returned to solution and recovered.

- **Polyacrylamide gel:**
  - Cross-linked polysaccharide gel are formed from the polymerization of acrylamide monomer in the presence of small amount of N,N'-methylene bis acrylamide (aka- bis-acrylamide).
  - Bis-acryl amide is basically two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent.
  - Acrylamide monomers is polymerized in head to tail fashion into long chain, thus introducing a second site for chain extension.
  - Proceeding in this way, a cross-linked matrix of fairly well-defined structure is formed.
  - The polymerization of acrylamide is an example of free radical catalysis and is initiated by the addition of ammonium persulfate and the base N, N, N', N'- tetra-methylene diamine (TEMED).
  - TEMED catalyses decomposition of the persulphate ion to give free radical.
    - $S_2O_8^{2-} + e^{-}$ à $SO_4^{2-} + SO^{\bullet}_4$
    - $R^{\bullet} + M$ à $RM^{\bullet}$
    - $RM^{\bullet} + M$ à $RMM^{\bullet}$
    - $RMM^{\bullet} + M$ à $RMMM^{\bullet}$ and so on…
  - Photopolymerisation is an alternative method that can be used to polymerize acrylamide gels.
  - The ammonium persulphate and TEMED are replaced by riboflavin and when the gel is poured, it is placed in front of a bright light for 2-3hrs.

- Photodecomposition of riboflavin generates a free radical that initiates polymerization.
- Acrylamide gels are defined in terms of the total percentage of acrylamide present, and the pore size in the gel can be varied by changing the concentration of both acrylamide and bis-acrylamide.
- The acrylamide gel can be made with a content between 3% and 30% acrylamide.
- Thus, the low percentage gels (e.g., 4%) have large pore size and are used for electrophoresis of protein- example flat bed isoelectric focusing, or stacking gel system of an SDS-PAGE.
- Low percentage acrylamide gels are also used to separate DNA.
- Gels between 10% and 20% acrylamide are used in techniques such as SDS-gel electrophoresis, where smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size.

2. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a technique used to separate nucleic acids primarily by size. Agarose (Fig 1) is a polysaccharide obtained from seaweeds (Figure 8.11). It can be dissolved in boiling buffer and poured into a tray, where it sets up as it cools (Figure 8.12) to form a slab. Agarose gels are poured with a comb in place to make wells into which DNA or RNA samples are placed after the gel has solidified. The gel is immersed in a buffer and a current is applied across the slab. Double-stranded DNA has a uniform negative charge that is independent of the sequence composition of the molecule. Therefore, if DNA fragments are placed in an electric field they will migrate from the cathode (-) towards the anode (+). The rate of migration is directly dependent on the ability of each DNA molecule to worm or wiggle its way through the sieving gel. The agarose matrix provides openings for macromolecules to move through. The largest macromolecules have the most difficult time navigating through the gel, whereas the smallest macromolecules slip through it the fastest (Fig 2).

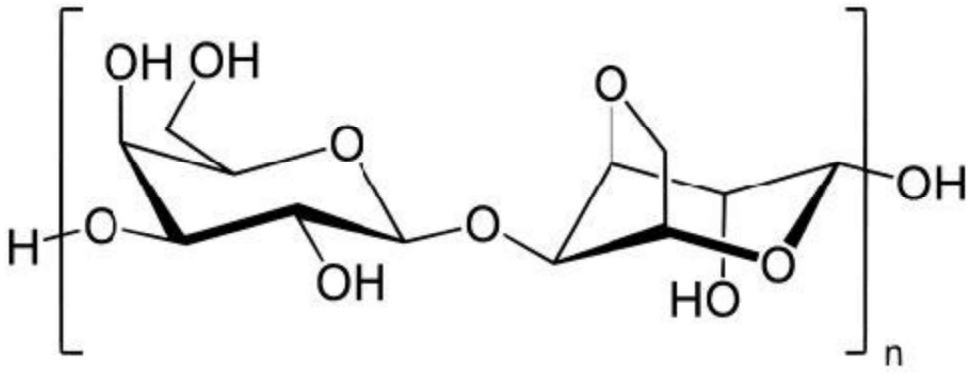

Figure 1 Structure of the agarose polysaccharide.

Because electrophoresis uses an electric current as a force to drive the molecules through the matrix, the molecules being separated must be charged. Since the size to charge ratio for DNA and RNA is constant for all sizes of these nucleic acids, the molecules simply sort on the basis of their size - the smallest move fastest and the largest move slowest.

All fragments of a given size will migrate the same distance on the gel, forming the so-called "bands" on the gel. Visualization of the DNA fragments in the gel is made possible by addition of a dye, such as ethidium bromide, which intercalates between the bases and fluoresces when viewed under ultraviolet light (Figure 8.13) By running reference DNAs of known sizes alongside the samples, it is possible to determine the sizes of the DNA fragments in the sample. It is useful to note that, by convention, DNA fragments are not described by their molecular weights (unlike proteins), but by their length in base-pairs(bp) or kilobases (kb) (Fig 3).

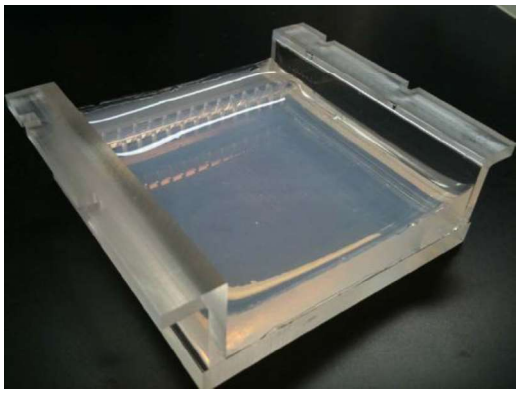

Figure 2 Agarose gel electrophoresis separation of DNA - orange bands are DNA fragments.

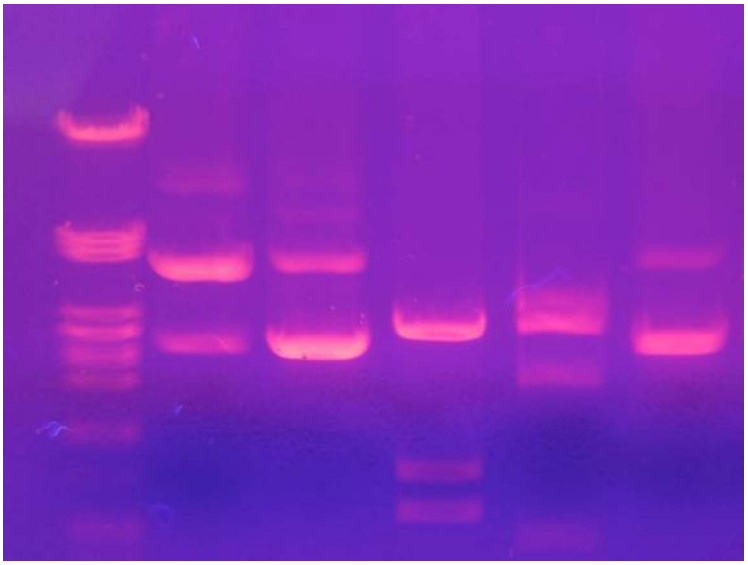

Figure 3 DNA bands visualized with ethidium bromide staining.

3. Polyacrylamide gel electrophoresis (PAGE)

Like DNA and RNA, proteins are large macromolecules, but unlike nucleic acids, proteins are not necessarily negatively charged. The charge on each protein depends on its unique amino acid sequence. Thus, the proteins in a mixture will not necessarily all move towards the anode.

Additionally, whereas double-stranded DNA is rod-shaped, most proteins are globular (folded). Further, proteins are considerably smaller than nucleic acids, so the openings of the matrix of the agarose gel are simply too large to effectively provide separation. Consequently, unaltered (native) proteins are not very good prospects for electrophoresis on agarose gels. To separate proteins by mass using electrophoresis, one must make several modifications.

3.1 Gel matrix

First, a matrix made by polymerizing and cross-linking acrylamide units is employed. A monomeric acrylamide (Fig 4) is polymerized and the polymers are cross-linked using N,N'-Methylene-bisacrylamide (Fig 5) to create a mesh-like structure. One can adjust the size of the openings of the matrix/mesh readily by changing the percentage of acrylamide in the reaction. Higher percentages of acrylamide give smaller openings and are more effective for separating smaller molecules, whereas lower percentages of acrylamide are used when resolving mixtures

of larger molecules. (Note: polyacrylamide gels are also used to separate small nucleic acid fragments, with some acrylamide gels capable of separating pieces of DNA that differ in length by just one nucleotide.)

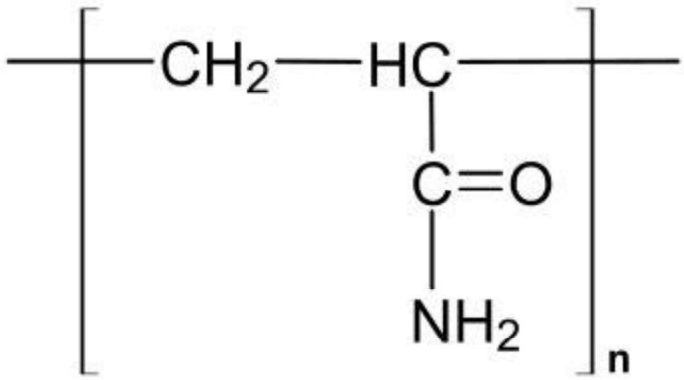

Figure 4 Acrylamide monomer.

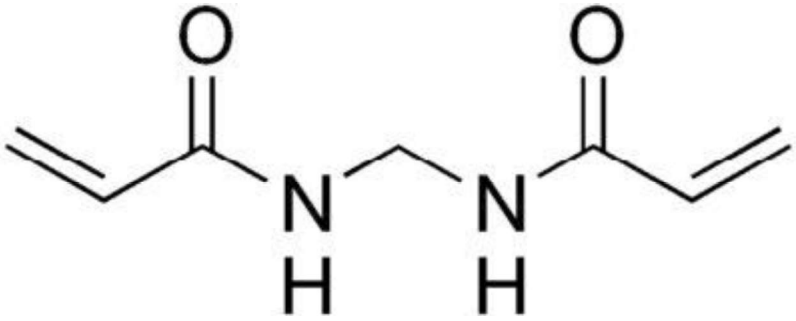

Figure 5 N,N'-Methylenebisacrylamide - acrylamide crosslinking reagent.

3.2 Charge alteration by SDS

A second consideration is that proteins must be physically altered to "present" themselves to the matrix like the negatively charged rods of DNA. This is accomplished by treating the proteins with the anionic detergent, SDS (sodium dodecyl sulfate). SDS denatures the proteins so they assume a rod-like shape and the SDS molecules coat the proteins such that the exterior surface is loaded with negative charges, masking the original charges on the proteins and making the charge on the proteins more proportional to their mass, like the backbone of DNA.

Since proteins typically have disulfide bonds that prevent them from completely unfolding in detergent, samples are boiled with mercaptoethanol to break the disulfide bonds and ensure the

proteins are as rod-like as possible in the SDS. Reagents like mercaptoethanol (and also dithiothreitol) are sulfhydryl-containing reagents that become oxidized as they reduce disulfide bonds in other molecules (Fig 6)

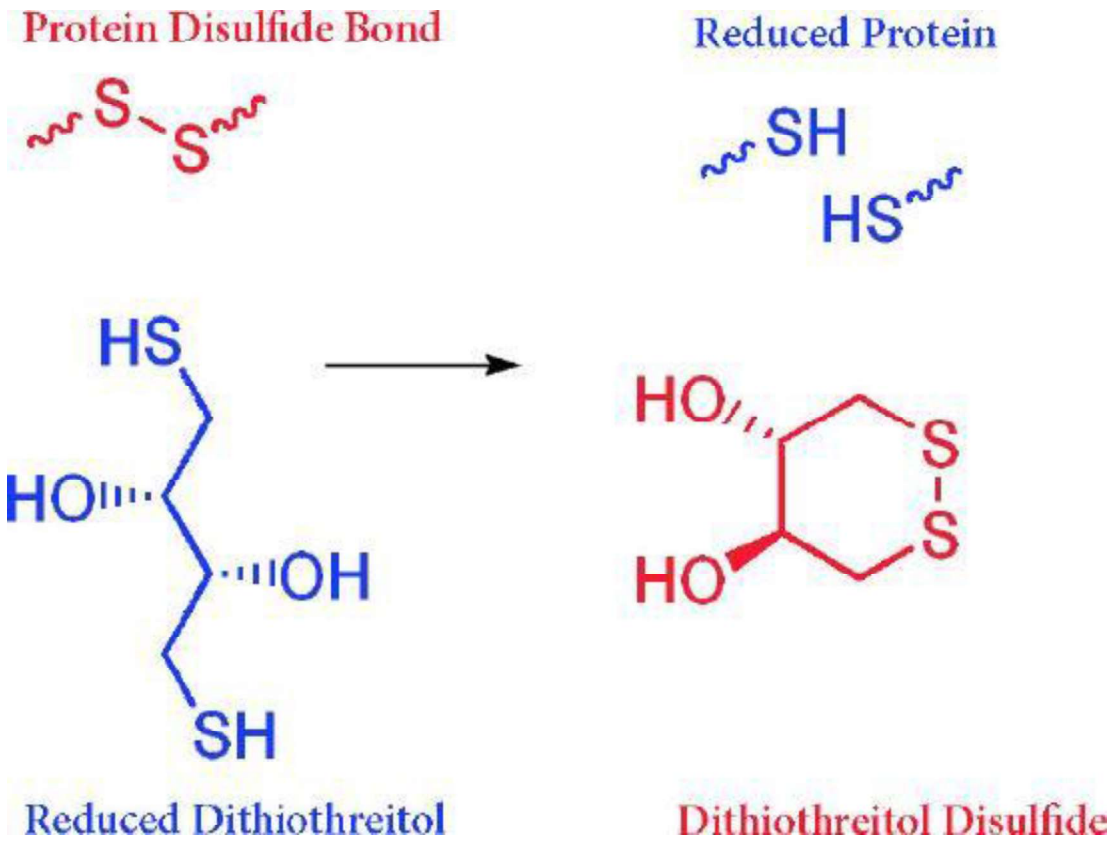

Figure 6 Reduction of disulfide bonds by dithiothreitol.

3.3 Stacking Gel

A third consideration is that a "stacking gel" may be employed at the top of a polyacrylamide gel to provide a way of compressing the samples into a tight band before they enter the main polyacrylamide gel (called the resolving gel). Just like DNA fragments in agarose gel electrophoresis get sorted on the basis of size (largest move slowest and smallest move fastest), the proteins migrate through the gel matrix at velocities inversely related to their size. Upon completion of the electrophoresis, proteins may be visualized by staining with compounds that bind to proteins, like Coomassie Brilliant Blue (Fig 7) or silver nitrate.

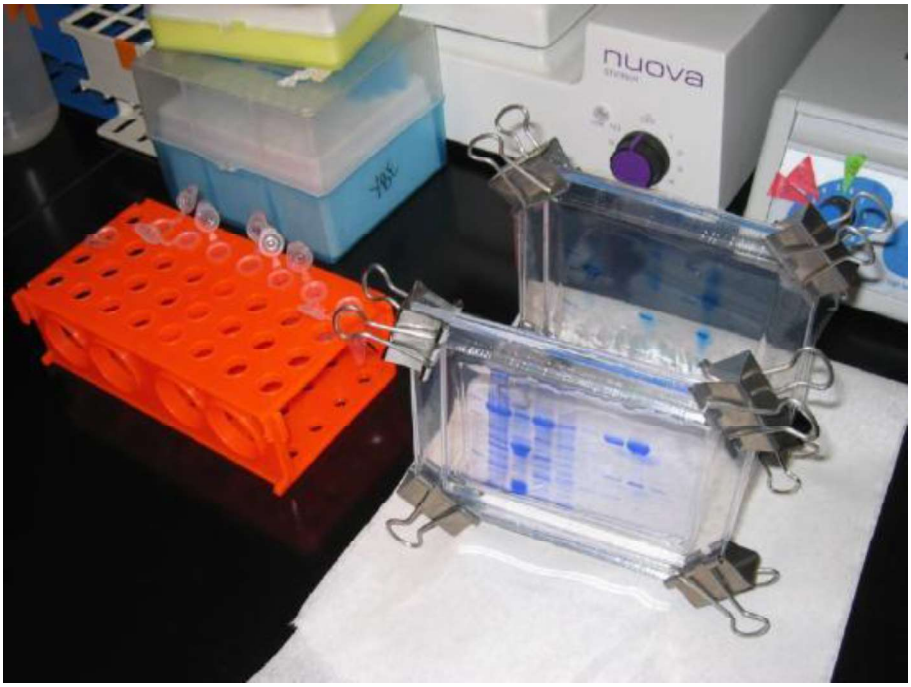

Figure 7 Two SDS-PAGE gels - Proteins are the blue bands (stained with Coomassie Blue).

3.4 **Non-denaturing gel electrophoresis**

The SDS_PAGE technique described above is the commonest method used for electrophoretic separation of proteins. In some situations, however, proteins may be resolved on so-called "native" gels, in the absence of SDS. Under these conditions, the movement of proteins through the gel will be affected not simply by their mass, but by their charge at the pH of the gel, as well. Proteins complexed with other molecules may move as single entity, allowing the isolation of the binding partners of proteins of interest.

4. Isoelectric focusing

Proteins vary considerably in their charges and, consequently, in their pI values (pH at which their charge is zero). This can be exploited to separate proteins in a mixture. Separating proteins by isoelectric focusing requires establishment of a pH gradient in a tube containing an acrylamide gel matrix. The pore size of the gel is adjusted to be large, to reduce the effect of sieving based on size. Molecules to be separated are applied to the gel containing the pH gradient

and an electric field is applied. Under these conditions, proteins will move according to their charge.

Positively charged molecules, for example, move towards the negative electrode, but since they are traveling through a pH gradient, as they pass through it, they reach a region where their charge is zero and, at that point, they stop moving. They are at that point attracted to neither the positive nor the negative electrode and are thus "focused" at their pI (Fig 8). Using isoelectric focusing, it is possible to separate proteins whose pI values differ by as little as 0.01 units.

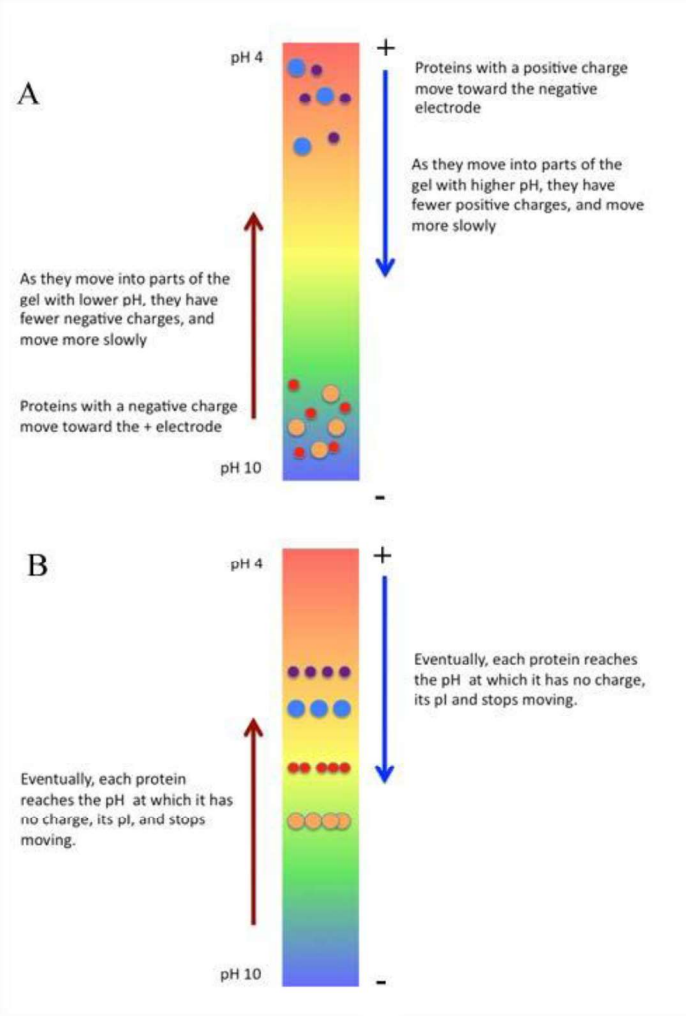

Figure 8 Isoelectric focusing: A. At the start of the run; B. at the end of the run

5. 2D gel electrophoresis

Both SDS-PAGE and isoelectric focusing are powerful techniques, but a clever combination of the two is a powerful tool of proteomics - the science of studying all of the proteins of a cell/tissue simultaneously. In 2-D gel electrophoresis, a lysate is first prepared from the cells of interest. The proteins in the lysate are separated first by their pI, through isoelectric focusing and then by size by SDS-PAGE.

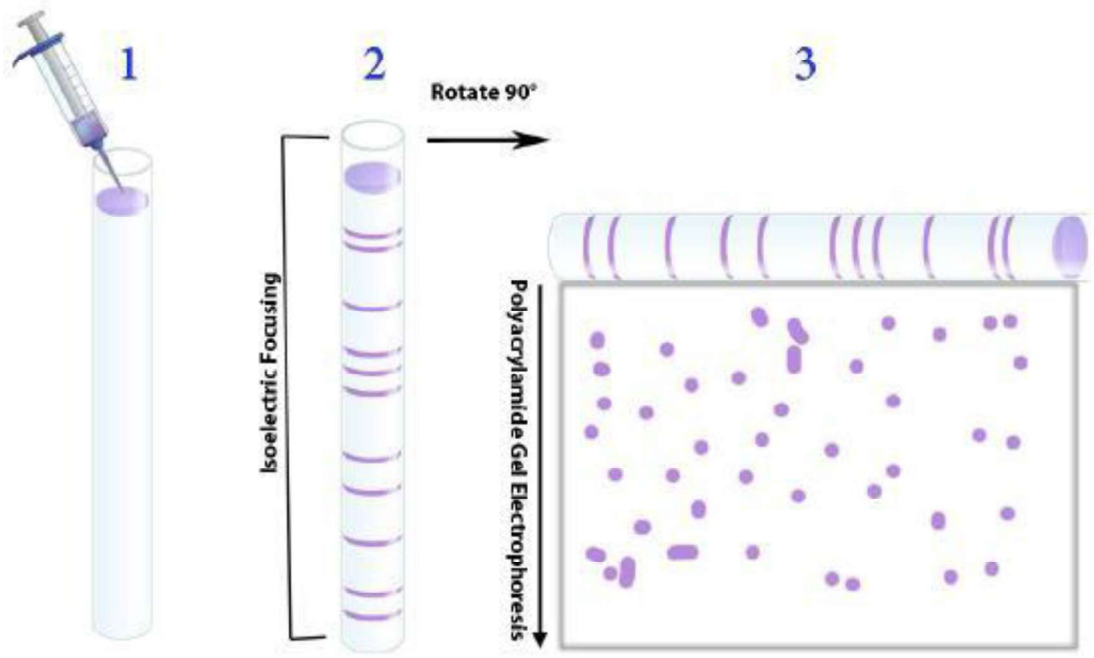

Figure 9 Scheme for performing 2-D gel analysis.

The mixture of proteins is first applied to a tube or strip (Figure 8.19, Step 1) where isoelectric focusing is performed to separate the proteins by their pI values (Step 2). Next, as shown in the figure, the gel containing the proteins separated by their pIs is turned on its side and applied along the top of a polyacrylamide slab for SDS-PAGE to separate on the basis of size (Step 3). The proteins in the isoelectric focusing matrix are electrophoresed into the polyacrylamide gel and separated on the basis of size (Fig 9). The product of this analysis is a 2-D gel as shown in Figure 10. The power of 2-D gel electrophoresis is that virtually every protein in a cell can be separated and appear on the gel as a spot defined by its unique size and pI. In the figure, spots in the upper left correspond to large positively charged proteins, whereas those in the lower right

are small negatively charged ones. Every spot on a 2-D gel can be eluted and identified by using high throughput mass spectrometry. This is particularly powerful when one compares protein profiles between different tissues or between control and treated samples of the same tissue.

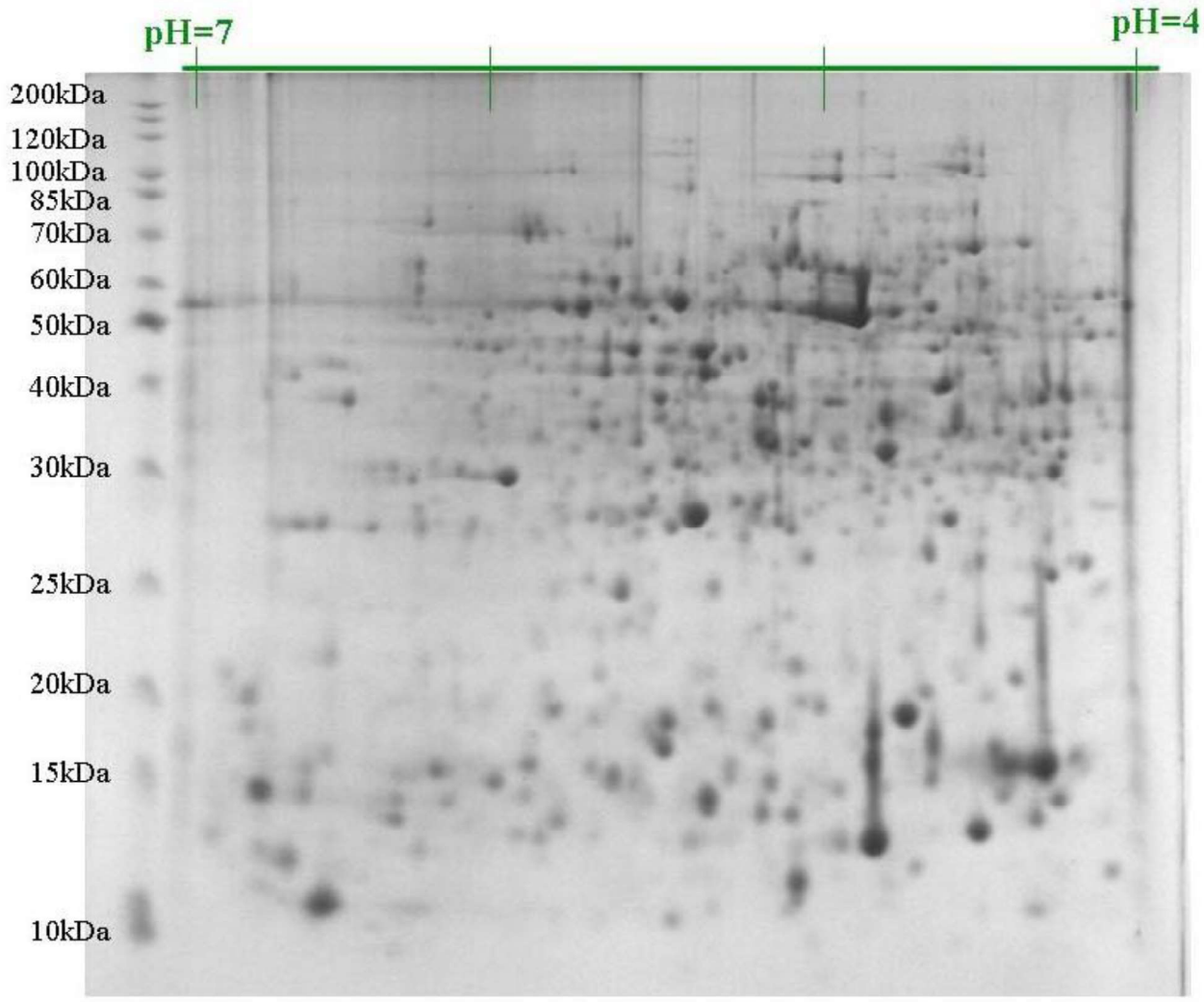

Figure 10 Result of 2-D gel electrophoresis separation.

5.1 Protein profiles comparison

Comparison of 2-D gels of proteins from non-cancerous tissue and proteins from a cancerous tissue of the same type provides a quick identification of proteins whose level of expression differs between the two. Information such as this can be useful in designing treatments or in understanding the mechanism(s) by which the cancer develops.