ELECTROPHORESIS

1. Introduction

Electrophoresis is a technique in which charged solute particle migrate under the influence of an electric field. Many important biological molecules, such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations or anions. Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge. Mainly protein and DNA mixtures are separated by their different rates of migration in an electric field. This is best done using an electrophoresis unit or instrument.

In Electrophoresis usually a sample is first placed in a container or support that also contains a background electrolyte (or "running buffer"). When an electric field is applied to this system, the ions in the running buffer will flow from one electrode to the other and provide the current needed to maintain the applied voltage. At the same time, positively charged ions in the sample will move toward the negative electrode (the cathode), while negatively charged ions will move toward the positive electrode (the anode). The result is a separation of these ions based on their charge and size.

In 1937 a scientist named **Arne Tiselius** used electrophoresis for the separation of serum proteins. He conducted this separation by employing a U-shaped tube in which he placed his sample and running buffer. When he applied an electric field, proteins in the sample began to separate as they migrated toward the electrodes of opposite charge. However, the use of a large sample volume gave a series of broad and only partially resolved regions that contained different mixtures of the original proteins. The method employed by Tiselius is now known as moving boundary electrophoresis, because it produced a series of moving boundaries between regions that contained different mixtures of proteins. Today it is more common to use small samples to allow analytes to be separated into narrow bands or zones, giving a method known as zone electrophoresis. An example of zone electrophoresis is shown in where DNA is sequenced by separating its strands of various lengths into narrow bands on a gel. There are many ways in which electrophoresis is used for chemical analysis. These include the sequencing of DNA, as well as the purification of proteins, peptides, and other biomolecules. In clinical chemistry, electrophoresis is an important tool for examining the patterns of amino acids, serum proteins, enzymes, and lipoproteins in the body.

2. GEL ELECTROPHORESIS:

One of the most common types of electrophoresis is the method of gel electrophoresis. This technique is an electrophoresis method that is performed by applying a sample to a gel support that is then placed into an electric field. In this type of system, several samples are usually applied to the gel and allowed to migrate along the length of the support in the presence of an applied electric field. The separation is stopped before analytes have left the end of the gel, with the location and intensities then being determined. It is important to remember in gel electrophoresis that the velocity of an analytes movement will be related to the distance it has travelled in the given separation time (the migration distance). The farther this distance is from the point of sample application, the higher the migration velocity is for the analyte and the larger its electrophoretic mobility. This migration distance will, in turn, be related to the size and charge of the analyte and can be used in identifying such a substance.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Polyacrylamide gel is the result of polymerizing acrylamide monomers into long chains and then cross-linking the chains with a bifunctional compound. A number of these bifunctional crosslinkering compounds are known including ethylene diacrylate, N,N'bisacrylycystamine (BAC), and N,N'diallyltartardiamide (DATD).

However, the most generally useful compound is N, N'-methylene bisacrylamide (bis meaning two). Polymerization of acrylamide and bisacrylamide is catalyzed in the presence of either Ammonium per sulphate (APS) or riboflavin. In addition, the compound N,N,N,N'-tetramethylethyldiamine (TEMED) or, less commonly, 3-dimethylamino proprionitrile (DMAPN), are introduced to accelerate the polymerization process.

In the ammonium persulphate-TEMED system that is conventionally employed, TEMED catalyzes the formation of free radicals from persulphate and these free radicals initiate polymerization. Since the free base of TEMED is required, polymerization can be slowed at lower pH and can be prevented entirely at very low pH.

Polymerization rates can be increased by increasing the TEMED or persulphate concentrations. Also, temperature has a direct relationship with speed of polymerization. For this reason, the persulphate and the acrylamides are usually stored at –20 C.

3. SDS-PAGE

In SDS-PAGE, the protein sample is first treated with SDS, an ionic detergent which causes the proteins to unfold, or denature. ßmecaptoethanol is also included to reduce disulfide bonds and cause separation of protein subunits. When a sample is heated (in a container, 90-95 ^{0}C for 5 min in a water bath) in the presence of SDS, the denatured proteins become long linear molecules completely coated with SDS and assume a uniformly negative ionic charge.

Whenever a porous support (Gel) is present in an electrophoretic system, it is possible that large analyte's may be separated based on their size as well as their electrophoretic mobilities.

This size separation occurs in a manner similar to that which occurs in size-exclusion chromatography and can be used to determine the molecular weight of biomolecules.

This is accomplished by SDS-PAGE. SDS-treated proteins are subjected to PAGE, the rate of migration is determined ONLY by the molecular weight of the protein. When mobility (Rf) of proteins during SDS-PAGE is graphed against the log of the protein molecular weight, a linear relationship is observed Thus, the rate of protein migration during SDS-PAGE is proportional to the log of the protein molecular weight. In practice, the molecular weight of an unknown protein is determined by comparing its movement during SDS-PAGE with protein standards of known molecular weights. SDS-PAGE, is a widely used technique for separating mixtures of proteins based on their size and nothing else. SDS, an anionic detergent, is used to produce an even charge across the length of proteins that have been linearized. By first loading them into a gel made of polyacrylamide and then applying an electric field to the gel.,

SDS-coated proteins are then separated. The electric field acts as the driving force, drawing the SDS coated proteins towards the anode with larger proteins moving more slowly than small proteins. In order to identify proteins by size, protein standards of a known size are loaded along with samples and run under the same conditions.

4. 2D-ELECTROPHORESIS

2-Dimensional Electrophoresis. Another way gel electrophoresis can be utilized is in two-dimensional (or 2-D) electrophoresis, which is a high-resolution technique used to look at complex protein mixtures. In this method, two different types of electrophoresis are conducted on a single sample. The first of these separations is usually based on a isoelectric point, as accomplished by using isoelectric focusing. The second separation method

(SDSPAGE) is according to size. First, a small band of sample is applied to the top of a support for use in isoelectric focusing. The support used in this case is typically agarose or a polyacrylamide gel with large pores. After this first separation has been finished, some proteins will have been separated based on their pI values, but there may still be many proteins with similar isoelectric points and overlapping bands. A further separation is obtained by turning this first gel on its side and placing it at the top of a second support (a polyacrylamide gel) for use in SDS-PAGE. This process gives a separation according to size, in which each band from the first separation has its own lane on the SDS-PAGE gel. The result is a series of peaks that are now separated in two dimensions (one based on pI and the other on size) across the gel. The fact that two different characteristics of each protein are used in their separation makes it possible to resolve a much larger number of proteins than is possible by either IEF or SDS-PAGE alone.

a. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is one of several physical methods for determining the size of DNA. In this method, DNA is forced to migrate through a highly cross-linked agarose matrix in response to an electric current. In solution, the phosphates on the DNA are negatively charged, and the molecule will therefore migrate to the positive (red Cathode) pole. There are three factors that affect migration rate through a gel; size of the DNA, conformation of the DNA, and ionic strength of the running buffer. Usually for DNA, TBE as a running buffer and therefore ionic strength will be constant throughout if the same buffer is used.

Electrophoresis is essentially a sieving process. The larger the fragment of DNA, the more easily wills it become entangled in the matrix and, therefore, the more slowly will it migrate. Small fragments, therefore, run more quickly than large fragments at a rate proportional to their size. The relationship of size to migration rate is linear throughout most of the gel, except for the very largest fragments. Large fragments have a more difficult time penetrating the gel and their migration, therefore, does not have a linear relationship to size. The matrix can be adjusted, though, by increasing the concentration of agarose (tighter matrix) or by decreasing it (looser matrix). A standard 1% agarose gel can resolve DNA from 0.2 - 30 kb in length. Agarose gels are referred to as submarine gels because the slab is laid horizontally and is completely covered by running buffer. There are number of different gel boxes in two basic sizes. The larger boxes have gel beds of approximately 11 x 14 cm (gel bed). The smaller boxes typically referred to as “baby gels” have gel beds of approximately 50 x 75 cm. In most cases, the gel tray is removable and the gel is poured outside of the box.

Each type of gel box has its own unique way of sealing the gel bed to prevent leakage of the agarose. Baby gels are used for quick checks. Their resolution isn't great but the gel runs within 30 to 40 minutes and is very useful for monitoring longer reactions. The larger boxes can be run either with a single comb at the top of the gel, or with a second set of combs in the middle. In this way, twice the number of samples can be run, but the resolution is similar to a baby gel.

5. SEPARATION OF NUCLEIC ACIDS

The gel electrophoresis as a method of separating and analyzing DNA has significantly influenced the progress achieved in molecular biology. The electrophoresis is a method that separates molecules on the basis of their size, electric charge, and other physical properties. The DNA gel electrophoresis refers to the technique in which DNA macromolecules are forced across a span of gel, which is a colloid in solid form, motivated by an electrical current. Two types of gel matrices, agarose and polyacrylamide gels are mostly used for DNA gel electrophoresis. In these gels the electrophoretic mobility of macromolecules is thought to be determined primarily by the volume fraction of pores within the gel that the macromolecules can enter. Hence, a mixture of DNA molecules of different sizes will separate into discrete blobs or bands in the process of electrophoresis. Since DNA is negatively charged because of its phosphate groups, it migrates in an electrical field. The electrophoretic mobility of DNA fragments in solution is independent of the molecular mass. However, electrophoresis of DNA in gel matrices (agarose or polyacrylamide) at constant field strength is an effective method of separating DNA molecules according to molecular mass. Important experimental parameters are gel concentration, field strength, temperature, and running time. Gels of 0.5–2% are usually employed for the separation of 50 – 20,000 base pairs at field strengths of 2 – 3 V/cm. The mobilities of linear and supercoiled DNA of equivalent molecular mass are different. Estimation of the molecular mass of supercoiled DNA molecules based on linear standards is therefore not possible. Polyacrylamide gels (4 – 12%) give a sharp separation of polynucleotides up to a length of about 600 base pairs. Special gels can separate polynucleotides with up to 2500 base pairs. Mobility varies logarithmically with the size of the DNA at field strengths below 1 V/cm. Separation efficiency is limited in the range of 10000 – 50000 base pairs, there is no resolution above 50,000 base pairs. Denaturing gels (with urea) are used in the sequencing and analysis of single stranded nucleic acids. Under special conditions, the behavior of individual DNA molecules in an electric field can be observed with a fluorescence microscope.

Samples of DNA, digested by specific restriction enzymes also can be separated by this method. The digested samples or DNA segments of different sizes are loaded in the separated wells at the front edge of the gel. After turning on the power supply each sample runs in its own trace (the so-called lane). A fluorescent dye is used for marking the positions of the DNA bands in the gel which is then photographed to provide a permanent record of the electrophoresis experiment. In the recent years a number of software systems have been developed for digitization of the photographs of electrophoretic gels, processing and analysis of the digitized images to yield the final information on DNA fragment mobility, i.e., the band positions in each individual lane given in molecular weight (DNA base pairs). Pulse-field electrophoresis is used to separate larger linear DNA molecules. Here the direction of the electric field is changed at intervals. The DNA molecules therefore have to change their orientation; the time required for this change depends on the size of the DNA. Unlike conventional electrophoresis (duration 30 – 60 min), a pulse-field run takes 20 – 140h. Usually, 1.5% agarose gels are used and 10 – 20 µg of DNA can easily be separated at field strengths of 2.5 – 10 V/cm. Separation can be achieved up to 12×106 base pairs. Separation is affected by DNA topology and sequence, pulse time (seconds to minutes), field geometry, field strength, gel composition, sample concentration, temperature, and running time. Electrophoresis in agarose or polyacrylamide gels is the most usual way to separate DNA molecules according to size. The technique can be used analytically or preparatively, and can be qualitative or quantitative. Large fragments of DNA such as chromosomes may also be separated by a modification of electrophoresis termed pulsed field gel electrophoresis (PFGE). The easiest and most widely applicable method is electrophoresis in horizontal agarose gels, followed by staining with ethidium bromide. This dye binds to DNA by insertion between stacked base pairs (intercalation), and it exhibits a strong orange/red fluorescence when illuminated with ultraviolet light. Very often electrophoresis is used tocheck the purity and intactness of a DNA preparation or to assess the extent of an enzymatic reaction during for example the steps involved in the cloning of DNA. For such checks ‘minigels’ are particularly convenient, since they need little preparation, use small samples and give results quickly. Agarose gels can be used to separate molecules larger than about 100 bp. For higher resolution or for the effective separation of shorter DNA molecules polyacrylamide gels are the preferred method. When electrophoresis is used preparatively, the piece of gel containing the desired DNA fragment is physically removed with a scalpel.

The DNA may be recovered from the gel fragment in various ways. This may includecrushing with a glass rod in a small volume of buffer, using agarase to digest the agarose leaving the DNA, or by the process of electroelution. In this method the piece of gel is sealed in a length of dialysis tubing containing buffer, and is then placed between two electrodes in a tank containing more buffer. Passage of an electrical current between the electrodes causes DNA to migrate out of the gel piece, but it remains trapped within the dialysis tubing, and can therefore be recovered easily. Gel electrophoresis remains the established method for the separation and analysis of nucleic acids. However a number of automated systems using pre-cast gels and standardized reagents are available that are now very popular. This is especially useful in situations where a large number of samples or high-throughput analysis is required. In addition technologies such as the Agilents' Lab-on-a-chip have been developed that obviate the need to prepare electrophoretic gels. These employ microfluidic circuits constructed on small cassette units that contain interconnected micro-reservoirs. The sample is applied in one area and driven through microchannels under computercontrolled electrophoresis. The channels lead to reservoirs allowing, for example, incubation with other reagents such as dyes for a specified time. Electrophoretic separation is thus carried out in a microscale format. The small sample size minimises sample and reagent consumption and the units, being computer controlled, allow data to be captured within a very short timescale. More recently alternative methods of analysis including high performance liquid chromatography based approaches have gained in popularity, especially for DNA mutation analysis. Mass spectrometry is also becoming increasingly used for nucleic acid analysis.

Conclusion:

The movement of small particles through a gel by the application of electricity. The particles can be DNA, RNA, or proteins. The matrix nature of the gel acts as a sieve to slow the progress of the molecules. Smaller molecules move more rapidly and thus further. Gel electrophoresis separates molecules on the basis of size, but is also influenced by molecule shape and charge density. The most common electrophoresis for proteins is SDS-PAGE and for DNA is Agarose electrophoresis. As per the availability of different form of electrophoresis it is possible to use this technique as an analytical as well as preparative tool in the laboratory analysis of bio molecules. Electrophoresis is familiar and generally used in genomics and proteomics based on the high resolution output separation and ease of use and preparation of required solution. A great variety of slab gel modules are available and it can

be used according to the requirement of the size, charge and activity of the biomolecules applied.