7

Use of Size Exclusion Chromatography in Biopharmaceutical Development

Tim Wehr and Roberto Rodriguez-Diaz

INTRODUCTION

Size exclusion chromatography (SEC, earlier referred to as gel filtration chromatography) has several uses in the development of biopharmaceuticals. It is used as a preparative tool to isolate biologically active species, often in concert with other chromatographic techniques in a multistage purification process. It is also used as an analytical tool to obtain information about analyte molecular sizes or shapes and aggregation states, or to determine the extent or kinetics of ligand–biopolymer binding. Preparative SEC often employs soft gels such as dextrans, agarose, or polyacrylamide.[1–3] These are compressible and only compatible with elution by the use of gravity or low-pressure pumps. They may be stabilized by cross-linking, in which case they can be eluted at higher flow rates using modest pressure. Analytical SEC is most often performed using rigid supports such as inorganic silica or cross-linked organic polymers. These materials are mechanically stable at high flow rates and pressures, and are used with HPLC systems.[4] This chapter will focus on analytical applications of SEC.

RETENTION MECHANISMS IN SEC

Size exclusion is the simplest form of chromatography, in which retention depends only on the permeation of analyte into and out of the pore system of the stationary phase. In contrast to other modes of chromatography, such as reversed phase or

ion exchange, in which analytes are retained by chemical interactions with the stationary phase, SEC (under ideal conditions) operates only by a molecular sieving mechanism. Molecules that are too large to enter the pores all elute in a volume of mobile phase equal to the interstitial volume between the stationary-phase particles (V_0). Molecules that are small enough to freely enter the pores all elute in a volume equal to the interstitial volume plus the volume of the pore system (V_i). Molecules of intermediate sizes sample different amounts of the pore system, depending on their size or shape, and elute between V_0 and $V_0 + V_i$. The total mobile-phase volume V_M can be expressed as the sum of the interstitial volume and the pore volume:

$$V_M = V_0 + V_i \tag{1}$$

The extent to which an analyte can penetrate the pore system is governed by its distribution coefficient K_D, which is related to its elution volume V_R by:

$$K_D = V_R - V_0/V_M - V_o \tag{2}$$

These equations can be combined:

$$V_R = V_0 + K_D\, V_i \tag{3}$$

From this it is readily apparent that molecules too large to enter the pores will all have K_D values of zero and will all coelute at V_0. Similarly, all molecules small enough to freely penetrate the pore system will have K_D values of one and coelute at V_M. Molecules of intermediate size will have K_D values between zero and one and will be separated according to size, with larger molecules eluting before smaller molecules.

The relationship between molecular size and elution behavior can be used to estimate the molecular weight of an analyte. A calibration plot of log molecular weight (MW) vs. retention volume (or K_D) exhibits a linear segment between V_0 and V_i (Figure 7.1). If the plot is constructed with standard proteins with shapes similar to that of an analyte protein, the molecular weight of the analyte can be estimated by interpolation of its retention volume on the plot. The relationship between log MW and K_D is linear for K_D values between about 0.2 to 0.8.

Although SEC is often used to estimate protein molecular weight, it should be understood that retention is governed by the hydrodynamic volume of the solute, which may not be closely related to molecular weight. Hydrodynamic volume is affected by the degree of solute hydration and molecular shape. The effective molecular size of a protein in solution depends on its radius of gyration or Stokes radius. Two proteins with similar molecular weights but different shapes (e.g., spherical vs. oblate vs. rod-like) will "carve out" different hydrodynamic volumes and could display significantly different retention volumes (Figure 7.2). To obtain accurate molecular weight estimates with SEC, it is necessary that the proteins used to construct the calibration plot and the analytes all have similar shapes. An alternative approach is to perform calibration and analysis under

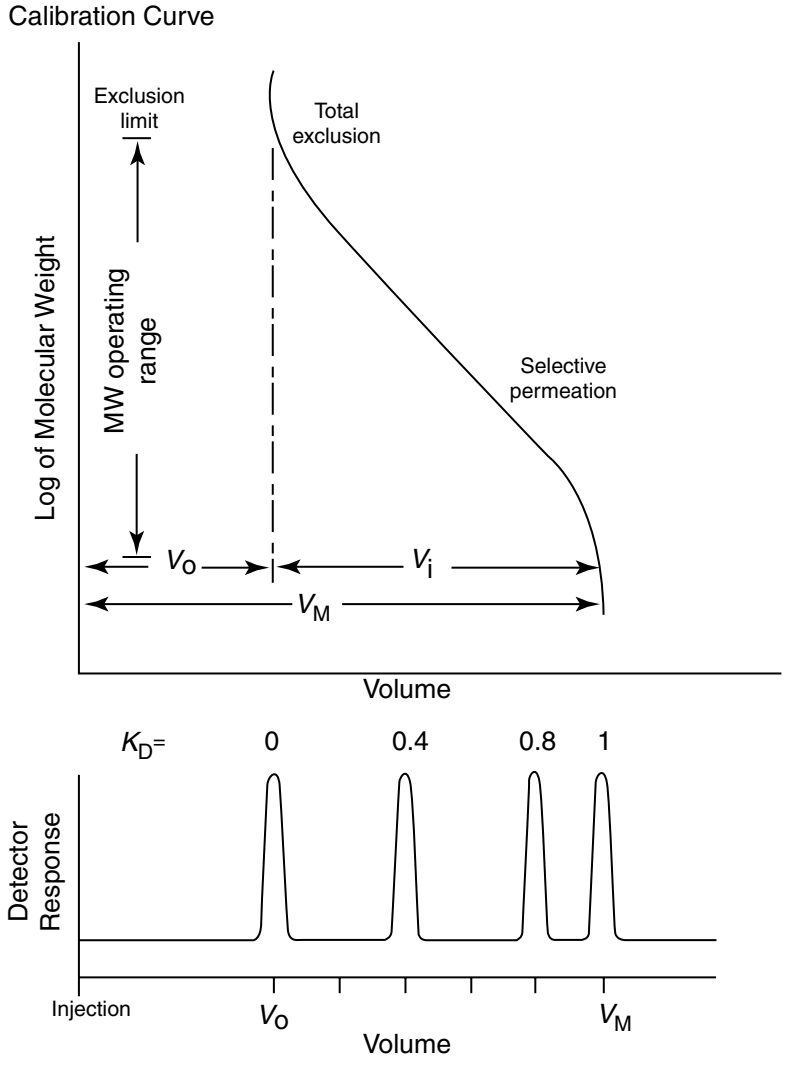

Figure 7.1 SEC chromatogram and calibration curve. (Adapted from E.L. Johnson and R.L. Stevenson, Exclusion chromatography, in *Basic Liquid Chromatography*, Varian, 1978. With permission of Varian, Inc.)

denaturing conditions so that both calibrant and analyte proteins are converted to linear random-coil conformations such that elution behavior is correlated to molecular weight.

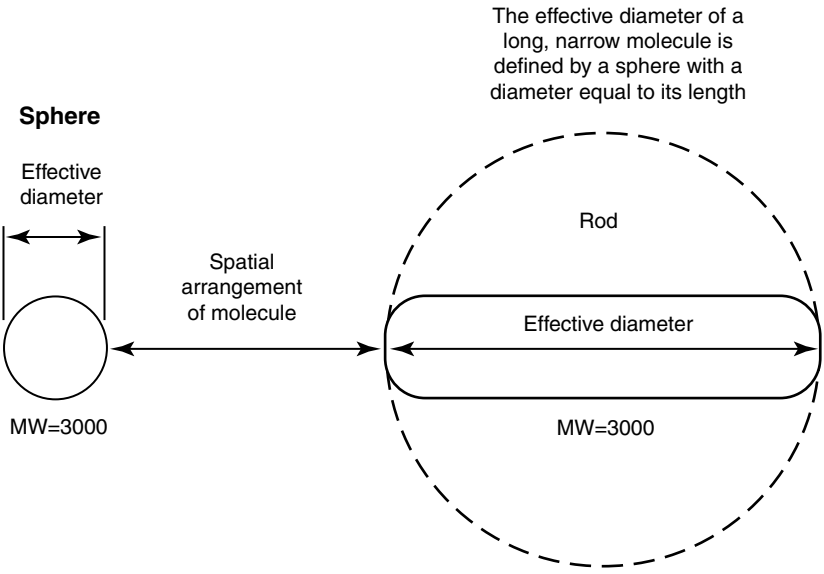

Figure 7.2 Molecular "size" vs. molecular shape. (Reprinted from E.L. Johnson and R.L. Stevenson, Exclusion chromatography, in *Basic Liquid Chromatography*, Varian, 1978. With permission of Varian, Inc.)

COLUMNS FOR SEC

The column packings used for SEC must be compatible with aqueous mobile phases and therefore must be hydrophilic in nature. The support surface must be inert to minimize interactions with protein analytes. The packing must be available in pore sizes suitable for permeation of a wide range of proteins, and the pores should be uniform in diameter. Because the separation only takes place within the pore system, the porosity of the packing should be as large as possible. The support material should be chemically compatible with SEC mobile phases and mechanically stable under high flow rates and pressures.

Support Materials

Two types of materials are used for SEC columns: bonded silicas and hydrophilic organic polymers. Silica is the most widely used material for HPLC packings in general, due to its good mechanical stability, high porosity, and availability in a range of pore sizes with closely controlled pore diameters. However, silica surfaces are highly interactive with proteins and must be derivatized to eliminate interactions. The most common approach is to react surface silanols with an organosilane reagent to introduce a diol-type or carbohydrate-like coating covalently attached to the silica. A limitation of silica-based SEC packings is their instability under alkaline conditions. Silica dissolves at pH values above 8,

leading to reduced column lifetime. One product (the Zorbax GF SEC columns from Agilent Technologies) uses a zirconyl cladding to stabilize the silica support.

Because of the limitations of silica, several manufacturers offer SEC columns based on hydrophilic organic polymer. These include polymethacrylate supports, proprietary hydrophilic polymers, and semirigid cross-linked agaroses and dextrans. These materials are more stable under high-pH operation.

Pore Size and Porosity

High-performance SEC packings are available in pore sizes ranging from 10 to 400 nm. A column should be selected with a pore size so that the analytes elute within the linear portion of the calibration plot, e.g., with K_D values between 0.2 and 0.8. Manufacturers provide calibration plots in their product literature for this purpose (Figure 7.3). However, the calibration plots used for column selection should be obtained using calibrants and elution conditions appropriate for the analysis. For example, calibration plots constructed with native proteins should be used for SEC of analyte proteins under physiological conditions. Similarly, column selection for chromatography of proteins under denaturing conditions should be done using calibration plots of denatured proteins or linear hydrophilic polymers (e.g., polyethylene glycols or sulfonated polystyrenes).

Columns with a narrow pore-size distribution will be characterized by high resolution over a narrow fractionation range, i.e., they exhibit a calibration plot with a shallow slope. Columns with a wide pore-size distribution will be characterized by a wider fractionation range, but poor resolution across that range (i.e., a steep calibration plot).

The porosity of an SEC column can be characterized by its phase ratio (V_i/V_0). Soft-gel SEC packings have high porosities with phase ratios of 1.5 to 2.4.[5] High-performance SEC packings have more modest phase ratios of 0.5 to 1.5.[6] This limitation is offset by the high efficiencies and rapid analysis times of high-performance SEC. It should be evident that as support pore diameter and pore volume increase, the amount of solid material in the particle will be reduced, compromising the mechanical strength of the support matrix.

Particle Diameter

As in interactive modes of chromatography, reduction in particle diameter reduces mass transfer effects and improves column efficiency in SEC. Column packings with particle diameters of 10 to 12 μm are available for less demanding applications, whereas SEC packings with particle diameters of 4 to 5 μm can be used for applications requiring higher resolution.

MOBILE PHASES FOR SEC

In contrast to interactive modes of chromatography where the mobile phase is an active participant in the separation process, the mobile phase in SEC is simply a

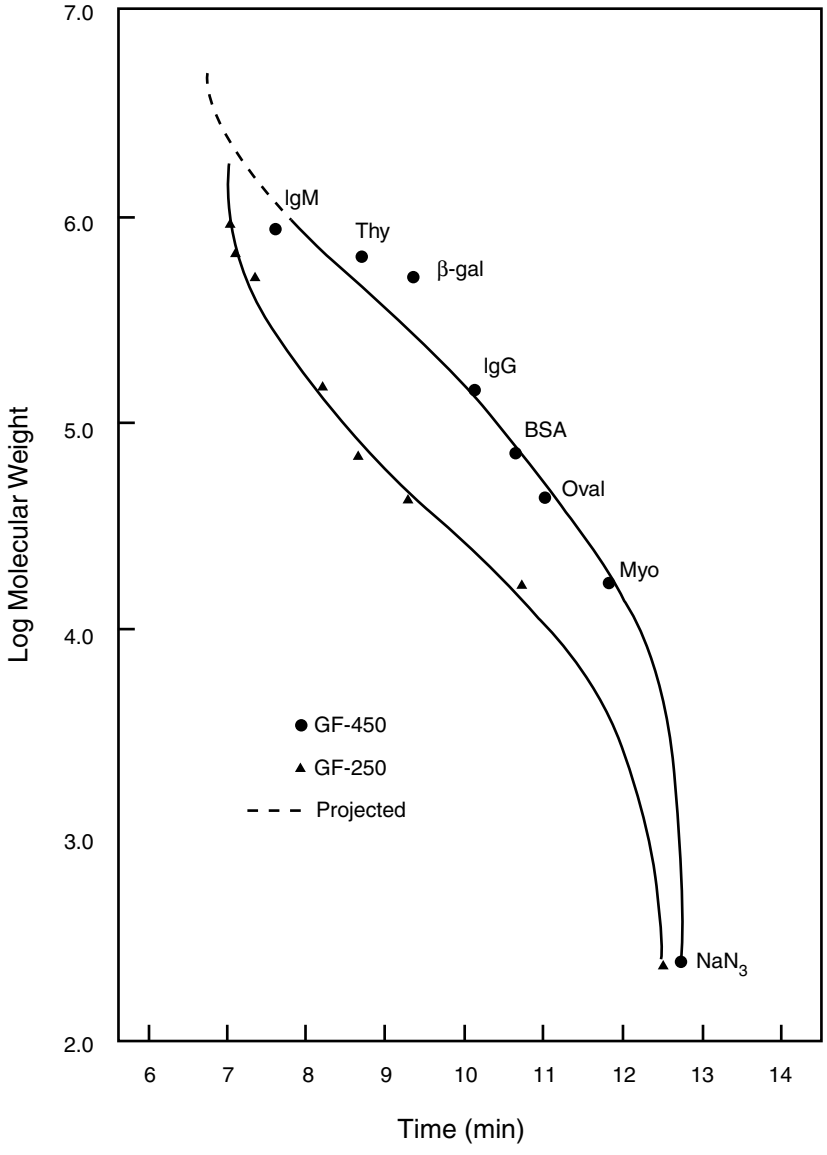

Figure 7.3 Calibration curves of proteins using a 0.2 *M* sodium phosphate (pH 7.5) mobile phase. (Printed with permission of Agilent Technologies, Inc.)

carrier to transport analytes through the column. In principle, the mobile phase chemistry is designed to keep the analyte in solution and in the appropriate conformation and to maximize column lifetime. In practice, it may contain additives to

suppress "nonideal" interactions of the analyte with the support matrix or the bonded stationary phase. These interactions may be electrostatic in nature and, in the case of silica-based columns, are most often due to residual silanols on the support. For cationic analytes such as basic proteins, this results in a cation-exchange contribution to retention that causes analytes to elute later than predicted by a solely sieving mechanism. For anionic analytes such as acidic proteins and nucleic acids, this results in an ion-exclusion phenomenon that causes analytes to elute earlier than predicted. In severe cases, analytes may elute after V_M (ion exchange) or before V_0 (ion exclusion). A second type of nonideal behavior in SEC is hydrophobic interaction. This may be due to hydrophobic sites on the support (polymer-based columns) or on the bonded phase (silica-based columns). A typical mobile phase for SEC is 100 m*M* potassium phosphate + 100 m*M* potassium chloride (pH 6.8). If nonideal behavior is observed, it can often be minimized by adjusting the salt concentration: increasing ionic strength reduces electrostatic interactions, and decreasing ionic strength reduces hydrophobic interactions. Hydrophobic interactions can also be reduced by adding a small amount (e.g., 5 to 10%) of an organic modifier (e.g., methanol, ethanol, and glycerol).

OPERATIONAL CONSIDERATIONS

Sample Capacity

The loading capacity of SEC columns is quite modest compared to interactive modes of chromatography. A rule of thumb dictates that the sample volume capacity is about 2% of the column volume. A typical analytical SEC column with dimensions of 8 × 300 mm has a V_M of 10 to 11 ml, providing a sample volume limit of about 200 µl. The mass loading limit for such a column is about 1 to 2 mg. Above these volume and mass limits, resolution will be compromised. Sample capacity will scale in proportion to column volumes for different column lengths and diameters.

Flow Rate

The low molecular diffusion coefficients of proteins and other biopolymers reduces the efficiency of mass transfer and compromises efficiency as flow rate is increased. Therefore, high-performance SEC columns are usually operated at modest flow rates, e.g., 1 ml/min or less. However, operation at very low flow rates is undesirable due to excessive analysis times, loss of efficiency due to axial analyte diffusion, and the risk of poor recovery due to analyte adsorption.

Use of Denaturants

As discussed above, accurate estimation of molecular weights may not be achieved under native conditions due to molecular shape effects. Performing

calibration and analysis under denaturing conditions may be desirable in this circumstance. Addition of a denaturant such as 4 to 6 *M* guanidinium hydrochloride, 4 to 6 *M* urea, or 0.1 to 1% sodium dodecylsulfate (SDS) to the mobile phase can be used to convert calibrants and analytes to random coil conformations. However, denaturants can reduce the effective porosity of the column. Also, surfactants such as SDS may bind strongly to the column and be difficult to remove. It is advisable to dedicate the column to such an application. High concentrations of chaotropic salts such as urea and guanidinium HCl can compromise pump and injector seals and should never be left standing in the HPLC system following use.

Use of Probes to Characterize Column Performance

When installing a new SEC column, the values of V_0 and V_M should be determined using appropriate probes. The value of V_0 can be measured using a large biopolymer outside the exclusion limit of the column; high-MW DNA (e.g., calf thymus DNA) is often used. The blue dextran used for measuring V_0 on soft-gel columns may give erroneous values on some high-performance SEC columns due to hydrophobic binding. The value of V_M is determined using a very hydrophilic small molecule that can be detected in the UV. Popular choices are cyanocobalamin (vitamin B_{12}) and glycyl tyrosine.[6]

Nonideal interactions can be also be characterized using small-molecule probes.[7] Ideally, these probes should elute at V_M. Cation-exchange interactions can be detected by excessive retention of arginine or lysine.[8] Ion-exclusion effects can be characterized by early elution of citrate or glutamic acid. Hydrophobic interactions can be detected by late elution of phenylethyl alcohol or benzyl alcohol.[8]

Exploiting Nonideal Interactions

While nonideal interactions can prevent accurate estimates of molecular weight, they may be exploited to achieve a separation if SEC is being used for preparative isolation or to profile a sample. The same techniques used to suppress nonideal interactions (modulating salt levels, adding organic modifiers, and manipulating pH) can be used to enhance these interactions.

Coupling SEC Columns

SEC columns may be coupled in series either to increase efficiency or to increase the fractionation range. Because resolution is increased by the square root of column efficiency, operating two columns in tandem to double the number of theoretical plates will increase the resolution by 2½, or 40%. As there are very few options in optimizing an SEC separation, coupling columns is often the only road to achieving the desired separation. In cases where the distribution of analyte sizes is larger than can be accommodated with a single pore-size column, coupling

columns of different pore sizes can increase the MW range of the separation. The order of coupling is not important. In either application of coupled columns, the columns should be closely matched in terms of efficiency.

ADVANTAGES AND LIMITATIONS OF SEC

SEC offers several advantages that make it a desirable technique for both preparative and analytical applications. First, separations are rapid: with an 8×300 mm analytical column operated at 1 ml/min, all analytes elute in about 10 min. Second, because the stationary phase is designed to eliminate interactions with the sample, SEC columns exhibit excellent recovery of mass and biological activity. Third, because all separations are performed under isocratic conditions, peak area and retention time precision are high.

There are four limitations to SEC. First, the resolving power is quite modest compared to interactive chromatographic modes. The peak capacity (i.e., the maximum number of baseline-resolved peaks in a separation) is approximately 5 to 10. For an SEC column with a fractionation range from 10 to 500 kDa, this implies that two proteins can be resolved if they differ in molecular weight by a factor of two. This means that SEC is useful as an analytical tool only for samples containing a limited number of components. The second limitation of SEC is the low volume and mass loading capacity. As a consequence of these two limitations, SEC is more likely to be used as a later step in a purification scheme. A third limitation of SEC is modest column lifetime, particularly for silica-based SEC columns. When operated with aqueous buffers at neutral pH, SEC-column lifetime is typically shorter than that of a silica-based reversed-phase column operated with aqueous–organic solvent systems. A final limitation of SEC is the accuracy of molecular weight estimates. Although SDS-PAGE and mass spectrometry provide more accurate values, the first technique is laborious and the second very expensive. SEC may be preferred when a quick and rough estimate of molecular weight is satisfactory.

APPLICATIONS

SEC can be used to accomplish a class separation in which one component of the sample elutes in either excluded volume or permeation volume; we term this application as *group fractionation*. Alternatively, SEC can be used to resolve two or more species within the included volume (e.g., between V_0 and V_P). We term this application simply as *fractionation*.

Although resolution in SEC is relatively low as compared with other techniques (e.g., SDS-PAGE), it allows analysis of the native protein. Thus, we can obtain a glimpse into the tertiary or quaternary structure of the molecule. Indeed, proteins such as bovine serum albumin (BSA) are usually resolved into monomer, dimer, tetramer, etc., by SEC. Caution should be exercised, as aggregates can go

undetected if sheer forces disrupt them. The Stokes radius increases for partially unfolded or denatured proteins, and SEC detects such changes as an increase in apparent MW.

Group Fractionation

SEC can be used for group fractionation when the molecule of interest has a significant MW difference relative to contaminants. The most common uses are desalting, buffer exchange, and the removal of excess reactants during protein modification. Buffer exchanges and desalting are performed when the sample is at a pH or has a composition that is not suitable for our purposes, e.g., when the sample is in a UV-absorbing buffer and we want to measure the UV absorbance of the protein. Protein treatment (e.g., reduction of S–S bonds) and modifications (e.g., acetylation) result in an excess of reactants. Often the reactants are much smaller than the modified protein, and they can be removed by group fractionation. Some molecules are purchased with specific protecting groups that need to be removed before they can be used for synthesis. In this regard, Figure 7.4 shows a typical chromatogram obtained when deprotecting oligonucleotides containing a modifiable chemical group. The oligonucleotide has a MW of approximately 7500 Da, whereas the protecting group and excess reagents have MWs below 1000 Da. The chromatographic packing is selected to exclude globular proteins with MW higher than 5000 Da. However, because oligonucleotides are usually linear (unless they have complementary sequences), their retention is lower and apparent MW in SEC is higher (more than 2 times) than that of a globular protein. In Figure 7.4A, the UV signal at 260 nm is totally saturated, and any resolution between the oligonucleotide and the low-MW contaminants is obscured. In such a case, the injection and collected volumes can be predetermined. As a rule of thumb, the injection volume should not exceed 20% of the column volume, and the collected volume should be approximately 1.5 times the injection volume. Monitoring at a wavelength that is not saturated during the procedure (i.e., a wavelength with less sensitivity) can be helpful in visualizing more detail of the chromatogram as shown in Figure 7.4B, in which the detector signal is collected at 300 nm. Of course, better resolution is obtained when the MW difference between the sample and the contaminants is greater (we routinely obtain baseline resolution when desalting proteins with MW higher than 40,000 Da). When desalting will be used as a routine technique, we find it useful to inject a smaller volume (2 to 3% of the column volume) during purification development to obtain better resolution and then evaluate the peak shape for tailing, which can severely affect sample recovery. If the peak tailing is excessive, other packings are tried or, if the application permits, the buffer composition is changed. It is necessary to keep in mind that in SEC, column length and mobile-phase flow can also be used to manipulate resolution.

There are several other techniques that can be used to perform group fractionation. These include dialysis, ultrafiltration, ultracentrifugation, tangential

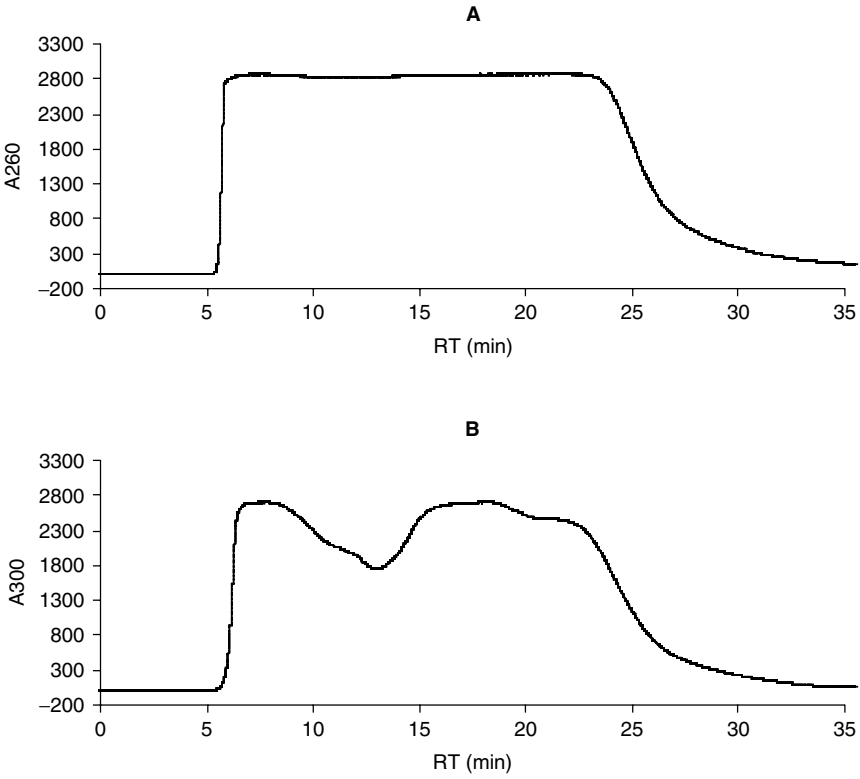

Figure 7.4 Preparative chromatogram showing SEC group separation of a 22mer phosphorothiolated oligonucleotide and its protecting group. Because of the high volume and concentration loaded, the UV signal at 260 nm is totally saturated (panel A), and no resolution is observed between the oligonucleotide and the low-MW contaminants. A second wavelength removed from the absorption maxima of the oligonucleotide is shown in panel B (300 nm).

flow filtration, etc. Selection of the appropriate method depends on variables such as sample volume (ultrafiltration requires larger volumes), sample stability (dialysis is a slow process), differences in MW (the chromatographic packings offer a wider selection of pore sizes), and cost. Ultrafiltration is more amenable and cost-effective for scaled-up processes (tens to thousands of milliliters).

Fractionation

During the development of sample-fractionation methods, the main goal is to achieve the highest resolution possible between the molecule of interest and the contaminants. Fractionation by SEC is used both as an analytical tool and as a

(preparative) purification step. The main differences between these two modes are the size of the column and the amount of sample loaded, which dictate a number of other parameters (e.g., mobile-phase flow, detection, etc.). Although the resolution is optimized, often the chromatograms resulting from preparative SEC are not much different from those obtained by desalting (see previous section). A major difference is that in fractionation, the peak of interest is not excluded by the chromatographic packing, and although the peak may be broad, the size of molecules across its width may not be homogeneous. When fractions are collected, they can be analyzed by various techniques, including analytical SEC. For example, Figure 7.5 shows the analysis of selected fractions collected during the synthesis of Ara h 2 and an oligonucleotide conjugate. Ara h 2 is one of the main allergens in peanuts that, when ingested by a peanut-allergic individual, often results in anaphylaxis. The oligonucleotide (ISS, immunostimulatory sequences) used to create this conjugate has immunoregulatory properties, which have been shown to redirect the allergic response to a normal response after treatment. Because peanut-allergic patients are extremely sensitive to the protein, a second function of the ISS is to block the epitopes on the surface of the polypeptide. Antibodies and receptors (e.g., mast cell receptors) do not as readily recognize the covered epitopes in the conjugate, and this increases the margin of safety during treatment. Figure 7.5 shows chromatograms obtained during analytical SEC analysis of fractions eluting early (panel A), in the middle (panel B), and toward the end (panel C) of a conjugate peak after preparative SEC purification. Notice that the first two fractions generate a single species, whereas the last fraction depicted shows the conjugate peak and excess ISS. SDS-PAGE clearly shows that these fractions are not composed of single species, but rather of protein containing discrete amounts of ISS; nevertheless, analytical SEC provides the data required for pooling of the final product for this and other conjugates. Notice that the mobile phase for the analytical SEC contains a small amount (10%) of methanol. The addition of the organic solvent was done with two purposes: reduced sample tailing and prevention of microbial growth in the HPLC. Before adding any additive to the mobile phase, it is important to show that the sample and the chromatography are not affected in undesirable ways. Because in many laboratories HPLC instruments are used in the "micro-preparative" mode (lab bench-scale purification), system sanitation must be performed routinely and microbial growth prevented to avoid interference in biological assays (*in vitro* and *in vivo*), which usually are performed with the HPLC-purified material. One of the most persistent effects of microbial growth is sample contamination with endotoxin.

Amb a 1 is another allergenic protein that we have conjugated to ISS. Amb a 1 is the main allergen of ragweed pollen, and when conjugated, also generates a family of molecules containing protein and various amounts of oligonucleotides (Amb a 1 immunostimulatory complexes, or AIC). Preparative SEC is used to purify the main product from excess reactants, and analytical SEC is used to evaluate the purity (and relative concentration by the deflection of the detector

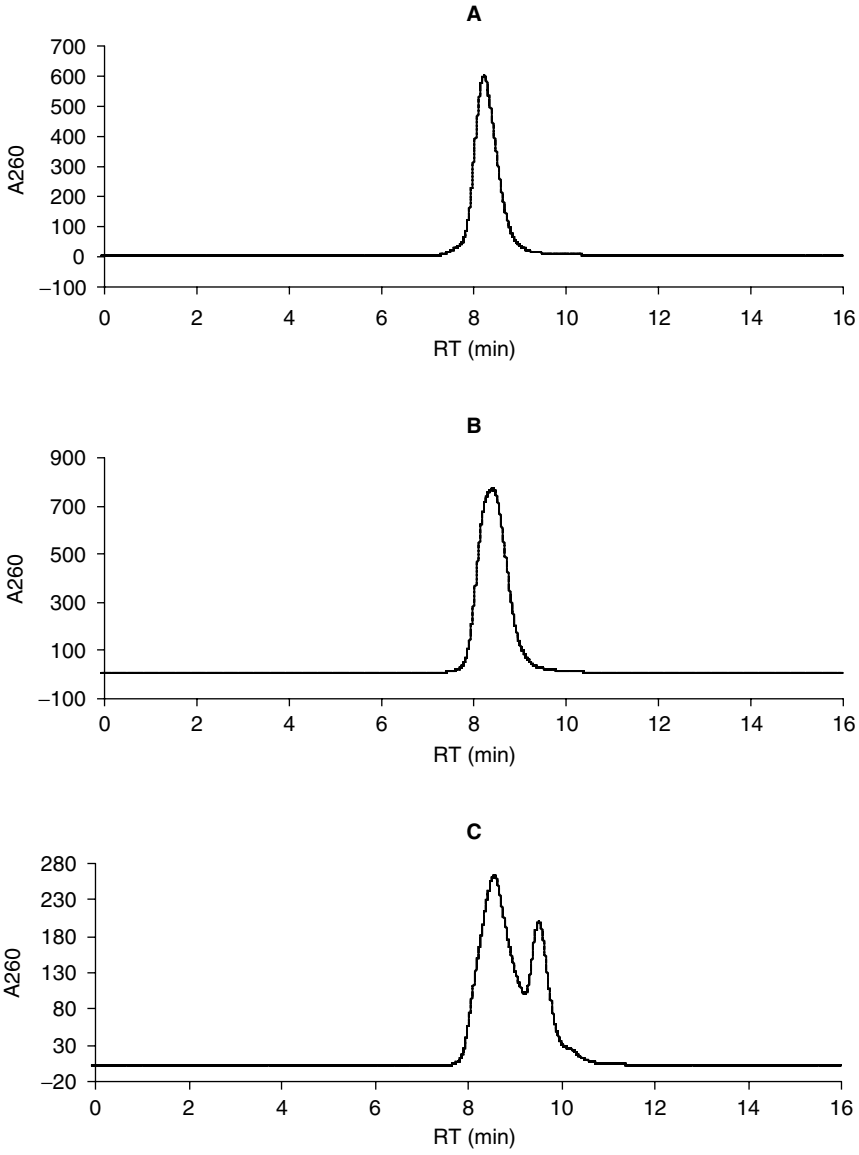

Figure 7.5 Analytical SEC of selected fractions collected during the synthesis of Ara h 2 and an oligonucleotide conjugate. The fractions were chosen to represent early eluting species (panel A, higher MW), middle of the peak (panel B), and late eluting (panel C, lower MW) of the conjugate peak. Notice that the first two fractions generate a single species, whereas the last fraction depicted shows the conjugate peak and excess ISS. Size exclusion chromatography was performed using a Biosep 3000 column (Phenomenex, Torrance, CA) 4.6 cm × 30 cm, using PBS + 10% methanol as mobile phase flowing at 0.35 ml/min. The injection volume was set to 20 µl, and detection was performed by UV absorption at 260 nm.

signal) of collected fractions, that define the pooling criteria and therefore the overall purity and composition of the conjugate.

AIC formulation and stability studies were initiated aiming to obtain a suitable formulation for AIC that provides at least a 1-year shelf life at 2 to 8°C. The originally recommended storage temperature was at or below 60°C. Although AIC is stable at that temperature, more convenient storage conditions are desirable because of transportation and on-site storage. Accelerated stability studies suggested that the PBS formulation for storage at below 60°C was inadequate for our purposes because product changes could be detected by various analytical techniques. Thus, a number of systematic experiments were designed to evaluate the stability of AIC under various conditions to elucidate the key parameters affecting the stability of AIC. SEC played an important role in monitoring AIC stability under accelerated and real-time conditions. The data collected by SEC were supported by other techniques such as SDS-PAGE, RALS, and intrinsic fluorescence. Early observations indicated that AIC stored for prolonged periods of time or subjected to freeze/thaw cycles developed a peak with a decreased retention time as compared to the monomer peak when analyzed by SEC. The change correlated with storage temperature and was not detected in samples stored at below 60°C. This peak, thus, had a larger hydrodynamic volume, which could be attributed to aggregation and/or protein unfolding.

A second profile change in accelerated stability samples resulted in peaks of low molecular weight (longer retention time in SEC), which increased with storage time but at a lower rate than the higher apparent MW peak. Focusing on these two forms of AIC changes, a series of experiments were performed to evaluate the stability of AIC at 2 to 8°C (the desired storage temperature) and accelerated stability at 30°C, using various buffers differing in pH, ionic strength, additives, and salt types. Because protein stability is often concentration-dependent, AIC was diluted to its product concentration of 30 μg/ml.

In the case of AIC, the behavior of a protein–oligonucleotide hybrid was largely unknown. However, because it is common knowledge that proteins are highly susceptible to their environment pH, we chose to evaluate the stability of the molecule varying this parameter first. As shown in Figure 7.6, there is a dramatic effect of pH on the stability of AIC. The samples depicted in this figure were incubated at 30°C for 12 h in citrate buffer with the pH adjusted to 5 (panel A), 6 (panel B), and 7.4 (panel C). Not only the degree of aggregation/unfolding, but the speed of the process also was surprising. At pH 5 there is less than 50% monomer remaining after incubation; at pH 6 the process is slower than at pH 5, but it is still significant. Notice that aggregation/unfolding practically stops at pH 7.4. The peak with a retention time >10 min is due to a buffer component. SDS-PAGE analysis indicated that the fronting peak was composed of aggregated and unfolded AIC. This was concluded because the relative area of the high-MW peak in SEC was much higher than the aggregate band in SDS-PAGE. Because proteins are denatured (unfolded) during SDS-PAGE, only chemically bound

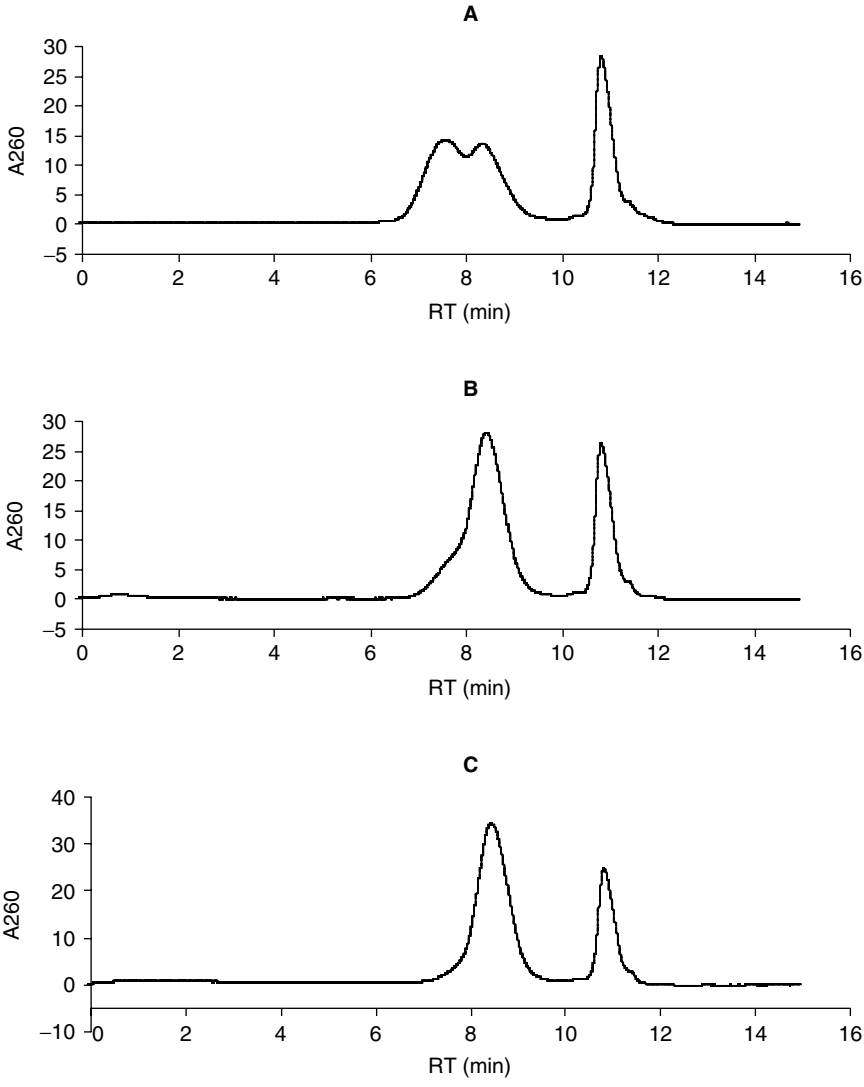

Figure 7.6 Effect of pH on the stability of AIC after incubation at 30°C for 12 h in citrate buffer with the pH adjusted to 5 (panel A), 6 (panel B), and 7.4 (panel C). The peak with a retention time >10 min is due to a buffer component. Panel C consist mainly of monomer, and the front peak observed in panels A and B is due to aggregation/unfolding of the conjugate. Analysis conditions as described in Figure 7.5.

aggregates remained. We conducted SEC in the presence of urea to unfold the protein and disrupt physical aggregates, and then a more consistent relative area between the two techniques was obtained for the aggregate.

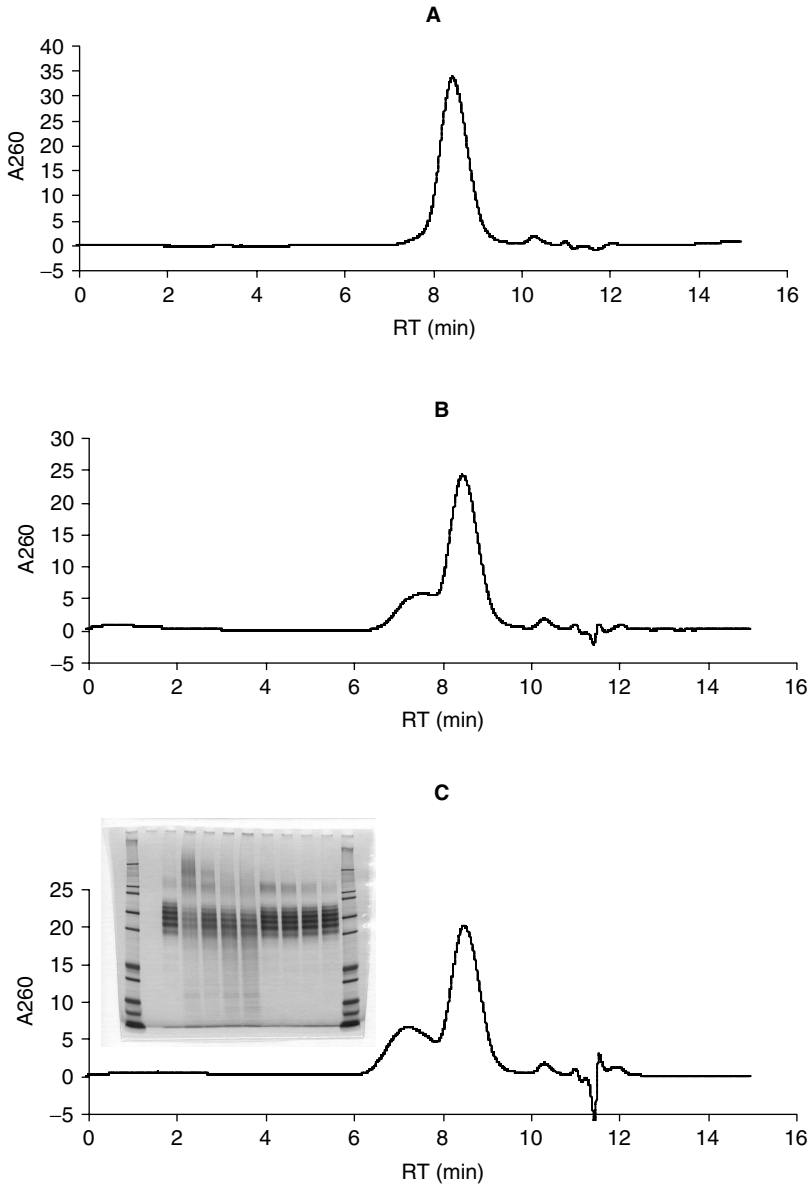

Figure 7.7 Effect of salt concentration on the stability of AIC after incubation for 24 h at 30°C in phosphate buffer pH 7.2 in the absence of salt (panel A), 0.5 *M* NaCl (panel B), and 1 *M* NaCl (panel C). AIC exposed to high concentration of NaCl shows a fronting shoulder. Analysis conditions as described in Figure 7.5. The gel shown as an inset in panel C also depicts AIC. From left to right, Lanes (1) MW standards, (2) blank, (3) reference AIC, (4) AIC incubated in the presence of 1 *M* NaCl, (5) AIC incubated in the presence of 0.5 *M* NaCl, (6–7) AIC incubated in the absence NaCl, (8–11) reference AIC, and (12) MW standards.

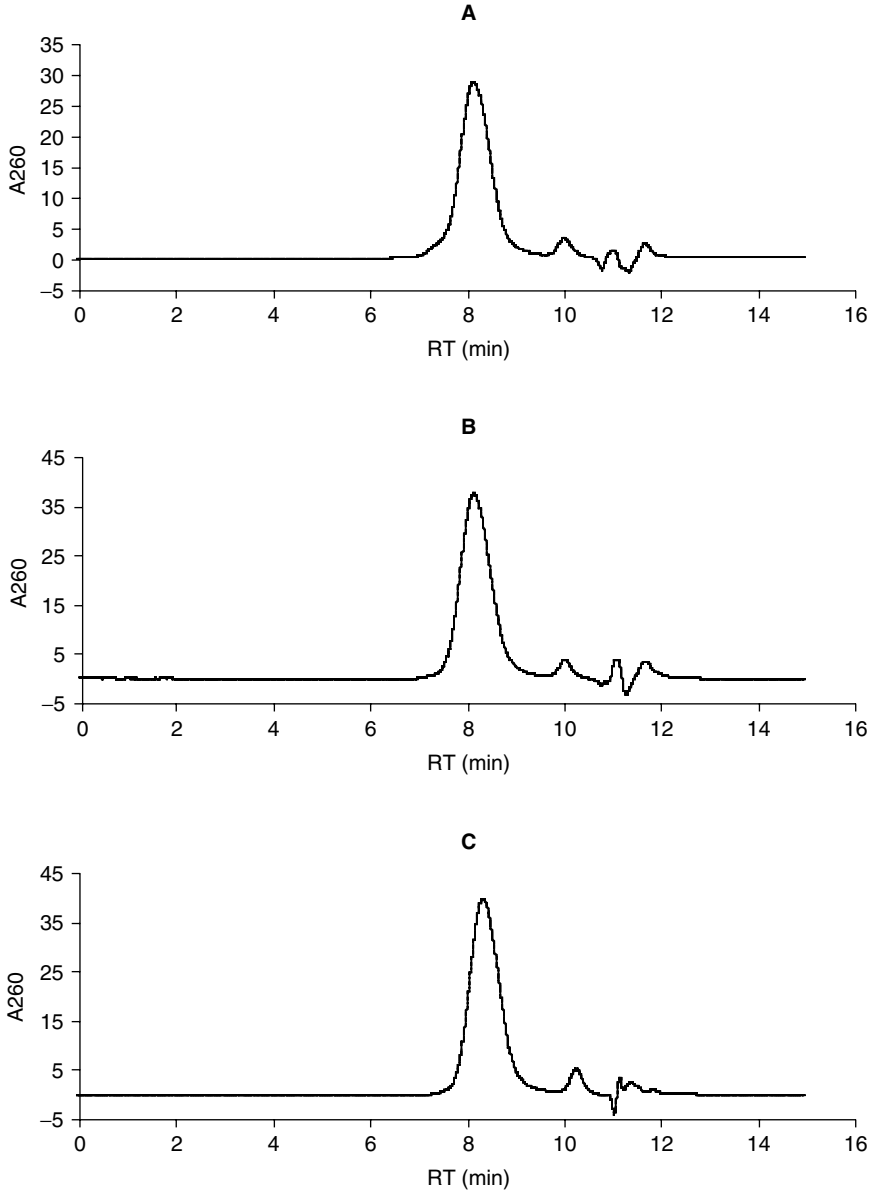

Figure 7.8 Stability of AIC in borate buffer containing sucrose (pH 8) after storage at 2 to 8°C for various time periods as analyzed by SEC. AIC reference material (panel A), AIC incubated 4 months (panel B), and 7 months (panel C) at 2 to 8°C are practically identical. Analysis conditions as described in Figure 7.5.

Next, the effect of salt concentration was evaluated. Figure 7.7 shows SEC analysis of AIC incubated for 24 h at 30°C in phosphate buffer pH 7.2 containing various amounts of NaCl. AIC incubated in the absence of NaCl (panel A)

generates a fairly symmetrical peak. Both samples incubated in high concentrations of NaCl (0.5 *M* for panel B and 1 *M* for panel C) show a fronting shoulder with an area that correlates with salt concentration. Although the effect of the salt concentration is significant, it is not as dramatic as the pH effect. Notice the appearance of an unfolded/aggregate peak in front of the monomer peak for the sample containing 0.5 and 1 *M* NaCl. Although we thought of the possibility that this effect could be due to salting-out, other experiments showed that the same phenomena was manifested in samples containing as little as 0.1 *M* salts, but aggregate accumulated at a lower rate.

Using these findings, a potential new formulation for AIC was tested, and the results are shown in Figure 7.8. A new formulation (a borate buffer containing sucrose, pH 8) was slightly alkaline and devoid of ionic salts (other than the buffer salts). After incubation at 2 to 8°C, AIC was analyzed by SEC and the chromatograms showed much improved stability. In Figure 7.8, AIC reference material (panel A) and AIC incubated 4 months (panel B) and 7 months (panel C) at 2 to 8°C are practically identical. At the end, it was concluded that changes in AIC are predominantly related to pH and salt concentration in the formulation buffer. These two parameters have strong influence on the ionic and hydrophobic forces that give proteins their particular shape. In the case of AIC, it was demonstrated that if the original conformation was preserved, other changes (e.g., chemical aggregation) were prevented.

References

1. J. Porath and P. Flodin, *Nature (London)*, 183, 1657 (1959).
2. S. Hjerten and R. Mosbach, *Anal. Biochem*., 3, 109 (1962).
3. S. Hjerten, *Arch. Biochem. Biophys*., 99, 466 (1962).
4. E.L. Johnson and R.L. Stevenson, Exclusion chromatography, in *Basic Liquid Chromatography*, Varian, 1978.
5. L. Hagel and J.C. Janson, Size-exclusion chromatography, in *Chromatography,* 5th ed. (E. Heftmann, Ed.), Elsevier, Amsterdam, 1992.
6. K.M. Gooding and F.E. Regnier, Size exclusion chromatography, in *HPLC of Biological Macromolecules,* 2nd ed. (K.M. Gooding and F.E. Regnier, Eds.), Marcel Dekker, New York, 2002.
7. R.L. Cunico, K.M. Gooding, and T. Wehr, Size exclusion chromatography, in *Basic HPLC and CE of Biomolecules*, Bay Bioanalytical Laboratory, 1999.
8. E. Pfannkoch, K.C. Lu, F.E. Regnier, and H.G. Barth, *J. Chromatogr. Sci.,* 18, 430 (1980).