High Speed and High Resolution Cation Exchange Chromatography for Biological Samples on Non-Porous Packings

Hiroyuki MORIYAMA, Mutsumi SHIMADA, Kazuaki MURANAKA,
Toshinao IWAEDA

Separation Center, TOSOH Corporation

www. tosohbioscience.com

Poster presented at HPLC 2008, Baltimore; session P-1405-M

Introduction

- In 1987 Tosoh introduced a series of mono-disperse, non-porous resin (NPR) columns for the rapid separation of biological samples. Non-porous columns packed with spherical 2.5µm particles provide high efficiency and rapid analyses. However, due to their small surface area 2.5µm NPR columns have much smaller loading capacities than porous resins. Also, 2.5µm particle size NPR columns require high operating pressures.

- Recently, Tosoh scientists developed novel non-porous cationic exchange (CX) resins, to be marketed later this year as TSK-GEL SP-STAT and TSK-GEL CM-STAT columns. The new cation exchange columns are packed with 7 and 10µm spherical, mono-disperse, non-porous particles of which the surface has been modified with open access multi-layered cation exchange groups.

- The novel CX resins show higher adsorption capacities and require lower pressures compared with conventional non-porous columns of the same column dimension. Rapid separations of proteins were achieved within 1 minute on short columns (3.5cm) packed with 10µm resin and high resolution analyses were achieved on a 10cm length column packed with 7µm resin.

- The basic properties of the novel cation exchange columns (TSK-GEL CM-STAT and TSK-GEL SP-STAT) and how they apply to the separation of proteins, antibodies and peptides are reported in comparison with commercially available non-porous CIEX columns.

Experimental

- **HPLC columns - Tosoh Corporation**
  - **TSKgel SP-STAT, 10µm, 3.0mm ID x 3.5cm**
  - **TSKgel SP-STAT, 7µm, 4.6mm ID x 10cm**
  - **TSKgel CM-STAT, 10µm, 3.0mm ID x 3.5cm**
  - **TSKgel CM-STAT, 7µm, 4.6mm ID x 10cm**
  - **TSKgel SP-NPR, 2.5µm, 4.6mm ID x 3.5cm**

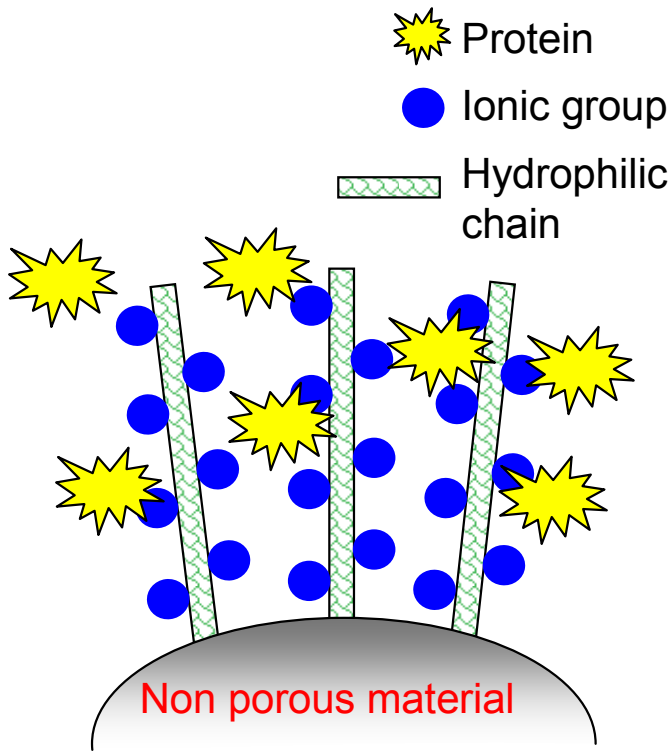
- **HPLC columns - Commercially available**
  - **Brand A: Non-porous WCX type, 4mm ID x 25cm (Dionex)**
  - **Brand B: Monolithic SCX type, 5.0mm ID x 5cm (Dionex)**

- **Reagents**
  - **All proteins were purchased from Sigma. Other reagents were purchased from Kishida Chemicals (Osaka). Antibody samples (mAb A~F) were generously donated by Dr. H. Kakitani of Sagani Chemical Research Center (Kanagawa, Japan). Samples of a pegylated protein-based pharmaceutical preparation were purchased from Pfizer. Pegylation reactions with .-lactoglobulin and IgG were performed in our laboratory.**

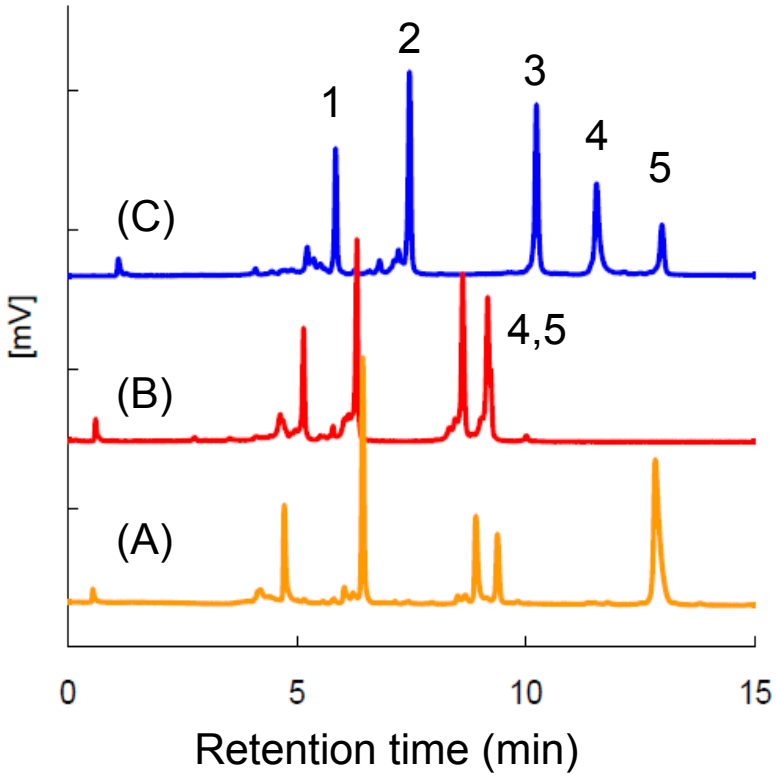
Basic Properties of TSK-GEL SP-STAT and CM-STAT Cation Exchange Columns

Property	TSK-GEL SP-STAT		TSK-GEL CM-STAT	
Base material	Cross-linked hydrophilic polymer (mono-disperse particles)			
Pore size	Non-porous			
Functional group	Sulfonate		Carboxymethyl	
Particle size	7µm	10µm	7µm	10µm
Column size	4.6mm ID x 10cm	3mm ID x 3.5cm	4.6mm ID x 10cm	3mm ID x 3.5cm
Application	High resolution (HR) protein separation	High throughput (HT) protein separation	High throughput (HR) protein separation	High throughput (HT) protein separation

Schematic Diagram of TSK-GEL STAT CIEX Series



Protein Separations on Non-Porous Cation Exchange Columns

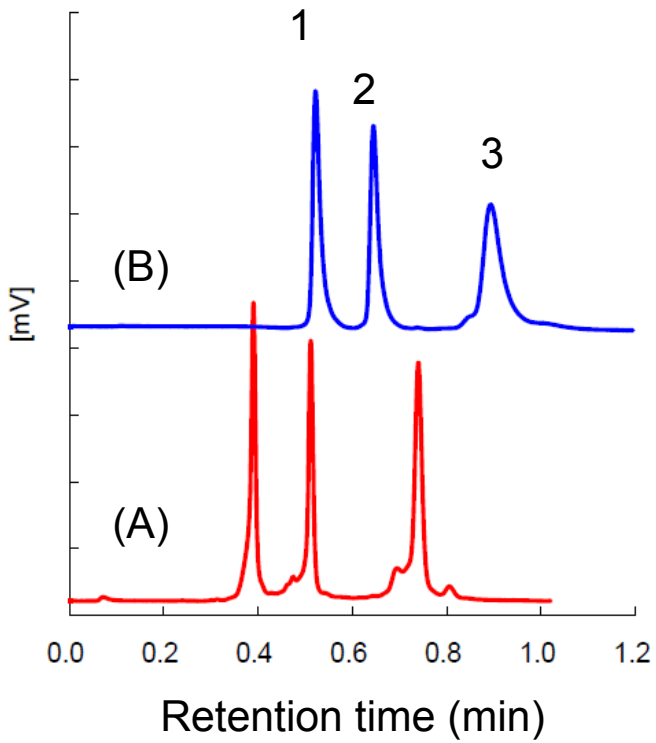


Columns: A: TSKgel SP-STAT, 7µm, 4.6mm ID x 10cm
B: TSKgel CM-STAT, 7µm, 4.6mm ID x 10cm
C: Brand A, Non-porous CM-type, 4mm ID x 25cm

Eluent: A: 20mmol/L MES buffer (pH6.0)
B: 1.0mol/L NaCl in buffer A (pH6.0)
Gradient: 0% B (0min), 100% B (60min)
Flow rate: 1.0mL/min
Detection: UV@280nm
Samples: 1. trypsinogen
2. alpha-chymotrypsinogen A
3. RNase A
4. cytochrome C
5. lysozyme

In this comparison of protein separations on various cation exchange columns, different selectivities were observed for each set of proteins on all three columns. The TSKgel SP-STAT column shows excellent resolution for cytochrome C and lysozyme.

Fast Protein Separations on Monolithic and Non-Porous Cation Exchange Columns



Columns: A: TSKgel SP-STAT, 10µm, 3.0mm ID x 3.5cm
B: Brand B, Monolithic SP-type, 5mm ID x 5cm

Eluent: A: 20mmol/L Sodium Acetate (pH5.0)
B: 1.0mol/L NaCl in buffer A (pH5.0) for column A
1.5mol/L NaCl in buffer A (5.0) for column B

Gradient: 0% B (0min), 100% B (1min)

Flow rate: A: 2.0mL/min
B: 4.73mL/min

Detection: UV@280nm

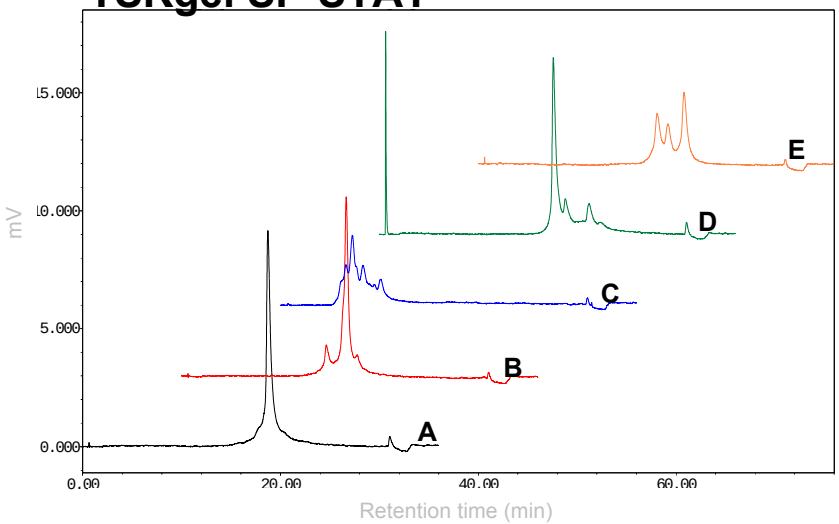
Samples: 1. alpha-chymotrypsinogen A
2. cytochrome C
3. lysozyme

The fast separation of standard proteins was investigated using short cation exchange columns. A TSKgel SP-STAT column shows superior resolution, better peak shape, and a shorter analysis time (< 60 seconds) compared to a monolithic SP-type column.

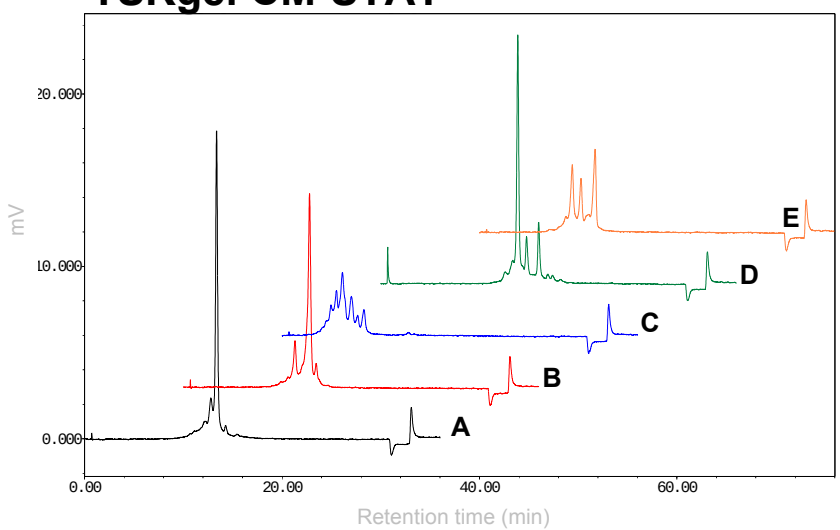
Separation of Antibodies on TSK-GEL SP-STAT and TSK-GEL CM-STAT Columns

Antibody Separation Profiles on TSK-GEL STAT Series Cation Exchange Columns

TSKgel SP-STAT



TSKgel CM-STAT



Columns: A: TSKgel SP-STAT, 7µm, 4.6mm ID x 10cm
B: TSKgel CM-STAT, 7µm, 4.6mm ID x 10cm

Eluent: A: 20mmol/L MES (pH6.0)
B: 20mmol/L MES + 0.5mol/L NaCl (pH6.0)

Gradient: 10% B (0min), 30% B (30min), 100% B (30min), 100% B (32min), 10% B (32min), 10% B (36min)

Flow-rate: 1.0mL/min

Temp.: Ambient

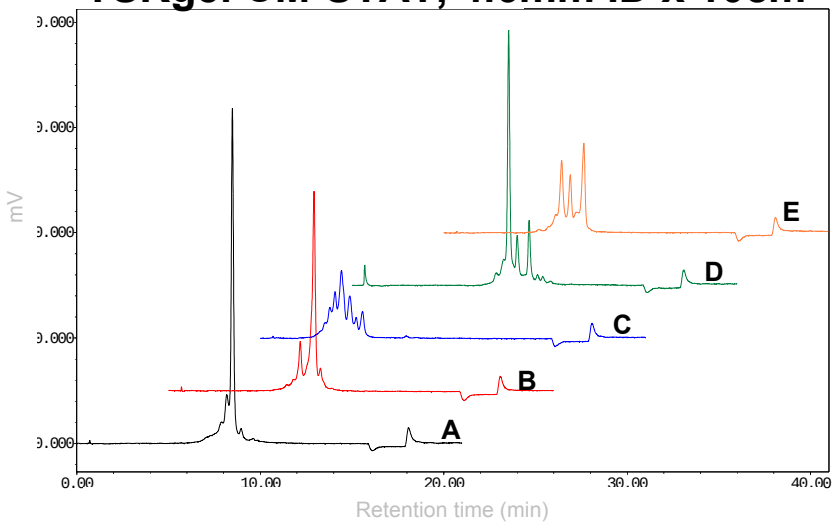
Detection: UV@280nm

Inj. Vol.: 20µL

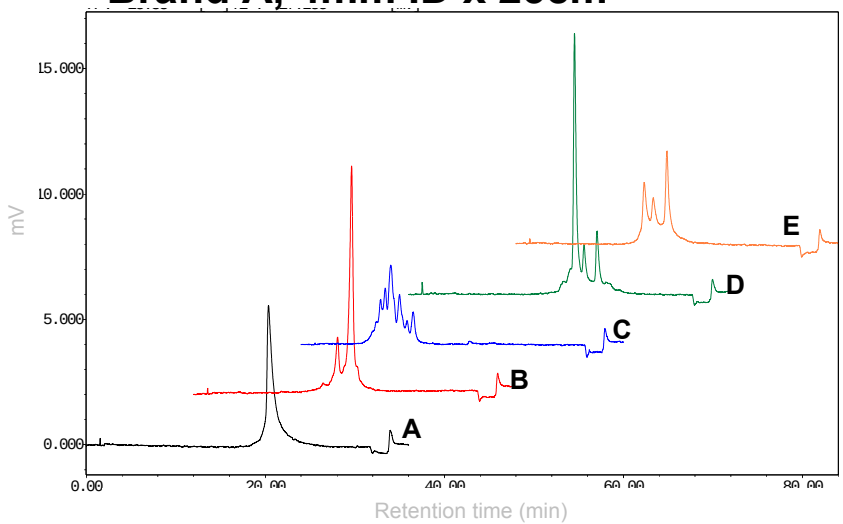
Five different antibodies were injected on TSKgel SP-STAT and TSKgel CM-STAT high resolution cation exchange columns. TSKgel CM-STAT provided better peak shape, higher resolution and shorter analysis time than could be obtained on the TSKgel SP-STAT column.

Antibody Separation Profiles on High Throughput Cation Exchange Columns

TSKgel CM-STAT, 4.6mm ID x 10cm



Brand A, 4mm ID x 25cm



Columns: A: TSKgel CM-STAT, 7µm, 4.6mm ID x 10cm
B: Brand A, WCX, 4mm ID x 25cm

Eluent: A: 20mmol/L MES (pH6.0)
B: 20mmol/L MES + 0.5mol/L NaCl (pH6.0)

Gradient: A: 10% B (0min), 30% B (15min), 100% B (15min), 100% B (17min), 10% B (17min), 10% B (21min)
B: 10% B (0min), 30% B (30min), 100% B (30min), 100% B (32min), 10% B (32min), 10% B (36min)

Flow-rate: A: 1.0mL/min
B: 2.0mL/min

Temp.: Ambient

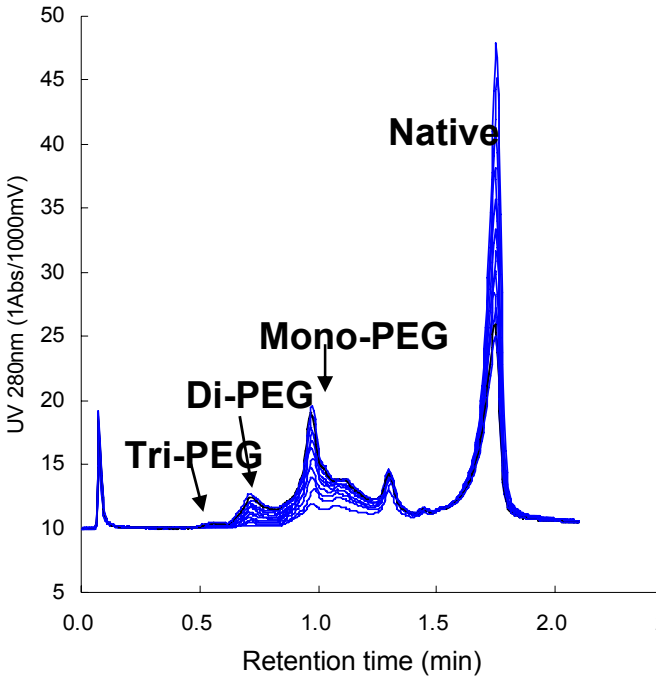
Detection: UV@280nm

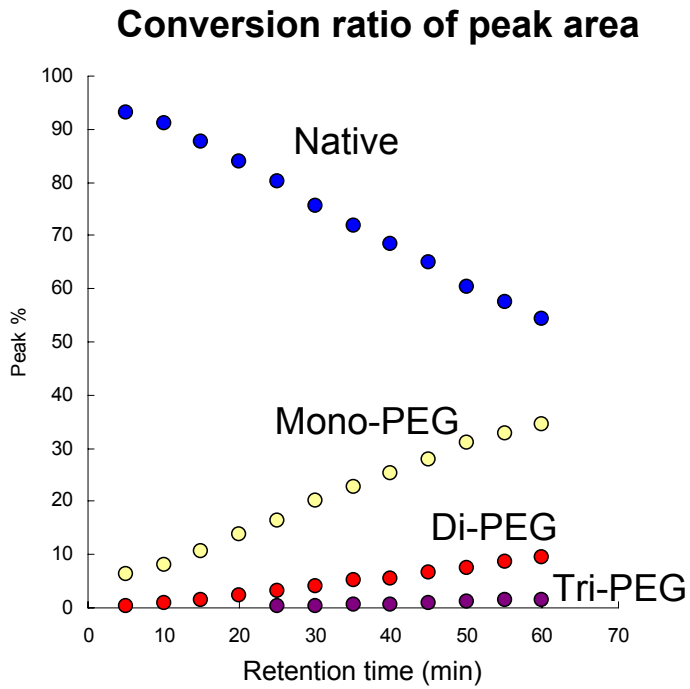
Inj. Vol.: 20µL

The analysis profiles for the five antibodies on CM-STAT were compared with the profiles obtained on a competitive non-porous-type cation exchange column. Similar or higher resolution profiles were obtained on TSKgel CM-STAT in approximately half the time.

Applications of Protein Separations on TSK-GEL SP-STAT Columns

In-Process Analysis of Pegylated β-Lactoglobulin on High Throughput TSKgel SP-STAT Column



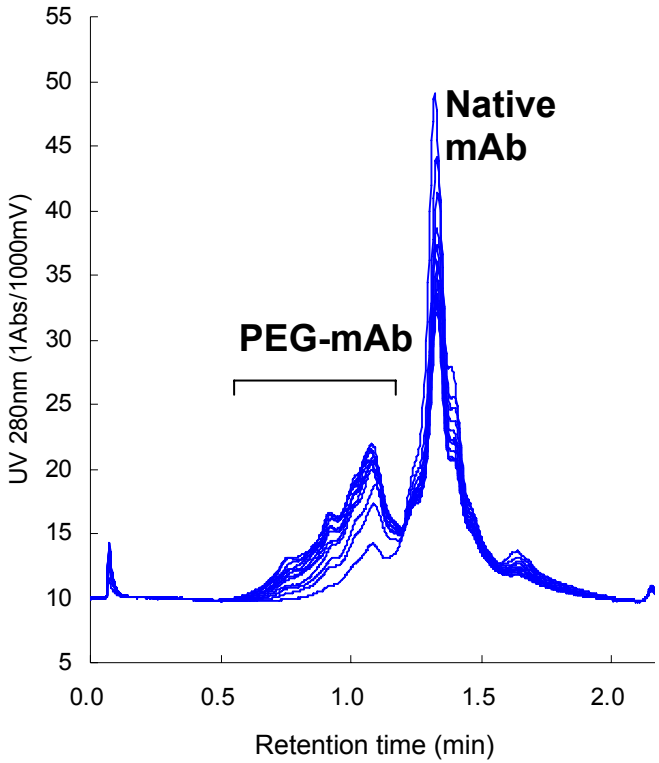


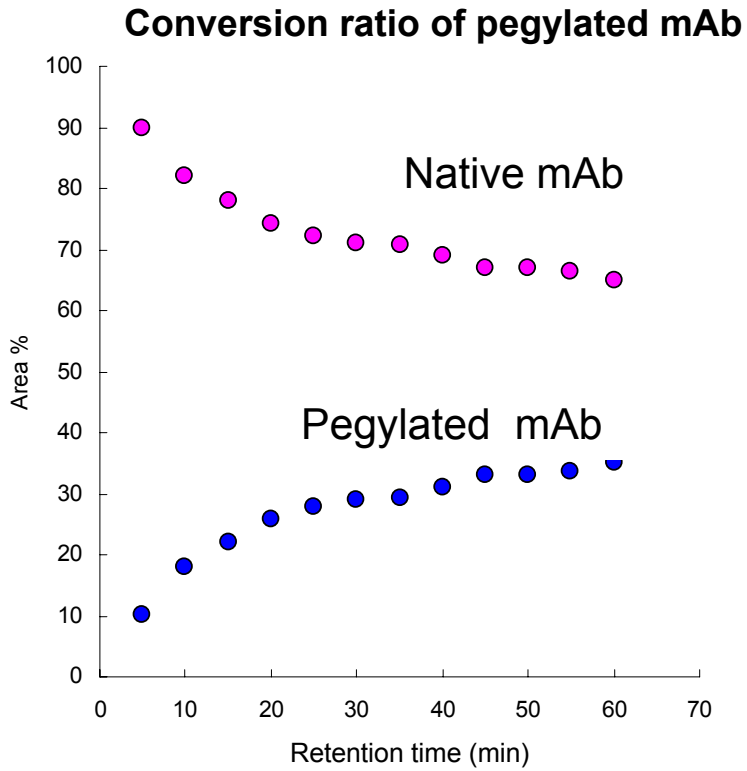
Column: A: TSKgel SP-STAT, 10µm, 3mm ID x 3.5cm

Eluent: A: 20mmol/L Sodium Acetate buffer (pH5.0)
B: 1.0mol/L NaCl in buffer A (pH5.0)
Gradient: 0% B (0min), 100% B (2min)
Flow-rate: 2.0mL/min
Detection: UV@280nm
Sample: Pegylated β-lactoglobulin

A sample of β-lactoglobulin (5mg/mL) was reacted with polyethylene glycol (5k Da) in a pH 6.5 phosphate buffer. The formation of pegylated proteins was monitored in 5 minute intervals on a 3.5cm TSKgel SP-STAT column. Peak areas of mono-, di-, and tri-pegylated proteins increased with reaction time, while the area of unreacted β-lactoglobulin declined.

In-Process Analysis of Pegylated mAb Sample on High Throughput TSKgel SP-STAT Column



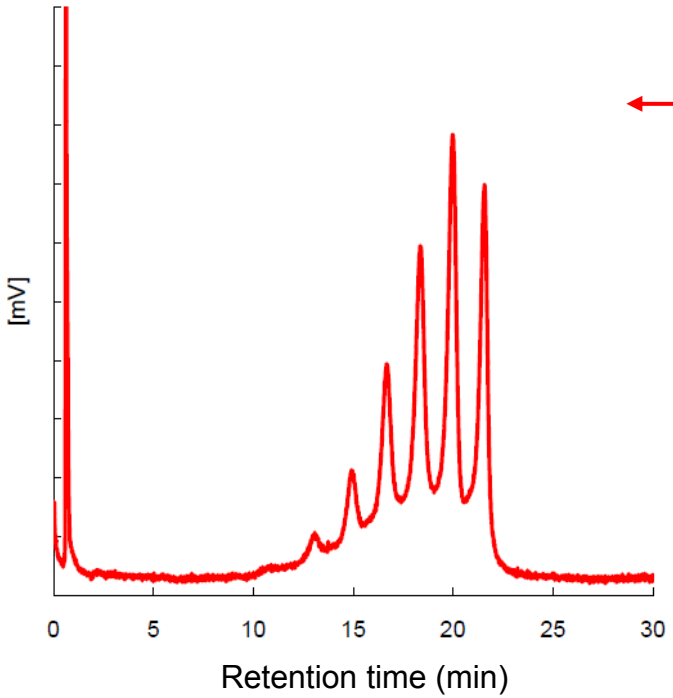


Column: **A: TSKgel SP-STAT, 10µm, 3mm ID x 3.5cm**

Eluent: **A: 20mmol/L Sodium Acetate buffer (pH5.0)**
B: 1.0mol/L NaCl in buffer A (pH5.0)
Gradient: **0% B (0min), 100% B (2min)**
Flow-rate: **2.0mL/min**
Detection: **UV@280nm**
Sample: **Pegylated monoclonal antibody**

A monoclonal antibody sample (5mg/mL) was reacted with polyethylene glycol (5k Da) in a pH 6.5 phosphate buffer. The formation of pegylated antibodies was monitored in 5 minute intervals on a 3.5cm TSKgel SP-STAT column. Peak areas of pegylated antibodies increased with reaction time, while the peak area of native antibody decreased.

Separation of mAb sample on TSKgel CM-STAT

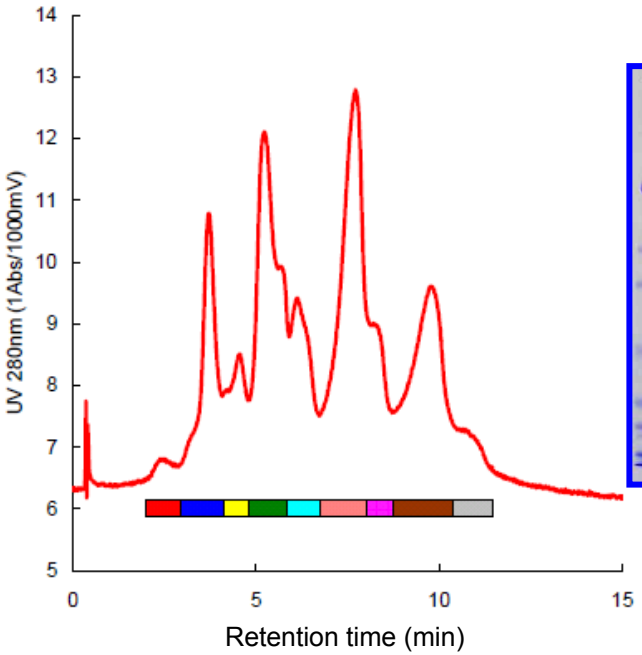


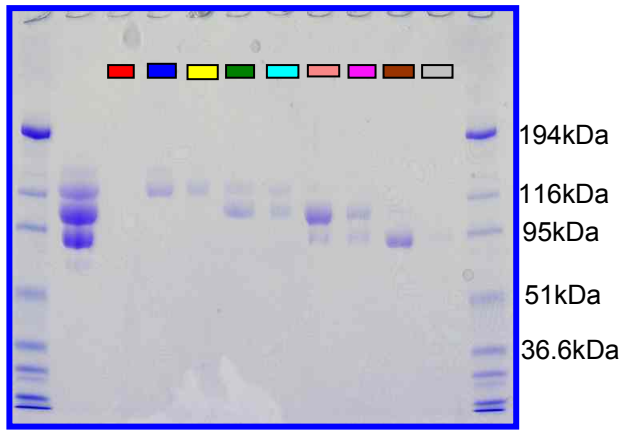
Non reduced SDS-PAGE

Column:	**A: TSKgel CM-STAT, 7µm, 4.6mm ID x 10cm**
Eluent:	**A: 20mmol/L MES buffer (pH6.0)**
	B: 0.1mol/L NaCl in buffer A (pH6.0)
Gradient:	**20% B (0min), 50% B (30min)**
Flow-rate:	**1.0mL/min**
Detection:	**UV@280nm**
Inj. Vol:	**20µL**
Sample:	**monoclonal antibody (mAb F)**

High resolution analysis of a monoclonal antibody can be successfully performed on a TSKgel CM-STAT, 10cm column.

Analysis of a Pegylated Protein Preparation using a High Resolution TSKgel SP-STAT Column





Non reduced SDS-PAGE

Column: A: TSKgel SP-STAT, 7µm, 4.6mm ID x 10cm

Eluent: A: 20mmol/L Sodium acetate (pH4.2)
B: 0.5mol/L NaCl in buffer A (pH4.2)
Gradient: 0% B (0min), 40% B (20min)
Flow-rate: 1.5mL/min
Detection: UV@280nm
Sample: pegylated protein (Pfizer)

A pharmaceutical preparation containing pegylated protein was analyzed on a 10cm TSKgel SP-STAT column. Label information claims that the pegylated protein sample consists of a mixture of 4, 5 and 6 PEG molecules attached to a 192 amino acid protein. As expected, molecular weights as determined by non-reduced SDS-PAGE are much higher than actual molecular weights for the various fractions. None of the fractions were further analyzed.

Conclusions

- Two new cation exchange columns, TSK-GEL SP-STAT and TSK-GEL CM-STAT, were evaluated for the analysis of biological samples.
- Short 3.5cm long columns, packed with 10µm particles, were very useful for high throughput separations requiring less than one minute analysis time, while, as expected, higher resolution protein separations were obtained on 10cm columns packed with 7µm particles.
- TSK-GEL CM-STAT and TSK-GEL SP-STAT cation exchange columns show excellent resolution and fast separations of protein samples compared to other non-porous and monolithic CIEX columns.
- TSK-GEL CM-STAT columns showed sharper peaks for mAb samples compared with other non-porous cation exchange columns.
- Pegylated proteins were analyzed on a short TSKgel SP-STAT column. The conversion ratios of pegylated and native proteins can be monitored by following the reaction using 5-minute intervals.
- TSK-GEL CM-STAT and TSK-GEL SP-STAT cation exchange columns can be powerful tools for fast and high resolution separations of proteins.