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Review

Unraveling the mysteries of modern size exclusion chromatography - the way to achieve 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 × 4.6 mm) packed with sub-3 µm particles, allowing a 3- to 5-fold increase in method throughput compared to that of conventional SEC. The quick success of the first sub-2 µm SEC column introduced in 2010 led 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 attributes (CQAs) of mAbs, as HMWS may decrease the product efficacy or cause immunogenicity effects. Therefore, the confident characterization of mAbs requires strong knowledge of not only modern SEC performance (i.e., selectivity and efficiency) but also the inherent limitations caused by non-specific interactions more likely to occur with complex antibody drug conjugates (ADCs) and some commercial mAb products. This review discusses the importance of liquid chromatographic (LC) instrumentation in order 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., mAbs, ADCs, Fc-Fusion proteins and bispecific antibodies) are presented. Finally, tips and tricks are provided to further optimize SEC separations and maintaining their performance over time with better understanding of unexpected SEC results.

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

Therapeutic proteins including monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), bispecific monoclonal antibodies (BsAbs), 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 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 the conformational structure and local environment of the protein. SEC is considered a non-denaturing chromatographic mode, as proteins are analyzed in 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 differences without any retention, using an aqueous buffer as the mobile phase [7,8]. SEC is mostly considered an entropy-controlled separation mode and requires the use of inert stationary phases to avoid physicochemical interactions between the protein and the stationary phase [7,8].

Beside the advantages of SEC, it has to be mentioned that SEC has the ability to retain individual aggregates, of different MWs by reversible or irreversible adsorption onto the surface of the stationary phase, so that they may not appear in the final chromatogram in their true quantity. Also, in SEC, an initial dilution of the sample into the

Abbreviations: 2D-LC, Two Dimensional Liquid Chromatography; ADC, Antibody Drug Conjugate; AEX, Anion Exchange Chromatography; BEH, Bridged Ethylene Hybrid (hybrid silica); BSA, Bovine Serum Albumin; BsAb, Bispecific Antibody; CQA, Critical Quality Attribute; EMA, European Medicines Agency; ESI, Electrospray Ionization; Fab, Fragment of Antigene Binding (antibody fragment); Fc, Fragment Crystallizable (antibody fragment); FDA, US Food and Drug Administration; FL, Fluorescence (detection); HMWS, High Molecular Weight Species; LMWS, Low Molecular Weight Species; mAb, Monoclonal Antibody; MS, Mass Spectrometry; PEEK, Polyether ether ketone; RP(LC), Reversed Phase (Liquid Chromatography); SEC, Size Exclusion Chromatography; UHPLC, Ultra High-Pressure Liquid Chromatography; UHP-SEC, Ultra High-Performance Size Exclusion Chromatography; USP, United States Pharmacopeia; UV, Ultra Violet (detection)

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mobile phase has to be performed, which can change the aggregate/monomer ratio and pattern.

In conventional SEC, large column volumes (e.g., 300 mm × 8 mm) have been used mainly at low flow rates (and low pressure). The analysis time for these columns usually ranged from 25 to 40 min. The reason for using such large columns on 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. However, from a technical point of view, it is easier to pack columns with large diameters with such highly porous particles (more porous than in other chromatographic modes and hence possessing lower mechanical stability), which are 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 mm × 4.6 mm, and these 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 columns was also strongly improved, as they can be operated at up to 250–480 bar. High mobile phase velocities can be applied, and the analysis time is decreased to 4–8 min [11]. Under 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 over the past few years [7–9], mostly dealing with conventional SEC applications and method development. The aim of this paper is 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 volumetric contributions of the instrumentation 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 also illustrated. Finally, the latest applications to modern therapeutic protein-based products are reviewed.

2. Stationary phases in modern UHP-SEC

2.1. Physicochemical properties of SEC columns

While cross-linked dextran particles, polyacrylamide-based gels and polystyrene resins were the first chromatographic media introduced for SEC, the limited mechanical stability of these materials led to the introduction of silica-based particles to perform SEC at higher flow rates and pressures (a 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 the proteins and the acidic silanols (Fig. 1) [12]. Currently, a diol-bonded silica stationary phase remains 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 intra-particle pores, the total porosity of the columns was increased from 60 to 70% (which is common in most sorptive modes of LC such as reverse phase /RP/) to approximately 80–90% for modern SEC columns. The pore size is another important feature in SEC, as the pore size determines the molecular weight (MW) range of proteins that can be analyzed successfully. The MW range of the proteins can be evaluated by injecting various protein markers possessing different MWs and plotting the log MW vs. the 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 MWs between 10 and 500 kDa and is thus well suited for the separation of mAb and ADC trimers, dimers, and 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 similarity was particularly true for the linear range of the calibration curves (which corresponds to the useful elution window), always corresponding to a 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. A large solute can travel through only the part of the individual pore that has a large enough diameter, while the thinnest parts of the pores are not accessible. In addition, pore size distribution also has a strong contribution on the shape of the calibration curves, and specifically, the slope of the linear part of the calibration curve is higher with a large pore size distribution. When proteins cover a broad range of MWs, 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 both the resolution between the largest proteins compared to the individual 200 Å column and the resolution between smaller proteins, in comparison with a large pore size column [13].

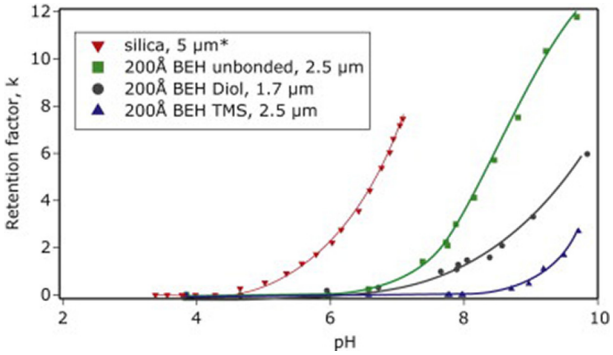

Fig. 1. Comparing the silanol acidity of different columns by plotting the retention factor of lithium ion as a function of pH based on the method of Mendez et al. [78]. The chemical modification of the silica surface allows the maintenance of negligible electrostatic interactions down to a pH value of approximately 6 compared to 4.5 with silica.
(Reprinted from Ref. [12]).

The BEH200 SEC column packed with 1.7 µm particles was the first sub-2 µm material launched in 2010 and can be considered the precursor of modern SEC. Obviously, the column allowed a significant increase in the method throughput, thanks to smaller column dimensions of 4.6 mm × 150 mm vs. 7.8 mm × 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 with 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 × 150 mm and 4.6 mm × 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 separation that was six times faster than that with conventional SEC was achieved (Fig. 2). Yang et al.