

Size Exclusion Chromatography (SEC) Separations Module

Goal: This module is designed to provide theory and practical use of size exclusion chromatography (SEC) for separation of proteins.

In-Class Questions:

1. What is an obvious physical difference between an immunoglobulin (antibody), which is a protein, and glucose, which is a monosaccharide?
2. When would it be useful to separate immunoglobulins from glucose in blood?
3. How could you devise an experiment to separate immunoglobulins from glucose utilizing the physical difference between the molecules determined in the first question?

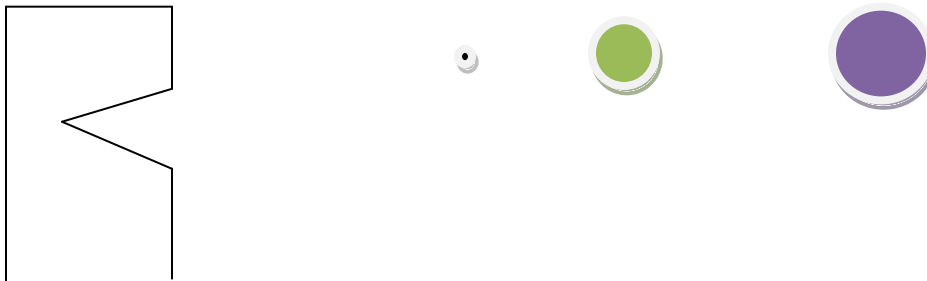
Introduction:

Size exclusion chromatography (SEC), also called gel filtration, gel permeation, molecular sieve, and gel exclusion chromatography, is a separation technique used to separate molecules on the basis of size and shape (hydrodynamic radius). Size exclusion chromatography is called gel filtration chromatography because the gel essentially allows for the filtering of molecules from a sample based upon molecular size. However, unlike other techniques, the larger molecules elute first. This technique is widely applicable to purification or desalting of proteins in complex samples such as blood, which contain molecules of widely different sizes.

Theory:

In-class question:

4. Depicted below are three different sized molecules. Next to them is shown a pore. What would happen as each molecule interacted with the pore? How would this effect the retention time of each molecule?



Molecules of different sizes can be separated by this technique because of differential time spent inside a solid phase particle which excludes entrance of relatively larger molecules, allows some entrance of medium-sized molecules, and allows free accessibility of the smallest molecules. The particles contain pores with tunnels in which the size can be controlled depending on the size of molecules to be separated. Smaller molecules experience a more complex pathway (like a maze) to exit the particle than do larger molecules. Because molecules that have a large size compared to the pore size of the stationary phase have very little entrance into the pores, these larger sized molecules elute first from the column. Medium sized molecules are relatively large compared to the pore size of the solid phase and therefore may find some pores in which they enter and spend some time. Smaller-sized molecules have more pores that are accessible to them and therefore spend more time inside the pores relative to larger-sized molecules. Therefore, smaller molecules elute last and larger molecules elute first in Size Exclusion Chromatography.

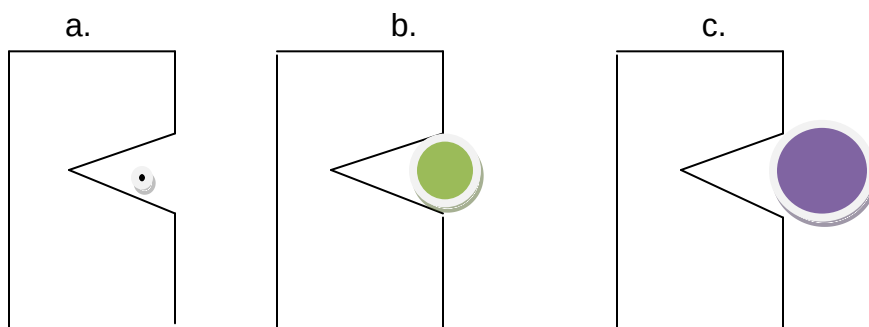


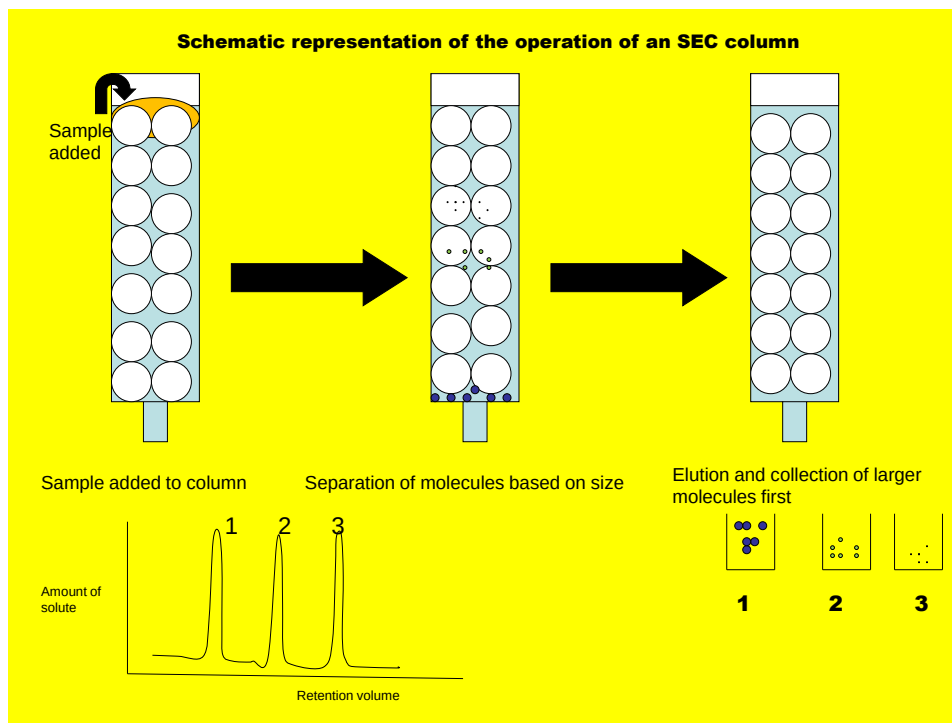
Figure depicting the separation of three molecules by Size Exclusion Chromatography (SEC):

a. The black molecule is much smaller in size compared to the pore size of the solid particle and has total access to the pores (not excluded).

b. The Green molecule is somewhat smaller in size compared to the pore size of the solid particle and has some access to the pores (partially excluded).

c. The purple molecule is larger in size than the pore size of the solid particle and does not have any access to the pores (totally excluded).

The figure below shows a schematic representation of the operation of a size exclusion chromatographic column:



The link below shows an animation of a SEC separation:

www.wiley.com/college/fob/quiz/quiz05/5-6.html

The Solid Phase Material:

Pore Size

Solid phase materials used in SEC are usually classified based on their ability to separate different sizes of proteins. Since size is a difficult item to accurately measure for a large molecule, the solid phase materials are identified with a molecular weight range instead and the weight is equated with size. All compounds with a molecular weight less than or equal to the lower number in the range will see the entire internal volume of the beads resulting in no selection and therefore no separation. All compounds with a molecular weight greater than or equal to the higher number in the range are completely excluded from the inside of a bead and therefore no separation is achieved. Molecules with weights or sizes between these two extremes of the range can be separated. This is the numerical pore size range reported for each solid phase material used in SEC. The pore size used for a separation is dependent on the size range of the particular set of molecules to be separated. Smaller pore sizes are used for rapid desalting of proteins or for protein purification. Intermediate pore sizes are used to separate relatively small proteins. Very large pore sizes are used for purification of biological complexes.

In-class Question:

5. Other chromatographic methods depend on specific interactions between the molecules being separated and the surface of the solid support. What effect might such specific interactions have on the method you came up with to separate glucose from immunoglobulins? Can you think of how you might limit specific interactions between the molecules and the surface?

Types of Material

Protein separations are generally performed using materials composed of dextrose, agarose, polyacrylamide, or silica which have different physical characteristics. Polymer combinations are also used. Ideally the materials will have no interaction with the proteins or maybe modified so that there is little interaction between the material and protein. The dextran sephadex is most commonly used in protein separations. Typical separation ranges that can be achieved using sephadex are given below. Molecules ranging from 100 to 600,000 Da can be separated depending on the type of sephadex chosen.

G10 (100-800Da), G15 (500-1500 Da), G25 (1000-5,000 Da), G50 (1,500-30,000 Da), G75 (3,000-80,000 Da), G100 (4,000-150,000 Da), G150 (5,000-300,000 Da), and G200 (5,000-600,000 Da).

Therefore, the solid phase packing material is selected based upon some knowledge of the size of the range of molecules to be separated.

Applications of SEC:

SEC is not a high resolution technique; generally the molecules to be separated must differ by at least two-fold in molecular weight. Therefore, SEC is useful for desalting or removal of small molecule contaminants from protein samples, determination of the solution subunit composition of a multimeric protein, and to isolate different multimers from each other.

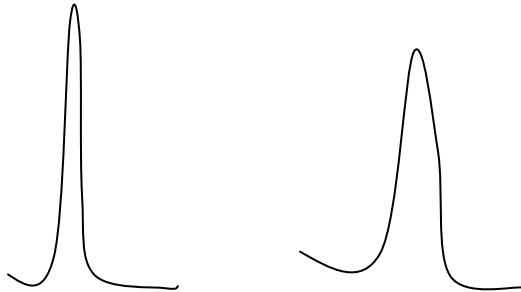
Size exclusion chromatography is generally used near the end of the purification process for a protein of interest, for example to desalt the protein or separate the correctly folded native protein from the denatured protein.

Other Important Aspects of SEC:

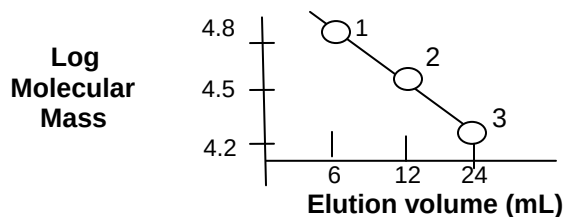
The ability to correctly pack a column is very important in obtaining the desired separation. Overpacking a column can lead to inaccessible pores and poor resolution. Underpacking a column can lead to surface area accessibility problems and cause poor resolution.

In-class Question:

6. Would you expect glucose or immunoglobulins to produce a broader peak profile when dissolved in buffer solution at a similar concentration? Which of the peaks below is most likely to belong to glucose? Which peak is most likely to belong to an immunoglobulin? Explain.

**Obtaining Size and Molecular Weight Data from SEC:**

Calibrations must be performed in order to get information about the size or molecular weight of a protein separated by SEC. Standard protein samples of known molecular size are separated on a SEC column and the retention volumes or elution volumes (i.e. the volume of eluting buffer necessary to remove a particular analyte from a packed column) are recorded. A calibration plot of log molecular mass (Y axis) versus elution volume (X axis) is prepared. The calibration plot can be used to estimate the molecular weight of a protein(s) by separating the protein(s) on the same column as the standards and recording its retention volume. The molecular weight can then be extrapolated from the calibration plot as shown below.



If standard proteins 1, 2, and 3 have molecular masses of 60,000, 30,000, and 15,000 Da, respectively, then an unknown protein which has an elution volume of 9mL will have an estimated log molecular mass of 4.7 and a molecular mass of 45,000 Da.

In-class Question:

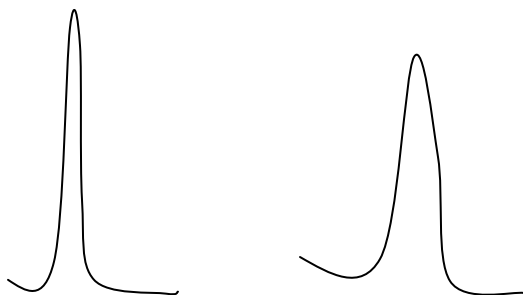
7. Would it be easier to separate glucose from an immunoglobulin or two immunoglobulins from each other? Explain.

Inquiry-based in class Questions: Designed to be presented to the students preceding the textual materials).

- (1) What is an obvious physical difference between an immunoglobulin (antibody), which is a protein, and glucose, which is a monosaccharide?
- (2) When would it be useful to separate immunoglobulins from glucose in blood?
- (3) How could you devise an experiment to separate immunoglobulins from glucose utilizing the physical difference between the molecules determined in (1)?
- (4) Depicted below are three different sized molecules. Next to them is shown a pore. What would happen as each molecule interacted with the pore? How would this effect the retention time of each molecule?



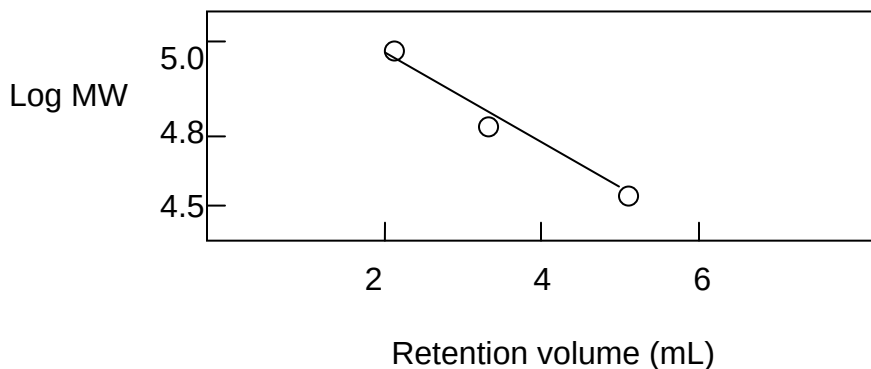
- (5) Other chromatographic methods depend on specific interactions between the molecules being separated and the surface of the solid support. What effect might such specific interactions have on the method you came up with to separate glucose from immunoglobulins? Can you think of how you might limit specific interactions between the molecules and the surface?
- (6) Would you expect glucose or immunoglobulins to produce a broader peak profile when dissolved in buffer solution at a similar concentration? Which of the peaks below is most likely to belong to glucose? Which peak is most likely to belong to an immunoglobulin? Explain.



- (7) Would it be easier to separate glucose from an immunoglobulin or two immunoglobulins from each other? Explain.

Out-of-class problem set:

- (1) Draw a chromatogram depicting separation of proteins 1, 2, and 3 (protein #1, mw 30,000, protein #2, mw 60,000, and protein #3, mw 90,000). Which type of packing material would you use to separate these proteins?
- (2) Indicate on the chromatogram in (1) where insulin would most likely elute. Would it have total accessibility, some accessibility or no accessibility to the particle pores on this column?
- (3) Referring to the size exclusion chromatogram in (1), Indicate on the chromatogram where you would expect a molecule with a size of 40,000 to elute. Would you expect the molecule to be well resolved, somewhat resolved, or not resolved from the peak for protein #1? Explain.
- (4) Use the SEC calibration plot shown below to estimate the molecular weight (MW) of an unknown protein with a retention volume of 3mL.



Instructor's Guide/Resource:

This module is designed to be used by instructors teaching undergraduate courses in which the concept of protein separation by size/shape is important (e.g. Biochemistry, Molecular biology, Biotechnology, Immunology, Bioanalytical chemistry, etc.). The students should have some basic knowledge of chemistry and biology. The in class activity portion of this module is designed to be completed by a small group of students in about a 1 hour time period.

Objectives:

After completing the module, the students will be able to:

- 1.Explain how size exclusion chromatography works
- 2.Illustrate (by drawing) the SEC separation process
- 3.Predict elution order of proteins
- 4.Interpret size exclusion chromatograms
- 5.Recognize when SEC would be useful
- 6.Estimate molecular mass using SEC

Inquiry-based in class questions:

(1) Most students will immediately answer this question correctly. They are aware that there is a large difference in size or mass between monosaccharides and proteins. If problems arise, the instructor may decide to ask them to find and compare the structures .

(2) Students will probably make the connection between glucose and diabetes. Therefore, they may say to get rid of the protein so that glucose can be measured in the blood. They should probably know what immunoglobulins do in the body. Guide them to think about what immunoglobulins are and what role they play in the body. Ask them if there may be a need for pure immunoglobulin (examples include use as a pharmaceutical or for development of an immunoassay).

(3) Students will know that small molecules move faster than larger molecules in solution. So, they may say something like put the sample in a flow system and the glucose molecules will move faster and can be separated first. Suggest to them what might happen if the sample is placed in a column which contains only buffer and the sample is added to the top and the solution allowed to flow through the column using a pump to continuously add buffer. They will most likely say that the glucose will separate out first. Now, draw some particles in the column with holes a little larger than glucose, but a lot smaller than an antibody. They will get that glucose can enter the holes (use the term pores) and that the antibody cannot. Now ask them what will happen to the rate at which glucose is able to exit (use elute) from the column. They will understand that glucose will be held up inside the pores. Now ask them what will happen to the antibody.

They will probably get that the antibody will not be held up much due to the presence of the particles and will elute before the glucose molecules. It is worth emphasizing at this point that SEC is a method that depends on the molecules having no attractive forces with the surface of the solid phase. Attractive forces, which are exploited in virtually all other chromatographic methods but SEC, could be different for glucose and an immunoglobulin and if present, could interfere with the separation scheme.

(4) The smallest molecule can diffuse through the entire pore. The largest molecule does not fit into any of the pore. The intermediate-sized one can only diffuse through part of the pores. Because of this, the smallest one should take the longest to come through the column. The largest one should come through fastest. Of course, this requires that the molecules have no attractive interaction with the surface of the solid particles.

(5) Dissolving the compounds being separated in an especially good solvent (one they are highly soluble in) and using a solid particle with a surface with properties very different from the nature of glucose and the proteins (so a non-polar surface) creates an environment where specific interactions with the surface can be eliminated.

(6) Students will generally know that larger molecules will produce broader peak profiles. So, they will most likely understand that peak 1 is most likely to belong to glucose and peak 2 is most likely to belong to an immunoglobulin.

(7) Students will probably not have too much difficulty in realizing that placing a smaller peak and larger peak in the same amount of space has less overlap (like resolution) than placing two larger peaks in the same amount of space. If there is any question, the instructor may have the students perform an exercise to illustrate this concept.

Out-of-class problem set:

(1) Students will refer to the schematic representation of the operation of an SEC column to draw this chromatogram. Ensure that they correctly show protein #1 eluting last and protein #3 eluting first. Based on the information in the module, sephadex G100 would be a good packing material.

(2) Students will look up the mass of insulin and realize that it is a lot smaller in mass (~6000 Da) than the other proteins depicted on the chromatogram, and therefore it will elute a lot later than other proteins depicted. Based on use of G100, insulin would have some accessibility to the pores.

(3) They will understand that for peaks to be well separated (resolved), there must be a two-fold difference in molecular mass. So, a peak of 40,000 Da eluting between proteins 1 and 2 may not be completely separated.

(4) Students should be able to extrapolate the corresponding Log MW associated with 3 mL retention volume:

Retention volume = 3mL yields a corresponding Log MW ~ 4.8
MW ~ 60,000Da

References

Snyder, L.R.; Kirkland, J.J.; Glajch, J.L.; *Practical HPLC Method Development*; Wiley : New York (1997).

<http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/E/ExclusionChrom.html>

<http://www.impactanalytical.com/applications/sec.aspx>

<http://faculty.tamu-commerce.edu/xli/files/541-19.pdf>