# BCHEM 365
# Lecture 4
# September 18, 2018

# Theory and methods of electrophoresis

- What is electrophoresis?
- It is the motion of charged particles in a colloid under the influence of electric field
- Particles with a positive charge move to the cathode and particles with a negative charge move to the anode
- The phenomenon was observed for the first time by F. Reuss in 1807
- In his experiment, he found that application of constant electric field caused clay particles dispersed in water to migrate
- Electrophoresis is therefore a technique for separating different types of molecules based on their patterns (driven by size, shape, charge) of movement in an electric field
- Many important biomolecules, such as amino acids, peptides, proteins, nucleic acids possess ionizable groups. At any given pH, these exist in solution as electrically charged particles. Under influence of electric field, the charged particles migrate either to the cathode or to the anode, depending on their net charge

# Theory and methods of electrophoresis

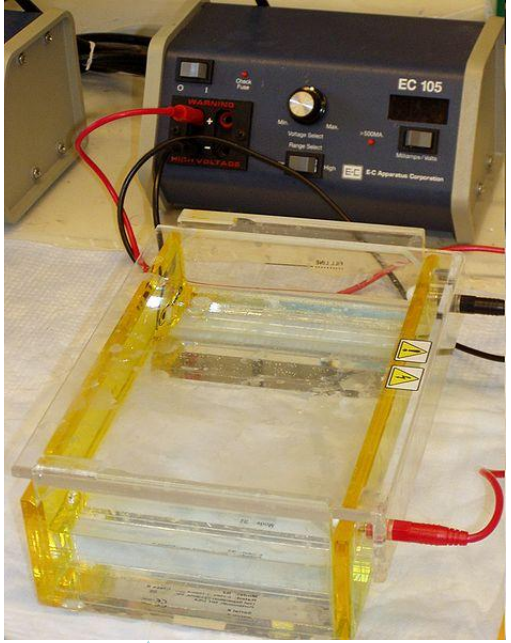
The equipment required for electrophoresis consists of two items:

1. A power pack
2. An electrophoresis unit

Electrophoresis units are available for running either **vertical** or **horizontal** gel system

Vertical gel systems are routinely used for protein separation on acrylamide gels

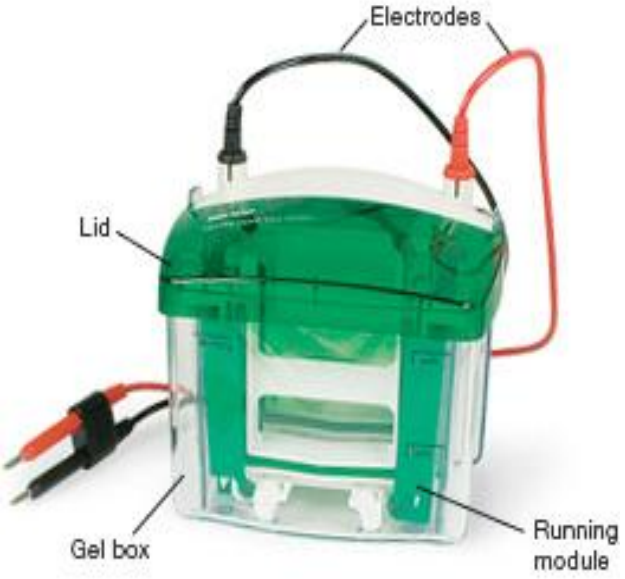
Horizontal gel systems are used for DNA separation on agarose gels




Power pack

Horizontal unit

Electrophoresis unit




Vertical unit

# Equations relating to electrophoresis

- When a potential difference (voltage) is applied across the electrodes, it generates an electric gradient, *E*
- *The gradient, E* is equivalent to the applied voltage, *V*, divided by the distance, *d*, between the electrodes ($E=V/d$)
- In the presence of this electric gradient *E*, the force on a molecule bearing a charge of *q* coulombs is given by *Eq* newtons.
- *Eq* is the force that drives a charged molecule toward an electrode

# Equations relating to electrophoresis

- As the charged particle moves toward the electrode, it encounters frictional resistance that retards its movement

This frictional force is a measure of the ff:

✓ Hydrodynamic size of particle

✓ Shape of the particle

✓ Pore size of the medium in which it is moving, and

✓ Viscosity of the buffer in which it is immersed

- The velocity, *v*, of a charged particle in an electric field is therefore given by the force on the particle divided by the resistance to movement, i.e.,

$$v = Eq/f$$

where *f* is the frictional coefficient

# Equations relating to electrophoresis

- A more common term used to describe the characteristic movement of a particle in electrophoresis is electrophoretic mobility, $\mu$,
- $\mu$ is the ratio of the velocity of the charged particle to the field strength (the electric gradient)

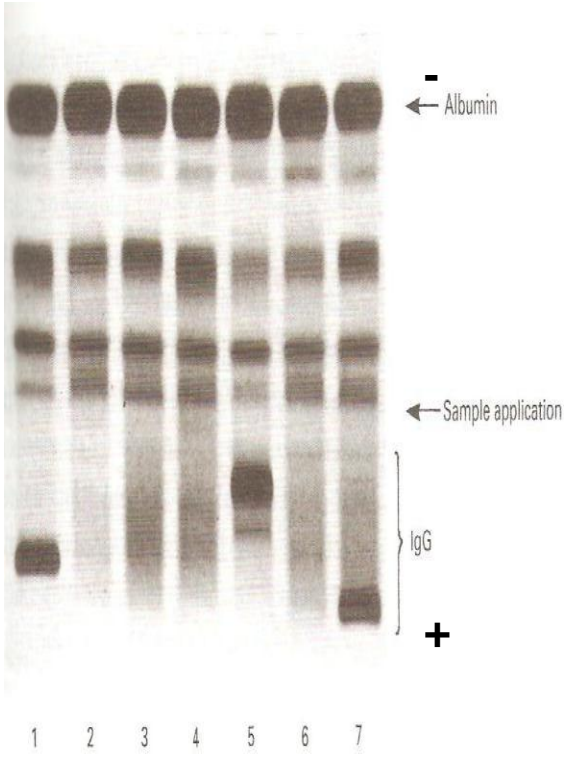
$$\mu = v/E$$

- When a potential difference is applied, molecules with different overall charges will begin to separate out owing to their different electrophoretic mobilities
- Molecules with similar charges will begin to separate if they have different sizes, since they will experience different frictional forces

# Equations relating to electrophoresis

- Some forms of electrophoresis rely totally on the different charges on the molecules to achieve separation
- Other methods exploit differences in molecular size and therefore encourage frictional effect to bring about separation
- Movement must be monitored to prevent particles from moving beyond the electrodes
- By the time the electric field is removed, mixtures of molecules would have moved at different velocities toward the electrodes, thereby separation is achieved

# Equations relating to electrophoresis




- The separated samples are then located by staining with an appropriate dye or by autoradiography if the sample is radiolabeled
- The current in the solution between the electrodes is conducted mainly by the buffer ions, with a small proportion conducted by the sample ions
- Ohm's law expresses the relationship between current, I, voltage V, and resistance R

# Some technical problems with electrophoresis (heating)

- Ohm's law: V=IR
- Increase in applied voltage leads to increase in current, hence acceleration of electrophoretic separation
- The velocity of migration of particles, hence the distance moved will be proportional to both current and time
- Increase in current causes faster movement of particles and ions, which leads to generation of heat, a major problem in electrophoresis

# Equations relating to electrophoresis

- During electrophoresis the power, W in watts, generated in the support medium is given by

$$W = I^2 R$$

- Most of this power is dissipated as heat
- Heating of the electrophoretic medium produces the following effects:
  1. Increased rate of diffusion of sample and buffer ions leading to broadening of separated samples
  2. Formation of convection currents, which leads to mixing of separated samples
  3. Denaturation of thermally unstable samples, such as proteins,
  4. Decrease in buffer viscosity, hence reduction in the resistance of the medium

# Elecctrophoresis and heating problems

- Constant heat generation is a problem. One way of overcoming the heating problem is to run electrophoresis at very low power, i.e. low current
- Running electrophoresis at low current slows down movement of ions, long separation times are required, poor resolution

# Electrophoresis and heating problems

- Compromise conditions have to be sought, with reasonable power settings in order to have acceptable separation times, and an appropriate cooling system to remove liberated heat
- With the use of such systems, heating is not always totally eliminated. With the use of cylindrical tubes or gel slabs, heat is removed only from the edges, resulting in a temperature gradient within the gel, with the center being hotter than the edges
- Since the warmer fluid at the center is less viscous, electrophoretic mobilities are greater in this region. Note that for every 1°C rise in temperature, electrophoretic mobility increases by 2%
- Electrophoretic zones develop a bowed shape, with the zone center migrating faster than  the edges

# Some technical problems with electrophoresis (electroendosmosis)

- Presence of charged groups on the surface of the support medium establishes a phenomenon known as electroendosmosis (or electroosmotic flow). For example:
- Paper support medium contains carboxyl groups on its surface
- Agarose, depending on the purity grade, contains sulfate groups
- The surface of glass walls used in capillary electrophoresis contains silanol (Si-OH) groups

# Electroendosmosis

- **Electroendosmosis (EEO)** - movement of liquid through the gel. This liquid originates from (1) the water used to prepare the gel, which indeed has to be trapped by the gel material, (2) movement of counter cations from both the gel and buffer toward the cathode, while anionic groups in the gel are affixed to the matrix and cannot move, giving rise to EEO.
- Since electrophoretic movement of biopolymers is usually toward the anode, EEO can disrupt separations because of internal convection

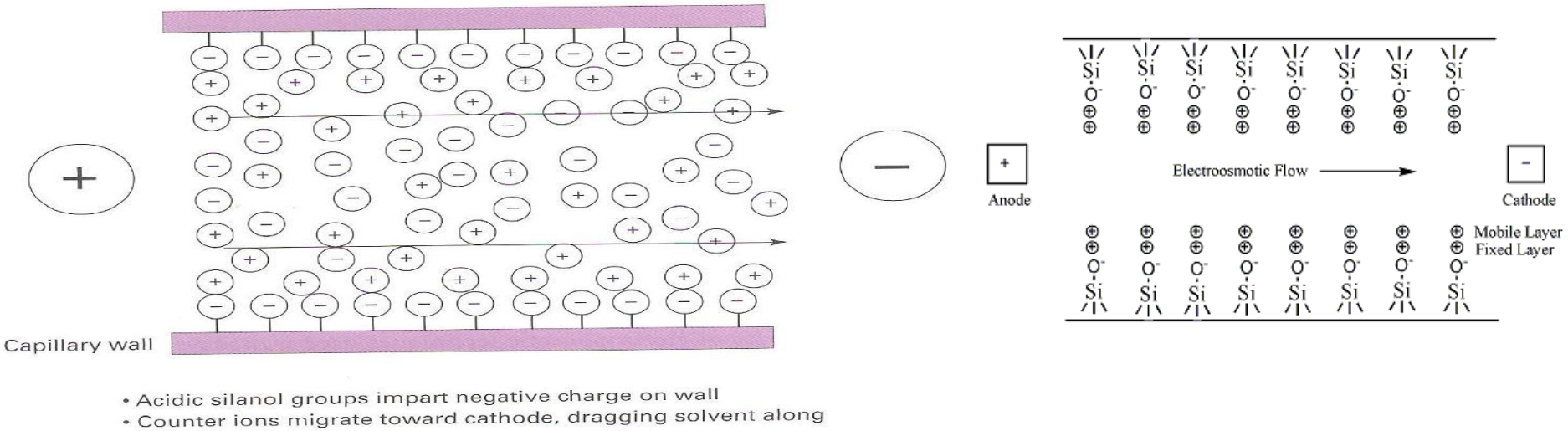



Fig. 1. Electroosmotic flow through a glass capillary. Electrolyte cations are attracted to the capillary walls, forming an electrical double layer. When voltage is applied, the net movement of electrolyte solution toward the cathode is known as endosmotic flow

# Electrophoresis and electroendosmosis

- Silanol groups on the surface of glass ionize above pH 3 generating negatively charged sites
- It is these charges that generate electroendosomosis. The ionized silanol groups create an electrical double layer, or a region of charge separation at the capillary wall/electrolyte interface. When voltage is applied, cations in the electrolyte near the capillary wall migrate towards the cathode, pulling electrolyte solution with them. This creates a net electroosmotic flow toward the cathode.
- It is a diffusion process which is undesirable as it affects the performance of electrophoresis

# Support media

- The pioneering work on electrophoresis by Arne Tiselius et al. (1930) was performed in free solution
- It was soon realized that many of the problems associated with this approach, particularly the adverse effects of diffusion and convective currents could be minimized by stabilizing the medium
- Stabilization was provided by use of a porous mechanical support
- The support medium cuts down convective currents and diffusion so that the separated components remain as sharp zones.

# Support media

- The earliest support materials were filter paper or cellulose acetate strips, wetted in electrophoresis buffer. Not in common use in recent times, though cellulose acetate still has its uses
- For many years, small molecules like amino acids, peptides and carbohydrates were separated and analyzed on supports such as paper or thin-layer plates of cellulose, silica or alumina, but presently analyzed by more sensitive methods such as high performance liquid chromatography (HPLC)
- Separation of macromolecules such as proteins and nucleic acids on paper or thin-layer plates was found to be poor
- **Gels** were therefore developed as support media for macromolecules.

# The gel

- The earliest gel system used was starch gel. Nowadays majority of electrophoretic techniques involve agarose or polyacrylamide gels as support media
- In most cases, the gel is a crosslinked polymer whose composition and porosity is chosen based on the specific weight and composition of the molecules being separated

- When separating **proteins**, small **nucleic acids** or oligonucleotides, different concentrations of **acrylamide** and a **cross-linker** molecule are used to produce varying- sized mesh of networks of polyacrylamide
- When separating larger nucleic acids, usually greater than a few 100 bases the preferred matrix is purified **agarose**

# Agarose gels

- Agarose is a linear polysaccharide (average molecular mass = 12,000 Daltons), made up of the basic repeating unit agarobiose
- Agarobiose comprises alternating units of galactose and 3,6-anhydrogalactose
- Agarose is one of the components of agar, a mixture of polysaccharides isolated from certain seaweeds

Gelling Temp: >35° C

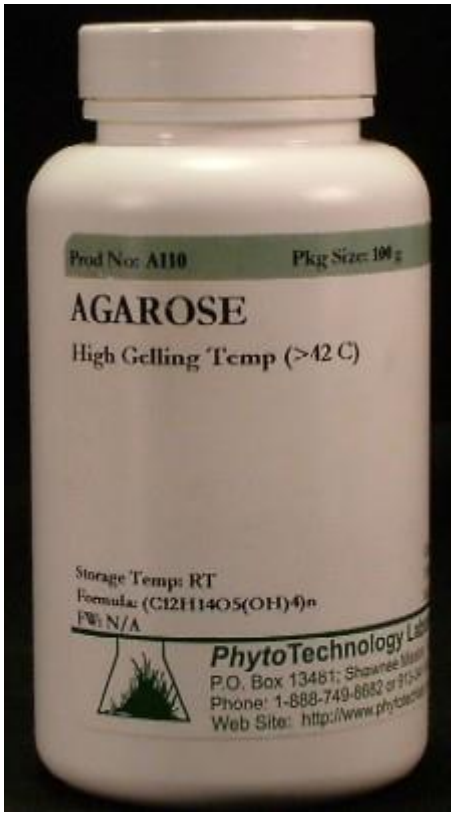
► Formula: $(C_{12}H_{14}O_5(OH)_4)_n$

► Soluble in Hot Water

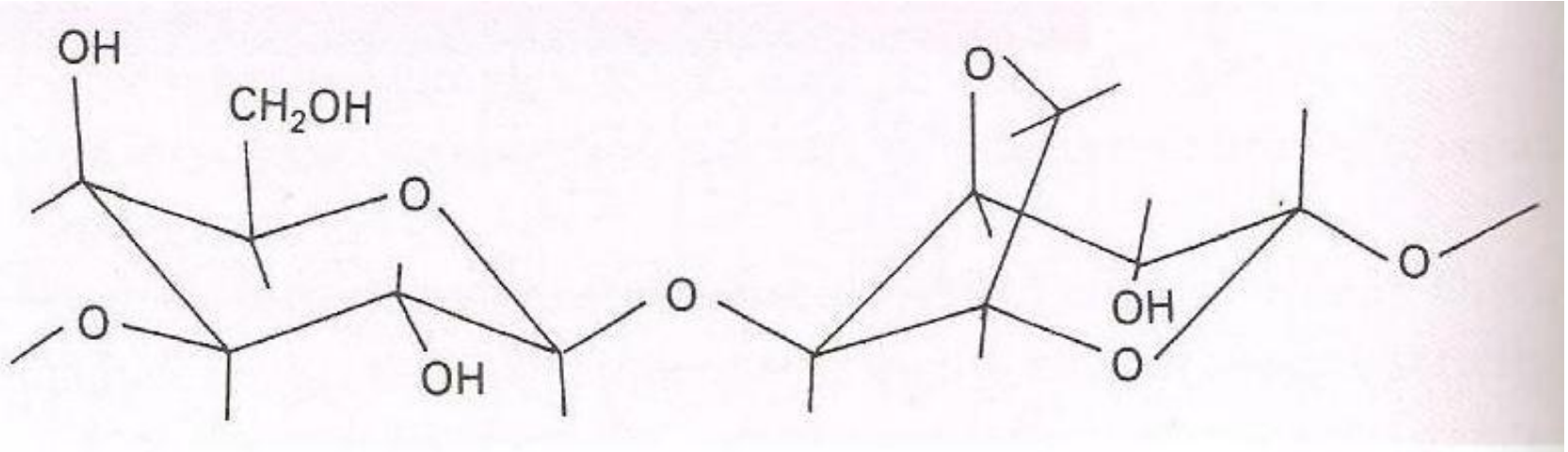
► 100 g costs $307.75

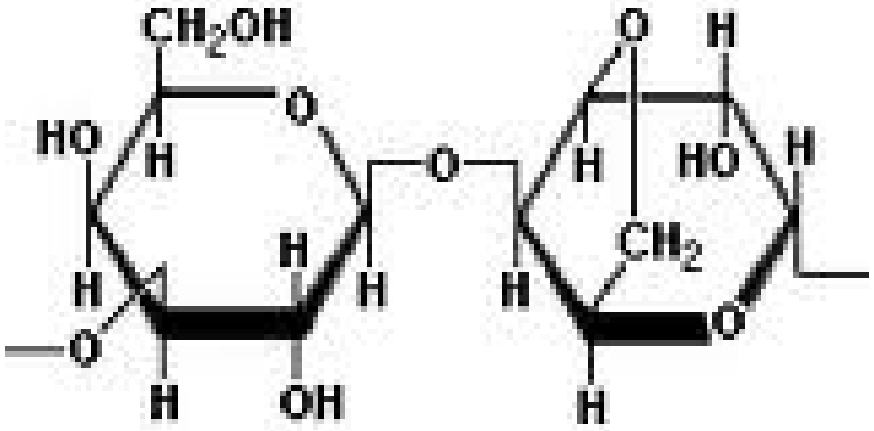
► Manufacturers prepare special grades of agarose for scientific experimentation

► Purified agarose is in powdered form, and is insoluble in water (or buffer) at room temperature



# Agarobiose - the repeating unit of agarose



# Preparation of Agarose gels

- Agarose is usually used at concentrations of between 1% and 3%. For large DNA fragments (5–10kb) 0.7% gels give good resolution. For small 0.2–1kb fragments 2% gels give good resolution
- Agarose dissolves in boiling water. When it starts to cool, it undergoes **polymerization,** where the long polysaccharides crosslink with each other, causing the solution to "gel" into a semi-solid matrix
- Increase in agarose concentration produces a firmer gel
- While the solution is still hot, we pour it into a mold called a "casting tray" so it will assume the shape of the tray
- To make 40 ml of 1% agarose: weigh 0.4 g of agarose, dissolve in 40 ml TAE buffer (Tris-Acetate-EDTA). Pour on gel plate and allow to cool to room temperature to form a rigid gel

# Preparation of Agarose gels

- Gelling properties of agarose attributed to both inter- and intramolecular hydrogen bonding within and between the long agarose chains: cross-linkages
- This cross-linked structure gives the gel good anticonvectional properties

casting tray

# Agarose gels

- Pore size of the gel is controlled by the initial concentration of agarose
- Low concentrations form large pore sizes while high concentrations form small pore sizes
- Also present on the agarose are substitutions of alternating sugar residues with carboxyl, methoxyl, pyruvate and sulfate groups to varying degrees
- This substitution can result in electroendosmosis during electrophoresis: this is undesirable
- Also ionic interactions between gel and sample may occur: unwanted
- Agarose is therefore sold in different purity grades, based on the sulfate concentration
- The lower the sulfate content, the higher the purity

# Agarose gels

- Agarose gels are used for electrophoresis of both proteins and nucleic acids
- For proteins, pore sizes of a 1% agarose gel are large relative to the sizes of proteins
- Agarose gels are therefore used in techniques such as immunoelectrophoresis or flat-bed isoelectric focusing, where the proteins are required to move unhindered in the gel matrix according to their native charge
- Such large pores of agarose gels are used to separate large molecules such as DNA and RNA because the pore sizes in the gel are large enough for these molecules to pass through the gel
- If molecular size increases, frictional effects begin to play a role in the separation of these molecules

# Agarose gels

- Advantage of using agarose is the availability of low melting temperature agarose (62-65°C) gels which can be reliquefied by heating to 65°C. After separation of the samples, DNA bands can be cut out from the gel, returned to solution and recovered
- Horizontal slab gels which make use of agarose are used for immunoelectrophoresis and isoelectric focusing, as well as routine separation of DNA and RNA