# Chromatographic Methods of Analysis

## Section: 6 Gel Chromatography

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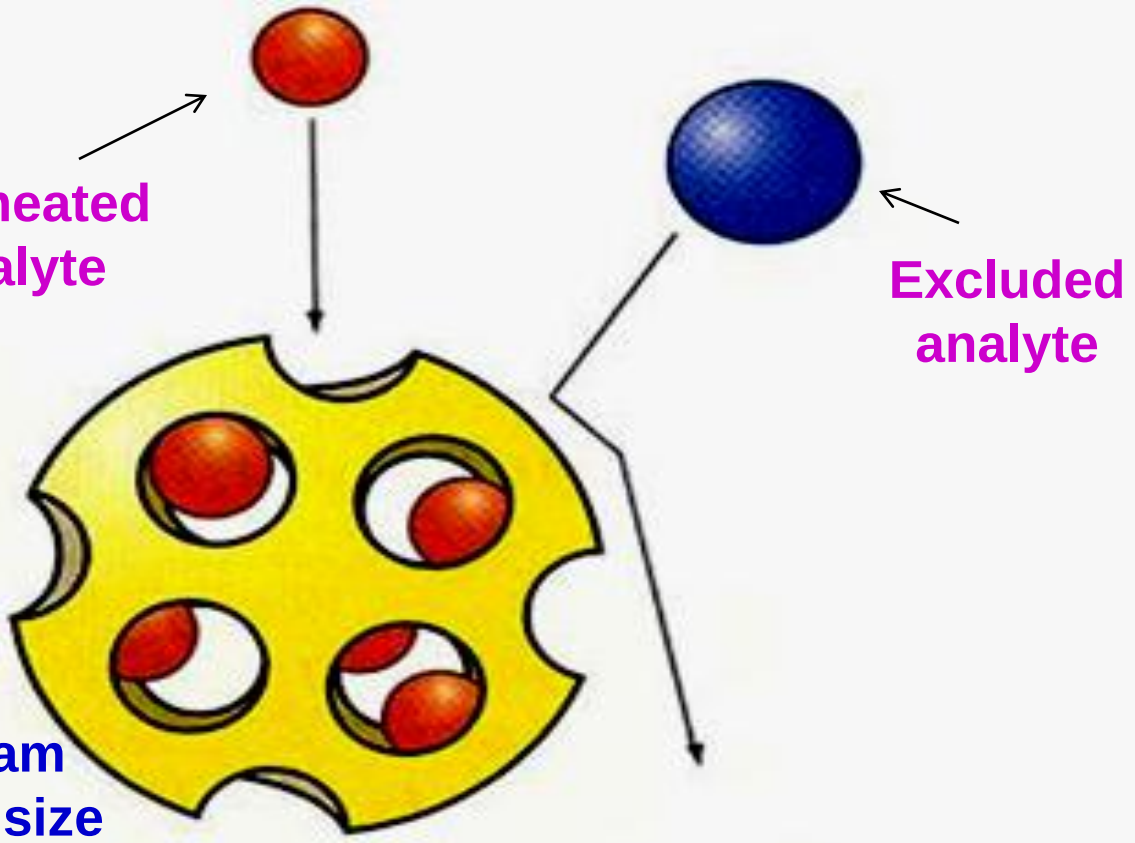
# Gel Chromatography

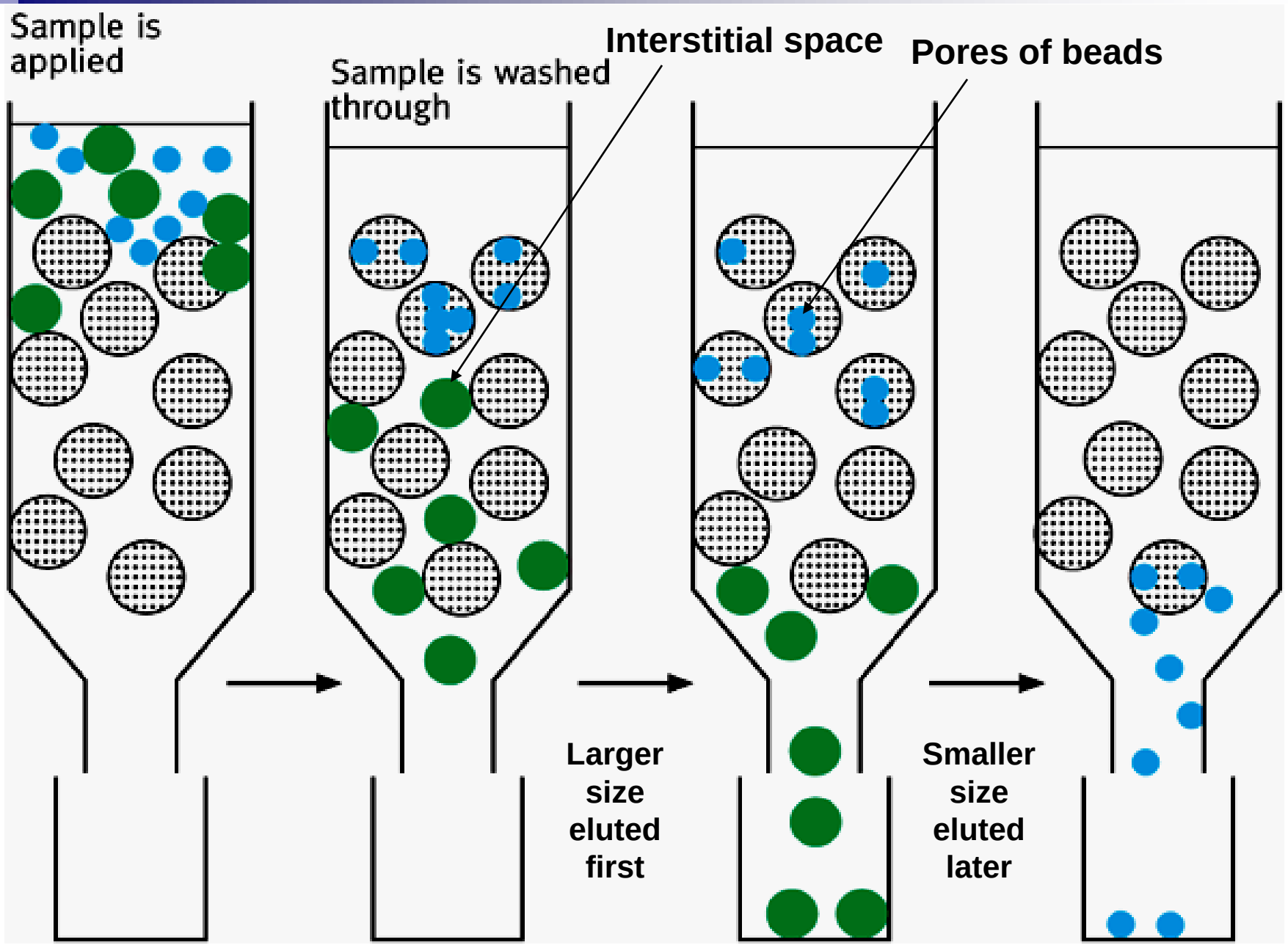
- **Gel Chromatography (also known as gel permeation, molecular sieving or size exclusion chromatography) is a chromatographic technique in which the separation of components is based on the difference of molecular weight or size, and is one of the effective methods used to isolate and analyze the bio-macromolecular substances.**
- **The stationary phase consists of beads containing pores that span a relatively narrow size range. When the gel is packed into a column and percolated with a solvent, it permits the large molecular weight components to pass faster without penetration of the pores (totally excluded).**
- **Smaller molecules spend more time inside the beads (are permeated) and therefore is eluted later (after a larger volume of the mobile phase has passed through the column).**

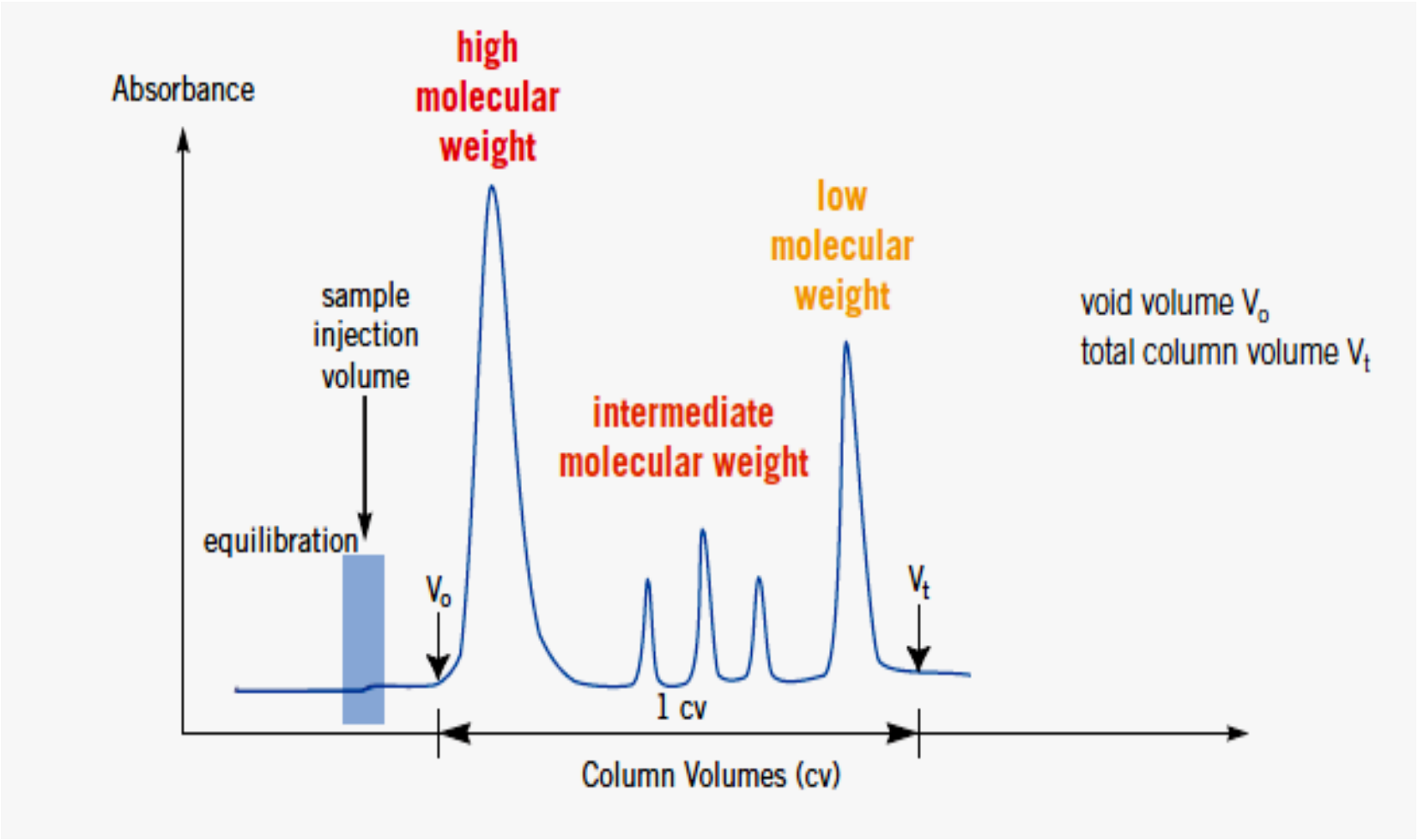
# Principle of separation




Schematic diagram of pore vs. analyte size

**Figure** **Gel filtration chromatography.** **(a)** Principle of the method. A resin bead is schematically represented as a "whiffle ball" (yellow). Large molecules (blue) cannot fit into the beads, so they are confined to the relatively small buffer volume outside the beads. Thus, they emerge quickly from the column. Small molecules (red), by contrast, can fit into the beads and so have a large buffer volume


Sample is applied
Sample is washed through
Interstitial space
Pores of beads
Larger size eluted first
Smaller size eluted later




**Theoretical chromatogram of a high resolution fractionation (UV absorbance detector of biomacro-molecules)**

# Properties and nature of the gel

**The gel should be:**

1- Chemically inert

2- Mechanically stable

3- Has ideal and homogeneous porous structure (wide pore size results in low resolution).

4- Uniform particle and pore size.

5- The pore size of the gel must be carefully controlled (via the degree of crosslinking).

## Types of gel:

### 1- Dextran (Sephadex) gel

- α 1-6-polymer of glucose is prepared by fermentation of sucrose (glucose + fructose). It is a natural gel.

### 2- Agarose gel

- Obtained from agar and composed of alternating units of 1,3 linked β-D-galactose and 1,4- linked 3,6-anhydro-α, L-galactose. It is a natural gel.

## 3- Acrylamide gels

It is not dextran polymer. It is polymerized acrylamide or methylen-bis-acrylamide. It is a synthetic gel.

**According to the swelling process, the gels are two types:**

**1- Soft gels (Xerogel, is gel only on swelling)**

**e.g. Polyacrylamide gels, dextran or agarose (used for separation of proteins in aqueous mobile phase).**

**2- Semirigid or rigid gels (aerogel, is gel in air)**

1) **Polystyrene gels (separation of non-polar polymers in non-polar solvents).**
2) **Porous glass gels (separation of polar systems)**

## Eluent

**The eluent (mobile phase) should be a good solvent for the polymer, should permit high detector response from the polymer and wet the packing surface. It may also buffer.**

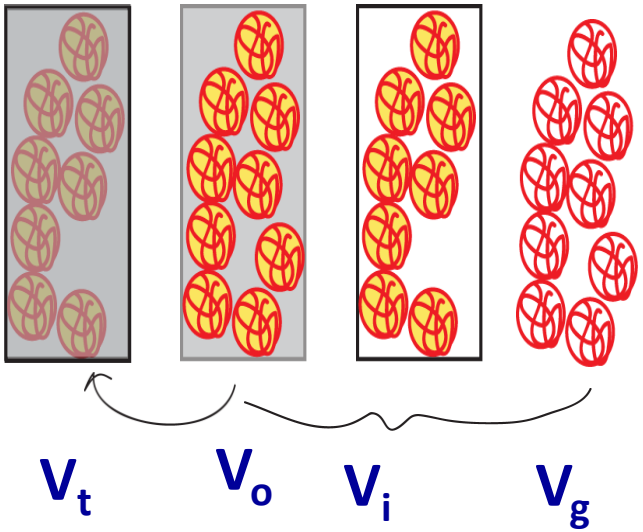
## Advantages of Gel Filtration

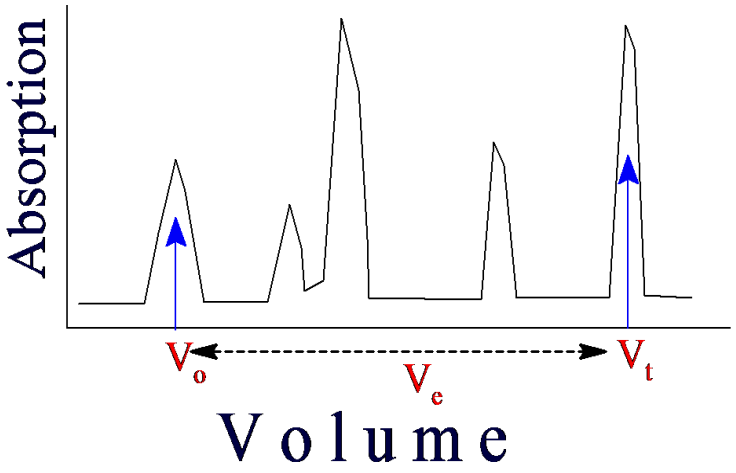
- Can handle biomolecules that are sensitive to changes in pH, concentration of metal ions or harsh environmental conditions (presence of conc. acids or bases).
- Separations can be performed in the presence of essential ions, detergents, at high or low ionic strength. At 37 °C or on cold according to the experimental requirements.

## Common terms in size exclusion chromatography

- **The total volume ($V_t$):** the sum of the volume of the gel matrix, the volume inside the gel matrix, and the volume outside the matrix. The total volume is also equal to the amount of the eluent required to elute a substance, through the column, which is small enough to completely penetrate the pores of the gel.
- **Inner volume ($V_i$):** the volume of the eluent inside the gel matrix. The volume inside the beads.

- **Void volume ($V_o$), the volume of eluent outside the gel matrix. This is the volume required to elute a large substance so that it cannot penetrate the pores at all. Such a substance is said to be completely excluded, such as dextran blue 2000.**
- **Elution volume ($V_e$), the volume of eluent required to elute any given substance.**
- **Gel volume ($V_g$), the volume of dry gel.**



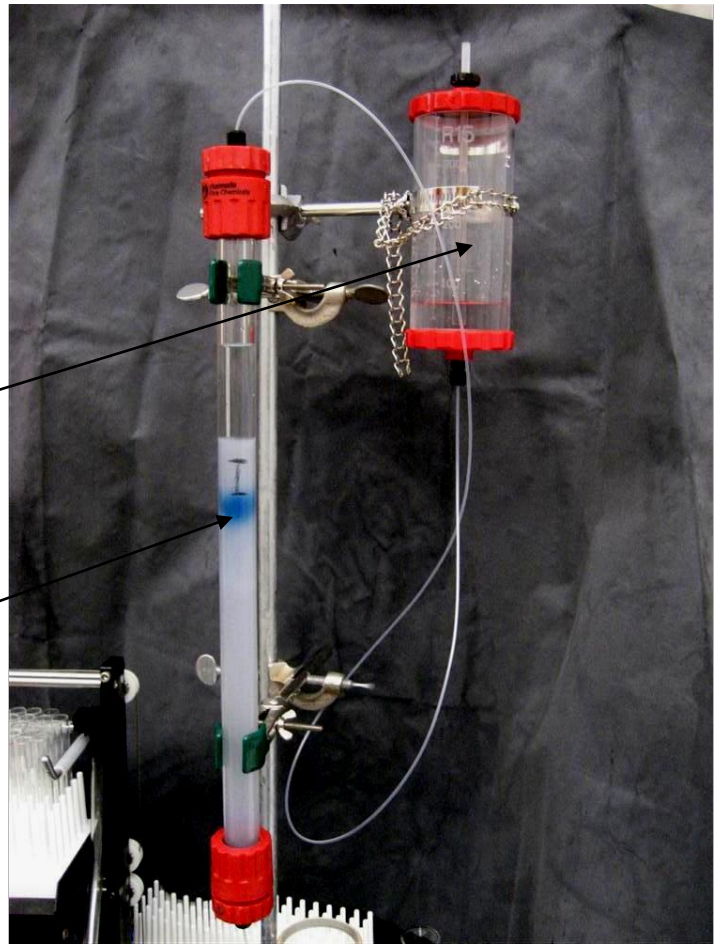
$$V_t = V_o + V_i + V_g$$

- In classical and more rigorous scince, elution position of any molecules should be reported as the partition coefficient ($K_{av}$) rather than volume.

$$K_{av} = \frac{(V_e - V_o)}{(V_t - V_o)}$$

For totally excluded;
$K_{av} = 0$ and $V_e = V_o$

For totally permeated;
$K_{av} = 1$ and $V_e = V_t$



# Separation procedures

**1- Preparation of column for gel filtration**

- **Swelling of the gel: some resin come in a powder form, these must be sonicated first in the eluent or the desired buffer to swell.**
- **Packing the column: make a slurry of gel plus buffer and pour it into column which is one third filled with the buffer.**
- **Washing the resin: after packing, pass several column volumes of the buffer through the column to remove any air bubbles and to test the column homogeneity.**

**2- loading the sample onto the column: the sample must enter the resin in the form of solution using a syringe.**

**3- eluting the sample and detection of components:**

**Fractions are collected as the sample elutes from the column.**

# Applications of gel chromatography

## 1.Molecular Weight Determination

**For spherical biomolecules like proteins, the elution volume ($V_e$) of the component is proportional to log of its molecular weight. So each component has its own $V_e$.**

**Log MW = A - B($V_e$/$V_o$)  A and B are constants**

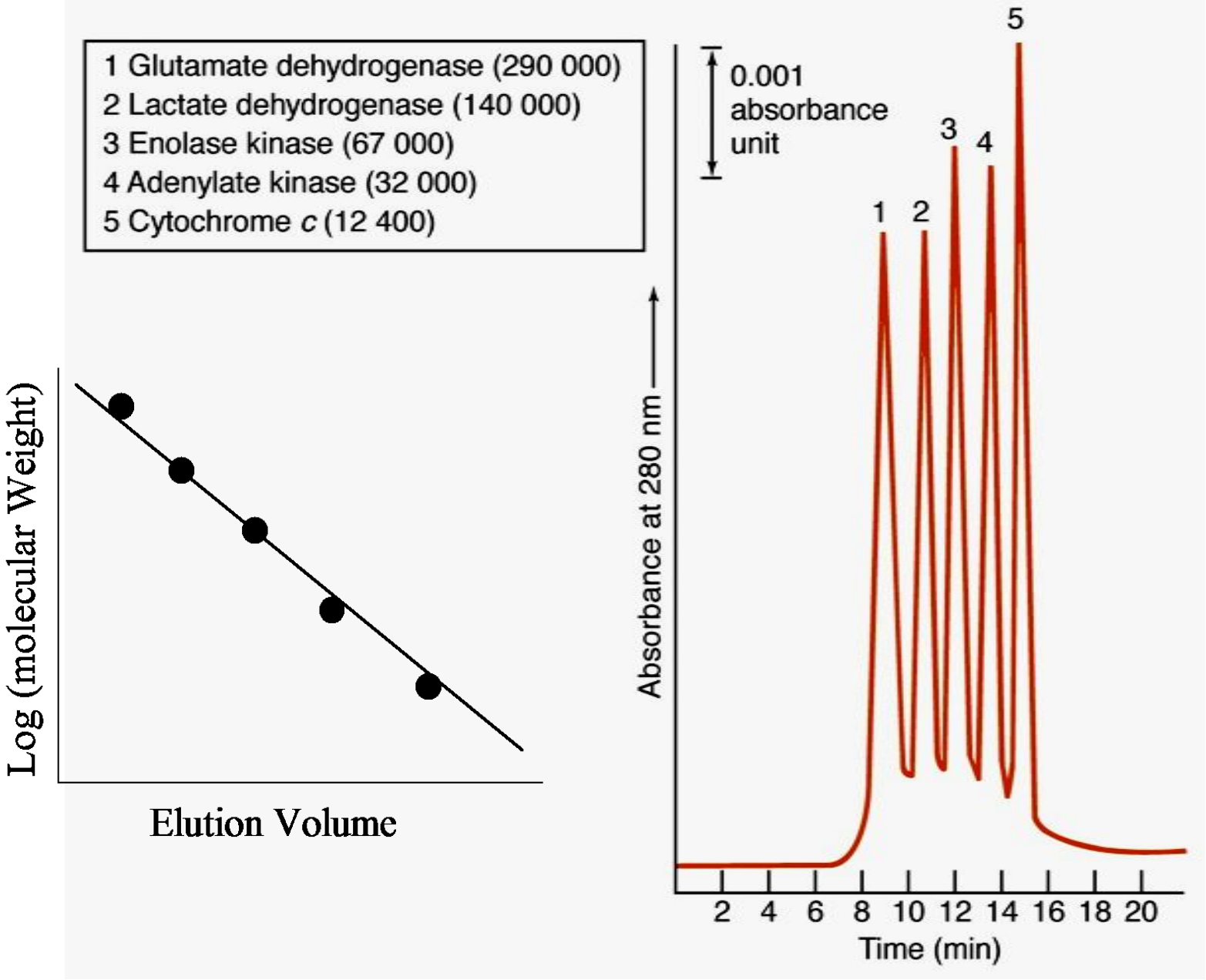
**Procedure:**

1- Calibrate the column with a series of polymers having known molecular weights.

2- Determine elution volumes of each polymer including the unknown.

3- Construct a calibration curve relating (known) molecular weight to (measured) elution volume specifically for that column.

4- Use this calibration curve to estimate the molecular weight of the unknown protein, based on its elution volume.


1 Glutamate dehydrogenase (290 000)
2 Lactate dehydrogenase (140 000)
3 Enolase kinase (67 000)
4 Adenylate kinase (32 000)
5 Cytochrome c (12 400)
Log (molecular Weight)
Elution Volume
0.001 absorbance unit
Absorbance at 280 nm
1
2
3
4
5
2 4 6 8 10 12 14 16 18 20
Time (min)

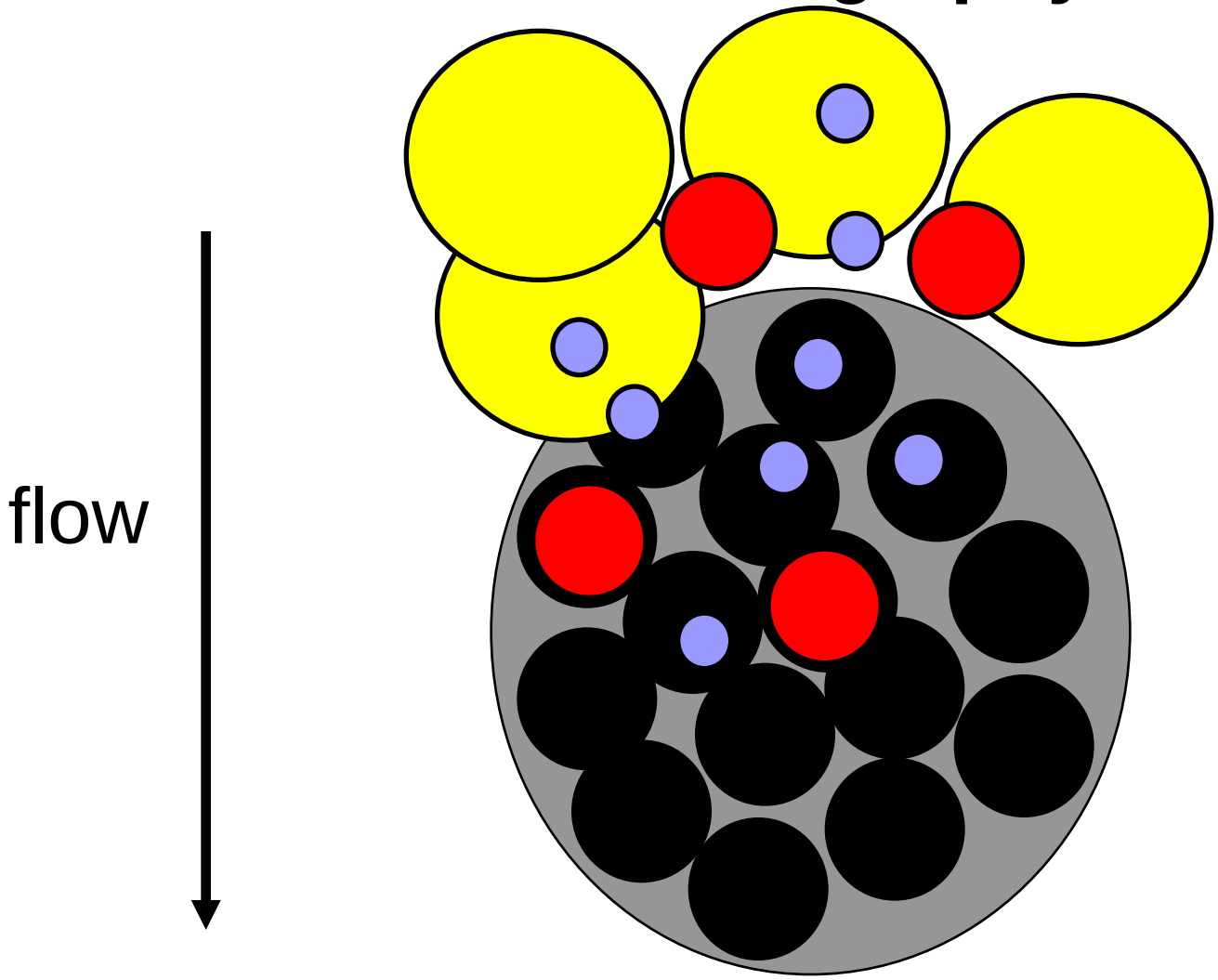
## 2. Fractionation of macromolecules

- Biological macromolecules are synthesized or extracted as a mixture of several components, and when scientists study macromolecules (protein, antibody, hormone, enzyme, or DNA) they need to separate it from the mixture.
- These macromolecules are of different sizes and shapes, and thus, can be separated using gel filtration chromatography.
- As an example is the purification of the synthesized proteins (to separate amino acids and peptide).

## 3- Desalting (Group Separation)

- Biological samples may contain unwanted substances such as: salts, buffer components, small ligands, cofactors and precipitating reagents. All of these can be removed by gel chromatography. Examples; are the removing of NaCl from hemoglobin, and phenol or unincorporated nucleotides from nucleic acid preparations..

# Gel filtration chromatography



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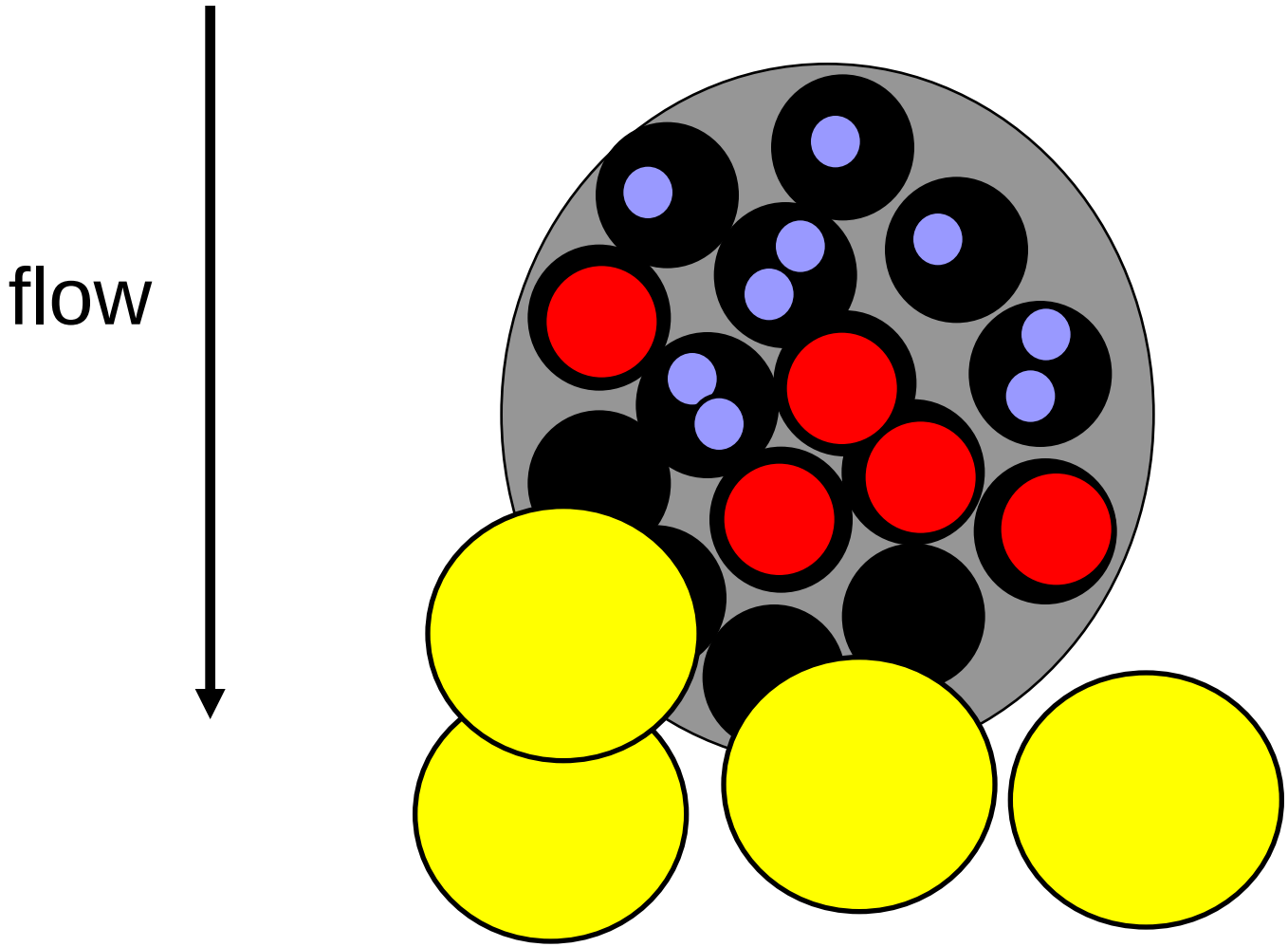
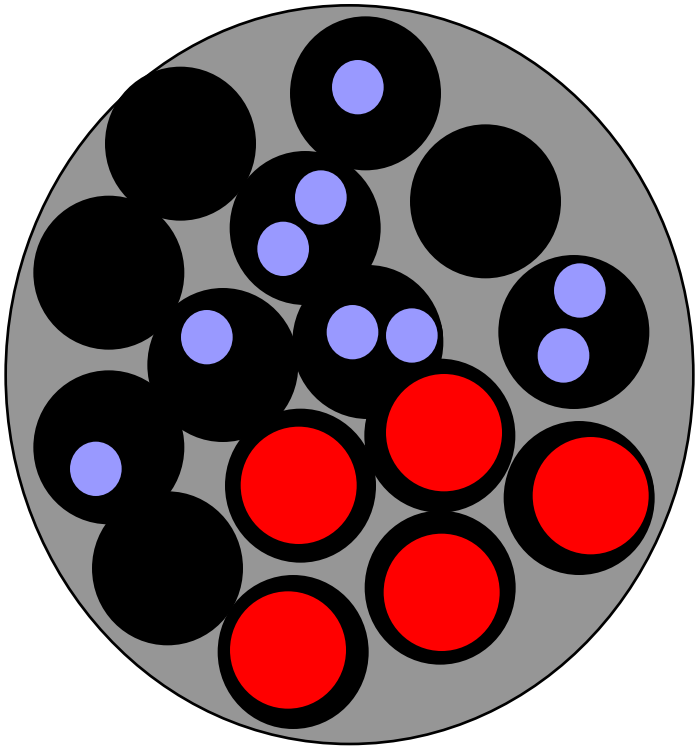


# Gel filtration chromatography

flow



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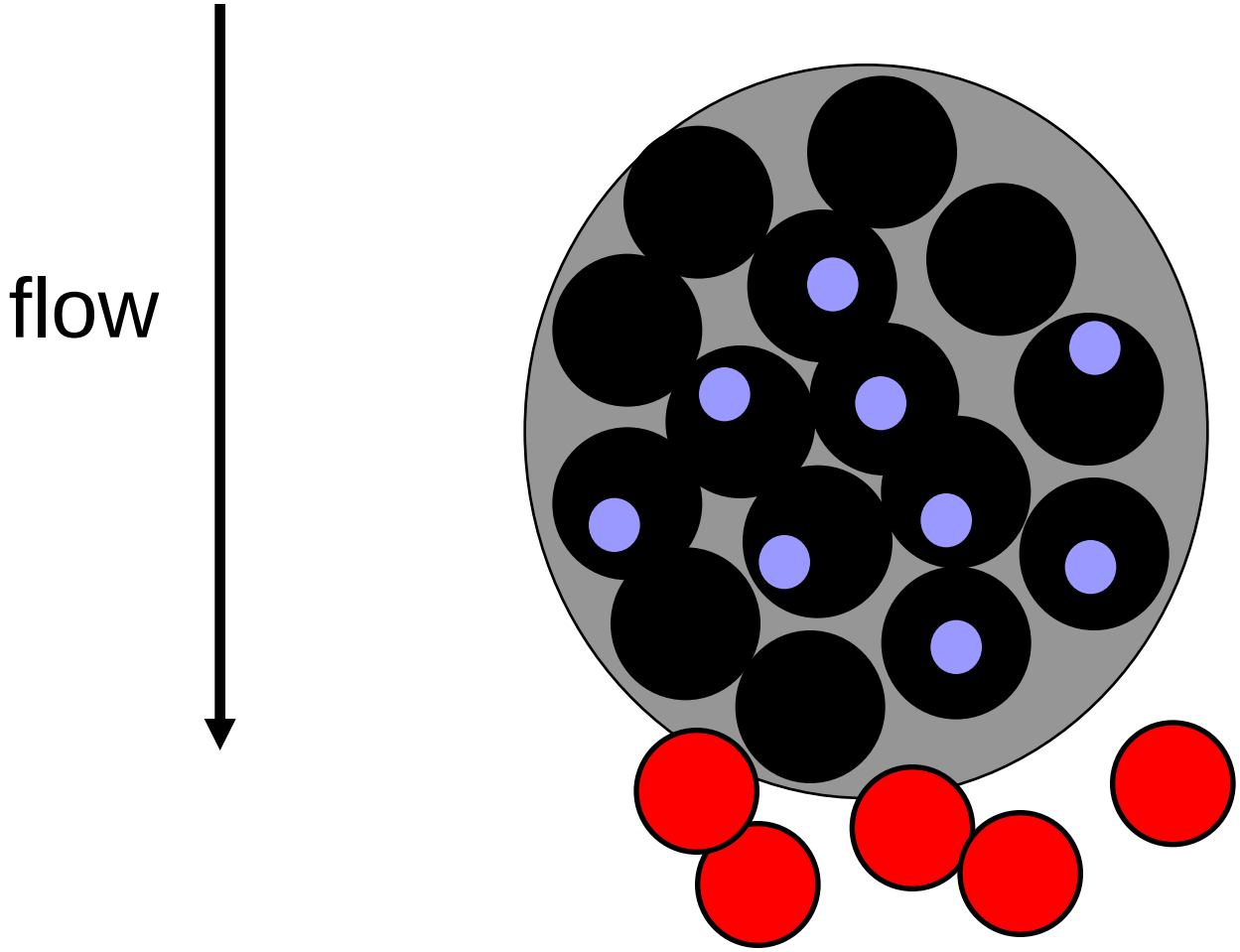

# Gel filtration chromatography