TOSOH

BIOPHARMACEUTICAL APPLICATION NOTEBOOK

(U)HPLC ANALYSIS OF BIOMOLECULES

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EDITORIAL
DEAR READER

High performance liquid chromatography (HPLC) and, increasingly, ultra-high performance liquid chromatography (UHPLC) are the analytical workhorses of the pharmaceutical industry. All stages of the product's lifecycle, from early development until production and stability testing need chromatographic analysis to characterize and quantify target molecules.

Today, biopharmaceuticals are the fastest growing product segment of the pharmaceutical industry. A thorough characterization of therapeutic biomolecules is a key task for the successful submission of data for regulatory approvals of new drugs, no matter whether biologic, biosimilar or biobetter. Quality control needs effective analytical tools that allow fast determination of critical quality attributes of the various kinds of biopharmaceuticals, such as monoclonal antibodies (mAbs) and other therapeutic proteins. With new biopharmaceutical formats, such as bispecific mAbs, antibody fragments and antibody-drug-conjugates (ADCs) in the pipeline, rapid and thorough characterization will become even more important.

Size exclusion chromatography (SEC) and ion exchange chromatography (IEC) are typical modes for the separation of proteins in native form. They are routinely used for the characterization of biotherapeutics. Especially SEC has become a Swiss-army knife for protein aggregate determination. It can virtually be considered a platform – quick and straightforward. Reversed phase (RPC) and hydrophilic interaction liquid chromatography (HILIC) are used to characterize peptides or oligosaccharide chains after enzymatic cleavage. Detection is usually performed by UV, fluorescence, light scattering or mass spectrometry.

TSKgel® UHPLC and HPLC columns are renowned for their quality and reliability and cover all common modes of liquid chromatography, including ion exchange (IEX), hydrophobic interaction (HIC), reversed phase, hydrophilic interaction (HILIC), size exclusion (SEC), and affinity. They are popular in the biotech and biopharmaceutical industry and are used in R&D, method development, production, quality control and stability testing.

This application notebook compiles more than twenty application notes covering important aspects of biopharmaceutical analysis such as protein aggregation, charge isoforms and glycosylation as well as determination of drug-to-antibody ratio (DAR) of ADCs. The last section of the notebook provides tips and tricks to improve chromatographic separation of biomolecules.

ENJOY READING AND STAY INFORMED.
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IMPRESSUM

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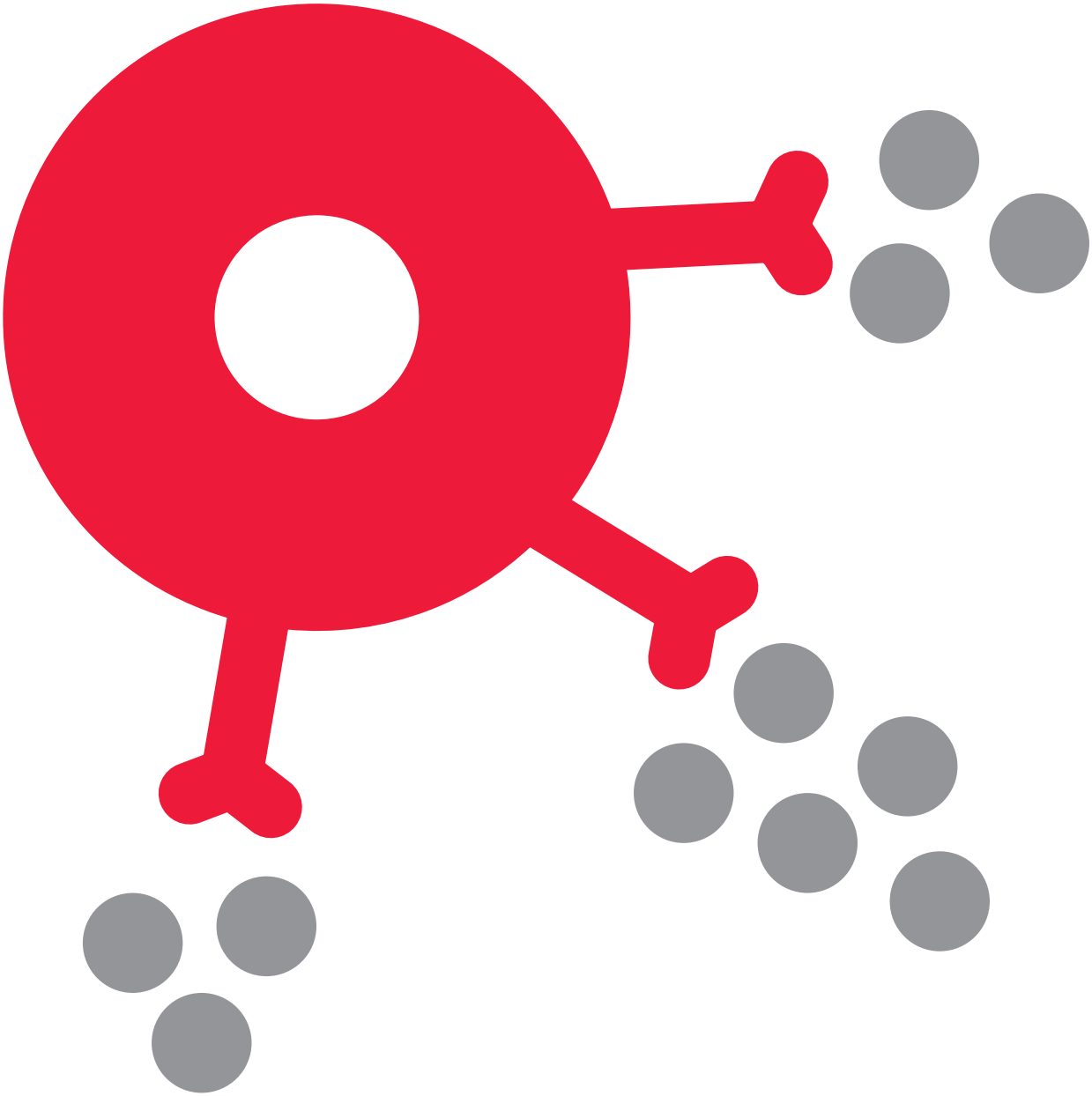

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APPLICATION NOTE

HIGH SPEED AND RESOLUTION SEC ANALYSIS OF mAbs USING TSKgel SuperSW mAb COLUMNS

Size exclusion chromatography (SEC) is the standard method for aggregate and fragment analysis of monoclonal antibodies in biopharmaceutical QC. A new series of silica-based SEC columns was engineered to provide shorter analysis time or higher resolution than standard columns for the separation of fragments, monomers and dimers.

Antibody therapeutics are enjoying high growth rates in the biopharmaceutical market, the major areas of therapeutic application being cancer and immune/inflammation-related disorders including arthritis and multiple sclerosis. In 2010, four of the top ten best-selling global drug brands were monoclonal antibodies (mAbs). The characterization of these complex biomolecules is a major challenge in process monitoring and quality control. The main product characteristics to be monitored are aggregate and fragment content, glycosylation pattern and charged isoforms. The standard method used in biopharmaceutical QC for mAb aggregate and fragment analysis is size exclusion chromatography (SEC). A new series of silica based HPLC columns can be applied to either increase speed or improve resolution of the separation of antibody fragments, monomers and dimers.

EXPERIMENTAL CONDITIONS

IgG was digested with papain over 24 hours. The fragmentation process was monitored by analyzing 10 or 5 µl aliquots of the sample.

Mobile phase:	200 mmol/L phosphate buffer + 0.05% NaN_3, pH 6.7
Flow rate:	A & B: 1.0 ml/min C: 0.35 mL/min
Injection vol.:	A & B: 10 µl; C: 5 µL
Temperature:	25°C
Detection:	UV @ 280 nm
Samples:	10 g/L IgG digested with papain for 0-24 hr
Columns:	A: TSKgel G3000SWXL, 7.8 mm ID x 30 cm B:TSKgelSuperSWmAbHR,7.8mmIDx30cm C:TSKgelSuperSWmAbHTP,4.6mmIDx15cm

SEPARATION OF mAb FRAGMENTS, MONOMERS AND DIMERS

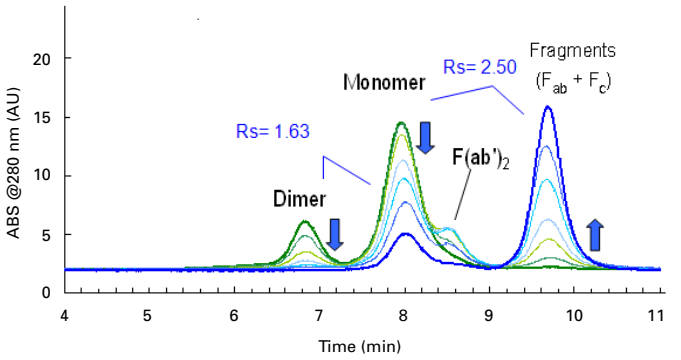

Figure 1A

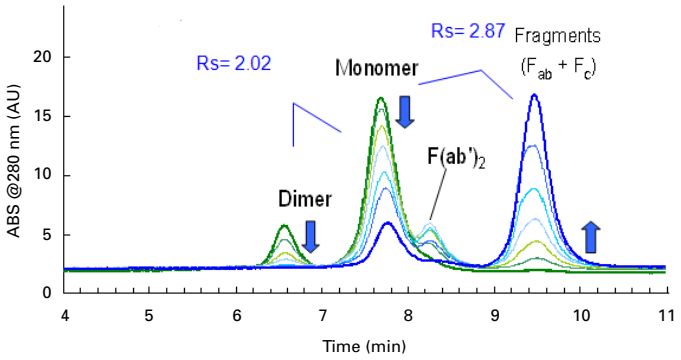

Figure 1B

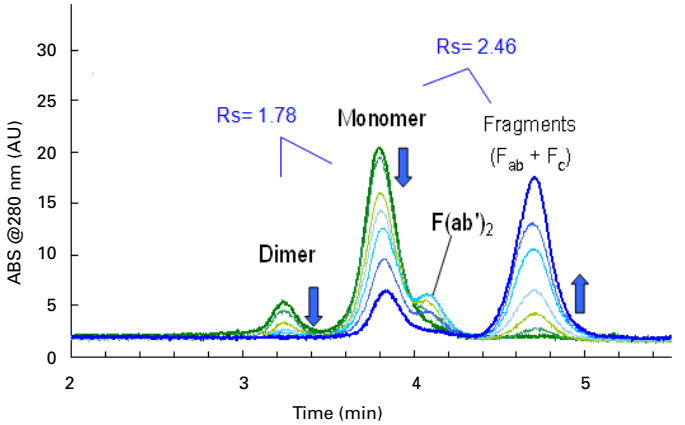

Figure 1C

Separation of antibody fragments, monomers and dimers by SEC A: TSKgel G3000SWXL, 7.8 mm ID x 30 cm; B: TSKgel SuperSW mAb HR, 7.8 mm ID x 30 cm C: TSKgel SuperSW mAb HTP, 4.6 mm ID x 15 cm

RESULTS

Figure 1A shows the separation of a papain digested immunoglobulin G sample on a TSKgel G3000SWXL column, which is applied as the standard SEC column in routine analysis of aggregates in many QC and R&D labs. Figure 1B demonstrates that the resolution of the separation can be improved by using the new TSKgel SuperSW mAb HR (HR stands for 'High Resolution') with 4 micron silica particles. This column provides higher resolution than the conventional column at the same analysis time. Using the TSKgel SuperSW mAb HTP (HTP stands for 'High Throughput'), a short semi-micro column packed with the same 4 micron particles as SuperSW mAb HR, dimer/ monomer/ and fragments were separated at the same resolving power as on the conventional column but in half the analysis time (Figure 1C).

SUMMARY

Size exclusion chromatography (SEC) is a common method for the separation of antibody monomer from dimer, aggregates, or degradation products on the basis of molecular size. Two novel SEC columns designed for antibody separation exhibit reduced analysis time while achieving baseline separation or enhanced resolution between monomer and dimer.

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APPLICATION NOTE

Increased Monoclonal Antibody Resolution with TSKgel® UP-SW3000 Columns

INTRODUCTION

The antibody therapeutics market is enjoying high growth rates, the major areas of therapeutic application being cancer and immune/inflammation-related disorders including arthritis and multiple sclerosis. In 2013, six of the top ten best-selling global drug brands were monoclonal antibodies (mAbs) and more than 400 mAbs were in clinical trials. The characterization of these complex biomolecules is a major challenge in process monitoring and quality control. The main product characteristics to be monitored are aggregate and fragment content, glycosylation pattern and charged isoforms.

The standard method used in biopharmaceutical QC for mAb aggregate and fragment analysis is size exclusion chromatography (SEC). TSKgel G3000SWXL columns have been the industry standard for quality control of mAbs by SEC for decades. With the introduction of TSKgel UP-SW3000, 2 µm silica-based UHPLC/HPLC columns, increased speed and higher resolution can be achieved for the separation of antibody fragments, monomers, and dimers. These columns feature the same pore size (25 nm) as the renowned TSKgel G3000SWXL columns while improving resolution through a smaller particle size.

RESULTS AND DISCUSSION

Figure 1 demonstrates the advantages of the TSKgel UP-SW3000 column for mAb analysis versus the use of a TSKgel G3000SWXL column. The TSKgel UP-SW3000 column offers higher resolution of both the high molecular weight (HMW) species and the Fab/c on the low molecular weight side. In addition, the analysis was completed in half the run time since the TSKgel UP-SW3000 column was used on a UHPLC system.

COMPARISON OF mAb ANALYSIS USING TSKgel G3000SWXL AND UP-SW3000 COLUMNS

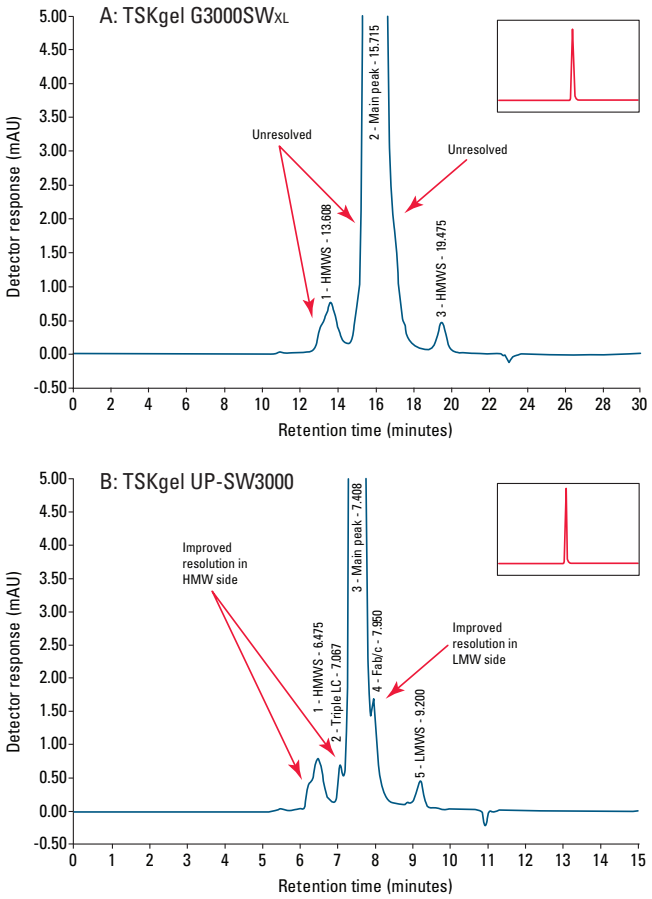

Figure 1

Columns: A. TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm
B. TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Instruments: A. Agilent 1260
B. Dionex UltiMate® 3000RS UHPLC System
Mobile phase: 0.2 mol/L potassium phosphate/0.25 mol/L KCl, pH 6.2
Flow rate: A. 0.5 mL/min, B. 0.35 mL/min
Detection: UV @ 280 nm
Temperature: A. and B. 25 °C
Injection vol.: A. 50 µL, B. 10 µL

The TSKgel UP-SW3000 column is suited for the separation of antibody dimer, monomer, and fragments in one run with ultra-high resolution, as shown in Figure 2. One TSKgel UP-SW3000 achieves even higher resolution than two TSKgel G3000SWXL columns connected in series.

COMPARISON OF mAb ANALYSIS USING TWO TSKgel G3000SWXL COLUMNS VERSUS ONE UP-SW3000 COLUMN

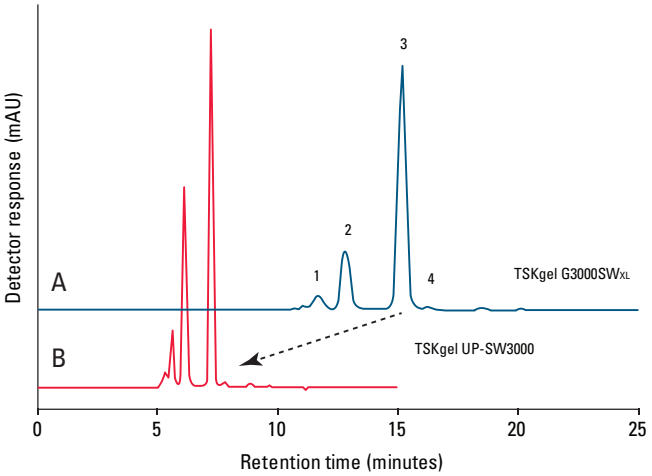

Figure 2

Column	Rs (peak 1/2)	Rs (peak 2/3)	Rs (peak 3/4)
A: TSKgel G3000SWXL x 2	1.60	3.63	1.77
B: TSKgel UP-SW3000	2.16	5.02	2.56

Columns: A. TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm × 2
B. TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase: 100 mmol/L phosphate buffer + 100 mmol/L sodium sulfate + 0.05% sodium azide, pH 6.7
Flow rate: A. 1.0 mL/min, B. 0.35 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C
Injection vol.: 10 µL
Samples: mouse-human chimeric IgG, monoclonal
1. trimer, 2. dimer, 3. monomer, 4. fragment

CONCLUSION

The TSKgel UP-SW3000 column is ideally suited for the analysis of aggregate and fragment contents of antibody preparations. It features the same pore size as the renowned TSKgel G3000SWXL column while improving resolution through a smaller particle size.

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APPLICATION NOTE

UHPLC ANALYSIS OF IMMUNOGLOBULINS WITH TSKgel® UP-SW3000 SEC COLUMNS

Antibody therapeutics are enjoying high growth rates, the major areas of therapeutic application being cancer and immune/inflammation-related disorders including arthritis and multiple sclerosis. In 2013, six of the top ten best-selling global drug brands were monoclonal antibodies (mAbs) and more than 400 monoclonals were in clinical trials. The characterization of these complex biomolecules is a major challenge in process monitoring and quality control. The main product characteristics to be monitored are aggregate and fragment content, glycosylation pattern and charged isoforms.

The standard method used in biopharmaceutical QC for mAb aggregate and fragment analysis is size exclusion chromatography (SEC). A new series of 2 micron silica based UHPLC columns with 25 nm (250 Å) pore size can be applied to either increase speed or improve resolution of the separation of antibody fragments, monomers, and dimers.

CALIBRATION CURVES

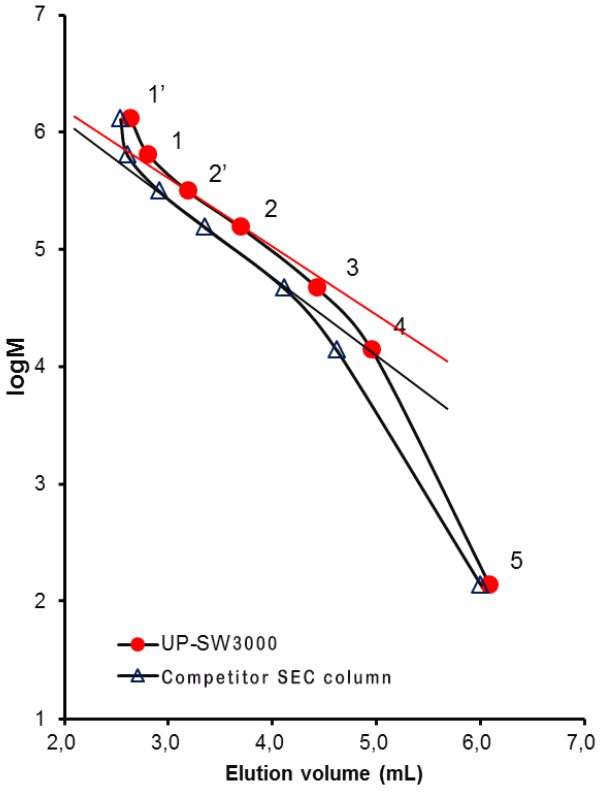

Figure 1

EXPERIMENTAL CONDITIONS

Columns:	TSKgel UP-SW3000 (P/N 0023449), 2 µm Competitor Protein SEC Column, 1.7 µm
Column size:	4.6 mm ID x 15 cm
Eluent:	100 mmol/L phosphate buffer (pH 6.7) + 100 mmol/L sodium sulfate + 0.05% NaN_3
Flow rate:	0.35 mL/min
Temperature:	25 °C
Detection:	UV @ 280 nm, micro flow cell
Sample (Calibration):	
	1. thyroglobulin, 640,000 Da (1' thyroglobulin dimer); 2. γ-globulin, 155,000 Da (2' γ-globulin dimer); 3. ovalbumin, 47,000 Da; 4. ribonuclease A, 13,700 Da; 5. p-aminobenzoic acid, 137 Da
Inj. Volume:	5 µL
Sample (mAb Analysis):	
	therapeutic mAb (mouse-human chimeric) 1: trimer; 2: dimer; 3: monomer ; 4: fragment
Inj. Volume:	10 µL

RESULTS

Figure 1 shows the calibration curves of the new TSKgel UP-SW 3000 2 µm column and a commercially available 1.7 micron UHPLC column. The calibration of TSKgel UP-SW3000 shows a shallower slope in the region of the molecular weight of γ-globulin. These differences in the separation range and steepness of the curves are related to a slight difference in pore size (25 nm for TSKgel versus 20 nm for the 1.7 µm material).

The separation of an antibody sample on the new 2 µm packing compared to the competitor UHPLC column is depicted in figure 2 . The difference in pore sizes results in a better separation in the molecular weight range of antibodies, fragments and aggregates. Based on the wider separation window, the resolution between monomer and dimer, as well as dimer and trimer is slightly higher with TSKgel UP-SW3000 although particle size is slightly larger than in the competitor column. Moreover, also the fragment peak is more clearly separated from the monomer peak.

CONCLUSION

TSKgel UP-SW 3000 is ideally suited for the analysis of aggregate and fragment contents of antibody preparations. It features the same pore size as the renowned TSKgel G3000SWXL and TSKgel Super mAb columns while improving resolution through a smaller particle size. Based on the optimized pore size and the high degree of porosity, the resolution in the molecular weight range of immunoglobulins is even superior to a competitive UHPLC column with slightly smaller particle and pore size.

COMPARISON OF ANTIBODY ANALYSIS RESULTS

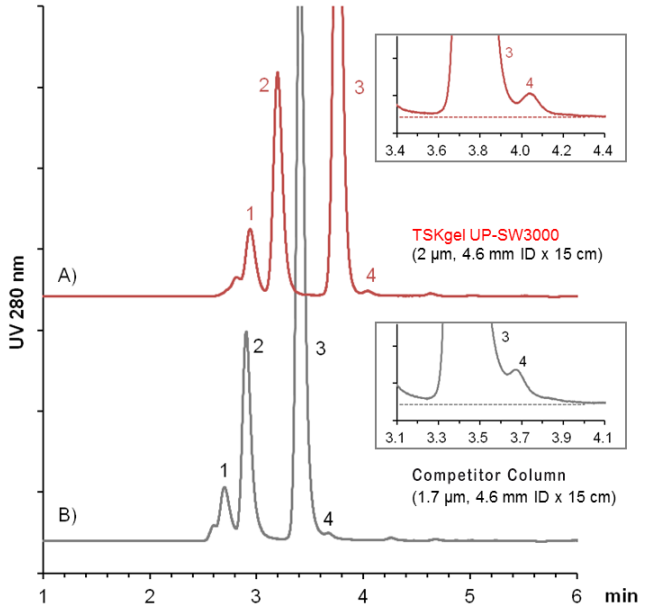

Figure 2

mouse-human chimeric mAb
1: trimer; 2: dimer; 3: monomer ; 4: fragment

Column	RS (peak 1/2)	RS (peak 2/3)
TSKgel UP-SW3000 2 µm	1.52	3.56
Competitor UHPLC-SEC 1.7 µm	1.25	3.47

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APPLICATION NOTE

RAPID AND ACCURATE THERAPEUTIC mAb AGGREGATE ANALYSIS USING TSKgel® UP-SW3000, 2 µm, SEC COLUMN

HPLC analytical size exclusion chromatography (SEC) columns are widely used to determine the ratio of aggregates, dimers, monomers, and fragments in monoclonal antibodies (mAbs). Columns are expected to deliver high resolution, excellent reproducibility in a short analysis time. In order to achieve these parameters, SEC columns must have the appropriate particle size, pore size, good bonding chemistry, and suitable column dimensions. In addition, the columns must be packed well. Traditionally, SEC columns with 30 cm length are used for high resolution analysis because the length allows different molecular sizes to be separated with a longer run time. However, because of the long length, a typical analysis can take up to 30-40 minutes for each analysis. With the demands for high sample throughput, there is a need for shorter analysis time. There are many available SEC columns with 15 cm length currently available for this usage. However, these columns typically suffer from low resolution.

This application describes the use of a 4.6 mm ID × 15 cm TSKgel UP-SW3000 SEC column for fast and accurate mAb aggregate analysis without compromising the quality of the aggregate determination or reproducibility. Unlike many other available 15 cm length SEC columns, these columns are packed such that they can be operated with both HPLC and UHPLC systems. The 4.6 mm ID × 15 cm TSKgel UP-SW3000 SEC column has a particle size of 2 µm and a 25 nm pore size. The particles are coated with a hydrophilic diol-type bonded phase in order to minimize the interaction between the silica surface and proteins. The column is designed to be operated with a simple and well-established method (sodium phosphate mobile phase, pH 6.8). A comparison study was done between a TSKgel UP-SW3000, 15 cm column and a 30 cm length column, both 4.6 mm ID. Results show that the run time of the 15 cm column was completed in 4 minutes without compromising the resolution of the chromatogram.

EXPERIMENTAL HPLC CONDITIONS

Columns:	TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm (0023448) TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm (0023449)
Mob. phase:	100 mmol/L sodium phosphate buffer, pH 6.7, +100 mmol/L sodium sulfate + 0.05% sodium azide
Gradient:	Isocratic
Flow rate:	As indicated in each chromatogram
LC system:	Ultimate® 3000RS UHPLC system
Detection:	UV @ 280 nmTemperature: 25 °C
Inj. vol.:	10 µL
Sample:	mAb (0.4 mg/mL)

RESULTS

Figure 1 shows the protein standard calibration curve data that was generated using the TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm SEC column. The column was run with a simple aqueous mobile phase (sodium phosphate buffer, pH 6.8) as typically reported in literature for SEC separations. The data demonstrates that the TSKgel UP-SW3000 column has a broad and linear resolving range of molecular weights. The shallow slope around the molecular weights of thyroglobulin, γ-globulin to p-aminobenzoic acid suggests that the particles of the column have an optimized pore size for separating aggregates, dimer, monomer, and fragments of proteins with a molecular weight of approximately 150 kDa such as mAb.

STANDARD CALIBRATION CURVE OF QC PROTEIN STANDARD MIXTURE GENERATED BY TSKgel UP-SW3000, 4.6 MM ID × 15 CM

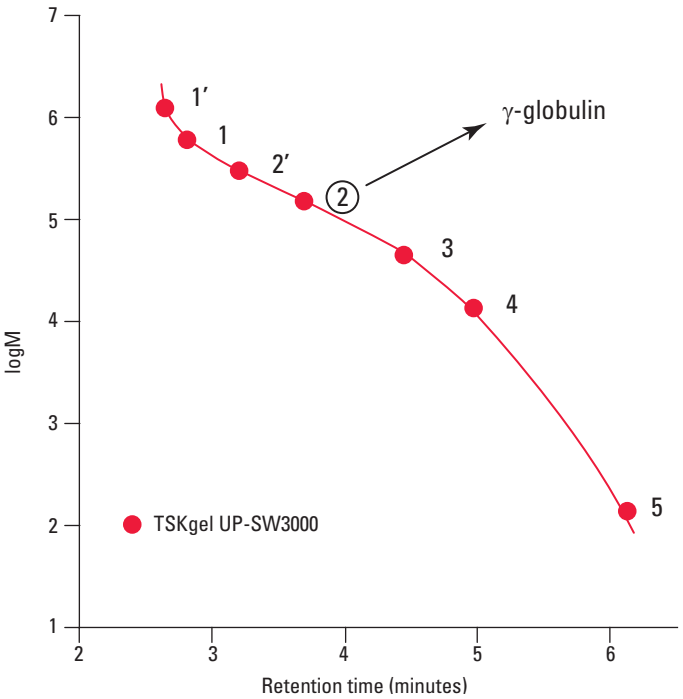

Figure 1

Column: TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm
Mobile phase: 100 mmol/L phosphate buffer, pH 6.7,
+ 100 mmol/L Na_2SO_4 + 0.05% NaN3
Flow rate: 0.35 mL/min; Detection: UV @ 280 nm
Temperature: 25 °C; Injection vol: 5 µL
Samples: 1′ Thyroglobulin dimer
1. Thyroglobulin, 640,000 Da
2′ γ-globulin dimer
2. γ-globulin, 155,000 Da
3. Ovalbumin, 47,000 Da
4. Ribonuclease A, 13,000 Da
5. p-aminobenzoic acid, 137 Da

Figure 2 shows the separation comparison data for mAb between a 30 cm TSKgel UP-SW3000 and a 15 cm length column. Both columns were operated under the same mobile phase conditions and flow rate. The results indicate that the 15 cm TSKgel UP-SW3000 column provides a similar profile to the 30 cm column with 50% less run time and 50% lower backpressure at a typical flow rate of 0.35 mL/min (See Figure 2). The resolution between dimer and monomer is slightly less with the 15 cm column but it is still above the resolution guidelines from the USP monogram (1.2 resolution is acceptable). In addition, when the 15 cm column is operated at the typical flow rate of 0.35 mL/min, the backpressure is only 11 MPa. Therefore, these columns can be used with both HPLC and UHPLC systems.

COMPARISON OF mAb AGGREGATES ANALYSIS BETWEEN TSKgel UP-SW3000, 15 CM AND 30 CM COLUMNS USING THE SAME MOBILE PHASE CONDITIONS AND FLOW RATE

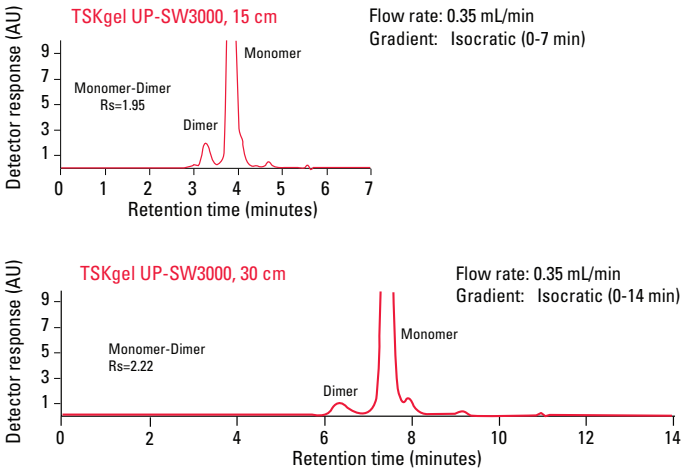

Figure 2

FAST ANALYSIS OF mAb SAMPLE USING TSKgel UP-SW3000, 4.6 MM ID × 15 CM

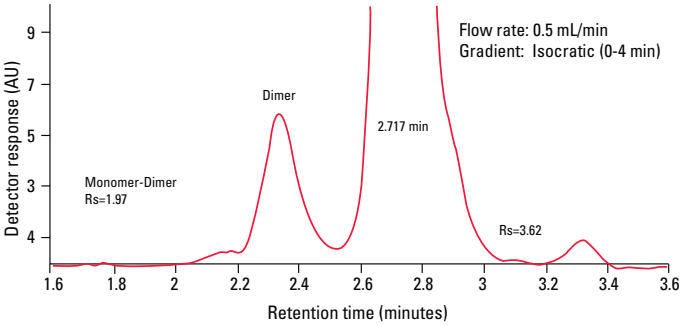

Figure 3

Figure 3 demonstrates the rapid aggregate determination of a mAb using a TSKgel UP-SW3000, 4.6 mm ID × 15 cm column operated at 0.5 mL/min. The figure shows that the analysis was completed in only 4 minutes, nearly a 4 times faster run time than the 30 cm length column (compare the run time of Figure 2, bottom panel to Figure 3). The resolution profile of the aggregates and monomer of mAb (Rs = 1.97) is still maintained at the acceptable range in the USP guideline. Results from 10 consecutive injections (Table 1) show that the TSKgel UP-SW3000, 15 cm column provides high reproducibility at a fast run time.

CONCLUSION

The above results demonstrate the broad and linear molecular weight resolving range of TSKgel UP-SW3000, 2 µm SEC columns. This, in turn, drives the accuracy, reliability and reproducibility for molecules of interest such as the monomer, dimer, and aggregates of mAbs. The comparison between a 15 cm and 30 cm TSKgel UP-SW3000 column using the same flow rate and operating mobile phase conditions showed that the 15 cm length column generates similar and acceptable resolution for aggregate analysis. At 0.5 mL/min flow rate, analysis can be completed within 4 minutes with acceptable resolution and at a low backpressure that allows TSKgel UP-SW3000 columns to be run in both HPLC and UHPLC systems.

10 CONSECUTIVE RUNS (OF mAb SAMPLE) YIELDED EXCELLENT REPRODUCIBILITY.

Monomer peak						
Injection #	Ret. time min.	Area mAU min	Height mAU	Width (50%) min	Asym. EP	Plates EP
1	2.717	16.72	155.460	0.093	1.26	4754
2	2.717	16.58	155.440	0.093	1.26	4762
3	2.717	16.62	155.780	0.093	1.26	4762
4	2.717	16.87	156.750	0.093	1.26	4740
5	2.717	16.91	157.360	0.093	1.26	4748
6	2.717	16.90	157.310	0.093	1.26	4749
7	2.717	16.75	157.190	0.093	1.26	4770
8	2.717	16.92	157.540	0.093	1.27	4758
9	2.717	16.94	157.910	0.093	1.27	4762
10	2.717	16.85	157.400	0.092	1.27	4780
11	2.717	16.77	156.840	0.093	1.28	4787
12	2.717	16.64	154.700	0.093	1.26	4748
13	2.717	16.73	155.360	0.093	1.26	4747
15	2.717	16.82	156.090	0.093	1.26	4742
Average	2.717	16.787	156.509	0.093	1.264	4758
Std Dev	0.000	0.119	1.014	0.000	0.006	13.907
%RSD	0.000	0.707	0.648	0.391	0.501	0.292

Table 1

APPLICATION NOTE

EFFICIENT SEC-UV-RI-MALS ANALYSIS OF PROTEIN AGGREGATES

Static light scattering in combination with size exclusion chromatography is a valuable tool for verifying purity of monoclonal antibodies (mAbs) as such, or as a quick check while the downstream processing takes place. Besides fluorescence detection, light scattering is one of the most sensitive methods to detect protein aggregates.

As a matter of their size, mAb aggregates produce scattered light more efficiently than they absorb UV light at 214 or 280 nm. Nevertheless, light scattering is not a plug-and-play technique, compared to simple UV detection. This application note provides some advice that may help to improve the signal to noise ratio and the robustness of the complete system. First of all, it is important to keep the whole system clean and to refresh the eluent frequently. Buffers without sodium azide should be changed daily. At least for protein applications, sodium azide does not cause any problems in the scattering signal. Therefore, it is recommended to add 0.05 % sodium azide to aqueous buffers in order to prevent bacterial growth. When working with organic solvents, proper degassing of the eluent is essential.

However, any eluent, either organic or aqueous, needs to be filtered thoroughly. Air bubbles and small particles will not be detected by UV but will disturb the light scattering signal. Changing in-line filters regularly, as well as filtering buffers through a 0.1 µm membrane prevent noisy baselines. Detector contamination due to impurities of the sample as such appears frequently. In this case, the system without the column should be rinsed with water containing 1 % SDS for 4-5 hours, followed by a quick purging step with water and an overnight washing step with a mixture of 20 % ethanol 80% water. Against persisting noise, the system can be flushed with a sample degrading solvent. Proteases for instance might help when working with proteins.

Noise generated by the HPLC column can be reduced by cleaning the column as described in the manual delivered with the column. Overloaded columns also release sample molecules independent from retention time, causing a noisy and drifting baseline. The TSKgel SWXL series can be cleaned efficiently by flushing overnight with 0.1 M Glycine/HCl buffer pH 3. The system flow rate also affects the noise level and should only be changed gradually. Equilibrating the column with 4-5 column volumes at operational flow stabilizes the baseline.

SEC-UV-RI-LS ANALYSIS OF A MONOCLONAL ANTIBODY

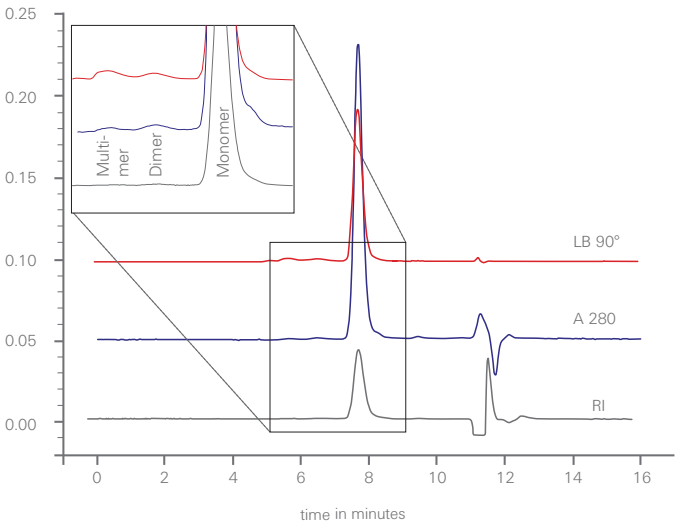

Figure 1

Column: TSKgel G3000SWxl, 5 µm, 7.8 mm ID x 30 cm L;
Flow rate 1 mL/min; Mobile phase: PBS; Inj. volume 20 µl;
Detection: 90° LS (red), RI (grey) & UV @ 280 nm (blue);
HPLC System: LC-20A prominence, Shimadzu;
MALS detector: minDAWNTM TREOS, Wyatt Technology Corp.

In fact, static light scattering is a multi-detector measurement. Therefore the concentration source, which is usually either UV absorption or the refractive index, is also important. Sodium azide absorbs UV at wavelengths typically used for protein detection. Hence, it is recommended to use the RI-Signal as source of concentration, also because the refractive index increment is approximately the same for all proteins. Nevertheless, the RI-signal does have disadvantages too. It is prone to solvent peaks, which disable measurements of molecules with the same retention time. Solvent peaks can be reduced by preparing samples in daily refreshed system solvent. Similar to the solvent peak, the injection peak is not related to the sample itself. The injection peak can be decreased by slowing down injection speed.

In general, the light scattering instrument is the first to indicate any unsteadiness in the system. System suitability tests with a standard sample can be used to check the system performance before starting an analysis sequence. Once the performance is ensured, an additional light scattering detector provides valuable information about the presence and structure of protein aggregates and the monomer itself as shown in Figure 1.

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APPLICATION NOTE

mAb AGGREGATE DETECTION - ANALYTICAL HIC AS AN ORTHOGONAL CHROMATOGRAPHIC APPROACH

ABSTRACT

During the last decades, mAbs have proved to be a very valuable medication for severe illnesses like autoimmune diseases and cancer. However, to ensure a successful therapy and the least possible side effects, a thorough investigation of potential aggregates is crucial. The quality of aggregates can be diverse in terms of physico-chemical and physiological properties. Besides a declined therapeutic effect, mAb aggregates may also be immunogenic. A detailed characterization of the different aggregate species requires resolution of the different species by an online analytical method, as aggregation is a dynamic process. Due to the rather hydrophobic nature of mAb aggregates, analytical HIC using 2.5 µm particles offers outstanding resolution of the different aggregates. Therefore, a targeted analysis of every single contained species is possible. Fluorescence detection and an applied light scattering device ensure maximum analysis sensitivity. Furthermore, we could show that the highly efficient non-porous resin allows a quantitative analysis, providing an actual back-up method for the verification of SEC results.

MATERIAL & METHODS

mAb AGGREGATION

MAb aggregation was performed by acidic incubation. The stock solution contained 5 mg mAb/ml, buffered in 0.1 M citrate, pH 6.1. To induce aggregation, the mAb was titrated to pH 2.7 using 1 M HCl. The solution was incubated for 1 h at room temperature.

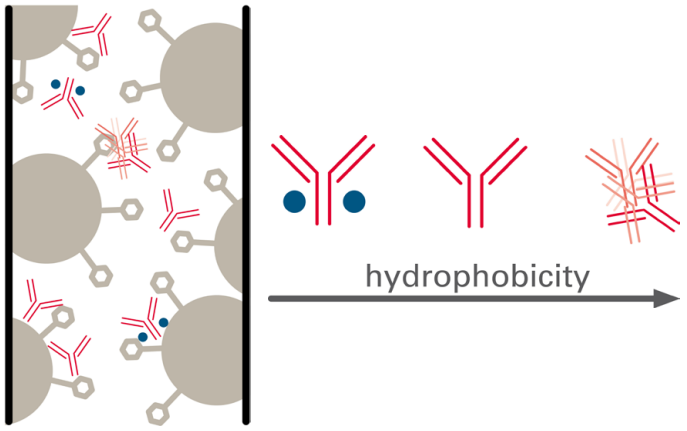


Figure 1

HIC column loaded with the mAb sample. The miscellaneous species behave differently in terms of hydrophobicity. Glycosylation decreases hydrophobicity, whereas aggregates are more hydrophobic than monomers.

Afterwards, the pH of the solution was increased to pH 6.5 by the addition 0.5 M disodium hydrogen phosphate. The aggregated mAb samples were stored at 4°C. Aggregation was accomplished on a daily base.

ANALYTICAL HIC

HIC was performed using two different column hardware formats: TSKgel Butyl-NPR 4.6 mm ID x 3.5 cm L and a 4.6 mm ID x 10 cm L. The shorter column was used in combination with fluorescence detection. The appropriate experiments were performed on a Dionex Ultimate 3000 RS system. A flow rate of 1 ml/min was applied. To induce hydrophobic interaction between the stationary phase and the mAb species, the loading buffer contained 3 M sodium chloride and 10 mM sodium phosphate, pH 7.0. 10 mM sodium phosphate, pH 7.0 comprised the elution buffer. 2 µg protein were injected. A linear gradient from 0% to 100% within 25 minutes was applied.

For HIC-MALS, the extended column hardware version was used. The column was connected to a Shimadzu Prominence HPLC system, including a Wyatt MiniDawn Treos light scattering device and a Wyatt Optilab TrEX refractometer. Flow rate was reduced to 0.7 ml/min. 10 µg mAb were injected. The sample was bound to the resin by applying either 3 M sodium chloride in 10 mM sodium phosphate buffer or 0.75 M ammonium sulfate, 0.5 M sodium sulfate and 10 mM sodium phosphate, both pH 7.0. 10 mM sodium phosphate, pH 7.0 or 2 M sodium chloride containing 30% methanol and 10 mM sodium phosphate, pH 7.0, were applied for the elution. The methanol containing elution buffer allowed a reproducible RI signal, which is necessary for molecular weight determination with static light scattering. This method is based on a two detector concept. On the one hand, the light scattering device provides the Rayleigh ratio, on the other hand a second device providing a concentration signal must be implemented into the system. Typical detectors measure the refractive index or the UV absorption. Both approaches have pros and cons; while the RI detector is a very general approach that is commonly used for isocratic chromatographic separations, using the UV signal might be more straightforward for non-isocratic chromatographic separations.

Both methods were employed to investigate the molecular weight. For the RI based approach, a linear gradient from 100% A to 60% A in 33 minutes was used, for the UV based approach a linear gradient from 100% A to 0% A in 40 minutes was used.

SEC

SEC was performed using a TSKgel G3000 SWXL column. 20 µg mAb or aggregated mAb, respectively, in a total volume of 20 µl were injected. A 0.1 M sodium phosphate buffer containing 0.1 M sodium sulfate and 0.05% sodium azide, pH 6.7 was used for the liquid phase. Applying 1 ml/min led to a analysis time of 15.5 minutes for one column volume.

DEGLYCOSYLATION

Deglycosylation was achieved by PNGase F from Elizabethkingia miricola. pH of the mAb stock solution was increased to 7.5 by addition of 0.1 M Tris/HCl, pH 8.0. 1 U was employed for 20 µg mAb. The reaction was incubated at 37 °C and 300 rpm. After every 24 hours aliquots of the reaction mix were analyzed using HIC with the fluorescence approach. For enzyme stability issues, the reaction was stopped after 72 hours. A blank mAb sample without enzyme was treated, respectively. Deglycosylation was also monitored by SDS-PAGE (data not shown).

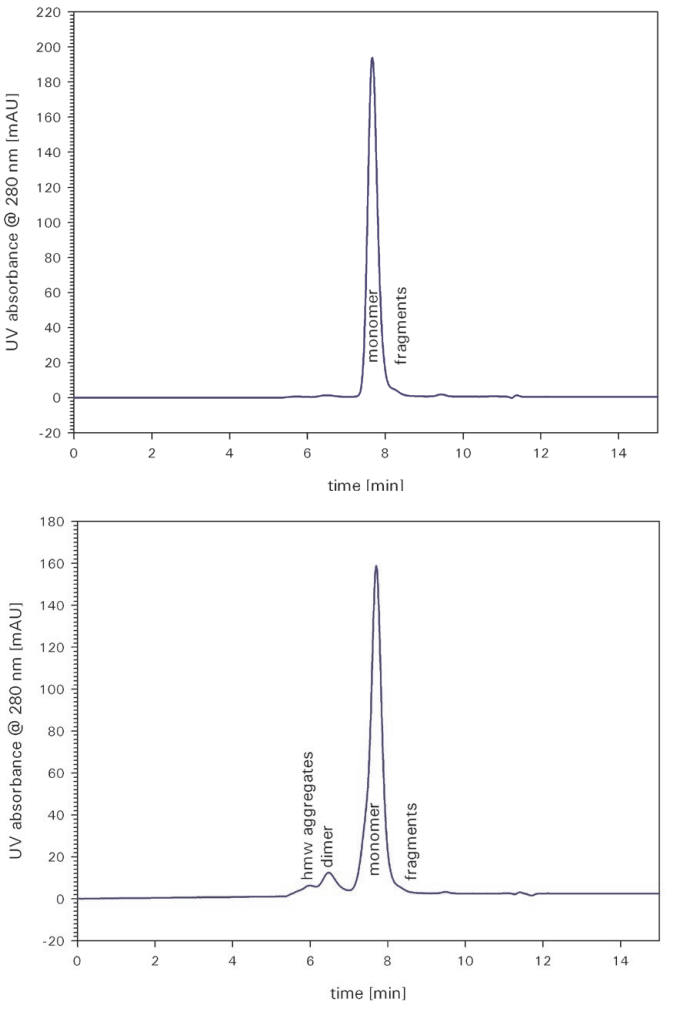


Figure 2 & 3

SEC chromatograms of the mAb and the aggregated mAb sample. Fragments, monomer and the various aggregate species can be seen.

RESULTS

SEC

SEC chromatograms of the mAb and the aggregated mAb sample are shown in Figure 2 and 3. The high molecular weight (hmw) aggregates elute first, followed by the dimer and the monomer.

FLUORESCENCE FOR SENSITIVE AGGREGATE DETECTION

As shown in Figure 4, the short column provides separation of fragments, a pre-monomer peak, the monomer peak and various aggregates. A first attempt to identifiy the various peaks separated by HIC was done according to the relative peak quantities resolved by SEC. Though, the pre-peak remains unresolved by SEC.

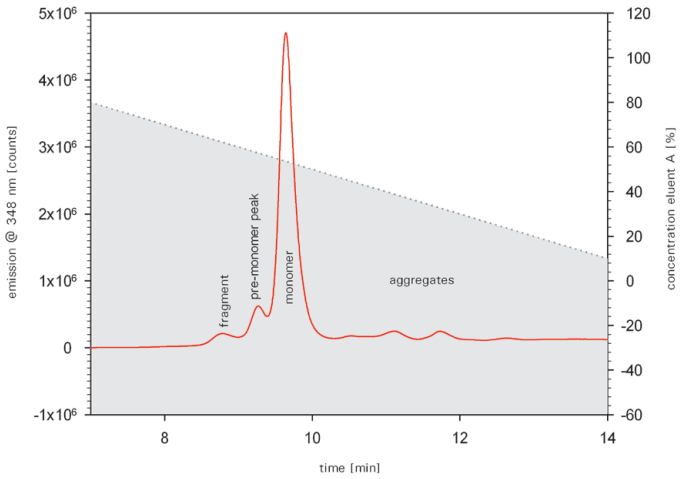


Figure 4

Aggregated mAb sample on TSKgel Butyl-NPR. Fragments, a pre-monomer peak, the monomer and aggregates can be resolved. A linear gradient was chosen.

MOLECULAR WEIGHT MEASUREMENTS

In order to investigate the pre-monomer peak more thoroughly, an online multi-angle light scattering detector was used. As this detector is less sensitive than a fluorescence detector, a bigger hardware version of TSKgel Butyl-NPR was chosen. The 4.6 mm ID x 10 cm L format allows crude protein loadings up to 10 µg, while the pre-monomer can still be observed as a shoulder of the monomer peak. An appropriate salt combination such as 0.75 M ammonium sulfate and 0.5 M sodium sulfate, pH 7 (Fig. 6) must be used. The UV-signal provides the concentration source for the molecular weight determination. These conditions do not allow RI based molecular weight determination, as the RI signal shows unsteadiness due to solvent mixing effects for above described conditions.

In consequence, aiming for the more generalized, RI based MALS approach requires an adjusted protocol. Relatively stable RI baselines can be achieved for a loading with 3 M sodium chloride and a salt containing elution buffer that consists of 2 M sodium chloride containing 30% methanol, both pH 7.0 (Fig. 5). However, resolution suffers from overloading and the detector caused conditions. The molecular weights determined with the RI and the UV based MALS measurements are shown in Table 1.

DEGLYCOSYLATION MONITORING

Aliquots of the deglycosylation reaction were analyzed using TSKgel Butyl-NPR. The appropriate chromatograms are shown in Figure 7 and 8. The blank control exhibits a reproducible chromatogram for aliquots of the sample before incubation was started and after 48 h at 37°C (fig. 8). Despite this behavior, a retention shift can be detected for the deglycosylation samples. The fragment peak, the pre-monomer peak and the monomer peak are shifted towards later elution, monitoring increasing hydrophobicity for all of the three species. No significant quantitative difference for the single mAb species can be obtained from these data.

DISCUSSION & CONCLUSION

HIC using nonporous resins offers another choice to the chromatographic toolbox for mAb characterization. Outstanding resolution of the miscellaneous mAb species potentially allows the detection of a glycosylation variant. A prototype TSKgel Butyl-NPR 4.6 mm ID x 10 cm L features higher capacity and therefore allows to apply light scattering as a detection method. Molar masses can be obtained from online measurements, providing information on the various mAb species.

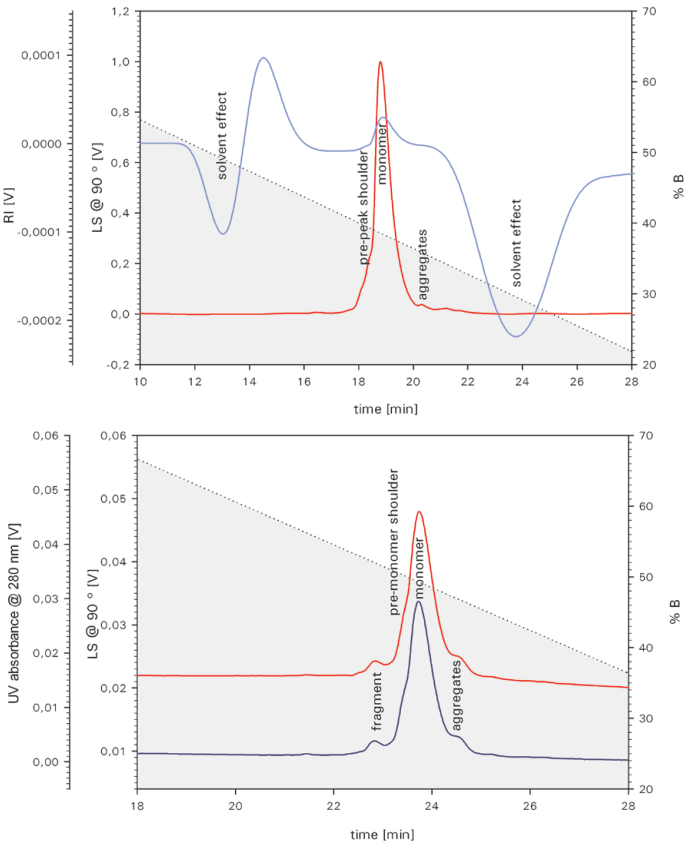


Figure 5 & 6

HIC-MALS chromatogram of the mAb. Fig. 5 shows the chromatogram for the RI based MALS, Fig. 6 shows the UV based MALS chromatogram. Different conditions were chosen due to varying detector requirements. Due to the relatively low RI response, the columns were slightly overloaded which causes the pre-monomer to affiliate to the monomer.

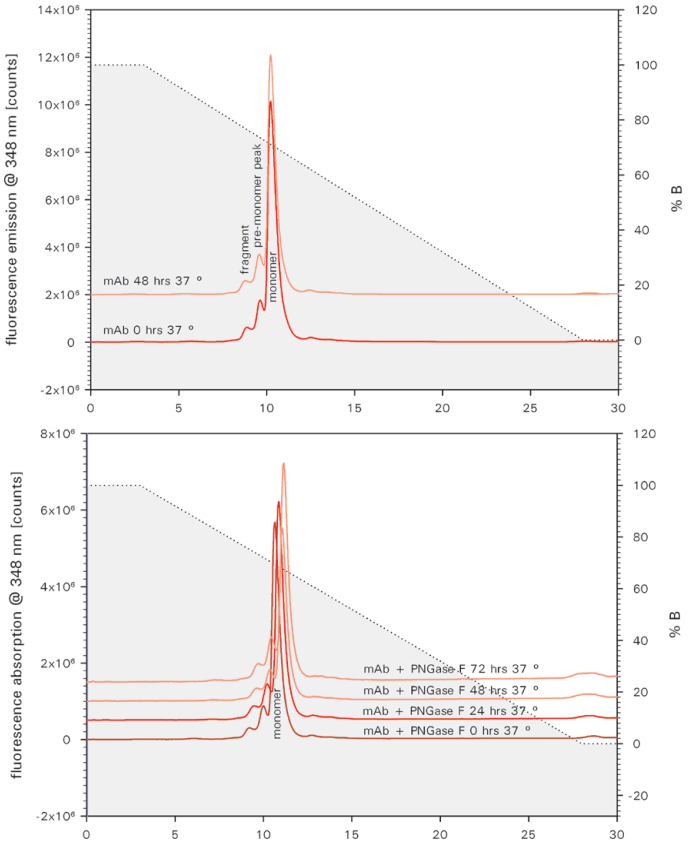


Figure 7 & 8

HIC was performed using two different column hardware formats: TSKgel Butyl-NPR 4.6 mm ID x 3.5 cm L and a 4.6 mm ID x 10 cm L. The shorter column was used in combination with fluorescence detection. The appropriate experiments were performed on a Dionex Ultimate 3000 RS system. A flow rate of 1 ml/min was applied. To induce hydrophobic interaction between the stationary phase and the mAb species, the loading buffer contained 3 M sodium chloride and 10 mM sodium phosphate, pH 7.0. 10 mM sodium phosphate, pH 7.0 comprised the elution buffer. 2 µg protein were injected. A linear gradient from 0% to 100% within 25 minutes was applied.

TABLE I

DETERMINED MOLECULAR WEIGHTS FOR THE MAB FRAGMENT, THE PRE-MONOMER AND THE MONOMER. MOLECULAR WEIGHTS WERE DETERMINED USING LIGHT SCATTERING. THE PRE-MONOMER CANNOT BE RESOLVED BY SEC.

Chromatography	Concentration Source	MW (kDa) Fragment	MW (kDA) pre-monomer	MW (kDa) Monomer
HIC				
TSKgel Butyl-NPR	UV-based detection	61	138	132
TSKgel Butyl-NPR	RI-based detection	99	151	150
SEC				
TSKgel G3000SWXL	RI-based detection	91	-	149

The necessary concentration source can be provided by a UV detector in case of a known extinction coefficient for the sample molecules. The results presented are predicated on an average IgG coefficient, which explains the deviation from the RI based results.

On the other hand, the RI based approach requires advanced fine tuning of the chromatographic separation to maintain resolution and peak shape, while doing the least possible changes to the liquid phase during sample elution. The herein presented multi-detector method leads to a resulting molecular mass of 150 kDa for the monomer peak and a molecular mass of 151 kDa for the potential glycosylation variant. A molecular weight of 99 kDa was determined for the fragment.

Retention times for all peaks are shifted towards higher hydrophobicity when PNGase F is used for glycosylation cleavage.

Concluding on this, the detected molecular weights and the qualitative SEC results of the mAb sample, the pre-monomer peak seems to be a glycosylation variant of the monomer. From these results, we deduce that the presented HIC analysis allows to separate certain glycosylation species of a mAb monomer. Further investigations on the cleaved glycosylation residues will be undertaken to confirm this.

TOSOH

APPLICATION NOTE

SEC/MS ANALYSIS OF A BISPECIFIC ANTIBODY

INTRODUCTION

More potent formats of monoclonal antibodies (mAbs), such as bispecific antibodies (bsAbs), are on the rise in the area of biotherapeutics. bsAbs recognize two different epitopes. This dual specificity increases the potency of these molecules compared to mAbs and expands the range of possible applications. bsAbs can be used to redirect T cells to tumor cells, block two different signaling pathways simultaneously, dually target different disease mediators, and deliver payloads to targeted sites. At this time, more than 50 bsAb products are currently undergoing clinical evaluation.

Characterization of bsAbs is essential to ensuring product safety and efficacy. Size exclusion chromatography (SEC) coupled with mass spectrometry (MS) is increasingly being used to identify the accurate molecular mass of biomolecules, including bsAbs. SEC/MS, however, requires the use of mobile phases that do not contain high concentrations of non-volatile salts and the use of columns that do not exhibit particle shedding which will interfere with the MS signal response.

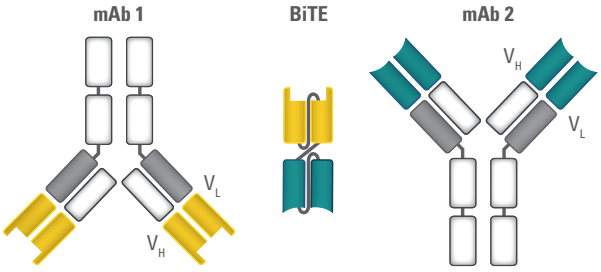
In this application note, a bispecific T cell engager (BiTE®) consisting of two single-chain variable fragments (scFvs) recombinantly linked by a nonimmunogenic five-amino-acid chain (Figure 1) was analyzed by SEC/MS using a TSKgel® UP-SW3000, 2 µm column.

EXPERIMENTAL HPLC CONDITIONS

Column:	TSKgel UP-SW3000 2 µm, 4.6 mm ID × 30 cm
HPLC Instrument:	Nexera® XR UHPLC system
MS Instrument:	Q Exactive™ Plus
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)
Ionization mode:	Electrospray ionization, positive mode
MS mode:	Scanning, m/z 800-6000

**SEC/MS analysis was performed by the Wistar Proteomics and Metabolomics Facility (Philadelphia, PA)*

FORMATION OF BISPECIFIC T CELL ENGAGER (BITE)



Formation of BiTE

Figure 1

COLUMN SHEDDING AND CARRYOVER ANALYSIS

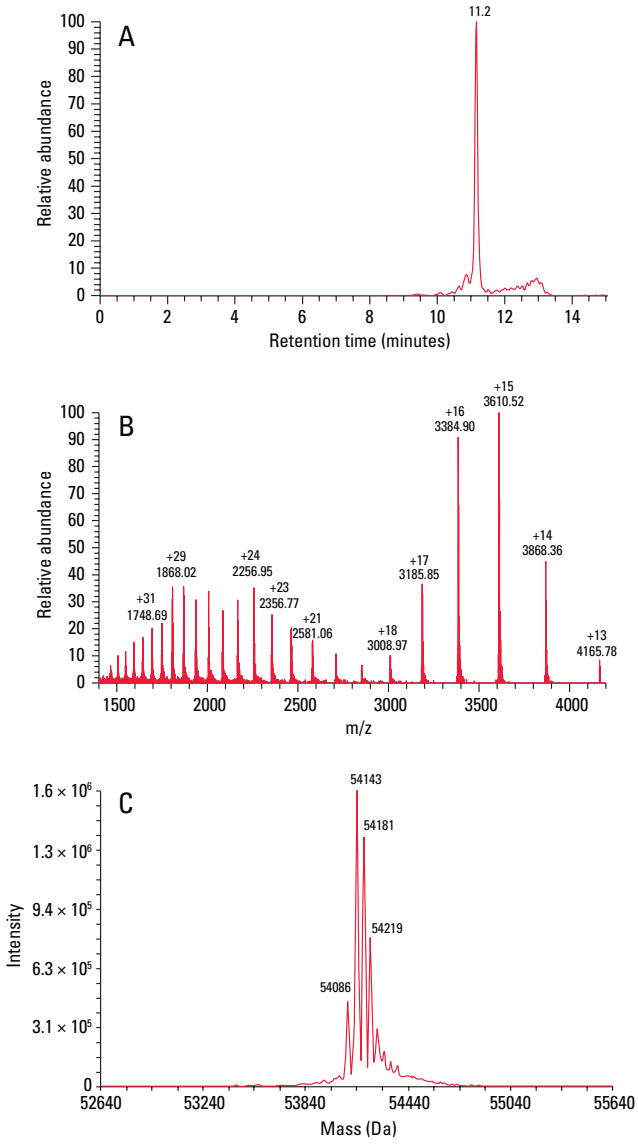


Figure 2

RESULTS AND DISCUSSION

The ~55 kDa BiTE and ~150 kDa parent mAbs were subsequently injected onto a TSKgel UP-SW3000 column coupled to a Q Exactive Plus mass spectrometer for molar mass determination. Figure 2 shows the (a) total ion chromatogram, (b) mass spectrum and (c) deconvoluted mass spectrum of the 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 3 shows the (a) total ion chromatogram, (b) mass spectrum and (c) deconvoluted mass spectrum of one of the parent mAbs. 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. Similar results (not shown) were reproduced for the other parent mAb.

These results demonstrate accurate molar mass determination for the BiTE and both parent mAbs utilizing a 20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate (pH 7.2) mobile phase with SEC/MS compatibility.

SEC/MS ANALYSIS OF THE PARENT MAB

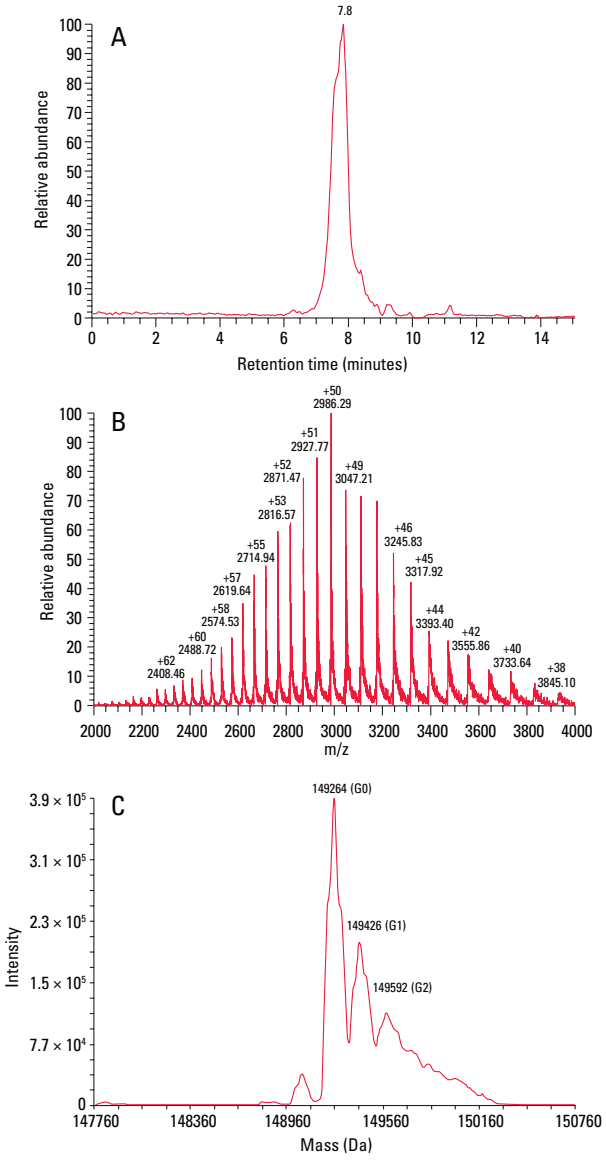


Figure 3

COLUMN SHEDDING AND CARRYOVER ANALYSIS

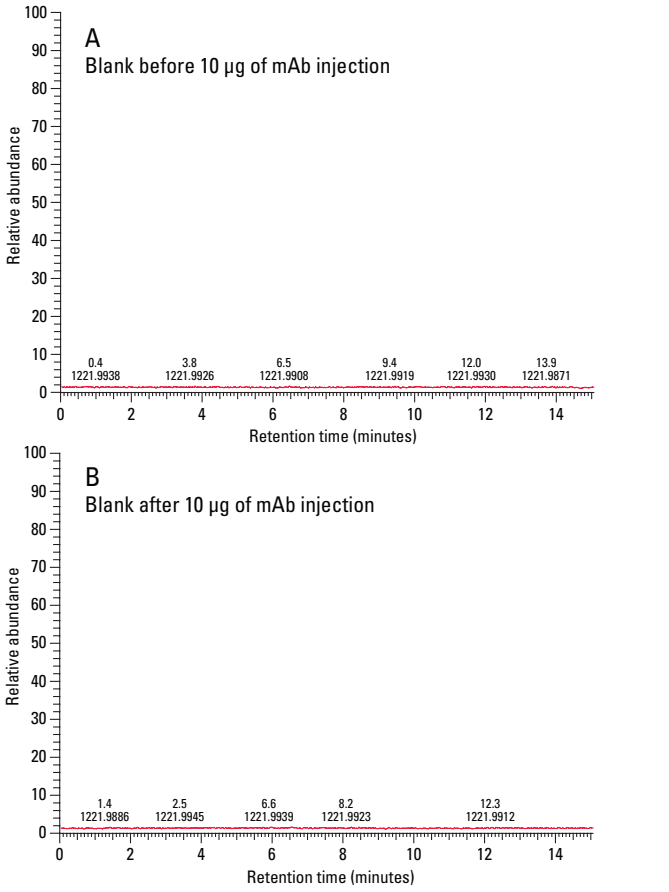


Figure 4

Prior to analysis, a blank injection was run in order to assess column particle shedding. Figure 4A shows the total ion chromatogram of a blank injection that was run on a new TSKgel UP-SW3000 column. MS data indicates 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. Figure 4B shows the total ion chromatogram of a blank injection run between the BiTE and parent mAb. No evidence of carryover can be seen in the run after sample injection. The lack of shedding and carryover indicate that the TSKgel UP-SW3000 column is suitable for use with MS.

CONCLUSION

The TSKgel UP-SW3000, 2 µm SEC column can be used as a platform method for bispecific antibody accurate mass determination using SEC/MS. A MS compatible mobile phase under non-denaturing condition was successfully used with the TSKgel UP-SW3000 column. No signs of particle shedding or sample carryover, which may interfere with MS signal response, were noted with the TSKgel UP-SW3000 column.

TOSOH

APPLICATION NOTE

DAR Analysis of Antibody-Drug Conjugate by HIC

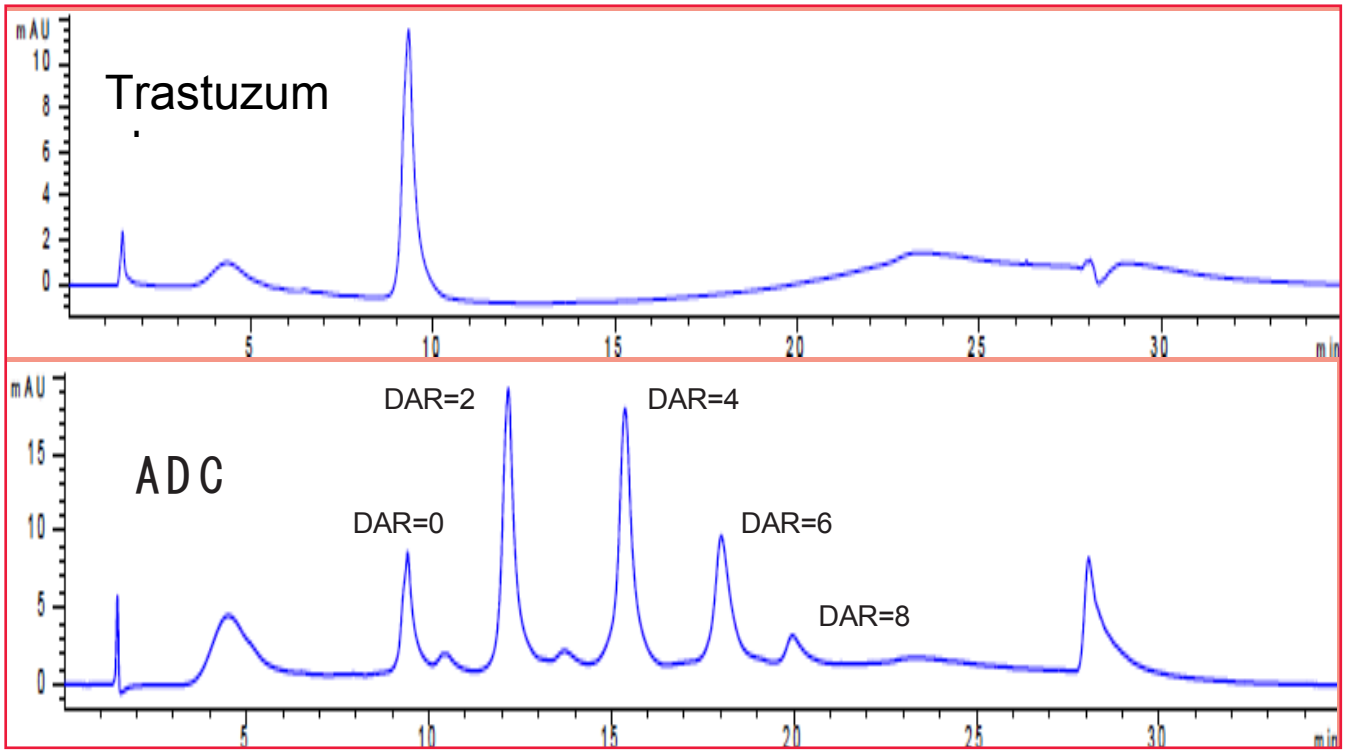
The antibody drug market has continued to expand in recent years, and antibody drugs held 7 of the top 10 blockbuster drug spots for 2012. The most promising antibody drug candidates for next-generation biopharmaceuticals are ADCs (antibody-drug conjugates). ADCs have a structure in which a low-molecular drug is chemically bonded to an antibody (IgG). Because there are numerous binding sites for a low-molecular drug on an antibody (Cys, Lys residues, etc.), heterogeneity arises with respect to the number of bonds and binding sites. Consequently, it is necessary to study in detail the effect that these heterogeneity have on the medicinal effects and safety of ADCs. Since low-molecular drugs are strongly hydrophobic compared with antibodies, differences arise in hydrophobicity when the bonding number of low-molecular drug differs. This property can be utilized to determine the drug-to-antibody ratio (DAR) by hydrophobic interaction chromatography (HIC).

Introduced here is an application in which an ADC was separated using a TSKgel Butyl-NPR column.

EXPERIMENTAL CONDITIONS

Column:	TSKgel Butyl-NPR (4.6 mm I.D. x 10 cm)
Eluent:	A) 25 mmol/L phosphate buffer (pH 7.0) including 1.5 mol/L ammonium sulfate B) 25 mmol/L phosphate buffer (pH 7.0) / 2-propanol = 8 / 2
Gradient:	0 -> 100 % B (20 minutes)
Flow rate:	0.5 mL/min
Detection:	UV 280 nm
Injection vol.:	10 µL
Sample Conc.:	Herceptin; 0.24 g/L, ADC(Herceptin-vcMMAE); 2.2 g/L

CHROMATOGRAMS OF TRASTUZUMAB AND ADC



An ADC (Trastuzumab-vcMMAE) in which an antineoplastic drug (monomethyl auristatin E, MMAE) is bonded via a linker to Trastuzumab was used. The results of HIC analyses using common ammonium sulfate gradient elution conditions showed that ADC could not be suitably eluted. Hence, an organic solvent (2-propanol) was added to eluent B, and by optimizing the organic solvent concentration, peaks exhibiting different DARs (DAR = 0 to 8) could be well separated (for the DAR, each peak fractionated and attributed by LC-MS/MS).

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TOSOH

APPLICATION NOTE

CHARACTERIZATION OF A NOVEL ANTIBODY DRUG CONJUGATE MIMIC BY SEC AND HIC

INTRODUCTION

Empowered antibodies, such as antibody drug conjugates (ADCs), continue to be investigated as biotherapeutic drug candidates. ADCs combine the tumor specificity and targeting capability of mAbs with the cytotoxicity of potent small molecule drugs into hybrid molecules that are promising anticancer therapeutics. These molecules are comprised of three components: a monoclonal antibody, a stable linker, and a cytotoxic small molecule drug. For the cysteine-linked ADC mimic used in this study, a dansyl fluorophore (~668 Da) is covalently bonded to an IgG_1 mAb (150 kDa) via a LC-SMCC crosslinker (Figure 1). This procedure results in a mixture of drug-loaded antibody species with 0 to 8 drugs (Figure 2).

ADC conjugation plays a role in both drug efficacy as well as clearance and must be well understood during drug development.

CYSTEINE-LINKED ADC MIMIC

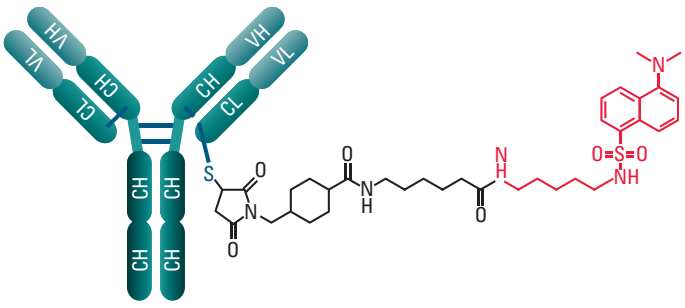

Figure 1

HETEROGENEITY OF CYSTEINE-CONJUGATED ADCS

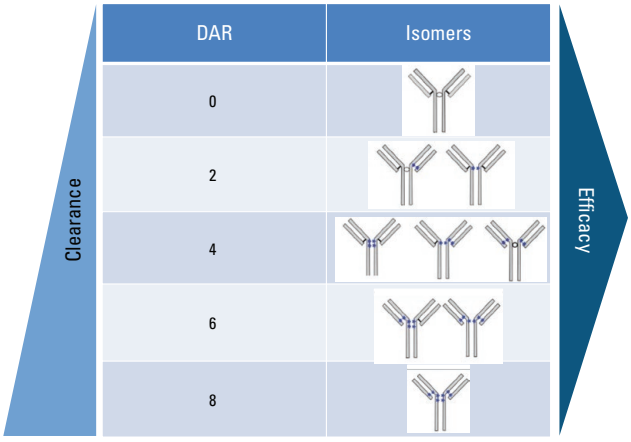

Figure 2

Size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) are two commonly employed techniques used to characterize the drug-to-antibody ratio (DAR) under native, physiological conditions. In this application note, the ADC mimic was analyzed by size exclusion chromatography/mass spectrometry (SEC/MS) using a TSKgel® SuperSW3000 column and by HIC using a TSKgel Butyl-NPR column. Combining these chromatographic techniques allowed elucidation and verification of the DAR profile for this model biomolecule.

EXPERIMENTAL HPLC CONDITIONS

SEC/MS CONDITIONS

Column:	TSKgel SuperSW3000, 4 µm, 2 mm ID × 30 cm
Mobile phase:	100 mmol/L ammonium acetate, pH 7.0
Gradient:	isocratic
Flow rate:	0.07 mL/min
Detection:	ESI-MS
Temperature:	35 °C
Injection vol.:	1.0 µL
Samples:	ADC mimic, 100 µg/mL (MilliporeSigma™), 100 mmol/L ammonium acetate, pH 7.0
MS mode:	Scanning, m/z 1000-8000

HIC/UV CONDITIONS

Column:	TSKgel Butyl-NPR, 2.5 µm, 4.6 mm ID × 10 cm
Mobile phase:	A. 50 mmol/L potassium phosphate, 1.5 mol/L ammonium sulfate, pH 7.0 plus 5% (v/v) isopropyl alcohol B. 50 mmol/L potassium phosphate, pH 7.0 plus 20% (v/v) isopropyl alcohol
Gradient:	0% B to 100% B in 50 min
Flow rate:	1.0 mL/min
Detection:	UV @ 215 nm
Temperature:	35 °C
Injection vol.:	5.0 µL
Samples:	ADC mimic, 100 µg/mL (MilliporeSigma), 100 mmol/L ammonium acetate, pH 7.0

RESULTS AND DISCUSSION

The ADC mimic was injected onto a TSKgel SuperSW3000 SEC column coupled to a mass spectrometer in order to examine the DAR profile. Figure 3 shows the deconvoluted mass spectrum of the ADC mimic. Main peaks can be seen at m/z 143,799; 145,135; 146,474; 147,812; and 149,147. The difference in molecular weight between each main peak is 1336 Da, corresponding to the molecular weight of two dansyl fluorophore molecules. The average DAR was found to be 3.9.

NATIVE SEC/MS SPECTRUM OF THE ADC MIMIC

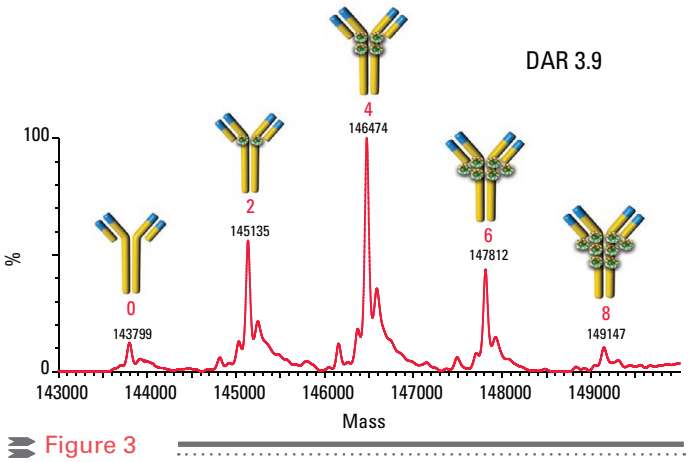

Figure 3

HIC/UV ANALYSIS OF NATIVE ADC MIMIC

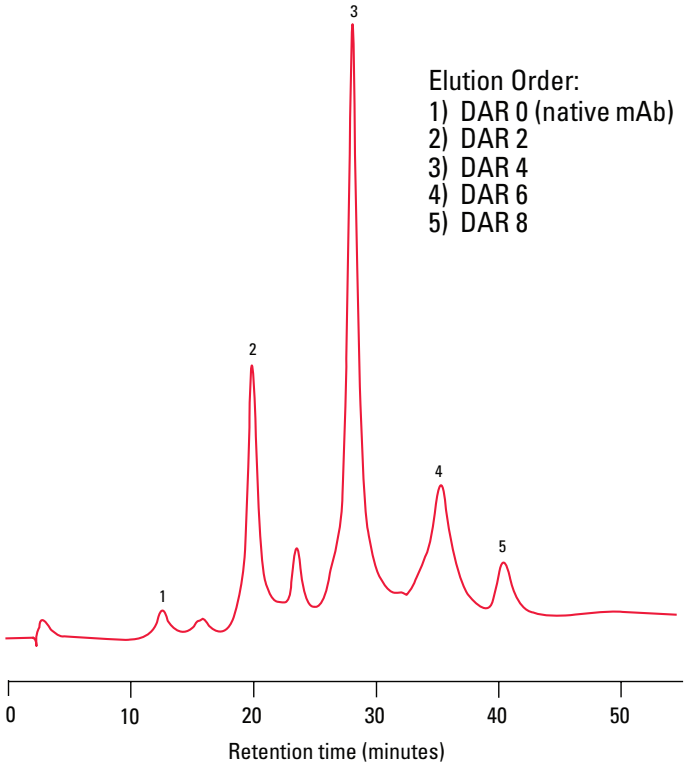

Figure 4

The DAR profile was then confirmed by HIC using a TSKgel Butyl-NPR column and UV detection. As more drug is conjugated to the mAb vehicle, the ADC becomes more hydrophobic and is retained longer by the HIC stationary phase, allowing resolution of the different drug loaded species. Figure 4 shows the DAR profile of the ADC mimic. The chromatogram shows well resolved peaks ranging from a DAR of 0 to 8.

CONCLUSION

SEC/MS and HIC/UV can be effectively used to characterize the DAR profile of ADCs. The mobile phase ensured a non-denaturing, MS compatible condition that was successfully used with the TSKgel SuperSW3000 SEC column to elucidate the molecular weight of the ADC species present in the drug mimic by high resolution ESI-MS detection; SEC/MS analysis indicated that the average DAR was 3.9. HIC/UV using a TSKgel Butyl-NPR column further confirmed the DAR profile by probing the hydrophobic character of the various antibody-payload combinations present in the sample. An average DAR of 3.9 was verified via HIC/UV analysis.

DATA CONTRIBUTED BY MilliporeSigma:

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TOSOH

APPLICATION NOTE

SEPARATION OF mAb MONOMER FROM ITS HALF-BODY USING SIZE EXCLUSION CHROMATOGRAPHY

INTRODUCTION

Monoclonal antibody (mAb) research continues to grow in an effort to develop effective biotherapeutics for a wide range of diseases. Recent research has shown an interest in mAb half-bodies as therapeutic vectors as they can be further targeted for conjugation, enzyme labeling, or antibody immobilization.

Monoclonal antibody half-bodies can be generated through the genetic engineering of cells or by selective reduction of hinge-region disulfide bonds present in the mAb by mild reducing agents, such as TCEP [tris(2-carboxyethyl) phosphine]. Due to its lack of odor and resistance to oxidation in the presence of air, TCEP is a stable reducing agent commonly used in mAb half-body formation.

A mAb half-body was generated through protein reduction using TCEP and subsequently identified by gel electrophoresins for use in this study. The superior resolution obtained between a monoclonal antibody monomer and half-body species using a TSKgel SuperSW mAb HR column is demonstrated in this application note.

EXPERIMENTAL CONDITIONS

Column:	TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm
Mobile phase:	0.1 mol/L phosphate/0.1 mol/L sulfate buffer + 0.05% NaN_3
Flow rate:	0.5 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	human IgG (4.6 g/L) – Sigma

RESULTS AND DISCUSSION

The complex and diverse nature of mAb structures make the reproduction of published methods difficult when using unique mAb samples. For this reason,multiple mAb reduction protocols were investigated for this study, all using TCEP Bond-Breaker® (Thermo Scientific).The use of 150 mmol/L TCEP with human IgG (4.6 g/L) incubated for 20 hours at 37 °C yielded the highest concentration of mAb half-body without excessive reduction of the protein into its low molar mass fragments. Predictably, the molar mass of the mAb half-body was approximately 70 kDa, or half that of the intact mAb.

Figure 1 illustrates the separation of human IgG monomer, half-body and fragment (1/3 mAb) formed using the TCEP reduction method discussed above using a TSKgel SuperSW mAb HR column. High resolution (Rs = 1.13) of the IgG monomer and half-body species was achieved.

SEPARATION OF HUMAN IgG MONOMER, HALF-BODY, AND FRAGMENTS USING A TSKgel SuperSW mAb HR COLUMN

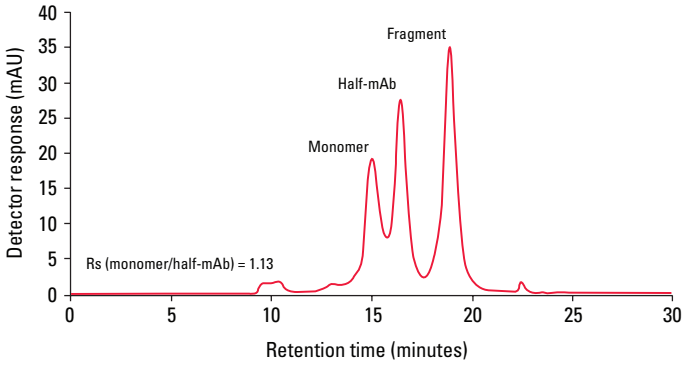


Figure 1

SDS-PAGE was used to confirm the identity of the mAb monome, half-body and fragment collected from the SEC separation on the TSKgel SuperSW mAb HR column. Fractions of each protein species were collected during the SEC separation and precipitated using acetone. The acetone was then removed and the protein precipitates were reconstituted in 100 µL of SDS-PAGE running buffer. The monoclonal antibody, half mAb and the fragment are clearly identified with the SDS-PAGE molar mass marker and transferrin (78 kDa) (Figure 2). This clearly shows that the half mAb could be generated using the TCEP reduction method and separated using the TSKgel SuperSW mAb HR column.

SDS-PAGE GEL OF HUMAN IgG MONOMER, HALF-BODY AND FRAGMENTS SEPARATED USING A TSKgel SuperSW mAb HR COLUMN.

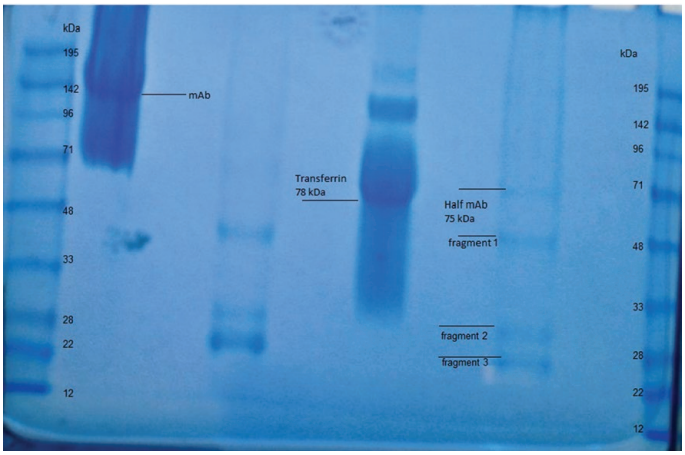


Figure 2

CONCLUSION

After investigation of multiple mAb reduction methods, it was determined that 150 mmol/L TCEP with human IgG incubated for 20 hours at 37 °C yielded a high concentration of IgG half-body. Separating the reduction products (IgG monomer, half-body and fragment) on the TSKgel SuperSW mAb HR column yielded high resolution (Rs of 1.13).

The TSKgel SuperSW mAb HR is able to achieve high resolution between the mAb and the mAb half-body due to its unique pore-controlled technology optimized for mAb analysis, as well as its smaller 4 µm particle size. Gel electrophoresis confirmed the identity of the reduction products separatedusing the TSKgel SuperSW mAb HR column. This study shows an excellent method for the separation of half-mAb or mAb half-body using the TSKgel SuperSW mAb HR column.

TOSOH

APPLICATION NOTE

FAST ANALYSIS OF IgG CHARGE HETEROGENEITY BY ION EXCHANGE CHROMATOGRAPHY IN HILIC MODE

EVALUATION OF STRONG AND WEAK CATION EXCHANGE COLUMNS AND DEPENDENCE OF SEPARATION ON MOBILE PHASE PH

ABSTRACT

Therapeutic antibodies are enjoying high growth rates in the pharmaceutical market. In 2013, seven of the top ten best-selling global drug brands were monoclonal antibodies (mAbs). The main product characteristics to be analyzed in process monitoring and quality control are aggregation, fragmentation, glycosylation and charge heterogeneity. We present the fast analysis of mAb charge heterogeneity by cation exchange chromatography on TSKgel STAT columns.

INTRODUCTION

Charge isoforms of proteins result from deamidation of asparagine or glutamine residues or from incomplete removal of the C-terminal lysine residue. Besides isoelectric focusing, ion exchange chromatography is the method of choice to analyze charge heterogeneity of proteins. TSKgel STAT columns are packed with a non-porous polymer based stationary phase. Proprietary surface modification technology ensures a high density of charged groups. Compared to traditional porous IEX phases this yields outstanding resolution of charged species at shorter analysis time. TSKgel STAT columns are available as weak cation-exchanger (WCX, carboxymethyl) and strong cation exchanger (SCX, Sulfopropyl). Best performance is achieved when the columns are used with low dead volume chromatography systems such as modern UHPLC systems.

RESULTS

The charge isoforms of two monoclonal antibodies were separated on a TSKgel CM-STAT weak cation exchange column and a TSKgel SP-STAT strong cation exchange column. Figure 1 shows the analysis of mAb A on both columns at pH 7. For this IgG the weak cation exchange column delivers a better separation of the basic variant from the main peak. As shown in Figure 2 this is not the case for mAb B, where the strong cation exchange column shows a better separation. Retention and resolution of the charged isoforms are dependent on the buffer pH as can be seen in Figure 3 for the analysis of mAb B on TSKgel SP-STAT.

DISCUSSION

Weak and strong cation exchange columns provide different selectivities for the analysis of charge heterogeneity of proteins. In order to reach the best separation of acidic and basic isoforms from the main peak, both types should be evaluated at various pH values of the mobile phase during method development. TSKgel STAT series columns provide a high resolution of isoforms in short analysis time and are ideally suited for the QC of biotherapeutics by UHPLC or HPLC.

ANALYSIS OF mAbA ON WEAK AND STRONG ION EXCHANGE COLUMNS AT PH 7.0

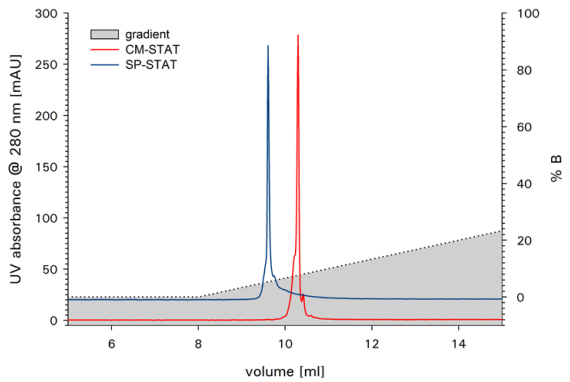


Figure 1

ANALYSIS OF mAbB ON WEAK AND STRONG ION EXCHANGE COLUMNS AT PH 7.0

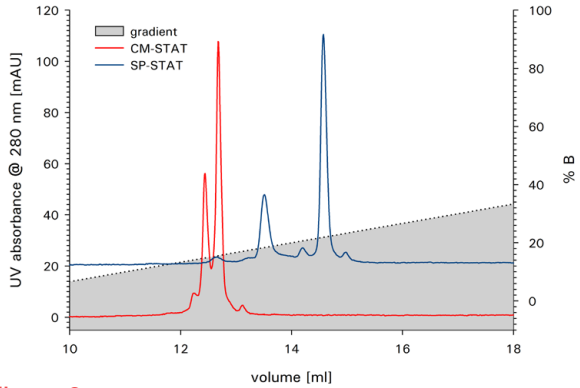


Figure 2

EXPERIMENTAL CONDITIONS

Columns:	TSKgel SP-STAT (7 µm, 4.6 mm ID x 10 cm); TSKgel CM-STAT (7 µm, 4.6 mm ID x 10 cm)
Mobile Phase	A: 10 mMol/L sodium phosphate buffer pH 7.0 (Figure 1, 2, 3) 10 mmol/L sodium phosphate buffer pH 6.0 (Figure 3) 10 mmol/L sodium acetate buffer pH 5.0 (Figure 3)
Mobile Phase	B: 100 mMol/L phosphate pH 7.0 + 500 mmol/L NaCl (Figure 1, 2, 3) 100 mmol/L phosphate pH 6.0 + 500 mmol/L NaCl (Figure 3) 100 mmol/L acetate pH 5.0 + 500 mmol/L NaCl (Figure 3)
Gradient:	0 - 100 % B in 30 min
Flow rate:	1 mL/min
Injection vol.:	10 µL
Detection:	UV @ 280 nm
Sample:	mAb A (2 g/L); mAb B (2 g/L)

DEPENDENCE OF CATION EXCHANGE SEPARATION ON MOBILE PHASE pH

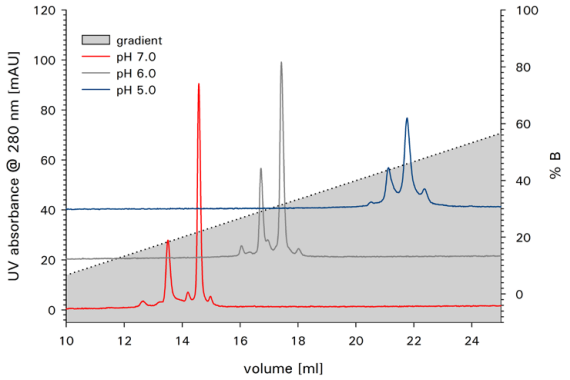


Figure 3

TOSOH

APPLICATION NOTE

UHPLC Glycosylation Analysis with TSKgel Amide-80 2 µm HILIC Columns

Glycosylation is one of the most common forms of post-translational modification of proteins. The polysaccharide side chains (Glycans) play critical roles in physiological and pathological reactions ranging from immunity to cell signaling. Besides the interest in characterizing glycosylation pattern of proteins for structure/function analysis, the thorough characterization of glycosylation is also a major quality parameter in the production of biotherapeutics. Hydrophilic interaction liquid chromatography (HILIC) is a well-recognized technique that effectively separates and quantifies isolated glycans.

Glycoprotein analysis involves characterizing complex N- and O-linked structures composed of sugar moieties. Besides mass spectrometric techniques, HILIC using amide-based stationary phases is a well-established, robust technique used by many laboratories to obtain high-resolution separation of N-linked glycans released from glycoproteins. Tagging the glycans with a fluorescent label such as 2-aminobenzamide (2AB) or aminopyridin (PA) allows the sugars to be detected at femtomole levels.

TSKgel Amide-80 column chemistry is ideally suited for the separation of charged and neutral fractions of glycan pools in one run. The retention of labelled polysaccharides by TSKgel Amide-80 enables the identification of glycan structures by comparison to a labelled dextran ladder that is used to normalize retention times in order to calculate the number of glucose units (GU values) of the separated glycans. The GU values obtained after separation of sequential exoglycosidase digests can be used to predict the glycan structure by database query (Glycobase, autoGU).

Packed with 2 micrometer spherical silica particles that are covalently bonded with non-ionic carbamoyl groups, TSKgel Amide-80 provides the same unique selectivity as TSKgel Amide-80 3 µm or 5 µm that are applied for glycan analysis in many QC labs for years. The new 2 µm material improves peak capacity and sensitivity for both, (U) HPLC and LC-MS analysis and allows a smooth transfer of established methods form HPLC to UHPLC. The columns are especially suited for use in UHPLC systems, as their reduced system volume and optimized detector specifications help to maintain the high resolution that can be achieved with 2 micron stationary phase.

RESULTS AND CONCLUSION

The new TSKgel Amide-80 2 µm phase shows a 1.4 fold higher resolution of PA-glycan peaks (Figure 1). Maximum pressure drops of TSKgel Amide-80 2 µm do not exceed 55 MPa during gradient at the conditions used (flow rate 0.5 mL/min). The suitability of the new 2 micron material for glycosylation analysis of labelled glycans by both fluorescence detection (Figure 2) and mass spectrometric detection (Figure 3) are demonstrated for various antibody samples.

COMPARISON OF TSKgel AMIDE-80 2 µm AND 3 µm

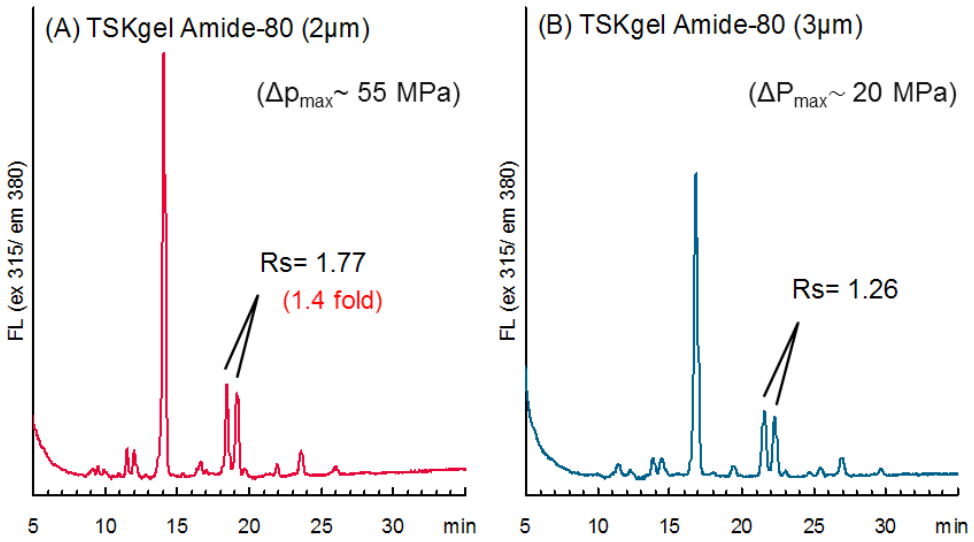


Figure 1

MATERIAL AND METHODS

UHPLC Analysis:

Columns:	TSKgel Amide-80 2 µm (2.0 mm ID x 15 cm) TSKgel Amide-80 3 µm (2.0 mm ID x 15 cm)
Mobile phase:	A: 200 mmol/L acetic acid + triethylamine (pH 7.3) B: acetonitrile
Gradient:	75%B (0-5min), 75-50%B (5-80 min, linear)
Flow rate:	0.5 mL/min
Temperature:	40 °C
Detection:	fluorescence (EX @ 315 nm, EM @ 380 nm Injection vol.: 50 µL
Sample:	Figure 1: pyridylaminated oligosaccharides released from mAb-1 (mouse) Figure2: (A) pyr dylaminated oligosaccharides released from mAb-1 (mouse) (B) pyridylaminated oligosaccharides released from mAb-2 (human) (C) PA-glucose ladder (3-22 mer) (TaKaRa Bio)

LC-MS Analysis:

Column:	TSKgel Amide-80 2 µm (2.0 mm ID x 15 cm)
Mobile phase:	A: 50 mmol/L HCOONH4, pH 7.5 B: acetonitrile
Gradient:	75 %B (0-5 min), 75-50 %B (5-30 min, linear)
Flow rate:	0.3 mL/min
Temperature:	40 °C
Detection:	(a) fluorescence (EX @ 315 nm, EM @ 380 nm) (b) LC/MS, ESI positive, SIM (Shimadzu LCMS-8030)
Injection vol.:	50 µL
Sample:	2-AB labelled N-glycans released from human IgG (Ludger, cat.# CLIBN-IGG-01)

GLYCOSYLATION ANALYSIS OF ANTIBODIES ON TSKgel AMIDE-80 2 µm

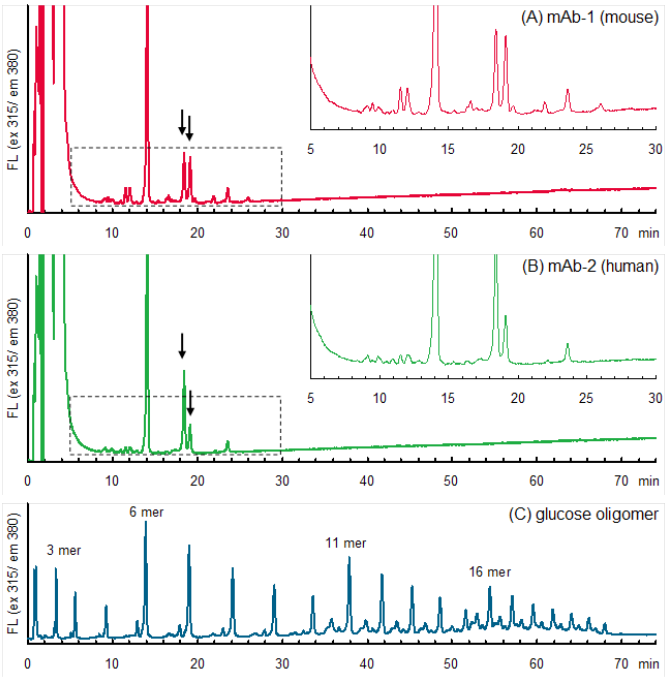


FIGURE 2

UHPLC-MS ANALYSIS OF 2-AB GLYCANS ON TSKgel AMIDE-80 2 µm

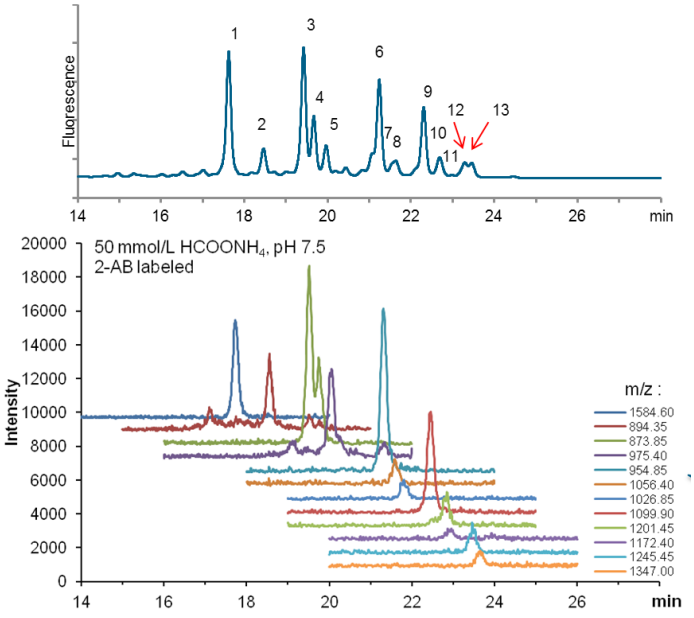


FIGURE 3

TOSOH

APPLICATION NOTE

FAST MONOCLONAL ANTIBODY TITER DETERMINATION WITH TSKgel® PROTEIN A-5PW

INTRODUCTION

The antibody therapeutics market is enjoying high growth rates, the major areas of therapeutic application being cancer and immune/inflammation-related disorders. Six of the top ten best-selling global drug brands are monoclonal antibody-based. This market is predicated to show continued growth for many years to come, with more monoclonal antibodies (mAbs) designed and produced for treatments of specific diseases.

Early in mAb development many harvested CHO cell supernatant samples must be screened for their mAb titers. Antibody titer determination by Protein A affinity HPLC is much more robust, reliable and reproducible than enzyme-linked immunosorbent assays (ELISAs). During upstream processing the optimal time for harvesting mAbs from cell culture supernatant can also be detected by using Protein A HPLC. In addition, partial purification of mAb can be accomplished using an Protein A affinity column initially to establish the right cell lines and to partially characterize a newly produced mAb. With many samples to be screened for different purposes, a reliable and high throughput column is needed for this workflow.

FAST CAPTURE OF IgG FROM CHO CELL SUPERNATANT

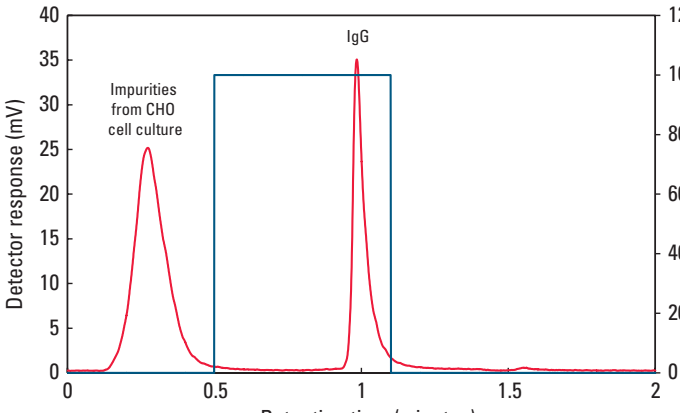


Figure 1

Column: TSKgel Protein A-5PW, 20 µm, 4.6 mm ID × 3.5 cm
Binding buffer: 20 mmol/L sodium phosphate buffer, pH 7.4
Elution buffer: 20 mmol/L sodium phosphate buffer, pH 2.5
Stepwise gradient: 0 – 0.5 min: binding buffer; 0.5 – 1.1 min: elution buffer; 1.1 – 2.0 min: binding buffer
Flow rate: 2 mL/min; Detection: UV @ 280 nm
Sample: 20 µL of CHO cell culture supernatant spiked with polyclonal IgG (0.5 mg/mL)

In this application note, the quick capture and accurate titer analysis over a wide concentration range of mAb is demonstrated using a TSKgel Protein A-5PW analytical column. Packed with 20 µm hydroxylated methacrylic polymer beads coupled with a recombinant Protein A ligand (a code-modified hexamer of the C domain), this 4.6 mm ID × 3.5 cm PEEK column can be used with high flow rates for high throughput analysis and still maintains chromatographic efficiency, peak width and resolution. In addition, the TSKgel Protein A-5PW column can perform for more than 2,000 injections with no sign of deterioration and without cleaning.

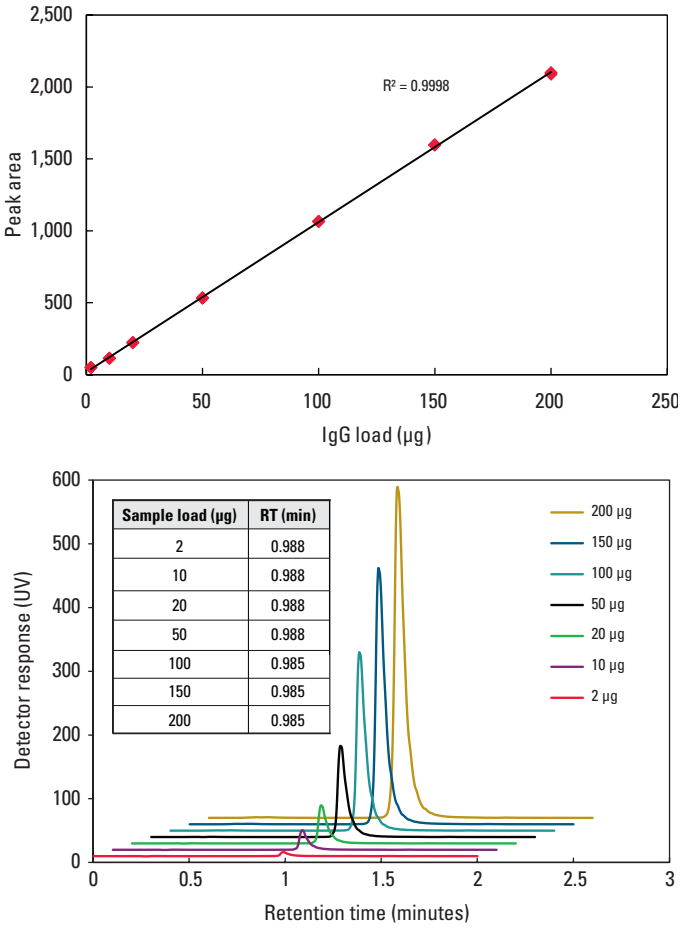
EXPERIMENTAL HPLC CONDITIONS

Columns:	TSKgel Protein A-5PW, 20 µm, 4.6 mm ID × 3.5 cm (PEEK), P/N 0023483
Binding and washing buffer:	20 mmol/L sodium phosphate buffer, pH 7.4
Elution buffer:	20 mmol/L sodium phosphate buffer, pH 2.5 Note: IgG can also be eluted with 12 mmol/L HCl, 20-100 mmol/L citric acid, pH 2.5-3.5, 20-100 mmol/L glycine, pH 2.5-3.5, 5-10% acetic acid
Stepwise gradient:	0 - 0.5 min: binding buffer 0.5 - 1.1 min: elution buffer 1.1 - 2.0 min: binding buffer
Flow rate:	2 mL/min
Detection:	UV @ 280 nm
Sample:	CHO supernatant and IgG as shown in the chromatograms

RESULTS AND DISCUSSION

Figure 1 shows the fast capture of mAb (human IgG) using a TSKgel Protein A-5PW column. The run was completed within 2 minutes, including bind, wash, elution, and re-equilibration steps. Host cell proteins from the supernatant were not absorbed by the column and so eluted as a flow-through peak. Only IgG was captured and then eluted from the column at approximately a 1 minute retention time. The IgG peak fraction was subjected to size exclusion chromatography using a TSKgel UP-SW3000 column for aggregate and monomer analysis. The result of that analysis indicated that the collected IgG consisted of more than 98% monomer (data not shown).

WIDE DYNAMIC RANGE OF TSKgel PROTEIN A-5PW



Sample load (µg)	RT (min)
2	0.988
10	0.988
20	0.988
50	0.988
100	0.985
150	0.985
200	0.985

Figure 2 & 3

Determination of mAb concentration from harvested cell culture supernatant requires a column with good linearity over a wide dynamic range so that the concentrations of mAb can be accurately determined. Figure 2 is a calibration curve with good linearity (R^2 >0.999) showing the wide dynamic loading range of the TSKgel Protein A-5PW column for a polyclonal IgG (0.1 - 10 g/L). Similar chromatograms from 2 to 200 µg without any change of peak profile or retention are produced by this column (Figure 3).

DURABILITY AND DYNAMIC RANGE OF TSKgel PROTEIN A-5PW

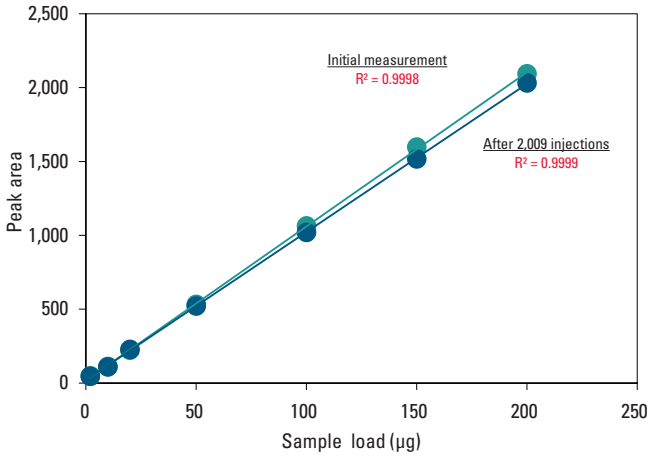
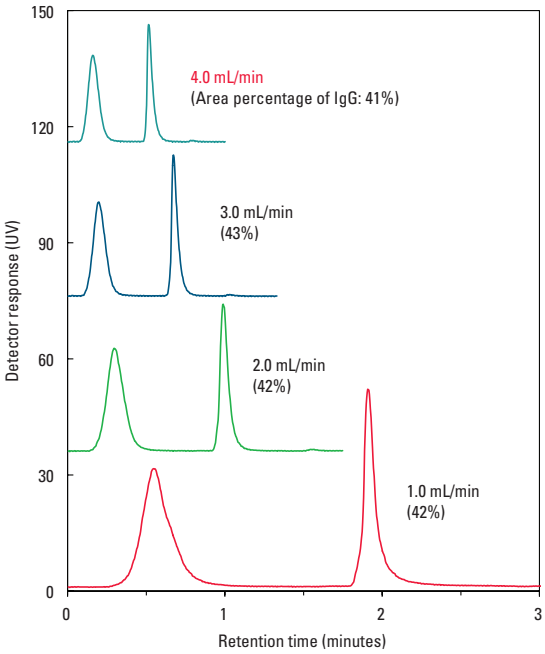


Figure 4

VARYING FLOW RATES USED ON TSKgel PROTEIN A-5PW



Gradient conditions

Flow rate (mL/min)	Binding buffer (min)	Elution buffer (min)	Binding buffer (min)
4.0	0-0.25	0.25-0.55	0.55-1.00
3.0	0-0.33	0.33-0.73	0.73-1.33
2.0	0-0.50	0.50-1.10	1.10-2.00
1.0	0-1.00	1.00-2.20	2.20-4.00

20 µL of CHO cell supernatant spiked with polyclonal antibody (0.5 mg/mL)

Figure 5

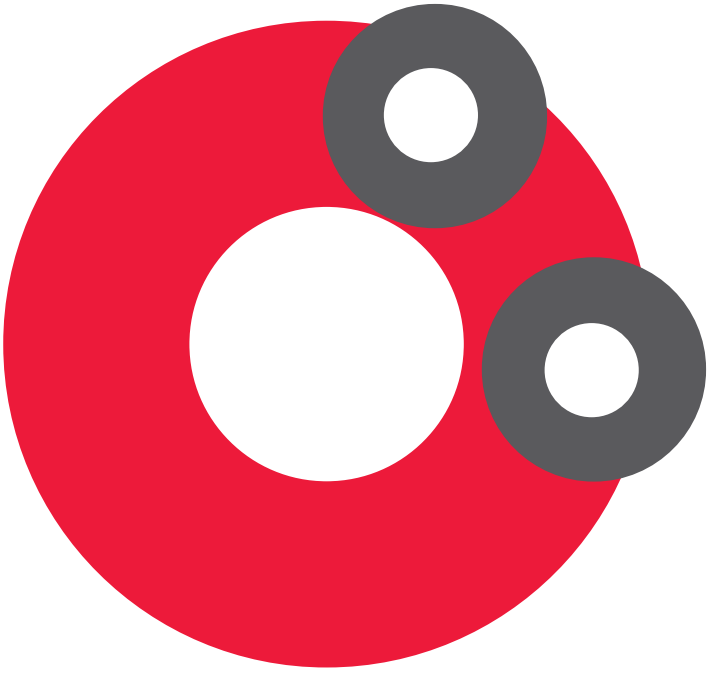
Figure 4 demonstrates the high durability and again the wide dynamic load range of the TSKgel Protein A-5PW column. The column was subjected to a linearity analysis test. Purified IgG was initially injected onto the column with subsequent injections of IgG made at different volumes. The column was then used up to 2,009 injections without being cleaned. A linearity analysis test was then repeated. No significant change in the calibration curve for IgG was seen. The column still maintained its high loading capacity with an excellent linearity (R^2 =0.9999).

Four different flow rates (1, 2, 3 and 4 mL/min), were used to demonstrate the high flow rate performance of the TSKgel Protein A-5PW column. Figure 5 shows there is a minimal effect of flow rate on IgG binding or absorbing onto the column. The relative peak area percentages of the unbound (flow-through) protein peak and the bound IgG remained unchanged at different flow rates.

CONCLUSION

The TSKgel Protein A-5PW column can capture and accurately quantitate monoclonal antibody from harvested cell culture media in less than 2 minutes. The wide range loading capacity of this column allows the titer of mAb to be determined at various stages of development. Because of the high flow rate tolerance and durability of the TSKgel Protein A-5PW column, high throughput analysis can be accomplished.

2 RECOMBINANT PROTEINS



APPLICATION NOTE

CHARACTERIZING BIOTHERAPEUTICS WITH HPLC SMART SOLUTIONS FOR LARGE MOLECULES

Regina Römling, Tosoh Bioscience GmbH, Griesheim, Germany

Development and production of biopharmaceuticals is a growing segment of pharmaceutical industry. Therapeutic proteins include blood factors, thrombolytic agents, growth factors, interferons, interleukins, therapeutic enzymes and, last but not least, monoclonal antibodies. More than twenty monoclonal antibody (mAb) therapeutics have been approved for the treatment of a broad range of diseases including autoimmune, cardiovascular, infectious diseases and cancer. A thorough characterization of therapeutic biomolecules is a key task for the successful submission of research and production data for regulatory approvals of new drugs. Due to its high degree of automation, robustness and reproducibility liquid chromatography is the workhorse of the pharmaceutical laboratory. Therefore, HPLC is also frequently used when establishing analysis methods for biotherapeutics. This article features some of the most frequently used HPLC applications for the characterization of biopharmaceuticals.

The typical chromatographic modes for separation of proteins in native form such as size exclusion chromatography (SEC) and ion exchange chromatography (IEC) are routinely used for the characterization of biotherapeutics. In addition, reversed phase (RPC) and hydrophilic interaction liquid chromatography (HILIC) are applied to characterize protein moieties such as peptides or oligosaccharide chains after enzymatic cleavage. Detection is usually performed by UV, fluorescence or light scattering. Additional structural information can be obtained by applying mass spectrometric detection. Some of the common modes of biochromatography are illustrated in this article by using the examples of important aspects of protein characterization such as analysis of protein aggregates, isoforms, modifications and glycosylation.

DETERMINATION OF PROTEIN AGGREGATES BY SEC

Protein aggregation is a common issue encountered during expression, purification and formylation of protein biotherapeutics. Proteins can aggregate as a function of temperature, pH, ionic strength and concentration[1]. Aggregation needs to be characterized and controlled during the development of protein pharmaceuticals such as monoclonal antibodies. Even small amounts of aggregates can alter the mAb's function as an effective therapeutic.

High-performance size-exclusion liquid chromatography on silica-based columns is the industry's workhorse for separating and quantifying soluble protein aggregates. Aggregation analysis of therapeutic proteins using SEC is almost always required for regulatory approval. Column-free techniques such as sedimentation velocity, analytical ultracentrifugation, and field-flow fractionation are complementary techniques suitable for aggregation analysis [2].

TSKgel SW series columns are based on highly porous silica particles. The surface has been shielded from interacting with proteins by derivatization. They contain a large pore volume per unit column volume, which results in high resolution when analyzing proteins. TSKgel G3000XL columns are the industry standard for quality control of monoclonal antibodies. When the sample is limited or the components of interest are present at very low concentration, TSKgel SuperSW3000 columns can be applied to achieve even higher performance. The internal diameter of these columns has been reduced from 7.8 mm to 4.6 mm and the particle size from 5 µm to 4 µm to provide higher sensitivity in sample-limited cases. It is important to employ an HPLC system that is optimized with regards to extra-column band broadening to take full advantage of the high column efficiency that can be obtained on these columns.

DETERMINATION OF mAb AGGREGATES ON SILICA-BASED SEC COLUMNS

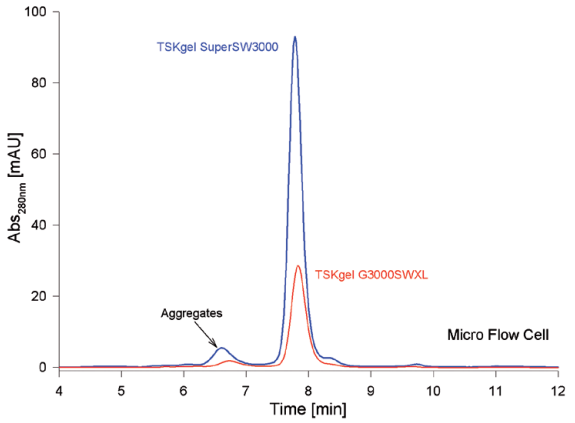


Figure 1

TSKgel G3000SWXL (5 µm, 7.8 mm ID x 30 cm), Flow rate: 1 mL/min; TSKgel Super SW3000 (4 µm, 4.6 mm ID x 30 cm); Flow rate 0.35 mL/min; Mobile phase: 0.1 M phosphate pH 6.8, injection volume 5 µL, detection: UV @ 280 nm (micro flow cell)

Figure 1 compares the analysis of mAb aggregates on both column types. Separations were performed on an optimized HPLC system equipped with a micro flowcell under identical conditions. Due to the different inner diameters of the columns the flow-rates must be different to apply the same linear flow, resulting in a flow-rate of 1 mL/min for the 7.8 mm ID G3000SWXL column and 0.35 mL/min for the 4.6 mm ID SuperSW3000 column. Figure 1 shows that resolution of monomer and aggregate peaks is higher on the SuperSW column, as is the detection sensitivity.

SEPARATION OF CHARGE VARIANTS OF mAbs ON TSKgel CM-STAT

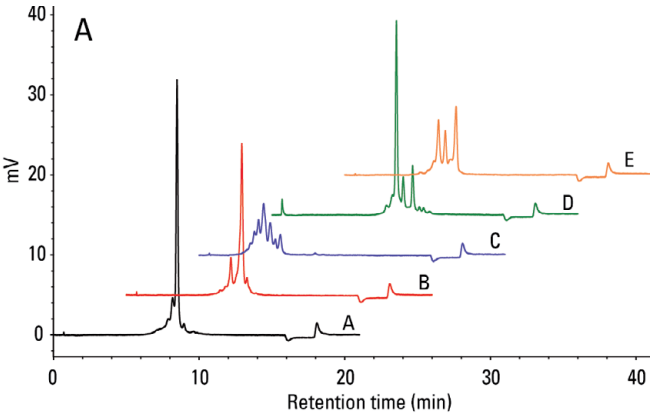


Figure 2

TSKgel CM-STAT column (7 µm, 4.6 mm ID x 10 cm L),
Mobile phase: A: 20 mM MES (pH 6.0), B: 20 mM MES + 0.5 M NaCl (pH 6.0), gradient 10% B to 15 % B in 15 minutes,
Flow rate: 1 mL/min, Detection: UV @ 280 nm, Inj. vol.: 20 µL

ANALYSIS OF CHARGE VARIANTS AND PEGylated PROTEINS BY IEC

Besides isoelectric focusing, cation exchange chromatography is the method of choice to analyse charge heterogeneity of proteins. Charge isoforms of proteins result from deamidation of asparagine or glutamine residues or from incomplete removal of C-terminal lysine residues[3]. Newly developed ion exchange columns can help to increase throughput in quality control (QC) of biopharmaceuticals. TSKgel STAT ion exchange columns are non-porous polymer columns with a high surface density of functional groups: quaternary ammonium for anion exchange (Q- and DNA-STAT), carboxymethyl (CM-STAT) and sulphopropyl (SP-STAT) for cation exchange. Particle sizes and dimensions of these IEC columns are optimized either for highest throughput or for highest efficiency. Applications for the TSKgel STAT cation exchange columns include the separation of peptides and proteins, PEGylated proteins and charge isomers of monoclonal antibodies. A TSKgel CM-STAT weak cation exchange (WCX) column was applied to separate charge variants of several monoclonal antibodies (Figure 2). The typical analysis time on conventional 25 cm long WCX columns of about forty minutes could be significantly reduced when separation was performed on a 10 cm CM-STAT column, filled with 7 µm particles. The analysis profiles for five antibodies show that high resolution analysis can be obtained in about 20 minutes analysis time.

PEGylation, the process by which polyethylene glycol (PEG) chains are attached to protein and peptide drugs is a common practice in the development of biopharmaceuticals to prolong serum half-life and improve pharmacokinetics of a drug[4]. Lysine residues of the protein typically serve as PEGylation sides. There is increasing demand for chromatographic methods to separate the modified isoforms from the native protein. The degree of PEGylation can be monitored by analyzing the increase in molecular weight by size exclusion chromatography, as every PEG group remarkably increases the apparent size of the protein. As every PEG residue also shields parts of the protein and, therefore, alters the overall surface charge, ion exchange separation can be used as well to analyze PEGylated proteins. SEC separates the native protein, mono- and di-PEGylated forms but because of the specific mode of interaction free separation it requires a certain analysis time and is, therefore, not well suited for high throughput applications. IEC, however, can be accelerated by using shorter columns and latest particle technology as shown on the left. In addition IEC has the ability to resolve isoforms differing in the position of PEGylated lysine residues, as the surface charge of the protein varies depending on the position of the modification.[5]

Figure 3 shows the monitoring of a PEGylation reaction of ß-Lactoglobulin in intervals of 5 minutes on a high-throughput TSKgel SP-STAT strong cation exchange (SCX) column. A fast gradient of sodium chloride in sodium acetate buffer at a high flow rate of 2 mL/min was applied to achieve fast separation. An efficient reaction monitoring has to follow the progress of the reaction in short intervals. Therefore, a high throughput column was applied here. For a more detailed characterization of the final product, a better separation of the different Mono-PEG isomers can be achieved by using a high resolution SCX column (10 cm length, smaller particles) and a more shallow gradient profile.

CHARACTERIZATION OF PROTEIN GLYCOSYLATION BY HILIC

Another important QC parameter of MAb characterization is the analysis of glycosylation. Glycosylation of MAbs may influence immunogenicity, pharmacokinetic and pharmacodynamic properties. According to the EMEA guideline on development, production, characterization and specifications for monoclonal antibodies and related products ‘glycan structures should be characterized, and particular attention should be paid to their degree of mannosylation, galactosylation, fucosylation and sialylation’.[6] Several complementary analytical techniques are routinely used to characterize, identify and quantify oligosaccharides isolated from glycoproteins.

The standard method for QC analysis of glycans is the HILIC/normal phase separation of AB-labeled glycans followed by fluorescence detection. Tagging the glycans with a fluorescent label such as 2-aminobenzamide (2-AB) allows the sugars to be detected at femtomole levels.

HIGH FREQUENCY MONITORING OF PEGylation REACTION

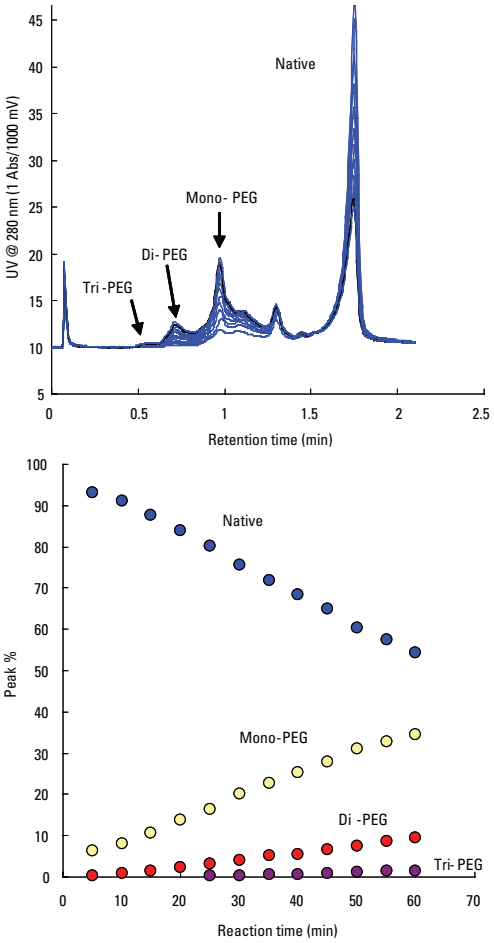


Figure 3

TSKgel SP-STAT column (10 µm, 3.0 mm ID x 3.5 cm L), Mobile phase: A: 20 mM sodium acetate (pH 5.0), B: 1.0 M NaCl in buffer A (pH 5.0), 2 minute gradient from 0 to 100% B, Flow rate: 2 mL/min, Detection: UV @ 280 nm

HILIC can separate structures with the same composition (isobaric glycoforms such as α-2,3- or α -2,6-sialic acid) on the basis of both sequence and linkage [7,8]. Complementary methods suitable for complete characterization of glycan structures comprise of HILIC-MS, weak anion exchange or porous graphite carbon separation of 2-AB labeled glycans, capillary electrophoresis of APTS-labeled glycans or the LC–MS peptide map of glycopeptides.

The chemistry of amide-bonded HILIC phases is ideally suited for the separation of charged and neutral fractions of glycan pools in one run. The retention of fluorescence labeled polysaccharides by TSKgel Amide-80 enables the identification of glycan structures by comparison to a labeled dextran ladder. The dextran ladder is used to normalize retention times to calculate the number of glucose units (GU values) of the separated glycans.

The GU values obtained after separation of sequential exoglygosidase digests can be used to predict the glycan structure by database query (Glycobase, autoGU). Figure 4 shows the fluorescence chromatograms of HILIC separations of 2-AB labeled N-glycans released from a recombinant ZP domain construct of murine transforming growth factor beta type 3 receptor (TGFR-3), compared with the dextran ladder.

HILIC CHROMATOGRAMS OF 2-AB LABELED GLYCANS

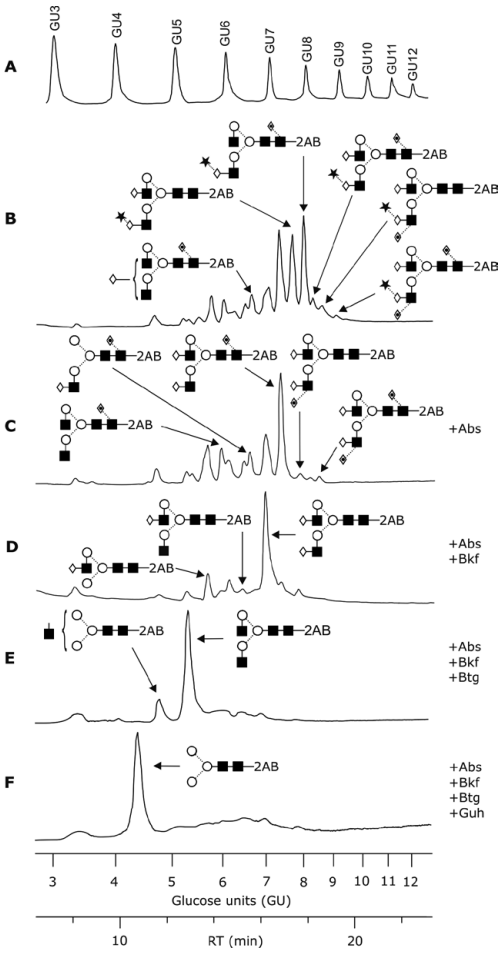


Figure 4

Dextran ladder (A), PNGaseF digest (B), sequential exoglycosidase digests (C–F). Used exoglycosidases: Sialidase A (Abs), α-Fucosidase (Bkf), β-Galactosidase (Btg), β-N-Acetylhexoamidase (Guh) TSKgel Amide-80 column (3 µm, 2 mm ID x 15 cm L); Mobile phase: A: 50 mM ammonium formate (pH 4.3), B: acetonitrile, 35 minutes gradient from 75 to 35 % B, Flow rate: 0.22 mL/min; Temperature: 50 °C; Detection: fluorescence; excitation @ 360 nm, emission @ 425 nm, Injection volume: 2 µL (approximately 300 fmol for GU3) *Courtesy of K. Darsow, S. Bartel & H. Lange, University of Erlangen-Nuremberg*

SUMMARY

As demonstrated here, there are a broad range of HPLC applications used in development, production, validation and release of protein therapeutics for human use. Some of the applications mentioned above are mandatory when submitting a new biopharmaceutical to approval at the regulatory agencies. The introduction of the first so-called biosimilars in Europe further increased the demand for highly efficient analysis methods. The continuous development of new stationary phases for the common modes of biochromatography, such as size exclusion, ion exchange and hydrophilic interaction chromatography support biochemists in increasing lab productivity and establishing high-throughput HPLC analysis not only for small molecules but also for large biopolymers.

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TOSOH

APPLICATION NOTE

Characterization Studies of PEGylated Lysozyme

ABSTRACT

PEGylation, the process by which polyethylene glycol (PEG) chains are attached to protein and peptide drugs is a common practice in the development of biopharmaceuticals to prolong serum half-life and improve pharmacokinetics of a drug. There is increasing demand for chromatographic methods to separate the modified isoforms from the native protein. This application note describes the use of size exclusion and ion exchange chromatography for the characterization of PEGylated lysozyme.

INTRODUCTION

Chemical modification of therapeutic proteins in order to enhance their biological activity is of increasing interest. One of the most frequently used protein modification method is the covalent attachment of poly (ethylene glycol) which is called PEGylation.This polymeric modification changes the biochemical and physicochemical properties of the protein, which decreases the in vivo clearance rate and reduces toxicity and immunogenicity of therapeutic proteins. After PEGylation the reaction mixture has to be purified in order to remove non-reacted protein and undesired reaction products. Chromatography as the most common purification method is influenced by PEGylation because of masking and shield effects of the covalently linked PEG molecule.

Lysozyme is a well known standard protein, which is often used to determine the dynamic binding capacity of Ion Exchange Chromatography (IEC) resins; therefore we decided to use PEG-lysozyme as a model protein in our study.

PEGylated lysozyme was produced out of methoxy-PEG-aldehyde (with a MW of 5 kDa, 10 kDa and 30 kDa) and chicken egg white lysozyme in phosphate buffer in presence of sodium-cyano-borohydrid (NaCNBH3) as reducing agent. The PEGylation reaction takes place between the aldehyde group of methoxy-PEG-aldehyde and free amino acid group (NH_2-group) of lysine residues within the lysozyme (Fig. 1).

The product mixture was analyzed by a TSKgel G3000SW$_{XL}$ SEC HPLC-column, SDS-PAGE (not shown), IEC (TSKgel SP-5PW (20) and TSKgel SP-NPR strong cation exchange (SCX)) and subsequent MALDI-TOF MS analysis (not shown).

METHODS

PEGylation of egg white lysozyme:

5, 10, 30 kDa methoxy PEG-aldehyde; 100 mM Phosphate buffer (Na_2HPO_4, NaH_2PO_4) pH 6.0; PEGylation by reductive alkylation; 20 mM $NaCNBH_3$ to reduce a Schiff base; 100 mM HCl to stop PEGylation reaction .

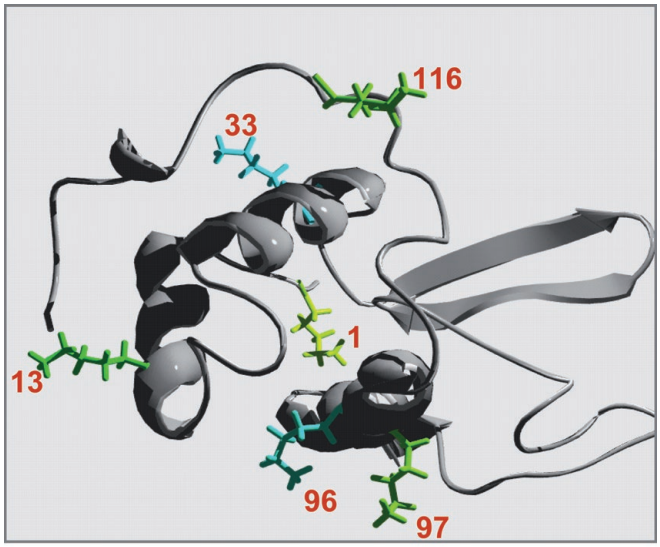

FIGURE 1

Lysozyme has six lysine residues as possible PEGylation reaction sides.

SEC-HPLC:

Column:	TSKgel G3000SW$_{XL}$ (7.8 mm ID x 30 cm, 5 µm, 250 Å)
HPLC-System:	Shimadzu Prominence
Flow rate:	1.0 mL/min
Mobile phase:	0.1 M Phosphate buffer 0.1 M Na_2SO_4, pH 6.7
Detector:	UV 280 nm
Injection vol.:	20 µL

IEC-FPLC:

Column:	TSKgel SP-5PW (20) (6.6 mm ID x 22 cm L, 20 µm, 1000 Å)
Flow rate:	0.85 mL/min
Buffer A:	25 mM Phosphate buffer 0.1 M Na_2SO_4, pH 6.0
Buffer B:	A + 0.5 M NaCl
Detector:	UV 280 nm
Injection vol.:	100 µL

IEC-HPLC:

Column:	TSKgel SP-NPR (4.6 mm ID x 3.5 cm, 2.5 µm)
Flow rate:	1.0 mL/min
Buffer A:	25 mM Phosphate buffer 0.1 M Na_2SO_4, pH 6.0
Buffer B:	A + 0.5 M NaCl
Detector:	UV 280 nm
Injection vol.:	5 µL

RESULTS

PEGylation of lysozyme

Figure 2 shows typical chromatogram pattern of a reaction mixture of PEGylated lysozyme separated on TSKgel SP-5PW. PEG chain lengths of 5kDa and 30kDa are shown from the left to right. The profiles indicate a similar reaction characteristic. Non-reacted lysozyme remained in the reaction mixture; mono-PEGylated lysozyme as well as poly-PEGylated lysozyme was formed during the reaction.

SEC was performed as shown in Figure 3. By the use of retention volumes from SEC analysis the viscosity radius of PEGylated was SEC-HPLC analysis of reaction mixes determined under assumption of being a globular protein.

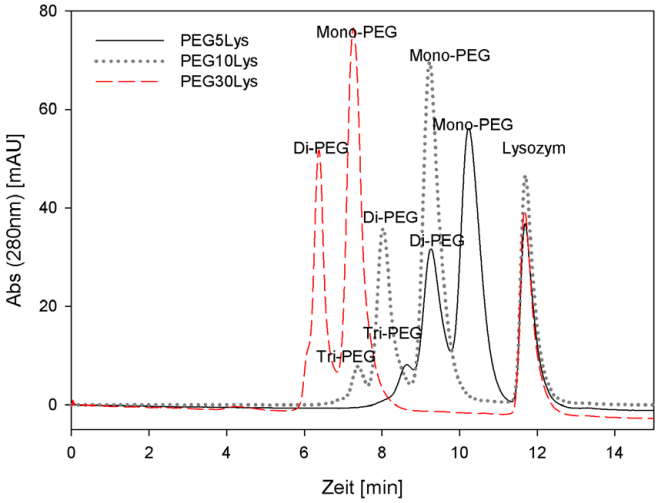

FIGURE 3

SEC analysis of reaction mixtures performed with a TSKgel G3000SWXL column. Lysozyme and PEGylated Lysozyme derivates for all tested sizes are shown.

SELECTIVITY

The particle size was of great importance for the selectivity. Especially the non-porous particle resin of the prepacked TSKgel SP-NPR column showed a very high resolution; with number of mono-PEGylated isoforms while two isoforms were visible for di-PEGylated lysozyme. TSKgel SP-5PW (20) is polishing resin with a particle size ten times bigger than the SP-NPR matrix. The resolution decreased, but two mono-PEGylated isoforms still remained visible (Fig. 4 A and B).

DISCUSSION

Lysozyme as model protein was PEGylated to examine the behaviour of PEGylated proteins in cation exchange chromatography. A random PEGylation of lysozyme using methoxy-PEG-aldehyde of sizes 5kDa, 10 kDa and 30 kDa was performed.

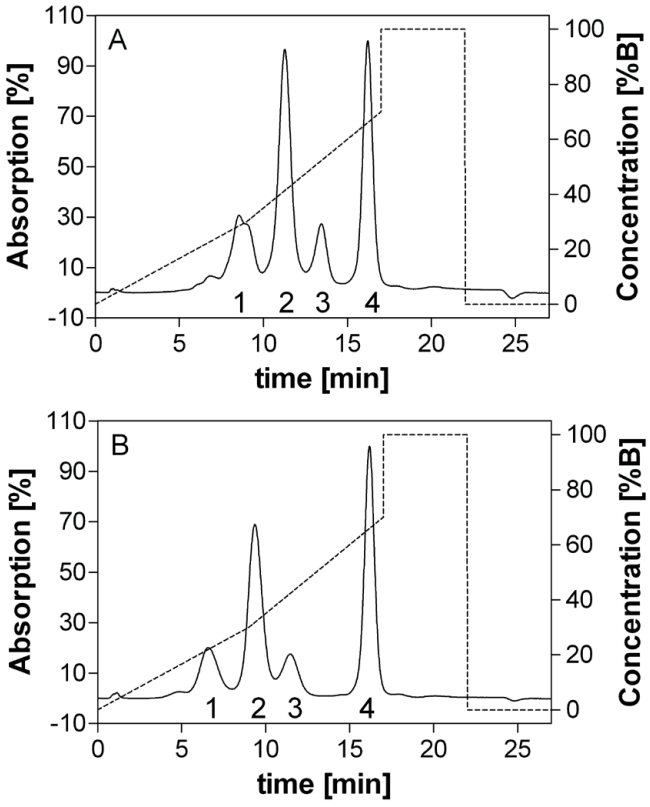

FIGURE 2

Separation of PEGylated lysozymes on an analytical TSKgel SP-5PW SCX column. Peaks were identifying by MALDI-TOF analysis, identical sizes were numbered consecutively.

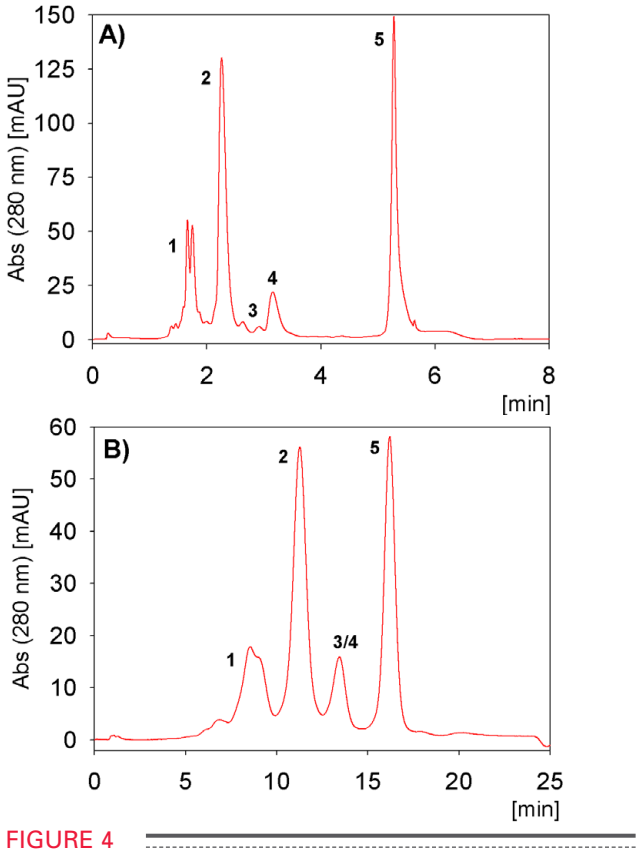

FIGURE 4

Resolution dependency on particle size shown with 5kDa PEGylated lysozyme reaction mixture. (A) TSKgel SP-NPR, (B) TSKgel SP-5PW; (1) poly-PEG5Lys, (2) 1-mono- PEG5Lys, (3) 2-mono- PEG5Lys, (4) 3-mono-PEG5Lys and (5) lysozyme

In Size Exclusion Chromatography, a massive increase of size by PEGylation was observed. The SEC elution behaviour of lysozyme modified with a 30 kDa PEG was equal to a 450 kDa globular protein. There was a linear correlation between the theoretical MW of PEGylated protein and the MW calculated via SEC. This result illustrates the influence of PEG on the hydrodynamic radius of PEGylated protein.

SELECTIVITY COMPARISON

Cation exchange chromatography was capable to resolve the PEGylated isomers which are the products of random PEGylation. The use of non-porous SP-NPR polishing resin leads to the best resolution. This is due to the better mass transfer kinetics for large molecules on small, non-porous particles.

Despite the loss in resolution, it was useful to use a porous resin with larger particle size for the first chromatographic step because of higher capacity and better pressure-flow-characteristics.

CONCLUSION

The selectivity of various cation exchanger resins were evaluated with random PEGylated lysozyme (chicken egg white). It is shown that the selectivity for PEG modified proteins depends on particle size of the resin. All PEGylated lysozyme species could be resolved on a TSKgel SP-NPR column with a particle size of 2.5 µm and on a TSKgel SP-5PW column with a particle size of 20 µm. A further increase of particle size leads to loss of resolution.

ACKNOWLEDGMENT

We thank the colleagues from Institute of Bioprocess Engineering, Friedrich-Alexander University Erlangen-Nuernberg, for carrying out the MALDI-TOF analysis. This work was supported by the Federal Ministry of Education and Research (BMBF), Germany.

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APPLICATION NOTE

ANALYSIS OF MONOCLONAL ANTIBODY AND PROTEIN AGGREGATES INDUCED BY DENATURATION

INTRODUCTION

Degradation studies of biotherapeutic proteins are necessary to test their stability. The best way to test the suitability of a method is the use of real-time stability samples containing all relevant degradation products that might occur over time. Certain factors, such as product development timeline, process characteristics, excipients, and other environmental factors, however, make the use of a forced degradation study necessary. The biological phenomenon of protein aggregation is a major issue in therapeutic protein development, since the presence of these impurities reduces the potency of the drug formulation, even if non-toxic. Monoclonal antibody proteins, widely being used in the field of biotherapeutics, with the potential to replace small molecules in the future, must be free from these aggregate impurities.

In order to fully evaluate the aggregates, a size exclusion column is needed which has a large enough molecular exclusion limit, so that the higher order aggregates are not excluded in the void but separated as a function of hydrodynamic volume. This application note will show the superior resolving powe,r of the TSKgel UltraSW Aggregate column for the analysis of monoclonal antibody and metalloprotein aggregates formed under forced denatured conditions, including acid and thermal denaturation.

EXPERIMENTAL CONDITIONS

Figure 1

Column:	TSKgel UltraSW Aggregate, 3 µm, 30 nm, 7.8 mm ID × 30 cm L
Mobile phase:	100 mmol/L potassium phosphate buffer, 100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	ambient
Injection vol.:	10 µL
Sample:	mAb-02
Concentr.:	4.5 g/L in glycine/Na phosphate, pH 6.0

Figure 2

Column:	TSKgel UltraSW Aggregate, 3 µm, 30 nm, 7.8 mm ID × 30 cm L
Mobile phase:	50 mmol/L potassium phosphate (monobasic), 50 mmol/L sodium phosphate (dibasic), 100 mmol/L sodium sulfate, 0.05% NaN_3, pH 6.7
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	10 µL
Sample:	Apoferritin Sigma, 5.0 mg/mL in 50% glycerol and 0.075 mol/L sodium chloride, stored at -20 °C

RESULTS AND DISCUSSION

Figure 1 shows the use of a TSKgel UltraSW Aggregate column for the analysis of mAb aggregates formed by the forced acid denaturation of a monoclonal antibody. After reducing the pH of the mAb-02 sample solution to 4.7 by dilute phosphoric acid, aliquots were analyzed at 5, 20 and 50 minutes and the response was compared to that of the original sample solution.

ANALYSIS OF mAb AGGREGATES FORMED BY FORCED DENATURATION BY ACID AT PH 4.7

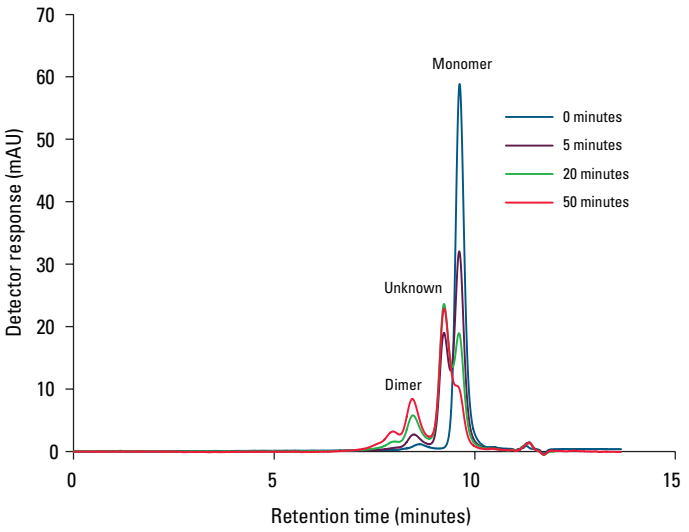
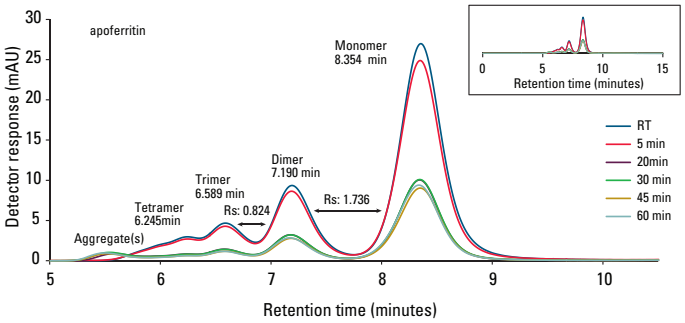


Figure 1

The degradation of the monoclonal antibody creates a larger MW entity (unknown) that elutes directly after the dimer and before the monomer. Continued decay increases both peaks, but more so for the dimer. Clearly, the dimer peak height increases while the peak height of the monomer decreases. Hints of higher order 'multimers' show between 7 and 8 minutes.

The analysis of a heat denatured, large hydrophobic metalloprotein, apoferritin, is shown in Figure 2. A set of six 0.3 mL HPLC vials each containing 100 µL stock solution of apoferritin was used for protein thermal denaturation. Thermal denaturation was carried out at 60 °C using an electric heating block. Individual sample vials were tightly capped and exposed to the heat for 5, 20, 30, 45, and 60 minutes. Samples were analyzed using a TSKgel UltraSW Aggregate column at the end of each incubation time period. The TSKgel Ultra SW Aggregate column yielded high resolution between the monomer and dimer. The trimer, tetramer and higher order aggregates of apo-ferritin were well separated.

ANALYSIS OF HEAT INDUCED FORCED DENATURED, LARGE HYDROPHOBIC METALLOPROTEIN, APOFERRITIN



Protein	Molecular weight (kDa)			
	Monomer	Dimer	Trimer	Tetramer
apoferritin	450	900	1350	1800

Figure 2

CONCLUSION

The results of both analyses demonstrate the high resolution separation of the aggregates of a monoclonal antibody and the large metalloprotein, apoferritin, generated by forced denaturation using a TSKgel UltraSW Aggregate column. With the increasing use of monoclonal antibodies in biotherapeutics, the TSKgel UltraSW Aggregate offers superior analysis of aggregates.

The TSKgel UltraSW Aggregate, 3 µm, SEC column with 30 nm pore size is specially designed with controlled pore technology which produces a shallow calibration curve in the molecular weight region of a typical monoclonal antibody. The larger pore size with an estimated exclusion limit of ~2 × 10^6 Da provides improved resolution and quantitation of mAb aggregates and oligomers. The TSKgel UltraSW Aggregate is an excellent choice for the analysis of monoclonal antibody protein aggregates, present in their native state or when induced by forced denaturation.

TOSOH

APPLICATION NOTE

GLYCOSYLATION ANALYSIS BY HYDROPHILIC INTERACTION CHROMATOGRAPHY (HILIC) – N-GLYCO MAPPING OF THE ZP-DOMAIN OF MURINE TGFR-3

Glycosylation is one of the most common forms of post-translational modification of eukaryotic proteins. Glycosylated proteins (glycoproteins) make up the majority of human proteins. The polysaccharide side chains (Glycans) play critical roles in physiological and pathological reactions ranging from immunity to cell signaling, development and death. Besides the interest of researchers in characterizing gylcosylation pattern of glycoproteins for structure/function analysis, the thorough characterization of glycosylation is a major quality parameter in the production of biotherapeutics. Hydrophilic interaction chromatography (HILIC) is a well-recognized technique that effectively separates and quantifies isolated glycans

HILIC

HILIC is used primarily for the separation of polar and hydrophilic compounds. It is commonly believed that in HILIC the aqueous content of the mobile phase creates a water rich layer on the surface of the stationary phase based. This allows for partitioning of solutes between the more organic mobile phase and the aqueous layer. Hydrogen bonding and dipole-dipole interactions are supposed to be the dominating retention mechanisms in HILIC mode (Figure 1).

GLYCAN ANALYSIS

Glycoprotein analysis involves characterizing complex N- and O-linked structures composed of frequently similar and repeating sugar moieties. Several complementary analytical techniques are routinely used to characterize, identify, and quantify oligosaccharides isolated from glycoproteins. Besides mass spectrometric techniques, HILIC using amide-based stationary phases is a well-established, robust technique used by many laboratories to obtain high-resolution separation of N-linked glycans released from glycoproteins. Tagging the glycans with a fluorescent label such as 2-aminobenzamide (2-AB) allows the sugars to be detected at femtomole levels.

HILIC PRINCIPLE

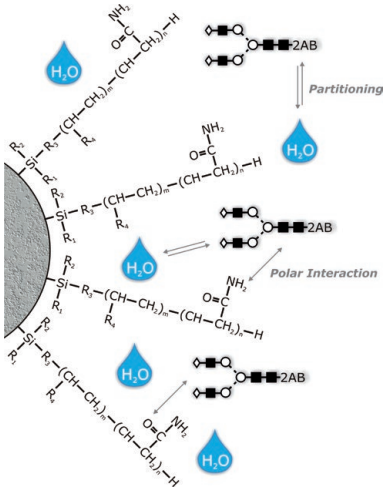


Figure 1

HILIC can separate structures with the same composition (isobaric glycoforms like 2,3- or 2,6-sialic acid) on the basis of both sequence and linkage. (1,2). TSKgel Amide-80 column chemistry is ideally suited for the separation of charged and neutral fractions of glycan pools in one run. The retention of fluorescence labelled (2-aminobenzamide, 2-AB) polysaccharides by TSKgel Amide-80 enables the identification of glycan structures by comparison to a labeled dextran ladder. The dextrane ladder is used to normalize retention times in order to calculate the number of glucose units (GU values) of the separated glycans. The GU values obtained after separation of sequential exoglygosidase digests can be used to predict the glycan structure by database query (Glycobase, autoGU).

TRANSFORMING GROWTH FACTOR RECEPTOR

Zona pellucida (ZP) is a glycoprotein matrix surrounding the plasma membrane of oocytes. Many eukaryotic extracellular proteins share a conserved sequence of 260 amino acids, called the ZP domain, which is an integral part of the ZP. The transforming growth factor beta (TGF-ß) signaling pathway is involved in many cellular processes including cell growth, cell differentiation, apoptosis, cellular homeostasis and other cellular functions.

PROTEIN CONSTRUCT OF THE ZP DOMAIN OF MURINE TGFR-3 EXPRESSED IN HEK293EBNA

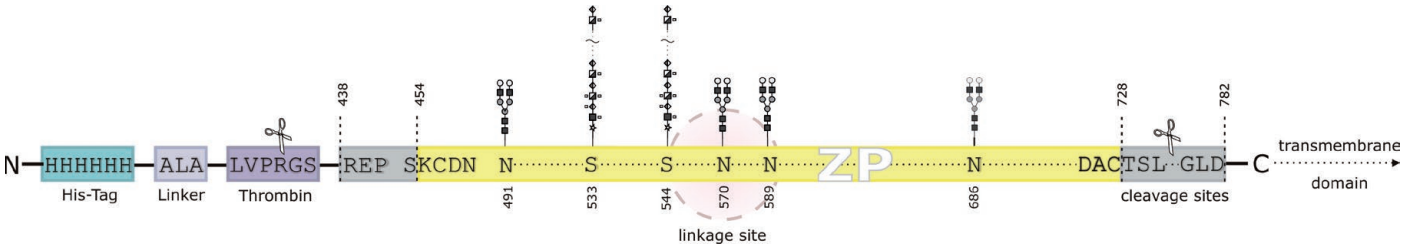


Figure 2

The cytokines of the TGF-ß family exist in different subtypes (TGF-ß1, TGF-ß2, TGF-ß3). The TGF-ß type 3 receptor (TGFR-3) shows a high affinity to these three subtypes of TGF-ß and other proteins of the TGF-ß super family. Compared to the wide range of cellular processes which are regulated by the TGF signaling pathway, the function of TGFR-3 ZP domain glycans is not fully determined yet. In order to achieve a better understanding of transduction pathways especially for fibrosis or neuro degenerative diseases the role of TGFR-3 ZP glycosylation has to be determined [3].

MATERIAL & METHODS

The application of HILIC for the characterization of a complex glycan structure is demonstrated using the example of N-glyco mapping of the ZP-domain of murine transforming growth factor beta type 3 receptor (TGFR-3). Figure 2 shows the protein construct of the ZP domain of murine TGFR-3 expressed in HEK293EBNA cells which was used for determination of the N- and O-glycosylation of the TGF receptor. Recombinant proteins were purified and submitted to endoglycosidase cleavage. Glycans were fluorescent labeled with 2-aminobenzamid and separated by HILIC. Figure 3 shows the fluorescence chromatograms of HILIC separations of 2-AB labeled N-glycans released from the recombinant ZP domain construct of murine TGFR-3, compared to the dextran ladder. The structure analysis was completed by high resolution mass spectra acquired on a MALDI QIT ToF MS instrument (Figure 4) (4).

CHROMATOGRAPHIC PARAMETERS

Column:	TSKgel Amide-80 3 µm (2 mm ID x 15 cm L)
HPLC:	Shimadzu Prominence
Flow rate:	0.22 mL/min
Mobile phase:	A: 50 mM ammonium formate (pH 4.3) B: acetonitrile
Gradient:	0 - 35 min: 75 - 35 % B
Temperature:	50 °C
Detection:	Fluorescence; excitation @ 360 nm, emission @ 425 nm
Injection:	2 µL, approximately 300 fmol for GU3 (Figure 3)

HILIC CHROMATOGRAMS OF 2-AB GLYCANS

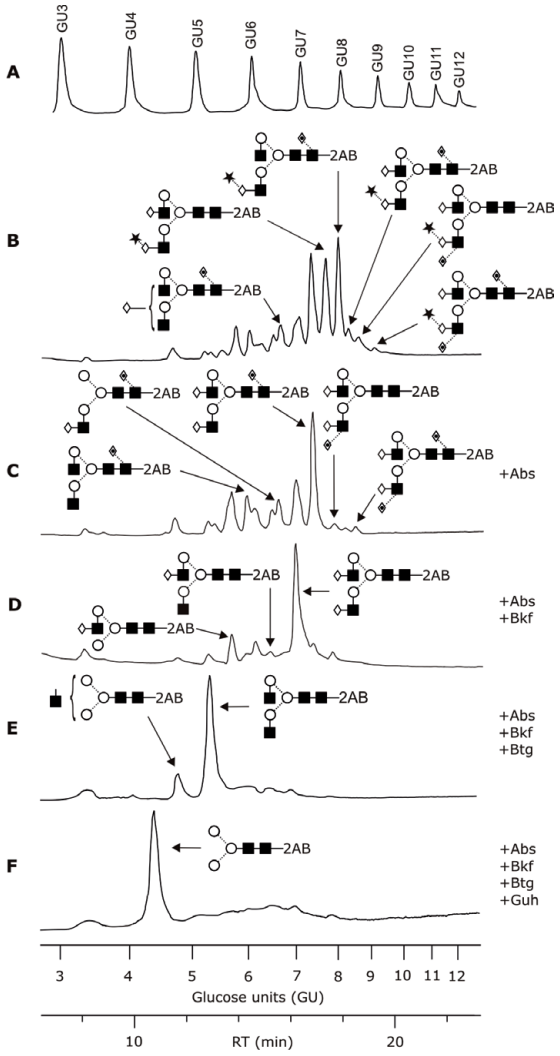


Figure 3

Dextran ladder (A), PNGaseF digest (B), sequential exoglycosidase digests (C-F). Used exoglycosidases: Sialidase A (Abs), α-Fucosidase (Bkf), ß-Galactosidase (Btg), ß-N-Acetylhexoamidase (Guh).

HILIC CHROMATOGRAMS OF 2-AB GLYCANS

MALDI mass spectrum of 2-AB-labeled glycans released from ZP domain construct of murine TGFR3

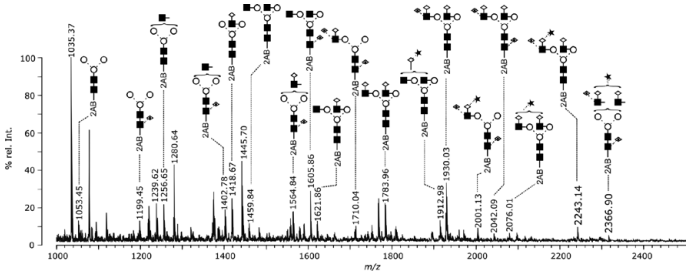


Figure 4a

MS2 (CID) mass spectrum of m/z 2243

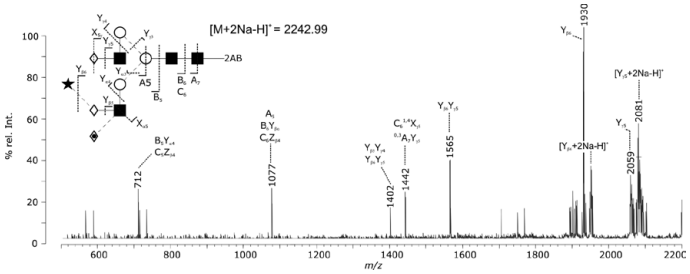


Figure 4b

MS3 (CID) mass spectrum of m/z 1930

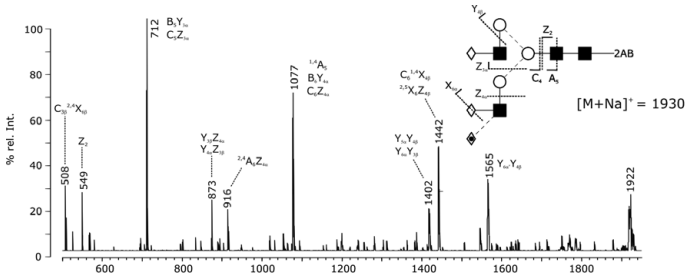


Figure 4c

CONCLUSION

Exoglycosidase sequencing in combination with HILIC separation on TSKgel Amide-80 and mass spectrometric analysis enables the determination of different N-glycan structures. Isobaric glycoforms have been identified by retention time (Glycobase) and MS/MS experiments.

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APPLICATION NOTE

AN IE-UHPLC METHOD FOR TESTING OF LONG-TERM ALCOHOL ABUSE

Carbohydrate deficient transferrin can be quantified using TSKgel Q-STAT

TRANSFERRIN IS A BIOMARKER

Transferrin is one of the most abundant plasma proteins in humans. The majority of transferrin is produced in the liver. It has two binding sites for $Fe3^{+}$ ions and plays an important role in the metabolism of iron. In human plasma, different glycosylation forms of transferrin are present, which may carry sialinic acid end-caps. In healthy humans, tetrasialo-transferrin is the most abundant transferrin. Long-term alcohol abuse leads to inhibition of glycosyltransferases in the liver. As a consequence, the relative concentration of disialo-, monosialo-, and asioalo-transferrin increases. Thus, the relative abundance of carbohydrate deficient transferrin (CDT) is used as a biomarker. A simplified illustration of the different glycoforms of Transferrin is shown in Figure 1.

At neutral pH, sialinic acid is a charged residue, which allows separation of the different sialo forms of transferrin based on charge. Anion exchange chromatography is a well-established method to quantify the relative plasma concentration of CDT[1]. With the published method, a single run can be accomplished within 30 minutes.

METHOD TRANSFER TO UHPLC

In the recent years, many routine applications have been transferred from HPLC to UHPLC. Adoption of UHPLC can improve a given separation and/or reduce the required analysis time. In biochromatography, the term UHPLC mainly refers to the use of low dead volume systems and small particles with excellent mass transfer properties. Based on UHPLC technology, separation of CDT from tetrasialo-transferrin could be accomplished in 6 minutes. Representative chromatograms of plasma samples from healthy donors are shown in Figure 2.

GLYCOSILATION OF TRANSFERRIN

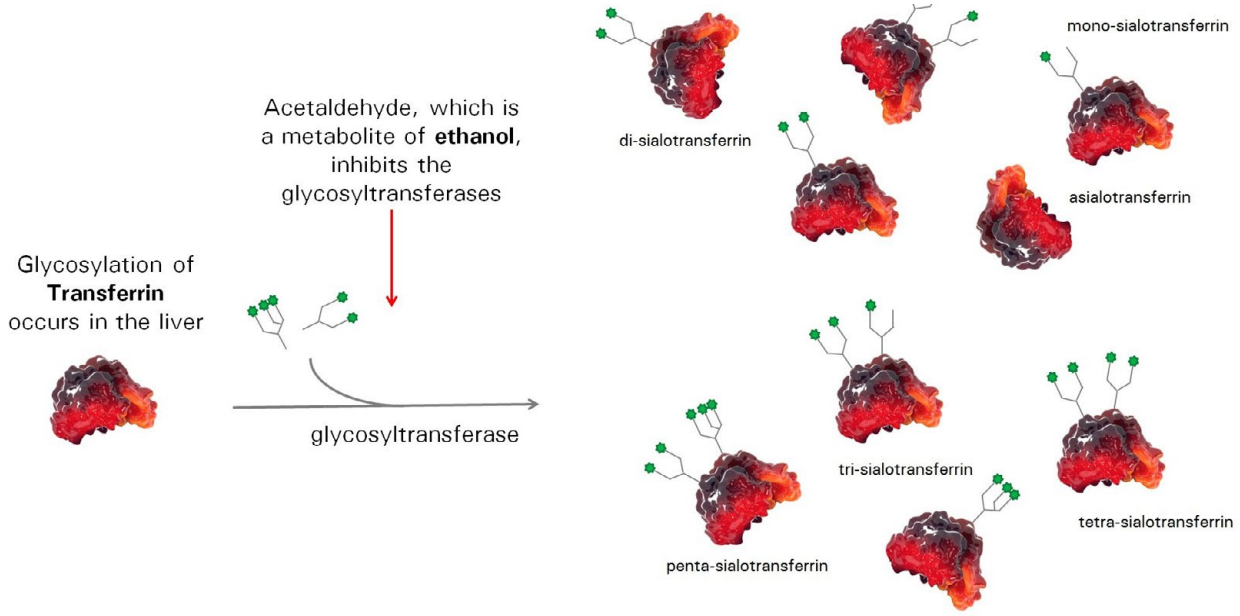


Figure 1

Glycosylation of Transferrin leads to various different glycosylated forms. The attached glycan structures may be end-capped by sialinic acid. The resulting charge variants can be distinguished by anion exchange chromatography.

SAMPLE PREPARATION

The sample preparation includes a lipoprotein precipitation step and iron-saturation of transferrin. 4 mL of a 10 mM nitrilo triacetic acid tri-sodium + 10 mM iron-(III) chloride solution at pH 6.5 were mixed with 2 mL of a 1.4 % (w/v) solution of dextran sulfate and 2 mL of a 0.7 mM solution of magnesium chloride directly prior to use. 50 µl of this reagent mix were added to 125 µl of plasma and thoroughly mixed. After addition of 700 µl distilled water, samples were incubated at 4 °C for 1 hour. Samples were centrifuged at 6400 g for 10 minutes. The supernatant was used for injection.

CONCLUSION

The presented method based on TSKgel Q-STAT reduces the required analysis time from 30 min to 6 min. This may lead to a fivefold increase of sample throughput compared to the conventional anion exchange method based on HPLC.

REFERENCE

1. A. Helander, A. Husa, J.O. Jeppsson, Improved HPLC Method for Carbohydrate-deficient Transferrin in Serum, Clin. Chem. 49 (2003) 1881–1890. doi:10.1373/clinchem.2003.023341.

SEPARATION OF PLASMA SAMPLES

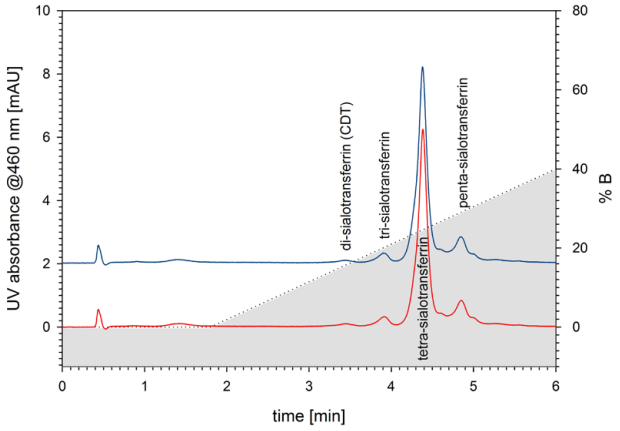


Figure 2

Figure 2 shows two plasma samples from healthy donors being separated on TSKgel® Q-STAT (4.6 mm ID x 10 cm L, P/N 0021961) at 1.0 mL/min. 100 µl of the samples were injected in 50 mM Tris/HCl, pH 7.4. The sialo-transferrin variants were separated in a 0-40 % B gradient in 4.2 min. Buffer B consisted of 50 mM Tris/HCl, pH 7.4 + 250 mM ammonium acetate. Transferrin specifically absorbs UV light at 460 nm.

APPLICATION NOTE

PURIFICATION OF ACTIVE PEPTIDASE A USING A SEMI-PREPARATIVE TSKgel® G3000SW

INTRODUCTION

Enzymes are a highly versatile group of proteins which find applications ranging from medicine through waste degradation to food technology. Their safe application requires thorough characterization, which needs sufficient amounts of purified enzyme. The purification process is both an important, yet complicated step in enzyme production, especially for unmodified proteins lacking any means of selective binding. On the other hand, the introduction of such domains (e.g.a His-tag) may influence the enzymes characteristics and is therefore often not desired. Such constraints leave a very limited choice of purification methods. In this study, recombinant aminopeptidase A (PepA) expressed in E.coli was purified. This enzyme forms dodecamers which suggested size exclusion chromatography (SEC) as a suitable separation technique. The high loading capacity of the TSKgel G3000SW columns series is demonstrated, as well its superior separation performance compared to a competitor column. Furthermore, a larger column of the G3000SW series was used for the semi-preparative amount of 14 mg of protein extract, showing that the G3000SW column is suitable for upscaling purposes. (For a detailed report see *Stressler et al., Protein Expression and Purification 131 (2017) 7-15).*

PURIFICATION OF PepA USING A TSKgel G3000SW (TOP) AND A COMPETITOR COLUMN (BOTTOM)

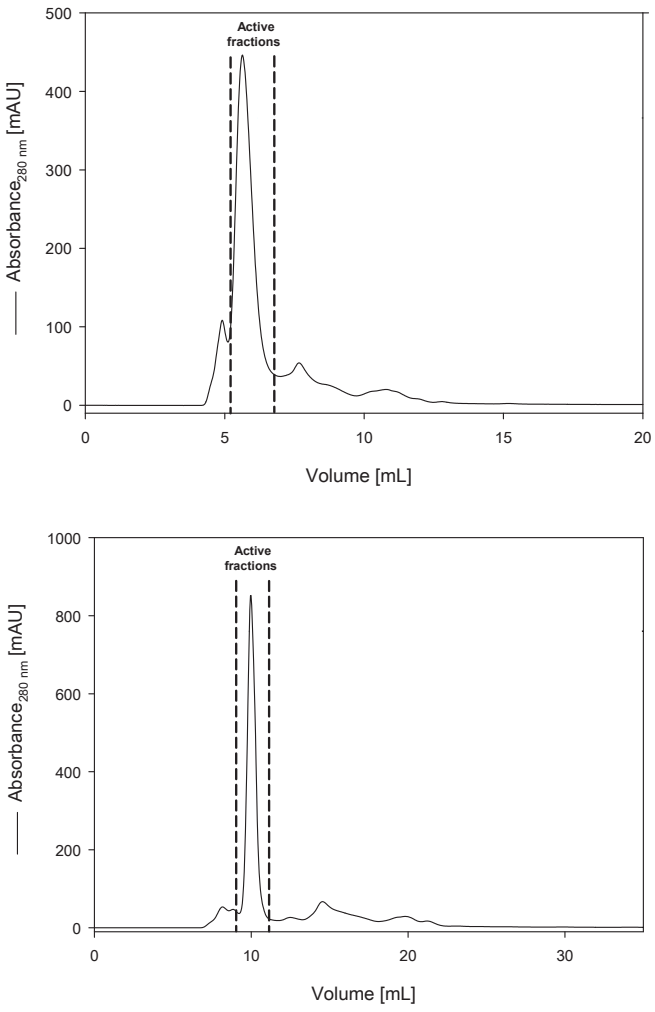


Figure 1

RESULTS AND DISCUSSION

After ammonium sulfate precipitation, the PepA showed a purity of approximately 95% (data not shown). For the subsequent purification SEC seemed promising due to the native molecular mass of approximately 480 kDa (homo dodecamer with ca. 40 kDa per subunit). Figure 1 shows the purification of PepA using the analytical TSKgel G3000SW column compared to a competitor column.

EXPERIMENTAL CONDITIONS

Columns:	TSKgel G3000SW (10 µm; 7.5 mm ID x 30 cm L; P/N 0005789) Competitor column (8.6 µm; 10 mm ID x 30 cm L) TSKgel G3000SW (13 µm; 21.5 mm ID x 60 cm L; P/N 0005147) and SW Guard (13 µm; 21.5 mm ID x 75 mm L; P/N 0005758)
Mobile phase:	50 mM Na/K-phosphate buffer pH 6.0
Flow rate:	Small scale: 0.5 mL/min Semi preparative scale: 6 mL/min
Detection:	UV @ 280 nm
Sample:	Recombinant aminopeptidase A (PepA) ammonium precipitate
Injected amounts:	Small scale: 0.3 mg/mL column resin (4.1 mg (TSKgel G3000SW) and 7.1 mg (competitor column) Semi preparative scale: 14 mg
System:	ÄKTA fast protein liquid chromatography
Detection:	UV @ 280 nm
Activity test:	H-Asp-pNA activity assay (data not shown)

As seen in Figure 1, a defined peak containing PepA was observed for both columns. To evaluate the particular purifications, a subsequent SDS-PAGE analysis was performed (see Figure 2).

SDS-PAGE ANALYSIS OF THE PepA ACTIVE FRACTIONS AFTER PURIFICATION USING AN ANALYTICAL TSKgel G3000SW (A) AND A COMPETITOR COLUMN (B)

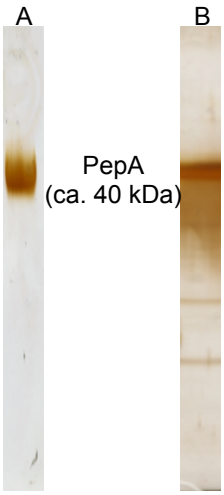


Figure 2

Although the PepA peak was sharper using the competitor column, the purity (see Figure 2) was higher for the TSKgel G3000SW column. Only a single band was observed when using the TSKgel G3000SW column. In contrast, besides PepA, additional bands were observed on SDS-PAGE after purification using the competitor column.

Due to the higher purity obtained with the analytical TSKgel G3000SW column, up-scaling of the purification process was performed with a preparative TSKgel G3000SW column.

PURIFICATION OF PepA USING A PREPARATIVE TSKgel G3000SW COLUMN

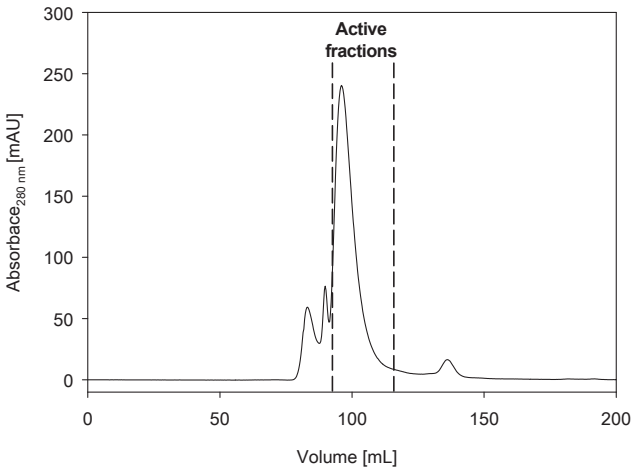


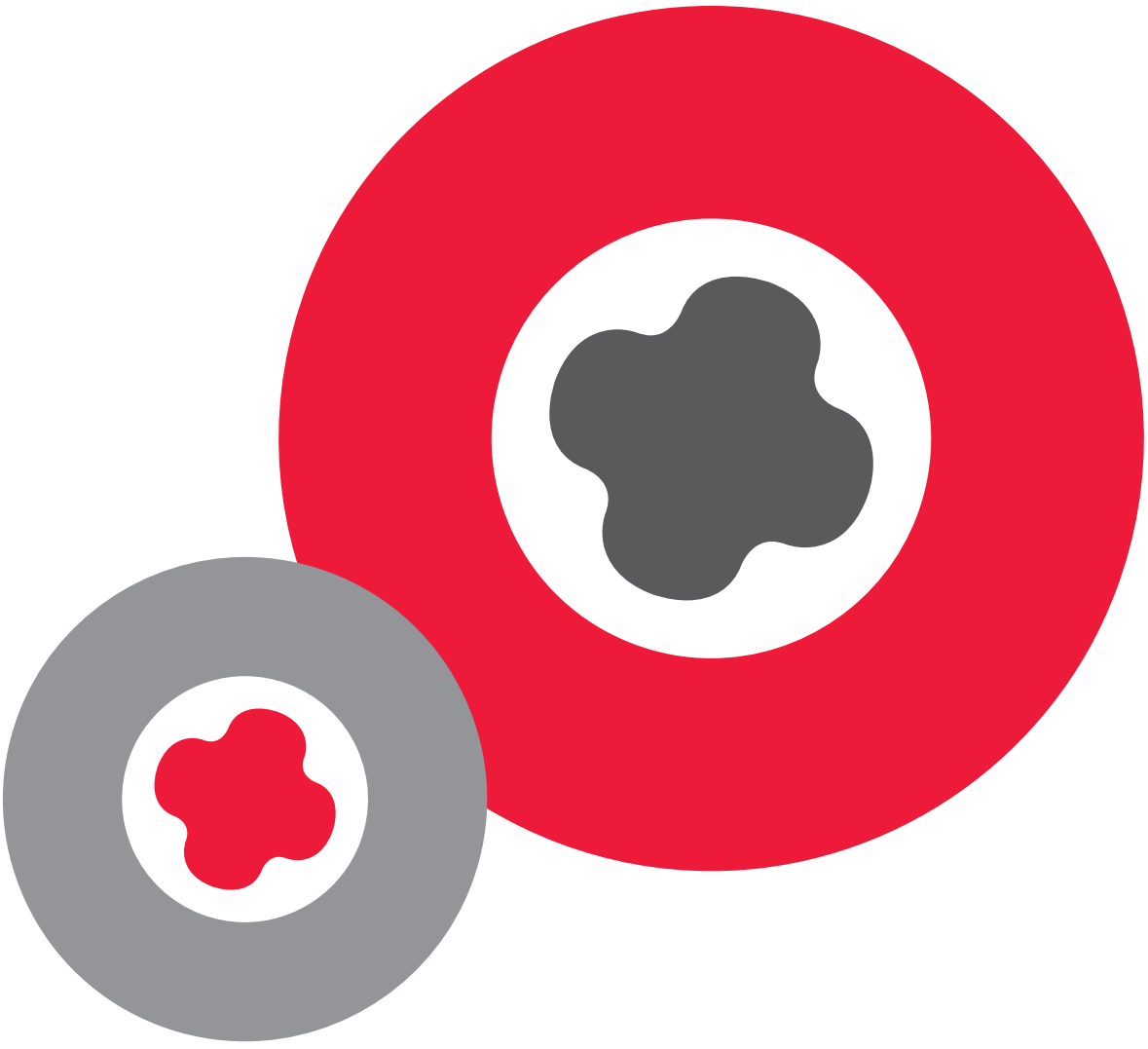
Figure 3

As seen in Figure 3, the purification chromatogram of the preparative TSKgel G3000SW was similar to that obtained using the analytical TSKgel G3000SW column (Figure 1). In addition, the subsequently performed SDS-PAGE was identical to Figure 2 (lane A). This indicates that up-scaling from the analytical to the preparative TSKgel G3000SW was possible without losing purity of the PepA.

CONCLUSION

This work illustrates that the series of TSKgel G3000SW columns was useful for the purification of an enzyme with a high molecular mass of approximately 480 kDa. The purity was higher than using a comparable competitor column. In addition, up-scaling of the purification process was possible without losing purity.

3
NUCLEIC ACIDS



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Application Note

HILIC SEPARATION OF NUCLEOBASES USING TSKgel® SuperSW mAb HTP COLUMN

INTRODUCTION

Hydrophilic Interaction Liquid Chromatography (HILIC) is one of the fastest growing modes of separation, in which any polar chromatographic surface can be used. Chemically bonded diol coated phases, as found in TSKgel SW size exclusion chromatography (SEC) columns, demonstrate high polarity and hydrogen bonding properties and do not contain ionizable groups other than the unreacted residual silanols, making them appropriate for 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 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.

The TSKgel SuperSW mAb HTP column is a newly introduced SEC column designed for the high throughput separation of monoclonal antibodies from their high and low molecular mass variants. TSKgel SuperSW mAb HTP has a diol coating to minimize secondary interactions which may occur in SEC separations. This note demonstrates the benefits of using a TSKgel SuperSW mAb HTP column in HILIC mode for the superior resolution of four nucleobases, as opposed to using the column in SEC mode or using a HILIC column.

EXPERIMENTAL CONDITIONS

Instrument:	Agilent 1100 HPLC system run by Chemstation (ver B.04.02)
Columns:	TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm L ; TSKgel Amide-80, 5 µm, 2.0 mm ID × 10 cm L
Mobile phase:	A: acetonitrile (HILIC mode); B: 15 mmol/L ammonium bicarbonate, pH 7.4 (HILIC mode)
Mobile phase:	100 mmol/L phosphate/100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3 (SEC mode)
Gradient:	Isocratic
Flow rate:	0.4 mL/min
Detection:	UV @ 280 nm
Injection vol.:	1 µL
Temperature:	ambient
Samples:	uracil (1.5 mg/mL), adenine (1.5 mg/mL), cytosine (1.5 mg/mL), cytidine (1.5 mg/mL) from Sigma Aldrich

SEPARATION OF FOUR NUCLEOBASES USING TSKgel SuperSW mAb HTP COLUMN IN HILIC MODE AT pH 7.4

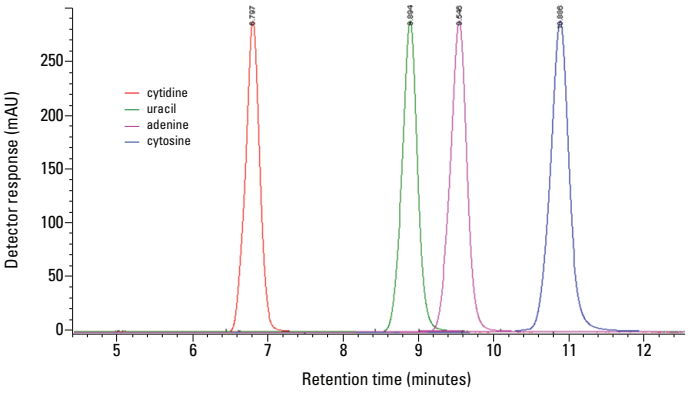

Figure 1

SEPARATION OF NUCLEOBASES USING THE TSKgel SuperSW mAb HTP COLUMN UNDER CONVENTIONAL SEC CONDITIONS

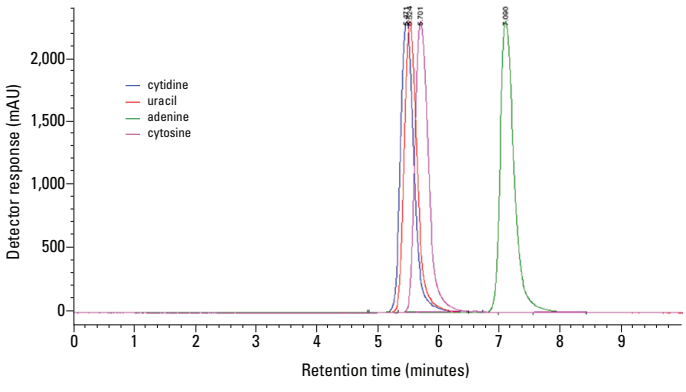

Figure 2

RESULTS AND DISCUSSION

Figure 1 illustrates the separation of 4 nucleobases using the TSKgel SuperSW mAb HTP column in HILIC mode with 15 mmol/L ammonium bicarbonate, pH 7.4 as mobile phase B. It is important to note that 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.

Figure 2 illustrates the separation of the four nucleobases on the TSKgel SuperSW mAb HTP column using conventional SEC conditions. As expected, due to the similarities in molecular masses between the four compounds, significant interference is observed amongst 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 3 compounds) may be attributed to possible interactions between the stationary phase and the derivatized purine compound, leading to a shift towards longer retention time.

In an effort to explore the novelty of the separation of nucleobases using the TSKgel SuperSW mAb HTP column in HILIC mode, the same separation was carried out using a TSKgel Amide-80 HILIC column. The use of the TSKgel Amide-80 column yields very poor separation of the four nucleobases with virtually no retention of any of the components (Figure 3).

SEPARATION OF NUCLEOBASES USING THE TSKgel AMIDE-80 HILIC COLUMN

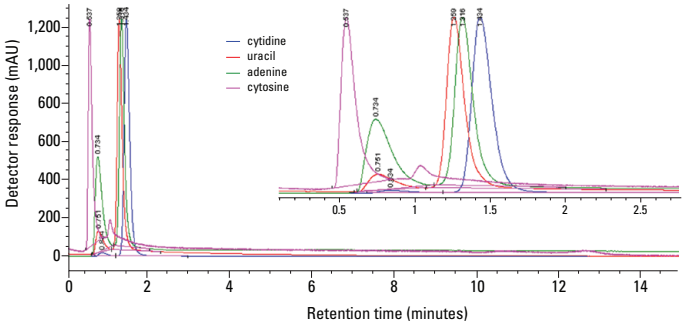

Figure 3

CONCLUSION

This work illustrates the novelty and utility of the TSKgel SuperSW mAb HTP column as a diol-functionalized HILIC column for the high resolution separation of nucleobases on the basis of their relative hydrophilicity, rather than differences in their relative molecular mass. As shown, markedly different separation profiles are observed with the use of the TSKgel Amide-80 HILIC column under identical chromatographic conditions. Additionally, nucleobase separation using the TSKgel SuperSW mAb HTP under conventional SEC conditions yielded poor resolution of all components, making it an ineffective mode of separation for this application. The TSKgel SuperSW mAb HTP column, while designed for SEC separation of monoclonal antibodies, is an extremely effective tool in HILIC mode that should be considered for the fast separation of nucleobases.

4
TIPS AND TRICKS

TOSOH

APPLICATION NOTE

UHPLC SEPARATIONS USING HPLC METHODS AND TSKgel® COLUMNS

INTRODUCTION

The use of UHPLC systems for small molecule analysis has gained widespread acceptance among researchers in recent years. A UHPLC system is a HPLC system optimized with regards to dead volume, injection performance, and detector sampling rate and is able to tolerate application pressures exceeding 1,000 bar. It is therefore advantageous to use UHPLC instrumentation for methods developed on conventional HPLC systems with HPLC columns. The use of HPLC columns with UHPLC systems offers the advantages of cost and time savings over having to purchase and develop new methods with UHPLC columns. This application note demonstrates the excellent performance of conventional TSKgel HPLC columns on a UHPLC system.

EXPERIMENTAL CONDITIONS

Column:	TSKgel SP-STAT, 7 µm, 4.6 mm ID × 10 cm
Systems:	Dionex UltiMate® 3000 HPLC System (equipped with Dionex UVD 170S Detector) Dionex UltiMate 3000RS UHPLC System
Mobile phase:	A: 10 mmol/L sodium phosphate, pH 7.0 B: 10 mmol/L sodium phosphate, pH 7.0, + 1 mol/L sodium chloride
Gradient:	0-50% B in 25 min
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Injection vol.:	5 µL
Sample:	monoclonal IgG, 1 mg/mL
Column:	TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm
Systems:	Dionex UltiMate 3000 HPLC System (equipped with Dionex UVD 170S Detector) Dionex UltiMate 3000RS UHPLC System
Mobile phase:	0.1 mol/L sodium phosphate, pH 6.7 + 0.1 mol/L sodium sulfate
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Injection vol.:	20 µL
Samples:	para-aminobenzoic acid, thyroglobulin, γ-globulin, ovalbumin, ribonuclease A, 2 mg/mL each

RESULTS AND DISCUSSION

Figure 1 shows the analysis of a monoclonal IgG using a TSKgel SP-STAT cation exchange column. The same column, sample, and method were used to verify the column-system compatibility on both the HPLC (blue chromatogram) and UHPLC (red chromatogram) systems. The column performs better in the UHPLC system than it does when connected to the HPLC system. The number of theoretical plates is 6% higher for the UHPLC setup.

Figure 2 shows a zoomed elution profile of the mAb charge variants on the TSKgel SP-STAT column, which provides better insight into what extent the elution profile benefits from using a UHPLC system.

ANALYSIS OF A MONOCLONAL IgG USING A TSKgel SP-STAT COLUMN AND A ZOOMED ELUTION PROFILE OF THE mAb CHARGE VARIANTS ON THE TSKgel SP-STAT COLUMN

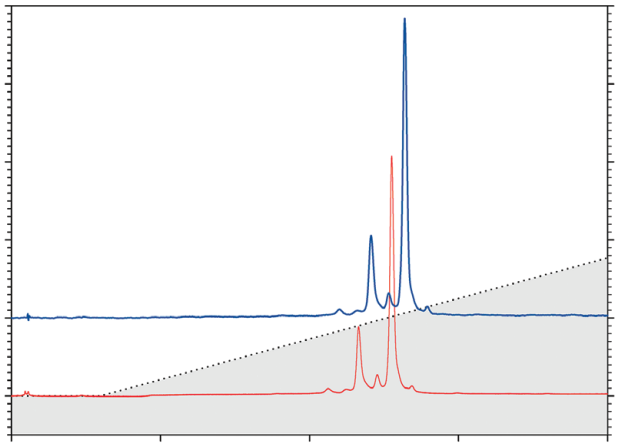

Figure 1

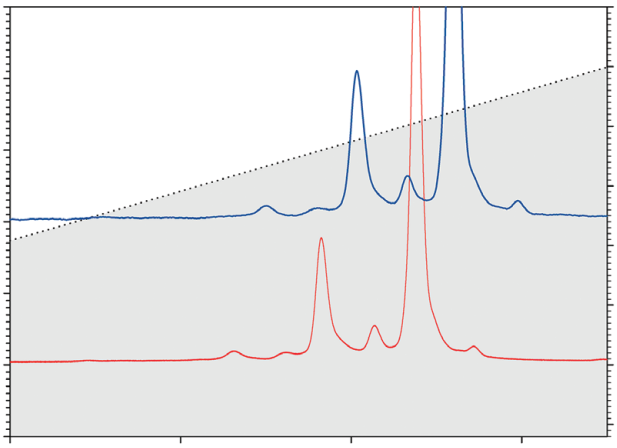

Figure 2

The peak width is smaller for the UHPLC chromatogram. The decreased system dead volume resulted in the peak elution to occur earlier than it did on the HPLC system.

Figure 3 shows a standard protein mixture analyzed using a TSKgel G2000SWXL size exclusion column. The number of theoretical plates exceeds 32,000 for para-aminobenzoic acid when connected to the UHPLC system (blue chromatogram), while the column connected to the HPLC system (red chromatogram) only reaches 29,000 for the same component.

Of course, resolution is an important factor when considering chromatographic performance. The HPLC data shows a resolution of 2.1 for the separation of γ-globulin and ovalbumin, and 10.2 for ribonuclease A and para-aminobenzoic acid. For the UHPLC data, the resolution factors increase to 2.2 and 10.9 for the respective peak pairs.

CONCLUSION

The results of both analyses using a TSKgel SP-STAT ion exchange column and a TSKgel G2000SWXL size exclusion column indicate excellent separation with high resolution when used on a UHPLC system. As a UHPLC system is simply an optimized HPLC system, bioseparation method transfer from HPLC to UHPLC is hardly more complicated than from one HPLC instrument to another. The applied pressure in these applications was far from exceeding the limit of a conventional HPLC system. This is the case in most bioseparation applications, since many biological sample molecules themselves cannot withstand very high pressure. Transferring established HPLC methods using HPLC columns to a UHPLC system is an economical and time-saving way to take advantage of this latest wave of development in HPLC technology.

SEPARATION OF A STANDARD PROTEIN MIXTURE USING A TSKgel G2000SWXL COLUMN

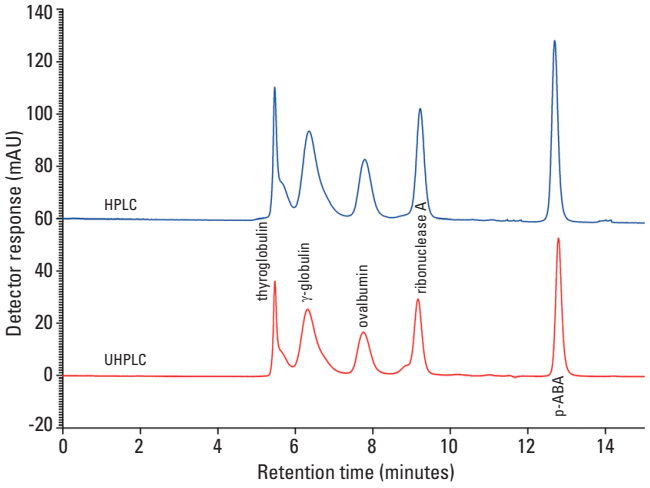

Figure 3

TOSOH

APPLICATION NOTE

A TOOLBOX OF AMINO ACIDS FOR OUT-OF-THE-BOX mAb SEPARATIONS

Size exclusion chromatography (SEC) is well established for mAb aggregate analysis. As the technique has been used since the early days of mAb development for pharmaceutical purposes, various method improvements have evolved. For instance, the benefits of arginine on analytical SEC of mAb aggregate samples are well-known. Herein, we present how SEC of mAb aggregate samples may take advantage of other amino acid additives in the mobile phase.

Recently, various approaches to improve analytical SEC have focused on reducing the analysis time. For instance, this can be achieved by staggered injection protocols or increased linear flow rates as possible for columns with outstanding packing quality. On the other hand, in the light of method optimization, the mobile phase composition leaves less room for improvement, compared to other chromatographic modes. As soon as a certain ionic strength to inhibit electrostatic interactions without causing hydrophobic interactions and the pH of the mobile phase which ensures structural integrity of proteins and the stationary phase are set, one might think that the analysis solely depends on the particle size, packing quality and column length. However, the mobile phase composition is not at the end of its rope, in case the mentioned parameters have been set. For example, Arakawa et al. have described the impact of arginine on aggregate recovery in SEC[1]. Confirming that this effect was not caused by an increased ionic strength, Yumioka et al. investigated the impact of sodium chloride as a rather chaotropic salt on mAb aggregate SEC. For increasing sodium chloride concentrations, protein recovery was decreased[2]. In fact, the arginine addition ensures proper aggregate elution. This is also true for other amino acids, as it can be seen in Fig. 1.

A mAb was aggregated by incubation at 75°C for 5 min. Subsequently, the sample was analyzed via TSKgel UltraSW Aggregate 7.8 mm ID x 30 cm L with different mobile phases, all of them using virgin columns. 0.2 M lysine, arginine, proline, glutamine or sodium sulfate were added to 0.1 M sodium phosphate buffer, pH 6.7, respectively. A flow rate of 1 mL/min was applied, 20 µl and 100 µg of the aggregated mAb sample were injected. The columns were equilibrated for at least 10 column volumes. Figure 1 illustrates the results on aggregate recovery. Glutamine and proline show a similar behavior: the aggregates are hardly recovered for the first two injections, while the aggregate peak suddenly appears for injection #3 and #4. The rise is not as sudden for sodium sulfate, but the aggregate peak will only achieve its full size for injection #10. In opposition to these results, lysine shows an even improved aggregate recovery compared to arginine. The inter-injection variability is low, depicting the complete aggregate content for all of the injections.

AGGREGATE RECOVERY IN ANALYTICAL SEC ON NEW COLUMNS.

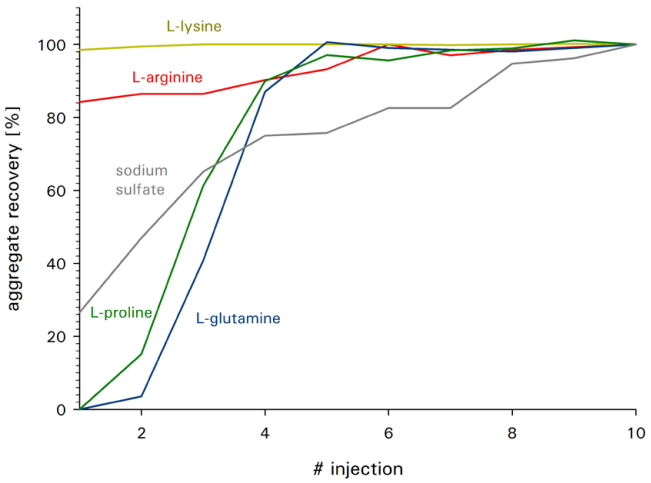

Figure 1

The mobile phases contain different amino acids: lysine (yellow), arginine (red), prolin (green) and glutamine (blue). Sodium sulfate instead of an amino acid was added as a reference. Lysine and arginine allow almost complete aggregate recovery starting with injection #1, while proline and glutamine lead to reduced aggregate recovery compared to sodium sulfate. Column: TSKgel UltraSW Aggregate, Flow: 1 ml/min, Injected volume: 20 µl, injected mass: 100 µg, Detection: UV @ 280 nm.

mAb SAMPLE ON TSKgel UltraSW AGGREGATE

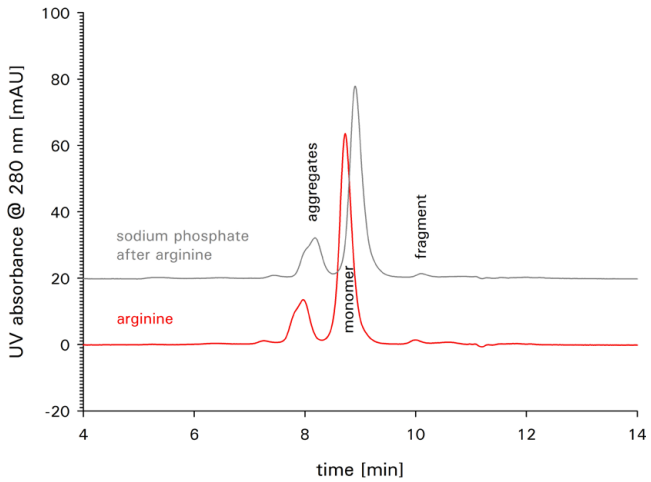

Figure 2

mAb sample on TSKgel UltraSW Aggregate with 0.1 M sodium phosphate buffer containing 0.2 M arginine in the mobile phase (red). After 10 injections, the mobile phase was switched to sodium phosphate buffer with an addition of 0.2 M sodium sulfate (grey). For both mobile phases, injection #10 is shown. Column: TSKgel UltraSW Aggregate, Flow: 1 ml/min, Injected volume: 20 µl, Injected mass: 100 µg, Detection: UV @ 280 nm.

Besides aggregate recovery, resolution of the different sample components, namely the monomer and the different aggregates, is crucial for accurate analysis. Clearly, there is motivation to increase resolution. If this was achieved with a simple and inexpensive mobile phase additive, many applications could potentially benefit from such an advanced buffer composition. The impact of arginine in the mobile phase for analytical SEC of mAb aggregates focusing on the separation performance has been investigated and reported in the literature [3]. Figures 2 & 3 depict the separation profile of an aggregated mAb sample on TSKgel UltraSW Aggregate using 0.1 M sodium phosphate buffer, pH 6.7 with an addition of either 0.2 M arginine, or 0.2 M proline.

Ten injections with the respective amino acid buffer were followed by 10 injections applying sodium phosphate buffer with an addition of 0.2 M sodium sulfate, in order to compare the two buffers. Monomer aggregate resolution, as well as monomer fragment resolution is slightly improved for the two amino acid buffers. Table 1 lists the resolutions for some amino acid buffers and the results for the corresponding columns applying sodium phosphate buffer containing 0.2 M sodium sulfate. New columns were used for every amino acid.

Arginine, proline and glutamine provide slightly increased monomer aggregate resolution. For arginine, the fragment monomer resolution is also improved. Although these increases in resolution are not drastic, they confirm that increased resolution due to the use of an advanced mobile phase is possible and that mobile phase testing can contribute to a more reliable and robust aggregate analysis. Depending on the attributes of a particular mAb, one might consider different amino acids. For mAbs which are especially prone to unspecific interactions, lysine might be the preferable option, as it provided the most reliable aggregate recovery beyond the tested amino acids in this study. Contrariwise, if an aggregated mAb would cause less severe problems due to unspecific interactions, arginine offers highest resolution of all the tested amino acids and a slightly decreased aggregate recovery for the first injections, compared to lysine.

REFERENCES

1. Arakawa T, et al., The critical role of mobile phase composition in size exclusion chromatography of protein pharmaceuticals. J. Pharmaceutical Sciences 99(4) 2010: 1674-1692
2. Yumioka R, et al., Mobile phase containing arginine provides more reliable SEC condition for aggregate analysis. J. Pharmaceutical Sciences, 99(2) 2010: 618-620
3. Ejima D, et al., Arginine as an Effective Additive in Gel Permeation Chromatography. J. Chromatography A, 1094(1-2) 2005: 49–55.

mAb SAMPLE ON TSKgel UltraSW AGGREGATE

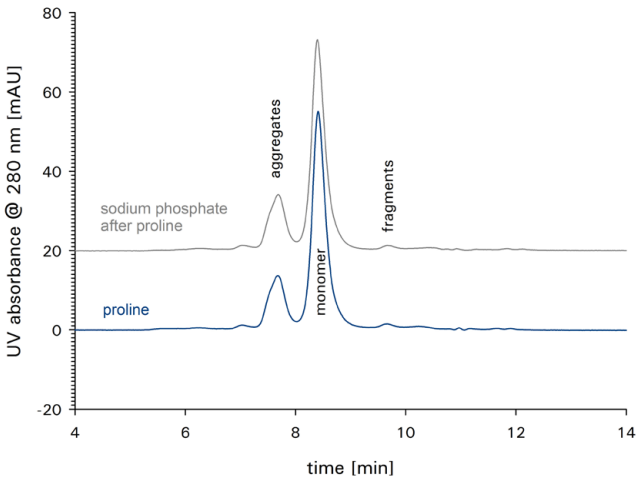

Figure 3

mAb sample on TSKgel UltraSW Aggregate with 0.1 M sodium phosphate buffer containing 0.2 M proline in the mobile phase (blue). After 10 injections, the mobile phase was switched to sodium phosphate buffer with an addition of 0.2 M sodium sulfate (grey). Injection #10 of the corresponding mobile phase is presented in the chromatogram. Column: TSKgel UltraSW Aggregate, Flow: 1 ml/min, Injected volume: 20 µl, Injected mass: 100 µg, Detection: UV @ 280 nm.

Buffer	Mean Rs Monomer - Aggregates	Mean Rs Monomer - Fragment
Arginine	1.6	3.2
NaP after Arginine	1.4	3.0
Proline	1.5	3.0
NaP after Proline	1.3	3.1
Glutamine	1.4	3.0
NaP after Glutamine	1.3	3.0
Lysine	1.3	3.0
NaP after Lysine	1.4	3.1

Table 1

The average resolution of 10 injections with the according mobile phase is listed in the table. Arginine proves to result in the highest resolution. Column: TSKgel UltraSW Aggregate, Flow: 1 ml/min, Injected volume: 20 µl, Injected mass: 100 µg, Detection: UV @ 280 nm.

The Usage of Isopropyl Alcohol in SEC for Monoclonal Antibody Separation

INTRODUCTION

Size exclusion chromatography (SEC) is widely used to quantitate monomers, dimers, aggregates, and fragments in antibody analysis. Due to a high demand for better resolution and faster analysis time, more well-designed SEC columns have been introduced. These are 2 µm and sub-2 µm particle size SEC columns with the appropriate pore size for analyzing antibodies with optimized particle chemistry. Despite this improvement, nonspecific absorption of antibodies onto the column gel matrix poses a challenge, with some newly engineered antibodies possessing a high degree of hydrophobicity. The use of organic 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.

In this application note, 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 were not impacted.

EXPERIMENTAL HPLC CONDITIONS

Column:	TSKgel UP-SW3000, 2 µm, 4.6 mm ID x 30 cm (PN 0023448)
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

RESULTS AND DISCUSSION

The excellent reproducibility of injection to injection of the USP mAb standard onto the TSKgel UP-SW3000, with a typical sodium phosphate buffer, pH 6.7, is shown in Figure 1. This figure is an overlay of 14 consecutive injections of the USP mAb standard sample at the flow rate of 0.3 mL/min. The retention times of monomer, dimer, aggregate, and fragment peaks are nearly unchanged. Peak width and peak shape are very consistent from injection to injection.

SEPARATION OF USP mAb STANDARD

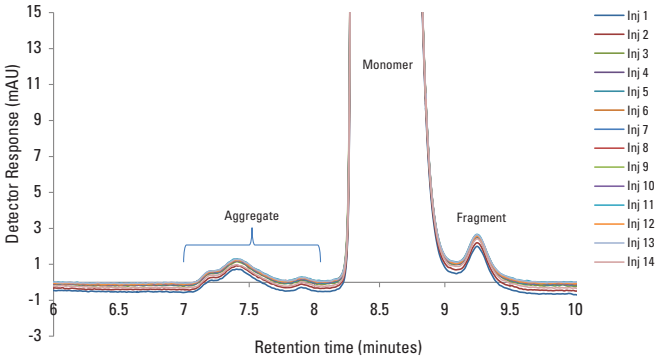

Figure 1

% RSD OF MONOMER AND DIMER PEAK OF 14 INJECTIONS: SEPARATION OF USP mAb STANDARD

	MONOMER PEAK		DIMER PEAK	
INJECTION	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 1

Table 1 consolidates the recorded calculated data from the monomer and dimer peaks of the 14 injections from Figure 1 with the % RSD of retention time and percent relative area below the allowance from the USP monograph guidance.

Figure 2 shows the overlay of 15 injections of the USP mAb standard sample onto the TSKgel UP-SW3000 column with the addition of 15% IPA. These injections are performed after the column is subjected to 15 injections of the USP mAb standard sample with sodium phosphate buffer, pH 6.7, without IPA. The baseline of the first injection (as shown in blue) indicates that the column takes only one to two injections to be stabilized. After that all subsequent injections are overlaid perfectly.

Table 2 lists the calculated data from the monomer and dimer peaks of the 15 injections from Fig. 2 with the % RSD of retention time and percent relative area. As shown, the % RSD is below the allowance from the USP monograph guidance.

The retention times and peak areas from the injections without IPA added are very similar to the retention times and peak areas with IPA added (compare Table 1 to Table 2).

At 0.3 mL/min, the pressure of the column is slightly higher when IPA is added to the mobile phase compared to when the column is operated without IPA. However, the pressure is only at 22 MPa with the IPA added. It is still far below the allowance of the maximum pressure of 34 MPa of the column's rating. With this low operating pressure, the TSKgel UP-SW3000 column can be operated with both HPLC and UHPLC systems. As the chromatograms indicate, all runs are completed within 15 minutes.

Figure 3 is an overlay of injections with and without IPA added to the mobile phase. The overlay indicates the similarities of peak retention times, peak width and peak height of dimer, monomer, aggregate and fragment peaks between the two different conditions.

CONCLUSION

An appropriate percentage of organic solvent such as isopropyl alcohol (IPA) does 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 indicates that the 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.

SEPARATION OF USP mAb STANDARD WITH 15% IPA ADDED

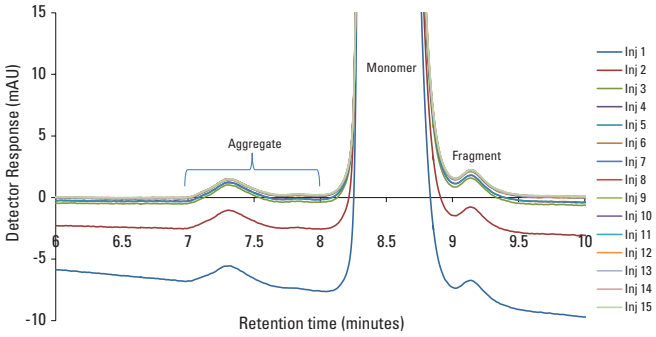

Figure 2

SEPARATION OF USP mAb STANDARD WITH AND WITHOUT 15% IPA ADDED

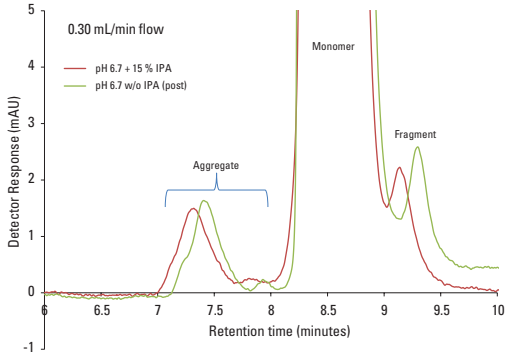

Figure 3

% RSD OF MONOMER AND DIMER PEAK OF 15 INJECTIONS: SEPARATION OF USP mAb STANDARD WITH 15% IPA ADDED

	MONOMER PEAK		DIMER PEAK	
INJECTION	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 2

TOSOH

APPLICATION NOTE

ANALYSIS OF MONOCLONAL ANTIBODY AGGREGATES BY SEC USING MS-FRIENDLY MOBILE PHASES

INTRODUCTION

The use of mass spectrometry as a means of detection is becoming increasingly more common among research laboratories in the field of proteomics. After more than 15 years of research, LC-MS systems are now more robust, and used more often for routine analyses which are nearly unachievable by any other mode of detection.

Separation of protein aggregates from their native species is often performed using Size Exclusion Chromatography (SEC), as this mode allows for the analysis of various components in a sample on the basis of their hydrodynamic radius in solution. Conventional SEC separations make use of relatively high ionic strength mobile phase compositions in an effort to minimize ionic interactions between the analyte and stationary phase. Due to the substantial amount of salt present in the mobile phase, on-line interfacing with mass spectrometry is not feasible due to the inevitable contamination of the MS ion source by the mobile phase salts. In order to make SEC-MS an applicable technique, volatile, MS-friendly mobile phase compositions must be implemented to avoid damage to the MS system. The challenge of using such mobile phase compositions exists due to the absence of salts which hinder ionic interactions during a separation.

This Application Note illustrates the effective use of MS-friendly mobile phase compositions in the analysis of monoclonal antibody aggregates using a TSKgel UltraSW Aggregate SEC column. The TSKgel UltraSW Aggregate column demonstrates high stability and low reactivity via ionic interactions even in low salt/no salt environments most likely due to the diol coating of the silica stationary phase.

EXPERIMENTAL CONDITIONS

Instrumentation:	Agilent 1200 HPLC system run by Chemstation® (ver. B.04.02)
Columns:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
Mobile phase:	100 mmol/L PO_4/100 mmol/L SO_4, pH 6.7 20% CH_3CN/0.1% TFA/0.1% FA 100 mmol/L ammonium bicarbonate, pH 7.0
Gradient:	Isocratic
Flow rate:	1 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	TBL mAb 1 (4.0 mg/mL)

SEPARATION OF mAb 1 USING VOLATILE AND SALT-BASED MOBILE PHASE COMPOSITIONS ON THE TSKgel UltraSW AGGREGATE COLUMN

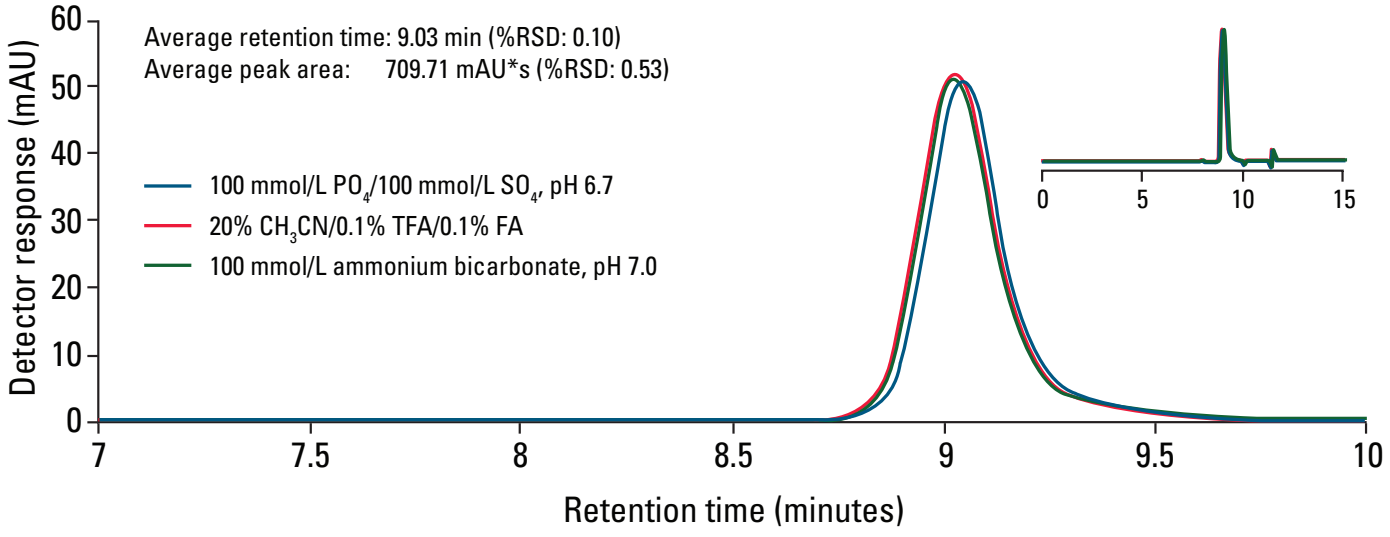

Figure 1

RESULTS AND DISCUSSION

Figure 1 illustrates the separation of mAb 1 using three different mobile phase compositions. All three mobile phase compositions yielded highly similar results for all peak parameters of the mAb 1 monomer. Additionally, slightly later elution of the mAb 1 monomer was observed when separated using the salt-based mobile phase. The observed shift in retention time of the mAb 1 monomer peak represents a %RSD of 0.1% among the three mobile phase conditions. Similarly average peak area of the mAb 1 monomer was found to be highly reproducible as well, yielding a %RSD of 0.53% across the three mobile phase systems evaluated in this work. This illustrates that the observed differences in retention time and peak areas obtained from the three mobile phase systems are not statistically significant.

As characterization of protein aggregates is of increasing importance in research, and with significant work being directed towards analysis by MS, the mAb 1 antibody was subjected to thermal stress for forced aggregation to evaluate the various mobile phase systems in this context. As shown in Figure 2, aggregates of mAb 1 are clearly separated from the monomeric species using all three mobile phase compositions.

Similar to Figure 1, results for critical peak parameters of the mAb 1 monomer are highly reproducible regardless of the mobile phase composition. Additionally, the total peak area (monomer and aggregate) obtained using the three mobile phase systems was highly similar to one another (avg: 671.04 mAu*s), and yielded a %RSD of 3.54%, illustrating the differences are not statistically significant. It is also of note that the observed shift in retention time of the mAb 1 monomer peak only corresponds to a %RSD of 0.12% for all three mobile phase compositions.

CONCLUSION

The growing interest in both protein aggregate analysis and mass spectrometry in the field of proteomics demand efective SEC-MS methods utilizing suitable mobile phases. The use of volatile mobile phase systems, such as 20% acetonitrile, 0.1% trifluoroacetic acid, and 0.1% formic acid or 100 mmol/L ammonium bicarbonate at pH 7.0 yield highly reproducible separation of mAb 1 aggregates, with similar or better performance over traditional salt-based mobile phase compositions. These results also show the effectiveness of the diol coating on the TSKgel UltraSW Aggregate column to assist in minimizing ionic interaction which are frequently present between the sample and stationary phase in low salt environments.

SEPARATION OF FORCED AGGREGATED mAb 1 USING VOLATILE AND SALT-BASED MOBILE PHASE COMPOSITIONS ON THE TSKgel UltraSW AGGREGATE COLUMN

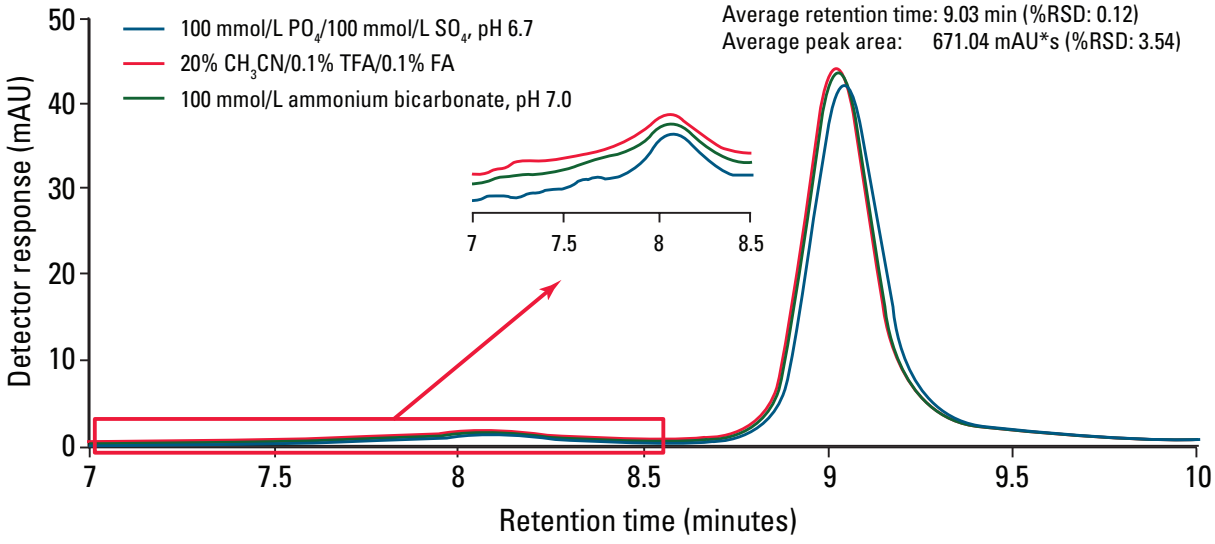

Figure 2

NOTES

NOTES

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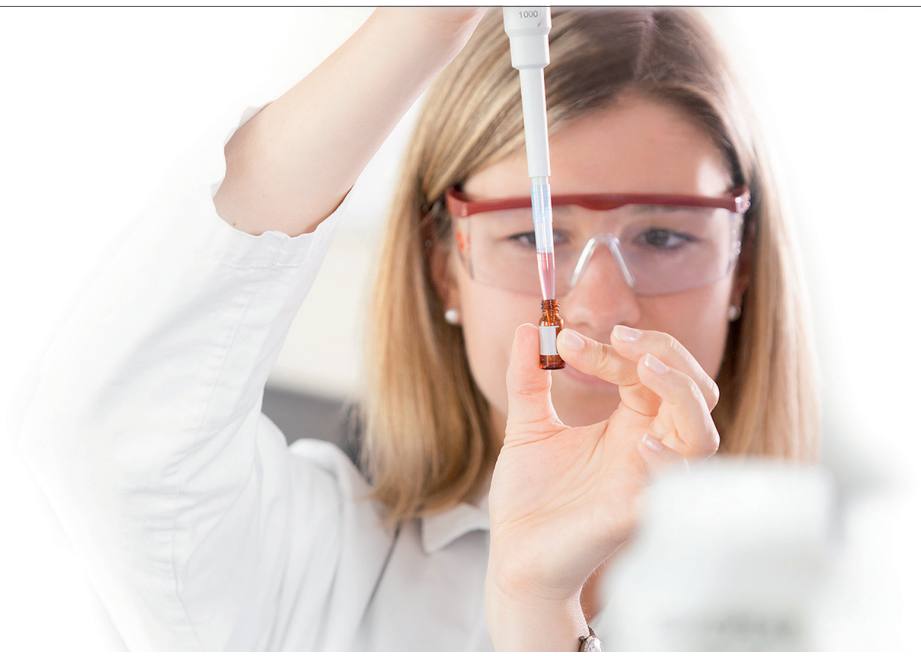

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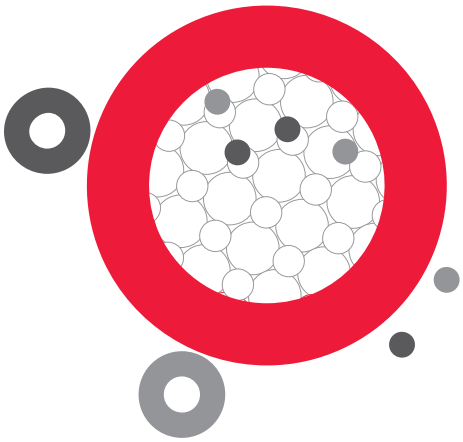

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