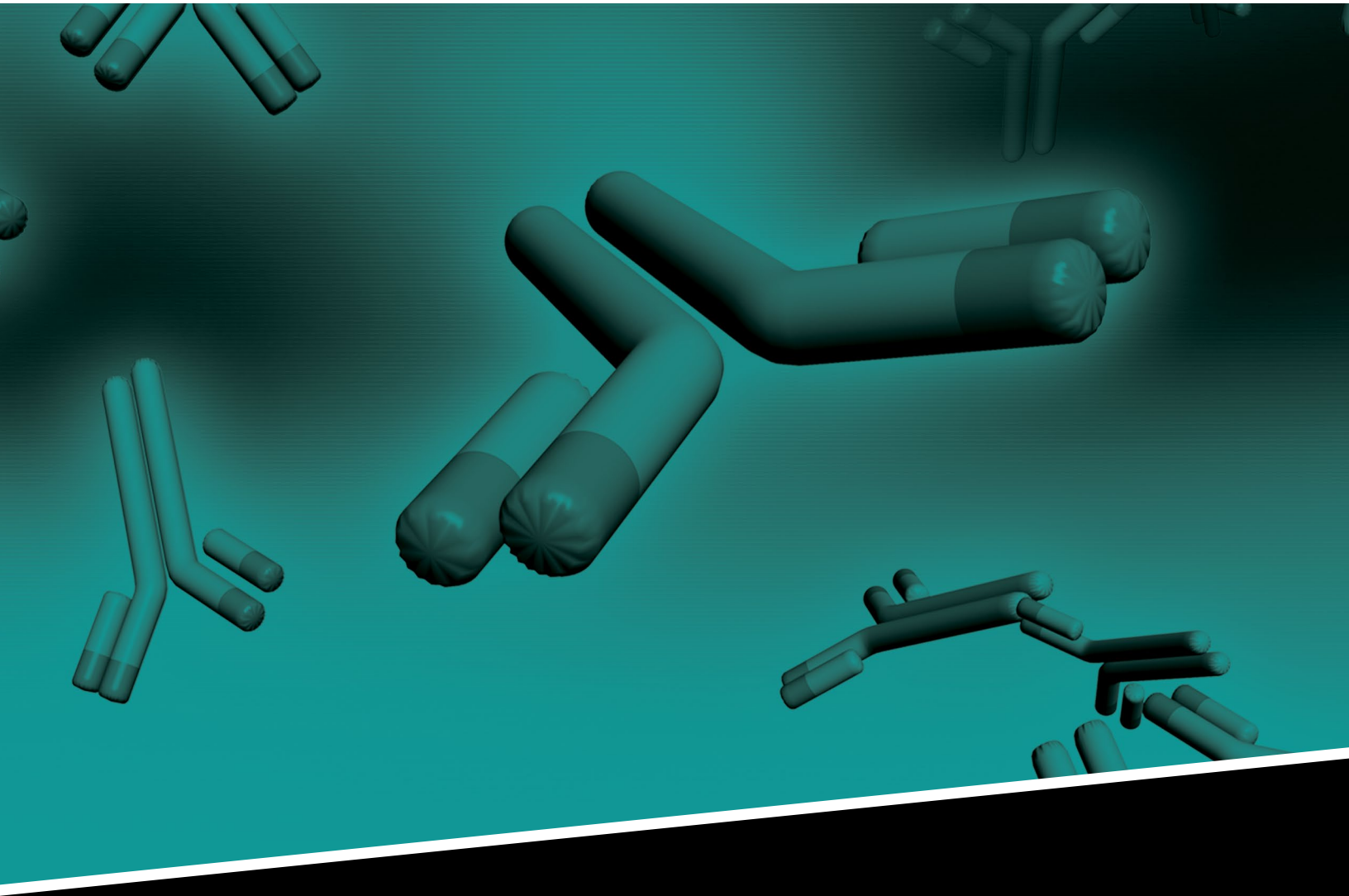
TOSOH

Solutions for Monoclonal Antibody Analysis and Characterization



TSKgel® U/HPLC Columns for Bioseparations

TOSOH BIOSCIENCE

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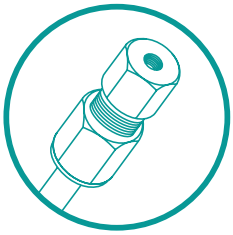
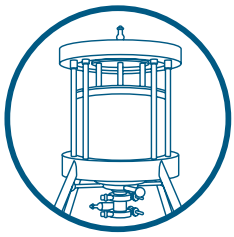
Solutions for Your Separation Tasks from Lab to Production

Tosoh Bioscience is a leading supplier of chromatographic columns, media and gel permeation chromatography (GPC) instruments with over 500 specialty products to meet your analysis and purification needs.



TSKgel® U/HPLC Columns

Extensively used in laboratories all over the world, our TSKgel columns are designed for researchers seeking the highest level of performance. Covering the total range of U/HPLC, these columns offer high resolution, excellent reproducibility, and long column life. Scaling up from analytical to preparative columns is made simple and easy.



TOYOPEARL® TSKgel & Ca^{++}Pure-HA® Resins

TOYOPEARL and TSKgel chromatography resins are specifically designed for the purification of biomolecules. Ca^{++}Pure-HA is a hydroxyapatite resin and has unique separation properties for biomolecules. These resins show excellent physical strength and ideal flow characteristics for industrial downstream processing.

EcoSEC® GPC Systems

The EcoSEC series of fully automated liquid chromatography systems for gel permeation chromatography is designed for robust polymer analysis. Both solutions, for ambient and for high temperature GPC, combine dual pump solvent systems, sophisticated heating and a highly efficient detection system to deliver the highest reproducibility.

This brochure will introduce you to TSKgel columns for monoclonal antiboday (mAb) analysis and characterization.

Solutions for Monoclonal Antibody Analysis and Characterization

TSKgel U/HPLC Columns for Bioseparations

Protein, monoclonal antibody (mAb) and antibody drug conjugate (ADC) biopharmaceuticals form a major part of the growing biologics drug market. During development and production of these products, it is essential to detect, characterize and quantify impurities as well as structural variants and modifications, and to monitor product stability. Figure 1 highlights several approaches to mAb and ADC characterization including size exclusion chromatography (SEC) for aggregate and fragment analysis, SEC/mass spectrometry (MS) for accurate mass determination, hydrophobic interaction chromatography (HIC) for ADC drug-to-antibody ratio (DAR) analysis, cation exchange (CEX) for charge variant analysis, and affinity chromatography (Protein A) for titer analysis.

Figure 1. LC and LC/MS protein analytical chemistry methods for testing biological products

Titer Analysis	**mAb titer determination (concentration) and screening**
Aggregate Analysis	**Screening for mAb aggregates**
Charge Variant Analysis	**Charge variant profiling/screening**
Drug-to-Antibody Ratio (DAR) Analysis	**ADC DAR analysis**
Intact mAb and Fragment Analysis	**Light chain (LC) and heavy chain (HC) analysis; Fab and Fc analysis; scFc and $F(ab')_2$ analysis**
Glycan Profiling	**Profiling of released glycans**
Accurate Mass Determination	**Mass spectrometry under native conditions**

Tosoh Bioscience offers innovative products that have been specifically engineered for these LC and LC/MS protein analytical chemistry methods for testing biological products. This specificity ensures high quality and consistent results.

Get Started

Additional resources are available for helping you implement TSKgel U/HPLC columns into your laboratory:

Web

Visit tosohbioscience.com for videos, product information and ordering.

Email

Our technical service staff is ready to answer questions: techservice.tbl@tosoh.com

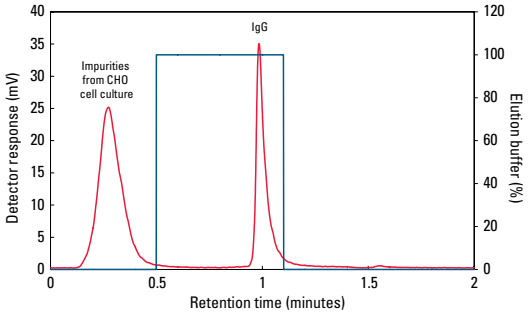
In Person

A technical seminar can be arranged on-site or via the web. Request via seminars@tosoh.com

Titer Analysis

The **TSKgel Protein A-5PW** affinity column has been designed for the rapid separation and robust quantification of a variety of antibodies. Monoclonal antibodies from harvested cell culture media can be captured and accurately quantitated in less than 2 minutes per injection (Figure 2).

Figure 2. Fast capture of IgG in the mixture of CHO cell supernatant spiked with IgG using a TSKgel Protein A-5PW column



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)

Figure 3 demonstrates the high durability and wide dynamic load range of the TSKgel Protein A-5PW column prior to and after 2,009 injections without being cleaned.

Figure 3. Durability and dynamic range of TSKgel Protein A-5PW column

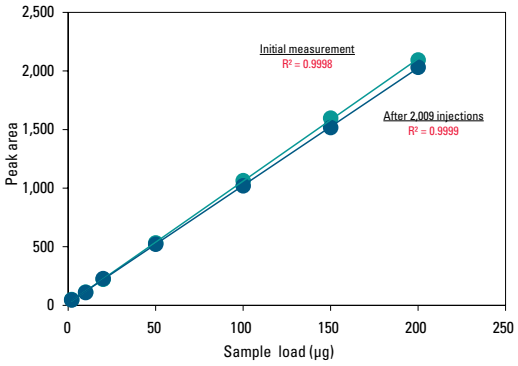
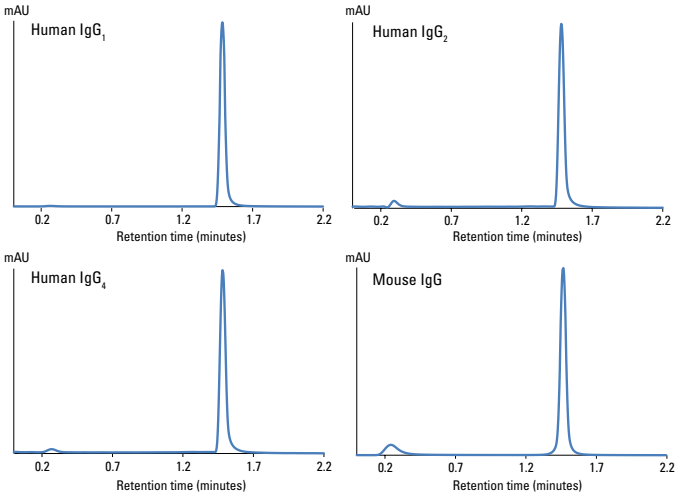


Table 1 demonstrates the wide range of species and IgG subclasses that the **TSKgel Protein A-5PW** column has affinity towards. The recombinant protein A ligand is a code-modified hexamer of the C domain which enables the column to bind more species and IgG subclasses than columns that contain native protein A ligands (Figure 4).

Table 1. The affinity of the Protein A ligand of Protein A-5PW columns versus native protein A ligand for various species' IgG subtypes

Species	Subclass	Protein A ligand of Protein A-5PW	Native Protein A
Human	IgG_1	+++++	++++
	IgG_2	+++++	++++
	IgG_3	-	-
	IgG_4	+++++	++++
Mouse	IgG_1	++++	+
	IgG_{2a}	+++++	++++
	IgG_{2b}	+++++	+++
	IgG_3	++++	++
Rat	IgG_1	++++	-
	IgG_{2a}	-	-
	IgG_{2b}	+++	-
	IgG_{2c}	++++	-
Goat	IgG_s	++++	-
Chicken	IgY	-	-
Rabbit	IgG	+++++	++++

Figure 4. Fast capture of various IgG subclasses using TSKgel Protein A-5PW columns

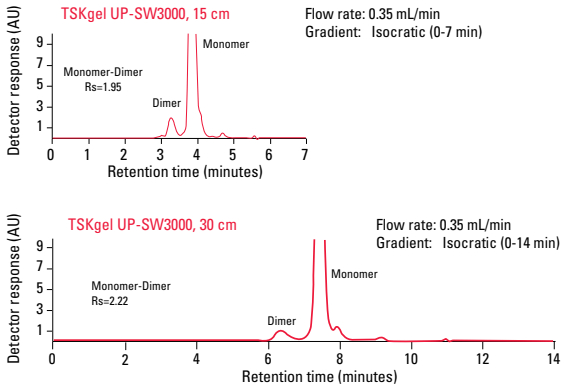


Aggregate Analysis

The **TSKgel UP-SW3000**, 2 µm SEC column provides fast and accurate mAb aggregate analysis with exceptional reproducibility.

Figure 5 compares the resolution between mAb aggregate and monomer achieved with a 15 cm and 30 cm **TSKgel UP-SW3000** column under the same operating conditions. The 15 cm **TSKgel UP-SW3000** column provides a similar profile to the 30 cm column with a 50% reduction in run time and 50% lower backpressure while still attaining acceptable resolution.

Figure 5. Comparison of mAb aggregate analysis between TSKgel UP-SW3000, 15 cm and 30 cm columns under equivalent conditions



Columns:	TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 15 cm TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase:	100 mmol/L sodium phosphate buffer, pH 6.8, + 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 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	mAb (0.4 mg/mL)

Figure 6 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 while still maintaining resolution in the range acceptable by USP guidelines.

Figure 6. Fast analysis of a mAb sample using TSKgel UP-SW3000

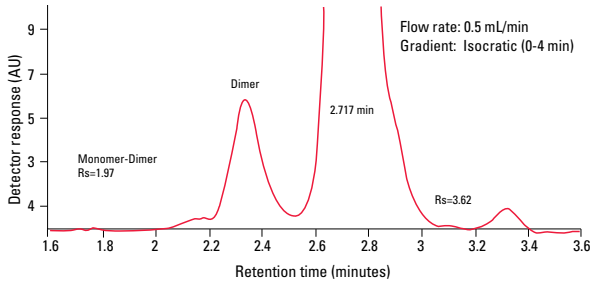


Table 2 demonstrates the reproducibility achieved with the **TSKgel UP-SW3000** column. Ten consecutive injections produce RSD values <1% for retention time, peak area, peak height, peak width, asymmetry, and efficiency.

Table 2. Ten consecutive runs of mAb sample yielded excellent reproducibility

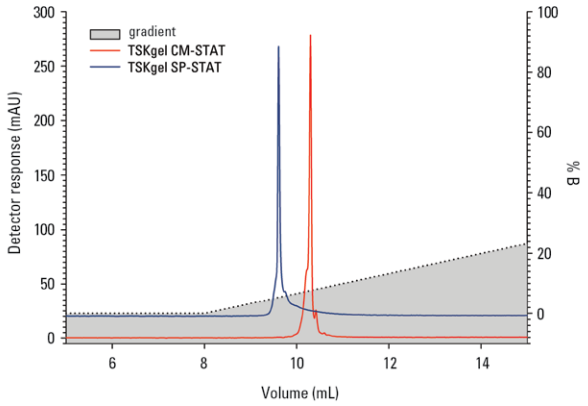
Monomer peak						
Injection #	Ret. time min.	Area mAU min	Height mAU	Width (50%) min	Asym. EP	Plates EP
1	2.717	16.72	155.460	0.093	1.26	4754
2	2.717	16.58	155.440	0.093	1.26	4762
3	2.717	16.62	155.780	0.093	1.26	4762
4	2.717	16.87	156.750	0.093	1.26	4740
5	2.717	16.91	157.360	0.093	1.26	4748
6	2.717	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

Charge Variant Analysis

TSKgel CM-STAT and **TSKgel SP-STAT** columns are designed for fast, high resolution analysis of mAb charge isoforms.

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 7 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.

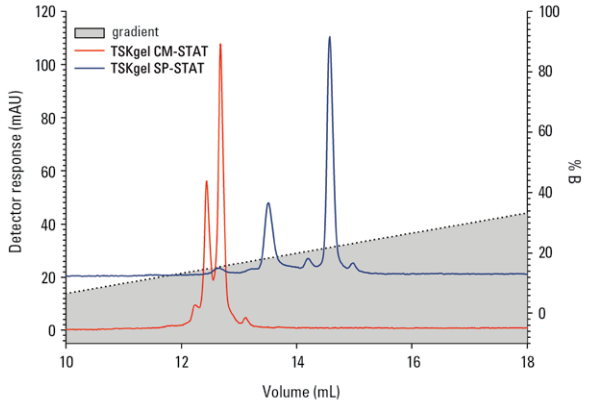
Figure 7. Analysis of mAb A on weak and strong ion exchange columns at pH 7



Columns:	TSKgel SP-STAT, 7 µm, 4.6 mm ID × 10 cm TSKgel CM-STAT, 7 µm, 4.6 mm ID × 10 cm
Mobile phase:	A: 10 mmol/L sodium phosphate buffer, pH 7.0 (Figures 7, 8, 9) 10 mmol/L sodium phosphate buffer, pH 6.0 (Figure 9) 10 mmol/L sodium acetate buffer, pH 5.0 (Figure 9) B: 100 mmol/L phosphate, pH 7.0 + 500 mmol/L NaCl (Figures 7, 8, 9) 100 mmol/L phosphate, pH 6.0 + 500 mmol/L NaCl (Figure 9) 100 mmol/L acetate, pH 5.0 + 500 mmol/L NaCl (Figure 9)
Gradient:	0 - 100% B in 30 min
Flow rate:	1 mL/min
Detection:	UV @ 280 nm
Injection vol.:	10 µL
Samples:	mAb A (2 g/L), mAb B (2 g/L)

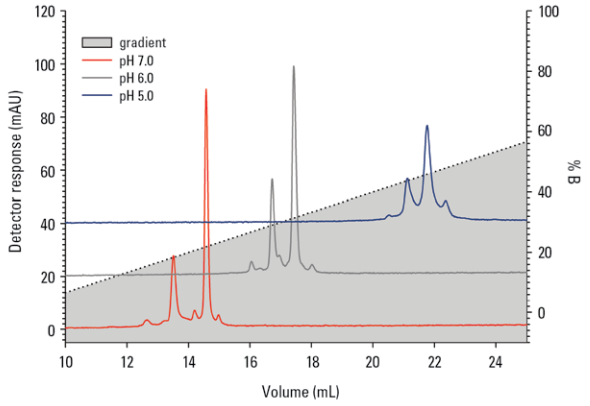
As shown in Figure 8, this is not the case for mAb B, where the strong cation exchange column shows a better separation. Retention and resolution of the charged isoforms are dependent on the buffer pH, as can be seen in Figure 9 for the analysis of mAb B on **TSKgel SP-STAT**.

Figure 8. Analysis of mAb B on weak and strong ion exchange columns at pH 7



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 a short analysis time and are ideally suited for the QC of biotherapeutics by UHPLC or HPLC.

Figure 9. Analysis of mAb B at various pH values using TSKgel SP-STAT column



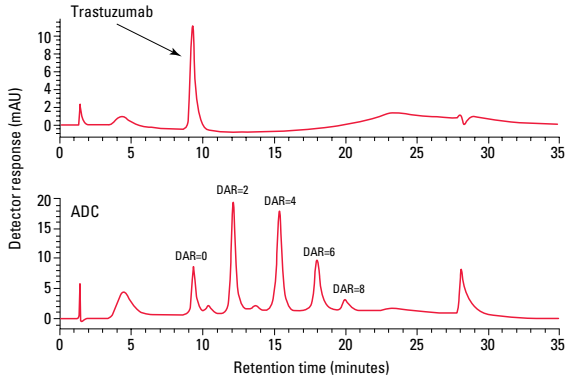
Drug-to-Antibody Ratio (DAR) Analysis

The **TSKgel Butyl-NPR** column is the best choice for high speed separations with excellent recovery, even for more hydrophobic samples. The non-porous resin requires lower sample loading and thus leads to faster analysis times. For this reason, the **TSKgel Butyl-NPR** is particularly popular for the analysis of drug to antibody ratios (DARs) of antibody drug conjugates (ADCs).

Both the unconjugated monoclonal antibody (Trastuzumab) and antibody drug conjugated (ADC) Trastuzumab (Trastuzumab-vcMMAE) samples were independently injected onto a **TSKgel Butyl-NPR** column. After injection, samples were eluted with organic solvent combined with sodium phosphate buffer as indicated in Figure 10.

The unconjugated Trastuzumab sample was eluted as a major single peak at approximately 9.5 minutes (Figure 10, upper panel). This single peak indicated that the unconjugated Trastuzumab consisted of mostly homogeneous molecules. The profile of the drug conjugated Trastuzumab exhibited well resolved peaks with different retention times than that of the unconjugated drug and with baseline separation (Figure 10, upper panel). 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. The chromatogram shows well resolved peaks ranging from a DAR of 0 to 8 based upon peak mobility (Figure 10, lower panel).

Figure 10. Analysis of unconjugated Trastuzumab (upper panel) and drug conjugated Trastuzumab (lower panel) using TSKgel Butyl-NPR column



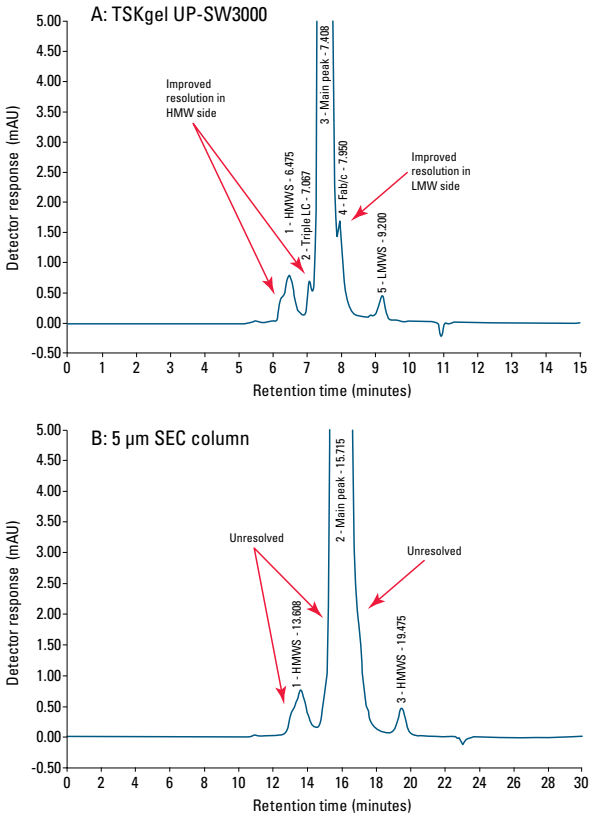
Column:	TSKgel Butyl-NPR, 4.6 mm ID × 10 cm
Mobile phase:	A: 25 mmol/L phosphate buffer, pH 7.0 including 1.5 mol/L ammonium sulfate B: 25 mmol/L phosphate buffer, pH 7.0 / 2-propanol = 8 / 2
Gradient:	0 → 100% B (20 minutes)
Flow rate:	0.5 mL/min
Detection:	UV @ 280 nm
Injection vol.:	10 µL
Sample Conc.: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

Intact mAb and Fragment Analysis

The **TSKgel UP-SW3000**, 2 µm SEC column provides fast, high resolution separation of antibody fragments, monomers and dimers.

Figure 11 demonstrates the advantages of the **TSKgel UP-SW3000** column for mAb analysis versus the use of 5 µm SEC columns. 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.

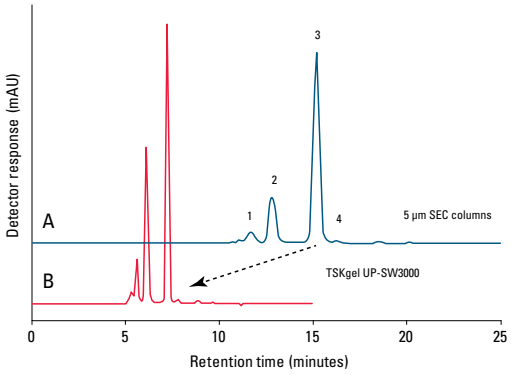
Figure 11. mAb analysis using a 5 µm SEC column versus TSKgel UP-SW3000 column



Columns:	A. TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm B. SEC column, 5 µm, 7.8 mm ID × 30 cm
Instruments:	A. 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. 0.35 mL/min B. 0.5 mL/min
Detection:	UV @ 280 nm
Temperature:	A. 40 °C B. 25 °C
Injection vol.:	A. 10 µL B. 50 µL

The **TSKgel UP-SW3000** column is capable of separating antibody dimer, monomer and fragments in one run with improved resolution compared to two 5 µm SEC columns connected in series.

Figure 12. mAb analysis using two 5 µm SEC columns versus one TSKgel UP-SW3000 column



Column	Rs (peak 1/2)	Rs (peak 2/3)	Rs (peak 3/4)
A: 5 µm SEC column × 2	1.60	3.63	1.77
B: TSKgel UP-SW3000	2.16	5.02	2.56

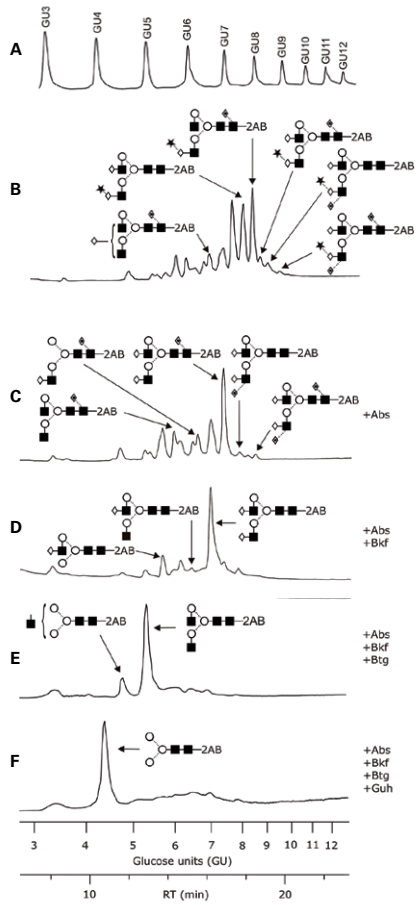
Columns:	A. SEC column, 5 µm, 7.8 mm ID × 30 cm × 2 B. TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase:	100 mmol/L phosphate buffer + 100 mmol/L sodium sulfate + 0.05% sodium azide, pH 6.7
Flow rate:	A. 1.0 mL/min B. 0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Samples: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

Glycan Profiling

The **TSKgel Amide-80** column is well suited for glycan analysis under HILIC conditions.

The application of HILIC for the characterization of a complex glycan structure is demonstrated in Figure 13 using the example of N-glyco mapping of the ZP-domain of murine transforming growth factor beta type3 receptor (TGFR-3). Recombinant proteins were purified and submitted to exoglycosidase cleavage. Glycans were fluorescently labeled with 2 aminobenzamid (2-AB), compared to the dextran ladder, and separated by HILIC.

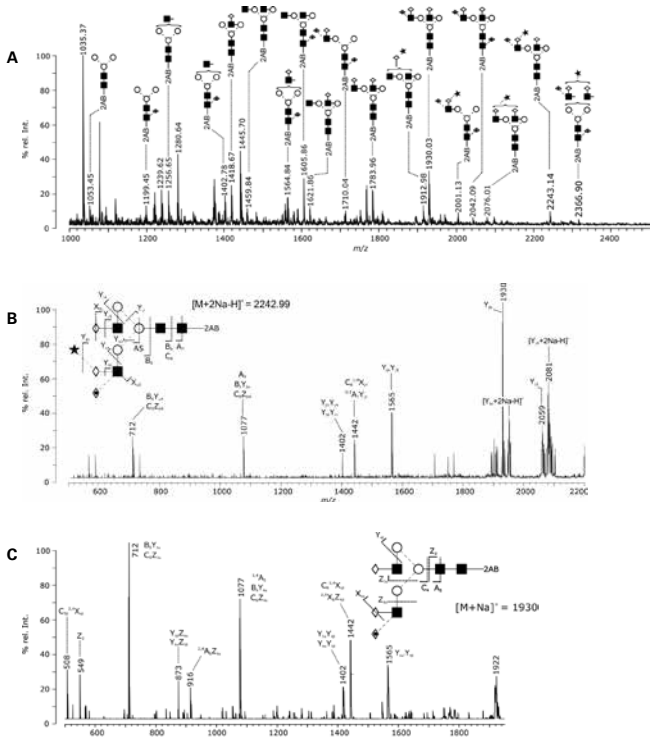
Figure 13. (A) Dextran ladder, (B) PNGase digest, (C-F) sequential exoglycosidase digests. Sequential exoglycosidase digest: Sialidase A (Abs), α-Fucosidase (Bkf), ß-Galactosidase (Btg), ß-N-Acetylhexoamidase (Guh).



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

The structure analysis of the glycans was completed by high resolution mass spectra acquired on a MALDI QIT ToF MS instrument (Figure 14).

Figure 14. (A) Mass spectrum of 2-AB-labeled glycans released from ZP domain construct of murine TGFR3, (B) MS2 (CIC) mass spectrum of m/z 2243, (C) MS2 mass spectrum of M/Z 1930



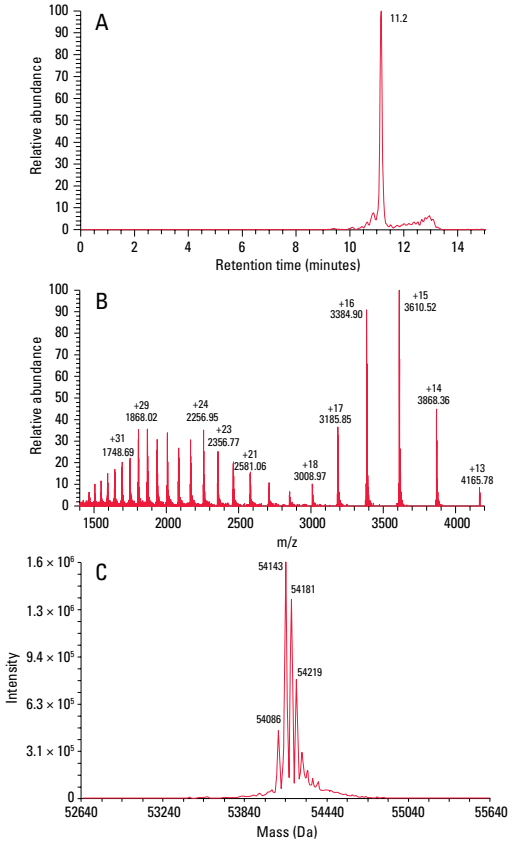
Accurate Mass Determination

The **TSKgel UP-SW3000**, 2 µm SEC column can be effectively used for accurate mass determination of monoclonal antibodies and related products using SEC/MS.

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

The ~55 kDa BiTE and parent mAbs (data not shown) were subsequently injected onto a **TSKgel UP-SW3000** column coupled to a mass spectrometer for molar mass determination. Figure 15 shows the (a) total ion chromatogram, (b) mass spectrum and (c) deconvoluted mass spectrum of the BiTE.

Figure 15. SEC/MS analysis of the BiTE

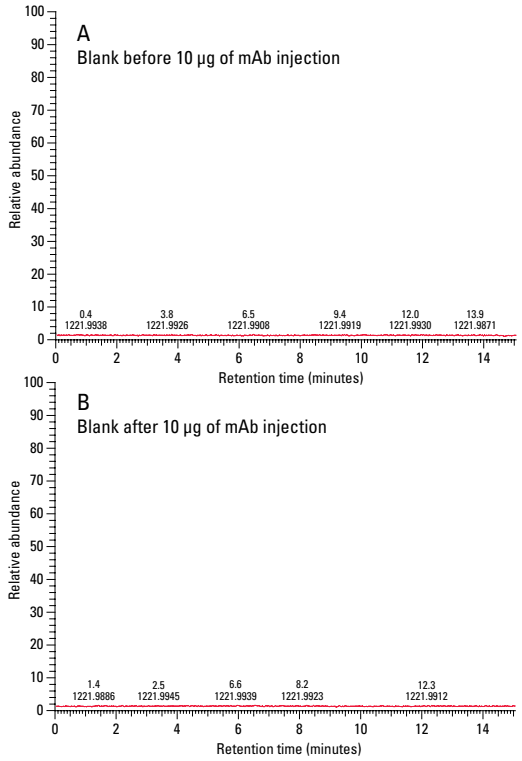


Column:	TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
HPLC Instrument:	Nexera® XR UHPLC system
MS Instrument:	Q Exactive™ Plus
Mobile phase:	20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate, pH 7.2
Gradient:	isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	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

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.

Prior to analysis, a blank injection was run in order to assess column particle shedding. Figure 16a shows the total ion chromatogram of a blank injection. 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 16b shows the total ion chromatogram of a blank injection run between the BiTE and parent mAb showing no evidence of carryover. The lack of shedding and carryover indicate that the **TSKgel UP-SW3000** column is suitable for use with MS.

Figure 16. Column Shedding and Carryover Analysis



Ordering Information

Titer Analysis

Part #	Description	Particle Size	ID (mm)	Length (cm)
23483	TSKgel Protein A-5PW	20 µm	4.6	3.5

Aggregate Analysis, Intact mAb and Fragment Analysis, Accurate Mass Determination

Part #	Description	Particle Size	ID (mm)	Length (cm)
23449	TSKgel UP-SW3000	2 µm	4.6	15
23448	TSKgel UP-SW3000	2 µm	4.6	30
23450	TSKgel guard column UP-SW3000	2 µm	4.6	2
23451	TSKgel guard column UP-SW3000 DC*	2 µm	4.6	2

*The guard column can be directly connected to the analytical column without tubing between the two columns.
A male-type outlet endfitting on the guard column enables the direct connection to the screw-type endfitting of the analytical column.

Charge Variant Analysis

Part #	Description	Particle Size	ID (mm)	Length (cm)
21965	TSKgel CM-STAT	10 µm	3	3.5
21966	TSKgel CM-STAT	7 µm	4.6	10
21963	TSKgel SP-STAT	10 µm	3	3.5
21964	TSKgel SP-STAT	7 µm	4.6	10

Drug-to-Antibody Ratio (DAR) Analysis

Part #	Description	Particle Size	ID (mm)	Length (cm)
14947	TSKgel Butyl-NPR	2.5 µm	4.6	3.5
42168	TSKgel Butyl-NPR	2.5 µm	4.6	10

Glycan Profiling

Part #	Description	Particle Size	ID (mm)	Length (cm)
23456	TSKgel Amide-80**	2 µm	2	15
21865	TSKgel Amide-80**	3 µm	2	15

**Columns are available with 2, 3, 5 and 10 µm particles and a wide range of column geometries

TOSOH BIOSCIENCE

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