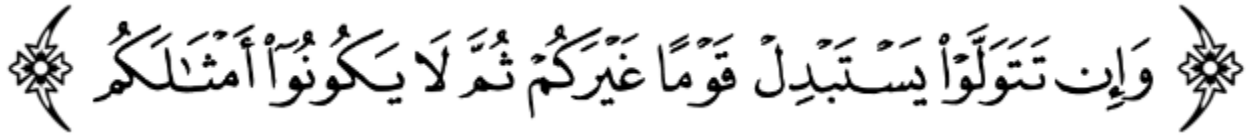
بسم الله الرحمن الرحيم

**BIOCHEMISTRY**




اللهم استعملنا لنصرة دينك.

Lecture 31

# Protein Purification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# Bases of protein separation

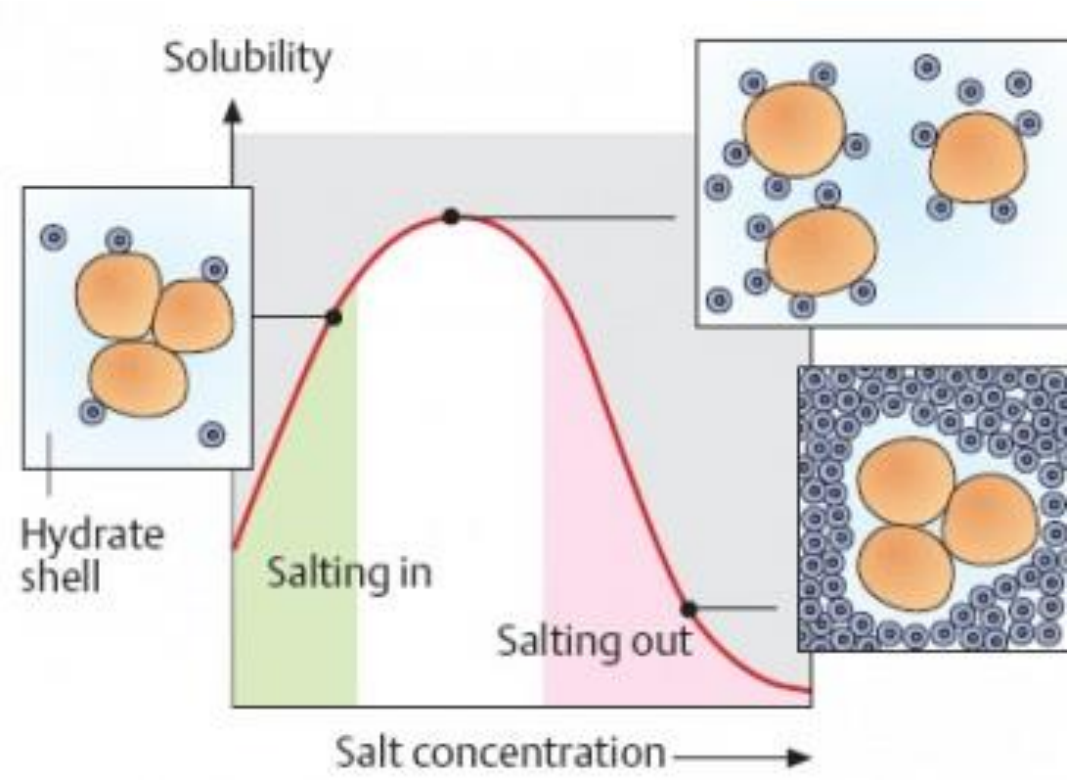
**Proteins can be separated from each other based on their chemical characteristics.**

- Proteins can be purified on the basis
  - Solubility
  - Size
  - Charge
  - specific binding affinity

# Salting in…salting out

The idea here is that we gradually add salt to a solution that contains different proteins, as we rise salt concentration, proteins will start to precipitate at different concentration of salt, thus leading to the separation of proteins into different fractions.

- Proteins are poorly soluble in pure water, but their solubility increases as the ionic strength increases.
  - salting in
- At very high concentrations of salt, most proteins become less soluble and precipitate.
  - salting out
- Why?



# Why?

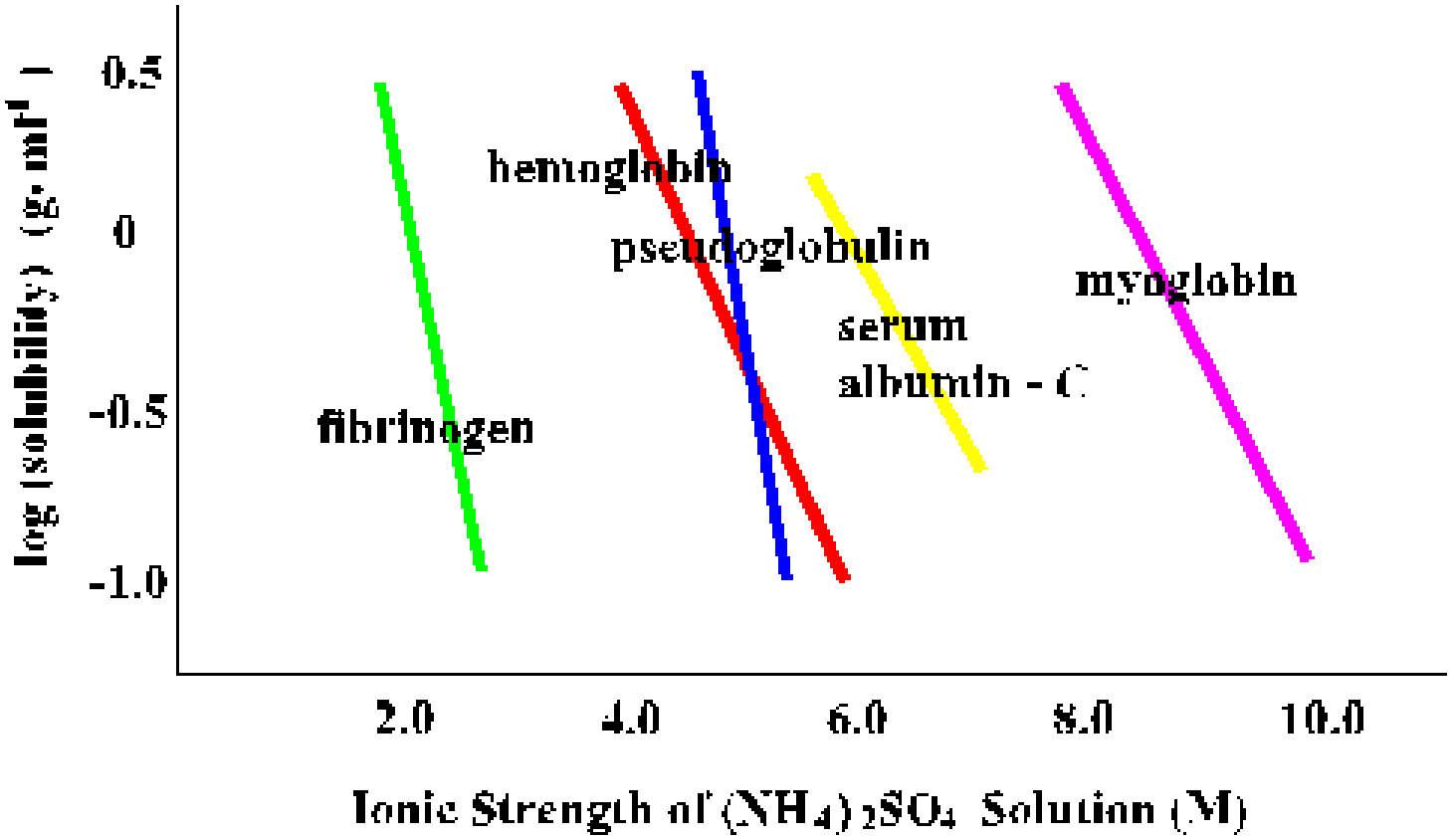
- When salt, like ammonium sulfate, is added to a protein solution, some of the water is taken away from the protein to make ion–dipole bonds with the ions.
- With less water available to hydrate the proteins, the protein molecules begin to interact with each other through hydrophobic bonds.
- Proteins precipitate at different and defined concentrations of the salt, resulting in precipitates that contain different proteins. These proteins can then be separated.

The solubility of proteins in water is significantly influenced by salt concentration. At low salt concentrations, salts can enhance protein solubility by shielding electrostatic interactions and reducing protein-protein interactions, thus facilitating better dissolution in water. However, at high salt concentrations, salts compete with proteins for water molecules, which diminishes proteins solubility. This competition results in the aggregation of proteins, causing them to cluster together and precipitate out of the solution. Consequently, while salts can initially improve protein solubility, excessive salt levels lead to precipitation due to increased protein aggregation.

# Uses of salting out

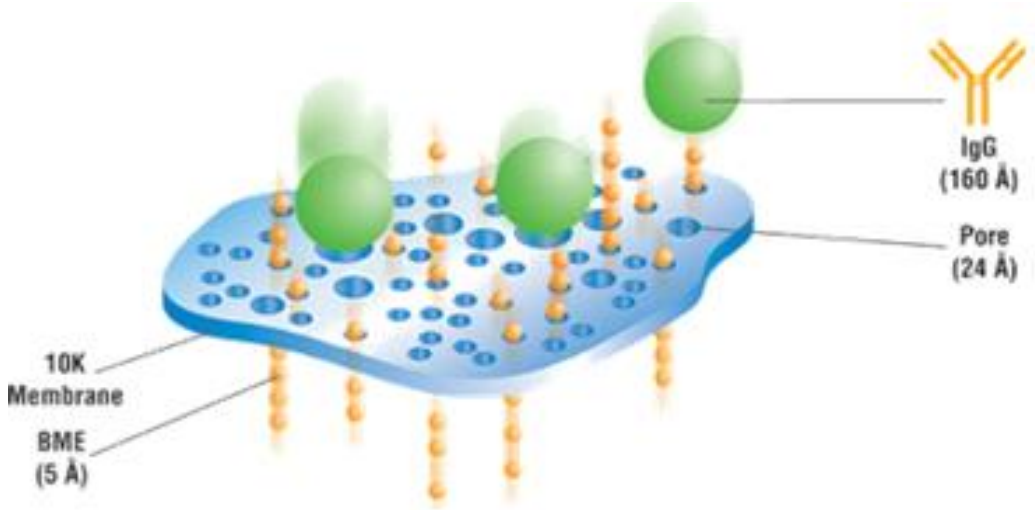
Proteins would precipitate at different concentrations of salt, when a specific protein precipitates, we separate it from the solution. This process is repeated until all the proteins in the solution are fractionised.

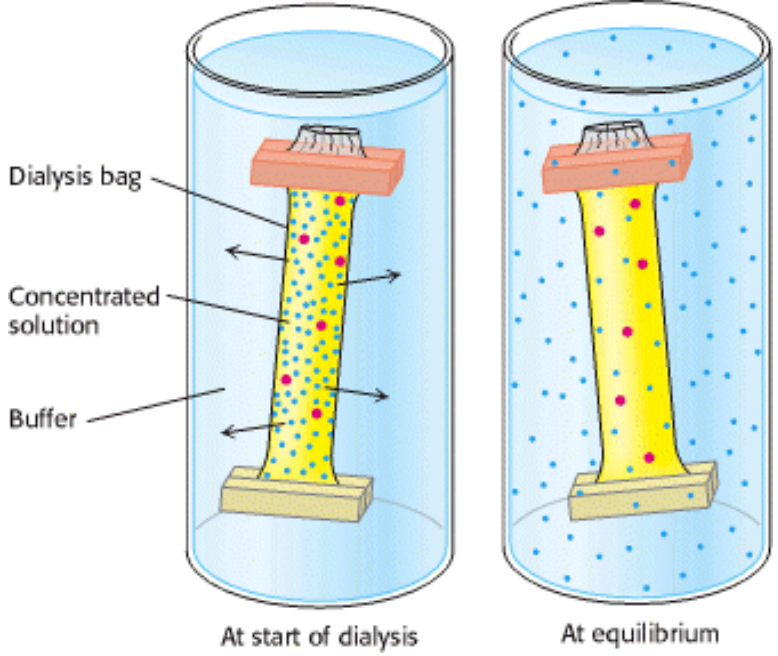
- Salting out can be used to fractionate proteins



# Dialysis

- Proteins can be separated from small molecules by dialysis through a semi-permeable membrane.



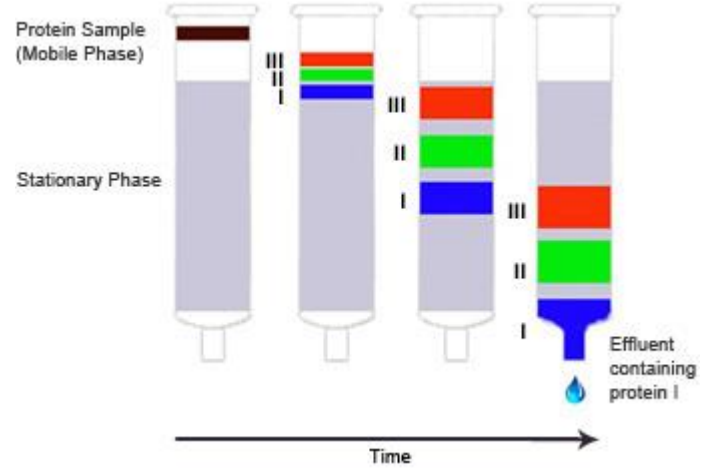

Disadvantage of dialysis

1. Large number of larger proteins will still exist.
2. Smaller proteins of significance are lost.

When we want to separate proteins from small molecules (e.g. sugars, salts), we use dialysis to reduce the concentration of the small molecules, the solution is placed in a dialysis bag and immersed in a large container of fresh solution. Over time, small molecules will diffuse out of the bag into the surrounding solution, while proteins will remain inside; as they can't diffuse through the small pores of the bag. This process will help in creating a better environment for the proteins by decreasing other molecules concentration.

# Chromatography techniques

- Separation of  molecules present in a liquid or gaseous environment (mobile phase) via passing through a column (long tube) that contains an immobile phase (stationary phase)
- Types:
  - Gel filtration
  - Ion-exchange chromatography
  - Affinity chromatography

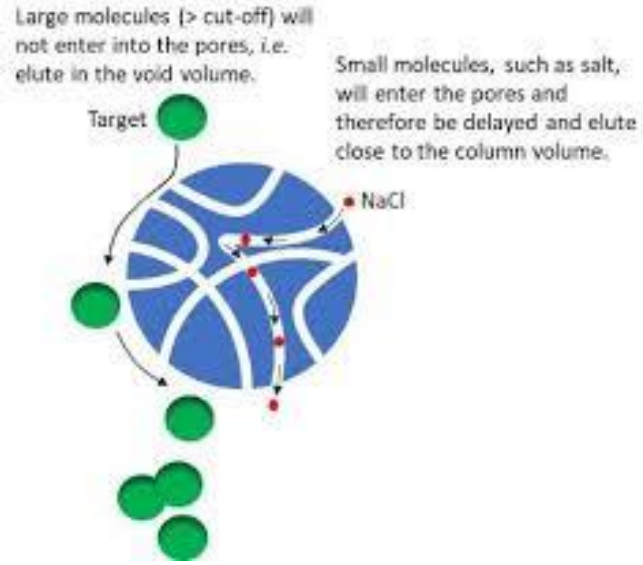


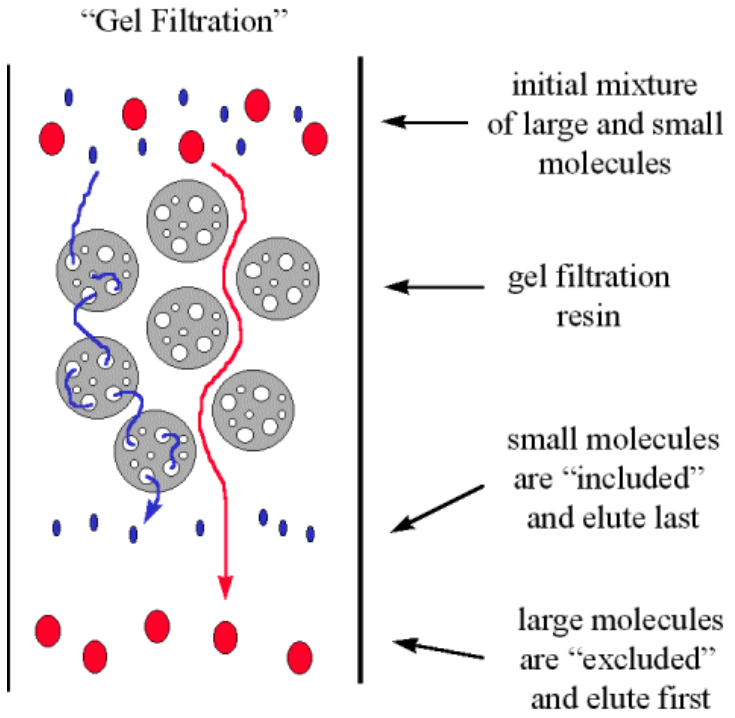

**In chromatography techniques**, We pack a column with stationary beads. When the solution is added, the molecules will move downward through the column (this is called the mobile phase). As the solution passes through the column, the molecules will be separated based on their size or charge by the stationary beads. At the bottom of the column, there is an opening to collect the separated molecules.

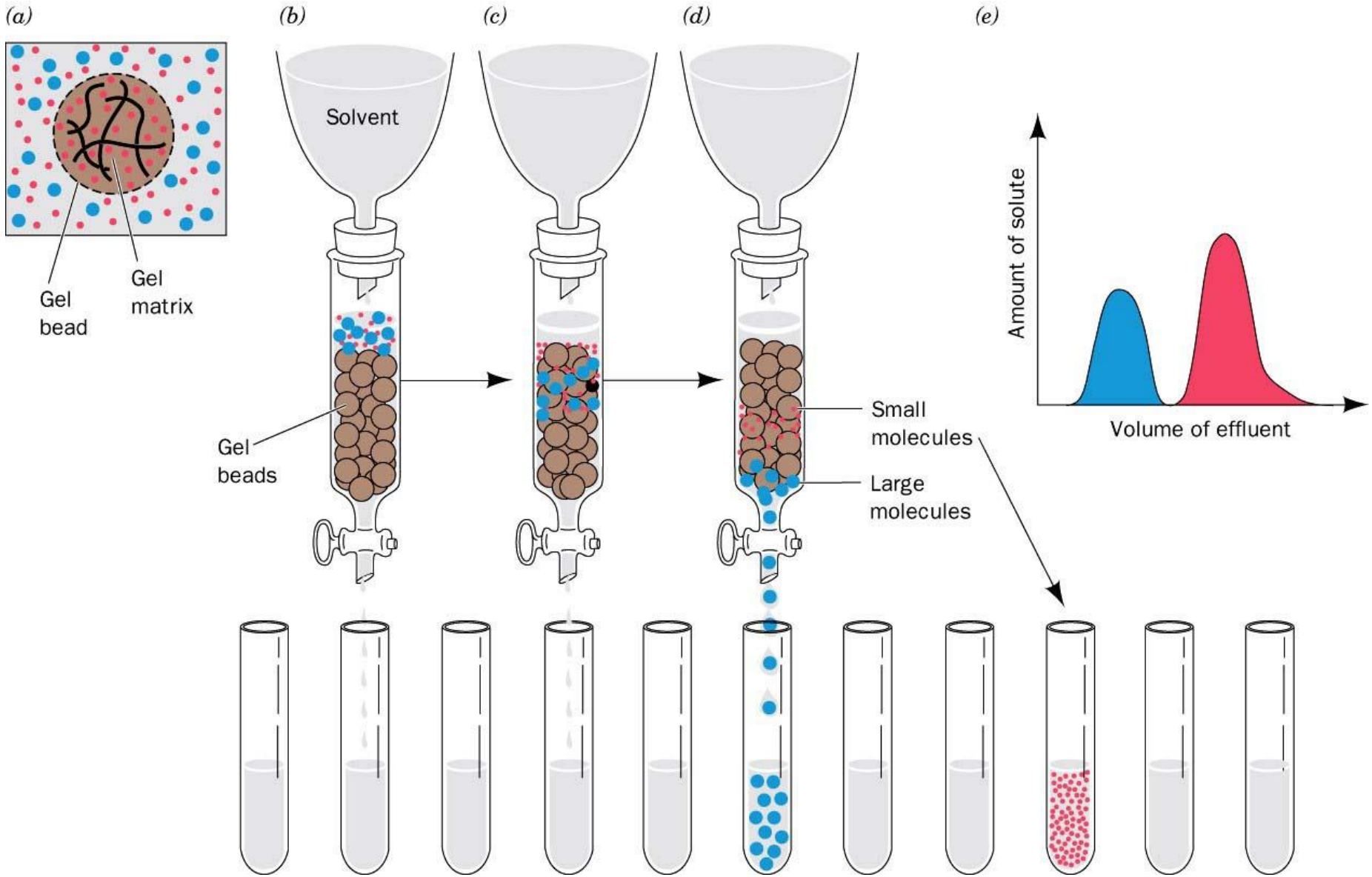
# Gel-filtration chromatography

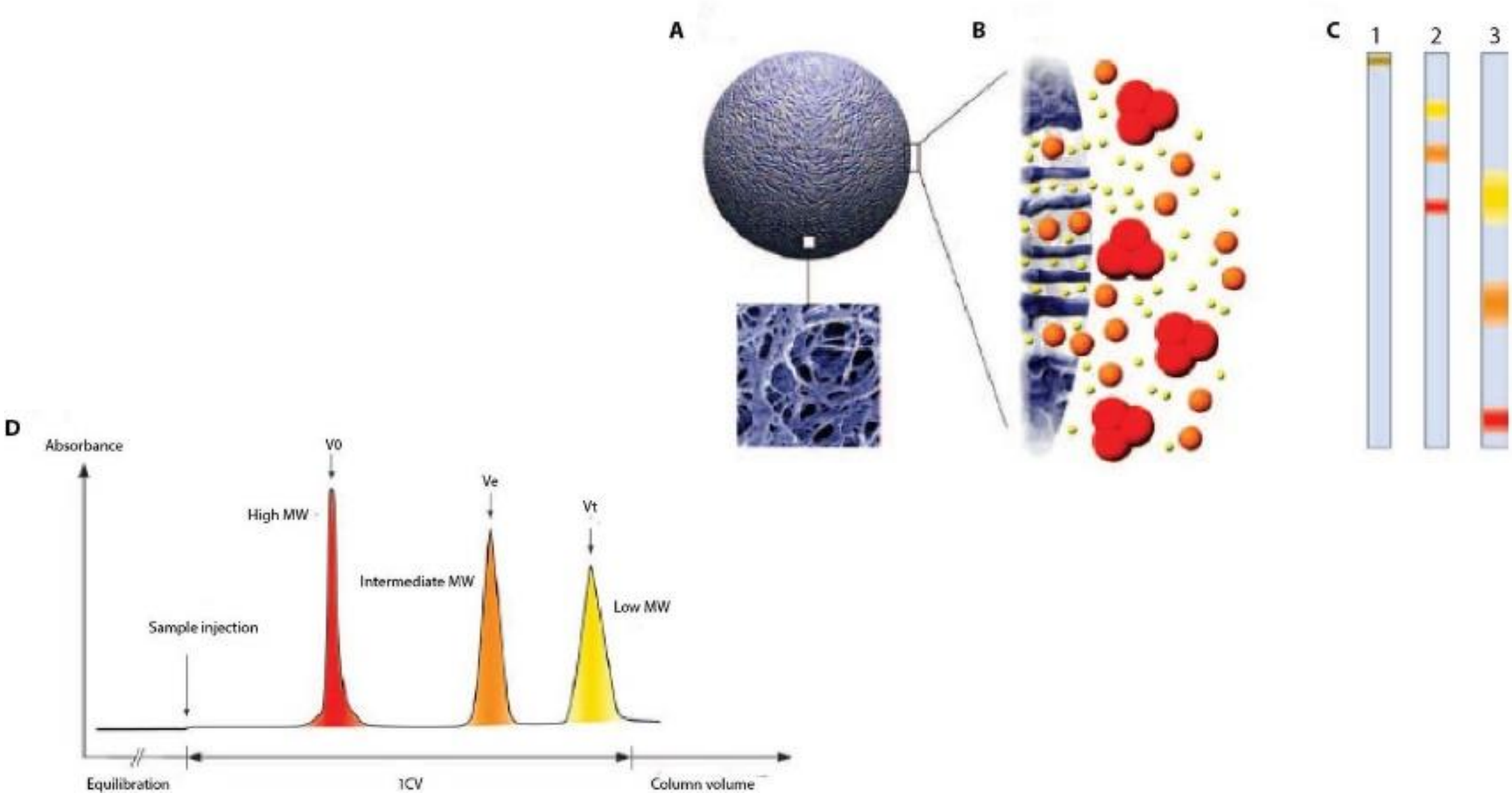
**Focus on the slide's notes.**

- Separations on the basis of size
  - Size-exclusion chromatography
  - Molecular sieve chromatography
- The stationary phase: porous beads
- Large molecules flow more rapidly and come out first
- Smaller molecules take a longer time in the column and exit late **because they go from bead to bead.**




(a)
Gel bead
Gel matrix
(b)
(c)
(d)
Solvent
Gel beads
Small molecules
Large molecules
(e)
Amount of solute
Volume of effluent

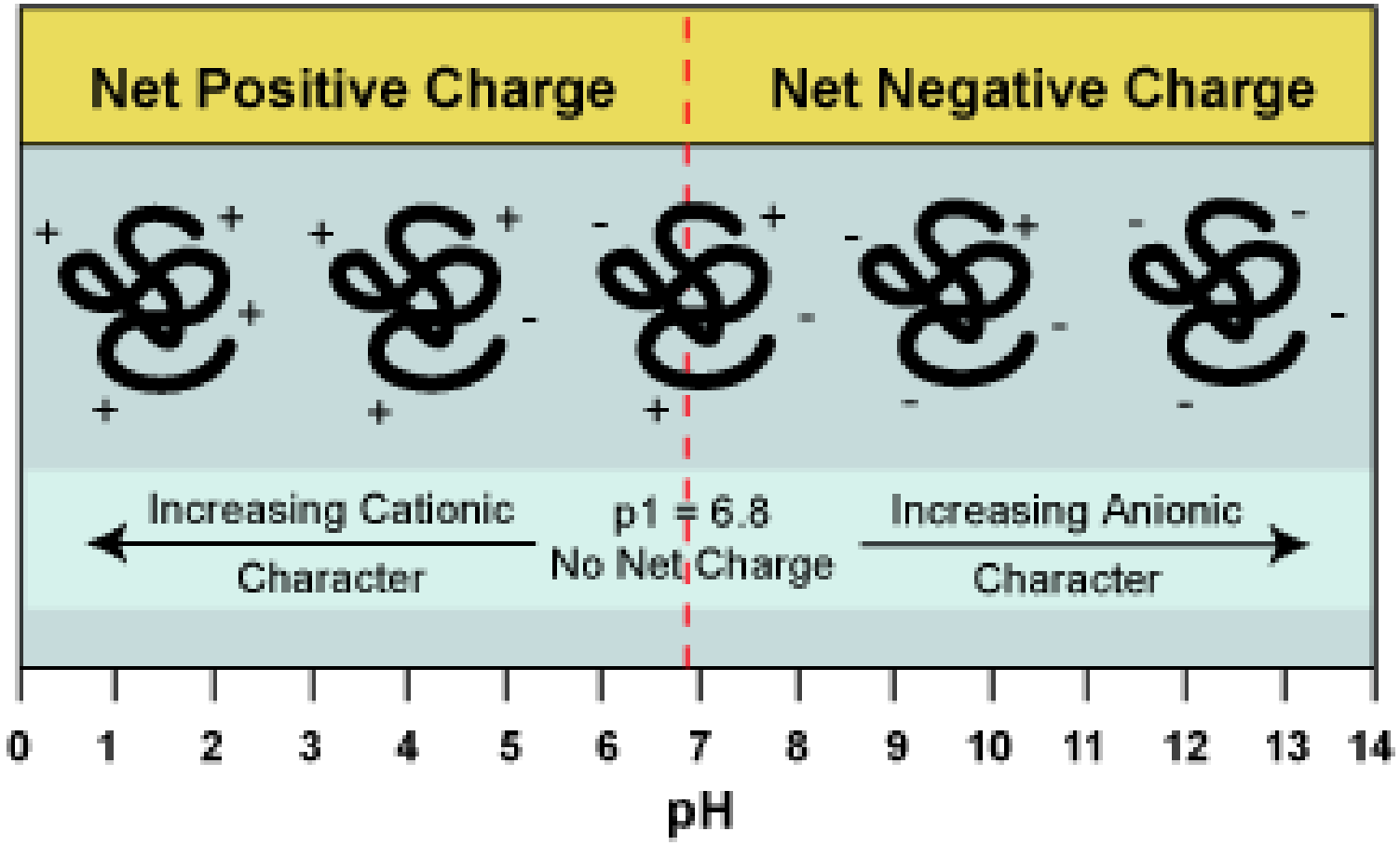

A
B
C
1
2
3
D
Absorbance
V0
Ve
Vt
High MW
Intermediate MW
Low MW
Sample injection
Equilibration
1CV
Column volume

# Ion-Exchange Chromatography

Here the separation is based on the charge of the proteins, as proteins would have different charges in different pH values due to different isoelectric points.

- Proteins have different pI's (and net charges at various pH's).

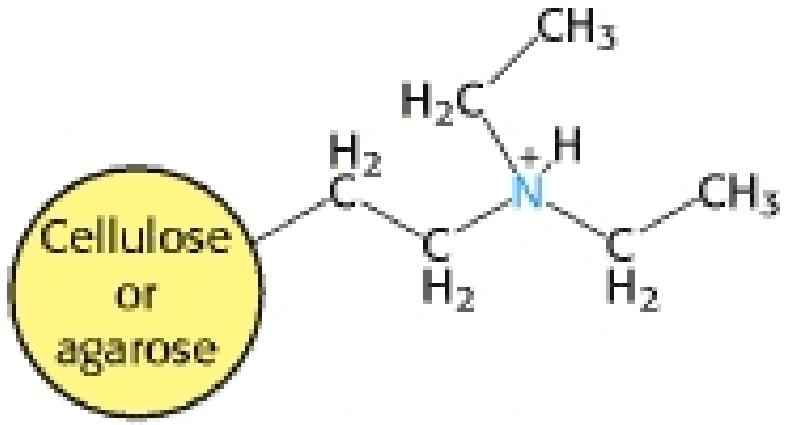
The figure shows a protein with isoelectric point of 6.8, notice how the overall protein charge changes when we change the pH of the solution.
If the pH is decreased, the positive charges will increase because of increased protonation of the groups.
If the pH is increased, more deprotonation will happen, leading to more negative charges.



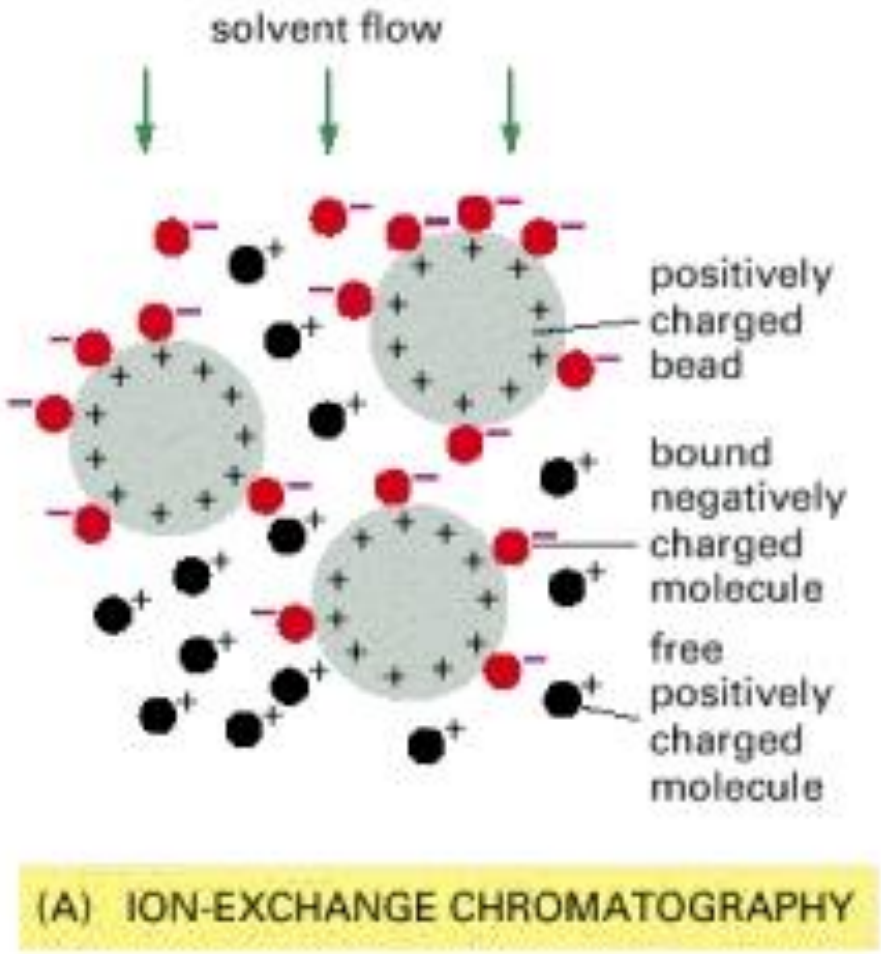
# Anionic-exchange chromatography

- The beads are positively-charged.
- Negatively charged proteins are separated.

Negatively charged Proteins will interact with the beads that have a positive charge, while proteins that are positively charged will continue to flow towards the opening of the column.



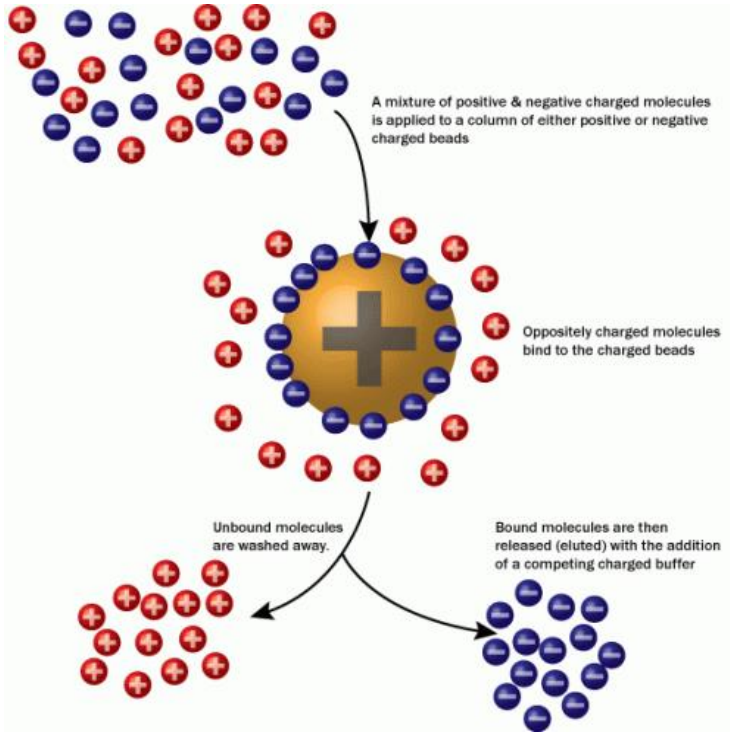

**Diethylaminoethyl (DEAE) group**
(protonated form)

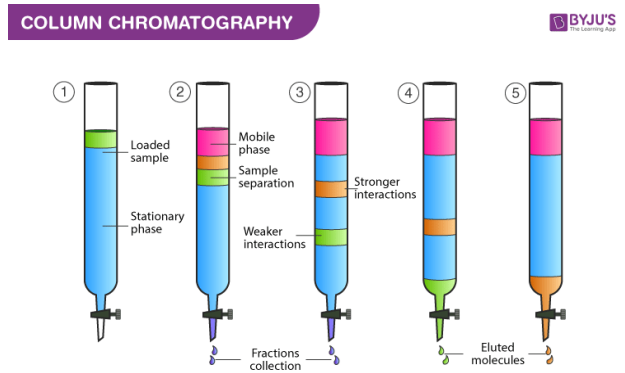


# Elution

- A negatively-charged protein bound to such a column can then be eluted (released) by adding increasing concentrations of sodium chloride. Why?
  - Because chloride ions compete with negatively-charged groups on the protein for binding to the column.

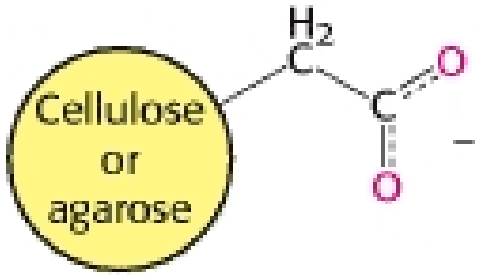
**In anionic exchange chromatography**, after collecting the positively charged proteins, we add increasing concentrations of sodium chloride.
The chloride ions will compete with the negatively charged proteins in binding with the beads, washing them out of the column.
The proteins that are washed first are the ones with the least negative charge density, following them the ones with the higher negative charge density as we increase the salt concentration.



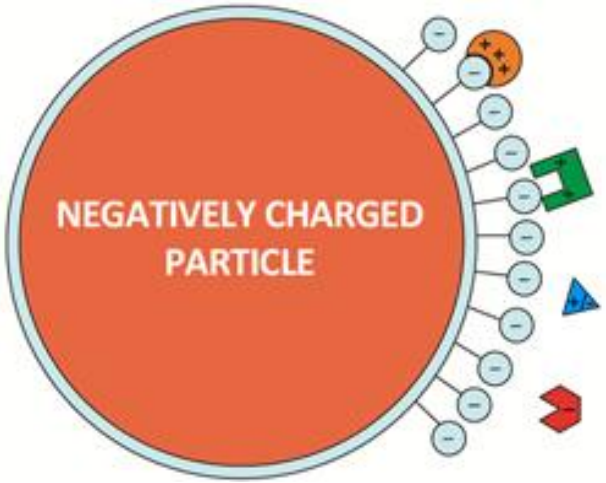
# Cationic-exchange chromatography

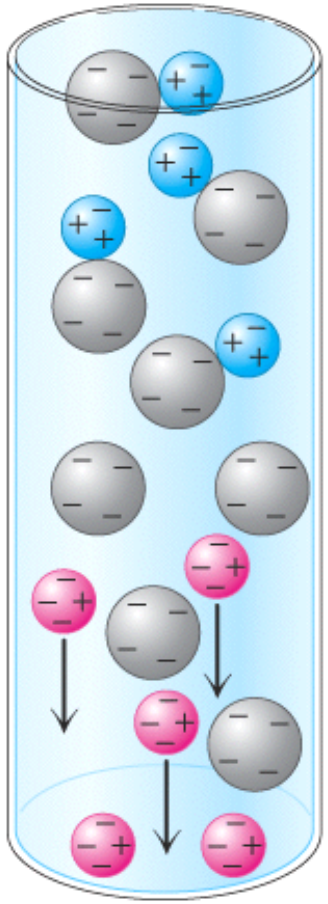
- The beads are negatively-charged.
- Proteins that have a net positive charge will tend to emerge first, followed by those having a higher positive charge density.




This is the structure of a bead, do not memorize the names.

**Carboxymethyl (CM) group** (ionized form)

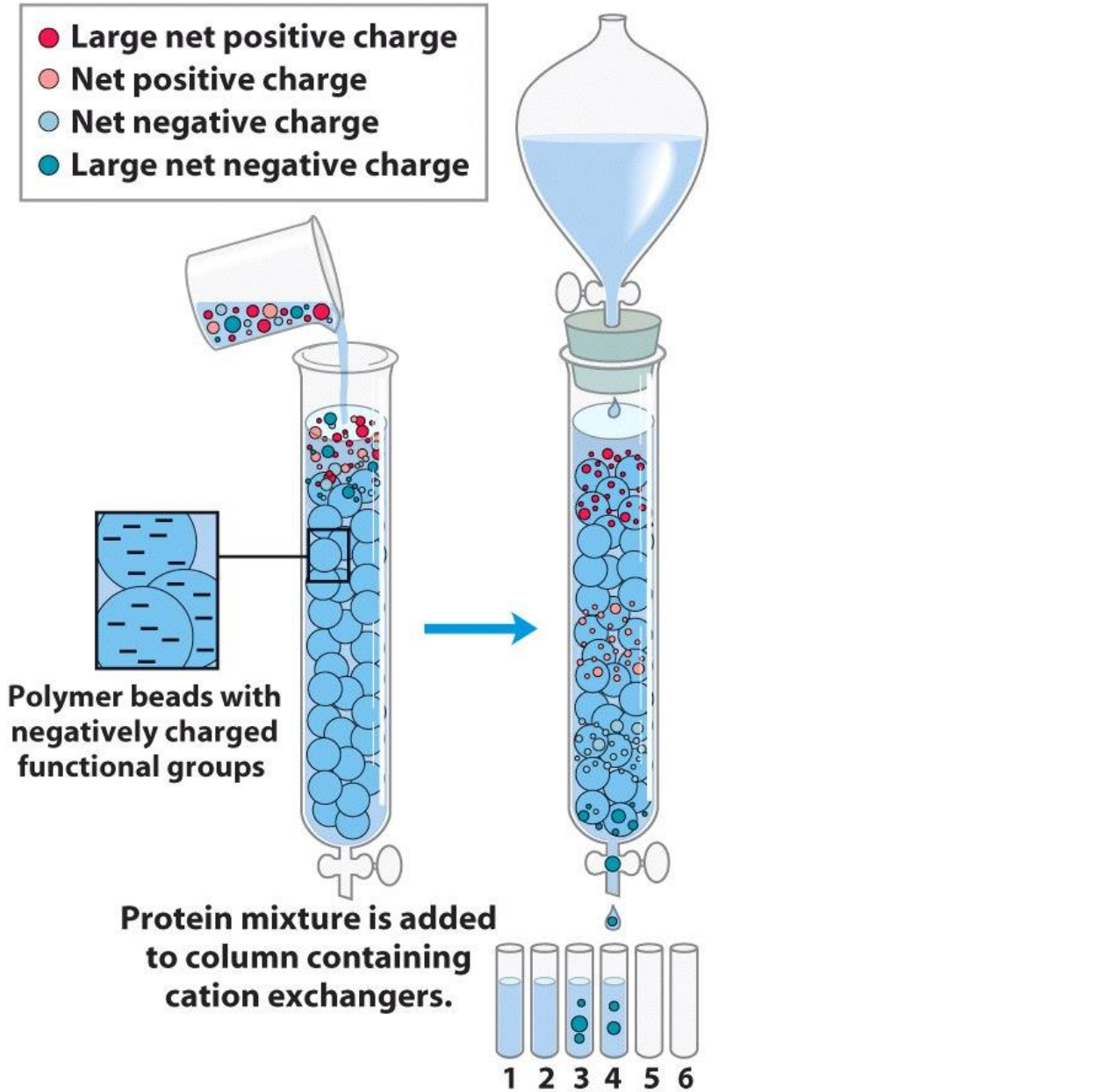


Positively charged protein binds to negatively charged bead

Negatively charged protein flows through

What happens here is the opposite, as the beads are negative, they will attract the positively charged proteins, negatively charged proteins will continue to flow towards the opening of the column.
When we wash the beads using sodium chloride, sodium ions will compete the positively charged protein in binding with the beads.




Proteins move through the column at rates determined by their net charge at the pH being used. With cation exchangers, proteins with a more negative net charge move faster and elute earlier.

# Problem

See the next slide for the solution.

- You have 5 different proteins (#1, #2, #3, #4, and #5), with different isoelectric points (pI's).
  - pI#5 = 2.3
  - pI#4 = 4.7
  - pI#1 = 7.2
  - pI#2 = 9.1
  - pI#3 = 12.1

Which one of the following proteins will have the largest number of negatively charged groups at pH of 6.5? 5
Which of the following proteins will have largest number of positively charged groups at pH of 6.5? 3

- Starting the column at pH 6.5, the sample is added and then washed to remove unbound molecules. What is the order of protein elution in
  - Cationic-exchange chromatography?
  - Anionic exchange chromatography?

First, determine each protein's charge at pH of 6.5.

- Protein 1 with isoelectric point of 7.2 will be positively charged.
- Protein 2 with isoelectric point of 9.1 will be positively charged.
- Protein 3 with isoelectric point of 12.2 will be positively charged .
- Protein 4 with isoelectric point of 4.7 will be negatively charged.
- Protein 5 with isoelectric point of 2.3 will be negatively charged.

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Let's arrange the proteins ascendingly depending on the density of the charge

- For positively charged proteins: 1 --> 2 --> 3 (increased positive charge density as the isoelectric point is further from 6.5).

- For negatively charged proteins: 5 will be more negative than 4.

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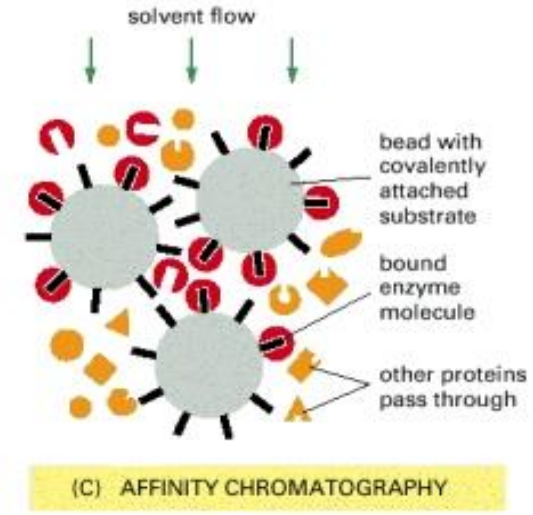
**In cationic-exchange chromatography**, the beads will be negatively charged, proteins 1,2 and 3 will be bound to the beads, protein 5 will leave the column first, then protein 4.
When the beads are washed with sodium chloride, protein 1 will be eluted first, then protein 2, and finally protein 3.

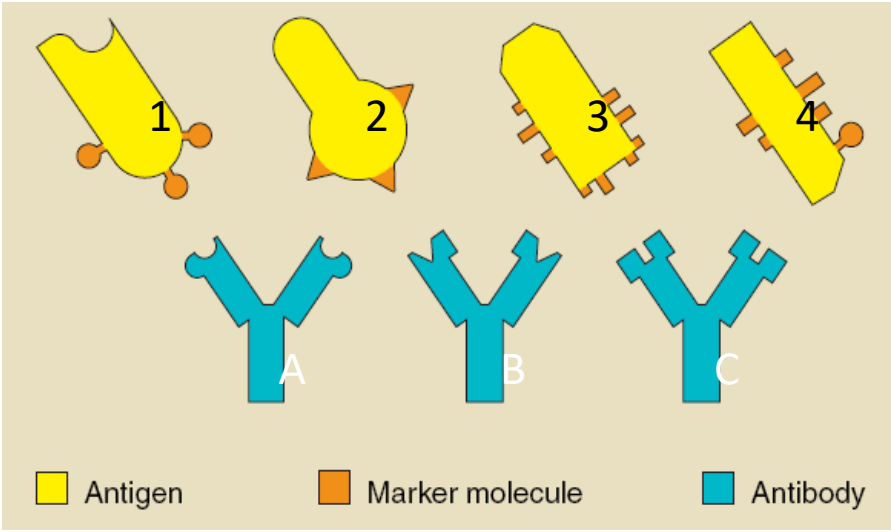
**In anionic-exchange chromatography**, the beads will be positively charged, proteins 4 and 5 will be bound to the beads, protein 3 will leave the column first, then protein 2 and finally protein 1.
When the beads are washed with sodium chloride, protein 4 will be eluted first, then protein 5.

# Affinity Chromatography

In this technique, we take advantage of specific protein-protein interaction, such interactions are found between a protein and its anti-body, so we put monoclonal anti-bodies that recognize a specific protein on the surface of the beads.

- Affinity: strength of binding between two molecules
- Affinity chromatography takes advantage of the high affinity of many proteins for specific chemical groups or other proteins (antibodies).
- Affinity chromatography is most effective when the interaction of the protein and the molecule that is used as the bait (trap) is highly specific.

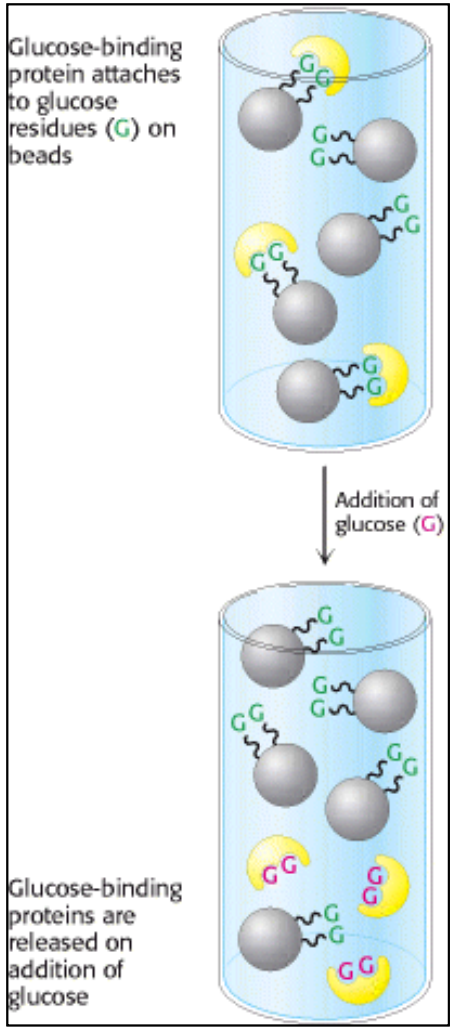


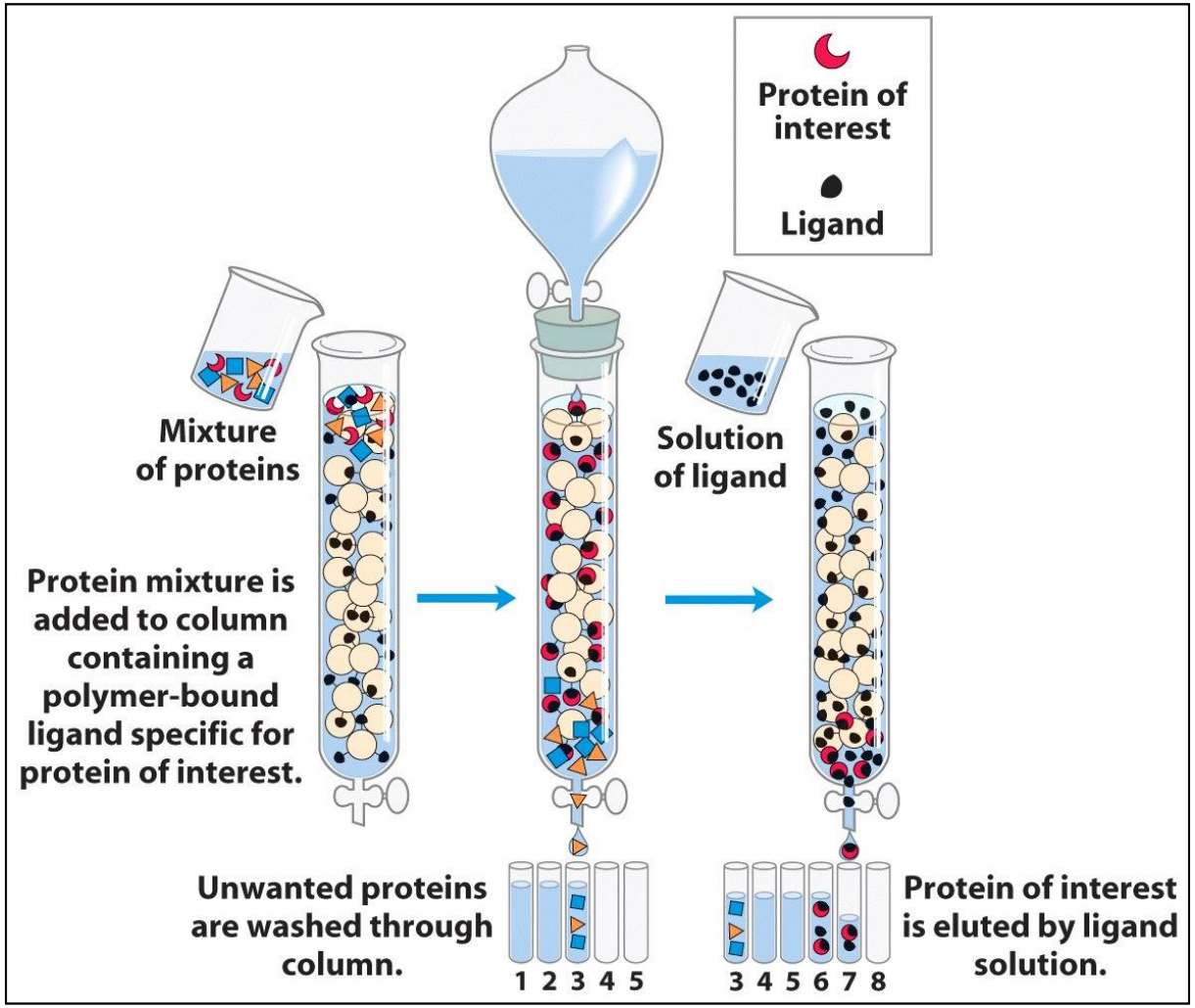




These monoclonal antibodies are highly specific to epitopes (marker molecules). Note that antibody A can interact with protein 1 and protein 4 as they both share the same epitope. Antibody C can interact with proteins 3 & 4 as they also share the same epitopes.

We pass the solution through the column; the desired protein will bind to the anti-bodies and the other proteins will be washed out. At the end, to get our desired protein that is bound to the anti-bodies we could:

- Change the pH.
- Put a competing molecule that competes with the protein in binding with the anti-body (as the bond between the protein and the anti-body is reversible, high concentration of the competing molecule will win the competition leaving the proteins free from the anti-bodies.

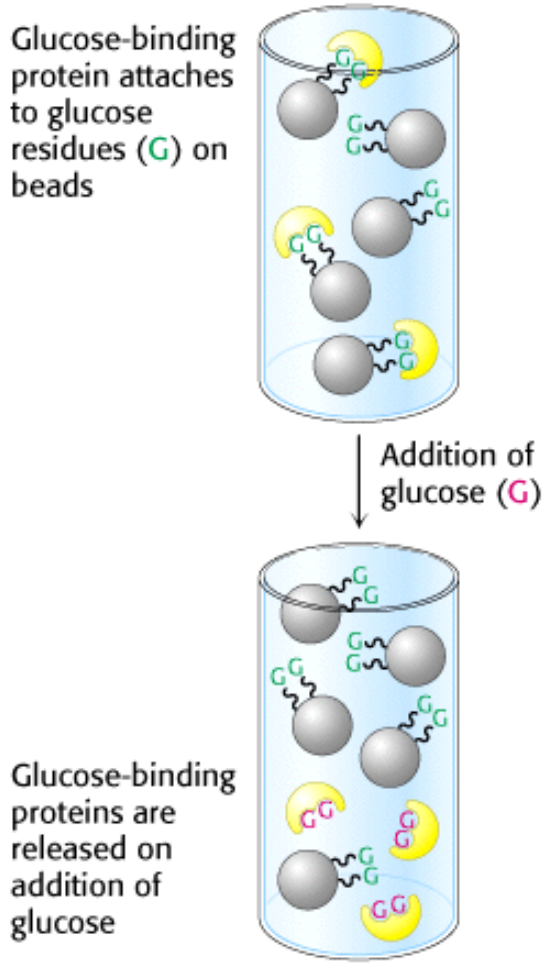


# Example

**Focus on the slide's notes**

- The plant protein concanavalin A, which binds to glucose with high affinity, can be purified by passing a protein mixture through a column of beads attached to glucose residues.
- Concanavalin A, but not other proteins, binds to the beads.
- **The bound concanavalin A can then be released by adding a concentrated solution of glucose.**




After separating concanavalin A from the beads using concentrated free glucose, is there a way to separate concanavalin A form glucose?
Yes, by dialysis we could separate concanavalin A from the bound glucose without ruining the structure of the protein.

We took a blood sample from a diabetic person, then we extracted the proteins from his RBC's and passed them in a column that has concanavalin A on its beads.
What protein would interact with the concanavalin A?
**Glycosylated Hemoglobin**

What kind of proteins would a concanavalin A-linked bead separate? Glycoproteins (more specifically, those with glucose).

# For any feedback, scan the code or click on it.

Corrections from previous versions:

| Versions | Slide # and Place of Error | Before Correction | After Correction |
|---|---|---|---|
| V1 → V2 | | | |
| V2 → V3 | | | |

# Additional Resources Used:

# رسالة من الفريق العلمي:

عن عمر بن الخطاب رضي الله عنه قال: سمعت رسول الله صلى الله عليه وسلم يقول: **"إنما الأعمال بالنيات، وإنما لكل امرئ ما نوى، فمن كانت هجرته إلى الله ورسوله، فهجرته إلى الله ورسوله، ومن كانت هجرته لدنيا يصيبها أو امرأة ينكحها، فهجرته إلى ما هاجر إليه"**

أخلصوا النوايا لله ... وهانت إن شاء الله