GPC/SEC Column Selection & Method Development

- LIVE WEBCAST: Tuesday April 27, 2010

Topics
- Introduction to GPC/SEC
- Column Characteristics & Column Selection
- Additional Method Development
- Troubleshooting
Introduction to GPC/SEC

- GPC: Gel Permeation Chromatography
- SEC: Size Exclusion Chromatography
- GFC: Gel Filtration Chromatography

GPC/SEC/GFC is pore-size limited, diffusion-controlled liquid chromatography (LC) method.
Introduction to GPC/SEC

Separation mechanism

**GPC/SEC Column**
Filled with porous particles; diffusion into the pores when size fits

**Sample Mixture**
- size: hydrodynamic radii
- color: different chemistry

**GPC/SEC Detector Elugram**
Signal Intensity vs. Elution Volume;
1) solvent elutes
2) large sizes
3) small sizes
Introduction to GPC/SEC

Required Items

Columns are the heart of the system! Without sufficient separation only limited information (even when intelligent detection is used).

Isocratic pump

Injection system

1 or more detectors (depending on application)

Calibration Standards, Reference Materials, Validation Standards

GPC/SEC data acquisition and evaluation software
Introduction to GPC/SEC

Applications
a) Polymer/Biopolymer characterization using various detectors
b) Separation of mixtures into discrete fractions

Typical samples
• Synthetic polymers, molar mass range 100 to 30 000 000 Da
  ▪ Synthetic copolymers
  ▪ Water soluble (charged) polymers, molar mass range 100 to 30 000 000 Da
  ▪ Proteins
  ▪ Polysaccharids
  ▪ Polypeptides
  ▪ ....
## Introduction to GPC/SEC

### Results

<table>
<thead>
<tr>
<th>GPC/SEC Results</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete Molar mass distribution (MMD)</td>
<td>One of a few methods!</td>
</tr>
<tr>
<td>$M_n$, $M_w$, $M_z$, PDI</td>
<td>+ other averages</td>
</tr>
<tr>
<td>% material above/below MM ranges</td>
<td></td>
</tr>
<tr>
<td>$[\eta]$, branching index $g'$, structure</td>
<td></td>
</tr>
<tr>
<td>Radius of gyration, branching, structure</td>
<td></td>
</tr>
<tr>
<td>Composition (Copolymers)</td>
<td></td>
</tr>
<tr>
<td>Concentration residual monomer, solvent, additive content</td>
<td></td>
</tr>
<tr>
<td>Substance Identification</td>
<td></td>
</tr>
<tr>
<td>On-line viscometer required</td>
<td></td>
</tr>
<tr>
<td>On-line MALLS required</td>
<td></td>
</tr>
<tr>
<td>2 concentration detectors required</td>
<td></td>
</tr>
<tr>
<td>Detector response calibration required</td>
<td></td>
</tr>
<tr>
<td>FTIR/NMR/ESI-MS/MS required</td>
<td></td>
</tr>
</tbody>
</table>
Introduction to GPC/SEC

From Detector Elugram to Molar Mass Distribution

Elugram

- Y: Signal Intensity
  X: Elution Volume

- Left: high molar masses
  Right: low molar masses

- Sample information AND system parameters contribute to $V_e$

- required for MMD: $(\log) M = f(V_e)$
Introduction to GPC/SEC

From Detector Elugram to Molar Mass Distribution

How to get the molar mass $M$ at a elution volume $V_e$:

- Calibration with narrow or broad standards, universal calibration with Mark-Houwink coefficients
- Universal calibration curve with on-line viscometer
- Measure $M$ online with on-line light scattering detector (LS, $dn/dc$ required)
Introduction to GPC/SEC

- Calibration Curve

Calibration Curve - (depends on accessible pore volume)
Introduction to GPC/SEC

From Detector Elugram to Molar Mass Distribution

Molar Mass Distribution

- Y: \( w(\log M) \) (transformed!)
  X: M (log scale, to compress)

- Left: low molar masses
  Right: high molar masses

- System parameters eliminated when using the calibration curve

- Only sample information present

Inter-laboratory sample comparison
Introduction to GPC/SEC

Calibration Curve

Has all information about the column material’s pore size distribution!

(Used in inverse SEC to characterize networks!)

Users can learn about

- Molar mass separation range
- Resolution in a molar mass range
Introduction to GPC/SEC

- Mixture Polystyrene injected onto 2 different column sets (overlaid with corresponding calibration curve)

- Slope of the calibration curve:
  - Steep: low resolution (in this molar mass area)
  - Shallow/Flat: high resolution (in this molar mass area)
Column Characteristics

Ideal GPC/SEC column

- Minimal/No interactions between sample and column material (stationary phase)
- High resolution (large pore volume)
- Minimal shear degradation (especially for high molar masses)
- Low column backpressure
- Good mechanical, chemical and thermal stability
Other important analytical tasks

- High throughput
- Time savings
- Save eluent
- High sample loading for fractionation
Column Characteristics

Optimize 4 Parameters to find the best column(s) for your application.

- Column Type/Dimensions
- Chemistry Column Material
- Particle Size Column Material
- Porosity Column Material
## Column Characteristics

- **Column Type/Dimensions**

Task: Find the column that meets your application needs

<table>
<thead>
<tr>
<th>Typical Dimensions [mm]</th>
<th>Preparative</th>
<th>HighSpeed</th>
<th>Analytical</th>
<th>Semi-micro</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID: 20</td>
<td>ID: 20</td>
<td>ID: 8 mm</td>
<td>ID: 4.6 mm</td>
<td></td>
</tr>
<tr>
<td>Length: 300/600</td>
<td>Length: 50</td>
<td>Length: 300</td>
<td>Length: 250</td>
<td></td>
</tr>
<tr>
<td>Flow-rate [mL/min]</td>
<td>3 - 10</td>
<td>3 - 10</td>
<td>0.5 – 1</td>
<td>0.2 - 0.5</td>
</tr>
<tr>
<td>Time consumption per column [min]</td>
<td>12.5</td>
<td>2</td>
<td>12.5</td>
<td>10</td>
</tr>
<tr>
<td>Eluent consumption per column [mL]</td>
<td>78.1 (6.25 mL/min)</td>
<td>12.5 (6.25 mL/min)</td>
<td>12.5 (1 mL/min)</td>
<td>3.5 (0.3 mL/min)</td>
</tr>
<tr>
<td>Maximum loading per column*</td>
<td><strong>10 mg/mL, Up to 1000 µL</strong></td>
<td>2 mg/ml, 20 µL</td>
<td>2 mg/ml, 20 µL</td>
<td>2 mg/ml, 5 µL</td>
</tr>
</tbody>
</table>

* Depends largely on molar mass and polydispersity, loading maximized (not separation)
Column Characteristics

- Chemistry: Column Material

Column materials:
- mostly designed either for organic solvents or aqueous solvents
- either polymer beads (copolymer networks, usually manufactured via suspension polymerization) or silica based
Column Characteristics

- **Chemistry: Column Material**

Task: GPC/SEC must be interaction free

Apply different solvent – interaction dominates
### Column Characteristics
- **Chemistry: Column Material**

<table>
<thead>
<tr>
<th></th>
<th>Polymer Packing</th>
<th>Inorganic packing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polarity</strong></td>
<td>Non</td>
<td>Medium</td>
</tr>
<tr>
<td><strong>Chemistry</strong></td>
<td>St-DVB</td>
<td>Acrylic, Polyester</td>
</tr>
<tr>
<td><strong>Solvents</strong></td>
<td>THF, TCM, Toluol</td>
<td>DMF, NMP, DMAc, DMSO, (all)</td>
</tr>
<tr>
<td><strong>Examples</strong></td>
<td>SDV, POLEFIN</td>
<td>GRAM</td>
</tr>
<tr>
<td><strong>Samples</strong></td>
<td>PS, PMMA PVC, PC, Resins, PE, PP, etc.</td>
<td>PU, Starch, Cellulose, Polyimide etc.</td>
</tr>
</tbody>
</table>
Column Characteristics

- Chemistry: Column Material
  Task: GPC/SEC must be interaction free

Balance Polarity:
- Sample
- Solvent
- Stationary Phase

To avoid doing HPLC on a GPC/SEC column!
Column Characteristics

Solvent: DMF

Polystyrene 1620 Da on divinylbenzene gel

Polystyrene 1620 Da on polyester gel

Calibration curves on divinylbenzene gel

Calibration curves on polyester gel
Column Characteristics

- Particle Size

Optimum packing and resolution:

- spherical particles with narrow particle size distribution

Lower particle size results in:

- higher plate count
- higher resolution
- higher column backpressure
- higher shear forces
- lower mechanical stability (large porosity)
Column Characteristics

- **Particle Size**

**High viscous solvents** (e.g. water, DMAc) use higher particle sizes to reduce back pressure and shear forces.

**High molar masses** use higher particle sizes to avoid shear degradation.

Typical particle sizes

- 5 µm (low viscous solvents)
- 10 µm (high viscous solvents)
- 10-20 µm (to avoid flow-induced degradation)
- 3 µm (reduce flow-rate to reduce column back pressure)
Column Characteristics

- Particle Size

Influence particle size on resolution:

Same protein mixture injected using the same chromatographic conditions but columns with different particle size:

- PSS SUPREMA 10µm (8x300 mm)
- PSS SUPREMA 5µm (8x300 mm)
Column Characteristics

- Porosity

Linear/Mixed Bed Column

Many different pore sizes in the same column (by synthesis or mixture of single porosity materials)

Constant resolution over a wide molar mass range, wide linear molar mass range

Single Porosity Column

100Å

Narrow pore size distribution

100 000Å

Highest resolution in a smaller molar mass range
Column Characteristics

- Porosity

<table>
<thead>
<tr>
<th>Pore type</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single porosity column</td>
<td>Efficient, optimized, low cost</td>
<td>Viscous fingering</td>
</tr>
<tr>
<td>Linear column</td>
<td>Fast, universal, inject band dilution</td>
<td>Low efficiency</td>
</tr>
</tbody>
</table>
Column Characteristics

- Porosity

Use column combination/bank to increase:

Resolution: Add column of the same porosity (increase accessible pore volume)

Doubling column length increases resolution by a factor of 1.4 \( \left( \sqrt{\text{Length}} \right) \).

Separation range: Add column of different porosity

Avoid: column mismatch

Disadvantages of column combinations:

- increased analysis time, eluent consumption, back pressure
Column Characteristics

- Porosity

Use multiple columns of the same porosity to increase resolution

Protein (Monomer/Dimer):

PSS SUPREMA 5µm, 100Å (8x300 mm)

PSS SUPREMA 5µm, 2x 100Å (8x300 mm each)
Column Characteristics

- Porosity

Increase separation range: add column of different porosity but be aware of column mismatch, do not confuse mismatch with higher resolution.

If the pore size distributions of the columns do NOT match: bimodal peaks, peak shoulders may occur.

Example data:
Dextrans on a column bank with porosity mismatch
Column Characteristics

- Porosity

Mismatch-free combination/bank

Example data:
Dextrans on a column bank
SUPREMA 1000 Å and 30 Å
Column Characteristics

- **Porosity**
  Calibration curve can show mismatch (2 slopes), but this must not be the case.

Potentially problematic are the following combinations:
- Linear column with low porosity single porosity column
  Example: Linear + 100 Å to increase resolution for oligomers

- Single porosity column combinations with a wide gap in porosity
  Example: 10 000 000 Å + 1000 Å
Column Characteristics

- Porosity

Calibration curve can show mismatch (2 slopes), but this must not be the case.

- linear and 100 Å
- $10^7$ Å and 1000 Å
- mismatch-free

Polystyrene: 48 000 g/mol
Column Characteristics

- Porosity

How to avoid column mismatch?

- Use column combinations/banks recommended by the manufacturer.
- Never combine linear columns with single porosity column.
- If in doubt (e.g. because of unexpected shoulders):
  - have a look at the calibration curve
  - overlay different samples and see if the shoulder appears always at the same elution volume
  - use broad standards to detect column mismatch
Additional Method Development

For macromolecules with or without functional groups
  • neutral polymers
  • negatively charged polymers
  • positively charged polymers

Application in
  • organic solvents
  • aqueous solvents


Additional Method Development

Why additional method development and solvent additives?

To overcome non-size exclusion effects.

Potential problems:  
- ion exchange
- ion inclusion/exclusion
- (intramolecular) electrostatic interactions
- adsorption
- sample aggregation

- Column material is made by suspension polymerization, there are potential residuals/charges on column and imperfections in surface chemistry.
- Adding electrolyte eliminates ion exchange/ion exclusion, aggregate formation and shields electrostatic interactions.
Additional Method Development

Salt effect

Norbonenderivative GRAM 10µm 100 Å

Method development includes variation of ionic strength.
# Additional Method Development

## Organic Solvents

<table>
<thead>
<tr>
<th>funct. Group</th>
<th>suitable additives</th>
</tr>
</thead>
<tbody>
<tr>
<td>-OH</td>
<td>Alcohol (Methanol, Ethanol)</td>
</tr>
<tr>
<td>-NH₂</td>
<td>Diethylamine</td>
</tr>
<tr>
<td>-COOH</td>
<td>Acetic acid, Trifluoro acetic acid (TFA)</td>
</tr>
</tbody>
</table>

**Solvent:**

+ 5g/L LiBr (DMF, DMAc, DMSO)

+ Additive: 0.05...2%

(lowest possible concentration)

**Columns:**

(SDV), GRAM, GRAL
Additional Method Development

Aqueous Solvents
Additional Method Development

Aqueous Solvents, Neutral Polymers:

- Polymer with functional groups: -CO-NH2, -OH, Polysaccharides

**Solvent (buffer):**

0.05M...0.1M NaNO₃, NaCl, Na₂SO₄

+ Additive

**highly unpolar parts:**

+ 20% Acetonitrile or
+ 20% Methanol

**Columns:** SUPREMA
Additional Method Development

Aqueous Solvents, Polyanions:

- Polymer with functional groups: -COO\(^{-}\) Na\(^{+}\), -SO\(_4\)\(^{-}\) Na\(^{+}\)

**Solvent (buffer):**

- 0,05M...0,1M NaNO\(_3\), NaCl, Na\(_2\)SO\(_4\) + Additive
- or
- 0,05M...0,1M Na\(_2\)HPO\(_4\) (pH = 9) (buffer)

+ Additive highly unpolar parts:
  + 20% Acetonitrile or
  + 20% Methanol

**Columns:** SUPREMA, MCX
Additional Method Development

Aqueous Solvents, Polycations:

- Polymer with functional groups: -NH4+, Cl-

**Solvent (buffer):** + Additive

0.1 Vol% - 1.5 Vol% Formic acid

to

0.1 Vol% - 1.5 Vol% Trifluoro acetic acid (TFA)

Columns: NOVEMA, SUPREMA-MAX
Troubleshooting

- Column Performance Tests:

Plate count (half height method):

\[ N = \left( \frac{V_p}{\sigma} \right)^2 = 5.54 \left( \frac{V_p}{w_{0.5}} \right)^2 \]

- Measured using monodisperse low molar mass sample.
- Depends on: sample, detector, loading, tubing length, column dimensions, packing, solvent, flow rate…

Determine plate count for your system and verify regularly.
Test single columns if column bank fails.

Theoretical plate counts/m

- 3µm 167 000
- 5µm 100 000
- 10µm 50 000
- 20µm 25 000
- 40µm 12 000
Troubleshooting

- Column Performance Tests:

Resolution:

\[ R_s = \frac{V_2 - V_1}{2 \cdot (\sigma_1 + \sigma_2)} = \frac{\log(M_1/M_2)}{2D \cdot (\sigma_1 + \sigma_2)} \]

- Value and system dependent, using different standards yield different results.

- Specific resolution (sample independent):

\[ R_{sp} = \frac{R_s}{\log(M_1/M_2)} \]

D: slope calibration curve
V: peak volume
\( \sigma \): peak variance

Determine the resolution and verify regularly.
Troubleshooting

- Installing new columns:

  1) Installation of a column from a different manufacturer: The distance from ferrule to the end of the column can be very different. Change all fittings and ferrules connected to the column, otherwise you may damage your column.

  2) Be aware of column flow direction. Operating the flow rate in the reverse direction is only part of troubleshooting or operating after a long storage time.

  3) Thread the column fittings finger tight into the system. Do not overtighten the fitting. Overtightening may damage the column and the column head.
Troubleshooting

- Storing columns:

To store columns:

1) Remove all salt solutions with pure solvents and plug tight with the original end plugs.

2) Keep columns with volatile mobile phases in a refrigerator (4°C) after use to prevent solvent evaporation. Never let the column temperature fall below the freezing point of the storage solvent. This can destroy the stationary phase.

3) Occasionally solvent is lost during long-term storage. When the expected pressure does not build up, it is an indication of a partially evaporated column solvent.
Troubleshooting

- Recovering partially dry columns:

To re-wet a partially dry column:
1) Install the column in the reverse direction.

2) Fill the column with solvent, using a flow rate of 0.1 ml/min, until no more bubbles appear at the column outlet.

3) Change the column to the correct flow direction and use 0.1 ml/min for 2h.

4) Increase the flow rate slowly to the operating flow rate with an increment of 0.2 ml/min every 5 minutes.
Troubleshooting

- Cleaning Columns:

When columns lose efficiency or you suspect the presence of foreign material adsorbed on the column:

1) Remove the column from the detector.
2) Install the column in reverse direction for clean-up.
3) Flush the column at 0.1 ml/min flow rate with a solvent that dissolves the suspect impurities and is fully compatible with your system.

Aqueous columns: use variation of pH, buffer concentration, or mixtures of organic and aqueous eluents.

Organic columns: use variation of eluent polarity. Use mixtures of appropriate solvents (e.g. THF in Toluene resp. chloroform) or solvent additives (TFAc in THF).