Chromatography

What is chromatography?

An analogy which is sometimes useful is to suppose a mixture of bees and wasps passing over a flower bed. The bees would be more attracted to the flowers than the wasps, and would become separated from them. If one were to observe at a point past the flower bed, the wasps would pass first, followed by the bees. In this analogy, the bees and wasps represent the analytes to be separated, the flowers represent the stationary phase, and the mobile phase could be thought of as the air. The key to the separation is the differing affinities among analyte, stationary phase, and mobile phase. The observer could represent the detector used in some forms of analytical chromatography. A key point is that the detector need not be capable of discriminating between the analytes, since they have become separated before passing the detector. - Wikipedia

Yeah???

History

- Russian botanist Mikhail Semyonovich Tsvet invented first chromatographic techniques for separation of pigments from plants
- Used calcium carbonate to separate plant pigments.

Chromatography

- Separation depends on differential partitioning between stationary phase (chromatography media) and mobile phase (buffer solution or gas)
    - Stationary phase can be packed into a tube
        - Resin can be mixed in with absorbent material in batch and pour slurry over filter to collect resin
- There are many different types of systems depending on the need
- Basic systems consist for proteins consist of pump column and detector (UV, IR or light scattering)

Stationary phase - Matrix properties

- hydrophilic
- should not contain functional groups that spontaneously bind protein
- chemically and physically stable
- rigid enough to stand up to high flow rates
- Types of media
  - Inorganic materials (Silica, glass, hydroxyapatite)
  - Synthetic organic polymers (polyacrylamide, polystyrene)
  - polysaccharides (cellulose, dextran, agarose or cross-linked agarose)

Agarose

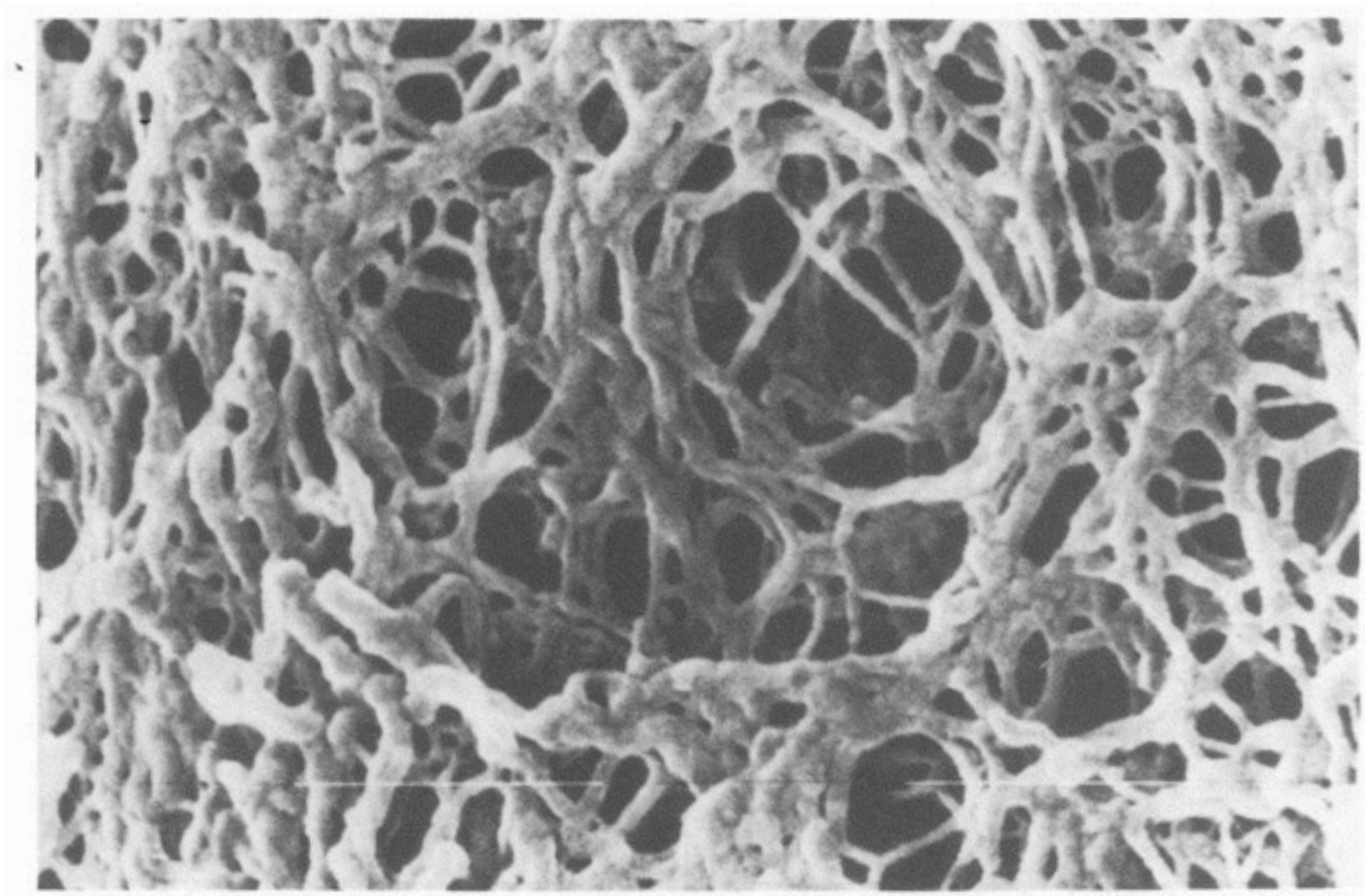
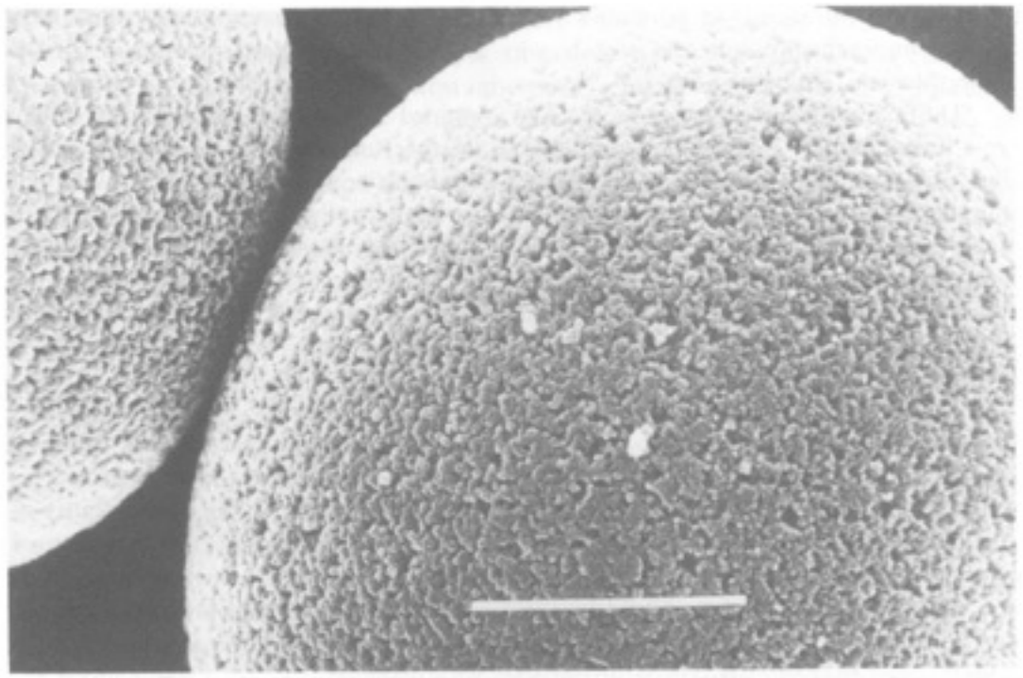


Figure 2-4. Scanning electron micrograph of 2% agarose gel. The white bar represents 500 nm. (Preparation and photo: A. Medin, Institute of Biochemistry, Uppsala University, Uppsala, Sweden).

Sephacryl - Gel filtration media



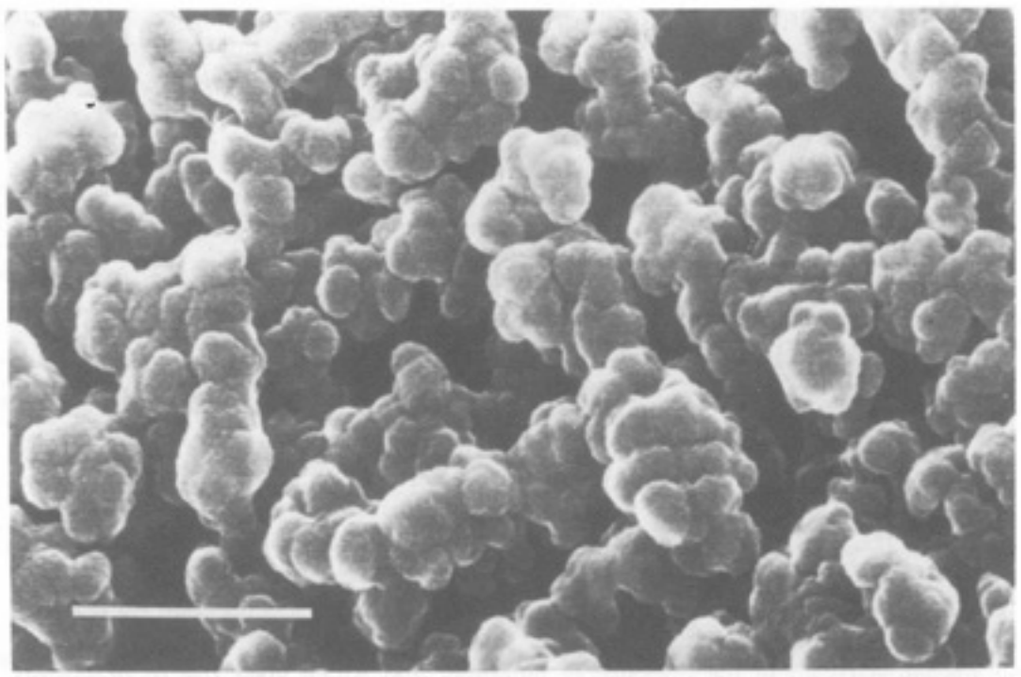


Figure 2-5. Scanning electron micrograph of Sephacryl® S-500. The white bars represent 10 μm (top picture) and 0.5 μm (lower picture). (Preparation and photo: A. Medin, Department of Biochemistry, Uppsala University, Uppsala, Sweden).

Mono Beads - Ion Exchange

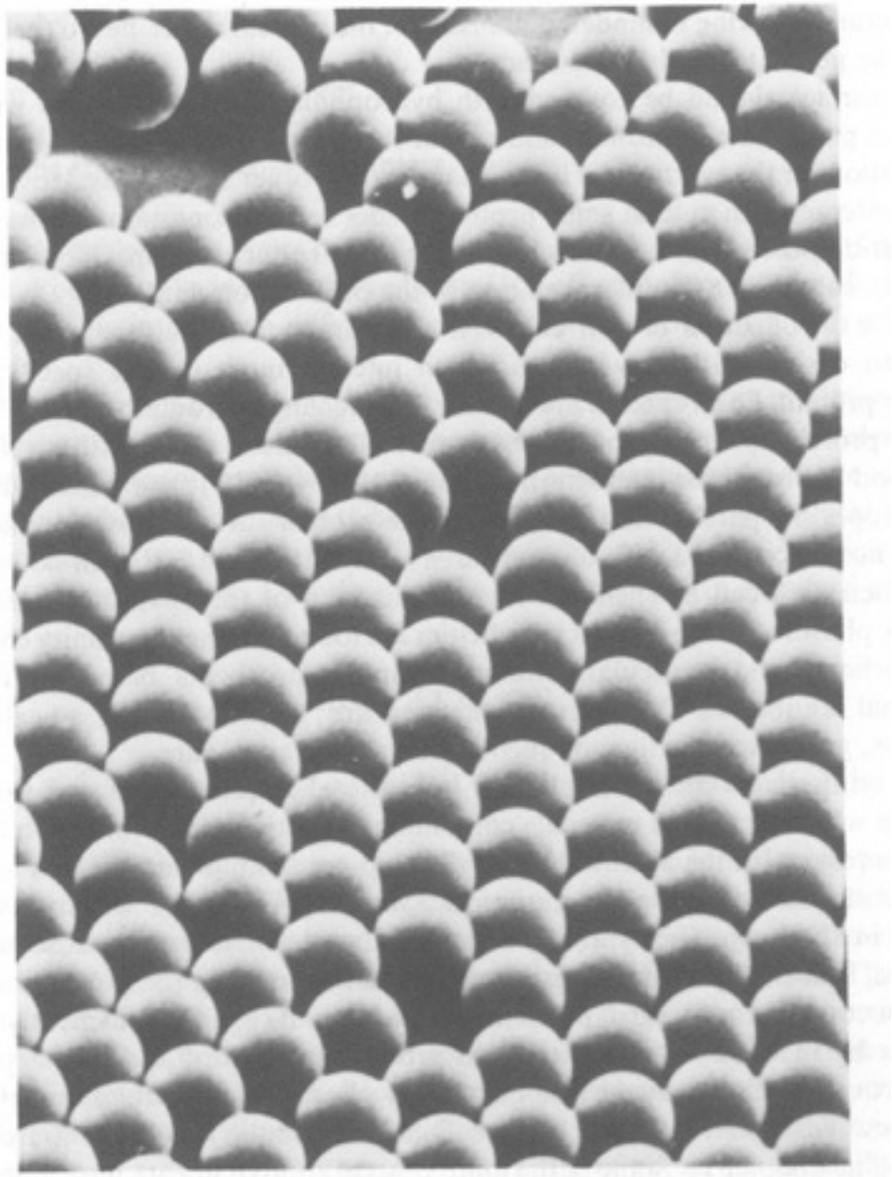


Figure 2-6. Scanning electron micrograph of MonoBeads™ [7].

Introduction to Ligand Groups

- this is the most dominant type of chromatography
- Matrix
  - should not have transport constraints allows for diffusion
  - should have an open, non-sieving structure
  - large surface area
  - examples ion exchange chromatography, hydrophobic interaction chromatography (HIC), affinity chromatography

Binding to an immobilized ligand

- several possible interactions
  - ion-ion or ion-dipole
  - hydrogen bonds
  - van der Waals forces
  - aromatic interactions
  - hydrophobic effect

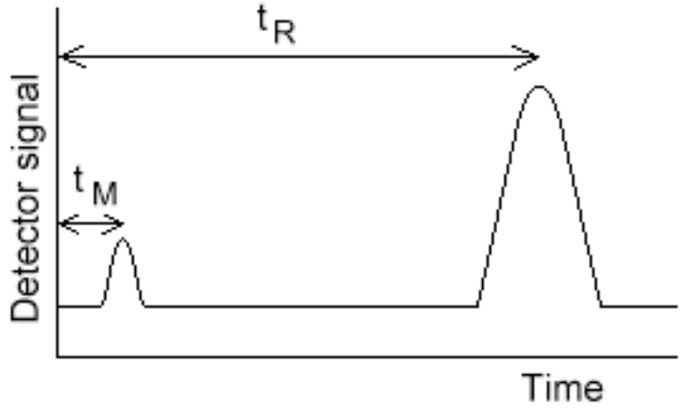
Binding to an immobilized ligand

- solution-protein-stationary phase minimizes free energy and give rise to binding
- each one of the previous interactions may have 1kcal/mole
- K_D of 10^{-5} M or less can be retarded by the column
  - K_D is the dissociation constant.

Column Parameters

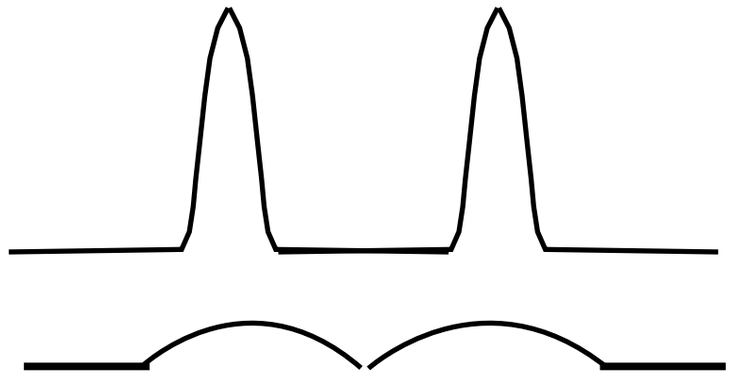
- V_o void volume - volume of the mobile phase in the column
  - t_M time in which the void volume comes out
- V_R retention volume -the volume that passes the column from injection until emergence of sample at the detector.
  - t_R time in which the retention volume comes out

Retention Parameters

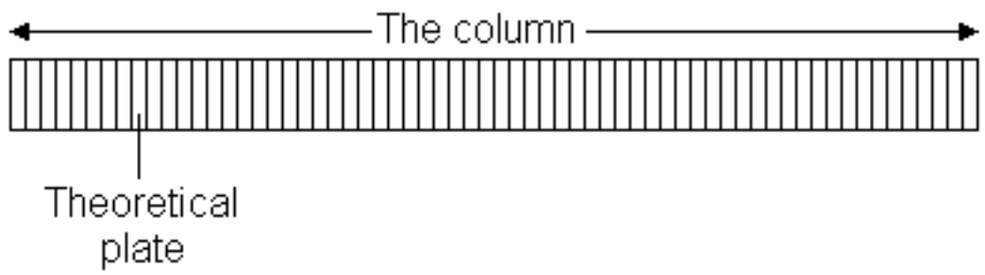


- *retention factor, k'*, is often used to describe the migration rate of an sample on a column
  - $k' = (t_R - t_M)/t_M = (t_R/t_M) - 1$
- Also called *capacity factor* when volumes are used instead of time. This is misleading because it is NOT the capacity of the column.
- retention factor is less than one, elutes really fast
- High retention factors (greater than 20) mean that elution takes a very long time
- Ideally, the retention factor for an sample is between one and five.
- Separation of two compounds is only possible if their *k'* or V_M are different.
- selectivity factor (α) for two peaks is $\alpha = k'_B / k'_A$

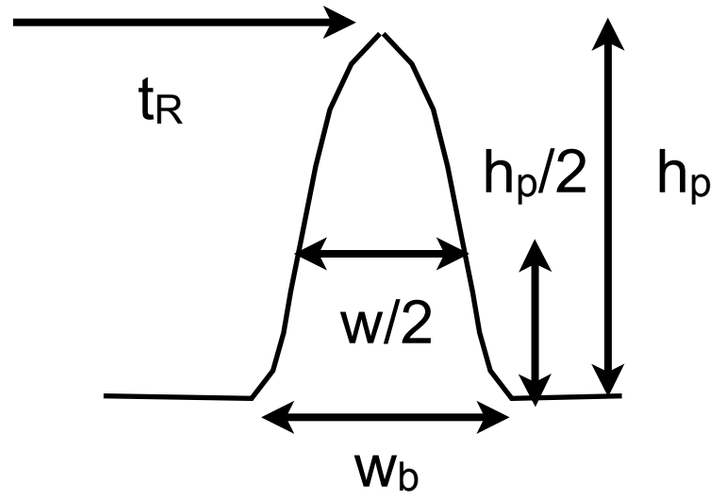
Peak Shape and Width; Theoretical Plates



- Peak shape matters
- To obtain optimal separations, sharp, symmetrical chromatographic peaks must be obtained.
- The Theoretical Plate Model of Chromatography
- the chromatographic column is contains a large number of separate layers, called theoretical plates.
- Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates"



Calculation of theoretical plates



- measure the efficiency of the column.
- THERE ARE NO SUCH THINGS AS PLATES: this is a calculation of how good the column is.
- calculate the number of theoretical plates (N) from a chromatogram
- $N=5.55\ (t_R / w/2)^2$
- $N= 16(t_R/W_b)^2$
- equations are based on gaussian probability curves
- the more plates the better
- the number of plates increase as the length of the column increases
- *Height Equivalent to a Theoretical Plate* (the smaller the better)
- column of length L HETP = L / N

The Rate Theory of Chromatography

- A more realistic description
- takes account of the time taken for the solute to equilibrate between the stationary and mobile phase
- the plate model, which assumes that equilibration is infinitely fast
- shape of a chromatographic peak is affected by the elution rate and the different paths available to solute molecules as they travel between particles of stationary phase
- Van Deemter equation HETP = $A + (B / u) + C u$ *where u* is the average velocity
    - A = **Eddy diffusion** Solute molecules will take different paths through the stationary phase at random. This will cause broadening of the solute band, because different paths are of different lengths.
    - B=**Longitudinal diffusion** The concentration of sample is less at the edges of the band than at the center. Sample diffuses out from the center to the edges. This causes band broadening. If the velocity of the mobile phase is high then the sample spends less time on the column, which decreases the effects of longitudinal diffusion.
    - **C=Resistance to mass transfer** The sample takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the sample has a strong affinity for the stationary phase, then the sample in the mobile phase will move ahead of the sample in the stationary phase. The band of sample is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

Van Deemter Plot

- Both A and C terms are proportional to the diameter of the column

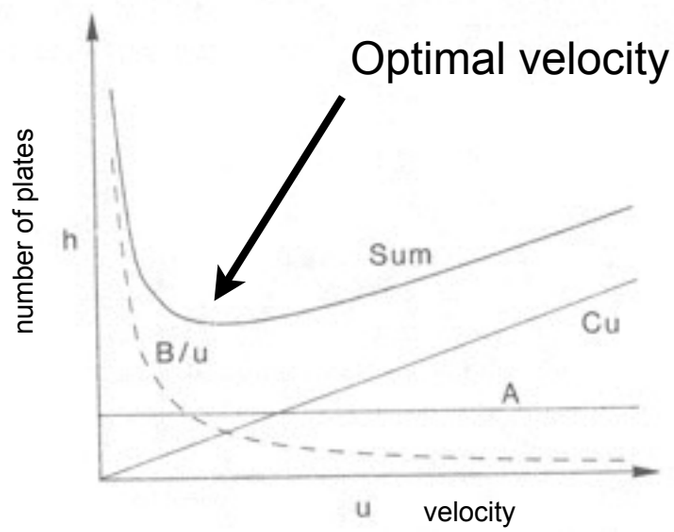
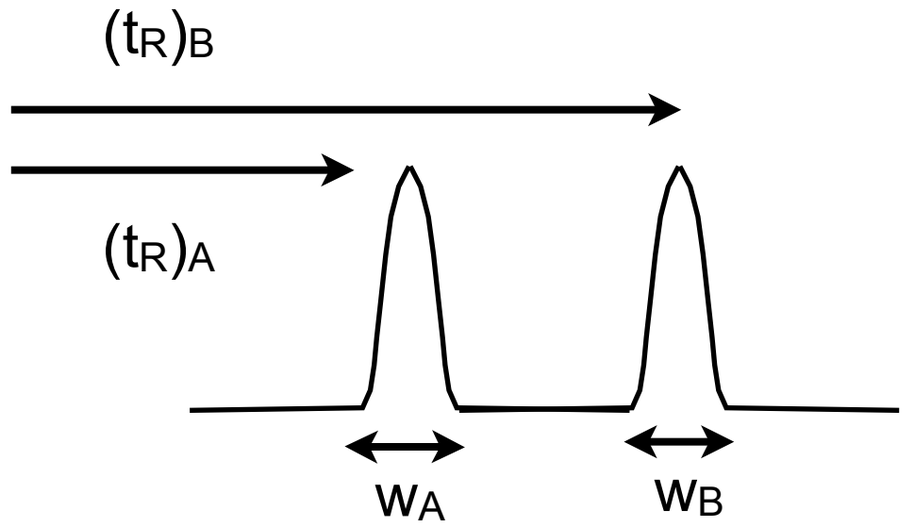


Figure 2-11. Plot of the three terms in the van Deemter equation and their sum.

Resolution



- Resolution between two peaks is given as

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

- for R<1 the peaks overlap
- R=1 they just touch each other
- R>1 optimal resolution
- baseline resolution when R≥1.5

High Resolution

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

- it is useful to think of resolution in terms of theoretical plates
- $N = 16(t_R/W_b)^2$

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right)$$

- α selectivity factor, k retention factor
- To obtain high resolution
- To increase the number of plates - increase length of column. Need a four fold increase to get 2 fold increase in R. Instead of increasing the column length reduce the resin size. More plates in the same size column
- to get best resolution need to optimize the selectivity factor - Get more selective
    - changing pH, salt concentration, type of column, type of salt

Ion Exchange

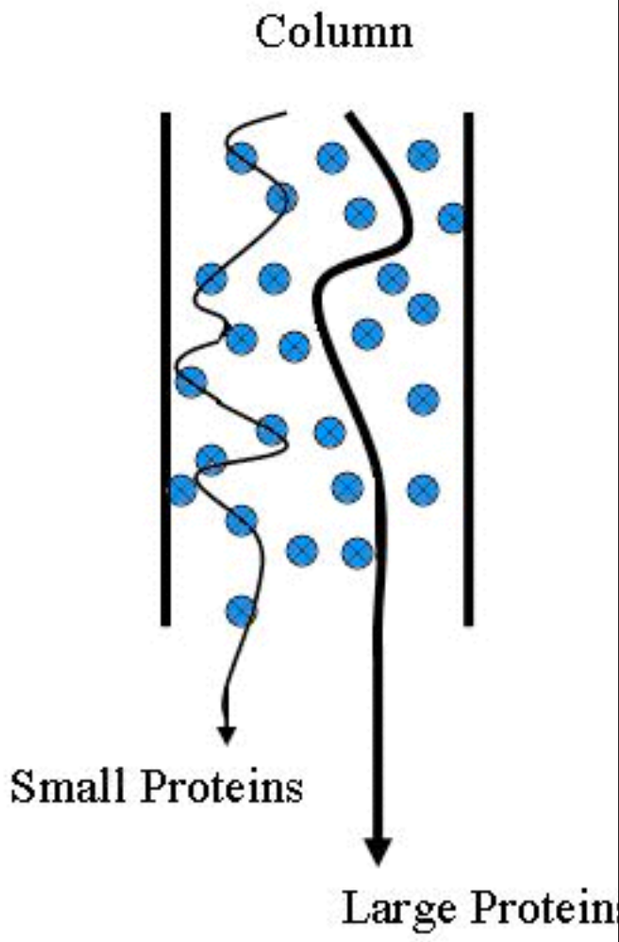
- Anion and Cation exchange
- Binding is dependent on the pI of the protein
- Anion exchange column is positively charged
- Cation exchange column is negatively charged
- Elute sample with increasing concentrations of salt

Affinity column

- A specific factor is bound to the column that a protein will bind
- antibodies, glutathione (Glutathione-S-transferase), nucleotides, DNA, Immobilized metal (Ni) His tags
- These tend to be more specific than ion exchange
- Many times this can be used as a first step

Gel Filtration

- Molecular sieves
- larger proteins will elute first, shorter path to travel
- vary pore size to get better selection
- can be used to estimate MW
- MW of unknown protein is made by a plot of V_R/V_o vs log MW for known protein standards.



Gel Filtration