

Lecture 5: Size Exclusion (Gel Filtration) Chromatography

Size exclusion chromatography is used for semi-preparative purifications and various analytical assays. It is a separation technique which takes the advantage of the difference in size and geometry of the molecules. The molecules are separated based on their size. Grant Henry Lathe and Colin R Ruthven was the pioneer of size exclusion chromatography who started this technique for separation of analytes of different size with starch gels as the matrix, later Jerker Porath and Per Flodin introduced dextran gels. Other gel filtration matrices include agarose and polyacrylamide.

Note: Unlike ion exchange chromatography, gel filtration does not depend on any chemical interaction with protein, rather it is based on a physical property of the protein - that being the **effective molecular radius** (which relates to mass for most globular proteins).

Principle: Size exclusion chromatography (SEC) is the separation of mixtures based on the molecular size (more correctly, their hydrodynamic volume) of the components. Separation is achieved by the differential exclusion or inclusion of solutes as they pass through stationary phase consisting of heteroporous (pores of different sizes) cross linked polymeric gels or beads. The process is based upon different permeation rates of each solute molecule into the interior of gel particles. Size exclusion chromatography involves gentle interaction with the sample, enabling high retention of biomolecular activity. For the separation of biomolecules in aqueous systems, SEC is referred to as gel filtration chromatography (GFC), while the separation of organic polymers in non-aqueous systems is called gel permeation chromatography (GPC).

Supplement 1: Gel filtration resin can be thought of as beads which contain pores of a defined size range. Large proteins which cannot enter these pores pass around the *outside* of the beads. Therefore, the volume of the column appears smaller to a large molecule. Smaller proteins which can enter the pores of the beads have a larger volume that they can explore, thus the volume of the column appear larger to a small molecule. Both large and small molecules experience the same flow rate of mobile phase (i.e. L/min). Thus, a sample of proteins passing through a gel filtration column will *separate based on molecular size*: the big ones will elute first and the smallest ones will elute last (and "middle" sized proteins will elute in the middle).

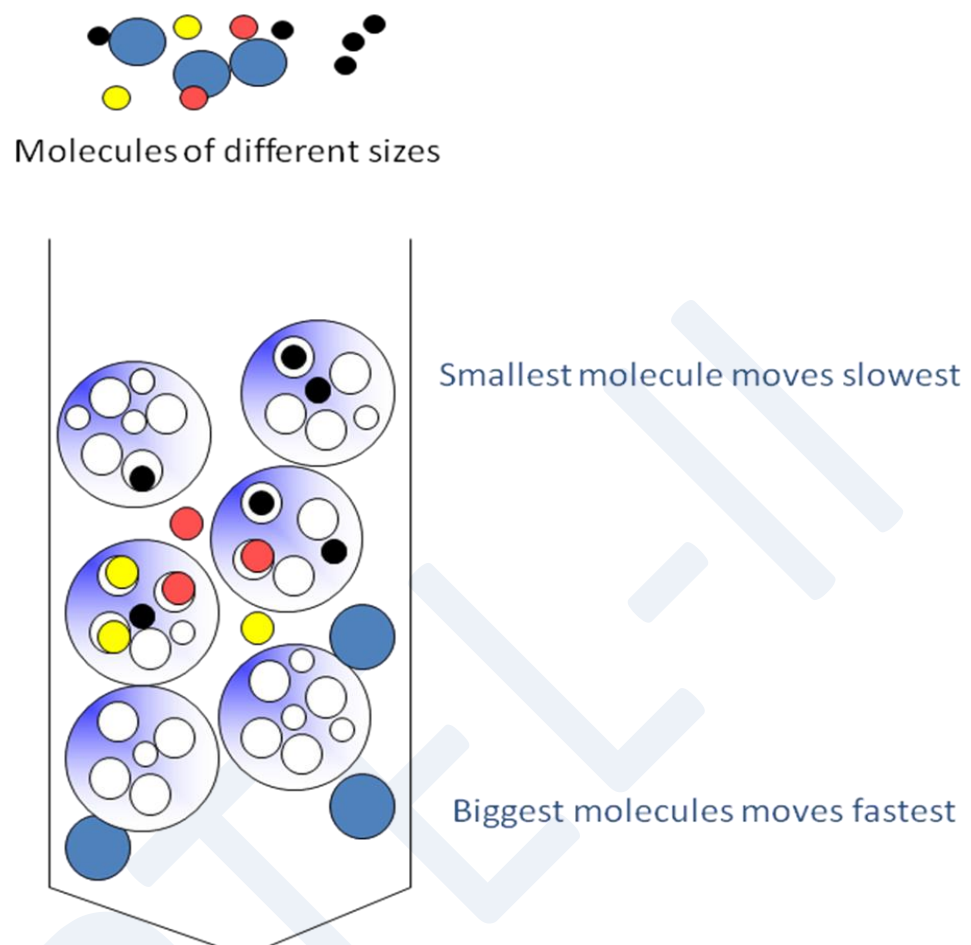


Figure 1: Theory of size exclusion chromatography. Please read details in supplement 1.

The basic principle of size exclusion chromatography is quite simple. A column of gel particles or porous matrix is in equilibrium with a suitable mobile phase for the molecules to be separated. Large molecules are completely excluded from the pores will pass through the space in between the gel particles or matrix and will come first in the effluent. Smaller molecules will get distributed in between the mobile phase of in and outside the molecular sieve and will then pass through the column at a slower rate, hence appear later in effluent (Fig. 1)

There are two extremes in the separation profile of a gel filtration column. There is a critical molecular mass (large mass) which will be **completely excluded** from the gel filtration beads. All solutes in the sample which are equal to, or larger, than this critical size will behave identically: they will all eluted in the excluded volume of the column. There is a critical molecular mass (small mass) which will be **completely included** within the pores of the gel filtration beads. All solutes in the sample which are equal to, or smaller, than this critical size will behave identically: they will all eluted in the included volume of the column. Solutes between these two ranges of molecular mass will elute between the excluded and included volumes (Fig. 2) Thus, while deciding a size exclusion matrix for protein purification, included and excluded range should be considered. For example: Sephadex G 75 matrix has fractionation range 3-80. This tells that the matrix has included volume range 3 kDa and excluded volume range 80kDa. If protein of interest and impurities both are close to 80 kDa or above they are likely to co-elute in excluded volume. Thus purification will not work. Now you can think what is the use of a size exclusion matrix Sephadax G25 (range 1-5kDa)? This is generally used for desalting as all proteins are above 5kDa and comes in excluded volume and salts are eluted late in included volume.

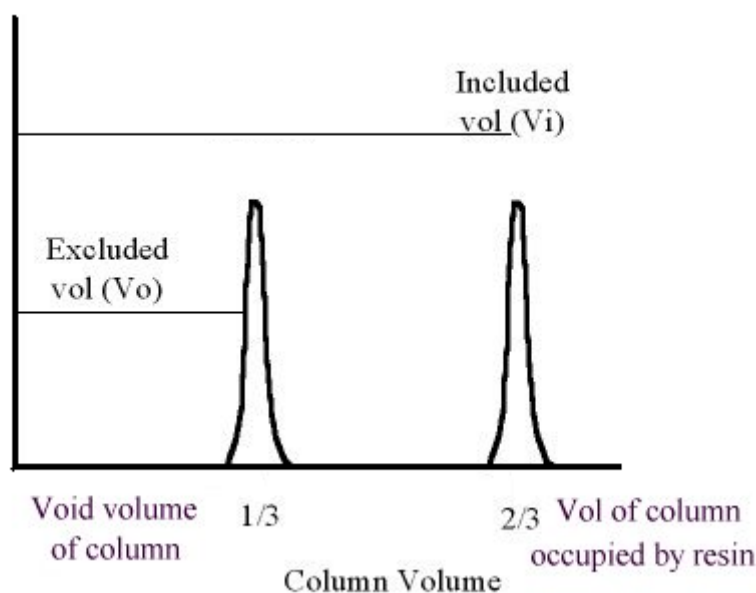


Figure 2: The excluded volume (V_o) is approximately equal to one third of the column volume, the included volume is approximately equal to two thirds of the column volume.

Students may visit following webpage for a nice animation

<http://people.virginia.edu/~dta4n/biochem503/Gel%20Filtration.html>

In gel filtration **the resolution is a function of column length** (the longer the better). However, one drawback is related to the maximum sample volume which can be loaded. The larger the volume of sample loaded, the more the overlap between separated peaks. Generally speaking, the sample size one can load is limited to about **3-5% of the total column volume**. Thus, gel filtration is best saved for the end stages of a purification, when the sample can be readily concentrated to a small volume. Gel filtration can also be used to remove salts from the sample, due to its ability to separate "small" from "large" components. Finally, gel filtration can be among the most "gentle" purification methods due to the lack of chemical interaction with the resin.

Mechanism of Size Exclusion Chromatography:

Size exclusion (also known as gel filtration chromatography) is a case of liquid-liquid partition chromatography, in which the solute molecules are get distributed in between two liquid phases, (i) liquid in the gel pores and (ii) liquid outside the gel.

The size exclusion may be explained by **Steric Exclusion Mechanism**. As the gel particles contain a range of pore sizes, small molecules can enter a large number of pores while the large molecules will get a small number of pores into which they can enter. Thus the different fractions of total pore volume are accessible to molecules of different sizes. Thus, molecules with different sizes will differ in distribution coefficient between these two liquid phases [As the small molecules can enter in more pores while larger molecules can enter in pores only larger than the molecular size]

The total volume (V_t) of a column packed with a gel that has been swelled by solvent is given by

$$V_t = V_g + V_i + V_o$$

Where V_g is the volume occupied by the solid matrix of gel, V_i is the volume of solvent held in the pores or interstices and V_o is the free volume outside the gel particles. When mixing or diffusion occurs, the diffusion equilibrium and the retention volume (V_R) of the given species is given by

$$V_R = V_{(int.)} + K_d V_{(int.)}$$

where distribution coefficient (K_d) is given by $K_d = V_{i(acc)} / V_{(total)}$

where $V_{i(acc)}$ is the accessible pore volume. $V_{(total)}$ is the total pore volume and $V_{(int.)}$ is the interstitial volume.

The other proposed mechanism is **Secondary Exclusion Mechanism**. This mechanism states that when a sample containing a mixture of small and large molecules is applied to a gel filtration column, the small molecules diffuse rapidly into the pores of gel, whereas large molecules will find relatively few unoccupied pores and move further down the column till they find the unoccupied pores. This results in the enhancement of separation of small and large molecules.

Applications:

- Purification.
- Desalting.
- Protein-ligand binding studies.
- Protein folding studies.
- Concentration of sample.
- Copolymerisation studies.
- Relative molecular mass determination

Quiz:

Explain how size exclusion chromatography may be used for protein folding/refolding studies?

Reading: Werner et al., (1994) Refolding proteins by gel filtration chromatography. FEBS Letters 345, 125-130.

<http://www.ncbi.nlm.nih.gov/pubmed/8200443>