Innovative particle and column technology for the high throughput LC-MS analysis of biomolecules

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Sample Characteristics and Mode of Chromatography

- Size (Molecular Weight)
- Molecular Charge
- Hydrophobic Regions
- Conformational Recognition Sites
- Size Exclusion
- Anion Exchange
- Cation Exchange
- Ion Pair
- Normal Phase
- Reversed Phase
- Hydrophobic Interaction
- Affinity
- Chiral
Liquid Chromatography and Mass Spectrometry

• Liquid chromatography
  ▪ Fundamental separation technique in bioscience, chemistry and related field
  ▪ Suitable for nonvolatile compounds
  ▪ Suitable for compounds prone to breakdown at high temperature
  ▪ Widely used for small molecules and macromolecules

• Mass spectrometry
  ▪ Signal strength
  ▪ Mass
Advantages of Mass Spectrometry

- High sensitivity
- High specificity
- Doesn’t need a chromophore in the analytes
- Able to analyze poorly resolved peaks also

Liquid chromatography coupled with mass spectrometry, is a very useful analytical tool not only for identification but also for quality control purpose in qualitative and quantitative analyses.

Mass spectrometry is used in forensic toxicological analysis.
Factors in analysis – a puzzle
Concerns for LC-MS

- Concentration of the sample

- Mobile phase compatibility
  - select the solvents and buffers carefully.

- Interference coming from mobile phase or sample preparation with regards to ionization and signal strength need to be optimized

- The column
Advantages of small particle size columns

● Higher efficiency
  ▪ Shorter diffusion path length through the particles

● Increased resolution

● Optimization of the extra-column components can significantly improve column performance.
  ▪ Reduce ID capillary tubing, detector cell volume
  ▪ Select a fast detector response time
  ▪ Reduce sample injection volume (≤ 2mm ID)
SEM of HPLC Column Packing materials

(3μm)  (4μm)  (6μm)
Advantages of small particle size columns

● Smaller particles are more efficient

  ▪ at the expense of higher back pressure compared to columns packed with larger particles.

  ▪ usually traded off by reducing column length
    o shorter analysis times
    o lower column back pressure

● Three micron packed columns are now a common tool in many LC-MS applications.
Bonded phase of HILIC Column

- Polar stationary phase similar to normal phase
- Mobile phase similar to reversed phase (high % organic)
- Elution in order of increasing hydrophilicity

TSKgel Amide-80
TSKgel NH₂-100
Bonded phase structure in Ion Exchange chromatography

TSKgel STAT columns

- Very efficient chromatography
  - for high as well as low MW solutes
  - novel bonding chemistry and the absence of micro-pores
- High speed and high resolution analysis of biomolecules
- Higher adsorption capacities and lower pressures compared with competitive non-porous columns
- 7 or 10µm particles for SP and CM chemistries
Bonded phases in Reversed phase Chromatography

Three micron packed TSKgel ODS-100V and ODS-100Z columns are effective tools to increase throughput and improve precision in LC-MS applications.
Bonded phases in Hydrophobic Interaction Chromatography (HIC)
Monoclonal antibodies (mAbs) have a MW of about 150,000 and would fall here on the calibration curves. Most of our customers use TSKgel G3000SW and TSKgel G3000SWxl columns for their mAb analysis.
### Gel Filtration Chromatography (GFC) Column Selection

#### Molecular mass separation ranges (Da) of TSKgel SW<sub>XL</sub> Series

<table>
<thead>
<tr>
<th>Column</th>
<th>Polyethylene glycol (linear)</th>
<th>Dextran (branched)</th>
<th>Protein (globular)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2000SW&lt;sub&gt;XL&lt;/sub&gt;</td>
<td>500~15,000</td>
<td>1,000~30,000</td>
<td>5,000~100,000</td>
</tr>
<tr>
<td>G3000SW&lt;sub&gt;XL&lt;/sub&gt;</td>
<td>1,000~35,000</td>
<td>2,000~70,000</td>
<td>10,000~500,000</td>
</tr>
<tr>
<td>G4000SW&lt;sub&gt;XL&lt;/sub&gt;</td>
<td>2,000~250,000</td>
<td>4,000~500,000</td>
<td>20,000~7,000,000</td>
</tr>
</tbody>
</table>
Proteins (general)
Select appropriate pore size based on knowledge or estimate of protein MW

Protein of unknown molecular weight
TSKgel G3000SW$_{XL}$
Ideal investigational column (Scouting column)

If peak elutes near the exclusion volume
Switch to TSKgel G4000SW$_{XL}$

If peak elutes near the end of the chromatogram
Switch to TSKgel G2000SW$_{XL}$
Analysis of purified mAb

Both runs are at same linear velocity

Mobile phase: 0.1mol/L Phosphate buffer (mono/dibasic), 0.1mol/L NaCl, 0.05% NaN₃, pH 6.8; Flow rate: 1mL/min; Detection: UV@280nm (micro flow cell); Injection volume: 5µL
Dependence of HETP on sample load

Sample: Bovine Serum Albumin (BSA)

~250µg
The Characterization of Biological Samples by Microbore TSKgel SuperSW3000 SEC Columns

Column:
TSKgel SuperSW3000 microbore, 1mm ID

• excellent choice for the rapid separation of proteins and enzymes at micro scale

• a great fit for the trace analysis of biological components by LC-MS.

Fraction of interest analyzed by off-line SELDI/TOF/MS to establish presence of BSA aggregates and IgG
LC/MS Chromatogram (TIC) of β-lactoglobulin Tryptic Digest

In this analysis we have used a highly consistent and chemically stable silica-based octadecylsilane stationary phase (TSKgel Super-ODS column) for LC-MS
TSKgel Super Series Columns

• Increased efficiency

• Reduction in analysis time

• Higher resolution

• TSKgel Super Series columns are available in:
  ▪ 1, 2, or 4.6mm ID
  ▪ 5 or 10cm length

• These ultra efficient columns are coupled with the specificity of Mass Spec, which results in superior analytical power.
Three micron packed TSKgel ODS-100V and ODS-100Z columns are effective tools to increase throughput and improve precision in LC-MS applications.
Rapid Identification of 20 Peptides

- Unwanted secondary ionic interactions from residual silanols can be eliminated by adding trifluoroacetic acid (TFA) to the mobile phase.

- The use of “mass-spec friendly TFA” eliminates extra steps involved with removing salts or non-volatile acids required by amino-bonded columns to eliminate ionic interactions.
Low level of background noise (indication of low bleeding) was observed in the total ion chromatogram (TIC) for LC/ESI/MS using an acidic mobile phase containing 0.1% formic acid with a gradient elution method. No sample was injected.

This data suggests that TSKgel ODS-100V, 3µm columns are well suited for LC-MS applications.
Bonded Phase HILIC Columns

- Polar stationary phase similar to normal phase
- Mobile phase similar to reversed phase (high organic)
- Elution in order of increasing hydrophylicity

TSKgel Amide-80
TSKgel NH₂-100
Both can be used for with evaporative light scattering (ELS) and mass spec (MS) detectors.

The **3μm material** – for use in LC/MS applications for the analysis of active pharmaceutical ingredients and their metabolites.
TSKgel NH$_2$-100 - Expanded HILIC Selectivity

Columns: TSKgel NH2-100, 3µm, 4.6mm ID x 15cm
TSKgel Amide-80, 3µm, 4.6mm ID x 15cm
Eluent: H$_2$O/ACN=10/90–90/10 (vol.%)
Flow rate: 1.0mL/min
Temp: 40ºC
Detector: RI
Sample: inositol
Injection vol.: 10µL
Identification of Isobaric Glycoforms by Retention Time (Glycobase) and MS/MS Experiments

Protein construct of the zp domain of murine tgifr-3 expressed in HEK293EBNA
Separations of 2-AB Labeled N-glycans

Fluorescence chromatograms of HILIC separations of 2-AB labeled N-glycans released from the recombinant ZP domain construct of murine TGFR-3, were compared to the dextran ladder.

<table>
<thead>
<tr>
<th>CHROMATOGRAPHIC PARAMETERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column: TSKgel Amide-80 3 µm (2 mm ID x 15 cm L)</td>
</tr>
<tr>
<td>HPLC: Shimadzu Prominence</td>
</tr>
<tr>
<td>Flow rate: 0.22 mL/min</td>
</tr>
<tr>
<td>Mobile phase: A: 50 mM ammonium formate (pH 4.3) B: acetonitrile</td>
</tr>
<tr>
<td>Gradient: 0 - 35 min: 75 - 35 % B</td>
</tr>
<tr>
<td>Temperature: 50 °C</td>
</tr>
<tr>
<td>Detection: Fluorescence; excitation @ 360 nm, emission @ 425 nm</td>
</tr>
<tr>
<td>Injection: 2 µL, approximately 300 fmol for GU3 (Figure 3)</td>
</tr>
</tbody>
</table>

The structure analysis was completed by high resolution mass spectra acquired on a MALDI QIT TOF MS instrument.
Separations of 2-AB Labeled N-glycans

Dextran ladder (A) →

PNGaseF digest (B) →

Sequential exoglycosidase digests (C-F) →

Used exoglycosidases:
Sialidase A (Abs),
α-Fucosidase (Bkf),
β-Galactosidase (Btg),
β-N-Acetylhexoamidase (Guh).
MALDI Mass Spectrum of 2-AB-labeled Glycans Released from ZP domain Construct of Murine TGFR3
MS2 (CID) Mass Spectrum of m/z 2243

[M+2Na-H]⁺ = 2242.99
MS3 (CID) Mass Spectrum of m/z 1930
Biogenic Amines in Tuna as Function of Storage

Spd
- 0.6µg/L

His
- 77.4µg/L
- 1.6µg/L

Put
- 3.6µg/L

Tyr
- 5.0µg/L
- 0.1µg/L

Cad
- 11.7µg/L

Trp
- 0.2µg/L
- 0.2µg/L

Stored at room temperature for 2 days
Stored frozen
TSKgel STAT Ion Exchange Columns

- Very efficient chromatography
  - for high as well as low MW solutes
  - novel bonding chemistry and the absence of micro-pores
- High speed and high resolution analysis of biomolecules
- Higher adsorption capacities and lower pressures compared with competitive non-porous columns
- 7 or 10µm particles for SP and CM chemistries
## Basic Properties of TSKgel STAT Columns

<table>
<thead>
<tr>
<th></th>
<th>TSKgel Q-STAT</th>
<th>TSKgel SP-STAT</th>
<th>TSKgel CM-STAT</th>
<th>TSKgel DNA-STAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Base materials</strong></td>
<td>Hydrophilic</td>
<td>Hydrophilic</td>
<td>Hydrophilic</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td></td>
<td>non-porous</td>
<td>non-porous</td>
<td>non-porous</td>
<td>non-porous</td>
</tr>
<tr>
<td></td>
<td>resin</td>
<td>resin</td>
<td>resin</td>
<td>resin</td>
</tr>
<tr>
<td><strong>Particle size</strong></td>
<td>7, 10µm</td>
<td>7, 10µm</td>
<td>7, 10µm</td>
<td>5µm</td>
</tr>
<tr>
<td></td>
<td>(mono-disperse)</td>
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<td>(mono-disperse)</td>
<td>(mono-disperse)</td>
</tr>
<tr>
<td><strong>Ligand</strong></td>
<td>Quaternary</td>
<td>Sulfopropyl</td>
<td>Carboxymethyl</td>
<td>Quaternary</td>
</tr>
<tr>
<td><strong>Column dimensions</strong></td>
<td>3.0 x 3.5</td>
<td>3.0 x 3.5</td>
<td>3.0 x 3.5</td>
<td>4.6 x 10</td>
</tr>
<tr>
<td>mm ID x cm</td>
<td>4.6 x 10</td>
<td>4.6 x 10</td>
<td>4.6 x 10</td>
<td>4.6 x 10</td>
</tr>
</tbody>
</table>
Protein Separations on Non-Porous Anion Exchange Columns

Commercial WAX column
4.0mmI.D. x 25cm
Rs = 10.1

TSKgel Q-STAT
4.6mmI.D. x 10cm (7um)
Rs = 15.3

Improved protein peak shapes on TSKgel Q-STAT vs. non-porous WAX column.
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

Commercial monolithic
5.0mm I.D. x 5cm

TSKgel SP-STAT
3.0mm I.D. x 3.5cm (10um)
Separation on Toyopearl GigaCap resins

Structure:

\[
\text{HW-65} \cdot \text{O-} \cdot \text{R'} \cdot \text{SO}_3^- \\
\text{strong cation exchanger}
\]
(Note: \(R'\) = proprietary polymer)

Structure:

\[
\text{HW-65} \cdot \text{O-} \cdot \text{R'} \cdot \text{N}^+ \cdot (\text{CH}_3)_3
\]
(Note: \(R'\) = proprietary)

Product Attributes:

<table>
<thead>
<tr>
<th>Pore size (mean)</th>
<th>1,000\AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (mean)</td>
<td>75\mu m (M-grade)</td>
</tr>
<tr>
<td>Pressure rating</td>
<td>3 bar</td>
</tr>
<tr>
<td>Shipping buffer</td>
<td>20% ethanol</td>
</tr>
<tr>
<td>pH stability</td>
<td>3-13</td>
</tr>
<tr>
<td>Shelf life (estimated)</td>
<td>10 years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pore size (mean of base bead)</th>
<th>1,000\AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (mean)</td>
<td>75\mu m (M-grade)</td>
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<tr>
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<td>Shelf life (estimated)</td>
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Toyopearl GigaCap S-650M is the first in a family of ion exchange resins optimized for high throughput chromatography of IgG.

The high capacity for lysozyme shows that Toyopearl GigaCap S-650M will be an excellent resin for smaller proteins as well.

* Toyopearl GigaCap is a registered trademark of Tosoh Corporation
The new Toyopearl GigaCap S-650M resin featured very high binding capacities with excellent binding kinetics and almost quantitative recoveries (data not shown).
Conclusions

- The new surface modifications improve not only chromatographic performance but also are effective tools to increase throughput and improve precision in LC-MS applications.
Innovative particle and column technology for the high throughput LC-MS analysis of biomolecules

Thank You for your attention

Atis Chakrabarti, Ph.D.
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