Determining column dead volume - Gel filtration chromatography

Supratim Datta
Biochemistry lab
LS2102

Gel Filtration

Molecular sieve chromatograpy
Gel permeation chromatography
Size exclusion chromatography

Separation of molecules on the basis of size (and shape)

Agarose Gel Electrophoresis

- Electrophoresis is a molecular technique that separates nucleic acids and proteins based on

Size

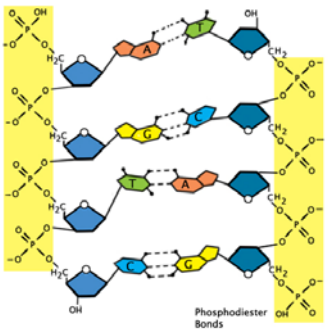
Shape

and

+-+Charge+-+

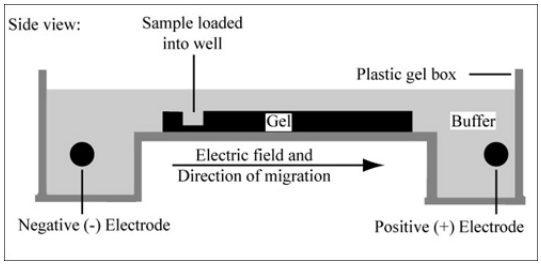
Agarose Gel Electrophoresis

- DNA is a negatively charged molecule and therefore is attracted to positive charges

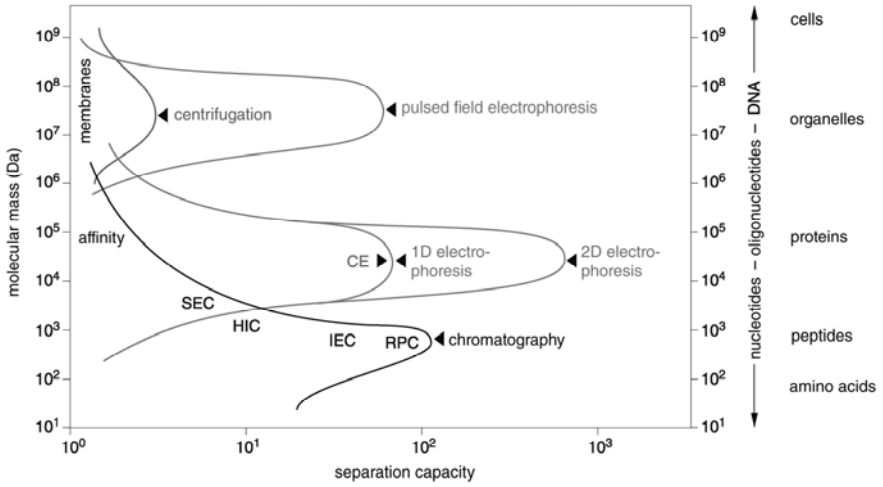


Agarose Gel Electrophoresis

- Agarose provides a matrix through which DNA molecules migrate.
  - Larger molecules move through the matrix slower than small molecules
  - The higher the concentration of agarose, the better the separation of smaller molecules

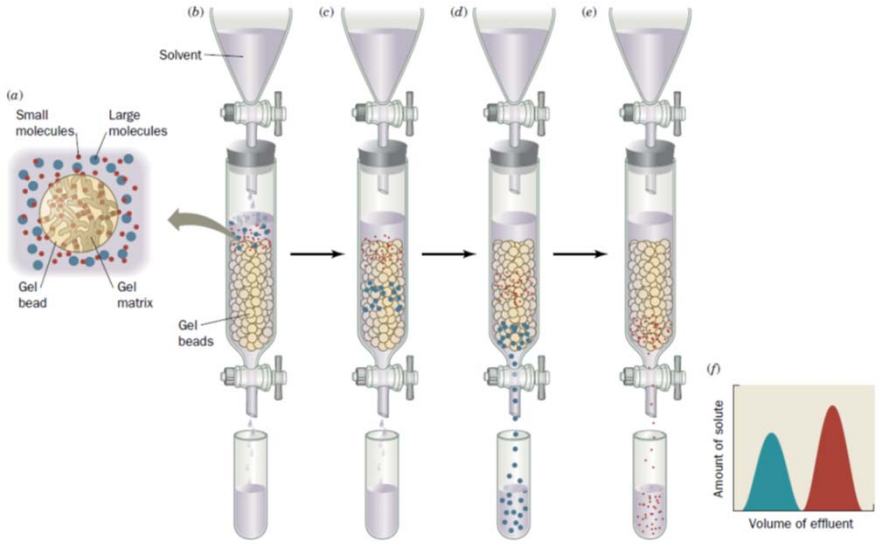


Various separation methods



The maximal number of compounds that can be separated in a single analysis of the various separation methods is very different for different molecular masses of the analyte.

Separation but of a different kind

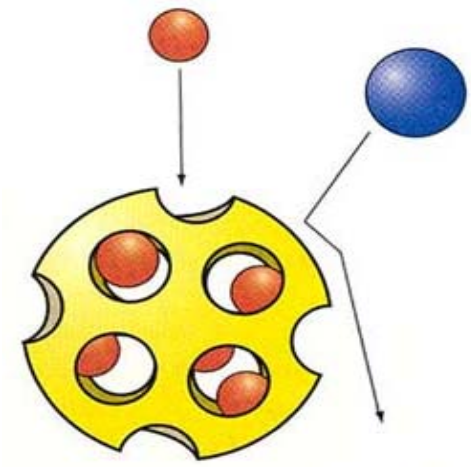


Basis

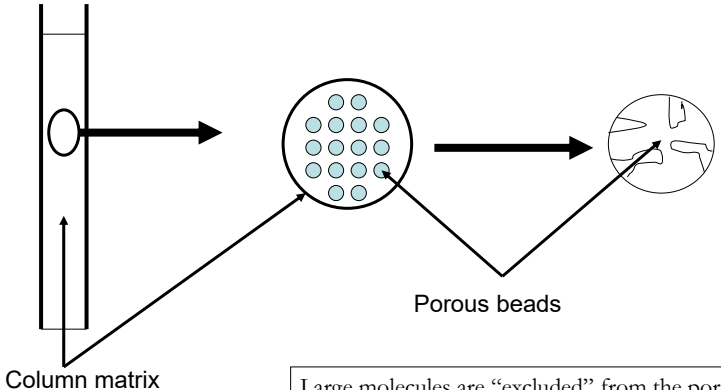
- Separation according to molecule size and shape.
- Stationary phase- beads made up of pores that span a relatively narrow size range in a long cylindrical column

  - Expensive and challenging to control pore size of beads
- Mobile phase - buffer

- Smaller molecules spend more time inside the beads than larger molecules
- therefore the small molecules elute later (after a larger volume of mobile phase has passed through the column).



To sum up



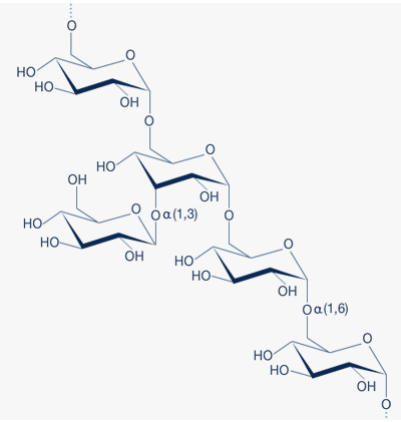
Large molecules are "excluded" from the pores and travel through the column fastest

Small molecules are "included" – can diffuse into the pores and elute later

Beads: the packing material

- The gels used as molecular sieves are cross-linked polymers
- They are uncharged and inert i.e. don't bind or react with the materials being analyzed
- Three types of gels are used under this technique

Dextran (Sephadex)

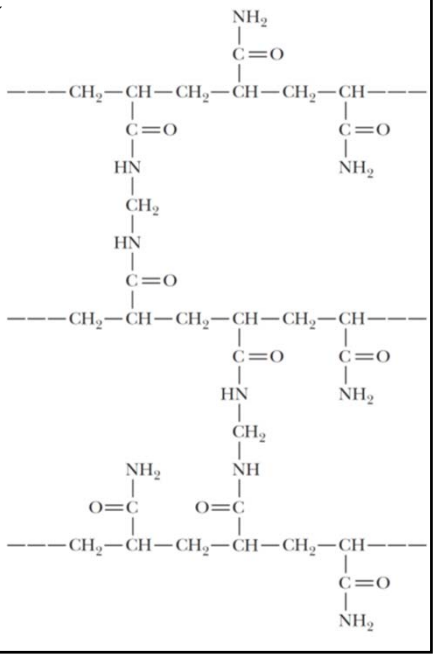


homopolysaccharide of glucose residues.

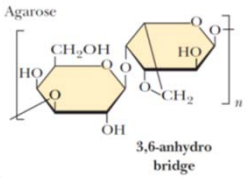
- various degrees of cross-linking to control pore size.
- the dry beads swell when water is added.
- It's mainly used for separation of small peptides and globular proteins with small to average molecular mass.

Polyacrylamide

- cross linking acrylamide with N,N-methylene bis acrylamide.
- The pore size is determined by the degree of cross-linking.
- The separation properties of polyacrylamide gels are mainly the same as those of dextrans.
- They are sold as bio-gel P. They are available in wide range of pore sizes.



Agarose



linear polymers of D-galactose and 3,6 anhydro-1-galactose

It forms a gel that's held together with H bonds. It's dissolved in boiling water and forms a gel when it's cold.

The concentration of the material in the gel determines the pore size.

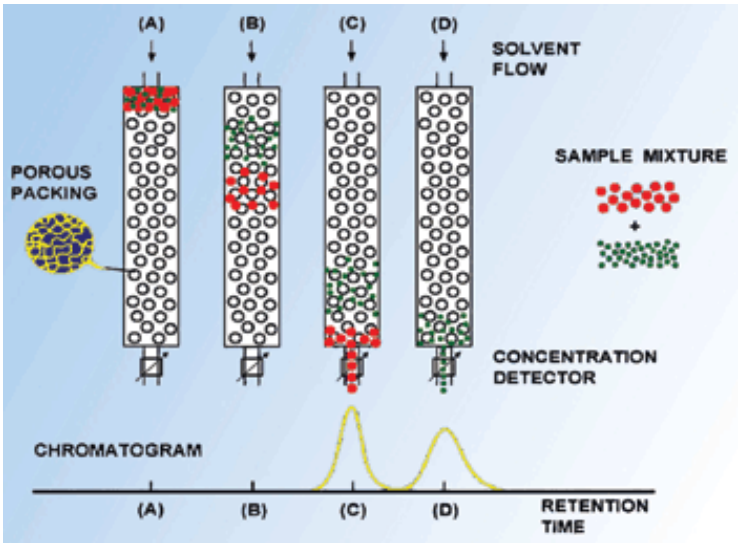
The pores of agarose gel are much larger than those of sephadex or bio-gel p.

It's useful for analysis or separation of large globular proteins or long linear molecules such as DNA

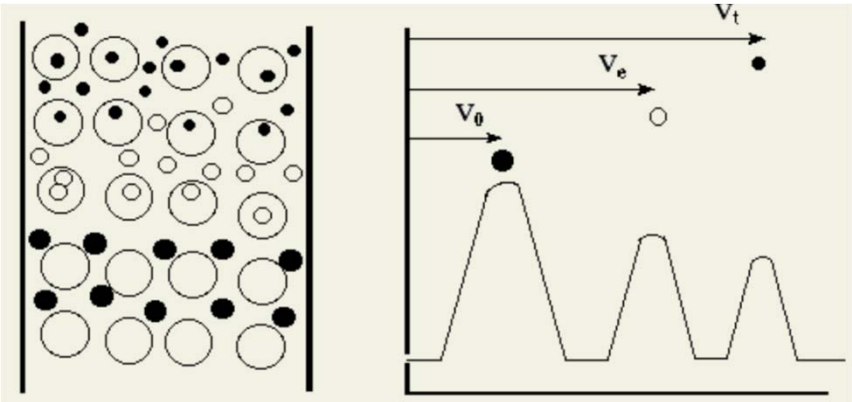
Our experiment

- The gel filtration material that will be used in the experiment below is called Sephadex G-75 and it will separate molecules with molecular weights from 3,000 to 70,000. Molecules with molecular weights larger than 70,000 will be excluded from the beads. You will use Blue Dextran to measure the column void volume

Another schematic: you could also use retention time instead of volume



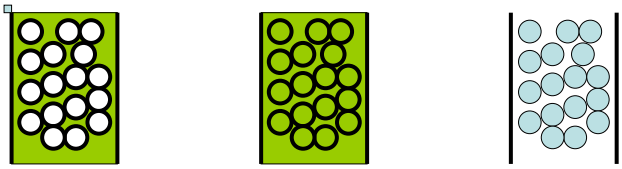
Elution Profile: three cases



V_e = Elution volume (volume of solvent between injection and elution).

Dictated by proportion of porous matrix available to molecules (K_d).

Column Parameters



V_o = void volume

V_t = total volume

V_s = volume of solvent held in the pores. This is normally approximated to

V_t-V_o = volume of beads

V_o = Elution volume of a large "totally excluded" molecule such as blue dextran

V_t = Physical volume of column

Behaviour of molecule on any column

$$K_{d\,(B)} = [B]_{\text{stationary phase}}/[B]_{\text{mobile phase}}$$

- K_d is the ratio of the concentrations of the component in the stationary and the mobile phases
- difficult to determine the concentrations of an analyte in the stationary and mobile phases.
- express K_d in terms, V_e and V_o

For example, the flow of component B through the column is retarded and it elutes at volume $V_{e\,(B)}$

$(V_{e(B)} - V_0)$ is the 'extra' stationary phase volume that B is able to access by virtue of its small size.

'extra' volume is a component of the internal pore volume V_i, and the partition coefficient of B can be expressed as a fraction of V_i.

$$\frac{(V_{e\,(B)} - V_0)}{V_i} = K_{d\,(B)}$$

$$\frac{(V_{e\,(B)} - V_0)}{V_T - V_0} = K_{d\,(B)}$$

V_T is the total solvent accessible volume, a parameter that is difficult to measure. Therefore, substitute V_T by the term V_c, which is the total geometric volume of the column:

$$\frac{(V_{e\,(B)} - V_0)}{V_c - V_0} = K_{av\,(B)}$$

$K_{av\,(B)}$ is not a true partition coefficient but is easily determined.

K_{av} = proportion of pores available to the molecule.
Totally "exclude" $K_{av} = 0$ and $V_e = V_o$
Totally "included" $K_{av} = 1$ and $V_e = V_t$

Matrix Types

Material

- Sephacryl
  - dextran
- Sephadex
  - dextran
- Sepherose
  - agarose
- Superdex
  - mixture

Sephacryl	Protein (kDa)	Dextrans (kDa)
S-100	1-100	NS
S-200	5-250	1-80
S-300	10-1500	2-400
S-400	20-8000	10-2000
S-500	NS	40-20,000

Types of Column Systems

Design of Column is important

- Column size
  - Analytical or preparative
- Solvent
  - Inert matrix most solvents OK
- Matrix
  - Most important consideration
  - Many different types
    - Material
    - Pore size

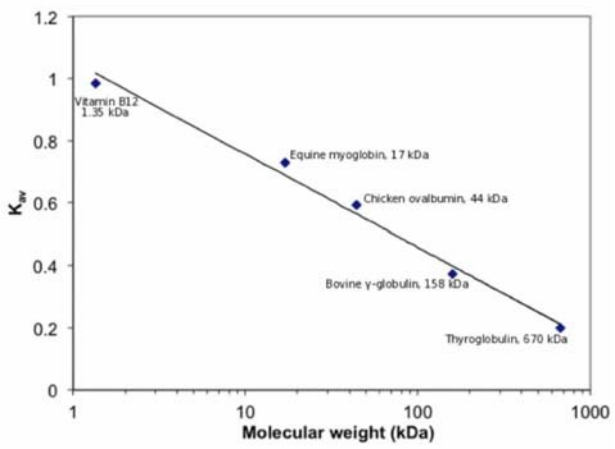
Running the column

- Sample size / Fraction size
  - 0.5 – 5% of total bed volume (Vt).
  - Concentration limited by viscosity
- Running time
  - Determined by "trial and error"
  - Slow rates allow efficient partitioning into pores and thus increase resolution
  - Slow rates increase diffusion of sample on column thus increasing peak width and reducing resolution.
  - Protein about 5 mL cm^{-2}. h^{-1}

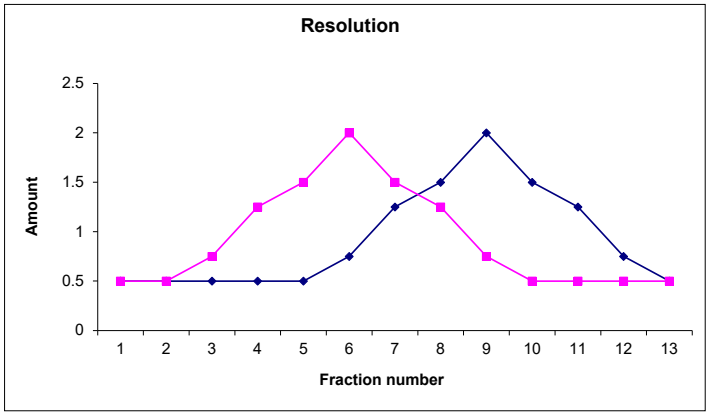
What are you running in the lab?

Determination of molecular weight possible

- Calibrate column with known standards
- Plot K_{av} against log Mol WT



Resolution is an imp term



Resolution proportional to square root of column length.

Also affected by rate at which column is run

Other Types of Column Chromatography

- Ion-Exchange Chromatography
  - Separation on basis of charge
    - DEAE- sephadex
- Hydrophobic Interaction Chromatography
  - Separation on basis of hydrophobicity
    - Phenyl-sepherose
- Affinity Chromatography
  - Affinity of enzyme for substrate or other ligand