Chapter 21

Size Exclusion Chromatography (SEC)

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21.1 INTRODUCTION

In SEC, molecules are separated based on differences in their apparent size, which is influenced by both molecular weight and shape [1]. The technique is also referred to as gel filtration or gel permeation chromatography, but size exclusion chromatography is used here because today, it is the most widely used term, and furthermore, it describes the separation principle. Although SEC is used in both analytical and preparative applications [1], this text focuses on the latter. The separation mechanism was first observed in the mid-1950s using swollen starch [2,3], and quickly gained popularity due to its gentle separation mechanism, preserving molecular structure and biological activity, and the commercial availability of suitable macroporous resins [4]. Resins for SEC may be constructed from different material, for example, natural polymers such as cross-linked dextran or agarose, synthetic polymers such as cross-linked polyacrylamide or polymethacrylate, composites of both natural and synthetic polymers, or silica. The separating properties of a SEC resin are based on pore size and pore-size distribution in the chromatography beads, and there are, in principle, no ligand interactions involved. Solutes that are sterically smaller than a given pore in the resin can diffuse into the pore, while larger solutes are excluded. When a sample is applied to a SEC column and eluent flow started, smaller solutes have access to a larger part of the total pore volume than larger solutes. As a result, solutes varying in size will gradually become separated as they pass along the column, and large solutes, which have access to a smaller part of the column volume (CV), will elute earlier than small solutes (Fig. 21.1). Since the main separation occurs in the pores of the chromatographic beads, and because ideally there is no binding of solute to the resin, capacity in SEC depends on the volume of the sample relative to the pore volume in the column, and not the quantity of solute in the sample relative to binding sites in the resin. More pores of useful size and size distribution, and a larger column volume relative to sample volume will provide more volume for separation. In practice, the sample volume in SEC ranges from approximately 2% to 30% of the CV, depending on the type of application (see Section 21.3).

In preparative chromatography, SEC usually delivers high product yields (>90%), but it suffers from the need for relatively small sample loads and, in most applications, low flow rates compared with those used in other types of chromatography. Small sample load and low flow rate result in low productivity. This limits the use of SEC in large-scale production processes; although, historically, SEC has had an important role in the production of some of the earliest biopharmaceuticals (e.g., insulin), and it is common to use SEC as a simple technique for purification and buffer exchange when preparing smaller amounts of biopharmaceuticals in early development phases.

21.2 BASIC THEORY

This section describes some theory and expressions used to define operating parameters in SEC. For a more extensive description, see reference [1].

Results from SEC experiments are usually expressed as an elution profile or chromatogram that illustrates the variation in concentration of eluted sample components (for proteins typically shown as UV absorbance, at wavelength 280 nm) as they elute from the column in order of their apparent size. Fig. 21.1D shows a hypothetical chromatogram of a SEC fractionation. The total volume of a column, CV, can be divided in different partial volumes which are conceptually depicted in Fig. 21.2.

Molecules that are too large to enter pores in the matrix are eluted together in the void volume, V_0 (Figs. 21.1, 21.2, and 21.4), as they pass directly through the column at the same speed as the flow of eluent. For a well-packed SEC column, V_0 is approximately 30% of the CV. Molecules with partial access to the pores of the matrix, elute from the column in order of decreasing size, that is, the smaller the molecule, the larger the accessible pore volume and the later the elution. Small molecules such as salts that have full access to the intraparticle pores, move through the column, but do not separate from each other. These molecules elute at the total liquid volume $CV - V_s = V_t$ (Figs. 21.1 and 21.4), just before one CV for resins that have a low matrix content (i.e., a very high proportion of intraparticle pore volume, V_i, relative to particle volume, V_p).

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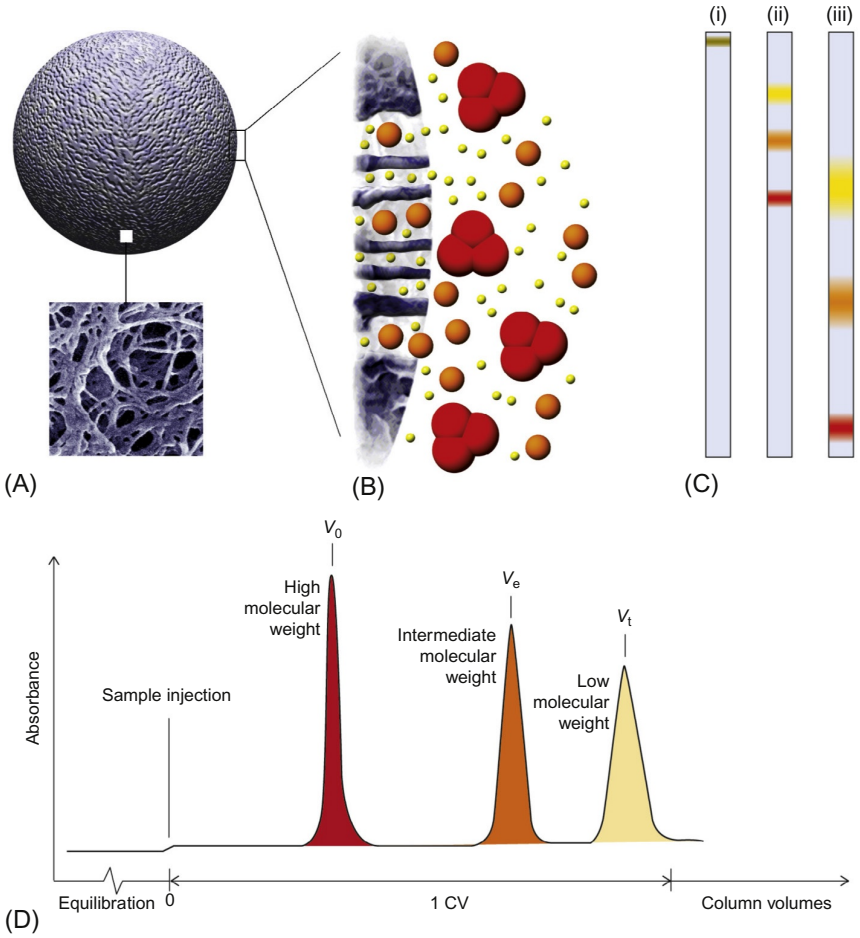

FIG. 21.1 SEC principle. (A) Schematic picture of a chromatography resin bead with an inserted electron microscopic enlargement. (B) Schematic drawing of sample molecules diffusing into or being excluded from the bead pores. (C) Graphical description of separation: (I) sample applied on the column; (II) the smallest molecule *(yellow)* is more delayed than the largest molecule *(red)*; (III) the largest molecule is eluted first from the column. Band broadening causes significant dilution of the protein zones during SEC. (D) Hypothetical chromatogram. *Courtesy of GE Healthcare. Size Exclusion Chromatography: Principles and Methods, GE Healthcare Life Sciences, 18-1022-18. http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/pdfs/Size%20Exclusion%20Chromatography.pdf.*

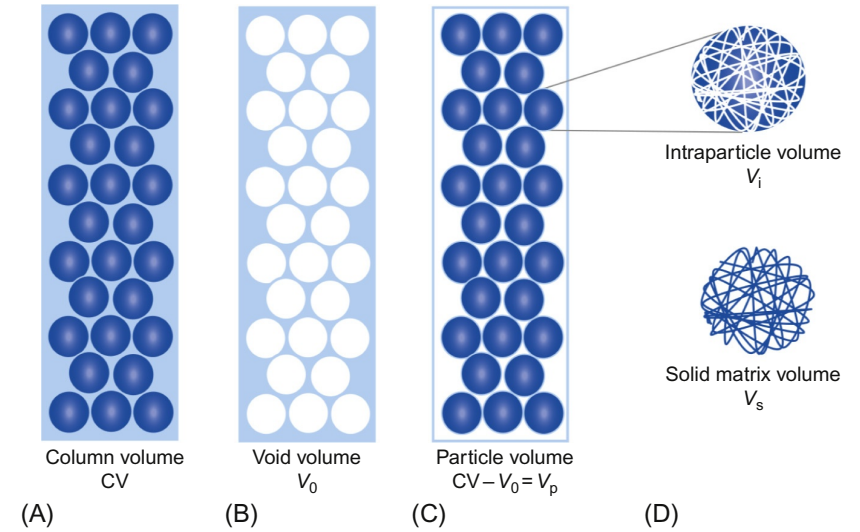

FIG. 21.2 Diagrammatic representation of a column and its subset of volumes indicated in *blue*. From left to right, the total column volume, CV (A), the void volume, V_0 (B), and the resin particles volume, V_p (C), where V_p is made up of the intraparticle pore volume, V_i (D upper), and the volume of the solid matrix, V_s (D lower). *Courtesy of GE Healthcare, adapted from L. Fischer, An introduction to gel chromatography, Laboratory Techniques in Biochemistry and Molecular Biology, vol. 1 part II, North Holland Publishing Company, Amsterdam, 1969.*

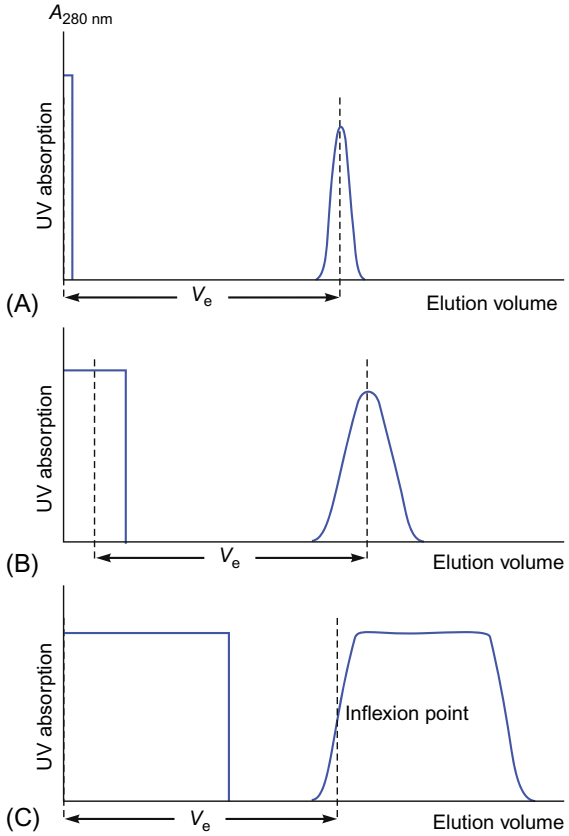

FIG. 21.3 Measurement of elution volume, V_e. (A) Sample volume negligible compared with volume of packed bed. (B) Sample volume not negligible compared with volume of packed bed. (C) Sample volume giving plateau elution curve. *Courtesy of GE Healthcare. Size Exclusion Chromatography: Principles and Methods, GE Healthcare Life Sciences, 18-1022-18. http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/pdfs/Size%20Exclusion%20Chromatography.pdf.*

The part of the column volume which is accessible for each component is expressed in terms of the component elution volume, V_e, which is measured/calculated from the chromatogram (Figs. 21.1, 21.3, and 21.4).

For SEC, this elution position is typically independent of the amount of solute or its concentration in the sample. However, there are exceptions, as for example a highly concentrated sample may have high viscosity relative to the eluent and this can lead to asymmetric peaks and various tailing and fingering phenomena which will distort the peak shape and make the estimation of V_e difficult. As shown in Fig. 21.3, there are three different ways of measuring V_e, dependent on the volume of sample applied to the column. The methods described in Fig. 21.3 are valid for symmetrical peaks and fronts, which are common in SEC at typical, rather low, flow rates and with low viscosity samples (relative to the eluent). V_e is a characteristic property of a sample component for a given SEC resin and sample in a column of fixed dimensions, and can therefore not be used for direct scale-up purposes, since it varies with the total volume of the packed bed and how well the column has been packed. Therefore, for scale-up, and/or comparison purposes, the elution of a component is best characterized by a distribution coefficient, K_d. K_d is independent of column dimensions and thus allows comparison and prediction between columns with different sizes, if the same resin and sample is used. The distribution coefficient, K_d, for a given sample component is defined as the fraction of the total intraparticle pore volume, V_i, accessible for that component. K_d is calculated as follows:

$$K_d = \frac{V_e - V_0}{V_t - V_0} = \frac{V_e - V_0}{V_i} \tag{21.1}$$

where V_e is the component elution volume, V_0 is the column void volume, V_t is the total liquid volume, and V_i is the intraparticle pore volume.

Since in practice, V_t, and therefore V_i, can be difficult to estimate due to weak interactions between small probes and groups on the surface of pores in the beads of the stationary phase, it is more convenient to employ the term $(CV - V_0)$ in calculations of the distribution coefficient. The distribution coefficient thus achieved is designated K_{av}.

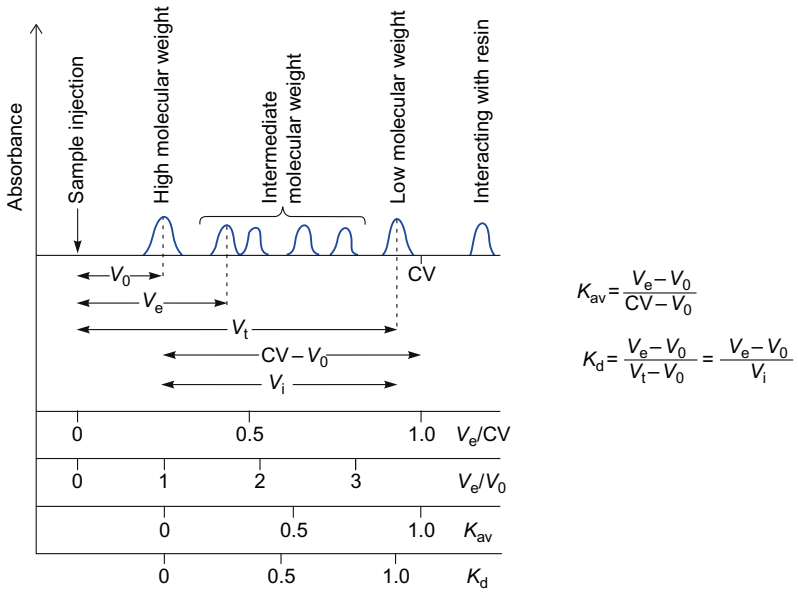

FIG. 21.4 Relationship between several expressions used for normalizing elution behavior. *Courtesy of GE Healthcare. Size Exclusion Chromatography: Principles and Methods, GE Healthcare Life Sciences, 18-1022-18. http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/pdfs/Size%20Exclusion%20Chromatography.pdf.*

$$K_{av} = \frac{V_e - V_0}{CV - V_0} \tag{21.2}$$

As seen from the above equation, for a solute that can access part of the intraparticle volume, K_{av} is a measure of the accessible pore volume relative to the total particle volume in the column. K_{av}, like K_d, defines sample behavior independently of the column dimensions and how tightly the beads are packed. The approximate relationships between various terms used in SEC are shown in Fig. 21.4.

Since $(CV - V_0)$ includes the volume of the matrix backbone, V_s, that is inaccessible to all solute molecules, K_{av} is not a true partition coefficient. However, for a given resin there is a constant ratio of K_{av} to K_d which is independent of the nature of the molecule or its concentration.

$$\frac{K_{av}}{K_d} = \frac{V_i}{V_s + V_i} \tag{21.3}$$

The partition coefficient, K_{av}, is related to the size of a molecule, and molecules of similar shape demonstrate a sigmoidal relationship between their K_{av} values and the logarithms of their molecular weights (M_r), termed a selectivity curve. This selectivity curve is useful for selection of SEC resin for the desired separation. Over a considerable range there is a linear relationship between K_{av} and $\log M_r$ for molecules of a similar type and shape (Fig. 21.5). The ability of each SEC resin to

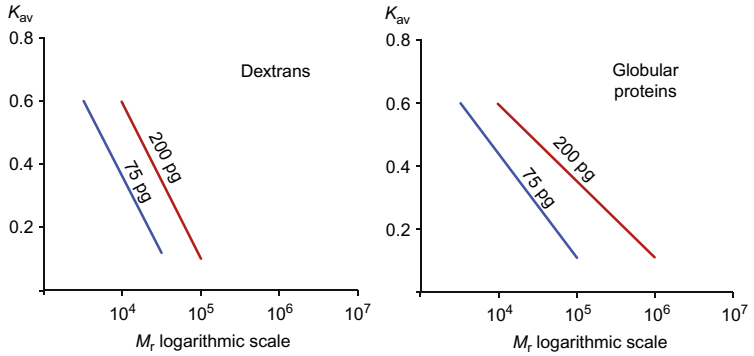

FIG. 21.5 Selectivity curves for dextrans and globular proteins on Superdex 75 and 200 pg resins. *Courtesy of GE Healthcare. Size Exclusion Chromatography: Principles and Methods, GE Healthcare Life Sciences, 18-1022-18. http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/pdfs/Size%20Exclusion%20Chromatography.pdf.*

separate molecules depends on its pore size distribution and is described by this selectivity curve. Plotting K_{av} against the log M_r for a set of standards (e.g., dextrans or globular proteins, Fig. 21.5), is helpful for predicting where a solute will elute on a specific SEC resin and for choosing the appropriate SEC resin for a separation.

SEC resins intended for fractionation (see Section 21.3) should be selected so that the important sample components are found in the most linear part of the selectivity curve. Comparing resins, the steeper a selectivity curve, the higher the resolution between two similarly sized solutes that can be expected, given similar performance regarding peak widths (column efficiencies). Under ideal conditions, no molecules can be eluted with a K_{av} greater than 1 or less than 0. If the K_{av} of a solute is greater than 1, molecules have interacted with the chromatographic resin. If the K_{av} of a solute is less than 0, then there is probably channeling or some other change in the chromatography bed packing, and the column must be repacked.

21.3 APPLICATION AREAS

There are two principal types of SEC in preparative process applications, defined by the purpose and difficulty of the separation: (1) buffer exchange/group separation where there is a large difference between the sizes of the target molecule and those molecules that need to be removed or exchanged, and (2) fractionation where molecules of similar sizes need to be separated [1]. A concentrated sample is preferred in both cases to increase the productivity, since the sample volume is limited to about 15%–30% of the CV in buffer exchange/group separation and about 2%–6% of the CV in fractionation. Thus, SEC is often preceded by a concentration step, for example, precipitation/re-dissolving, ultrafiltration, or bind/elute chromatography, although consideration must be given to the viscosity of the sample relative to the eluent. In view of the sample volume restriction with SEC, a large sample can be applied in several cycles to the column, thereby reducing the size of column required. The choice of flow rate in SEC (usually expressed as flow velocity cm/h) is influenced by the size of the solutes. Considering solutes that enter pores in the SEC resin, lower flow velocities are required for large solutes that diffuse slowly, and higher flow velocities can be used for separation of small solutes that diffuse more rapidly. High flow velocities can frequently be used for group separations and buffer exchange, because the small molecules or buffer components diffuse rapidly and the solute of interest elutes in the V_0 (i.e., without diffusing into the pores). There is always dilution of solutes in a SEC step, and due to this, its limitations regarding sample loads, and the nature of its selectivity, SEC is often used late in a process as a polishing step, for example, for separation of monomers from dimers or aggregates.

In group separations, such as buffer exchange and desalting, the molecules to be separated must differ substantially in size, for example, by a factor of 10 more [1]. Buffer exchange and desalting typically involve separation of large biomolecules from much smaller salts and buffer components, where the biomolecules are completely excluded from the pores of the beads and elute in the void fraction, and the small buffer components enter the pores [5]. As the biomolecules pass through the column, they are excluded from the resin pores and elute from the column at V_0 in the "new" buffer (with low salt content for "desalting" applications), while salts and "old" buffer components elute around V_t. In general, the dilution of target solutes is moderate in group separations; the molecules which do not enter the pores can usually be collected in approximately 1.2–2 times the sample volume.

In fractionation, the molecules to be separated are similar in size, for example, 2–5 times size difference, such as in the separation of monomers from dimers and aggregates [1]. The dilution of target solutes is usually quite high in fractionation, with product recovery frequently in 4–6 times the sample volume.

There should be no ligand interactions or adsorption of solutes to the resin in pure SEC. However, a recently introduced core bead resin is based on a combination of SEC and adsorption principles, where large molecules are excluded from the pores as in conventional SEC, but smaller solutes which enter the pores are adsorbed to ligands which are present only in the core of the beads [6]. See also Chapter 20.

21.4 EXAMPLES

SEC is used in many different applications, such as purification of plasma proteins, recombinant proteins, monoclonal antibodies, nucleic acids, carbohydrates, viruses, etc. The examples presented here are not from large-scale biopharmaceutical processes, but they demonstrate SEC principles in different preparative applications.

21.4.1 Group Separation—Buffer Exchange/Desalting

A sample of human blood plasma was subjected to buffer exchange on a column packed with Sephadex G-25 Coarse, see Fig. 21.6. The pore size of the resin allows very small molecules such as salts to enter the pores of the beads, while the plasma proteins are excluded. The rapid mass transfer of small salt molecules enables high flow rates and a quick process step in buffer exchange. The separation in Fig. 21.6 was performed at 300 cm/h, and the run took 8 min. A plasma sample is relatively viscous, and this can lead to broad peaks of the plasma proteins (which are excluded from the pores) and the salts (which enter the pores).

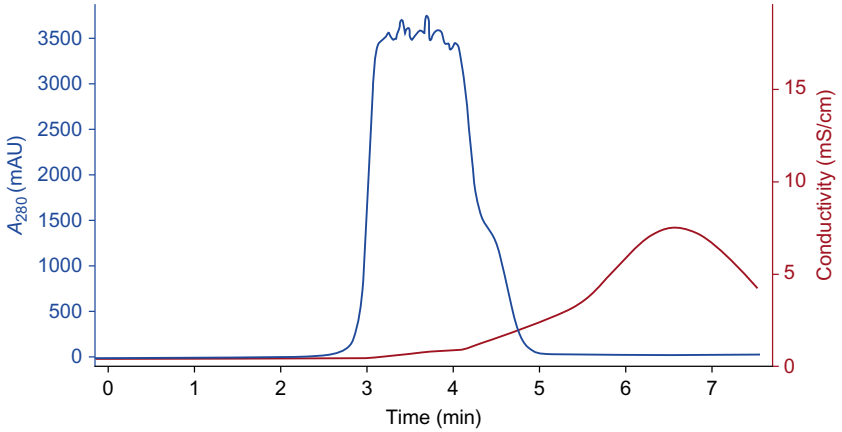

FIG. 21.6 Buffer exchange (desalting) chromatogram. 363 mL of a plasma sample (14% of the CV) was applied to a column with Sephadex G-25 Coarse (diameter 10 cm, bed height 33 cm, CV 2590 mL). *Blue curve*: absorbance at 280 nm. *Red curve*: conductivity. *Courtesy of GE Healthcare.*

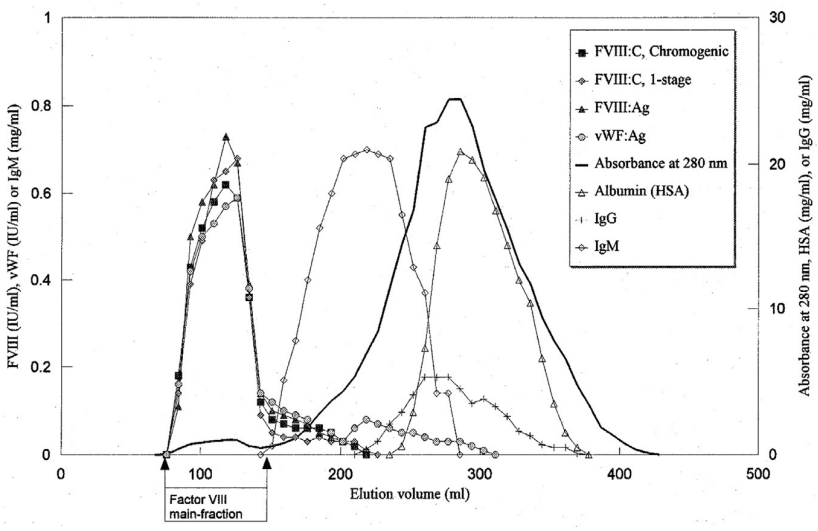

FIG. 21.7 Group separation chromatogram. 50 mL of plasma (17% of the CV) was applied to a column with Sepharose 4 Fast Flow (diameter 2.6 cm, bed height 60 cm, CV 300 mL). The absorbance at 280 nm was recorded to monitor the protein peaks, fractions were analyzed for presence and activity of selected proteins. *With permission from P. Kaersgaard, K.A. Barington, Isolation of the factor VIII-von Willebrand factor complex directly from plasma by gel filtration, J. Chromatogr. B 715 (1998) 357–367.*

The sample volume was therefore limited to 14% of the CV to achieve adequate resolution. The purpose of the buffer exchange was to remove salts (desalting) and transfer the plasma proteins to conditions suitable for the subsequent process step.

21.4.2 Group Separation—Large Proteins

The example in Fig. 21.7 shows group separation of very large plasma proteins, for example, coagulation Factor VIII-von Willebrand Factor complex (FVIII/vWF), from the bulk of plasma proteins [7]. FVIII/vWF is much larger than the most plasma proteins, which makes this group separation possible. The pore size distribution of the resin will allow salts and the bulk of plasma proteins, for example, albumin and IgG, to enter the pores, while the large FVIII/vWF is excluded and elutes in the void fraction. The flow velocity for this separation is limited by the slow diffusion of the bulk of the plasma proteins, and the run was performed at 40 cm/h. The separation also results in buffer exchange of the FVIII fraction in preparation for the subsequent process step.

21.4.3 Group Separation—Virus

The example in Fig. 21.8 shows a group separation of cell culture-derived influenza virus from impurities [8]. The virus particles are much larger than the impurities, which makes group separation possible. The porosity of the resin will allow

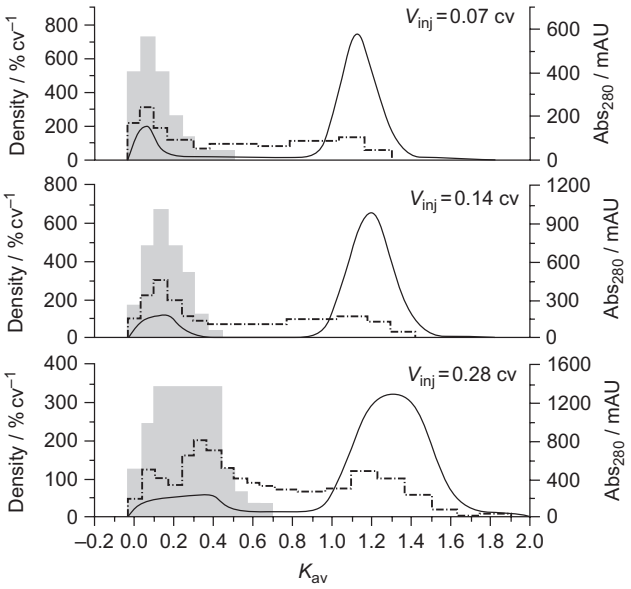

FIG. 21.8 Loading study of an agarose based SEC resin using concentrated influenza virus preparation. Column was loaded with 7%, 14%, and 28% of the CV. Eluates were analyzed for hemagglutinin (HA) activity *(shaded)* and total protein *(dotted line)* and UV absorbance at 280 nm *(solid line)*. *With permission from B. Kalbfuss, M. Wolff, R. Morenweiser, U. Reichl, Purification of cell culture-derived human influenza A virus by size-exclusion and anion-exchange chromatography, Biotechnol. Bioeng. 96 (2007) 932–944.*

salts and the bulk of the impurities to enter the pores, while the large virus particles are excluded. The flow velocity is limited by the diffusion rate of the protein impurities, and this run was performed at 60 cm/h. The separation also results in buffer exchange of the virus fraction.

Another example of a group separation of large entities is the preparation of an adeno-associated viral (AAV) vector for gene therapy. The material was intended for use in clinical phase I/II studies, so the process was run under GMP conditions. The chromatography part of the process contained two SEC steps and one ion exchange step [9].

21.4.4 Fractionation—Monoclonal Antibody (mAb)

The example in Fig. 21.9 shows fractionation of dimers (early small peak in absorbance at 280 nm) and monomers (subsequent large peak) of a mAb sample. In this example, none of the molecules is totally excluded from the beads; that is, both dimers and monomers enter the pores where the separation takes place. The separation requires not only accessible pore volumes for both types of molecules but also the mass transfer (intraparticle diffusion) of the dimers and monomers. Therefore, the run was performed at the low flow velocity of 25 cm/h. The separation also results in buffer exchange of the antibody fractions (the dip in conductivity at the end of the chromatogram indicates the elution position of the original

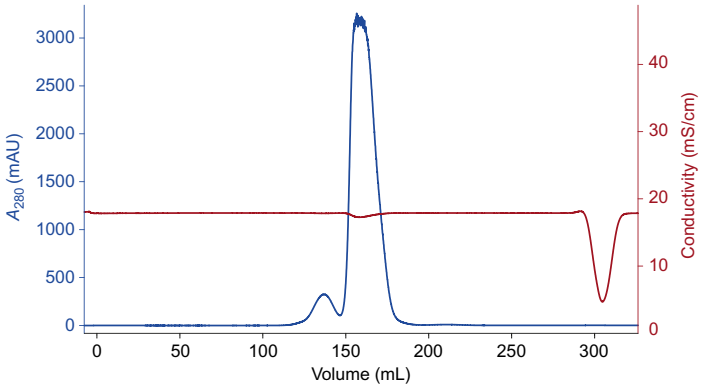

FIG. 21.9 Fractionation chromatogram. 13 mL of a mAb sample (4% of the CV) was applied to a column with Superdex 200 prep grade (diameter 2.6 cm, bed height 60 cm, CV 319 mL). *Blue curve*: absorbance at 280 nm. *Red curve*: conductivity. *Courtesy of GE Healthcare.*

buffer of the antibody sample at approximately V_t). In fractionation, relatively small sample volumes are applied (4% of the CV in Fig. 21.9), which results in low productivity. To circumvent this disadvantage, samples can be applied at frequent intervals, exploiting "transport zones" in the chromatogram where nothing significant happens, see Fig. 21.11.

21.5 STANDARD METHODS

The standard SEC protocol is straightforward, as it requires only one buffer and a simple sequence of steps: equilibrate the column with buffer, apply the sample, switch back to buffer, and collect the eluted product peak. The procedure is suited for repeated cycles to handle large sample volumes, especially since there is no binding of components to the resin (at least in theory). Once all cycles are complete, the column and system can be subjected to cleaning-in-place (CIP) and sanitization.

21.6 BUFFERS

In theory, SEC operates independently of the composition of the buffer used for elution, unless the conditions affect solute molecular size or shape (or the pore dimensions in the resin beads), or promote interactions with the resin. The buffer should provide appropriate conditions for target solute stability and SEC resin stability, and in most cases, adjust to conditions suitable for the subsequent process step. Addition of electrolytes, for example, 50–150 mM NaCl, to the buffer may be needed to prevent interactions with the small amounts of ionic groups commonly present on SEC resins [1]. Buffer consumption can be large in a SEC process step due to a large CV, and several cycles can be needed to process the entire sample. One approach is to use buffer concentrates, for example, 5× or 10×, and use the chromatography equipment for inline dilution of the buffer concentrate with water. However, the volume of waste buffer will still be the same.

21.7 CIP/SIP

A SEC process step is often performed by running several cycles on the same column(s), whereas cleaning-in-place (CIP) and sanitization-in-place (SIP) are typically performed once, after all cycles are finished. In many cases CIP and SIP can be combined. The solutions used for CIP and SIP depend on the types of contaminants, and the chemical stability of the resin and the equipment. Alkaline CIP and SIP with NaOH (0.1–1 M) is the most common choice. In some cases, acidic conditions or different additives might be needed. In theory, there should not be any interactions between the SEC resin and the components of the sample, which might imply that there is no need for a CIP procedure. However, unexpected interactions can cause some fouling, and a CIP is therefore recommended, preferably with reversed flow direction, as fouling is more likely to occur at the column inlet on the resin or column net. Note that the CIP and SIP solution(s), and the concentrated release of contaminants might cause an increase in back pressure, and it can be necessary to reduce the flow velocity during these steps.

21.8 PROCESS DEVELOPMENT (PD) WORKFLOW

A SEC separation is difficult to mimic in high throughput process development formats such as 96-well plates or mini-columns. On the other hand, SEC separations are relatively simple to scale up, provided the resin can be packed and operated in columns of the required size. PD in SEC is therefore performed using lab-scale columns, typically with bed heights of 10–90 cm, depending on the application area (buffer exchange, group separation, or fractionation). Taller bed heights are often needed for fractionation applications to get sufficient resolution (doubling of bed height increase resolution by the square root of 2). Column packing and evaluation of column packing is important at all scales from PD to final process scale, since a badly packed column will result in broadening of the sample zone, which in turn results in more diluted fractions and impaired separation. The temperature of the full-scale process step must be considered during PD, as temperature can affect the diffusion rate of the solutes and the viscosity of the liquids—relatively important in SEC.

SEC resins cover a range of pore dimensions and are sometimes available with different bead diameters. Resins with small beads give better resolution, due to shorter diffusion distances, and offer higher yields for challenging separations or higher flow velocities when resolution is adequate. However, small beads also result in increased column backpressure which can be problematical at large scale, especially for soft resins that can operate without problem at lab scale in narrow columns that give wall support, but might fail in wide diameter columns, and also due to pressure limitations of large-scale equipment. One must therefore consider flow velocity and back pressures for the estimated column diameter and bed height to be used in the full-scale process, when evaluating SEC resins. Some large-scale applications overcome such problems by running several shorter columns in series; in place of one single tall column, for example, running 3 columns with 30 cm bed height instead of one column with 90 cm bed height.

It is the pore dimensions which determine the fractionation range of SEC resin. In buffer exchange/group separation, where the size difference is large, one should aim for a resin that has a pore size that excludes the target molecules but not the contaminants, or vice versa. In fractionation, the size difference between the target and contaminants molecules is small. In this case, the choice of a resin with suitable pore dimensions and fractionation range is critical, and a guideline value is that the target molecule should elute at approximately half a CV [1]. The examples in Fig. 21.10 demonstrate how the V_e of the sample proteins gradually shift from close to V_0 in the top chromatograms, to close to V_t in the bottom chromatograms, as the pores of the resins become larger [10].

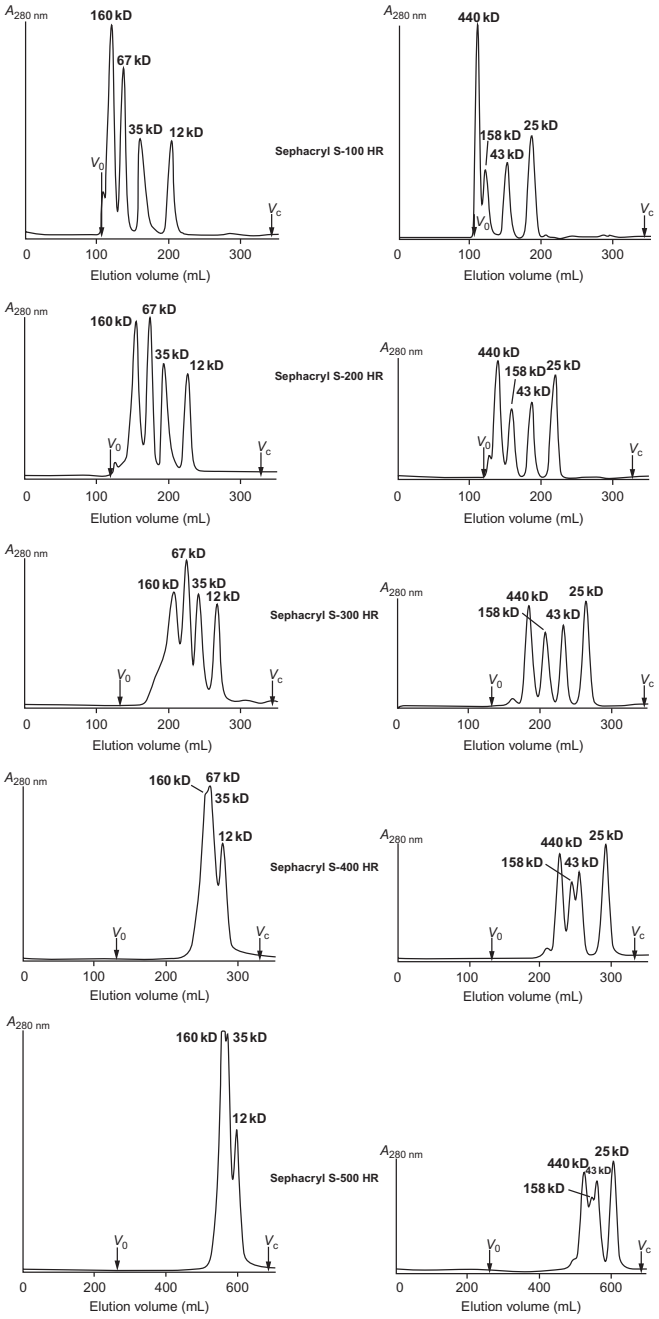

FIG. 21.10 Pore size effect on fractionation range. Two protein mixtures (left: smaller proteins, right: larger proteins) were applied to columns packed with different SEC resins. The pore size of the resins increase from top to bottom, where Sephacryl S-100 HR at the top has the smallest pore size, and Sephacryl S-500 HR at the bottom has the largest pore size. *With permission from L. Hagel, H. Lundström, T. Andersson, H. Lindblom, Properties, in theory and practice, of novel gel filtration media for standard liquid chromatography, J. Chromatogr. 476 (1989) 329–344.*

The resins are evaluated by running the chromatography step and varying factors such as sample concentration, sample volume, and flow velocity [1,5,11]. For high productivity, the aim is a process step with the desired resolution using as high a sample concentration, as large a sample volume, and as high a flow velocity as possible. However, high sample concentration will increase the viscosity (which in turn might result in peak broadening), large sample volume will certainly increase the peak width and impair the resolution, and high flow velocity will lead to zone broadening of large solutes and cause high column back pressure. The final conditions for a SEC process step are usually a compromise between these different factors in order to achieve the required separation at the best productivity.

Once the conditions have been defined, it is recommended to perform tests under process-like conditions (e.g., apply a relevant sample from the process, perform the required number of cycles, or perform CIP/SIP) to confirm that the results from the PD work are valid. It is also important to consider the extra-column dead volumes of the chromatography systems in for example, pumps, connections and tubing/pipes during scale-up tests. This can be critical if it is necessary to use for example, three columns connected in series to get the desired bed height, where each connection between the columns will add dead volume to the system. Increased dead volumes compared to the column volume (CV) will have negative effect on the separating power of the SEC column.

21.9 CRITICAL AND KEY PROCESS PARAMETERS (CPP AND KPP)

Process parameters such as flow velocity, column bed height, column performance, sample volume, sample viscosity, buffer conditions, temperature, and product collection breakpoints are all important in SEC and need to be monitored/controlled. The designation of these parameters as CPPs or KPPs is not strict, and can be application and product specific. CPPs are critical for the product quality, for example, purity, and/or various product modifications, while KPPs more affect the process performance, for example, product yield. As mentioned earlier, SEC is a gentle separation method (e.g., no bind/elute, buffer conditions chosen for product stability), and has usually no modifying effect on the target molecule itself. This means that the CPPs in SEC tend to focus on the product purity, rather than product modifications. CPPs affecting the purity are typically flow velocity, column bed height, and sample volume. Some process parameters, such as product collection breakpoints and column efficiency (number of plates), can be CPP or KPP, as they can affect both purity and yield. Buffer conditions and temperature are often considered as KPPs.

21.10 METHODOLOGY

A process step using SEC normally has a clearly defined task, for example, desalting to a conductivity of <5 mS/cm, or removal of aggregates and dimers to <5%. This task sets the requirements for the SEC step, and can be used, for example, to define breakpoints for peak collection, or values for buffer composition.

The resin and the chromatographic conditions will be determined by the PD work, and the final process parameters will be set during the scale up.

For achieving an efficient SEC process, it is crucial that the column is properly packed. The experimental conditions for packing SEC resins will mainly depend on bead rigidity, bead size, and column design. Most vendors provide detailed information on how to pack the resin, and it is recommended to use these protocols.

To ensure that the column is properly packed, one needs to test its performance. See Chapter 26.

21.11 ECONOMY/OPTIMIZATION

The price per liter of SEC resin is low compared to many other resins, but large column volumes are often needed to compensate for the sample volume limitations (low capacity). A SEC resin can typically be in use for several hundreds of cycles, so the total resin cost spread out over the amount of product processed will be low in most cases. The cost for buffers can be relatively high, since large columns require large buffer volumes, especially in fractionation applications. Standard chromatography equipment can be used; there are no special requirements for SEC, except that the SEC separation might require a large/tall column, or two or three shorter columns connected in series. The estimated costs for labor depend on time spent with the process step, which can vary considerably depending on the application area. Buffer exchange applications are usually quick operations, while fractionation applications are usually more time consuming.

Most of the process parameters like sample concentration, sample volume and flow velocity, are determined from the PD work and scale-up tests, and changing parameters to improve the economy might jeopardize the whole separation. However, the separation in buffer exchange applications allows some variation in flow velocity and column bed height, which might be used to optimize the conditions in the final process step. In fractionation applications, large savings in

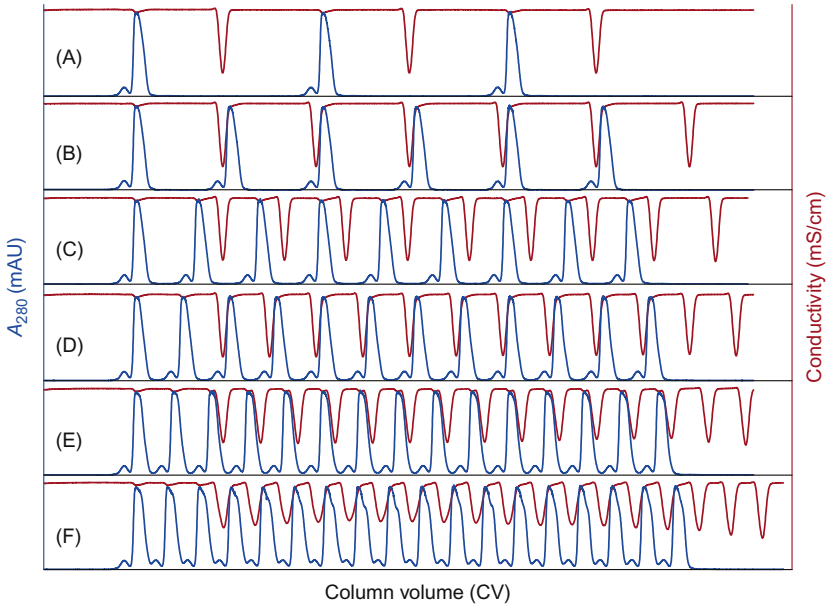

FIG. 21.11 Chromatograms (A–F) with increasing number of sample applications per CV (1–6) on a Superdex 200 prep grade column (diameter 2.6 cm, bed height 60 cm, CV 319 mL). A sample application consisted of 13 ml (4% of the CV) of a monoclonal antibody sample, concentration 16 mg/mL. Total chromatogram length was 4 CVs, sample applications were made during the first 3 CVs. *Blue curve*: absorbance at 280 nm, *red curve*: conductivity. *Courtesy of GE Healthcare.*

time and buffer consumption can be made by optimizing the sample volume and the number of sample application cycles needed to handle a batch. When resolution is adequate but only occupies a small region of the separation volume (CV $- V_0$), a second or third sample application can be made prior to elution of the full CV (not easily applicable if the SEC step is also used for buffer exchange). See example in Fig. 21.11 using a monoclonal antibody sample.

Each sample application results in absorbance peaks (a small dimer peak and a large monomer peak) and a conductivity dip (sample conductivity lower than SEC buffer conductivity), as already shown in Fig. 21.9. If the aim is to purify the monomer only, and the buffer composition is not critical, it might be possible to make four or five sample applications per CV, as seen in Fig. 21.11D–E, where the dimer and monomer peaks remain separated. With six sample applications per CV, as in Fig. 21.11F, the dimer and monomer peaks from two different sample applications begin to overlap, and the purification will not be sufficient. If the aim is to purify monomer and transfer the monomer to the SEC buffer, then three sample applications per CV, as shown in Fig. 21.11C, seem the best, and the monomer can be collected in the SEC buffer.

The timing of the preceding and the subsequent process steps can be adjusted to shorten the overall process time. For example, if a SEC step is run in several cycles, it is possible to start the subsequent process step as soon as there is sufficient target fraction from the SEC step available, before all SEC cycles are finished.

21.12 PRODUCTIVITY

The productivity of SEC operations can vary greatly. It is governed by the maximum sample volume, the maximum sample concentration and the cycle time (flow velocity). Buffer exchange applications can have high productivity due to the relatively large sample volume and high flow rate. Calculations show that buffer exchange at favorable conditions can approach 150 g/h and liter resin [5]. Group separation applications such as in Figs. 21.7 and 21.8 (large proteins or virus separated from smaller proteins) have relatively large sample volumes but usually deliver relatively lower productivity due to the lower flow velocity with soft SEC resins. Fractionation applications have even lower productivity due to small sample volumes and low flow velocity.

21.13 FUTURE

Continuous manufacturing is drawing a great deal of attention for production of biopharmaceuticals, and SEC is one of the chromatographic techniques which can be adopted for continuous processing [12,13]. SEC is also suited for use in generic

approaches for purification of different target molecule variants, since the separation, for example, is not affected by charge variants with the same molecular size. This has been demonstrated in automated small-scale preparation of monoclonal antibodies for screening of drug candidates [14].

Resins where the beads that have solid cores and a porous outer shell are commonly used in analytical SEC to enable higher flow rates and speed up analyses. This has not been introduced in large-scale preparative SEC, but theoretical calculations suggest that this design of chromatography resin could improve preparative SEC [15].

Another variant of utilizing differences in the core and the outer shell is core bead technology, where SEC and adsorptive chromatography are combined in one resin [6]. This technique is mentioned in Section 21.3, and Chapter 20. The core bead technology could also open possibilities for new resins based on the same principle, perhaps with other combinations of size exclusion and ligands.

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