ENCYCLOPEDIA OF BIOPROCESS TECHNOLOGY: FERMENTATION, BIOCATALYSIS, AND BIOSEPARATION

VOLUME 2

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VOLUME 2

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ENCYCLOPEDIA OF BIOPROCESS TECHNOLOGY: FERMENTATION, BIOCATALYSIS, AND BIOSEPARATION

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OUTLINE

INTRODUCTION

Size-exclusion chromatography (SEC) is a widely used means for separation of macromolecules of different sizes. Porath and Flodin in 1959 first demonstrated the use of SEC, or gel filtration chromatography, for separations by resolving glucose from two size fractions of dextrans (1). In addition, they applied the technique to desalting of serum proteins. Remarkably, these two applications—crude separations and desalting—remain the primary uses of SEC today. A wide range of materials, including sugars, proteins, and nucleic acids, have been analyzed or separated using this technique. In addition, SEC is used extensively in the characterization of polymers. However, in this article, the emphases are on analysis and on separations involving biological macromolecules.

Basic Principle

The essential principle of size exclusion chromatography is partitioning of species on the basis of preferential sieving into the pores of support particles. Sample is applied to the top of a bed packed with porous stationary phase particles. Elution results from flow of a fixed composition buffer through the bed (isocratic elution), in contrast with other modes of chromatography in which the buffer composition is varied (gradient elution). Molecules that are larger than the pores of the support access only the interstitial space between particles and therefore pass through the column quickly, whereas smaller particles access the volume within the pores and are retained for a longer time by the column (Fig. 1). Therefore, a support must be selected with a pore size distribution so that there exists a differential partitioning of the molecules to be separated.

Lexicon

In any chromatographic process, sample is injected into the column and eluted by continuous flow of a buffer through the column. The time between injection and the peak of any eluted solute is termed its retention time (t_R). An equivalent measure of retention is the retention volume (V_R), which, for the usual case of constant flow rate, is merely the product of the flow rate and retention time. The terms *elution time* and *elution volume* are used interchangeably with *retention time* and *retention volume*. For solute molecules larger than the size of the pores, the elution volume represents the volume of the column between the support particles plus that of extracolumn tubing between the injection loop and the column and from the column to the detector. This is known as the void volume (V_0), and it is frequently estimated using large, inert tracer solutes. The pore volume (V_p) is the volume within the particles occupied by fluid and, in principle, accessed entirely by very small particles, for which the retention volume is maximized:

$$V_{R,max} = V_0 + V_p \tag{1}$$

In general, the elution volume is intermediate between the void volume and the sum of the void and pore volumes and thus defines a distribution coefficient (K_d) through the relation

$$V_R = V_0 + K_d V_p \tag{2}$$

By definition, the distribution coefficient assumes values only between zero and unity. The other relevant volume is that of the support particles or chromatographic matrix (V_m). In principle, one can calculate the pore volume based on the amount and density of the support through the relation

$$V_p = \frac{W_r \rho_{gel}}{1 + W_r \rho_{H_2O}} (V_t - V_0) \tag{3}$$

where V_t is the total volume (sum of void, pore, and matrix volumes, the first corrected for the volume of extracolumn tubing), ρ represents mass density, and W_r is the water

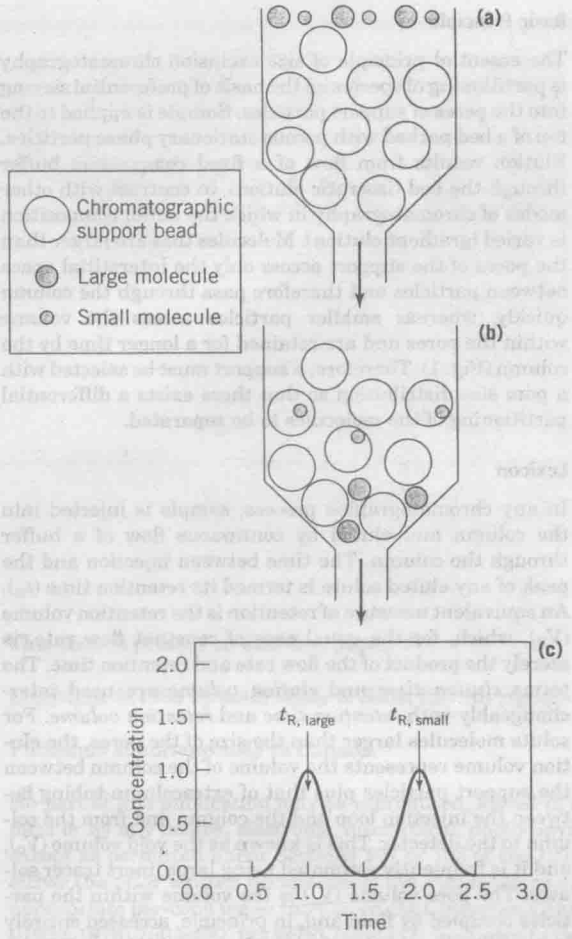

Figure 1. Principle of SEC. (**a**) A mixture of large and small particles is introduced together into the top of the column. (**b**) As the particles move down the column, the small particles are able to access the volume within the pores of the particles. Therefore, the larger particles are eluted from the column before the smaller ones. (**c**) Schematic chromatogram showing the elution profiles of the large and small particles.

regain of gel (grams of water per gram of dry gel divided by the density of water). Experimentally, V_p can be measured as the retention volume of a small molecule that does not interact with the support. With determination of V_0 and V_p, the distribution coefficient of any solute can be determined from its retention and equation 2; conversely, the retention time of a solute could be predicted from a model for its distribution coefficient (see "Process Variables") and from the column parameters.

The typical chromatographic peak is Gaussian in shape. The quality of separation of two (or more) species in SEC is characterized by differences in their retention times and by certain measures of the distribution of material in each peak about the mean. The resolution (R_s) is a measure of separation of two bands, defined by

$$R_s = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_2 - w_1)} \tag{4}$$

where w_i is the baseline width of peak i, related to the standard deviation of the Gaussian peak through $w_i = 4\sigma_i$, and t_{Ri} is the retention time of the ith peak. Another measure of separation is the selectivity, α, between two bands. It is defined by

$$\alpha = \frac{k'_2}{k'_1} \tag{5}$$

where k′ of species i is its retention factor (or capacity factor). The retention factor is a relative retention time defined as

$$k' = \frac{t_R - t_0}{t_0} = \frac{V_R - V_0}{V_0} \tag{6}$$

For non-Gaussian bands, t_R and σ can be determined via statistical analysis of the chromatogram from the first and second moments, respectively, of a trace.

MATERIALS

Equipment

A typical laboratory setup for SEC is depicted in Figure 2. The equipment required includes solvent and reservoir, valves and tubing, pump, packed column, detector, fraction collector, and recording device. For the most part, this equipment is universal to the various modes of chromatography. The precolumn setup for SEC, especially at low pressure, is relatively straightforward. Solvent delivery is simpler in SEC than for other modes of chromatography because there is no need for gradient formation and the only pump requirement is for stable flow. Peristaltic pumps are adequate for low-pressure work, whereas piston or diaphragm pumps are used for high pressures (2).

The most important aspect of the equipment is the column packing. The gel must be packed uniformly throughout the column to avoid channeling or other heterogeneous effects that act to blur separation of the solutes. Frequently, the gel is supplied as a powder or as a suspension in a storage solvent. In the latter case, this solvent is filtered off, and in both cases the gel is then suspended in a packing solvent, which is usually an aqueous buffer with some NaCl added. A stable, uniform packing is attained by degassing the gel slurry before packing, pouring it at once, and then packing at a flow rate greater than used in normal operation.

Detection of proteins and nucleic acids is most commonly accomplished with UV absorbance detectors, although more sensitive techniques such as light-scattering and mass spectrometry are also possible (3). The detection limit (C^*) is dictated by the absorbance sensitivity (A^*) of the instrument, the extinction coefficient (ϵ) of the solute, and the path length (L) of the detector, according to

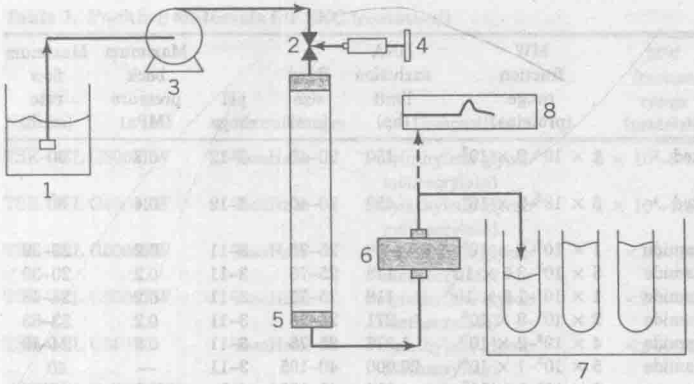

Figure 2. Laboratory SEC setup. Solvent (*1*) is pumped through a three-way valve (*2*) by a peristaltic pump (*3*). The desired volume of sample is introduced by syringe (*4*) through one port of the valve while the solvent flow is temporarily blocked. The valve is repositioned to allow elution of the sample though the packed column (*5*). The eluting solution is monitored by a detector (*6*) and collected in a fraction collector (*7*). A permanent record of the chromatogram is obtained from a recorder (*8*) or computer.

$$C^* > \frac{A^*}{\epsilon l} \qquad (7)$$

The sensitivity of absorbance detectors is usually about 5×10^{-4}, and their path lengths can be up to 1 cm. Typical proteins have extinction coefficients on the order of 1 cm^2/mg; therefore, the detection limit is on the order of 1 μg/mL. Nucleic acids exhibit strong absorbance, peaking around 260 nm, with extinction coefficients on the order of 25 to 50 cm^2/mg; consequently, lower concentrations down to 20 ng/mL may be detected. Carbohydrates, on the other hand, exhibit no significant absorbance in UV or visible light; refractive index detectors are often used for their quantitation.

Packing Materials

A wide variety of packing materials are available for use in SEC. The choice of gel for a particular application depends on a number of factors, including materials compatibility, operating conditions, and particularly the sizes of the molecules to be separated. Manufacturers typically provide a good deal of information about their packing materials, including fractionation range, maximum flow rate or pressure drop, and stable operating ranges of pH and temperature.

Properties. The key feature of the packing material with respect to a particular separation is the pore size distribution, because this determines the range of particles that may be fractionated. Very small pores are required for desalting applications, so that all macromolecules are excluded and low molecular weight species are retained. Materials possessing much larger pores are used for analysis and purification of biological macromolecules. Because the pore sizes are normally inferred from the sieving of test solutes, the useful molecular weight fractionation range, rather than the actual pore size, is generally reported. It is important to note that different types of molecules exhibit different fractionation ranges; therefore, a fractionation range reported using protein standards will not be identical to that of, for example, nucleic acids. Some manufacturers report two fractionation ranges: one using protein standards and another using nucleic acids or dextrans. Usually, these two values will be sufficient to at least select an appropriate gel for the application of interest.

Once a fractionation range is determined, a material appropriate for the solutes of interest must be identified. Although the gel materials used in SEC have been developed to exhibit minimal adsorption, biological macromolecules inevitably interact to some extent with these materials. Proteins in particular will adsorb to a variety of surface functionalities. A general rule is that the gel material should be hydrophilic and uncharged. However, most hydrophilic materials exhibit at least a weak charge. Undesirable electrostatic interactions caused by this charge can be minimized by eluting with a buffer of moderate ionic strength, 0.3 to 0.5 M. In addition, the column must be operated under conditions at which the material is stable. Packing materials can deteriorate when subjected to extremes of pH, ionic strength, flow rate, and temperature. For some materials, enzymatic degradation is also possible in the event of bacterial contamination.

The size of the particles and their degree of compressibility determine the pressure drop across a bed and limit the flow rate that may be used. Particles ranging from 5 to 600 μm are available (4,5). Some SEC media, particularly cross-linked materials, are rigid over their normal operating range (Table 1). For these rigid materials, the pressure drop (Δp) across a gel of length (L) is inversely proportional to the square of the particle diameter (d_p) and proportional to velocity (v_0) (flow rate divided by cross-sectional column area), in accordance with Darcy's law:

$$\Delta p = \frac{\mu v_0 L}{k d_p^2} \qquad (8)$$

where k is a constant and μ is the fluid viscosity. However, as the particles compress, the pressure drop increases dramatically, effectively setting an upper limit on the maximum attainable velocity. From the standpoint of materials selection, manufacturers supply information regarding particle size, fraction range, and maximum allowable flow.

Available Materials. Materials comprised of dextran, agarose, acrylamide, or combinations have traditionally been the workhorses of SEC. However, novel polymeric materials and polymer-coated silica media have been de-

Table 1. Packing Materials for SEC

Name	Manufacturer	Composition	MW fraction range (proteins)	DNA exclusion limit (bp)	Bead size (μm)	pH range	Maximum back pressure (MPa)	Maximum flow rate (cm/h)
Superose 12 prep grade	Pharmacia	Highly cross-linked agarose	1×10^3–3×10^5	150	20–40	3–12	0.7	30
Superose 6 prep grade	Pharmacia	Highly cross-linked agarose	5×10^3–5×10^6	450	20–40	3–12	0.4	30
Sephacryl S-100 HR	Pharmacia	Dextran/bisacrylamide	1×10^3–1×10^5	—	25–75	3–11	0.2	20–39
Sephacryl S-200 HR	Pharmacia	Dextran/bisacrylamide	5×10^3–2.5×10^5	118	25–75	3–11	0.2	20–39
Sephacryl S-300 HR	Pharmacia	Dextran/bisacrylamide	1×10^4–1.5×10^6	118	25–75	3–11	0.2	24–48
Sephacryl S-400 HR	Pharmacia	Dextran/bisacrylamide	2×10^4–8×10^6	271	25–75	3–11	0.2	33–63
Sephacryl S-500 HR	Pharmacia	Dextran/bisacrylamide	4×10^4–2×10^7[c]	1,078	25–75	3–11	0.2	24–48
Sephacryl S-1000 SF	Pharmacia	Dextran/bisacrylamide	5×10^5–1×10^8[e]	20,000	40–105	3–11	—	40
Sepharose 6B	Pharmacia	Agarose	1×10^4–1×10^6	194	45–165	4–9	2.8	14[a]
Sepharose CL-6B	Pharmacia	Cross-linked agarose	1×10^4–1×10^6	194	45–165	3–13	2.8	30[a]
Sepharose 4B	Pharmacia	Agarose	6×10^4–2×10^7	872	45–165	4–9	0.9	11.5[a]
Sepharose CL-4B	Pharmacia	Cross-linked agarose	6×10^4–2×10^7	872	45–165	3–13	1.7	26[a]
Sepharose 2B	Pharmacia	Agarose	7×10^4–4×10^7	1,353	60–200	4–9	0.6	10[a]
Sepharose CL-2B	Pharmacia	Cross-linked agarose	7×10^4–4×10^7	1,353	60–200	3–13	0.9	15[a]
Sephadex G-10	Pharmacia	Cross-linked dextran	<700	—	40–120	2–13	DL	2–5
Sephadex G-25	Pharmacia	Cross-linked Dextran	1×10^3–5×10^3	—	20–150	2–13	DL	2–5
Sephadex G-50	Pharmacia	Cross-linked dextran	1×10^3–3×10^4	—	20–80	2–10	DL	2–5
Sephadex G-75	Pharmacia	Cross-linked dextran	3×10^3–7×10^4	—	10–120	2–10	0.016[b]	18
Sephadex G-100	Pharmacia	Cross-linked dextran	4×10^3–1.5×10^5	—	10–120	2–10	0.0096[b]	12–50
Sephadex G-150	Pharmacia	Cross-linked dextran	5×10^3–3×10^5	—	10–120	2–10	0.0036[b]	6–23
Sephadex G-200	Pharmacia	Cross-linked dextran	5×10^3–6×10^5	—	10–120	2–10	0.0016[b]	3–12
Superdex 75 HR 10/30	Pharmacia	Dextran/Cross-linked agarose	3×10^3–7×10^4		13–15	3–12	1.8	110
Superdex 200 HR 10/30	Pharmacia	Dextran/cross-linked agarose	1×10^4–6×10^5	200	13–15	3–12	1.5	76
Bio-Gel P-2	Bio-Rad	Polyacrylamide	1×10^2–1.8×10^3	—	45–90	2–10	—	5–10[d]
Bio-Gel P-4	Bio-Rad	Polyacrylamide	8×10^2–4×10^3	—	45–180	2–10	—	10–20[d]
Bio-Gel P-6	Bio-Rad	Polyacrylamide	1×10^3–6×10^3	5	45–180	2–10	—	10–20[d]
Bio-Gel P-10	Bio-Rad	Polyacrylamide	1.5×10^3–2×10^4	—	45–180	2–10	—	10–20[d]
Bio-Gel P-30	Bio-Rad	Polyacrylamide	2.5×10^3–4×10^4	20	45–180	2–10	—	6–13[d]
Bio-Gel-P-60	Bio-Rad	Polyacrylamide	3×10^3–6×10^4	55	45–180	2–10	—	3–6[d]
Bio-Gel P-100	Bio-Rad	Polyacrylamide	5×10^3–1×10^5	—	45–180	2–10	—	3–6[d]
BioGel A 0.5m	Bio-Rad	Agarose	$<1 \times 10^4$–5×10^5	—	38–300	4–13	—	7–20[e]
BioGel A 1.5m	Bio-Rad	Agarose	$<1 \times 10^4$–1.5^10^6	—	38–300	4–13	—	7–20[e]
BioGel A 5m	Bio-Rad	Agarose	1×10^4–5×10^6	—	38–300	4–13	—	7–20[e]
BioGel A 15m	Bio-Rad	Agarose	4×10^4–1.5×10^7	—	38–300	4–13	—	7–20[e]
BioGel A 50 m	Bio-Rad	Agarose	1×10^5–5×10^7	350	75–300	4–13	—	5–25[e]
Macro-Prep SE 100/40	Bio-Rad	Cross-linked Agarose	5×10^3–1×10^5	—	40	1–14	4	240
Macro-Prep SE 1000/40	Bio-Rad	Cross-linked Agarose	1×10^4–1×10^6	—	40	1–14	3	240
Hydropore-5-SEC	Varian/Rainin	Polymer-coated silica	5×10^3–1×10^6	—	5	—	—	—
Toyopearl HW-40	TosoHaas	Poly(ethylene glycol/ methacrylate)	1×10^2–1×10^4	—	20–60	2–12	0.3	DL
Toyopearl HW-50	TosoHaas	Poly(ethylene glycol/ methacrylate)	5×10^2–8×10^4	—	20–60	2–12	0.3	DL
Toyopearl HW-55	TosoHaas	Poly(ethylene glycol/ methacrylate)	1×10^3–2×10^5	—	20–60	2–12	0.3	DL
Toyopearl HW-65	TosoHaas	Poly(ethylene glycol/ methacrylate)	4×10^4–5×10^6	—	20–60	2–12	0.3	DL
Toyopearl HW-75	TosoHaas	Poly(ethylene glycol/ methacrylate)	$5 \times 10^5 \times 10^7$	—	30–60	2–12	0.3	DL
TSK-GEL G2000SW	TosoHaas	Bonded silica	5×10^3–1.5×10^5	55	4–13	2.5–7.5	1–12	DL
TSK-GEL G3000SW	TosoHaas	Bonded silica	1×10^4–5×10^5	110	4–10	2.5–7.5	1–12	DL
TSK-GEL G4000SW	TosoHaas	Bonded silica	2×10^4–1×10^7	375	8–17	2.5–7.5	1–3.5	DL
TSK-GEL G1000PW	TosoHaas	Poly(ethylene glycol/ methacrylate)	$<2 \times 10^3$	—	10	2–12	2	DL
TSK-GEL G2000PW	TosoHaas	Poly(ethylene glycol methacrylate)	$<5 \times 10^3$	—	10–20	2–12	2–4	DL
TSK-GEL G2500PW	TosoHaas	Poly(ethylene glycol/ methacrylate)	$<8 \times 10^3$	—	6–20	2–12	2–4	DL

Table 1. Packing Materials for SEC *(continued)*

Name	Manufacturer	Composition	MW fraction range (proteins)	DNA exclusion limit (bp)	Bead size (μm)	pH range	Maximum back pressure (MPa)	Maximum flow rate (cm/h)
TSK-GEL G3000PW	TosoHaas	Poly(ethylene glycol/ methacrylate)	5×10^2–8×10^5	—	6–20	2–12	2–4	DL
TSK-GEL G4000PW	TosoHaas	Poly(ethylene glycol/ methacrylate)	1×10^4–1.5×10^6	—	10–22	2–12	1–2	DL
TSK-GEL G5000PW	TosoHaas	Poly(ethylene glycol/ methacrylate)	$<1 \times 10^7$	1,500	10–22	2–12	1–2	DL
TSK-GEL G6000PW	TosoHaas	Poly(ethylene glycol/ methacrylate)	$<2 \times 10^8$	—	13–25	2–12	1–2	DL
TSK-GEL GMPW	TosoHaas	Poly(ethylene glycol/ methacrylate)	$<2 \times 10^8$	—	13–17	2–12	1–2	DL
TSK-GEL G-Oligo-PW	TosoHaas	Poly(ethylene glycol/ methacrylate)	$<3 \times 10^3$	—	6	2–12	4	DL
TSK-GEL G-DNA-PW	TosoHaas	Poly(ethylene glycol/ methacrylate)	$<2 \times 10^8$	7,000	10	2–12	2	DL

Note: Properties described are as stated by the manufacturers. In many cases, several sizes of particles are available for a given material; these are indicated as a range. DL indicates that the material obeys Darcy's law (i.e., flow rate proportional to pressure drop per bed length) over the normal operating range.
[a]Determined using distilled water at room temperature on a 2.5- by 30-cm column.
[b]Determined using distilled water at room temperature on a 2.6- by 30-cm column.
[c]The indicated fractionation range was determined for dextrans rather than proteins.
[d]Typical flow rates determined using a 1.5- by 70-cm column.
[e]Typical flow rates determined using a 1.5- by 20-cm column.

veloped. Silica possesses excellent mechanical strength, but it is prone to adsorption of biomolecules. Materials are available from a number of manufacturers, and many are summarized in Table 1. Although this list is not exhaustive, it represents many of the commonly used materials, and it is notable that these have not changed significantly over the past 15 years (5–7).

Dextrans are used in a wide variety of protein and nucleic acid separations. They are polysaccharides that are made into a porous gel by cross-linking with epichlorohydrin (8). These materials are quite stable for pH > 2 and for temperatures up to at least 120 °C and can be used with either aqueous or organic solvents. Dextran gels with small pore sizes exhibit low compressibility, but versions with larger pore sizes are quite compressible (9). As a result, some dextran gels are limited to flow rates that can be less than 1 mL/min. Because dextrans are polysaccharides, they are susceptible to bacterial degradation; however, because of their thermal stability, they can be autoclaved. A variation of the traditional dextran gel is provided by cross-linking dextran with *N,N'*-methylenebisacrylamide. This provides a material that is substantially more rigid but has a smaller stable pH range and is more susceptible to adsorption of proteins. The latter obstacle can often be overcome by eluting with a higher ionic strength solvent.

Acrylamide gels are formed by cross-linking acrylamide monomer with *N,N'*-methylenebisacrylamide. The resulting matrix is quite stable over the pH range from 2 to 10 and to high temperatures and is not prone to bacterial degradation. It is less adsorptive than the corresponding dextran material. Consequently, polyacrylamide gels are commonly used for protein separations. Their main disadvantage is that they are incompatible with organic solvents.

A copolymer of D-galactose and 3,6-anhydro-L-galactose is used to form an agarose gel. The composition and degree of cross-linking have a profound effect on the properties of agarose-based media. With less cross-linking, the gels are typically stable only in the pH range of 4 to 9, are compressible at flow rates as low as 1 mL/min, and must be sterilized chemically, but they exhibit little adsorption of macromolecules and broad fractionation ranges that make them much more suitable than acrylamide or dextran for the separation of large proteins and nucleic acids. With a higher degree of cross-linking, the material is stable over a wider range of pH and flow rate, and it can be autoclaved; however hydrophobic interactions may occur between proteins and the support, and the available fractionation range is reduced.

A number of variations on traditional materials have been established. Generally, these are produced to combine the desirable sieving properties of one material with the mechanical or chemical stability of another. The three most commonly used materials—dextran, acrylamide, and agarose—have been combined in all possible pairs. The dextran–bisacrylamide material (Sephacryl) mentioned earlier is one such example. Another is a composite of dextran and cross-linked agarose, covalently bound together to form a material (Superdex, [9]) that has the fractionation range of a dextran support with the rigidity of agarose. The resulting material is autoclavable and stable in the range 3 < pH < 12 and to a variety of strong solvents. Acrylamide has also been entrapped in an agarose gel to form the commercial Ultrogel matrix (7).

Other supports, based on materials such as silica and hydrophilic polymers, have been produced for SEC (5). Although porous silica is inherently rigid and can be manufactured in a variety of pore sizes, it exhibits marked ad-

sorption of proteins and is unstable at basic pH. Other polymer gels exist commercially, such as Toyopearl and Spheron, which are based on polyethers and poly(2-hydroxyethylmethacrylate), respectively. The latter is a material approved by the Food and Drug Administration and can thus be used to produce biocompatible supports.

APPLICATIONS

A number of applications exist for SEC in the research laboratory and in industrial processes. These can be divided into the two categories of sample analysis and purification. The former is most often performed on small columns with high resolution, and the latter is a bulk operation in which the separations are usually fairly crude. A summary of some examples of SEC applications is provided in Table 2.

Analysis

At the analytical level, SEC is used as a tool in characterizing the size, shape, and interactions of biological macromolecules. For example, SEC has been used to study the kinetics and equilibrium dimerization of proteins (10,11). Likewise, it can be used to study the hybridization of nucleic acids (12). Similar applications have been made to reversible interactions between macromolecules and low molecular weight species (13,14). It has also been used to identify and characterize molten globule states in studies of protein unfolding (15). In addition, it is an excellent tool for identification and separation of small molecules that are conjugated to biomolecules, such as probes attached to antibodies or drugs conjugated to targeting molecules (16–18).

SEC is often used for molecular weight determination, although this is an approximate method. Molecules that are similar to the solute of interest but with known molecular weight are used to create a calibration curve of retention time versus molecular weight. The unknown molecular weight of the solute is then estimated from its retention time. The degree of success of this approach is highly dependent on how similar the unknown solute is to the molecular weight standards. That is, a calibration curve prepared with globular proteins will give an inaccurate molecular weight for an oligonucleotide or even for a glycoprotein. Nonetheless, for similar molecules, molecular weights can be successfully estimated. For example, the retention times of a number of globular proteins correlate well with log(MW), as shown in Figure 3. Under the conditions of Figure 3, the retention time of α_1-acid glycoprotein is 8.70, from which an apparent molecular weight of 57 kDa is calculated, significantly greater than its actual molecular weight of 40 kDa.

For molecules of similar chemistry, such as globular proteins, the deviation of the observed elution volume from the calibration curve can be an indication of molecular shape. Deviation from spherical to ellipsoidal shape results in increased molecular friction, and thus the molecule sieves as if its apparent molecular weight is greater than its actual value. Although exact molecular weight determination is complicated by the shape and chemical nature of different protein molecules, changes in a particular structure can be discerned with high resolution. For instance, the extent of modification of a protein by a dye or other ligand can be monitored by the relatively small decrease in retention time if the same column, buffer, and operating conditions are used (16).

Table 2. Examples of SEC Applications

Analysis
Molecular weight estimation
Identification and analysis of protein dimers
Analysis of reversible protein–small molecule interactions
Studies of protein unfolding
Identification of products in molecular conjugates
Evaluation of the stability of macromolecular complexes
Separations
Desalting
Crude protein separations
Purification of molecular conjugates
Removal of endotoxins and viruses from pharmaceutical formulations

Separations

By design, SEC separates molecules based on their size, so it is not possible to achieve well-resolved separations of biomolecules from a mixture of similar species using this technique. Although fair discrimination of molecules according to molecular weight was seen in the previous section, small differences in retention time are insufficient for resolution in purification (see "Dependence of Separation Parameters on Operating Variables"). Nonetheless, crude fractionation of a particular size range is possible with

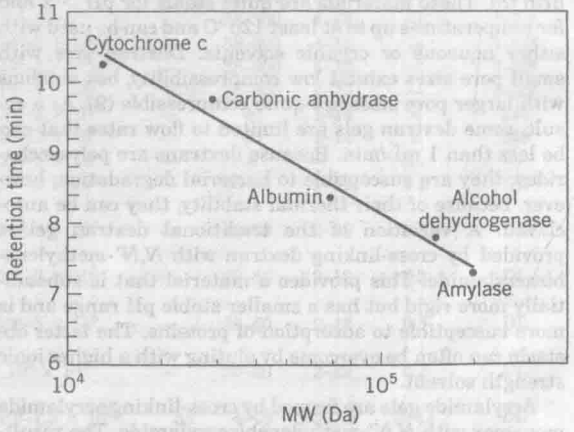

Figure 3. Calibration curve of an analytical SEC column. The elution volumes of several globular proteins were determined on a TSK-GEL G3000SW$_{XL}$ column, 7.8 mm i.d. and 30 cm length. Twenty microliter aliquots (concentration approx. 1 mg/mL) were injected and elution was performed in a buffer of 0.1 M KH_2PO_4 + 0.1 M Na_2SO_4, pH 7. The eluate was monitored by UV absorbance detection at 280 nm.

SEC. Thus, it is best suited either for purification of one molecule of interest from a small set of dissimilar contaminants in the laboratory or as an early or late step in conjunction with other steps in an industrial process.

SEC finds many laboratory applications in manipulations of biological molecules. For example, unreacted species in molecular conjugates are often removed via SEC (18). Cleanup of materials involved in the synthesis of peptides and oligonucleotides is also performed frequently via SEC (19). It is also used for removal of aggregates resulting from a variety of biochemical preparations. For example, an intermediate or final step in purification of a monoclonal antibody is often removal of dimers and aggregates that have been generated as a result of prior processing. In the production of recombinant proteins, a certain fraction may be clipped by proteolysis; these smaller molecules can be separated by SEC (20).

The most common application of SEC at both laboratory and process scales is desalting or buffer exchange. Many processing steps (e.g., extraction, crystallization, other modes of chromatography) involve the use of a solvent that is undesired for future processing or formulation steps. Very small pore SEC columns are used to replace the undesired buffer by the desired one. Usually, a wide disparity in molecular sizes exists between the biological solute and the buffer salts, so short columns can be used, limiting the dilution to twofold or less (21).

PROCESS VARIABLES

Selection of a support material for an application of interest is only the first step in performing an analysis or separation. The amount of sample to be analyzed or purified must be considered. The column length and total amount of material to be injected into the column must be determined. Finally, a flow rate must be found that will provide adequate throughput and resolution. In the process of designing a configuration, convective dispersion, transport of the solutes to and within the particles, and partitioning of the solutes between the mobile and gel phases must all be considered. For these reasons, a substantial body of literature has been devoted to the engineering issues associated with SEC. The most general and applicable models are discussed in this section.

Plate Theory

An operational description of chromatography can be obtained from plate theory, which is a phenomenological description of zone spreading in chromatography, first proposed by Martin and Synge in 1941 (22). The essential concept is that as the solute traverses the column, it undergoes repeated equilibration with the stationary phase, so that each equilibration defines one stage, or plate. The column length over which equilibration occurs is known as the height of an equivalent theoretical plate (HETP), or simply plate height. The development of a solute peak zone is shown schematically in Figure 4. In each stage, fluid in the mobile phase is mixed, and partitioning occurs between the stationary and mobile phases. A mass balance on a particular stage takes the form

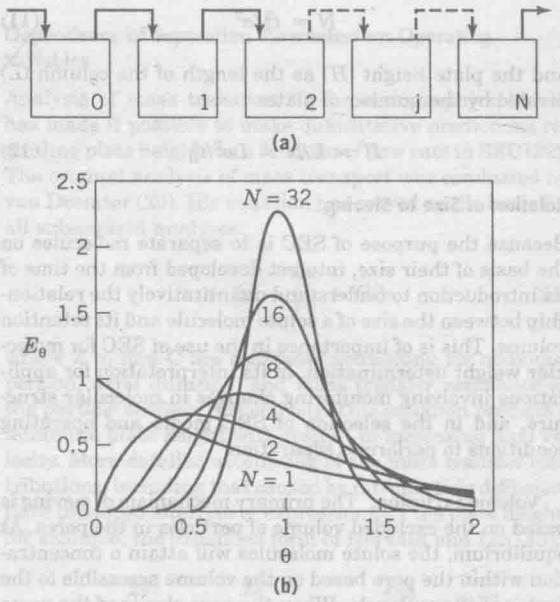

Figure 4. (**a**) Plate theory. The progression of solute through the column can be viewed as passing through a number of stages. In each stage, fluid in the mobile phase is mixed and partitioning occurs between the stationary and mobile phases. (**b**) Residence time distribution from N successive plates. The residence time distribution E, which is the output from a pulse input, in terms of the dimensionless variable $\theta = t/\tau$, is shown as a function of θ for various numbers of plates (N). It can be seen that as the number of plates exceeds approximately 10, the residence time distribution begins to assume a Gaussian shape, which is enhanced as N increases further.

$$Q(C_{j-1} - C_j) = v_m \frac{dC_j}{dt} + v_p \frac{dC_{pj}}{dt}, \tag{9}$$

where Q is the flow rate, C_j is the concentration of solute in the jth stage, v_m is the mobile phase volume per stage, v_p is the pore volume per stage, and C_{pj} is the average pore concentration in the jth stage. For linear chromatography, the average pore concentration is proportional to the bulk concentration, and equation 9 becomes directly analogous to the mass balance on stirred tanks in series (23).

The input to a chromatography column is essentially a pulse; consequently, the output from a series of N stages is analogous to the residence time distribution (E) from N tanks in series. The output from a single stage is an exponential distribution, but, as the number of plates increases, the residence time distribution takes on a Gaussian shape, as shown in Figure 4. It can be shown that the variance σ^2 of the output peak is dependent only on the retention time and number of stages through

$$\sigma^2 = t_R^2/N \tag{10}$$

Therefore, the number of plates (N) in chromatography is defined as

$$N = t_R^2/\sigma^2 \tag{11}$$

and the plate height (H) as the length of the column (L) divided by the number of plates

$$H = L/N = L\sigma^2/t_R^2 \tag{12}$$

Relation of Size to Sieving

Because the purpose of SEC is to separate molecules on the basis of their size, interest developed from the time of its introduction to understand quantitatively the relationship between the size of a solute molecule and its retention volume. This is of importance in the use of SEC for molecular weight determination, in its interpretation for applications involving monitoring changes in molecular structure, and in the selection of SEC media and operating conditions to perform a separation.

Volume Exclusion. The primary mechanism of sieving is based on the excluded volume of particles in the pores. At equilibrium, the solute molecules will attain a concentration within the pore based on the volume accessible to the center of the molecule. When the pore size is of the same order as the solute molecular size, the accessible volume becomes significantly less than the pore volume (Fig. 5). As a result of the excluded volume effect, even a monodisperse pore size distribution will produce a continuum of distribution coefficients with increasing molecular size.

To develop this concept into a quantitative model, a geometry is usually assumed. An early result was that of Laurent and Killander (24), who used the expression of Ogston (25) for the available volume in a network of rigid rods to develop an expression for the distribution coefficient:

$$K_d = \exp[-\pi L(r_s + r_r)^2] \tag{13}$$

where L is the total length of rods per volume, r_s is the radius of the (assumed) spherical particles, and r_r is the radius of the rods. The parameters L and r_r are generally not known and must be fit to experimental data. Other expressions were derived for cylindrical, conical, and slitlike pores (26). The problem of ascribing a particular pore geometry was circumvented by Ackers (27), who represented a pore by the maximum characteristic radius particle that could pass through it and postulated that the distribution of pore sizes is distributed normally. For this model, the distribution coefficient takes the form

$$K_d = \text{erfc}\left[\frac{a - a_0}{b_0}\right] \tag{14}$$

where a is the radius of the solute particle, a_0 is effectively an average pore radius, and b_0 is a measure of the variance of the distribution. These parameters must still be determined for each gel from sieving experiments on at least two (preferably more) solutes.

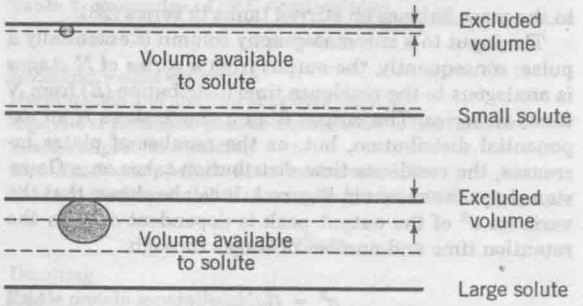

Figure 5. Volume exclusion of solutes in pores. Solutes assume concentrations within pores of varying sizes according to the volumes accessible by the centers of its molecules.

Rates of Pore Diffusion. The models based on volume exclusion (see previous section) are entirely thermodynamic; that is, they do not include any transport limitations and do not predict a dependence of distribution coefficient on flow rate. In practice, a slight dependence is observed, which led to the development of theories that account for transport of solute molecules into the pores. An approach to accounting for diffusion is to model the distribution of molecules between the mobile and gel phases using the one-dimensional (1-D) unsteady-state diffusion equation (28), which has the solution

$$C_g = C_m \text{ erfc}\left[\frac{x}{2(Dt_d)^{1/2}}\right] \tag{15}$$

where C_g and C_m are concentrations in the gel and mobile phases, respectively, x is distance into the 1-D gel phase, t_d is the diffusion time, and D is the diffusion coefficient of the solute. With t_d inversely proportional to the mobile phase velocity (v_0) and b representing the power to which diffusion coefficient is inversely proportional to molecular weight (M), the distribution coefficient can be determined to be (28)

$$K_d = \frac{k}{(\pi v_0 M^b)^{1/2}}(1 - e^{-v_0 M^b/k^2}) + \text{erfc}\left[\frac{(v_0 M^b)^{1/2}}{k}\right] \tag{16}$$

where k is a grouping of constants fit to experimental data.

Purely Empirical Correlations. The equations initially used to correlate SEC data were essentially empirical. The most commonly used forms are

$$V_R = A - B \log(M) \tag{17}$$

and

$$\log(V_R) = A' - B' \log(M) \tag{18}$$

where M is the molecular weight of the solute and A, B, A', and B' are empirical constants for the particular column configuration. As shown in Figure 3 and discussed in "Analysis", very good correlations of data for similar species are possible, but a priori prediction or extrapolation beyond the measured range of molecular weights or to mol-

ecules of different chemistry is problematic. Note that the more sophisticated theoretical models discussed earlier also make use of essentially empirical constants that require calibration of the column using proteins (or other molecules) of known molecular weight. Calibration of the column must be repeated if the pH, ionic strength, or temperature is changed significantly or if the column is repacked. Furthermore, the calibration standards must be well characterized and of a similar nature to the solutes of interest. Nonetheless, the use of theoretical models attaches a physical significance to the parameter's fit from experimental data and provides some insight into the relative contributions of the volume-exclusion versus pore-diffusion mechanisms (28).

Transport Issues

The previous section dealt with the retention time, which mostly results from the equilibrium partitioning of species into the gel support. For separations, it is necessary to consider that chromatography is conducted in a packed bed, under flow, sometimes at high pressures. The configurational aspects of the chromatography column lead to band broadening, or spreading of the eluted solute band. The main contributions to band broadening are eddy diffusion, axial dispersion, and mass transfer resistance at the surface of the particles and within the pores. Eddy diffusion results from the tortuosity of extraparticle paths that solute particles take from the top to the bottom of the column. Axial dispersion is essentially molecular diffusion along the length of the column. The mass transfer resistance at the surface arises from the boundary layer, or film, through which solute must pass to reach the gel particle, and the mass transfer resistance of the pores is due to diffusion of solute into them and is enhanced by their nonuniformity.

A chromatography column is a packed bed of gel particles and is subject to the same types of scaling analysis as for packed beds in other chemical processes. Most of the resistance to mass transfer is at the level of the particle; consequently, the particle diameter is the characteristic length for scaling. Hence, a reduced velocity or Péclet number, *(Pe)* can be defined in terms of the particle diameter as

$$Pe = \frac{d_p u}{D_0} \tag{19}$$

where u is the interstitial velocity of the fluid and D_0 is the bulk solute diffusion coefficient. Likewise, the reduced plate height (h) is

$$h = \frac{H}{d_p} \tag{20}$$

where H is the plate height defined in equation 12.

The relation of interest is that between plate height and flow rate (or velocity) or, equivalently, between reduced plate height and Péclet number. This is obtained from solution to the governing differential mass balance equations in lesser or greater complexity (29–31).

Dependence of Separation Parameters on Operating Variables

Analysis of mass transport in chromatography columns has made it possible to make quantitative predictions regarding plate heights as a function of flow rate in SEC (32). The original analysis of mass transport was conducted by van Deemter (29). His equation has served as the basis for all subsequent analyses:

$$h = 2\lambda + \frac{2\gamma}{Pe} + CPe \tag{21}$$

where λ, γ, and C are constants accounting for eddy dispersion, axial diffusion, and mass transfer resistance at the particle surface, respectively. This equation predicts a minimum plate height with respect to increasing fluid velocity. More detailed accounting of the mass transfer contributions, including that caused by intraparticle diffusion, have produced alternative expressions for the plate height, for example, the expanded form of Horváth and Lin (33):

$$h = \frac{2\gamma}{Pe} + \frac{2\lambda}{1 + \omega Pe^{-1/3}} + \frac{\kappa k_0^2}{(1 + k_0)^2} Pe^{2/3} + \frac{\theta k_0}{30(1 + k_0)^2} Pe \tag{22}$$

where θ is a geometric factor for a given particle, λ, γ, ω, and κ are parameters dependent on the details of the particular column packing, and k_0 is the ratio of intraparticle to interstitial volumes available to the solute of interest. The third and fourth terms represent mass transfer resistances at the particle surface and within the pores, respectively.

For liquid chromatography involving macromolecules, the contributions from axial dispersion and resistance at the particle surface are small, and the largest contribution is from pore diffusion. Consequently, the minimum observed by van Deemter for GLC (29) is typically not observed in SEC of biomolecules, and the plate height is roughly proportional to velocity (32,34,35). Plate height also depends on particle size:

$$H \sim \frac{u d_p^2}{D_0} \tag{23}$$

From this approximate equation, two practical points can be inferred. First, because plate height is a measure of zone spreading and should generally be minimized, smaller particles are preferred because of the strong dependence of plate height on diameter. However, pressure drop is inversely proportional to the square of particle diameter, so the mechanical properties of the material limit the extent to which particle size can be reduced. Second, lower velocities, that is, slower flow rates, will result in less zone spreading. This advantage may be offset where the speed of a separation is important. Furthermore, the flow rate cannot be reduced too drastically or the effects of axial diffusion will become limiting.

An important measure of the separation is the resolution between two or more solutes to be separated. The de-

pendence of the resolution on column variables can be determined from the equations already developed. Combining equations 4, 6, and 12 gives an expression for resolution in terms of capacity factors and plate heights (36):

$$R_s = \frac{L^{1/2}}{2} \frac{k_2' - k_1'}{H_2^{1/2}(1 + k_2) + H_1^{1/2}(1 + k_1')} \quad (24)$$

Employing the approximation that the plate heights of the two solutes are equal, an expression for resolution as a function of retention, selectivity, and column efficiency can be derived by combining the definition of selectivity (equation 5) with equation 24:

$$R_s = \frac{1}{2}\left(\frac{\alpha - 1}{\alpha + 1}\right) \frac{\bar{k}'}{1 + \bar{k}'} \frac{L^{1/2}}{H^{1/2}} \quad (25)$$

where $\bar{k}' = (k_1' + k_2')/2$ is the average retention time of the solutes.

Equation 25 expresses resolution as a function of three terms representing selectivity, retention, and zone spreading, respectively. Selectivity is the most important determinant of resolution, up to a point. An increase in α from 1.1 to 1.2 results in an 83% increase in resolution, all other factors being equal; however, an increase from 2.1 to 2.2 results in only a 6% further increase in resolution. Selection of a column best suited to fractionate the species of interest is thus critical to obtaining high resolution. Because the capacity factor is greater than or equal to unity, the middle term takes values only between 0.5 and 1; nonetheless, some improvement in resolution is seen to be possible by increasing the retention time. Because of the weak dependence of resolution on length, significant increases in column length are often required to effect a modest improvement in resolution. The inverse relationship between resolution and plate height suggests that minimizing zone spreading by the methods discussed earlier—reducing particle diameter or flow rate—will concomitantly improve resolution. This is true, subject to the same limitations on flow rate and pressure drop. Furthermore, if speed of separation is important, a trade-off exists.

In addition to the particle size and composition, column length, and operating flow rate, the SEC practitioner must choose column diameter, sample volume, and buffer composition. Column diameter does not appear explicitly in the equations describing resolution, but they are derived with the assumption of a uniform packing, which is increasingly difficult to achieve as the diameter-to-length ratio increases. Therefore, columns used for fractionation are prepared with a length-to-diameter ratio of about 10:1. But in industrial scale desalting applications, where the selectivity is great and resolution not an issue, much wider columns can be used. Sample volumes are usually chosen to be less than 5% of the total column volume, because values greater than a couple of percent tend to produce significant increases in zone spreading. In principle, the solute molecules should not interact with the stationary phase in SEC, but in practice biological molecules exhibit some interaction with a variety of materials, including gels used for SEC (37). Variation of buffer composition can be used to modulate adsorptive interactions. For example, adsorption is often mediated by charge interactions between solute and sorbent and can be reduced by increasing the ionic strength of the buffer, which provides double-layer screening of the electrostatic interactions.

CONSIDERATIONS FOR LARGE SCALE

SEC is used in large-scale industrial processes for the production of proteins for agricultural and pharmaceutical use. At this scale, economic considerations become imperative, and in particular it is important for the chromatographic operation to achieve a high level of productivity and throughput while maintaining high resolution.

General Strategies

For large-scale operation, the throughput required necessitates reuse of chromatography columns in a repeated-batch operation. Usually, the primary goal is to maximize productivity, which is defined as the amount of purified material per column area per time. This can be achieved by maximizing flow rate and minimizing the amount of time during which the column is not actively performing a separation. From a productivity standpoint, the time between elution of the valuable solute and all others is wasted time. One route to improving productivity is to reduce the resolution of the solutes to the minimum necessary (Fig. 6). Likewise, the time after elution of all solutes before the next injection can be used more efficiently by applying the next sample before the column volume of the present run is finished (21). These general guidelines govern both desalting and fractionation; further optimization depends on the particular application.

Another strategy to maximize throughput is to maximize the sample size. However, dispersion effects are enhanced for large sample volumes. Consequently, for fractionation applications, it is advisable to use sample sizes less than 3% of the total column volume (38). Although it is often desirable to have highly concentrated samples, higher concentrations result in higher viscosities, which result in slower diffusion into and equilibration with the gel. The requirement of sample equilibration with the gel also limits the flow rate that may be applied.

For desalting, on the other hand, larger sample volumes can be applied, because the sample elutes in the void volume and the salts are retained in the pore. Because of this large discrepancy in elution volumes, the increased dispersion resulting from large sample sizes is not much of an issue, and sample sizes as great as 25 to 30% of the column volume may be used. Furthermore, small molecules (e.g., ions) equilibrate quickly with the pores because of their small excluded volume and high diffusion coefficients, and macromolecules are completely excluded, so high flow rates may be used without compromising the separation.

Scale-Up Guidelines

The principle of scale-up is to determine the values of new process variables that will allow a desired change in one

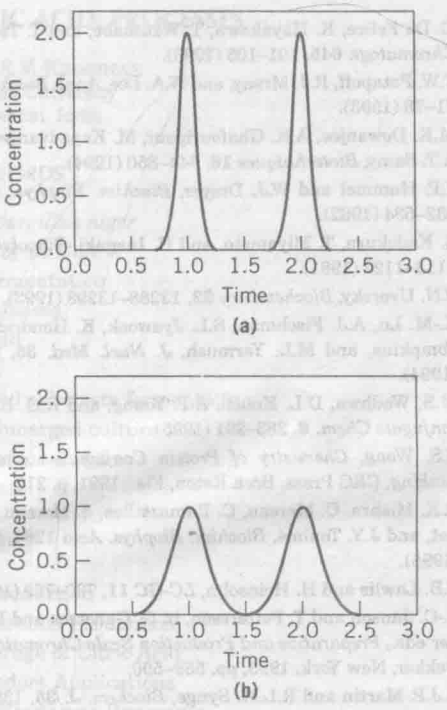

Figure 6. Optimization of resolution for large-scale SEC. (**a**) A small injection of solutes at low flow rate results in well-resolved bands. (**b**) The productivity is increased by increasing flow rate, increasing sample size, or decreasing column length to the point where the solutes are just resolved.

process variable while keeping others constant. Generally, it is desired to increase the throughput while maintaining the same resolution, but sometimes it is necessary to change resolution while keeping throughput constant. In any case, the key is to have a description of the relationship among process variables. It is here that the analysis of the preceding section is most useful.

Scaling rules for isocratic elution chromatography, which includes both SEC and adsorption chromatography, have been described by Wankat and Koo (36). The starting point is the definition of scaling parameters describing the ratios of process variables in the new and old designs, that is,

$$\delta = \frac{d_{p,new}}{d_{p,old}}$$

$$\lambda = \frac{L_{new}}{L_{old}}$$

$$\rho = \frac{D_{new}}{D_{old}}$$

$$\pi = \frac{\Delta p_{new}}{\Delta p_{old}}$$

$$\theta = \frac{Q_{new}}{Q_{old}} \tag{26}$$

where D is the column diameter and Q is the buffer flow rate. The capacity factor in SEC is a thermodynamic quantity representing the partitioning of solutes in the pores of the support particles and therefore should not vary with column configuration or process variables. Therefore, the description of column resolution in equation 24 can be used for both the new and old designs to define a ratio of design resolutions, Ψ (36):

$$\Psi \equiv \frac{R_{s,new}}{R_{s,old}} = \left(\frac{L_{new}}{L_{old}}\right)^{1/2} \frac{[H_2^{1/2}(1 + k_2') + H_1^{1/2}(1 + k_1')]_{old}}{[H_2^{1/2}(1 + k_2') + H_1^{1/2}(1 + k_1')]_{new}} \tag{27}$$

The difference between plate heights of the different solutes is usually ignored (see equation 25) and equation 27 is greatly simplified, that is,

$$\Psi = \lambda^{1/2}\left(\frac{H_{old}}{H_{new}}\right)^{1/2} \tag{28}$$

As discussed in "Dependence of Separation Parameters on Operating Variables", transport resistances at the particle surface and within the pores usually dominate, with the result that the plate height can be represented by a scaling of the form equation 21. Substituting in equation 28 gives a simple design equation:

$$\Psi = \left(\frac{\lambda}{\theta}\right)^{1/2} \frac{\rho}{\delta} \tag{29}$$

Note that more complete plate height expressions (derived from either theoretical or empirical correlations) may be substituted into equation 28 to develop more sophisticated design equations.

Equations such as equation 29 can be used to derive new sizes and operating conditions for a given change in any of the others. As a simple example, suppose that a design involving a reduction in particle size is desired, while maintaining constant column dimensions and throughput. In this case, λ, ρ, and θ are equal to unity, and the resolution is inversely proportional to the particle size

$$\Psi = \frac{1}{\delta} \tag{30}$$

Alternatively, the objective of reducing particle size may be to allow a greater flow rate while maintaining constant resolution. In this case, Ψ, ρ, and λ are equal to unity, and the flow rate scales as the inverse square of particle diameter

$$\theta = \frac{1}{\delta^2} \tag{31}$$

However, this is only valid in the regime where the particles are rigid, and it may not be feasible because of the large increase in pressure drop that would result.

The application of scaling rules to the interrelationship of process variables in equation 24 is an idealization that is subject to a number of constraints. Arbitrary column diameters and lengths are not possible, and certain length-

to-diameter ratios (on the order of 10:1) are desirable to achieve a uniform packing. Also, only certain particle sizes are available, and the range in which they are manufactured is dependent to a large degree on their resistance to flow (i.e., pressure drop). As discussed in "Packing Materials", many packings are rigid only within a certain range of pressure drops. If a new column design takes the pressure drop out of this range, the performance may be drastically altered. For rigid particles, the pressure drop is described by equation 8; therefore, its scaling is

$$\pi = \frac{\theta\lambda}{\rho^2\delta^2} \tag{32}$$

For compressible particles, the same procedure may be used with a correlation that describes the relationship between pressure drop and flow rate (39).

SUMMARY

SEC has been applied extensively to biological macromolecules in the laboratory and has found utility as a step in the production of proteins at large scale as well. Further growth of the biotechnology industry and the ease and applicability of the technique ensure that SEC will be used widely in the future as well. Many packing materials have been developed to enable the chromatographer to perform an analysis or separation quickly, efficiently, and unambiguously through improvements in the inertness and mechanical stability of the support. Engineering analysis has enabled quantitative descriptions to be made regarding the influence of mass transfer resistances to the efficiency (plate height) of the column. These can be incorporated into simple design equations for the development and optimization of column configurations and operating conditions for a number of industrially important separations.

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See also ADSORPTION, PROTEINS WITH SYNTHETIC MATERIALS; CHROMATOGRAPHY, ION EXCHANGE; MEMBRANE CHROMATOGRAPHY.