Effects of Secondary Interactions in Size Exclusion Chromatography

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Size-based separations are a form of chromatography which, unlike many other chromatographic techniques, separates molecules without any chemical interaction with the stationary phase. The concept was first postulated by Synge and Tiselius [1] when investigating the properties of zeolites, with the first examples being demonstrated by Wheaton and Bauman [2], and the first application to the analysis of proteins being demonstrated by Lindqvist and Storgårds [3]. There are many names that are given to a sized-based separation process; gel permeation chromatography (GPC) is common when using organic solvent as the mobile phase with a hydrophobic stationary phase, while gel filtration chromatography (GFC) [4] or more recently size exclusion chromatography (SEC) [5] is used for separations in an aqueous mobile phase with a hydrophilic stationary phase.

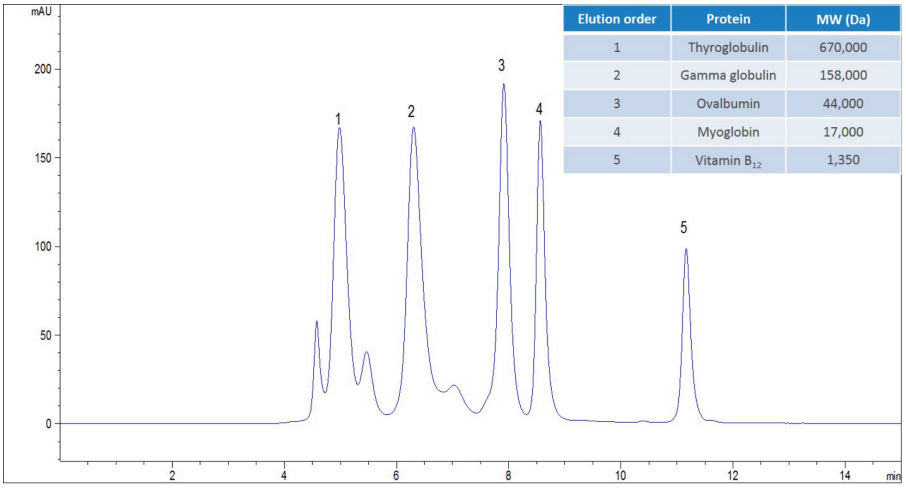
An example of a size based separation is given in Figure 1, which shows a chromatogram of a mixture of different molecular weight proteins, obtained on an Agilent AdvanceBio SEC column.

Due to the aqueous mobile phase used in SEC, it is particularly beneficial for the quantitative analysis of proteins in their native state. The addition of organic solvent, as used in GPC, will cause changes in the protein's conformational structure. These changes in shape and size will result in the protein eluting at a different elution volume and consequently the size of the native protein will be incorrectly determined. GPC is specifically useful for the analysis of polymers and plastics, as these tend to dissolve readily in organic mobile phases [6,7].

For the analysis of large protein molecules in their native state, SEC is a powerful technique and is of particular use to the biopharmaceutical industry to ensure the purity of the biotherapeutic. During the manufacturing process, the proteins may undergo aggregation within the cell culture during several of the production stages including;

- product expression,
- product purification in downstream processing,
- or in the drug during storage.

Aggregates found in monoclonal antibodies may cause an immune response and so accurate quantification, using techniques such as SEC, of these moieties is essential.



Elution order	Protein	MW (Da)
1	Thyroglobulin	670,000
2	Gamma globulin	158,000
3	Ovalbumin	44,000
4	Myoglobin	17,000
5	Vitamin B_{12}	1,350

Figure 1. AdvanceBio SEC 2.7µm, 300 Å, 4.6 x 300 mm (Agilent Technologies) analysing gel filtration standard, (BioRad PN: 151-1901) with a flow rate of 350 µL/min, 150 mM sodium phosphate at pH 7.0.

This article will focus on the analysis of protein molecules and their aggregates/impurities using SEC, and the effect that secondary interactions can have on the analysis.

The separation process in size exclusion chromatography is based on the hydrodynamic size/Stokes radius of the molecule [8] as it naturally occurs in the aqueous buffer solution, which is used as the mobile phase. The stationary phase used is typically a hydrophilic inert material, polymer or silica based, with a narrow size distribution of pores. The mode of separation occurs when the analyte moves into the pores by differing degrees based on their hydrodynamic size.

The first peak to elute will be the analytes that are too large to enter into any of the pore structure, this will be in the same retention volume (V_0) as the interstitial space between particles, or the exclusion limit. From here, the larger analytes elute, as they are able to penetrate only a few pores in the stationary phase. Referring to Figure 1 it can be seen that thyroglobulin is the largest protein and elutes first. Analytes that are smaller are able to visit more of the pore's infrastructure and therefore take longer to elute. Small molecules, including the solvent front, will elute at the end in a permeation limit, in Figure 1 it is Vitamin B_{12}, representing the total volume of solvent in both the interstitial space and the particle pores (V_{tot}). The exact relationship between a molecule's size and its retention is dependent on the physical characteristics of the stationary phase, and in particular

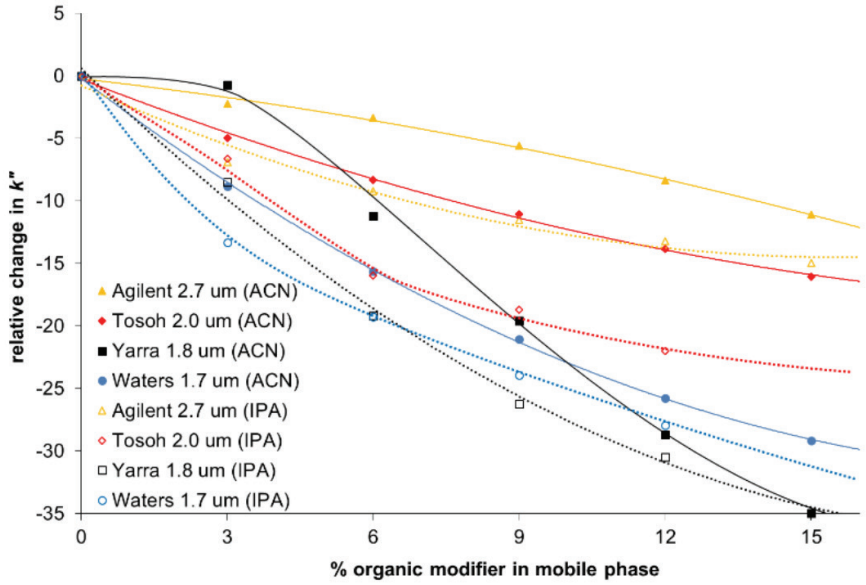


Figure 2. Relative change of zone-retention factor vs. organic modifier for ipilimumab. Inspired by A. Goyon, A. Beck, O. Colas, K. Sandra, D. Guillarme, S. Fekete, Separation of protein biopharmaceutical aggregates using size exclusion chromatographic columns packed with sub-3 µm particles, J. Chromatogr. A., [submitted] JCA-16-1083.

the pore structure. Since the process of retention is dependent on the hydrodynamic size of the molecule, it is possible to relate the retention times of individual analytes to their molecular mass [9] (the assumption being that the mass of a molecule is related to the hydrodynamic radius).

Assuming that the retention mechanism is purely based on the Stokes radii, then it has been shown that the retention time for an individual analyte is directly proportional to the log of the relative molecular mass [10], for molecules that are neither completely excluded or for molecules that can penetrate all of the pore network, (Equation 1).

$$logM = m.K_D + b$$

Where;

m and b are the slope and intercept of the linear part of the calibration line, and K_D, the thermodynamic retention factor, is given by the following expression (Equation 2);

$$K_D = \frac{V_R - V_0}{V_{tot} - V_0}$$

Where;

V_R – retention volume of the analyte/protein

V_0 – retention volume of the column

V_{tot} –total solvent volume of the column

While theory predicts a perfectly linear correlation, the variability in exact pore structure often leads to a degree of nonlinearity. Calibrations are run using a series of known molecular weight/ size samples, of a similar nature to the unknown, to allow identification of precise retention times for that particular molecular weight, MW. Hence, when determining the molecular mass of unknowns, comparison of retention times with known standards will give a good indication of the size of the unknown molecule. The calibration curve provides an upper and lower MW limit that the column is able to separate, where a sharp upward and downward deviation from a linear response obtained the for the calibration range.

SEC should ideally have no chemical interaction between the analyte and the stationary phase, since the separation mechanism is based on size only. It is, therefore, a purely entropy driven process from a thermodynamic point of view. It is interesting to note that using a modified van 't Hoff expression [11] (Equations 3 to 5) it is readily seen that temperature should not affect the separation mechanism. Examining the kinetics, however, the temperature does affect the diffusion rates, solvent viscosity, and also potentially affect the structure of proteins, which will effectively alter the radius of the molecule. Consequently, it should be expected that the retention would vary with temperature.

$$RTlnk = -\Delta H^0 + T\Delta S^0 \quad \text{(Equation 3)}$$

$\Delta H^0 = 0$, due to no chemical interactions

$$RTlnk = T\Delta S^0 \quad \text{(Equation 4)}$$

$$k = e^{\wedge}\left(\frac{\Delta S^0}{R}\right) \quad \text{(Equation 5)}$$

Where:

R- universal gas constant

T – thermodynamic temperature

k – retention factor, for a size exclusion process this would be replaced with the thermodynamic retention factor, K_D.

ΔH^0 – standard heat of enthalpy

ΔS^0- standard heat of entropy

A practical investigation of this phenomena will be detailed later in this article, comparing the temperature sensitivity of retention times for reversed phase columns and for SEC.

As well as considering secondary interactions and the pore volume/diameter of the stationary phase, another approach to improve overall chromatographic performance is to reduce the particle size. Smaller particles form a tightly packed bed in the column on loading, providing a more homogenous, tortuous path for the smaller molecules, this can provide better efficiency with narrow, sharper peaks. A reduction in particle size, however, results in an increase in column back pressure, and this may induce conformational changes to the protein. For SEC of larger protein structures, high pore volumes are required for good separation, but this same porosity makes the smaller particles much weaker than the less porous particles typically used for reversed phase chromatography. As the particles deform or break, due to high column back pressures, so the uniformity of the packing bed structure will decrease resulting in an increase in band broadening.

As with all chromatographic systems it is important to be aware of band broadening, which also affects peak width. Tubing and fittings should be short and narrow to provide the least dead volume in the system, injection volumes and detector volumes need to be minimised. The flow rates should also be optimised [12] to ensure that the dispersion processes are minimised. The optimal flow is dependent on the temperature, the viscosity of the mobile phase and the molecular mass of the analyte. Thus for size exclusion chromatography, optimisation of the flow rate is something that has to be considered much more than in other forms of chromatography, where the range of molecular masses tend not to be so varied.

It has already been stated that with true SEC, chemical interactions between the stationary phases and the analytes should not contribute to the separation mechanism, however this can be very difficult to control

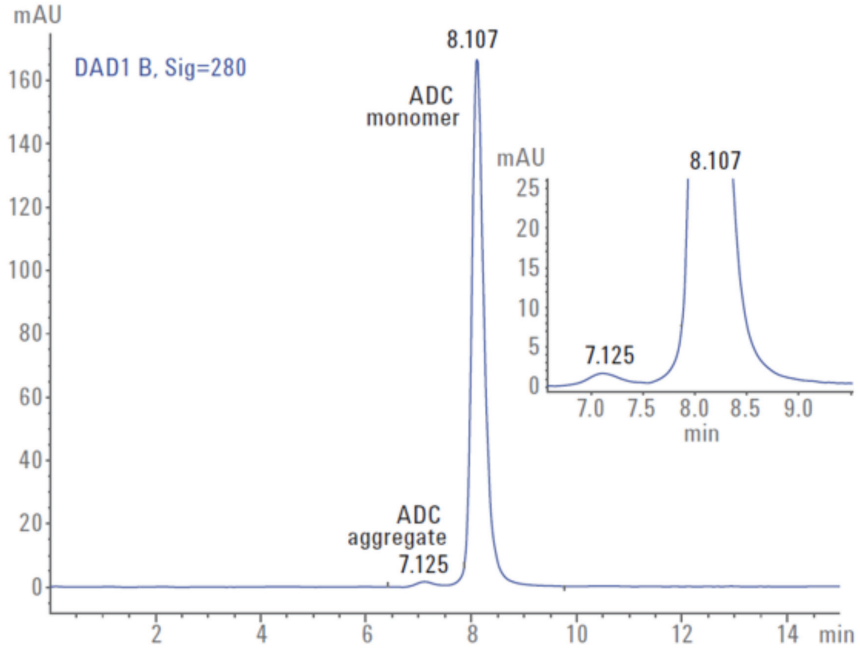


Figure 3. SEC profile of intact T-DM1 (ADC) on an Agilent AdvanceBio SEC 300 Å, 7.8 x 300 mm, 2.7 µm column at flow rate of 800µL/min using PBS, pH 7.4 (M.Sundaram Palaniswamy, Agilent Technologies).

from a practical aspect. Biomolecules often contain both hydrophilic and hydrophobic groups, which easily interact with traditional packing sorbents. Secondary interactions can shift the retention times, causing incorrect prediction of MW values. They also significantly impact peak shape, which will have a pronounced effect on the resolving ability of the process. Increasing the ionic strength/salt concentration can minimise polar interactions through electrostatic screening, and peak shapes can be improved, however altering the salt concentration may also affect the form of the molecule being analysed and reduces the compatibility of the technique with some detectors such as mass spectrometry.

Hydrophobic interactions can be overcome by the addition of an organic component to the mobile phase, however this can cause protein molecules to denature, which will alter their hydrodynamic size, and so this should be avoided if the native size of the protein is being determined.

There are a variety of methods to test the nonspecific interactions of a SEC column, and a recent study by Goyon [13] demonstrates how this can be used to compare the performance of a variety of columns. The next section looks at the experimental data obtained from this publication and also looks at the practical implications when analysing real proteins.

Table 1 Commercially available SEC columns included in the study.

Column Manufacturer	Column name	Stationary phase	Guard column	Column dimensions	Particle size	Pore size
Agilent	AdvanceBio SEC	Proprietary bonded silica	50 x 4.6 mm	150 x 4.6 mm	2.7 µm	300 Å
Phenomenex	Yarra SEC-X150	Proprietary bonded silica	Not used	150 x 4.6 mm	1.8 µm	150 Å
Tosoh	TSKgel-UP-SW3000	Silica, diol bonding	20 x 4.6 mm	150 x 4.6 mm	2.0 µm	250 Å
Waters	Acquity UPLC BEH 200 SEC	Ethylene bridged hybrid based particle, diol bonding	30 x 4.6 mm	150 x 4.6 mm	1.7 µm	200 Å

Experimental

The investigation [13] was performed using ten monoclonal antibodies (mAbs) covering a broad range of isoelectric points (*pI* = 6.7 to 9.2) and hydrophobicity, along with two antibody-drug conjugates, Trastuzumab-emtansime and Brentuximab-Vedotin. The data presented here will be representative of this exhaustive data set.

A range of SEC columns were evaluated as shown in Table 1:

Goyon et al. performed SEC experiments on a two UHPLC instrumentation with the average extra-column peak variance of the two systems being and (depending on flow rate, mobile phase composition and solute), respectively.

The standard SEC mobile phase was 100 mM disodium hydrogen-phosphate buffer and 200 mM sodium chloride in water, pH 6.8 (adjusted with hydrochloric acid). Variations in the mobile phase composition were made dependent on the experiment being performed. Measurements were performed at a flow rate of 350 µL/min and temperature of 25°C. The mAb and antibody-drug conjugate (ADC) samples were diluted from the concentrated commercial solutions to 0.5 mg/mL in water. Typical injection volume was 1.0 µL, which corresponded to 0.5 µg mass injected onto the column. The secondary hydrophobic and electrostatic interactions between the stationary phase and proteins were evaluated by measuring the change of elution time when varying the organic modifier or salt concentration in the mobile phase.

The second set of results shows the AdvanceBio SEC column range from Agilent Technologies, firstly analysing an ADC and then in a comparison with a C18 reversed phase chromatography column. The effect of temperature is investigated using a reversed phase arrangement and a SEC column. The SEC method used AdvanceBio SEC, 2.7 µm, 300 Å (Agilent Technologies) 4.6 x 300 mm, using 150 mM sodium phosphate buffer at pH 7.0. The temperature was varied in the range 30°C to 40°C.

The reversed phase study used nine model drug compounds; antipyrine, aminohippuric acid, paracetamol, hydroxyantipyrine, aminoantipyrine, atenolol, aminobenzoic acid, theophylline, phenacetin and caffeine. The analysis employed was a standard C18 column, 100mm x 2.1mm, capable of running at elevated temperatures, with the chromatographic system comprising of an HPLC system with a GC oven used to obtain higher temperatures up to 180°C.

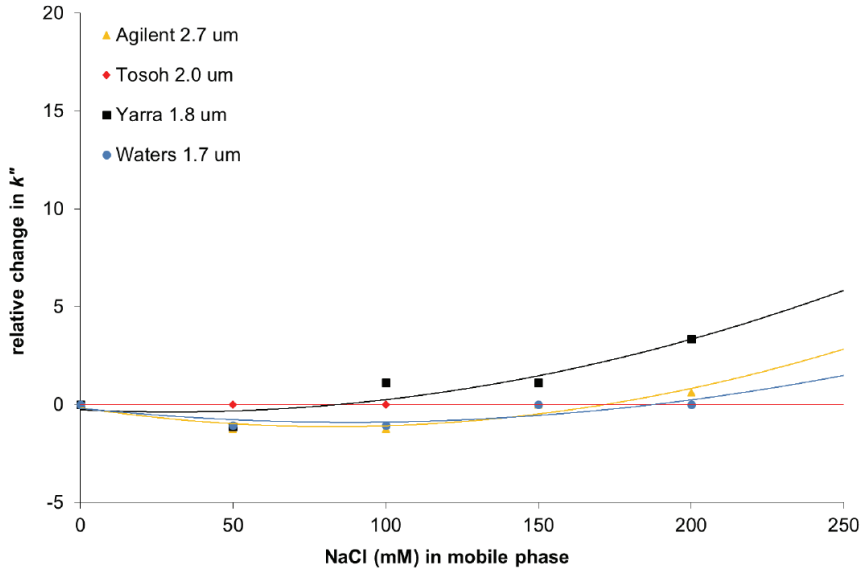


Figure 4. Relative change of zone-retention factor vs. salt concentration for rituximab. Inspired by A. Goyon, A. Beck, O. Colas, K. Sandra, D. Guillarme, S. Fekete, Separation of protein biopharmaceutical aggregates using size exclusion chromatographic columns packed with sub-3 µm particles, J. Chromatogr. A., [submitted] JCA-16-1083.

Results and Discussion

The hydrophobic interactions, when analysing the mAbs, were determined by the addition of an organic solvent either; acetonitrile (ACN), an aprotic solvent or isopropanol (IPA), a protic solvent, with the amount varied between 0 and 15% (w:w) by 3% steps. As well as reducing the hydrophobic interactions this approach can also result in changes to the molecular structure of the proteins, which in itself can result in a retention time shift, so some care has to be taken when interpreting the data.

Figure 2 shows plotted the relative change in the zone retention factor, k", against the percentage of organic modifier (either ACN or IPA). The zone retention factor was expressed by Engelhardt in the following (Equation 6) [14]:

$$k'' = \frac{V_e - V_i}{V_i}$$

Where;

Vi is the interstitial volume of the column

Ve is the volume that the analytes are in while visiting the pore structure of the column

Figure 2 shows the data obtained by the Geneva University team for the retention of ipilimumab, which showed the most significant changes as the organic content increased. Ipilimumab is one of the most hydrophobic components evaluated and as such is sensitive to the amount of organic modifier in the system. The resulting relative decrease of k" was significant when increasing the content of ACN and IPA with all columns tested. The largest change seen (over 25%) was with the Yarra and Acquity columns at 15% addition of organic modifier.

The decrease in elution time with the addition of organic modifier highlights the occurrence of hydrophobic interactions. Consideration is required when carrying out SEC in the presence of organic solvents, as this example illustrates, retention times change as a result of selectivity between the stationary phase and the analyte. The protein structure may also alter with increasing levels of organic solvent within the mobile phase, due to denaturing. Modern SEC columns are developed to minimise the degree of interactions but the user must be aware of the potential limitations.

To show specifically the inertness of the Agilent column, an antibody drug conjugate (ADC) was analysed using 150mM sodium phosphate buffer at pH 7.0. The drug conjugate provides a notable increase in hydrophobicity of the antibody, which with some columns, will result in secondary interactions occurring between the hydrophobic ADC and the bonded phase. In order to minimise these hydrophobic interactions [15], the addition of an organic modifier was necessary to improve peak shape and aid resolution between aggregate and the ADC monomer.

Figure 3 shows, that using the AdvanceBio SEC column, the addition of IPA, to reduce hydrophobic interactions, was not necessary. The peak shape looks good, with a tailing factor of 1.35, and there is resolution of 1.9 between the aggregate and monomer indicating that there were no non-specific interactions occurring even without modifier. The Agilent column allows analysis of the highly hydrophobic trastuzumab emtansine using non denaturing conditions. This is clearly a technological improvement as it was necessary for Wakankar et al [15] to add IPA for the analysis of the same sample 5 years ago.

To investigate the occurrence of any electrostatic interactions, the Geneva team varied the salt concentration (NaCl) between 0 and 200 mM by 50 mM steps. The mobile phase pH of 6.8 was kept constant. The relative change in *k"* (Equation 6) was plotted as a function of mobile phase salt (NaCl) concentration (Figure 4).

Figure 4 indicates that the electrostatic interactions have less of an effect than the hydrophobic ones, as the relative changes in retention time seen for each of the mAbs are less pronounced. Goyon et al, again showed a shift in the zone retention factor when using sodium chloride to change the ionic strength of the mobile phase. Increasing the salt concentration within the mobile phase will reduce any potential ionic interactions, which can result in reduced repulsive or attractive forces between the stationary phase and the analyte. The Yarra column shows the most significant increase in zone retention factor when the concentration of NaCl was increased above 150mM.

The data in Figure 5 shows a comparison between the van 't Hoff plots obtained for Vitamin B_{12} on the SEC column and one of the nine model compounds separated in reversed phase. It is evident, by the greater slope on the curve, that the retention factor response to variations in temperature is substantially more significant on reverse phase columns than that obtained using the SEC column. The solubility of the analytes in the organic mobile phase and the interactions with the stationary phase are changing significantly with temperature. It is also interesting to note that the reversed phase curve has a positive slope which tends to suggest an exothermic reaction, due to a positive enthalpy change compared to the negative slope of the SEC data, indicating an endothermic process where a negative enthalpy change occurs.

Both secondary interactions and reverse phase chromatography are affected by variations in temperature and so can be used as a good indicator of the inertness of the SEC stationary phase. If a degree of interaction between analyte and

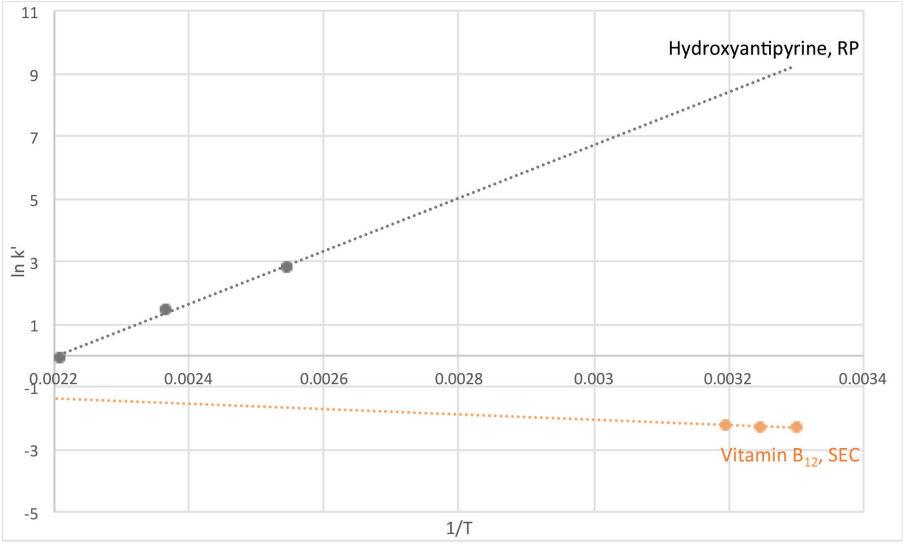


Figure 5. A comparison of the Van't Hoff plot for a C18 reversed phase column and an AdvanceBio SEC column. A linear relationship between the log of the retention time and the reciprocal temperature is observed for both separation mechanisms, however the gradient differs significantly.

the stationary phase is expected and acceptable, then it is important to be aware of the temperatures that will be employed in the methods. As a column approaches the ideal SEC mechanism, temperature changes will not affect the separation power of the stationary phase. There should be minimal effect on the retention time with increases in temperature for a particular molecule when using size-based separations and any deviation from this suggests secondary interactions are present. The changes with variation in temperature will instead be isolated to the mobile phase viscosity and back pressure, column efficiency, and the conformation of the proteins being analysed.

Conclusions

SEC is a well-established chromatographic method, but still poses a challenge. Development of stationary phases to increase inertness, and reduction in particle size to increase efficiency, are two ways in which manufacturers are optimising the separations. Looking at some of the commercially available columns on the market, the optimum particle size of the stationary phase is decreasing. A reduction in particle size does, however, result in an increase in column back pressure, which can lead to practical difficulties.

If the column is more inert, the fewer mobile phase additives are required, preventing generation of aggregates, giving a more accurate determination of MW for large protein molecules in their native state. Another advantage of running in a purely aqueous phase is the ability to couple SEC columns to MS, which is an area that is becoming more popular, with the need to accurately determine low-level aggregates/ fragments in mAbs. This method of detection is also one of the most convenient and reliable ways to identify the analytes as they elute from the column.

In modern chromatography, method optimisation goes beyond just the column and eluent. Band broadening must be minimised in all areas, with the system, flow rate, temperature, and plumbing all needing to be considered.

So it seems that there is often a compromise required with SEC in order to achieve the desired outcome of separating a protein, while preserving its native conformation. We want to keep the mobile phase aqueous, and use temperatures and flow rates that will not affect the protein's structure. While modifications to the aqueous mobile phase may improve peak shape and reduce secondary interactions, we run the risk of altering the very molecule that is being analysed.

SEC is an extremely useful technique, moving forward, it is becoming essential to quantitatively determine aggregates within the protein's structure. Practical considerations for column stationary phases are crucial. The phase needs to present a tool avoiding nonspecific interactions, possess a narrow pore size and particle size distribution, and provide a uniformly packed bed that is able to reproducibly separate large molecules based on size.

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