New silica-based SEC columns designed for the separation of mAb monomers and their impurities

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Introduction

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- Monoclonal antibodies (mAbs) are widely used as biopharmaceuticals and new mAbs are still being developed by modifying the complementarity determining regions.
- mAbs easily undergo structural and chemical changes during preparation and storage processes and such denaturation may cause loss of therapeutic efficacy or manifestations of toxicity.
- Therefore, therapeutic mAbs must be subject to strict quality control.
- Size exclusion chromatography (SEC) is a powerful and convenient tool for determining mAb monomers and their impurities, including aggregates, oligomers, and mAb fragments.

Introduction

We have developed three silica-based SEC columns designed especially for mAb analysis:

1) A 4.6 mm ID x 15 cm semi-micro column packed with 25 nm pore size, 4 µm particles, which is designed for high throughput analysis of mAbs.

2) A 7.8 mm ID x 30 cm analytical column packed with the same particles as mentioned above. The column dimension is compatible with conventional LC systems with relatively large extra-column dead volume and is suitable for high resolution analysis of mAb monomers and dimers.

3) A 7.8 mm ID x 30 cm analytical column packed with newly developed 30 nm pore size, 3 µm particles. Larger pore size with the estimated exclusion limit of ~$4x10^6$ Da provides improved separation and quantitation of mAb aggregates and oligomers.

We report here the features of these new SEC columns and their superior performance of mAb separation in comparison to conventional columns.

Experimental

Experimental

Columns

- TSKgel® SuperSW mAb HTP, 4.6 mm ID x 15 cm, 4 µm particle*
- TSKgel SuperSW mAb HR, 7.8 mm ID x 30 cm, 4 µm particle*
- TSKgel UltraSW Aggregate, 7.8 mm ID x 30 cm, 3 µm particle*
- TSKgel G3000SW$_{XL}$, 7.8 mm ID x 30 cm, 5 µm particle
- All of TSKgel columns were manufactured by Tosoh (Tokyo, Japan).
  * prototype columns

Instrumentation

- The HPLC system was a Tosoh liquid chromatograph equipped with pump (DP-8020), column oven (CO-8020), UV detector (UV-8020), and data processor (LC-8020 model II).

Reagents

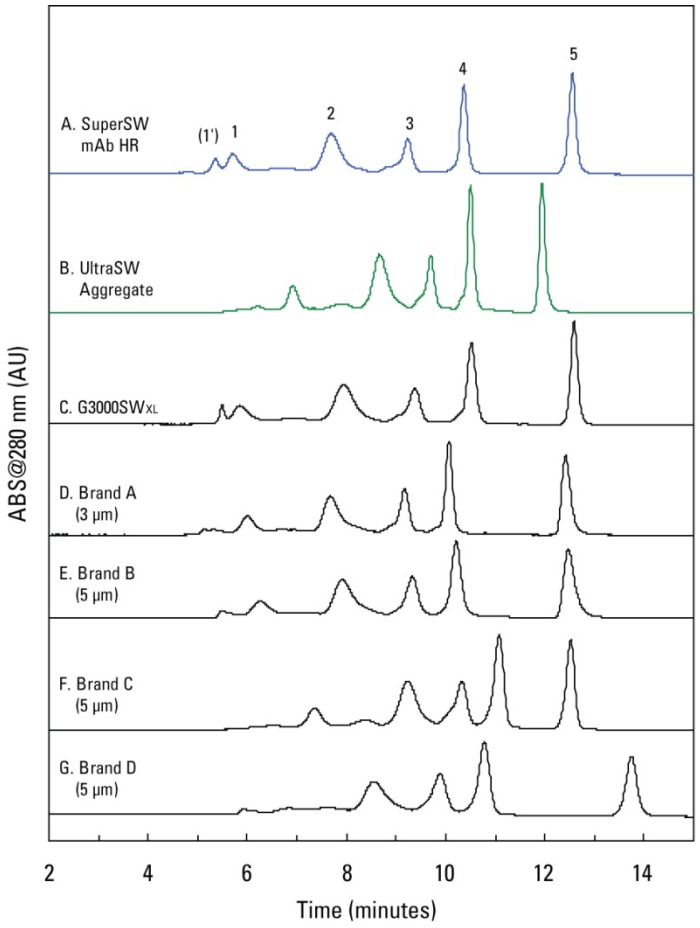
- Disodium hydrogenphsophate 12-water, potassium dihydrogen phosphate, sodium azide, sodium nitrate, and p-aminobenzoic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan).
- Pullulan standards were obtained from Showa Denko (Tokyo, Japan).
- IgG was obtained from Kaketsuken (Kumamoto, Japan).
- All other proteins were obtained from Sigma-Aldrich (St. Louis, MO, USA).
- Water was purified with the Milli-Q® system (Merck Millipore, Darmstadt, Germany).

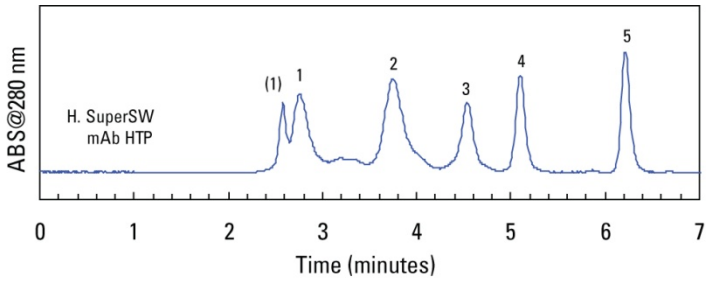
Characteristics of the Columns

Table 1: Specifications of the Columns

Column	TSKgel SuperSW mAb HTP	TSKgel SuperSW mAb HR	TSKgel UltraSW Aggregate
Column dimension	4.6 mm ID x 15 cm	7.8 mm ID x 30 cm	7.8 mm ID x 30 cm
Base material	Silica gel		Silica gel
Functional group	Diol		Diol
Particle size	4 µm		3 µm
Pore size	25 nm		30 nm
Separation range (for globular proteins)	10,000 - 500,00 Da		10,000 - 2,000,000 Da
Applications	Fast separation of mAb monomer and dimer (UHPLC compatible)	Separation of mAb monomer and dimer (conventional LC compatible)	Separation of mAb aggregates

Figure 1: Chromatograms of Standard Proteins

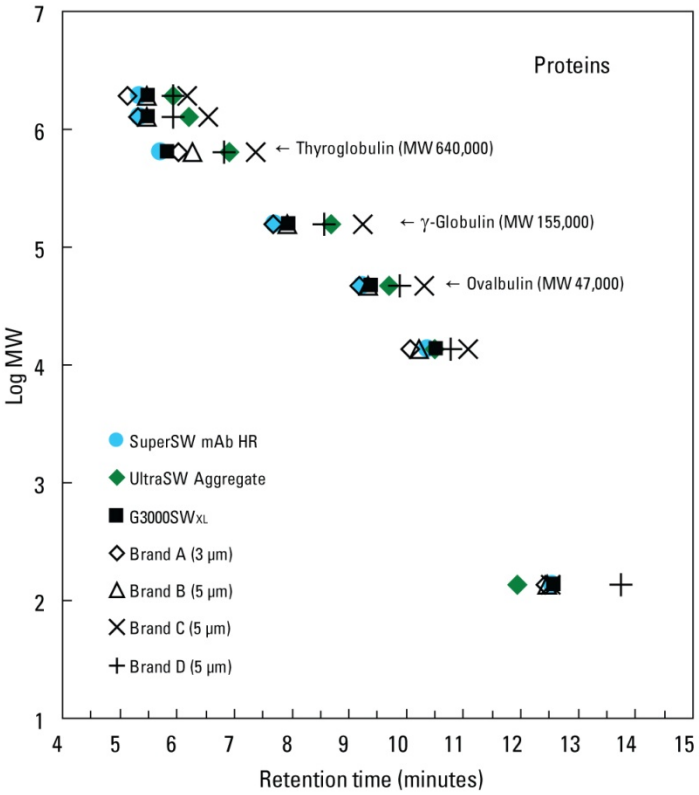


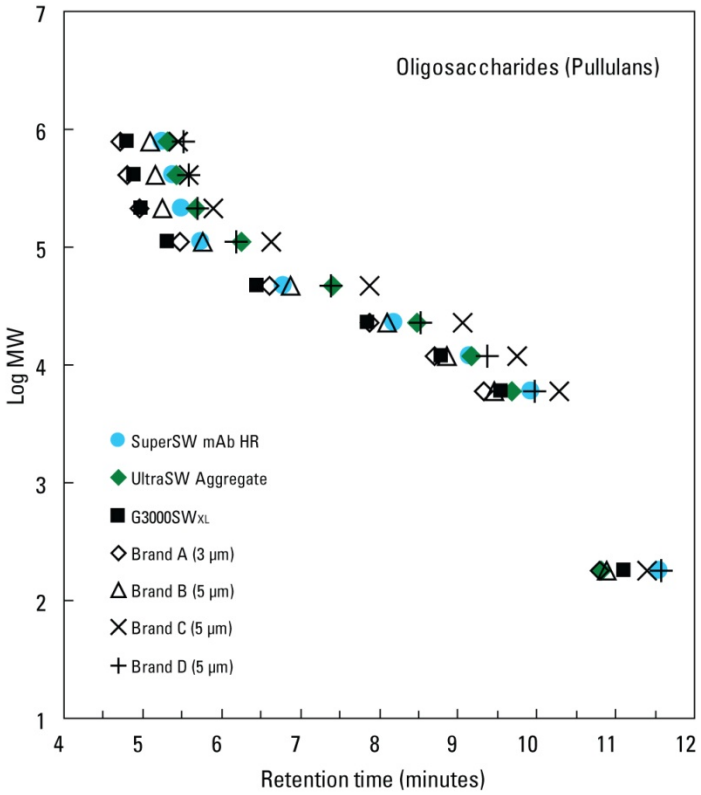


Column dimension:	A-E: 7.8 mm ID x 30 cm
	F&G: 8.0 mm ID x 30 cm
	H: 4.6 mm ID x 15 cm
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	A-G: 1.0 mL/min H: 0.35 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	A-G: 10 µL H: 3.5 µL
Samples:	1 thyroglobulin (MW 640,000) (A-G: 0.5 g/L H: 2.0 g/L)
	(1) thyroglobulin oligomer
	2 γ-globulin (MW 155,000) (A-G: 1.0 g/L H: 1.5 g/L)
	3 ovalbumin (MW 47,000) (A-G: 1.0 g/L H: 1.5 g/L)
	4 ribonuclease A (MW 13,700) (1.5 g/L)
	5 p-aminobenzoic acid (MW 137) (0.01 g/L)

New TSKgel SEC columns show their superior performance over other columns.

Figure 2: Calibration Curves

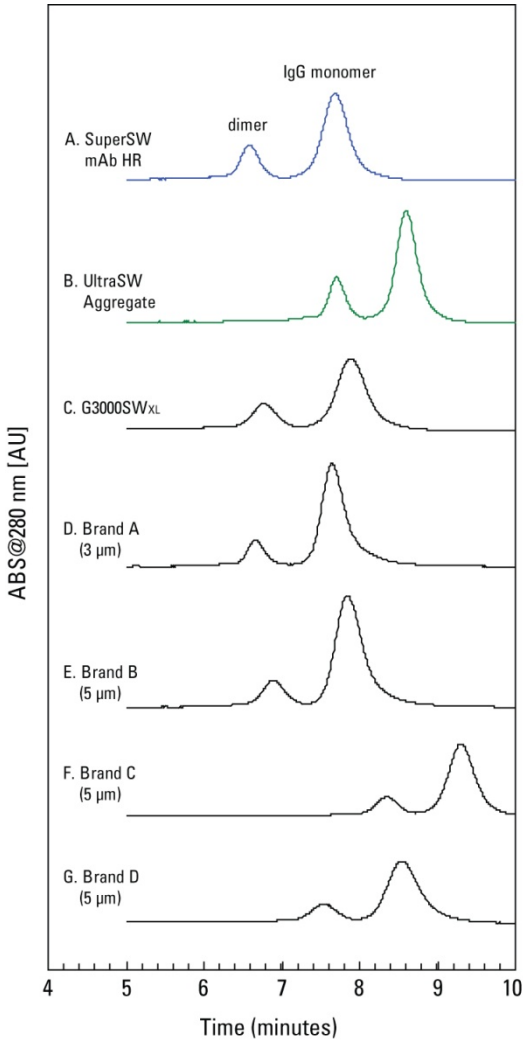


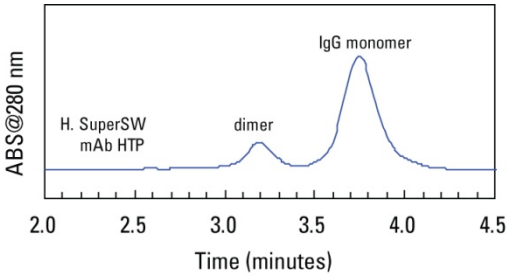


Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	10 µL
Samples:	thyroglobulin, γ-globulin, ovalbumin, ribonuclease A, p-aminobenzoic acid

Mobile phase:	50 mmol/L $NaNO_3$
Flow rate:	1.0 mL/min
Detection:	RI
Temperature:	25°C
Injection vol.:	10 µL
Samples:	pullulans (MW 788,000/ 404,000/ 212,000/ 112,000/ 47,300/ 22,800/ 11,800/ 5,900), glucose

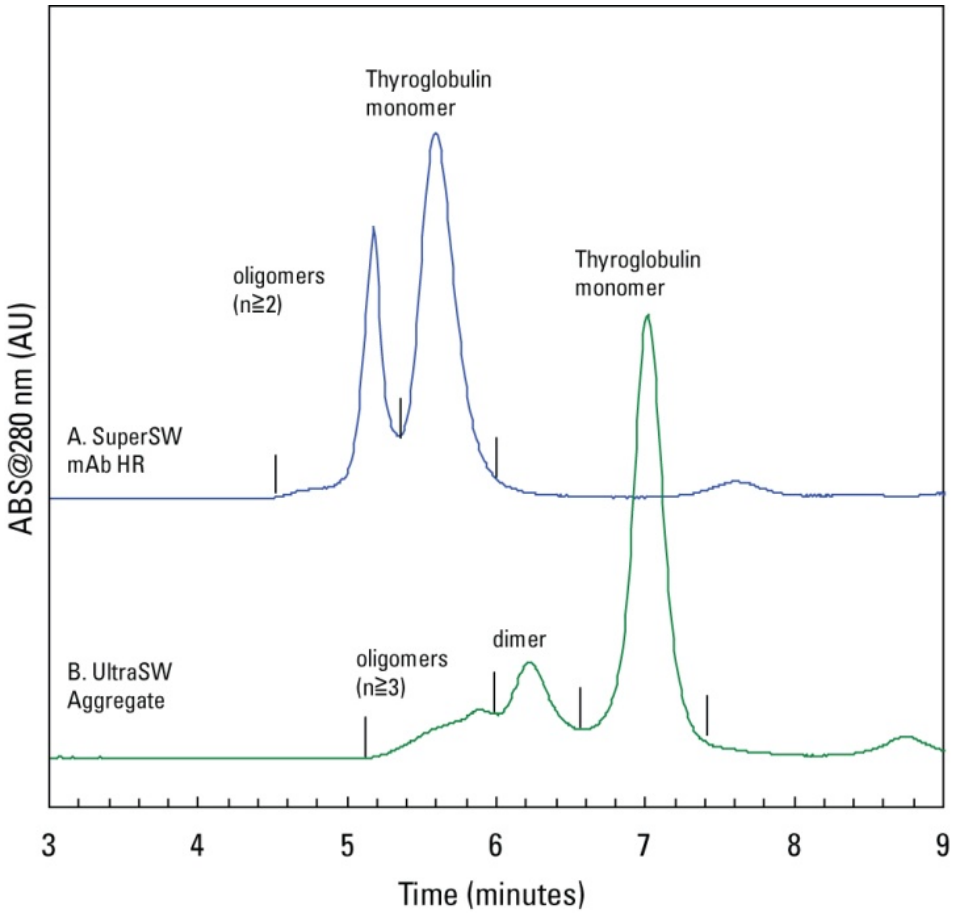
Figure 3: Chromatograms of IgG Dimer/Monomer





Column dimension: A-E: 7.8 mm ID x 30 cm
F&G: 8.0 mm ID x 30 cm
H: 4.6 mm ID x 15 cm
Mobile phase: 200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate: A-G: 1.0 mL/min H: 0.35 mL/min
Detection: UV@280 nm
Temperature: 25°C
Injection vol. : A-G: 10 µL H: 3.5 µL
Sample: IgG (A-G: 1.0 g/L H: 4.5 g/L)

Figure 4: Chromatograms of Thyroglobulin



Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	10 µL
Sample:	thyroglobulin (2.0 g/L)

Table 2: Summary of Column Performance

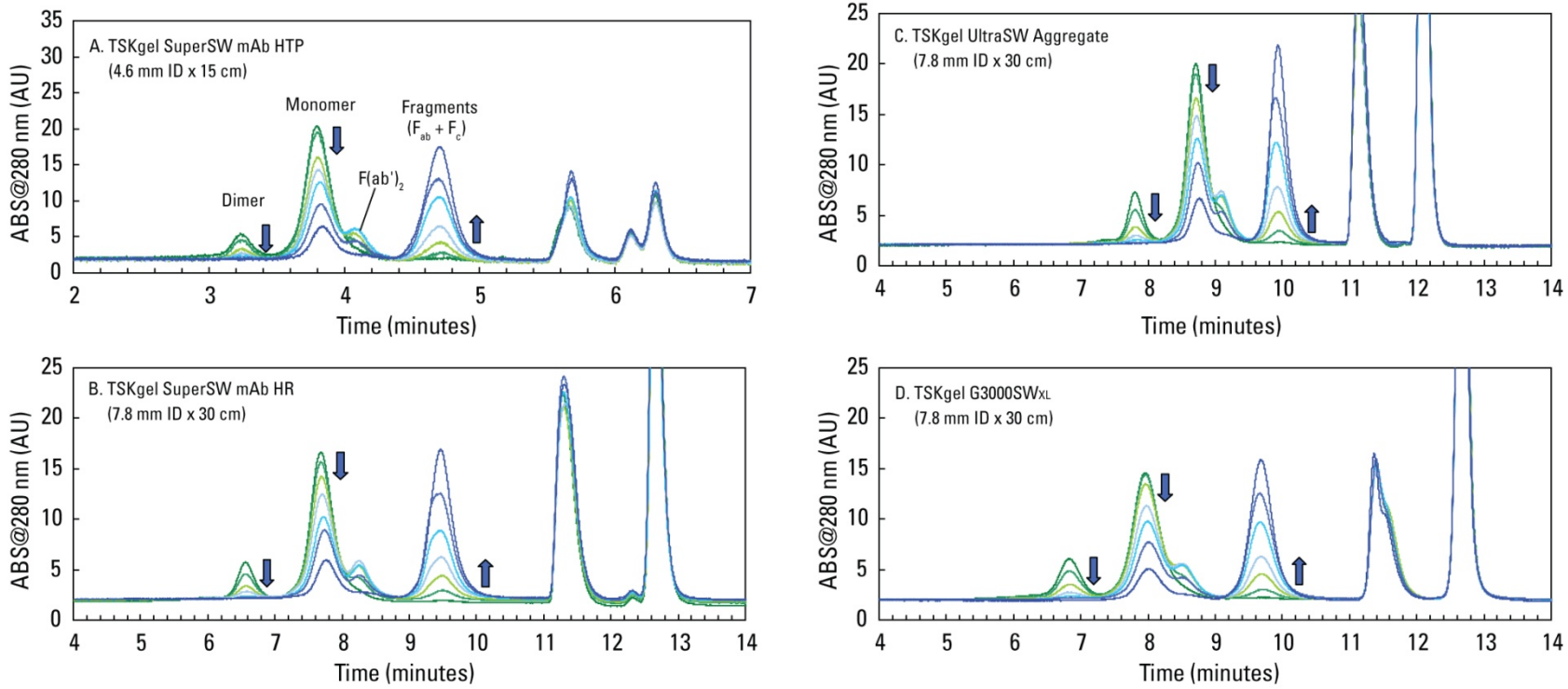
Column	Slopes of calibration curve		MW exclusion limit for proteins (Da)	Separation of IgG		Separation of thyroglobulin	
	thyroglobulin /γ-globulin	γ-globulin /ovalbumin		TP (monomer)	Rs (dimer/monomer)	TP (monomer)	Rs (dimer/monomer)
4.6 mm ID x 15 cm column:							
TSKgel SuperSW mAb HTP	-0.618	-0.659	8.4×10^5	1,765	1.69	1,238	0.76
7.8 mm ID x 30 cm columns:							
TSKgel SuperSW mAb HR	-0.309	-0.335	8.4×10^5	2,338	1.97	2,463	1.00
TSKgel UltraSW Aggregate	-0.351	-0.506	$3.6 \times 10^{6(*)}$	4,446	1.91	5,155	1.34
TSKgel G3000SW$_{XL}$	-0.294	-0.360	8.6×10^5	1,835	1.64	1,421	0.87
Brand A (3 µm)	-0.370	-0.345	$3.0 \times 10^{6(*)}$	2,888	1.95	2,165	n.d.
Brand B (5 µm)	-0.370	-0.368	1.2×10^6	2,270	1.56	1,133	1.15
8.0 mm ID x 30 cm columns:							
Brand C (5 µm)	-0.328	-0.474	$3.6 \times 10^{6(*)}$	3,304	1.59	2,894	n.d.
Brand D (5 µm)	-0.353	-0.390	1.4×10^6	1,868	1.33	n.d.	n.d.

(*) V_0 was estimated by elution times of pullulans

- **The TSKgel SuperSW mAb HTP exhibited equal separation between IgG monomer and dimer in half the analysis time compared to the conventional SEC column, TSKgel G3000SW$_{XL}$, 5µm particle, 7.8 mm ID x 30 cm.**
- **The TSKgel SuperSW mAb HR exhibited superior resolving power for IgG monomer and dimer compared to other SEC columns.**
- **The TSKgel UltraSW Aggregate, which possesses a larger MW exclusion limit, exhibited superior resolving power for thyroglobulin oligomers with high molecular weight.**

Applications: Separation of IgG

Figure 5: Separation of Papain-Digested IgG



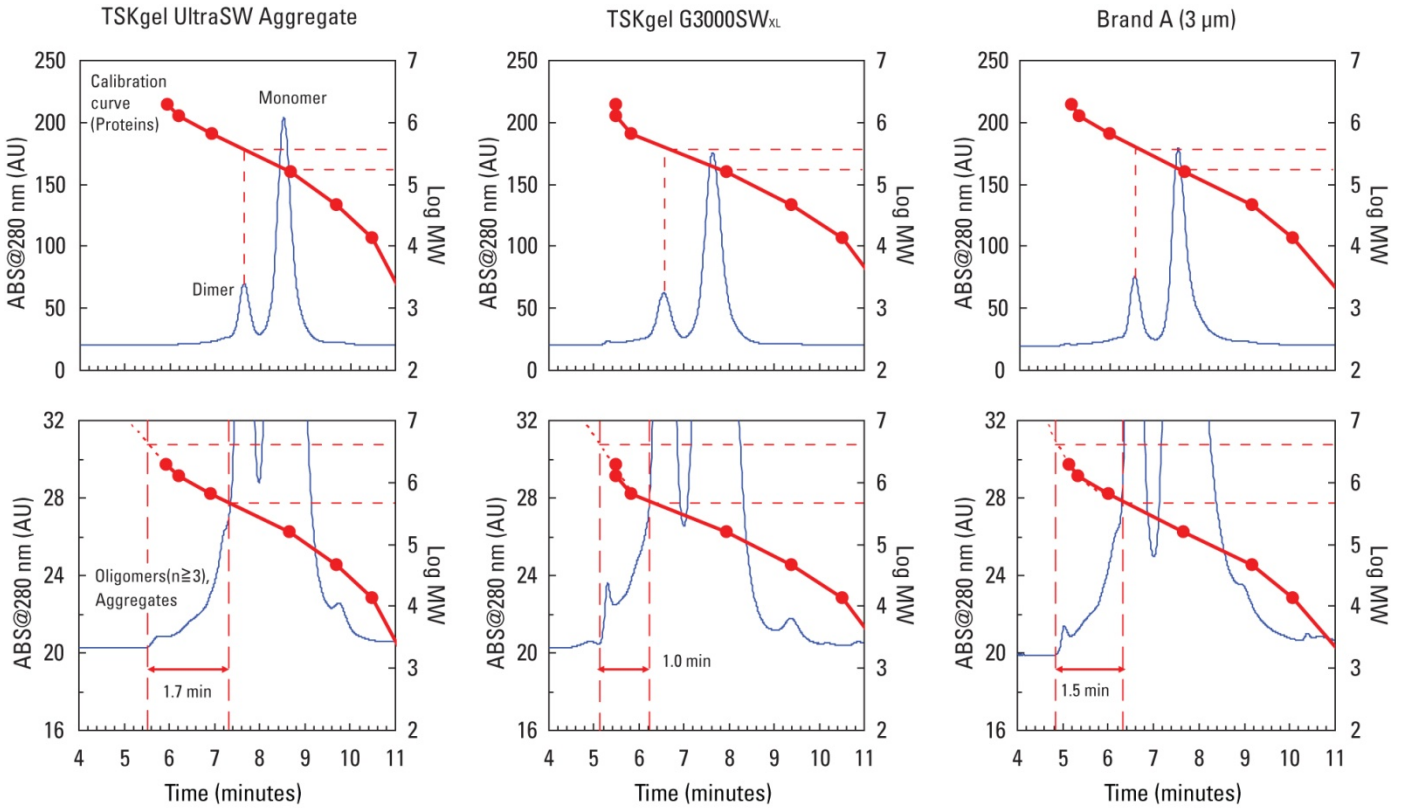
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	A: 0.35 mL/min B-D: 1.0 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	A: 5 µL B-D: 10 µL
Samples:	IgG (10 g/L) digested with papain for 0, 5, 15, 30, 60, 120, and 1,440 min

Table 3: Summary of Separation of Papain-Digested IgG

Column	Undigested IgG				IgG digested with papain for 1,440 min	
	ET (min) (monomer)	TP (dimer)	TP (monomer)	Rs (d/m)	TP (fragments)	Rs (m/f)
TSKgel SuperSW mAb HTP, 4.6 mm ID x 15 cm	3.798	2,005	1,909	1.78	2,489	2.46
TSKgel SuperSW mAb HR, 7.8 mm ID x 30 cm	7.683	2,895	2,320	2.02	3,826	2.87
TSKgel UltraSW Aggregate, 7.8 mm ID x 30 cm	8.710	5,563	4,279	1.90	7,807	2.49
TSKgel G3000SW$_{XL}$, 7.8 mm ID x 30 cm	7.963	1,912	1,781	1.63	3,883	2.50

- **TSKgel SuperSW mAb HTP reduced the overall analysis time in half compared to that using a conventional TSKgel G3000SW$_{XL}$ SEC column without any compromise in resolutions between monomer/dimer in undigested IgG or monomer/fragments.**
- **TSKgel SuperSW mAb HR exhibited superior resolving power for monomer/dimer and monomer/fragment separation.**

Figure 6: Separation of IgG Aggregates



Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	50 µL
Sample:	IgG (1.0 g/L)

The TSKgel UltraSW Aggregate possesses a wider separation window for IgG oligomers and aggregates with high MW ($5x10^5$~$5x10^6$ Da) than other SEC columns.

Conclusions

Conclusions

The following three novel SEC columns have been developed:

- TSKgel SuperSW mAb HTP exhibited equal separation between IgG monomer and dimer in half the analysis time compared to the conventional SEC column, TSKgel G3000SW$_{XL}$ (5µm particle, 7.8 mm ID x 30 cm).
- TSKgel SuperSW mAb HR exhibited superior resolving power for IgG monomer and dimer compared to other SEC columns.
- TSKgel UltraSW Aggregate, which possesses a larger MW exclusion limit, exhibited superior resolving power for oligomers and aggregates of large proteins, including thyroglobulin and IgG.

The performance of these columns was demonstrated by the separation of IgG fragments generated by papain digestion and separation of IgG aggregates.