We are IntechOpen,
the world's leading publisher of
Open Access books
Built by scientists, for scientists

6,900
Open access books available

186,000
International authors and editors

200M
Downloads

154
Countries delivered to

Our authors are among the

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS
BOOK CITATION INDEX
INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Separation of Monoclonal Antibodies by Analytical Size Exclusion Chromatography

Atis Chakrabarti

Additional information is available at the end of the chapter

Abstract

Size exclusion chromatography (SEC) is a powerful tool for the separation of biotherapeutics such as monoclonal antibodies (mAb) and others such as antibody drug conjugates (ADCs), biosimilars, and bi-specific mAbs as well as other therapeutic proteins. Detection of purified protein heterogeneity is essential. Heterogenic impurities cause immunogenic response. More than 99% purity is needed for the medicinal purpose. Size exclusion chromatography (SEC) is used to monitor this purity level in the quality control (QC) process of the biopharmaceutical industry. With the increased use of ultra-high-performance liquid chromatography (UHPLC) instruments in QC laboratories today, instead of the conventional HPLC, it is important to have a size exclusion chromatography (SEC) column which is compatible with both UHPLC and conventional HPLC instruments. Orthogonal and complimentary modes such as reversed phase chromatography (RPC), hydrophobic interaction chromatography (HIC), and ion exchange chromatography (IEC) can also be used along with SEC. SEC columns are generally modified with diol groups on the surface to prevent a secondary interaction. Surface and pore characteristics of the SEC columns are critical for the separation. Pore characteristics need to be optimized to have high resolution of mAb monomer from dimer and higher order aggregates as well as from fragments. Shallow calibration curve is necessary for the best resolution. Overall, the separation of monoclonal antibodies from the impurities by analytical size exclusion chromatography column is primarily discussed in this chapter. The evaluation of the different peak parameters such as retention time, peak asymmetry, column efficiency, peak resolution, run time, and loading capacity is also briefly discussed. Finally, the tips and tricks for the best separation and maintaining the column health are also discussed.

Keywords: mAb, monoclonal antibody, heterogeneity, aggregate, SEC, size exclusion, ADC, bi-specific antibody, mass spectroscopy

1. Introduction

Antibodies belong to a family of globular proteins called immunoglobulins [1]. Immunoglobulin G (IgG) is the most common. Eighty percent of all the antibodies present in the blood are IgG [2]. IgG is a relatively large molecule (approx. 150 kDa). It has four subclasses, which are IgG1, IgG2, IgG3, and IgG4 [3]. Monoclonal antibodies are antibodies derived from one unique B cell clone [4]. They have single antigenic determinant specificities [5]. Monoclonal antibodies are screened and isolated by special procedures, expressed, and purified [6]. Monoclonal antibodies, particularly IgG1, have tremendous application in biotherapeutics. Other subclasses such as IgG2 and IgG4 are also used as biotherapeutic, and interest in these two antibody classes is also increasing. As for today, IgG1 comprise most of the mAb biotherapeutic drugs in the market. Twenty-nine new mAbs are presently undergoing late-stage clinical trials, including human and humanized IgG1, IgG2, and IgG4 molecules [7]. Few IgG2 and IgG4 drugs are already available such as OKT3 (Muronomab-CD3), a murine IgG2a drug from Johnson & Johnson (1986), Bexxar (Tositumomab-I-131), and a murine IgG2a drug radiolabeled with I-131 from Corixa/GSK (2003). IgG4 antibodies are evolving as an important class of cancer immunotherapies [8].

Size exclusion chromatography (SEC) is a powerful analytical tool for the separation of monoclonal antibodies and other proteins [9]. SEC, as a strategy for the isolation and purification of antibodies, is not new; in 1989, high-resolution Superose 6 HR 10/30 fast protein liquid chromatography (FPLC) columns were used. [10]. Since then, many researchers used SEC for the purification of antibodies. The literature search for the number of "publications on the purification of monoclonal antibodies by size exclusion chromatography" [11] shows that between 1983 and 2003, there was a surge of research in this regard (**Figure 1**).

For the large-scale purification of monoclonal antibody biotherapeutics, Protein A is commonly used as the primary capture step. Following the use of Protein A chromatography, SEC is used to characterize the Protein A purified fractions. Size exclusion chromatography (SEC) is primarily used for the separation in analytical HPLC and for routine quality control
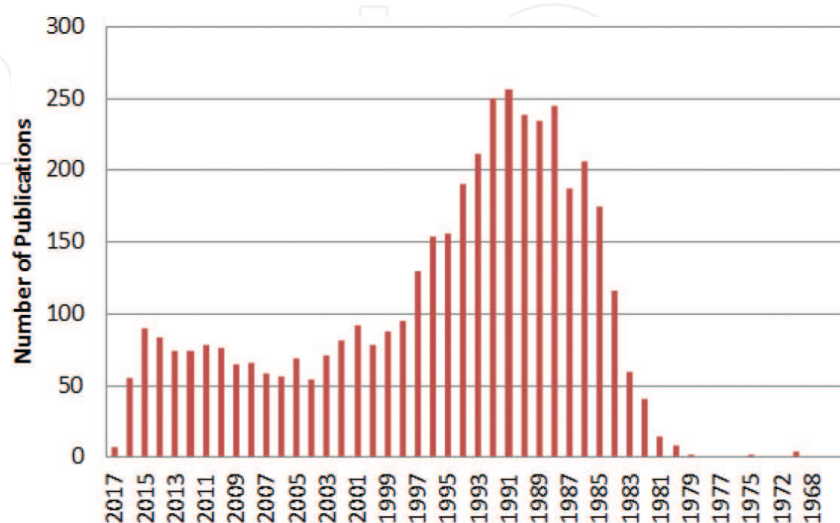
Figure 1. Number of publications dealing with size exclusion chromatography over the years.

analysis of the mAb. Detection wavelength of 280 nm is commonly used. SEC combined with multi-angle light scattering is one method for the characterization of the molar mass distribution of mAb, ADC, and other biomolecules [12].

In this chapter, certain aspects of size exclusion chromatography and its use in the analytical purification or separation of monoclonal antibodies are discussed. Secondary interactions, effects of particle size and pore size on the SEC separation, particularly in resolving monomer peak from dimer and fragment peaks with a better resolution are also discussed. Selection of the right SEC column is critical to achieve the goal of separation. The calibration curve has a very important role in this regard. Since there are different kinds of proteins differing in structure and shape, many vendors provide calibration curves using globular protein standards, branched standards, and linear standards, so that the separation range can ideally be interpreted under the chromatographic conditions.

Reproducible separation of a monomer from its dimer and other high molecular weight (HMW) impurities, fragments, and other low molecular weight (LMW) impurities is needed during the purification of the mAb biotherapeutic. Optimized particle chemistry and packing of SEC column help in this regard. mAb analysis using a mobile phase containing an appropriate amount of selected organic solvents such as isopropyl alcohol (IPA) may be needed to prevent alteration of peak retention time, poor peak shape, or resolution. Few examples are shown to elaborate this. Digestion with papain or pepsin is commonly applied to obtain antibody fragments without the loss of activity—this is discussed in the context of selecting right SEC columns from a variety of particle sizes, pore sizes, and dimensions. Forced degradation studies are needed to assess the stability of the protein, to understand the mechanism of degradation by oxidation, heat, light, or hydrolysis. Forced degradation study by SEC is separately discussed in Section 2.7. Interest in the accurate molecular weight analysis of intact monoclonal antibody IgG1 by SEC using MS-friendly mobile phases is increasing, and it is discussed in Section 4. Section 5 focuses mostly on the use of chromatographic methods which are orthogonal or complimentary size exclusion and useful to detect the protein heterogeneity. The use of ultra HPLC is needed for fast separation, and many methods already developed in HPLC need to be easily transferred to UHPLC. Section 6 briefly discusses the usefulness of a SEC column compatible to both HPLC and UHPLC instruments by easy method transfer. Section 7 is about desalting of mAb or any biopolymer solution, not by dialysis membrane or spin column but by using an analytical SEC HPLC column. The use of SEC column in hydrophilic liquid chromatography (HILIC) mode is interesting and can separate the nucleobases (Section 8). This chapter ends with few remarks about the tips and tricks for size exclusion chromatography.

2. Size exclusion chromatography and purification of monoclonal antibodies

2.1. Size exclusion chromatography

Size exclusion chromatography uses a molecular sieving retention mechanism [13], based on differences in the hydrodynamic radii or differences in size of analytes such as proteins. Large

sample molecules cannot penetrate or only partially penetrate the pores of the stationary phase. So, the larger molecules elute first and smaller molecules elute later, the order of elution being a function of the size.

SEC is the only mode of chromatography where theoretically there is no interaction of the analyte with a stationary phase. The whole process of partitioning or separating the different molecular species is due to the entropy factor and not due to adsorption, ΔH being equal to zero. Pure Silica particles are most commonly used as base material in this type of chromatography of biomolecules. Since the separation of the biomolecule by SEC will depend on its hydrodynamic radii, two proteins of the same molecular weight (such as 70 kDa) may elute at two different retention times, if there is a difference in their hydrodynamic radii (**Figure 2**). So, any factor at any stage of purification, affecting the shape of the protein, will affect the elution volume or retention time.

Unless otherwise mentioned, SEC analyses discussed in this chapter were carried out using the mobile phase 100 mM KH_2PO_4/Na_2HPO_4, pH 6.7, 100 mM Na_2SO_4, 0.05% NaN_3. Agilent 1100, Agilent 1200 HPLC and Thermo Ultimate 3000 UHPLC systems and associated software were used for integration and peak analysis. Sodium azide (NaN_3) was used as an antibacterial agent to prevent fouling of the phosphate buffer. Detection wavelength was 280 nm unless otherwise mentioned. Flow rates and injection volume of the sample are varied as needed. Reproducibility for calculation of % relative standard deviation (RSD) was based on 10 consecutive injections. Linearity of both monomer and dimer during loading study was calculated based on peak areas versus total material loaded during injection. Please refer to individual chromatograms for the respective chromatographic conditions.

2.2. SEC and secondary interaction

As mentioned earlier, among all modes of chromatography, it is only during the size exclusion chromatography where the analyst does not demonstrate any kind of interaction with the stationary phase. During all other chromatographic modes, an analyst demonstrates some kind of interaction between the protein and the stationary phase, followed by the elution using
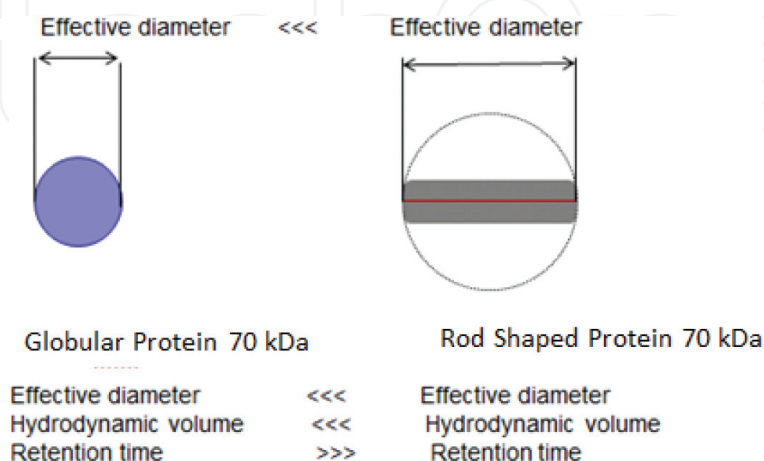

Figure 2. Comparison of globular and rod-shaped proteins.

a stronger solvent. This is because SEC is purely a separation technique based on the permeability of the protein through the pores. Any factor which will prevent the free passage through the pores will result into a nonideal SEC condition. These interactions between proteins and the stationary phases are called secondary interactions. The secondary interactions are based on charge-related interactions or hydrophobicity-related interactions. The secondary interaction coming from the free silanol groups present on the silica surface of the stationary phase is often protected by a diol-bonded coating on the stationary phase which shields the silica surface from such action [14].

In a real-world situation, any secondary interaction between the stationary phase and the proteins including monoclonal antibodies needs to be taken care of by selecting the right column where the stationary phase is effectively coated, and free silanol groups are inaccessible to proteins. Further method development by optimizing the chromatographic conditions may be needed to get the best separation. Ionic and hydrophobic interactions between the sample and the column packing material can be avoided by controlling the ionic strength. A general rule of thumb is that low ionic strength (<0.1 M) may induce charge-related secondary interactions and high ionic strength (>1.0 M) may lead to hydrophobicity-related secondary interactions, while the concept of low or high concentration may vary from mAb to mAb depending on the nature of the individual one. For each protein sample, there will be an optimal buffer type and salt concentration for the best separation that results in the highest resolution and recovery. This can be found out by trial and error approach only. If a sticky protein comes into contact with the stationary phase (dotted line in the figure below), it may undergo a conformational change; the binding constant of the conformationally changed protein can be so strong that it would not elute out of the column (**Figure 3**). The use of additives may be needed. Arginine prevents binding to the surface [15]. Similarly, a number of other additives may also be used to minimize the secondary interactions. The use of isopropyl alcohol (IPA) as another additive to avoid nonspecific interaction and the reproducibility of the analysis is somewhat discussed in the Section 2.5.

There is no universal protocol for working with additives to avoid nonspecific interactions for all proteins. Chromatographic conditions preventing secondary interactions need to be found out by trial-and-error type of experiments with a variety of additives.
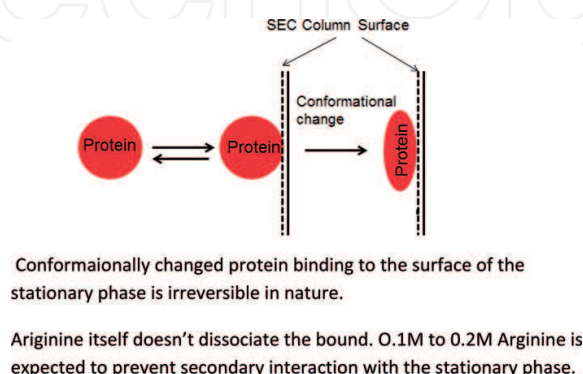

Figure 3. Binding of a protein to the stationary phase and the influence of arginine.

2.3. Effect of particle size and pore size of the stationary phase on SEC separation

Particle size and pore size are important factors for better peak shape, peak sensitivity, and resolution. SEC columns with smaller particle size yield better separation and resolution but with higher back pressure as expected from the basic chromatography theory.

Pore characteristics need to be optimized for SEC separation of biomolecules. To have the high resolution of a mAb monomer from dimer and higher order aggregates as well as from fragments, permeability through the pores of the SEC stationary phases is needed. This is similar to accessibility of the pore needed in reverse phase chromatography (RPC) for better mass transfer kinetics. The larger pore size helps in more efficient permeation of the biomolecules inside the pores, resulting in better size-based separation (**Figure 4**); 8–14 nm pores are not suitable for the separation of large intact biomolecules and its aggregates since they are too large to enter into the pores. SEC columns with 25 nm pore size are widely available and popular for SEC separation of biomolecules. In the last few years, even larger pore size, such as 30 nm SEC columns, became available in the market, which enables increased permeability of the higher order aggregates of large biomolecules.

Increase in the pore volume of the packing material results into a shallower slope in the calibration curve and increases the spread in elution time between compounds with different molecular masses and improves resolution. But there is a limit to what extent the pore of a particle of definite size can be stretched to its maximum. This is because as the pore volume of a packing material increases, the strength of the packing material generally decreases, making the material more fragile. **Figure 5** [16] illustrates the relation between the particle and pore characteristics to the resolution and other peak parameters.

It is important to compromise between the particle sizes and pore volume in order to get the desired separation. Pore characteristics of the SEC column need to be optimized to have a high resolution of a mAb monomer from a dimer and higher order aggregates, as well as from fragments. For large biomolecules, such as therapeutic proteins and monoclonal antibodies (mAbs), a larger exclusion limit will yield better separation particularly of the dimer and higher order aggregates from the monomer.
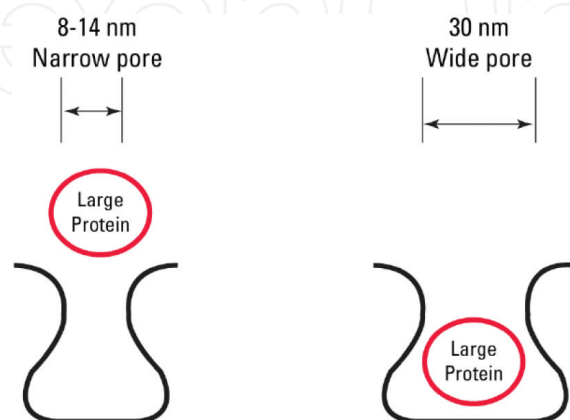

Figure 4. Permeation of large molecules into pores depends on the pore size.
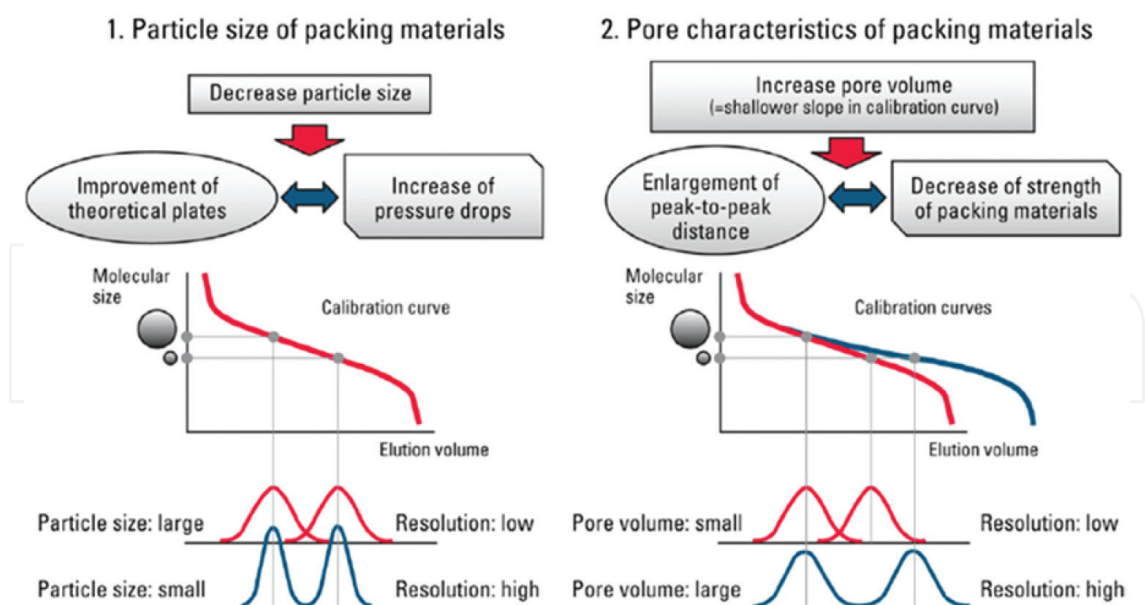
Figure 5. Relation between the particle and pore characteristics to the resolution and other peak parameters.

2.4. SEC column selection and calibration curve

In the absence of any secondary or non-SEC retention mechanism, the calibration yields an S-shaped curve containing a linear portion in between the total exclusion and total inclusion limits. In **Figure 6** [17], the calibration curve was generated using three different types of standards (globular, branched, and linear).

The red line in **Figure 6** represents the average molecular weight of the monoclonal antibody (mAb) IgG (150 kDa). If we follow the calibration curve generated by globular proteins (•), mAb is eluting very close to the total exclusion limit of TSKgel G2000SW, while it is eluting very close to the total inclusion limit of TSKgel G4000SW, resulting into poor separation of the monomer from its impurities in both the cases. But in the middle panel, as seen in the case of TSKgel G3000W, the monomer is eluting around in the middle of the linear range of the calibration curve, so the monomer peak can clearly be separated from its dimer and higher order aggregates and fragment impurities way better than with the other two columns. It is the pore volume between total exclusion and total inclusion volume which is important. The greater the pore volume per unit column volume, the better the separation. In other words, the shallower the calibration curve, the better the separation. All SEC columns with the same dimension, particle size, and pore size from different vendors otherwise may look identical, but the pore volume per unit column volume may not be the same. An analyst can gain advantage by selecting a column with larger pore volume per unit column volume.

Different SEC columns obtained from different vendors, having the same dimensions and particle size as labeled on the individual columns, may apparently look alike. The difference is in the pore volume per unit column volume due to the differences in particle size and pore size distributions, packing quality, and so on. In general, the better the pore volume per unit
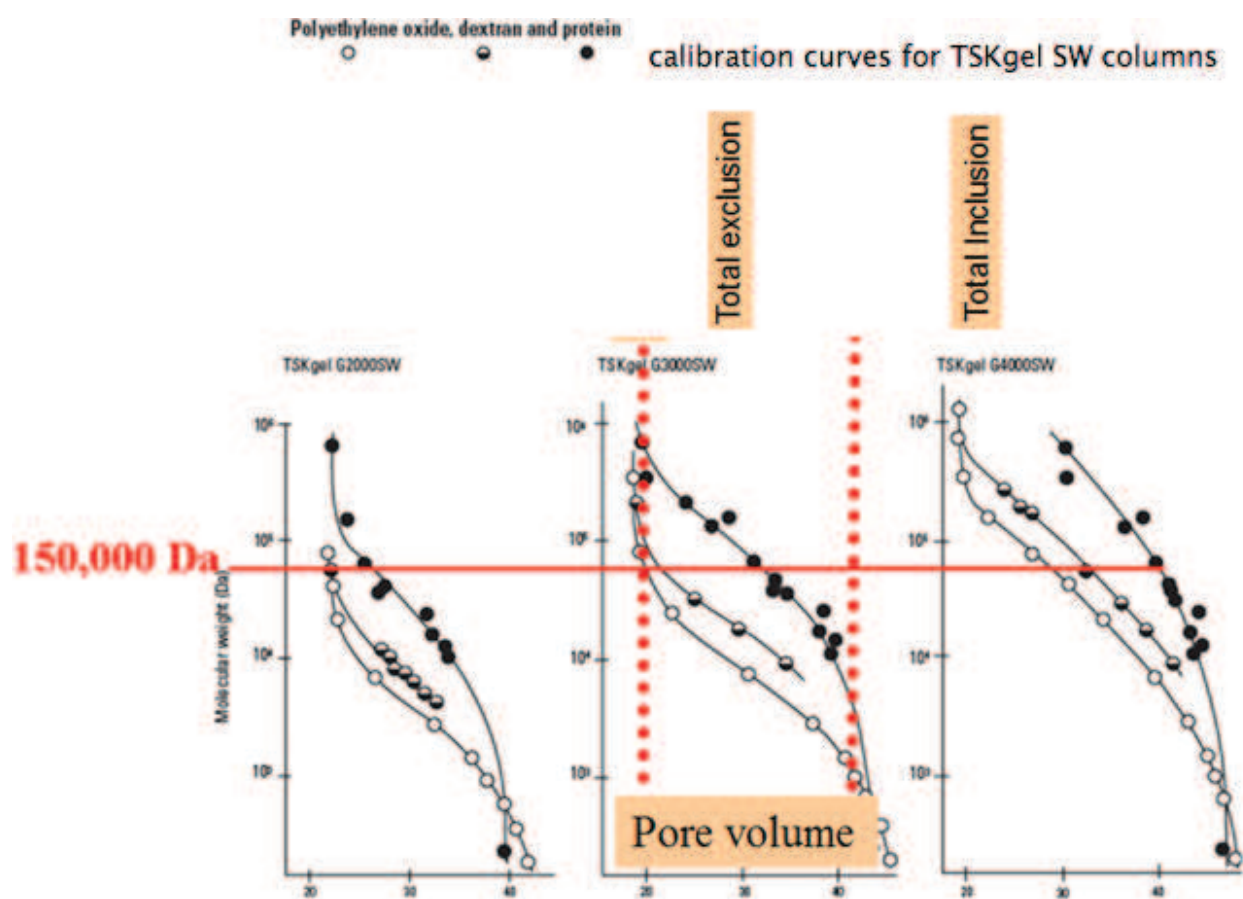
Figure 6. Elution profile of mAb IgG obtained with TSKgel G2000SW, TSKgelG3000W, or TSKgel G4000SW columns.

column volume, the better the separation and resolution of proteins from a SEC column. This is a very critical criterion when selecting a SEC column for better separation.

2.5. Separation of HMW and LMW species by SEC and reproducibility

A chromatographer uses SEC primarily to separate monoclonal antibodies from its impurities or heterogeneities. The monomer IgG1 peak (150 kDa) needs to be purified from its dimer, trimer, or higher order aggregates popularly known as high molecular weight (HMW) species and the fragments which are known as the low molecular weight (LMW) species. SEC is widely accepted as a work horse for routine quality control with its purpose to monitor these HMW and LMW impurities from a mAb monomer. Protein aggregation of biotherapeutics is a common issue. Even a very small amount of aggregates may cause an immunogenic response in the human body and needs to be removed from the monoclonal antibody monomer. More than 99% purity is needed for medicinal purpose [17]. The formulation containing the pure monomer monoclonal antibody needs to be monitored for its stability. The purified protein in the formulation may also undergo aggregation over time. Protein aggregation can happen at any stage during expression and purification. Temperature, pH, ionic strength, concentration and many other factors can give rise to protein instability, leading to aggregation. Effect of ions

on protein agitation and temperature-induced aggregation of mAbs has been reported in the literature [18]. Specific racemization of Heavy-Chain Cysteine-220 in the hinge region is identified as a possible cause of degradation during storage. Increased hydrophobicity can increase the likelihood of aggregate formation during manufacturing and storage [19]. Freeze-thaw cycles too can cause an aggregation of an already purified protein [20]. Formation of aggregates may be induced by light as well [21]. That is the reason why all registration applications for new molecular entities and associated drug products require photo-stability data [22]. Increased resolution between the monomer peak and other HMW and LMW impurities is the key for the purification of monoclonal antibodies. SEC can monitor the stability during storage. The removal of HMW and LMW impurities is equally important for application of newly emerging biotherapeutics such as ADCs, bi-specific antibodies, biosimilars, and bio betters as well. As an example from the recent literature report, aggregate and fragment levels were determined by SEC-HPLC for the characterization of bi-specific antibodies [23]. A single chain variable fragment (scFv), composed of the variable regions of the heavy chain (V_H) and the light chain (V_L), is gaining interest too as it retains the specificity of the original IgG. The scFv format is often used, but one problem that cannot be easily solved by purification is the fact that hybridomas can secrete different monoclonal antibodies [24]. Literature reports a few interesting articles about the concept of mAbs being a perfectly defined entity to researchers. The following is an excerpt from the commentary as shown here [25]. "most researchers consider monoclonal antibodies to be perfectly defined reagents with single specificities" but "Hybridomas frequently secrete more than one light and/or heavy chain." So "the problem is probably best summarized thus: antibodies sold as different are often identical, while antibodies sold as identical are often different (thanks to Natalie de Souza (editor Nature Methods) for this pithy insightful observation), and the customer does not know which is which." In another interesting article, the authors have discussed that "two kappa immunoglobulin light chains are secreted by an anti-DNA hybridoma" and its implications for isotypic exclusion are discussed [26]. Biotherapeutic scientist needs to be aware of these facts while analyzing mAb. The separation of scFvs from dimers and aggregates is also important; most of the affinity purified scFv fractions are monitored by SEC.

Different mAbs may have different amount of impurities under native conditions. Representative chromatograms of the separation of four different monoclonal antibodies (IgG1) at 0.75 mL/min using a 4 μm; 4.6 mm ID × 15 cm TSKgel SuperSW mAb HTP column are shown in **Figure 7** [27]. This analysis clearly showed that under native conditions different monoclonal IgG1 antibodies had different extent of HMW and LMW species based on the individual % peak area analysis (data not shown here). The monomer peak eluted as fast as in 2 min.

A chromatographer needs to choose a suitable column based on the separation criteria the chromatographer is looking for. Comparison of the analysis of mAb aggregates using 15 and 30 cm long TSKgel UP-SW3000, 2 μm columns using the same mobile phase and flow rate is shown below [28].

Fast separation of the HMW and LMW species is important. The effect of the column length should be taken into account when selecting a column in this regard. The results indicate that the TSKgel UP-SW3000 column with a shorter length yielded a similar profile to the 30-cm
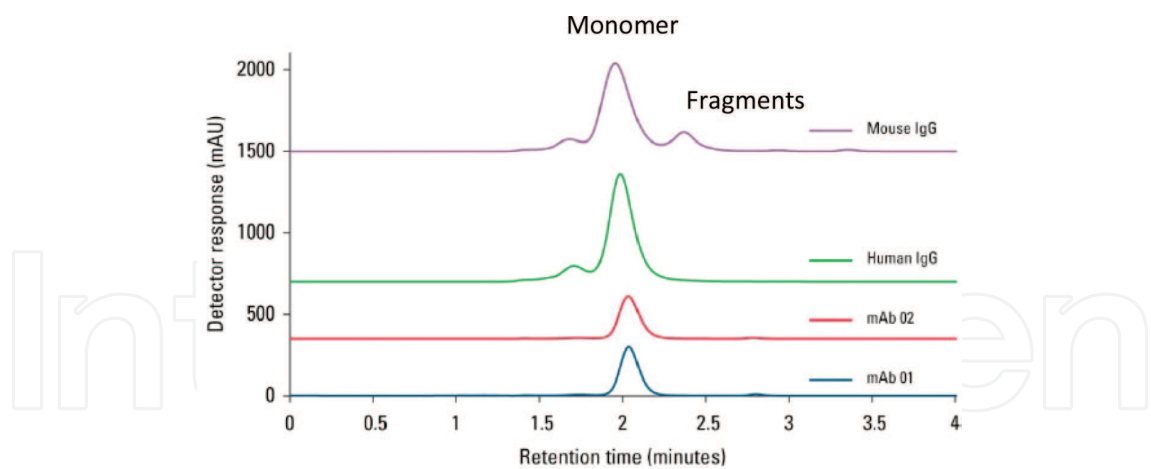
Figure 7. Separation of four different mAbs on a 4 µm; 4.6 mm ID × 15 cm TSKgel SuperSW mAb HTP column.

column with 50% less run time and 50% lower backpressure at a typical flow rate of 0.35 mL/min (**Figure 8**). The resolution between dimer and monomer is still maintained within the acceptable range. Thus, a shorter column could be successfully used for the separation of the dimer and monomer, reducing the overall runtime by half but the resolution of the fragment on the LMW side of the monomer slightly decreased. The longer column yielded a better resolution. As long as the resolution is 1.5 and above yielding a baseline resolution of the two species, the method may remain acceptable. The selection of the correct length of the column should be based on the goal of the separation. The 15-cm column operated at the typical flow rate of 0.35 mL/min yielded a backpressure of 11 Mpa, which was well within its maximum operable pressure and thus could be used in both HPLC and UHPLC systems.
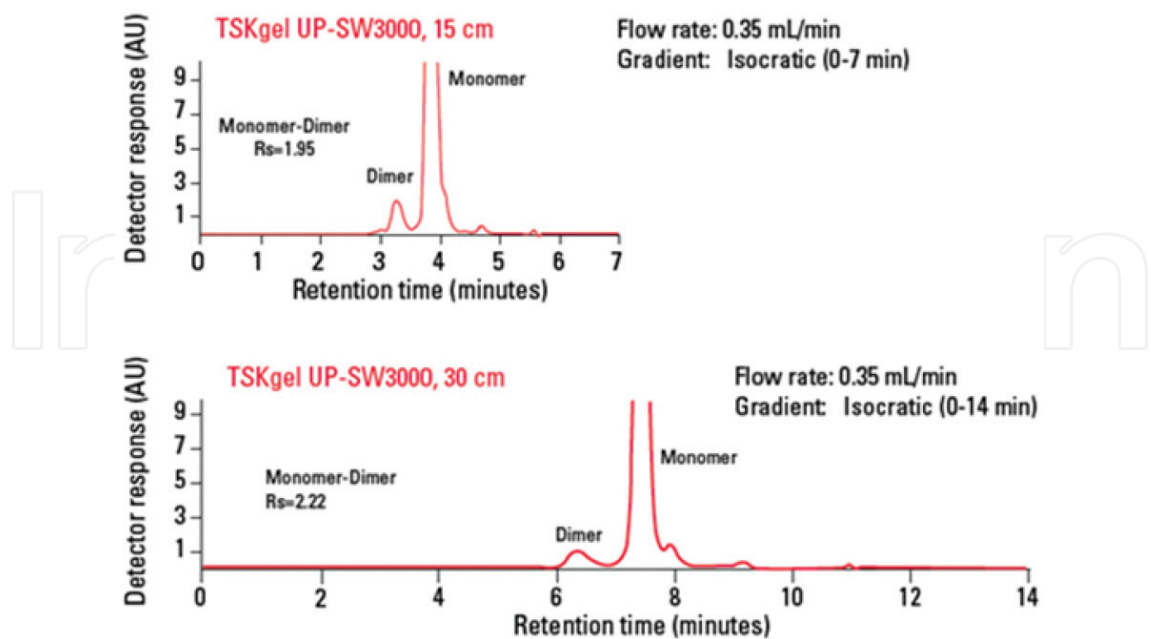

Figure 8. Effect of protein separation on the column length of SEC columns.

Factors considered when selecting an HPLC column supplier			
Factor	**Respondents (Normalized %)**		
	2007	**2009**	**2011**
Column-to-column reproducibility	21 ⎤ 37%	21 ⎤ 39%	19 ⎤ 34%
Column lifetime	16 ⎦	17 ⎦	15 ⎦
Price	12	14	13
Reputation of company	14	12	9.6
Column plate number	8.7	9.1	9.1
Technical assistance	5.7	5.8	6.7
Variety of phases available	4.5	4.0	6.4
Tailing factor	6.3	5.4	5.0

Ref: LCGC: Jan 1, 2012; Article: Current trends in HPLC column usage – By: Ron Majors

Table 1. Factors that play a role in selecting a suitable HPLC column.

Reproducibility is important in SEC analysis of mAbs. The survey clearly showed the importance of column lifetime and reproducibility for column selection [29, 30]. The survey also shows how the same topic developed over a number of years. Factors which should be considered to select an HPLC column supplier are shown in **Table 1**.

Two different sources of silica can be a factor in lot-to-lot reproducibility. Vendors always maintain a strict quality control passing criteria if the silica source is different.
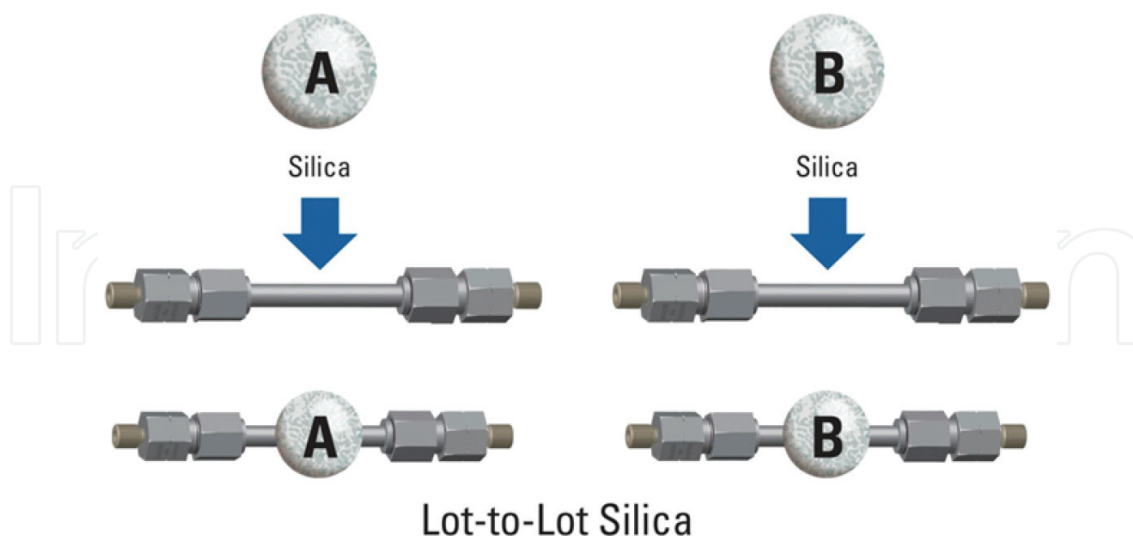
Bonding chemistry developed on the same silica at different times can also be a factor in lot-to-lot reproducibility.

Lot-to-Lot Bonding Chemistry
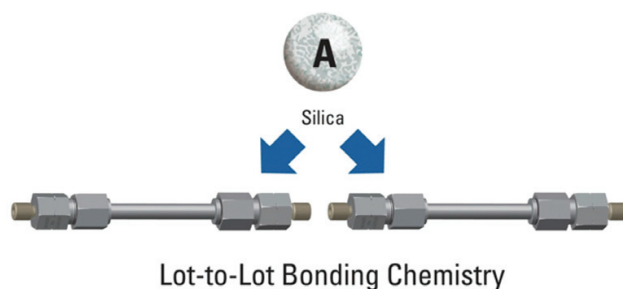
A robust method with a good SEC analytical column is critical for the analyst. A protein standard mixture can be used to confirm the lot-to-lot reproducibility of an SEC column. The protein standards are chosen to cover the whole range of calibration curve from total exclusion limit to total inclusion limit. A representative chromatogram of the analysis of a protein standard mixture using a TSKgel G3000SWXL, 5 µm, 7.8 mm ID ×30 cm column is shown in **Figure 9**. Thyroglobulin (700 kDa) is close to the total exclusion limit and para-amino-benzoic acid (PABA–137 Da) is near the total inclusion limit. Chromatographers use also vitamin B12 (1.4 kDa) in place of PABA. Vendors generally pass the packed SEC columns using a protein standard mixture and establish a QC pass criteria. For example, the specification for TSKgel G3000SWXL column passing QC is N (PABA) > 20,000 and As (PABA) = (0.7–1.6) where N represents the number of theoretical plates and As represents the peak asymmetry.
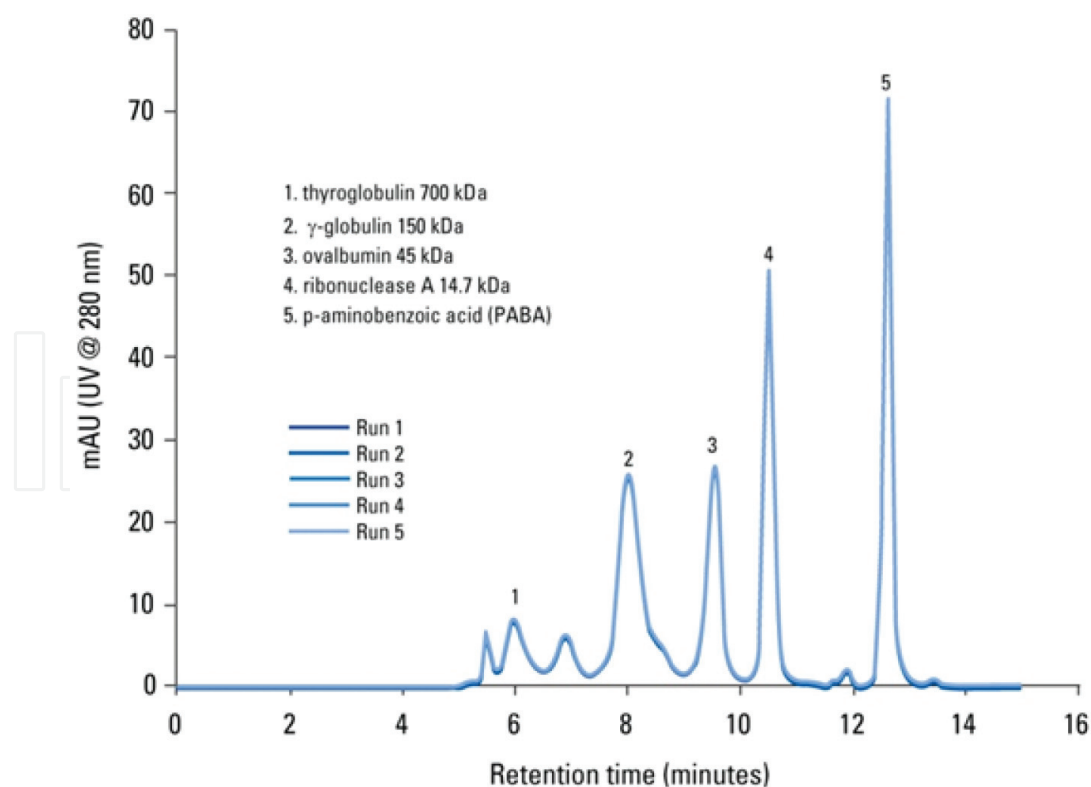

Figure 9. Retention time of five different proteins analyzed with a TSKgel G3000SWXL column was high reproducible. An overlay of five injections is shown.

Figure 9 [31] shows the reproducibility of five consecutive injections with low %RSD of all the peak parameters (data not shown here).

Reproducibility of the analysis of a protein standard mixture with low %RSD of the peak parameters such as retention time, peak area, peak asymmetry and number of theoretical plates and passing the vendor-defined QC criteria can be used for monitoring the column health. Routine users purifying a particular mAb with a particualr SEC column may use the same mAb as their internal standard to monitor the column quality and lifetime over a number of injections. Nowadays, mAb standards from USP and NIST are available for similar purpose.

Reproducibility of 15 consecutive injections during the analysis of a USP mAb using a 15-cm TSKgel UP-SW3000 column at 0.5 mL/min flow rate and phosphate buffer at pH 6.7 is shown in **Figure 10**.

The mAb monomer peak eluted at 2.717 min with good resolution between monomer and the dimer peaks as well as the fragments. Similar reproducibility is noticed in case of pH 6.2 (data not shown here).

Reproducibility in the analysis of the USP mAb in pH 6.2 conditions with 250 mM KCl at 0.3 mL/min using a 30 cm column is shown below—the overlay of the 15 consecutive injections demonstrated consistency (**Figure 11**) (all USP Reference Standards are provided as delivered and specified by the US Pharmacopeia). The monomer peak elution time was 8.367 min. Similar reproducibility was obtained using a 15-cm column at pH 6.7 (data not shown here).

Nonspecific absorption of antibodies onto the column gel matrix poses a challenge, and some newly engineered antibodies possess a high degree of hydrophobicity. The use of organic
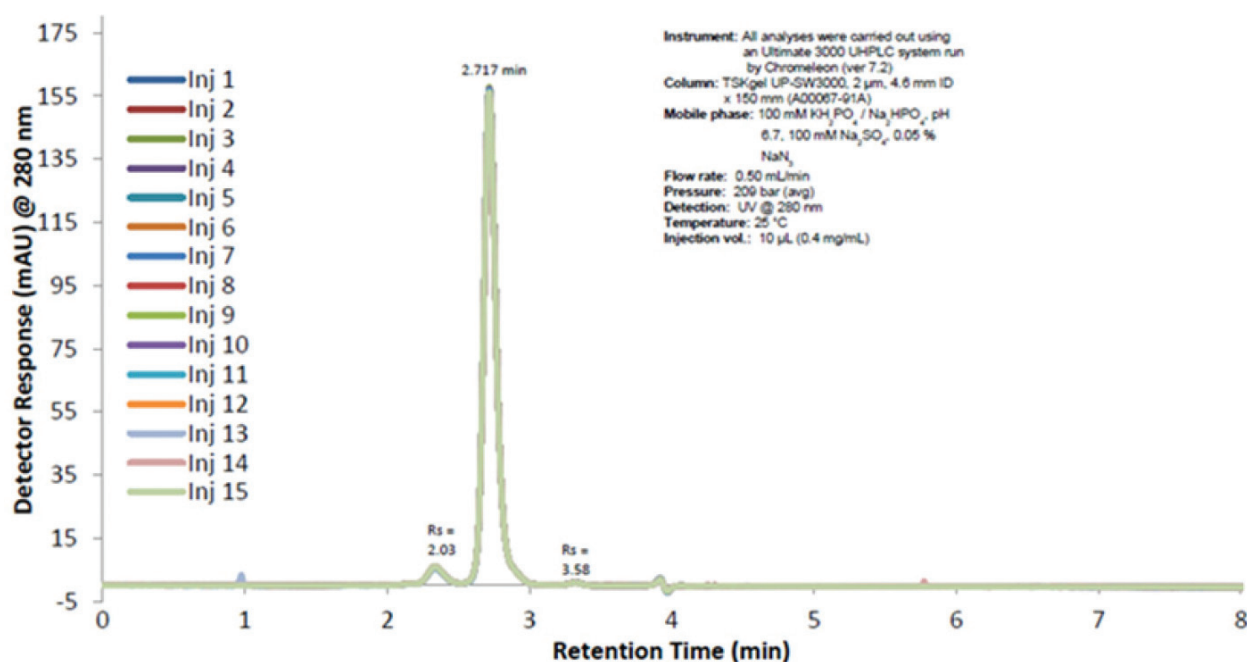

Figure 10. Reproducibility of 15 consecutive analytical injections of a USP mAb using a 15-cm TSKgel UP-SW3000 column at phosphate buffer pH 6.7. The overlay of 15 injections is shown.
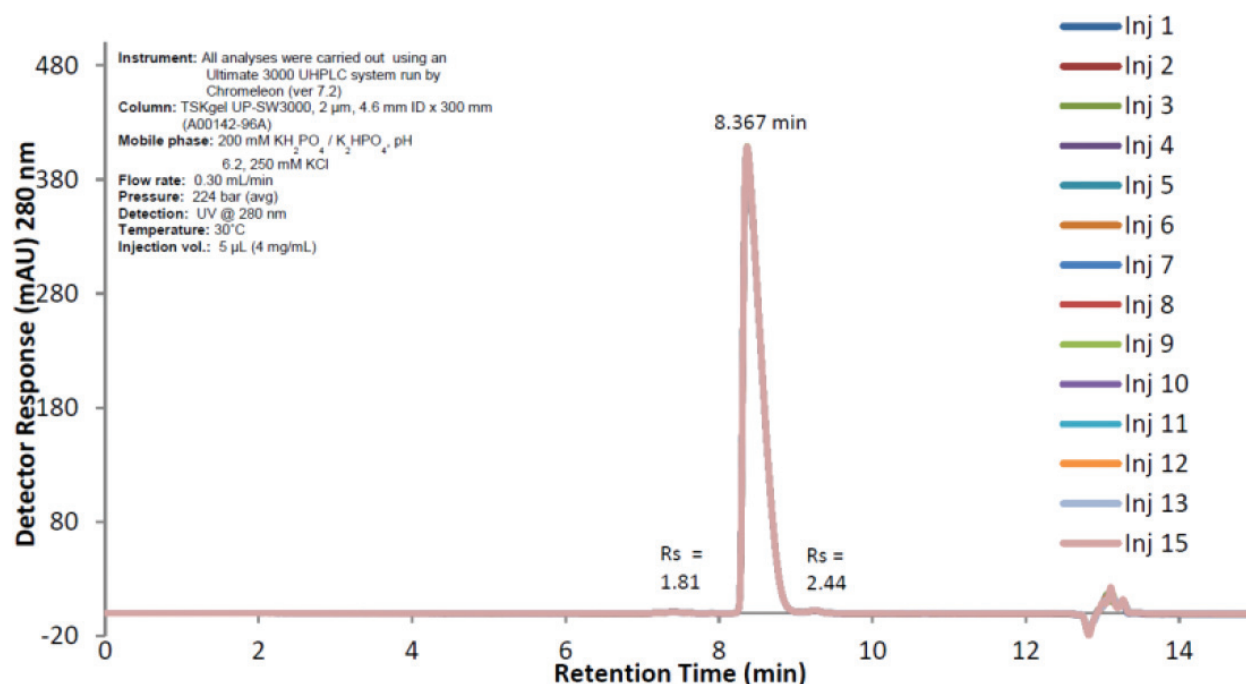
Figure 11. Reproducibility of 15 consecutive analytical injections of a USP mAb using a 30-cm TSKgel UP-SW3000 column at pH 6.2. The overlay of 15 injections is shown.

solvents such as isopropyl alcohol (IPA) or salts can decrease this interaction as reported by many scientists. However, the additives may alter the diffusion of these molecules, which results in retention time shift and poor peak resolution that did not occur in a typical aqueous buffer system, such as sodium phosphate buffer at neutral pH. The **Figure 12** and **Table 2** show that a TSKgel UP-SW3000, 2 μm SEC column was used for analyzing monoclonal antibodies (mAbs) with the addition of 15% IPA in sodium phosphate buffer, pH 6.7. As demonstrated, peak resolution and retention time shift were not impacted.

Similarly, **Figure 13** shows the overlay of the 15 consecutive injections of USP mAb at pH 6.7 with 15% IPA using a 30-cm column (**Figure 13** and **Table 3**). Improvement in the baseline was noticed after the first two injections. Overall, the analysis yielded excellent reproducibility. The monomer peak elution time was 8.338 min. As expected, there was no considerable difference here compared to the retention time obtained earlier at pH 6.2. The presence of IPA as additive obviously will yield a higher back pressure, and so long as the column is operated within its maximum operable pressure, this should not be an issue. The retention times of monomer, dimer, aggregates, and fragment peaks are nearly unchanged. Peak width and peak shape are very consistent from injection to injection. The baseline of the first injection (as shown in blue) indicate that the column takes only 1–2 injections to be stabilized. After that, all subsequent injections are overlaid perfectly.

The overlay indicates the similarities of peak retention times, peak width and peak height of dimer, monomer, aggregates and fragment peaks between the two different conditions. An appropriate percentage of organic solvent such as isopropyl alcohol (IPA) did not alter the diffusion of mAb molecules using a TSKgel UP-SW3000 column. As demonstrated, this column can be successfully operated with the addition of 15% IPA. Data indicate that the

Column: TSKgel UP-SW3000, 2 µm, 4.6 mm ID x 30 cm
Instrument: UltiMate® 3000 UHPLC system run by Chromeleon® (ver 7.2)
Mobile phase: 15% IPA in 100 mmol/L KH_2PO_4 / Na_2HPO_4, pH 6.7,
                  100 mmol/L Na_2SO_4, 0.05% NaN_3
Flow rate: 0.30 mL/min
Detection: UV @ 280 nm
Temperature: 30 ˚C
Pressure: 22 MPa (maximum column pressure is 34 MPa)
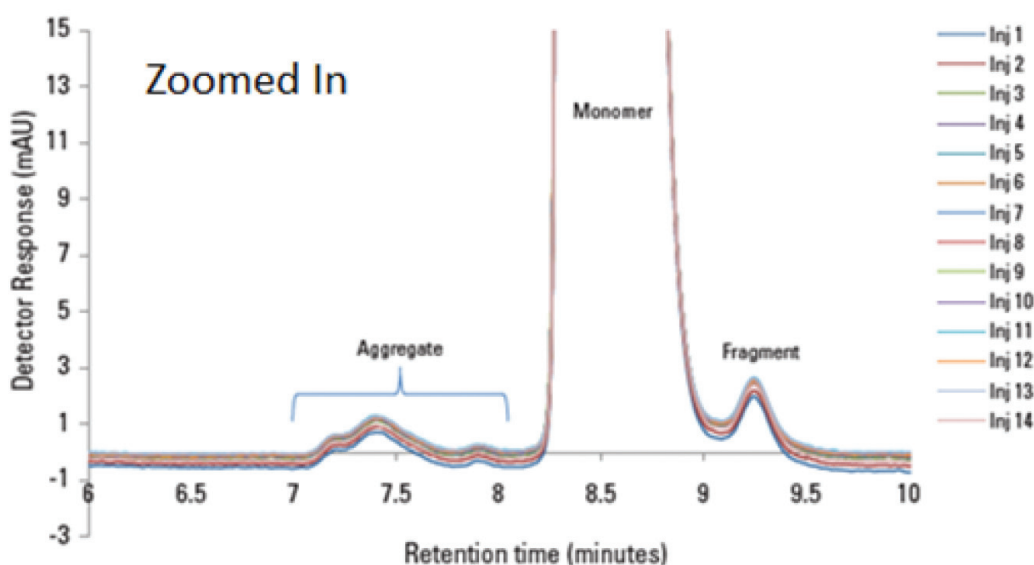Injection vol.:   5 µL, 4 mg/mL
Sample: USP mAb standard

Figure 12. Reproducibility of 14 consecutive analytical injections of a USP mAb using a 30-cm TSKgel UP-SW3000, 2 µm SEC column at pH 6.7 with isopropyl alcohol. The overlay of 14 injections is shown.

column's particle chemistry and packing are optimized so that with the addition of an appropriate amount of selected organic solvents, there is no alteration of peak retention time or poor peak resolution [32].

2.5.1. Loading study

Sample load, in both volumetric load and absolute load, may affect SEC separation. Mass overload takes place when sample molecules no longer have free access to diffuse into and out of the pores, thus bypassing part of the column and thereby effectively reducing the length of the column that remains to fractionate the sample. If the height equivalent theoretical plates (HETP) are plotted against the load amount, the HETP values should remain constant as long as the column efficiency is not compromised. The loading capacity is the maximum load beyond which the HETP value starts increasing, as shown in **Figure 14** [33].

To obtain the capacity of an analytical SEC column as often the chromatographers like to do, a loading study plot HETP vs. load amount is shown above (**Figure 14**). The loading capacity of a SEC column with defined dimensions depends on the sample. The loading capacity can be

Injection	Monomer peak		Dimer peak	
	Ret. time (min)	Area (mAU*min)	Ret. time (min)	Area (mAU*min)
1	8.370	99.300	7.403	0.380
2	8.370	99.290	7.400	0.400
3	8.367	99.250	7.407	0.400
4	8.367	99.270	7.433	0.390
5	8.367	99.260	7.423	0.400
6	8.367	99.270	7.413	0.390
7	8.367	99.260	7.403	0.400
8	8.367	99.260	7.403	0.400
9	8.367	99.090	7.420	0.390
10	8.367	99.270	7.427	0.390
11	8.367	99.260	7.400	0.400
12	8.367	99.250	7.407	0.400
13	8.367	99.250	7.397	0.400
14	8.367	99.260	7.403	0.390
Average	8.367	99.253	7.410	0.395
Std Dev	0.001	0.049	0.011	0.007
%RSD	0.013	0.049	0.153	1.647

Table 2. Retention time and peak areas of the monomer and dimer peak (**Figure 12**).

increased by increasing the column length or diameter. Increasing column length also increases resolution and retention time, leading to an additional separation time and amount of mobile phase.

Below is the loading study of γ-globulin (150 kDa) using the TSKgel UP-SW3000 column. It is necessary to know the experimental range of loading where the retention time, peak shape, separation efficiency, etc., remain nearly unchanged over varying load concentrations. Please note that when a loading study is carried out, both volumetric loading and absolute loading amounts should be studied. In **Figure 15**, a volumetric loading study is shown. In any SEC analysis, by theory, the total volume injected should not be more than 3% of the column volume to avoid the effect of band broadening.

Figure 15 shows that even at larger volumetric load containing up to 160 μg proteins, the monomer peak remains well resolved from its dimer. Retention time is remaining constant over the experimental range. Excellent linearity of both monomer peak areas and dimer peak areas versus total load were obtained (data not shown here). So if the primary interest of the analyst is to separate the monomer from the dimer, 160 μg loading in 40 μL volume can be used. Now if the monoclonal antibody concentration can be increased so that 160 μg can be loaded in lower volume (e.g., 10 μL), then the peak shape can further be improved, if needed.
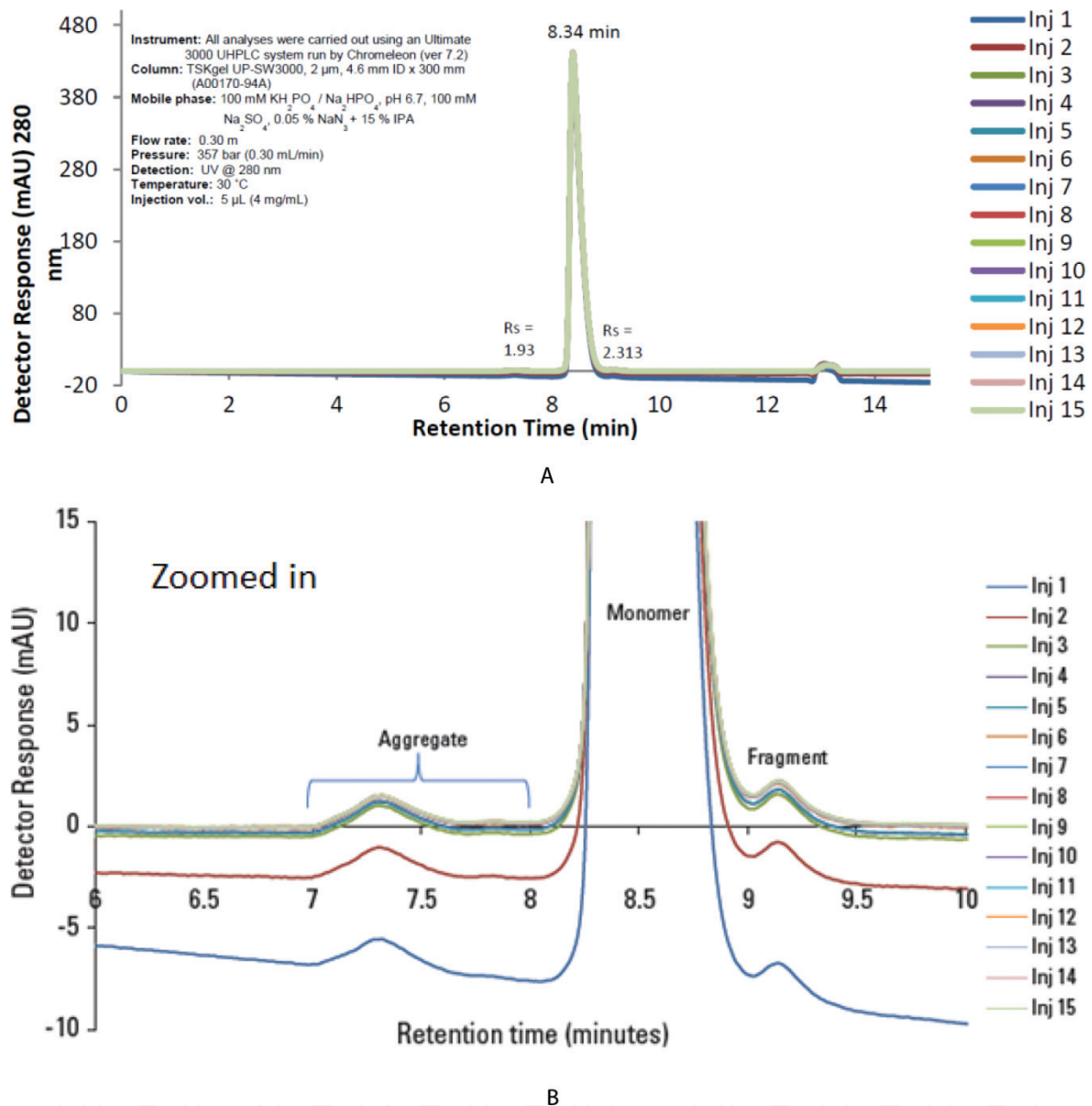
Figure 13. (A) Reproducibility of 15 consecutive analytical injections of a USP mAb using a 30-cm TSKgel UP-SW3000, 2 μm SEC column at pH 6.7 with 15% IPA. The overlay of 15 injections is shown. (B) Chromatogram of 13 a zoomed in.

Peak shape and efficiency were not affected when injecting 400 μg of a monoclonal antibody preparation during a loading study of a monoclonal antibody using a TSKgel G3000SWXL, 5 μm, 7.8 mm ID × 30 cm column (**Figure 16**). A 10-fold increase in total protein content did not affect the retention time, peak symmetry, or separation efficiency of the column [30]. Similar study using a TSKgel G2000SWXL, 5 μm, 7.8 mm ID × 30 cm analytical column even with a high load of Bovine Serum Albumin also yielded a well-resolved peak without any splitting [34].

As mentioned earlier, having an idea about the sample loading capacity will provide analyst the knowledge about the load range within which the desired sensitivity and resolution can be

Injection	Monomer peak		Dimer peak	
	Ret. time (min)	Area (mAU*min)	Ret. time (min)	Area (mAU*min)
1	8.340	97.110	7.417	0.470
2	8.340	98.280	7.410	0.460
3	8.340	98.420	7.410	0.470
4	8.340	98.400	7.407	0.490
5	8.340	98.440	7.417	0.470
6	8.340	97.940	7.413	0.500
7	8.337	98.010	7.420	0.470
8	8.337	98.030	7.437	0.470
9	8.337	98.110	7.407	0.470
10	8.337	98.110	7.423	0.470
11	8.337	98.120	7.413	0.460
12	8.337	98.220	7.417	0.470
13	8.337	98.130	7.420	0.480
14	8.337	98.220	7.413	0.460
15	8.367	98.260	7.413	0.390
Average	8.338	98.120	7.416	0.471
Std Dev	0.002	0.317	0.008	0.012
%RSD	0.018	0.323	0.102	2.598

Table 3. Retention time and peak areas of the monomer and dimer peak (**Figure 13**).
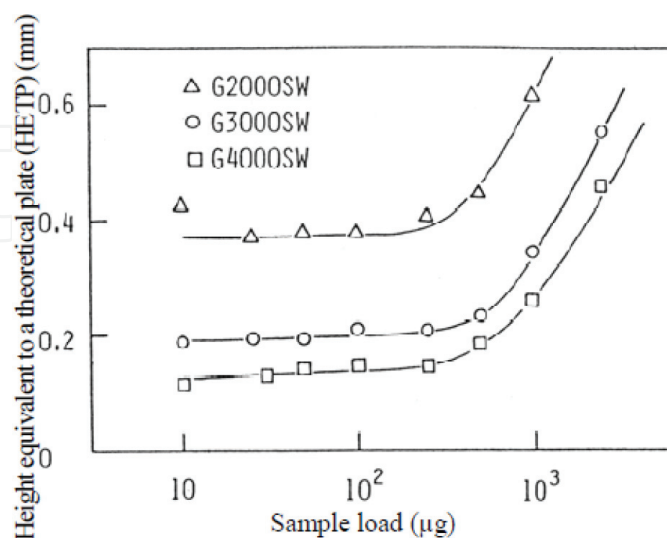

Figure 14. Influence of sample load on height equivalent theoretical plates (HEPT) using three different columns (G2000SW, G3000SW, G4000SW).
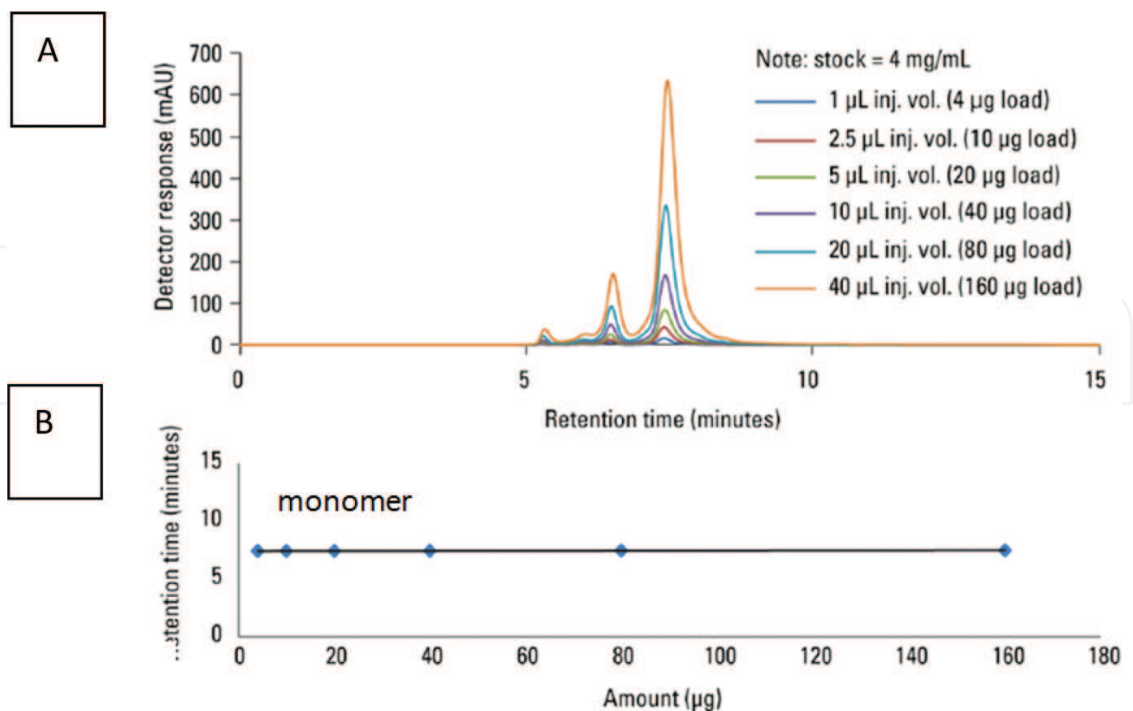
Figure 15. (A) Influence of peak resolution and retention time on the amount of γ-globulin loaded on a TSKgel UP-SW3000 column; (B) monomer retention time in dependence of the amount of sample.
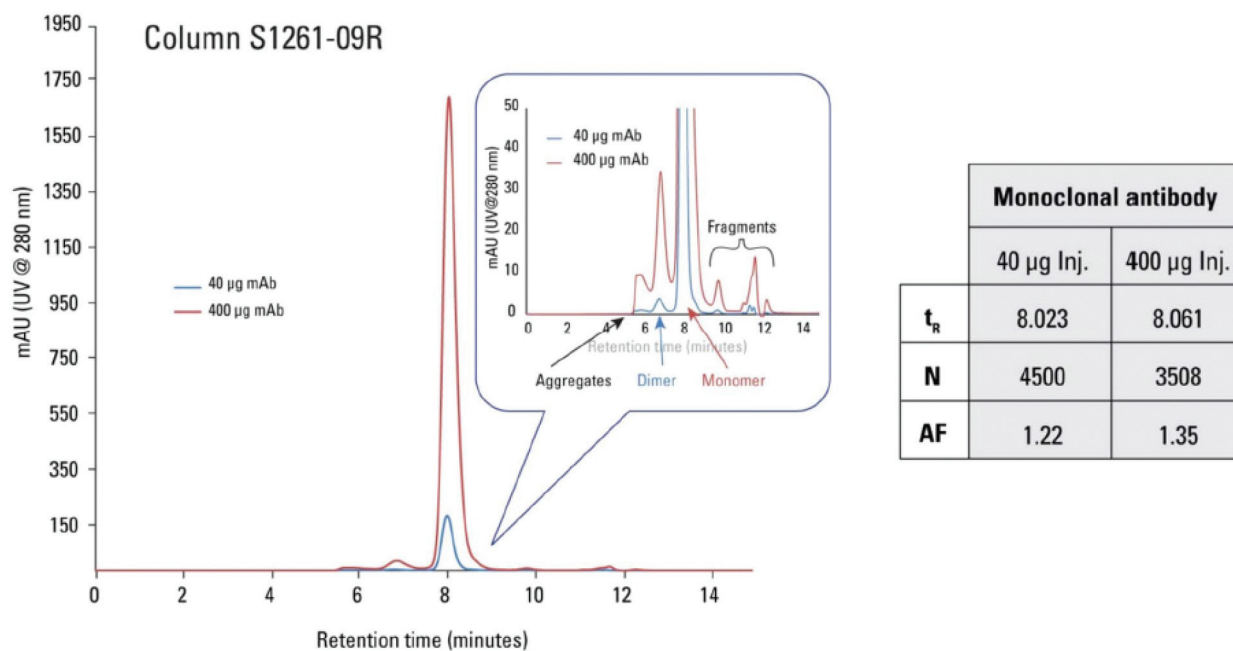
Figure 16. Influence of mAb loading on retention time, peak symmetry, or separation efficiency using a TSKgel G3000SWXL, 5 μm, 7.8 mm ID × 30 cm column.

achieved. The loading study can be extended to a lower range of detection to get the limit of detection (LOD) and limit of quantitation (LOQ) values for the column in the analysis of mAbs (data not shown here). Similarly, aggregation pattern of mAbs as a function of concentration

can be monitored using SEC columns if a mAb is susceptible to aggregation at higher concentration.

2.6. Separation of digestion products of mAbs by size exclusion chromatography

IgG is a relatively large molecule (approx. 150 kDa), and in order to improve the penetration to the tissue, fragmentation is carried out. Digestion with papain or pepsin is commonly applied to obtain antibody fragments without the loss of activity. When papain is used for the antibody digestion, 2 Fab (50 kDa each) and 1 Fc (50 kDa) are obtained from one antibody (**Figure 17**).

When pepsin is used, a F(ab')2 is obtained. SEC can be used to analyze the separation of these fragments. The scope of this analysis by SEC is taken as an opportunity to explain how to select a column with right particle size, pore size, column dimensions, and so on.

In **Figures 18** and **19**, a set of four different SEC columns are compared during the separation of papain digestion products of a mAb to explain how to select the right SEC column for the right purpose [35].

For analyzing monoclonal antibody and other biopolymers 250 Å pore size, 5 μm, 7.8 mm ID × 30 cm SEC columns are widely considered. For example, TSKgel G3000SWxl columns
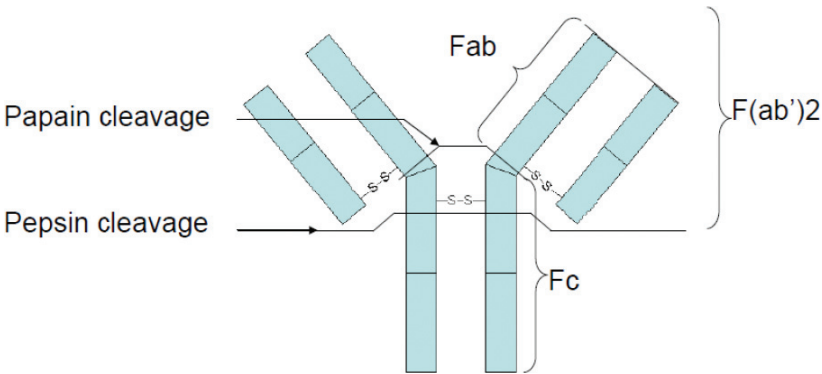
Figure 17. Cleavage of an IgG with papain or pepsin.

Columns	TSKgel SuperSW mAb HR	TSKgel SuperSW mAb HTP	TSKgel UltraSW Aggregate	TSKgel G3000SWxL
Column size	7.8 mm I.D.×30 cm	4.6 mm I.D.×15 cm	7.8 mm I.D.×30 cm	
Base material	Silica			
Ligand	Diol			
Particle size	4 μm		3 μm	5 μm
Pore size	25 nm		30 nm	25 nm
Exclusion limit (globular proteins)	800,000 Da		2,500,000 Da[*]	800,000 Da
Separation range (globular proteins)	10,000−500,000 Da		10,000−1,500,000 Da	10,000−500,000 Da
Applications	High resolution of mAb dimer/monomer/fragments	High speed separation of mAb dimer/monomer	High resolution of mAb aggregates	Separation of proteins

*) Estimated value

Figure 18. Characteristics of the SEC columns used in the analysis of **Figure 19**.

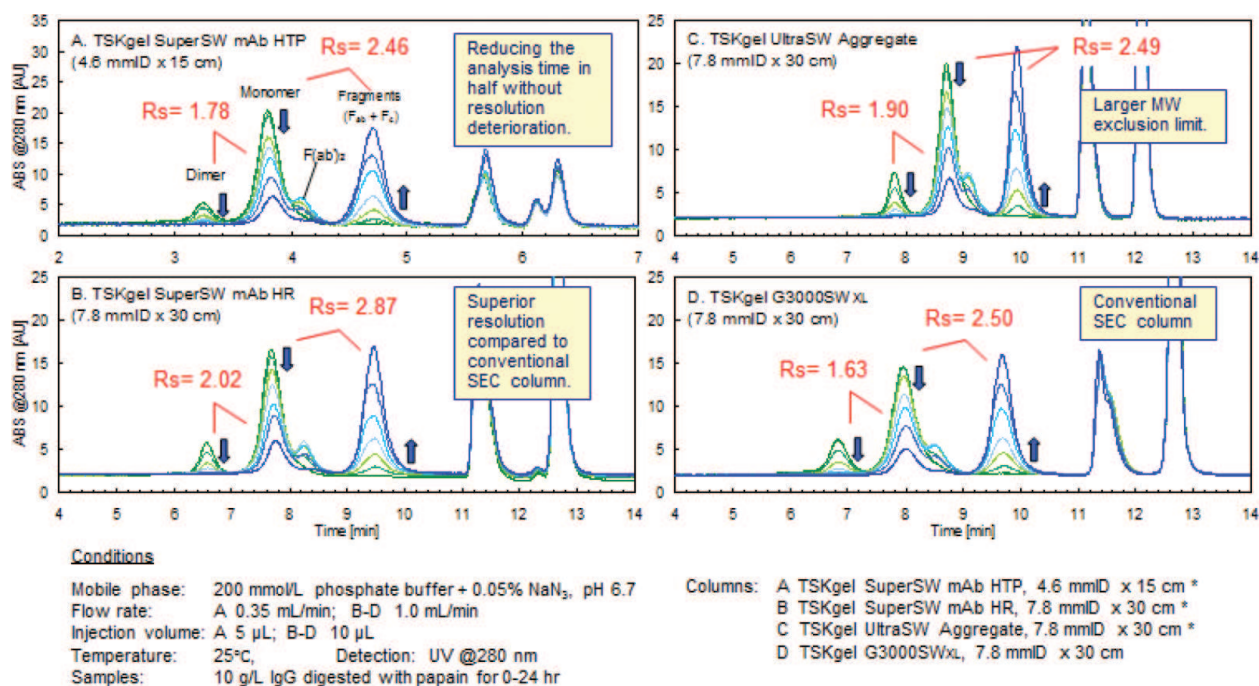**Figure 19.** Separation of papain digestion products of a mAb using a set of four different SEC columns. Columns in panel A, B, and D are of 250 Å pore sizes, while column in panel D is of 300 Å pore size [35].

(Panel D) have a separation range for globular protein samples up to 500 kDa. The other SEC column is a 4-μm, 7.8 mm ID × 30 cm TSKgel SuperSW mAb HR SEC column (Panel B). It is smaller than the conventional 5 μm TSKgel G3000SWXL column. Smaller particle size and the optimized packing are expected to yield high-resolution analysis of mAb monomers, dimers, and fragments due to shallow calibration slope at the corresponding molar mass region of the mAb monomer (150 kDa). Monomer – dimer resolution increased from 1.63 to 2.02. Another SEC column (Panel A) is a 4-μm TSKgel SuperSW mAb HTP column which is smaller in dimensions, length, and ID (4.6 mm ID × 15 cm). This column offers high throughput analysis, separating the dimer and monomer in half the run time compared to all the other three columns in panels B, C, and D. Results are similar to the analysis of mAbs with a 5-μm conventional column (panel D). The fourth column (Panel C) discussed here is of even smaller particle size (3 μm) with higher molar mass exclusion limit (2500 kDa, globular proteins) than all other three columns (500 kDa, globular proteins). Due to the higher exclusion limit, this TSKgel UltraSW aggregate column (Panel C) is expected to yield higher resolution of mAb multimers and aggregates. Please see further discussion about this in Section 2.7.

2.7. Forced degradation study by SEC

Stability of the biotherapeutic proteins in formulation is very critical for candidate selection, characterization of the biotherapeutic, formulation and assay development, and so on. A forced degradation study, popularly known as stress testing, is considerably a faster way to monitor the stability of the therapeutic proteins. Stress is provided by increasing the temperature, changing the pH or a combination of both. Depending on the nature, individual mAbs can be susceptible to light, freeze–thaw conditions, mechanical stress, oxidation and so on. So,

monitoring the stability from time to time is important. Literature reports: "Whereas stability-testing requirements are defined in regulatory guidelines, standard procedures for forced degradation of therapeutic proteins are largely unavailable, except for photo stability" [36]. Stress conditions induce unfolding of the native protein structure. As a result, the exposed hydrophobic patches may be able to interact with each other, leading to aggregation [37]. Protein degradation can also be measured using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), and the protein structure can be characterized by other techniques using Fourier transform infrared spectrometry and circular dichroism [38]. SEC is widely accepted as a great tool to monitor the forced degradation. Forced degradation by acid denaturation and heat denaturation analyzed using a TSKgel UltraSW Aggregate, 3 μm, 30 nm, 7.8 mm ID × 30 cm column is used as an example below [39] (**Figure 20**).

Acid denaturation: After reducing the pH of the IgG1 sample solution down to 4.7 by adding phosphoric acid, aliquots were analyzed at 5, 20, and 50 min, and the response was compared to that of the original sample solution. The blue trace shows the intact mAb and what is (presumably) its dimer eluting at 8.65 min when analyzed at flow rate of 1 mL/min. The degradation of the monoclonal antibody creates a larger MW entity (unidentified) that elutes directly after the dimer and before the monomer. Continued decay led to increase of both peaks. Clearly the dimer increases in size, while the peak height of the monomer decreases. Hints of higher order "multimers" are detected between 7 and 8 min [31].

Heat denaturation: Degradation of mAb at pH 5.5 and a temperature of 60°C were monitored. Fifty microliters of antibody in 0.1 M phosphate buffer (pH 6.0) was mixed with 50 μL of 0.1 mol/L phosphate buffer, pH 4.65; the final pH was 5.5; and 20 μL was injected [31]. Heating for 1 h at 60°C results in almost complete breakdown of the monoclonal antibody and the formation of very large aggregates (multimers) that extend to the exclusion volume of the column. The intensity of the multimer peaks increased as a function of the incubation period at 60°C. The larger the total exclusion volume of the column, the better the resolution of higher order aggregates. An SEC column with a 500-kDa exclusion limit may not be able to resolve the individual multimers such as trimers and tetramers. An SEC column with larger exclusion limit, for example, 2500 kDa, may be useful in such a case [31].
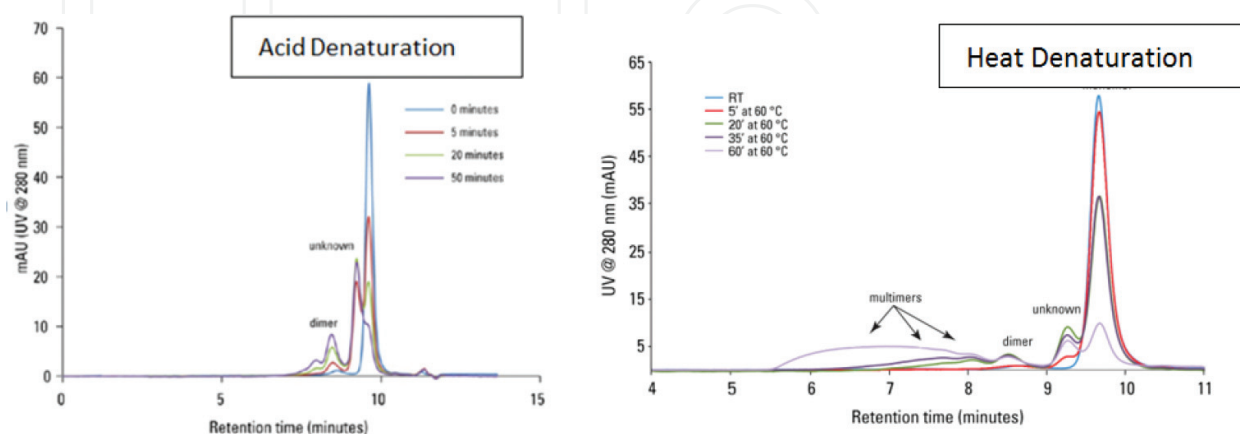

Figure 20. Analysis of forced degradation of IgG after acid and heat denaturation using a SEC TSKgel UltraSW aggregate, 3 μm, 30 nm, 7.8 mm ID × 30 cm column.
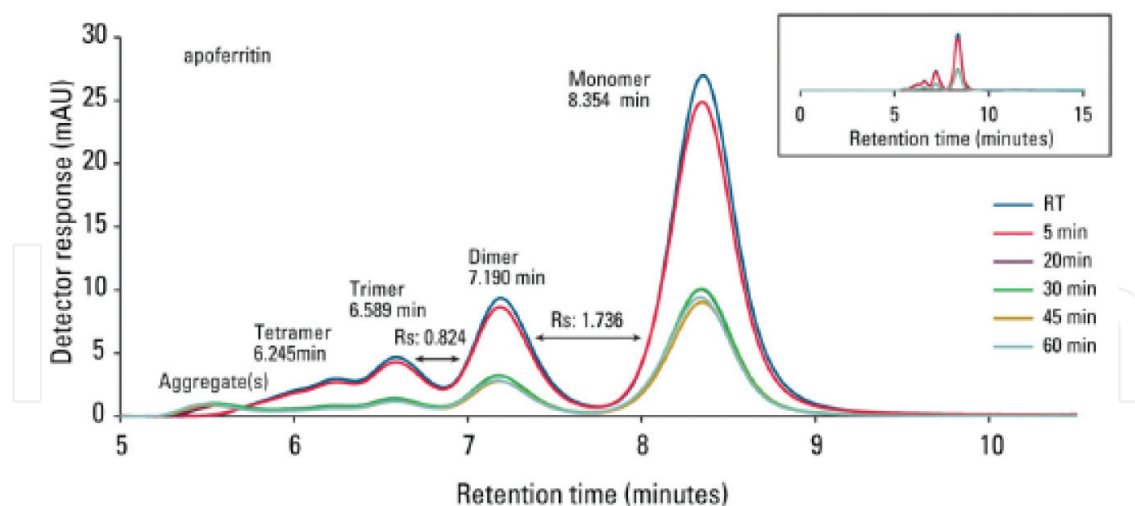
Figure 21. Analysis of Apo ferritin after heat denaturation using a TSKgel UltraSW aggregate column.

The analysis of a heat denatured, large hydrophobic metalloprotein, Apo ferritin, analyzed using a TSKgel UltraSW Aggregate column yielded high resolution between the monomer (450 kDa) and dimer (900 kDa) (**Figure 21**). The trimer (1350 kDa), tetramer (1800 kDa), and higher order aggregates of Apo ferritin were well separated. Tetramer of Apo ferritin is approximately equivalent to 13 mer of a mAb. Larger exclusion limit yielded better resolution of the higher order aggregates. [40].

3. Effect of mobile phase additives on the separation of monoclonal antibodies

The use of organic solvents such as isopropyl alcohol (IPA) or salts can decrease the secondary interaction as reported by many scientists and mentioned earlier briefly in this chapter in Section 2.5. Peak resolution and retention time shift were not impacted with the use of 15% IPA as demonstrated in the two examples (**Figure 12** and **Figure 13**). It is necessary to evaluate the individual SEC column regarding the impact of the additives since a stationary phase with minimum impact is always a favorable choice. After important mobile phase parameters have been set, such as pH, stationary phase, and ionic strength, significant improvements can, in fact, be made to separate mAb monomers from aggregates and fragments. There are no universal additives which can be applied for every mAb or protein purification. Other common additives are methanol and ethanol. Use of sodium perchlorate may also improve the separation and resolution. By switching from 0.2 mol/L sodium chloride to 0.2 mol/L of the more chaotropic sodium perchlorate salt, together with a twofold reduction in the buffer concentration, less peak tailing and distinct peaks for the dimer and trimer could be noticed [41]. Sodium dodecyl sulfate (SDS), urea, guanidine hydrochloride, etc., are sometimes used when the proteins need to be solubilized, leading to denaturation of the protein and breakage of the noncovalent bonds. The use of additives should be considered only when needed. In many cases, the performance of the column is irreversibly changed when a column is subjected

to particular additives. Retention time may shift when the additive is added and the resolution may change, expectedly to a better resolution but these values should remain constant and the analysis should be reproducible. It is always better to dedicate the SEC column if the column is subjected to additives, since we still have no clear idea how the pore characteristic of the stationary phase may behave with and without additives. Analysts should check the operational and conditions (OCS) sheet for the column as provided by the vendor to make sure that additives are compatible with the stationary phase. If compatible the analyst should be aware of the percentage of organic solvent the column is compatible with. Generally, when the organic solvent is used, the column may need a slower ramping rate for proper equilibration of the column with the mobile phase containing the additive, generally by using gradual solvent changes using a shallow gradient at low flow rate.

4. Analysis of monoclonal antibody IgG1 by SEC using MS-friendly mobile phases

The use of mass spectrometry is becoming increasingly popular for scientists dealing with biomolecule separation to identify the individual peaks by molecular weight. The liquid chromatography-MS (LC–MS) system available nowadays is very robust and useful for routine mass determination. Reversed phase LC–MS or SEC-MS using organic solvents such as acetonitrile can be used for the mass spectrometric characterization of mAbs. But mAbs get denatured under these conditions.

There is a growing interest in the analysis of mAbs by online-SEC-MS under native conditions. Conventional SEC analysis of mAbs use phosphate buffers at pH 6.7—for example, the most common one is composed of 100 mmol/L phosphates (monobasic + dibasic) as buffering salts +100 mM Na_2SO_4 as neutral salt to adjust the ionic strength +0.05% NaN3 (as antibacterial agent). Both the buffering salts (phosphates) and neutral salt (sodium sulfate) are helpful in preventing secondary interaction of the proteins with the stationary phase. The concentration of these salts may need further optimization depending on the individual properties of the mAbs. But phosphate buffer is not suitable for the mass spectrometer and yields substantial noise and damage the MS system. So online SEC-MS is not possible in the presence of phosphate and other non-volatile salts. Use of volatile salts at lower concentration, which do not interfere with the MS system, can be applied and the method needs to be optimized as well. SEC columns should not exhibit particle shedding which will interfere with the MS signal.

The data below illustrate the effective use of MS-friendly mobile phase compositions in the online SEC-MS analysis of a monoclonal IgG1, IgG2 antibody, ADC and Bi-specific mAb using volatile salt environments (**Figure 22**).

The online LC–MS compatible chromatographic conditions used for the analysis of IgG1, IgG2, ADC, and a Bi-specific mAb is shown below.

Following the development of an optimized separation, liquid chromatography mass spectrometry (LC–MS) analysis was performed using a Q Exactive Plus mass spectrometer

- Column: TSKgel UP-SW3000 4.6mm ID x 30cm, 2μm

- Instrument: UHPLC Ultimate 3000

- Mobile Phase: 20mM Ammonium Acetate, 10mM Ammonium Bicarbonate; pH 7.2

- Gradient: 15 Minute Isocratic

- Flow rate: 0.350 mL/min

- Detection: UV@280nm

- Temperature - 30°C

- Sample: IgG1, IgG2, ADC, BI-Specific mAb
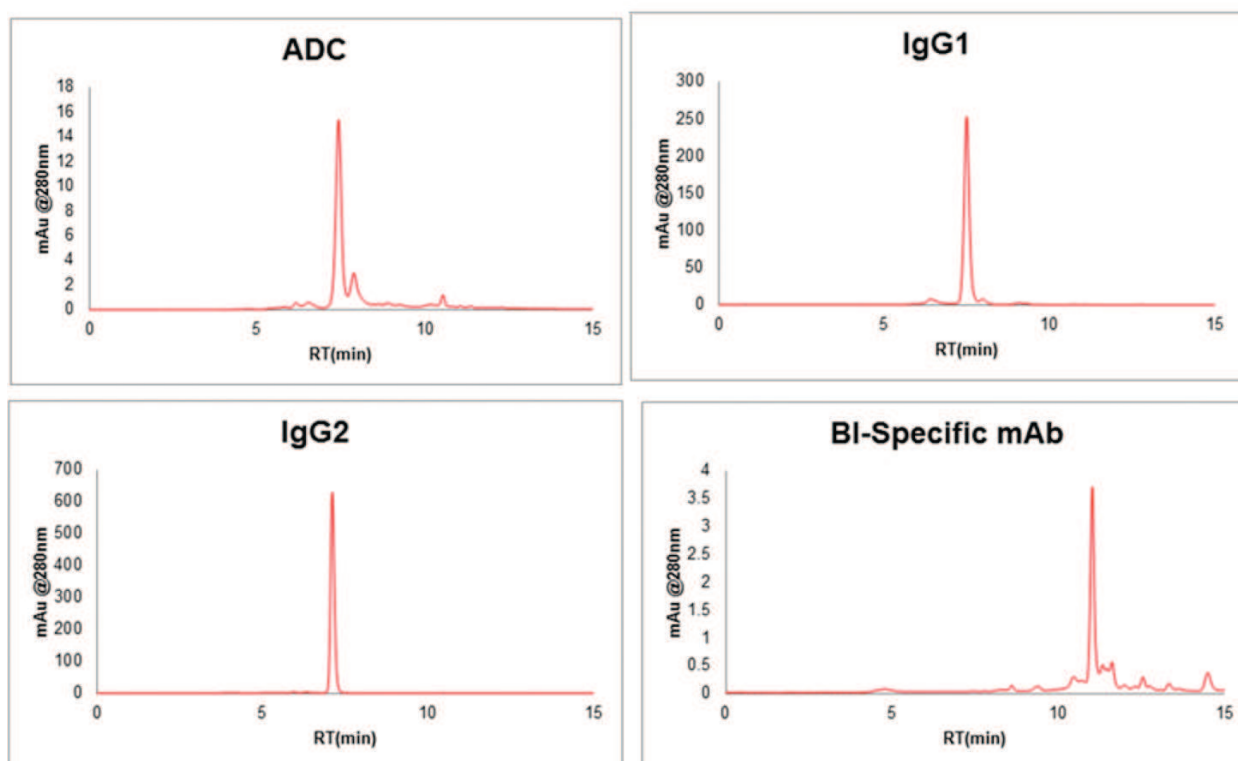
Figure 22. Separation of an ADC, two IgG1 and IgG2 mAbs, and the corresponding bi-specific mAb using a TSKgel UP-SW3000 column (2 μm, 4.6 mm ID x 30 cm).

(ThermoFisher Scientific) coupled to a Shimadzu Nexera XR UHPLC system. Samples were injected onto a TSKgel UP-SW3000 column (2 μm, 4.6 mm ID x 30 cm) and isocratically separated at 0.350 ml/min for 15 min with a mobile phase comprising 20 mM ammonium acetate and 10 mM ammonium bicarbonate, pH 7.2. A 15-min blank isocratic gradient was run between sample injections. No carryover was observed in the blank runs. Eluted proteins were analyzed by the mass spectrometer set to repetitively scan m/z from 800 to 6000 in a positive ion mode. The full MS scan was collected at 17,500 resolution, with spray voltage 4 kV, S-Lens RF 75, and in-source CID 80 eV. Protein mass deconvolution was performed using ProMass (Novatia). The (1) total ion chromatogram, (2) mass spectrum, and (3) deconvoluted mass

spectrum of one mAb was evaluated. A main peak can be seen at m/z 149,264; adjacent peaks at m/z 149,426 and 149,592 correspond to different glycoforms.

Here we report the use of a TSKgel® UP-SW3000, 2 μm column for the separation of a bispecific antibody and the two parent mAbs (IgG1) followed by MS analysis. The Bispecific T cell Engager (BiTE®) technology was used in this study. BiTE is a fusion protein consisting of two single-chain variable fragments (scFvs)–CD19, a biomarker for normal and neoplastic B cells and CD3 (on T cells) – recombinantly linked by a nonimmunogenic five-amino-acid chain (**Figure 23**). BiTE is approximately 55 kDa in size. SEC/MS analysis was performed by the Wistar Proteomics and Metabolomics Facility (Philadelphia, PA) using a Nexera® XR UHPLC system (Shimadzu) coupled to a Q Exactive™ Plus mass spectrometer (Thermo Fisher Scientific) (**Figures 24–26**).

Prior to analysis, a blank injection was run in order to assess column particle shedding. The total ion chromatogram of a blank injection was run on a new TSKgel UP-SW3000 column. MS data indicate that there is no shedding from the TSKgel UP-SW3000 column prior to sample injection. Additionally a blank injection was run between each of the sample injections in order to monitor sample carryover.

Each mAb is different, and a method with the use of volatile salts needs to be optimized for reproducibility. There was a difference between the retention time of mAb1 under the isocratic mobile phase 20 mM ammonium acetate and 10 mM ammonium bicarbonate, pH 7.2 compared to 100 mM phosphate buffer containing 100 mM Na_2SO_4 and 0.05% NaN_3 pH 6.8. In an attempt to look for the condition where a MS compatible buffer yields a retention time similar to phosphate buffer, a comparison of elution profiles under 100 mM phosphate buffer and 100 mM ammonium acetate buffer both at pH 6.8 is shown below (**Figure 27** and **Table 4**).

Monomer peak areas remain constant under both conditions with high reproducibility of all the peak parameters. % RSD deviations of all the peak parameters were low. Mass spectrometric analysis under this chromatographic condition will be reported elsewhere.
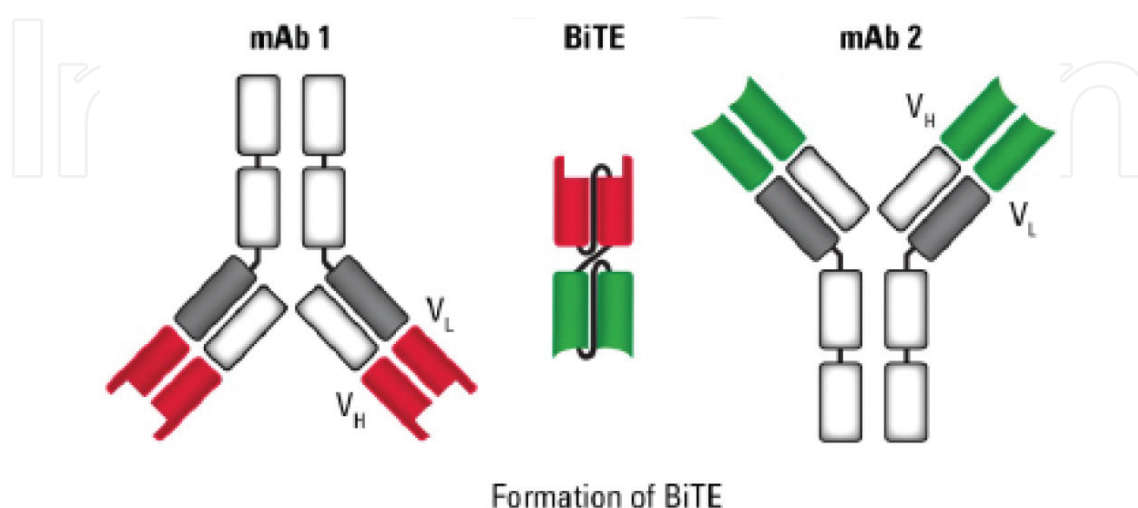

Figure 23. Scheme of a BiTE and corresponding original mAb 1 and mAb 2.

Column:	TSKgel UP-SW3000, 2 μm, 4.6 mm ID × 30 cm
Instrument:	Dionex UltiMate® 3000 UHPLC system run by Chromeleon® (ver 7.2)
Mobile phase:	20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate; pH 7.2
Gradient:	isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	5.0 μL
Samples:	BiTE, 0.3 mg/mL (*Creative Biolabs*) parent mAb shown, 0.5 mg/mL (*Creative Biolabs*)

Mass Spectrometric Conditions

- Eluted proteins were analyzed by the mass spectrometer set to repetitively scan m/z from 800 to 6000 in positive ion mode.

- The full MS scan was collected at 17,500 resolution, with spray voltage 4 kV, S-Lens RF 75, and in-source CID 80 eV. Protein mass deconvolution was performed using ProMass® (*Novatia*).

- Total ion chromatograms were collected; mass spectrum and peak deconvolution for the accurate mass are shown.
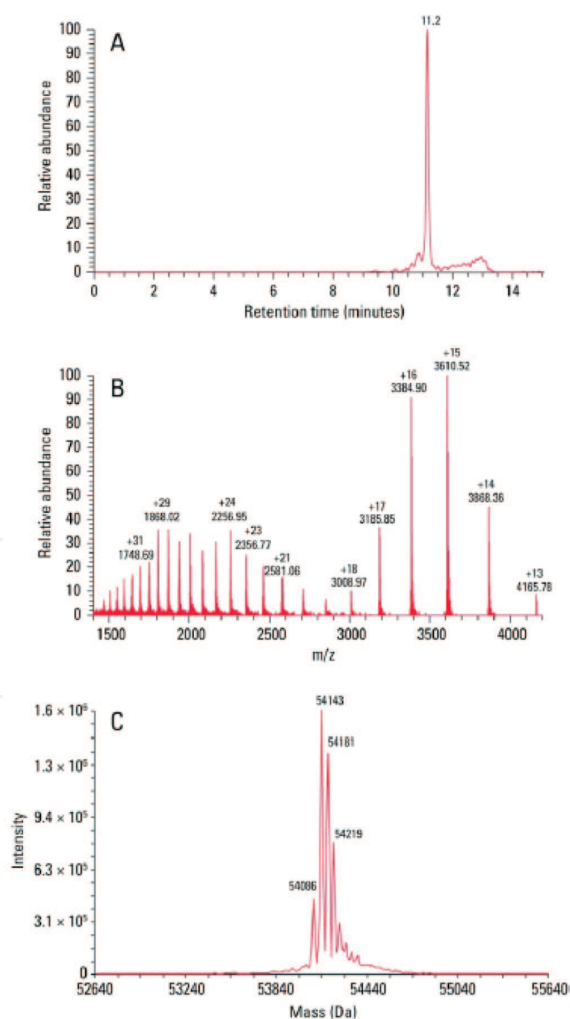
A: total ion chromatogram
B: mass spectrum
C: deconvoluted spectrum of BiTE

A main peak can be seen at m/z 54,143; adjacent peaks at m/z 54,181, 54,219 and 54,086 correspond to different salt adducts.

Figure 24. SEC/MS analysis of the CD19 X CD3 BiTE antibody.
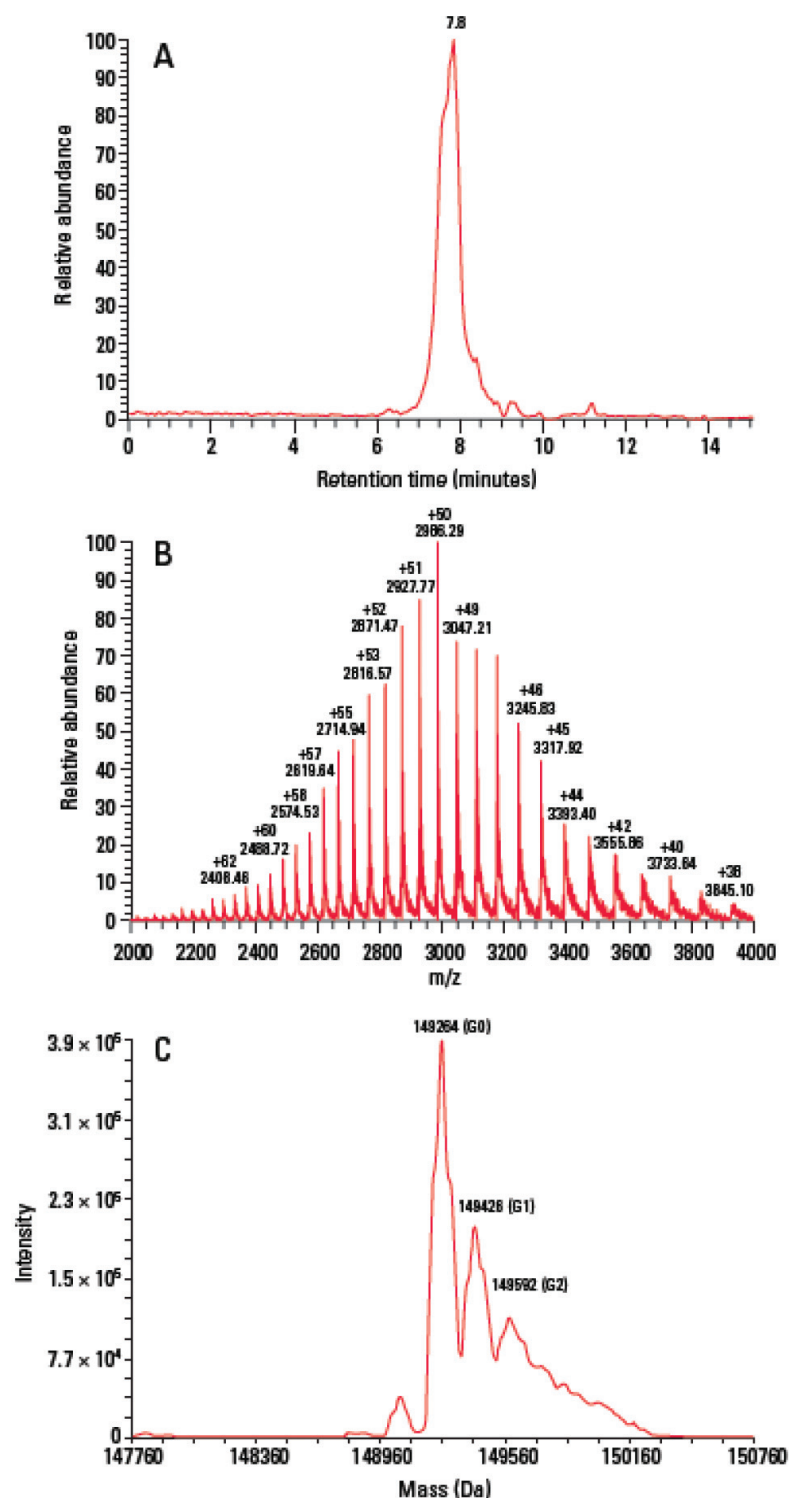
A: total ion chromatogram
B: mass spectrum
C: deconvoluted spectrum of BiTE

A main peak can be seen at m/z 149,264; adjacent peaks at m/z 149,426 and 149,592 correspond to different glycoforms.

Figure 25. SEC/MS analysis of the original IgG1 mAb1.

Figure 26. Analysis of blank injections in order to assess column particle shedding using the TSKgel UP-SW3000 column.

Figure 27. Comparison of elution profiles of IgG1 under 100 mM phosphate buffer and 100 mM ammonium acetate buffer, pH 6.8 using a TSKgel UP-SW3000 column.

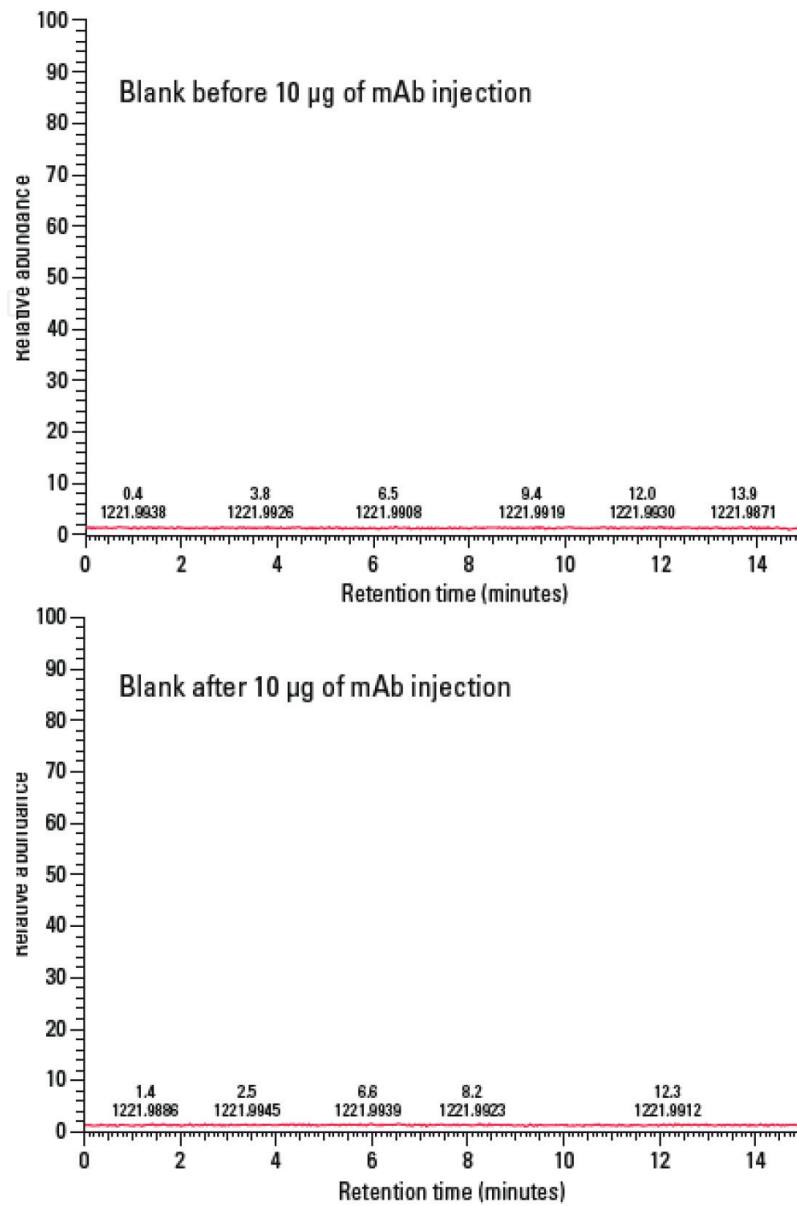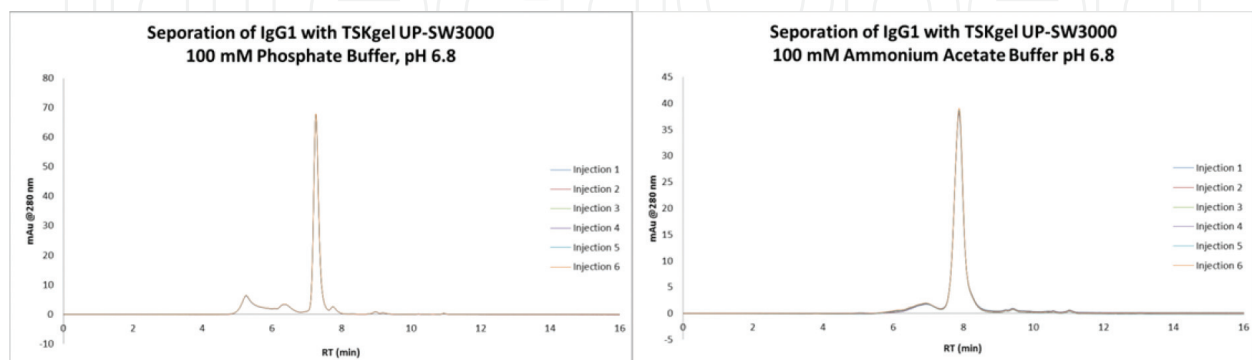| Monomer | | | | | | Monomer | | | | | |
| Phosphate Buffer IgG1: 2 g/L | | | | | | 100 mM Amm Ace pH 6.8 LC/MS Buffer IgG1: 2 g/L | | | | | |
Injection	RT	Area	Height	As	N	Injection	RT	Area	Height	As	N
1	7.258	11.6	66.7	1.3	12104	1	7.872	11.9	37.3	1.2	4623
2	7.258	11.6	66.5	1.3	12083	2	7.877	11.9	37.4	1.2	4628
3	7.258	11.6	66.6	1.3	12058	3	7.875	11.9	37.7	1.2	4637
4	7.260	11.6	66.6	1.2	12074	4	7.875	12.0	37.9	1.2	4656
5	7.258	11.6	66.4	1.3	12042	5	7.878	12.0	38.2	1.2	4657
6	7.260	11.7	66.9	1.3	11974	6	7.878	12.1	38.5	1.2	4651
Avg	7.259	11.6	66.6	1.3	12056	Avg	7.876	12.0	37.8	1.2	4642
Std	0.0	0.0	0.2	0.0	45.3	Std	0.0	0.1	0.5	0.0	14.7
%RSD	0.0	0.4	0.3	0.5	0.4	%RSD	0.0	0.8	1.2	1.2	0.3

Table 4. Analysis of retention time, peak area, peak height, as (peak asymmetry) and N (theoretical plates) of the monomers. The average (Avg), standard deviation (Std) and relative standard deviation (RSD) are also shown.

5. Size exclusion chromatography and its orthogonal and complimentary modes for detection of heterogeneity

Two chromatographic modes are considered orthogonal techniques if the selectivity of the two modes are significantly different. Under ideal conditions without any secondary interaction, SEC should yield a characteristic Gaussian-shaped peak without the presence of any heterogeneity.
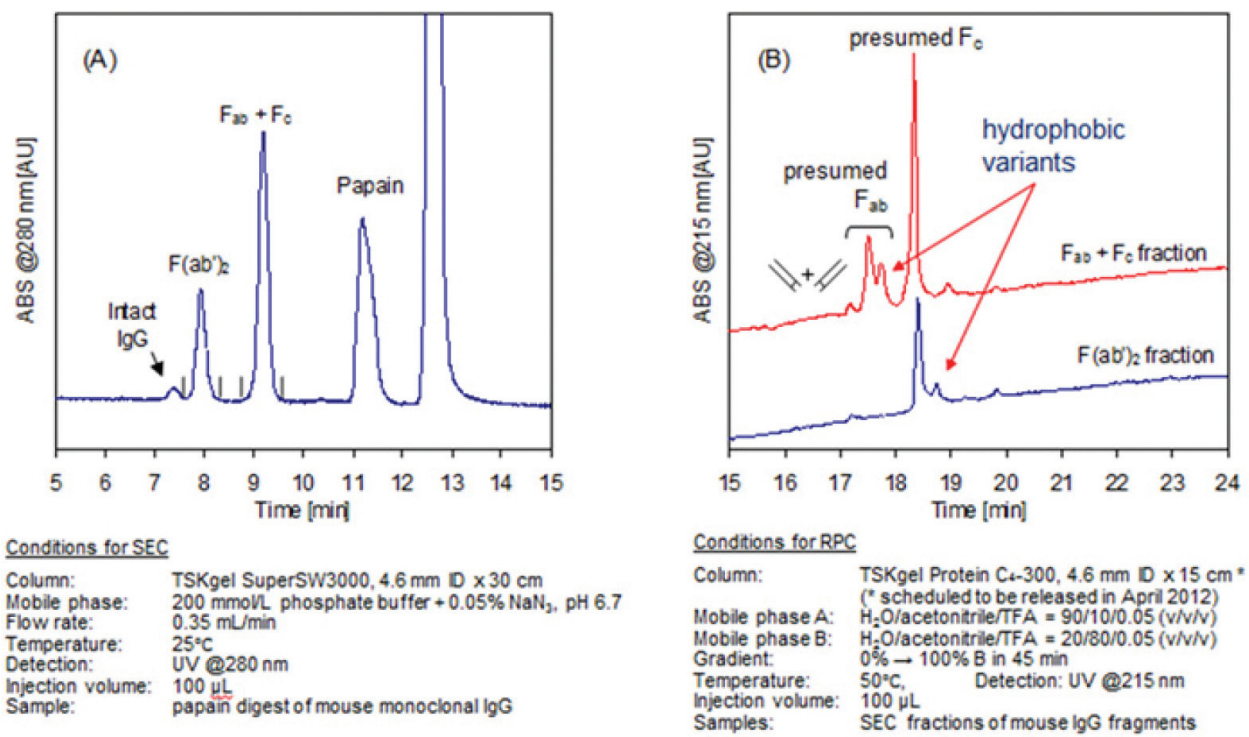

Figure 28. Analysis of papain digested IgG1 fragments. (A) Chromatogram of papain digested IgG1 fragments separated on a TSKgel SuperSW3000 column. (B) Individual peaks F (ab) 2 and Fab + Fc from the SEC separation (panel A) were applied to a reversed-phase chromatographic (RPC) TSKgel protein C4–300 column.

As shown in the figure below when individual peaks F (ab) 2 and Fab + Fc from SEC separation (panel A) were applied to the reversed-phase chromatographic (RPC) column, a number of hydrophobic variants eluted (panel B) in the increasing order of their hydrophobicity (**Figure 28**). Mechanism of papain digestion is discussed in Section 2.6. Though papain digestion yields primarily the Fab fragments, F(ab')2 fragment can be generated if the papain is first activated with 10 mM cysteine. Following the completion of the reaction, the excess needs to be removed by gel filtration. Size exclusion chromatography cannot differentiate these heterogenic impurities or hydrophobic variants, which are not sufficiently different in the size or hydrodynamic radii from each other. Similarly, a number of other chromatographic modes, other than RPC, can also be used as an orthogonal technique. The extent of the heterogeneity present in the SEC peak can only be confirmed by an orthogonal analysis.

Similarly, a reversed-phase chromatography column can also be used as a complimentary chromatography column along with SEC as shown below (**Figure 29**). The elution order of elution of the peaks is simply reversed as expected.

The PEG-conjugated species were more strongly retained by RPC, than the different forms of intact lysozyme. The order of elution in RPC is opposite to the order of size-based separation in SEC [35].

Conditions for SEC

Column:	TSKgel SuperSW3000, 4.6 mm ID × 30 cm × 2
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN$_3$
Flow rate:	0.35 mL/min
Temperature:	25 °C
Detection:	UV @ 280 nm
Injection vol.:	50 μL
Samples:	5 g/L PEG(MW 5,000)-Lysozyme, 5 g/L PEG(MW 30,000)-Lysozyme

Conditions for RPC

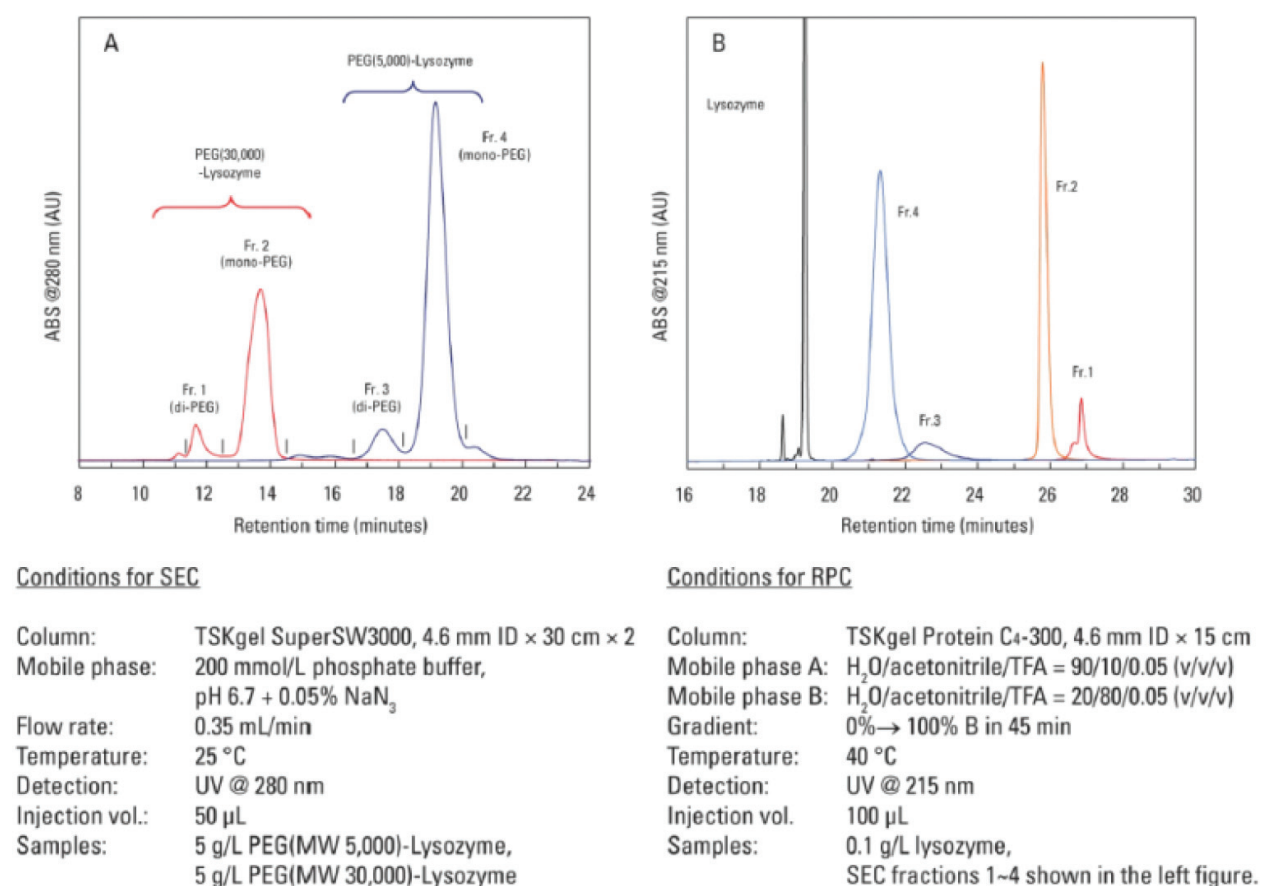| Column:	TSKgel Protein C$_4$-300, 4.6 mm ID × 15 cm
Mobile phase A:	H$_2$O/acetonitrile/TFA = 90/10/0.05 (v/v/v)
Mobile phase B:	H$_2$O/acetonitrile/TFA = 20/80/0.05 (v/v/v)
Gradient:	0%→ 100% B in 45 min
Temperature:	40 °C
Detection:	UV @ 215 nm
Injection vol.	100 μL
Samples:	0.1 g/L lysozyme, SEC fractions 1~4 shown in the left figure.

Figure 29. Separation of PEG (MW 5000)-lysozyme and PEG (MW 30,000)-lysozyme on a SEC TSKgel SuperSW3000 column (A) followed by chromatography of the SEC fractions 1–4 using a reverse phase TSKgel protein C$_4$–300 column (B).

6. Method transfer

With the advancement of the liquid chromatographic instruments from HPLC to UHPLC and with the advancement in column packing technology with smaller particle sizes, an easy method transfer from HPLC to UHPLC is becoming necessary. UHPLC has much lower extra column volume and can withstand more than 1000 bar besides other optimized instrumental features while conventional HPLC has maximum operable pressure of 400 bars. Differences between HPLC and UHPLC instruments give rise to many challenges when a method needs to be transferred from one system to another and if equivalent separation profiles are obtained. On the other hand, there are analysts who do not possess the UHPLC but still want to get advantage of smaller particle size SEC columns while using a traditional HPLC instrument. The effect of extra column volume in band broadening in conventional HPLC needs to be reduced and optimized by reducing the diffusion in the tubing between the injection valve and the column and between the column and the UV cell by using smaller ID tubing and micro flow cell. A column compatible with both UHPLC and conventional HPLC instruments may be helpful for easy method transfer in both cases.

As an example, an SEC HPLC method for the separation of a mAb using 5 μm, 7.8 mm ID × 30 cm SEC column (TSKgel G3000SWXL) was transferred to a 2 μm, 4.6 mm ID × 30 cm SEC column (TSKgel UP-SW3000 SEC) on a UHPLC instrument (**Figure 30**). The mobile phase and other chromatographic parameters were not changed except the flow rate reduced for the 2-μm column.
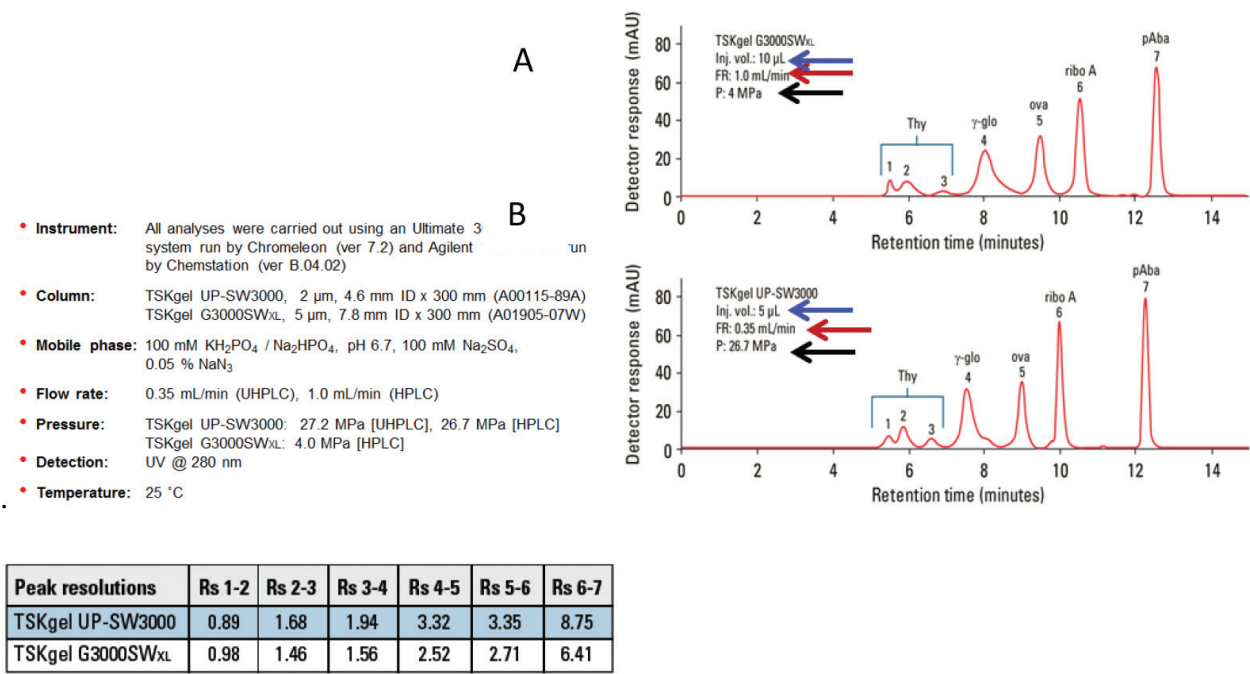

- **Instrument:** All analyses were carried out using an Ultimate 3 system run by Chromeleon (ver 7.2) and Agilent   un by Chemstation (ver B.04.02)
- **Column:** TSKgel UP-SW3000, 2 μm, 4.6 mm ID x 300 mm (A00115-89A)
  TSKgel G3000SWXL, 5 μm, 7.8 mm ID x 300 mm (A01905-07W)
- **Mobile phase:** 100 mM KH$_2$PO$_4$ / Na$_2$HPO$_4$, pH 6.7, 100 mM Na$_2$SO$_4$, 0.05 % NaN$_3$
- **Flow rate:** 0.35 mL/min (UHPLC), 1.0 mL/min (HPLC)
- **Pressure:** TSKgel UP-SW3000: 27.2 MPa [UHPLC], 26.7 MPa [HPLC]
  TSKgel G3000SWXL: 4.0 MPa [HPLC]
- **Detection:** UV @ 280 nm
- **Temperature:** 25 °C

Peak resolutions	Rs 1-2	Rs 2-3	Rs 3-4	Rs 4-5	Rs 5-6	Rs 6-7
TSKgel UP-SW3000	0.89	1.68	1.94	3.32	3.35	8.75
TSKgel G3000SWXL	0.98	1.46	1.56	2.52	2.71	6.41

Figure 30. Comparison of the SEC HPLC method with the SEC UHPLC system. Separation efficiency of column TSKgel G3000SWXL (A) and column TSKgel UP-SW3000 SEC (B) was compared by loading a mixture of standard proteins (Thy = thyroglobulin bovine, γ-glo = γ-globulins from bovine blood, ova = albumin chicken egg grade VI, riboA = ribonuclease A type I-A from bovine pancreas, pAba = p-aminobenzoid acid) on both columns.
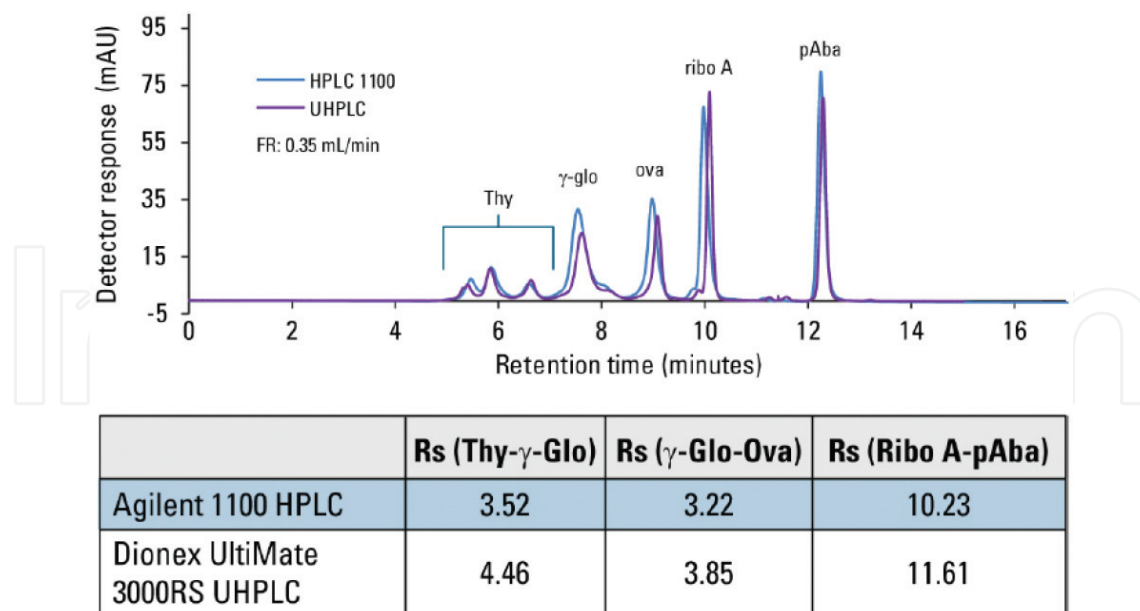
Figure 31. Comparison of the separation efficiency of a 2-μm TSKgel UP-SW3000 column applied in HPLC and UHPLC mode. Conditions were the same as in **Figure 30**. The same standard protein mixture as in **Figure 30** was used.

TSKgel UP-SW3000 columns feature the same pore size as the well-established TSKgel G3000SWxl columns. With the use of the 2-μm column, the resolution between peaks 2–3, 3–4, 4–5, 5–6, and 6–7 increased respectively by 15, 25, 33, 24, and 37% compared to the 5-μm column. Retention time consistency was maintained, and similar separation profile was obtained at lower flow rate. A smaller 2-μm particle size column yielded a twofold higher sensitivity. The 2-μm columns yielded a back pressure acceptable for use in both HPLC and UHPLC.

Similarly, a method can be transferred directly from HPLC to UHPLC, without any change in conditions using 2 μm TSKgel UP-SW3000 columns as shown in **Figure 31** [42].

7. SEC for desalting

Desalting is a process to remove or reduce salt from the liquid, such as protein sample solution. Desalting by gel filtration chromatography (GFC) is the preferred method in biochemical laboratories to reduce the salt concentration or to exchange the buffer of a biopolymer solution. The main advantage of desalting by GFC over dialysis is the faster analysis time. Desalting may be needed for various reasons. Proteins eluting at high or elevated salt concentrations may need to be desalted to lower salt concentration prior to its use for the next step. Protein samples may also contain denaturants such as sodium dodecyl sulfate (SDS), guanidine hydrochloride, and urea which need to be removed. Desalting and buffer exchange of proteins or polynucleotides can also be performed by dialysis, ultra-filtration, or by using spin

columns. Desalting columns are characterized by a low exclusion limit and a large pore volume. Salts can fully access all pores, while proteins and other high MW species are excluded. Analytical columns packed with conventional packing materials such as dextran, cellulose, and polyacrylamide have limited physical stability and are not suitable when fast desalting is desired. Requirements for a fast desalting SEC column are [1] an inert matrix, [2] a large pore volume that is fully accessible to common salts and buffer components, [3] a pore size distribution that excludes the component(s) of interest from accessing the pores, and [4] sufficient mechanical strength to allow the use of the column in standard HPLC equipment. As an example, a 15-μm particle size TSKgel BioAssist DS column is composed of a stationary phase where the mechanical strength of the polyacrylamide gel is fourfold higher as compared to conventional gel by urea cross-linking. Conventionally, polyacrylamide beads have been prepared by reversed-phase suspension polymerization or by using a spray dry method. The uniform and more pressure-stable polyacrylamide beads packed in TSKgel BioAssist DS columns were prepared using a normal phase suspension method as shown in **Figure 32** [43].

Fast desalting with excellent reproducibility could be carried out within 5 min using conventional HPLC system and TSKgel BioAssist DS Columns (4.6 mm ID and 10 mm ID) (**Figure 33**). All the proteins (see table below) eluted with the same retention time closer to void volume irrespective of their size (see the figures below), while salt and other small impurities eluted at longer retention time as a function of their size. Refractive index was used as a detector in this study since salts do not have any chromophore.

SEC columns designed for desalting using a HPLC instrument can be useful for the desalting of proteins and polynucleotides at analytical and semi-preparative scale.
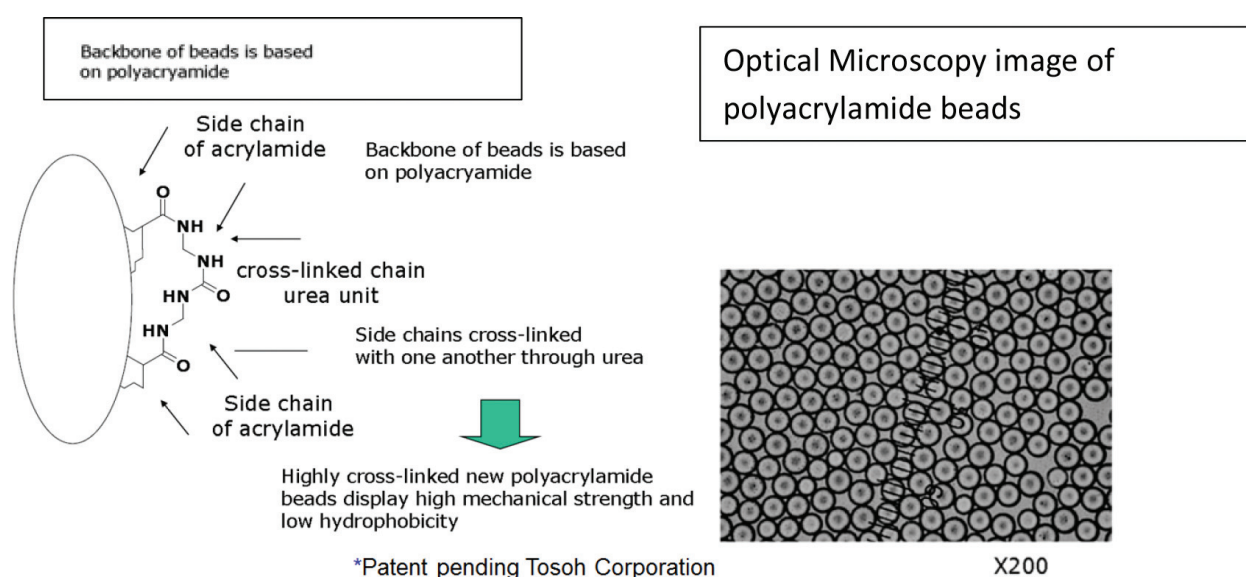

Figure 32. Principle of the generation of pressure-stable polyacrylamide beads which can be packed in TSKgel BioAssist DS columns.

Protein	MW (kDa)	Concentration* (g/L approx.)
ribonuclease A	14.7	19.5
thyroglobulin	670	11.3
γ-globulin	150	14.5
ovalbumin	45	13.1
α-chymotrypsinogen	25.6	13.1
β-lactoglobulin	18.4	10.8
lysozyme	14.7	11.6
myoglobin	16.7	14.5
cytochrome C	12.3	11.0
hemoglobin	68	11.9

*in 100 mmol/L Phosphate buffer, pH 6.7

Proteins in 0.1mol/L phosphate buffer, pH 6.7
Mobile phase: 0.01mol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 0.01mol/L Na_2SO_4 + 0.05% NaN_3

Proteins in 0.1mol/L phosphate buffer, pH 6.7
Mobile phase: 0.01mol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 0.01mol/L Na_2SO_4 + 0.05% NaN_3
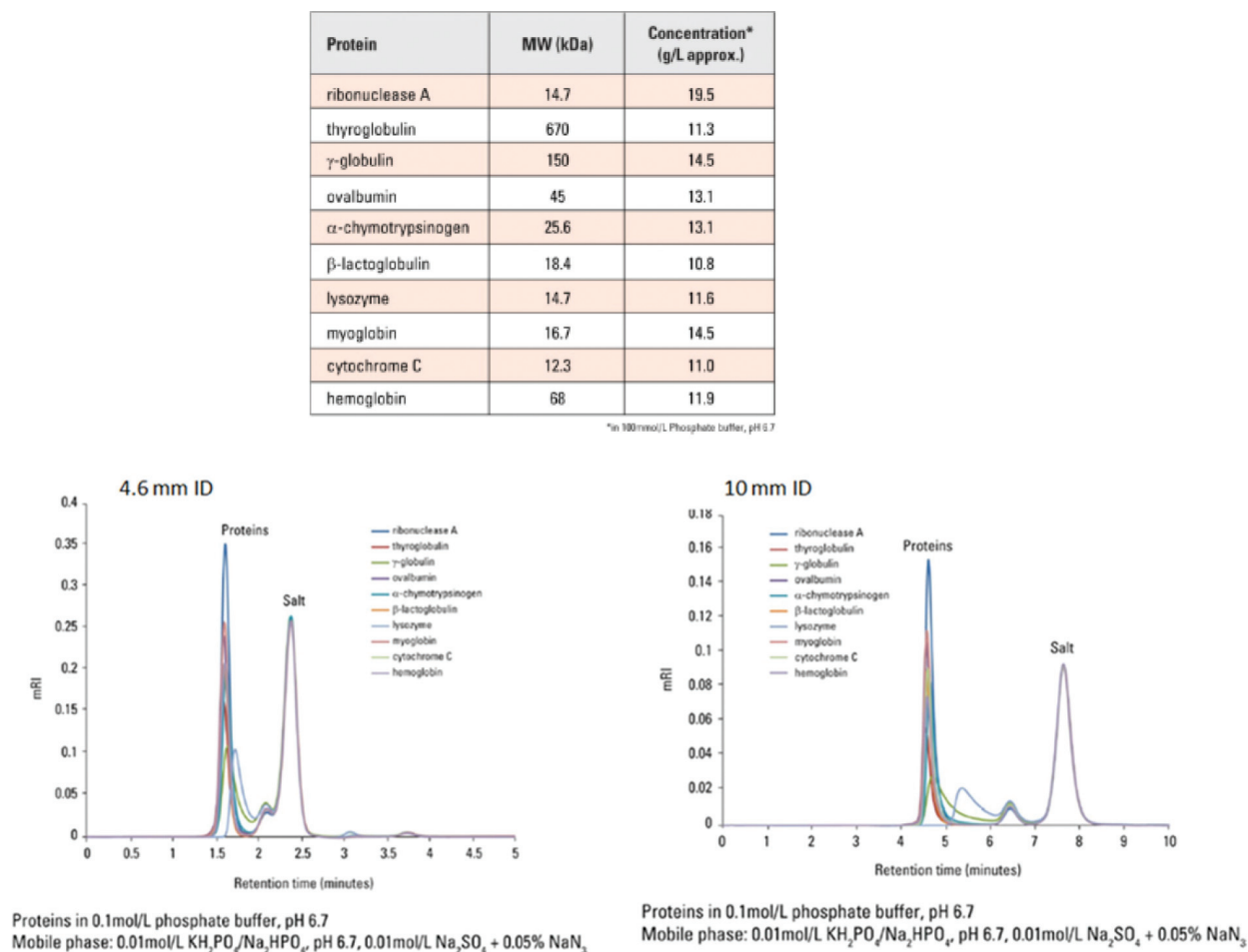
Figure 33. Desalting of proteins using a TSKgel BioAssist DS column with 4.6 mm ID or 10 mm ID packed with pressure-stable polyacrylamide beads. The retention times of standard proteins and salt are shown.

8. Size exclusion chromatography column in the HILIC mode

For many years, SEC columns have been used to separate various nucleic acid species such as DNA, RNA, and tRNA as well as their constituent bases, adenine, guanine, thymine, cytosine, and uracil. In medicine, several primary nucleobases are the basis for the nucleoside analogues and other synthetic analogs which are used as anticancer and antiviral agents. Nucleobase modifications are the basis of oligonucleotide-based therapeutics, making their purification very important.

Hydrophilic interaction chromatography (HILIC) is a variant of normal phase liquid chromatography which uses hydrophilic stationary phases with reversed-phase type eluents. It is applied for the separation of polar hydrophilic compounds.

Chemically bonded diol-coated phases in size exclusion chromatography (SEC) columns demonstrate high polarity and hydrogen bonding properties. They do not contain ionizable groups compared to the unreacted free residual silanols, making them appropriate for the HILIC mode.
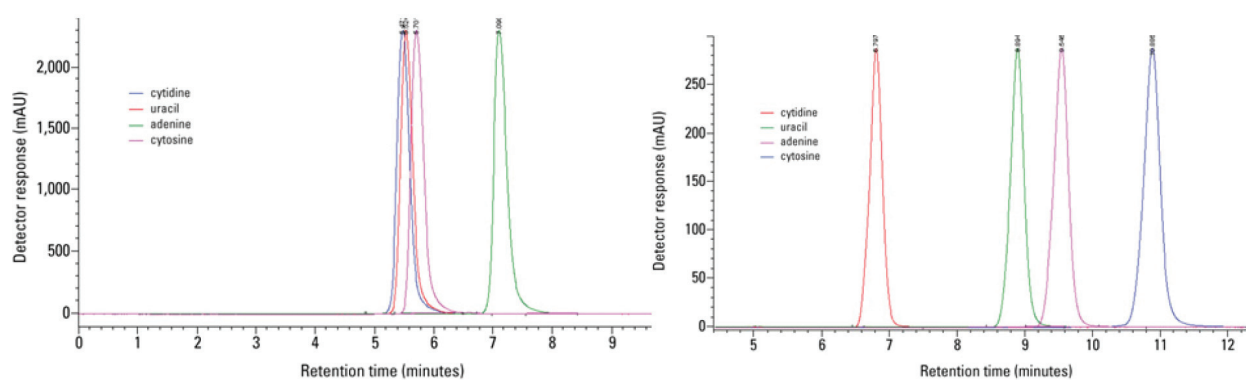
Figure 34. Separation of four nucleobases using a TSKgel SuperSW mAb HTP column under SEC condition (left panel) and in HILIC mode (right panel) at pH 7.4.

Figure 34 [44] shows the separation of four nucleobases using a TSKgel SuperSW mAb HTP Column under SEC condition and in HILIC mode at pH 7.4 (**Figure 34**).

As expected, due to the similarities in molecular masses between the four compounds, significant interference is observed among the peaks of interest, particularly the three pyrimidine derivatives, when separated on the TSKgel SuperSW mAb HTP column under SEC conditions. The late elution of adenine (relative to the other three compounds) may be attributed to possible interactions between the stationary phase and the derivatized purine compound, leading to a shift toward a longer retention time. When the same SEC column is used in the HILIC mode, the order of elution of the analytes does not correlate with their molecular mass (as in SEC separations), but instead is based on their relative hydrophilicity. This note demonstrates the benefits of using a SEC column in HILIC mode for the superior resolution of four nucleobases, as opposed to using the column in the SEC mode or using another type of a HILIC column.

9. Tips and tricks for size exclusion chromatography

It is a recommended practice to protect the column from potential sources of contamination during the SEC separation of mAbs and other proteins. Standards and mobile phases should be filtered through a 0.45-μm syringe filter. A frit filter used between injector and column will also be an additional help. The use of guard columns is highly recommended. Guard columns being short, of similar ID and with the same stationary phase do not possess any separation power. The slight change in the retention time due to small increase in length remains constant in consecutive injections. Using guard columns can prolong the lifetime of the analytical column. The guard column needs to be changed before the dirty material spills over to the analytical column. Frits at different parts of the HPLC instruments need to be changed intermittently. Please refer to the picture below which clearly shows how much dirty materials are trapped by these frits (**Figure 35**). Frequent changes of the frits are necessary to avoid spillage of the dirty materials to the columns. Phosphate buffer pH 6.8, very commonly used for the protein analysis by SEC is prone to bacterial growth. The column will get clogged and dirty, eventually leading to failure of the analysis and breakdown of the system. Previously I
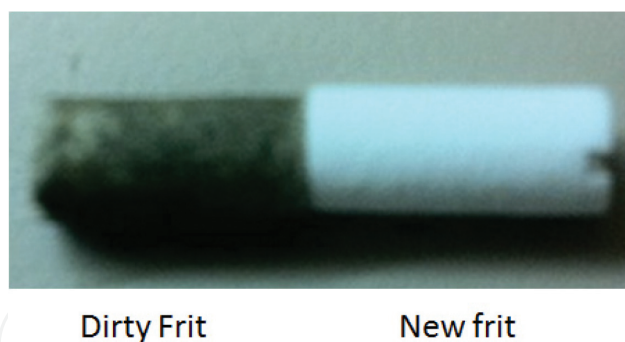
Figure 35. Comparison of a dirty frit with a new frit.

mentioned that sodium azide (NaN$_3$) was used as an antibacterial agent to prevent fouling of the phosphate buffer.

During the SEC of mAbs and proteins, mobile phases containing salts are constantly used and can deposit on the different parts of the HPLC system (**Figure 36**). The pump head assembly may get affected from the deposition of salts, resulting into a rise of back pressure as well as causing damage to the sapphire piston. The pump head assembly can be cleaned with distilled water and the back pressure issue is resolved. Mobile phase containing high salt or a combination of high salt and organic solvent can create this problem more than any other low salt aqueous mobile phases. The system should not remain idle with these types of mobile phases. The deposition may happen anywhere within the HPLC system and also inside the column, or in the pump, the injector, the in-line filter or the tubing.

The use of a surfactant is necessary for certain SEC applications as discussed earlier, but surfactants may change the bonding phase, so it may be necessary to dedicate the column for that particular application.

Occasionally protein samples are adsorbed onto the packing material. When this occurs, it is time to clean the SEC column. At the beginning of the separation, when the SEC column is new and operating correctly and the mAb monomer is yielding a well-resolved peak, it is better to establish baseline data and acceptable running conditions criteria. Then, if one of the performance

Figure 36. Salt depositions at screws of the column.

characteristics of the SEC column changes by 10% or more, it is prudent that cleaning is necessary. The acceptance criteria can be more stringent as needed. Similar cleaning of the whole system including the flow cell may be necessary.

If cleaning is necessary, it is better to try cleaning the column in reversed direction at half the flow rate while the column is detached from the detector to prevent detector damage since the proteins have a tendency to adhere to the quartz material of the detector. Since the dirty materials may remain trapped over the frit outside the column, cleaning in reversed flow direction may be the easiest way to clean the column. Once the column is cleaned using the vendor suggested protocol, the analyst must qualify the column using the QC method and QC pass criteria under normal flow direction.

It is always recommended to read the operational conditions and specification sheet (OCS) before the column is used. Occasionally, we may intend to use the column outside the specification for a short period of time, but it is the responsibility of the analyst to monitor if the column is functioning properly. For example, in Section 2.5, it has been shown that an analysis could be repeated to monitor the stability of the antibody over time using a TSKgel SuperSW mAb-HTP column at 0.75 mL/min. The used flow rate (0.75 mL/min) was higher than the recommended maximum flow rate of 0.5 mL/min, as mentioned in the operational conditions and specification (OCS) sheet for this column. Though the column could successfully be used at this higher flow rate without compromising the column health, the effect of the higher flow rate on the column lifetime for prolonged use was not investigated. It is always wise to operate within recommended maximum flow rate to remain in safe side in order to prolong the lifetime of the column. It is always necessary to keep an eye on the back pressure, peak parameters, and so on to monitor the column health.

HPLC system and analytical chromatography columns are costly. Method development is time-consuming and costly too. It is important to employ an HPLC system that is optimized with regard to extra-column band broadening to take full advantage of the high column efficiency that can be obtained on analytical columns. For any troubleshooting situation, the problem is one of the following: [1] the column, [2] the sample, [3] the mobile phases, [4] the instrumentation, and [5] personal errors. The same is true for size exclusion chromatography related troubleshooting issues too. Proper maintenance of the column and instrument is much more needed and important to avoid the troubleshooting in the beginning. Many troubleshooting issues can be avoided by taking proper handling the top three major components of any HPLC analysis. The importance of the use of a protein standard mixture and the standard mAb to monitor the column health from time to time is always a good idea as mentioned in brief in Section 2.5. Maintaining a SEC column is an art and good performance, which will result in a prolonged column lifetime.

10. Conclusions

Size-exclusion chromatography is a great tool for the purification of monoclonal antibodies. The secondary interaction of the stationary phase needs to be taken care of by controlling the

stationary phase as well as by optimizing the mobile phase. The effect of particle size and pore size of the stationary phase on SEC separation needs to be clearly understood. A right column selection for the purification will depend on the separation range and the slope of the calibration curve. Separation of HMW and LMW species to purify the monomer is critical to get the pure mAb without heterogenic impurities, which might be immunogenic to human. Enzymatic digestion and forced degradations are necessary to characterize the monoclonal antibodies, and SEC columns need to be rightfully selected based on the separation goal. Mobile phase additives may be necessary to improve the separation, and few examples are discussed. Since online LC/MS is becoming popular, a mobile phase compatibility is needed as discussed in this chapter. Since size exclusion chromatography cannot separate the heterogenic species without much difference in hydrodynamic radii, an orthogonal technique is necessary. A similarly complimentary technique is also helpful for complete characterization of the monoclonal antibodies. SEC column developed, and optimization of surface and pore characteristics is critical for the separation of monoclonal antibodies and other proteins by HPLC and UHPLC instrument. With the advancement of UHPLC, an easy method transfer between HPLC and UHPLC is becoming necessary. Ease of method transfer using a column with dual functionality for its use in both HPLC and UHPLC is helpful. Desalting using a conventional SEC HPLC column can be very useful in removing the unwanted salts and additives. A robust separation with excellent reproducibility needs a size exclusion chromatography column with optimized packing as well as the knowledge about the tips and tricks to maintain the column lifetime.

Acknowledgements

Author details

Atis Chakrabarti

Address all correspondence to: atis.chakrabarti@tosoh.com

Tosoh Bioscience LLC, USA

References

[1] Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th ed. New York: Garland Science; 2001

[2]   Elgert KD. Immunology: Understanding the Immune System, Chapter 04 Copyright ©. John Wiley & Sons, Inc; 1998

[3]   Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: From structure to effector functions. Frontiers in Immunology. 2014 Oct 20;**5**:520

[4]   Starkie DO, Compson JE, Rapecki S, Lightwood DJ. Generation of recombinant monoclonal antibodies from immunised mice and rabbits via flow Cytometry and sorting of antigen-specific IgG+ memory B cells. PLoS One. March 29, 2016

[5]   Morrow PR, Rennick DM, Leung CY, Benjamini E. The antibody response to a single antigenic determinant of the tobacco mosaic virus protein: Analysis using monoclonal antibodies, mutant proteins and synthetic peptides. Molecular Immunology. Apr 1984;**21**(4):301-309

[6]   Beerli RR, Bauer M, Buser RB, Gwerder M, Muntwiler S, Maurer P, Saudan P, Bachmann MF. Isolation of human monoclonal antibodies by mammalian cell display. PNAS. September 23, 2008;**105**(38):14336-14341

[7]   Reichert JM. Antibodies to watch in 2013: Mid-year update. MAbs. 2013;**5**:513-517

[8]   Yang X, Wang F, Zhang Y, Wang L, Antonenko S, Zhang S, Zhang YW, Tabrizifard M, Ermakov G, Wiswell D, Beaumont M, Liu L, Richardson D, Shameem M, Ambrogelly A. Comprehensive analysis of the therapeutic IgG4 antibody Pembrolizumab: Hinge modification blocks half molecule exchange in vitro and in vivo. Journal of Pharmaceutical Sciences. Dec 2015;**104**(12):4002-4014

[9]   Hong P, Koza S, ESP B. Size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. Journal of Liquid Chromatography and Related Technologies. Nov 2012;**35**(20):2923-2950

[10] Coppola G, Underwood J, Cartwright G, Hearn MT. High-performance liquid chromatography of amino acids, peptides and proteins. XCIII. Comparison of methods for the purification of mouse monoclonal immunoglobulin M autoantibodies. Journal of Chromatography. 1989 Aug 4;**476**:269-290

[11] https://www.ncbi.nlm.nih.gov/pubmed

[12] Haberger M, Leiss M, Heidenreich A-K, Pester O, Hafenmair G, Hook M, Bonnington L, Wegele H, Haindl M, Reusch D, Bulau P. Rapid characterization of biotherapeutic proteins by size-exclusion chromatography coupled to native mass spectrometry. MAbs. 2016;**8**(2):331-339

[13] Ackers GK. Molecular exclusion and restricted diffusion processes in molecular-sieve chromatography. Biochemistry. 1964;**3**(5):723-730

[14] Eksteen R, Pardue KJ. Modified silica-based packing materials for size exclusion chromatography. In: Wu C-s, editor. Handbook of Size Exclusion Chromatography. Vol. 69. NY: Marcel Dekker; 1995. p. 453

[15] Arakawa T, Philo JS, Ejima D, Tsumoto K and Arisaka F. Bioprocess International, 2006 v4. (10) 42–43 (November 2006)

[16] Benner C, Chakrabarti A. Size exclusion chromatography column with larger exclusion limit useful for the separation of biomolecules using UHPLC and HPLC. Abstract #120–1, Session: Bioanalytical: LC Techniques; PITTCON 2016 Oral Presentation

[17] Separation Report SR 074, Tosoh Bioscience; visit: https://www.separations.us.tosohbioscience.com/literature

[18] Fesinmeyer RM, Hogan S, Saluja A, Brych SR, Kras E, Narhi LO, Brems DN, Gokarn YR. Effect of ions on agitation- and temperature-induced aggregation reactions of antibodies. Pharmaceutical Research. April 2009;**26**(4):903-913

[19] Wakankar A, Chen Y, Gokarn Y, Jacobson FS. Analytical methods for physicochemical characterization of antibody drug conjugates. MAbs. March/April 2011;**3**(2):161-172

[20] Amano M, Hasegawa J, Kobayashi N, Kishi N, Nakazawa T, Uchiyama S, Fukui K. Specific racemization of heavy-chain Cysteine-220 in the hinge region of immunoglobulin gamma 1 as a possible cause of degradation during storage. Analytical Chemistry. 2011; **83**:3857-3864

[21] Stathopulos PB, Scholz GA, Hwang Y-M, JAO R, Lepock JR, Meiering EM. Sonication of proteins causes formation of aggregates that resemble amyloid. Protein Science. Nov 2004;**13**(11):3017-3027

[22] https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm073373.pdf

[23] Fischer N, Elson G, Magistrelli G, et al. Exploiting light chains for the scalable generation and platform purification of native human bispecific IgG. Nature Communications. 2015;**6**:7113

[24] Blatt NB, Bill RM, Glick GD. Characterization of a unique anti-DNA hybridoma. Hybridoma. Feb 1998;**17**(1):33-39

[25] Kungulovski G, Jeltsch A. Quality of histone modification antibodies undermines chromatin biology research. Protein Engineering, Design & Selection. 2015;**28**(10):303-305

[26] Zack DJ, Wong AL, Stempniak M, Weisbart RH. Two kappa immunoglobulin light chains are secreted by an anti-DNA hybridoma: Implications for isotypic exclusion. Molecular Immunology. Dec 1995;**32**(17–18):1345-1353

[27] Steve J, Chakrabarti A. High throughput and highly reproducible sub-separation of proteins and antibodies using size exclusion chromatography. Tosoh Bioscience LLC; Poster #212, Session – HPLC of Peptides, Proteins and Other Biomolecules Eastern Analytical Symposium 2014

[28] Application Note AN95 Tosoh Bioscience; https://www.separations.us.tosohbioscience.com/HPLC_Columns/id-8306/TSKgel_UP-SW3000

[29] Majors R. Current trends in column usage. LCGC. Jan 1, 2012

[30] Chakrabarti A. Troubleshooting tricks and tips about column maintenance and Improving column lifetime of aqueous size exclusion chromatography columns m Poster. HPLC 2012. Anaheim, CA

[31] Chakrabarti A. Three novel prototype SEC columns for the separation of an antibody monomer from its dimer, higher aggregates, and antibody fragments Poster – Fall ACS. 2012

[32] Application note: AN 97; visit: https://www.separations.us.tosohbioscience.com/HPLC_Columns/id-8306/TSKgel_UP-SW3000 Tosoh Bioscience

[33] Separation Report SR 046. visit: https://www.separations.us.tosohbioscience.com/literature

[34] Gayen-Betal S, Chakrabarti A. Separation of BSA monomer from its dimer and aggregates using a 125 Å pore size diol coated 7.8 mm ID × 30 cm, 5 μm gel filtration column. Poster, HPLC 2012 Anaheim, CA

[35] Kawai Y, Yamasaki H and Moriyama H. Separation Center, TOSOH Corporation, Kaisei-cho 4560 Shunan, Yamaguchi 746–8501, Japan; New silica-based SEC columns designed for the separation of mAb monomers and their impurities; Poster – PITTCON 2012

[36] Hawe A, Wiggenhorn M, van de Weert M, Garbe JH, Mahler HC, Jiskoot W.; Forced degradation of therapeutic proteins, Journal of Pharmaceutical Sciences Mar 2012;**101**(3): 895-913

[37] Hughes H, Morgan C, Brunyak E, Barranco K, Cohen E, Edmunds T, Lee KA. Multi-tiered analytical approach for the analysis and quantitation of high-molecular-weight aggregates in a recombinant therapeutic glycoprotein. The AAPS Journal. June 2009;**11**(2):335-341

[38] Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. Nature Protocols. 2006;**1**(6):2876-2890

[39] Chakrabarti A et al. Separation of Monoclonal Antibody and Protein Aggregates Induced by Thermal Denaturation, using a Novel Size Exclusion Chromatography Column, Poster Fall ACS. 2013

[40] Chakrabarti A et al Characterization of Two Novel Analytical Chromatographic Columns for Orthogonal Analysis of Monoclonal Antibodies and Protein Aggregates and Their Isoforms, Poster ISPPP 2013

[41] Chakrabarti A. A brief overview of the analysis of biomolecules using gel filtration chromatography (GFC): common issues in method development and troubleshooting Oral Presentation, PITTCON 2012

[42] Benner C, Chakrabarti A, et al. Characterization Of New 2 μm Particle Size, 25 nm Pore Size Analytical Size Exclusion Chromatography Column With Larger Exclusion Limit Useful For The Separation Of Biomolecules Using UHPLC And HPLC–Poster, ESF New York Biotechnology Symposium 2016

[43] Hashimoto Y, Nishi T, Muranaka K, Araki K, Mitoma Y, Higley TJ, Nakatani S. The Characteristic of New Desalting Column for Biomolecule. PITTCON: Poster; 2005

[44] Application Note AN064, Separation of Nucleobases using TSKgel® SuperSW mAb HTP column in HILIC mode, visit https://www.separations.us.tosohbioscience.com/literature