Journal of Pharmaceutical and Biomedical Analysis 101 (2014) 161–173

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Review

Theory and practice of size exclusion chromatography for the analysis of protein aggregates

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ARTICLE INFO

Article history:
Received 12 February 2014
Received in revised form 3 April 2014
Accepted 8 April 2014
Available online 19 April 2014

Keywords:
Size exclusion chromatography
Therapeutic proteins
Aggregates
Biopharmaceuticals

ABSTRACT

Size exclusion chromatography (SEC) is a historical technique widely employed for the detailed characterization of therapeutic proteins and can be considered as a reference and powerful technique for the qualitative and quantitative evaluation of aggregates. The main advantage of this approach is the mild mobile phase conditions that permit the characterization of proteins with minimal impact on the conformational structure and local environment. Despite the fact that the chromatographic behavior and peak shape are hardly predictable in SEC, some generic rules can be applied for SEC method development, which are described in this review.

During recent years, some improvements were introduced to conventional SEC that will also be discussed. Of these new SEC characteristics, we discuss (i) the commercialization of shorter and narrower columns packed with reduced particle sizes allowing an improvement in the resolution and throughput; (ii) the possibility of combining SEC with various detectors, including refractive index (RI), ultraviolet (UV), multi-angle laser light scattering (MALLS) and viscometer (IV), for extensive characterization of protein samples and (iii) the possibility of hyphenating SEC with mass spectrometry (MS) detectors using an adapted mobile phase containing a small proportion of organic modifiers and ion-pairing reagents.

Contents

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http://dx.doi.org/10.1016/j.jpba.2014.04.011
0731-7085/

1. Introduction

The clinical use of therapeutic proteins has enabled the treatment of a wide range of life-threatening diseases. Many of these diseases were considered incurable or untreatable only a few decades ago. Almost half of the new drugs recently approved by the United States Food and Drug Administration (FDA) are therapeutic proteins, and the drug-pipeline landscape is now shifting even more toward this drug class of drugs [1]. There are several hundred potential biopharmaceuticals that have been evaluated in clinical trials for the treatment of genetic diseases, cancer and infectious diseases [2–4]. Because the development of biopharmaceuticals and biosimilars is complex, regulatory bodies such as the FDA and European Medicines Agency (EMA) require comprehensive drug substance characterization, lot-to-lot and batch-to-batch comparisons, stability studies, impurity profiling, glycoprofiling and related protein and excipient determination, such as polysorbate or protein aggregate elucidation [5,6].

Due to the unique physical and chemical properties of therapeutic proteins, they are prone to a number of changes during their preparation, formulation or storage [7]. These changes include several possible modifications, such as oxidation, deamination, glycosylation, aggregation, misfolding, or adsorption [8–11]. These modifications could lead to the potential loss of therapeutic efficacy or unwanted immune reactions [7]. Aggregate levels in protein-based drugs are a critical quality attribute due to their potential immunogenicity [12,13]. In recent studies, cytotoxic effects have also been observed with several biotherapeutic proteins due to significant denaturation or chemical alterations of the native protein [8,9,14]. In this context, the characterization of protein modifications requires a number of analytical methods because one single technique does not allow for the assessment of all the required parameters [6].

Protein aggregates are generally characterized using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [15], size-exclusion liquid chromatography (SEC-HPLC) [16–18], asymmetrical field flow fractionation (AF4) [19], fluorescence spectroscopy [20], circular dichroism (CD) [21] or light-scattering-based methods, such as multi-angle laser light scattering (MALLS) [22]. In addition, native mass spectrometry appears to be an alternative, emerging method to identify and characterize small oligomeric protein aggregates [23–25].

Among these techniques, SEC-HPLC is employed today as the standard separation technique for the quantification of protein dimers, trimers and oligomers. The main advantage of this approach is the mild elution conditions that allow for the characterization of the protein with minimal impact on the conformational structure and the local environment. Recently, some developments have been made in SEC-HPLC via the introduction of columns packed with sub-2 μm particles or the tandem and parallel interlaced approaches that enable a new level of chromatographic performance or throughput [26,27]. Conversely, several detection techniques are now available, ensuring improved sensitivity. The aim of this study is to review the possibilities and new developments in SEC-HPLC applied to the analysis of protein aggregates.

2. Theory of SEC separation

SEC separates biomolecules according to their hydrodynamic radius. The stationary phase consists of spherical porous particles with a carefully controlled pore size, through which the biomolecules diffuse based on their molecular size difference using an aqueous buffer as the mobile phase.

Basically, SEC is an entropically controlled separation process in which molecules are separated on the basis of molecular size differences (filtering) rather than by their chemical properties [28]. In SEC, the partitioning is ideally driven by entropic processes without any adsorption, thus the generally used Gibbs free-energy equation becomes

$$\ln\ K_D = -\frac{\Delta S_0}{R} \tag{1}$$

where K_D is the thermodynamic retention factor, ΔS_0 is the change in the system entropy and R is the gas constant. However, it is important to notice that in practice, temperature can indirectly impact the elution time to a small degree by altering the conformations of the proteins and affecting mobile phase viscosity and solute diffusivity. The thermodynamic retention factor in SEC is different from other chromatographic modes. Here, the thermodynamic retention factor is the fraction of the intraparticle pore volume that is accessible to the analyte [29].

Conversely, an analogous retention factor, k^*, was suggested by Engelhardt as the ratio of the probabilities of the sample staying in the stagnant mobile phase inside the pores and in the moving mobile phase between the particles (interstitial volume) [30]. In SEC, this k^* value is limited, and its maximum value is given by the ratio of the pore volume (V_p) and the interstitial volume (V_z) of a column or similarly by the pore porosity (ε_p) and the interstitial porosity (ε_z):

$$k^* = \frac{V_{elu} - V_z}{V_z} \tag{2}$$

$$k^*_{\max} = \frac{V_p}{V_z} = \frac{\varepsilon_p}{\varepsilon_z} \tag{3}$$

Therefore, the ratio of the pore porosity to the interstitial porosity determines the achievable retention range for a given separation. Using currently available state-of-the-art columns, this maximum retention factor is around $k^* \sim 1.6$–1.8. These parameters – and the associated selectivity – can be altered by packing a column more densely (or decreasing particle size) or by using particles with larger pore volumes.

In SEC, the size-based separation allows the construction of a calibration curve based on a set of known analytes, which can be used to estimate the molecular weight of an unknown analyte. Typical calibration curves are based on proteins or polymers of known molecular weights. By plotting $\log M$ vs. the retention volume, one typically obtains a third order polynomial with a linear region providing the highest resolution and molecular weight accuracy [29]. The largest proteins, which are excluded from the pores, elute first. Subsequent proteins elute in order of decreasing size. As proteins shapes could also vary (e.g., globular, rod-like or flexible chains), their Stokes radii do not correlate exactly with molecular weight. Another source of error in the calibration curve is that non-ideal adsorption may alter the retention volume [31–33].

The slope of the line in the linear portion of the calibration curve is a measure of the selectivity of the stationary phase, which can be defined by the relationship

$$\log\ M = m \cdot K_D + b \tag{4}$$

where m and b are the slope and intercept of the line, respectively. As the pore size distribution of the particle becomes narrower, the slope becomes less steeper, which results in a greater selectivity to discriminate analytes of similar size. A typical SEC calibration curve is shown in Fig. 1.

Similarly to other separation techniques, the main interest of modern SEC is to improve the analysis throughput by reducing the analysis time. The analysis time in SEC is mostly determined by the mobile phase flow rate with a given column configuration because all of the analytes of interest are eluted before the total void time (volume) of the column (there is no partition and retention) [34]. To shorten the separation time in SEC, the ratio of the column void

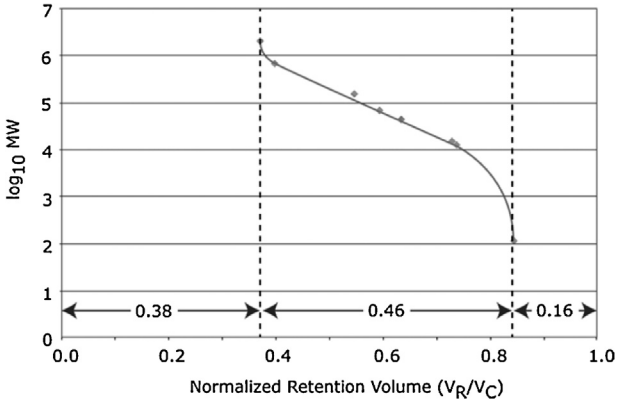

Fig. 1. Typical calibration curve in SEC.
(Reprinted from Ref [29]).

volume to the flow rate needs to be decreased. Reducing the column size and increasing the flow rate are the straightforward ways for performing fast SEC analysis [34]. Shortening the column length has been applied in fast SEC analysis to keep the backpressure at a reasonable level even at high flow rate when separating synthetic polymers [35,36]. However, decreasing the column length proportionally reduces the number of theoretical plates.

Popovici and Schoenmakers reported several considerations for fast separations using different commercial SEC columns at room temperature [36]. The effects of particle size, column length, and mobile phase flow rate on SEC separation were studied and reported in the past [36,37]. The main difficulty in achieving both high-speed and high-resolution separation in SEC is the slow mass transfer of the large solutes between the interstitial space and the pore space [37]. To increase the mass transfer rate, the temperature could be a valuable parameter. As the temperature increases, the mobile phase viscosity decreases, and the analytes diffusivity improves. Furthermore, this approach allows the use of higher flow rates for given column dimensions. As an example, Park et al. showed some very fast high temperature SEC separations, performed at 110 °C [34]. Therefore, and according to theory, the analysis time in SEC can be shortened by applying small particles, short columns and elevated column temperatures. Engelhardt proposed the following semi-empirical relationship to describe the column performance in SEC [30]:

$$H = 3.5 \cdot d_p + 1.3\frac{(1+k^*)D_M}{u} + 0.6\frac{k^*}{(1+k^*)^2}\frac{d_p^2}{D_M}u \quad (5)$$

where H is the plate height, d_p is the particle diameter, D_M is the molecular diffusion coefficient and u is the mobile phase linear velocity. Based on this model, some predictions have been made to estimate the impact of the particle size and mobile phase temperature (D_M depends on the temperature) on the H–u plot as shown in Fig. 2.

Today, various particles of 3–20 μm are routinely applied with different pore-sizes [28]. The most common column dimension in analytical SEC is 30 cm column length and 4.6–8 mm internal diameter. With these "conventional" SEC columns, the analysis time is generally between 15 and 50 min.

When high-resolution separation is required, the separation power (resolution) of an SEC column increases in direct proportion to the square root of the column length; thus, the separation of complex samples requires long columns that can be obtained by joining multiple columns in a series.

3. Occurrence of protein aggregation in pharmaceutical formulations

The control of protein aggregation is always a concern during the purification, formulation and manufacturing of therapeutic protein products [37–40]. The kinetics or rates of aggregate formation are of primary interest because they determine the aggregate levels over a given period of time in different physical environments and at different protein concentrations and temperatures experienced by a protein during its processing, shipping, or storage.

At a minimum, aggregates represent a process related impurity and/or degradation product that must be controlled at relatively low levels of concentration throughout the manufacturing and during the product storage [41]. Recent concerns have also been raised regarding the potential immunogenicity of aggregates, particularly those that are composed of multiple folded or partially folded monomers, although the precise mechanisms that make a particular aggregate size, morphology, and/or structure more or less immunogenic remain uncertain [42]. Therefore, it is also of interest to differentiate between the rates of formation for aggregates ranging from small oligomers to soluble high molecular-weight (HMW) aggregates and larger, effectively insoluble particles.

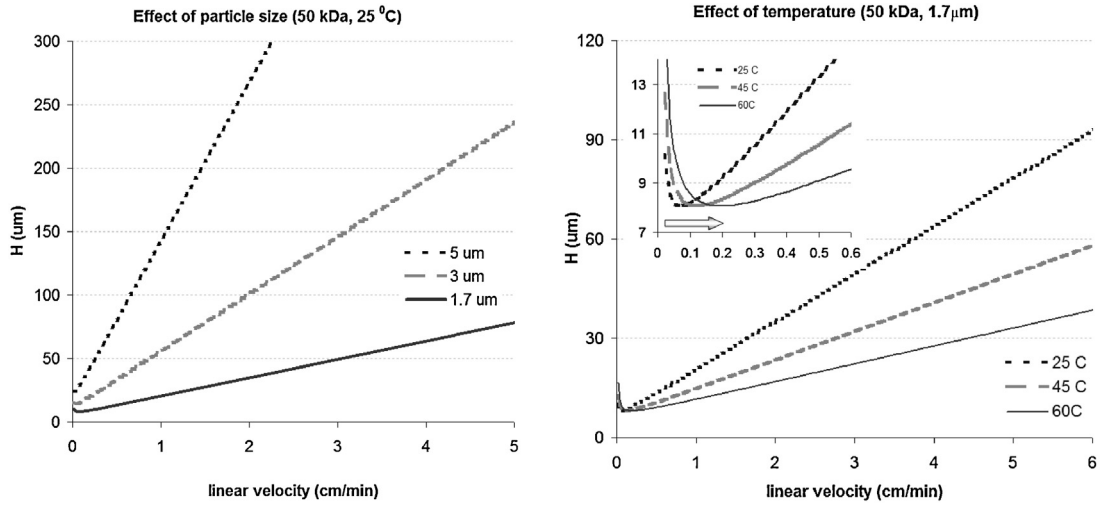

Fig. 2. Theoretically expected impact of the particle size and mobile phase temperature on column performance. (For the calculations, a 50 kDa protein was assumed.).

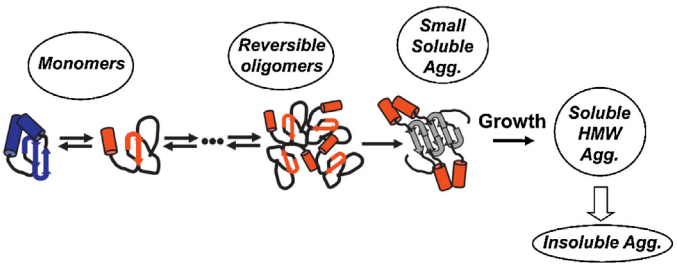

Fig. 3. Schematic representation of some of the key steps in non-native aggregation. (Reprinted from Ref [48]).

In this context, the term soluble denotes species that are molecularly dispersed in solution, while insoluble is used to denote aggregates that have coalesced into a macro- or micro-phase separated domains or particles. It has also been shown recently that protein aggregates can reversibly transition between soluble and "insoluble" or "condensed" states [43,44]. Alternatively, one can differentiate aggregates based on size measurement using other analytical techniques [38,45].

Assuming aggregation is the fastest degradation route, product shelf life and the effective rate coefficient of monomer loss (k_{obs}) are equivalent quantities when one is concerned with relatively low conversion percentages of monomer to aggregate (<approx. 10%) [46]. Aggregation pathways, such as thermal unfolding and structural perturbations, were reviewed across a wide range of proteins by Roberts et al. [47]. Fig. 3 shows a schematic view of protein aggregation based on the work of Li et al. [48].

All therapeutic proteins have an inherent propensity to aggregate. This process can be accelerated by various external factors, such as temperature variations, freezing–thawing, agitation, presence of impurities, formulation changes, exposure to interfaces or light, and long-term storage [38,40]. Aggregates may vary in many properties, such as size, type of intermolecular bonds, reversibility, morphology, and hydrophobicity. They may also contain proteins with different physical and chemical degradation states.

The United States, European and Japanese Pharmacopeias (USP, PhEur and JP, respectively) are harmonized regarding the requirements for visible and sub-visible particles in parenteral formulations. For sub-visible aggregates, there are two main SEC strategies: (1) observe the aggregation profile and quantify the percentage of high-molecular-weight species eluted from the column or (2) indirectly estimate the fraction of large aggregates (typically >100 nm) as the loss of total peak area [49]. However, there is no clear definition for aggregate acceptance limits in biopharmaceutical products. The FDA and International Conference on Harmonization (ICH) consider aggregates to be process or product related impurities [50,51]. In the particular case of a biosimilarity study, the FDA guidelines recommend the following: "If comparative physicochemical analysis reveals comparable product-related impurities at similar levels between the two products (originator's and biosimilar), pharmacological/toxicological studies to characterize potential biological effects of specific impurities may not be necessary." However, if the manufacturing process used to produce the proposed biosimilar product introduces different impurities or higher levels of impurities than those present in the reference product, additional pharmacological/toxicological or other studies may be required [50].

The main challenge in analyzing the degraded/aggregated protein formulations lies in the complex, dynamic, heterogeneous and transient nature of degraded/aggregated species. The number of chemical and physical degradation pathways is very high and many of them are intrinsically related.

4. Separation of aggregates in SEC

4.1. Possible interactions with the stationary phase

Proteins tend to interact with the charged surface sites of the stationary phase [29,31–33,52–54]. Non-binding interactions are dominated by two types of interactions: (1) electrostatic interactions and (2) hydrophobic interactions [32].

Due to the ionic interactions, protein adsorption can occur, inducing elution time shifting, band tailing or asymmetrical band elution. The three-dimensional conformation of the proteins can also be changed [31,55]. Electrostatic interactions can be classified into two groups. (1) If the protein and the stationary phase surface are identically charged, "ion-exclusion" can occur due to the electrostatic repulsion. In this way, the protein is prevented from entering the pores of the particle and, therefore, elutes faster (decrease in elution time). (2) If the protein and the particle are oppositely charged, the adsorption of protein on the stationary phase surface may result in increased elution time.

Possible hydrophobic interactions between the protein and hydrophobic sites of the stationary phase can also lead to increases in elution time.

The last generation of SEC materials is much more inert than the older versions, minimizing the possible electrostatic and hydrophobic interactions between the proteins and stationary phase surface. This behavior could be attributed to the regular improvement of silica materials (type B silica or hybrid silica) and the use of diol bonding. Except the nature of the stationary phase bonding and the interactions between the proteins and the stationary phase can also be minimized by proper adjustment of the mobile phase composition.

4.2. Mobile phase composition

To reduce electrostatic interactions, a common approach in SEC is to increase the ionic strength or salt concentration of the mobile phase. In this way, the unwanted secondary interactions can be reduced, and the peak shapes can be improved and quantification can become more precise. The recovery of aggregates can also be increased by the addition of salts (e.g., ≥100 mM sodium chloride) [54,56]. It was demonstrated that by varying the mobile phase ionic strength, the retention time of some monoclonal antibodies (mAbs) was shifted, and poor peak shapes were observed at low sodium chloride concentration [54]. The aggregate recovery was also improved when increasing the salt concentration in the mobile phase [56]. In addition to the ionic strength increase, another valuable approach consists of increasing the concentration of a counter ion in the mobile phase. Indeed, very high concentrations of the same ions can increase the hydrophobic or ion-exclusion effects [57]. To mitigate the undesired secondary ionic interactions in SEC, it is also recommended to use some additives in the mobile phase. Arginine is commonly used as an SEC additive [58,59]. The addition of arginine reduces the possible secondary interactions between the protein and the stationary phase as it can bind to the protein, leading to an improvement in protein aggregates quantitation and peak shape [29,58,59]. As an example, Fig. 4 shows the SEC chromatographic profiles of recombinant human basic fibroblast growth factor (bFGF) in the presence and absence of arginine. However, arginine shows significant UV absorbance at low wavelengths (below 220 nm) therefore can drastically decrease sensitivity.

Some organic modifiers (e.g., methanol and ethanol) could be used to reduce the strength of hydrophobic interactions due to their higher eluent strength compared to water [60]. Therefore, the peak shapes can be improved in some cases by the addition of 5–10% of an organic modifier.

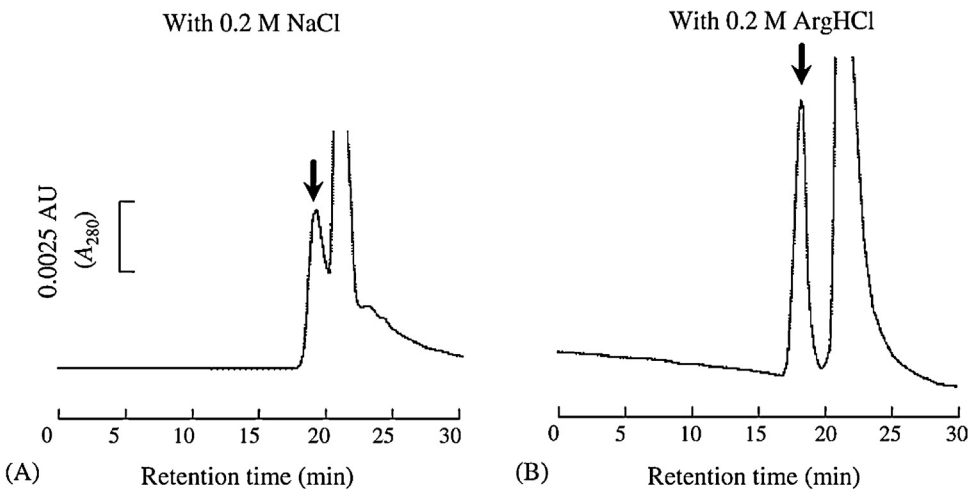

Fig. 4. SEC chromatographic profiles of recombinant human basic fibroblast growth factor (bFGF) in 0.2 M NaCl (A) and 0.2 M arginine (B). There is little resolution in the separation of bFGF (arrow) from the salt in (A), while baseline resolution is observed in (B).
(Reprinted from Ref [58]).

The pH of the mobile phase can also affect the interactions between proteins and the stationary phase. In an optimal case, the pH should be close to the isoelectric point (*pI*) of the protein to limit secondary interactions. Indeed, there is a risk for ion-exchange interactions when the pH is lower than the *pI*, while ion-exclusion effects can occur at pH values above the *pI*. This effect becomes especially important when working at low ionic strengths [33].

4.3. Columns

There are mostly two types of SEC packing materials: (1) silica, with or without surface modification, and (2) cross-linked polymeric packings, which possess non-polar (hydrophobic), hydrophilic, or ionic character [28]. The most common silica packings consist of chemically bonded 1,2-propanediol functional groups that provide a hydrophilic surface. This stationary phase blocks or reacts with many of the acidic silanol groups and neutralizing the surface. Therefore it makes it ideal for SEC separation of biopolymers [28]. Bare silica is also a useful packing material for non-aqueous polar or non-polar organic mobile phases; however, it is not recommended with aqueous mobile phases because of the presence of active silanol sites. The latest type of silica-related packing is an ethylene-bridged hybrid inorganic–organic (BEH) material that is currently available at sizes of 1.7 μm – the first sub-2 μm SEC packing – and 2.5 μm [26,61]. Compared to regular silica packings, BEH particles have improved chemical stability as well as reduced silanol activity [61].

There have been a number of different hydrophilic cross-linked packings developed for the SEC of biopolymers. Most of these packings are proprietary hydroxylated derivatives of cross-linked polymethacrylates [28]. Unusual polymeric packings for aqueous SEC include sulfonated cross-linked polystyrene, polydivinylbenzene derivatized with glucose or anion-exchange groups, a polyamide polymer, and high-performance, crossed-linked agarose [28]. However, when using organic or polar SEC packings for aqueous SEC, one must prevent the adsorption that is possible if there are extensive hydrophobic regions on the packing or polymer.

For interested readers, the most important SEC column providers and products were recently reviewed in Ref. [28].

4.4. Method development

In any SEC method, a variety of parameters can be adjusted to improve the resolution between the native protein and aggregates and make the method more robust and reliable.

First, an appropriate pore size has to be selected. The pore size selection depends on the size (or molecular weight) of the protein and its aggregates to be separated. Molecules with a diameter larger than the largest pores in the stationary phase are unable to enter the porous volume of the particles. Therefore, they pass through the column and elute first with the interstitial volume (V_z). Smaller molecules enter the pores within the particles and depending on their apparent size they elute later in order of decreasing size. For protein characterization, typical pore sizes between 150 and 500 Å are applied. For common therapeutic proteins (MW ~ 15–80 kDa), a pore size of 150–200 Å is well suited, while a 200–300-Å pore size is usually applied for mAbs (MW ~ 150 kDa). For very large proteins (MW > 200 kDa, e.g., Pegloticase or PEGylated proteins), the 500–1000 Å phases offer the best selectivity.

Resolution between the aggregates and native protein peaks is enhanced when decreasing particle size. The achievable plate numbers are inversely proportional to the particle size, while the resolution is inversely proportional to the square root of particle size. Historically, 5–10 μm particles are commonly used in routine SEC applications, but several providers offer now 3 μm particles or even sub-2 μm packings. By using these state-of-the-art columns packed with very fine particles, the resolution can be significantly improved or the analysis time can be shortened by applying shorter columns. The use of very fine particles in SEC has shown clear advantages in resolution [30,62].

Because SEC is an entropically controlled separation where no retention occurs, large pore volumes (high porosity) are required to ensure appropriate selectivity. Generally, this large pore volume is provided by long- and wide-bore columns. In routine SEC applications, a 30 cm column length with internal diameters (I.D.) of 6, 7.8, 8 or 10 mm is generally employed. These SEC columns are referred to as standard bore columns. Now, several vendors offer narrow bore columns with 4.6-mm I.D. and 15 cm lengths that are packed with very efficient, small particles. By using these columns, similar separation power can be attained as with 5 μm particles in 30 cm standard bore columns, but the analysis time can be reduced by a factor of 3–4 [26].

After selecting the appropriate column, the mobile phase composition has to be optimized. As mentioned in Section 4.2, an appropriate pH and salt concentration are required to ensure correct peak shape and resolution. Generally, the pH should be close to the isoelectric point of the protein, and the protein should be soluble and stable in the buffer. It is good practice to use a buffered solution with an ionic strength of 50–200 mM. This condition helps to reduce non-specific interactions between the protein and the stationary phase. The most commonly used buffer is phosphate at 10–200 mM concentration and pH between 6 and 7.2. When the isoelectric point of the protein is unknown, a pH of 6.5 may be a good starting point. To improve the peak shape, sodium chloride, potassium chloride or sodium sulfate is often added into the mobile phase at a concentration of 10–250 mM. However, when working at low ionic strength, non-ideal conditions can improve selectivity in some cases. Enhancement in selectivity and resolution was reported for model proteins in the sorptive mode with mobile phase pH below the *pI* [63]. In some cases, the addition of organic modifiers, such as methanol or ethanol, can also help to improve the resolution and peak shape due to a reduction of possible hydrophobic interactions between proteins and the surface of the stationary phase. Please note that buffers with high salt concentration and organic modifiers can be hazardous for the pump and system. Salt precipitation can block the tubing, seals or plungers. Therefore it is suggested to flush the salts from the system regularly with water or with a high aqueous (e.g. 5% acetonitrile or methanol) mobile phase.

In terms of mobile phase flow rate, it is common to work at 0.2–1.0 ml/min with standard bore columns. In most cases, the resolution can be improved by decreasing the flow rate, albeit the throughput is reduced.

As in other chromatographic modes, sample loading (both volume and mass) affects SEC chromatographic resolution and sensitivity [64,65]. In the case of column overloading, resolution between analytes can be decreased. The ideal volume loads correspond to sample volumes lower than 1–5% of the accessible column volume. If the sample volume increases beyond this cut-off value, the resolution may be reduced, and peak tailing may be observed.

To conclude the discussion of SEC method development, it remains partly empirical because chromatographic behavior and peak shape are hardly predictable. Therefore, the optimization of an SEC separation is sometimes time-consuming.

5. Detection modes in SEC

In SEC separations of proteins, UV is still the predominant detection mode [66,67]. UV wavelengths of 270, 275 and 280 nm give specific response for aromatic amino acids (tryptophan, tyrosine, phenylalanine) and are commonly used to assess protein aggregation [68]. At lower UV wavelengths (210, 214 or 220 nm), the amide peptide bond has a relatively high absorbance. Therefore, these lower UV wavelengths offer enhanced sensitivity but also lower specificity. Finally, higher wavelengths provide a greater linear dynamic range. Dual wavelength detection including both low and high detection wavelengths can be useful for purity profiling in SEC [22,69]. The lower wavelength provides the sensitivity for the low abundance species (aggregates), while the higher wavelength provides a greater linear range for the major species (native monomer).

Using a fluorescence detector, both sensitivity and selectivity can be significantly improved [70–73]. Fluorescence detection enables the measurement of low levels of aggregates. Intrinsic protein fluorescence is generally observed at an excitation wavelength of 280 nm and up to 295 nm. The emission is mostly due to excitation of tryptophan residues, and scarcely attributed to tyrosine and phenylalanine residues [74]. The maximum emission occurs between 300 and 370 nm, depending on the folding, conformation and mobile phase properties. It is also important to notice that proteins can be covalently labeled with various fluorophores, thus producing fluorescent protein conjugates. The emission from these attached tags is called extrinsic fluorescence. Tagging a protein with fluorescent labels is an important and valuable tool for studying structure and microenvironment [74].

Modern analytical SEC platforms are capable of more than just sample detection with a single concentration detector. The combination of multiple detectors including refractive index (RI), ultraviolet (UV), light scattering (LS) – or multi-angle light scattering (MALS), multi-angle laser light scattering (MALLS) – and viscometer (IV) allows extensive characterization of protein samples [75]. RI and UV both allow accurate concentration measurements. Light scattering detectors allow the measurement of the molecular weight without the need for column calibration [76]. Intrinsic viscosity is a measure of the molecular density and enables structural changes to be assessed. A combination of light scattering and intrinsic viscosity allows the determination of the size of the molecules. This set of detection is called tetra-detection [75]. A typical tetra-detector analytical SEC system is shown in Fig. 5.

MALS detectors are generally more sensitive to the high MW aggregates and enable their confirmation and analysis.

Mass spectrometry (MS) is another possibility for obtaining molecular weight information. However, there are challenges to interfacing SEC with MS due to the inherent incompatibility of the mobile phases containing high concentrations of non-volatile salts [77]. SEC mobile phases are typically non-denaturing aqueous solutions close to the physiological pH range. Such mobile phases made with phosphate buffer lead to ion suppression and contamination of the mass spectrometer. Ammonium formate or ammonium acetate at reasonable ionic strengths could be used as an alternative to phosphate buffer, but physiological pH conditions may be difficult to achieve because these buffers are adapted for acidic conditions (pK_a of 3.75 and 4.75 for formic and acetic acid, respectively) [78]. To overcome this difficulty, SEC-MS methods have been developed using denaturing mobile phases containing organic solvents and ion-pairing reagents instead of large amounts of non volatile salts [79–82]. Acetonitrile and trifluoroacetic acid (TFA) in the mobile phase allows for successful SEC-MS experiments. Another possible method for avoiding the problems encountered with the mobile phase is to use off-line MS detection. As an example, Matrix-assisted Laser Desorption Ionization (MALDI) combined with Time of Flight (TOF) analyzer has been used in the past for off-line SEC [83].

Comprehensive two dimensional LC (2D-LC) is most likely a good alternative to make SEC separations compatible with MS detection and to improve the separation power. Using SEC in the first dimension and reversed-phase LC mode as the second dimension would be ideal because this strategy provides a high peak capacity, even for fast gradient separations, in addition to straightforward compatibility with MS.

6. Possible pitfalls of modern SEC

Today, a wide variety of porous packing materials with different particle sizes are available for the separation and characterization of macromolecules [84]. The pore volume and the pore size distribution determine the molar mass range that can be evaluated, whereas the particle size determines the diffusion processes of proteins through the column [84]. Smaller particles lead to narrower peaks due to better column efficiency. In terms of efficiency and achievable analysis time, porous materials with a small particle sizes seem to be the most favorable column material for SEC

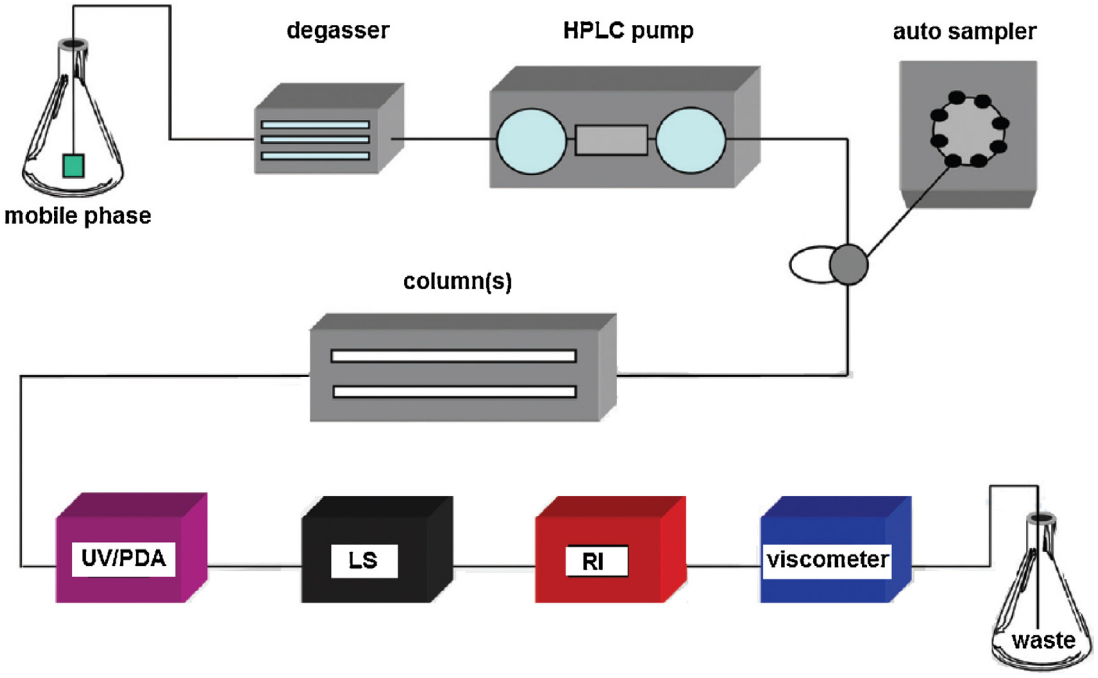

Fig. 5. Schematic view of a multiple-detector SEC system equipped with ultraviolet UV/PDA), light scattering (LS), refractive index (RI) and viscometric detectors. (Adapted from Ref [73]).

[26]. However, with very fine particles (sub-2 μm particles), the separation quality is improved at the cost of pressure. It has been established in reversed phase liquid chromatography (RPLC) that high pressure (i.e., >500 bar) might cause important frictional heating effects inside the column, and this heating is also likely true for SEC [85]. Therefore, temperature sensitive proteins might suffer from on-column aggregation or denaturation [86]. In summary, reducing the particle size to the sub-2 μm range increases the risk of shear degradation [87].

In general, SEC separations are performed at low to moderate pressures (typically $P < 100$ bar) using conventional SEC columns packed with 5–10 μm particles and operating at relatively low flow rates [28]. Under these conditions, the thermal effects (longitudinal or radial temperature gradients) inside the columns as well as the shearing forces are negligible. However, when applying very fine particles and/or high flow rates, these adverse effects could become important and could have a strong impact on several chromatographic properties, such as retention, peak shape, efficiency or selectivity [88,89]. Using the recently introduced 1.7 μm particles in SEC (aqueous mobile phase, 15 cm column length), very high pressures (up to 500–600 bar) can be generated. Therefore, thermal effects and shearing forces might become critical for temperature or pressure sensitive proteins [86,87]. As explained previously, elevated temperatures in SEC could be an interesting tool to increase the efficiency and are often used in ultra high-pressure liquid chromatography (UHPLC) to decrease the mobile phase viscosity. Under such conditions, the generated pressure drop is lower, thus allowing work at higher flow rates. However, elevated temperatures might also cause some changes in protein structure, and there is an additional risk of on-column protein denaturation and aggregation.

One of our recent studies systematically investigated the effects of pressure and temperature on the observed amount of aggregates when using SEC columns packed with sub-2 μm particles [26]. Fig. 6B shows the effect of the column head pressure on the measured amount of IgG aggregates. As the pressure increased, the amount of measured aggregates also increased. A similar trend was observed when the aggregate amount was evaluated as a function of the mobile phase temperature. As shown in Fig. 6A, the measured amount of aggregates changed from ~4% to ~10% when increasing the temperature from 30 to 60 °C.

To conclude, using very fine particles (high pressure) and elevated temperature in SEC can generate serious artifacts in the quantitation of protein aggregates. The induction of protein subunit associations and aggregations at high pressure is well documented [90–92]. In addition, the possible changes in aggregate solubility caused by the very high pressure cannot be ignored. However, some contradictory results can be found in the literature. Both increases and decreases in protein aggregate solubility caused by high pressure were reported [93,94]. The adverse effects of UHPLC-SEC approach are most likely strongly protein dependent [26].

The other important issue in SEC is the column lifetime. In biopharmaceutical formulations, several excipients and stabilizers (e.g., polysorbates, salts, PEG) are added to minimize the possible chemical and physical modification of the proteins during storage. Unfortunately, these additives decrease the long-term column stability and result in changes in elution, peak shape or recovery. Various time-consuming cleaning protocols are suggested by column suppliers to restore the SEC columns, but in most cases, they are ineffective. The use of guard columns is a valuable strategy to improve column lifetime. However, the expected lifetime of SEC columns is clearly shorter than for reversed phase or ion-exchange columns when analyzing therapeutic protein formulations. Typically, only a few hundred injections (e.g., 300) can be performed without significant loss in efficiency or peak symmetry.

7. Applications of modern SEC to the characterization of biopharmaceuticals

In this section, some important SEC applications were selected from the biopharmaceutical industry to show the potential of this analytical technique.

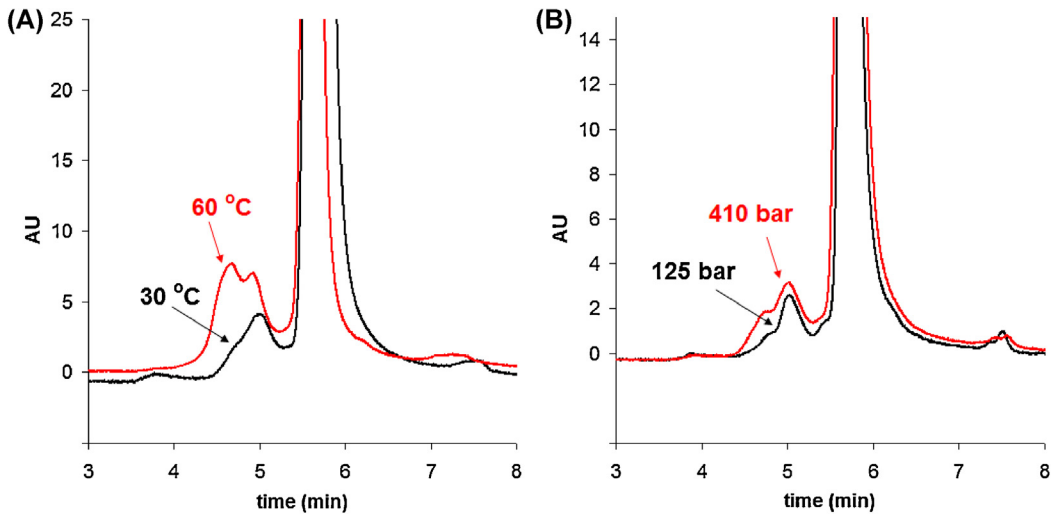

Fig. 6. Representative chromatograms on the effect of column temperature (A) and pressure (B) on the observed amount of antibody aggregates. (Reprinted from Ref [26]).

7.1. Application of SEC for the characterization of mAbs

Recently, Latypov et al. showed an impressive high-throughput method that is able to differentiate candidate recombinant human monoclonal IgG1 and IgG2 antibodies based on their propensity to form aggregates when subjected to agitation (vortexing) stress [95]. Intact mAbs were separated from soluble and insoluble aggregates using size exclusion chromatography, under non-denaturing conditions, and the individual components of the mixture were identified with a UHPLC-TOF-MS platform and quantified vs. an unstressed control. An internal standard was added to the mixture after stress, and used to correct for ionization differences between samples. Treatment of the samples with the IdeS (Fabricator) enzyme significantly reduces sample complexity, and allowed for a large number of candidate molecules to be assessed in a single analysis. The IdeS enzyme generated $F(ab')_2$ (fragment of antigen binding) and Fc (fragment crystallizable) domains of the antibody. A volatile ammonium acetate salt was used to generate an MS compatible mobile phase matching the pH formulation. The mobile phase was 25 mM ammonium acetate and 5% acetonitrile in water, adjusted to pH 5.2. Separation was performed using an Acquity UPLC BEH200 SEC 1.7 μm × 4.6 mm × 150 mm column at ambient temperature. A mixture of 8 intact mAbs at $t = 0$ h, 1 h and 4 h of agitation and the fragments generated by digestion with IdeS, were analyzed at the same time points. The growth of HMW species did not account for the overall monomer loss because of the formation of insoluble aggregates [95]. Fig. 7 shows the corresponding SEC chromatograms obtained at different agitation times.

Another study reported an online fluorescent dye detection method suitable for SEC and asymmetrical flow field flow fractionation (AF4) [96]. The noncovalent, extrinsic fluorescent dye (Bis-ANS) was added to the mobile phase or the sample, and the fluorescence emission at 488 nm was recorded at an excitation wavelength of 385 nm. By combining SEC and AF4 with online dye detection, it was possible to simultaneously detect heat-induced aggregation and structural changes of monomeric and aggregated IgG [96].

Wätzig et al. showed a 15 min SEC separation of IgG1 antibody aggregates using a conventional 30 cm long column packed with 5 μm particles and demonstrated the precision and repeatability of monomer and aggregate quantitation [97]. However, by using state-of-the-art column technology and UHPLC instrumentation, it was demonstrated that the analysis time can be reduced to less than 3 min [26].

A highly sensitive capillary SEC methodology was developed for Fab aggregate analysis in human vitreous humor [98]. The capillary SEC method enabled picogram sensitivity with an RSD of less than 8% for the relative peak area of HMW of the Fab fragments. Fig. 8 shows representative chromatograms obtained with capillary SEC by injecting antibody Fab fragments. Another study also

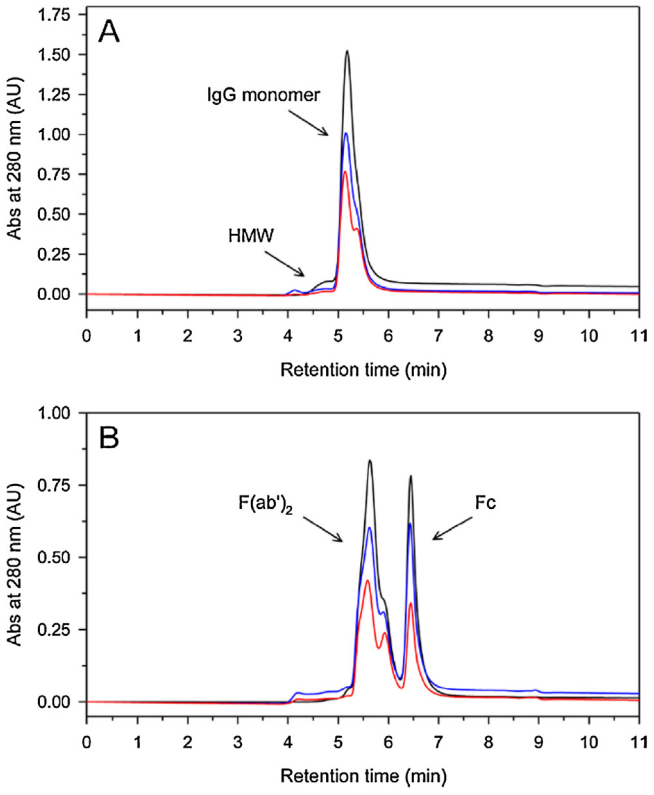

Fig. 7. UV chromatograms at 280 nm of a mixture of (A) 8 intact mAbs at $t = 0$ h (black), 1 h (blue), and 4 h (red) of agitation, and (B) the fragments generated by digestion with Fabricator, at the same time points. The growth of HMW did not account for the overall monomer loss because of the formation of insoluble aggregates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
(Reprinted from Ref [93]).

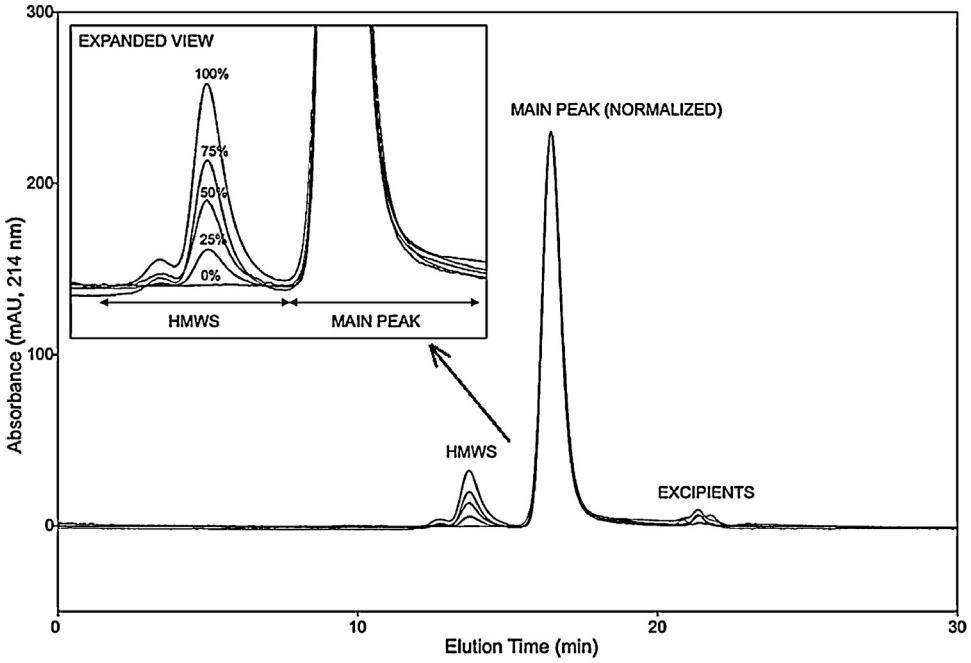

Fig. 8. Representative chromatograms of co-mixed oxidized/control samples of Fab1 obtained by capillary SEC. The main peak height is normalized across samples. The percentage of oxidized sample in the sample mixture corresponding to each chromatogram is indicated.
(Reprinted from Ref [96]).

demonstrated the enhanced sensitivity of capillary SEC for the analysis of mAbs purified from harvested cell culture fluid [99].

The development of a fast and easy SEC method under moderately hydrophobic conditions (mixed mode) to monitor the heterogeneity in drug product samples was recently reported [100]. The best separation was obtained on a column packed with sub-2 μm particles along with a mobile phase consisting of sodium acetate and sodium sulfate that separates IgG into aggregates, monomer, and fragments. The moderate salt concentration resulted in a secondary separation mode based on hydrophobicity, resolving a monomer pre-peak from the monomer main peak. MALS determined that the pre-peak had a similar mass as the IgG monomer. Characterization of the purified pre-peak fraction using MS, and bioactivity revealed this degradant to be a tryptophan-oxidized IgG monomer, with significantly reduced bioactivity [100].

Similar to what is commonly performed for intact mAbs, the determination of Antibody Drug Conjugates (ADC) is carried out using SEC. However, it is worth mentioning that regular SEC using a phosphate-buffered mobile phase provides a poor peak shape of ADC and unacceptable resolution between aggregates and monomeric ADC products [101]. This result could probably be explained by non-specific interactions between the hydrophobic cytotoxic drugs and the surface of the stationary phase. To solve this problem and improve peak shape, various organic modifiers were added to the SEC mobile phase, such as 25% propylene glycol [102] or 10% DMSO [103]. It is also conceivable that alcohol-type organic modifiers could be successfully employed to improve SEC performance of ADC products. In addition, the smaller charge variants, namely reduced fragments (i.e., light chains and heavy chains with different drug loads) were successfully characterized by RPLC using generic conditions [104]. As for naked mAbs, mass spectrometry is a key technique to gain insights into the oligomeric nature of the SEC-isolated "multimeric" fraction of ADCs [105].

7.2. Application of SEC for the characterization of other proteins

The gain in analysis time and resolution power achieved using narrow-bore SEC columns packed with 1.7 μm particles was demonstrated for insulin aggregate analysis [106]. Compared to a regular 300 mm × 7.8 mm, 10 μm standard SEC column, the analysis time was shortened by a factor of 3 when using a 300 mm × 4.6 mm, 1.7 μm column.

The development and validation of a sensitive SEC method for the quantitation of r-HuEPO aggregates in formulations containing 0.03% polysorbate 80 were described by Gunturi et al. [73]. A conventional column (300 mm × 7.8 mm, 250 Å) packed with 5 μm particles and fluorescence detection was used. The mobile phase consisted of isopropyl alcohol–potassium phosphate (0.1 M)/potassium chloride buffer (pH 6.8, 0.2 M) (25:75, v/v). The method was shown to be specific for r-HuEPO total aggregates (dimer and oligomers) and allowed for their quantitation at 80 ng/ml in the presence of r-HuEPO monomer and the pharmaceutical excipients, namely glycine (5 mg/ml), sodium chloride (4.3 mg/ml) and 0.03% polysorbate 80 [73].

The interest in SEC–MALLS to investigate aggregation and degradation of glycosylated and non-glycosylated proteins, under various conditions, such as addition of detergent, pH modification, variation of the protein concentration and heat stress temperature was systematically examined by Ye [107]. The characterization of proteins and their aggregates was performed by online UV, refractive index, and MALLS detectors. Aggregation and degradation were examined under various conditions and quantitative results were presented for bovine serum albumin, choriogonadotropin, glyceraldehyde-3-phosphate dehydrogenase, Herceptin, and ReoPro. The reported method could simultaneously determine both the quantities and the molecular weights of macromolecules from a single injection [107].

A methodology based on on-line coupling of SEC with mixed-mode liquid chromatography has been reported by He et al. [108]. The method allowed for the simultaneous measurement of a wide range of components in biopharmaceutical drug products, including the active pharmaceutical ingredient (protein) and various types of excipients, such as cations, anions, nonionic hydrophobic surfactant and hydrophilic sugars. Dual, short SEC columns were used to separate small molecule excipients from large proteins. The separated protein was quantified by UV detection at 280 nm.

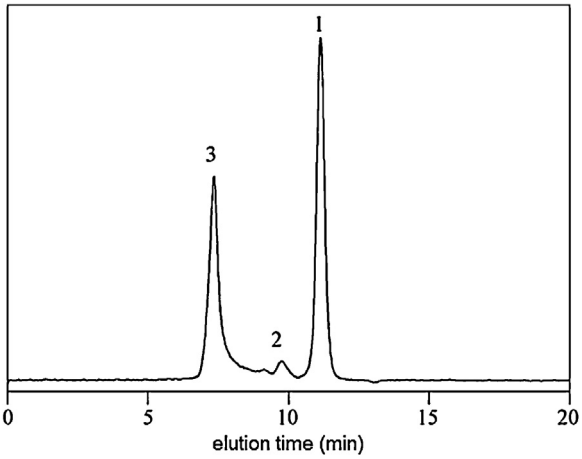

Fig. 9. SEC analysis of thermally aggregated interferon alpha-2b sample. Separation of interferon alpha-2b (0.1 mg/ml) containing monomer (peak 1, 53%), dimer (peak 2, 6%) and HMW aggregate (peak 3, 38%). The sample was incubated in a water bath at 70 °C for 30 min. Fluorescence detection was performed. (Reprinted from Ref [7]).

The isolated excipients were switched online, to a mixed-mode column for separation, and detected by an evaporative light scattering detector (ELSD). A volatile buffer was used for both SEC and mixed-mode separation. This method facilitated the detection of different excipients by ELSD and provided potential for online characterization of the protein by mass spectrometry (MS). The method was finally applied to quantify protein and excipients in different biopharmaceutical products, including antibody drug conjugates (ADC) and vaccines [108].

SEC methods coupled with intrinsic fluorescence detection were developed for evaluating the stability and degradation profiles of interferon alpha-2 drug substances and drug products [7]. The method allowed baseline resolution of the active ingredient from the excipients contained in the final product, including large amounts of albumin. HMW aggregates with apparent molecular weight of ~650 kDa, as well as dimers and denatured and reduced variants, were successfully identified and separated from the native proteins. This chromatographic method, which quantitatively measured physical and chemical changes taking place in solution formulations, was found to be capable of monitoring interferon alpha-2 stability [7]. Fig. 9 shows the SEC profiles of thermally aggregated interferon samples.

8. Some future trends in SEC

8.1. Interlaced and parallel interlaced SEC

Using two SEC columns in parallel can significantly increase the analysis throughput. A further reduction of analysis time can be obtained by combining interlaced sample injections with parallelization of two narrow bore columns packed with sub-2 μm particles. With this strategy, both lag times before and after the peaks of interest can successfully be eliminated, resulting in an assay time below 2 min [27].

Farnan et al. [109] have described the methodology of interlaced SEC in detail. In summary, the methodology is based on injecting a new sample before the ongoing analysis of a previous sample has ended. The subsequent information phase begins immediately after the salt fraction of the preceding sample has eluted. Fig. 10A shows the schematic view of an interlaced SEC injection. The total time required for the analysis of n samples can be expressed as:

$$t_{total} = t_{lag} + n \cdot (t_{\inf} + t_{hold} + t_{salt}) \quad (6)$$

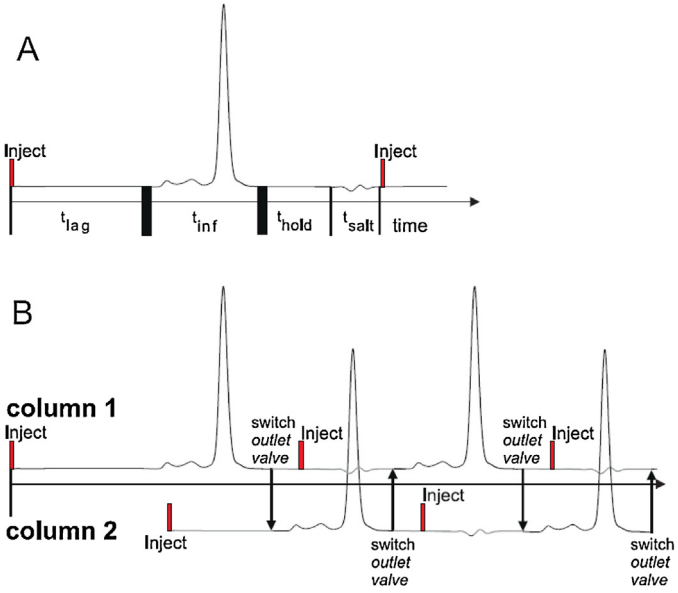

Fig. 10. Schematic view of interlaced (A) and parallel interlaced (B) SEC separation. (Reprinted from Ref [27]).

where t_{total} is the time required for the whole analysis, t_{lag} is the first part of the elution window after the injection (without any compound of interest), t_{inf} is the information phase that corresponds to the time window in which aggregates and monomer elute, t_{hold} is the third part of the chromatogram between the monomer and salt peaks and t_{salt} is the elution region of salt species (low molecular weight compounds eluting at t_0).

A further increase in throughput can be achieved when applying interlaced injections on two columns operating in parallel (Fig. 10.B). Compared to interlaced chromatography, the assay time in parallel interlaced SEC is further reduced by t_{hold}. Two switching valves are used to direct the flow alternately between the autosampler, the two columns and the detector, thus enabling the elimination of t_{lag}, t_{hold} and t_{salt}. The use of two columns and switching valves require two distinct programs on which pumps, autosampler and column compartment, including the switching valves, are controlled.

8.2. Sub-3 μm particles, ultrahigh-pressure SEC

Since the introduction of ultra-high-performance/pressure liquid chromatography (UHPLC) systems and columns, which enabled higher resolution, sensitivity and peak capacity, there has been a variety of new analytical advancements in LC and LC–MS based on the use of low dispersion systems [110]. Because column peak dispersion – and therefore the apparent efficiency – depends on the column volume, the achievable plate numbers and solute retention factor (k), the importance of system dispersion is worth mentioning. In the SEC mode, there is no retention; thus, relatively low column peak variance is expected. However, achievable plate numbers with large molecules are relatively low and moreover the volume of 150 mm × 4.6 mm columns is reasonably large (~1700 μl). Therefore, these two contributions compensate for the low retention. Finally, the expected column peak variance is approximately 200–400 μl^2. Using state-of-the-art UHPLC instruments (possessing extra-column peak variance between 2 and 30 μl^2), the system dispersion is negligible and does not impact the column efficiency.

The UHPLC technology was originally developed for RPLC applications, but it is now also available for SEC operation due to the availability of 1.7 and 2.5 μm BEH silica particles with varying pore

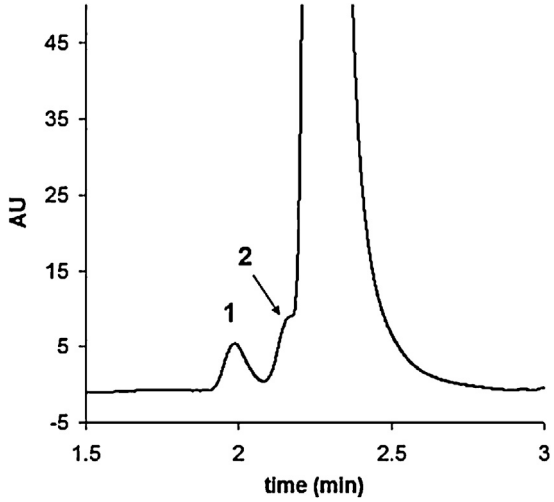

Fig. 11. A very fast SEC separation of panitumumab aggregates by using narrow-bore column packed with 1.7 μm particles. Peaks 1 and 2 are aggregated forms of the native antibody.
(Reprinted from Ref [21]).

sizes. Today, columns for aqueous and non-aqueous SEC applications with pore sizes of 125–900 Å are commercially available [110]. Very fast separations of peptide, myoglobulin and insulin aggregates were demonstrated with 1.7 μm SEC columns [111]. These very efficient columns were also applied for the characterization of recombinant mAbs [26]. Fig. 11 presents a 4 min separation of panitumumab aggregates.

It appears that applying 1.7 and 2.5 μm particles in SEC opened a new level of SEC separations, but it has to be kept in mind that on very fine particles, the separation quality is improved at the cost of pressure (and frictional temperature gradients). Therefore, there is a risk of creating on-column aggregates when analyzing sensitive proteins under high pressure (i.e., >200 bar) conditions [26] as discussed in Section 6. The other disadvantage of sub-2 μm SEC separations is that currently there are only a very limited number of commercially available stationary phases.

8.3. Capillary SEC

To improve the sensitivity of aggregate determination or handle very small amounts of available samples, the use of capillary columns in SEC appears to be a promising approach. However, several key modifications to a commercially available liquid chromatography system are required to reduce the system volume and associated extra-column band broadening, which could be critical for capillary SEC operation. Until now, the number of applications in this field is rather limited, but 300 mm × 300 μm I.D. SEC capillary columns were successfully applied for the separation of mAbs fragments [98,99].

9. Conclusion

To conclude, SEC remains an important strategy for the detailed characterization of protein aggregation. Indeed, due to their potential immunogenicity, the amount of aggregates has to be thoroughly controlled during the production and storage of therapeutic proteins.

During recent years, there have been a number of advances in SEC that improve the quantity of information that can be gained from a single injection. Among them, we can cite the use of shorter and narrower columns packed with smaller-sized particle to improve the throughput and resolution, but care should be taken to avoid (or at least limit) the risk of shear degradation. To further decrease analysis time, parallel interlaced SEC can also be implemented.

Various detectors may be multiplexed in SEC, including refractive index (RI), ultraviolet (UV), multi-angle laser light scattering (MALLS) and viscometer (IV), for extensive characterization of protein samples. It is also important to note that mass spectrometry (MS) plays a pivotal role in the structural elucidation; for this reason, some efforts were recently made to successfully couple SEC to MS by changing the nature of mobile phase constituents.

The sale of biologics grew at an incredible rate during the last three years. The new drugs currently in preclinical and clinical phases are very difficult to characterize; for example, the case of therapeutic mAbs or ADCs as well as bispecific antibodies [112–114]. Thus, there will be an increasing need for highly efficient SEC methods. In this context, the use of a 2D-LC platform including SEC in the first dimension and RPLC in the second dimension may be of interest to (i) further improve the resolving power of the chromatographic step and (ii) improve the compatibility of SEC with MS due to the intermediate RPLC step.

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