From Proteins to Polymers- GPC/SEC

Understanding column selection and method considerations for your sample

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Agenda

- GPC/SEC
  – A quick review of terms and an overview of the separation mechanism

- Sample type
  – What type of sample do we have and how they differentiate
  – And **why** do we do GPC /SEC

- Solvent and mobile phase
  – Considerations for solvent selection and why solvent choice IS important

- Column selection
  – Important points to consider for making your column selection
  – How to maximize your column performance thru selection

- Detector and instrument considerations
  – Concentration detectors and advanced detection
  – Easy ‘fixes’ for ensuring optimal chromatography

Terminology

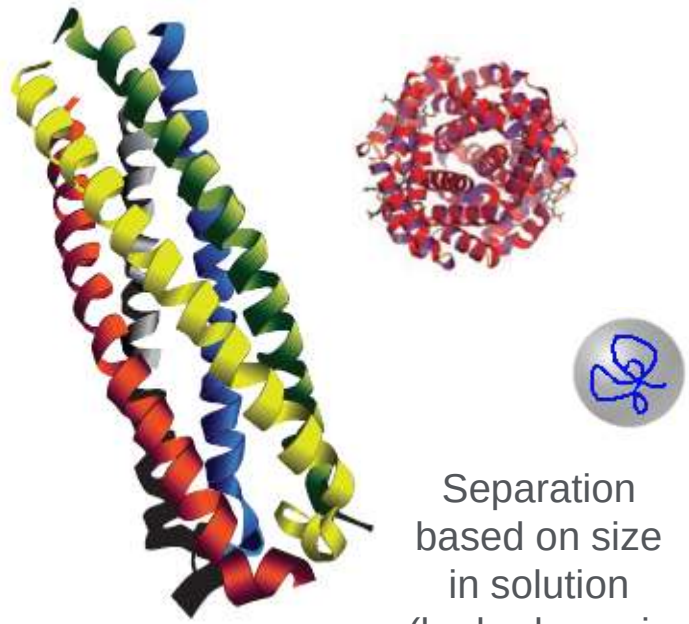
Same technique, but different acronyms:

- **GPC** – Gel permeation chromatography
  - Organic solvents like THF and methylene chloride
- **SEC** – Size exclusion chromatography
  - Primarily water and buffer mobile phases
- **GFC** – Gel filtration chromatography
  - Water and buffer mobile phases; common term for industrial purification step in the life sciences industry

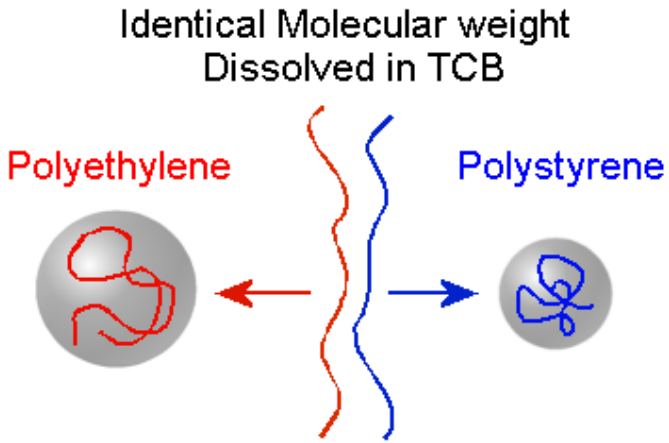
GPC/SEC refers to the chromatographic technique that separates samples by their size.

Hydrodynamic Volume

- The size of a polymer/protein coil in solution
- Measure of molecular size in solution

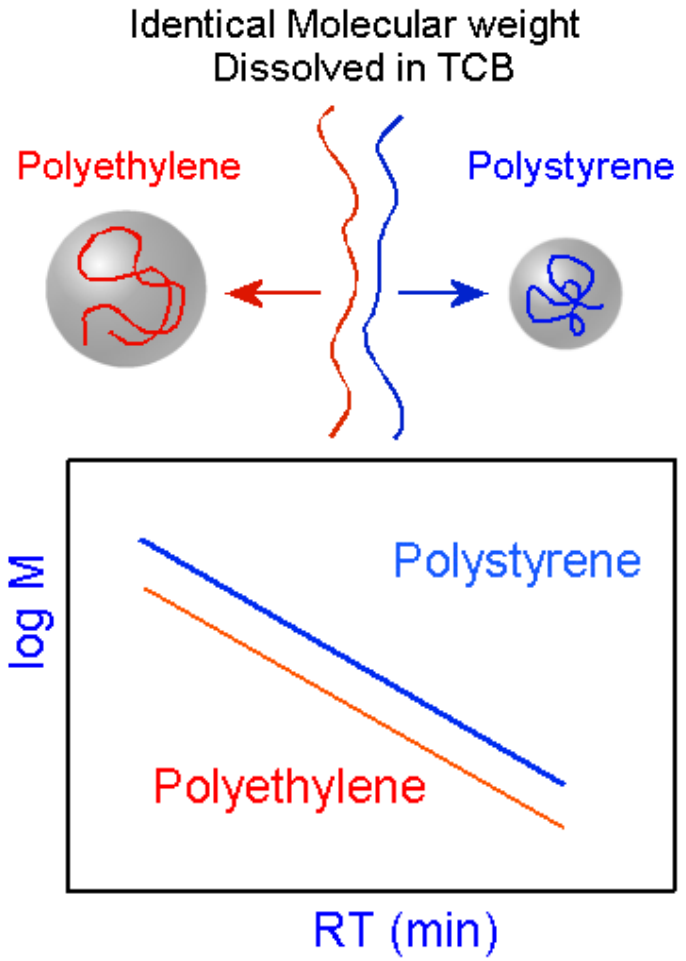


Separation based on size in solution (hydrodynamic volume)



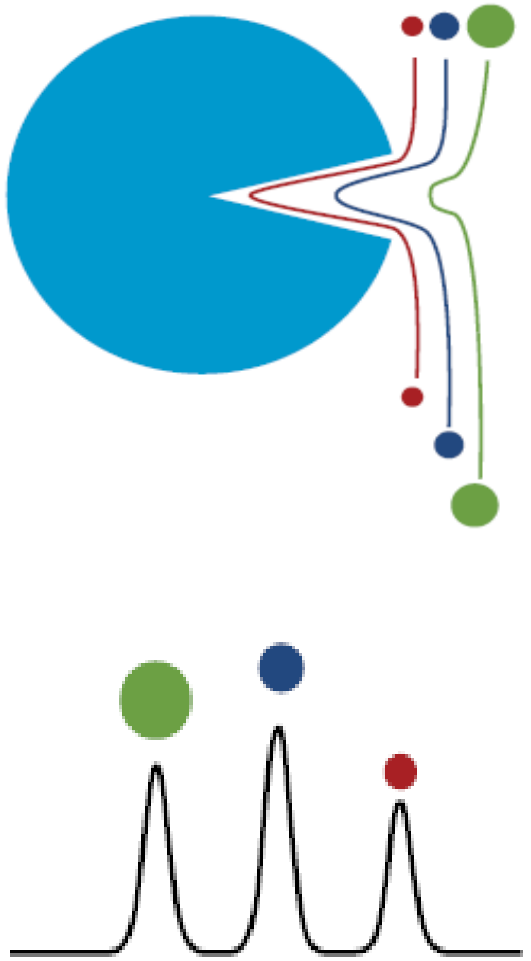
Hydrodynamic Volume: Expect differences

- Two different polymers will behave differently with solvent
- Column separates on basis of molecular size NOT molecular weight
- At any molecular weight, the two polymers will have different sizes in solution



GPC/SEC Separation Mechanism

- A GPC/SEC column is packed with porous beads of controlled porosity and particle size
- Sample is prepared as a dilute solution in the eluent and injected into the system
- Large molecules are not able to permeate all of the pores and have a shorter residence time in the column
- Small molecules permeate deep into the porous matrix and have a long residence time in the column
- Sample molecules are separated according to molecular size, eluting largest first, smallest last



Sample Type – Polymer or BioMolecule

Polymer

Questions that you need to ask

What type of polymer do I have?

Organic soluble → GPC

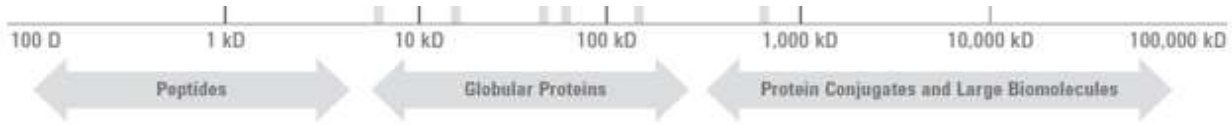
Aqueous soluble → SEC

BioMolecule

Questions that you need to ask

What type of sample do I have?

Peptides
Proteins/globular proteins
mAbs
Protein conjugates
Large BioMolecules



Sample Type

Polymers are showing up in more and more pharmaceuticals

Polymers are found as part of:

- Gel coatings

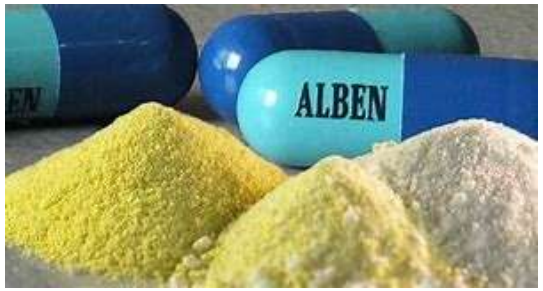
  (For example, ethyl cellulose)

- Drug encapsulation

  (For example, PEG)

- Excipients (stabilization, fillers, and adsorption facilitation)

  (For example, polyvinyl pyrolidone)



Sample Type

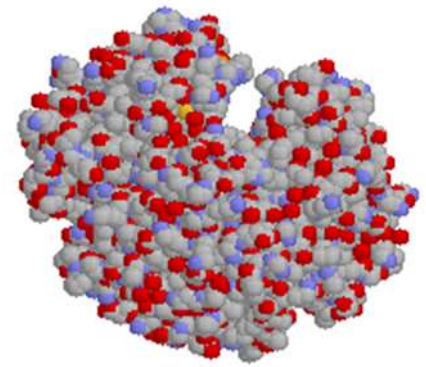
Why GPC/SEC is done?

- **Plastics**
  - Mol wt dictates polymer strength, flexibility, and physical properties

- **Sample cleanup**
  - Separates target molecules from large molecules that fragment in MS and cause interference

- **Water soluble polymers**
  - Mol wt impacts viscosity, surfactant effects, dissolution, and chemical characteristics

- **BioMolecules**
  - Mol tw is often known
  - Can be run on intact molecules
  - Aggregation can be dangerous



Solvents

- Selecting a solvent system is one of the first steps in developing a GPC method.
- Some polymers or biomolecule samples are easy to dissolve, some are much harder.
- The solvent conditions must be appropriate for the sample to prevent any unwanted interactions between that of the sample with the packing particle. Interactions will give a false mol wt result.
- Agilent's range GPC/SEC columns are available with phase chemistries that are optimized for all types of solvents that may be required: aqueous and organic, polar and nonpolar solvents.

Criteria for Solvent Selection

- Solvent must be able to fully solubilize the sample
- True sample solubility to avoid non-size exclusion effects
- Compatibility with column(s) and packing
- Permit adequate detection (for example, refractive index, UV cutoff)
- Safety (for example, toxicity, elevated temperature, and so on)

Solvent Considerations

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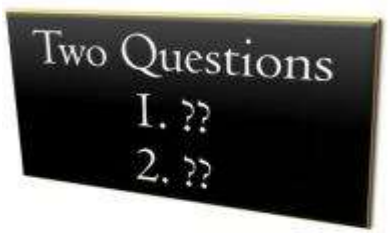
Question: What solvent is your sample soluble in?

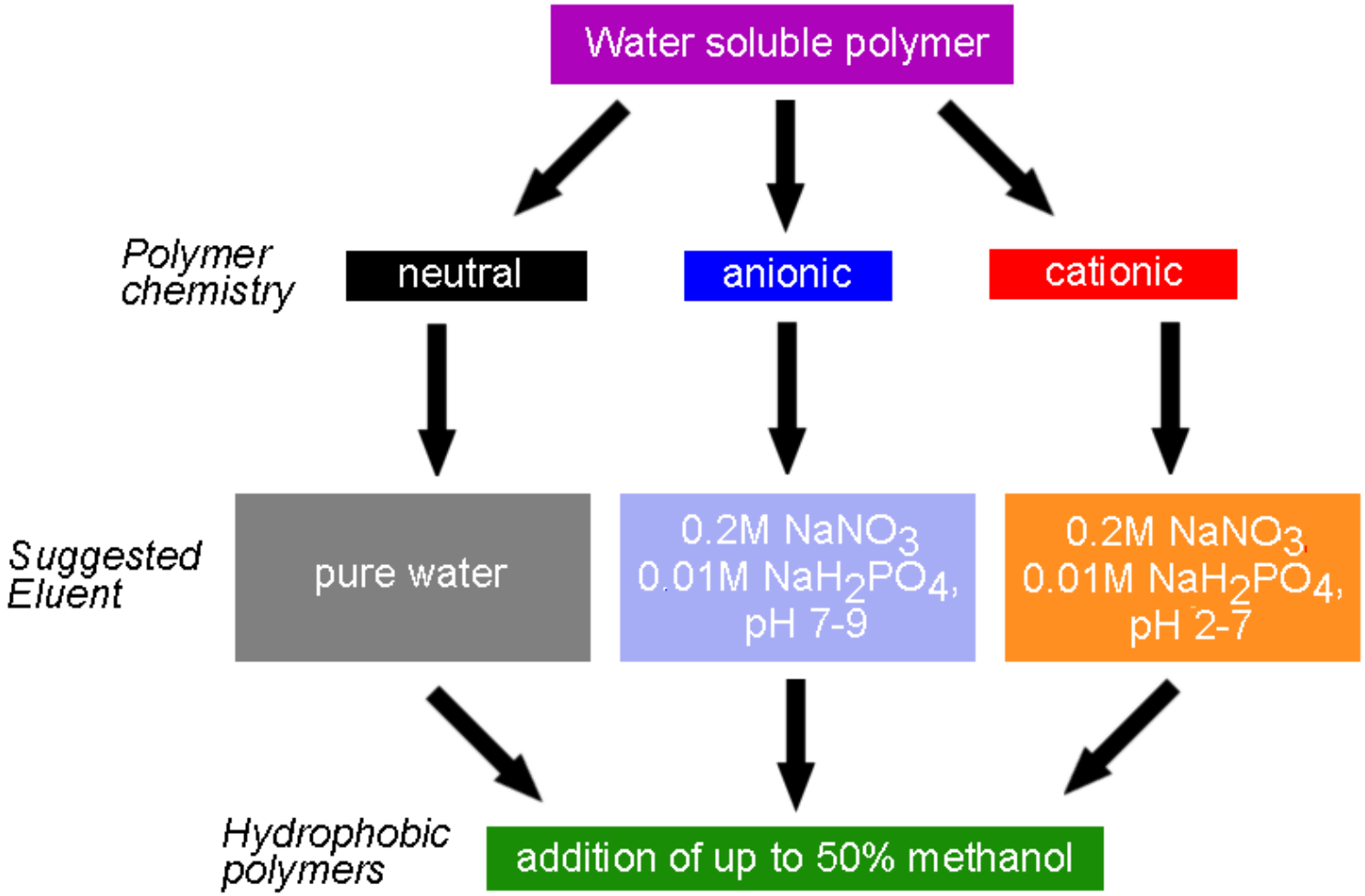
Type	Typical Solvents
Organic	• THF • Chloroform • Toluene • TCB
Mixed or polar organic	• THF/water • DMF • NMP
Aqueous	• Water • Buffer in water • Water/methanol (up to 50%) • Water/buffer, ACN



Additives can be employed:

- Minimize nonsize exclusion interactions between the sample and the column
- Stabilize the solution of the polymer (ionic aggregation)

Solvent Considerations and Optimizing for Aqueous SEC



Guide to eluent selection for PL aquagel-OH applications

Solvent Considerations: Importance in Method Development

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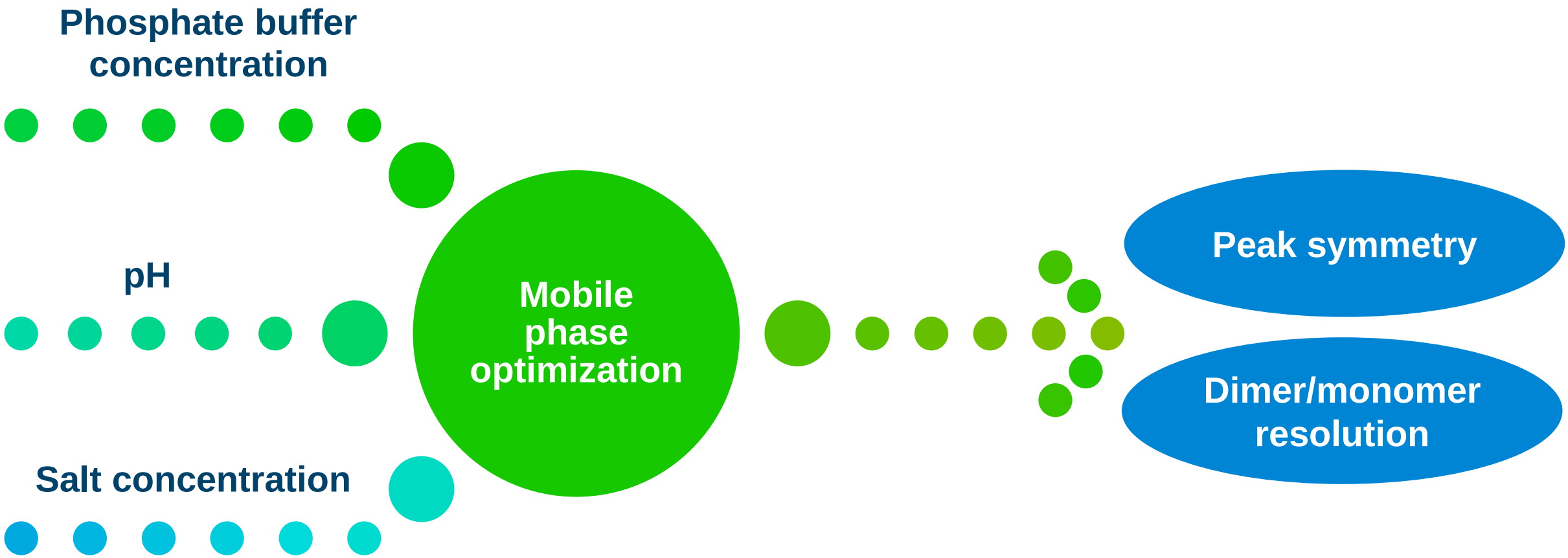
Biomolecules



Recommended Starting Conditions

For AdvanceBio SEC columns, we recommend starting with 150 mM sodium phosphate, pH 7.0

Peptides, polypeptides, proteins, mAbs
MW >0.1-1,250 kDa

AdvanceBio SEC (2.7 µm)	
Pore Size	**MW Range (kDa)**
130Å	0.1-100
300Å	5-1,250

Recommended Initial Separation Conditions

Column: AdvanceBio SEC or Agilent Bio SEC-5

Mobile phase: 150 mM phosphate buffer, pH 7.0*

Gradient: Isocratic in 10-30 min range

Temperature: Recommended: 10-30 °C, Maximum: 80 °C

Flow rate: 0.1-0.4 mL/min for 4.6 mm id columns
0.1-1.25 mL/min for 7.8 mm id columns

Sample size: ≤ 5% of total column volume

*Other aqueous buffers with high and low salt can be used

Buffer concentration and ionic strength can impact retention time, peak shape, and resolution. Adjustments can be made depending on your sample requirements.

Buffers and SEC: Criteria for Optimal Mobile Phase

- Mobile phase should contain enough buffer/salt (to overcome ionic interactions).
- Mobile phase should not contain too much buffer/salt (to prevent hydrophobic interactions).
- Mobile phase should not alter the analyte (cause degradation/aggregation, and so on).
- Mobile phase should be made up fresh and used promptly (bacterial growth is rapid in dilute buffer stored at room temperature).
- Buffer shelf life <7 days unless refrigerated.
- Mobile phase should be filtered before use. Particulates may be present in water (less likely) or in buffer salts (more likely).

Selecting a GPC/SEC Column

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Points to consider when making a column choice:

- Organic or aqueous eluents being used
- What is the expected mol wt range of your sample
- What type of column chemistry
- What are your **key** requirements for your GPC/SEC analysis?
  i. Resolution is important
  ii. Reproducibility of sample chromatography and results
  iii. Speed of analysis and sample throughput is something to improve on

Key Requirement Might Be

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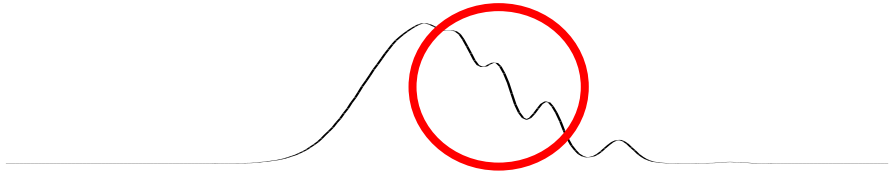
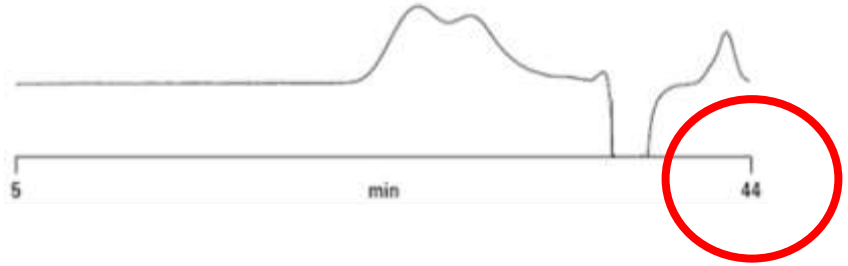
Resolution is too low

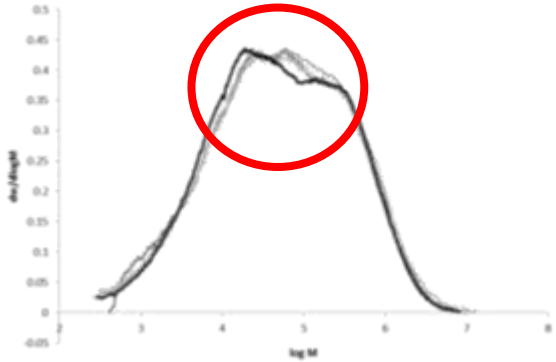


Analysis time is too long



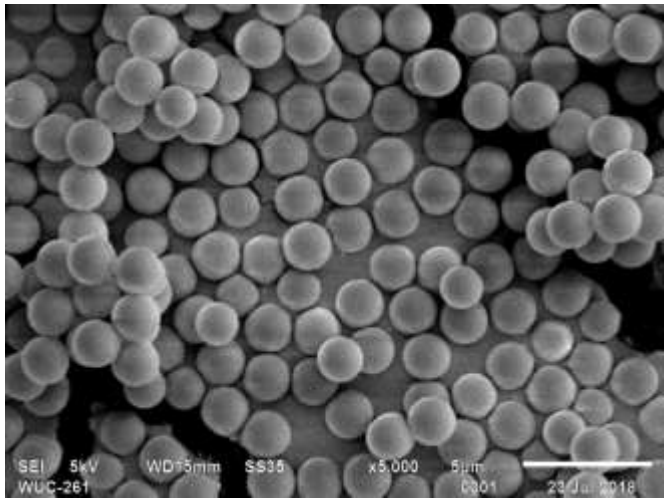
Peak shapes are poor

Results are not reproducible



Column Chemistries

AdvanceBio SEC 200Å 1.9 µm particles have a very narrow size distribution, which provides high efficiency.



Polymer chemistries

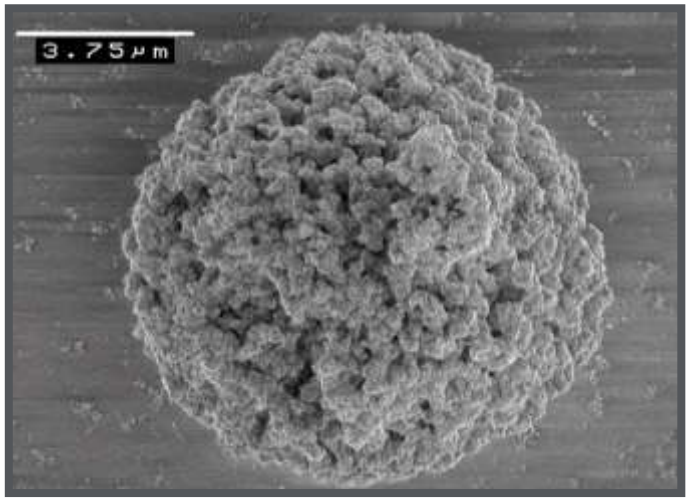
Common types:

Polymethacrylate packings

Polyester copolymers

DVB, divinylbenzene

PS-DVB, polystyrene divinylbenzene



Silica chemistries

Common types:

Diol

Surface modified hydroxyl

Surface modified polymeric

Correctly Choosing a Column

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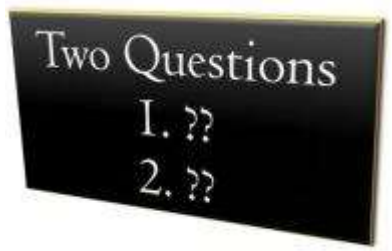
Question: What is the expected molecular weight range of your polymer sample or your protein sample?

Mol Wt	Mol Wt Range (g/mol or Da)
High	Up to several millions
Intermediate	Up to hundreds of thousands
Low	Up to tens of thousands
Very low	A few thousand



Column Selection

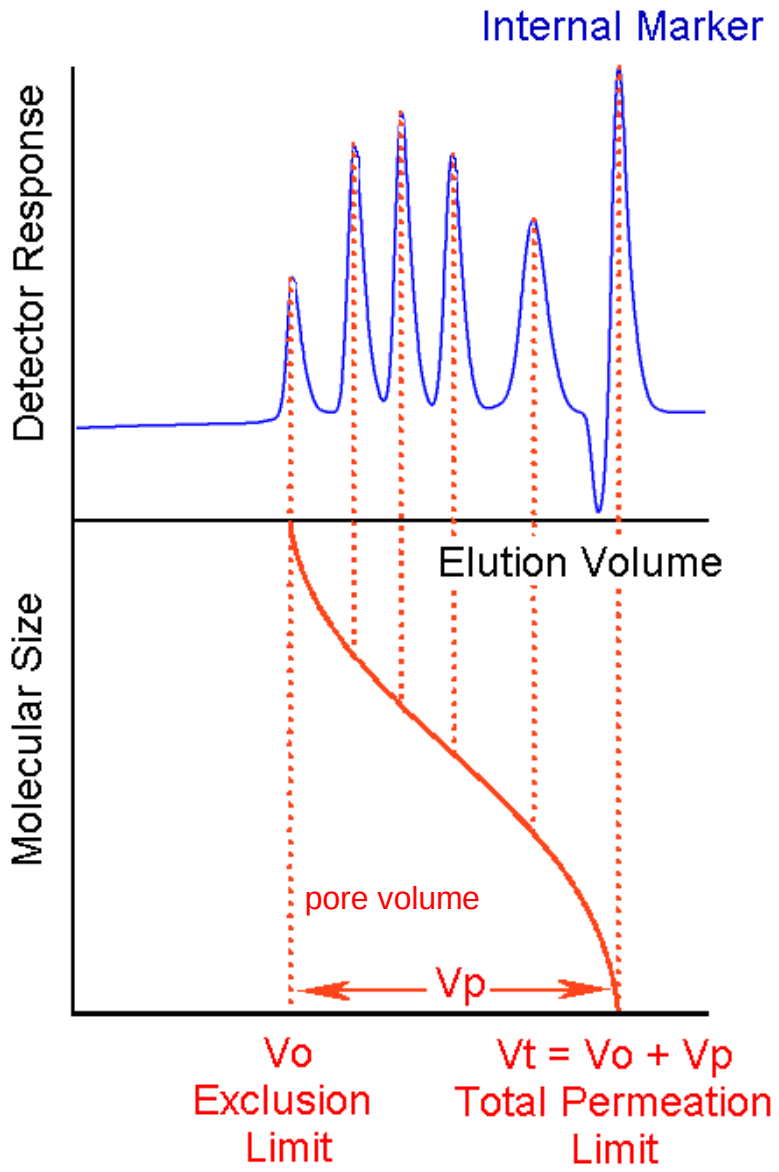
Choose the right pore size

- Ensure you select a column with large enough pores to allow your molecule to permeate into the pore structure of the stationary phase and not be excluded.
- It should provide complete coverage for the mol wt range of your sample and for your calibration standards
- It is also essential to choose a pore size that is not too large

For example, for monoclonal antibodies the optimum pore size is around 300 Å.

Choose the Right Pore Size

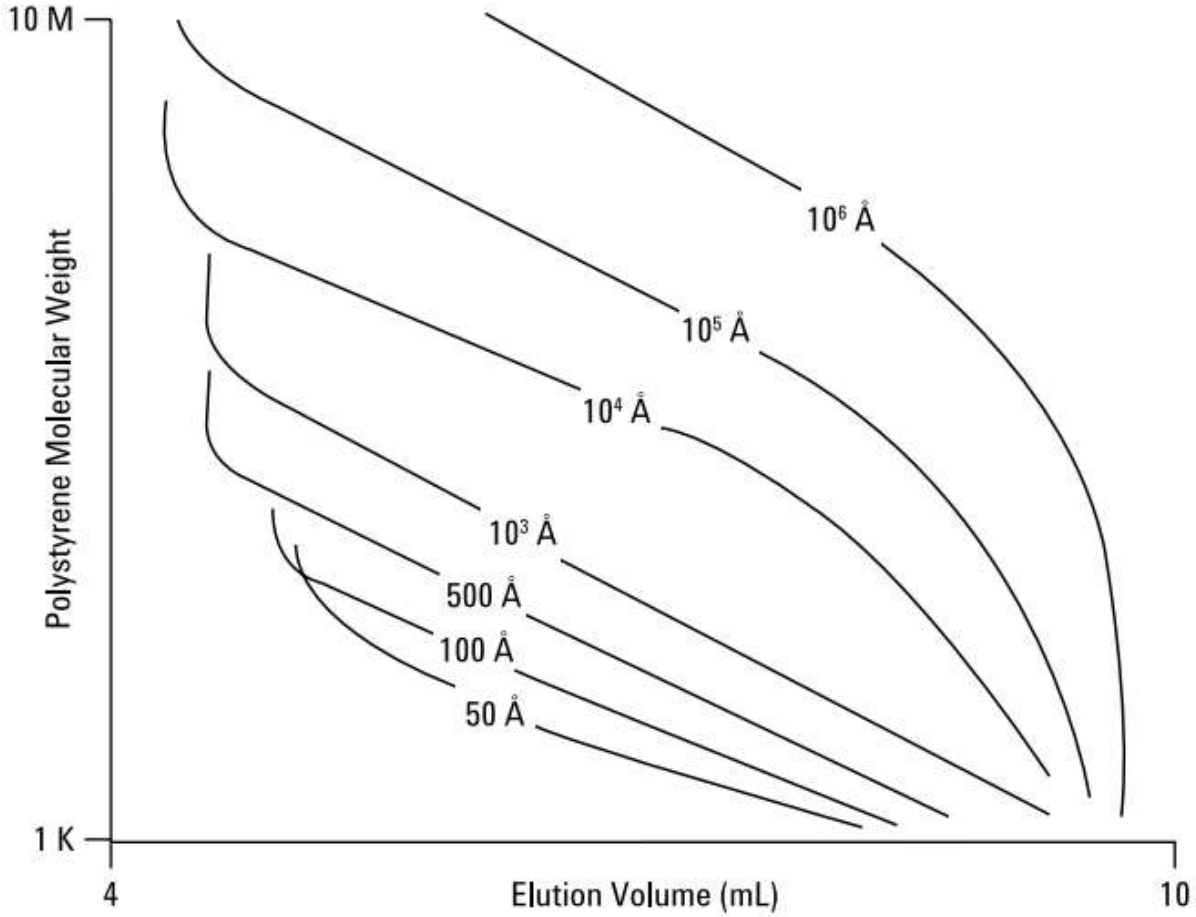
- The example chromatogram and calibration curve illustrate how different size molecules elute from the column.
- Choose a pore size that allows you to work in the linear portion of the calibration curve.



Column Types: Individual Pore Size

- All particles have the same pore size
- Good separation, but narrow range of mol wt
- Very nonlinear curve; linear only over a narrow mol wt range
- Oldest technology, but still popular, and useful for separating very small and very large compounds
- Wider mol wt range possible by combining different columns in series, but need to select carefully so not to have column ‘mismatch’

Calibration curve

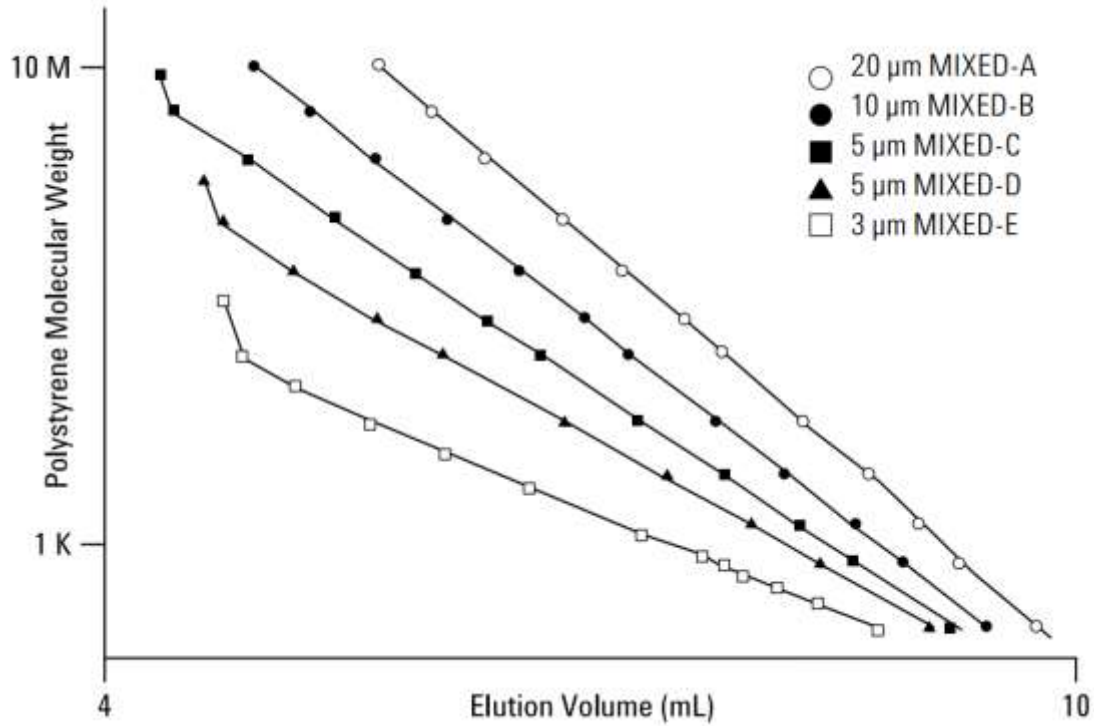


PLgel individual pore size calibration plots

Column Types: MIXED

- Individual pore size particles are mixed together/blended to make a linear curve
- Very wide ranges possible, but only a small amount of separation of each mol wt
- Linear curve makes chromatogram easy to read and analyze
- Most popular technology, well established and widely used
- Columns in series of same type are still linear

Column family: PLgel

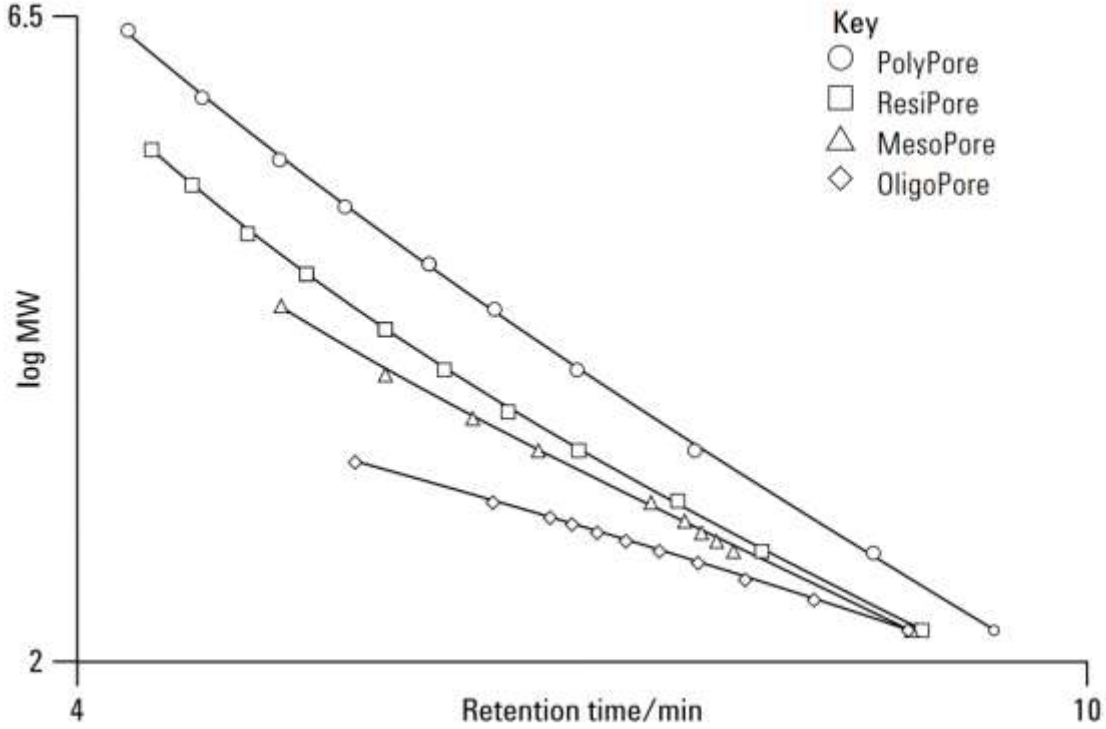


PLgel MIXED calibration plots

Column Types: Multi-Pore Particle

- Newest, fastest growing technology
- Each particle has multiple pore sizes
- Increased pore volume
- Highest resolution and efficiency
- Best performance for most common mol wt ranges

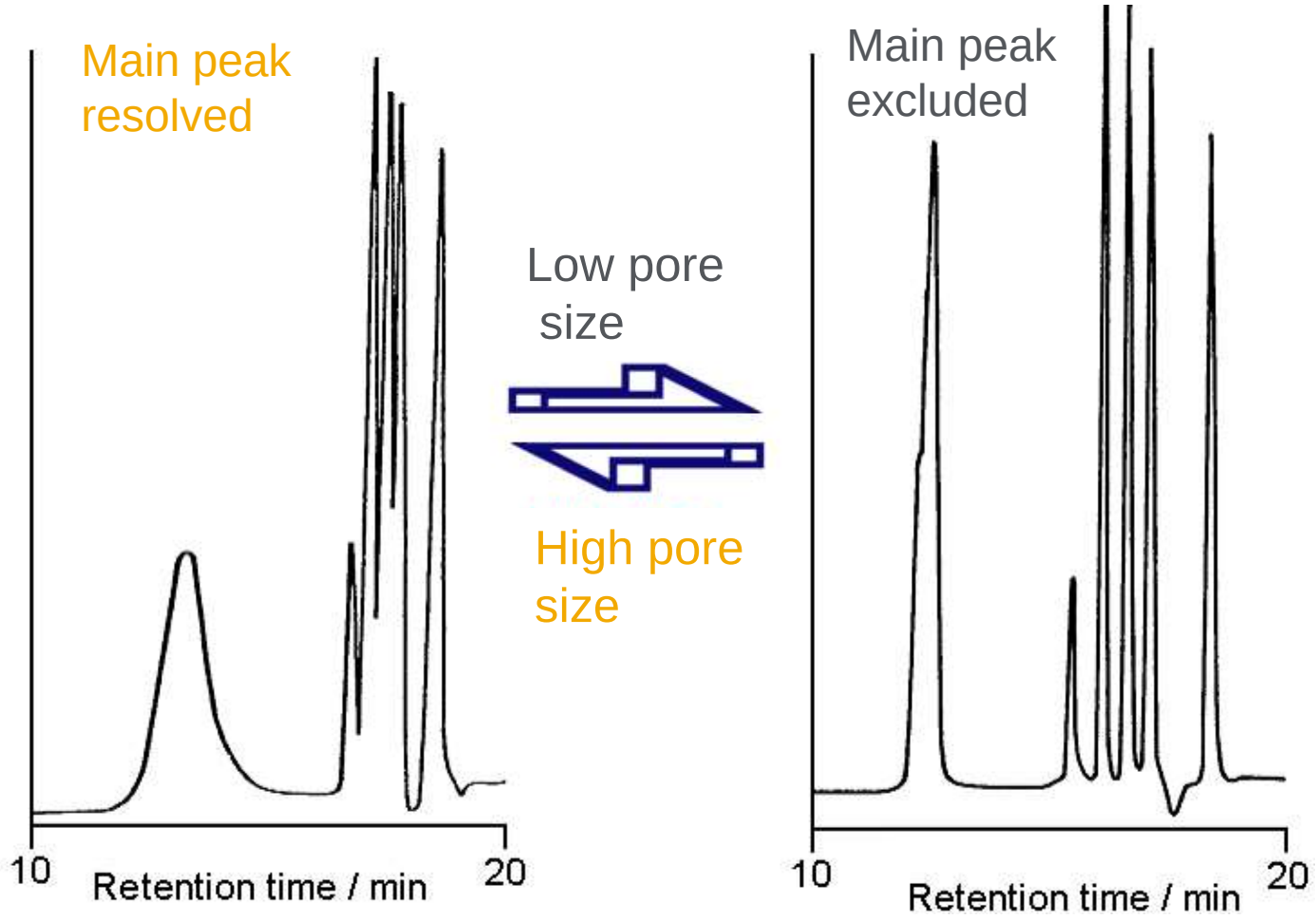
Column family: PlusPore



PlusPore calibration plots

Column Selection

Effect of pore size



* Samples run using PLgel individual pore size columns

Importance of Pore Size Selection
Calibrants

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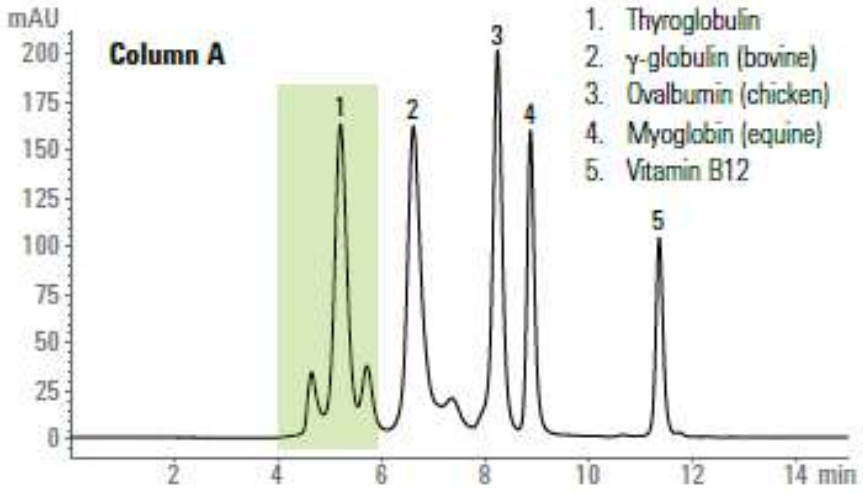
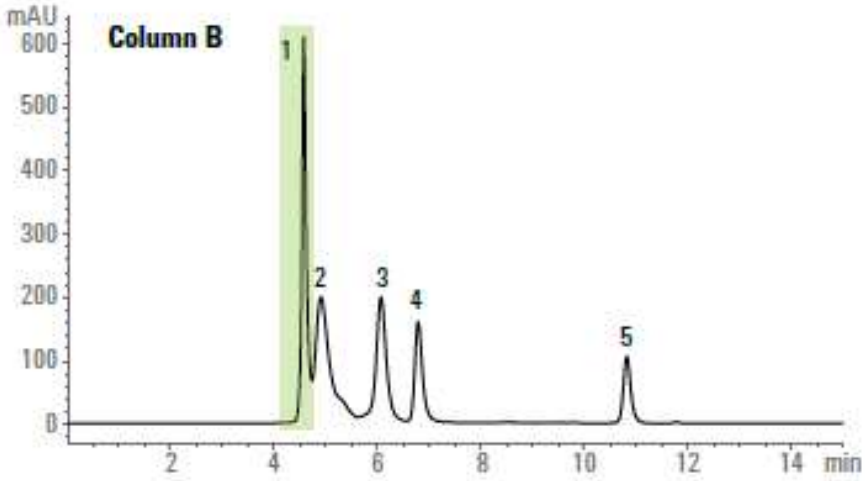
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm
Sample:	BioRad gel filtration standards mix

Column A: **AdvanceBio SEC 300Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)

Column B: **AdvanceBio SEC 130Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)

BioRad gel filtration standards mix





Importance of Pore Size Selection
Sample

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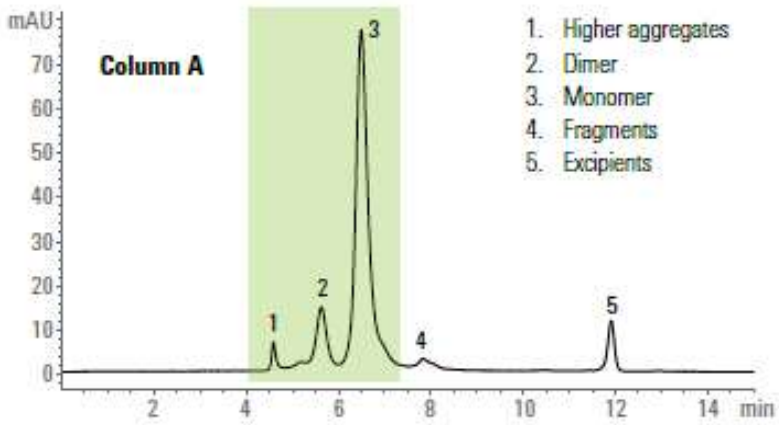
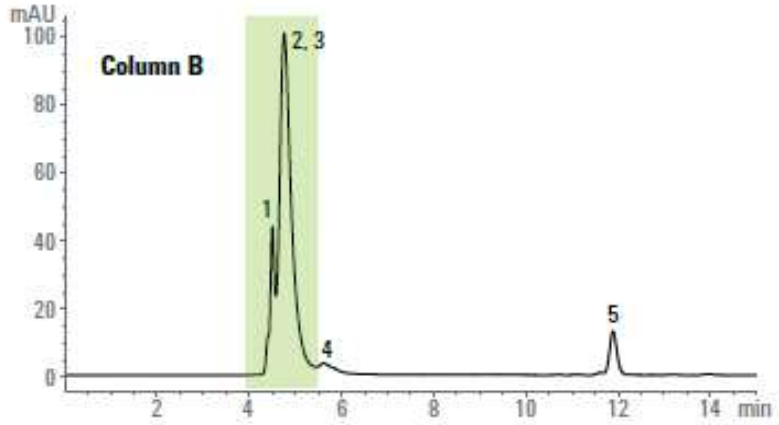
Polyclonal IgG separation





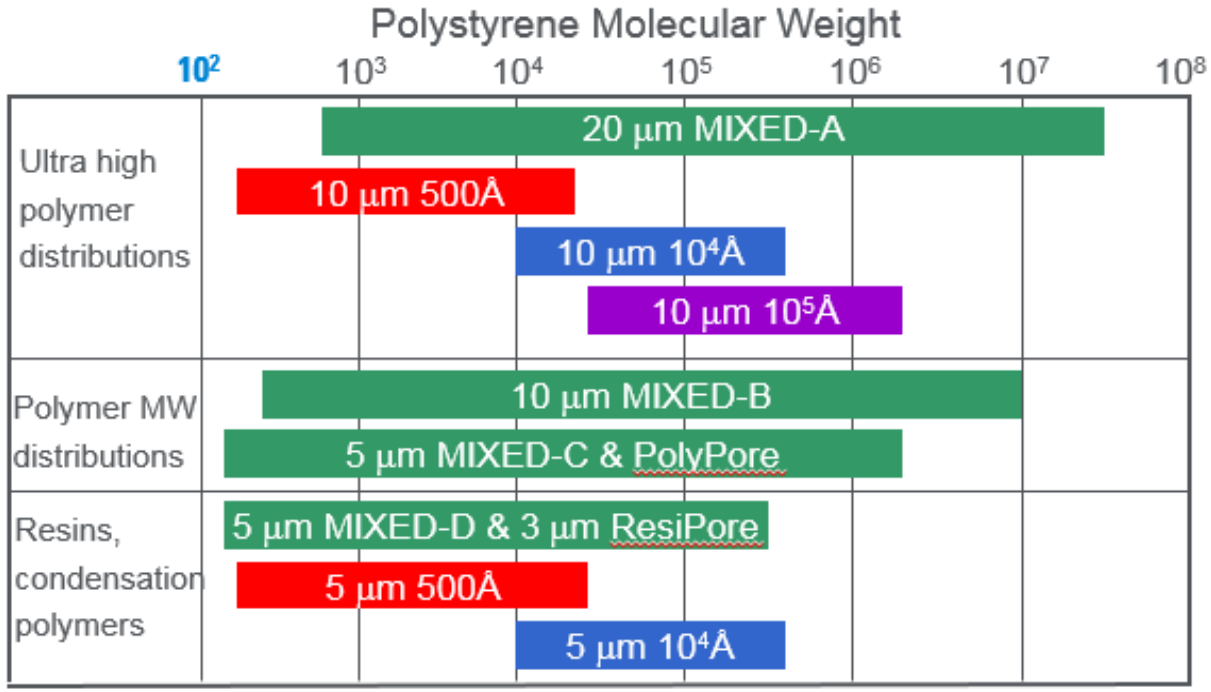
Column A: **AdvanceBio SEC 300Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)

Column B: **AdvanceBio SEC 130Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)

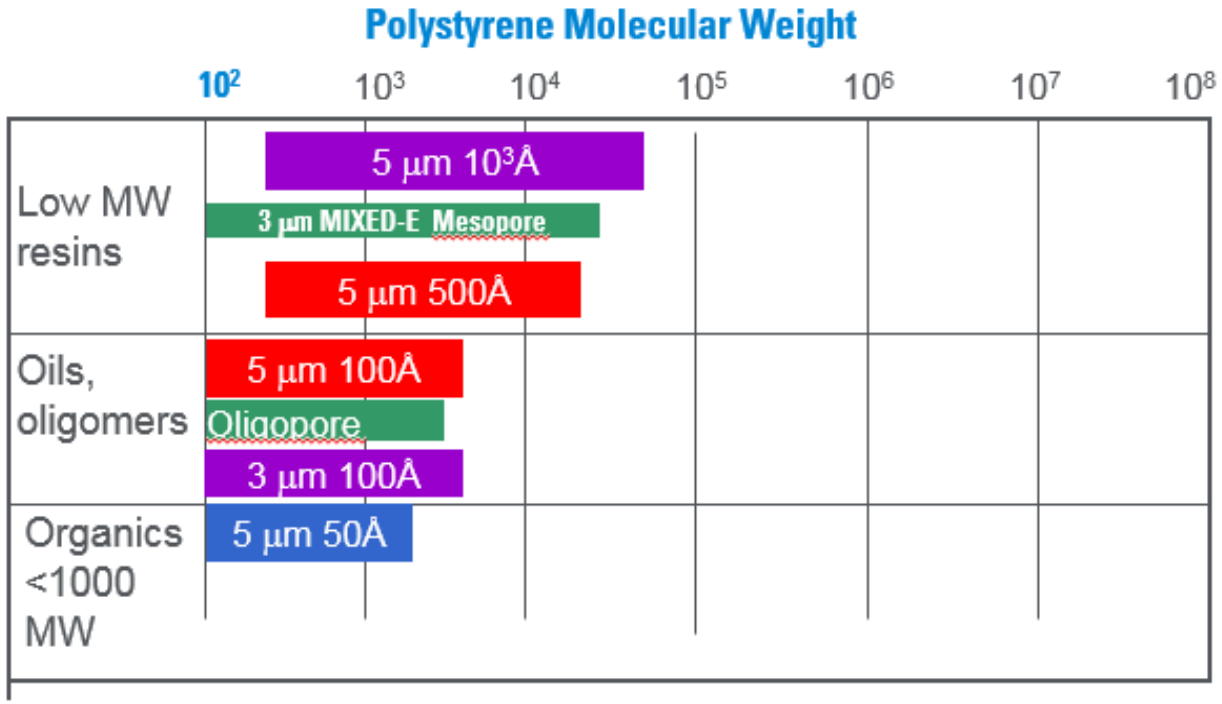
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm
Sample:	Polyclonal IgG

Agilent GPC Columns

Separation ranges and column choices for organic soluble polymers

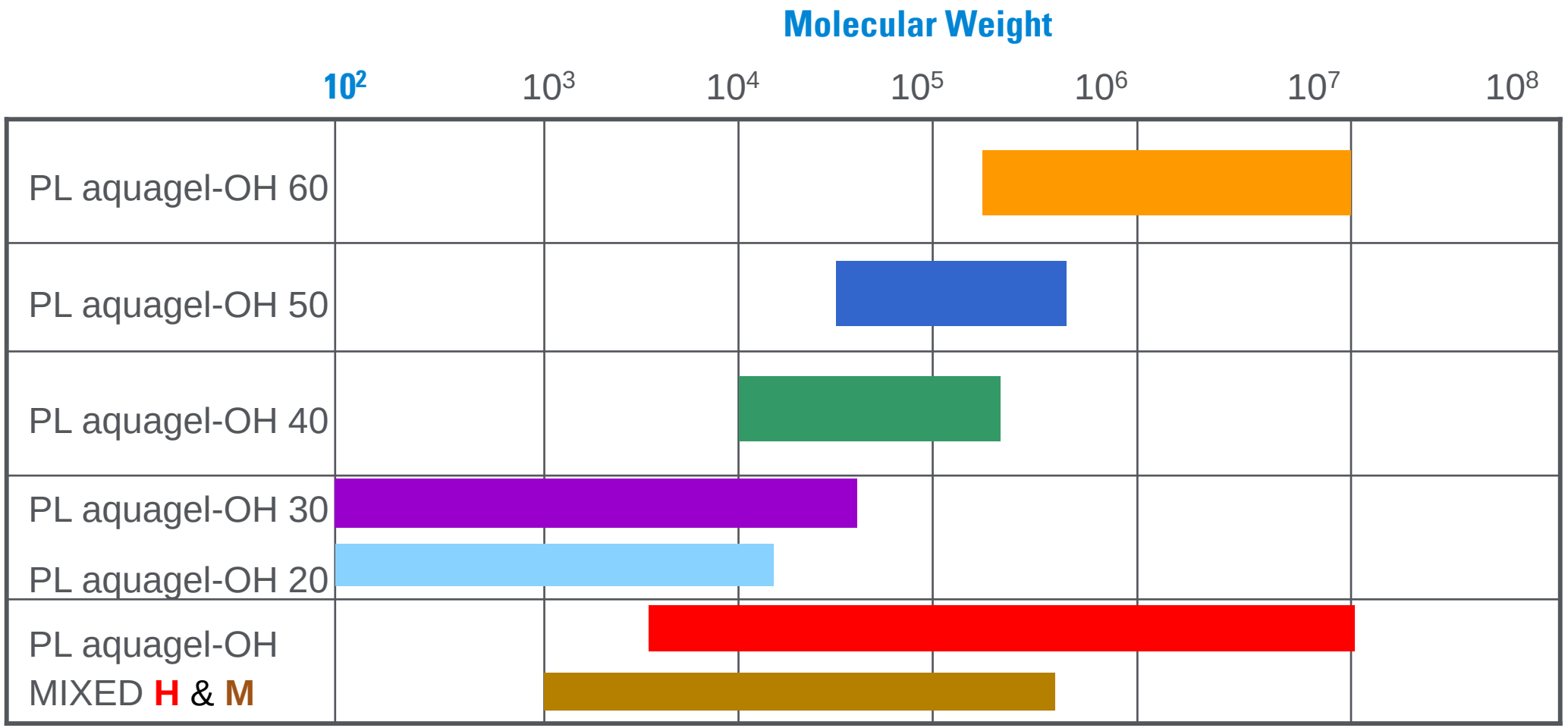


Column families:
PLgel
PlusPore



Agilent PL Aquagel-OH Columns

SEC analysis of water soluble polymers



Agilent Size Exclusion Columns

SEC of biopolymers, proteins, mAbs

AdvanceBio SEC	AdvanceBio SEC	Bio SEC-3	Bio SEC-5	ProSEC 300S	ZORBAX GF-250 and GF-450
1.9 µm	2.7 µm	3 µm	5 µm	5 µm	4 µm, 6 µm
200 Å	130 Å, 300 Å	100 Å, 150 Å, 300 Å	100 Å, 150 Å, 300 Å, 500 Å, 1000 Å, 2000 Å	Nominal 300 Å (linear resolving range)	150 Å, 300 Å
Coated silica (USP L59)	Coated silica (USP L59)	Coated silica (USP L59)	Coated silica (USP L59)	Silica Diol (USP L20)	Zirconium stabilized silica diol (USP L35)
• mAb and ADC analysis • Dimer/monomer • LMW mAb fragments	• mAb and ADC analysis • Higher-order aggregates • Dimer/monomer	• Polypeptide to small proteins • MS capable separations	• Broadest range of pore sizes for wide variety of biomolecules	• Unique linear resolving range • 30 cm and 60 cm column lengths	• Legacy product • Larger column dimensions • Ideal for GF-450 and GF-250 in series

Increasing Resolution in GPC/SEC

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Running two columns in series, same pore size

- Increase pore volume, increases resolution

Running two columns in series, different pore size

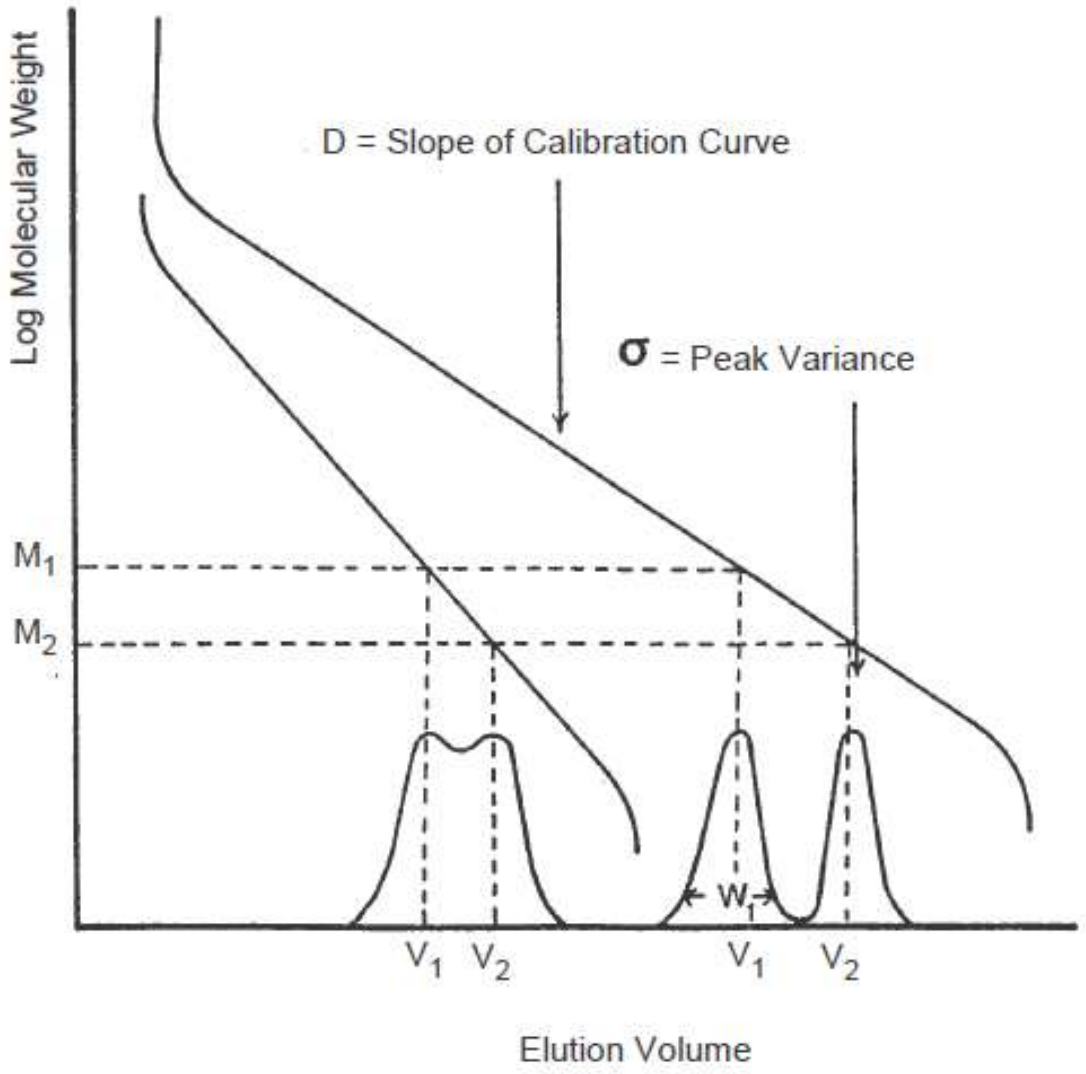
- Extends the resolving range and enables analysis of multiple attributes in one run

Use a packing with a smaller particle size

- Decrease particle size, increase column efficiency

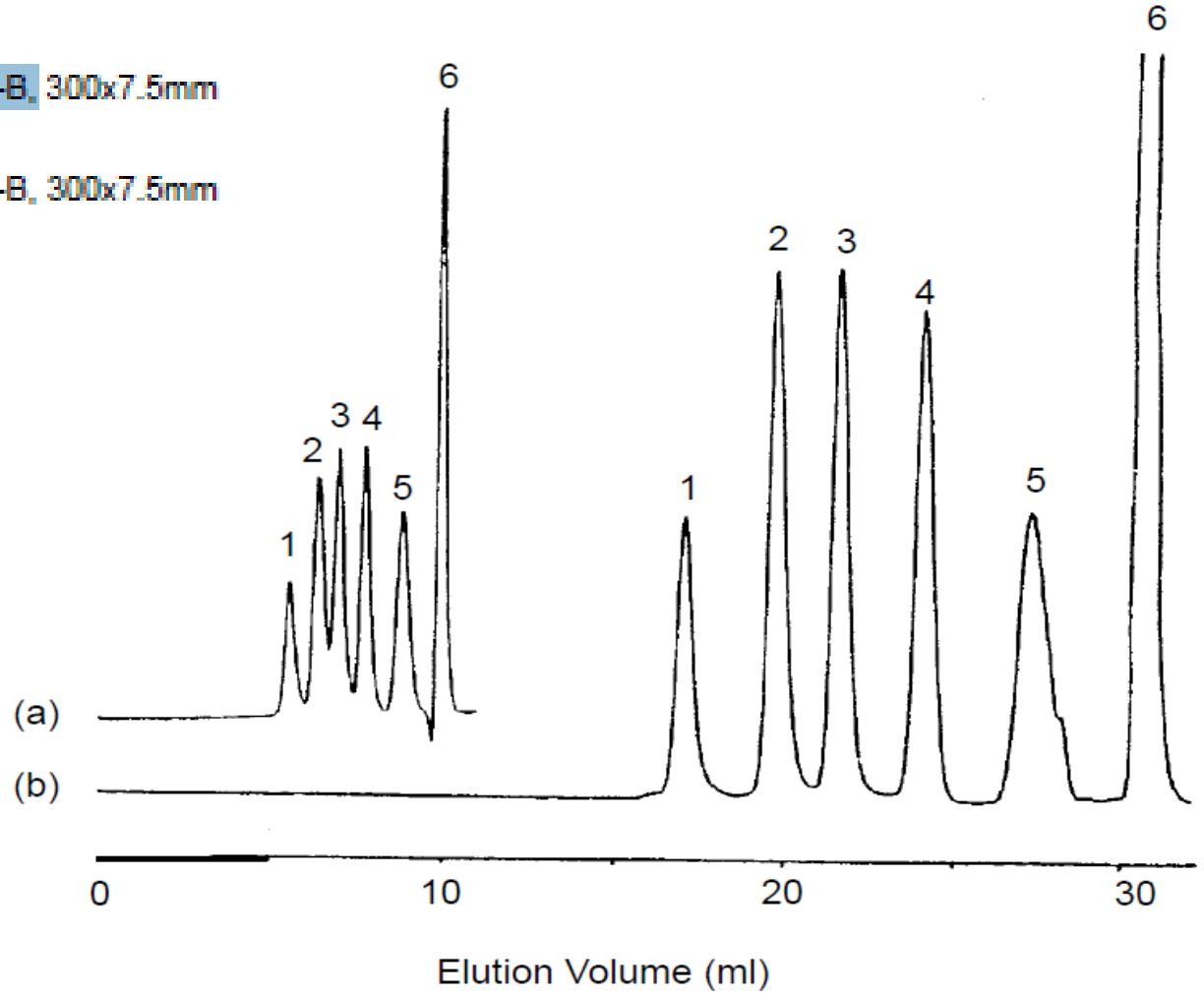
Resolution in GPC

Add a column to improve resolution



Effect of Column Length on Resolution

Columns:	1xPLgel 10µm MIXED-B, 300x7.5mm (1110-6100) 3xPLgel 10µm MIXED-B, 300x7.5mm (1110-6100)
Eluent:	THF
Flow Rate:	1.0ml/min
Detector:	RI



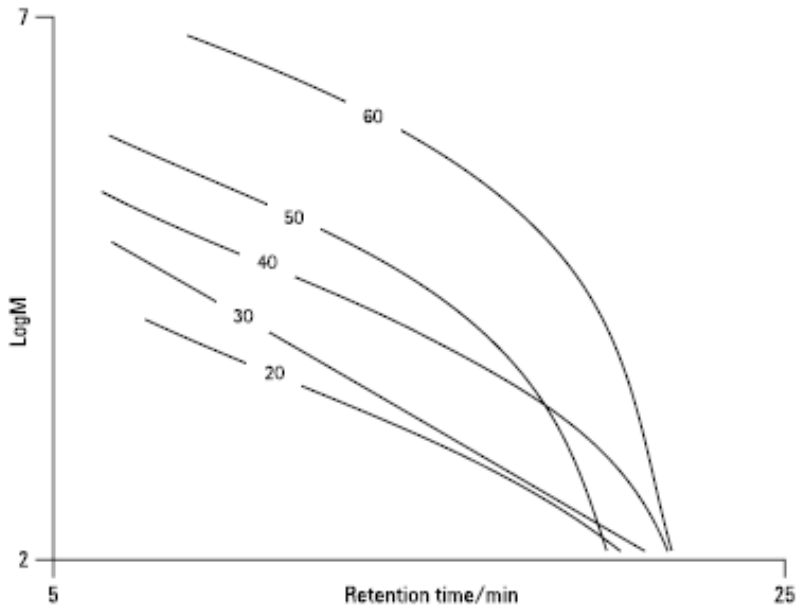
Polystyrene Standards (EasiCal)

1. 3,040,000
2. 330,000
3. 66,000
4. 9,200
5. 580

Column in Series

Extend the mol wt resolving range

PL aquagel OH columns
Individual pore sizes



Conditions

Samples:	Four samples of hyaluronic acid
Columns:	1 x PL aquagel-OH 60 15 µm, 300 x 7.5 mm (p/n PL1149-6260) + 1 x PL aquagel-OH 40 15 µm, 300 x 7.5 mm (p/n PL1149-6240)
Eluent:	0.2 M $NaNO_3$ + 0.01 M NaH_2PO_4 at pH 7
Flow Rate:	1.0 mL/min
Detection:	RI

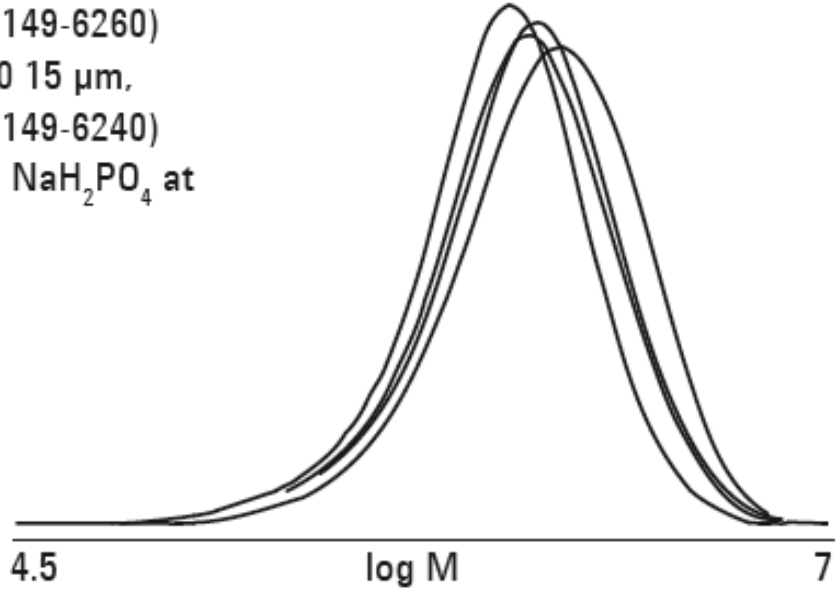


Figure 3. Overlay of the molecular weight distributions of four hyaluronic acid samples

Effect of Particle Size on Resolution

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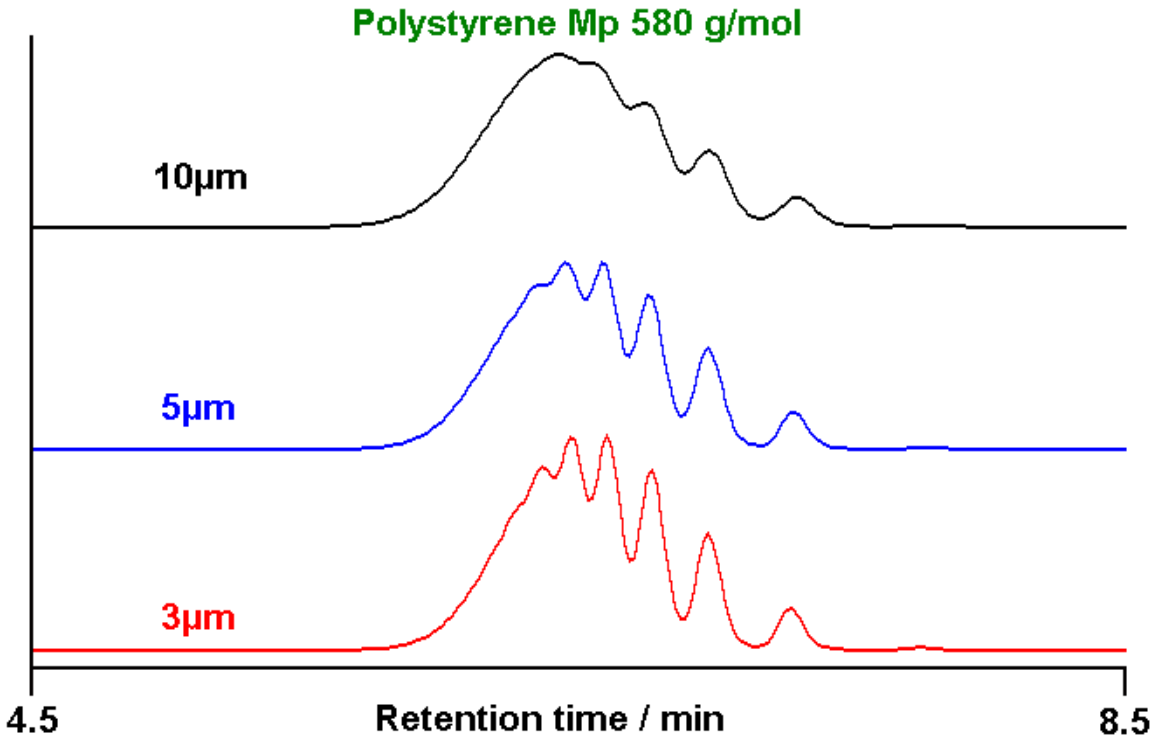
Column:	PLgel 100 Å 300 x 7.5 mm
Eluent:	THF
Flow rate:	1.0 mL/min
Inj vol:	20 µL
Detector:	DRI



Comparison of 3 µm vs 5 µm Particle Size

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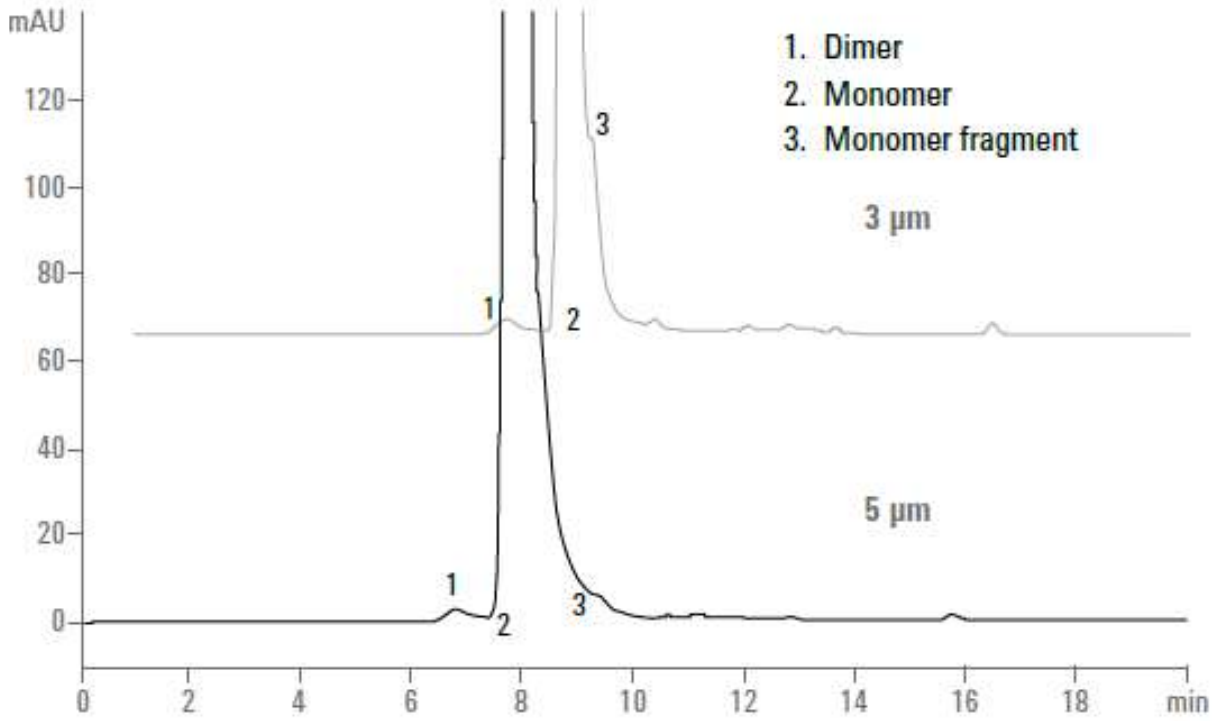
Analysis of monoclonal antibody

Column:	**Bio SEC-3, 300Å 7.8 x 300 mm, 3 µm (p/n 5190-2511)**
Column:	**Bio SEC-5, 300Å 7.8 x 300 mm, 5 µm (p/n 5190-2526)**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM sodium phosphate, pH 7
Flow rate:	1 mL/min
Detector:	UV, 220 nm
Sample:	Humanized monoclonal antibody



Sub-2 µm Particle Size Comparison

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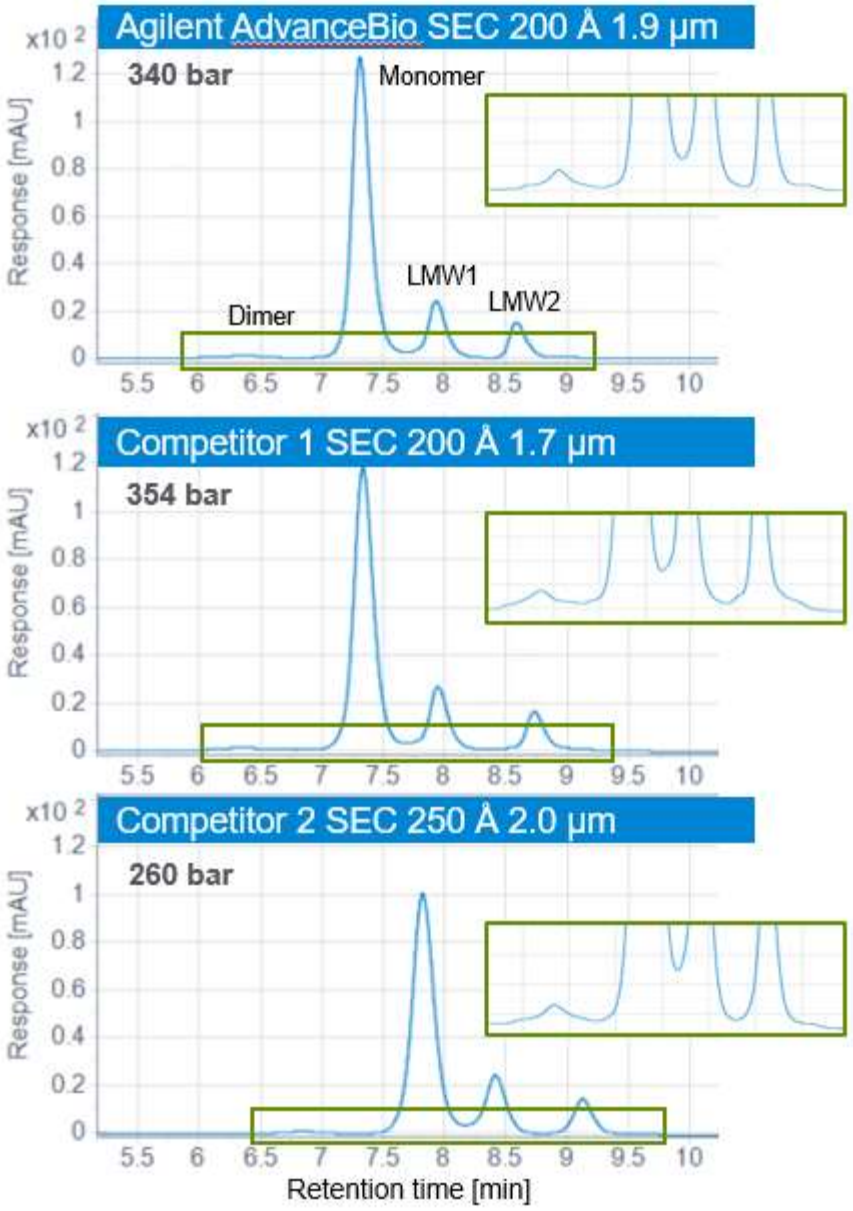
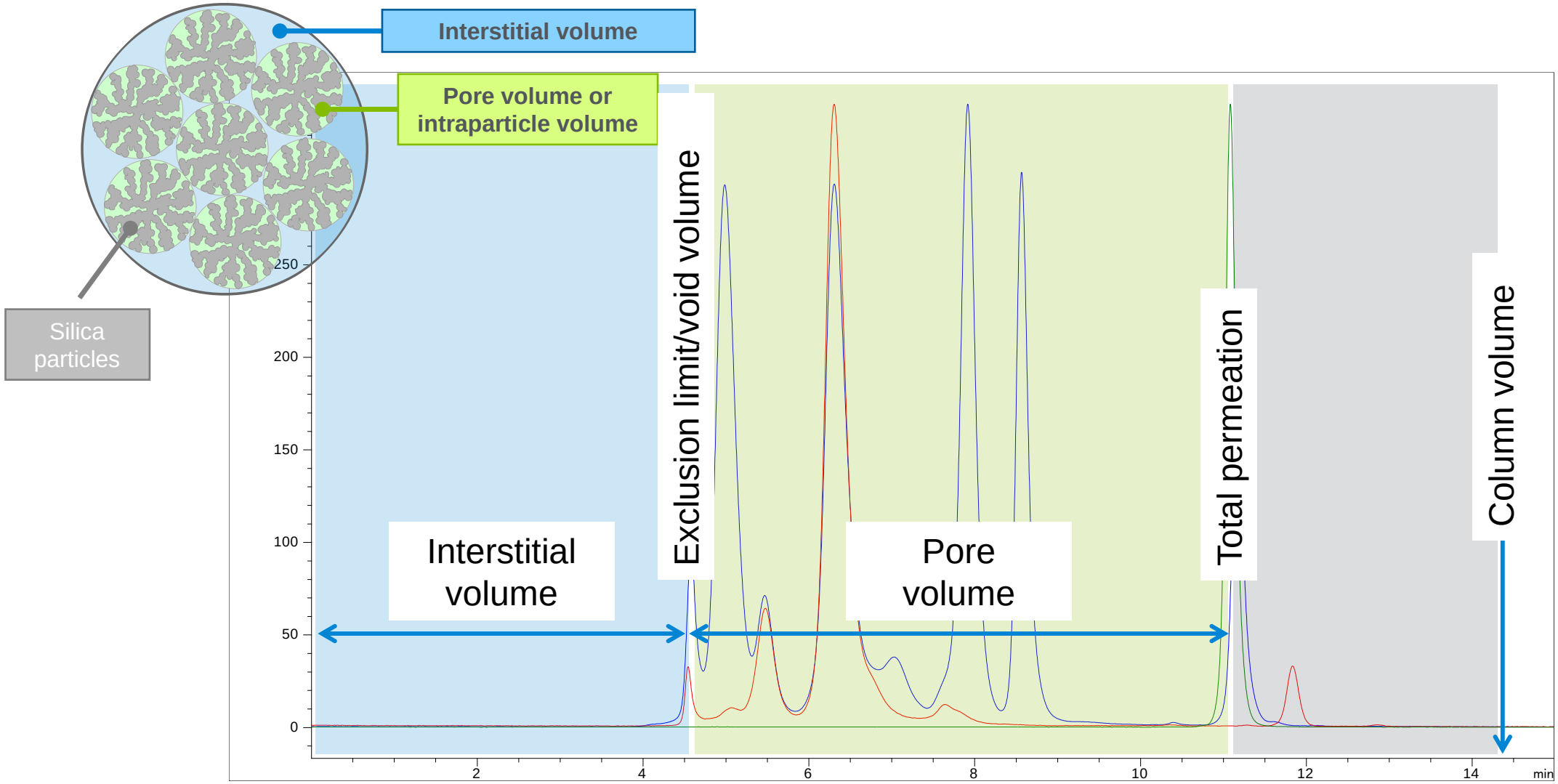


LC Conditions	1260 Infinity II Bioinert LC System
Column dimension	4.6 x 300 mm
Mobile phase	50 mM sodium phosphate, 200 mM NaCl, pH 7.0
Temperature	25 °C
Sample	Sigma mAb (spiked with its $F(ab')_2$ and Fc fragments)
Flow rate	0.35 mL/min
UV detection	220 nm

	Peak Width 50%			Resolution		
	Monomer	LMW1	LMW2	Dimer/Monomer	Monomer/LMW1	Back Pressure (bar)
gilent Advance Bio SEC 1.9 µm	0.159	0.154	0.148	2.79	2.28	340
ompetitor EC column 1	0.172	0.166	0.160	2.46	2.09	354
ompetitor EC column 2	0.194	0.182	0.169	2.49	1.83	260

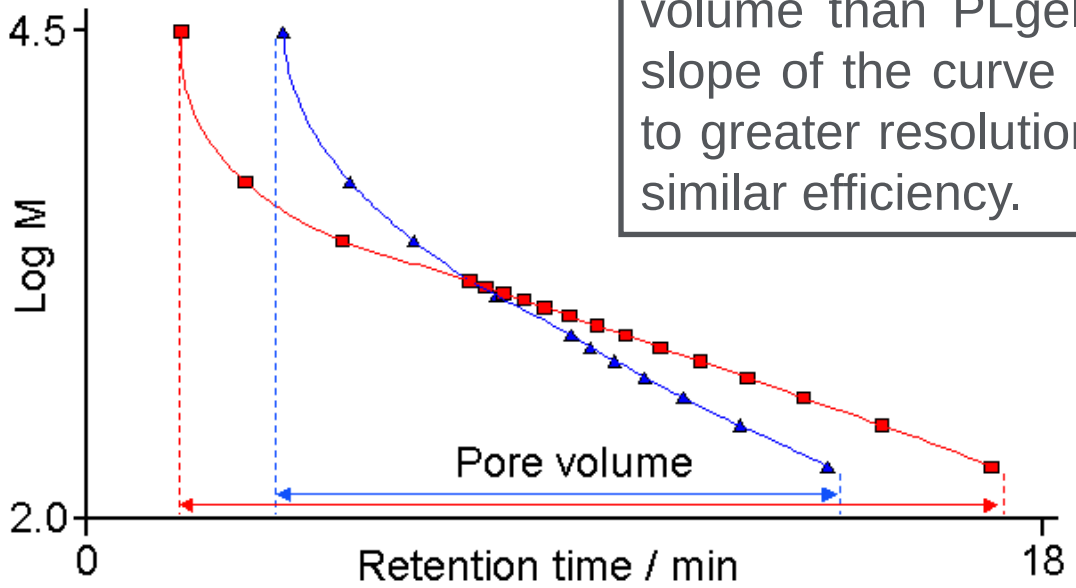
Product Name	Particle	Column Hardware	Column Dimensions	Part Number
AdvanceBio SEC 200Å 1.9 µm	1.9 µm 200Å (coated silica)	RRHD	4.6 x 300 mm 4.6 x 150 mm 4.6 x 30 mm guard	PL1580-5201 PL1580-3201 PL1580-1201

A Story Inside Every SEC Chromatogram



Effect of Increased Pore Volume

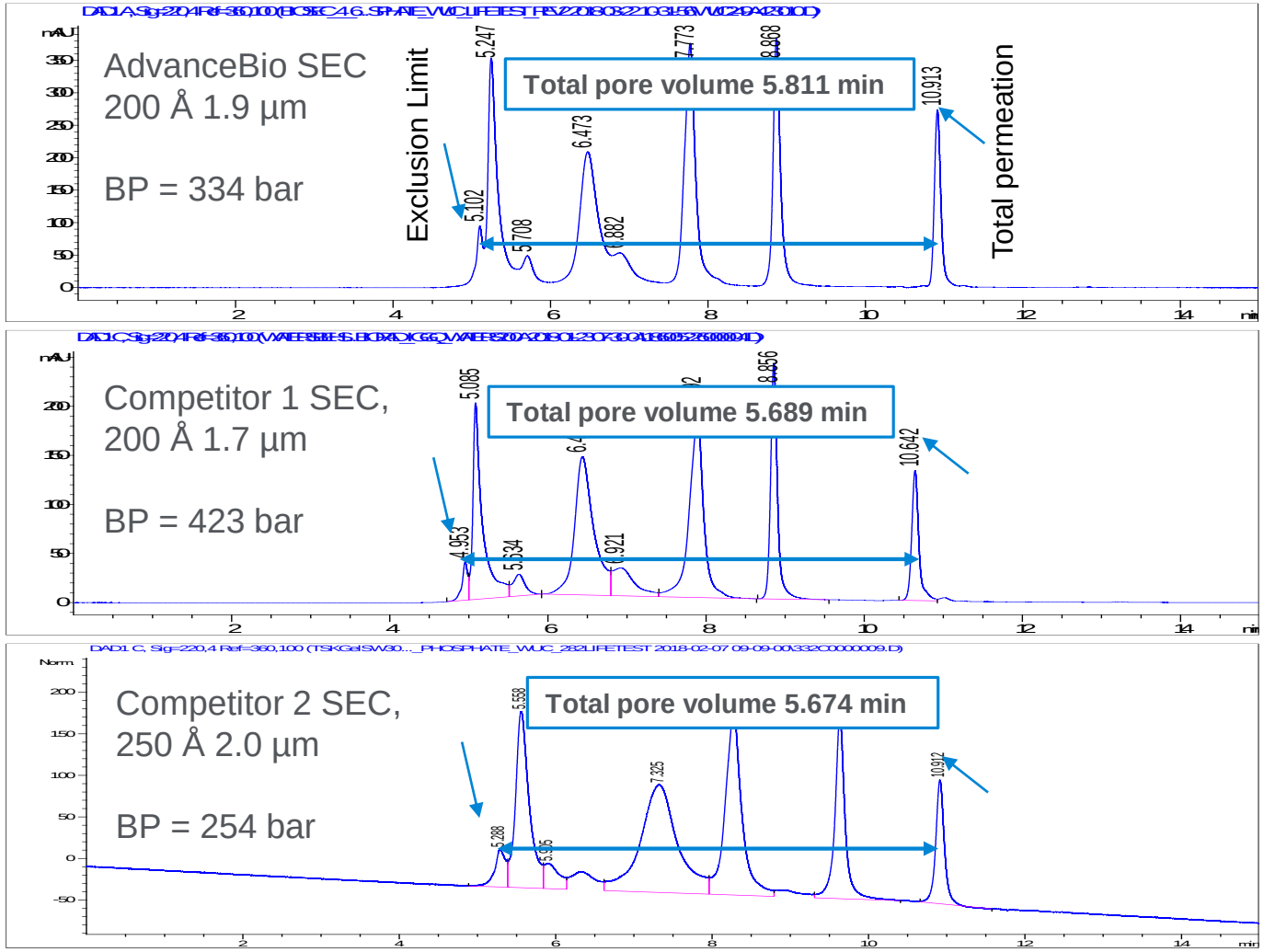
Columns	2 x PLgel, 3 µm, 100 Å, 300 x 7.5 mm 2 x OligoPore, 300 x 7.5 mm
Eluent	THF
Flow rate	1.0 mL/min



Both columns have a similar exclusion limit but OligoPore has greater pore volume than PLgel 100 Å. Hence the slope of the curve is shallower leading to greater resolution for columns of the similar efficiency.

Column Pore Volume Analysis

(4.6 x 300 mm, 0.35 mL/min, pH 7.0 phosphate buffer, BioRad sample)

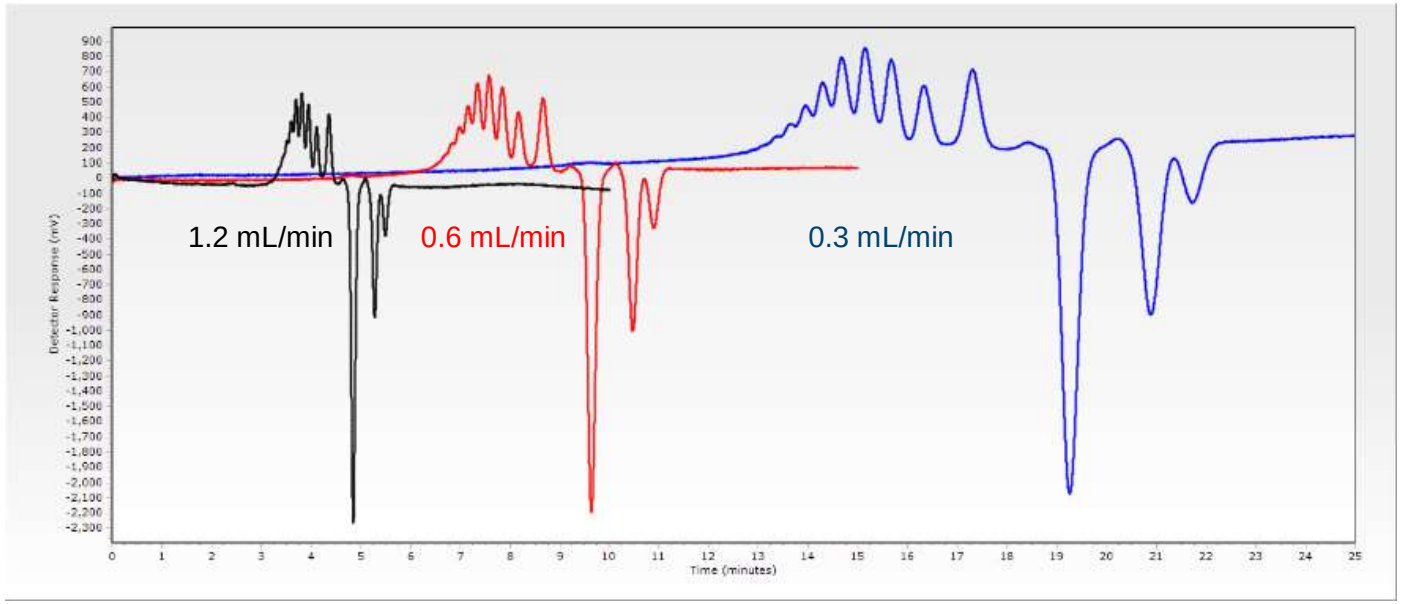


BioRad # 151-1901	Molecular Weight
Thyroglobulin	670,000
γ-globulin	158,000
Ovalbumin	44,000
Myoglobin	17,000
Vitamin B12	1,350

Column	Exclusion Limit (min)	Total Permission (min)	Total Pore Volume (min)
AdvanceBio SEC 200 Å 1.9 µm	5.102	10.913	5.811
Competitor 1 SEC, 200 Å 1.7 µm	4.953	10.642	5.689
Competitor 2 SEC, 250 Å 2.0 µm	5.238	10.912	5.674

AdvanceBio SEC 200 Å 1.9 µm columns provide widest separation window.

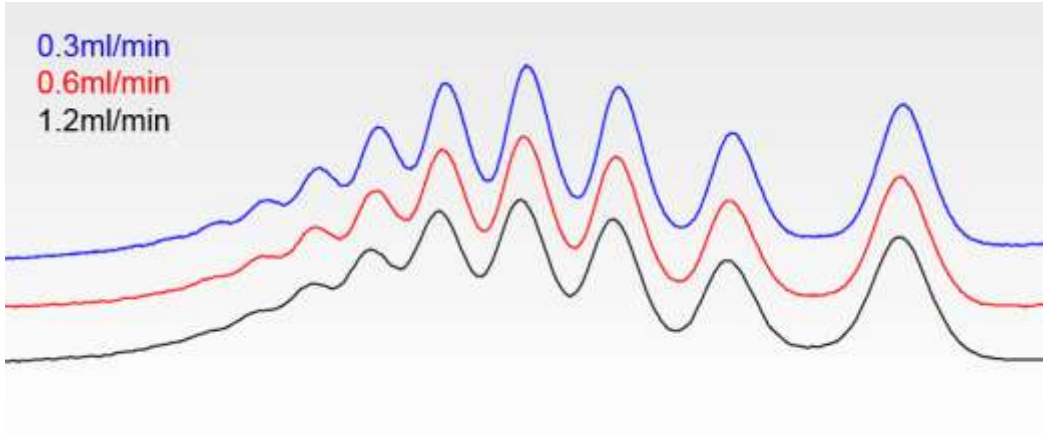
Improving Speed for Analysis Without Sacrificing Resolution



Sample: Polystyrene mol wt 580
Column: OligoPore 250 x 4.6 mm

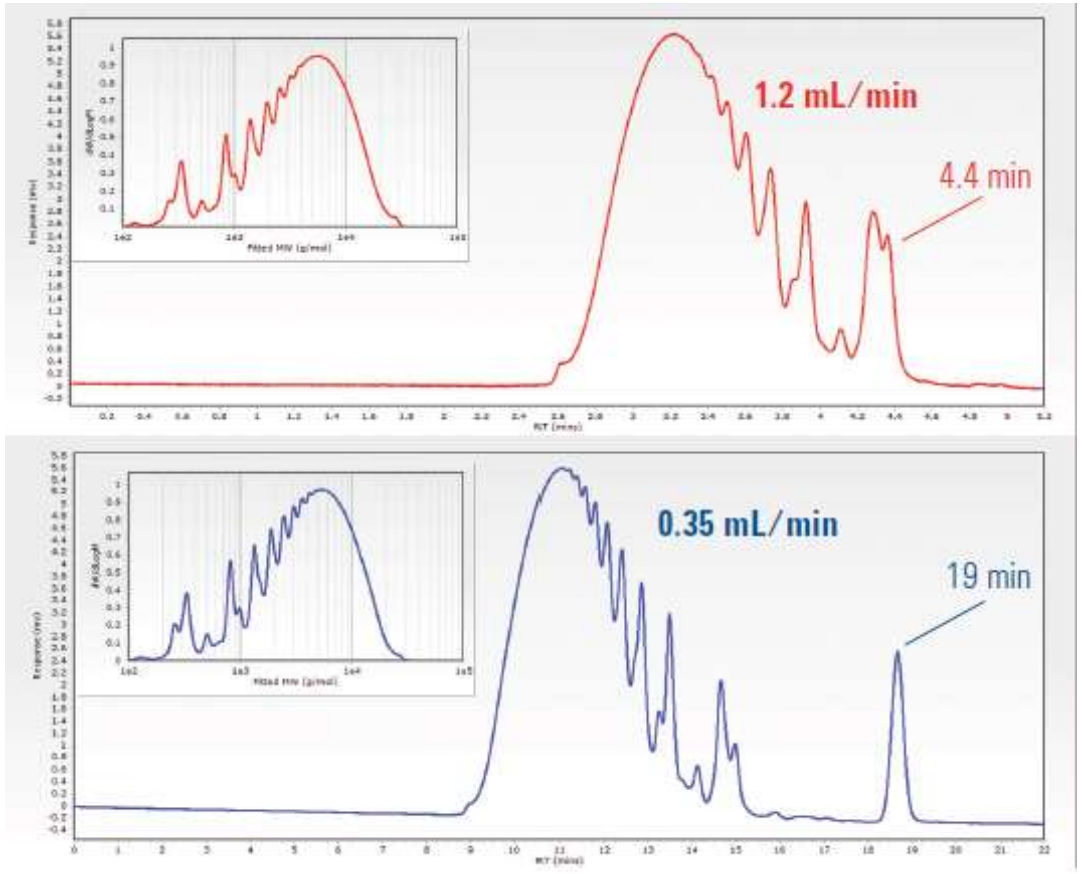
MW Range: up to 3,300 (g/mol)
Nominal Particle Size: 6 µm
Typical Efficiency: >55,000 p/m

The diagrams show different flow rates overlaid to show that faster doesn't sacrifice resolution. The chromatograms have been normalized to better illustrate the differences.



Fast GPC

Example with MesoPore columns



Conditions

Column:	2 x MesoPore, 4.6 x 250 mm (PL1513-5325)
Sample:	Epoxy resin
Eluent:	THF
Flow rate:	0.35 and 1.2 mL/min
Inj vol:	4 µL
System:	1260 Infinity GPC/SEC System, UV, 254 nm

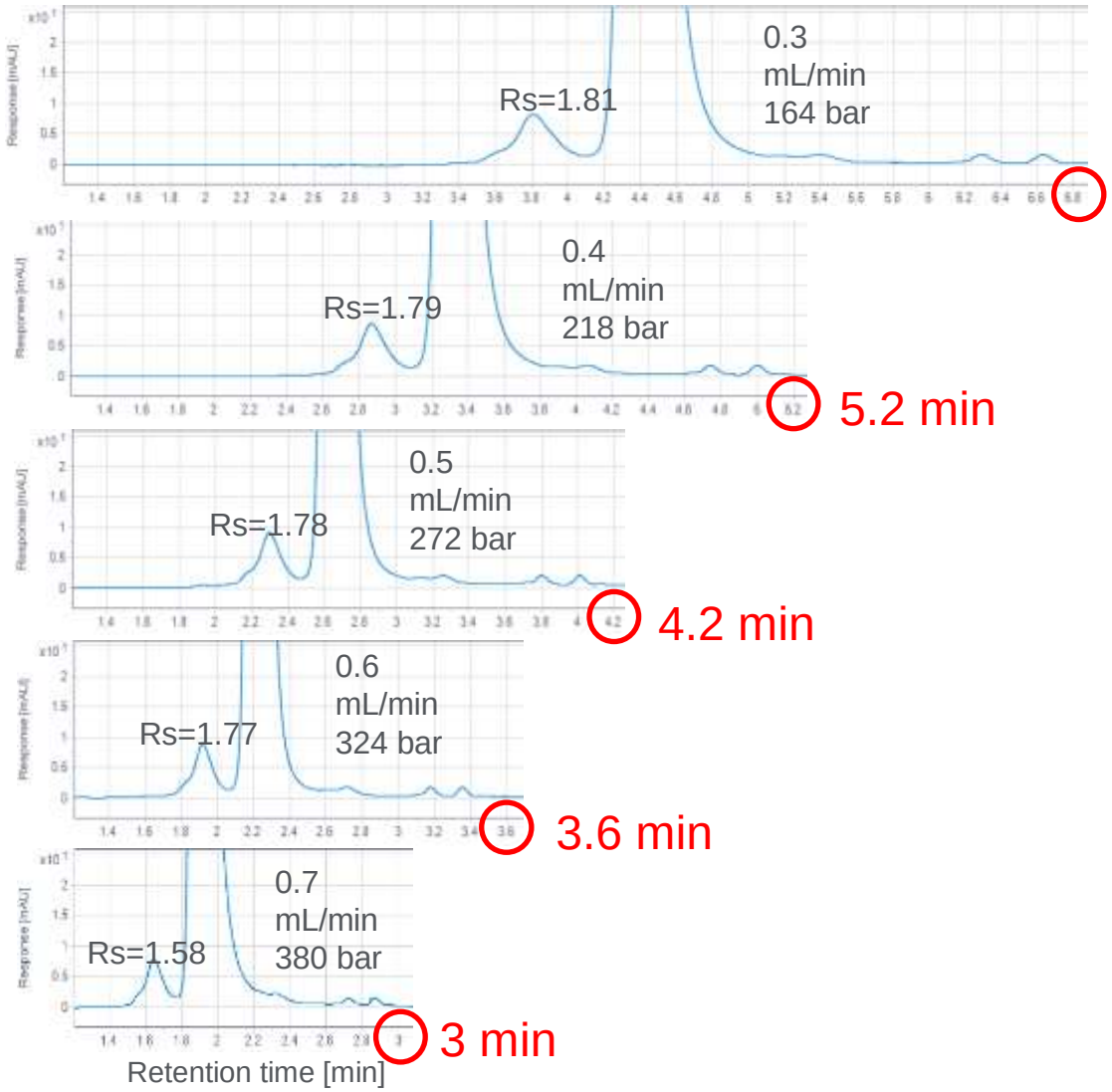
Easy method transfer from standard to rapid GPC on MesoPore 250 x 4.6 mm GPC columns

MW Range: up to 25,000 (g/mol)

Nominal Particle Size: 3 µm

Typical Efficiency: >80,000 p/m

Fast SEC AdvanceBio SEC 200 Å 1.9 µm



LC Conditions	1260 Infinity II Bioinert LC System
Column used	Agilent AdvanceBio SEC 200 Å, 1.9 µm 4.6 x 150 mm
Mobile phase	50 mM sodium phosphate, 200 mM NaCl, pH 7.0
Temperature	25 °C
Sample	Sigma mAb
UV detection	220 nm

Fast Analysis

150 mm
0.3 mL/min

2.3 times faster

150 mm
0.7 mL/min

300 mm
0.3 mL/min

4.6 times faster

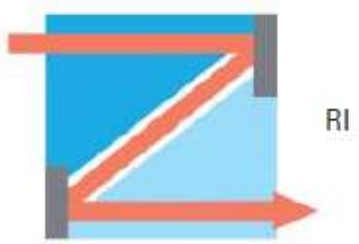
150 mm
0.7 mL/min

Flow Rate (mL/min)	Dimer Area (%)	Samples Per Hour	Samples Per Day (24 h)
0.3	2.33	8-9	211
0.4	2.35	11-12	276
0.5	2.35	14	342
0.6	2.39	16-17	400
0.7	2.30	20	480

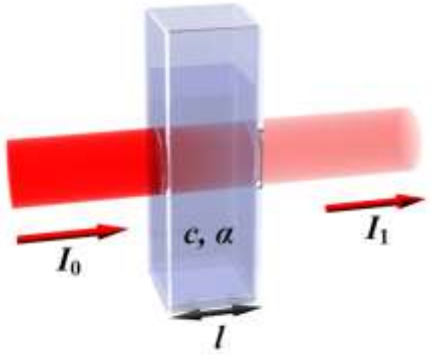
Detectors and Instrument Considerations

Concentration detectors

- Most common detectors for GPC/SEC are *concentration* detectors:



RID

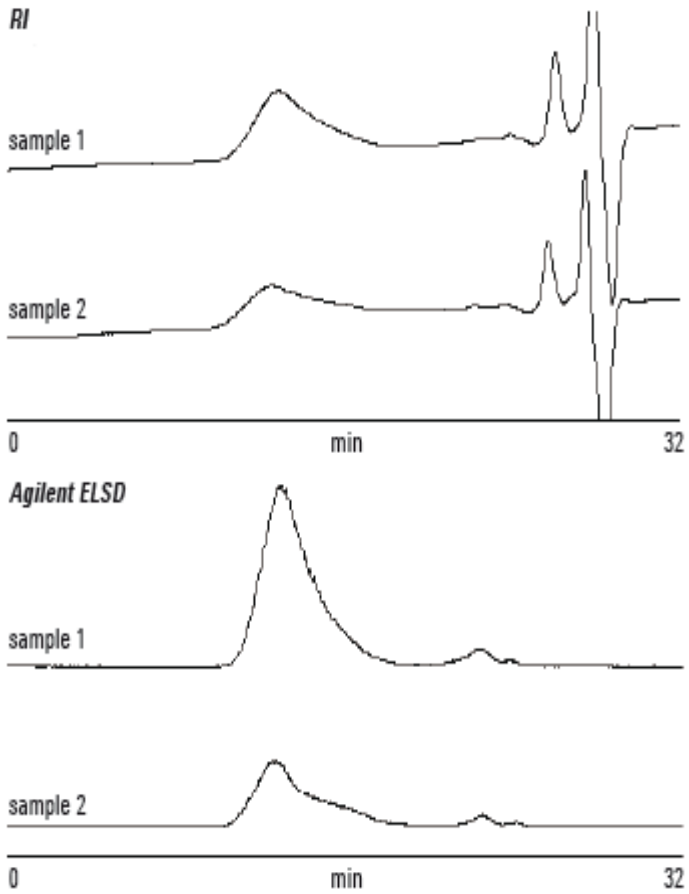


UV/DAD

ELSD

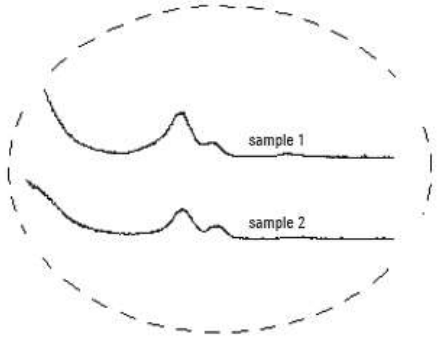
- These provide information on the amount of polymer or sample eluting from the column at any given time.

Detector Selection
Refractive Index vs ELSD



RI:
- Low response for sample
- Unable to detect additives
- System interference peaks present

ELSD:
- Improved response
- Additives detected
- No system interference peaks



Expanding Conventional GPC/SEC

Viscometer and light scattering detectors

Agilent CrossLab
From Insight to Outcome

Advanced detectors give a greater understanding of the analyte as well as overcoming the limitations of conventional GPC.

GPC/SEC Technique	Molecular Weight	Molecular Size	Information
Conventional (RI or UV)	Relative to standards used for calibration	No	Molecular weight distribution, concentration
Viscometry	More accurate from universal calibration	Yes, hydrodynamic radius (Rh).	Conformation, branching. Works with copolymers
Light scattering	Absolute determination	Yes, radius of gyration (Rg) directly.	Conformation, branching
Triple	Absolute determination	Yes, Rg and Rh, directly.	The ultimate configuration for comprehensive polymer characterization

Agilent InfinityLab II 1260 with MDS

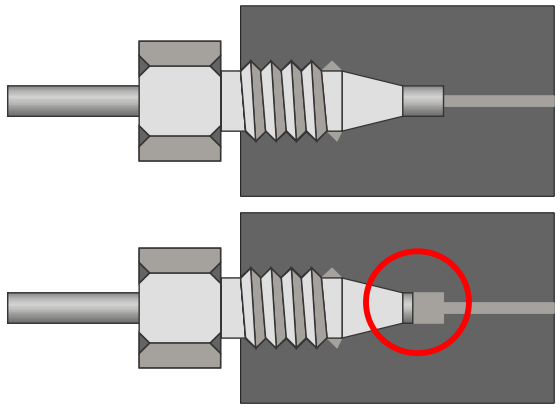
Advanced Detection for Proteins and Biomolecules

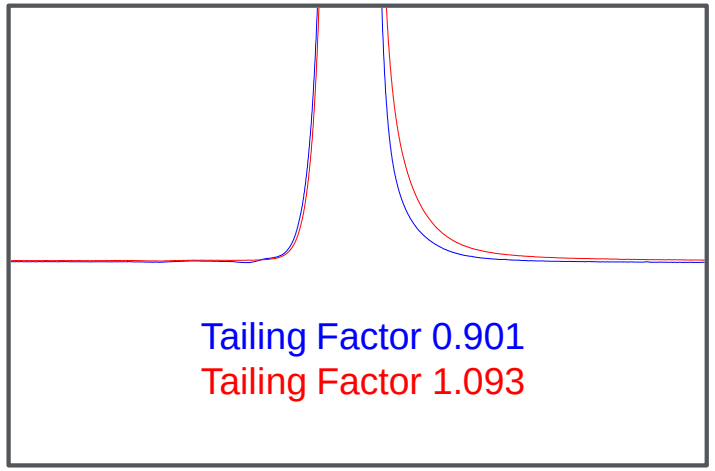
Agilent 1260 Infinity with Multi-Detector suite

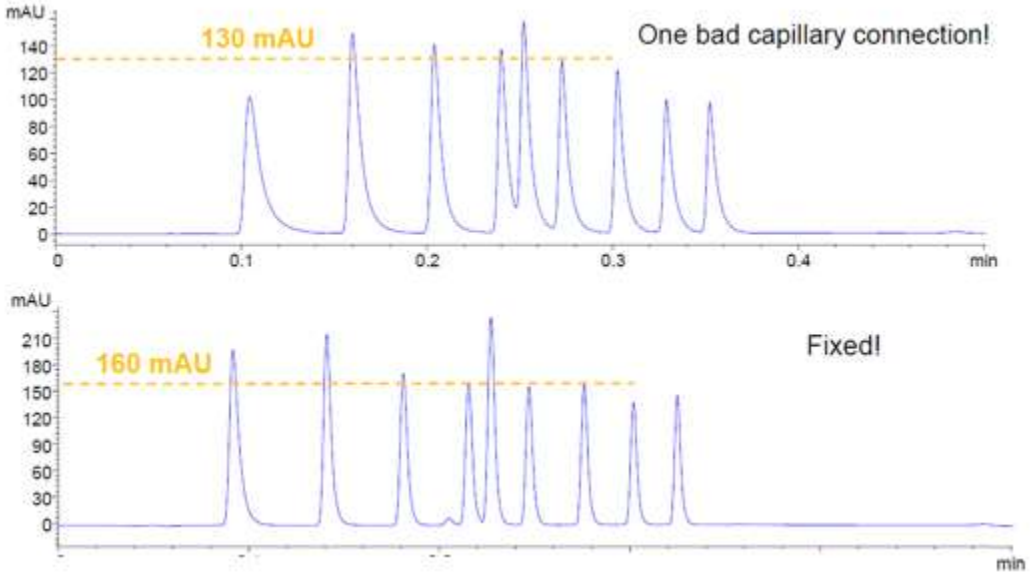
- Combined static and dynamic light scattering detector simultaneously determines the absolute molecular weight (mol wt), as well as the size of the molecule (Rh)
- A solution for sizing and aggregation studies of proteins and other large biomolecules using size exclusion chromatography

Best Practice

AVOID BAD CONNECTIONS !
Ensure column connections do not leave dead spots/voids.
Use proper fittings/ferrules to ensure correct connections







Agilent quick Connect &
Quick Turn fittings

Agilent Quick Turn

Instrument considerations
Best practices

Low dispersion LC
Use optimal tubing ID and minimize length to reduce extra-column volume and band broadening

Use Correct Data Collection Rates
Data collection rates of 10 – 20 Hz could result in 4 – 5% reduction in column efficiency compared to 40 or 80 Hz.*

* - if working with a sub 2um SEC column

In Summary

Agilent CrossLab
From Insight to Outcome

Sample type and solvent selection	Consider you choice of solvent carefully for the type of sample, conditions, and columns required for analysis.
Column selection	Organic or aqueous. Polymer or protein. Look to make the appropriate selection based on expected mol wt range, but also be sure to ask ‘what is it that I want or need for my analysis’?
Column selection and key requirement of analysis	Choose the right pore size packing for your sample. Multiporous, MIXED,or individual. Keeping mind your key requirement, be sure to also consider particle size, pore volume, and # of columns needed
Detectors and instrument considerations	Concentration type detectors for conventional GPC/SEC or look to Multi-Detector SEC to get additional information for your polymer or protein sample

Resources for Support

- GPC/SEC Columns and Standards Product Guides: 5990-7994EN, 5990-7995EN, 5990-7996EN
- Agilent AdvanceBio SEC columns webpage: https://www.agilent.com/en/products/liquid-chromatography/lc-columns/biomolecule-separations/advancebio-sec
- Size Exclusion Chromatography for BioMolecule Analysis: A “How To” guide: https://www.agilent.com/cs/library/primers/public/5991-3651EN_LR.pdf
- Agilent Community for Liquid Chromatography: https://community.agilent.com/community/technical/lc
  - LC Documents
  - LC Helpful Links
  - LC Videos
- Consumables Chemistry and Supplies Resource page: https://community.agilent.com/docs/DOC-1952-collection-of-consumables-resources.
  - Quick reference guides
  - Catalogs, consumables supplies guide, column user guides
  - Online selection tools, how-to videos

Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 Option 3, Option 3:

Option 1 for GC and GC/MS columns and supplies

Option 2 for LC and LC/MS columns and supplies

Option 3 for sample preparation, filtration and QuEChERS

Option 4 for spectroscopy supplies

Option 5 for chemical standards

Available in the USA and Canada 8-5 all time zones

gc-column-support@agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

chem-standards-support@agilent.com

Thank you for attending

Any questions?