APPLICATION MONOCLONALS

MAB AGGREGATE ANALYSIS BY UHPLC

ANTIBODY THERAPEUTICS ARE ENJOYING HIGH GROWTH RATES. IN 2016, FIVE 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 ISO-FORMS.

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.

Compared to a commercially available 1.7 micron UHPLC column the calibration curves of the new TSKgel UP-SW3000 2 µm column (see Figure 1) shows a slightly 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 shows that the small difference in pore sizes results in a better separation in the molecular weight range of antibodies, fragments, and aggregates. Due to 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.

TSKgel UP-SW3000 is ideally suited for the analysis of the 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.

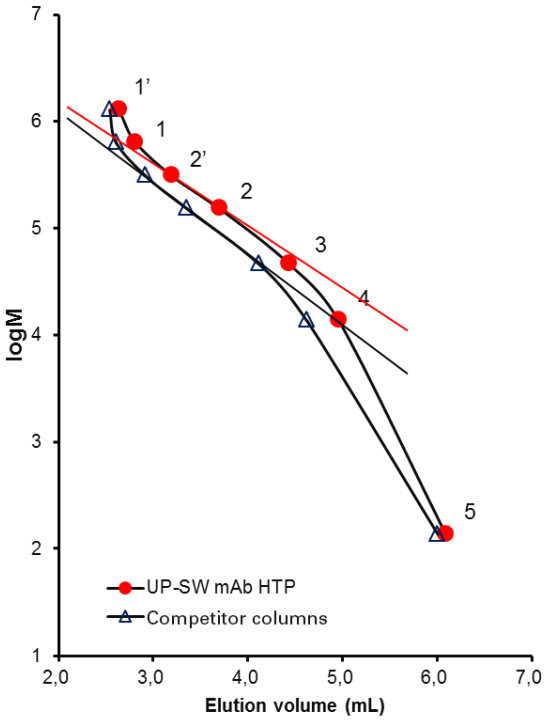


FIGURE 1 **CALIBRATION CURVES**
PROTEIN STANDARD ON 2 µm TSKgel UP-SW3000 AND 1.7 µm COMPETITOR COLUMN

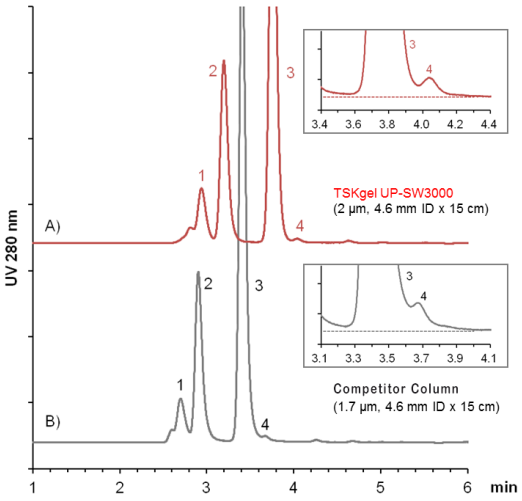
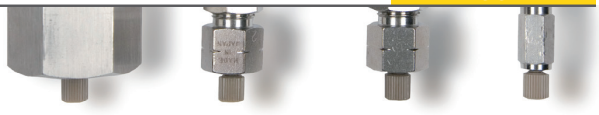


FIGURE 2 : **COMPARISON OF ANTIBODY ANALYSIS RESULTS**
MOUSE-HUMAN CHIMERIC mAb; 1: TRIMER; 2: DIMER; 3: MONOMER ; 4: FRAGMENT



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

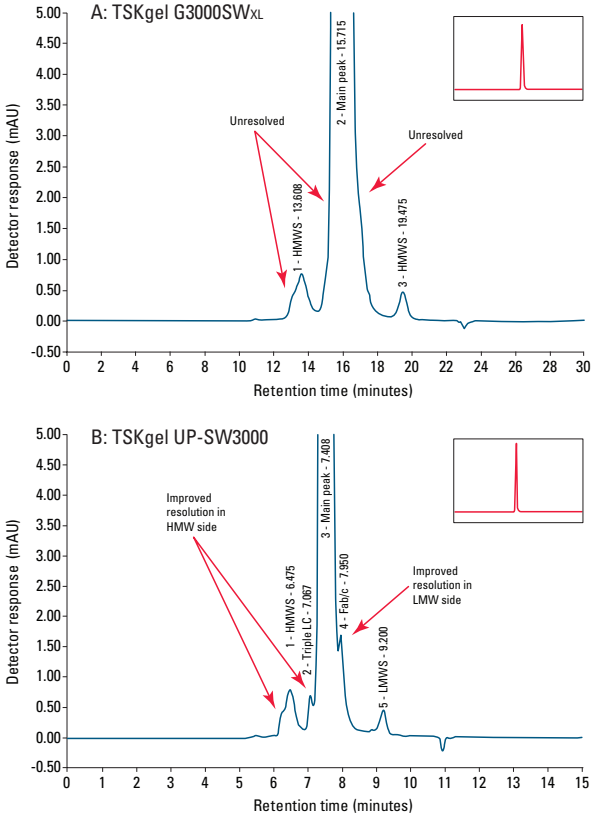


Figure 1

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. Dionex UltiMate® 3000RS UHPLC System
B. Agilent 1260
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

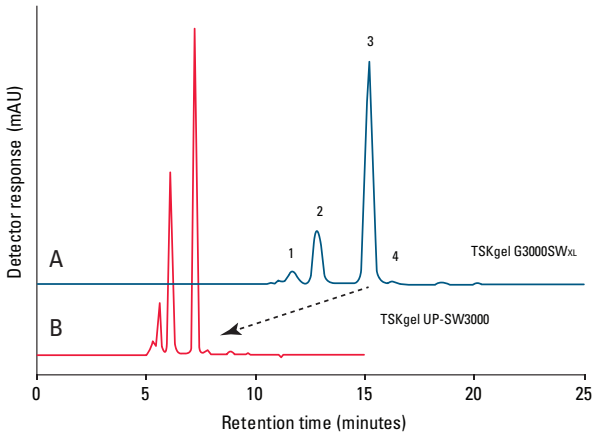


Figure 3

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.

TOSOH

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

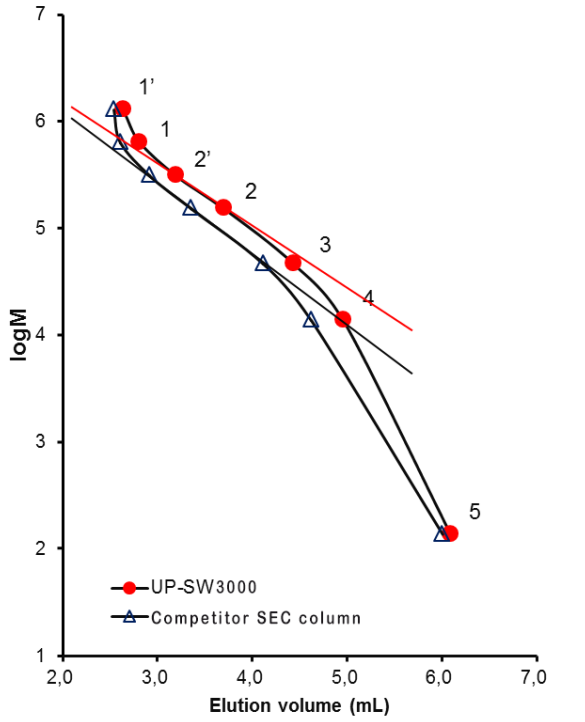


Figure 1

EXPERIMENTAL

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

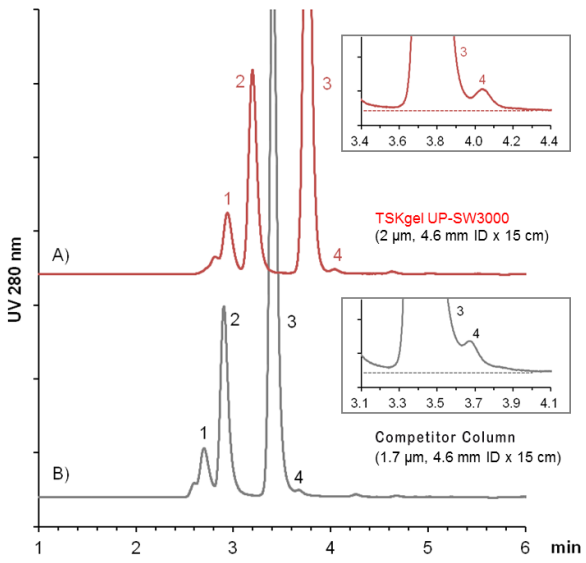


Figure 2

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

TOSOH

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

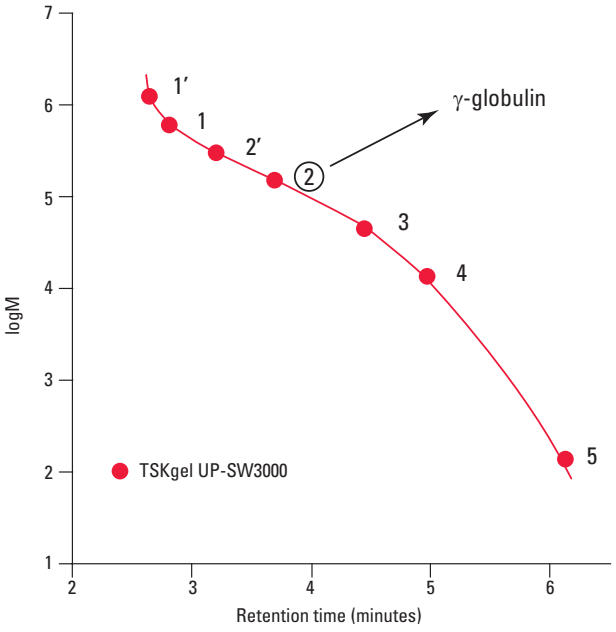


Figure 1

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

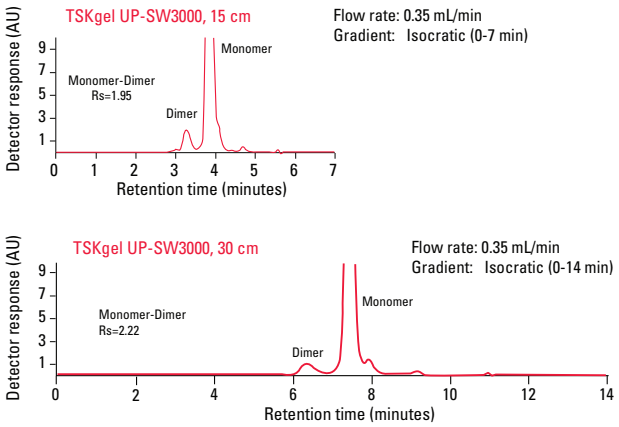


Figure 2

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

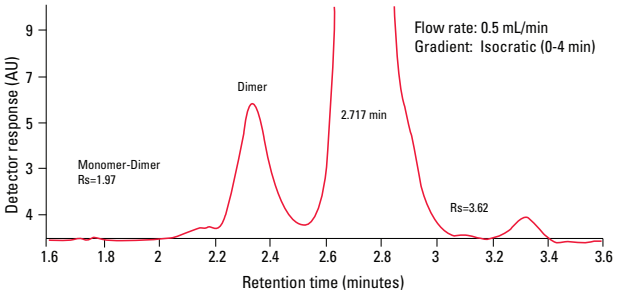


Figure 3

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

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

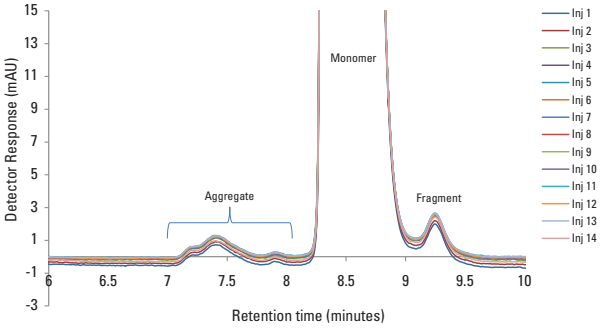


Figure 1

% 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

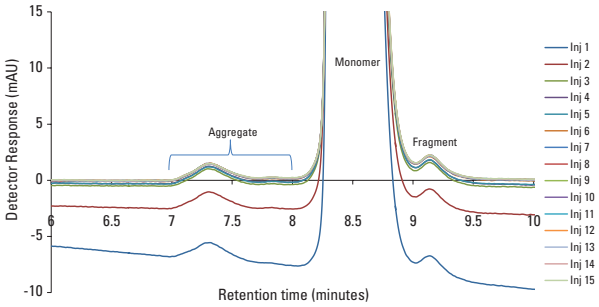


Figure 2

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

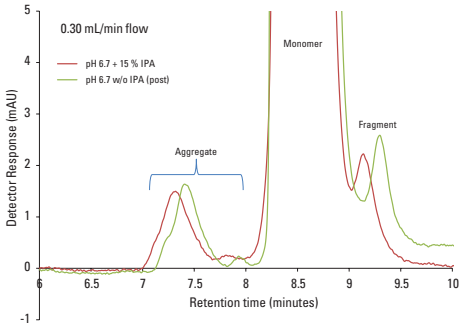


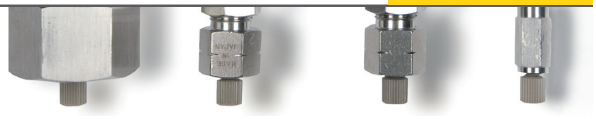
Figure 3

% 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

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.

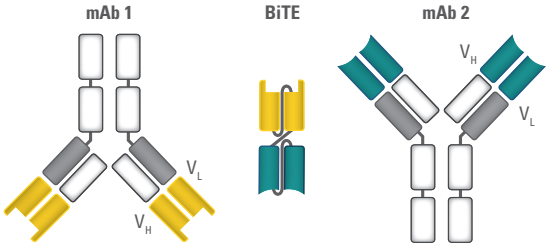
In this application note, a bispecific T cell engager (BiTE®) consisting of two single-chain variable fragments (scFvs) recombinantly linked by a nonimmunogenic five-amino-acid chain (Figure 1) was analyzed by SEC/MS using a TSKgel® UP-SW3000, 2 µm column.

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

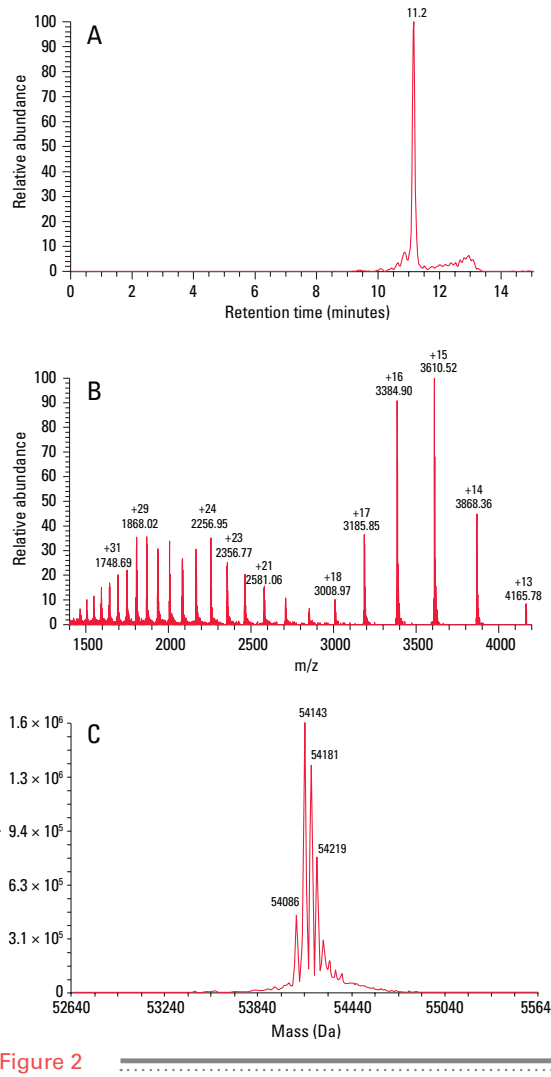


Figure 2

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

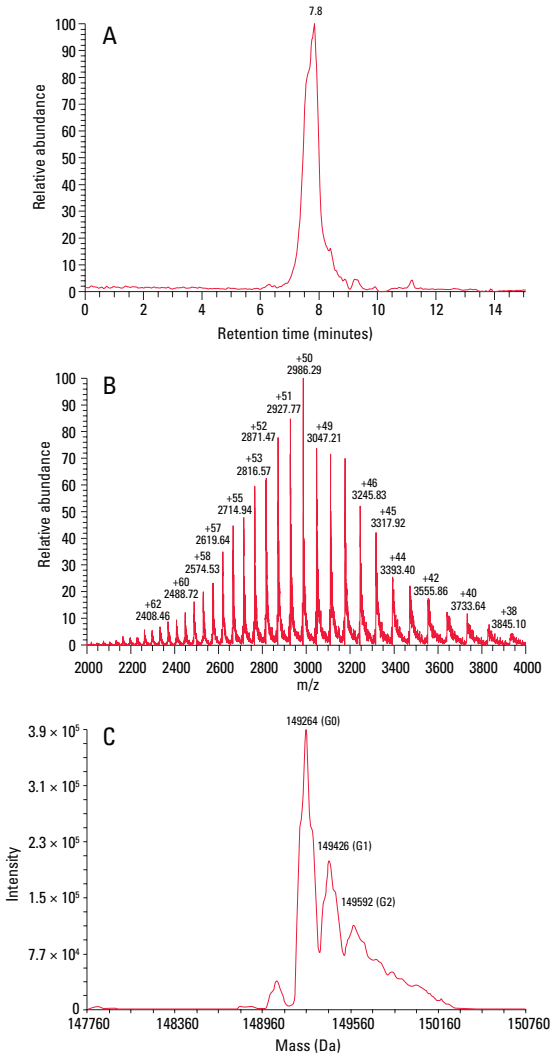


Figure 3

COLUMN SHEDDING AND CARRYOVER ANALYSIS

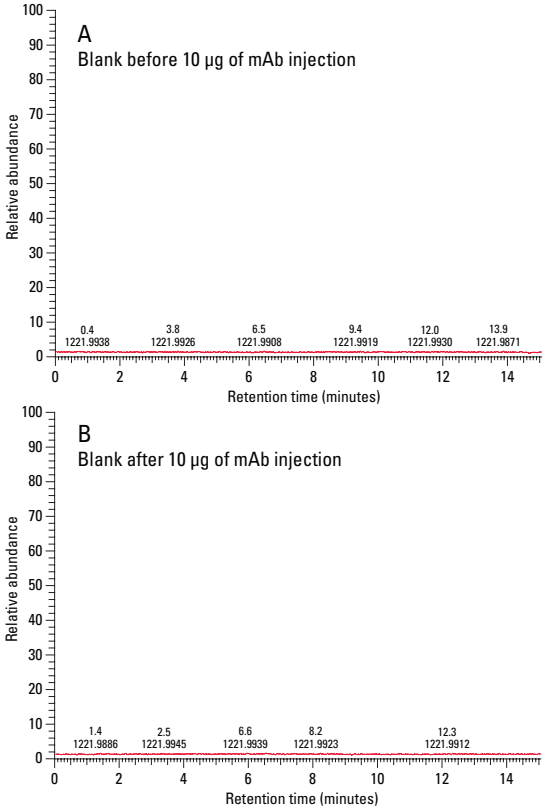


Figure 4

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.