Three Novel Size Exclusion Chromatography Columns Designed for the Separation of a Monoclonal Antibody Monomer from its Impurities

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Introduction

- Monoclonal antibodies (mAbs) are widely being used in the field of biotherapeutics.
- Separation of the pure antibody monomer needs to be very well resolved from its dimer and higher aggregates.
- Similarly, for quality control and regulatory purposes the separation of antibody fragments is also very much essential.
- The species other than the monomer might induce toxic side effects to the body if not removed.
- 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 prototype TSKgel® SEC columns designed especially for mAb analysis:
  - a 4.6 mm ID × 15 cm semi-micro column packed with 25 nm pore size, 4 µm particles, which is designed for high throughput analysis of mAbs.
  - a 7.8 mm ID × 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.
  - a 7.8 mm ID × 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.
- Here we report the features of these new SEC prototype columns and their superior performance of mAb separation in comparison to conventional columns.

Specifications of Three New Prototype Columns

Column	TSKgel UltraSW Aggregate	TSKgel SuperSW mAb HR	TSKgel SuperSW mAb HTP
Dimension	7.8 mm ID × 30 cm	7.8 mm ID × 30 cm	4.6 mm ID x 15 cm
Base material	Silica gel		
Functional group	Diol		
Particle size	3 µm	4 µm	
Separation range for globular proteins	10 kDa - 2000 kDa	10 kDa - 500 kDa	
Applications	Separation of aggregates	Separation of mAb monomer and dimer Conventional HPLC compatible	Fast separation of mAb monomer and dimer UHPLC compatible

Material and Methods

Columns

- TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID x 30 cm
- TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID x 30 cm
- TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID x 15 cm

Instrumentation

- Agilent 1100 HPLC system using Agilent Chemstation (Rev B.04.02)

Samples

- Human IgG (4.6 mg/mL)
- BI mAb 2 (4.6 mg/mL)

Papain Digestion protocol:

Please refer to the EAS 2012 poster from this author.

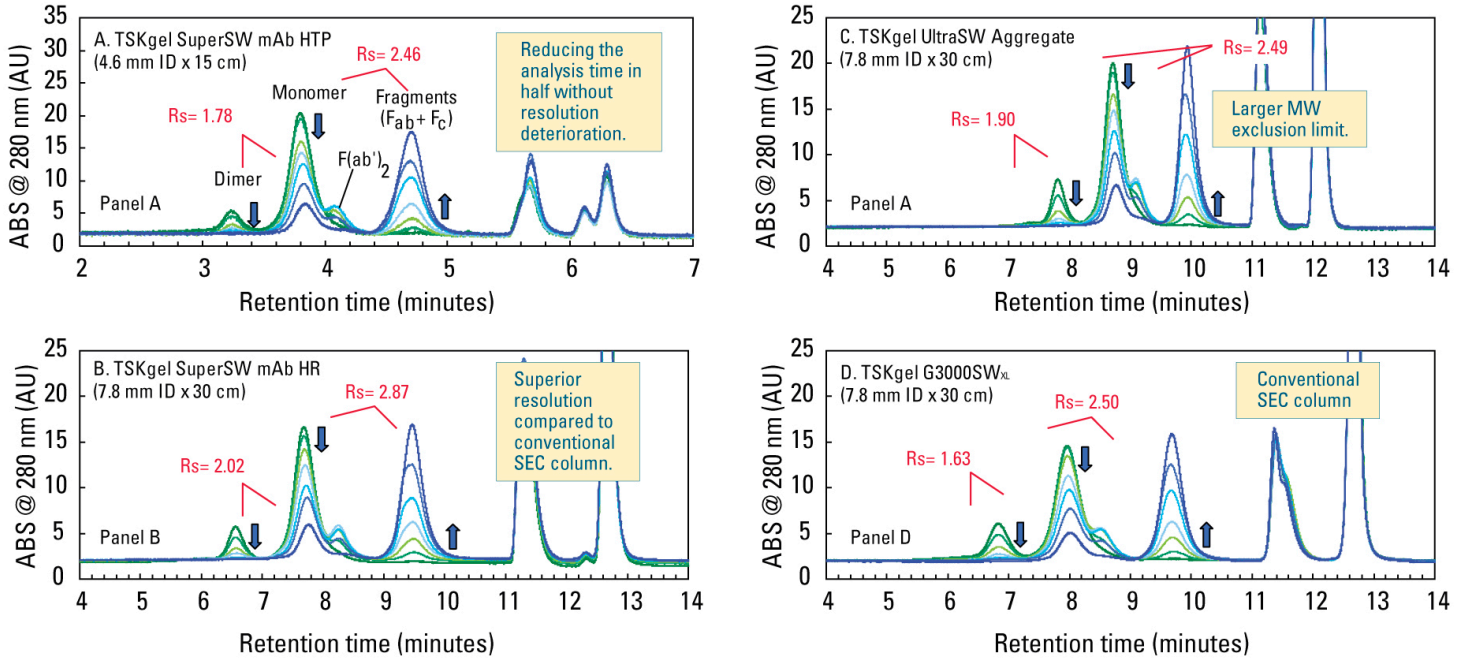
Chromatographic Conditions

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, unless mentioned otherwise
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL unless mentioned otherwise

High purity HPLC grade Sigma Aldrich chemicals were used in this study.

High purity 18.2 m.Ohm-cm quality water was used to make buffer and samples.

Figure 1: Separation of IgG Monomer, Dimer, and Fragments from Papain Digested IgG by: TSKgel SuperSW mAb HTP, SuperSW mAb HR, UltraSW Aggregate Columns



Columns:
A: TSKgel SuperSW mAb HTP, 4.6 mm ID × 15 cm*
B: TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm*
C: TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm*
D: TSKgel G3000SWXL, 7.8 mm ID × 30 cm
(*prototype columns)

Mobile phase:	200 mmol/L phosphate buffer + 0.05% NaN_3, pH 6.7
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:	10 g/L IgG digested with papain for 0-24 hr

Figure 1: Discussion

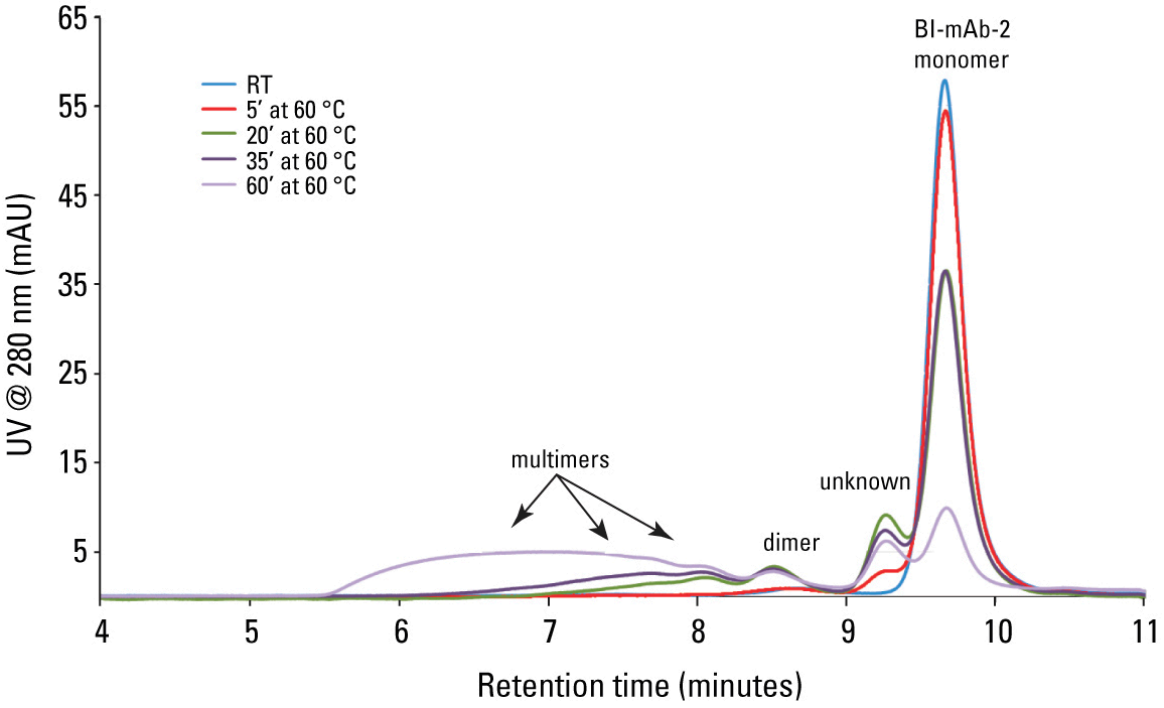
We have developed three silica-based SEC columns designed especially for mAb analysis:

- a 4.6 mm ID × 15 cm semi-micro column packed with 25 nm pore size, 4 µm particles (Panel A) is designed for high throughput analysis of mAbs for separation within half the analysis time compared to the conventional TSKgel G3000SW$_{XL}$, 5 µm, 7.8 mm ID x 30 cm SEC column without losing resolution.
- a 7.8 mm ID × 30 cm analytical column packed with the same particles (Panel B) is suitable for high resolution analysis of mAb monomers and dimers and is compatible with conventional LC systems with relatively large extra-column dead volume.
- a 7.8 mm ID × 30 cm analytical column packed with newly developed 30 nm pore size, 3 µm particles (Panel C) with larger pore size with an estimated exclusion limit of ~4 x 10^6 Da. A larger exclusion limit provides improved separation and quantitation of mAb aggregates and oligomers.

The results show efficient separation of aggregates and the separation of fragments generated by the digestion of the antibody by papain.

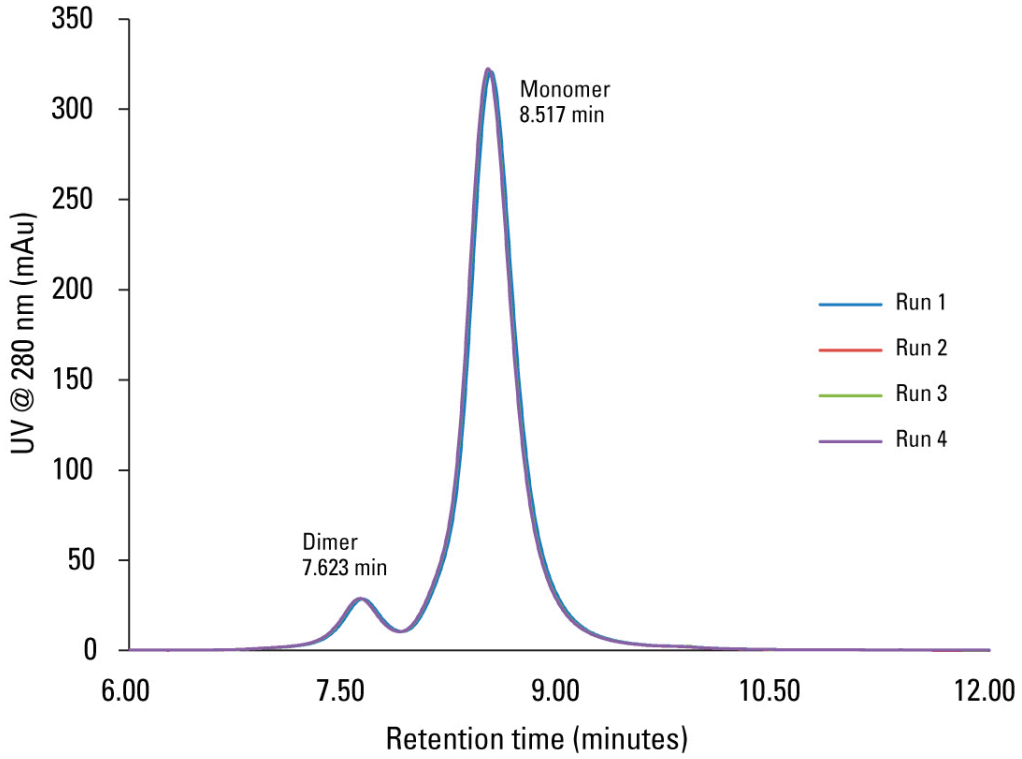
The results also show that these three new SEC columns have superior performance of mAb separation in comparison to conventional TSKgel G3000SW$_{XL}$ column (Panel D).

Figure 2: Heat Denaturation Study of Monoclonal Antibody (BI-mAb-02) Using a TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm Column



- Heat denaturation of mAb degradation is monitored at pH 5.5 and at temperature of 60 °C
- 50 µL of antibody (pH 6.0) was mixed with 50 µL of 0.1 mol/L phosphate buffer ,pH 4.65; final pH is 5.5; 20µL was injected.
- In addition to the presumed dimer peak at 8.5 minutes, an ‘unknown’ aggregate peak of intermediate molecular weight between the monomer and dimer and several higher order aggregate peaks were seen.
- Heating for one hour at 60 °C results in almost complete breakdown of the monoclonal antibody and the formation of very large aggregates that extend to the exclusion volume of the column.

Figure 3: Analysis of Human IgG Using a TSKgel UltraSW Aggregate (Y0007T), 3 µm, 7.8 mm ID × 30 cm Column



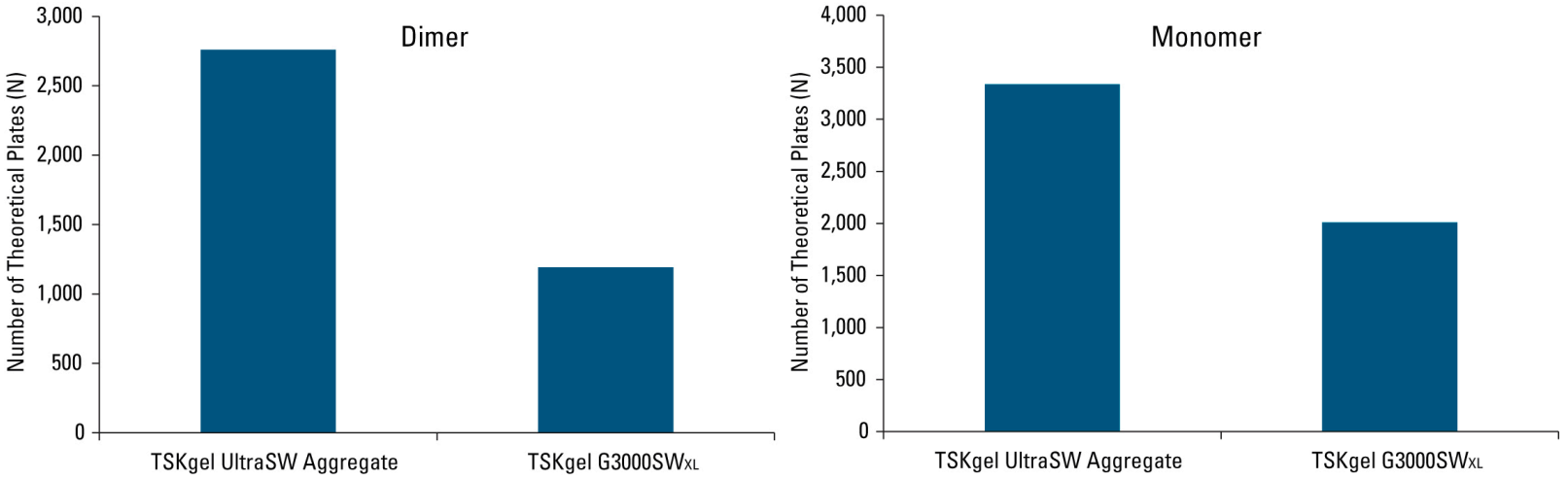
- Monomer and dimer peak of Human IgG could be baseline resolved.
- Retention times of monomer and dimer peaks shown above are an average of 4 consecutive injections.
- 4 consecutive injections illustrating excellent retention time stability (%RSD = 0.09) of monomer peak and (%RSD = 0.1) of dimer peak.
- Similarly the other peak parameters such as peak area, As, number of theoretical plates, etc. also showed low %RSD values as shown in the table below.

Figure 3: Results

	Dimer						Monomer						
Injection	RT	Area	Height	As	Width	Plates	RT	Area	Height	As	Width	Plates	RS
1	7.64	673.18	27.97	0.77	0.34	2799.00	8.53	8471.17	315.90	1.23	0.35	3301.00	1.52
2	7.62	676.74	27.79	0.75	0.34	2746.00	8.51	8491.78	318.33	1.25	0.34	3381.00	1.53
3	7.62	678.02	27.85	0.75	0.34	2747.00	8.51	8493.89	318.28	1.25	0.34	3382.00	1.53
4	7.62	680.87	27.90	0.75	0.34	2745.00	8.51	8485.33	318.63	1.25	0.35	3287.00	1.52
Avg	**7.62**	**677.20**	**27.88**	**0.75**	**0.34**	**2759.25**	**8.52**	**8485.54**	**317.79**	**1.25**	**0.35**	**3337.75**	**1.53**
S.d.	**0.01**	**2.76**	**0.07**	**0.01**	**0.00**	**22.96**	**0.01**	**8.88**	**1.10**	**0.01**	**0.00**	**44.03**	**0.01**
%RSD	**0.10**	**0.41**	**0.24**	**1.32**	**0.32**	**0.83**	**0.09**	**0.10**	**0.35**	**0.54**	**0.69**	**1.32**	**0.33**

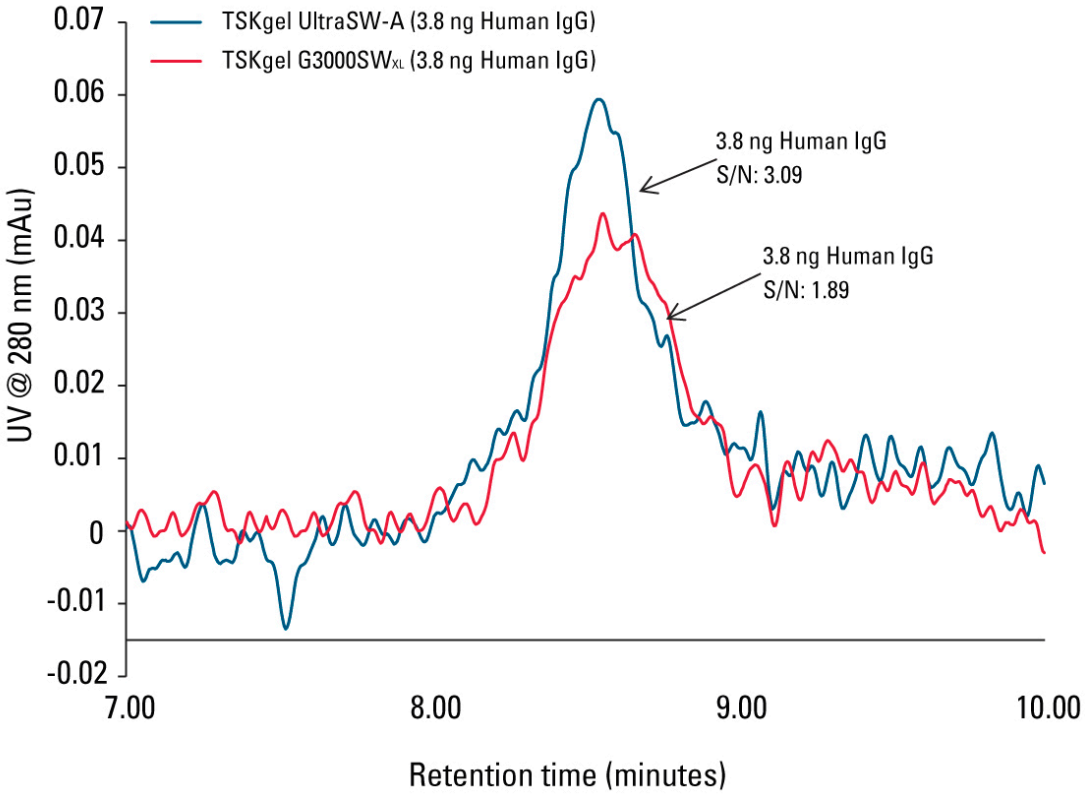
The table shows the excellent reproducibility among critical peak parameters for the monomer and dimer peaks.

Figure 4: Comparison of Column Efficiencies between TSKgel UltraSW Aggregate and TSKgel G3000SWXL Columns During the Analysis of Human IgG



The results show that the TSKgel UltraSW Aggregate column has a greater efficiency than the TSKgel G3000SWXLcolumn.

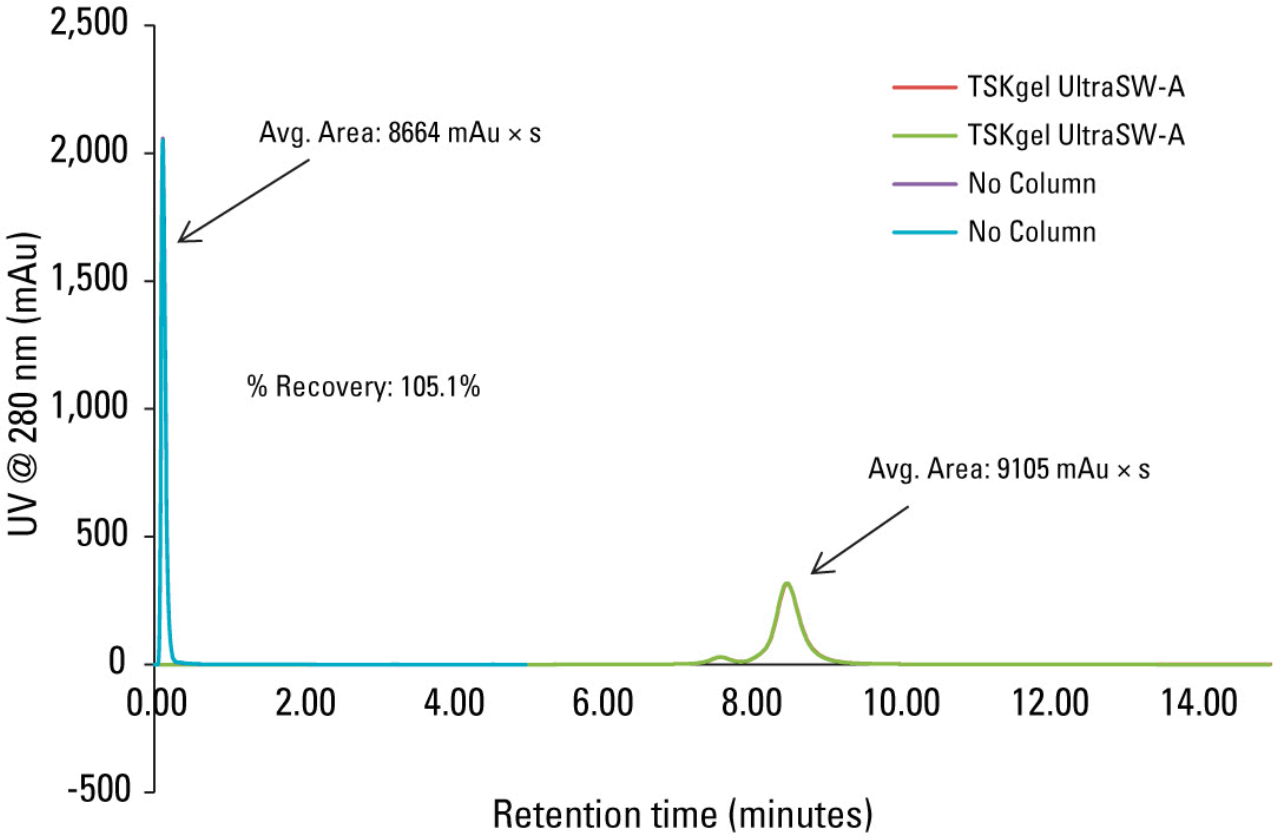
Figure 5: Comparison of LOD/LOQ Values of TSKgel UltraSW Aggregate and TSKgel G3000SWXL Columns



This study shows:

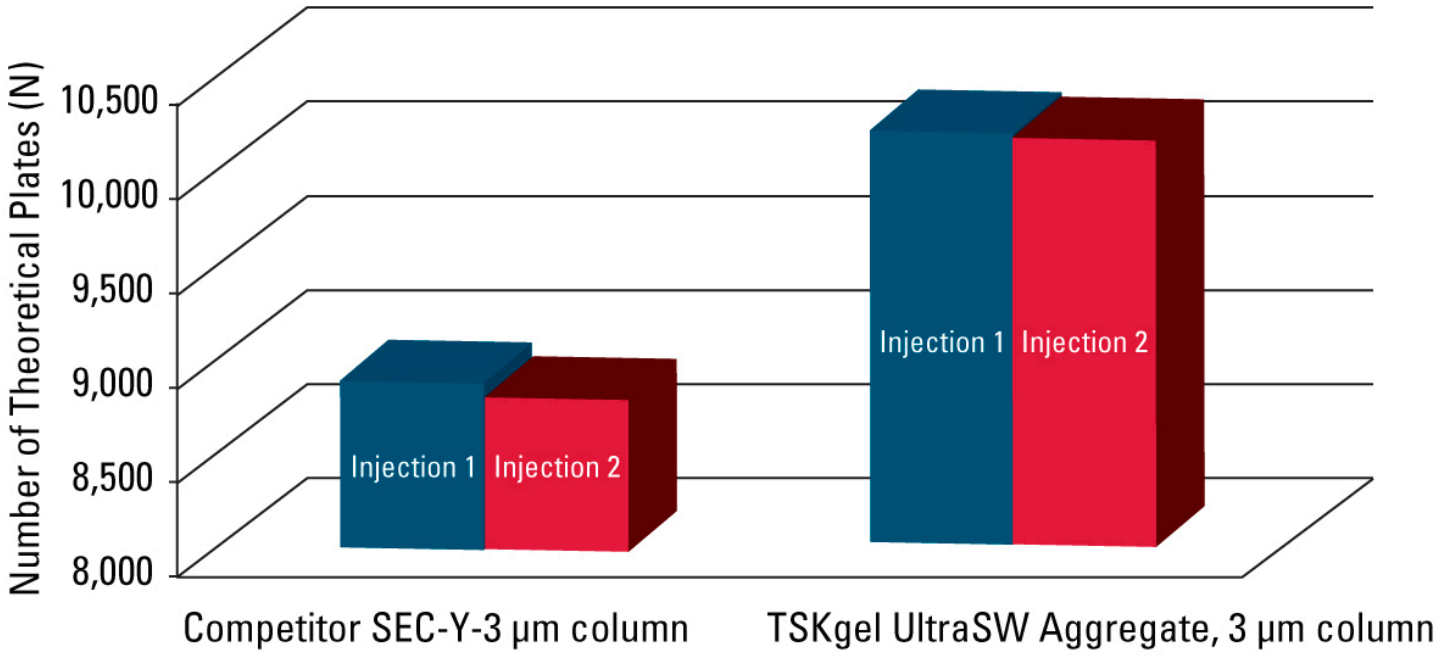
1. The LOD value of the TSKgel UltraSW Aggregate column is approximately 3.8 ng.
2. The sensitivity of the TSKgel UltraSW Aggregate column is more than the TSKgel G3000SWXL column.
3. At the LOD concentration, the dimer peak was not visible in either of the columns.
4. The LOQ for the TSKgel UltraSW Aggregate column is 38 ng.

Figure 6: Recovery of Human IgG During the Analysis Using a TSKgel UltraSW Aggregate (Y0007T), 3 µm, 7.8 mm ID × 30 cm Column



- Overlay of two injections with and without the TSKgel UltraSW Aggregate column is shown above.
- Excellent protein recovery (105.1%) was observed.
- Similar studies with a number of other monoclonal antibodies are in progress.

Figure 7: Comparison of Column Efficiency of TSKgel UltraSW Aggregate, 3 µm, 30 nm Column with a Competitor SEC Column with Same Particle and Pore Size



- The new TSKgel UltraSW Aggregate column yielded much higher efficiency during the analysis of BI-Mab-02. The two injections were consecutive injections.
- Both the Tosoh and the competitor column have the same particle size (3 µm) and same pore size (30 nm).

Conclusions

The following three novel prototype SEC columns have been developed:

- TSKgel SuperSW mAb HTP, 4.6 mm ID × 15 cm, exhibited equal separation between IgG monomer and dimer in half the analysis time compared to the conventional SEC column, TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm.
- TSKgel SuperSW mAb HR exhibited superior resolving power for IgG monomer and dimer compared to other SEC columns.
- TSKgel UltraSW Aggregate possesses a larger MW exclusion limit and superior resolving power for oligomers and aggregates with high molecular weight compared to the conventional column.

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

Conclusions (continued)

- TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column can successfully be used for the separation of the dimer from the monomer peak of IgG with high resolution.
- TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm column can be used for fast separation of dimer/monomer and fragments with the same resolving power and in half the analysis time compared to the conventional column.
- The efficient separation of aggregates induced by heat denaturation could be achieved using these columns.
- TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column is more efficient compared to a competitor 3 µm column.
- This study shows that these three novel SEC columns are suitable for the analysis and purification of antibodies.
- Please see the posters presented previously at EAS 2012 and ACS 2012 for further information.