Bioseparation

محاضرة 4

Assistant Professor Dr. Ahmed Aljanaby

Ionic Charge

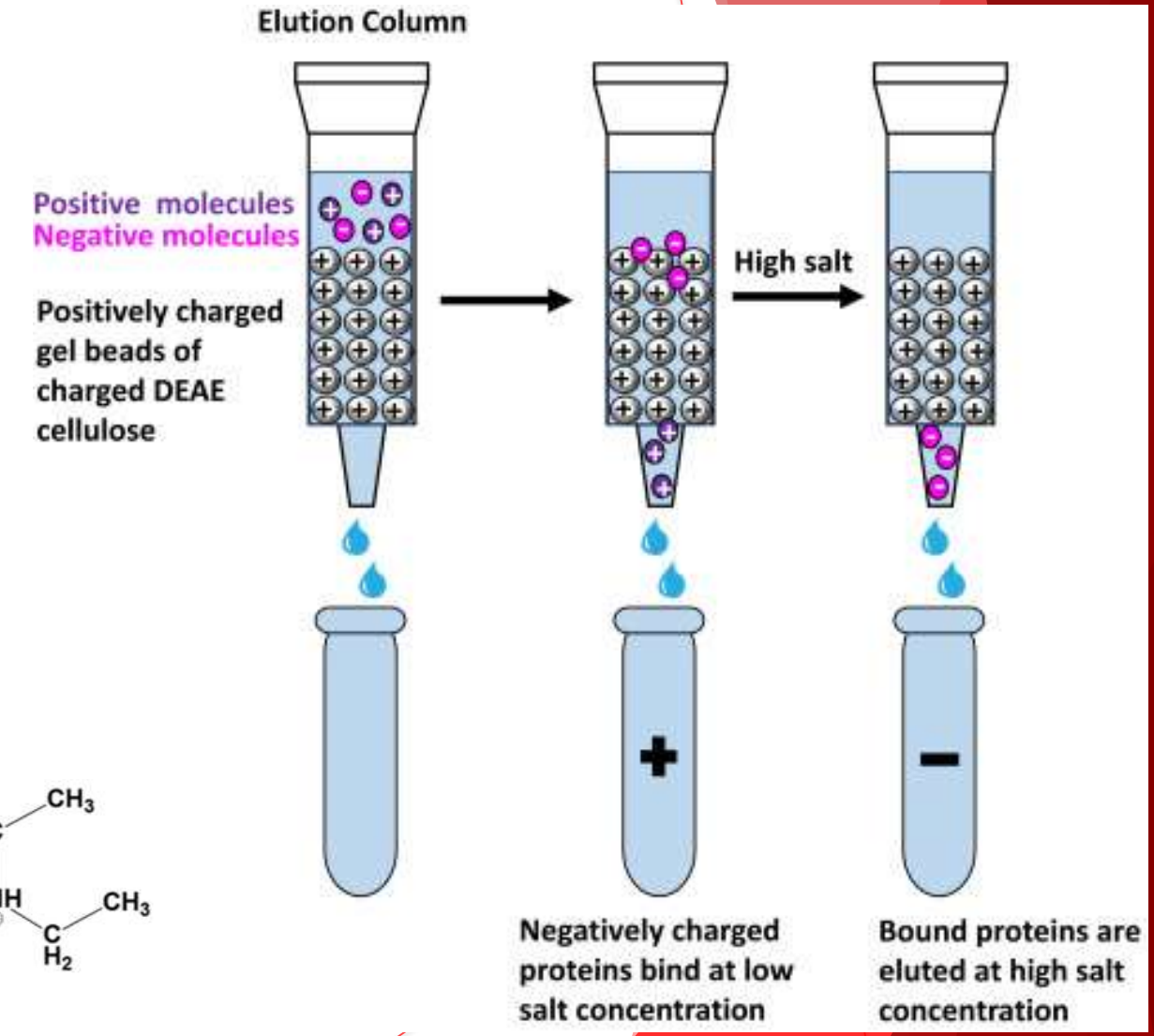
- **Ion-exchange chromatography**

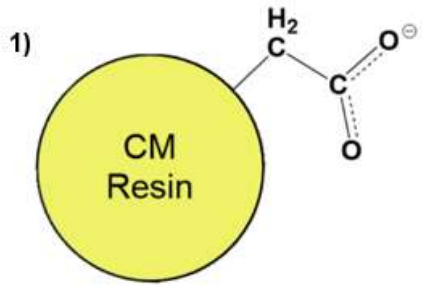
•Stationary phase: Ion-exchangers

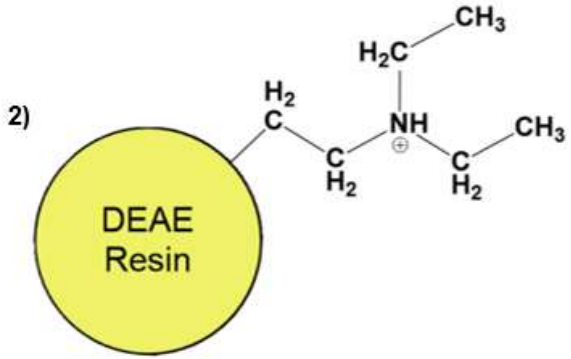
• Mobile phase: Buffers of variable concentrations

• Principle: Ion-exchange chromatography relies on the attraction between oppositely charged ion-exchangers and analyte (protein sample).

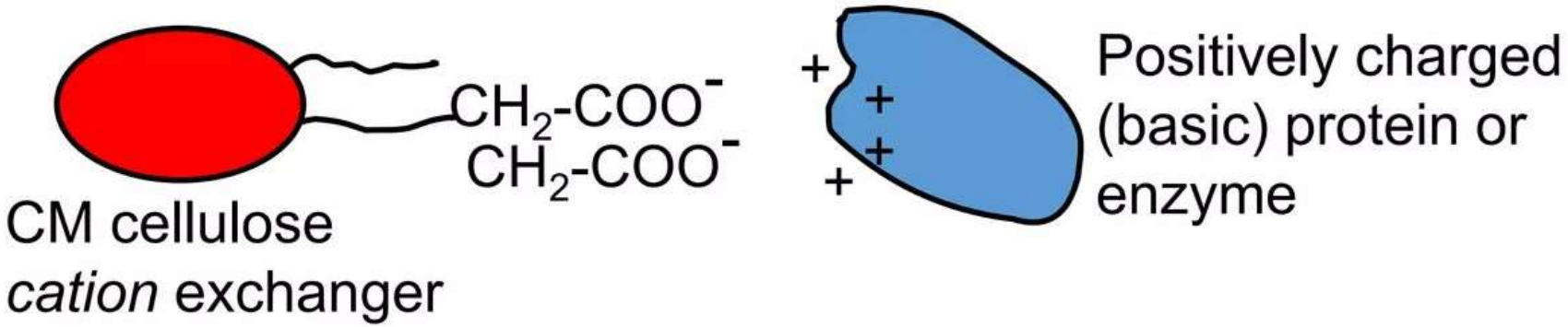
• Ion-exchangers: Carboxymethyl (CM) group (cation-exchanger) Diethylaminoethyl (DEAE) group (anion-exchanger)







- Ion exchange resins contain charged groups.
- • If these groups are acidic in nature they interact with positively charged proteins and are called cation exchangers.



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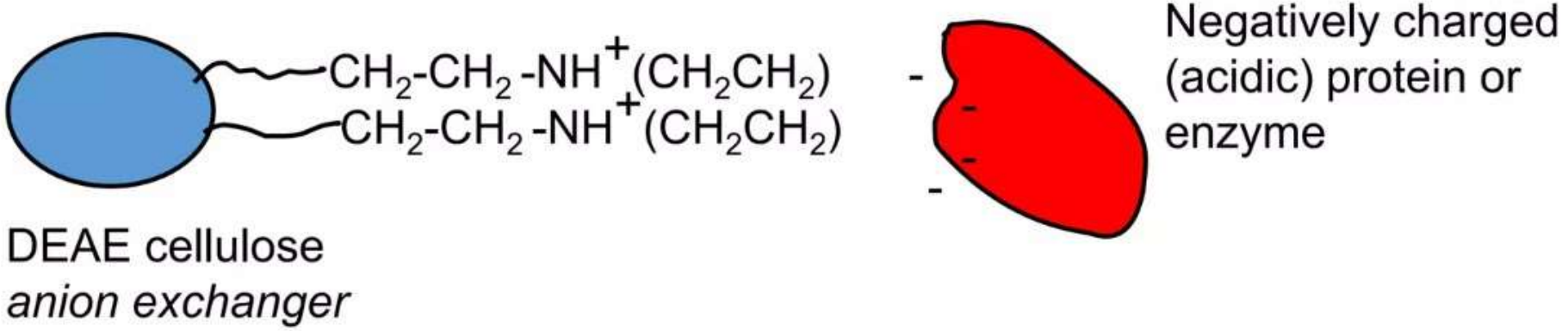


Ion-exchange chromatography

- There are four steps of ion-exchange chromatography:

1. Equilibration - stabilization of the ion-exchangers with oppositely charged ions in the buffer. E.g: Na+Cl-

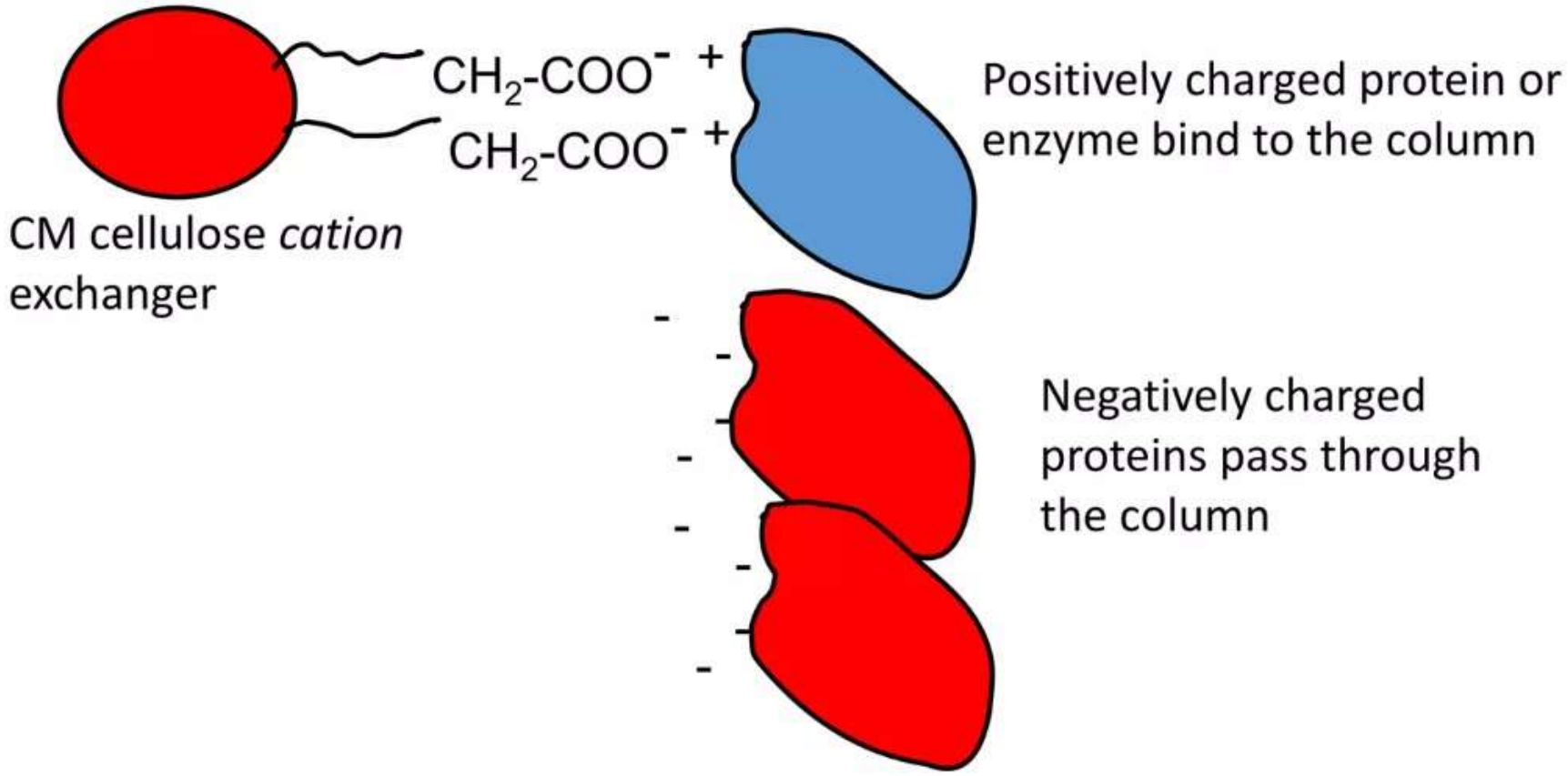
2. Sample application and wash (Protein bound to the ion-exchangers remain attached while other gets removed during wash)

3. Elution - Removal of bound protein from the ion exchangers with the help of increased concentration of elution buffer.

4. Regeneration - Preparing the ion exchangers for the next round of protein purification.

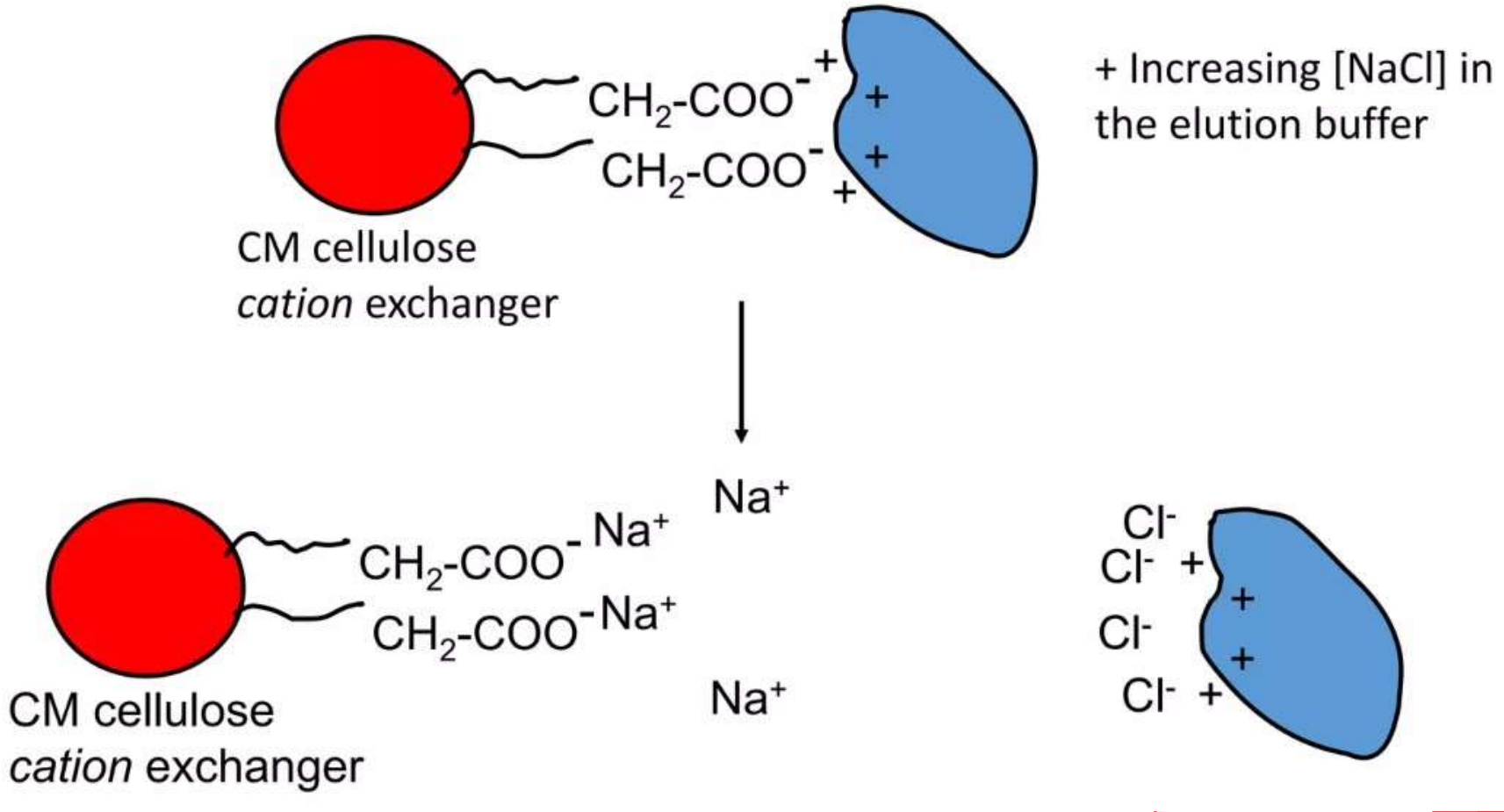
Ion-exchange chromatography

- If a protein has a net positive charge at pH 7, it will usually bind to a column of beads containing carboxylate groups (cation-exchangers).



Ion-exchange chromatography

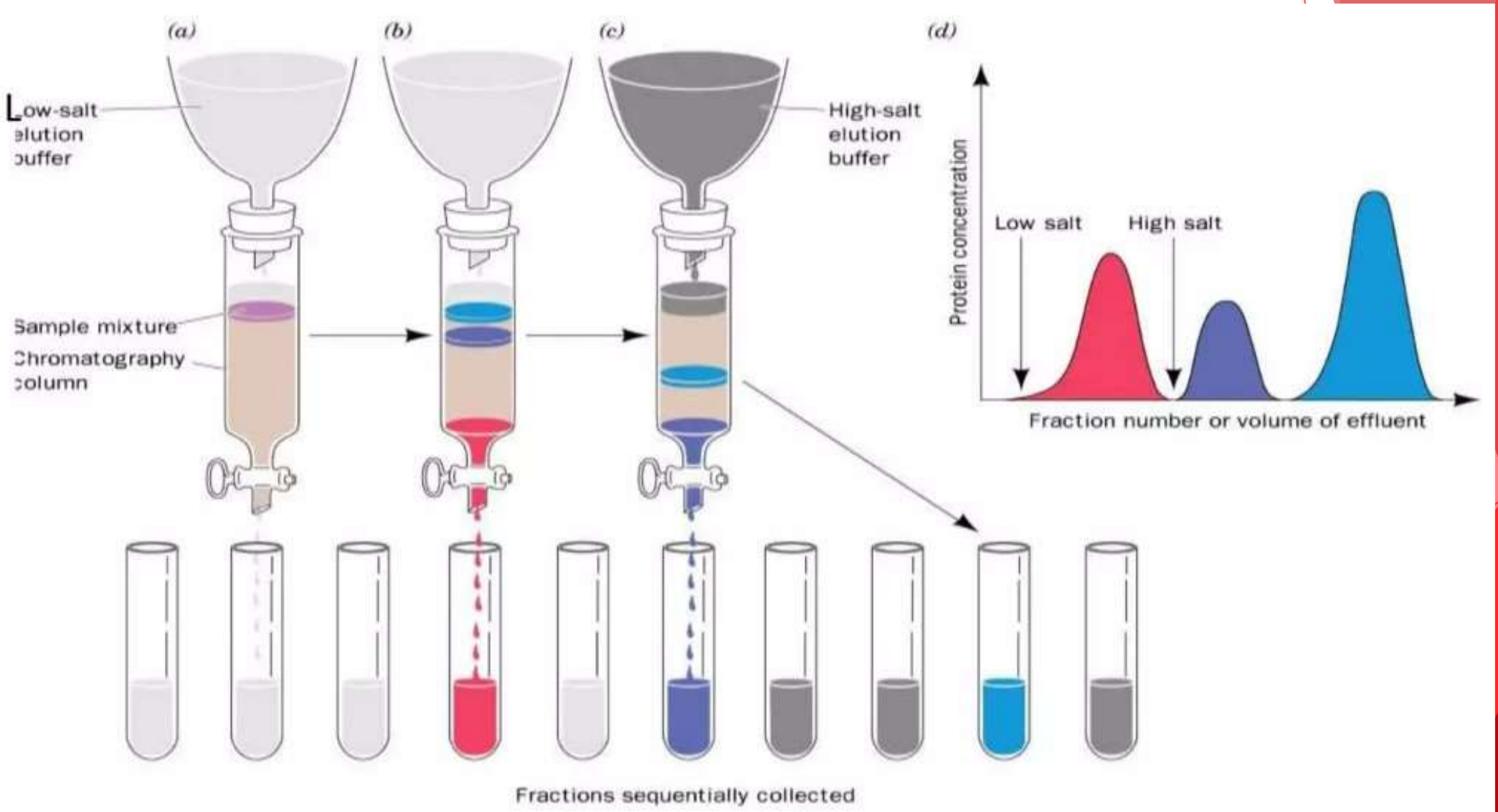
- To elute our protein of interest, add buffer of higher salt concentartion. Na+ will interact with the cation resin and Cl- will interact with our positively charged protein to elute off the column.



Ion exchange chromatography

- • Proteins will bind to an ion-exchanger with different affinities.
- • As the column is washed with buffer, proteins which have relatively low affinities for the ion-exchange resin will move faster through the column than the proteins that bind tightly to the column to the ion-exchangers.
- • The greater the binding affinity of a protein for the ion exchange column, the more it will be slowed in eluting off the column.
- • Proteins can be eluted by changing the elution buffer to one with a higher salt concentration and/or a different pH (stepwise elution or gradient elution).

Ion exchange chromatography using stepwise elution



Some Biochemically Useful Ion Exchangers

Name[a]	Type	Ionizable group	Remarks
DEAE-cellulose	Weakly basic	Diethylaminoethyl $-CH_2CH_2N(C_2H_5)_2$	Used to separate acidic and neutral proteins
CM-cellulose	Weakly acidic	Carboxymethyl $-CH_2COOH$	Used to separate basic and neutral proteins
P-cellulose	Strongly and weakly acidic	Phosphate $-OPO_3H_2$	Dibasic; binds basic proteins strongly
Bio-Rex 70	Weakly acidic, polystyrene-based	Carboxylic acid $-COOH$	Used to separate basic proteins and amines
DEAE-Sephadex	Weakly basic cross-linked dextran gel	Diethylaminoethyl $-CH_2CH_2N(C_2H_5)_2$	Combined chromatography and gel filtration of acidic and neutral proteins
SP-Sepharose	Strongly acidic cross-linked agarose gel	Methyl sulfonate $-CH_2SO_3H$	Combined chromatography and gel filtration of basic proteins
CM Bio-Gel A	Weakly acidic cross-linked agarose gel	Carboxymethyl $-CH_2COOH$	Combined chromatography and gel filtration of basic and neutral proteins

Ionic Charge

- Gel Elecrophoresis

Apparatus:

1. Buffer tank
2. Buffer
3. Electrodes
4. Power supply
5. Support media
6. Tracking dye

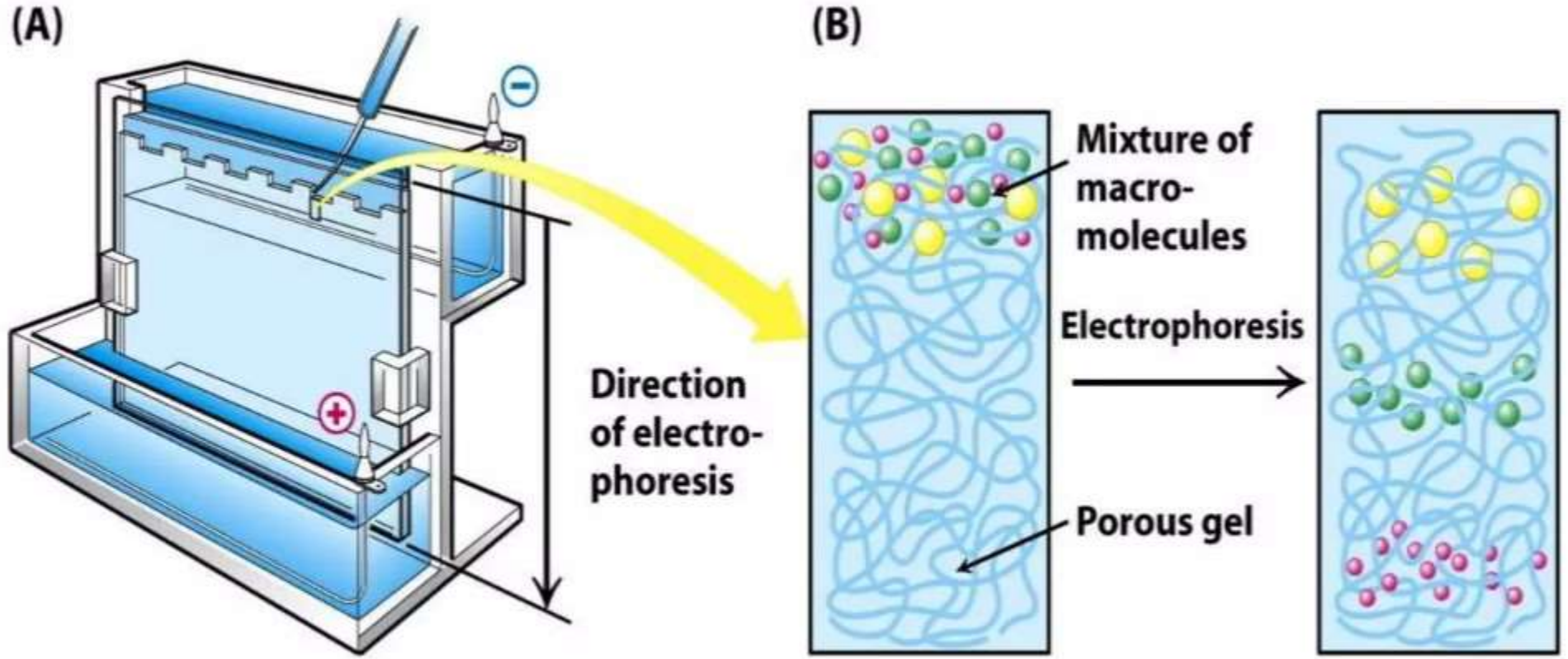


Figure 3-7
Biochemistry, Sixth Edition

Gel Electrophoresis

- **Principle:**

Any charged ion or molecule migrates when placed in an electric field, the rate of migration depend upon its net charge, size, shape and the applied electric current.

- **Can be represented by following equation:**

$$V = \frac{E * q}{f}$$

- V = velocity of migration of molecule
- E = electric field in volts per cm
- q = net electric charge on the molecule
- f = frictional coefficient

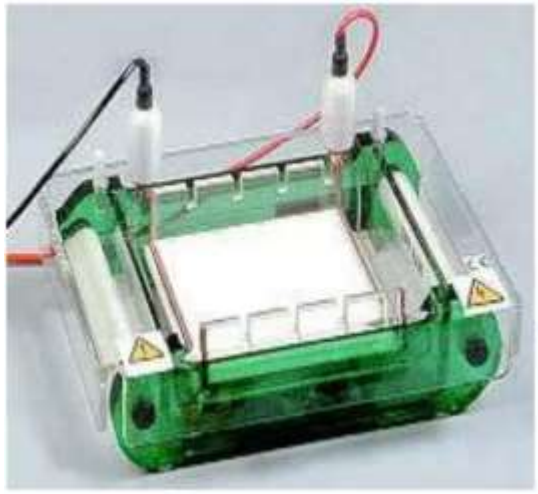
Gel Electrophoresis

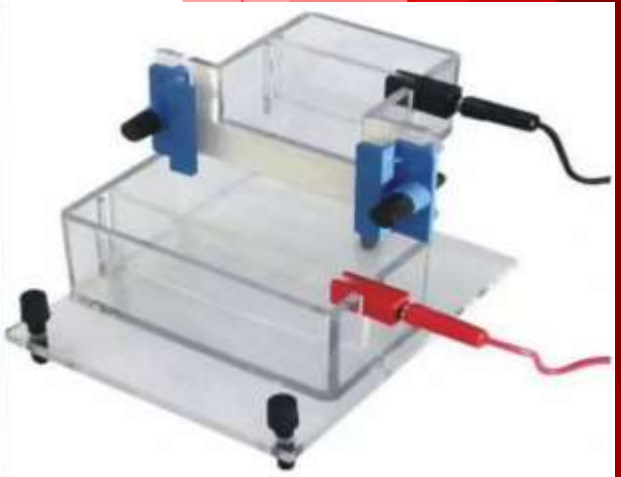
- **Electrophoresis is the movement of charged particles through an electrolyte when subjected to an electric field.**

- **Cations move towards cathode.**
- **Anions move towards anode.**
- **Commonly used in biological analysis, particularly in the separation of proteins, peptides and nucleic acids.**
- **Polyacrylamide is used as supporting media for the separation of proteins.**

- Based on gel casting technique, classified into:

1. Horizontal
2. Vertical





Principle of Polyacrylamide Gel Electrophoresis (PAGE)

Is an analytical method used to separate components of a protein mixture based on their size.

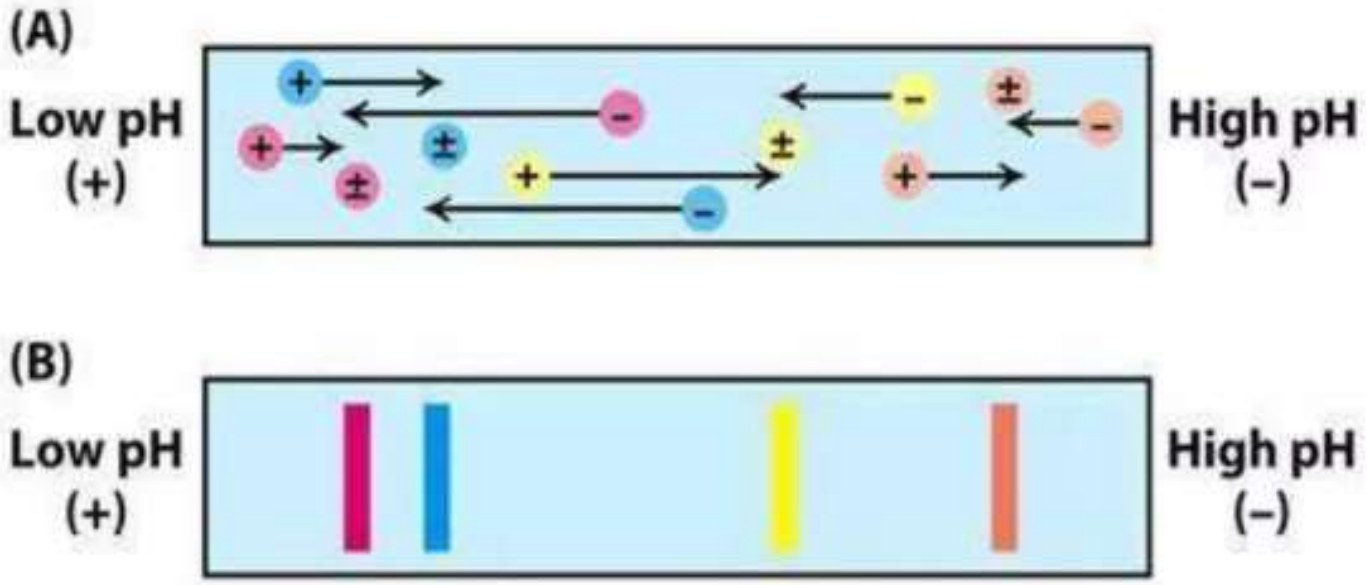
The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign.

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 and 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 lose 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).

Isoelectric Focusing

- • Isoelectric focusing is an electrophoretic method in which proteins are separated on the basis of their isoelectric point (pI).
- • It makes the use of the property of proteins that their net charge are determined by their local environment.
- • Principle: At the isoelectric point, the net charge of the protein become zero. Therefore, mobility of proteins becomes zero in an electric field.



Isoelectric Focusing

- Procedure of isoelectric focusing:
- cell extract is fully denatured by high concentration of urea (8M).
- - Denatured cell extract is poured on the polyacrylamide gel layered with ampholytes.
- - When placed in an electric field, the ampholytes will separate and form a continuous pH gradient based on their net charge.
- Charged proteins migrate through the gradient until they reach their isoelectric point.
- - At Ip, its net electrical charge becomes neutral and stops migrating.

Binding Affinity

- • **Affinity chromatography:**

Principle:

- Many proteins can bind specific molecules very tightly but non covalently.
- The substance to be purified is specifically and reversibly adsorbed to a ligand (binding substance), immobilized by a covalent bond to a chromatographic bed material.
- **Steps:**

1. Incubate crude sample with the immobilized ligand.

2. Wash away non bound sample components from solid support .

3. Elution of the molecules of interest in a purified form.

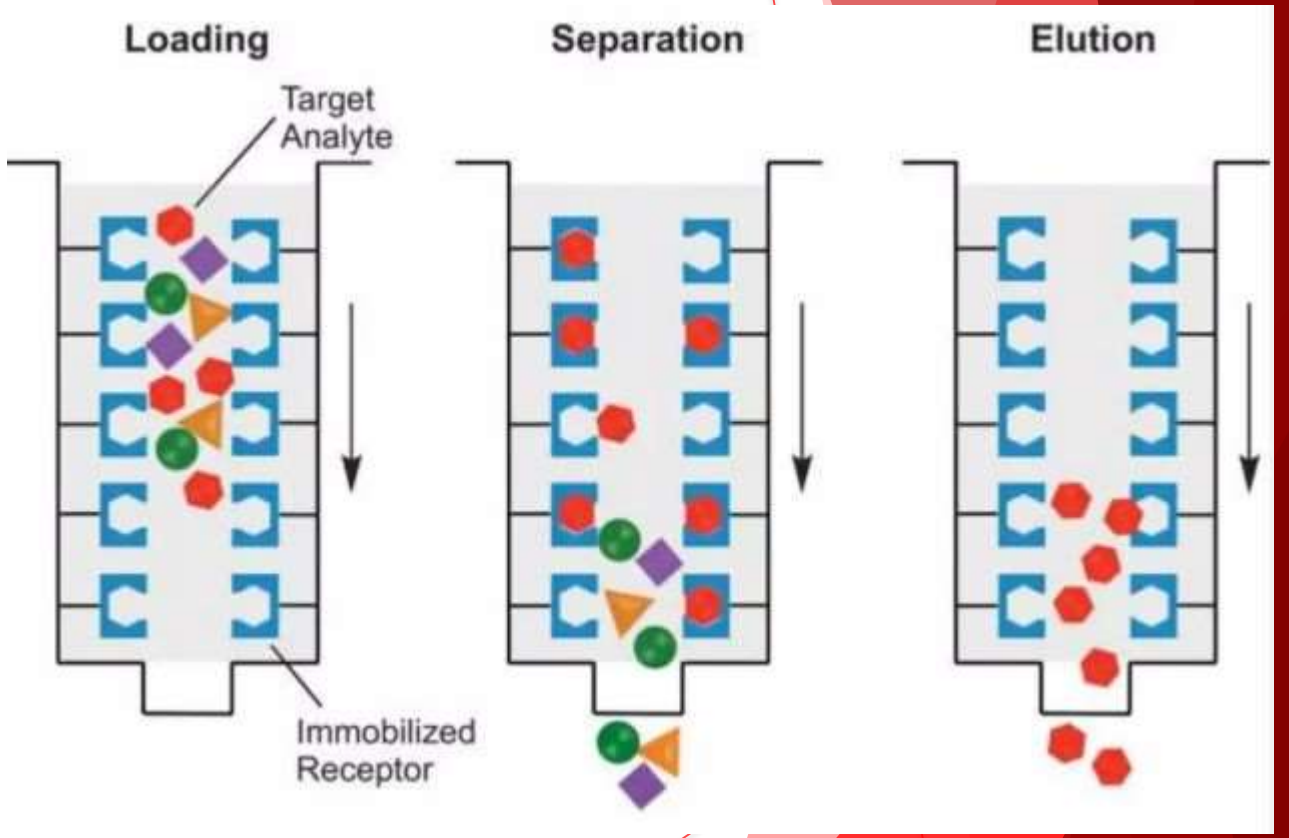
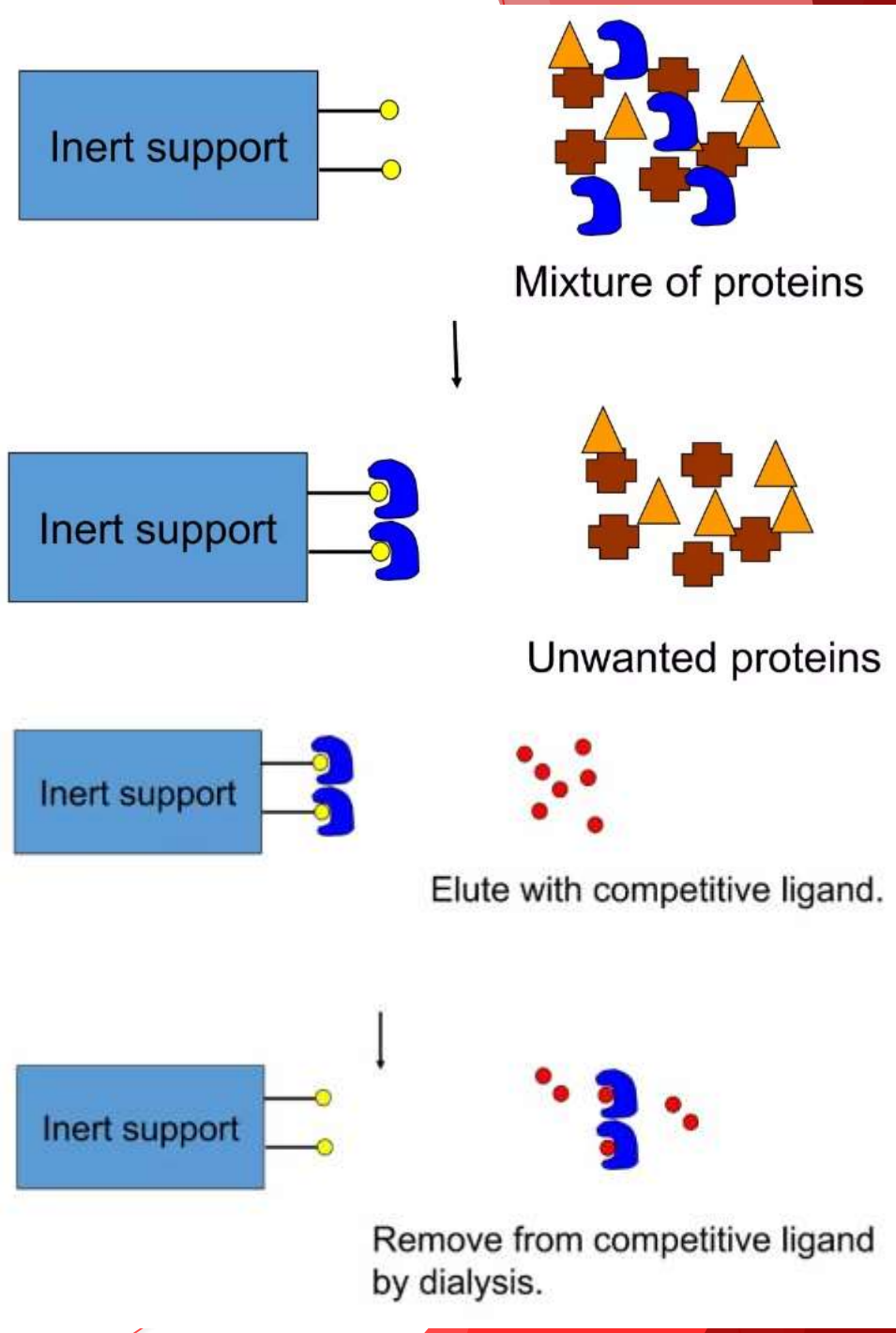


Table 1.3 Typical biological interactions used in affinity chromatography

Types of ligand	*Target molecules or molecules of interest*
Enzyme	Substrate analogue, inhibitor, cofactor
Antibody	Antigen
Lectin	Polysaccharide, glycoprotein, cell surface receptor, cell
Nucleic acid	Complementary base sequence, nucleic acid binding protein
Hormone	Receptor
Avidin	Biotin
Calmodulin	Calmodulin-binding molecule
Poly(A)	RNA containing poly(U) sequences
Glutathione	Glutathione-S-transferase or GST fusion proteins
Proteins A and G	Immunoglobulins
Metal ions	Poly (His) fusion proteins, native proteins with histidine

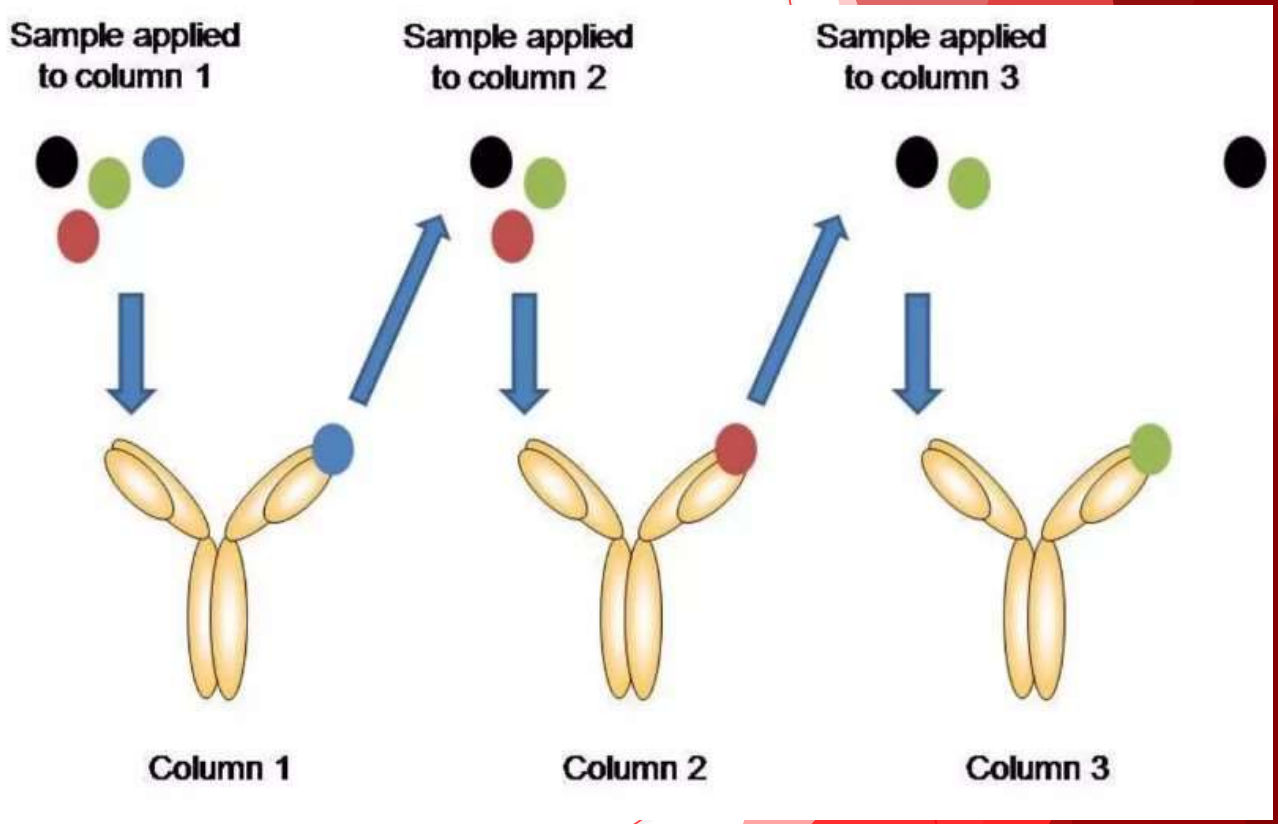
Affinity chromatography

- • To remove the protein of interest from the column, you can elute with a solution of a compound with higher affinity than the ligand (competitive)
- • You can change the pH, ionic strength and/or temperature so that the protein-ligand complex is no longer stable.



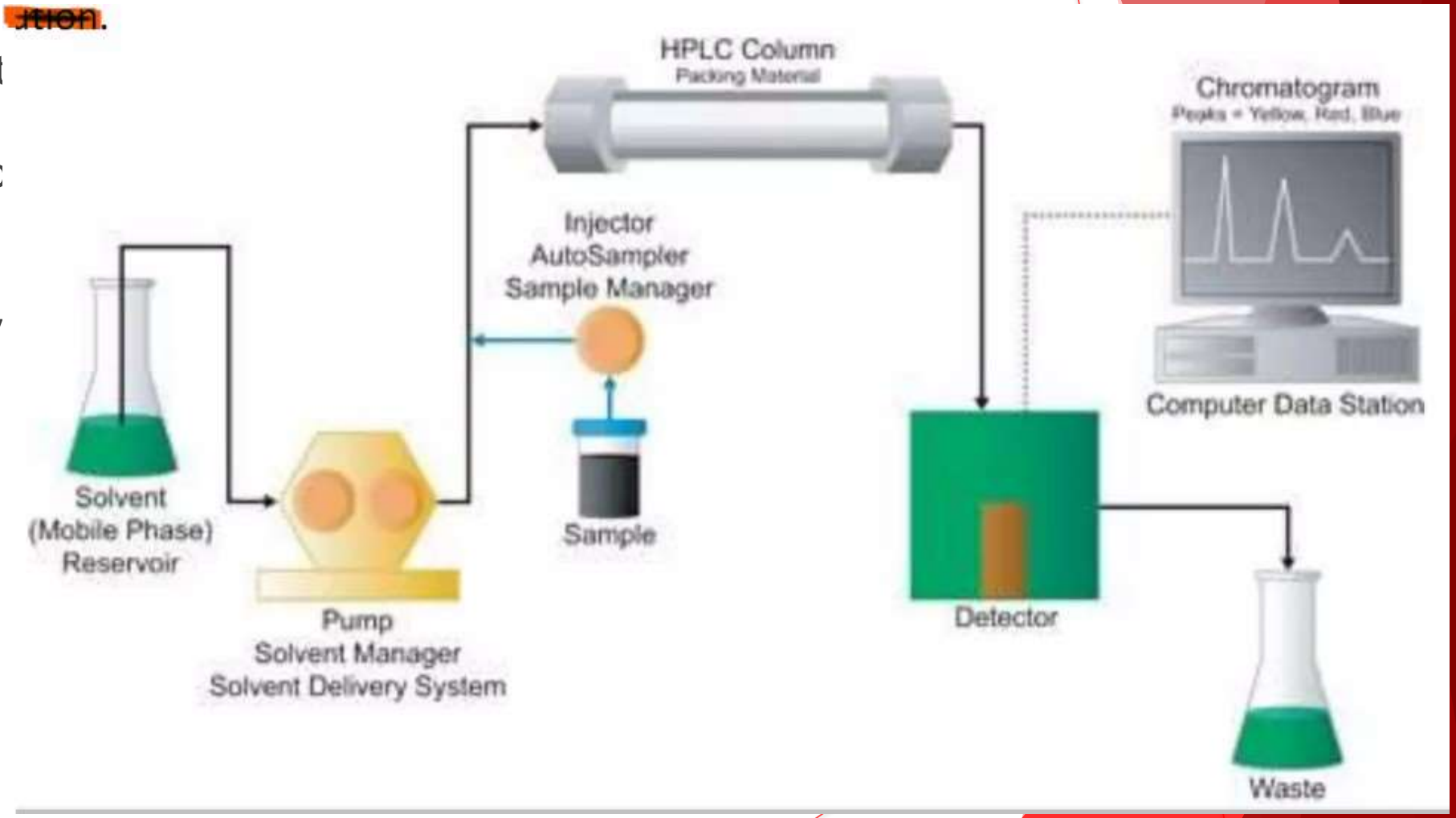
Immunoaffinity chromatography

- Monoclonal antibodies can be attached to the column material.
- • The column only binds the protein against which the antibody has been raised.
- • 10,000-fold purification in a single step!
- • Disadvantages: • Difficult to produce monoclonal antibodies
  • Cost-effective



HPLC

- • Type of liquid chromatography that use high pressure & small particle size to push a mobile phase solution through a column of stationary phase allowing separation of complex mixtures with high resolution.



HPLC

- • Principle: Separation of compounds occur on the basis of polarity and solubility.
- The interaction between stationary and mobile phase allow the separation of compounds in the analyte from each other.
- - Like dissolve like i.e., polar molecules binds with polar stationary phase while non-polar molecules binds with the non-polar stationary phase.
- • Stationary Phase: small diameter particles
- • Mobile Phase: Solvent
- • On the basis of polarity of stationary phase & mobile phase, HPLC can be of two types:

	Normal Phase HPLC	Reverse Phase HPLC
Stationary Phase	Polar	Non-polar
Mobile Phase	Non-polar	Polar