Comparison of Optimized Wide Pore Superficially Porous Particles (SPPs) Synthesized By One-Step Coating Process With Other Wide Pore SPPs For Fast And Efficient Separation Of Large Biomolecules

Wu Chen, Anne Mack, and Xiaoli Wang
Agilent Technologies, Inc.
Outline

1. Introduction of wide pore superficially porous particles (SPPs)
2. Performance of optimized SPPs for large molecule separation
3. Comparison of wide pore totally porous particles (TPPs)
4. Comparison of other wide pore SPPs
5. Conclusion
## Current Status of Superficially Porous Particles

<table>
<thead>
<tr>
<th>Status in 2000</th>
<th>Status in 2010</th>
<th>Status in 2015</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Vendors</td>
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</tr>
<tr>
<td>Small molecules</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Large molecules</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

- Ron Majors: “In my recent Pittcon 2014 article, in terms of new product introduction, it seems like the entire columns world has turned to SPP as the favored type of HPLC or UHPLC column, with SPP introductions exceeding sub-2-um columns by a 10:1 margin”.

Bell, LC-GC, 2015 June
Majors, LC-GC, 2014 Nov
Layer-by-Layer Process for Producing Superficially Porous Particles

1. Raise pH
2. Add cationic polymer, rinse
3. Add silica sol, rinse
4. Add more cationic polymer, rinse
5. Add more silica sol, rinse
6. Repeat coating steps
7. Burnoff polymer, Sinter
8. Add more cationic polymer, rinse
9. Add silica sol, rinse

Solid silica cores made by modified Stöber process

Superficially Porous Particles
Coacervation Process for Producing Superficially Porous Particles

Urea, formaldehyde polymerization coats sol and core. Coated sol then adsorbs to coated core.

Nearly monodisperse solid silica cores made by modified Stöber process.

Burnoff polymer, Sinter.

Superficially Porous Particles.
# Wide Pore SPPs Made by Coacervation Process

<table>
<thead>
<tr>
<th>Particle Size (µm)</th>
<th>Core Size (µm)</th>
<th>Shell Thickness (µm)</th>
<th>SA (m²/g)</th>
<th>BET Pore Size (Å)</th>
<th>Pore Volume (cm³/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.72 - 3.77</td>
<td>1.69 - 2.95</td>
<td>0.20 - 0.51</td>
<td>10 - 31</td>
<td>253 - 460</td>
<td>0.07 - 0.19</td>
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**HPLC 2016**

2.7 µm  
3.5 µm
# Wide Pore SPPs Made by Coacervation Process

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![Image of Wide Pore SPPs](image.png)

**Particle Size:** 2.7 µm

**Core Size:** 3.5 µm

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**Agilent Technologies**
### Effect of Shell Thickness

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<tr>
<td>2.70</td>
<td>2.25</td>
<td>0.23</td>
<td>10</td>
<td>281</td>
<td>0.07</td>
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### Effect of Pore Size

<table>
<thead>
<tr>
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</tr>
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<td>3.46</td>
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<td>454</td>
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### Effect of Particle Size

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<tr>
<td>2.67</td>
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<td>0.21</td>
<td>10</td>
<td>452</td>
<td>0.11</td>
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Summary of Physical Property Study on Large Molecule Separation

Effect of physical property on large molecule separation

Pore size $>>$ shell thickness $>$ particle size
(450 Å $>$ 300 Å) (0.25 µm $>$ 0.50 µm) (3.5 µm $>$ 2.7 µm)

Pore size has the biggest effect on large molecule separation. Large pores results in better resolution and narrow peak width.

The particles with thinner shell have narrow peak width at high flow rate.

The particle size seems not affect peak width, but large particles have lower back pressure.

Effect of Pore Size-Large Molecule Separation

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<td>0.15</td>
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Column: 3.5 µm, SB-C18, 2.1 x 100 mm; flow rate: 1.5 mL/min; gradient: mobile Phase: A: 90% water/10% ACN, 0.1% TFA, 0.3% Polyethylene glycol, B: 90% ACN/10% water, 0.1% TFA, 0.3% PEG; hold 19% B for 0.5 min and then 19%-41% B in 11.5 min; injection: 1 µL; detector: 220 nm; temperature: 80 °C; sample: intact IgG2 lambda.
Optimized Wide Pore SPPs for Large Molecule Separation - AdvanceBio RP-mAb

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Si ligand type</th>
<th>End-capping</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4</td>
<td>n-butyldimethyl</td>
<td>Yes</td>
</tr>
<tr>
<td>SB-C8</td>
<td>n-octyldiisopropyl</td>
<td>No</td>
</tr>
<tr>
<td>Diphenyl</td>
<td>diphenylmethyl</td>
<td>Yes</td>
</tr>
</tbody>
</table>

450Å pore size
Difference Selectivity of A Protein Standard

Columns: 2.1 x 100 mm; gradient: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, 20-50% B in 15 min, 3 min wash at 95% B, 2 min re-equilibration at 20% B; flow rate: 0.3 mL/min; temperature: 60 C; detector: 220, 8 nm; Ref = Off; injection: 5 µL injection of Protein Standard; samples: 1. ribonuclease A (14 kDa), 2. cytochrome C (12 kDa), 3. holo-transferrin (80 kDa), 4. α-lactalbumin (14 kDa), 5. catalase (240 kDa), 6. carbonic anhydrase (30 kDa)
Difference Selectivity of Intact IgG2

Columns: RP-mAb, 2.1 x 100 mm; mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile; gradient: 34-38% B in 3 min, 3 min wash at 95% B, 2 min re-equilibration at 34% B; flow rate: 1.0 mL/min; injection: 5 µL; temperature: 85 °C; detector: 215 nm; sample: intact IgG2 lambda from human myeloma plasma.
Selectivity Comparison of Intact IgG1

Column: 2.1 x 100 mm; A: 0.1% TFA in water/IPA (98/2), B: IPA/acetonitrile/M.P. A (70/20/10), 1.0 mL/min, 10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B, 5 µL injection of Humanized Recombinant Herceptin IgG1 Intact from Creative Biolabs (1 mg/mL), 80 C, 254, 8 nm; Ref = Off.
Effect of Temperature on Separation of Intact IgG1

Column: SPP, C4, 3.5 µm, 450 Å, 2.1 x 100 mm; mobile phase A: 0.1% TFA in water:IPA (98:2), mobile phase B: IPA:acetonitrile:mobile phase A (70:20:10); flow rate: 1.0 mL/min; gradient: 10-70% B in 5 min; detection: UV at 254 nm; sample: 5 µL humanized recombinant Herceptin IgG1 (1 mg/mL).
Carryover Study of A Protein Standard

1) (yellow): blank injection before real sample injection;
2) (blue): real sample injection;
3) (red): blank sample injection after real sample injection.

Column: SPP, C4, 3.5 µm, 450Å, 2.1 x 100 mm; mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile; gradient: 20-50% B in 10 min, 3 min wash at 95% B, 2 min re-equilibration at 20% B; flow rate: 0.5 mL/min; temperature: 60 °C; detector: 220, 8 nm; Ref = off; injection: 2 µL. 1. Ribonuclease A (14 kDa), 2. Lysozyme (14.3 kDa), 3. Cytochrome c (12 kDa), 4. α-Lactalbumin (14 kDa), 5. Catalase (240 kDa), 6. Carbonic anhydrase (30 kDa);
Comparison of Totally Porous Particles-Separation of Intact IgG2 Isomers

Schematic drawing of IgG2 disulfide isoforms from J. Sep. Sci. 2010, 33, 2671–2680

Column: 2.1 x 100 mm; gradient: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile; 34-38%B in 8 min, 3 min wash at 95%B, 2 min re-equilibration at 34%B; flow rate: 1.0 mL/min; injection: 5 µL injection; temperature: 85 degree; detector: 215 nm, 8 nm, Ref = Off; sample: intact IgG2 Lambda from human myeloma plasma
Comparison of Totally Porous Particles-Separation of Intact IgG1

A: 0.1% TFA in water/IPA (98/2), B: IPA/acetonitrile/M.P. A (70/20/10), 1.0 mL/min, 10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B, 5 µL injection of Humanized Recombinant Herceptin IgG1 Intact from Creative Biolabs (1 mg/mL), 80 C, 254, 8 nm; Ref = Off

Best peak shape and resolution →

PW = 0.013
SPP, C4,
2.1 x 100 mm, 3.5 µm,
Pmax = 490 bar

PW = 0.025
Hybrid TPP, C4,
2.1 x 100 mm, 1.7 µm,
Pmax = 910 bar

High Pressure
Comparison of Other Wide Pore Superficially Porous Particles-Physical Properties

<table>
<thead>
<tr>
<th></th>
<th>Particle Size (µm)</th>
<th>Average Pore Size (Å)</th>
<th>Surface Area (m²/g)</th>
<th>Shell Thickness (µm)</th>
<th>Pore Volume (cm³/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other SPP</td>
<td>3.6</td>
<td>200</td>
<td>25</td>
<td>0.20</td>
<td>0.12</td>
</tr>
<tr>
<td>Other SPP</td>
<td>3.4</td>
<td>400</td>
<td>15</td>
<td>0.20</td>
<td>0.11</td>
</tr>
<tr>
<td>SPP</td>
<td>3.5</td>
<td>450</td>
<td>12</td>
<td>0.25</td>
<td>0.12</td>
</tr>
</tbody>
</table>

3.6 µm, 200 Å, SPP

3.4 µm, 400 Å, SPP

3.5 µm, 450 Å, SPP
Comparison of Other Wide Pore Superficially Porous Particles-Pore Size Distribution

- The volume percentage of pores smaller than 200 Å is 0.8% for the 450 Å SPP, 12% for the 400 Å SPP, and 66% for the 200 Å SPP.
Comparison of van Deemter Plot

Columns: 2.1 x 100 mm; mobile phase A: 0.1% TFA in water; mobile phase B: 0.1% TFA in ACN; 43.7% mobile phase B; 41.6% B for the 200Å, 43.3% B for the 400Å and the 450Å SPPs; injection: 1 µL; detector: 220 nm; temperature: 60 °C; sample: carbonic anhydrase (30 kDa).
Comparison of Protein Separation

<table>
<thead>
<tr>
<th>Peak Widths (min)</th>
<th>RP-mAb, 3.5 µm, 450 Å, C4</th>
<th>3.4 µm, 400 Å, C4</th>
<th>3.6 µm, 200 Å, C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A (14 kDa)</td>
<td>0.035</td>
<td>0.036</td>
<td>0.040</td>
</tr>
<tr>
<td>Cytochrome C (12 kDa)</td>
<td>0.035</td>
<td>0.038</td>
<td>0.041</td>
</tr>
<tr>
<td>Holo-transferrin (80 kDa)</td>
<td>0.106</td>
<td>0.126</td>
<td>0.121</td>
</tr>
<tr>
<td>α-Lactalbumin (14 kDa)</td>
<td>0.035</td>
<td>0.034</td>
<td>0.041</td>
</tr>
<tr>
<td>Catalase (240 kDa)</td>
<td>0.059</td>
<td>0.068</td>
<td>0.074</td>
</tr>
<tr>
<td>Carbonic Anhydrase (30 kDa)</td>
<td>0.052</td>
<td>0.054</td>
<td>0.060</td>
</tr>
</tbody>
</table>

A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, 0.3 mL/min, 20-50% B in 15 min, 3 min wash at 95% B, 2 min re-equilibration at 20% B, 5 µL injection of Protein Standard: Ribonuclease A (14 kDa), Cytochrome C (12 kDa), Holo-transferrin (80 kDa), α-Lactalbumin (14 kDa), Catalase (240 kDa), Carbonic Anhydrase (30 kDa), 60 C, 220, 8 nm; Ref = Off
Comparison of Digested IgG1 Separation

SPP, C4, 2.1 x 100 mm, 3.5 µm
Pmax = 570 bar

Good selectivity and resolution

Other SPP, 3.4 µm, 400 Å, C4
Pmax = 400 bar

Other SPP, 3.6 µm, 200 Å, C4,
Pmax = 480 bar

Poor peak shape and resolution

Column: 2.1 x 100 mm; A: 0.1% TFA in water, B: n-propanol/acetonitrile/M.P. A (80/10/10), 0.8 mL/min, 5-40% B in 5 min, 1 min wash at 95% B, 1 min re-equilibration at 5% B, 1 µL injection of Fc/Fab, Papain Digested Humanized Recombinant Herceptin IgG1 from Creative Biolabs (2 mg/mL), 60 C, 220, 8 nm; Ref = Off
Intact IgG2

High resolution for intact mAb

A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, 1.0 mL/min, 30-40%B in 8 min, 85°C, 215 nm
5 µL injection of IgG2 Lambda (Sigma I5279-1MG)
2.1 x 100 mm,

SPP, 3.5 µm, 450 Å, C4

Other SPP, 3.4 µm, 400 Å, C4

Other SPP, 3.6 µm, 200 Å, C4
Comparison of Other Wide Pore SPPs-Fast Separation of Intact IgG1

**Method Parameters**
- Column dimensions: 2.1 x 100 mm
- Mobile phase A: 0.1% TFA in water/IPA (98/2)
- Mobile phase B: IPA/acetonitrile/MPA (70/20/10)
- Flow rate: 1.0 mL/min
- Gradient: 10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
- Sample: 5 μL injection of Humanized Recombinant Herceptin Variant IgG1 Intact from Creative Biolabs (1 mg/mL)
- Temperature: 80 °C
- Detection: UV @ 254nm
Conclusion

3.5 µm SPPs with a 0.25 µm shell thickness and a 450 Å pore size were chosen as optimized base particles for large biomolecule separation.

The three phases (C4, SB-C8 and Diphenyl) of SPPs were evaluated with no carryover.

3.5 µm, 450 Å SPPs were compared with other commercially available wide pore SPPs with similar particle sizes and sub-two micron wide pore TPPs, and showed better performance for proteins and large biomolecules such as IgG1 and IgG2.