**CHEM 132 Lab #6**

**Chromatographic Techniques: Ion Exchange Chromatography and Gel Filtration**

# Introduction

Chromatography of both small molecules and macromolecules is widespread in biochemistry. Two of the most common techniques used for the separation of macromolecules are size exclusion chromatography (SEC, also called gel filtration) and ion exchange chromatography (IEC).

**SEC separates molecules on the basis of their size.** Formally, SEC is a separation based on hydrodynamic radius; however, for similarly shaped molecules hydrodynamic radius is proportional to molecular weight. Therefore we generally talk about SEC as a mass based separation, even though this is not strictly true. Many different chromatographic materials for SEC are available. Each material is characterized by an “exclusion limit” which specifies an approximate upper limit for the size of molecules able to be separated using the material. Separation in SEC is based on the partitioning of molecules between the bulk fluid between the beads of chromatographic media and the aqueous space with the pore of these beads. The smaller the molecule the larger fraction of the total volume it “sees” and the slower it moves through the column. Thus, **the largest molecules elute first while the smallest elute last**.

SEC separations are performed by placing the appropriate chromatographic medium in a long thin column. The sample is introduced at the top of the column and material eluted by flowing an appropriate buffer through the column. SEC columns are characterized by two parameters, the void volume (VO) and the total volume (VT). VO is essentially the volume of the space between the “beads” of chromatographic medium; molecules larger than the exclusion limit elute in the VO. VT is the volume of all of the liquid within column (i.e. both within the porous beads, as well as between them). The smallest molecules appear in the VT.

**IEC separations are based on molecular charge.** Chromatographic materials for the separation of both cations (cation exchange media) and anions (anion exchange media) are available. A cation exchange medium contains anionic functional groups while an anion exchange medium is cationic. Thus **these materials bind molecules with a charge opposite to their own, while allowing similarly charged molecules to pass through. Changing the composition of the buffer elutes materials bound to the column.** In some cases the ionic strength of the buffer is increased, in others, the pH is changed in order to elute material from the column.

In this experiment you will be given a mixture of four colored compounds that vary in their size and charge, a number of different chromatographic media and an assortment of solutions for eluting columns. Your task is to devise a method for the separation of all four components by performing a number of trial separations using test mixtures of the compounds.

# Materials

## **Samples**

Note: Use 200 µL of these samples for each column you run.

Trial Mixture #1: blue dextran (3 mg/mL), cytochrome C (3 mg/mL), DNP-glycine (1 mg/mL)

Trial Mixture #2: blue dextran (3mg/mL), cytochrome C (3 mg/mL), vitamin B12 (0.2 mg/mL)

## **Chromatographic Media**

Sephadex G25, equilibrated in 0.1 M KAc, pH 6

This SEC medium has an exclusion limit of about 25,000 Da. Use this medium in a column with a bed height of about 12 cm.

CM-Sephadex, equilibrated in 0.1 M KAc, pH 6

This ion exchange medium contains carboxymethyl [-CH2COO-] groups **for binding cations**. It can be eluted by using KAc, pH 6 buffers of increasing concentration.

DEAE-Cellulose, equilibrated in 0.1 M KAc, pH 6

This ion exchange medium contains diethylaminoethyl [-CH2CH2N+H(CH2CH3)2] groups **for binding anions**. It can be eluted by using KAc, pH 6 buffers of increasing concentration. Solutions of HCl (1.0 and 6.0 M) can also be used to elute compounds that remain bound at pH 6.

## **Eluting Solutions**

* 1. M potassium acetate (KAc), pH 6

Use for elution of the SEC column and as the initial buffer for IEC columns.

The following solutions are for elution of IEC columns. They are listed in order of increasing elution power. Use HCl only on DEAE-cellulose.

0.5 M KAc, pH 6

1.0 M KAc, pH 6

1.0 M HCl

6.0 M HCl

**Methods**

General Procedure

1. Examine the components of each trial mixture. Devise a plan to separate the individual components based on their molecular weights and ionic characters.
2. Based on your plan, load 200 µl of the sample onto the respective column.
3. Collect the fractions as they elute off the column.
4. Feel free to experiment through trial and error. Use the two test mixtures to determine the chromatographic properties of the various compounds on the various media.
5. Use this information to devise a separation procedure involving the use of two different columns that will result in the separation of all four compounds.
6. Hand in (before you leave the lab) a Flowchart that describes how you could separate a single sample mixture that had all 4 components in it.
7. Hand in (before you leave the lab) 4 test tubes, each containing (hopefully!) a single component of the original sample.

Helpful Hints:

1. Collect fractions of equal volume (1 mL is recommended) while eluting columns during the trial phase. During the separation of the “real” sample, act as a “smart” fraction collector and try to collect each component of the mixture in a single test tube.
2. Remember how colors combine (e.g. **purple** = red + blue ; **green** = yellow + blue) and the effect of dilution on color (e.g. red diluted out = **pink**) and make careful observations of the colors of the various fractions.
3. After finishing an IEC experiment, remember to re-equilibrate the column back to its original state before running another sample on it. Re-equilibration is accomplished by running 3 to 5 column volumes of “starting” buffer (0.1 M KAc, pH 6 in this case) through the column.
4. Properties of the compounds to be separated:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Compound** | **Color** | **MW (Da.)** | **Ionic Character at pH 6** |
|  | Blue dextran | Blue | > 500,000 | Very strong anion |
|  | Cytochrome C | Red-orange |  12,400 | Strong cation |
|  | Vitamin B12 | Cherry red |  1,357 | Weak cation |
|  | DNP\*-glycine | Yellow |  241 | Weak anion |
|  |  |  |  |  |

 \*DNP = dinitrophenyl

**Data Sheet**: Chromatography

1. Draw the flow chart that describes how you separate a single sample mixture that had all 4 components in it.

**Pre-lab**: Chromatography

1. What is similar and what is different between SEC and IEC?