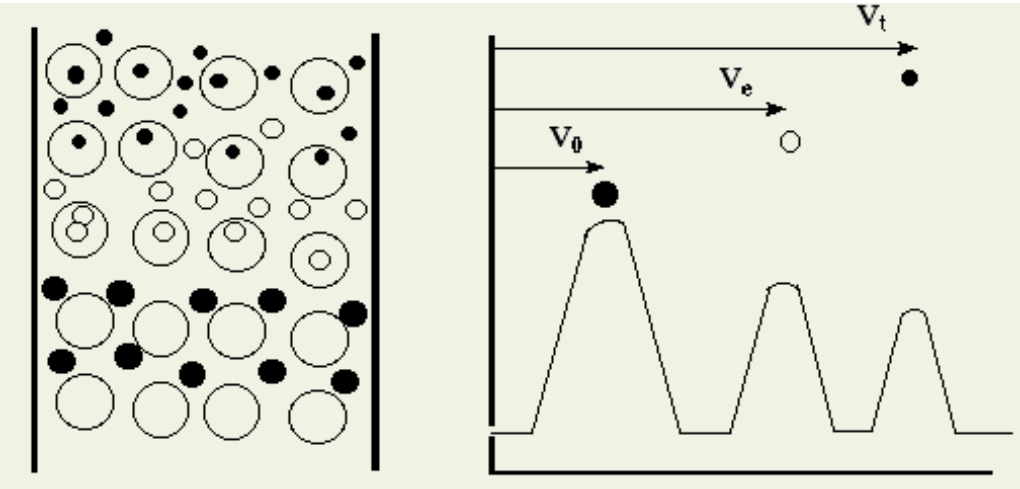
# Experiment #4
# Gel Filtration Chromatography

## Introduction

Gel-filtration chromatography is a form of column chromatography in which molecules are separated on the basis of their molecular mass or, more properly, their Stokes radius. The Stokes radius is the effective radius a molecule has as it tumbles rapidly in solution. A long, extended molecule has a larger Stokes radius than a compact molecule of the same molecular mass. The stationary phase in gel-filtration chromatography consists of fine beads that contain pores of controlled size. The space between the beads is referred to as the void space ($V_0$). The space within the beads is referred to as $V_i$. The inert volume of the stationary phase is referred to as $V_g$. The mobile phase fills all of the space between the beads and within the beads. The chemical and physical properties of both the stationary and mobile phases in gel-filtration are chosen to prevent, as nearly as possible, interactions of proteins with the beads that are due to properties other than molecular size. This is in direct contrast to other forms of chromatography in which interactions with the stationary phase provide the basis of separation.

The sample is applied in a narrow band at the top of the column and then it is washed through the column by the mobile phase. Large molecules in the sample that cannot pass through the pores of the beads are excluded from the beads and are restricted to the outer voids. They elute from the column after an amount of the mobile phase equal to $V_0$ has passed through the column. Molecules that are much smaller than the pores equilibrate with the entire liquid volume and elute at a volume equal to $V_t$, which is the sum of $V_0$ and $V_i$. Molecules that are small enough to pass through some of the pores of the beads, however, elute at various volumes ($V_e$), depending on how small they are and what fraction of the pores of the beads are accessible to them. Such molecules are referred to as partially included. Molecules in the sample can be separated in order of their size by collecting fractions as the mobile phase is eluted through the column, with the largest molecules eluting first and the smallest last.

The figure below represents the principle of gel-filtration chromatography.




A partition coefficient can be calculated from the values above using the equation $K_{av}$ =

$(V_e-V_0)/(V_t-V_0)$.

A semilogarithmic plot of the relationship of $K_{av}$ to molecular weight can elucidate the efficiency of separation of molecules. Assuming that all molecules are of similar shape, the separation of molecules on the basis of molecular weight will be greatest in the linear range of the curve.

In this experiment you will prepare your own Sephadex G-75 column and separate three substances based upon molecular weight. You will be given a mixture containing three colored molecules: blue dextran (MW> 100,000 Da), cytochrome c (a red protein, MW = 12,400 Da), and an unknown.

## Experiment

Obtain a column from your TA and pack it using the guidelines below using 50 mM Tris, pH 8.0 buffer. You should pack a height of 20 cm or more to get an efficient separation of your sample. Once the column is packed, equilibrate with 40ml of buffer.

Following are some important guidelines for preparing the gel-filtration chromatography columns:

- The column should be vertical. After setting up the column, confirm this by looking at it from two directions.
- Prevent air bubbles from forming in the column bed, and pack the matrix material uniformly with no troughs or channels. The bed material should never be allowed to go dry. If the column has bubbles or does go dry it should be repacked.
- Choose a column of appropriate size for the application. The volume of the column should be 20 to 50 fold that of the sample to be applied. Usually tall thin columns with lengths 20-40 times their diameter are used.
- Try to maintain a constant temperature without any drafts, so that turbulence or thermal gradients do not effect the flow.
- When packing the column, fill the column about one third full with elution buffer. Open the valve at the bottom of the column so the liquid starts to drip out. Then, using a pipette, add a dilute slurry of the column matrix material to the top of the column while stirring gently. Keep adding a slurry to the column while gently stirring intermittently. The goal is to produce a uniform bed of desired height using a minimal amount of Sephadex G-75. After the desired height has been reached, equilibrate the column with several column volume of elution buffer.
- The sample should be applied in a narrow band at the top of the column. At some point stop adding buffer to the column and allow the buffer to flow until it reaches the top of the bed. Stop the flow by closing the valve, and gently add the sample against the wall of the column without disturbing the bed. Once added, open the valve and let sample flow into the bed. Now, close the valve and gently add a small amount of elution buffer using the same technique you used for sample loading. Repeat this procedure with buffer and finally add a larger quantity of buffer without disturbing the bed.

After equilibration, use 0.5 ml of the sample provided by your TA and add it to the column as indicated in the guidelines. You are now ready to elute the fractions using your elution buffer (50mM Tris, pH 8.0). Collect 2 ml fractions in small test tubes, until all three samples have eluted. You can measure 2 ml of distilled water into a test tube and use it as a reference for fraction collection. Measure the absorbance of each fraction at 550 nm and 450 nm. Be sure to zero the spectrophotometer with elution buffer for each wavelength.

## Data Analysis

You should create 2 plots. Plot the absorbance at each wavelength versus collected volume on the same graph. This will elucidate the elution profile of each of the known components of your mixture. You should also comment on the elution profile of your unknown. Also, give a rough estimate of the unknown protein's molecular weight.