Characterization of Two Novel Analytical Chromatographic Columns for Orthogonal Analysis of Monoclonal Antibodies and Protein Aggregates and Their Isoforms

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Introduction

- Multiple chromatographic modes can be used to separate complex mixtures, variants or impurities otherwise inseparable by a single mode of chromatography.
- A very favorable combination is Size Exclusion Chromatography (SEC) and Reversed Phased Chromatography (RPC).
- SEC and RPC are some of the most frequently used chromatographic modes for analytical separations of biomolecules, particularly monoclonal antibodies.
- The separation mechanism is orthogonal and the mobile phases are compatible.
- A fraction from SEC can directly be transferred to RPC.
- Orthogonal separation is basically an additional mode of chromatography complimentary to the primary method.
- The two different modes of chromatography, used for orthogonal separation, are very different in separation mechanism or selectivity.

Introduction

- The orthogonal method can be used to verify the primary method.
- The orthogonal method can be useful to separate the impurities, degradation products or variants of monoclonal antibodies, otherwise co-eluting or not separable by a single, primary mode of chromatography.
- For a biotherapeutic application, separation of the pure monomer needs to be very well resolved from its dimer and higher order aggregates.
- Similarly for quality control and regulatory purposes, the separation of antibody fragments is also very much essential.
- A well resolved symmetric pure protein peak from size exclusion chromatography needs to be verified by another mode for the presence of any variants.
- The species other than the monomer might induce toxic side effects to the body if not removed.

Introduction

- The new TSKgel® UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm analytical SEC column packed with 30 nm pores has an estimated exclusion limit of ~$2x10^6$ Da and provides improved separation and quantitation of mAb aggregates and oligomers.
- The 3 µm silica-based butyl (C4) TSKgel Protein C4-300 column, with a wide pore size of 30 nm, is optimized for the separation of large biomolecules such as proteins.
- Here we report the use of these two columns from two different modes of chromatography in the separation of the impurities and variants of proteins and monoclonal antibodies.

TSKgel UltraSW Aggregate Column – Product Attributes

Column	TSKgel UltraSW Aggregate
Column dimension	7.8 mm ID × 30 cm
Base material	Silica gel
Functional group	Diol
Particle size	3 µm
Pore size	30 nm
Separation range (for globular proteins)	1×10^4 - 2×10^6 Da
Applications	Separation of mAb aggregates

- TSKgel UltraSW Aggregate 7.8 mm ID × 30 cm analytical columns are novel columns packed with 30 nm pore size, 3 µm silica particles.
- The larger pore size with the estimated exclusion limit of ~2×10^6 Da provides improved separation and quantitation of protein aggregates and oligomers, particularly for the separation of the monoclonal antibody aggregates.

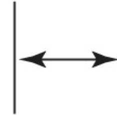
TSKgel Protein C4-300 – Product Attributes

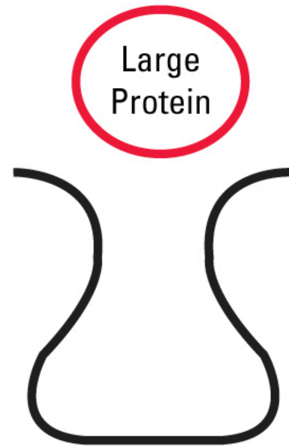
Column	TSKgel Protein C4-300
Pore size (silica):	30 nm
Particle size:	3 µm
Endcapped:	Yes (Trimethylsilyl)
pH stability:	1.5 - 7.5
Ligand:	C4 (butyl)
Specific surface area:	100 m^2/g
% carbon	3%

- The TSKgel Protein C4-300 column offers a 3 µm particle resulting in high resolution and increased column efficiencies.
- The short C4 (butyl) alkyl ligand allows for optimal recovery and retention of proteins.
- The large 30 nm pore size leads to higher sample capacity and more efficient mass transfer compared to conventional reversed phase columns with 10 nm pores.
- Controlled bonding density of the C4 short alkyl chain provides moderate hydrophobicity to the stationary phase, suitable for protein separation with high recovery.

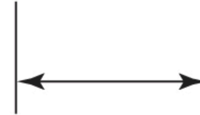
TSKgel Protein C_4-300 Column

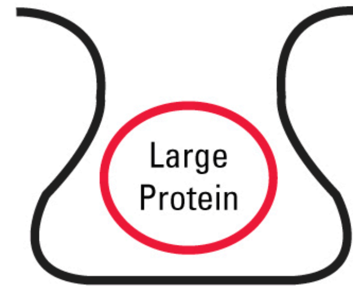
8-14 nm
Narrow pore





30 nm
Wide pore





Restricted mass transfer

More efficient mass transfer

- The larger pore size of the TSKgel Protein C_4-300 column helps in more efficient mass transfer during chromatographic analysis.
- The large pore size, allowing macromolecules to enter the interior of the pore, provides higher peak capacities than reversed phase columns with 10 nm pore size.

Material and Methods

Columns

- TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
- TSKgel Protein C_4-300, 3 µm, 4.6 mm ID × 10 cm
- TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm
- TSKgel SuperSW3000, 4 µm, 4.6 mm ID × 30 cm

Instrumentation

- Agilent 1200 system with Chemstation (Rev B.04.02)

Samples

- Ferritin (4.6 mg/mL, Sigma F4503-100MG)
- Apoferritin (5.0 mg/mL, Sigma A-3660)
- mAb 02 (4.5 mg/mL) – A gift from Tosoh Bioscience GmbH

Chromatographic Conditions

SEC Conditions (unless mentioned otherwise):

- Mobile phase: 0.1 mol/L phosphate buffer/0.1 mol/L sulfate buffer + 0.05% NaN_3
- Gradient: Isocratic
- Flow rate: 1.0 mL/min
- Detection: UV @ 280 nm
- Temperature: 30 °C
- Injection vol.: 10 μL

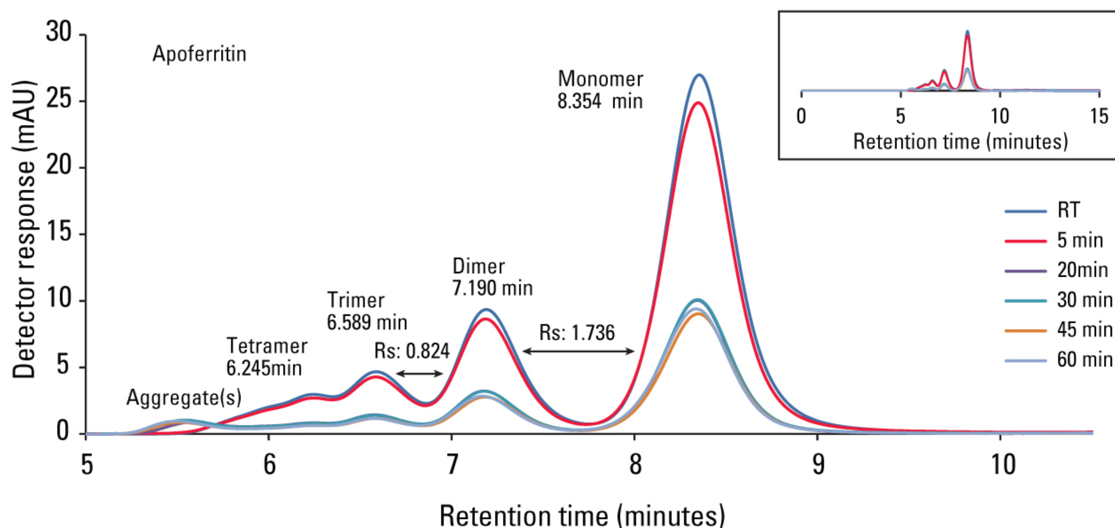
RPC Conditions:

- As mentioned in the respective chromatograms

Analysis of Heat Induced, Forced Denatured Large Hydrophobic Metalloprotein, Apoferritin, using a TSKgel UltraSW Aggregate Column

- Ferritin is a 450 kDa globular protein complex consisting of 24 protein subunits and is the primary intracellular iron-storage protein in both prokaryotes and eukaryotes, keeping iron in a soluble and non-toxic form.
- Ferritin that is not combined with iron is called apoferritin.
- Structurally, the molecule resembles a small icosahedral virus, shaped from a multimeric protein shell, apoferritin, within which variable amounts of iron may be stored.
- Ferritin and apoferritin are widely used for the calibration of gel filtration columns.
- In the following slide, we show the separation of monomer and higher order aggregates using a 30 nm pore size TSKgel UltraSW Aggregate column.

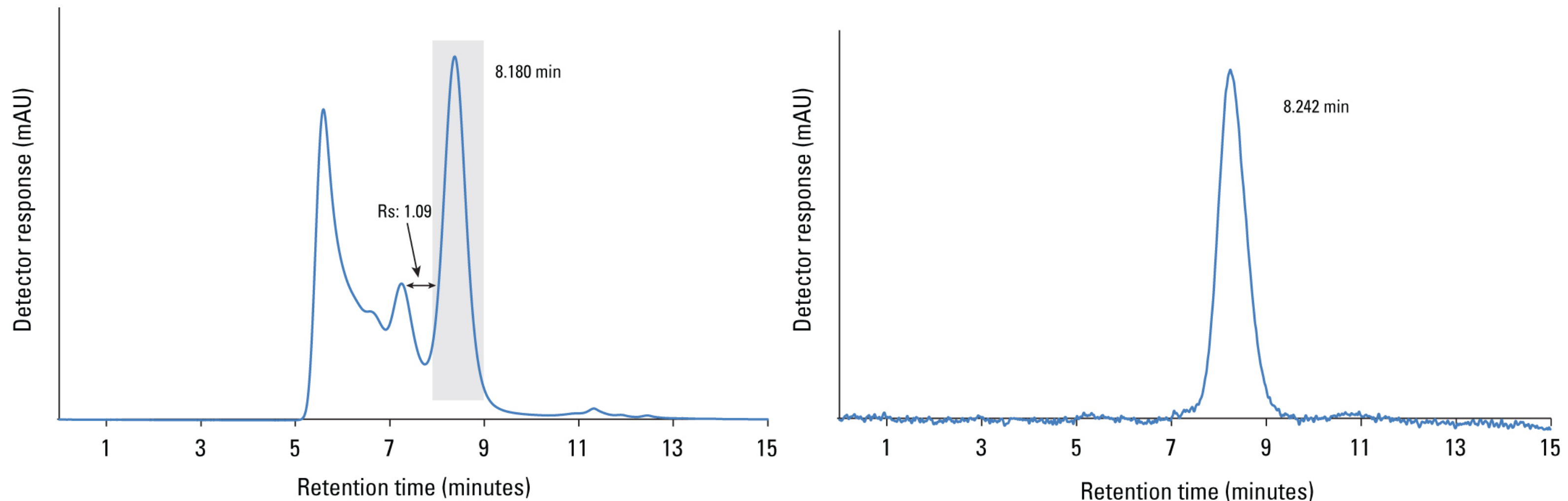
Analysis of Heat Induced, Forced Denatured Large Hydrophobic Metalloprotein, Apoferritin, using a TSKgel UltraSW Aggregate Column



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

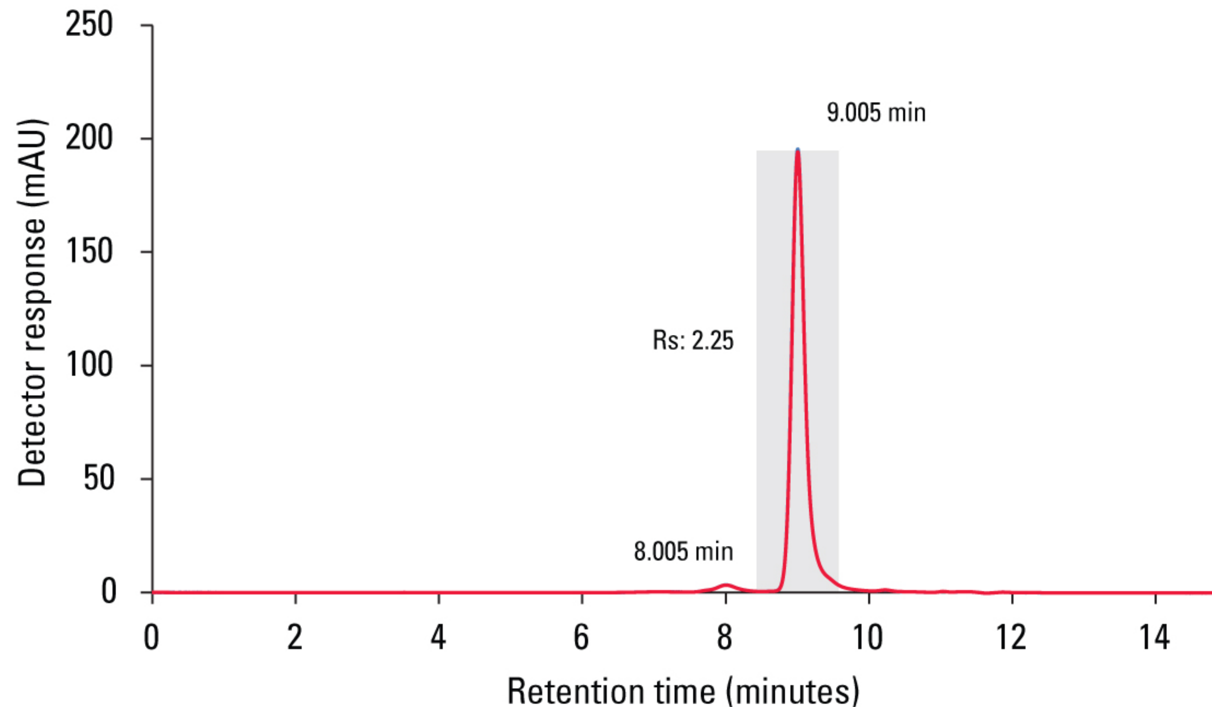
The result shows that the TSKgel UltraSW Aggregate column, containing a large exclusion limit of 2,000 kDa, is suitable for high resolution analysis of apoferritin aggregates containing multimers larger than trimer.

Analysis of Monomer Fraction of Ferritin using the TSKgel UltraSW Aggregate Column



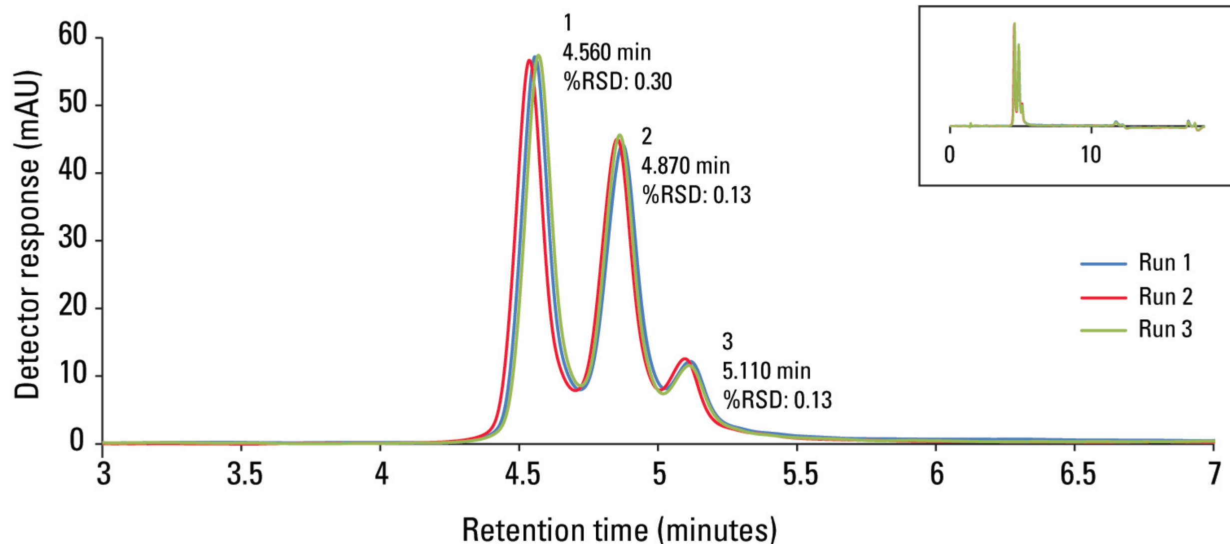
- **Left panel**: Analysis of ferritin using a 30 nm pore size TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column (shaded region represents the collected fraction).
- **Right panel**: Collected ferritin fraction re-analyzed on TSKgel UltraSW Aggregate column illustrating presence of only the monomeric species
- The results show that the monomer peak of ferritin could be completely resolved using the TSKgel UltraSW Aggregate column.

Separation of Hydrophobic Variants in a Monoclonal Antibody Sample – SEC Separation by TSKgel UltraSW Aggregate



- Analysis of mAb 02 by the TSKgel UltraSW Aggregate yielded nice separation between the monomer and dimer peaks.
- The monomer fraction was collected and loaded onto the TSKgel Protein C4-300 column as shown in the next slide.

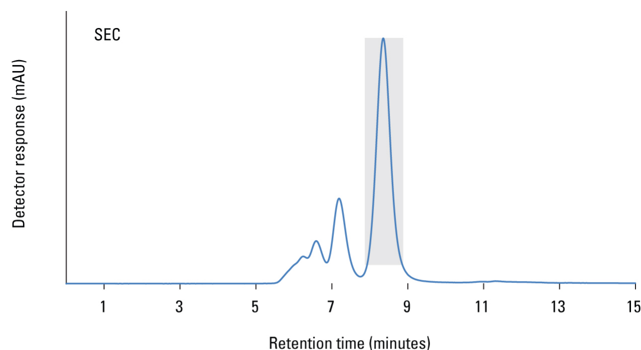
Separation of Hydrophobic Variants in a Monoclonal Antibody Sample – RPC Separation by TSKgel Protein C4-300

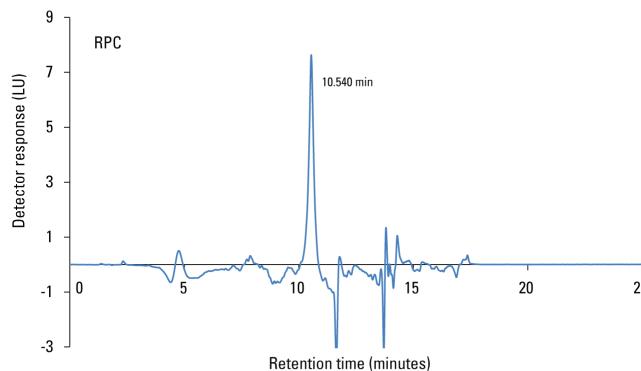


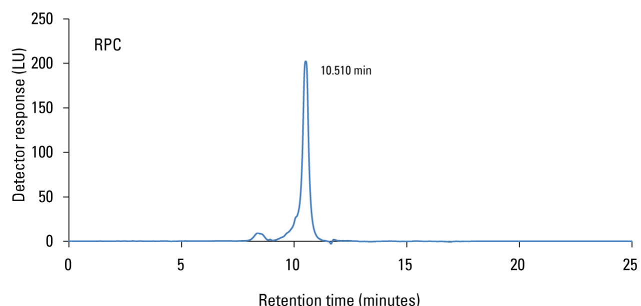
Mobile phase:	A: H_2O + 0.1% TFA
	B: ACN + 0.1% TFA
Gradient:	30-50% B over 10 minutes
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	70 °C
Injection vol.:	10 μL
Sample:	mAb 02

- TSKgel Protein C4-300 yielded the presence of 3 hydrophobic variants.
- 3 consecutive injections yielded low %RSD for k’ for all peaks (%RSD < 0.5).

TSKgel Protein C4-300 and TSKgel UltraSW Aggregate for Orthogonal RPC-FLD Separations

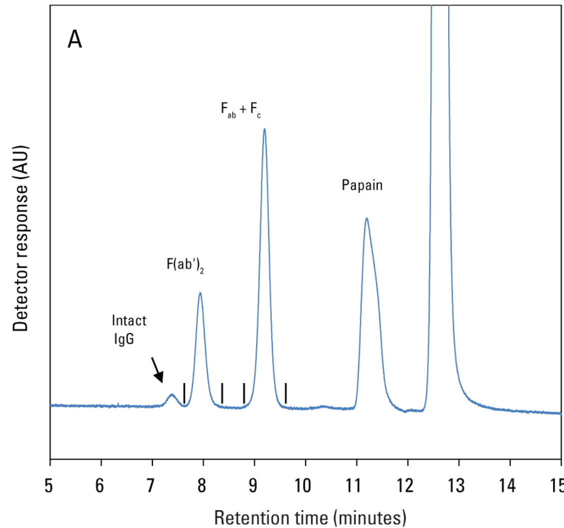


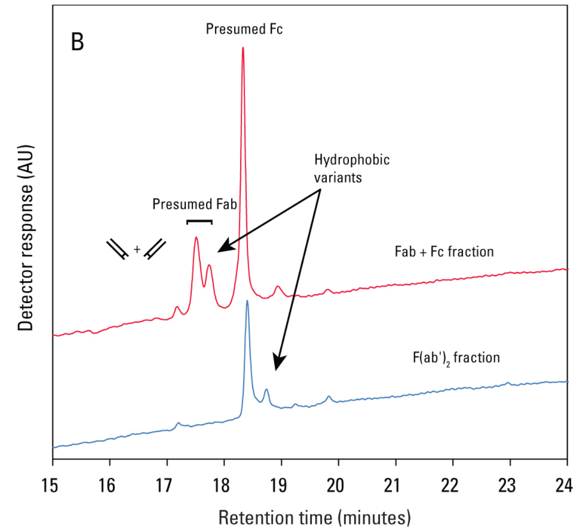




- **Top Panel**: Separation of apoferritin using the TSKgel UltraSW Aggregate SEC column (shaded region depicts collected fraction).
- **Middle Panel**: Analysis of apoferritin monomer fraction (collected from SEC separation) on TSKgel Protein C4-300 column using FLD detection.
- **Bottom Panel**: Separation of apoferritin on TSKgel Protein C4-300 using FLD detection.
- SEC-RPC-FLD of apoferritin illustrates the absence of hydrophobic variants present in the collected monomer fraction.

Separation of Papain-Digested IgG Fragments by RPC using a TSKgel Protein C4-300 Column





Conditions for SEC

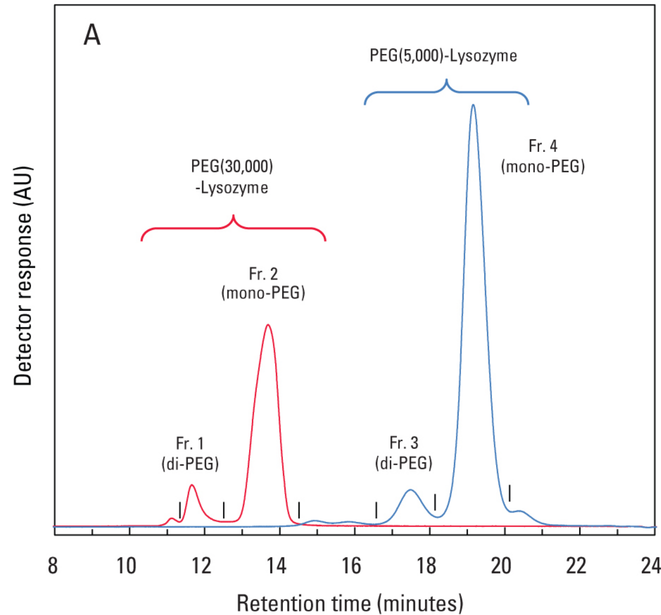
Column:	TSKgel SuperSW3000, 4 µm, 4.6 mm ID × 30 cm
Mobile phase:	200 mmol/L phosphate buffer + 0.05% NaN_3, pH 6.7
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	100 µL
Sample:	papain digest of mouse monoclonal IgG

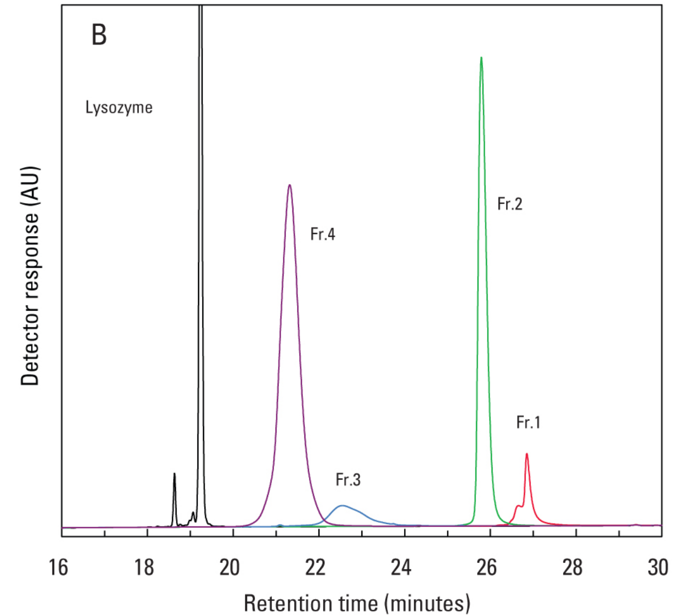
Conditions for RPC

Column:	TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 15 cm
Mobile phase A:	H_2O/ACN/TFA = 90/10/0.05 (v/v/v)
Mobile phase B:	H_2O/ACN/TFA = 20/80/0.05 (v/v/v)
Gradient:	0% → 100% B in 45 min
Detection:	UV @ 215 nm
Temperature:	50 °C
Injection vol.:	100 µL
Samples:	SEC fractions of mouse IgG fragments

A well resolved, symmetric, pure protein peak from size exclusion chromatography yielded a number of hydrophobic variants when separated by reversed phase chromatography..

Separation of PEGylated Lysozymes by RPC using a TSKgel Protein C4-300 Column





<u>Conditions for SEC</u>

Column:	TSKgel SuperSW3000, 4.6 mm ID × 30 cm × 2
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	50 μL
Samples:	5 g/L PEG(MW 5,000)-lysozyme, 5 g/L PEG(MW 30,000)-lysozyme

<u>Conditions for RPC</u>

Column:	TSKgel Protein C4-300, 4.6 mm ID × 15 cm
Mobile phase A:	H_2O/ACN/TFA = 90/10/0.05 (v/v/v)
Mobile phase B:	H_2O/ACN/TFA = 20/80/0.05 (v/v/v)
Gradient:	0%→ 100% B in 45 min
Detection:	UV @ 215 nm
Temperature:	40 °C
Injection vol.	100 μL
Samples:	0.1 g/L lysozyme, SEC fractions 1~4 shown in the left figure.

Conclusions

- Protein characterization by orthogonal chromatography using the TSKgel UltraSW Aggregate and TSKgel Protein C_4-300 columns effectively separates hydrophobic variants which cannot be detected by a single mode of chromatography.
- The new TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm analytical SEC column packed with 30 nm pore size has an estimated exclusion limit of ~2×10^6 Da and provides improved separation and quantitation of protein and mAb aggregates.
- The superb performance of these columns was demonstrated by the separation of aggregates and the variants.