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# Size Exclusion Chromatography of Protein Biopharmaceuticals: Past, Present and Future

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Biopharmaceuticals, size-exclusion chromatography, aggregate, monoclonal antibody, antibody drug conjugate, fusion proteins

## Abstract

Size exclusion chromatography (SEC) is a historical technique, routinely applied for the separation of species possessing different molecular masses (sizes). It is considered as a reference method for the qualitative and quantitative analysis of protein aggregates. In the last few years, several improvements were brought to high performance SEC (HP-SEC or conventional SEC) in terms of particle size, inertness and column dimension. Today, SEC columns of smaller dimensions packed with sub-3  $\mu\text{m}$  particles (also known as ultra-high performance SEC (UHP-SEC)) are used, to obtain faster and better separation compared to HP-SEC. This paper discusses the possibilities and limitations of both HP-SEC and UHP-SEC and also describes some possible directions for future applications related to protein biopharmaceuticals analysis.

## Introduction

Today, there is a need for more targeted therapies and more clinically efficacious drugs. Therefore, biopharmaceutical companies have increased their efforts to develop several biologics. Emerging classes of therapeutic agents now include monoclonal antibodies (mAbs), antibody-drug conjugates (ADC), bispecific monoclonal antibodies (BsAb), fusion proteins and related products.<sup>1-4</sup> Such complex molecules are inherently heterogeneous and their characterization requires an arsenal of analytical tools.<sup>5,6</sup> The different methods provide

complementary information about the compound of interest to build up the puzzle and understand the chemical structure of the protein biopharmaceutical product. Among the analytical techniques, size exclusion chromatography (SEC) is a reference technique for the qualitative and quantitative evaluation of protein aggregates.<sup>7,8</sup> The main advantage of this approach is the mild mobile phase conditions that permit the characterization of proteins with minimal impact on its conformational structure and local environment. 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 the biomolecules diffuse based on their molecular size difference without any retention, using an aqueous buffer as the mobile phase. SEC is an entropy controlled separation and requires the use of inert stationary phases to avoid physico-chemical interactions between the protein and the stationary phase.<sup>7,8</sup>

## Conventional Size Exclusion Chromatography (HP-SEC) – The Past

In traditional SEC, large column volumes (typically 30 cm long and 6 – 8 mm I.D. columns) 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 for using such large columns at the analytical scale, is that peaks elute before the column dead volume in SEC. Therefore, the expected peak volume (or variance) is very low and should be strongly affected by the system dispersion. To avoid serious efficiency loss on classical HPLC systems (possessing large extra-column volume), the solution was to work with relatively large columns. On the other hand, from a technical point of view, it was

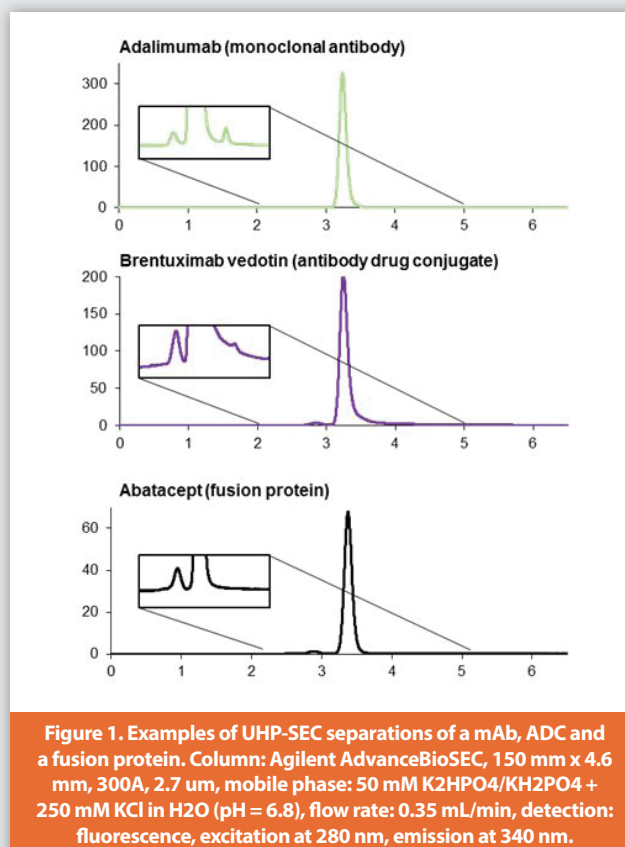
easier to pack columns of large diameters with such highly porous particles (more porous than in other chromatographic modes and hence possessing lower mechanical stability), which were less resistant to high packing pressure.

Those columns were packed with 5 – 10  $\mu\text{m}$  particles and their performance was consequently limited. Conventional SEC was indeed considered as a low performance chromatographic mode. However, resolution and throughput were not the only concerns. Indeed, old generation SEC stationary phases were not inert and proteins were often eluted as tailed (distorted) peaks at shifted elution time and aggregates recovery were not appropriate (irreversibly bound to the stationary phase), leading to inaccurate quantitative results.<sup>6</sup> The two main interactions that can occur between the solvent accessible part of the proteins and the active sites of the stationary phase are A) electrostatic and/or ionic interactions and B) hydrophobic interactions.<sup>7</sup> Undesired electrostatic interactions can be limited by the addition of a high concentration of salts (e.g. 0.2 M NaCl or more) whereas hydrophobic interactions can be decreased by the reduction of the amount of salts and/or addition of organic modifiers (e.g. 10 – 15 % IPA) into the mobile phase. Finally, column lifetime and batch-to-batch reproducibility were also often problematic with old generation SEC columns.

## Modern Size Exclusion Chromatography (UHP-SEC) – The Present

To achieve faster separations, the volume of conventional SEC columns was significantly reduced. The standard dimensions of modern columns are 150 x 4.6 mm and those columns are typically packed with sub-3  $\mu\text{m}$  particles ( $1.7 \leq d_p \leq 3 \mu\text{m}$ ).<sup>9</sup> The first sub-3  $\mu\text{m}$  SEC column was released in 2009, but it has not seen great success at that time. However, a few years later, its potential was recognized and various column providers have launched their own sub-3  $\mu\text{m}$  SEC materials since 2016.<sup>9</sup> The pressure stability of these packings was strongly improved as they can be operated up to 250 – 480 bar. Thanks to the good mechanical stability of UHP-SEC columns, relatively high mobile phase velocities can be applied and the analysis time can be decreased down to the 4 – 8 minutes interval.<sup>10</sup> In these conditions, the peak capacity for a 150 kDa antibody monomer ranges between  $n_c = 15$  and 25.<sup>10</sup> Table 1 lists the commercially available sub-3  $\mu\text{m}$  SEC columns.

However, the full advantage of 150 x 4.6 mm SEC columns can only be taken when operating them on a fully optimized UHPLC systems possessing very small extra-column volume ( $V_{ec}$ ).<sup>11,12</sup> A key aspect to consider here is the band dispersion due to the chromatographic system itself. Indeed, it is important to keep in mind that the band variance ( $\sigma_{col}^2$ ) depends on the solute retention factor ( $\sigma_{col}^2 \sim (k + 1)^2$ ). Since the compounds are excluded from the internal pores in SEC, the retention factor is extremely low (between  $-1 < k < 0$ ) and so does the  $(k + 1)^2$  contribution, resulting in a small column band variance. The most important source of extra-column band broadening for



SEC separations is probably the connection tube.<sup>12,13</sup> Therefore, when analyzing a mAb product with a very efficient 150 x 4.6 mm SEC column, only 15–40% of the column's potential is used on most of UHPLC systems. The shortest and narrowest connector tubing have to be used and PEEK tubing has also to be preferentially selected. By removing the column pre-heater from the LC instrument (SEC of proteins is performed at ambient temperature), a further gain in apparent column efficiency can be expected. Another important feature is that conventional HPLC systems have a huge impact on the apparent elution time of proteins – and then on mass-calibration curve – when working with 150 x 4.6 mm I.D. columns. Then calibration data are not reliable, except if corrected for extra-column residence time.

Besides the efficiency improvement and analysis time decrease in modern SEC, the inertness of state-of-the-art SEC phases was also drastically improved compared to old generation of HP-SEC phases, thanks to new hydrophilic surface modifications, such as chemically bonded 1,2-propanediol. These ligands block or react with most acidic residual silanol groups and neutralize the surface. The peaks corresponding to the mAb or ADC monomer elute mostly as symmetrical peak even with common mobile phases. However, to limit the adsorption of high molecular weight protein species (HMWS) - which are more hydrophobic and carry more charges than monomers - the use of high ionic strength mobile phases or addition of organic modifiers could still be required. Better HMWS recovery was observed with potassium-based SEC mobile phases versus sodium-

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

| column name                       | i.d. (mm) x length (mm) | d <sub>p</sub> (µm) | pore size (Å) | permeability (m <sup>2</sup> ) | max. P (bar) |
|-----------------------------------|-------------------------|---------------------|---------------|--------------------------------|--------------|
| Agilent AdvanceBioSEC             | 4.6 x 150, 4.6 x 300    | 2.7                 | 130, 300      | 10.1x10 <sup>-15</sup>         | 400          |
|                                   | 7.8 x 150, 7.8 x 300    |                     |               |                                |              |
| Agilent Bio SEC-3                 | 4.6 x 150, 4.6 x 300    | 3.0                 | 100, 150, 300 | n.a.                           | 240          |
|                                   | 7.8 x 150, 7.8 x 300    |                     |               |                                |              |
| Phenomenex Yarra SEC X-150, X-300 | 4.6 x 150, 4.6 x 300    | 1.8                 | 150, 300      | 2.3x10 <sup>-15</sup>          | 480          |
| Sepax SEC-300 Unix, Unix-C        | 4.6 x 150, 4.6 x 300    | 1.8                 | 300           | n.a.                           | 310          |
| Tosoh TSKgel UP-SW3000            | 4.6 x 150, 4.6 x 300    | 2.0                 | 250           | 5.9x10 <sup>-15</sup>          | 250          |
| Waters Acquity BEH SEC            | 4.6 x 150, 4.6 x 300    | 1.7                 | 125, 200, 300 | 3.7x10 <sup>-15</sup>          | 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          |

based buffers/salts, which were historically used.<sup>14,15</sup> Therefore, potassium-based salts should be preferred, to reduce possible secondary electrostatic interactions between the dimeric forms of biopharmaceuticals and SEC materials, as well as to lower protein-salts interactions (conformational changes, salting-out).

## Other Possibilities of SEC – The Future

Operating SEC columns at higher pressures is not desired, as high pressure conditions can lead to artefacts such as on-column protein aggregation or dissociation of reversible aggregates. In this section, we tried to summarize some possible future developments which are expected in SEC.

In SEC, only approximately one-third of the chromatogram acquisition time is interesting for proteins' analysis, as we mostly focus on the monomer and HMW peaks 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 decrease the analysis time, as additional interlace time can be applied to cut-out the acquisition time between the injection time and the total-exclusion elution time (the time before the HMW peaks). This approach was called "parallel interlaced SEC",<sup>16,17</sup> and allows decreasing the analysis time by a factor of 2-3. However, it 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. However, with capillary columns, several key modifications to a commercially available LC system are required to reduce the system volume and associated extra-column band broadening. Despite this limitation, 300 mm × 300 µm I.D. SEC capillary columns were successfully applied for the separation of mAbs fragments.<sup>18,19</sup>

In the past 10 years, columns packed with superficially porous particles (SPPs) have been successfully applied in several modes of liquid chromatography, as they provide much 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 evaluated in SEC, and the analysis time was somewhat decreased. Due to the lower porosity of SPP materials, the elution window is expectedly narrower and the probability to separate compounds becomes lower. However, the higher efficiency of the 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.<sup>20-22</sup>

SEC is considered as an inherently MS incompatible mode of liquid chromatography, since non-volatile buffers and salts are used as mobile phase additives (phosphate buffer and KCl or NaCl salts), leading to ion suppression and MS contamination. Ammonium formate or ammonium acetate at reasonable ionic strengths and slightly acidic pH of around 5-6 to minimize the presence of residual silanols of the stationary phase could be used as an alternative to the phosphate buffer. Besides the nature of the buffer, SEC-MS methods have also been developed using denaturing mobile phases containing organic solvents (as "pre-" or "post-column" additive), ion-pairing reagents (TFA) or charge-modifiers (e.g. m-nitro-benzyl alcohol).<sup>23-26</sup> Using such mobile phases is always a compromise between good chromatographic performance, MS sensitivity and protein denaturation. To avoid the problems associated with the mobile phase, an off-line MS detection can also be performed.<sup>27</sup>

Another solution to make SEC compatible with MS is to work with two-dimensional (2D) LC setup. A SEC-RP 2D-LC method was recently developed for the direct analysis of free drug and related small molecule impurities in ADC products without the need for sample preparation.<sup>28,29</sup> The SEC column in the first dimension provided information on the size variants and also separated the small molecular weight species from the protein. The small molecule peaks from the 1st dimension were trapped and further analyzed by reversed-phase (RP) LC in the 2nd dimension for identification and quantification. This SEC-RP approach allowed comprehensive profiling of both size variants and small molecule impurities of ADCs, and provided a powerful tool in gaining insight into ADC degradation mechanisms. Very recently, SEC has also recently been coupled to ion-exchange (IEX) chromatography and applied to mAb characterization.<sup>30</sup> Complementary (orthogonal)

information could be provided by the two modes (size variants and charge variants separations). Other combinations of SEC in 2D setups are expected in the future.

## Conclusion

As illustrated, the current trend in SEC is to use 150 x 4.6 mm columns packed with sub-3 µm particles. By using these columns, the analysis times now range from 4 to 8 minutes. The state-of-the-art SEC stationary phases also show excellent inertness, even if some non-specific interactions can still be observed with the most hydrophobic or basic therapeutic biopharmaceuticals. It often results in tailed peaks and poor recovery of the HMWs. Extra-column band broadening is another issue in UHP-SEC, thus only optimized UHPLC systems are adequate to operate those columns. Finally, the best way today to make generic SEC methods compatible with MS, is to perform 2D-LC using SEC in the first dimension and an MS compatible mode (e.g. RP) in the second one.

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