CHAPTER

7

Size-exclusion chromatography (SEC) in biopharmaceutical process development

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7.1 Introduction

Size-exclusion chromatography [1], SEC, was first used to separate biomolecules in the 1950s using columns packed with starch to separate peptides from free amino acids [1]. However, with the landmark paper by Porath and Flodin in 1959 [2], the introduction of cross-linked dextran as a chromatographic material led to the first successful commercial development of SEC material called Sephadex® by Pharmacia. With the introduction of Sephadex®, SEC became a workhorse chromatographic tool in the early days of molecular biological research [3]. Over the ensuing years, many different chromatography support materials, e.g., derivatives of polyacrylamide, agar, agarose, and porous silica particles to name a few, as well as chromatography particles of different sizes and configurations (e.g., monoliths and membranes) have been employed to improve SEC separation [4,5].

As a chromatographic technique, SEC isocratically separates biomacromolecules and synthetic macromolecules based on their size and/or shape of molecules (i.e., hydrodynamic size or volume) in solution [3,6]. Like all modes of chromatography, separation is achieved via a partitioning effect. In SEC, this partitioning is due to differences in the hydrodynamic volume of molecules in a sample between two unique liquid phases: (1) a stationary phase whose access is critically controlled by the pore structure of the chromatography beads or particle and (2) a mobile phase, which is the mobile liquid found between the chromatography beads. In SEC the stationary phase corresponds to the stagnant liquid contained within the pores of the chromatography particles that interestingly, has the same chemical composition as the mobile phase. Unlike other modes of chromatography, the basis for SEC's separation mechanism is dependent on entropic factors as appose to in enthalpic interactions differences between the

Biophysical Characterization of Proteins in Developing Biopharmaceuticals, Second Edition
https://doi.org/10.1016/B978-0-444-64173-1.00007-X

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different molecules in a sample and the two phases (stationary vs. mobile phase) within the chromatography column. In the biopharmaceutical industry, SEC finds its greatest use in its ability to analytically assess the level of aggregation (i.e., high molecular weight species, HMW) present in nearly all types of biopharmaceuticals. Nevertheless, early in SEC's history, the technique was primarily used for preparative purification and desalting, especially in the molecular biological sciences in academia, where it was heavily employed to produce pure proteins for research purposes [7–10]. Although this preparative mode of SEC is still employed in the biopharmaceutical industry, to some extent, the poor capacity of SEC to process large amounts of protein material in a given column run (without incurring very significant loss in resolution and/or the need for very large columns) makes its use for commercial purification purposes of biopharmaceuticals very unattractive. Nonetheless, the use of SEC for laboratory small-scale purification purposes, still finds utility in the analytical characterization and problem-solving areas, where fractionation by size still plays a role in generating material for further analytical characterization work.

7.2 Basic theory of normal or ideal SEC

Of the many forms of chromatography, SEC is by far the simplest to understand. The basis for its separation is illustrated in Fig. 7.1, which shows a cartoon depiction of a section of an SEC column with its packed chromatography particles. Within an SEC column, its total

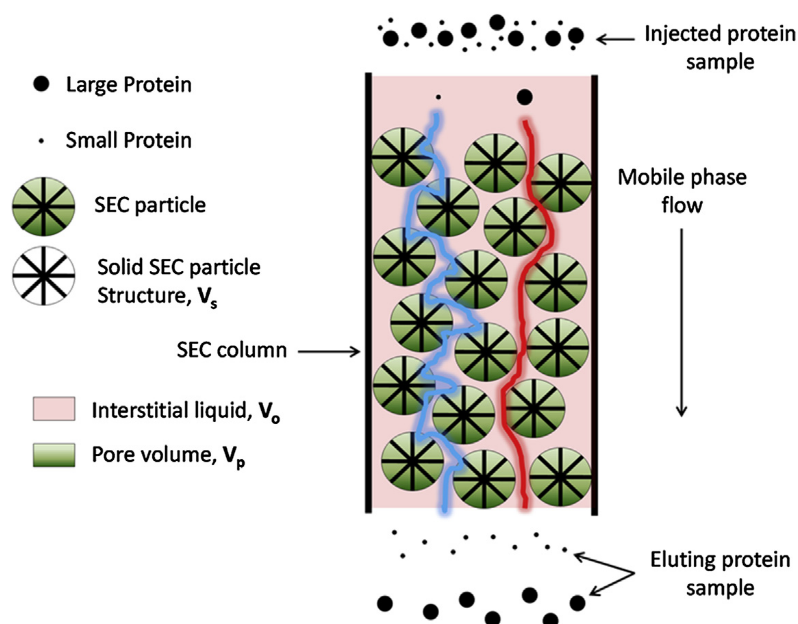

FIG. 7.1 A graphical picture illustrating the SEC separation mechanism by showing a cross section of an SEC column packed with spherical porous chromatography particles. Sample enters the column via the mobile phase flow at the top. Small proteins, indicated as a *small solid black spheres*, that can enter and explore the entire pore volume of the chromatography particles (as a result of diffusion) take the longest path through the column (as illustrated by the *blue line (light gray line in print version)*, line on the left). Large proteins, indicated as *large solid black spheres*, that cannot enter any of the pores, thus take the most direct and shortest path through the column (as illustrated by the *red line (dark gray line in print version)*, line on the right). Since the large proteins' path is shorter than the small protein's path, the large proteins elute from the column first. Those proteins having a hydrodynamic size between these two class sizes of proteins elute in the order of largest to smallest.

internal column volume, V_t, comprises three unique volume regions. The first volume region, V_o, contains the interstitial liquid, which is the bulk liquid within the SEC column, that does not include the liquid that is inside the pores of the chromatography particles. This volume region is shown as the light red area in Fig. 7.1 (i.e., the liquid region outside of the chromatography particles) and is also referred to as the void or excluded volume of the column. The liquid within this region corresponds to the SEC's column's mobile phase. The second volume region, V_p, corresponds to the total pore volume contained within all the chromatography particles packed inside the column. This is shown as the light green area (i.e., the liquid region inside of the chromatography particles) in Fig. 7.1. The liquid contained within this region corresponds to the SEC column's stationary phase, and as already mentioned has the same chemical composition as the mobile phase (note, this definition of stationary phase is very different from that commonly corresponding to the solid surface of the chromatography particles in other modes of chromatography). Finally, the third volume region, V_s, corresponds to the volume (or space) occupied by the solid support structure of all the chromatography material within the column. This is shown as the black area in each chromatography particle in Fig. 7.1. As a result, V_t is equal to:

$$V_t = V_o + V_p + V_s \tag{7.1}$$

In conducting normal SEC, V_s plays no role in the separation and ideally should be made as small as possible, within the limits of providing a strong and stable structure to maintain the integrity of the chromatography particle and its pore structure.

For biopharmaceuticals that are too large to enter the pores of the chromatography particles, these molecules can only occupy the interstitial volume during its migration through the SEC column. As a result, they will elute in a peak whose peak maximum will not appear until a V_o volume of liquid has passed through the SEC column. Consequently, large particles will be the first material to elute during SEC chromatography. All biopharmaceuticals that are completely excluded from the pore volume will elute at the same retention time, RT, (or in the same retention or elution volume, V_R, that equals V_o), in terms of their peak maximum, in one peak (see the SEC chromatogram in Fig. 7.2).

For biopharmaceuticals that are small enough to enter and freely explore (penetrate or permeate without encountering any repulsion or retardation in move through this liquid containing region of the column) the entire pore volume (V_p), they will elute in a peak whose peak maximum will correspond to the total liquid volume contained within the SEC column (V_c). Thus, V_c is equal to the following: $V_o + V_p$ (this volume region is also referred to as the permeation volume of the SEC column). Consequently, molecules that are equal to or smaller then this overall hydrodynamic volume (and that do not contain any physicochemical properties that would lead to negative or positive interactions) will also elute at the same RT (or in the same V_R that equals V_c), in terms of their peak maximum, in one peak (see the SEC chromatogram in Fig. 7.2).

Finally, for biopharmaceuticals that partially penetrate the pore volume of an SEC column, these molecules will elute in a peak whose peak maximum will correspond to a V_R, which is equal to $V_o + K_{sec}$ (V_p) as shown in Eq. (7.2):

$$V_R = V_o + K_{sec}(V_p), \text{which when rearranged shows that } K_{sec} = (V_R - V_o)/V_p \tag{7.2}$$

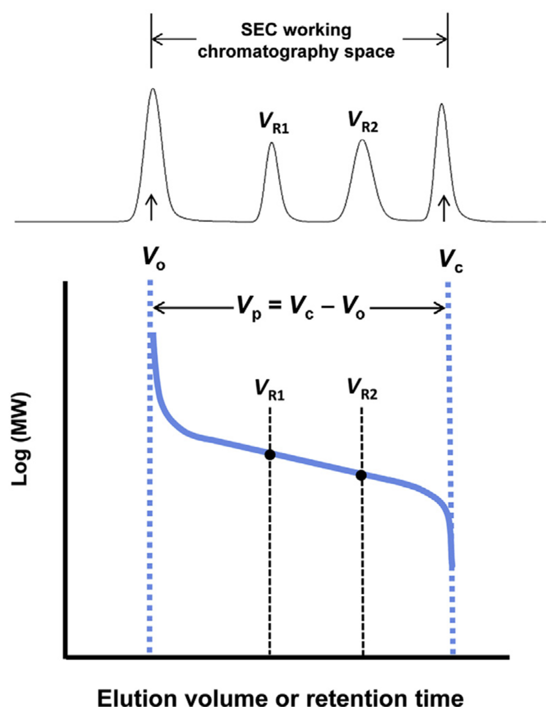

FIG. 7.2 On injecting a series of different size (MW) and homogeneous globular proteins on an SEC column, the plotting of each protein's elution or retention volume (V_R) or their corresponding retention time of their peak maximum (which is used as their x-axis co-ordinate) versus log of their MW (which is used as their y-axis co-ordinate) a MW calibration curve is generated (see *solid blue [dark gray in print version] curved line*). All proteins that are too big to enter any of the pores in the SEC chromatography particles will elute at the same elution volume called the void volume (V_o). All proteins small enough to enter and explore the entire pore volume in the SEC chromatography column will elute in the same elution volume called the column volume (V_c). Those proteins having a hydrodynamic size in between these two sizes of proteins will elute between V_o and V_c from the largest to the smallest (e.g., protein with the retention volume V_{R1} has a hydrodynamic size larger than the protein with the retention volume V_{R2}). The SEC working chromatographic space where all normal SEC chromatographic separation takes place is located between V_o and V_c.

As a result, K_{sec} is a parameter that is analogous to a partition or distribution coefficient, which indicate how a molecule partitions itself between the SEC column's mobile and stationary phases during SEC. For normal SEC, K_{sec} will take on values that range from 0, for an excluded molecule (since $V_R = V_o$) to 1, for molecules capable of permeating the entire column pore volume freely within the SEC column (since $V_R = V_o + V_p$). From this relationship we see all normal SEC chromatography is contained within the limiting experimental work space defined by the difference between V_c and V_o, which equals V_p. Consequently, in normal SEC, separations are dependent on the magnitude and properties of V_p. Furthermore, just as V_s has no functional role in SEC separation, the same can be said about V_o. In fact, V_o is just dead space that only contributes to the band-broadening effects and delay time, which lengthens an SEC experiment. Hence, conditions or factors that increase V_p and decrease V_o will generally improve SEC.

As mentioned in the introduction, normal SEC separation of macromolecules is based on their hydrodynamic volume, which in the case of the biopharmaceutical industry is always

conducted in an aqueous environment. Unfortunately, the size of a protein is typically not a common parameter that is known, in comparison to its molecular weight (MW). However, in general, globular proteins can be represented as a family of similar sphere-like shaped molecules. For such a family of similarly shaped molecules, as their MW increases their size also tends to increase and can be estimated via a power relationship like that shown in Eq. (7.3) [8]:

$$\text{Protein Size} = c(\text{MW})^{a} \tag{7.3}$$

where "a" and "c" are just constants for a given family of similarly shaped molecules.

Thus, for any SEC column, analyzing several homogeneous globular proteins with different but known MWs allows one to generate a MW calibration curve. This is achieved by determining the peak maximum V_R (or RT) for a given flow rate (FR, noting that $RT = V_R/FR$) for each protein, then taking the log of both sides of Eq. (7.3) and plotting log MW versus V_R or RT. A plot of such data can be seen in Fig. 7.2, which yields a sigmoidal shape curve with a linear or an almost linear region for a significant region of space between V_o and V_c. From this plot, we can predict the elution position of a protein by knowing its MW value (assuming that its shape falls into the same family of like shaped proteins that were used to generate this calibration curve). Having such calibration curve, which are usually provided for each specific type of SEC column by its manufacturer, is very helpful in enabling the experimenter to generally pick the right SEC column to achieve a given separation, if the MW of the proteins to be separated are known. However, this is only a first approximation, as we will see several factors can come into play that can alter the separation. Nevertheless, this capability to estimate the elution time of a macromolecule by knowing its MW is a unique property of SEC. Another unique feature is the rather limited working separation space that one has, in comparison to other forms of chromatography. In the cases of other forms of chromatography, the separation space can be extending over many equivalent column volumes, thus offering much higher resolution as a result of a much larger range of separation space to achieve a given separation.

For synthetic polymers, a calibration curve, such as the one shown in Fig. 7.2, plays a very significant role in calculating several different MW averages that are used to characterize different lots of polymer material. Such calculations are virtually never carried out in the biopharmaceutical industry (nevertheless it should be pointed out that such calculated information could be very useful for indicating subtle changes in a biopharmaceutical's conformation or surface chemistry or in assessing the quality of the SEC packing material and its packing). Rather, what is commonly assessed in the biopharmaceutical industry is the total amount of HMW (aggregates) and low MW (LMW) material (fragments) present in the biopharmaceutical sample. This assessment is simply performed via chromatographic integration of the eluted peaks to express fractional ratios of each peak or peaks of interest over the total integrated peak area (which when multiplied by 100 would yield a percentage, e.g., % total aggregation). This quantitative approach effectively comes down to using SEC to assess the purity of the monomeric form of a biopharmaceutical, in terms of its molecular size (hydrodynamic volume, which is a function of its MW and conformation). More definitive information about the nature of a drug's related impurities (as revealed by the presence of other eluting peaks or shoulders associated with the main peak) can be accessed via the

use of multiple on-line detectors and the preparative trapping of the material to conduct further off-line characterization work (which will be discussed in Sections 7.7 and 7.8).

7.3 Maximizing SEC separation by enhancing the usage of pore volume and pore structure

As noted in the previous section, the separation achieved by SEC is contained and determined by the SEC column's V_p. Consequently, maximizing V_p, with appropriate properties (i.e., pore opening size and pore opening size distribution) in an SEC column and maximizing ones use of the linear portion of the V_p region shown in Fig. 7.2 (a SEC column's plot of log MW vs. retention time) are very critical factors in achieving optimum performance from a given SEC column. Efforts to increase V_p and its usage are unfortunately limited, being confined by the column's internal volume space and the need to assure structural stability of the chromatographic beads packed within the column. Nevertheless, several factors or parameters can be maximized to increase V_p and its use. These factors include the following:

1. Reduction in the solid space (or solid volume) occupied per chromatography bead and its replacement with more pore space. This increases the porosity of the chromatography bead thus increasing V_p. However, as already mentioned, there are significant limitations to this approach. Reducing the solid will reduce the stability of the chromatography particle and its associated pore structure making the fracturing, collapse or deformation of the chromatography particle much easier. Such reduced structural stability will have a negative impact on the chromatography and its robustness as a useable analytical tool. In addition, it should be noted that simply increasing the porosity can also lead to internal pore cavity structure that is complex in shape, which could easily cause molecules to spend excess amounts of time inside the pore cavity, causing excessive band-broadening and lose of resolution. As a result, the shape or form of the pore structure is also important.
2. Reduction in the chromatography particle size so more chromatography material can be packed into a column to increase V_p. Ideally, this would be achieved if the ratio of V_p to chromatography, particle volume, V_p:($V_s + V_p$), is maintained or improved (increased). Unfortunately, in making chromatography particles smaller this ratio could become worse (decreased). Consequently, the gain in V_p may not be that great or effectively offset by other factors. Nevertheless, even if the increase in V_p is marginal this approach has other benefits. For one thing, as more chromatography material is packed into the same column, the void volume, V_o, is reduced. This will lead to sharper eluting peaks due to reduced band broadening effects in the mobile phase, which occurs as a band of macromolecules migrates between the smaller channels created by the chromatography particles. In addition, as the SEC chromatography particle is reduced in size, the average pore depth is decreased. This will lead to much higher efficiency in mass-transfer, allowing a biopharmaceutical to maximize their interrogation of available pore volume while reducing band broadening effects resulting from the excessive time spent within the pore volume. This increase in mass-transfer may in turn also be helpful in allowing the use of higher flow rates thus reducing the total time required to perform an SEC experiment

thereby increasing sample throughput. The reduced band broadening in both scenarios will translate into higher chromatographic resolution. However, it should be noted that these changes will likely lead to higher pressure if the column size and flow rate are maintained (note: since column pressure is inversely proportional to the square of the chromatography particle, a reduction in the particle diameter by a factor two, will, under the conditions mentioned, lead to a four-fold increase in pressure [11]).

3. Maximize the packing efficiency of the chromatography material within the SEC column in order to pack as much chromatography material into the column as possible (without physical altering the chromatography beads). This process is important in reducing the volume of V_o in the SEC column and typically increases the stability of the chromatography material from settling over time, which would lead to the formation of voids (note: voids increase band-broadening and therefore, negatively impact chromatographic resolution).
4. Increase column length by using a longer column or attaching two or more columns in tandem. However, it should be realized that increasing a column's length increases column pressure (note: the pressure on a column is directly proportional to its length [12]). Such an increase pressure may require a reduction in the mobile phase flow rate, due to pressure limits of the chromatography particles, causing a significant increase in chromatography time per sample.
5. Ensure the flow rate is slow enough to allow those biopharmaceutical molecules that are small enough to enter the chromatography have optimal time to equilibrate or partition between the mobile and stationary phases. Under optimum conditions, maximum V_p usage will be achieved with a minimum band-broadening effect. However, if the flow rate is too slow, excess thermodynamic band-broadening, due to diffusion, will occur.
6. Increasing the running temperature of the SEC chromatography. The use of higher temperatures helps in attaining efficient equilibrium transport or partitioning of macromolecules between the mobile and stationary phases, maximizing the usage of V_p. This is achieved by the direct thermal enhancing effect on the diffusion of the molecules being separated and an indirect effect of reducing the viscosity of the liquid in both mobile and stationary phases. However, in the case of proteins and therefore most biopharmaceuticals, significant care must be taken when increasing temperature as elevated temperatures can impact and alter the molecules higher-order structure, HOS. Such a HOS change could alter a molecule's hydrodynamic volume or induce aggregation or the interaction with the SEC chromatography material. In addition, aggregates that are initially present in a sample can potentially disassociate with increasing temperature. In these cases, increased temperature will lead to erroneous information about the biopharmaceutical.
7. The injected sample should not be analyzed at a concentration where molecular crowding effects and increased sample viscosity will interfere with the transport properties of the biopharmaceutical in or out of the pores of the stationary phase. In general, sample concentrations that are adequate in providing the necessary signal to quantitate aggregate or LMW levels should be used. At the same time, if sample concentrations of the biopharmaceutical are low, care will also be needed when increasing the volume of sample injected on to the column to achieve adequate signal quantitation. This care is

required because increasing the injected sample volume to column volume ratio negatively impacts chromatographic resolution.

8. The presence of excipients such as glycerol that give samples high viscosity may interfere with their transport properties into the pores of the stationary phase and should thus be avoided. If a sample contains high concentration of such excipients, it should be diluted with the same formulation buffer, but without the excipient (assuming the biopharmaceutical is still adequately stable without the excipient).

The reader should note that in most of the cases above, the factors/parameters listed are controlled by the manufacturer of the SEC column. Nevertheless, there are a few areas where the experimenter does have some control.

7.4 Characteristics of pore structure

In addition to finding ways to maximize the V_p in an SEC column, factors associated with the physical characteristics of the SEC chromatography bead particle e.g., geometry of the pore structure as well as the distribution of pore size openings, play important roles in the quality of the SEC data. In terms of the former, pore openings can have the same physical size opening but can have different internal complex geometries. This can lead to macromolecules becoming trapped or spending too much time inside the pore (e.g., due to its complex or unusual structure relative to a simple cylindrical structure). Regarding the distribution of the pore sizes in a given SEC column, it turns out that having a narrow distribution offers the maximum chromatographic resolution. However, the maximum resolution is limited to a narrow size range. In comparison, a wide distribution of pore sizes will offer greater capability in separating a wider size range of macromolecules in a given solution, but the level of separation that can be achieved will be limited. These characteristics are important to the experimenter and will influence their SEC column selection process depending on the information being sought. For example, if determining the total level of aggregation and LMW material in a sample is the goal, then the smallest aggregate and the largest fragment present in solution must be resolved from the biopharmaceutical monomer so that accurate quantification can be achieved. To best achieve this, one should use a SEC column that has a very narrow pore size distribution and it should be centered at an appropriate average pore size that chromatographically places the biopharmaceutical monomer in the linear range of the SEC column. An example of this is illustrated in Fig. 7.3, which shows the separation of a 150 kDa protein from an aggregate. In the bottom chromatographic trace of Fig. 7.3, the separation was performed on an SEC column having an average pore size of 150 Å. Here, the two proteins extensively overlap and elute near the void volume of the column. When an SEC column with an average pore size of 300 Å pore size was used, see the top chromatographic trace in Fig. 7.3, baseline resolution is achieved between the 150 kDa protein and its aggregate. In this case, the latter SEC column would be more appropriate to use, while the former 150 Å pore size would most likely be better for a smaller protein (e.g., 20 kDa protein). However, one should realize that while this will help achieve part of the initial goal, the separation of the 150 kDa protein from its aggregates, the separation of the 150 kDa protein from the LMW material present may be reduced. If the experimenter's goal is to acquire a better

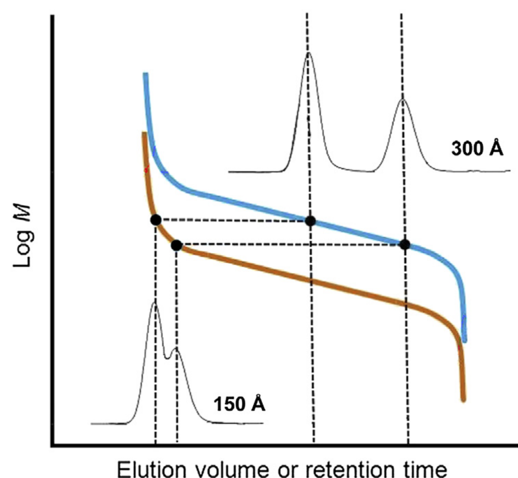

FIG. 7.3 The impact of different SEC columns with different pore sizes (150 Å or 300 Å) on the separation of a 150 kDa protein from its aggregate. In this figure, the MW calibration curve for the SEC column with an average pore size of 150 Å and 300 Å is shown in orange (dark gray in print version) (bottom) and blue (light gray in print version) (top), respectively. The resulting chromatogram for the 150 Å pore column is shown on the bottom left corner, which indicated that the proteins elute very close to the column's void volume where resolution is very poor. While the resulting chromatogram for the 300 Å pore column is shown on the upper right corner, indicates that both protein and aggregate elute in the linear region of the calibration curve where resolution is much better.

assessment of the polydispersity of different size aggregates and LMW material that are present in the same 150 kDa protein sample run in Fig. 7.3, separate large and small pore size SEC columns may be required. In the case of better separating LMW material from the 150 kDa protein, the SEC column with the average pore size of 150 Å would likely be a better choice.

7.5 Nonideal SEC chromatography

In the above sections, we have discussed SEC in terms of ideal or normal SEC. However, there are several situations where an observed separation is due to either a completely different mechanism or more likely, is a result of more than one separation mechanisms.

7.5.1 Attractive interactions—enthalpic effects

One of the biggest problems when performing SEC on biopharmaceuticals (and proteins in general) in assessing the level of aggregation is the interaction of these molecules with surfaces of the chromatography particles (both external and internal to the pores). Such interactions can result in misleading information as illustrated in Fig. 7.4. Manufacturers try to work with appropriate chromatography material or chemical bonded phases (i.e., the coating that makes the chromatography support surface inert) so that there is no interaction (or enthalpic interactions) between these materials and the biopharmaceutical. Nevertheless, the enormous diversity of chemical groups on a biopharmaceutical along with its intrinsic heterogeneity, in

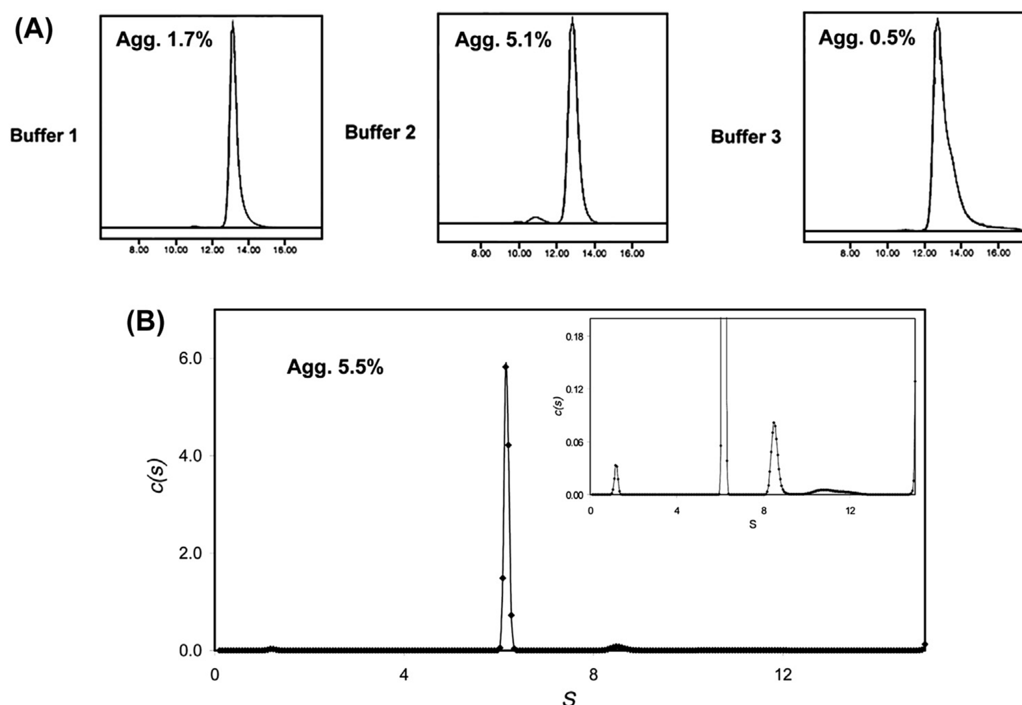

FIG. 7.4 (A) SEC chromatograms (OD_{280} vs. time) showing the effect of three different SEC mobile phases using the same protein sample and SEC column. All three buffers produced different peak shapes and yielded different aggregation values. (B) Supporting analytical ultracentrifugation data for these SEC experiments showing a plot of the distribution of sedimentation coefficients, $c(s)$ as a function of sedimentation coefficient (expressed in Svedbergs unit, S) for the same protein sample in its formulation buffer. The computed aggregation level for this plot was 5.5% indicating that buffer 2 is a likely good candidate SEC buffer for this biopharmaceutical–SEC column combination.

terms of primary structure and HOS, make chemical interactions extremely difficult to avoid. Two of the most direct signs that a biopharmaceutical is interacting with the SEC chromatographic surface is when the injected sample does not elute from the SEC column or elutes with K_{sec} values (see Section 7.2) that are greater than 1 (effectively eluting at V_R values greater than V_c). Another sign of interaction is the observation of an eluting peak that tails toward longer RT or V_R values. Yet another sign, concerns an unusual RT or V_R values relative to its known MW and the MW calibration curve generated for the SEC column being used. Critical to the use of this situation as a sign of interaction, is the established confidence that the test biopharmaceutical has the same general shape as the proteins used to generate the SEC calibration curve. Nevertheless, it should also be noted that anomalous RT or V_R values could also be an indicator of a change in the conformation of a biopharmaceutical (see discussions in Section 7.8.1). Approaches for attempting to eliminate these interactions include the following:

1. Find another SEC column that is packed with chromatography particles made of different material that has a different chemical surface, which will not interact with the biopharmaceutical of interest.

2. Condition the SEC column with several large injections of the biopharmaceutical or with another readily available pure protein (e.g., a 1% BSA solution is common). Here, the hope is that the binding sites on the SEC column can be masked by allowing the column to absorb the blocking protein. What is important for this approach to work is that the binding is very tight and the number of binding sites on the column is very low. This approach is often used when working with a new column and the initial injection(s) of the protein do not elute from the column. When this approach is observed to work, it is not uncommon that while using the conditioned column, additional rounds of conditioning will be needed from time to time to maintain the masking of these interaction sites. Hence, monitoring the RT, peak shape, and the number of theoretical plates (calculated using Eq. (2.6b) [13]) determined for the monomer peak of the biopharmaceutical's reference standard or control sample is very important.
3. Alter the mobile phase (within the stability limits of the biopharmaceutical being evaluated and the SEC column established by the manufacturer). In using this approach, many possibilities exist. These include the following [14]:
   a. Changing the pH
   b. Increase the ionic strength
   c. Introduction of a modifier, e.g., arginine
4. Increase (or possibly reduce) the temperature. If the interaction of the biopharmaceutical with the chromatographic surface is weak, increasing the temperature 10–20 °C might be enough to remove the interaction. However, if the interaction is a result of hydrophobic interactions, lowering the temperature will likely weaken these interactions potentially eliminating them [15]. However, when lowering the temperature, one needs to be aware that the viscosity of the mobile phase will increase, and the diffusion rate of macromolecules will with decrease. As a result of both effects, the lower temperature can negatively affect the quality of the resulting chromatography because both effects will reduce mass transfer of the macromolecules between the mobile and stationary phases reducing chromatographic separation and resolution. Although reduced mobile phase flow rates could offset some of this negative impact it will come at a cost of an increase in run time.

In developing a viable SEC method, it is not that uncommon that the above-mentioned approaches are investigated and used, especially the approach outlined in point 3. Unfortunately, in using approaches 3 and 4 above to develop an SEC aggregation method, some potential but important concerns need to be assessed. One problem is that in some cases, the approach may shorten the lifetime of the SEC column. However, a more important issue is that any of these changes to eliminate enthalpic effects can easily alter the state of aggregation present in the biopharmaceutical or alter its HOS. Hence, the SEC data generated would not be relevant to the biopharmaceutical sample being tested, resulting in misleading information that could lead to erroneous conclusions. To avoid this situation, appropriate testing using positive and negative controls (each with established levels of aggregation) should be used along with an appropriate orthogonal biophysical technique, such as analytical ultracentrifugation (AUC).

7.5.2 Repulsive interactions and hydrodynamic chromatography

In addition to the frequent observation of enthalpic effects, two other less common effects exist, which can also alter SEC separations. These include repulsive interactions [16], due mostly to charge effects [7,17,18] and the concurrent contributing effects from hydrodynamic chromatography (HDC) [19,20]. In the case of the former, the presence of charges on the surface of an SEC chromatography particle (both in terms of its external surface and internal pore surface) that is like the biopharmaceutical being analyzed, can impact the ability of the macromolecule to enter the chromatographic particle pores, leading to reduced chromatographic resolution. In such cases, a simple increase in salt concentration or change in pH of the mobile phase should effectively screen or eliminate these charge effects on either the surface of the chromatographic particle, the biopharmaceutical molecule, or both.

In the case of HDC, which is predominately used in separating small micron and submicron particles, the basis of its separation is achieved by taking advantage of the parabolic laminar flow velocity profile that occurs when liquid is forced to flow through an open tube (under nonturbulent conditions). Under this flow regime, the flow velocity of the liquid goes from zero, at the wall of the tube, to a maximum flow velocity, at the center of the tube (see Fig. 7.5A). For very large macromolecular particles that cannot approach the slower flow velocity regions near the tube's wall, they will spend the bulk of their time in the higher flow velocity regions at or near the center of the tube. As a result, they will move faster than smaller macromolecular particles that can approach the walls of the tube. The larger macromolecular particles will thus elute earlier from the tube than the smaller size macromolecular particles. HDC separations are classically conducted in either open small internal diameter capillaries [22,23] or in chromatography columns packed with nonporous chromatography particles [24,25]. In the latter case, the interstitial liquid region, V_o, of these columns created by the packed nonporous chromatography particles can be thought of as a simple collection of interconnected arrays of individual and twisting capillaries or channels (as illustrated in Fig. 7.5B), much like what exists in our circulatory system in terms of the interconnected arrangement of capillaries within our bodies. As macromolecular particles move through the column packed with nonporous particles, HDC separation is achieved that is anomalous to what occurs using only a single twisted capillary as shown in Fig. 7.5C and D. This latter form of HDC is equivalent to the situation that is encountered when SEC is performed on macromolecules that are too big to enter any of the pores of the chromatography particles. HDC separations can therefore occur during normal SEC for very large macromolecular particles that are too big to enter the pores of the SEC chromatography material. Consequently, those very large particles that are not trapped in the SEC column frits or in the SEC chromatography bed (due to steric reasons) can experience some separation from each other via HDC and can elute at V_R values that are less than V_o. It should be noted that this type of separation will increase in term of its contribution to the separation of particles or macromolecules as the effective channels in the SEC column get smaller [20]. This will occur as the SEC chromatography particles are reduced in size to improve SEC chromatographic separations. Given the nature of HDC separation mechanism, it should not interfere with SEC mechanism of separation. Rather, HDC contribution should only enhance the total separation that can be achieved using an SEC column [6].

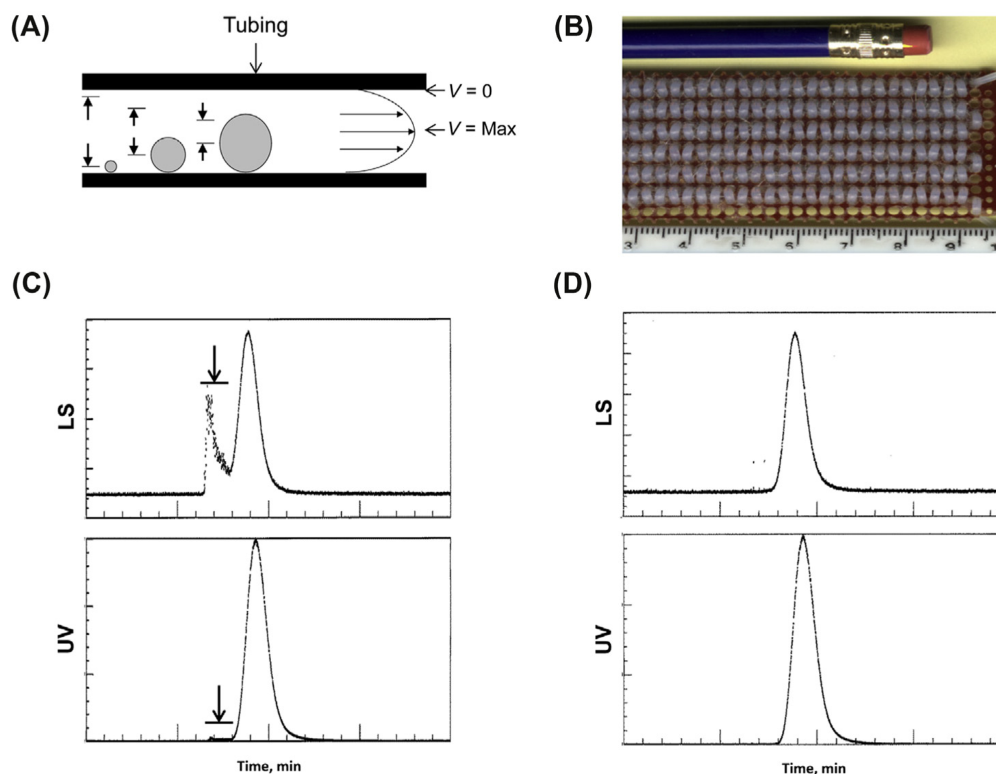

FIG. 7.5 (A) A graphical picture illustrating the separation mechanism of hydrodynamic chromatography (HDC), which occurs inside a narrow diameter tube during laminar flow. The parabolic velocity flow gradient created as liquid moves through a tube is maximum at the center of the tube ($V = \max$) and zero at the tube's wall ($V = 0$). Under these conditions, large particles become restricted to the higher flow velocity field of the liquid and move ahead of the smaller particles, which have the ability to explore the slower move liquid and therefore spend more time within the tube due to their average lower velocity in comparison to the larger particles. As a result, large particles will elute first followed by a stream of particles in decreasing size. (B) A woven portion of a 50 ft piece of Teflon tubing having an inside diameter of 0.01 mm used in place of an SEC column to create a flow-injection system. A pencil is shown to provide a prospective of the woven tubing. (C) Using the flow-injection system described in "B", which contains both LS and UV detectors, protein samples were injected into the tubing with a flow rate of 0.2 mL/min and LS and UV measurements were made. The LS (top) and UV (bottom) elution profiles are shown for a sample that contained particles. The *arrow* in the LS (measured at 90 degrees) elution profile shows the large particle peak separated by from the monomer peak. The corresponding UV profile showed only a trace of the particle peak. (D) In this case, the protein sample was clarified by centrifugation before it was injected onto the same flow-injection system run under the same experimental conditions, showing the effective removal of the particulate material initially present in the sample (see Ref. [21] for further discussion concerning this work).

7.6 Assessing and maintaining an optimum SEC chromatography method

In all phases of biopharmaceutical development (from research to process development to quality control), a heavy reliance is put on SEC to provide reliable, accurate, precise, and fast data with minimum sample consummation to characterize and monitor the amount of

aggregation present in biopharmaceutical samples. From drug candidate selection, formulation development, assessment of stability, comparability studies, to drug product (DP) release testing, SEC analysis will be part of the analytical testing package. This heavy dependence on SEC is due to the unique concern associated with aggregation and immunogenicity [26–28]. As a result, it is of great importance to monitor and understand the limits surrounding an SEC method. In so doing, this will assure its proper operation in developing confidence in the data it provides. To achieve this goal, the following suggestions and assessments are recommended when employing this technique:

1. Establish an appropriate SEC control sample for the biopharmaceutical being developed (preferably a DP or drug substance (DS) in its formulation buffer). This control should contain an appropriate level of stable aggregate to monitor the performance of the SEC column. Ideally, an aggregation level of 2% or greater would be good to enable an orthogonal biophysical technique, such as AUC (using sedimentation velocity, SV-AUC), to independently assess the aggregation level in the SEC control. This will assure the control's stability and therefore, its use in assessing the utility of the SEC methods. Additional parameters that should be followed, in terms of the SEC column's performance with this SEC control, is the resolution between the biopharmaceutical's monomer peak and some other appropriate peak in the SEC chromatogram of the control (i.e., possibly the dimer peak) using Eq. (2.2) in Ref. [29], the RT for the monomer peak maximum and the number of theoretical plates measured for this peak using Eq. (2.6b) in Ref. [13].
2. Assess and monitor the SEC column's pressure under running conditions.
3. Evaluate the chromatographic performance of several different lots of an SEC column selected for use. They should also be packed with different production lots of the SEC chromatography material before making a final decision to use a specific SEC column. This will allow one to better assess the variability of the vendor's SEC columns in terms of making the chromatography material and their ability to reproducibly pack the column.
4. Dedicate separate SEC columns to each biopharmaceutical. This is also important for DS and DP testing relative to other SEC sample testing conducted on the same biopharmaceutical, e.g., process development samples. The high purity and matrix consistency of these samples provides a very constant environment that the SEC column is exposed to. By maintaining this environment, problems are less likely to occur in comparison to exposing the same column to other matrices, pHs, or less pure samples of the same biopharmaceutical, e.g., during formulation development, stress stability testing, and in-process sample testing during purification development.
5. If the mobile phase of the SEC method is different from the formulation buffer of the sample, assess the impact of this change by comparing the aggregation level obtained by SEC with the aggregation level determined using an orthogonal biophysical technique such as AUC where there is no change in buffer (see Fig. 7.4).
6. Assess contributions made to an SEC chromatogram(s) from a biopharmaceutical's formulation buffer by making formulation buffer blank injections. This is particularly important for excipients such as Tween, which can also show variability in their contribution in term of lot to lot variations [30,31] and for situations where very dilute biopharmaceuticals are being tested.

7. If any sample preparation work is needed, assess the impact of this work on the aggregation level by comparing the SEC chromatograms before and after such sample preparation work. Also, repeat this experiment using an orthogonal biophysical technique (e.g., AUC or field flow fractionation, FFF).
8. Store the column in stable conditions. This can be critical for columns that require a protein conditioning step [32].

7.7 Detectors

A wide range of detectors can be employed to monitor the eluting material from an SEC column [33]. The only real limitation is the nature of the mobile phase in terms of its transparency and/or compatibility in relation to the mode of detection. While there are many detectors that can be used, the use of multiple detectors in tandem or in series is also common. This is important in characterization work, where the use of multiple detectors can significantly enhance the amount of information obtained from a single SEC experiment offering many advantages. These advantages include a higher level of homogeneity or heterogeneity assessment, a much more accurate assessment of MW, size, shape, and a better ability to detect changes in a biopharmaceutical's conformation, especially in dealing with more complex biopharmaceuticals, e.g., glycoproteins, pegylated proteins and other complex conjugated biopharmaceuticals, also see Chapter 15.

Biopharmaceutical scientists using these detectors or multidetector systems should be aware of the subtleties concerning their operation to fully maximize the information generated and its interpretation. A brief discussion of each of the most common detectors used with SEC in the biopharmaceutical industry is provided below. This will then be followed with more detailed discussions concerning the usage of some of the more common multidetector combinations.

7.7.1 Ultraviolet

The dominate mode of detection in SEC is ultraviolet (UV) absorbance using single-wavelength, multiwavelength, or photodiode array (PDA) detectors. These detectors primarily take advantage of the aromatic residues: tryptophan (Trp), tyrosine (Tyr), and/or phenylalanine (Phe) that are commonly found on most proteins that absorb light at or near 280 nm. In some cases, the more universal absorption of the peptide bond at or near 215 nm can be used if the concentration of the protein drug is low and if the mobile phase is sufficiently transparent at this wavelength. It should also be noted that although single wavelength detectors generally tend to be more sensitive than multiwavelength or PDA detectors, the ability to obtain full or partial UV spectrum data at each data point in an SEC chromatogram can be very beneficial. For example, some biopharmaceuticals are being constructed with small molecules attached to them (conjugated proteins, ADCs, etc.) that absorb UV light at different wavelengths. By recording absorbance readings at more than one wavelength at the same time, the amount and ratio of small molecule drug to protein on the conjugated protein can be determined across the eluting chromatogram. Such information will be helpful in assessing DP consistency and to assess the level of

homogeneity or heterogeneity of the conjugation as a function of size. The collection of such simultaneous information can help characterize these complex drugs. Additionally, detection of the UV spectrum can also help identify small molecule impurities from leachables derived from the container closures.

7.7.2 Fluorescence

One of the main reasons for using fluorescence (FL) detection is its high sensitivity. In situations where test samples are very dilute, a fluorescence detector could help overcome sensitivity issues. There is, however an important caveat: the biopharmaceutical must contain a fluorophore(s). In general, for protein biopharmaceuticals, the amino acids that absorb UV radiation are also responsible for protein fluorescence (which is referred to as intrinsic fluorescence). As described in Chapter 5, Section 5.2.3, fluorescence is a good indicator of a biopharmaceutical's HOS, thus conducting SEC with both UV and FL detection can be very informative. Coupling an FL detector with a UV detector and taking a ratio of the same total peak area for both UV and FL can provide a useful parameter for comparison purposes (comparability studies). Such ratios determined across an SEC chromatogram could also be used to assess, in more detail, a sample's heterogeneity. Another use for FL detection is the detection of glycation (a nonenzymatic post-translational modification (PTM) where a glycan is chemically linked to an amino acid, typically Lys [34]). Such modifications are often responsible for the observation of yellowish or light brown color seen in concentrated biopharmaceutical samples. The chemistry involved in these modifications can proceed through a string of reactions, which can give rise to varying chemical groups that will fluoresce at different excitation and emission wavelengths [34,35] (Ex at 340 nm/Em at 420 nm) from the intrinsic fluorescence common to proteins (Ex at 280 nm/Em at 340 nm). Again, FL/UV ratios can be computed in terms of the total peak area, point by point or over entire individual peak area to provide useful characterization information concerning consistency or variation in this type of PTM.

7.7.3 Refractive index (via differential refractive index detector)

The use of refractive index (RI) detection, with SEC (using a differential refractive index detector, DRI) is not that widely used in the biopharmaceutical industry. This is due, predominately, to the superior detection sensitivity and ease of operation of the UV detector. Nevertheless, RI detectors are universal detectors capable of detecting any material if the RI of the injected sample differs from the RI of the mobile phase (the greater the difference the greater the sensitivity). Consequently, RI detection is very useful in situations where the sample being tested does not have a UV chromophore or a FL fluorophore or is a conjugate or composite biopharmaceutical that is made of more than one chemical component that does not have any unique spectral properties. This latter situation can arise in the biopharmaceutical industry, particularly in terms of glycosylation and pegylation (PEG). Since many biopharmaceuticals in development are glycoproteins, the consistency of in vivo glycosylation (i.e., the carbohydrate to protein ratio) across the different species observed in an SEC chromatogram is often of significant interest. Similarly, for pegylation, the consistency in coupling

the PEG material to a biopharmaceutical and its resultant heterogeneity are important for assessing DP quality. Therefore, conducting SEC with both UV and DRI detection can be very informative in understanding and characterizing DP consistency and heterogeneity.

An additional feature of RI detection (in the case of proteins) is the minimum variation from one protein to another, in terms of the change in the response in a RI detector output relative to a change in protein concentration. This is mainly due to the minimum variation in the parameter called the refractive index increment, $\partial n/\partial c$, between different proteins [36,37]. This property enables better detection and assessment of the amount of material present in a given sample, in comparison to UV detection at 280 nm where the number and type of aromatic amino acids and the total number of amino acids in a given protein can greatly vary from one protein to another protein. As an example, the generation of cleavage fragments from a biopharmaceutical could yield material with significant differences in their UV extinction coefficient in comparison to the intact biopharmaceutical. On using UV detection at 280 nm, if the fragment has no aromatic amino acids the fragment will not be detected. However, in terms of a DRI detector, the response factor from both the intact biopharmaceutical and the fragment will likely be very similar allowing for the fragment's detection and quantification.

7.7.3.1 Unique troublesome properties of differential refractive index detectors

The signal output from a DRI detector is very sensitive to temperature fluctuations. In addition, subtle changes in mobile phase composition, e.g., due to evaporation or difference in dissolved gas, will generate a signal offset. It is not uncommon to see such offsets result in a DRI detector signal output that will constantly drift with time, unless temperature fluctuations and evaporation are properly controlled. In addition, these detectors can require excessively long times to equilibrate or re-equilibrate between SEC injections. Finally, it should be noted that these detectors, relative to other detectors, are very sensitive to pressure changes and are therefore sensitive to high-performance liquid chromatography (HPLC) pump fluctuations. Because of this sensitivity, in any series arrangement of HPLC detectors the DRI detector must be the last detector in the series, otherwise one might permanently damage its flow cell [38].

7.7.4 Light scattering

The coupling of online light scattering (LS) detection to SEC was initiated in the early 1970s. The key to this coupling was the development of a low angle laser light scattering (LS) instrument with a flow cell by Kaye and co-workers [39]. The first application of this instrument involved the characterization of synthetic polymers [40,41]. Eventually this instrument was applied to the characterization of proteins and other biological macromolecules via the efforts of Takagi and co-workers [42–46]. By coupling the LS detector with a DRI detector, MW information at different RT values (M_{RT}) across the SEC chromatogram could be calculated by simply taking the ratio indicated in Eq. (7.4) using the excess LS reading (corrected for buffer baseline LS) at RT, $[LS]_{RT}$, and the corresponding DRI (concentration) reading at RT, $[DRI]_{RT}$, after taking into account the inter-detector volume between the

LS and DRI detectors so the correct LS and DRI values at the same RT are used in these calculate:

$$M_{RT} = K([LS]_{RT}/[DRI]_{RT}) \quad (7.4)$$

where K in Eq. (7.4) is a collection of constant terms that includes the calibration constant for the DRI and LS detectors, K_{DRI} and K_{LS}, respectively, and the refractive index increment ($\partial n/\partial c$) was shown in Eq. (7.5):

$$K = K_{DRI}/[K_{LS}(\partial n/\partial c)] \quad (7.5)$$

The derivation of K and its experimental assessment using appropriate protein MW standards is outlined by Wen et al. [36]. However, it should be noted that for a given angle at which LS is measured, the K_{LS} is equal to a few constants itself that includes the refractive index of the SEC mobile phase, Avogadro's number, wavelength (in vacuum), the intensity of the light source in the LS detector, and the distance the LS detector is from the sample. (It should be noted that the basis of Eq. (7.4) is Eq. (8.1) in Chapter 8, Section 8.2.1 of this book.)

The inherent simplicity of Eq. (7.4) holds for SEC analysis conducted on nonglycosylated protein biopharmaceuticals (or proteins biopharmaceuticals where the level of glycosylated or other PTMs is low, amounting to only a few percent of the total mass of the protein) for several reasons [36].

First, by the time the injected biopharmaceutical reaches the LS it should have been diluted to a concentration level where nonideality effects are negligible (second and higher virial coefficients should equal zero).

Second, the monomeric biopharmaceutical and its small oligomeric aggregates (such as dimers, trimer, etc.) should behave as isotropic scatters (the resulting LS intensity is independent of scattering angle), hence LS readings at any angle will yield the correct MW (there is no need to extrapolate LS readings at different angles to zero angle to obtain the correct MW, see Chapter 8).

Third, the refractive index increment, which appears in the constant term K for nonglycosylated proteins or for proteins where the level of glycosylation or other PTMs is very low is also a constant [36,37].

A further simplification for extracting MW data from SEC-LS-DRI measurements that avoids the need to determine K (for many cases) is to simply use the MW of the SEC nonglycosylated protein biopharmaceutical monomer peak as an internal MW standard. The MW of this material should be accurately known from its amino acid sequence or mass spectrometry (MS). The critical step in using this approach is the normalization of the LS and DRI chromatograms so the monomer peak maximum reading in both the LS and DRI chromatograms are the same (i.e., so their ratio will equal one). Once the LS and DRI chromatograms are normalized, one can access M_{RT} at any RT value in the SEC chromatogram by simply determining the $[LS]_{RT}$ to $[DRI]_{RT}$ ratio at the same RT and multiply it by the monomer's MW.

A similar approach that yields an anomalous equation to Eq. (7.4) can be derived for using a UV detector instead of a DRI detector:

$$M_{RT} = K'([LS]_{RT}/[UV]_{RT}) \quad (7.6)$$

However in Eq. (7.6) the constant K' is given by Eq. (7.7):

$$K' = [\varepsilon_p K_{UV}] \Big/ \left[K_{LS}(\partial n/\partial c)^2\right] \tag{7.7}$$

where the term K_{UV} corresponds to the UV calibration constant and ε_p corresponds to the protein extinction coefficient for a 1 mg/mL solution in a 1 cm path length cell, which is also a constant for a given protein. Thus, given the constant nature of these parameters we can apply the same relative approach concerning the use of the internal monomer MW of the protein biopharmaceutical in the SEC chromatogram to compute the MW of any other material in the chromatogram. This is done by simply rationing the LS and UV values obtained at the same RT and multiplying this value by the monomer's MW. This is illustrated in Fig. 7.6. Here, the LS chromatogram was shifted, to account for the physical inter-detector volume due the serial arrangement of the LS and UV detectors, so that the LS maximum of the biopharmaceutical monomer peak is aligned with the UV concentration peak maximum of the corresponding biopharmaceutical monomer peak. The LS and UV concentration

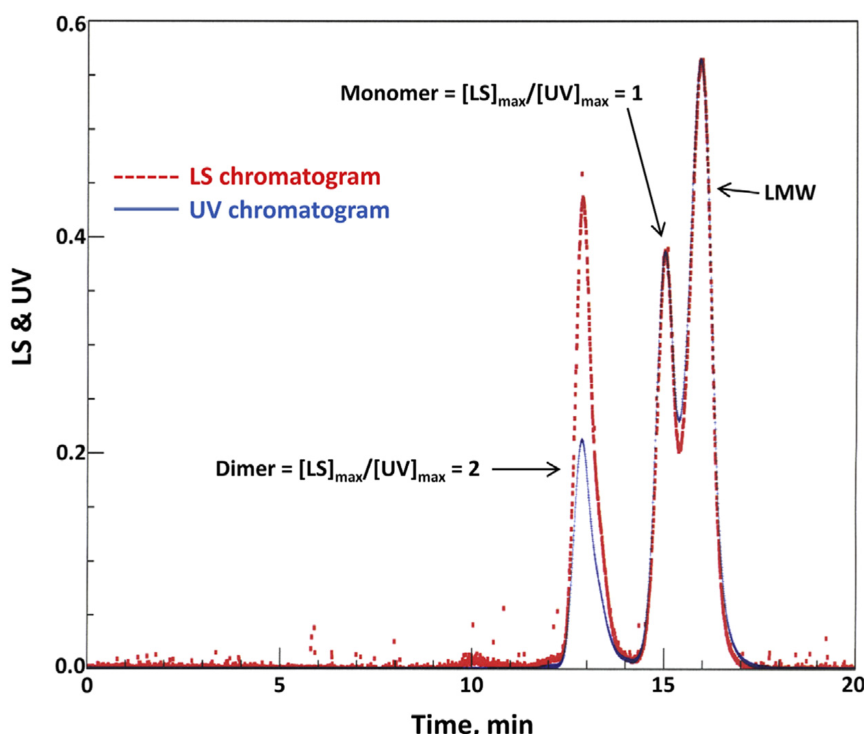

FIG. 7.6 An SEC chromatogram with LS and UV detection of a recombinant protein enriched in dimer and LMW material. The LS chromatogram is shown in red (*gray dotted line in print version*) and the UV chromatogram is shown in blue (*dark gray solid line in print version*). The chromatograms were corrected for the inter-detector volume and appropriately normalized so both the LS and UV monomer peak maximums overlaid and the ratio was equal to 1. The dimer peak maximum showed a relative LS to UV peak ratio of about 2, which confirmed this material as a dimer. The same ratio for the LWM, however, indicated that this material was not LMW, but is in fact another form of the recombinant protein monomer (see Section 7.8.1 for further discussion).

chromatograms are then appropriately normalized to the monomer biopharmaceutical peak maximum so that the monomer peaks in each SEC chromatogram are equal. After doing this, it then becomes immediately obvious that the earlier eluting peak at the RT of about 13 min corresponds to a dimer, since the ratio of $[LS]_{RT}/[UV]_{RT}$ at the peak maximum is very close to 2.

The ability to generate absolute M_{RT} data across an SEC chromatogram as a function of RT not only offers MW characterizing information about the biopharmaceutical independent of MW calibration curves, it also provides useful information about the conformation of the molecule or the performance of the SEC method. This is achieved by comparing these absolute M_{RT} values with elution position MWs obtained from a MW calibration curve (using proteins that have the same MW to size relationship). If a large difference is observed, then the likely explanation is either a change in conformation (and therefore a difference in the MW to size relationship relative to the protein standards used) or the presence of some form of interaction (giving rise to nonideal SEC chromatography, sections 7.5.1 and 7.5.2) between a biopharmaceutical or variant forms of the biopharmaceutical with the SEC chromatography material [47].

As noted in the discussion above, the assumption that the protein biopharmaceutical is nonglycosylated or glycosylated to a very low level by mass (which also applies to other PMTs) can be very limiting. Several protein biopharmaceuticals can be highly glycosylated or purposely modified with HMW polymers, e.g., polyethylene glycol. Such situations will give rise to heterogeneous biopharmaceutical products, even if the biopharmaceutical protein is highly pure and monodisperse. This can make SEC characterization very complex. However, by coupling both UV and DRI detectors with a LS detector, the characterization of these complex proteins can be made much more manageable and informative [36]. For those complex biopharmaceuticals that consist of additional chemical adducts that to not absorb UV light, this three detector SEC system is very helpful in assessing the protein MW (M_P) and therefore the weight fraction of protein material that is present in a complex biopharmaceutical as a function of RT, $(M_P)_{RT}$. This is achieved using Eq. (7.8), and the signal readings from the LS, UV, and DRI detectors at the same RT, or as integrated peaks where [LS], [UV], and [DRI] are the integrated peaks readings from the respective detectors integrated over the same range of RT values (the latter being less influenced by the effect of the inter-detector volume, see Section 7.8.2):

$$(M_P)_{RT} = \left(K''[LS]_{RT}\left([UV]_{RT}\right)^2\right) \Big/ \left(\varepsilon_P[DRI]_{RT}\right) \tag{7.8}$$

where K'' is a constant like K that can be determined using protein standards having known M_P and ε_P values (see Ref. [36] for details).

7.7.4.1 Unique and troublesome properties of light scattering detectors

One of the most troublesome aspects of using LS detectors is their high sensitivity to particulate material in the mobile phase. These particles generate interfering noise in the LS data making it very difficult to use. Sources of this annoyance could be intrinsic to the mobile phase itself, or could be due to the flaking, shedding or bleeding of material from the HPLC hardware and/or the solid chromatographic particle itself. In the case of the mobile phase, it is very common that it will almost always need to be filtered through an appropriate

in-line filter containing a 0.1 μm filter, located between the HPLC pump and the injector. Unfortunately, positioning this in-line filter in this location leaves the injector as a possible source of particulate material. However, if one were to position an in-line filter between the injector and the HPLC SEC column, two factors make this a problem: (1) the in-line filter would need to be a zero-dead volume filter, to avoid its negatively impact on SEC resolution due to excessive band broadening, which could easily result in column clog (due to the very small surface area of the filer) leading to frequent high column pressure problems and (2) more importantly, this zero-dead volume filter could now remove material, especially aggregate material, present in the injected sample that would be important for the SEC system to detect.

As noted in the above paragraph, SEC columns can be a source of particulate material (by leaching small particle "fines") that can give rise significant light scattering noise. This problem is particularly concerning when using a new SEC column for the first time [36]. In this situation, it is frequently found that the SEC column will require a fair amount of mobile phase to be pumped over it to reduce the slow bleeding of small particles from the column. These particles appear because they were not adequately removed by the manufacturer during their production, the material is unstable, and/or were generated during column packing. Nevertheless, one should be aware that additional particles from the SEC column can be generated over time due to wear and tear or instability of the chromatography material. The commercial availability of special SEC columns that are free or contain very low amounts of such debris can be obtained from Polymer Laboratories (a division of Agilent Technologies), Sepax, TOSOH biosciences, Wyatt technology, and others. Also, close attention should be given to all in-line filters used to clarify SEC mobile phases to make sure they are adequately free of shredding or bleeding of material and are properly functioning. Additional sources of such material may come from sample processing and or container closures (e.g., vials).

Additional source of LS detector problems concerns the buildup of material that sticks and accumulates on the walls of the LS cell, which can give rise to a large background signal. Special cleaning solutions (including contact lens cleaning solutions that contain enzymes that can digest biological material stuck to the cell wall) may be periodically required to appropriately clean the LS cell. Wyatt sells a special sonicator attachment (COMET™) for their LS instrument that can vibrate the cell to clean it with normal SEC mobile phase or in combination with appropriate cleaning solutions. Under extreme conditions, however, the LS cell may require the attention of the manufacturer to achieve proper performance.

Finally, although LS detectors respond linearly to sample concentration (where there is no concentration-dependent or non-ideality effects), their total response is dependent on the product of the sample's concentration multiplied by the sample's MW. Hence, the final signal output from a LS detector is not a linear function. Molecules with a MW of 150 kDa will give a LS signal that is 15 times greater than a molecule that is only 10 kDa, when both molecules are at the same concentration in solution. This can be a benefit or a problem. In Fig. 7.7C, a large LS peak is seen effectively in the void volume of the SEC column at about 5.5 min, while in Fig. 7.7D, the corresponding UV chromatogram only shows a trace of a UV signal at that RT. In comparison, in Fig. 7.7D, the very large UV signal from the two overlapping peaks at about 9.5 min shows a small LS signal at this RT in Fig. 7.7C. Consequently, for LMW material, much higher amounts of injected samples are required to increase LS signal to assess

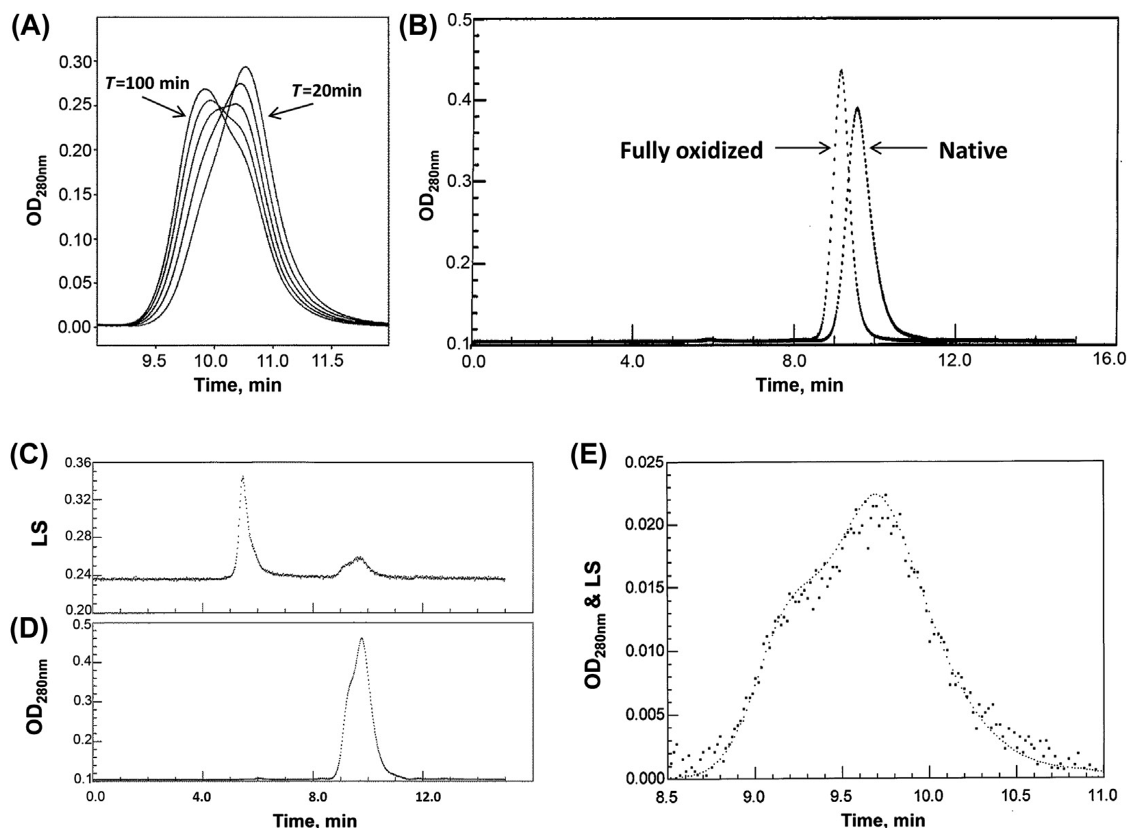

FIG. 7.7 A 23 kDa protein was reacted with a weak solution of H_2O_2 to oxidize it. (A) This reaction was set up and followed by SEC chromatography with UV detection by making injections every 20 min. (B) An overlaid of UV chromatograms of the fully reacted and unreacted protein showing the RT shift to shorter times upon oxidation. (C & D) A mixture of the unreacted and fully reacted protein with LS (top) and UV (bottom) detection. (E) After correction for the inter-detector volume and appropriate normalization, the results show that both the fully reacted (oxidized) and unreacted protein have effectively the same MW as indicated by the good overlay of LS and UV chromatograms giving a fairly constant LS to UV ratio of 1 across the elution profile.

accurate MWs. While for HMW material, much higher amounts of injected material may be required to obtain better signal from the concentration detector (e.g., UV or DRI detector) to assess accurate MWs. Hence, the task of obtaining accurate MWs of LMW and HMW material when both types of material are present in a sample may be a significant challenge depending on the spread in their MWs. In addition, the detection of large LS peaks in the void volume region of an SEC chromatogram may not be related to drug material but may be due to extraneous material in the sample, such as silicon oil, nonionic detergent impurities or even from the HPLC hardware. If buffer blank injections and repeat injections of different prepared solutions of the same material do not show the presence of this large LS peak, further investigation into the source of this signal may be warranted. In the case of Fig. 7.7C, the source of the large LS peak is from the oxidized sample, which is made unstable on oxidation resulting in the formation of the HMW species easily seen by the light scattering detector, but barely seen by the UV detector.

7.7.5 Mass spectrometry

While mass spectrometry will be covered in detail in Chapter 12, it is worth mentioning that SEC separation combined with native electrospray ionization mass spectrometry (ESI-MS) has and continues to be developed for the simultaneous identification and quantification of size variants in recombinant biopharmaceutical proteins. Advances in native MS have enabled the analysis of intact protein and protein complexes under more physiologically representative conditions. Thus, native SEC-UV/MS not only facilitates the detailed analysis of low-abundant and non-covalent size variants during process characterization and validation studies, but also has promise for SEC-UV method validation. The coupling of SEC with MS, however, is not necessarily straightforward. There are many solvent excipients and additives that are simply not compatible with MS (e.g., salts and detergents). As a result, MS compatible buffers such as ammonium acetate are required, which may or may not alter the HOS or other physiochemical properties of the protein. As a result, critical evaluation of data obtained from coupling a MS to an SEC will be needed [48–51].

7.8 Multidetector SEC

In discussing the most commonly used SEC detectors employed in characterizing biopharmaceuticals, we have pointed out on several occasions the common practice of using multidetector arrangements. In these SEC experiments, a series (or tandem) arrangement of detectors is used. Such an arrangement of multiple detectors is primarily applied to characterization work. Rarely are they employed in biopharmaceutical release testing. In the following sections, we will look at additional examples that further illustrate the type of expanded information that can be extracted from these types of SEC experiments, which goes beyond just measuring the level of aggregation in a biopharmaceutical sample.

7.8.1 Detecting conformational change and/or microheterogeneity by SEC

The data presented in Fig. 7.6 illustrate how SEC with LS and UV detection can be used to characterize and confirm the MW of material in a given observed peak. In discussing the data in Fig. 7.6 (in Section 7.7.4), there was an additional piece of information that concerns the peak labeled LMW that was not discussed and has bearing on revealing additional information about the biopharmaceutical, in the injected sample, and its biophysical properties and behavior. Without the use of the LS data, this material would, in fact, be referred to as LMW, given its longer RT. However, the relative ratio of the LS signal to concentration yields a ratio of 1, indicating that the MW of this material is the same as the biopharmaceutical monomer MW. However, for some reason it eluted at a different RT from that of the normal biopharmaceutical monomer peak. There are several explanations that can be envisioned, which predominately focus on the idea that the monomeric biopharmaceutical can take on more than one stable conformation. These explanations include the following: (1) A noncovalent change leading to a more compact conformation (2) a covalent change (e.g., PTM), leading to a more compact conformation, or (3) a small conformational change or just a simple surface modification (due to a PTM) that leads to a surface change on the monomer (rather than to a

significant reduction in hydrodynamic volume) that causes the biopharmaceutical to interact with the chromatography material. To determine which of these explanations might be the correct, additional characterization will be necessary. This will most likely require the use of the same SEC column to trap this material to further characterize it.

In another example, a 23 kDa protein was chemically stressed with a weak hydrogen peroxide (H_2O_2) solution to induce PTMs (oxidation). As a result, a unique reduction in the RT of the biopharmaceutical is observed, see Fig. 7.7A and B. Again, without LS detection this change could easily be interpreted as aggregation, possibly the conversion of the monomer to a dimer. However, a simple SEC run with LS and UV detection on a mixture of the unreacted and fully reacted material, shown in Fig. 7.7C and D, indicates that when the LS and UV SEC chromatograms are appropriately aligned and normalized as discussed for Fig. 7.6, the MW of both the fully reacted (oxidized) and unreacted protein are effectively the same. This is indicated by the good overlay of the appropriated aligned and normalized LS and UV chromatograms that yields a constant LS/UV ratio across the SEC chromatogram, see Fig. 7.7. In addition, preliminary AUC data appear to indicate that the monomeric sedimentation coefficients of the oxidized and normal samples are also the same. As a result, the most likely interpretation of the data is that, in this case the unreacted protein was slightly interacting with the SEC chromatography material and upon reaction with H_2O_2, this interaction was eliminated allowing it to elute earlier.

7.8.2 Critical assessment of multidetector SEC (inter-detector volume effect)

From examples given in this chapter, in addition to the numerous examples that exist in the literature, multidetector SEC is a powerful tool for enhancing our knowledge about the biochemical and biophysical properties of biopharmaceuticals. However, in critically assessing and interpreting data acquired from these multidetector systems, care must be taken in understanding the limitation of the data acquired at different RT values relative to integrated peak areas (where in the latter case, the entire LS and corresponding UV or DRI resolved peaks are integrated and compared). This limitation is due to the band-broadening effect that occurs when an eluted sample passes from one detector to the next. The physical separation of these detectors leads to chromatographic inter-detector volume that causes a time delay, lag, or offset in the recorded data acquired from these detectors, see Fig. 7.8A. This offset is typically assessed by injecting a monodispersed sample into the SEC system that is run under the same conditions as the experimental samples with the same inter-detector volume between the detectors [52]. Assessment of the time difference between the peak maximum is then used to account for the chromatographic time delay or offset to realign the chromatograms, see Fig. 7.8B. On normalizing these chromatograms so the peak maximums are the same, as indicated in Fig. 7.8C, it turns out there is a distinct broadening of the downstream detector chromatogram relative to the upstream detector chromatogram. It is this band-broadening, which if not corrected, will introduce bias into the computed results leading to inaccurate data (at least on a data point-to-data point basis). However, when integrated peaks are used this error will be significantly reduced. Depending on the sequential ordering of the detectors, the results obtained will yield different point-by-point ratio values, even when the eluting peak is known to be truly homogeneous, if this band broadening is not corrected (see Fig. 7.8C). Over the years, there have been many investigations

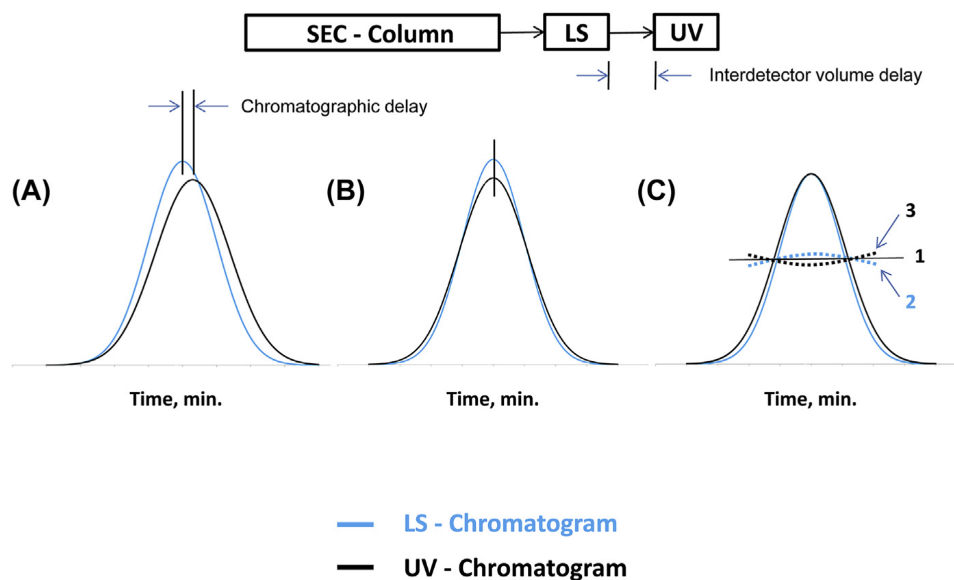

FIG. 7.8 The impact of the physical inter-detector volume, shown in the simple Chromatographic setup at the top of this figure, in conducting real multidetector SEC using a model situation where two identical detectors (called detector 1, which is the closest to the SEC column, and detector 2, which the furthest from the SEC column) are used in tandem. (A) The raw output from the two detectors as a function time. In this case the response from detector 1 is the light blue (light gray in print version) trace (i.e., taller peak in (A) and (B) and narrower peak in (C)) and the response from detector 2 is the black trace. The observed time delay or shift between these two chromatograms is due to the increased distance the sample must travel from detector 1 to reach detector 2. In addition to the time shift in the output from detector 2 the response from detector 2 is also lower and the peak is broader. This reduction in height can be better seen when the two chromatograms are properly aligned (to account for the inter-detector volume) as shown in part (B). (C) Normalizing these two chromatograms to their peak maximums reveals the incurred chromatographic band-broadening effect (in going from detector 1 to detector 2). If the corresponding data points for both chromatograms are rationed, the resulting ratios will not be the same across the chromatogram. In a real SEC-LS-UV setup, like that indicated at the top of this figure, if a homogeneous biopharmaceutical is used the LS/UV ratio values will be bias high around the peak maximum and become progressively biased low at the tails regions of the chromatogram yielding a plot of LS/UV ratios that is convex, as indicated by the *light blue dotted curve* (light gray in print version) labeled 2, instead of a constant LS/UV ratio indicated by the *black line* labeled 1. However, if the detector order is reversed (LS-UV is changed to UV-LS) the plot of their LS/UV ratios would be bias low at the peak maximum and progressively bias higher at the tail region of the plot to give a concave *dotted black curve* labeled 3.

and attempts to try to more effectively deal with this inter-detector volume and band-broadening problem [53–56] and in some case there have been patented and commercialized [57] procedures that were developed to minimize its effect.

7.8.3 Protein extinction coefficient

The use of LS detection to assess the MW of a complex biopharmaceutical was presented in Section 7.7.4, Eq. (7.8). In many cases, the MW of the biopharmaceutical monomer is accurately known via its amino acid sequence. However, what is unknown is the protein's extinction coefficient (ε_p). Although theoretical calculations using empirical equations [58–60] have been developed to predict this parameter from a protein's amino acid composition with reasonable accuracy of about ±5%, in some cases the uncertainty could approach ±10% or

more. Consequently, experimental verification of ε_p would be useful. This is particularly important in the case of assessing comparability between a biosimilar and its innovator drug molecule, as well as for complex biopharmaceuticals, e.g., pegylated or highly glycosylated proteins. Given the important role of concentration, which is usually assessed via a protein's UV extinction coefficient, the ability to assess such a parameter directly from an experimental technique, such as SEC−LS−UV−DRI can be very powerful. The technique requires nothing more than the injection of the sample onto an SEC column, which contains LS, UV, and DRI detectors. Furthermore, if these measurements are conducted with a highly purified biopharmaceutical material that yields a well-resolved SEC monomer peak, measuring the same monomer peak area (which will reduce erroneous contributions from band-broadening effects) for each detector enables the biopharmaceutical's ε_p value to be determined (independent of values calculated from their amino acid sequence) with an experimental uncertainty of about ±5% using Eq. (7.9) and a calibration procedure to obtain the value for K' outlined by Wen et al. [36]. However, if all that is needed is a relative head-to-head comparison, which can be done by taking the ratio of the measured extinction coefficient of the two samples being compared and asking how close to 1 is the ratio (as would be the case in comparability studies, e.g., between a biosimilar and the innovator biopharmaceutical), then the experimental uncertainty drops to about ±1−2% or better since the evaluation of constant K' is not required.

$$\varepsilon_p = \left(K'[\mathrm{LS}][\mathrm{UV}]^2\right) \Big/ (M_P[\mathrm{DRI}]) \tag{7.9}$$

7.8.4 Characterizing the concentration-dependent behavior of biopharmaceuticals

One of the more difficult issues surrounding biopharmaceutical development, which has attracted a get deal of attention recently, concerns the self-association of these molecules at high concentrations [61−63]. Given the high interest in developing high concentration biopharmaceuticals, methods are needed in the drug screening process of finding candidates and formulations that will yield a drug product that can successfully achieve these high concentrations in an efficient and economic manner. Methods that can find these drug candidates with favorable properties and formulations quickly with minimum amounts of material are highly desirable. In the past, there have been reports where SEC has been used to assess information on concentration-dependent aggregation and determine second virial coefficients [64−66]. However, these SEC methods have limited sample throughput, the ability to cover a limited range of concentrations, can consume a large amount of material, and are time consuming. Work from Kalonia's laboratory at the Univ. of Conn. has shown that SEC with LS and UV detection can offer an accurate approach to study the solution behavior of biopharmaceuticals by measuring the second virial coefficient using a specially designed cell [67,68]. Although LS is a classical technique for measuring second virial coefficients, a few issues have made this approach not as attractive. These issues include: (1) The need to adequately remove the presence of irreversible aggregates, especially large and high scattering aggregate material that does not play any role in the biophysical properties of the molecule being studied, but rather just interferes with the process of acquiring good LS data. (2) The

time-consuming need to make many solutions of different concentration and buffer composition solution to assess second virial coefficients under different solution conditions. (3) The need for a fair amount of material to make these measurements. Using the approach outlined by Kalonia and co-workers, a single high concentration sample is injected onto an HPLC system containing an SEC column with LS and UV detection. The SEC system then simultaneously buffer exchanges the sample, removes the interfering aggregates, and generates a peak that intrinsically provides a range of concentrations on both sides of the peak. This essentially treats the samples as different concentrations that passes through a single cell in which both LS and UV measurements are made on effectively the same sample (avoiding issues associated with inter-detector delay and band-broadening effects as discussed in Section 7.8.2). Hence, in one injection all the necessary data can be acquired to assess the second virial coefficient. Different experimental conditions could then be investigated by simply changing, for example, the mobile phase and/or temperature. At present, however, it is not clear how high a concentration one could go to using this type of system.

7.9 Aggregation

Given the use of SEC as the key biophysical technique for assessing the level of aggregation in biopharmaceuticals, SEC is shouldering a very significant responsibility in the development of these drugs. When SEC is working well, its ability to service this area is very good. Typically, SEC can monitor aggregation with an estimated limit of quantification (LOQ) that ranges from 0.1% to 0.5% total aggregation [69,70]. However, the ability to verify this accuracy is very difficult. Although orthogonal biophysical techniques such as AUC and FFF (the latter of which includes asymmetric flow field flow fractionation (AF4) [71,72] and more recently hollow fiber flow field flow fractionation (HF5) [73]) can be used to help in this assessment, these techniques have their own limitations. In the case for AUC its LOQ is more likely in the range of 2% at best, see Chapter 9, Section 9.5.6.1, while in the case of AF4 and HF5, there are potential unknown issues surrounding the possibility that aggregates present in a biopharmaceutical could be binding to the membrane or tubing used in these techniques.

Furthermore, while SEC can separate aggregates present in a biopharmaceutical, care is needed in interpreting the nature of these apparent aggregates based on their RT. There are many reasons that can lead to the misinterpretation of SEC data, including: (1) the intrinsic heterogeneity of fragments, (2) altered conformations and degeneracy, in terms of the number of different configurations an aggregate of two or more monomers can come together, (3) the microheterogeneity of the biopharmaceutical, (4) the interactions between the biopharmaceutical and/or its variant forms with the chromatography material, HPLC system, and/or sample vials, (5) the presence of leachable and excipient impurities, and (6) HMW impurities from excipients. This is where multidetector SEC systems can make a significant contribution.

7.10 Technology advances

Areas for improving SEC have generally focused on increasing chromatographic resolution, increasing sample throughput and in reducing the amount of sample required per

analysis. At present, the only advances in SEC that have made progress in these areas have been associated with the reduction in particle size of the SEC chromatography material. Chromatography particles as small as 1.7 μm are now available with a few different pore sizes that can be purchased in packed columns of varying lengths [74]. Using columns packed with these small chromatography particles has reduced analysis times by as much as 20-fold, significantly reduced the amounts of the sample required to be injected on to these columns, and it has greatly improved chromatographic resolution. The implementation of these SEC columns, however, requires the use of an HPLC system capable of handling very high pressures (e.g., >10,000 psi) where careful attention is required to minimize dead volume by using optimum fitting connections, biocompatible materials, and appropriate detector flow-cells to maximize the high efficiency of these SEC columns. In running these "ultra-high pressure" columns, one must also be aware of the potential impact of the required high pressures needed to push liquid through these columns, since for a given column length pressure scales inversely with the square of the chromatography particle diameter. Reports have appeared indicating that increases in pressure can induce aggregation [74–76], as well as disassociate aggregates [77–79], and can promote conformational changes. The presence of such effects on the biopharmaceutical being tested, if not realized, accounted for or removed, will result in misleading data and conclusions.

It should also be mentioned that reports have appeared concerning the investigation into the opportunities of using monolith column structure [4] and capillary format [80] for SEC columns, as well as a novel use of two 1.7 μm packed SEC columns to conduct interlaced and parallel SEC. In the case of the latter, SEC analysis times of a biopharmaceutical were reduced to less than 2 min per sample [75]. In the case of capillary SEC, material requirements were reduced to picogram levels, allowing SEC analysis to be carried out on very little material [80]. This latter capability is particularly useful in the early phase of drug development, where only limited amounts of material exist to conduct drug candidate selection.

7.11 Conclusion

Irrespective of the limited working separation space available to SEC, and notwithstanding its potential problems, many of which we have discussed in this chapter and by others [69,81–83]. SEC is still the principal tool for assessing aggregation for nearly all biopharmaceuticals. The positive advantages of a well-working SEC method have yet to be matched or exceeded by any other biophysical technique(s). Thus, the cautious use of SEC is a necessary and critical element of its success. Key to this is the supplementing of SEC results with other biophysical measurements to validate its proper operation. Once validated and optimized, this simple tool can provide a wealth of additional information about the biophysical properties and behavior of biopharmaceuticals that goes beyond just measuring the level of aggregation. In this chapter, we have touched on some of these capabilities, but by no means do we claim to have covered all the opportunities SEC has, can and will contribute to the science of developing biopharmaceuticals.

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