Non-Porous Ion-Exchange HPLC columns for High Speed and High Resolution Analysis & Purification of Biomolecules

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Abstract

- New ion-exchange HPLC columns for high-throughput protein separations were developed. They include two cation-exchange columns, one containing a sulphonic acid (SP) ligand, the other a carboxymethyl (CM) ligand. A strong anion-exchange column with quaternary ammonium groups was also developed.
- In all case, the ligand was bonded to methacrylate-based non-porous resin particles of 5, 7 or 10 micron particle size.
- The basic properties of the new columns and their application for the analysis of biomolecules such as proteins, peptides or nucleic acids were studied.
- The pressure drops of the new ion-exchange columns were examined at various flow rates in comparison with commercial TSK-GEL DEAE-NPR and SP-NPR columns packed with 2.5 micron non-porous resin particles. As expected, all new columns exhibited substantially lower back pressures compared with the commercial NPR columns.
- Even with particle sizes above 5 micron, the new ion-exchange columns were found to provide very narrow peak width for proteins. High-throughput separation for proteins on the ion-exchange columns were readily achievable with protein analysis times or less than 60 seconds.

Properties of Non-Porous Ion Exchange columns

- Particle chemistry: methacrylate polymer
- Particle type: non-porous
- Stationary phase ligands: Q, SP and CM
- Particle sizes of 10, 7 and 5µm
  - 5µm for Q-type only
- Column hardware and dimensions
  - Stainless steel tubing and fittings
  - 3.0mm ID x 3.5cm (10µm) for high-throughput separation
  - 4.6mm ID x 10cm (7 & 5µm) for high resolution

Benefits of Non-Porous Ion Exchange Columns

- **10µm: SP**
  - Low pressure drop
  - For high throughput separation of proteins, peptides and nucleic acids
  - Applicable to protein separation times of less than 60 seconds
  - Assay of reaction process for protein pegylation
- **7µm: SP, CM, and Q**
  - High resolution of proteins, peptides and nucleic acids
  - Separation of monoclonal antibody (mAb) aggregates
  - Separation of charged mAb isomers
  - Analysis of extend of protein pegylation
- **5µm: Q**
  - High resolution of nucleic acids
  - Separation of DNA oligomers
  - Separation of siRNA

Characteristics of column Types Investigated

Stationary phase	Particle size (µm)	column size (mm ID x cm)	Features	Application
Q	10	3.0 x 3.5	• High-throughput separation of proteins, peptides and Nucleic acids • Low pressure drops	• Proteins, peptide, LMW nucleic acids
Q	7	4.6 x 10	• High resolution analysis of proteins, peptides	• Aggregates of Mab, charge isomer of mAb, PEGylated proteins
Q	5	4.6 x 10	• High resolution analysis of nucleic acids	• Oligo DNA, siRNA
SP	10	3.0 x 3.5	• High-throughput separation of proteins and peptides • Low pressure drops	• Proteins, peptide digests
SP	7	4.6 x 10	• High resolution analysis of proteins, peptides	• Aggregates of Mab, charge isomer of Mab, PEGylated proteins
CM	10	3.0 x 3.5	• High-throughput separation of proteins and peptides • Low pressure drops	• Proteins, peptide digests
CM	7	4.6 x 10	• High resolution analysis of proteins, peptides	• Aggregates of Mab, charge isomer of mAb, PEGylated proteins

Features of Non-Porous Resin columns

Benefits of typical non-porous resin columns

- High resolution
- High speed

Challenges of typical non-porous resin columns

- High backpressure due to small particle resin
- Low loading capacity

***Experimental* non-porous resin columns:**

⇨ **Much higher resolution**

⇨ **Faster separation (within 1 min.)**

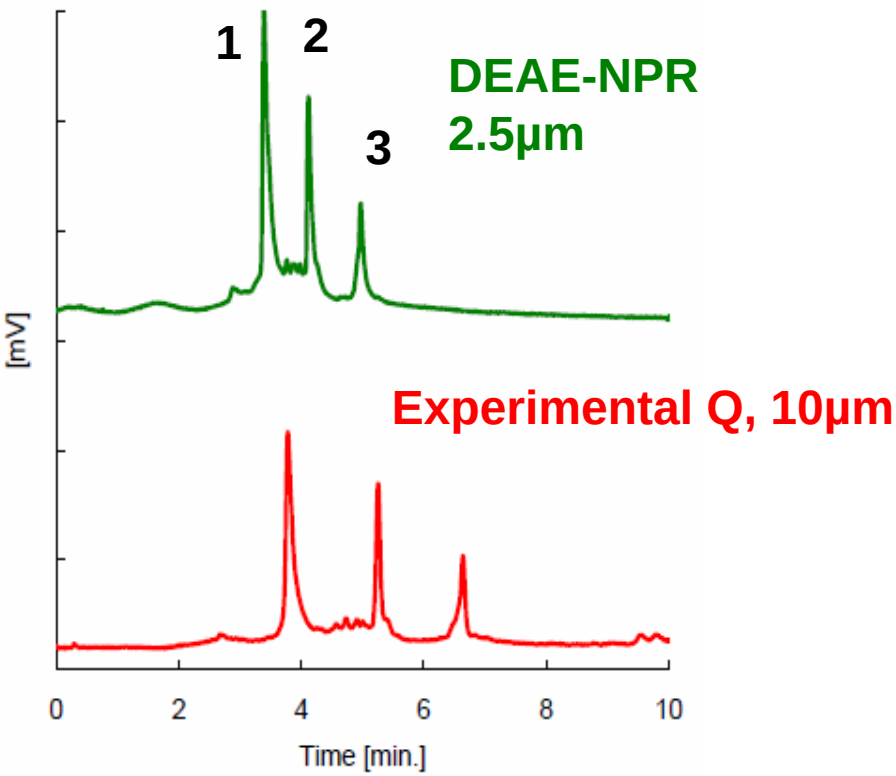
→ **Suitable for high throughput analysis**

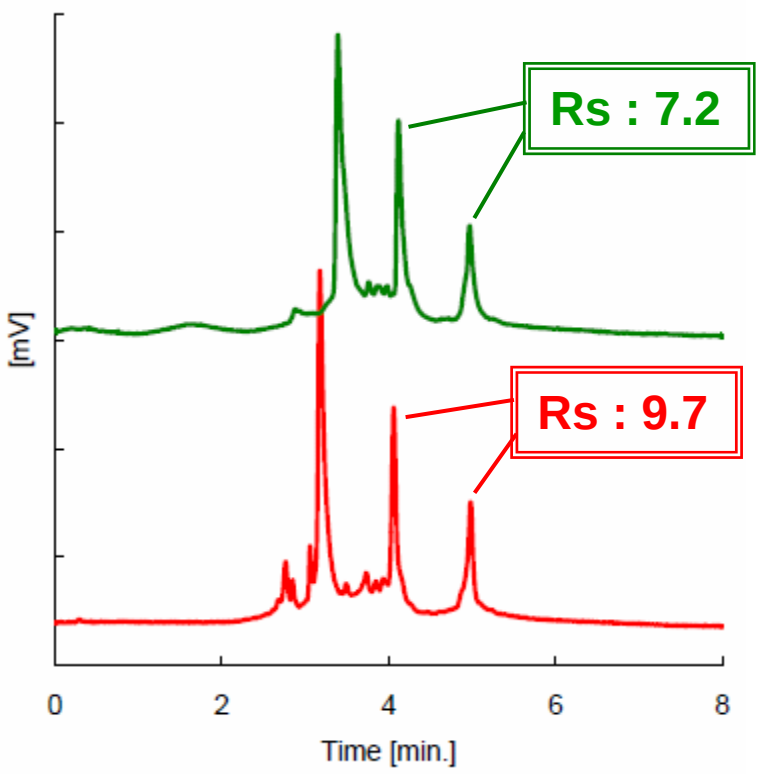
⇨ **Low backpressure**

⇨ **High capacity**

→ **Suitable for minor protein fraction**

Comparison of Resolution: Standard Protein Analysis by Anion Exchange columns





The same gradient condition:

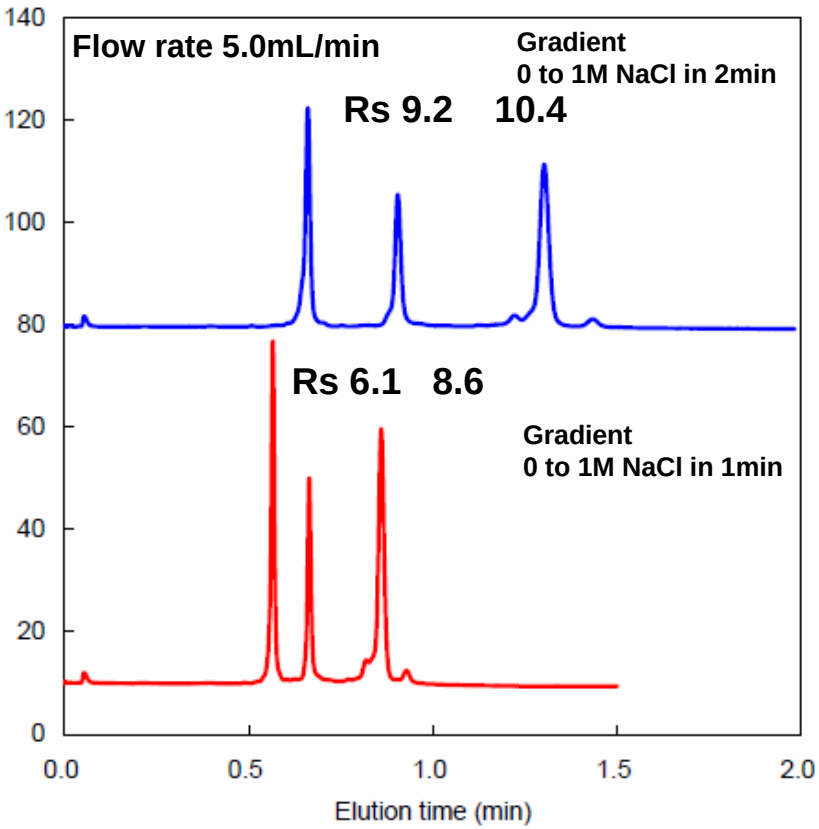
Gradient : B) 0 to 100% (10min.)

The same retention time for STI:

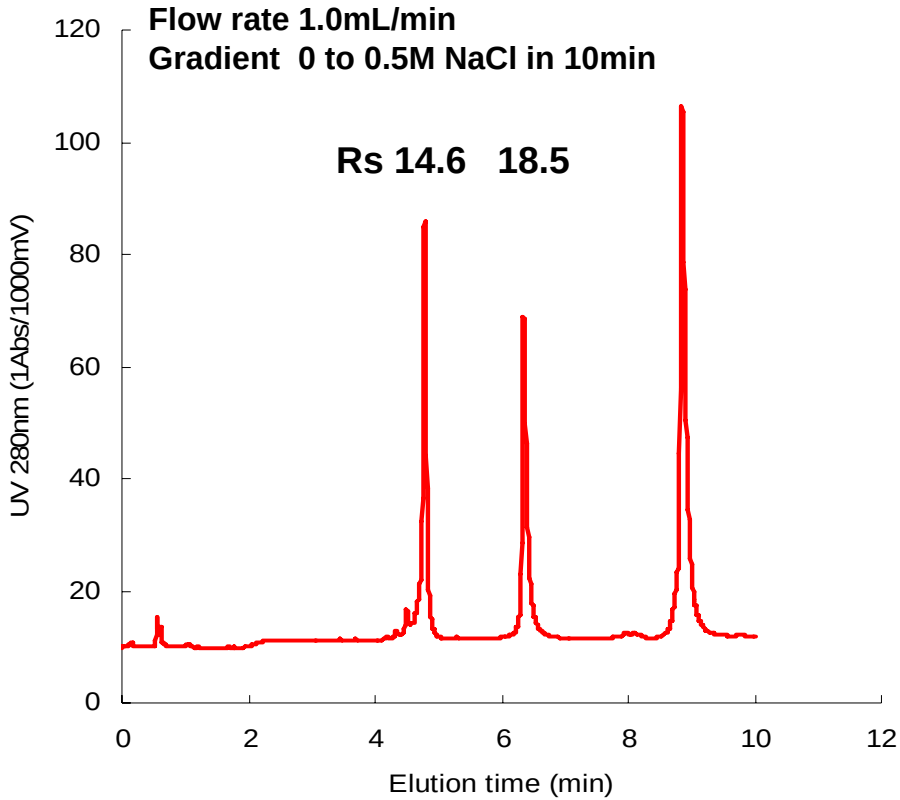
Gradient : B) 0 to 100% (10min.) for DEAE-NPR
B) 0 to 100% (6.5min.) for Q-type trial column

Column Size: 4.6mm ID x 3.5cm, Eluent: A) 20mmol/L Tris-HCl (pH8.5), B) 0.5 mol/L NaCl in A (pH8.5)
Flow rate: 1.0mL/min, Samples: 1) conalbumin 2) ovalbumin 3) soybean trypsin inhibitor

Resolution of TSK-GEL SP-NPR Strong Cation Exchange columns with Standard Protein Analysis



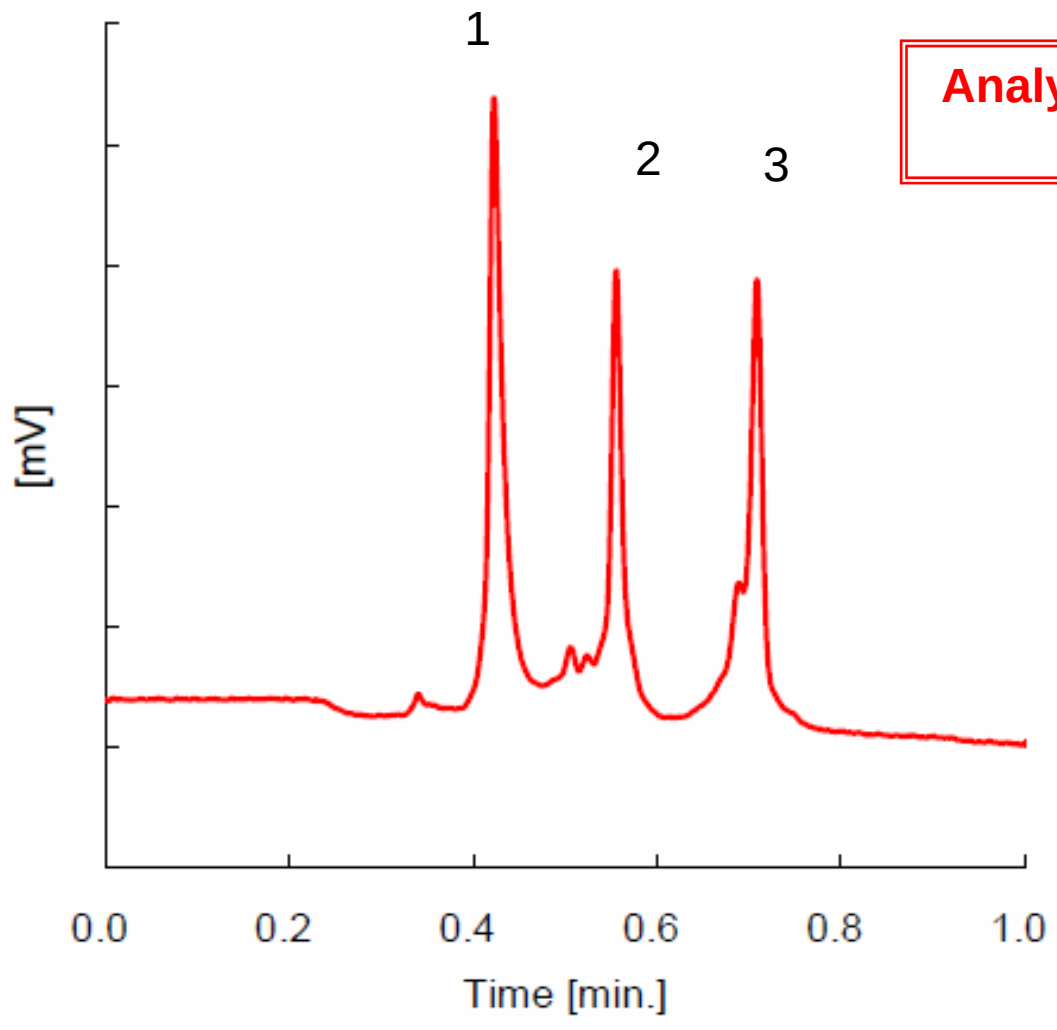
High throughput analysis by Experimental 10µm SP column



High resolution analysis by Exp. 7µm SP column

Eluent : A) 20mmol/L sodium acetate buffer (pH5.0) B) 1.0M NaCl in 20mM sodium acetate buffer (pH5.0)
Gradient : described in Figure Flow-rate : described in Figure
Sample 1) alpha-chymotrypsinogen A 2) cytochrome C 3) lysozyme Each protein 1mg/mL, 2.5uL injected

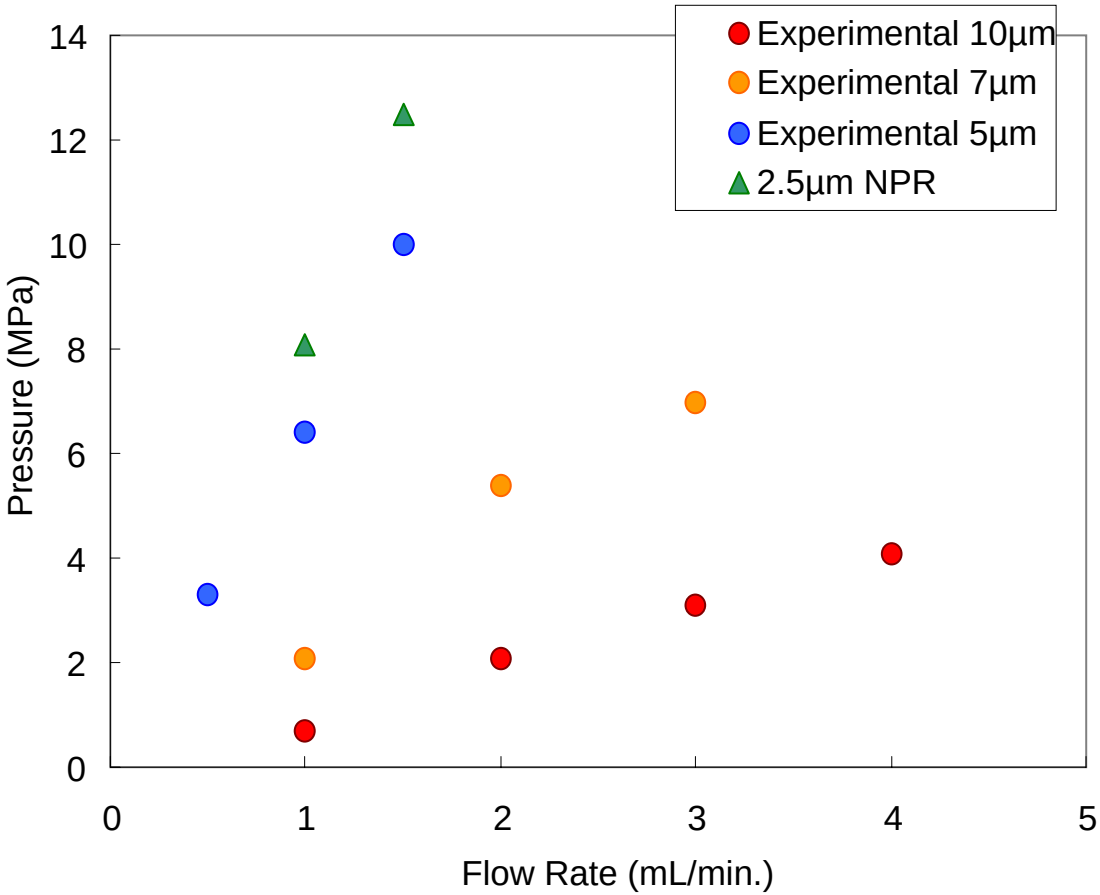
High Speed Analysis on Experimental Q column



Analysis within 1 minute is achieved at a flow rate of 2mL/min.

Exp. Q column, 10µm, 3.0 mm ID x 3.5cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5)
B) 0.5mol/L NaCl in A (pH8.5)
Gradient: B) 0% to 100% (1min.)
Flow-rate: 2.0mL/min
Sample: 1) conalbumin
2) ovalbumin
3) trypsin inhibitor
Detection: UV@280nm

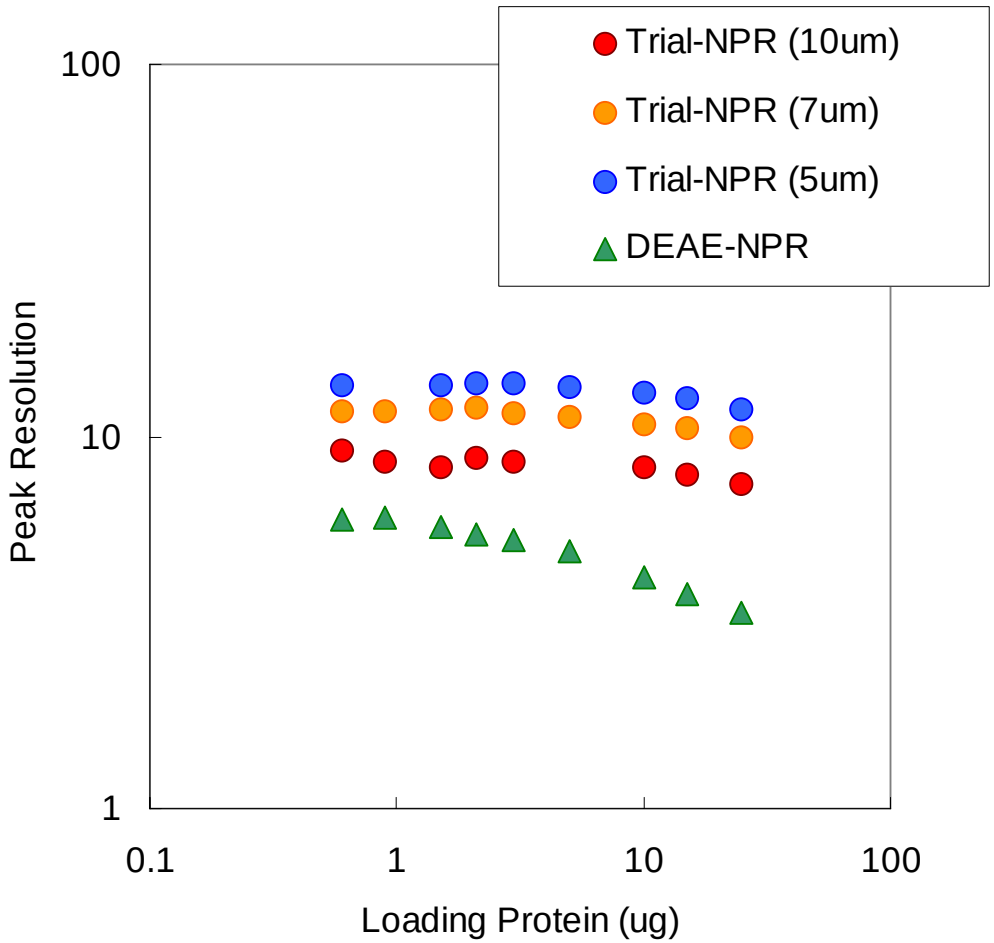
Pressure as a Function of Flow Rate



Column Size: 4.6mm ID x 3.5cm
Eluent: 20mmol/L Tris-HCl (pH8.5)

Comparison of pressure drops between each particle size of the experimental columns with a conventional NPR column of the same column dimension

Comparison of loading capacity

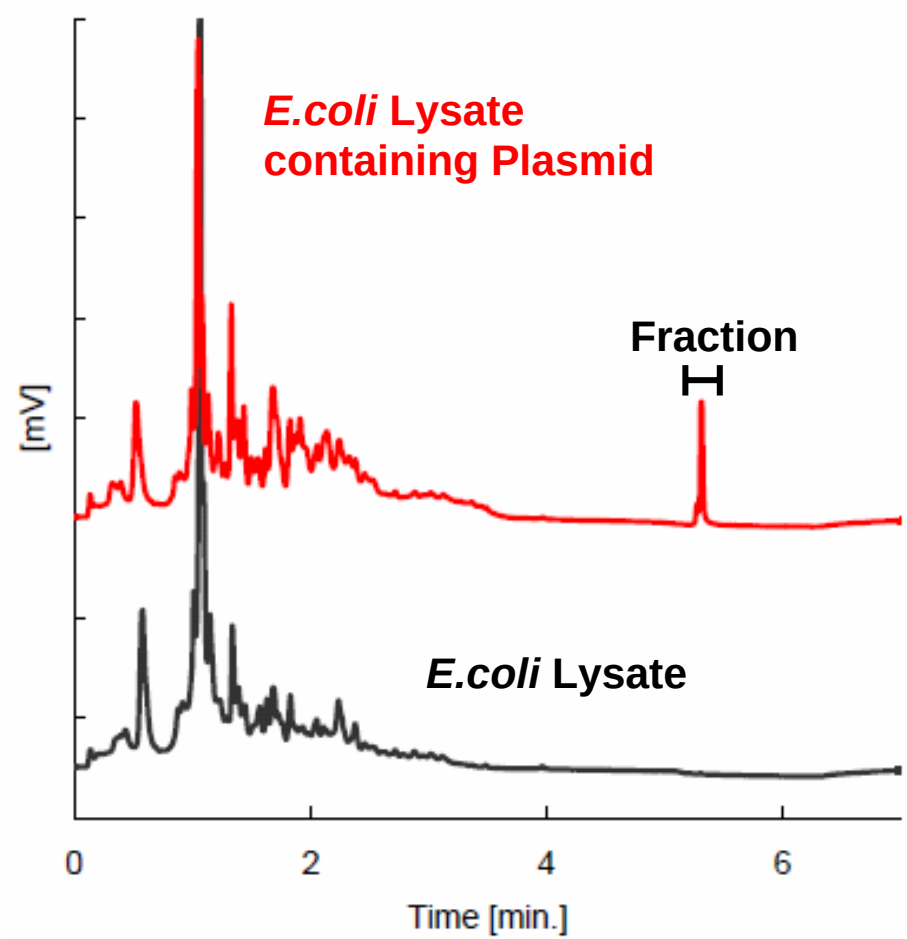


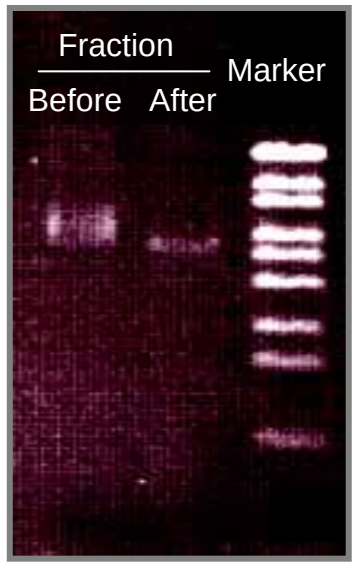
Column Size: 4.6mm ID x 3.5cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5)
B) 0.5mol/L Tris-HCl in A (pH8.5)
Flow rate: 1.0mL/min
Gradient: B) 0 to 100% (10min.)
Samples: 1) conalbumin, 2) ovalbumin
Detection: UV@ 280nm

Comparison of loading capacity between each particle size of the experimental (trial) NPR columns with a conventional NPR column of the same size

Applications of
10µm Non-porous Resin columns

Purification of Plasmid from *E.coli* Lysate using Experimental High Throughput Anion Exchange column



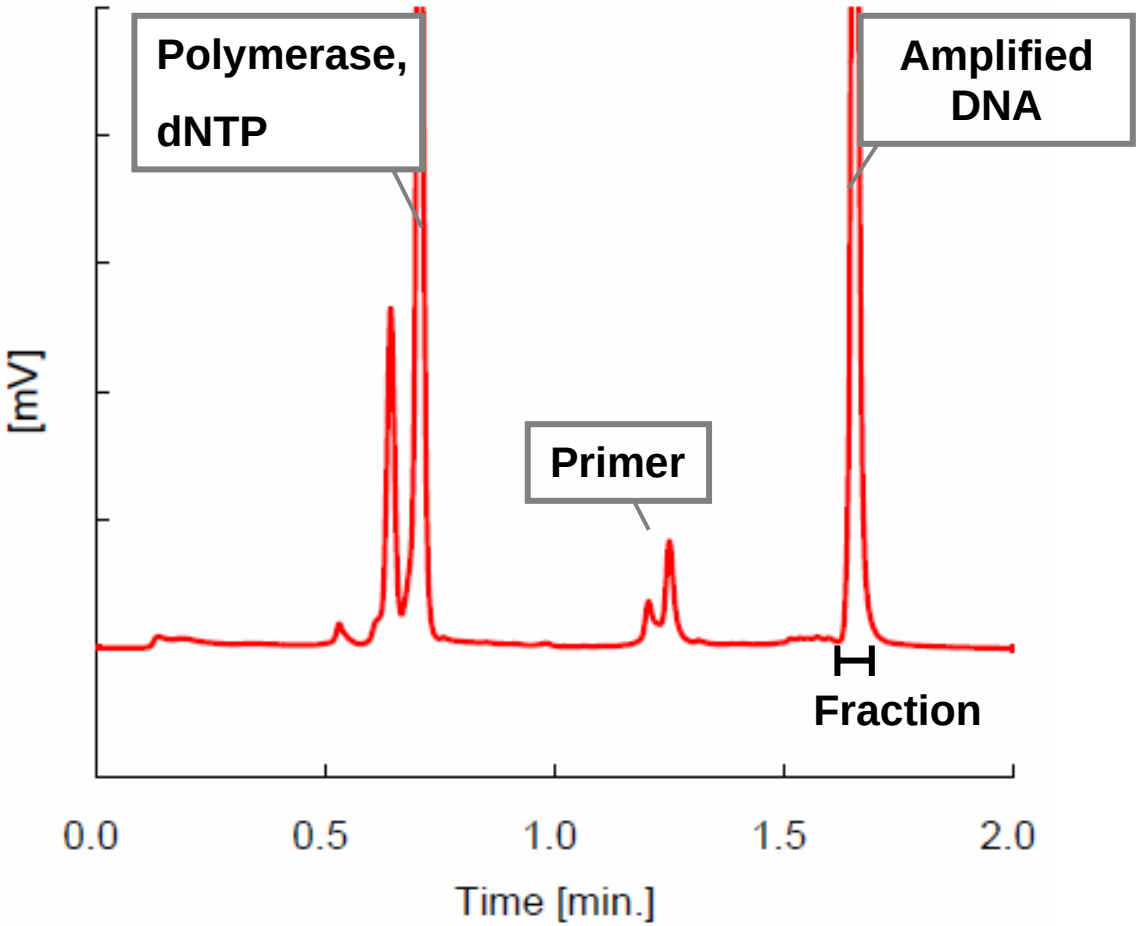


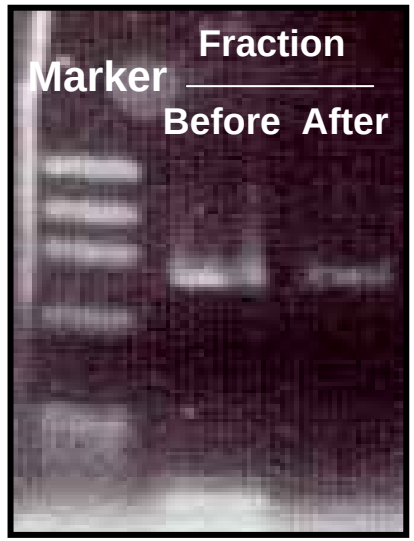
Agarose Gel Electrophoresis

Non-reduced SDS-PAGE

Exp. Q column,10µm, 4.6mm ID x 3.5cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5) B) 1.0mol/L NaCl in A (pH8.5)
Gradient: B) 80% to 100% (5min.) Flow-rate: 2.0 mL/min Detection: UV@280nm

Purification of PCR Products using experimental Q-type High Throughput Anion Exchange column

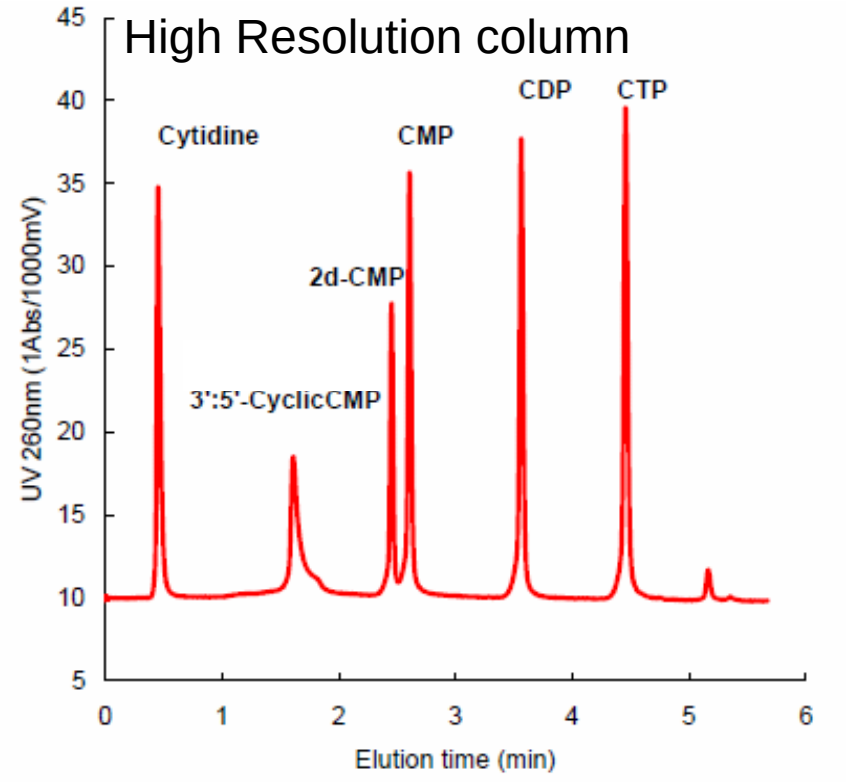




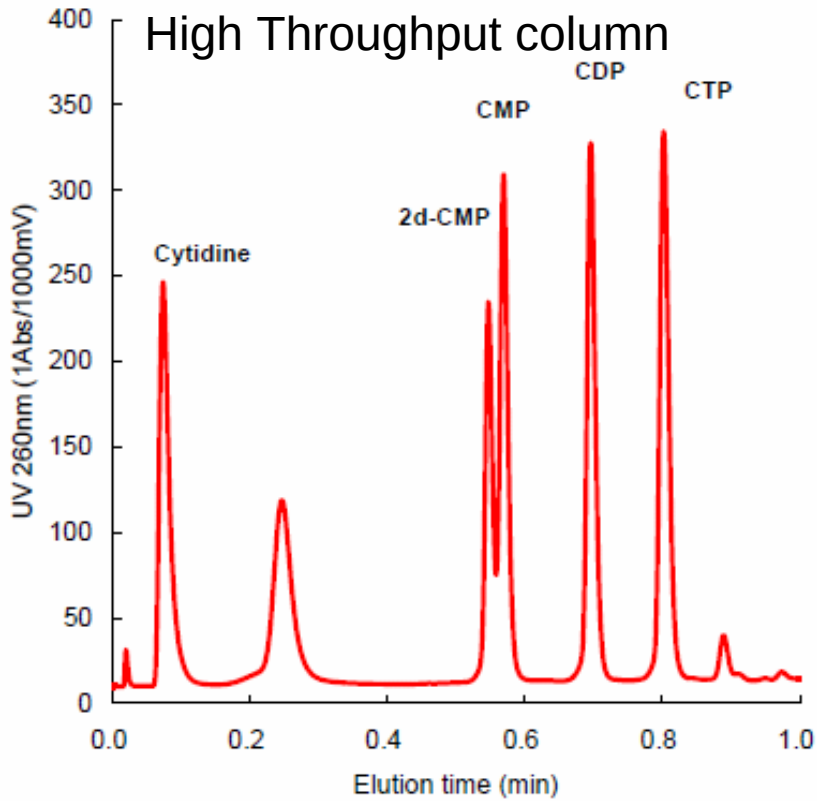
Agarose Gel Electrophoresis

Exp. Q column, 10µm, 4.6mm ID x 3.5cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5), B) 1.0mol/L NaCl in A (pH8.5)
Gradient: B) 0% to 100% (1.5min.) Flow-rate: 2.0 mL/min, Detection: UV@260nm

Analysis of Nucleotides using Experimental Q-type Anion Exchange columns

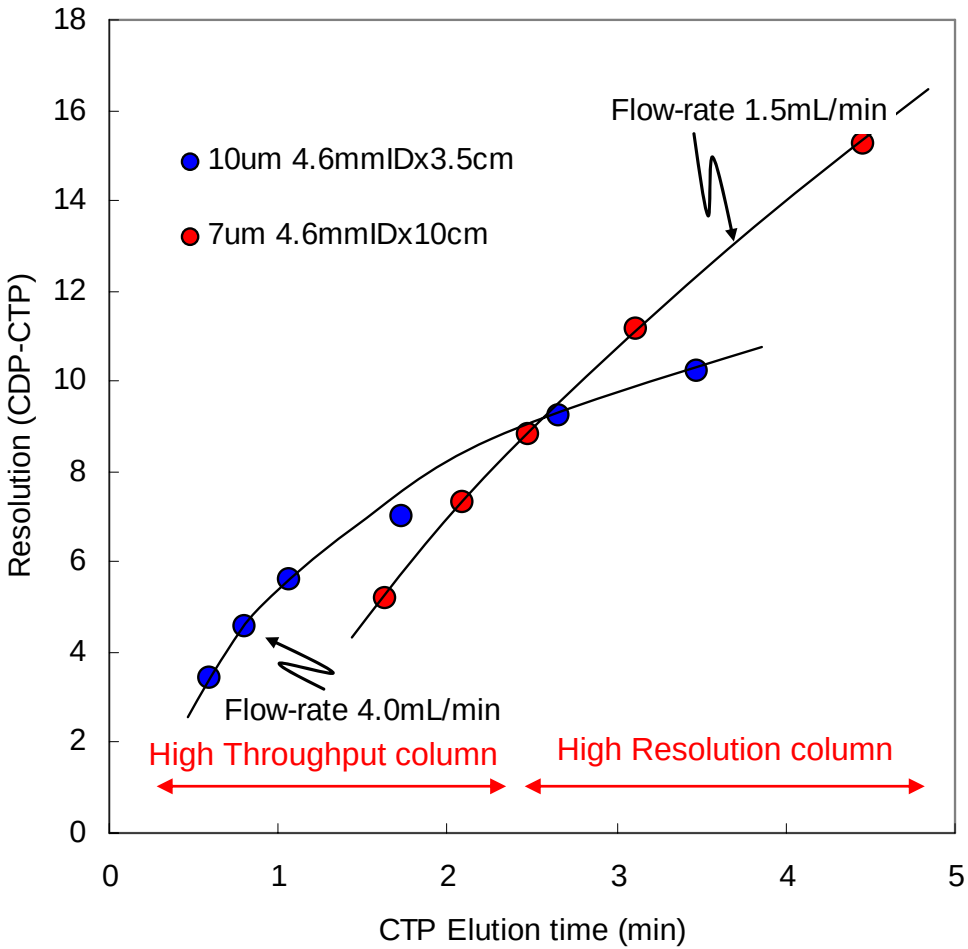


Exp. Q column, 7µm, 4.6mm ID x 10cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5)
B) 0.5mol/L NaCl in A (pH8.5)
Gradient: B) 0 to 100% (10min.)
Flow-rate: 1.5mL/min.
Detection: UV@260nm



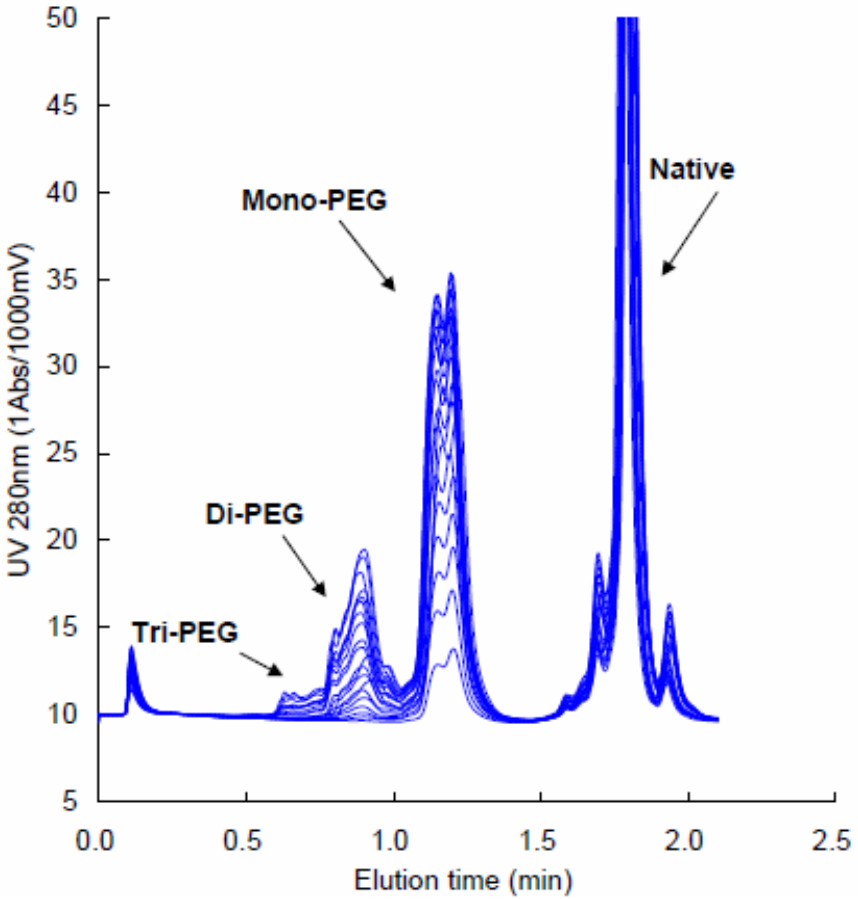
Exp. Q column, 10µm, 4.6mm ID x 3.5cm
Eluent : A) 20mmol/L Tris-HCl (pH8.5)
B) 0.5mol/L NaCl in A (pH8.5)
Gradient : B) 0 to 100% (1min.)
Flow-rate : 4.0mL/min.
Detection : UV@260nm

Comparison of Experimental Q-type columns: High Throughput vs. High Resolution



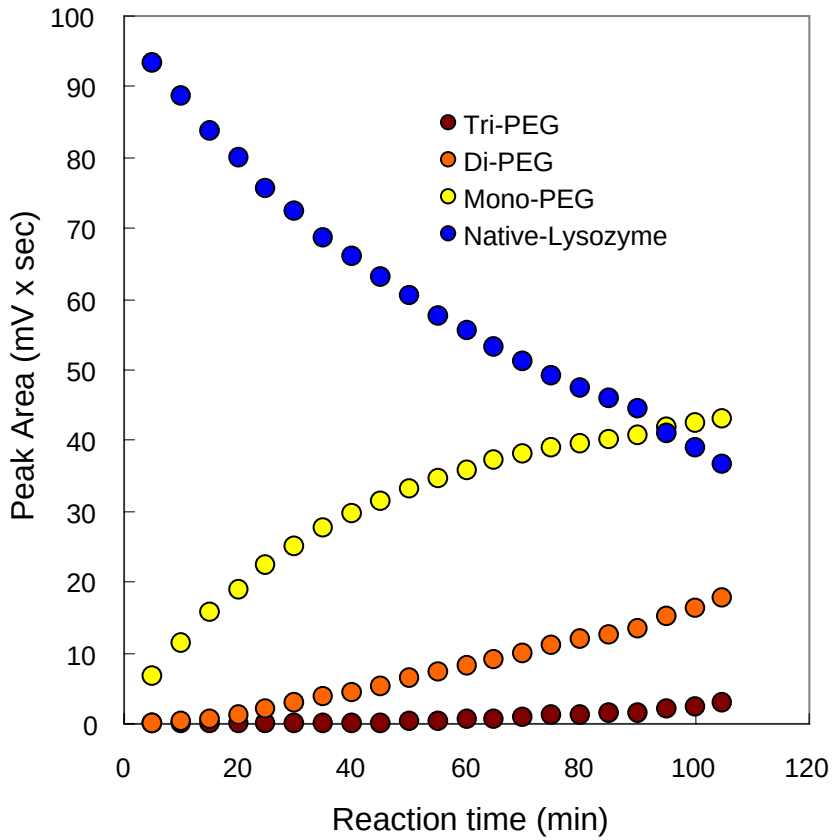
Exp. Q column, 10µm, 4.6mmID x 3.5cm
Exp. Q column, 7µm 4.6mm ID x 10cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5)
B) 0.5 mol/L NaCl in A (pH8.5)
Gradient: B) 0 to 100% (various time)
Flow-rate: 4.0mL/min for 4.6mmIDx3.5cm (10µm)
1.5mL/min for 4.6mmIDx10cm (7µm)
Sample: CDP, CTP
Detection: UV@260nm

Assay of Lysozyme PEGylation using an Experimental SP-type High Throughput Cation Exchange column



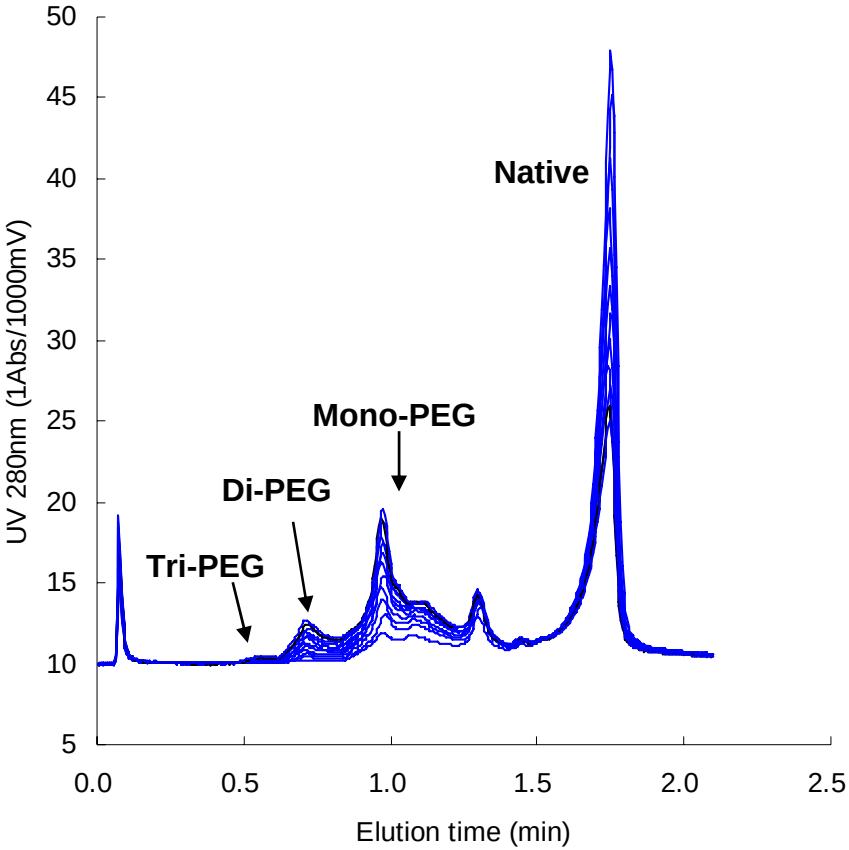
PEGylation

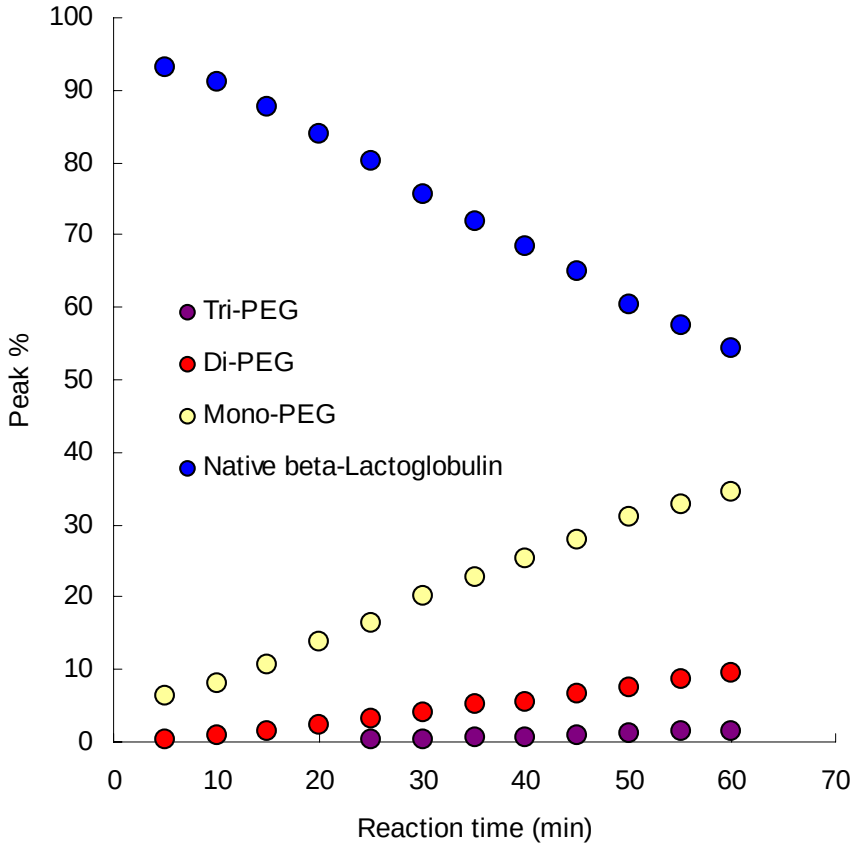
5mg/mL lysozyme in phosphate buffer pH 6.5
Lysozyme / PEG (Mw = 5,000) = 1 / 3



Exp. SP column, 10µm, 4.6mm ID x 3.5cm
Eluent: A: 20mmol/L Na acetate buffer pH5.0
B: 1.0mol/L NaCl in A pH5.0
Gradient: B: 0 to 100 % 2min
Flow-rate: 4.0mL/min
Real-time Analysis at 5-minutes intervals

Assay of beta-Lactoglobulin PEGylation using an Experimental High Throughput Cation Exchange column



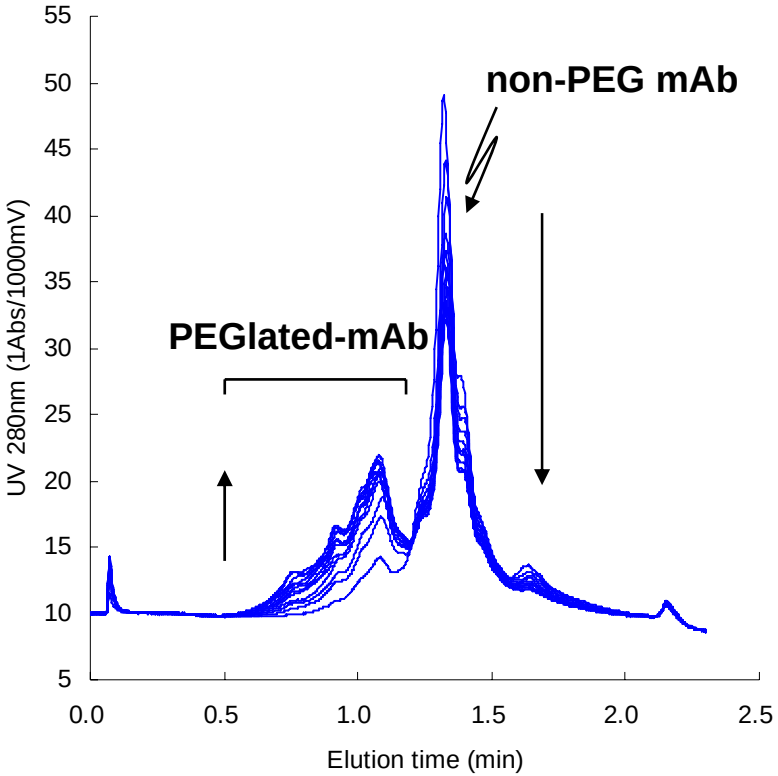


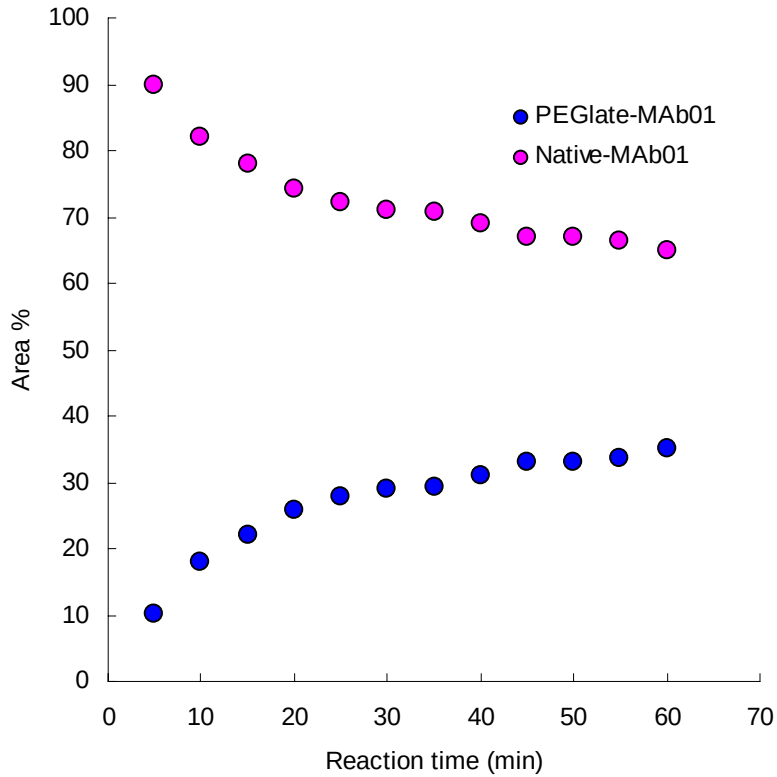
PEGylation

5mg/mL beta-lactoglobulin in phosphate buffer pH6.5
Lysozyme / PEG (Mw =5,000) = 1 / 5

Exp. SP column, 10µm, 4.6mm ID x 3.5cm
Eluent: A: 20mmol/L Na acetate buffer pH4.5
B: 0.8mol/L NaClO4 in A pH4.5
Gradient: B: 0 to 30 % 2min linear
Flow-rate: 4.0mL/min
Real-time Analysis at 5-minutes intervals

Assay of mAb PEGylation (1) using an Experimental High Throughput Cation Exchange column



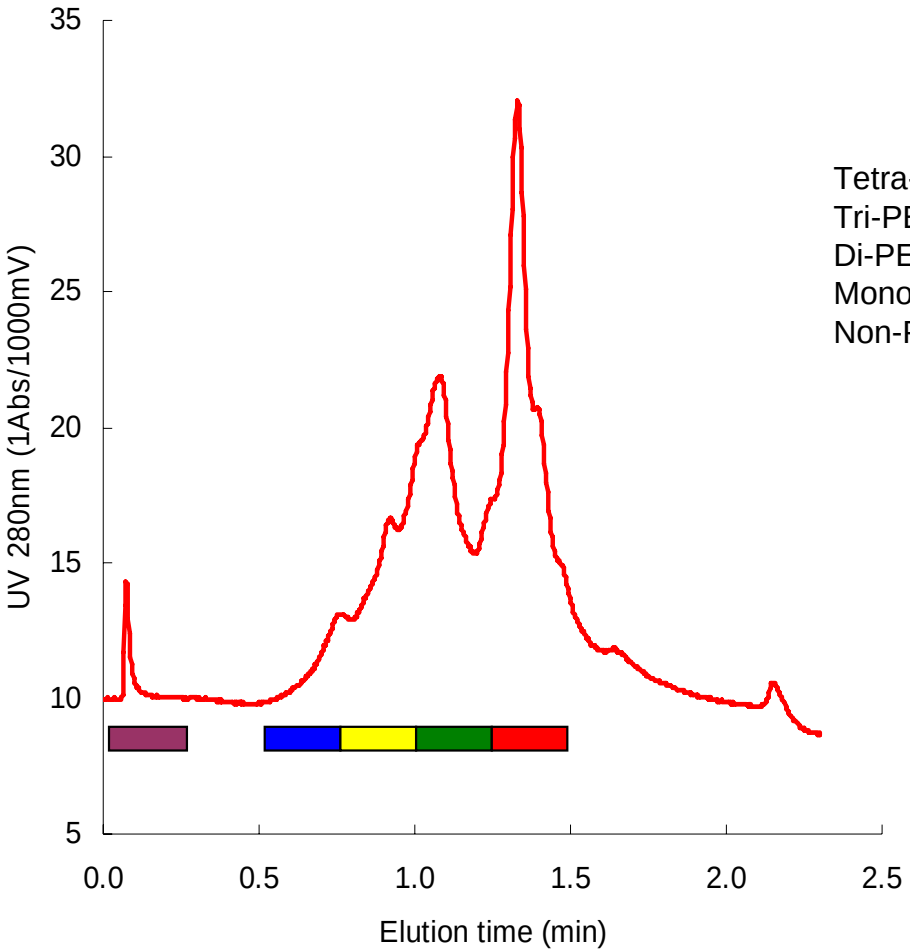


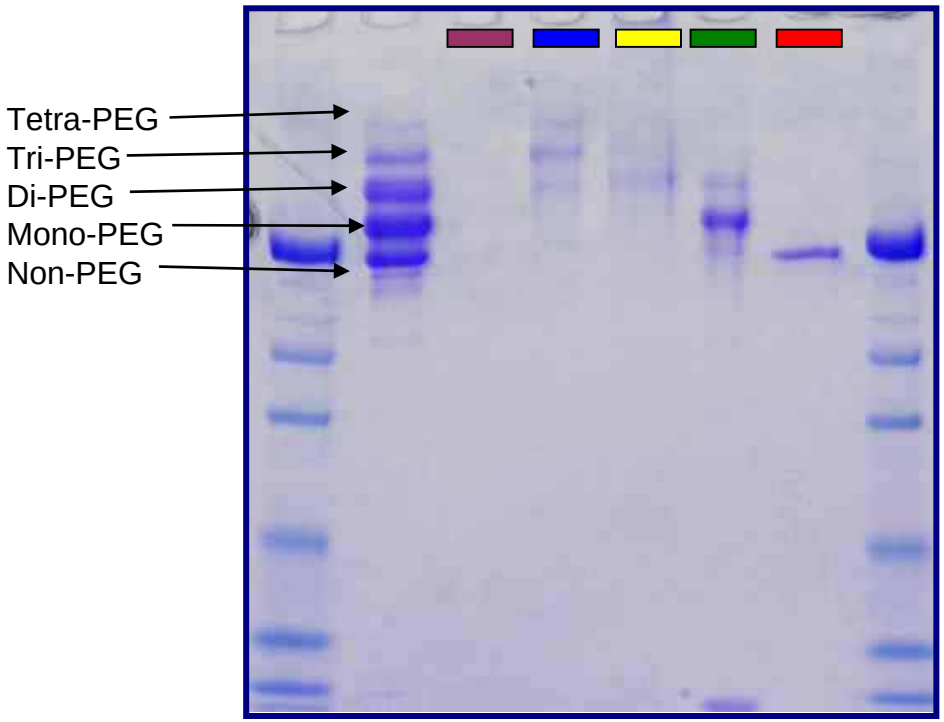
PEGylation

5mg/mL mAb in phosphate buffer pH6.5
Lysozyme / PEG (Mw =20,000) = 1 / 5

Exp. SP column, 10µm, 4.6mm ID x 3.5cm
Eluent: A: 20mmol/L Na acetate buffer pH5.0
B: 1.0mol/L NaClO4 in A pH5.0
Gradient: B: 10 to 40 % 1.8min linear
Flow-rate: 4.0mL/min
Real-time Analysis at 5-minutes intervals

Assay of mAb PEGylation (2) using an Experimental High Throughput Cation Exchange column

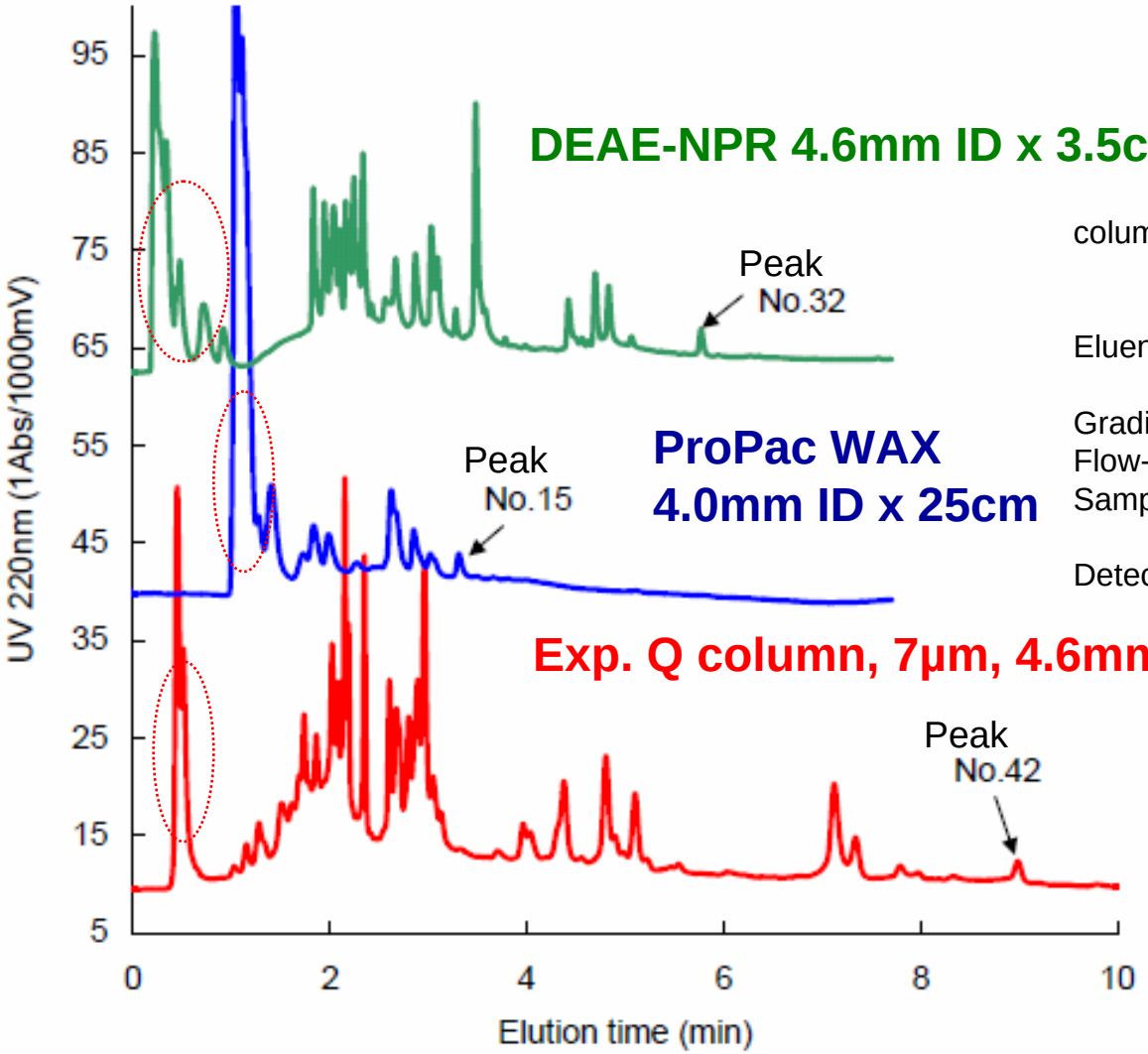




Non-reduced SDS-PAGE

Applications of
7µm Non-Porous Resin columns

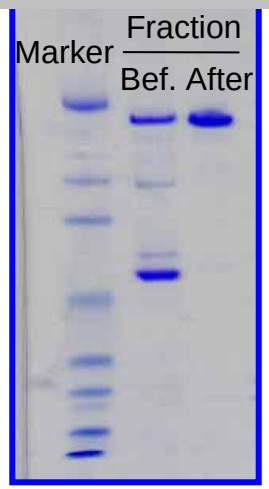
Comparison of Anion Exchange columns for the Analysis of BSA Digest



columns: DEAE-NPR, 2.5µm, 4.6mm ID x 3.5cm
ProPac WAX, 10µm, 4.0mm ID x 25cm
Exp. Q column, 7µm, 4.6mm ID x 10cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5)
B) 0.5mol/L NaCl (pH8.5)
Gradient: B) 0 to 100% (30min.)
Flow-rate: 1.5mL/min.
Sample: BSA digest (Waters MassPREP BSA Digestion Standard)
Detection: UV@280nm

Comparison of Anion Exchange columns for the Separation of Mouse Ascites containing IgG (1)

Non-reduced SDS-PAGE



Fraction

Exp. Q column
4.6mm ID x 10cm

Mouse Ascites

Purified IgG

[mV]

0 2 4 6 8 10

Time [min.]

ProPac WAX
4.0mm ID x 25cm

Mouse Ascites

Purified IgG

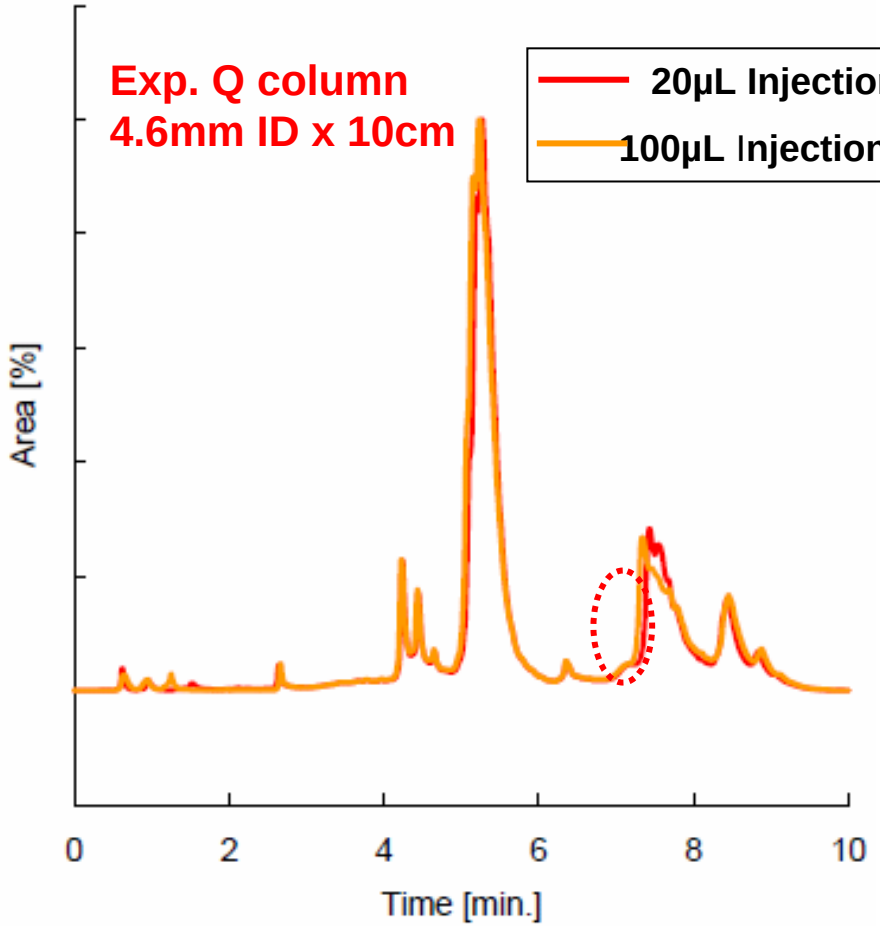
[mV]

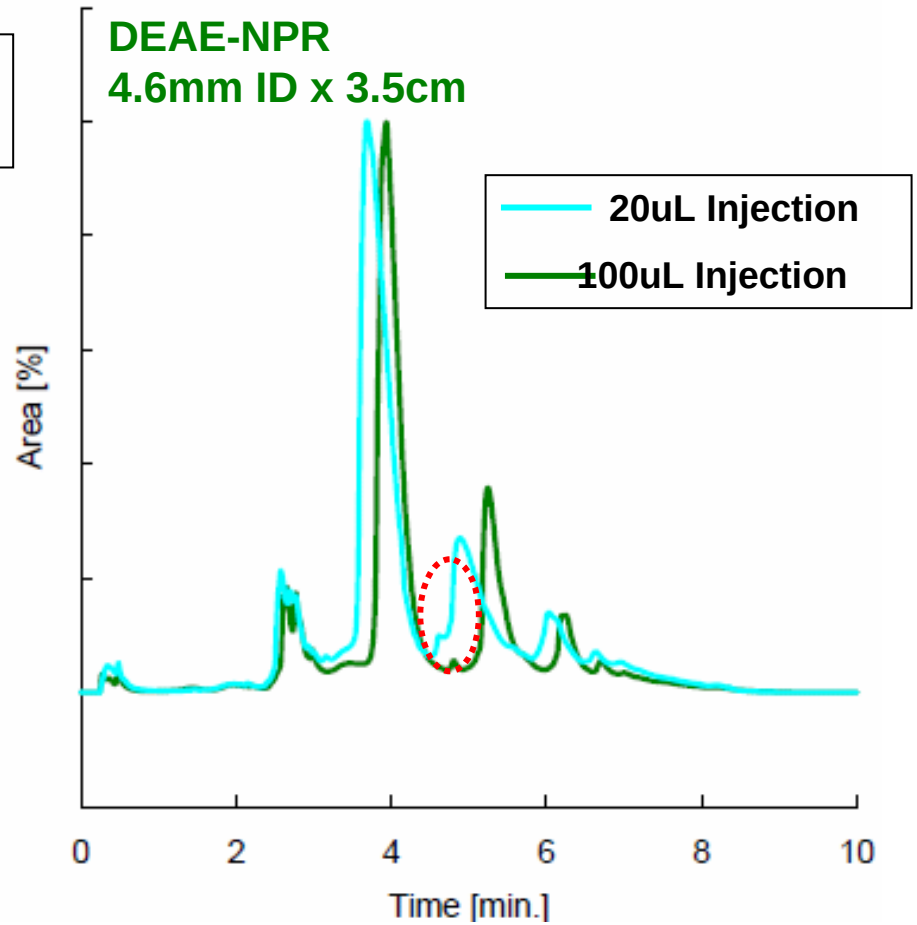
0 2 4 6 8 10

Time [min.]

columns: Exp. Q column, 7µm, 4.6mm ID x 10cm, ProPac WAX, 4.0mm ID x 25cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5) B) 0.5mol/L NaCl in A (pH8.5)
Gradient: B) 0 to 100% (10min) Flow rate: 1.0mL/min Detection: UV@ 280nm

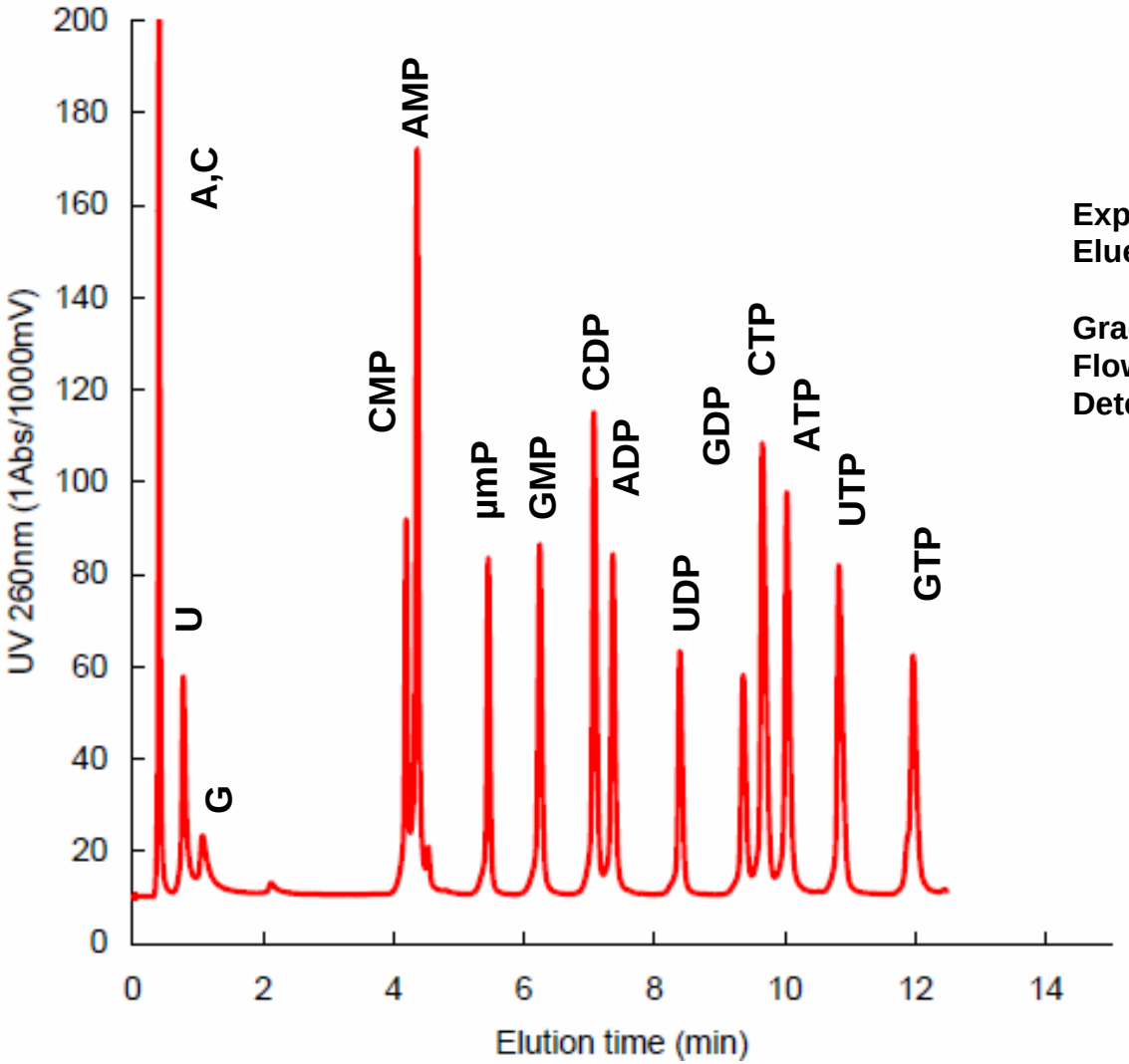
Comparison of Anion Exchange columns for the Separation of Mouse Ascites containing IgG (2)





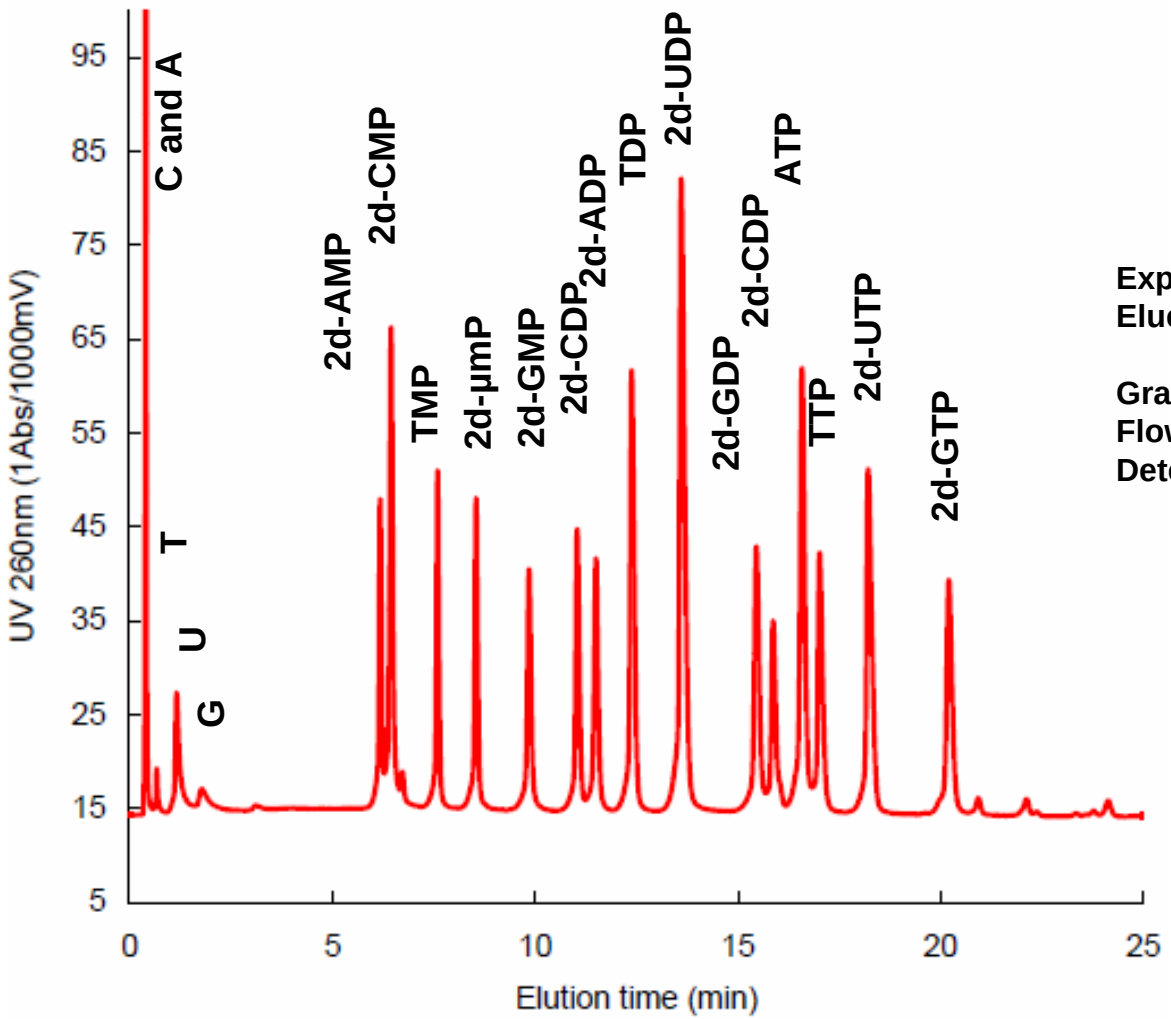
Exp. Q column, 7µm, 4.6mm ID x 10cm, DEAE-NPR, 4.6mm ID x 3.5cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5) B) 0.5mol/L NaCl in A (pH8.5)
Gradient: B) 0 to 100% (10min) Flow rate: 1.0mL/min Detection: UV@280nm

Simultaneous Analysis of Nucleotides using an Experimental High Resolution Anion Exchange column



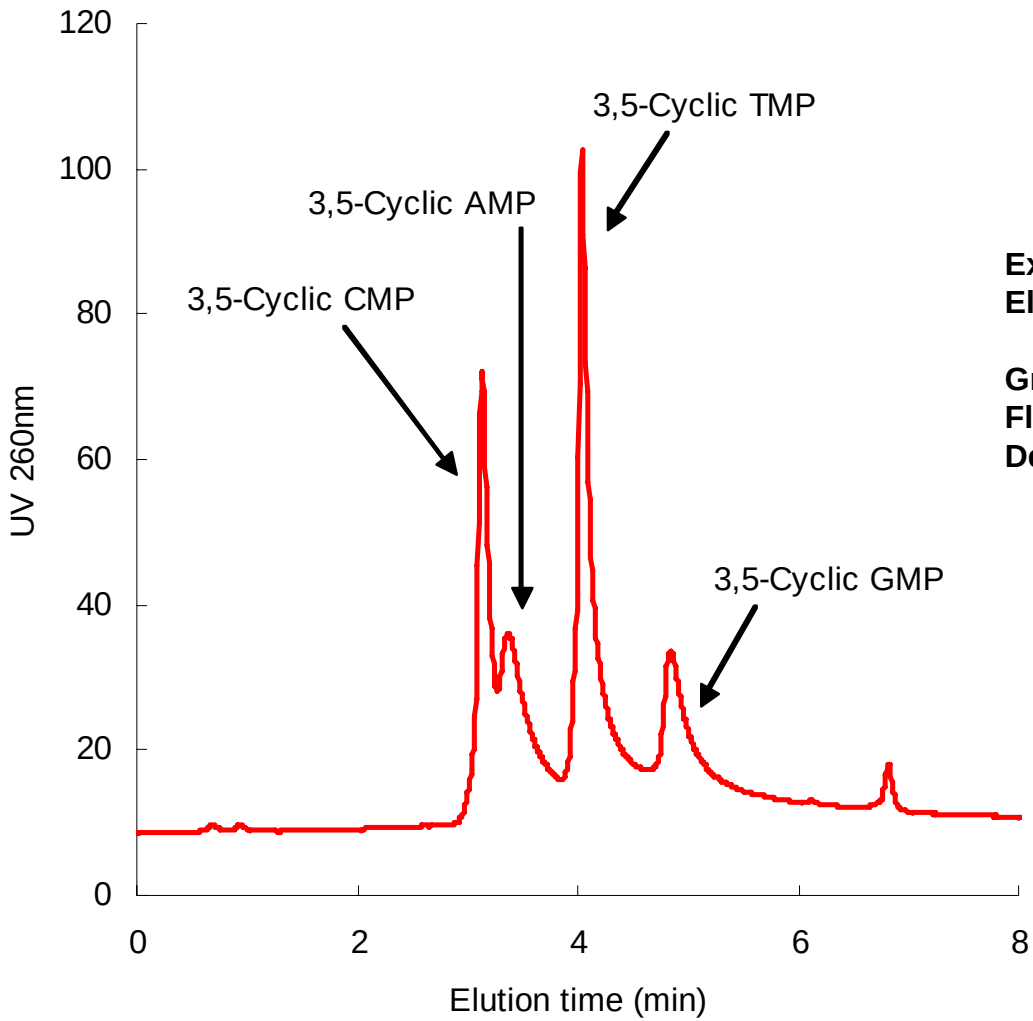
Exp. Q column, 7µm, 4.6mm ID x 10cm
Eluent: A) 20mmol/L Tris-HCl (pH8.7)
B) 0.5 mol/L NaCl in A (pH8.7)
Gradient: B) 0 to 50% 15min Linear
Flow-rate: 1.5mL/min
Detection: UV@260nm

Simultaneous analysis of Deoxynucleotides using an Experimental High Resolution Anion Exchange column



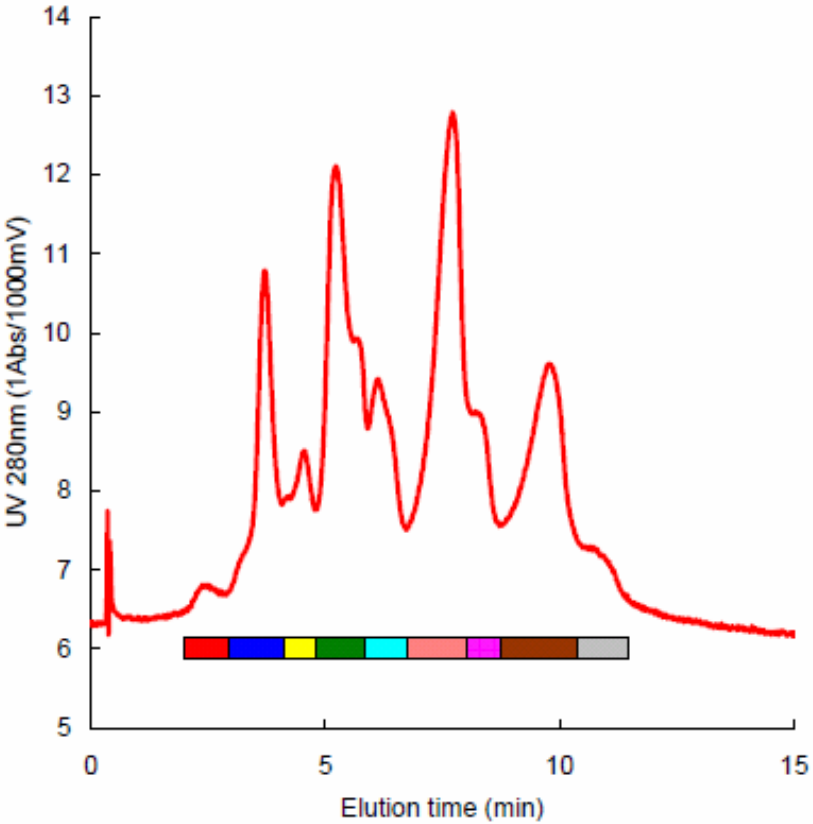
Exp. Q column, 7µm, 4.6mm ID x 10cm
Eluent: A) 20mmol/L Tris-HCl (pH8.6)
B) 0.5 mol/L NaCl in A (pH8.6)
Gradient: B) 0 to 50% 30min Linear
Flow-rate: 1.5mL/min
Detection: UV@260nm

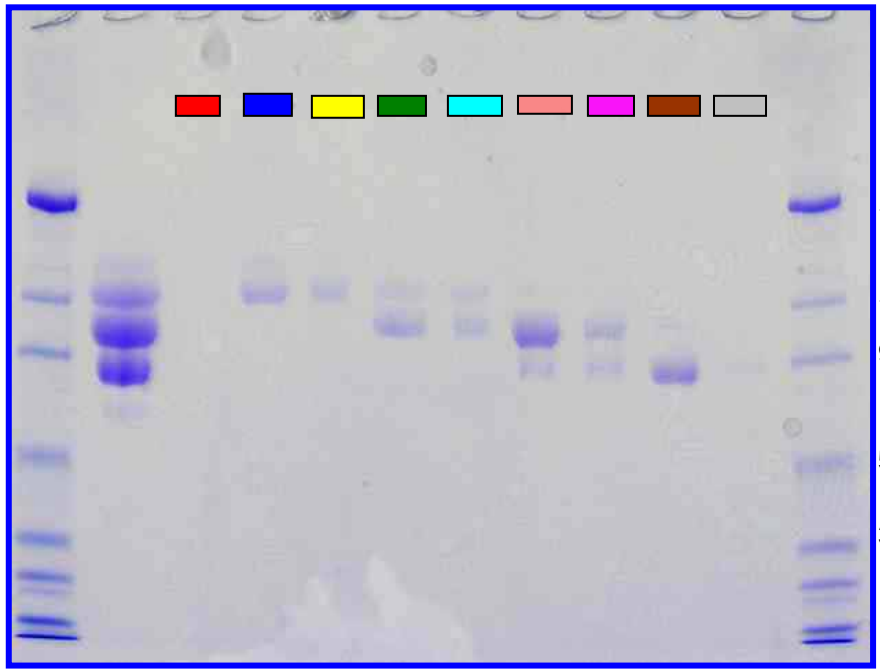
Simultaneous analysis of cyclic-NMP using an Experimental High Resolution Anion Exchange column



Exp. Q column, 7µm, 4.6mm ID x 10cm
Eluent: A) 5% AcCN in 5mmol/L Tris-HCl (pH8.6)
B) 0.15 mol/L NaCl in A (pH8.6)
Gradient: B) 0 to 100% 10min Linear
Flow-rate: 1.0mL/min
Detection: UV@260nm

Separation of PEGylated Protein using an Experimental High Resolution Cation Exchange column

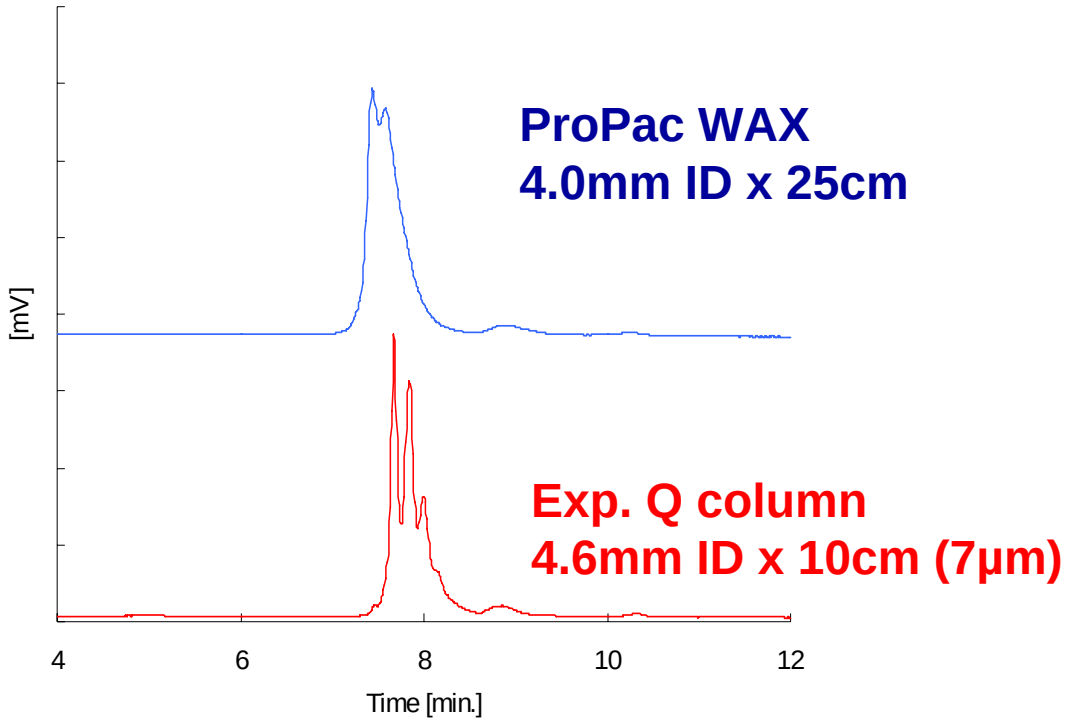




Non-reduced SDS-PAGE

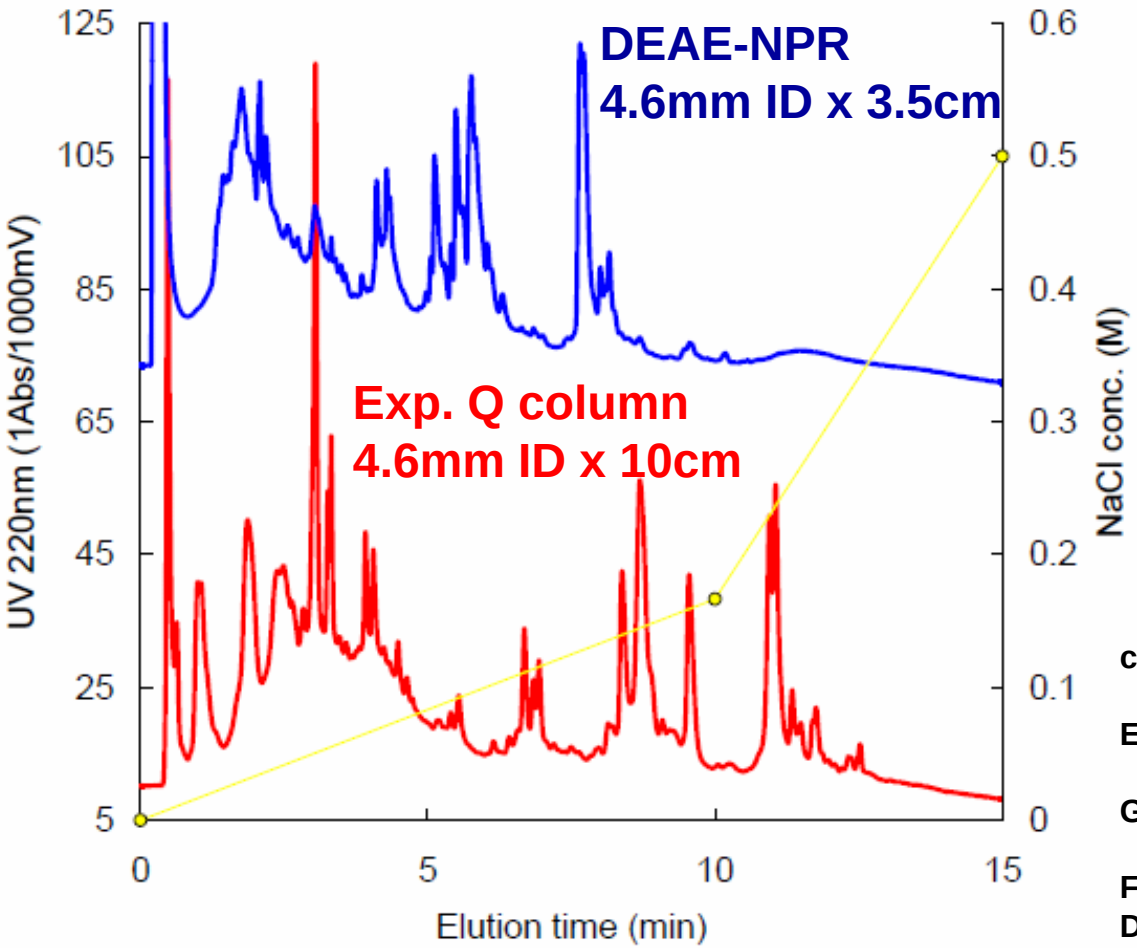
Exp. SP column: 7µm, 4.6mm ID x 10cm
Eluent: A) 20mmol/L Na acetate (pH4.2)
B) 0.5mol/L NaCl in A (pH4.2)
Gradient: B) 0 to 40% (20min.)
Sample: Somavert (Pfizer)
Flow rate: 1.5mL/min. Detection: UV@280nm

Comparison of Anion Exchange columns for the Analysis of a mAb Sample



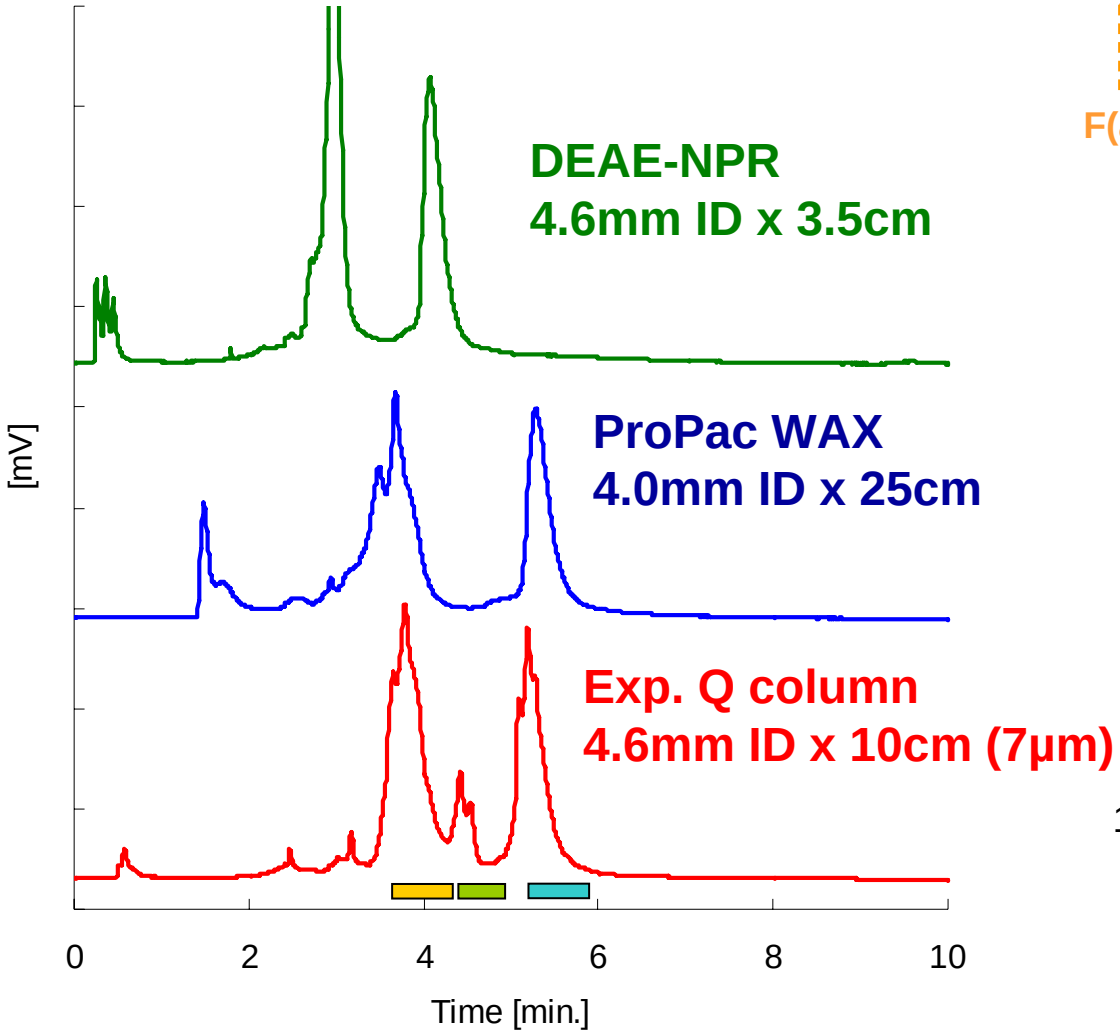
Eluent: A) 20mmol/L Tris-HCl (pH8.5), B) 0.5mol/L NaCl in A (pH8.5)
Gradient: B) 0 to 100% (20min.)
Flow-rate: 0.77mL/min for ProPac WAX, 1.00mL/min for Exp. Q column
Sample: MAb sample Detection: UV@280nm

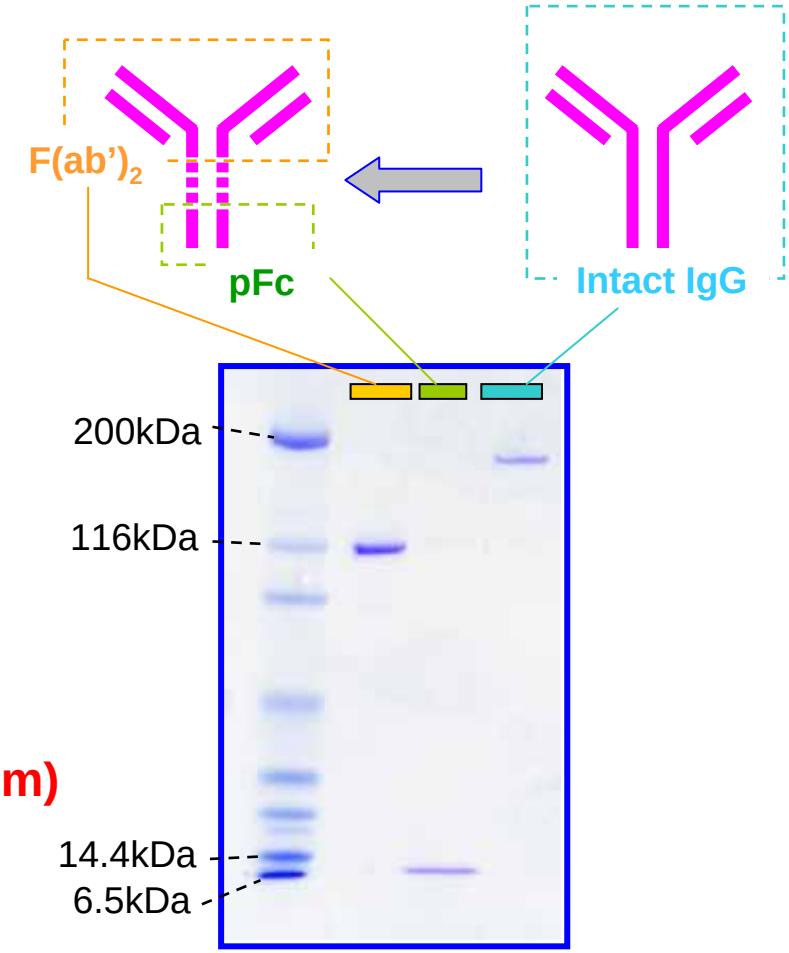
Comparison of Anion Exchange columns for the Analysis of beta-Lactoglobulin Digest



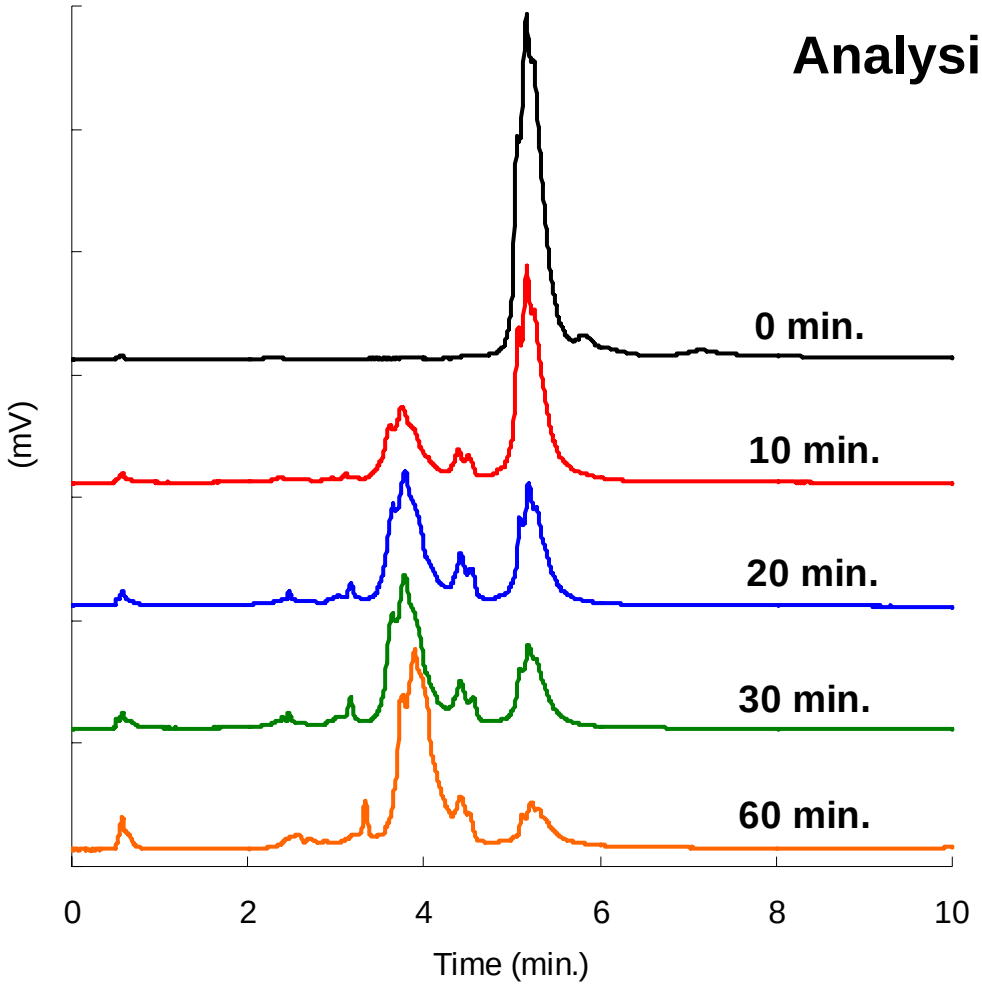
columns: DEAE-NPR, 2.5µm, 4.6mm ID x 3.5cm
Exp. Q column, 7µm, 4.6mm ID x 10cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5)
B) 0.5mol/L NaCl in A (pH8.5)
Gradient: B)0 to 33% (0-10min.)
33 to 100% (10-15min.)
Flow rate: 1.5mL/min.
Detection: UV@280nm

Comparison of Anion Exchange columns for the Analysis of mAb Digested with Pepsin (1)





Comparison of Anion Exchange columns with the Analysis of mAb Digested with Pepsin (2)

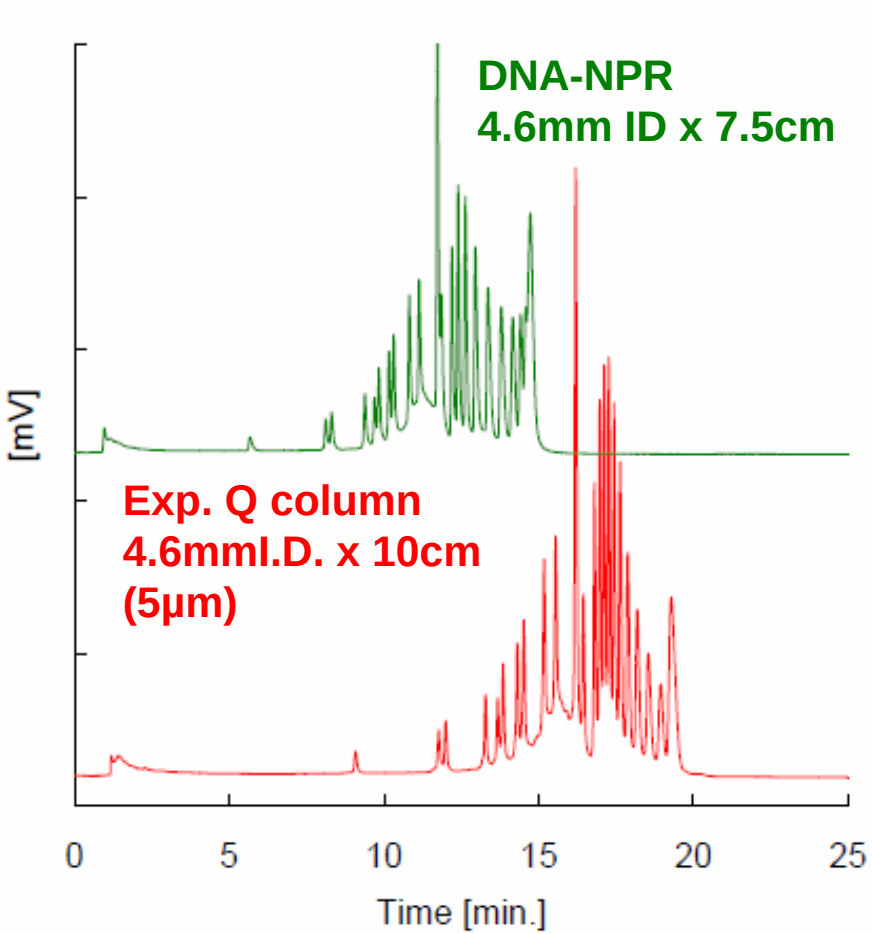


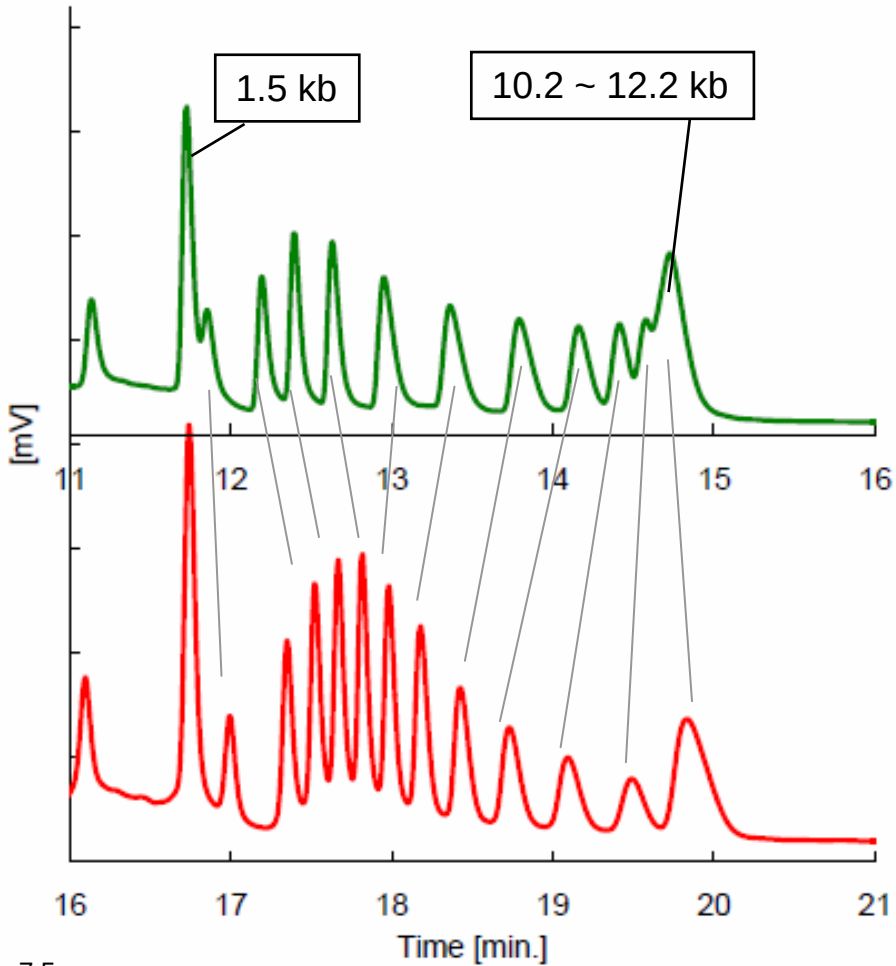
Analysis of Reaction Conversion

Exp. Q column, 7µm, 4.6mm ID x 10cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5)
B) 0.5mol/L NaCl in A (pH8.5)
Gradient: B) 0 to 100% (10min)
Flow rate: 1.0mL/min
Detection: UV@280nm

Applications of 5µm Non-Porous Resin columns for Nucleic Acid Separations

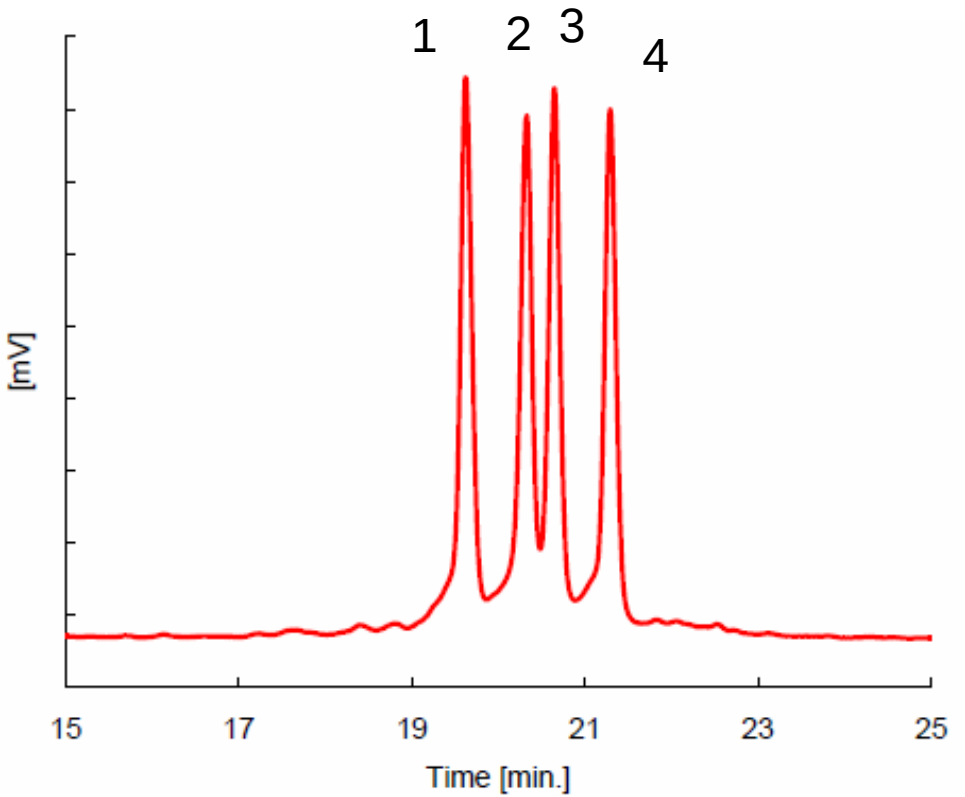
Separation of 1kb DNA Ladder using an Experimental Q column





columns: Exp. Q column, 5µm, 4.6mm ID x 10cm, DNA-NPR, 4.6mm ID x 7.5cm
Eluent: A) 20mmol/L Tris-HCl (pH8.5).B) 1.0mol/L NaCl (pH8.5) Flow rate: 0.5mL/min
Gradient: B) 50 to 75% (20min.) for DNA-NPR, B) 75 to 100% (20min.) for Q-NPR column
Sample: 1kb Ladder Detection: UV@260nm

Separation of Same Size DNA Oligomers (26mer) using an Experimental Q column



Exp. Q column, 5µm, 4.6mm ID 10cm
Eluent: **A) 20mmol/L Tris-HCl (pH8.5)**
B) 0.75mol/L NaCl in A (pH8.5)
Gradient: **B) 50 to 75% (25min)**
Flow-rate: **0.8mL/min**
Detection: **UV@260nm**

Sample: 26mer Oligomers

1. 5'-TAATTAAGGACTCCGTTCTTCTATAT-3'-NH_2
2. 5'-TCTTTACTTTAGTCACAAAGCGATAA-3'-NH_2
3. 5'-GACTCCGTTCTTCTATATTTTCGAGG-3'-NH_2
4. 5'-GGACGTGCTGGGTGTCTTCTCCGTCG-3'-NH_2

Summary

Experimental Non-Porous Resin Ion Exchange columns

- Packing Materials
  - Improved core particle
    - – High resolution of biomolecules over a wide range of MW
  - Improved surface chemistry
    - – Higher loading capacity and stronger retention
- Various Applications
  - High throughput analysis
    - – Maintains high resolution at fast flow rates
    - – Low backpressure
      - Analysis times of less than 1 minute are feasible
  - High resolution analysis
    - – Much higher resolution than conventional NPR columns
  - Nucleic acid analysis
    - – Excellent resolution for large DNA fragments