

## GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography is a technique which separates molecules on the basis of their physical dimensions. The chromatography medium consists of swollen gel beads of controlled pore size. The beads are produced by dispersing an aqueous solution of dextran (a polymer of glucose units linked  $\alpha$ -1,6) in an immiscible organic solvent. The dispersed droplets are in the form of tiny spheres. If epichlorohydrin is added to this dispersion, cross links are formed between adjacent strands of polymer. This "locks" the glucose strands into one giant molecule whose shape when fully hydrated resembles that of the aqueous droplet in which the dextran is currently dissolved. The average size of the pores (which are the "holes" defined by the now linked strands) can easily be controlled by the amount of epichlorohydrin added: more crosslinker gives smaller holes; less gives larger holes.

The beads so produced are not soluble, due to their great size, but are still very hydrophilic due to the large number of exposed -OH groups. Because of this they avidly take up water to the maximum extent permitted by the degree of crosslinking, and assume spherical shapes in aqueous media.

A chromatographic column packed uniformly with beads of a given pore size presents two distinct environments to molecules percolating through the bed:

(a) a moving phase, consisting of the solution outside the beads that moves in response to any pressure difference across the length of the column, and

(b) a stationary phase, which is the solution found inside the beads. It doesn't move since the strands composing the bead offer too much resistance to straight line flow. The only motion possible inside the bead is that due to diffusion, which occurs randomly in all directions.

Passage of dissolved molecules between the two phases is controlled by the relative sizes (hydrodynamic radii) of

(a) the dissolved molecules and

(b) the pores in the walls of the beads.

Molecules smaller than the pores enter and exit the beads freely, their motion caused by thermal agitation (diffusion). As long as the molecules are inside the beads, they are in the stationary phase and so do not migrate along the column (but they do spread out due to diffusion). Naturally, if they exit the bead they become entrained in the moving phase and are swept along just like everything else. Because they spend some time "motionless" inside the beads, small molecules take a relatively long time to pass through the column. Molecules larger than the pores cannot enter the beads, so are always in the moving phase. Thus they pass through the column relatively rapidly. Molecules whose hydrodynamic radius is the same as or somewhat smaller than that of the pores will bounce off the edges of the pores frequently, but also have a finite chance of entering. The chance of entering increases as the molecule becomes smaller compared to the pore. Thus sorting occurs depending on hydrodynamic radius: the largest molecules appear first in the elution, followed by smaller and smaller molecules. Beads of a single pore size can sort only a limited range of molecule sizes, so a variety of pore sizes is available on the market. One chooses the pore size suited to the range of particle radii that need to be separated.

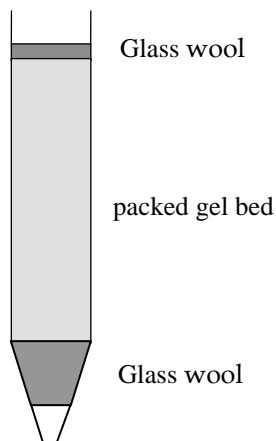
The physics of the sorting process has been described mathematically. There are two limiting cases, each measured from the point at which a sample of solute is loaded onto the top of the column:

(a) completely excluded solutes emerge when an amount of solvent equal to about 30% of the packed bed volume has passed through the column;

(b) solutes that enter beads freely behave as if the beads were not there at all, and so emerge when an amount of solvent equal to the bed volume has passed through.

The objectives of this experiment are to learn how to pour and use a gel-filtration column to separate solutes of different sizes and to quantitate input and recovery.

## EXPERIMENTAL SECTION



1. A disposable pipet will serve as the chromatography column. Put a small wad of glass wool, tamped firmly but not too tightly, at the bottom. Try to get a flat surface. Fill the column about half full of water and check the flow (want ~2 drips per sec - can be slowed by tamping glass wool more firmly). Temporarily stop the flow.

2. Swirl the Sephadex suspension and add it to the column while allowing the water to flow again until the volume of the settled gel is about 10 mL. Suck off clear liquid as gel settles and replace with more gel suspension. **AVOID AIR BUBBLES!** Put a small flat layer of glass wool on top.

3. Allow the surface of the glass wool to just barely dry – **DO NOT LET AIR ENTER THE BED!** – then stop the flow (you can lay the column on its side).

4. Fill 25 small test tubes with 3.0 ml of water each and set the rack aside. This can be done while packing the column.

5. Measure into a small test tube 0.90 ml of the Blue Dextran solution and 0.30 ml of the bromophenol blue solution. Mix well.
6. Put an empty 10 mL graduated cylinder under the column outlet to catch drops temporarily. Carefully load 0.50 ml of your blue mixture onto the dry surface of the column. Allow the sample to run into the gel until the surface is again barely dry, then wash in (rinse around the sides of the column) with a **few drops** of water. **Let these run in.** Repeat with another few drops of water, letting them run in, then carefully fill the column with water to the top. Keep adding water as necessary to maintain flow.
7. While the colored molecules are migrating down the column, determine the number of drops in 1.0 mL using a graduated cylinder. Collect effluent in the graduated cylinder until just before the first blue band starts to emerge. At this point change to the test tube rack and begin collecting 6-drop fractions into your 25 test tubes. Collect fractions of 6 drops until all the trailing blue band is eluted.

*(If you wanted to be able to reproduce this run many times and/or know the exact elution volume of each species, you would start collecting fractions in test tubes as you began to load the column. We don't do this here because it would use up too many test tubes.)*

8. Mix each collected fraction well, then read its absorbance at 590 nm (use water as your blank). Save each fraction in its original tube if possible. If reading is  $> 1.1$ , dilute appropriately; record how you made the dilution, and keep all such dilutions.
9. By trial and error, prepare separate dilutions of Blue Dextran and bromophenol blue stock solutions so as to give individual  $A_{590}$  between 0.4 and 0.9. Record how you made these dilutions and the absorbance value for each.
10. Combine (pool) the fractions containing Blue Dextran in a graduated cylinder. *Water clings to glass and drips down the outside of the test tube after you pour, so touch the lip of each tube to the cylinder before returning it to the rack. You'll get the best results if you transfer the last bits with a Pasteur pipette.* Mix cylinder contents well, and record the pool volume and its  $A_{590}$ . Repeat for the bromophenol blue fractions. (If you diluted any fractions, thoroughly mix the saved dilutions with the appropriate pool before measuring.)

11. Your report should contain:

- 1) identification of the kind of Sephadex used and the dimensions of the column
- 2) a plot of  $A_{590}$  vs. fraction number, with fractions that you pooled clearly shown
- 3) amounts of Blue Dextran and bromophenol blue loaded ( $A_{590} \times$  volume loaded). *Caution: what volumes of Blue Dextran and of bromophenol blue did you actually load? What values must you use as the  $A_{590}$  for each?*
- 4) amounts of (a) Blue Dextran and (b) bromophenol blue recovered: either

**[ $A_{590}$  of pool] x [ml of pool]**

OR

**[sum of  $A_{590}$  for all fractions containing desired compound] x [volume of one fraction]**

*Note that the volume of one fraction = 3.0 mL + volume of 6 drops.*

- 5) percent recovery of each compound

Discuss your results. If recoveries were low, suggest reasons which might account for this. *Keep in mind that nothing gets trapped inside gel beads (unless it physically precipitates). What did you observe that verifies this claim?*