POLYACRYLAMIDE GEL ELECTROPHORESIS

- **Polyacrylamide gel electrophoresis (PAGE), describes a technique widely used in biochemistry, forensics, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility.**

- **Mobility is a function of the length, conformation and charge of the molecule.**

- **PAGE (Polyacrylamide Gel Electrophoresis), is an analytical method used to separate components of a protein mixture based on their size.**

- **The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign.**

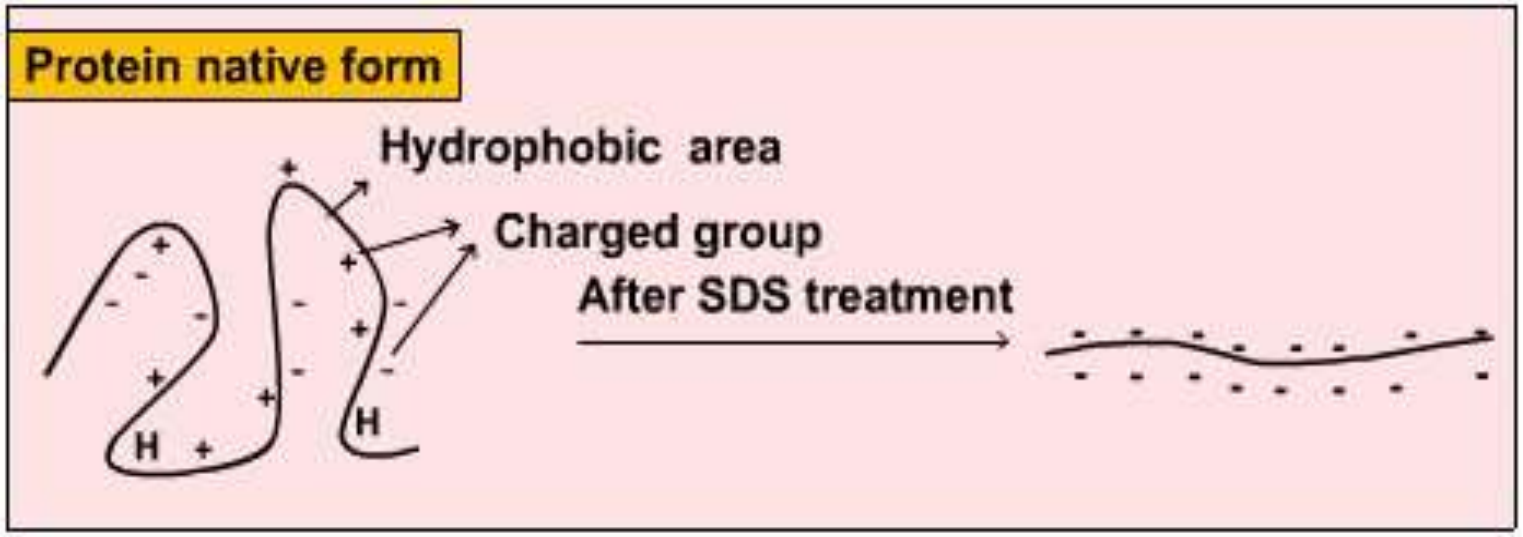
- **The general electrophoresis techniques cannot be used to determine the molecular weight of biological molecules because the mobility of a substance in the gel depends on both charge & size.**

- **To overcome this, the biological samples needs to be treated so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size.**

- **For this different protein molecules with different shapes and sizes, needs to be denatured(done with the aid of SDS) so that the proteins lost their secondary, tertiary or quaternary structure .**

- **The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, it will migrate towards the anode (positively charged electrode) are separated by a molecular sieving effect based on size.**

- **After the visualization by a staining (protein-specific) technique, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder(marker).**


Protein native form
Hydrophobic area
Charged group
After SDS treatment
H
H

Principle behind separation:

- **Separation of charged molecules in an electric field is based on the relative mobility of charged species which is related to frictional resistance.**

Charge of the species:

- **PAGE is working upon the principle in which, the charged molecule will migrate towards the oppositive charged electrode through highly cross linked matrix.**

- **Separation occurs due to different rates of migration occurs by the magnitude of charge and frictional resistance related to the size.**

Relative Mobility:

$$R_f = \frac{ZE}{F}$$

where,

Z = charge on the molecule

E = Voltage applied

and ,

f = frictional resistance

Rf is measured by:

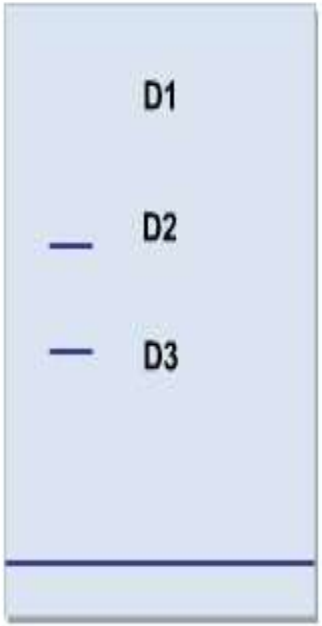
$$R_f = \frac{\text{Distance protein band moves}}{\text{Distance dye front moves.}}$$

Direction of movement is determined from Z: -

if Z < 0, then →+

if Z > 0, then → -

if Z = 0, then no movement



R_f = D(1)/D(dye) = [Z(1)V]/f

•The gel used is divided into an upper "stacking" gel of low percentage (with large pore size) and low pH (6.8), where the protein bands get squeezed down as a thin layer migrating toward the anode and a resolving gel (pH 8.8) with smaller pores. Cl^- is the only mobile anion present in both gels.

•When electrophoresis begins, glycine present in the electrophoresis buffer, enters the stacking gel, where the equilibrium favors zwitter ionic form with zero net charge. A zwitterion is a compound with no overall electrical charge, but which

contains separate parts which are positively and negatively charged.

•The glycine front moves through the stacking gel slowly, lagging behind the strongly charged, Cl- ions.

•Since these two current carrying species separate, a region of low conductivity, with high voltage drop, is formed between them. This zone sweeps the proteins through the large pores of the stacking gel, and depositing it at the top of the resolving gel as a narrow band.

Stacking gel interactions:

- **Stacking occurs by the differential migration of ionic species, which carry the electric current through the gel.**

- **When an electrical current is applied to the gel, the negatively charged molecules start migrating to the positively charged electrode.**

- **Cl- ions, having the highest charge/mass ratio move faster, being depleted and concentrated at anode end.**

SDS coated proteins has a higher charge/mass ratio than glycine so it moves fast, but slower than Cl-. When protein encounters resolving gel it slows the migration because of increased frictional resistance, allowing the protein to stack in the gel.

Resolving Gel Interactions:

When glycine reaches resolving gel it becomes negatively charged and migrates much faster than protein due to higher charge/mass ratio. Now proteins are the main carrier of current and separate according to their molecular mass by the sieving effect of pores in gel.

charge-to-mass ratio

- this ratio significantly affects the mobility of a macromolecule through a solution when driven by an electric field (two molecules of identical mass but different charge will move at different rates in an electric field).

- Since at neutral pH, the majority of the net charge on DNA is derived from the negatively charged phosphate groups in the DNA backbone, as DNA increases in size, the total charge increases at the same rate.

- **The resulting charge-to-mass ratio therefore remains constant, and DNA fragments of different sizes all move at about the same rate in an electric field.**

- **For separation of the fragments according to size, it is necessary to force the fragments to migrate through a molecular sieve or matrix of many small pores that allows the smaller fragments to move faster than the larger fragments. agarose and polyacrylamide gels are most commonly used as a matrix for size separation of DNA and RNA fragments**

Sample preparation

- Samples may be any material containing proteins or nucleic acids.

- The sample to analyze is optionally mixed with a chemical denaturant if so desired, usually SDS for proteins or urea for nucleic acids.

Preparing acrylamide gels

- The gels typically consist of acrylamide, bisacrylamide, the optional denaturant (SDS or urea), and a buffer with an adjusted pH.

- A source of free radicals and a stabilizer, such as ammonium persulfate and TEMED are added to initiate polymerization. The polymerization reaction creates a gel because of the added bisacrylamide, which can form cross-links between two acrylamide molecules.

Electrophoresis

- Various buffer systems are used in PAGE depending on the nature of the sample and the experimental objective. The buffers used at the anode and cathode may be the same or different.

- An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative electrode and towards the positive electrode (the anode). Depending on their size, each biomolecule moves differently through the gel matrix

- **The gel is run usually for a few hours, though this depends on the voltage applied across the gel; migration occurs more quickly at higher voltages, but these results are typically less accurate than at those at lower voltages.**

- **After the set amount of time, the biomolecules have migrated different distances based on their size.**

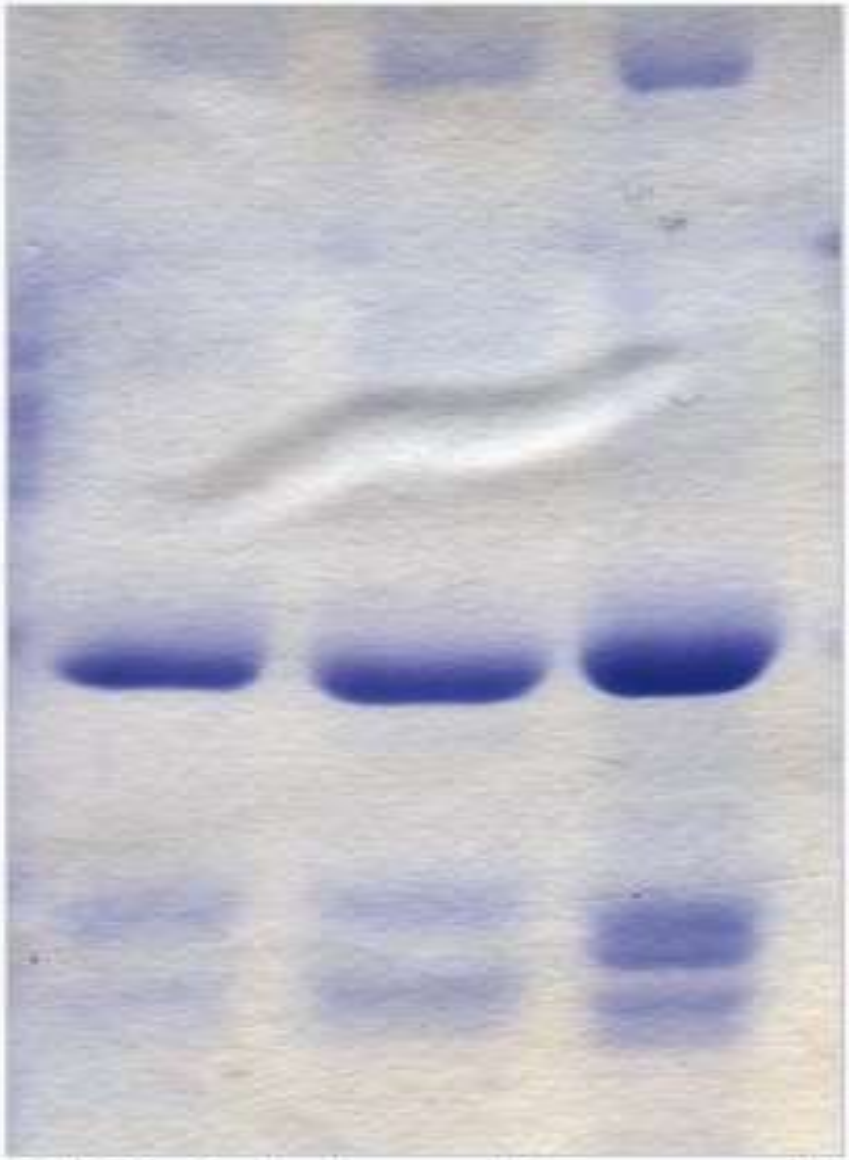
- **Smaller biomolecules travel farther down the gel, while larger ones remain closer to the point of origin.**

- **Biomolecules may therefore be separated roughly according to size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions.**

Further processing:

- Following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue R-250; for nucleic acids, ethidium bromide; or for either, silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot).

- After staining, different species biomolecules appear as distinct bands within the gel.



PAGE of rotavirus proteins stained with Coomassie blue

SDS PAGE

- 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).

- SDS (also called lauryl sulfate) is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range.

- **The movement of any charged species through an electric field is determined by its net charge, its molecular radius and the magnitude of the applied field.**

- **But the problem with natively folded proteins is that neither their net charge nor their molecular radius is molecular weight dependent.**

- **Instead, their net charge is determined by amino acid composition i.e. the sum of the positive and negative amino acids in the protein and molecular radius by the protein's tertiary structure.**

- **So in their native state, different proteins with the same molecular weight would migrate at different speeds in an electrical field depending on their charge and 3D shape.**

- **To separate proteins in an electrical field based on their molecular weight only, we need to destroy the tertiary structure by reducing the protein to a linear molecule, and somehow mask the intrinsic net charge of the protein. That's where SDS comes in.**

The Role of SDS

- 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.

•SDS also coats the protein with a uniform negative charge, which masks the intrinsic charges on the R-groups (amino acids).

•SDS binds fairly uniformly to the linear proteins (around 1.4g SDS/ 1g protein), meaning that the charge of the protein is now approximately proportional to its molecular weight.

•The SDS-treated proteins will now move toward the positive anode at different rates depending on their molecular weight. These different mobilities will be exaggerated due to the high-friction environment of a gel matrix.

- **The dominant factor in determining an SDS-coated protein is it's molecular radius.**

- **SDS-coated proteins have been shown to be linear molecules, 18 Angstroms wide and with length proportional to their molecular weight, so the molecular radius (and hence their mobility in the gel) is determined by the molecular weight of the protein.**

- **Since the SDS-coated proteins have the same charge to mass ratio, there will be no differential migration based on charge.**

- **A polypeptide chain binds amounts of SDS in proportion to its relative molecuar mass.**

- **The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.**

- **Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules**

- **Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides.**

- **In a gel of uniform density the relative migration distance of a protein (Rf, the f as a subscript) is negatively proportional to the log of its mass.**

- **If proteins of known mass are run simultaneously with the unknowns, the relationship between Rf and mass can be plotted, and the masses of unknown proteins estimated.**

- **Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions.**

- **The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed.**

- **Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties**

Molecular mass versus molecular weight

- Molecular mass (symbol m) is expressed in Daltons (Da). One Dalton is defined as 1/12 the mass of carbon 12. Most macromolecules are large enough to use the kiloDalton (kDa) to describe molecular mass. Molecular weight is not the same as molecular mass. It is also known as relative molecular mass (symbol Mr, where r is a subscript). Molecular weight is defined as the ratio of the mass of a macromolecule to 1/12 the mass of a carbon 12 atom. It is a dimensionless quantity.