Chromatographic Methods of Analysis
Section: 3 Electrophoresis

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What is Electrophoresis?

- It is a chromatographic technique used in molecular biology to separate macromolecules based on charge, size and shape, under the effect of electric field.
- The migration of charged components (like proteins) through a stationary phase under the effect of an electric field, toward the oppositely charged electrode, depends on the difference of their electrophoretic mobility (net effect of charge and size). The electrophoretic mobility of an analyte μ_e is proportional to the electric field strength (E).

$$\mu_e = \nu/E$$

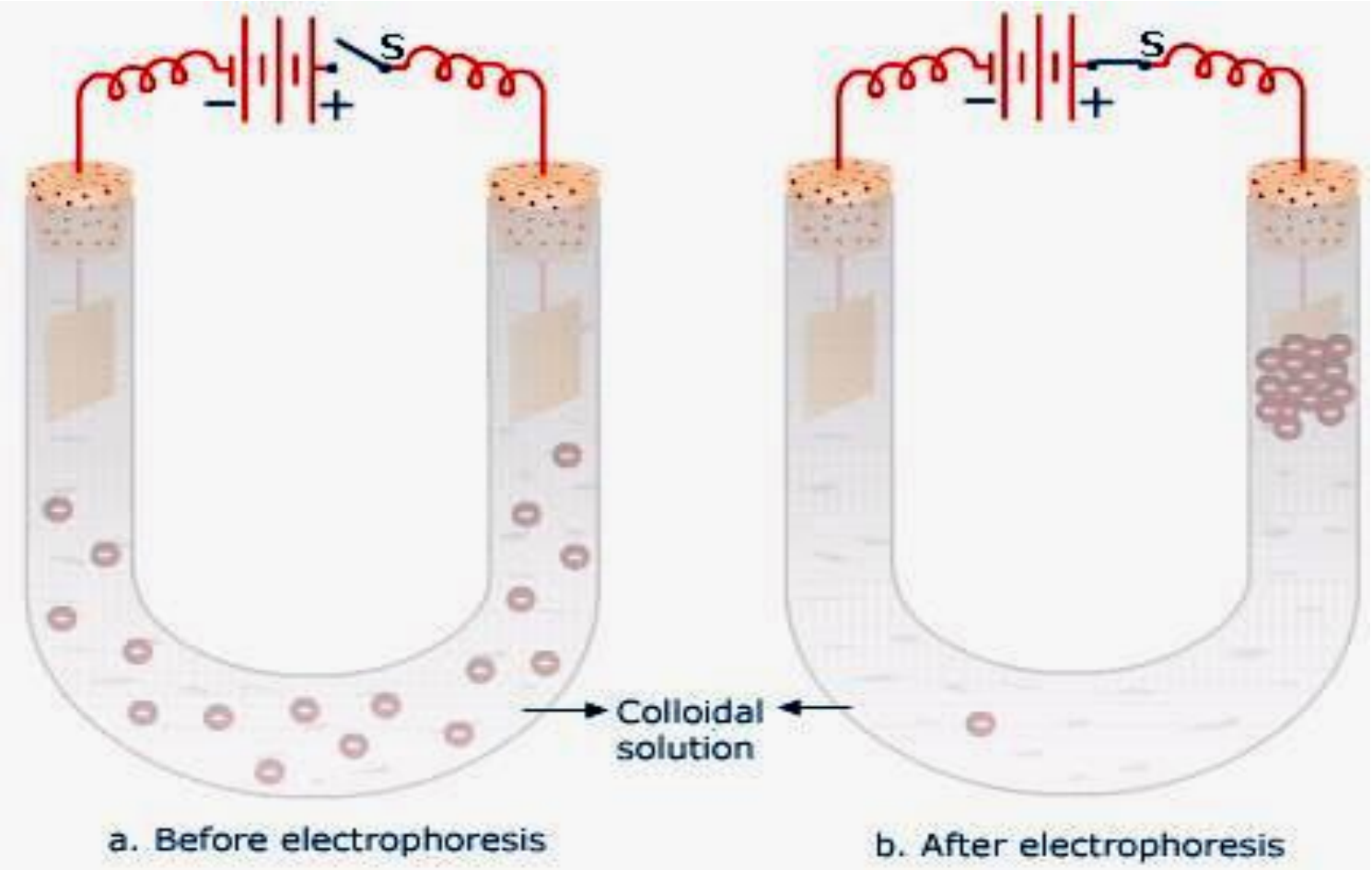
where ν is the velocity of migration of ions

Separation techniques

There are to types

1- Moving Boundary Electrophoresis (free electrophoresis):

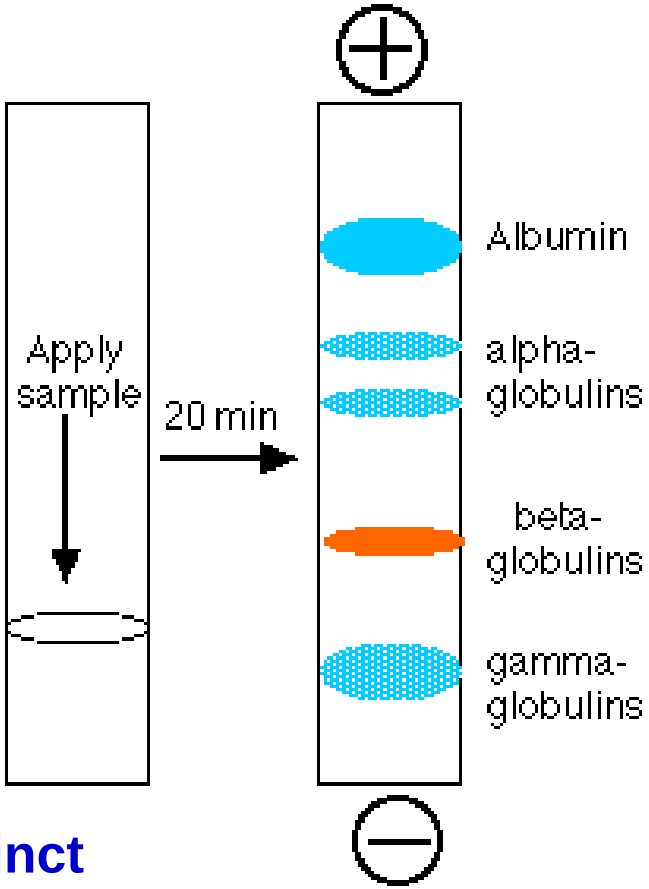
- Electrophoresis in a free solution in which the separated components are in solution, and are therefore free to diffuse away the moment at which the current is switched off.
- The apparatus includes a U-shaped cell filled with buffer solution and electrodes immersed at its ends. On applying voltage, the compounds will migrate to the anode or cathode depending on their charges.
- It is used for the separation of colloids.


S
−
+
S
−
+
Colloidal solution
a. Before electrophoresis
b. After electrophoresis

2- Zone Electrophoresis

In which the separation is carried out on a supporting medium, such as a starch gel or strips of filter papers.

- **The stationary phase could be a paper strip, thin layer sheet or a gel in the form of capillary or a slap on a solid support.**
- **The gel is like: aggarose, starch or poly-acrylamide gel.**
- **The mobile phase is a buffer solution.**



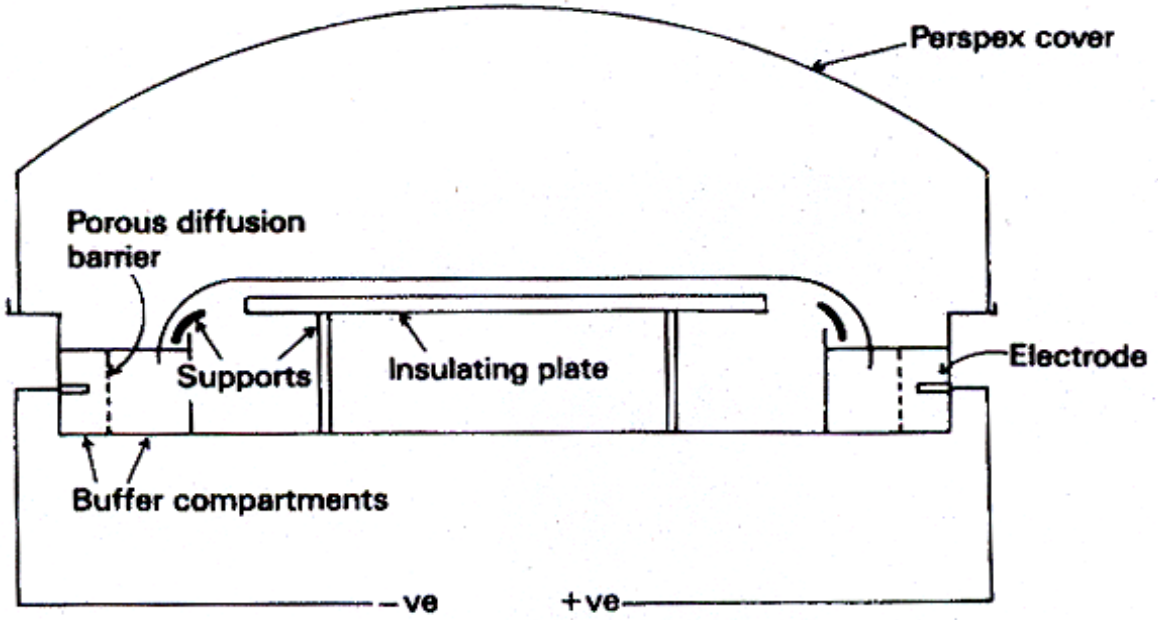
Separating serum proteins by electrophoresis

- **the proteins stained to make them visible**
- **The separated proteins appear as distinct bands .**
- **serum albumin moves closest to the positive electrode, why?**

General technique

On application of an electric current;

- **Cations move toward the cathode (-) while anions move toward the anode (+).**
- **Amphoteric substance (have a positive/negative/zero charge) has zero electrophoretic mobility, i. e does not move.**
- **The applied voltage is removed before the ions of analytical interest reach the electrodes (circuit is off).**



Setup of electrophoresis technique

- During the course of electrophoresis current flows, and as in electrolysis; the products are oxygen and hydrogen

$H_2O + 2e \rightarrow 2OH^- + H_2\uparrow$ at cathode (So, pH increases)

$H_2O - 2e \rightarrow 2H^+ + 0.5O_2$ at anode, (So, pH decreases)

To keep the pH constant during separation, a porous diffusion barrier or diaphragm is used near the electrodes.

Factors affecting mobility (sensitivity of technique)

1. Electric Field:

- **Voltage:**

The velocity of migration of a molecule is directly proportional to the potential gradient (V/d) across the stationary phase;

$$\nu \alpha (V/d)$$ **V= volt, d= distance from the electrode**

For low voltage electrophoresis (LVE); V = 200-500 V and for high voltage electrophoresis (HVE); V = 500-1000 V

- **Current:**

The current is carried mainly by the buffer but small amount is carried by the sample components. Due to resistance of the supporting medium heat is evolved. So the current intensity has to be controlled.

2. The sample

- **Size and molecular weight:**

  smaller molecules migrate faster than larger molecules carrying the same charge.

- **Shape:**

  molecules with lots of side-chains experiences more frictional resistance than a linear molecule of the same mass and charge, and will therefore move more slowly.

- **Charge:**

  The type and number of net charge determine the direction and mobility of components.

3- solid support (stationary phase):

It could affect the mobility via adsorption (as in paper or thin layer), molecular sieving (as in gel) or electro-osmosis (due to adsorption of ions on the surface of stationary phase). Both adsorption and molecular sieving causes decrease of mobility, while electro-osmosis Increase mobility.

4- Buffer:

- **pH (protonation - deprotonation process):**

  proteins and amino acids exist as zwitter ions, and can be either positively or negatively charged because they contain both acidic and basic groups. The extent, and direction, of ionization depends on the pH of the buffer.

- at low pH; $R\text{-}CH(COOH)NH_3^+ \rightarrow$ cation moves toward cathode
- at high pH; $R\text{-}CH(COO^-)NH_2 \rightarrow$ anion moves toward anode
- at neutral pH; $R\text{-}CH(COO^-)NH_3^+ \rightarrow$ zwitter ions do not move (iso-electric point).

- **ionic strength (μ):**
  - low μ
    - few counter-ions
    - low charge shielding for current carriers.
  - high μ
    - many counter-ions
    - high charge shielding for current carriers.

$$\mu = \frac{1}{2}\left([A]\cdot Z_A{}^2 + [B]\cdot Z_B{}^2 \ldots\right)$$

- **Therefore, the rate of migration depends on:**
  - **Net electrical charge on molecules**
  - **Size & shape of molecules**
  - **Electric field strength**
  - **Properties of supporting medium**
  - **Temperature of operation**

Procedures of separation by electrophoresis

1- Preparation and saturation of the supporting medium:

- If the supporting medium is not a gel, it should be saturated with buffer before separation is started to conduct the electric current.
- Saturation is best done before the sample is applied.

2- Sample application:

The sample is applied as a solution with a micro-pipette, as a small spot or narrow band on th stationary phase.

- Sample containing positively charged ion, is applied near the anode, but negatively charge ions are applied near the cathode.
- Mixed ions sample (containing positive and negative ions) is applied to the middle.

3- Running of sample

- After sample application, the power is switched on at the required and adjusted potential for a period of time (usually 2 hours). The current has to be constant.

4- Removal of supporting medium

After running of the ample and elution process, the supporting medium is removed and dried in an oven at 110 °C to remove the buffer. Gels are squeezed by hypodermic syringe.

5- Staining of components:

Most biological compounds are colorless, and therefore it is necessary to detect them and determine their position on the supporting medium by using coloring reagents. Organic dyes are used for this purpose.

6- Identification and determination of components:

Using of specific coloring reagents helps in identification of components (RNA gives blue color with pyronine, while DNA gives red color).

For estimation of components, complete elution and estimation using spectral methods is used.

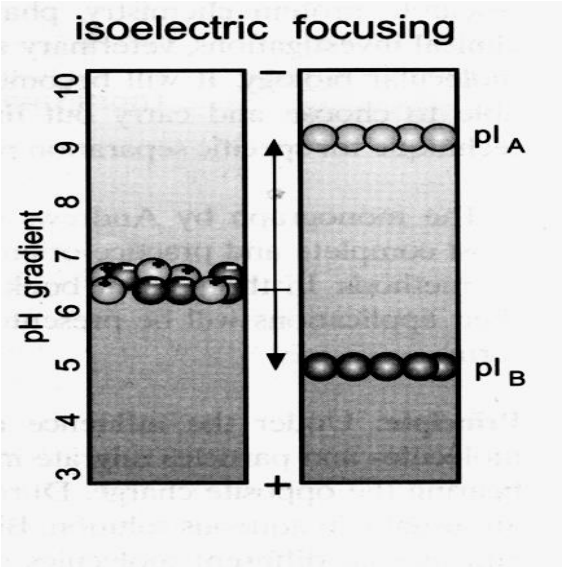
electrophortic techniques:

1- Isoelectric Focusing

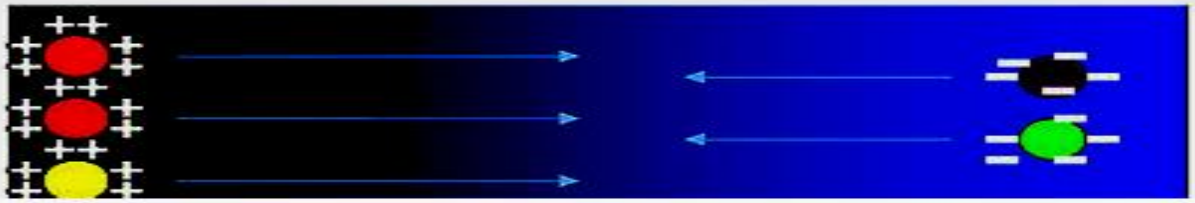
It is a technique sued for separation of amphoteric compounds eg. amino acids and Proteins under potential as well as pH gradients. Proteins moves to zone where:

pH of the medium = pI protein (iso-electric point) => charge = 0

- **Iso-electric focusing can be used for separation of protein confined in a narrow (pI) pH range (with 0.01 to 0.02 pH unit differences) -> sharp protein zones.**



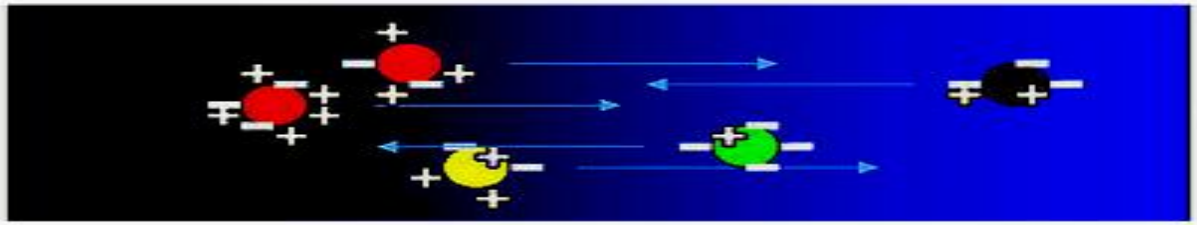
Stable pH gradient along the gel stationary phase



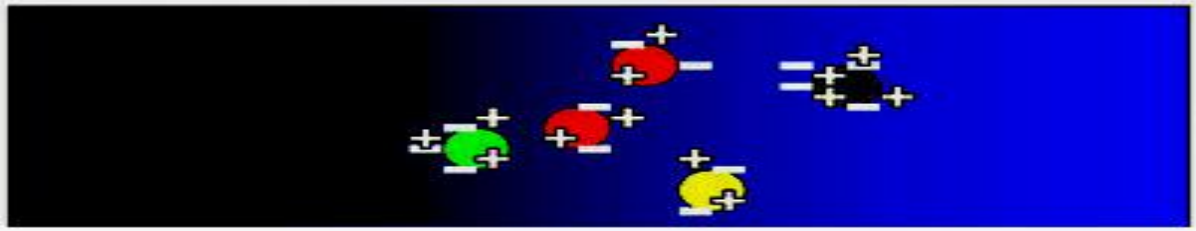
Anode

Cathode

At low pH, most amino acids and protein have a positive charge, while at high pH, they have a negative charge



When an electric field is applied, the cathode and anode pull the protein or amino acids to their iso-electric point where each component becomes neutral in charge



The amino acids and proteins stopped migration (moving) when they reached their iso-electric point at certain pH-value

=Isoelectric point at pH 7.5

=Isoelectric point at pH 6.8

=Isoelectric point at pH 8.5

=Isoelectric point at pH 10.1

=Isoelectric point at pH 5.6

Method of separation:

- use horizontal gels on glass/plastic sheets.
- introduce ampholytes into gel and create pH gradient (keep the anode area at lowest pH and cathode area at highest pH).
- apply a potential difference across gel for running of the sample proteins migrate until it arrives at pH = pI (zwitter ion).
- wash with fixing solution to remove ampholytes.
- stain, destain, visualise and determine the components by usual methods (IR, UV, Fluorescence or NMR.

2- High-voltage electrophoresis (HVE).

- It is used for separation of medium to low molecular weight charged compounds with improved resolution in shorter analysis times. However, though the rate of migration increases linearly with increase in voltage gradient the heat generated increases. Thus, heat dissipation for the control of evaporation of solvent is of great importance to the development and application of HVE.
- The applied potential is 500-10000 V, potential gradient 100-200 V/cm.

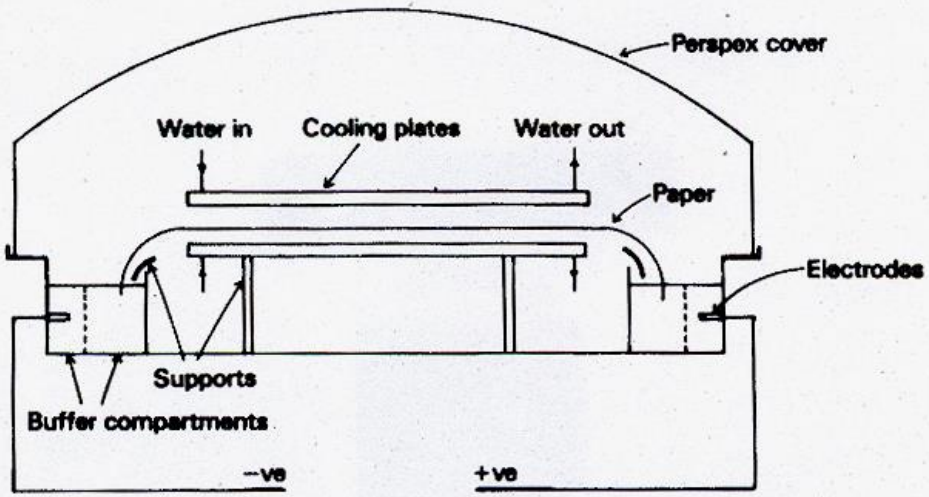
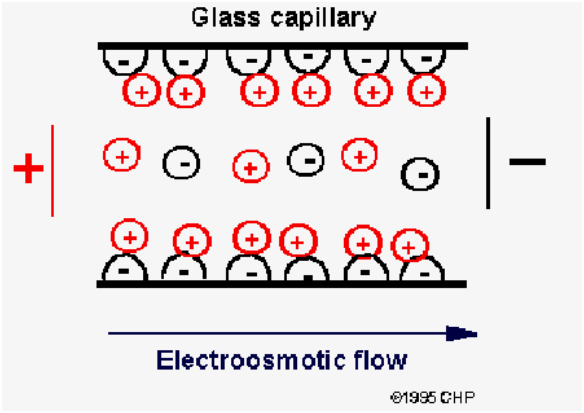
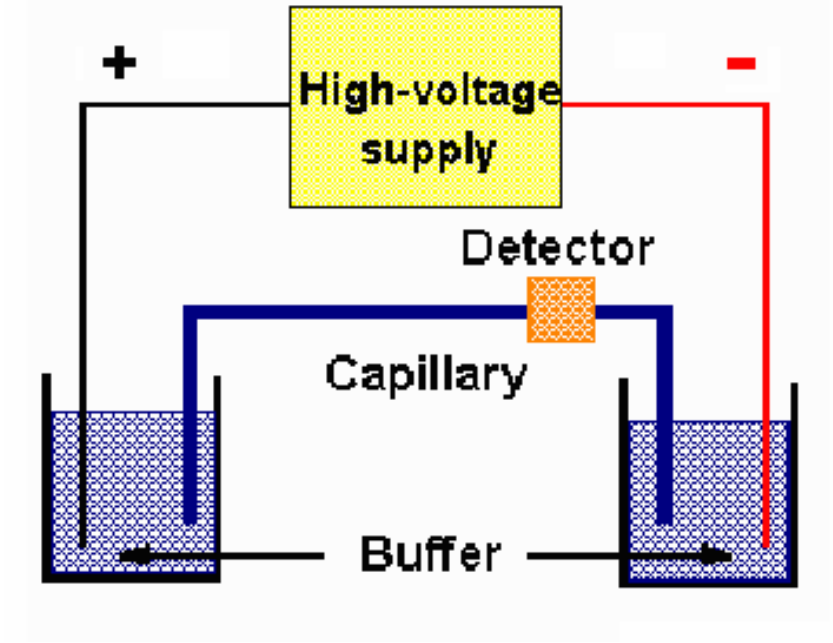


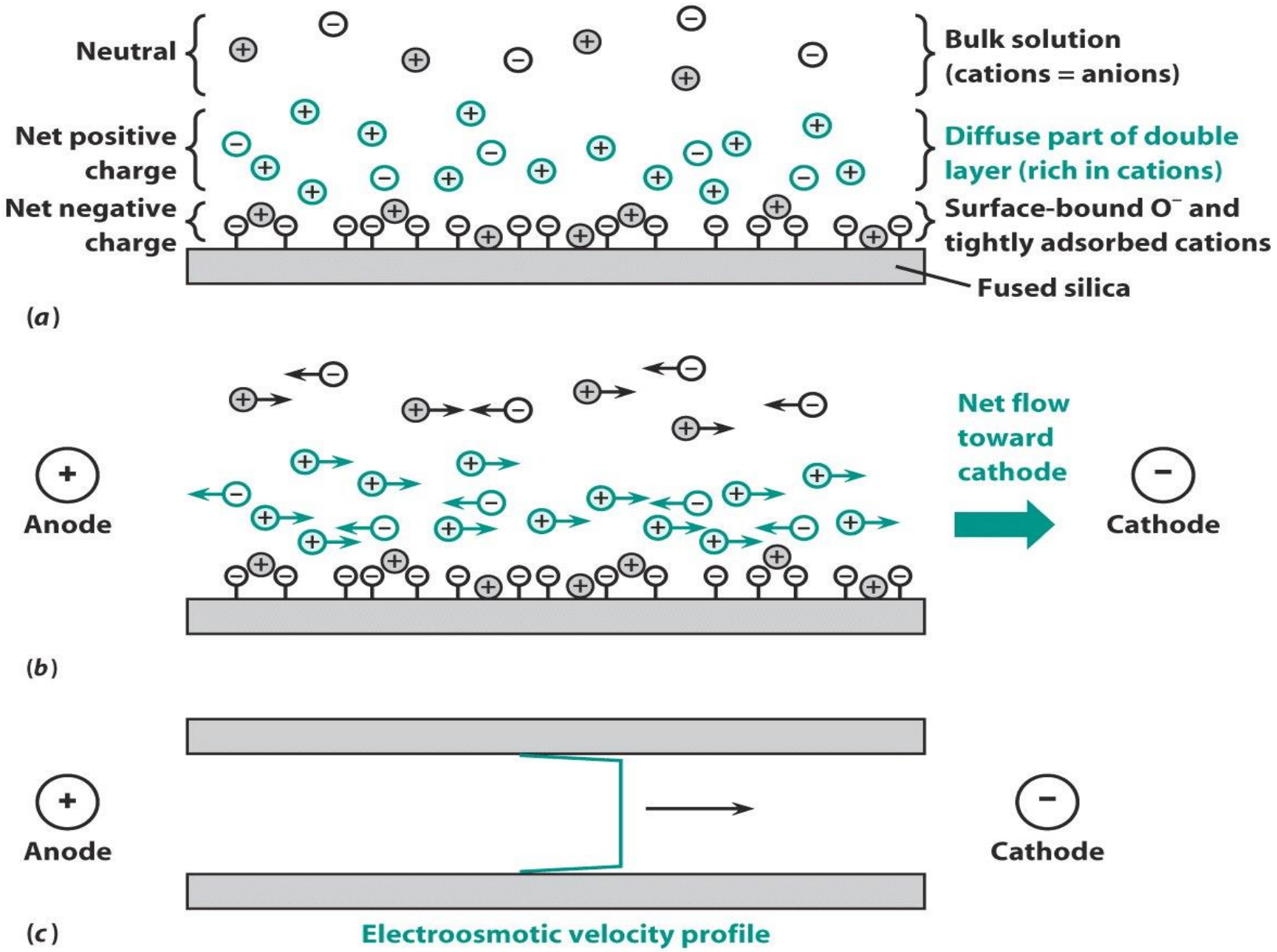
Figure 3.24 HVE apparatus.

3- Capillary electrophoreis:

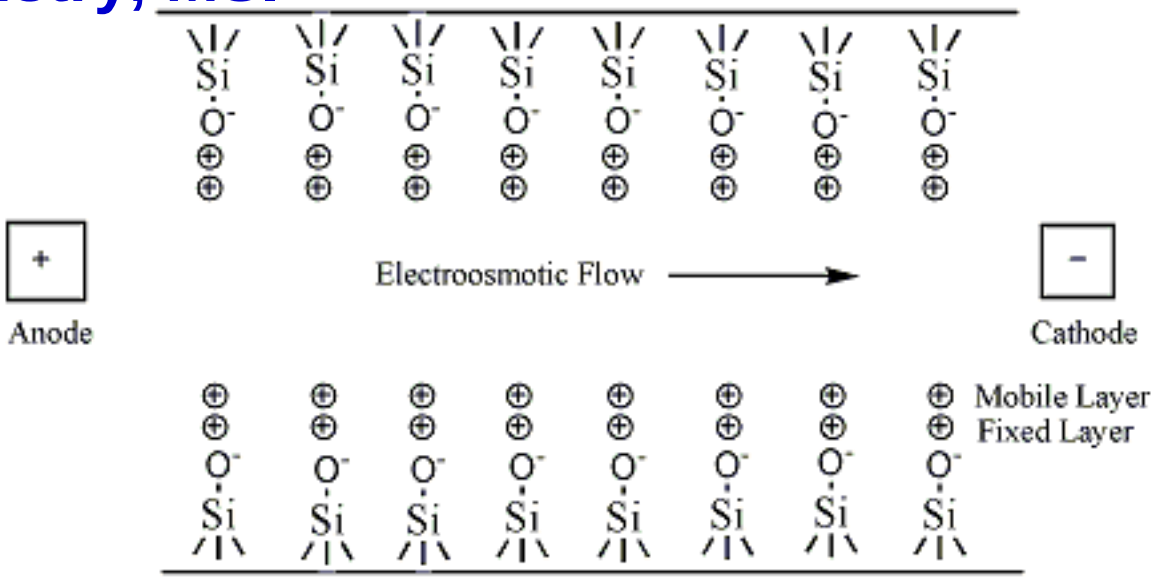
- Small-diameter capillaries (25-50 μm inner diameter, 0.5-1m length) allow use of very high electric fields, 20-30kV applied potential.
- Efficiently dissipate heat, Increasing electric fields, produces efficient separations, reduces separation times.
- The inner surface of the silicate glass capillary contains negatively-charged functional groups that attract positively-charged counter ions.





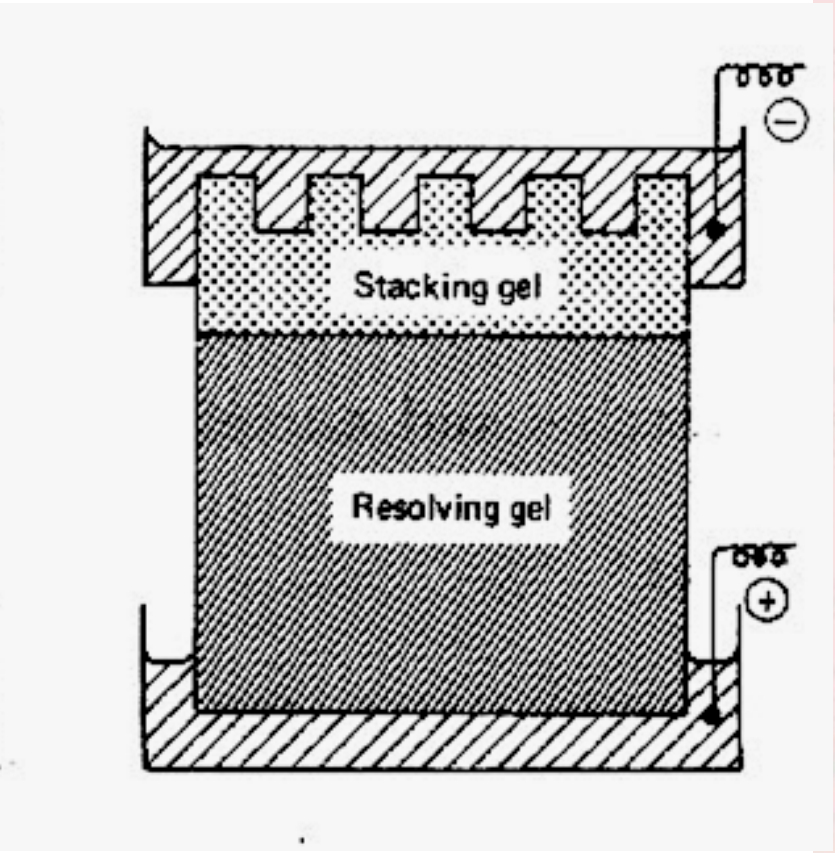

Neutral
Bulk solution
(cations = anions)
Net positive
charge
Diffuse part of double
layer (rich in cations)
Net negative
charge
Surface-bound O⁻ and
tightly adsorbed cations
Fused silica
(a)
Net flow
toward
cathode
Anode
Cathode
(b)
Anode
Cathode
(c)
Electroosmotic velocity profile

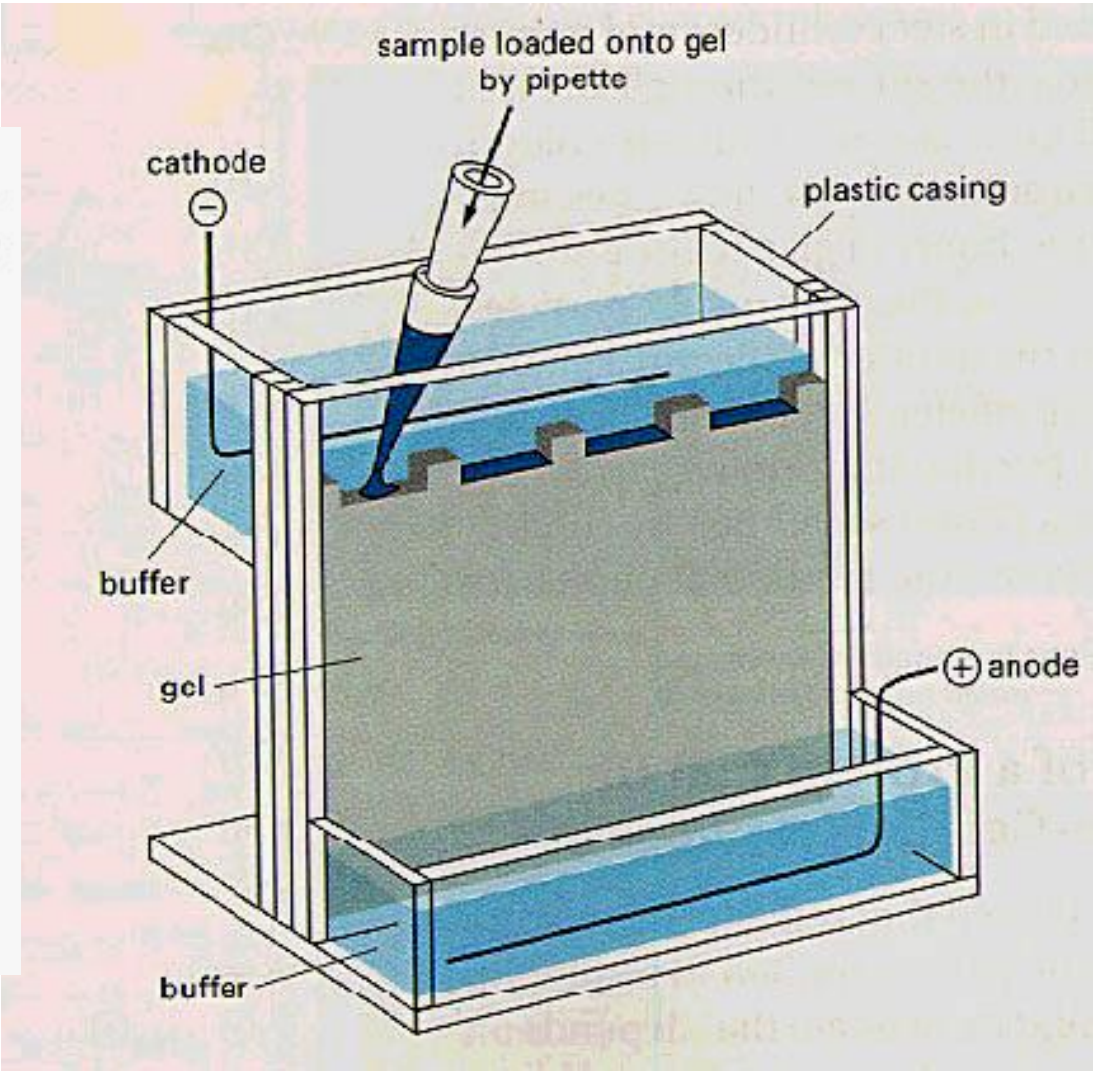
- **Positively-charged ions migrate towards the negative electrode and carry solvent molecules in the same direction. They move faster while negatively-charged ions move slower, but all migrate towards cathode. Small volume sample (10 nl) injected at anode end, and detected near cathode end.**
- **Detection can be carried out by several methods, including absorbance or fluorescence measurement, electrochemistry, MS.**



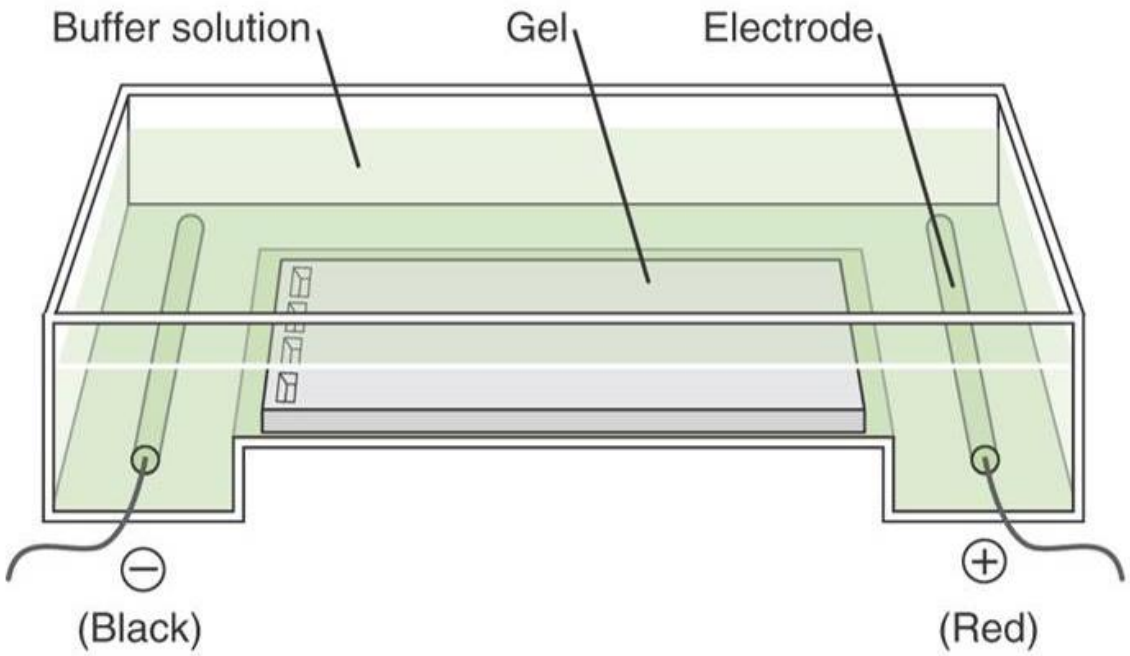
4- Continuous and Discontinuous electrophoresis

- In a continuous system, only a single separating gel is used with the same buffer in the tanks.
- In a discontinuous system a nonrestrictive large pore gel (as agarose gel), called a stacking gel, is layered on top of a separating small pore gel (as polyacrylamide gel).
- The stacking gel don't restrict the migration of the proteins, and deposits proteins in a stack at top of separating gel
- The resolution obtainable in a discontinuous system is much greater than that obtainable in a continuous one. However, the continuous system is a little easier to set up, but discontinuous gels are most common.


Stacking gel
Resolving gel


sample loaded onto gel
by pipette
cathode
plastic casing
buffer
gel
anode
buffer

Horizontal or Submarine Gel



DNA/RNA is negatively charged: RUN TO RED

Applications:

- Forensics, Molecular biology, Genetics,
- Biochemistry, Microbiology

- Electrophoresis is employed in biochemical and clinical fields: in the study of mixtures such as blood, serum, urine, cerebrospinal fluid analysis (CSF).
- Excellent method for fractionation of protein mixture without denaturation (expanding of helix).
- Analysis of lipoproteins.
- Separation of hemoglobin mixtures.
- Separation of mixtures of organic acids.
- Separation of DNA fragments.

How Capillary Electrophoresis Works

(2) Why electroosmosis?

(a)Wall is covered with silanol,

pH > 2, Si-OH $\rightarrow$ Si-O^- $\Rightarrow$ Electric double layer

(Diffuse part of the double layer ~1 nm)

(b) Electric field $\uparrow\Rightarrow$ flow $\uparrow$

(c) electroosmotic flow (electric field) v.s. hydrodynamic flow (pressure difference)