GEL FILTRATION CHROMATOGRAPHY

Lab#5

BCH 333

Gel Filtration Chromatography:

- Also called ***Molecular-sieve*** or ***Size-exclusion chromatograph***.

- The partition (separation) between a mobile phase and a stationary phase based on ***molecules sizes.***

- **Well suited for biomolecules that:**

  - Very sensitive to the changes in pH and harsh environmental factors.
  - If the protein is relatively large (over 100 KDa), gel filtration could be used as an initial step in the purification.

bromophenol blue[dye]
m.wt.= 669.99 Da .

blue dextran [sugar] with
m.wt.= 2,000,000 Da

After few mins.

mixture of
blue dextran
+
bromophenol
blue

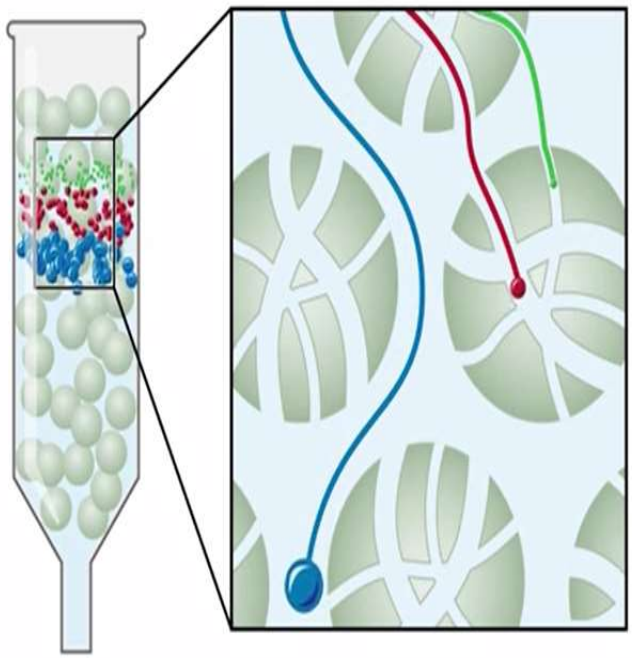
At the beginning

Gel filtration chromatography

Mobile phase:

(Buffer)

Is the liquid fills the space inside the beads and between the bead particles and this liquid occupies most of the bed volume.



Stationary phase:

(Column matrix)

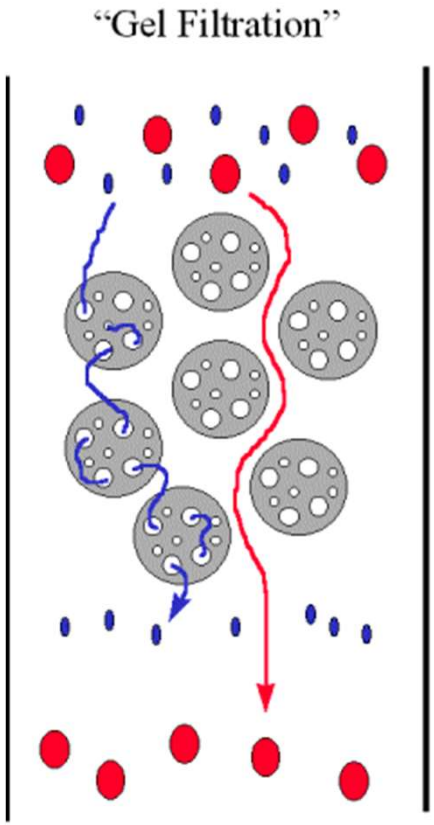
"Beads" of hydrated, porous polymer. The gel beads used as molecular sieves consist of cross-liked polymers that are generally inert, do not bind or react with the material being analyzed, and are uncharged.

Stationary phase

Principle:

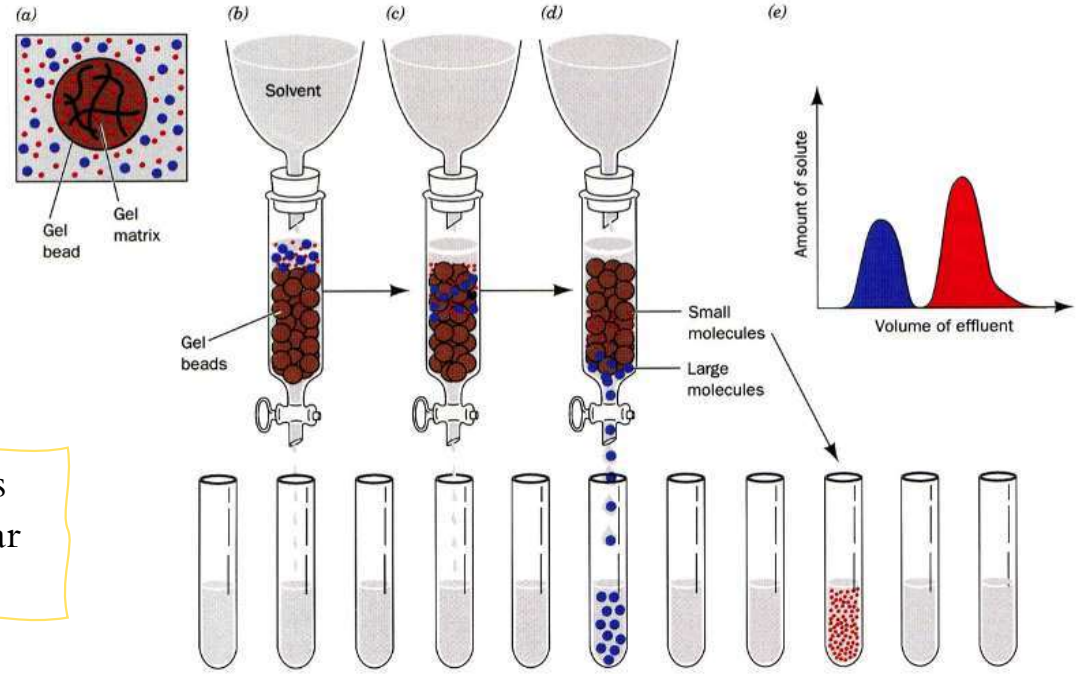
- Gel filtration chromatography separates molecules according to their ***size*** ➔ the molecules are ***filtered*** through the porous beads "sieve".

- The gel filtration matrix [stationary phase] contains pores which permit the buffer, small and medium sized molecules to pass through them, ***SO:***
- ***Large molecules***, can't get through any pores in the beads and move more rapidly through the column, emerging (eluting) sooner. ➔[elute from the column before the smaller proteins].
- ***Medium-sized molecules***, can enter the larger size pores in the matrix, and so they reach the end of the column later.
- ***Small molecules***, can enter through all pores of the beads and they have the largest volume to pass through before emerging from the column last. ➔elute last.

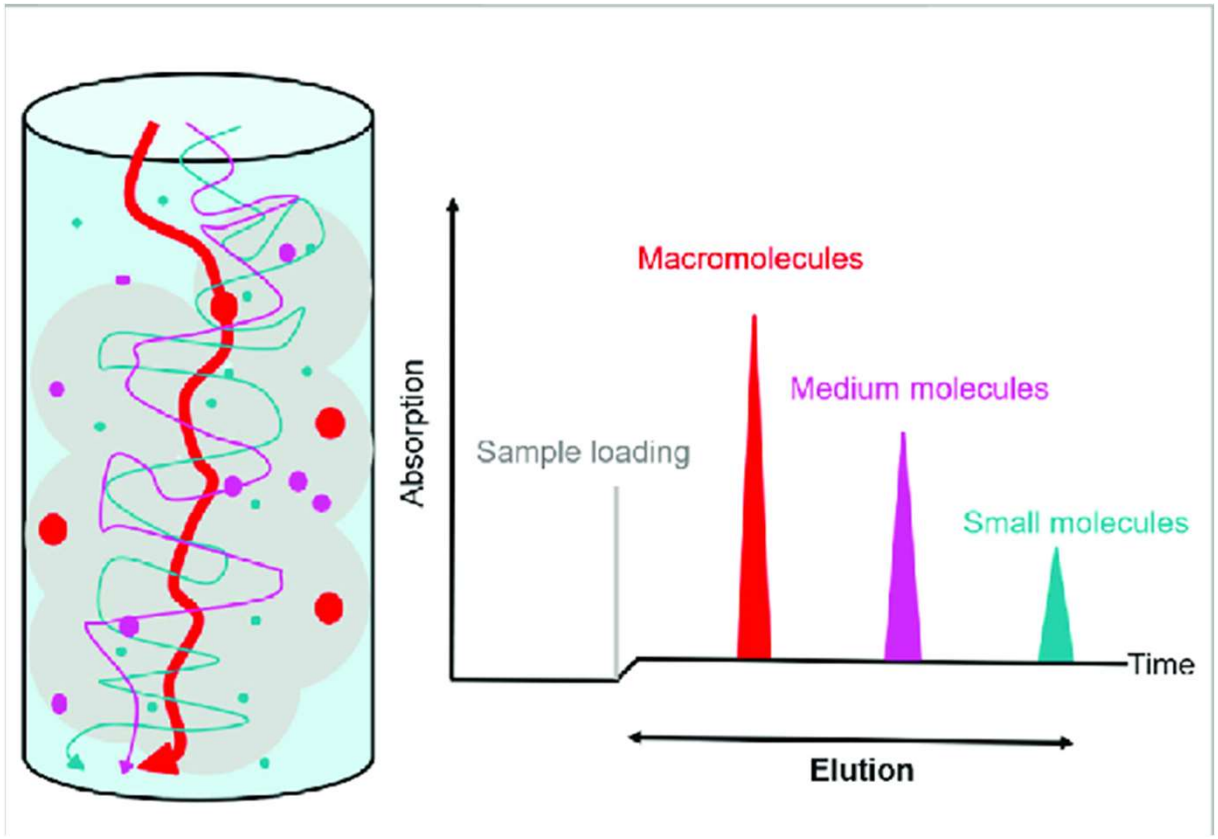


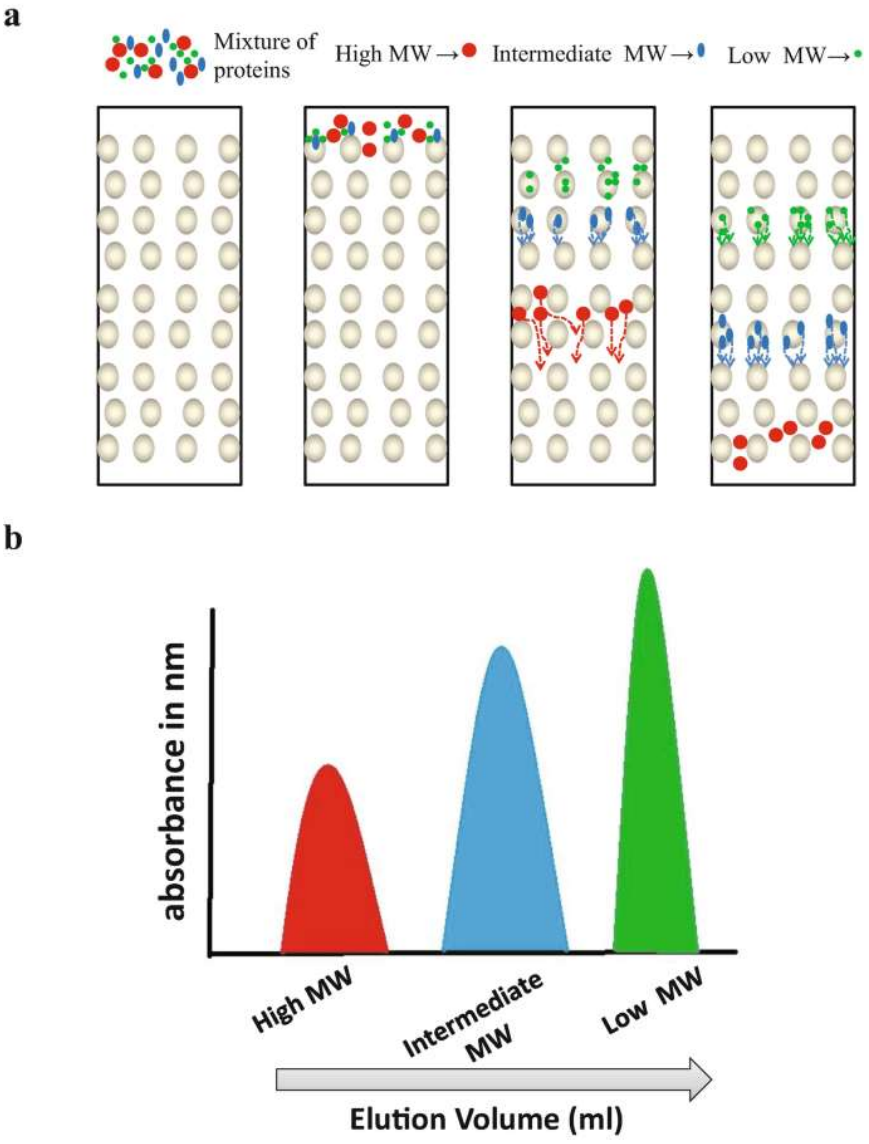
Principle Cont':

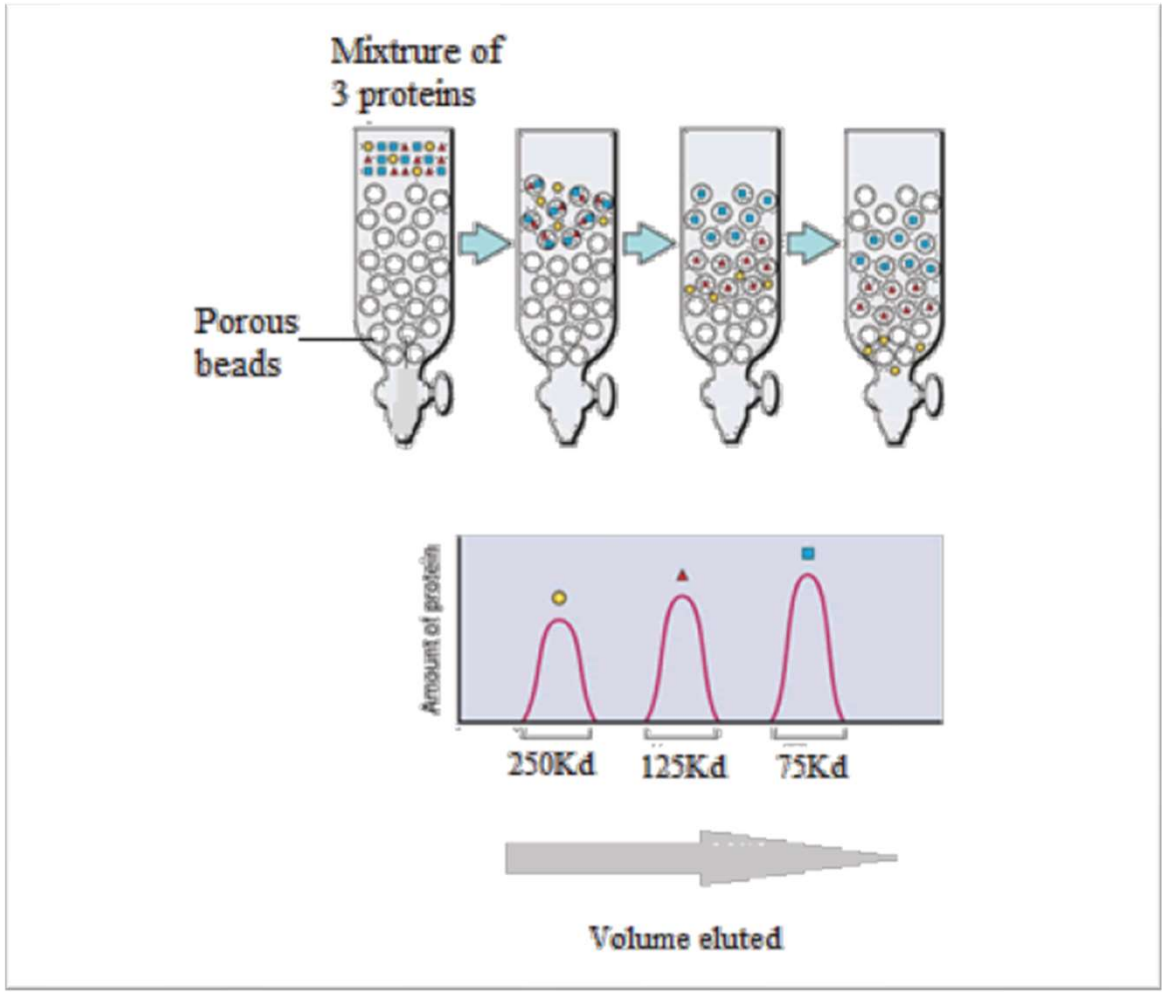
- Molecules and complexes that can enter the stationary phase will be fractionated according to their sizes ➔Smaller molecules will migrate deep into the pores and will be ***retarded more*** than larger molecules that ***do not so easily enter*** the pores ➔ and are thus eluted from the column more ***quickly***. This difference in pore migration leads to fractionation of components by size with the largest eluting first.



The elution volume is related to the molecular weight.


Macromolecules
Medium molecules
Small molecules
Sample loading
Absorption
Time
Elution


a
Mixture of proteins
High MW →
Intermediate MW →
Low MW →
b
absorbance in nm
High MW
Intermediate MW
Low MW
Elution Volume (ml)



The principle of gel filtration chromatography

Three volumes should be distinguished:

1. The Void volume, Vo:

- The volume of the mobile phase in the space between the beads [which is the volume external to the beads].
- Can be determined by the elution of High MW molecules which cannot enter any pores.

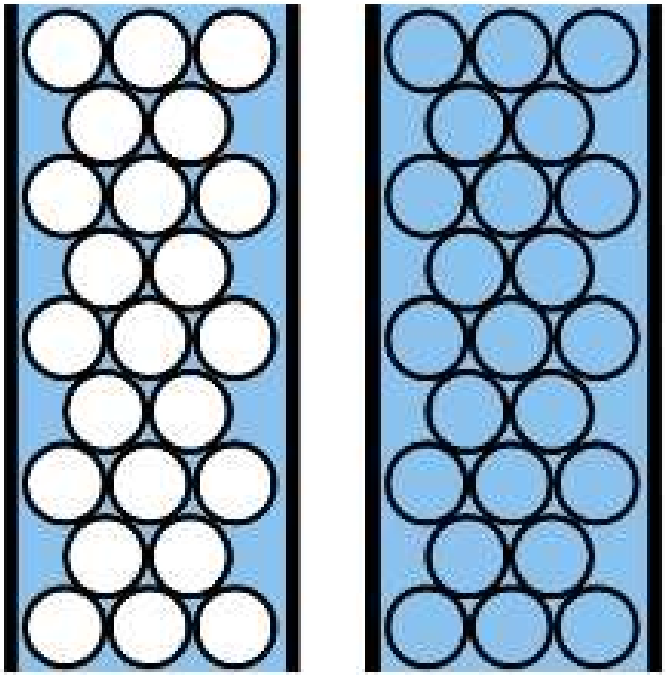
2. The Total volume, Vt (bed volume):

- The total volume of material in the column (both solid and liquid).

➔ can be calculated from the dimension of the column.

3. The Elution volume, Ve, of molecules:

- The amount of liquid(mobile phase) that must be added to produce a ***peak*** of a particular solute in the effluent.
- Or the volume required for completely eluting the solute from column.



Void volume V_0 Total volume V_t

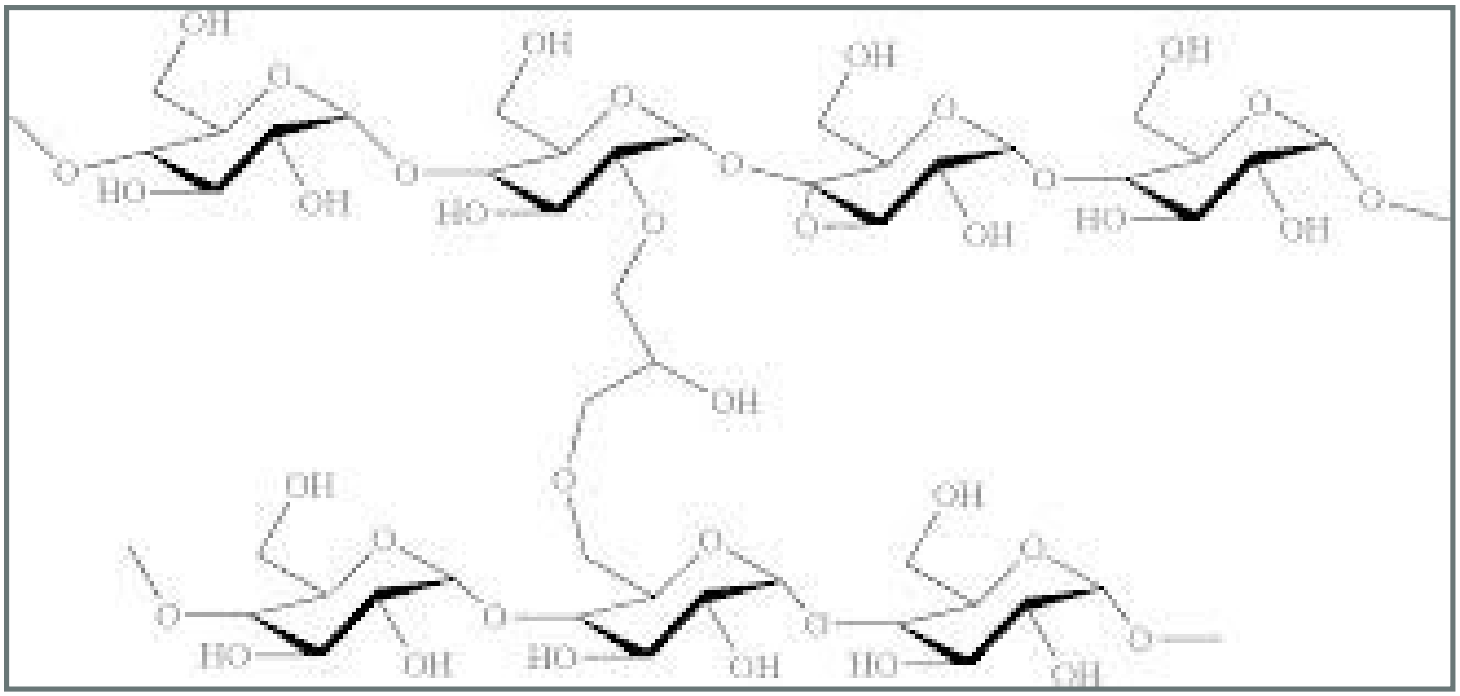
Gel Filtration Resins

Gel Filtration Resins:

- ***Dextran [e.g. Sephadex]:***

Which phase ?

- Is a polysaccharide composed of glucose residues.
- Supplied in the form of dry beads that swell when water is added.
- Prepared with various degrees of cross-linking ➔ to control pores size.
- It is mainly used for separation of small peptides and globular proteins with small to average molecular mass.



Dextran: polysaccharide composed of glucose residues.

Gel Filtration Resins Sizes:

- ***Gel beads come in various sizes:*** large, medium, fine, and superfine.

- All consist of semi-permeable, porous gels of cross-linked polymers with a range of pore sizes.

Cross linking degree ➔different pore sizes

Resins Size and Resolution:

- ***The larger the beads*** ➔ the more rapid the flow rate and the poorer the resolution. *(WHY?)*

➔Because as the flow rate increases, the time available for the molecules to equilibrate between the mobile phase and the pore space in the stationary phase decreases.

- ***The larger beads*** used in very large preparation in which resolution is less important than time.
- ***Super fine*** is used if maximum resolution is required, and the time is less.

Advantages and Application of Gel Filtration

Advantages of gel filtration:

- ***It is the best method for separation of molecules differing in molecular weight because:***

1. It doesn't depend on temperature, pH, ionic strength and buffer composition, so, separation can be carried out under any conditions.
2. There is less zonal spreading than in other techniques.
3. Important method in protein purification.
4. This separation method is unique in fractionating without requiring protein binding, thus significantly reducing the risk of protein loss.

Application of gel filtration in biochemistry:

- Fractionation of macromolecules.
- Purification.
- Molecular weight determination.

➔ HOW?

Determination of Protein Molecular Weight by Gel Filtration

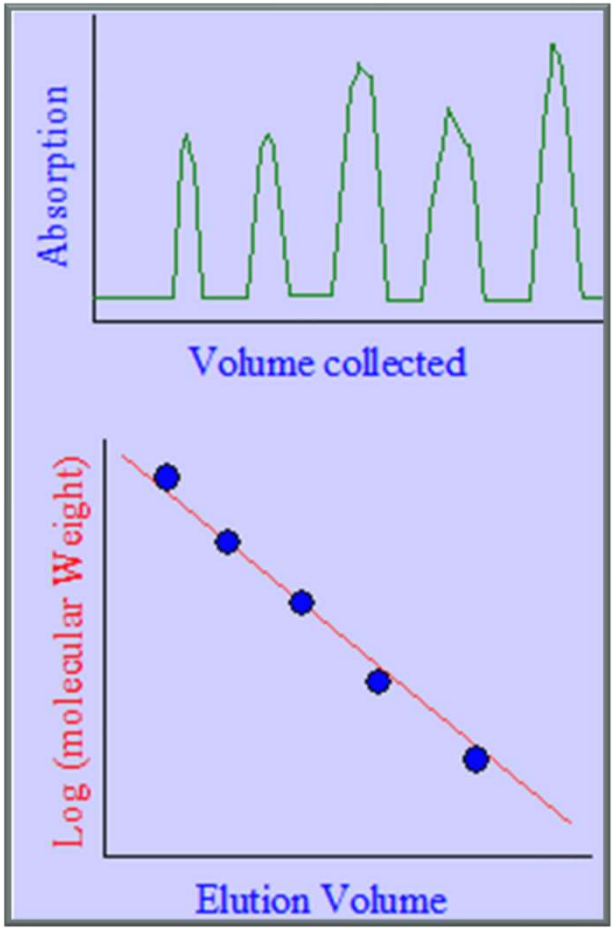
To estimate the molecular weight for a protein:

Several proteins with known molecular weights are run on the column and their elution volumes determined.

The elution volumes are then plotted against the log molecular weight of the corresponding proteins.

A straight line is obtained for the separation range of the gel being used.

The elution volume of a protein of unknown molecular weight is then found (it can be compared to the calibration curve and the molecular weight determined).



Inverse relationship between molecular size and elution volume.

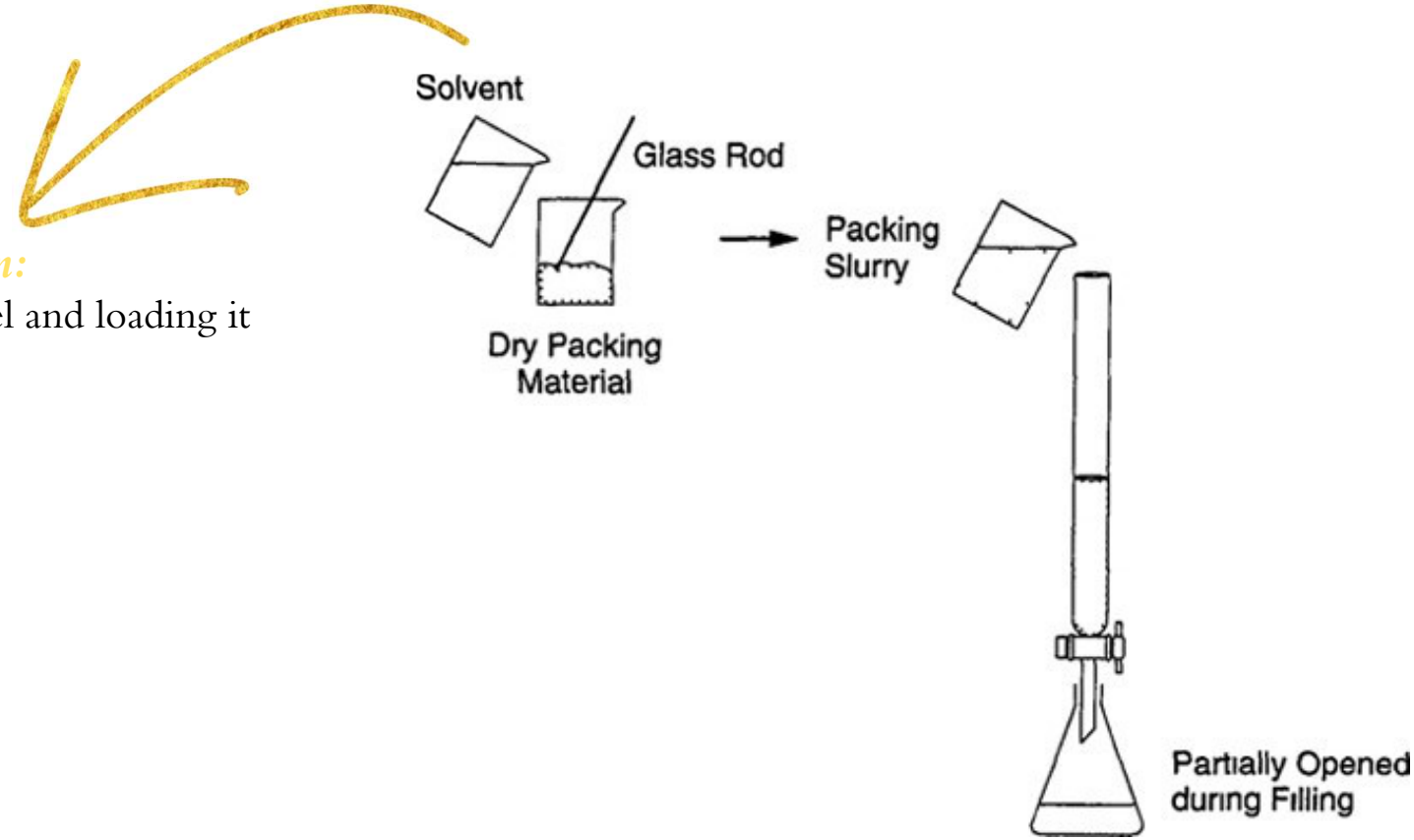
Elution volume, V_e is proportional to log of molecular weight.

➔ For high molecular weight less elution volume is needed, and for small molecular weight the large elution volume is needed to elute the sample.

Column Packing and Considerations

Packing the column:

Preparation of the gel and loading it in the column.



How to Pack a Column (for Column Chromatography):

https://www.youtube.com/watch?app=desktop&v=8Ff1Xx-On-g

Cautions must be taken while packing the column:

- ***It is important that the gel should be:***

- Homogenous.
- Free from bubbles.
- Free from cracks.
- Free from spaces between the walls.
- And it should be covered by the liquid "mobile phase" all the time (should not be dry).

Considerations while choosing the gel:

- Gels of various pore size are available ➔ choose the one with the best fractionation range for your sample.

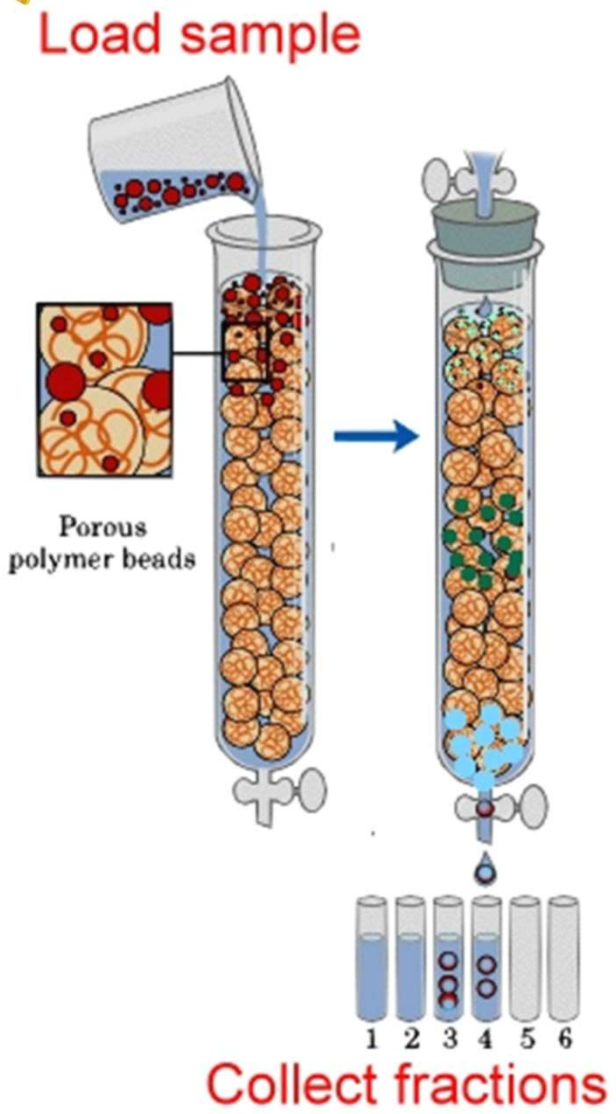
Material	*Fractionation range (globular proteins)*
Dextran	Sephadex G-10: 0–700 Da Sephadex G-25: 1000–5000 Da Sephadex G-50: 1500–30,000 Da Sephadex G-100: 4000–150,000 Da
Agarose	Sepharose 6B: 10,000–4,000,000 Da Sepharose 4B: 60,000–20,000,000 Da
Allyl dextran-bis-acrylamide	Sephacryl S-200 HR: 5000–250,000 Da Sephacryl S-300 HR: 10,000–1,500,000 Da

Examples

- Good separation usually require ***long columns*** and ***slow flow rate***.
- Gel filtration is NOT recommended for separating proteins with only a small difference in molecular weight .

Loading the sample :

small volume of sample is placed on the stationary phase and allowed to enter the column.



Practical part

OBJECTIVE:

- Separating mixture of blue dextran (BD) and bromophenol blue (BPB) based on their molecular weights by gel filtration chromatography.

METHOD:

- The gel filtration material that will be used in the experiment is called ***Sephadex G-100*** and it will separate molecules with molecular weights from 4,000 to 150,000 Da.
- So, Those molecules which are with molecular weight ***larger than 150,000 Da*** will be excluded from the beads, because of their huge size they can not get through the pores of the beads, and elute ***first.***

- In your experiment:

You will separate a mixture of blue dextran [sugar] with M.W.= 2,000,000 Da and bromophenol blue[dye] M.W.= 669.99 Da.

METHOD:

1. Remove the excess buffer.

2. add 1 ml of your sample

3. Open the screw clip and start to collect fractions of about 3 ml each.
4. Read the absorbance for each fraction at 560nm.

:RESULTS

Fraction number	*Absorbance at 560 nm*

- Plot the absorbance at 560nm against fraction number.
- Identify the peaks and determine the elution volume of blue dextran and bromophenol blue.

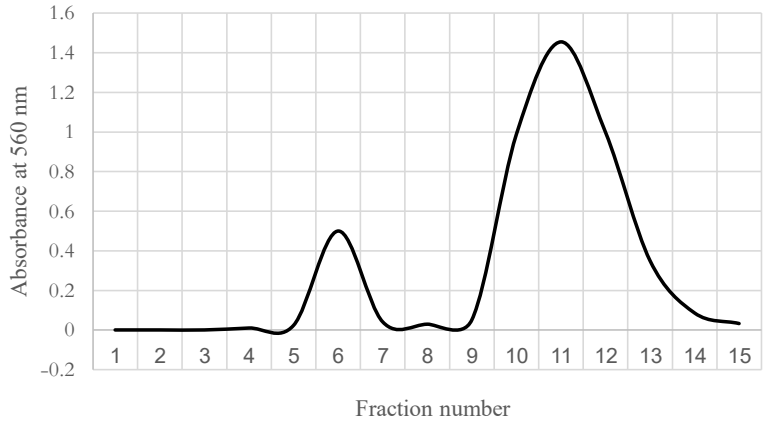


Figure 1. Gel filtration chromatography of separation of Bromophenol blue and blue dextran.

Diffusion