Short Monolithic Columns – Novel Technology Platform for the Analytics and Isolation/Purification of Human Plasma Proteins

Aleš Štrancar
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Outline

- Design and Properties of Short Monolithic Layers
- Design and Properties of Multiuse Column Housings
- Use of Short Monolithic Columns for Plasma Protein Purification and IVIG Process
- PAT using HPLC Monolithic Columns
- Take Home Message
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Conventional Liquid Chromatography Media

- **Interparticle void volume**
  - (preferential flow path)

- **Intraparticle void volume**
  - (contains majority of binding sites: > 90%)
Diffusion Limitations to the Pore

Binding capacity at high flow rate: $f_3 > f_2 > f_1 > f_0$

Larger the molecule, faster the capacity drop with the increased flow rate
Diffusion Limitations Out of the Pore

Resolution @ linear gradient elution at high flow rate: \( f_2 > f_1 \)

Larger the molecule, wider the peak with the increased flow rate, lower the resolution. Also one of the main reasons for carry-over.
Another Challenge – the Size of the Molecule of Interest

<table>
<thead>
<tr>
<th>Molecule</th>
<th>nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>1-3</td>
</tr>
<tr>
<td>IgM</td>
<td>25</td>
</tr>
<tr>
<td>Plasmids</td>
<td>150-250</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>130</td>
</tr>
<tr>
<td>Poxvirus</td>
<td>200 x 500</td>
</tr>
<tr>
<td>T4</td>
<td>220 x 85</td>
</tr>
</tbody>
</table>

Courtesy P. Gagnon [www.validated.com](http://www.validated.com)
Eddies create shear forces that damage labile biomolecules like IgM.

Eddy-generated shear is proportional to flow rate.

Gray areas indicate particles.
The white area indicates the void space between particles.
Black arrowheads indicate primary flow.
Red arrowheads indicate counter current flow.

**Yet Another Challenge – Shear Forces**

The frictional differential between particle surfaces and the deep void space creates eddies — areas of persistent countercurrent flow.
Monoliths

Single piece continuous units with a homogeneous open pore structure in all 3 directions (flow through channels).

Membranes – Stack of very thin Monoliths

Stacks of thin polymeric layers – supplied in single piece but in fact they are discontinuous unit.

Problems with:
- resolution due to void volumes
- shear forces due to eddies.
Monolith Channel Structure is Ideal to Perform Chromatography of Very Big Biomolecules

Traditional approach - Porous particle:
1. Diffusive mass transport - slow process or lower resolution
2. Pores too small – very low capacity
3. Counter current flow - shear forces – lower yields

Novel approach – Monolithic columns:
1. Convective mass transport – flow independent resolution and capacity, very fast processes
2. Accessible surface for big molecules – high capacity
3. Laminar flow - No shear forces – better yields of e.g. IgM

The high mass transfer in the large channel of the monolith is completely convective and not limited by the biomolecule size.

Mass transfer within the pore is almost exclusively by diffusion. Diffusion is slow and slower with increasing biomolecule size.
CIM Monolith Chemical Structure Realized via Bulk Polymerization in a Column Shape Mold

Made of highly cross-linked porous rigid monolithic poly(glycidyl methacrylate-co-ethyleneglycol dimethacrylate) or poly(styrene-divinylbenzene) polymers

Well proven and biocompatible:
- Toyopearl® from TosoH
- Fractogel® from Merck / EMD

Poly(glycidyl methacrylate-co-ethyleneglycol dimethacrylate)
CIM Monoliths - the Only Material Engineered to Address the Needs of Large Molecule and Nanoparticle Chromatography

CIM monolithic supports are highly porous rigid polymers with:

- High porosity (over 60%)
- Flow-through channels (“pores”) having large diameter (1.5 µm), for Vaccinia special monolith (3-4 µm)
- Biocompatible with uniform channel connectivity in 3D (homogeneous structure)
- Ligands (active groups) for **AEX, CEX, HIC, RPC, Affinity, Activated, Bioreactor**.
High Capacity for Large Biomolecules and Nanoparticles Due to Fast Surface Accessibility

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Column</th>
<th>Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>CIM QA, SO3</td>
<td>25-50 mg/ml</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>CIM DEAE</td>
<td>8 mg/ml</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>CIM DEAE</td>
<td>15 mg/ml</td>
</tr>
<tr>
<td>Endotoxins</td>
<td>CIM QA</td>
<td>&gt;115 mg/ml</td>
</tr>
<tr>
<td>ToMV</td>
<td>CIM QA</td>
<td>2.0E+14 vp/ml</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>CIM QA</td>
<td>2.0E+10 vp/ml</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>CIM QA</td>
<td>3.0E+12 vp/ml</td>
</tr>
<tr>
<td>Ad3 VLPs</td>
<td>CIM QA</td>
<td>7.3E+16 VLP/mL</td>
</tr>
</tbody>
</table>

Due to high capacity and/or high speed especially suitable for purification and/or removal of (large) human plasma proteins like IgA, IgM, FVIII/vWF, Fibrinogen, Thrombin, Inter-alpha-inhibitor.
Due to an almost rectangular adsorption isotherm, macromolecules remain adsorbed on the column almost irreversibly.

They are eluted by changing the mobile phase composition commonly applying linear or step gradients.

“Theory of short chromatographic layers”: Protein remains adsorbed at the top of the column until the eluting power of the mobile phase reaches the point at which a small change in the composition of the mobile phase causes the movement of the protein without any retention (Yamamoto, 1988). As a result, even very short columns can provide very good separations.
Short Monolithic Layer Design Allow for High Volumetric Flow Rates - High Productivity

Disk dimensions

V = 0.34 ml

Disks used with housing

Typical flow rates
Anion Exchange Semi-Preparative Purification of a 16-mer Oligodeoxynucleotide on a 0.34 ml CIM® DEAE Disk Monolithic Column (3 mm long x 12 mm ID column)

Even Very Short Monolithic Columns Offer Outstanding Resolution
Tubular Format Enables Short Monolithic Columns Design at Industrial Scale

80, 800, and 8,000 ml CIM Monoliths
1. Piston – Collector with flow-out
2. Seal
3. Frit
4. Monolith
5. Housing - Distributor
6. Upper plate with flow-in

CIM Tube Monolithic Column Structure
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Disadvantages of Traditional Housings Made of Stainless Steel or Plastics

Stainless steel:
- expensive
- difficult to handle
- difficult to transport, delivery of pre-packed columns quite impossible

Plastics:
- problem to reuse (cleaning)
- problems to run with higher backpressures
- in some cases leaking problems
Introduction of Composite Materials to Combine Advantages of SS and Plastics

- Epoxy thermoset composite
- Re-inforced with carbon fibers
- Coated pin-hole free with - USP Class VI Parylene C

- Disposable but multiuse
- Stainless steel performance characteristics
- cGMP compliant
Pin-hole Free Parylene Coating to Allow Regeneration and Sanitization Using 1M NaOH

Parylene C

- Transparent
- Low dielectric permittivity
- Excellent thermodynamic stability (resistant to the solvent and thermal endurance).
- Biocompatible and biostable as well.
- Parylene C used extensively for coating permanent medical devices implanted in humans

Certifications

- USP 29 Class VI package for FDA
- ISO-10993
- Applied pin-hole free

NO LEACHABLES
Composite Materials – Matching Stainless Steel Performance

<table>
<thead>
<tr>
<th>Type of column</th>
<th>1 ml</th>
<th>8 ml</th>
<th>80 ml</th>
<th>800 ml</th>
<th>8000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max pressure</td>
<td>18 bar</td>
<td>20 bar</td>
<td>20 bar</td>
<td>20 bar</td>
<td>7 bar</td>
</tr>
<tr>
<td>Recommed flow rates</td>
<td>1-10</td>
<td>1-10</td>
<td>2-60</td>
<td>2-60</td>
<td>20-300</td>
</tr>
<tr>
<td>Max. flow rate (ml/min)</td>
<td>16</td>
<td>16</td>
<td>100</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>Max. operating temperature</td>
<td>50 °C</td>
<td>50 °C</td>
<td>50 °C</td>
<td>50 °C</td>
<td>50 °C</td>
</tr>
<tr>
<td>L-t storage conditions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sanitization for IEX, C4 HLD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**BUT:**
- 3 times cheaper
- 5 times lighter
- allow for pre-packed column transport
- customer decides to use disposable column as single or multi use unit

www.biaseparations.com
CIMmultus Family of Composite Housings Packed with Monolithic Tubes

Flu vaccine 24,000 doses/run
pDNA .48 g/run

240,000 doses/run
4.8 g/run

2,400,000 doses/run
48 g/run

40 L unit available in 2014
CIMmultus Fits Perfectly with Multicolumn and Continuous LC Systems (BioSMB™)
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Separation of IgG, IgA, IgM and Cohn Paste Sample on CIM AEX Monolithic Column

Mobile phase A: 20 mM Tris pH 7.2; Mobile phase B: 20 mM Tris + 1 M NaCl pH 7.2.
Linear gradient from 0 – 45% buffer B in 6 min. Flow rate 3 ml/min.
Design of an IVIG Purification Process to Allow for IgA and IgM Production at the Later Point

1. **Cohn I+II+III**
   - Dissolve 10g in 100ml Buffer A
   - Hold 24h at 4 degC; centrifuge 20 min
   - Add 5% EtOH; centrifuge 20 min

2. **Anion exchange chromatography: CIM QA**
   - collect FT, pH adjusted with NaAc pH 5; add 1% Triton X100+0.3% TNBP
   - 16h viral inactivation

3. **Cation exchange chromatography: CIM SO3**
   - TFF 5X
1. step: Separation of Cohn Paste (I+II+III)
Sample on 80 mL CIM AEX Monolithic Column

IgA and IgM collected for further processing
## 1. step: Excellent Removal of the IgA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample Volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>IgA (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before 5% EtOH</td>
<td>950</td>
<td>21.73</td>
<td>5.14</td>
</tr>
<tr>
<td>After 5% EtOH (column load)</td>
<td>970</td>
<td>22.60</td>
<td>1.57</td>
</tr>
<tr>
<td>1. consecutive run - FT fraction</td>
<td>105</td>
<td>12.83</td>
<td>3.05</td>
</tr>
<tr>
<td>2. consecutive run - FT fraction</td>
<td>120</td>
<td>11.38</td>
<td>4.21</td>
</tr>
<tr>
<td>3. consecutive run - FT fraction</td>
<td>113</td>
<td>12.25</td>
<td>5.59</td>
</tr>
<tr>
<td>4. consecutive run - FT fraction</td>
<td>125</td>
<td>11.96</td>
<td>5.11</td>
</tr>
<tr>
<td>5. consecutive run - FT fraction</td>
<td>120</td>
<td>12.98</td>
<td>5.82</td>
</tr>
<tr>
<td>6. consecutive run - FT fraction</td>
<td>120</td>
<td>11.67</td>
<td>6.20</td>
</tr>
</tbody>
</table>
2. step: Consecutive Separations on 80 mL CIM CEX Monolithic Column
2. step: SD agents and aggregate removal

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>IgA (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. consecutive run</td>
<td>101</td>
<td>10,890</td>
<td>3,83</td>
</tr>
<tr>
<td>2. consecutive run</td>
<td>107</td>
<td>12,290</td>
<td>6,03</td>
</tr>
<tr>
<td>3. consecutive run</td>
<td>100</td>
<td>14,550</td>
<td>5,60</td>
</tr>
<tr>
<td>4. consecutive run</td>
<td>105</td>
<td>13,510</td>
<td>5,38</td>
</tr>
<tr>
<td>5. consecutive run</td>
<td>110</td>
<td>12,980</td>
<td>5,36</td>
</tr>
<tr>
<td>6. consecutive run</td>
<td>105</td>
<td>14,030</td>
<td>5,10</td>
</tr>
</tbody>
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Upstream Processing

Downstream Processing
Monolithic Analytical Columns for In-Process Control (PAT)

Developed in partnership with Agilent Technologies

10 ml/min = 2800 cm/h = 100 CV/min (res. time: 0.5 s) = faster than biosensor
Separation of IgG, IgA and IgM on a CIM QA (Strong AEC) Monolithic Analytical Column

Column: CIMac QA Monolithic Analytical Column (5,2 mm I.D. x 4,95 mm L; V = 100 µl); Sample: A mixture of human IgG, IgA and IgM dissolved in 20 mM Tris-HCl buffer, pH 7.4
Mobile phase A: 20 mM Tris-HCl buffer, pH 7,4; Mobile phase B: 20 mM Tris-HCl buffer + 1.0 M NaCl, pH 7,4; Flow rate: 1,0 ml/min; Gradient: A linear gradient from 0 % buffer B to 35 % buffer B in 4 min (40 column volumes); Injection volume: 50 ml; Column back-pressure: 15 bar (1,5 MPa)
CIM ImmunoDisk Allows Rapid Quantification of Biomarkers

MAb 69.31 ImmunoDisk standard curve
Fresh Frozen Human Plasma - 250 mg/L of INTER-ALPHA INHIBITOR

Courtesy of Prof. Yow-Pin Lim, ProThera Biologics, Providence, USA
Results on Progress of Sepsis Within Few Minutes by Using a CIM ImmunoDisk

SERIAL PLASMA STUDY OF SEVERE SEPTIC PATIENTS

Courtesy of Prof. Yow-Pin Lim, ProThera Biologics, Providence, USA
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Major Certifications and Approvals are in Place

- At present 8L monolith columns are available and already used in several projects to purify products for CP studies, 40L column will be available in 2014.

- Drug Master Files (DMF) for CIM DEAE, QA and SO3 monolithic columns are in place, HIC in preparation.


- First CIM monolith used for the industrial cGMP purification for plasmid DNA at Boehringer Ingelheim in 2004 provided 15-fold increase in productivity.

- Since 2008 in partnership with Agilent Technologies to produce analytical monolithic columns (Bio-monolith™).

- Partnerships with multinational companies JSR and SDK secure long term company and production stability.
Monolithic Columns are Becoming Industry Standard for Production of Complex Biomolecules

- First drug purified using CIM monoliths passed CPIII trial (pDNA for gene therapy).
- More than 50 projects in CPI – CPIII trials (various Influenza, various Adenovirus, bacteriophages, various IgMs and several human plasma proteins; e.g. Thrombin and FVIII/vWF).
- More than 300 projects in pre-clinical trials (Influenza A and B virus (eggs, Vero and MDCK cells), Rabies virus, Rotavirus, AAV, various Adenovirus subtypes, Hepatitis A, Vaccinia, Mulv, MVM, Feline calicivirus, Japanese encephalitis, Crimean-Congo hemorrhagic fever, Hantaan virus, VLP (Hepatitis B, HPV, Influenza, Adenovirus), bacteriophages (Lambda, T4, VDX10, Pseudomonas phage), Tomato and Pepino Mosaic virus, pDNA, IgM, various proteins).
Thank You for Your Attention!