14th Week

25-3 Molecular Exclusion Chromatography

In **molecular exclusion chromatography** (also called *size exclusion* or **gel filtration** or *gel permeation chromatography*), molecules are separated according to size.[11] Small molecules penetrate the pores in the stationary phase, but large molecules do not (Figure 22-6). Because small molecules must pass through an effectively larger volume, *large molecules are eluted first* (Figure 25-14). This technique is widely used in biochemistry to purify macromolecules.

Salts of low molecular mass (or any small molecule) can be removed from solutions of large molecules by gel filtration because the large molecules are eluted first. This technique, called *desalting*, is useful for changing the buffer composition of a macromolecule solution.

Large molecules pass through the column faster than small molecules do.

Gel filtration: usually refers to a hydrophilic stationary phase and aqueous eluent

Gel permeation: usually refers to hydrophobic stationary phase and organic eluent

The Elution Equation

The *total* volume of mobile phase in a chromatography column is V_m, which includes solvent inside and outside the gel particles. The volume of mobile phase *outside* the gel particles is called the **void volume**, V_o. The volume of solvent *inside* the gel is therefore $V_m - V_o$. The quantity K_{av} (read "K average") is defined as

$$K_{av} = \frac{V_r - V_o}{V_m - V_o} \qquad (25\text{-}6)$$

In pure molecular exclusion, all molecules are eluted between $K_{av} = 0$ and $K_{av} = 1$.

where V_r is the retention volume for a solute. For a large molecule that does not penetrate the gel, $V_r = V_o$, and $K_{av} = 0$. For a small molecule that freely penetrates the gel, $V_r = V_m$, and $K_{av} = 1$. Molecules of intermediate size penetrate some gel pores, but not others, so K_{av} is between 0 and 1. Ideally, gel penetration is the only mechanism by which molecules are retained in this type of chromatography. In fact, there is always some adsorption, so K_{av} can be greater than 1.

Void volume is measured by passing a large, inert molecule through the column.[12] Its elution volume is defined as V_o. Blue Dextran 2000, a blue dye of molecular mass 2×10^6, is commonly used for this purpose. The volume V_m can be calculated from the measured column bed volume per gram of dry gel. For example, 1 g of dry Sephadex G-100 occupies 15 to 20 mL, when swollen with aqueous solution. The solid phase of the swollen gel occupies only ~1 mL, so V_m is 14 to 19 mL, or 93–95% of the total column volume. Equal masses of different solid phases produce widely varying volumes when swollen with solvent.

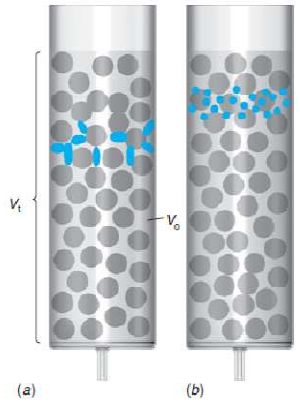

FIGURE 25-14 (*a*) Large molecules cannot penetrate the pores of the stationary phase. They are eluted by a volume of solvent equal to the volume of mobile phase. (*b*) Small molecules, which can be found inside or outside the gel, require a larger volume for elution. V_t is the total column volume occupied by gel plus solvent. V_o is the volume of solvent outside the gel particles. V_m is the total volume of solvent inside and outside the gel particles.

Stationary Phase[13]

Gels for open-column, preparative-scale molecular exclusion include Sephadex (Table 25-4), whose structure was given in Figure 25-2, and Bio-Gel P, which is a polyacrylamide cross-linked by *N,N'*-methylenebisacrylamide (Figure 25-15). The smallest pore sizes in highly cross-linked gels exclude molecules with a molecular mass ≥700, whereas the largest pore sizes exclude molecules with molecular mass $\geq 10^8$. The finer the particle size of the gel, the greater the resolution and the slower the flow rate of the column. Hydrophilic HPLC packings for molecular exclusion are made of poly(vinyl alcohol), polyacrylamide, and sulfonated polystyrene. Silica (Table 25-4) with controlled pore size provides 10 000–16 000 plates per meter. The silica is coated with a hydrophilic phase to minimize solute adsorption. A hydroxylated polyether resin with a well-defined pore size can be used over the pH range 2–12, whereas silica phases generally cannot be used above pH 8. Particles with different pore sizes can be mixed to give a wider molecular size separation range.

TABLE 25-4 Representative molecular exclusion media

Gel filtration in open columns		TSK SW silica for HPLC		
Name	Fractionation range for globular proteins (Da)	Name	Pore size (nm)	Fractionation range for globular proteins (Da)
Sephadex G-10	to 700	G2000SW	13	500–60 000
Sephadex G-25	1 000–5 000	G3000SW	24	1 000–300 000
Sephadex G-50	1 500–30 000	G4000SW	45	5 000–1 000 000
Sephadex G-75	3 000–80 000	G5000SW	100	>1 500 000
Sephadex G-100	4 000–150 000			
Sephadex G-200	5 000–600 000			

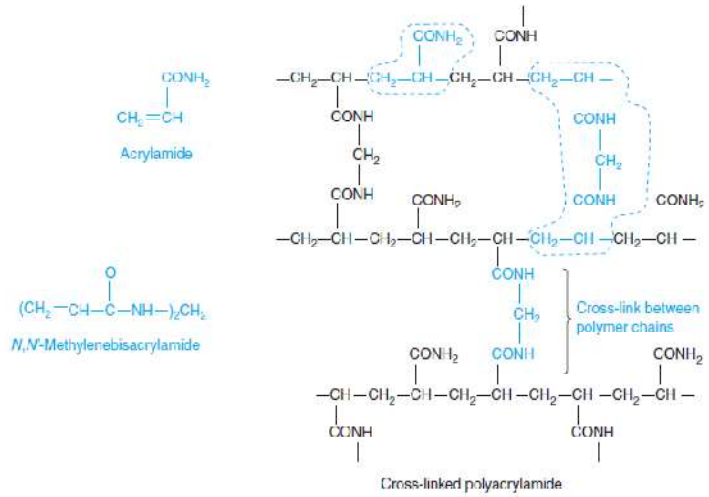

FIGURE 25-15 Structure of polyacrylamide.

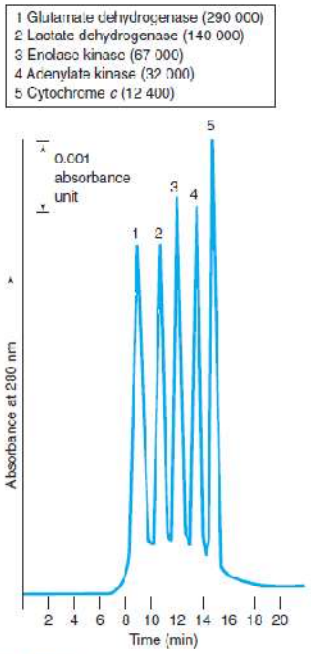

FIGURE 25-16 Separation of proteins by molecular exclusion chromatography with TSK 3000SW column. [Courtesy Varian Associates, Palo Alto, CA.]

For HPLC of hydrophobic polymers, cross-linked polystyrene spheres are available, with pore sizes ranging from 5 nm up to hundreds of nanometers. Particles with a 5-μm diameter yield up to 80 000 plates per meter of column length.

Molecular Mass Determination

Gel filtration is used mainly to separate molecules of significantly different molecular sizes (Figure 25-16). For each stationary phase, we construct a calibration curve, which is a graph of log(molecular mass) versus elution volume (Figure 25-17). We estimate the molecular mass of an unknown by comparing its elution volume with those of standards. We must exercise caution in interpreting results, however, because molecules with the same molecular mass but different shapes exhibit different elution characteristics. For proteins, it is important to use an ionic strength high enough (>0.05 M) to eliminate electrostatic adsorption of solute by occasional charged sites on the gel.

Nanoparticles can be separated by molecular exclusion chromatography just as proteins are separated. Figure 25-18 shows the relation between measured size and retention time of

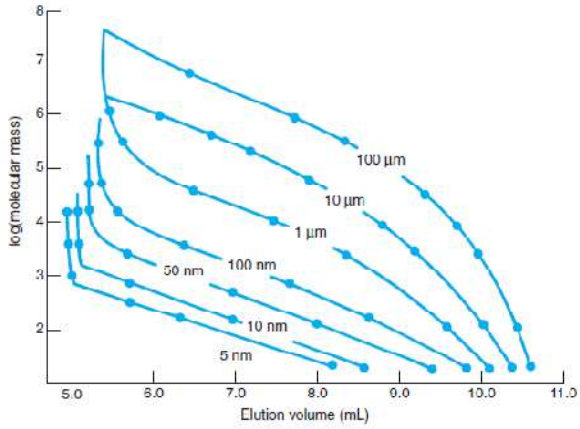

FIGURE 25-17 Molecular mass calibration graph for polystyrene on Beckman μSpherogel molecular exclusion column (0.77 × 30 cm). Resin pore size labeled on the lines ranges from 5 nm to 100 μm. [Courtesy Anspec Co., Ann Arbor, MI.]

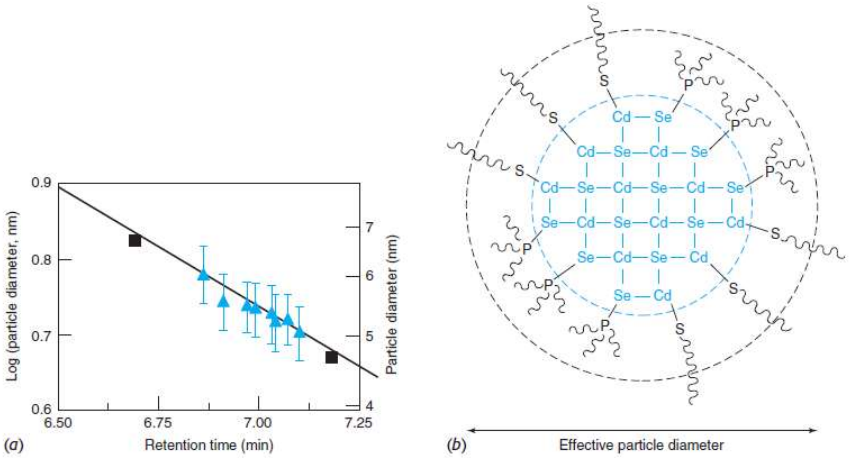

FIGURE 25-18 Larger CdSe quantum dots are eluted before smaller quantum dots by 0.1 M trioctylphosphine in toluene at 1.0 mL/min in size exclusion chromatography on a 7.5 × 300 mm cross-linked polystyrene column of Polymer Labs PLgel 5 μm with 100-nm pore size. Triangles are CdSe, and squares are polystyrene calibration standards. The size of the CdSe core was measured with a transmission electron microscope, and the length of 1-dodecanethiol endcaps (0.123 nm) was added to the radius. [Data from K. M. Krueger, A. M. Al-Somali, J. C. Falkner, and V. L. Colvin, "Characterization of Nanocrystalline CdSe by Size Exclusion Chromatography," *Anal. Chem.* **2005**, *77*, 3511.]

CdSe *quantum dots*. These are particles containing ~2 000 CdSe units in a dense, crystalline core capped by alkyl thiol (RS) groups on Cd and trialkylphosphine (R_3P) groups on Se. Quantum dots are useful because the wavelength of their visible emission depends on their size. Size is controlled during synthesis of the quantum dot by reaction time or other conditions. Quantum dots with different sizes can be used as spectroscopic labels in biological experiments.[14]