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Unravelling the mysteries of modern size exclusion chromatography - way to confident characterization of therapeutic proteins

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ABSTRACT

Modern size exclusion chromatography (SEC) can be defined by the use of relatively small columns (e.g. 150 x 4.6 mm) packed with sub-3 µm particles, allowing a 3- to 5-fold increase in method throughput compared to conventional SEC. The quick success of the first sub-2 µm SEC column introduced in 2010 lead to the development of numerous ultra-high performance (UHP)-SEC columns for the analysis of therapeutic monoclonal antibody (mAb) based products. Aggregates also known as high molecular weight species (HMWS) are indeed one of the most important critical quality attribute (CQA) of mAbs, as they may decrease the product efficacy or cause immunogenicity effects. Therefore, their confident characterization requires strong knowledge on modern SEC performance (i.e. selectivity and efficiency), but also on the inherent limitations caused by non-specific interactions more likely to occur with complex antibody drug conjugates (ADC) and some of the commercial mAb products. This review discusses the importance of the liquid chromatographic (LC) instrumentation, to exploit the full potential of modern SEC columns and current trends to hyphenate SEC to mass spectrometry (MS). Recent applications for antibody-based products (i.e. mAb, ADC, Fc-Fusion protein and bi-specific antibody) are presented. Finally, tips and tricks are provided, to further optimize SEC separations as well as maintaining their performance over time and better understand unexpected SEC results.

1. General introduction to SEC and UHP-SEC of therapeutic proteins

Therapeutic proteins including monoclonal antibodies (mAbs), antibody-drug conjugates (ADC), bispecific monoclonal antibodies (BsAb), Fc-fusion proteins and related products are inherently heterogeneous and their characterization requires an arsenal of analytical tools [1-6]. The different methods provide complementary information about the compounds of interest to build up the puzzle and understand the chemical structure of the protein biopharmaceutical product. Among all the analytical techniques, size exclusion chromatography (SEC) is a reference method for the qualitative and quantitative evaluation of protein aggregates [7,8]. The main advantage of SEC is the mild chromatographic conditions that permit the analysis of proteins with minimal impact on its conformational structure and local environment. SEC is considered as a non-denaturing chromatographic mode, as proteins are analyzed under their native (physiological-like) form. SEC separates biomolecules according to their hydrodynamic diameter. The stationary phase consists of spherical porous particles with a carefully controlled pore size and pore size distribution, through which biomolecules diffuse based on their molecular size difference without any retention, using an aqueous buffer as the mobile phase [7,8]. SEC is mostly considered as an entropy controlled separation mode and requires the use of inert stationary phases to avoid physico-chemical interactions between the protein and the stationary phase [7,8].

In conventional SEC, large column volumes (e.g. 300 x 8 mm) have been mainly used at low flow rate (and low pressure). On those columns, the analysis time usually ranged from 25 to 40 minutes. The reason of using such large columns at the analytical scale, is simply that peaks elute before the column dead volume and therefore, the expected peak volume (or variance) is inherently low and strongly affected by the system dispersion if the column volume is small. On the other hand, from a technical point of view, it was easier to pack columns of large diameters with such highly porous particles (more porous than in other chromatographic modes, and hence possessing lower mechanical stability), which were less resistant to high packing pressure [9].

Today, the trend in SEC is to decrease column volumes and particle sizes, to increase throughput. The standard dimension of modern SEC columns is 150 x 4.6 mm and those columns are typically packed with sub-3 µm particles ($1.7 \leq d_p \leq 3$ µm) [10]. The potential of such columns was recently recognized and various column providers have launched their own sub-3 µm SEC materials since 2016 [10]. The pressure stability of these packings was also strongly improved as they can be operated up to 250 – 480 bar. High mobile phase velocities can be applied and the analysis time decreased down to 4 – 8 minutes [11]. In these conditions, the peak capacity for a 150 kDa mAb monomer typically ranges between n_c = 15 and 25 [11]. To distinguish this new SEC approach, the term ultra-high performance SEC (UHP-SEC) is often used.

Several reviews have been published on SEC during the past few years [7-9], mostly dealing with conventional SEC applications and method development. The aim of this paper was to review the potential and limitations of modern UHP-SEC. Possible issues related to undesired secondary interactions with state-of-the-art stationary phases and instrumentation's volumetric contributions are discussed. Several practical tips and tricks are also described, to determine protein aggregates with high confidence. Possible solutions to couple SEC and mass spectrometry (MS) are illustrated too. Finally, the latest applications on modern therapeutic protein based products are reviewed.

2. Stationary phases in modern UHP-SEC

2.1 Physico-chemical properties of the SEC columns

While cross-linked dextran particles, polyacrylamide-based gels and polystyrene resins were the first chromatographic media introduced for SEC, their limited mechanical stability leads to the introduction of silica-based particles to perform SEC at higher flow rates and pressures (few hundred bars) [12]. Further surface modifications, i.e. derivatization with hydrophilic silanes or diol functionalization were then performed to minimize secondary electrostatic interactions occurring between the charged amino acids of proteins and the acidic silanols (Figure 1) [12]. Diol bonded-silica stationary phase remains today the most prevalent SEC material due to its high chemical stability and hydrophilic character compared to trimethylsilyl (TMS) groups.

To improve the efficiency of size-based separations occurring almost entirely within the intra-particle pores, the total porosity of the columns was increased from 60-70% (which is common in most sorptive modes of LC, such as RPLC) to around 80-90% for modern SEC columns. The pore size is another important feature in SEC as it determines the molecular weight (MW) range of proteins that can be successfully analyzed. The latter can be evaluated by injecting various protein markers possessing different MW and plotting the log MW *vs.* elution volume (or normalized elution volume). Then, the slope of the fitted calibration curve is determined by the pore size distribution, while the position of the curve (average log*MW*) is the function of the average pore size of the stationary phase. A pore size of 200 - 300 Å generally allows the separation of proteins with MW comprised between 10 and 500 kDa and is thus well suited for the separation of mAb and ADC trimers, dimers, monomers as well as fragments having sizes of 25 - 100 kDa, whereas higher orders of aggregates would elute together with the trimers through the interstitial column volume without entering the internal pores [10]. Surprisingly, very similar calibration curves were obtained using four different state-of-the-art UHP-SEC columns possessing nominal pore sizes between 150 and 300 Å. This was particularly true for the linear range of the calibration

curves (which corresponds to the useful elution window), corresponding always to protein MW between 10 and 500 kDa for all columns [10]. Indeed, there is no consensus on the pore size definition (mean, median or mode) and the determination of the pore volume is generally performed by low-temperature gas adsorption-desorption (BET method), which is unlikely to mimic the behavior of proteins in aqueous solution. Moreover, small gas molecules can interact with all the surfaces of the pores even if the pore diameter of the individual channels is unequal along the travel of the solute inside the pore. This is probably not the case for a large solute which can travel only a part of the individual pore having a large enough diameter, while the thinnest parts of the pores are not accessible. In addition, it is important to mention that pore size distribution has also a strong contribution on the shape of the calibration curves and in particular, the slope of the linear part of the calibration curve is higher with a wide pore size distribution. When proteins cover a broad range of MW, SEC columns with a wide pore size distribution or combination of SEC columns with different pore sizes can be of interest to extend the MW range of the SEC separation. For example, the combination of 200 and 450 Å SEC columns was tested on a protein mixture and improved the resolution between the largest proteins compared to the individual 200 Å column as well as the one between smaller proteins, in comparison with the large pore size column [13].

The BEH200 SEC column packed with 1.7 µm particles was the first sub-2 µm material launched in 2010 and can be considered as the precursor of modern SEC. Obviously, the column allowed to significantly increase method throughput thanks to smaller column dimensions of 4.6 x 150 mm *vs.* 7.8 x 300 mm for conventional SEC columns, without sacrificing performance. In comparison to columns packed with 3 and 5 µm particles, 2 to 5 times lower plate heights were achieved on the 1.7 µm packing and the analysis times could be cut by a factor of 2 to 4 [14]. In 2014, a study evaluated thirteen 4.6 mm x 150 mm and 4.6 mm x 300 mm UHP-SEC columns packed with sub-3 µm particles and concluded that the BEH200 SEC column achieved better SEC separation of various mAb products within 5 min [15]. The method was successfully implemented in bioprocess development and analytical testing, and six times faster separation was done compared to conventional SEC (Figure 2). *Yang et al.* found that the throughput was three times higher with the BEH200 SEC column in comparison to conventional SEC [16].

Following the successful application of mAbs for several diseases and the approval of more complex antibody formats such as ADCs, additional sub-2 µm and sub-3 µm UHP-SEC columns listed in Table 1 were introduced by other suppliers. Similar efficiencies were obtained on columns packed with 1.7, 1.8 and 2.0 µm particles and they were 1.5 to 2 times higher compared to the UHP-SEC material packed with 2.7 µm particles (Figure 3) [10]. In addition, flatter plate-height curves were obtained with the sub-2 µm packings allowing the increase of the flow rate without losing significant performance.

2.2 Possible secondary interactions

Ideally, only the hydrodynamic radius of proteins should contribute to their separations in SEC (entropy controlled separation). However, secondary interactions can occur between the proteins and the stationary phase. In practice, electrostatic, hydrophobic and hydrogen bonding interactions can all contribute to the elution and separation of proteins. Electrostatic interactions can be sorted in two categories depending on the charge of the proteins: i) ion exchange when the protein carry a positive charge and the stationary phase possesses a negative one and ii) ion exclusion with negatively charged proteins and stationary phase functional groups. Hydrophobic interactions can occur between the hydrophobic parts of the proteins and active site of the stationary phase (e.g. linker of functional groups or short alkyl chains). For example, He *et al.* suggested that the ethyl (C2) group in the BEH particle of the BEH200 SEC columns was likely to be responsible of hydrophobic interactions with polysorbate 80 [17]. Hydrophobic interactions can become critical on some sub-2 µm columns, particularly with ADCs, which are often highly hydrophobic, due to the high lipophilicity of the drug and linker attached to the mAb [6,10]. As reported elsewhere, two sub-2 µm UHP-SEC columns with a diol bonding were not suitable for the analysis of hydrophobic ADC products when using purely aqueous mobile phase, while the columns packed with 2.0 µm (diol bonding) and 2.7 µm particles (alternative proprietary bonding) allowed a baseline resolution between the HMWS and the main peak of two FDA- and EMA-approved ADCs [18]. Interestingly, the last generation of TSK UP-SW3000 columns packed with 2 µm particles did not require the addition of organic solvent (which is often used to limit or eliminate undesired hydrophobic interactions) in the mobile phase, contrary to the former generation of TSK 3000SW$_{XL}$ columns packed with 5 µm particles, meaning that column chemistry has also been improved by the provider [6,10].

3. Mobile phase in UHP-SEC

Once a suitable SEC column has been selected based on physical (i.e. dimensions, pore and particle sizes) and chemical (i.e. limited non-specific interactions) properties, the mobile phase composition has to be optimized. Of particular concern, protein aggregates can sometimes show a greater tendency than monomeric species to bind to the stationary phase resulting in the underestimation of their content, and proving the critical role of mobile phase on SEC quantitative measurements [19,20]. Historical native SEC involves the use of moderate amounts (between 0.1 and 0.4 M) of phosphate salts and neutral pH comprised between 6.2 and 7.4. Potassium cations are generally preferred to sodium ions due their better ability to shield the residual silanolates of silica-based SEC supports and therefore reducing potential electrostatic interactions [21]. In addition, at least 0.2 M of potassium-

based salts were required to effectively limit electrostatic interactions at pH = 6.8 with a wide range of FDA- and EMA-approved therapeutic proteins even when using the last generation of UHP-SEC columns [21]. Yang *et al.* also observed a better performance for a mAb with several mobile phases containing more than 0.15 M potassium-based salts at pH = 6.2 (Figure 4) [16]. However, the counter anion can also affect non-specific interactions. Indeed, the phosphate buffer has a strong salting-out effect that may promote hydrophobic interactions even on the most inert SEC supports. [19]. As summarized by Arakawa *et al.*, electrostatic interactions dominate at low salt concentrations, whereas hydrophobic interactions are promoted with the use of high ionic strength mobile phase [19]. Based on our experience, the combination of 50-100 mM phosphate buffer and 200-250 mM potassium chloride is a good compromise at pH = 6.8 with modern UHP-SEC columns for the analysis of therapeutic proteins. However, six different mAbs were still retained on a UHP-SEC column packed with 2.7 µm particles under these conditions [22]. A useful strategy to reduce hydrophobic interactions with the most critical mAbs can consist in reducing the ionic strength down to 50 mM, and in the same time to decrease mobile phase pH below 5 (to limit the most the presence of ionized silanolates and their interactions with proteins, which may become critical at low ionic strength) [23]. However, Mou *et al.* reported strong denaturing effects when the mobile phase pH was lower than 6.8 with loss of aggregates, increased peak tailing and reduced resolution between aggregates and fragments [15].

As an alternative, organic modifiers can be added to the SEC mobile phase up to 15-20 %, to limit hydrophobic interactions, which seem to be more critical than electrostatic interactions (Figure 5) [10]. This strategy is commonly applied in the industry with former generation of 300 x 7.8 mm SEC columns especially for the analysis of ADCs, which can be considered as highly hydrophobic species, due to the extreme lipophilicity of the attached drug [6]. However, it has to be kept in mind that the use of organic solvents may denature the non-covalent aggregate or/and change the proteins conformation, resulting in the underestimation of HMWS and change in the elution time. In addition, some organic additives such as acetonitrile are expected to enhance electrostatic interactions due to their low dielectric constant [24].

Finally, amino additives have been used in the past to limit secondary electrostatic interaction in SEC. While glycine and alanine greatly improved the recovery of BSA on a controlled-pore glass (CPG) column [25], arginine has attracted a major interest as a more effective co-solvent, to improve protein recovery without changing the aggregate content [19]. With a heat- and acidic stressed mouse mAb sample, Ejima *et al.* found 22% and 67% HMWS using a silica-based TSK G3000SWXL column in absence and in presence of 0.2 M arginine, respectively [24]. Similar results were observed with other proteins both on Superdex-75HR 10/30 and G3000SWXL columns, and confirm the tendency of aggregates

to bind more strongly to the SEC stationary phases in comparison with the monomeric species [24]. The authors indicated that arginine suppresses non-specific adsorption of proteins and does not change their aggregate content, while only slightly changing their melting temperature.

4. Importance of instrumentation in UHP-SEC

It is well-known that extra-column band broadening can be critical in modern ultra-high pressure liquid chromatography (UHPLC), as it can drastically decrease the apparent efficiency of small columns, when analyzing low molecular weight compounds [26-30]. In usual UHPLC conditions, the column volume and related volumetric band variance are essentially low, due to the column dimensions (e.g. 50 x 2.1 mm), and performance may be significantly affected by the extra-column band variance. In modern UHP-SEC, the typical column dimension is 150 x 4.6 mm, but band variance (σ_{col}^2) also depends on the solute retention factor (proportional to $(k+1)^2$). Since there is theoretically no adsorption in SEC and compounds are partially excluded from the internal pores, the retention factor is $k \leq 0$, resulting in small column band variance. As reported, around 20 – 200 μL^2 column peak variance is expected when working with 150 x 4.6 mm sub-3 μm UHP-SEC columns [7,8].

In conventional SEC (300 x 6.8 mm columns), it was showed that extra-column band broadening of regular HPLC systems was still acceptable [31,32], and the most important source of extra-column band broadening for the SEC separation of polymers was the tube dispersion [32].

In a recent study carried out in modern UHP-SEC (150 x 4.6 mm columns), the system variances of uridine, a monoclonal antibody (mAb) and blue-dextran were evaluated on conventional HPLC and optimized UHPLC systems [10]. It was found that for a totally permeable small analyte, only 25 – 60 % of the intrinsic efficiency of a 150 x 4.6 mm column was maintained on conventional HPLC systems. The situation was even worse with a partially excluded solute. When analyzing a mAb with the same UHP-SEC column, only 15 – 40 % of the column potential remained. Optimized UHPLC systems having very low extra-column volumes (typically V_{ec} < 10 μL) have then to be used, to properly operate these columns. Another interesting finding was that conventional HPLC systems also had an impact on the apparent elution time of proteins - and therefore on mass-calibration curve, when working with 150 x 4.6 mm I.D. columns. Then, calibration data were not reliable, except if corrected for extra-column residence time.

The optimization of UHPLC systems is important to take the full benefits of state-of-the-art 150 x 4.6 mm modern UHP-SEC columns. The possible shortest and narrowest connector tubing have to be used and PEEK tubing have to be preferentially selected [33]. Indeed, stainless steel tubing showed larger band broadening probably due to adsorption process.

By removing the column pre-heater from the UHPLC instrument (SEC of proteins is performed at ambient temperature), a further gain in apparent column efficiency was observed [33]. After a thorough system optimization, 20-40 % increase of apparent column efficiency can be achieved compared to a non-optimized UHPLC system, for a 150 x 4.6 mm UHP-SEC columns packed with sub-3 µm particles [33]. Figure 6 shows an example on UHPLC system optimization by replacing tubing and removing column pre-heater. Significant increase in plate numbers and shift of elution time can be visualized.

5. Are we always performing size-based separation in SEC?

In 2016, the United States Pharmacopeia (USP) published a general chapter <129> named analytical procedures for recombinant therapeutic monoclonal antibodies, which defines the HMWS as proteins species eluting before the main peak and similarly the LMWS corresponding to those eluting after the main protein peak. [34]. The USP definition of HMWS (or aggregates) and LMWS (or fragments) relies on a solely size-based separation of the proteins in SEC, which assumed the unlikelihood of SEC to separate monomeric species with similar molecular weight. However, different monomeric species may have different hydrodynamic radius (conformation) leading to size-based separations of monomeric species in SEC. Indeed, Philo *et al.* suspected the presence of a partially denatured monomer eluting before the main peak of a recombinant antigen, which was confirmed by LS/RI and sedimentation velocity experiments [35]. Furthermore, it has been shown that nonspecific interactions between the proteins and the SEC material can significantly modify their elution/retention time, particularly when analyzing hydrophobic mAbs [10]. Interestingly, the secondary hydrophobic interactions that may occur in SEC were successfully exploited in two studies for the separation of mAb-oxidized variants eluting before the main peak which was slightly retained on the SEC support thanks to its higher hydrophobicity (Figure 7) [23;36]. This approach called mixed mode chromatography, including size- and hydrophobic-based separation mechanisms was further optimized by adding high amount of salts (> 0.5 M), inducing protein salting-out effects and promoting hydrophobic interactions. However, the retention of proteins in SEC can also occur with common mobile phases. Such behavior was observed for seven FDA-approved mAbs out of 24, with both a historical SEC mobile phase containing 0.05 M potassium phosphate and 0.25 M potassium chloride at pH = 6.8 and an MS compatible mobile phase containing only 0.1 M of ammonium acetate at pH = 6.9 [22]. Interestingly, six mAbs out of the seven identified by the study possess the highest hydrophobicity, as measured by hydrophobic interaction chromatography (HIC). Therefore, these results suggested possible hydrophobic interactions with some mAbs in SEC and imply the possibility of separating less hydrophobic protein species from the main peak, such as oxidized variants in SEC. At the end, it is worth mentioning that antibody products containing

various species of different hydrophobicity may be therefore slightly separated in SEC, and induce peak tailing as observed for the highest DAR species of brentuximab vedotin [37].

6. Hyphenating SEC with MS

SEC is usually hyphenated to MS for performing an online desalting of the protein samples and enhance the quality of mass spectrum [38]. The approach significantly improves method throughput by avoiding the time consuming off-line use of desalting spin columns. The rise of 2D-LC methodologies in biopharmaceutical applications also promoted SEC as a powerful non-denaturing desalting step allowing the coupling of generally non-MS compatible techniques such as HIC to MS [39,40]. In particular, non-denaturing conditions are preferable for the analysis of the interchain cysteine-linked brentuximab vedotin, to avoid the dissociation of the conjugated heavy-chain (hc) and light-chain (lc) during the LC separation that would result in more challenging interpretation [41]. For these reasons, SEC is generally more suitable than RPLC for desalting proteins at the intact level as the latter involves the use of organic solvents, acidic additives and elevated column temperature. While the primary interest of SEC-MS is intended for protein desalting, Haberger *et al.* demonstrated the value of UHP-SEC coupled to native ESI-MS for the simultaneous quantification and identification of HMWS and LMWS of a bi-specific CrossMAb (Figure 8) [42]. The authors even mentioned the superiority of native SEC-MS for the analysis of HMWS against direct infusion in MS that often leads to the formation of oligomers artefacts based on their experience. However, they also pointed out that care must be taken to ensure the ESI process does not artificially form the species observed in SEC-MS. Indeed, aggregate may be formed in the ESI source according to the literature [43]. MS also suffers from poor sensitivity compared to UV or FL detection, in particular for the HMWS, and generally only the mAb dimers and trimers can be detected. Therefore, high injection volumes are often required to compensate for the lack of MS sensitivity, but they are likely to decrease the chromatographic performance. Otherwise, a post SEC column additive (such as m-nitrobenzyl alcohol) has been reported to significantly increase MS sensitivity and allowed the detection of size variants at levels down to 0.4%, but the approach was only applied until now for reduced mAb samples using denaturing conditions [44]. Alternatively, denaturing SEC-MS was also applied for the separation of hc and lc and also for a thioether linked hc and lc from free hc and lc from reduced mAbs without the need of post column derivatization [45].

It is also important to notice that MS-compatible mobile phases, which typically contain relatively low amounts of ammonium acetate (< 0.2 M), may only be suitable for the acidic proteins having pI below 7 [22]. Under these conditions, the authors reported strong peak tailing for the main peak (asymmetry factor > 1.5) and significant difference in HMWS amounts for proteins with pI higher than 7, in comparison with the historical SEC involving

the use of phosphate and potassium salts. The secondary electrostatic interactions between the proteins and the SEC stationary phase were indeed clearly more pronounced with the MS compatible mobile phases, thus resulting in protein adsorption. In another work, the HMWS of an ADC product were adsorbed onto the stationary phase even at 0.2 M ammonium acetate and could only be eluted by adding ammonium bicarbonate in the mobile phase, but such conditions are unlikely to be applied for routine SEC-MS analysis [46].

7. Application of modern UHP-SEC to real case study

MAbs are the largest class of biopharmaceutical products with more than 70 candidates that have been approved up to now, and therefore most SEC applications deal with mAbs analysis. A recent study evaluated the applicability of SEC for the analysis of different classes of FDA- and EMA-approved therapeutic proteins, i.g. mAbs, ADCs, Fc-fusion proteins and a bispecific antibody [22]. The HMWS were successfully separated from the main peak whatever the mAb formats of MW ranging from 54 to 150 kDa. This confirms the applicability of current sub-3 µm UHP-SEC columns with 150 – 300 Å pore size to separate species with a relatively large MW comprised between around 10 to 500 kDa [10]. Besides the determination of dimers and higher order aggregates, Boyd *et al.* recently reported the first SEC separation of an antibody species consisting in the non-covalent association of a mAb monomer with a heavy-light chain Fab arm (200 kDa) from the canonical antibody [47]. In another study, Yang *et al.* were able to consistently separate the minor “Fab/c” variants, which is an antibody fragment that has lost one arm of Fab (100 kDa), and the complementary Fab (50 kDa) from a mAb monomer [16]. As discussed in Section 2, non-specific interactions between the proteins and the SEC stationary phase are the most critical measures of the applicability of SEC independently on the antibody type. To the best of our knowledge - and surprisingly -, very few SEC studies have been published for other therapeutic proteins than mAbs and ADCs, except Waxman *et al.* who performed stability testing for Fc-fusion proteins and a cytokine [48] as well as Paul *et al.* who evaluated the efficacy of a BsAb formation from two parental mAbs [49]. Interestingly, the two parent mAbs had different SEC elution/retention times, while the BsAb had an elution/retention time in between the two mAbs. As discussed in Section 5, the significant difference of hydrophobicity between the two parent mAbs (HIC retention times of around 15 min vs. 45 min observed by running a generic linear inverse salt gradient) was likely to explain the unexpected SEC behavior [49]. Last but not least, biosimilarity evaluation also often includes SEC comparability studies for mAb [50,51], ADC [52], granulocyte colony stimulating factor (G-CSF) [53] and erythropoietin (EPO) [54] products.

As reported in section 6, SEC can act solely as an online desalting step in various 2D-LC combinations prior to MS analysis, but it can also add a true size-based separation

dimension. Despite a few applications reported for 1D-SEC analysis of mAbs originating from crude Chinese Hamster Ovary (CHO) cell culture [55], numerous Protein A x SEC 2D separations have been described for the analysis of mAb originating from complex matrices, i.e. cell cultures [56,57,58]. As example, Williams *et al.* developed an automated Protein A x SEC method with an on-line fraction collection device allowing the purification of the mAb originating from a complex bioreactor that may contained media components, host cells and DNA through Protein A chromatography, prior to the analysis of mAb aggregates by SEC (Figure 9) [59]. Heart cutting Protein A x SEC methods were also applied for the evaluation of mAb clones originating from cell culture supernatants, in comparison to the originator [51].

The optimization of antibody formulation also involves a set of analytical tools among which SEC is often used to evaluate the stability of the active principle ingredient (API). Furthermore, He *et al.* have developed a comprehensive excipient profiling by online coupling SEC and mixed-mode LC to separate the hydrophilic excipients eluting together in SEC at the total column volume, whereas the hydrophobic polysorbate 80 was already separated from the antibody and other excipients in SEC due to secondary hydrophobic interactions (Figure 10) [17]. Weisbjerg *et al.* developed a serial coupling of anion exchange (AEX) and SEC to retain a protein excipient (human serum albumin), which has a relatively similar hydrodynamic radius as the mAb. The separation was performed on the AEX column and the basic mAb was directly sent to the SEC column for HMWS determination [60]. Next to the presence of protein excipients in some formulations, the unwanted presence of free lipophilic linker-payloads related species in ADC products has to be assessed because the free cytotoxic payload exhibit very high cytotoxicity, which would cause off-target effects for the patients [1]. SEC x RPLC heart cutting methodologies have thus been developed to separate the lipophilic free linker-payload related species from the antibody in a first SEC dimension, while additional impurities from the small molecules were separated by RPLC in the second dimension [61,62]. Lipophilic linker-payload related species were characterized, but the use of 20 % acetonitrile in the mobile phase (to elute the lipophilic payload) may denature non-covalent aggregates. Therefore, an alternative approach was recently proposed to separate the HMWS from the ADC main peak under non-denaturing conditions prior elution of the free payload-related species by applying an acetonitrile gradient (Figure 11) [18]. This allowed the simultaneous determination of two CQAs, using a standard 1D LC instrumentation.

Besides the measurement of size variants, additional information can be obtained with SEC thanks to post column reaction. As example, Printz *et al*. added an extrinsic fluorescent dye latter than the SEC elution and UV detection, to observe changes in protein structure by fluorescence detection. [63] In another work, Furuki *et al*. added 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) to determine the mAb thiol-to-protein ratio. [64].

Finally, it is worth noting that oxidized variants might be relatively easily determined in SEC using high ionic strength (> 0.5 M) in particular for the most hydrophobic mAbs [23,36] and IgG4 BsAb [65], due to a mixed-mode separation mechanisms involving size- and hydrophobic-based separations.

8. Tips and tricks in SEC

In 2017, two protocol papers has been published to share the in-house knowledge related to the analysis of therapeutic proteins by chromatographic techniques [66,67]. First and as discussed in Section 4, SEC is a non-retentive chromatographic method, which may suffers from system band broadening especially when using modern 4.6 x 150 mm columns., Therefore, suitable chromatographic setup with minimized void volume should be used to ensure optimal SEC performance (typically extra-column system variance lower than 10 μL^2). Once an appropriate setup is employed, the LC system has to be systematically washed with water at the end of SEC experiments especially with 2D-LC setups, comprising additional valves more prone to salt clogging issues. Additional wash with a mixture of acetonitrile/water or ethanol/water (20:80, v/v) should also be carried out when the system is in standby for more than one week, to avoid the development of algae and bacteria. Besides the instrumentation, the SEC columns lifetime is known to be much lower compared to other LC modes. In addition to the mobile phase filtration through 0.22 µm membrane filters and care to avoid sudden change of column pressure, the column storage conditions can critically affect the lifetime of SEC columns. Indeed, column storage with a mixture containing an organic additive (typically water/acetonitrile (80:20, v/v)), significantly deteriorate the performance of several SEC columns, evidenced by a strong peak tailing observed after some weeks. Therefore, SEC columns should be ideally stored with 0.02 to 0.05% of sodium azide in water, which is generally used as column shipping solvent for long-term storage (> one week).

Besides column care, the risk of protein adsorption at the surface of the stationary phase is much higher on a brand new column. This adverse effect is more likely observed when injecting low protein amounts of around 1 µg. Therefore, pre-conditioning of the column with high amount of basic proteins (25 – 50 µg) such as cytochrome C may be carried out to feed the column before use. In addition, it has to be kept in mind that an increase of the mass load with the analyte of interest will decrease peak efficiency [16] and this effect is more critical when increasing the injection volume rather than sample concentration. The injection volume should thus be maintained below 2.5 µL when working with 150 x 4.6 mm SEC columns, to avoid significant peak efficiency loss.

At the end, SEC results should ideally be compared to an orthogonal technique such as analytical ultra-centrifugation (AUC), to confirm the data accuracy as i) SEC columns frits or

the column bed itself can filter some HMWS [19], ii) sample dilution prior to injection may results in potential dissociation of reversible aggregates [19], iii) oxidized variants may be separated from the main peak and considered as HMWS [23]. Prior to the analysis of antibody-based products, it is recommended to gently handshake the samples to homogenize the solution and avoid on-vial adsorption, as it can affect the reproducibility of measurements. This can be further limited by using appropriate vial shape and type [68]. In addition, polypropylene vials instead of glass containers are generally used to prevent potential adsorption issues. It is also possible to change the UV wavelength to 235 nm, to avoid sample dilution in case of UV detector saturation [69]. Finally, as suggested in a recent study, most mAbs that were retained in SEC had elution times correlating well with HIC retention times [22]. Indeed, when hydrophobic/hydrophilic variants of hydrophobic mAbs are already separated by HIC, this could indicate the potential separation of monomeric species in SEC.

9. Future perspectives

A common way to reduce solvent and sample consumption is to use small column dimensions such as 3 or 2.1 mm ID columns. However, in SEC it is probably not the future, as very low retention factors ($-1 < k < 0$) have a huge impact on peak variances and the observed peak variance is strongly affected by extra-column band broadening even on totally optimized UHPLC systems when working with 150 x 4.6 mm columns. Further decrease of column dimension such as 3 mm ID columns would result in > 50 % loss of the column intrinsic efficiency and this situation would be worse with 2.1 mm ID columns.

In the past 10 years, columns packed with superficially porous particles (SPPs) have been successfully applied in several LC modes, as they provide higher efficiency compared to fully porous particles (FPPs), especially for large molecules possessing low diffusivity. Despite the fact that SPP morphology may not be advantageous for SEC, columns packed with SPPs were recently evaluated in SEC, and the analysis time was somewhat decreased. Due to the lower porosity of SPP materials, the elution window is expectedly to be narrower and the probability to separate compounds becomes lower. However, the higher efficiency of SPPs can compensate for the decreased elution window. Indeed, peaks are sharper, and if compounds possessing large differences between their mass (size) have to be separated, then the use of SPPs could make sense in SEC [70,71,72]. Moreover, Brooks *et al.* described earlier that SEC does not really require porous stationary phase [73]. They suggested a chromatographic media based on solid particles bearing surface polymer layers. The partition of macromolecules into a layer of terminally attached polymer chains grafted onto a solid bead was fundamentally derived.

An interesting possibility to increase throughput is the so-called parallel interlaced SEC [74,75]. The main idea is that only approximately the one-third of the chromatogram acquisition time in SEC is interesting for proteins' analysis, as we mostly focus on the monomer and HMWS which elute in the middle of a SEC chromatogram. Thus, one can inject a new sample before the ongoing analysis of a previous sample has ended (interlaced injection). In addition, using two columns in parallel can further increase throughput, as additional interlaced time can be applied to cut-out the acquisition time between the injection time and the total-exclusion elution time (i.e. the time before the HMWS). This approach allows decreasing the analysis time by a factor of 2-3, but requires more sophisticated instrumentation (two columns, two valves, two pumps).

To improve the sensitivity of aggregate determination or handle very small amounts of samples, the use of i) fluorescence instead of UV detection and ii) capillary SEC columns appear as promising approaches [76,77]. However, with capillary columns, important loss of column efficiency is expected on current LC systems and therefore several key modifications to the best commercially available LC system are required to reduce the system volume and associated extra-column band broadening, as previously mentioned.

10. Conclusion

This article reviews the current possibilities of modern size exclusion chromatography applied for the characterization of therapeutic proteins.

Small columns (150 x 4.6 mm) packed with sub-3 µm particles indeed offer faster separations of protein aggregates than conventional SEC columns. The analysis time typically ranges between 4 – 8 minutes. The pore size of the SEC materials should be carefully selected and fitted for the purpose. It seems that nominal pore size values – provided by the vendors – are not always meaningful and it is better to experimentally measure a calibration curve based on the elution time of a set of test proteins.

The inertness of modern SEC phases is clearly better than of conventional stationary phases however secondary interactions (mostly hydrophobic interaction) may still exist with hydrophobic therapeutic proteins (e.g. ADCs). By adding a low amount of organic modifier, these interactions can be limited or eliminated. Potassium-based salts seemed to better limit possible electrostatic interactions than sodium salts especially for large aggregates carrying huge number of charges at the given pH set for the mobile phase. Amino additives can also be used to limit secondary electrostatic interactions. Glycine, alanine and arginine can improve protein recovery without changing the aggregate content.

To take the full benefits of state-of-the-art 150 x 4.6 mm modern UHP-SEC columns the UHPLC systems need to be optimized to avoid important extra-column band-broadening effects. The possible shortest and narrowest connector tubing have to be used and PEEK

tubing have to be preferentially selected. Mobile phase pre-heater can also be removed to further decrease system volume.

Unfortunately the definition of observed pre- and post-peaks in SEC is still not straightforward. In contrast with the definition of USP, pre-peaks are not always high molecular weight species. It was reported that partially denatured monomer species or oxidized protein monomers can elute before the main monomer peak due to conformational changes and/or decrease of hydrophobicity - compared to the native monomer.

SEC is inherently not compatible with MS but thanks to the more inert stationary phases some volatile buffers (e.g. ammonium-acetate) can be used to perform direct SEC-MS analysis for some proteins (acidic proteins possessing p*I* < 7). Post column additives (charge-modifiers) such as m-nitrobenzyl alcohol can also be used to improve MS sensitivity. In addition, 2D LC setups also make possible to use MS detection when putting an MS compatible mode in the second dimension (e.g. SEC x RPLC heart cutting).

Despite that lots of improvements have been seen in SEC column technology in the past few years, new developments are still expected. It now seems to be promising to use superficially porous particles or perform interlaced injections in SEC. The development of more inert stationary phases is also required.

Acknowledgements

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Figures captions

Figure 1. Comparing the silanol acidity of different columns by plotting retention factor of lithium ion as function of pH based on the method of Mendez *et al.* [78]. The chemical modification of the silica surface allowed to maintain negligible electrostatic interactions down to pH around 6 against 4.5 with silica (Reprinted from Ref. [12])

Figure 2. SEC profiles of a mAb control and same mAb after a 3-month thermal stability and another mAb control and in-process mAb sample using UHP-SEC (A,B) and HP-SEC (C,D) methods. Very similar separation performance were obtained by SEC and UHP-SEC using YMC Diol 200 and BEH200 columns, respectively. (Reprinted from Ref. [15])

Figure 3. Plate heights (H) *vs.* linear velocity (u_0) plots of adalimumab. Columns: AdvanceBioSEC 2.7 µm 300 Å (yellow curve with triangles), TSKgel UP-SW3000 2.0 µm 250 Å (red curve with diamonds), Yarra SEC X-150 1.8 µm 150 Å (black curve with squares) and Acquity BEH200 1.7 µm 200 Å (blue curve with rounds). (Adapted from ref. [10])

Figure 4. Mobile phase salts comparison for UHPSEC separation of a mAb sample using a Waters ACQUITY UPLC BEH200 (4.6 × 300 mm, 1.7 µm). Minimal differences in separation were observed except for the mobile phase containing only 50 mM potassium phosphate and 150 mM potassium chloride for which the post-monomeric peak was much less resolved. (Reprinted from Ref. [16])

Figure 5. Impact of the amount of acetonitrile in the mobile phase on the recovery of ADC (trastuzumab emtansine). By increasing the acetonitrile content, the recoveries of both monomer and dimer species increase and peak symmetry improves. (Reprinted from Ref. [79])

Figure 6. SEC chromatograms and plate numbers using different configurations of Acquity I-Class UHPLC system, using a 150 × 4.6 mm, 1.8 µm Yarra SEC-X300 column at 0.3 mL/min. Solute: IgG1 mAb.

Figure 7. Analysis of the molecular weights of the prepeaks and main peaks of a mAb by SEC-MALS. Tert-butyl hydroperoxide (TBHP) was used to generate oxidized species of the mAb sample, which separated from the main peak using a common mobile phase containing PBS (8.1 mM Na2HPO4 and 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Light

scattering overlay and mass distribution across the peaks are shown (Adapted from Ref. [23])

Figure 8. Comparison of the SEC profiles obtained using the BEH 200 SEC column with UV trace (A) and total ion current chromatogram (B). Chromatograms show the bi-specific antibody 'stability' (red trace, 24 months at 5°C) and temperature stressed (blue trace, 3 months at 40°C) samples. (Adapted from Ref. [42].)

Figure 9. (A) Protein A purification of a thermally-aggregated mAb with multiple fractions simultaneously collected from the aggregate peak and monomer peak via the in-line fraction collection device. (B) SEC of each of the collected fractions after sequential loading onto the second dimension column every 10 min. (Reprinted from Ref. [59])

Figure 10. On-line SEC-UV (BEH200 SEC column) with mixed-mode LC-ELSD (Acclaim Trinity P1 column) for the separation of protein and excipients in different drug products. (A) model mAb, (B) mAb, (C) ADC, and (D) vaccine. Insert is UV for protein. Peak ID: Na (sodium), K (potassium), Cl (chloride) (Reprinted from Ref. [17])

Figure 11. 1D-LC gradient SEC method for the analysis of an ADC at different stages of the purification process. Several impurities were separated and eluted after the column dead time at 5.8 min (Adapted from Ref. [18])

Table captions

Table 1. Physico-chemical properties of commercially available sub-3 μm SEC columns

Figure 1.

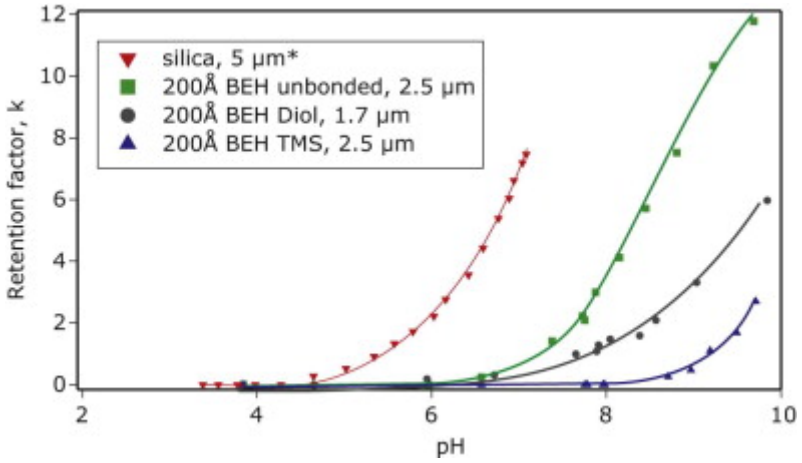
silica, 5 µm*
200Å BEH unbonded, 2.5 µm
200Å BEH Diol, 1.7 µm
200Å BEH TMS, 2.5 µm
Retention factor, k
pH
0
2
4
6
8
10
12

Figure 2.

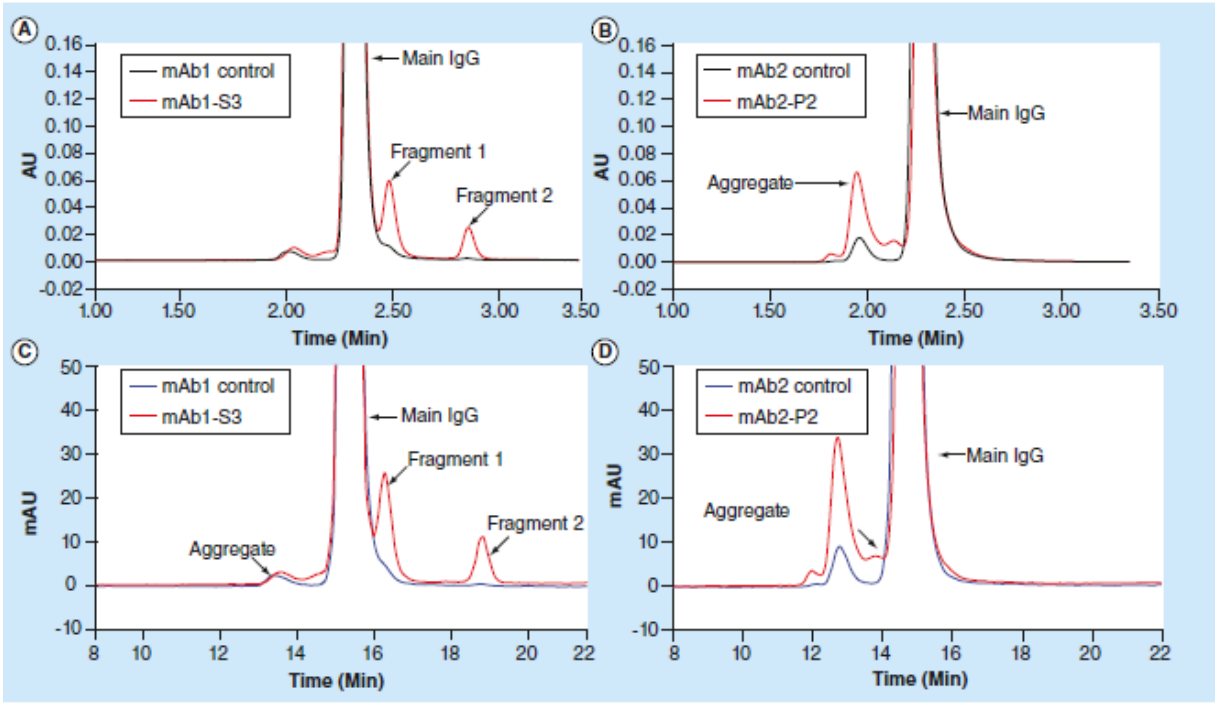
A
mAb1 control
mAb1-S3
Main IgG
Fragment 1
Fragment 2
AU
Time (Min)
B
mAb2 control
mAb2-P2
Main IgG
Aggregate
AU
Time (Min)
C
mAb1 control
mAb1-S3
Main IgG
Fragment 1
Fragment 2
Aggregate
mAU
Time (Min)
D
mAb2 control
mAb2-P2
Main IgG
Aggregate
mAU
Time (Min)

Figure 3.

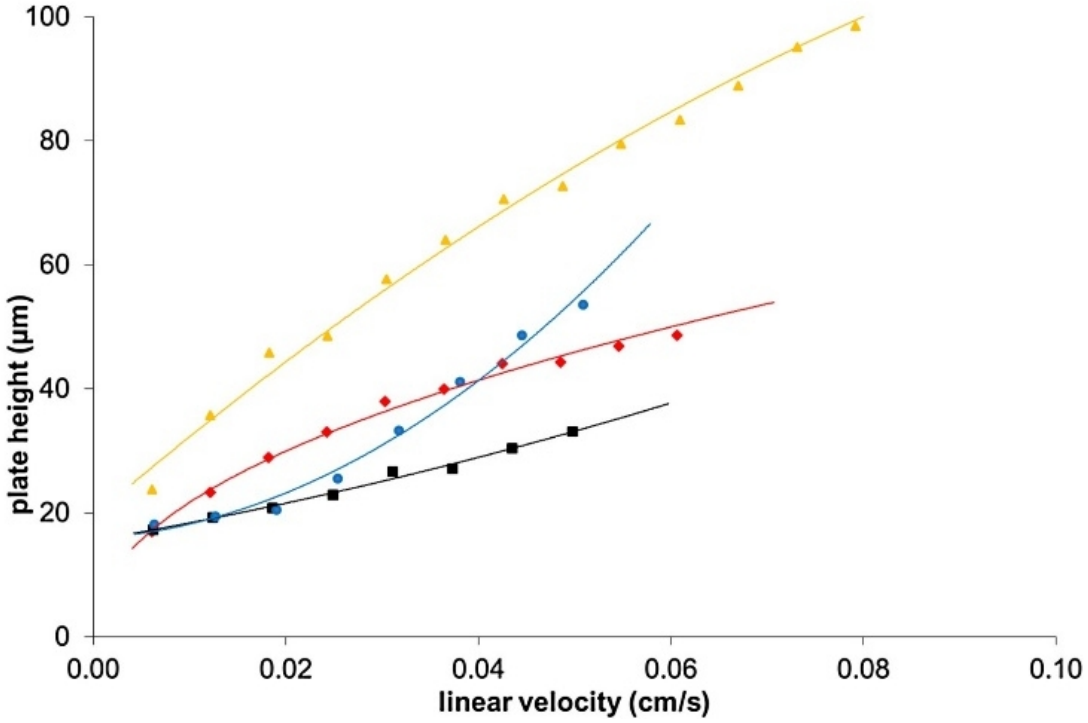
100
80
60
40
20
0
plate height (µm)
0.00
0.02
0.04
0.06
0.08
0.10
linear velocity (cm/s)

Figure 4.

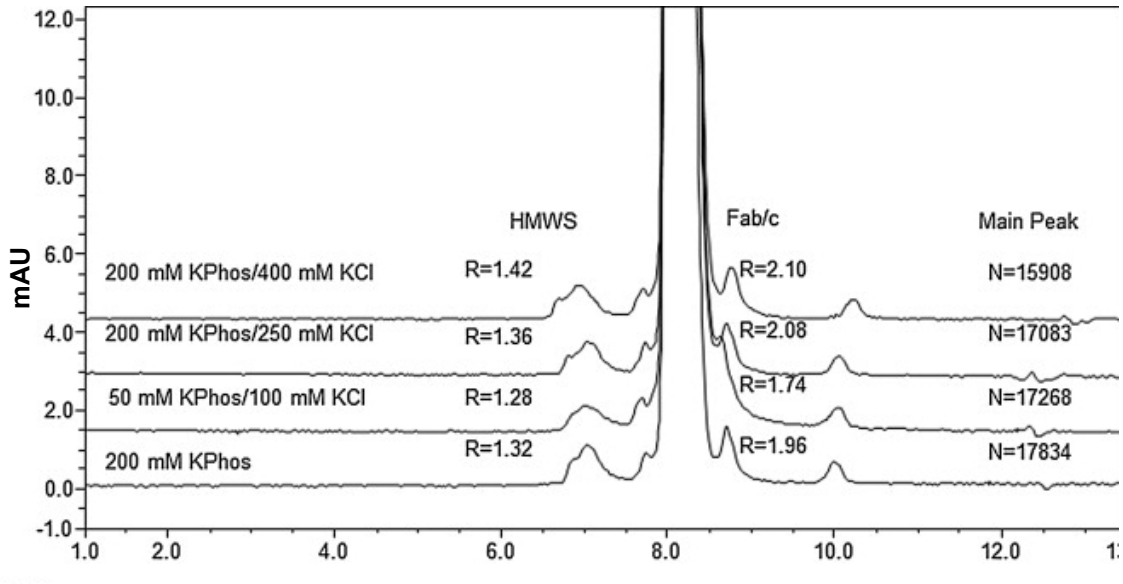
mAU
12.0
10.0
8.0
6.0
4.0
2.0
0.0
-1.0
1.0
2.0
4.0
6.0
8.0
10.0
12.0
HMWS
Fab/c
Main Peak
200 mM KPhos/400 mM KCl
R=1.42
R=2.10
N=15908
200 mM KPhos/250 mM KCl
R=1.36
R=2.08
N=17083
50 mM KPhos/100 mM KCl
R=1.28
R=1.74
N=17268
200 mM KPhos
R=1.32
R=1.96
N=17834

Figure 5.

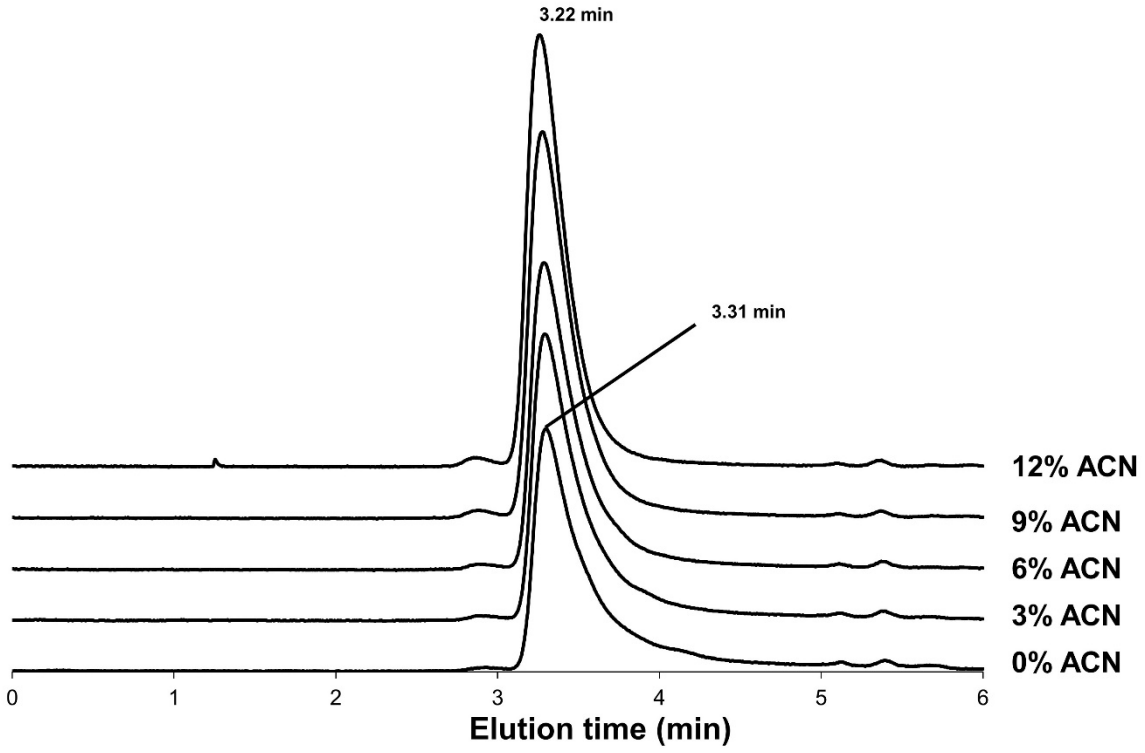
3.22 min
3.31 min
12% ACN
9% ACN
6% ACN
3% ACN
0% ACN
0
1
2
3
4
5
6
Elution time (min)

Figure 6.

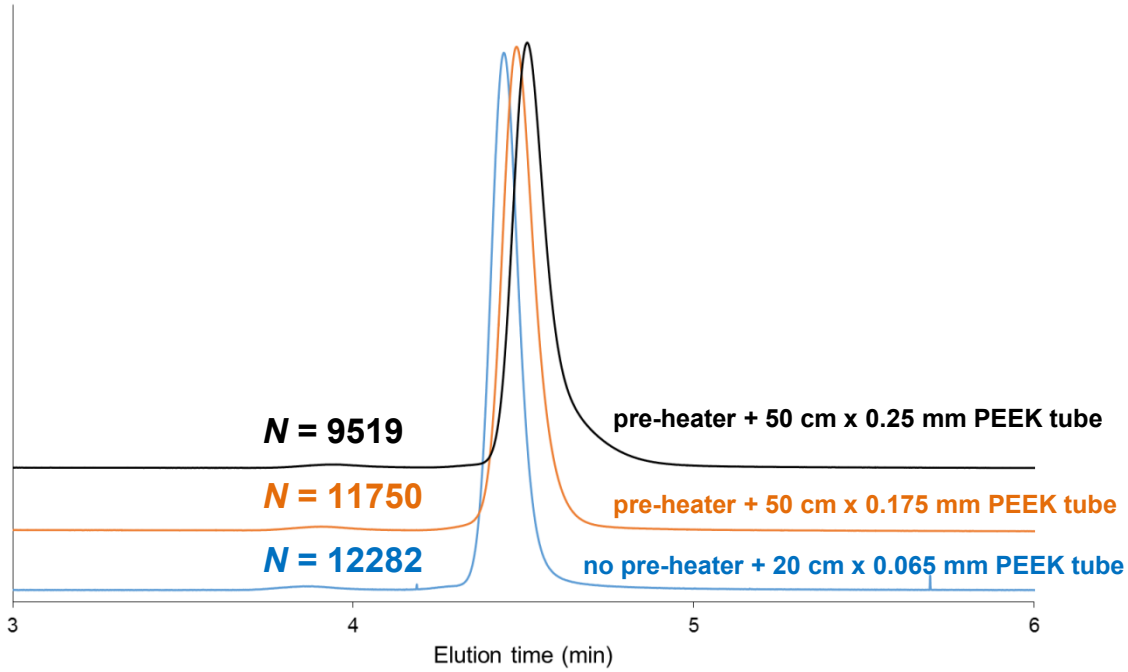
N = 9519
pre-heater + 50 cm x 0.25 mm PEEK tube
N = 11750
pre-heater + 50 cm x 0.175 mm PEEK tube
N = 12282
no pre-heater + 20 cm x 0.065 mm PEEK tube
3
4
5
6
Elution time (min)

Figure 7.

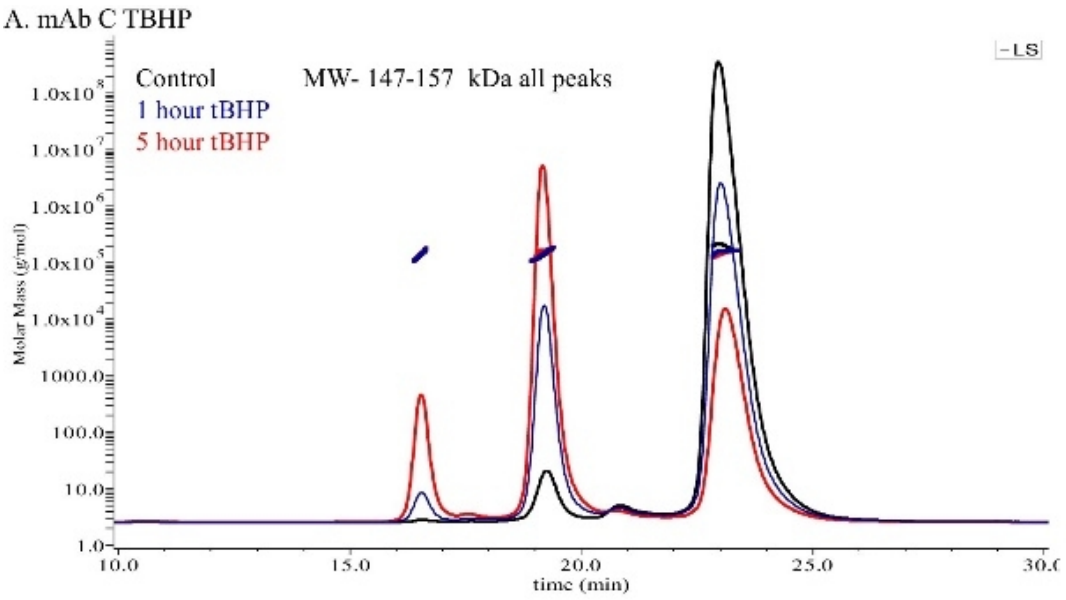
A. mAb C TBHP
Control
1 hour tBHP
5 hour tBHP
MW- 147-157 kDa all peaks
LS
Molar Mass (g/mol)
1.0x10^8
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
1000.0
100.0
10.0
1.0
10.0
15.0
20.0
25.0
time (min)

Figure 8.

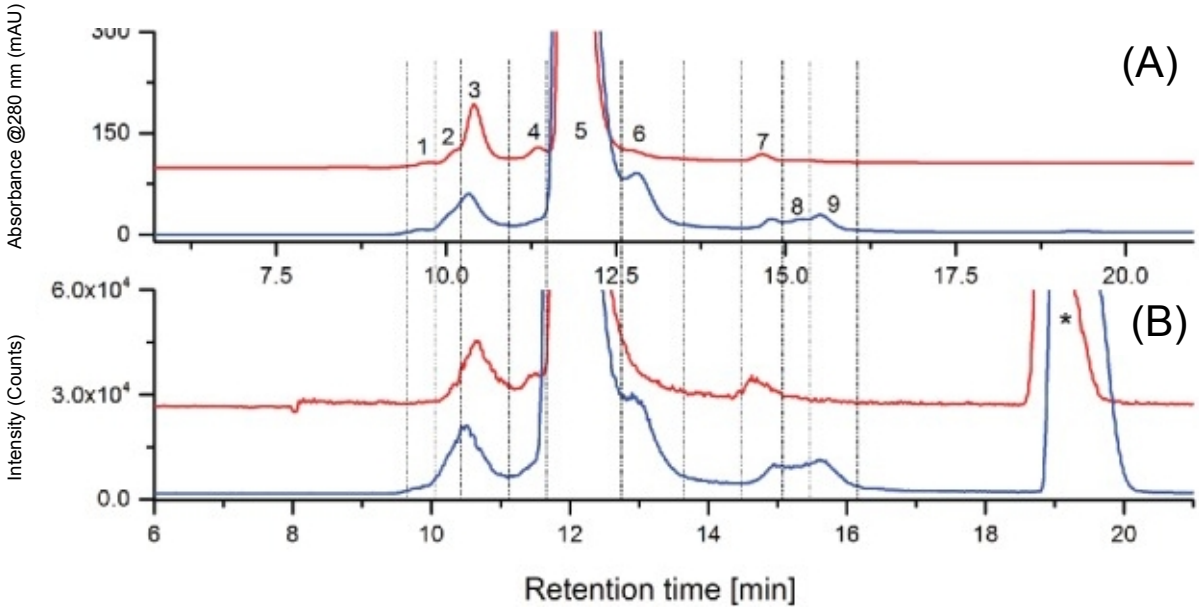
Absorbance @280 nm (mAU)
300
150
0
1
2
3
4
5
6
7
8
9
(A)
7.5
10.0
12.5
15.0
17.5
20.0
Intensity (Counts)
6.0x10⁴
3.0x10⁴
0.0
*
(B)
6
8
10
12
14
16
18
20
Retention time [min]

Figure 9.

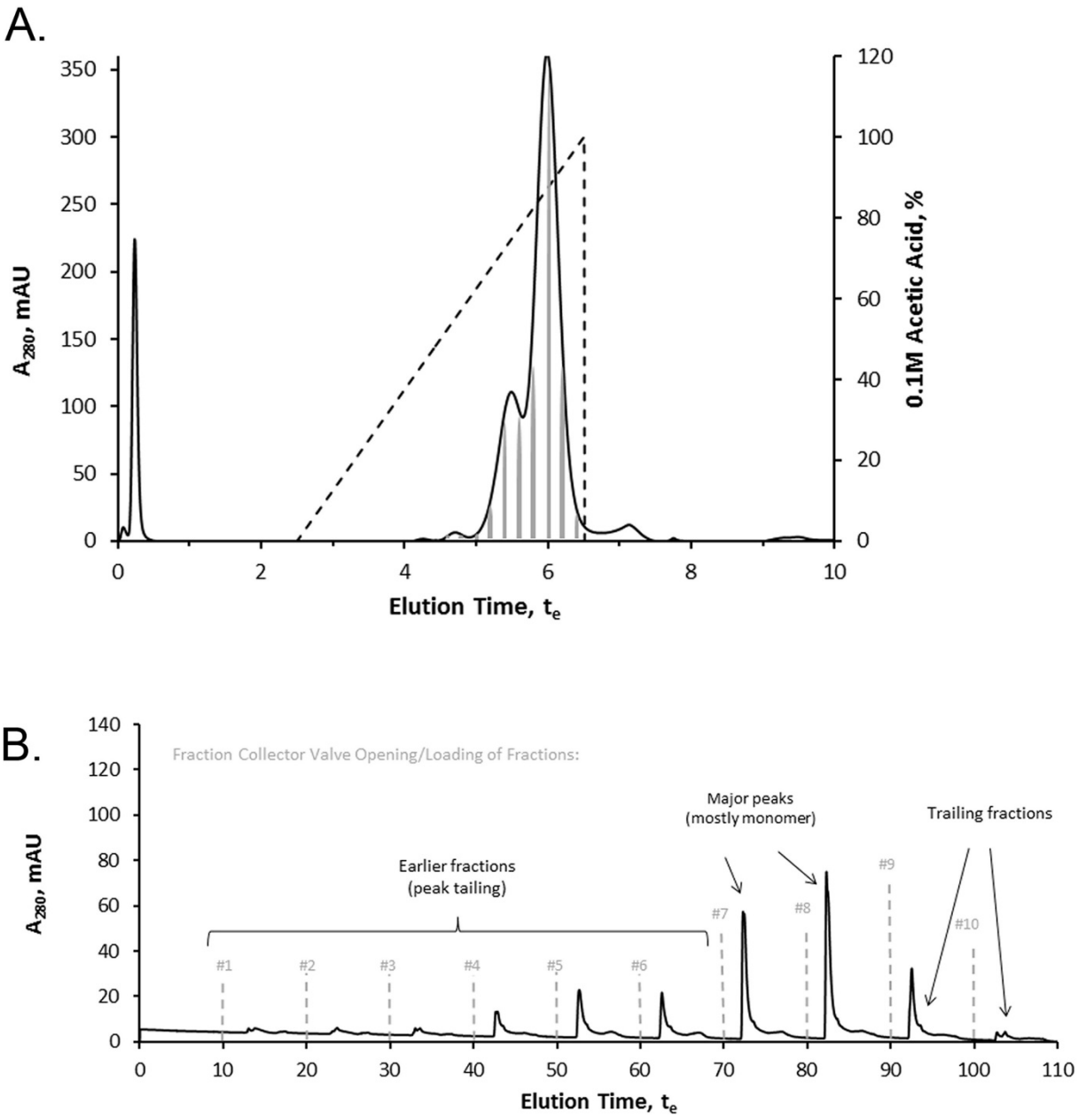
A.
350
300
250
200
150
100
50
0
120
100
80
60
40
20
0
0
2
4
6
8
10
A280, mAU
0.1M Acetic Acid, %
Elution Time, te
B.
Fraction Collector Valve Opening/Loading of Fractions:
Earlier fractions
(peak tailing)
Major peaks
(mostly monomer)
Trailing fractions
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
110
A280, mAU
Elution Time, te

Figure 10.

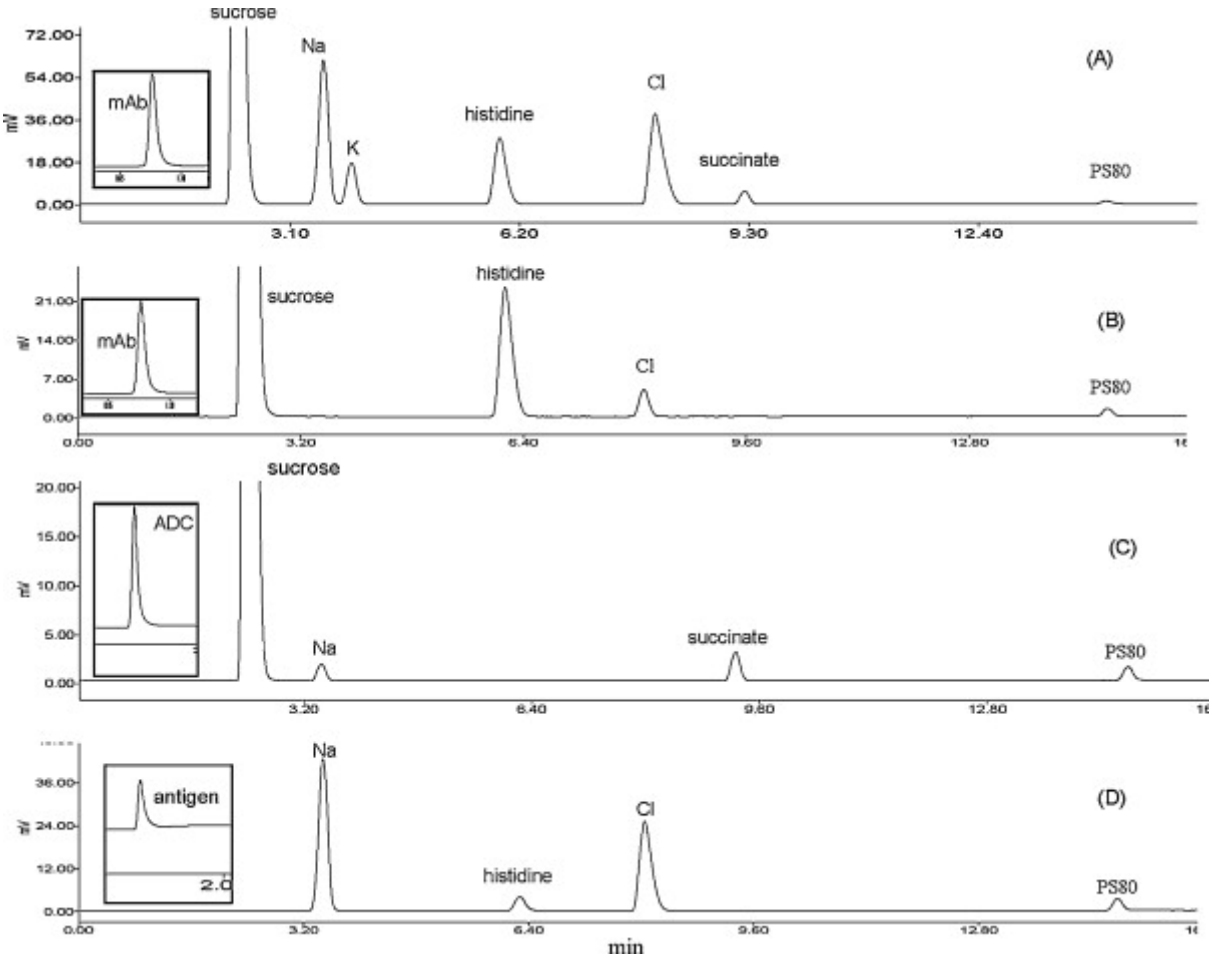
sucrose
Na
K
histidine
Cl
succinate
PS80
mAb
(A)
72.00
54.00
36.00
18.00
0.00
mV
3.10
6.20
9.30
12.40
(B)
21.00
14.00
7.00
0.00
3.20
6.40
9.60
12.80
(C)
ADC
20.00
15.00
10.00
5.00
antigen
(D)
24.00
12.00
min

Figure 11.

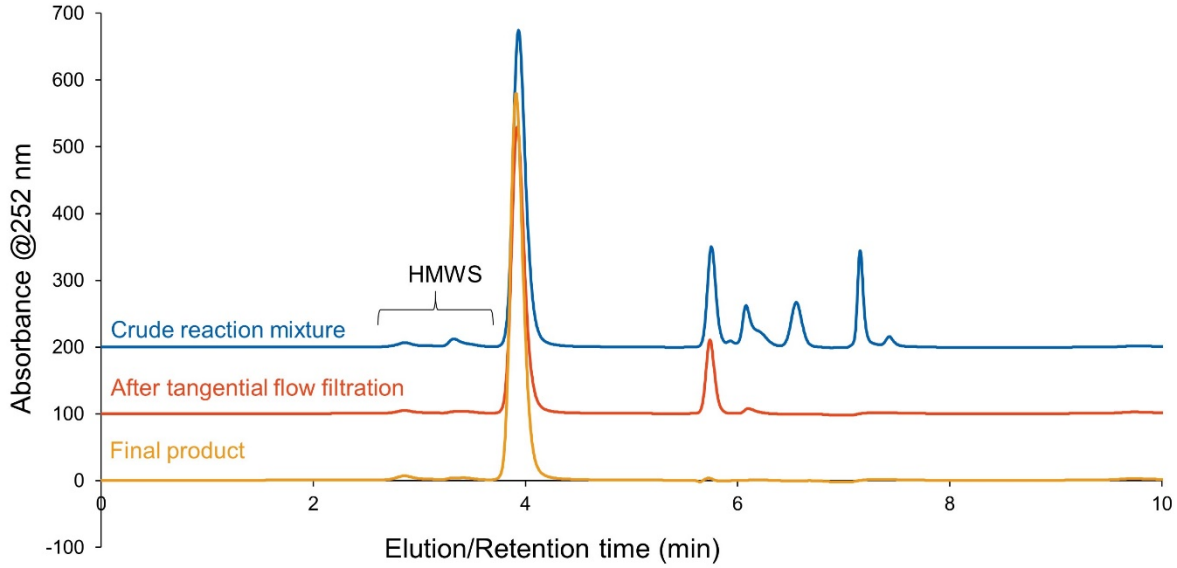
700
600
500
400
300
200
100
0
-100
Absorbance @252 nm
HMWS
Crude reaction mixture
After tangential flow filtration
Final product
0
2
4
6
8
10
Elution/Retention time (min)

Table 1.

column name	i.d. (mm) x length (mm)	d_p (µm)	pore size (Å)	permeability (m^2)	max. P (bar)
Agilent AdvanceBioSEC	4.6 x 150, 4.6 x 300 7.8 x 150, 7.8 x 300	2.7	130, 300	10.1×10^{-15}	400
Agilent Bio SEC-3	4.6 x 150, 4.6 x 300 7.8 x 150, 7.8 x 300	3.0	100, 150, 300	n.a.	240
Tosoh TSKgel UP-SW3000	4.6 x 150, 4.6 x 300	2.0	250	5.9×10^{-15}	250
Phenomenex Yarra SEC X-150, X-300	4.6 x 150, 4.6 x 300	1.8	150, 300	2.3×10^{-15}	480
Waters Acquity BEH SEC	4.6 x 150, 4.6 x 300	1.7	125, 200, 300	3.7×10^{-15}	480
YMC-Pack Diol-SEC 200, 300	4.6 x 150, 4.6 x 300	2.0	200, 300	n.a.	450
	4.6 x 300	3.0	200, 300	n.a.	200
YMC-Pack Diol-SEC 60, 120	4.6 x 300	3.0	60, 120	n.a.	200