# Basics about CIM® technology and key applications

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March, 2011

BIA separations

# Leaders in Monolith Chromatography

- BIA Separations was founded in September 1998 as a spin-off from BIA d.o.o. founded in 1989. Headquartes in Austria, R&D and Production in Slovenia.
- BIA Separations USA established in September 2007 - sales and tech support office.
- BIA Separations China established in January 2011 - sales and tech support office.
- Main focus: To develop and sell methacrylate monolithic columns & develop methods and processes for large biomolecules separation and purification.
- Pioneers and leaders in proprietary monolithic technology (CIM®). 4 USA patents granted including their foreign equivalents, more pending.

# Important Milestones

- 2002: **First Drug Master File (DMF) for CIM® DEAE supports**.
- 2002: Pass first FDA audit for one of the projects.
- 2004: First monolith used for the industrial cGMP purification for plasmid DNA at Boehringer Ingelheim provide 15-fold increase in productivity
- 2006: **Drug Master File (DMF) for CIM® QA supports.**
- 2006: First cGMP production of a vaccine (influenza) using CIM®.
- 2008: Partnership with Agilent Technologies – develop and produce analytical monolithic columns
- 2009: Pass second FDA audit for one of the projects.
- 2010: **Drug Master File (DMF) for CIM® SO3 supports.**
- 2001 - 2010: Pass many audits by Novartis, Boehringer Ingelheim, Octapharma, ...

# Moving to the new facility in summer

# BIA Separations CIM® Monolithic Columns are Becoming Industry Standard for Production of Complex Biomolecules

- Drug Master Files (DMF) for CIM® DEAE, QA and SO3 columns in place, HIC in preparation.
- First drug purified using CIM monoliths passed CPIII trial (pDNA for gene therapy).
- More than 15 projects in CPI – CPIII trials (various Influenza, various Adenovirus, bacteriophages, various IgMs, Inter-alpha-inhibitors).
- More than 200 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).

# Short Monolithic Columns Technology

# Many novel drug targets are large and complex, and in some cases used live

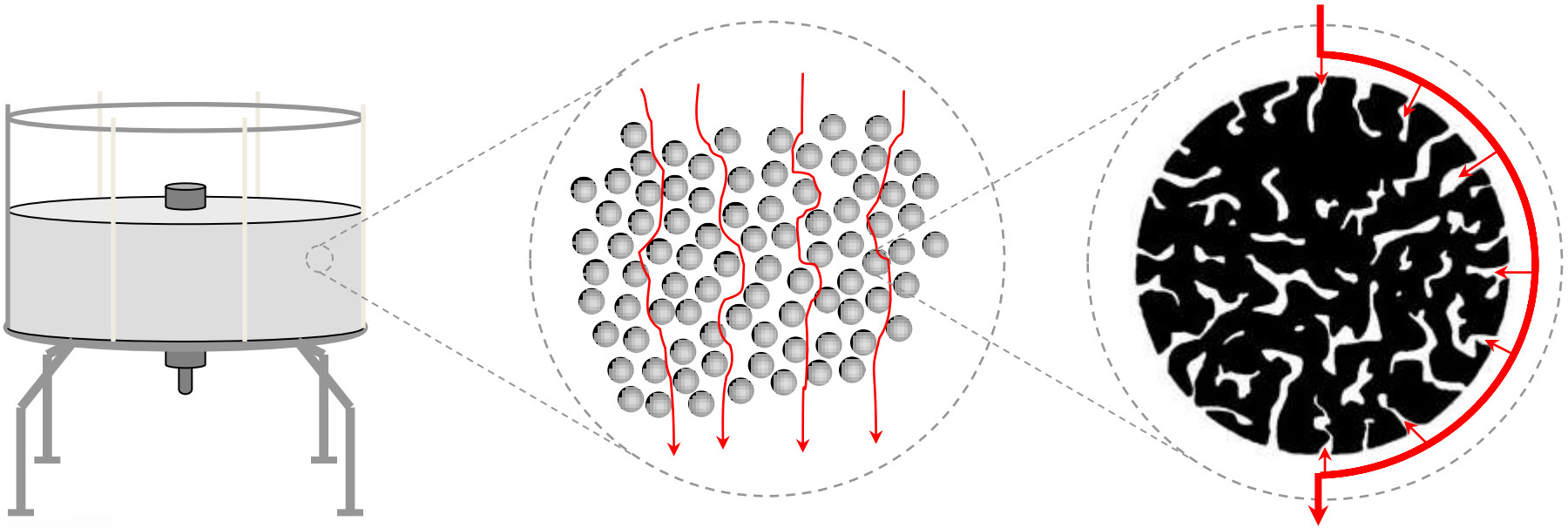
- These include different viral particles, pDNA, protein complex, IgM.

- ”Whilst highly effective for the purification of proteins and smaller molecules, chromatographic techniques are not necessarily well suited to purification of these newer, larger targets.“ (N. Willoughby, J Chem Tech & Biotech, 84, 2008, 145).

- Why?

# Conventional Liquid Chromatography Media

**Packed bed of porous particles - two types of void volume exist!**

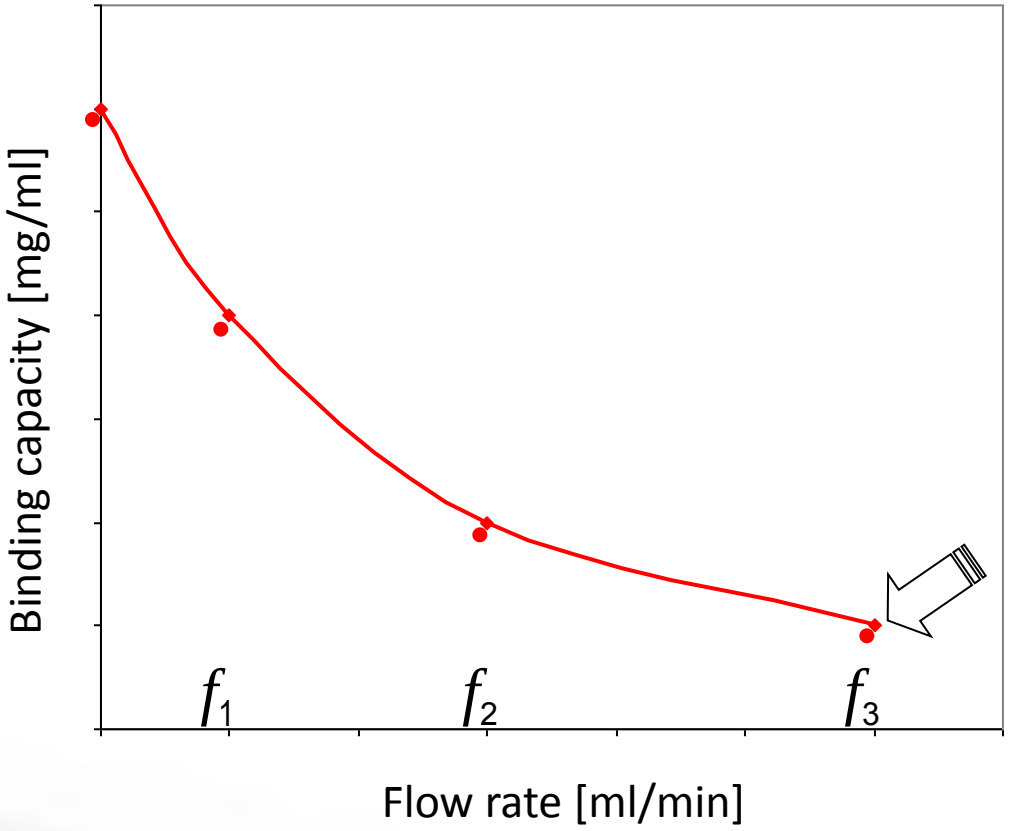


**Interparticle void volume**
(preferential flow path)

**Intraparticle void volume**
(contains majority of binding sites: > 90 %)

# Diffusion Limitations

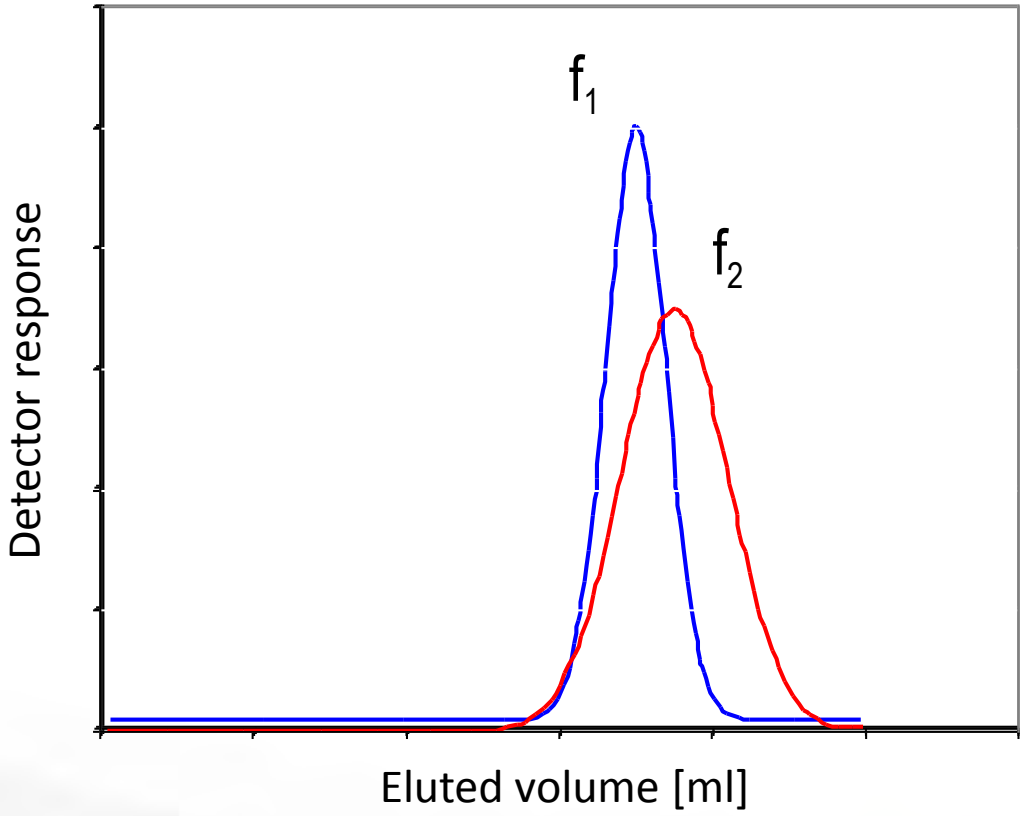
Binding capacity at high flow rate: f3 > f2 > f1 > f0




**Larger the molecule faster the capacity drop**

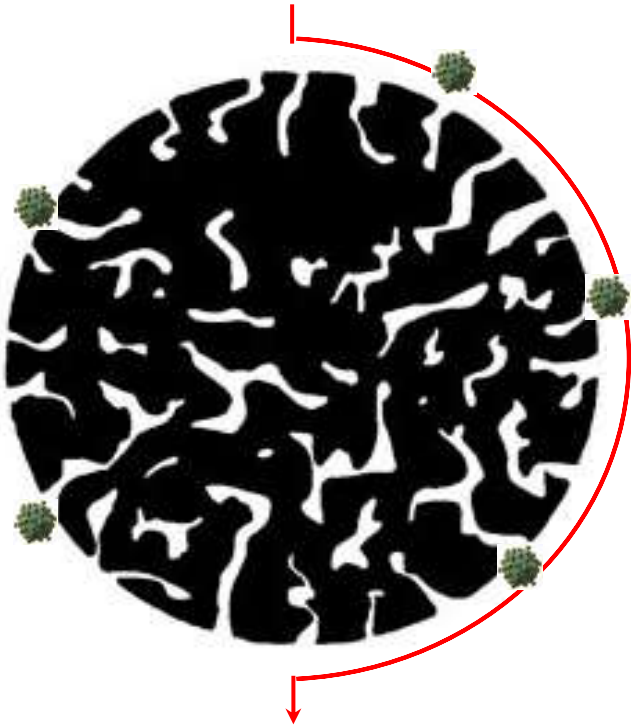
# Diffusion Limitations

Resolution @ linear gradient elution at high flow rate: f2 > f1




**Larger the molecule wider the peak – lower the resolution**

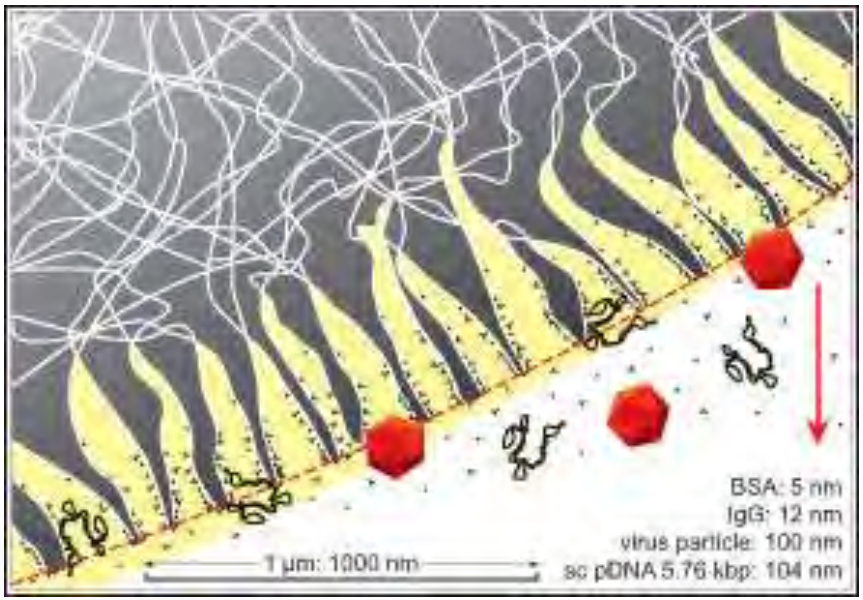
# Another Challenge – the Size of the Molecule of Interest



**Pores too small for large solutes!**
(Binding mostly on outer surface)
results in
**Very low binding capacity for large solutes**
**(behave like nonporous particles)**

# Working with big molecules

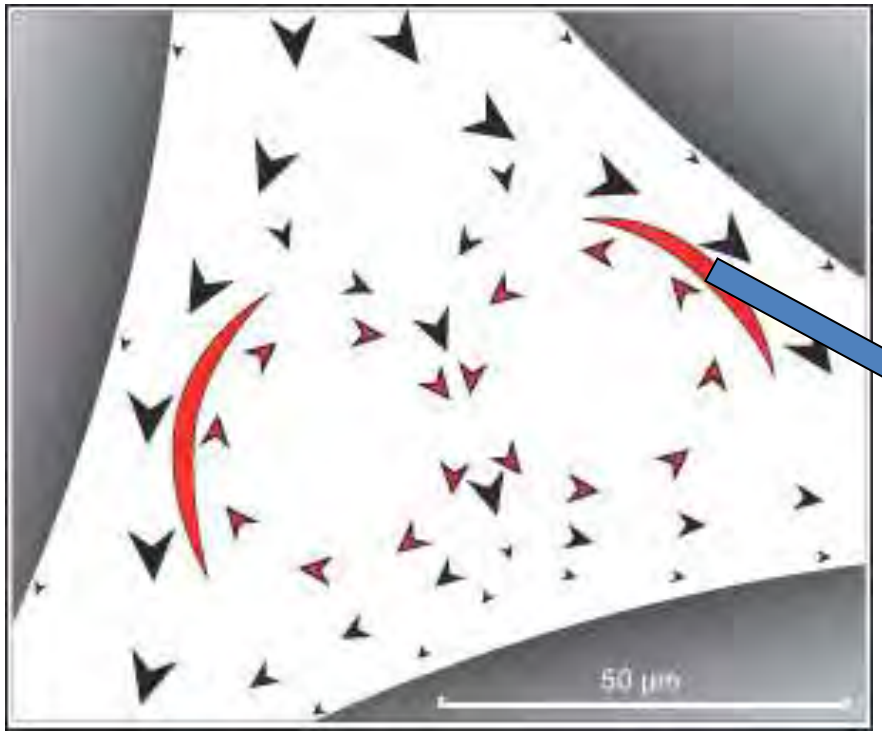
- Molecule size: surface accessibility




| Molecule | nm |
|---|---|
| Proteins | 1-3 |
| IgM | 25 |
| Plasmids | 150-250 |
| Rotavirus | 130 |
| Poxvirus | 200 x 500 |
| T4 | 220 x 85 |

*Courtesy P. Gagnon www.validated.com*

# Yet Another – Shear Forces




Gray areas indicate particles.

The white area indicates the void space between particles.

Black arrowheads indicate primary flow.

Red arrowheads indicate countercurrent flow.

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

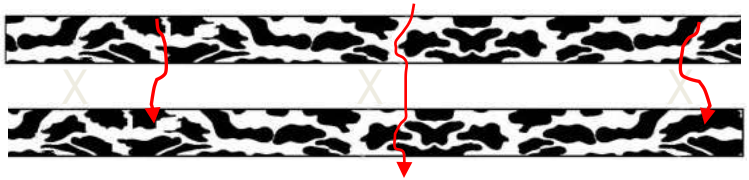
**Eddies create shear forces that damage labile biomolecules (<u>similar to the effect when using ultracentrifugation</u>).**

**Eddy-generated shear is proportional to flow rate.**

*Courtesy of Pete Gagnon, Validated Biosystems, USA – details at [www.validated.com](http://www.validated.com)*

# What are the Alternatives?

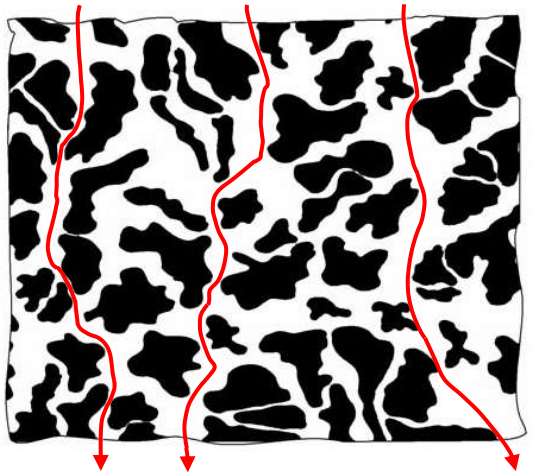
## Membranes – Stack of very thin Monoliths



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

**Problems with:**
- **resolution due to void volumes**
- **share forces due to eddies.**

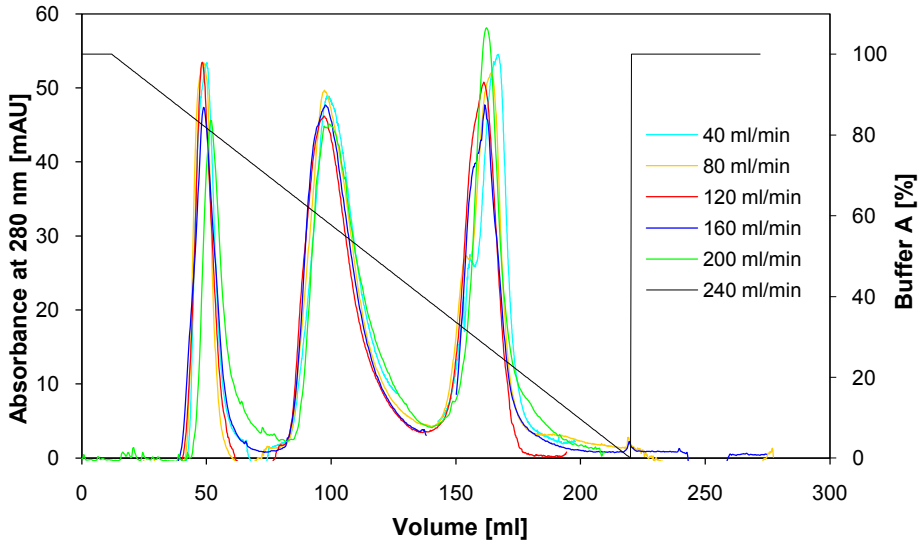
## Monoliths

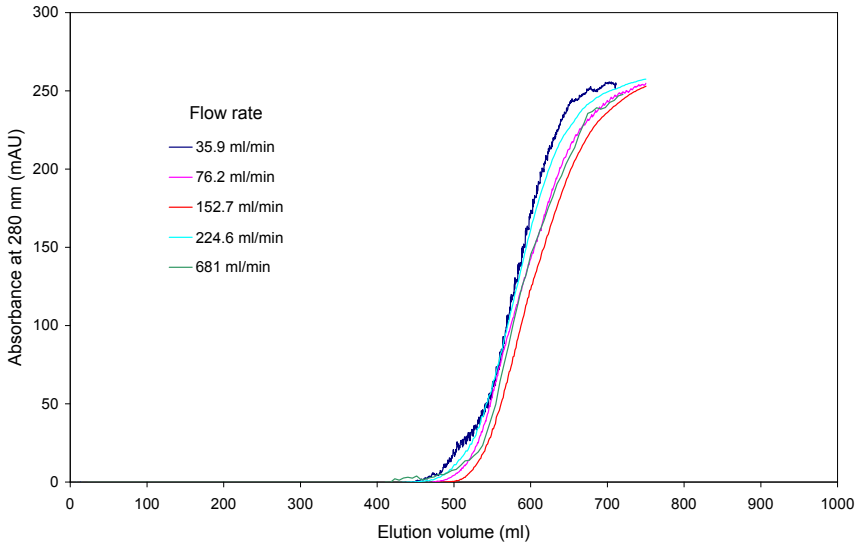


Single piece continuous units with a homogeneous open pore structure in all 3 directions (flow through channels).

# Convective Transport: Consequences

- Flow independent properties

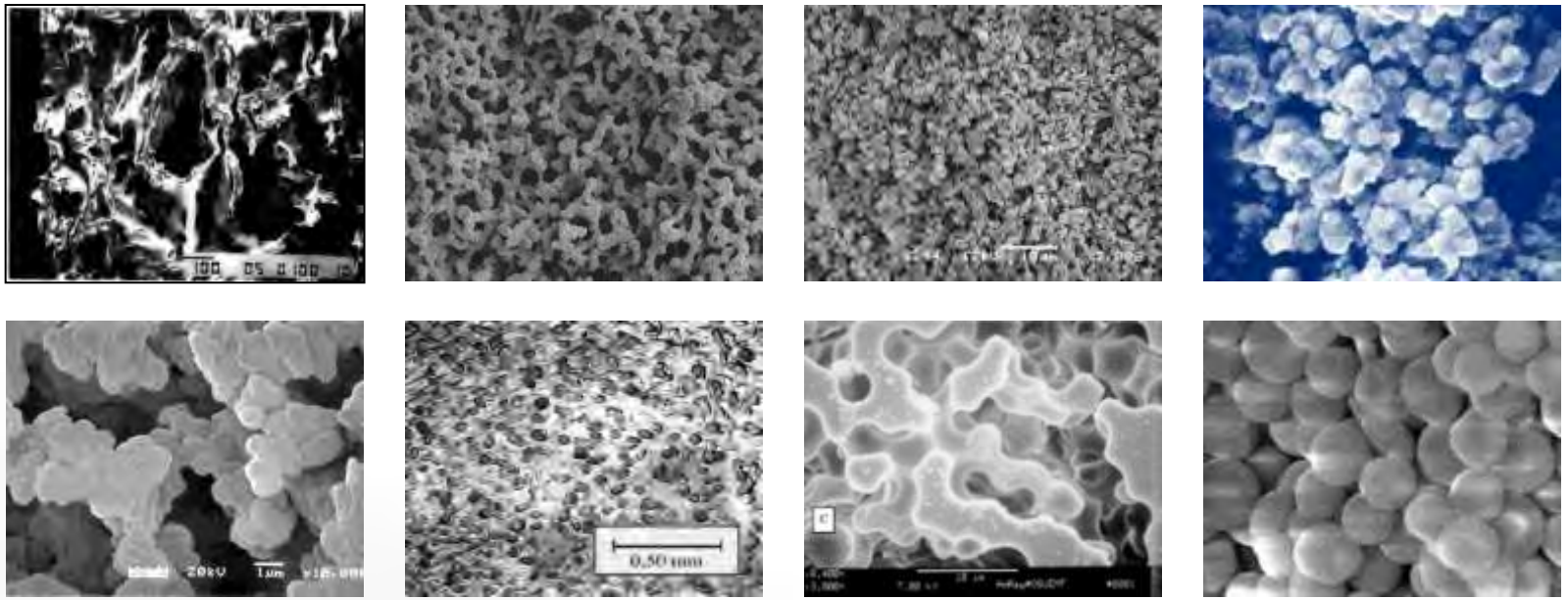


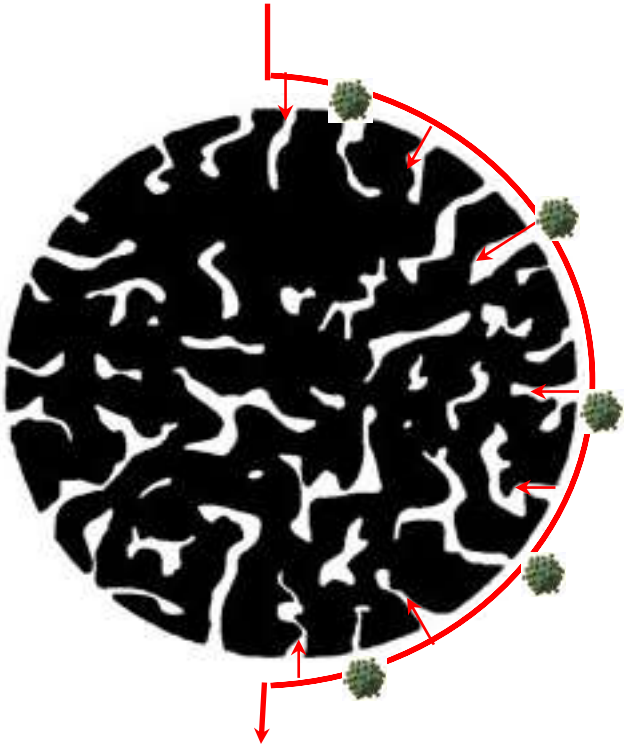

*Podgornik et al., Anal. Chem. 72 (2000) 5693*

# Monoliths

Monoliths are chromatography media that are cast as a single block and inserted into a chromatography housing. They are characterized by a highly inter-connected network of channels, sometimes compared to a sponge.

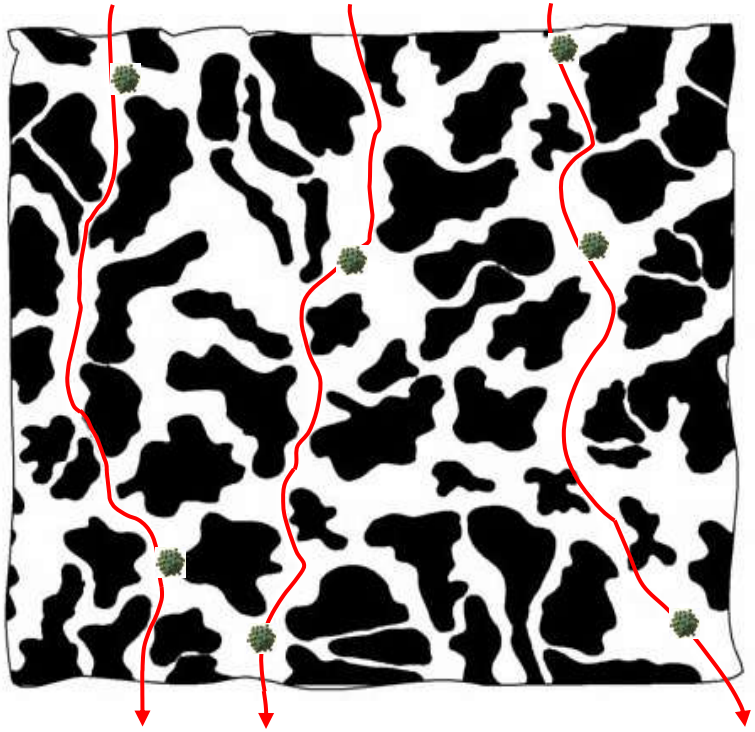


# Sum up: CIM® Monolithic Columns are purpose designed for the chromatography of big biomolecules



**Traditional approach - Porous particle:**

1. Diffusive mass transport – slow process or **lower resolution**
2. Pores too small – very **low capacity**
3. Countercurrent flow - shear forces – **lower yields**



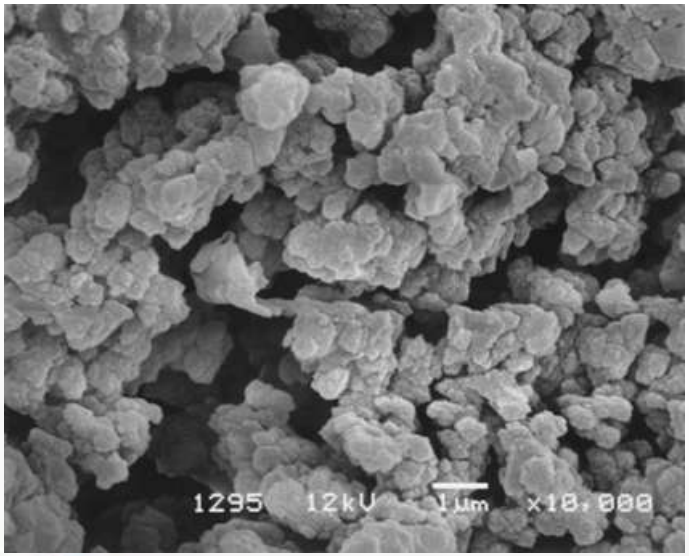
**Novel approach – CIM monoliths:**

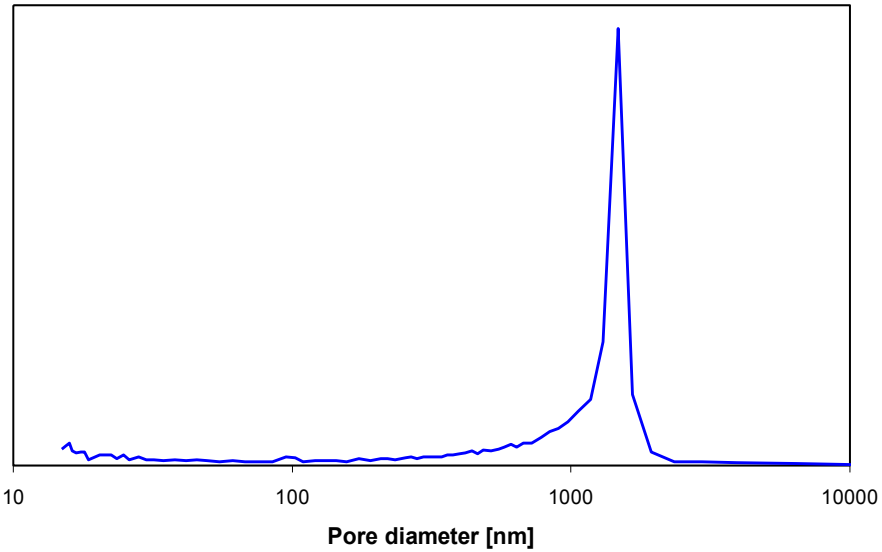
1. Convective mass transport – **flow independent resolution and capacity,** very fast process
2. Big channels – **high capacity**
3. Laminar flow - No shear forces – **better yields**

# CIM® Monoliths - the Only Material Engineered to Address the Needs of Large Molecule Separation

CIM® monolithic supports are highly porous rigid polymers with:

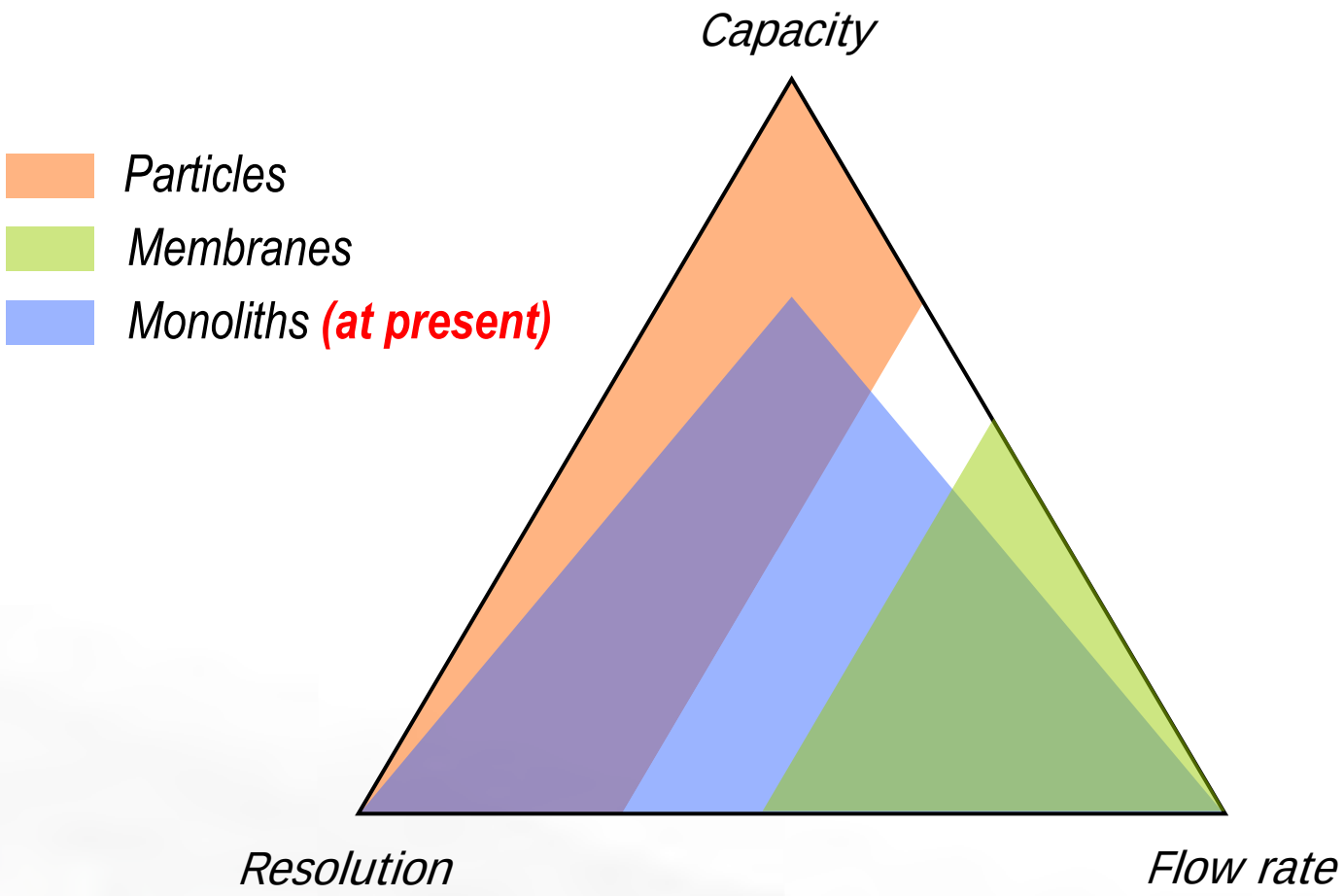
- High porosity (over 60 %)
- Flow-through channels ("pores") having large diameter (1.5 µm), for Vaccinia special monolith (3-4 µm)
- Biocompatible with uniform channel connectivity in 3D (homogeneous structure)
- Ligands (active groups) for **AEX, CEX, HIC, RPC, Affinity, Activated, Bioreactor.**



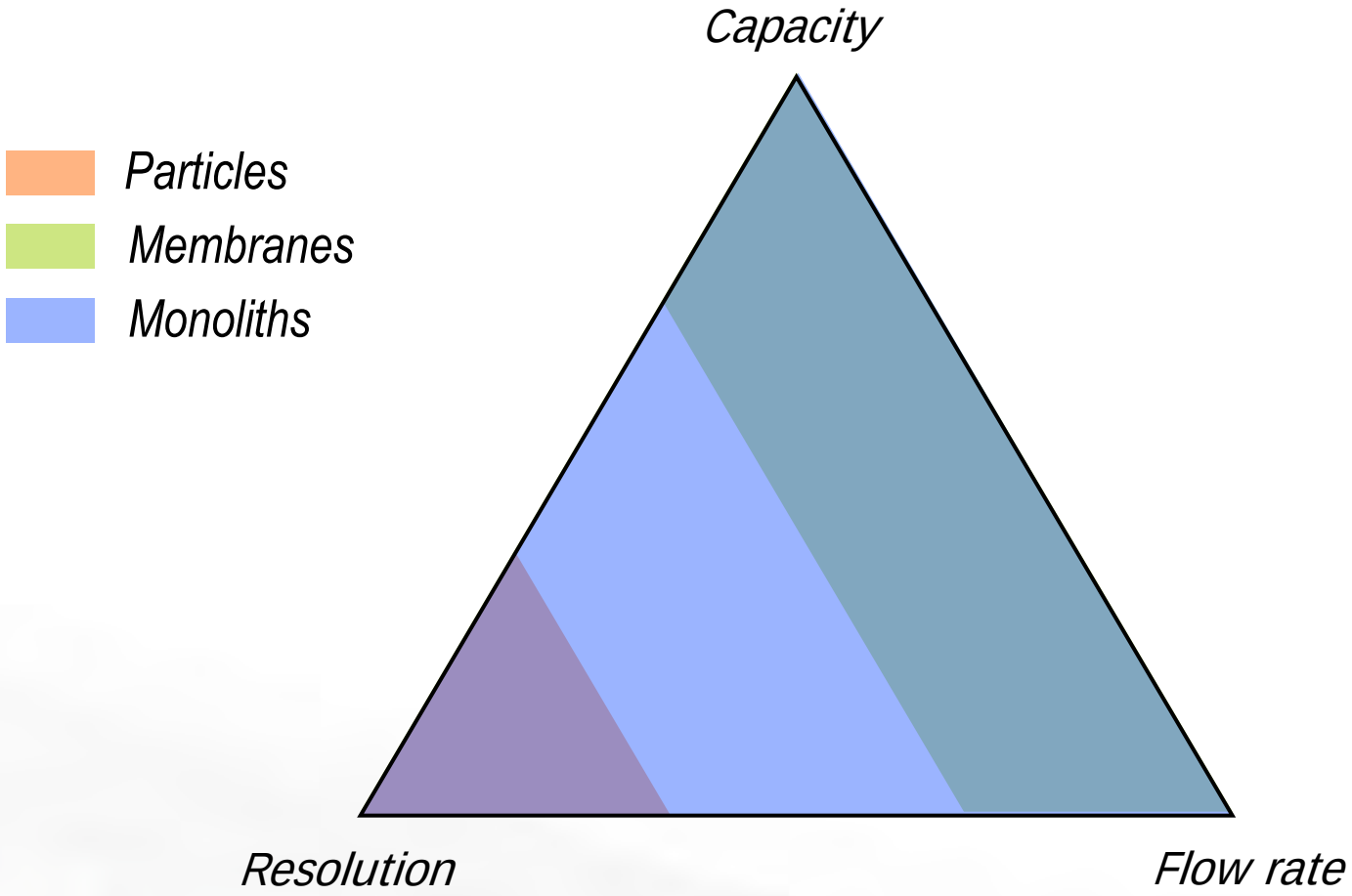
# Comparison of support performance

- **For proteins & peptides**

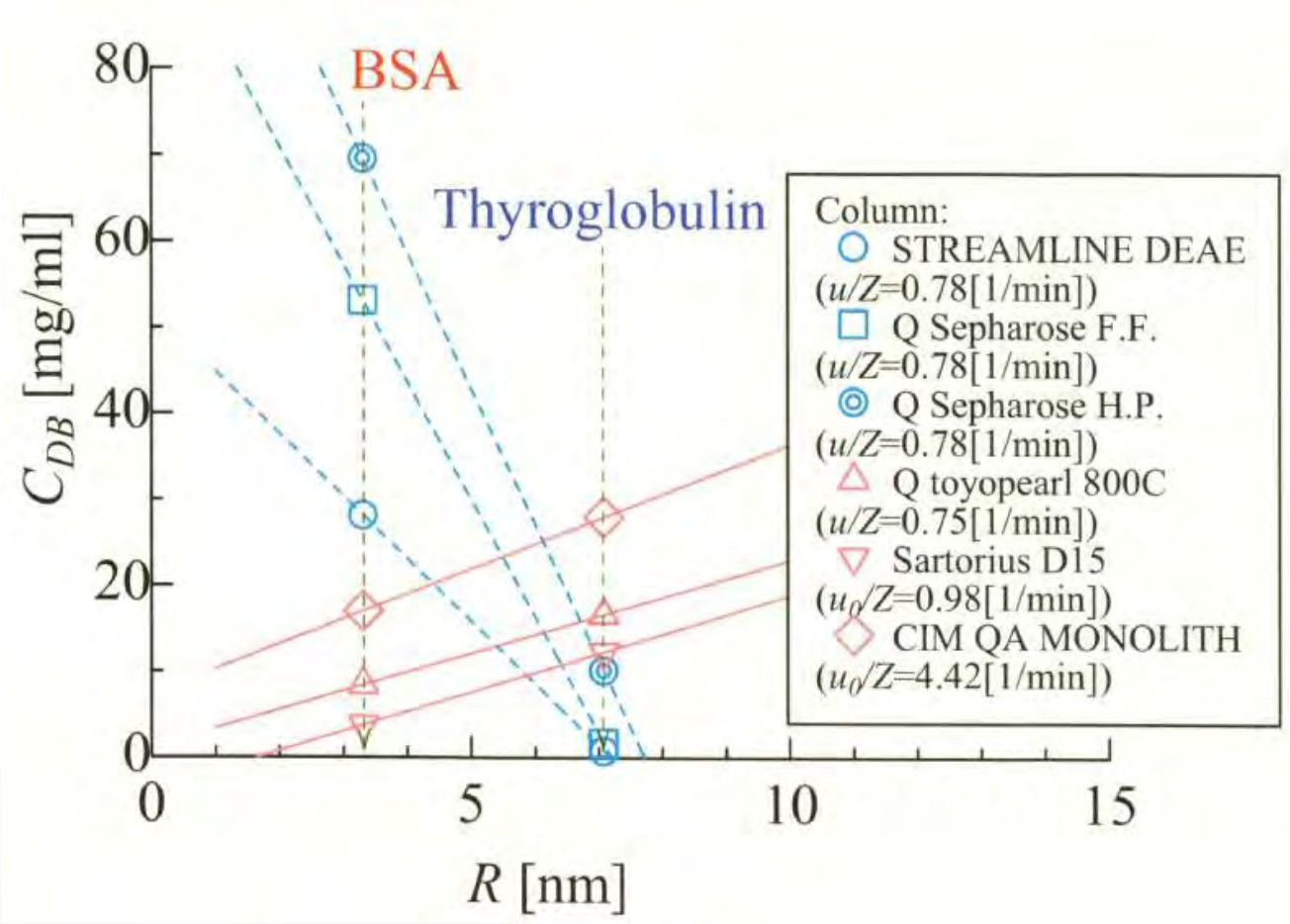


# Comparison of support performance

- **For large proteins, DNA & viruses**

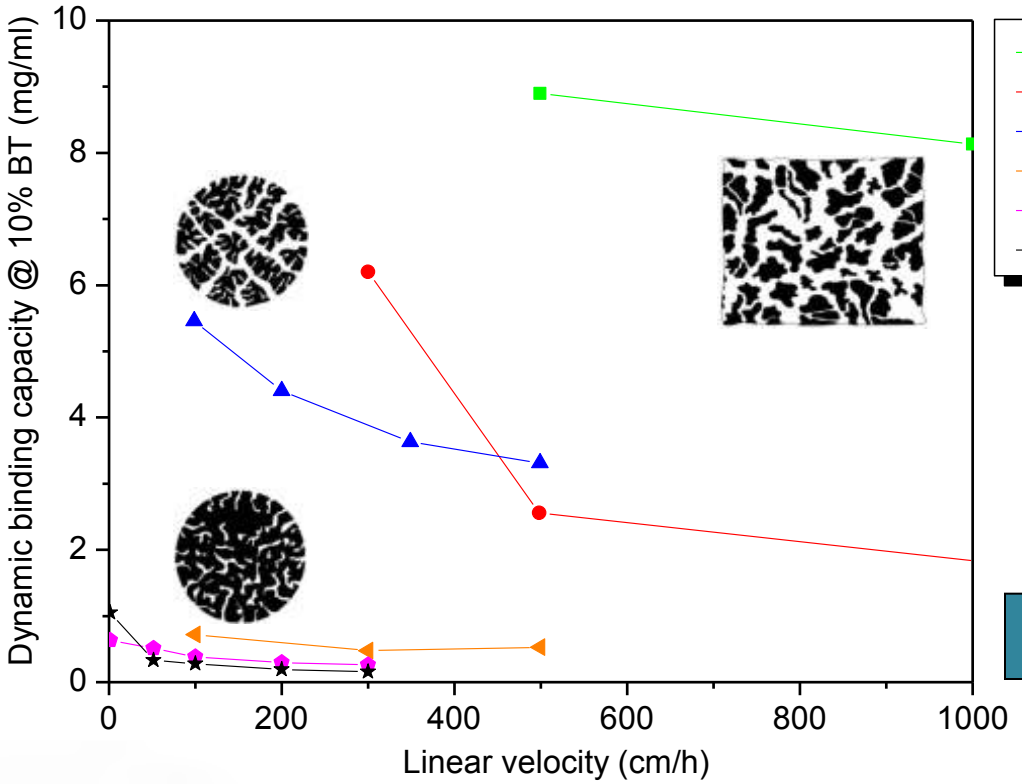


# Effect of the Molecule Size on Dynamic Binding Capacity




*Yamamoto S. and Kita A., Trans IChemE, Part C, Food and Bioproducts Processing, 84 (2006) 72-77.*

# Plasmid DNA Binding Capacity




**CIM DEAE binding capacity = ~10 mg/ml**

**Currently used for CP III trials**

## 15-fold increase in productivity

- High binding capacity at relevant flow rates
- High elution concentration - pDNA eluted in lower volume (important for SEC!)
- Fast process (no product loss due to oxidative degradation or enzymatic attack)

*Urthaler et al., J.Chrom. A, 1065 (2005), 93-106*

# Surface accesibility for CIM® Monoliths

High capacity for IgM, viruses and DNA

| Molecule | Column | Capacity |
|---|---|---|
| IgM | CIM QA, SO3 | 25-50 mg/ml |
| Plasmid DNA | CIM DEAE | 8 mg/ml |
| Genomic DNA | CIM DEAE | 15 mg/ml |
| Endotoxins | CIM QA | >115 mg/ml |
| ToMV | CIM QA | 2.0E+14 vp/ml |
| Influenza virus | CIM QA | 2.0E+10 vp/ml |
| Adenovirus | CIM QA | 3.0E+12 vp/ml |
| Ad3 VLPs | CIM QA | 7.3E+16 VLP/mL |

# CIM® Chemical Structure

Made of

**highly cross-linked**

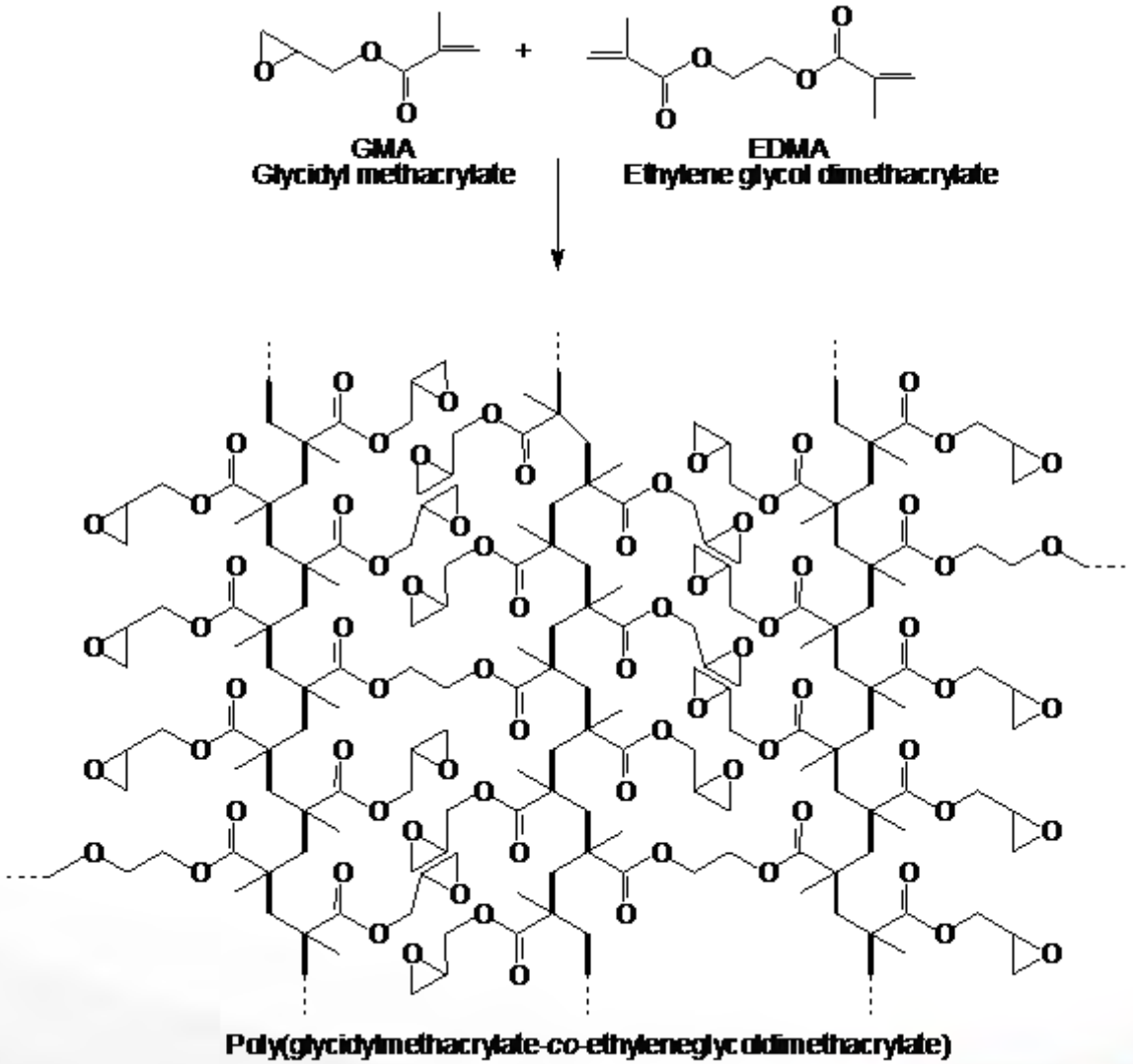
**porous rigid monolithic**

*poly(glycidyl methacrylate-co-ethyleneglycol dimethacrylate)*

or

*poly(styrene-divinylbenzene)*

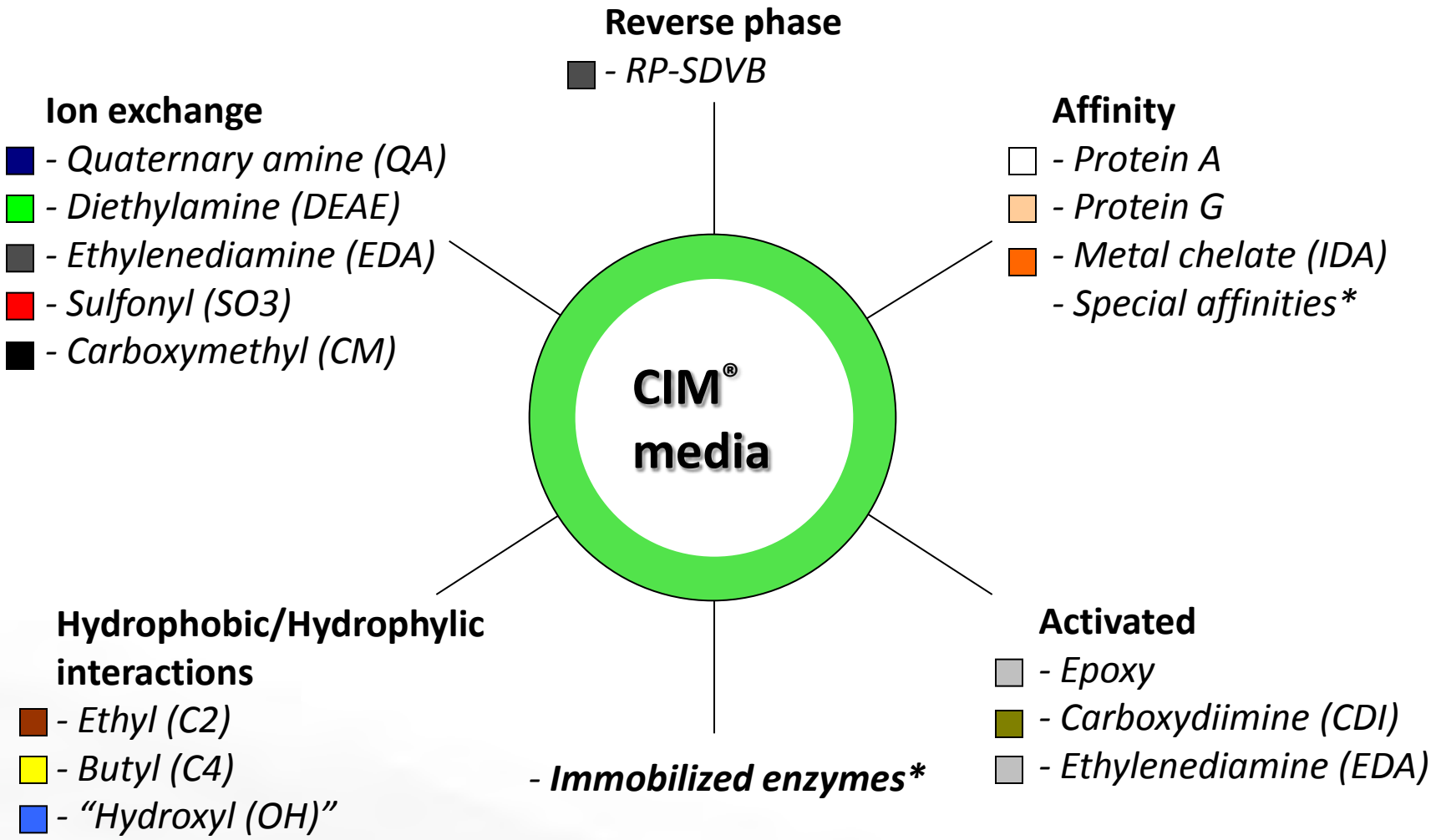
polymers




**Well proven and biocompatible**:

- Toyopearl® from TosoH
- Fractogel® from Merck / EMD

# Available Chemistries

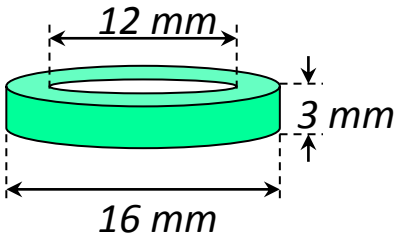



* *on request*

# CIM® Columns Design to Allow High Volumetric Flow Rates - High Productivity

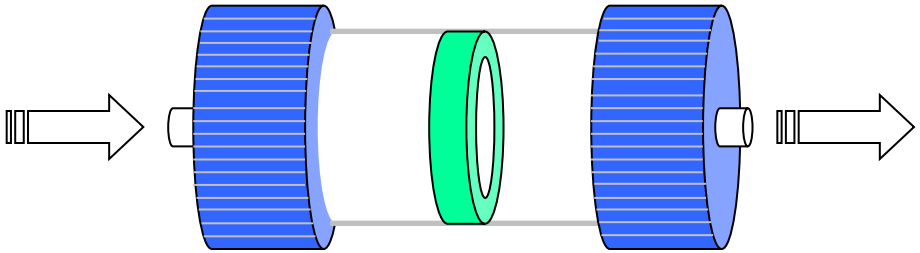
## Designed as Short Chromatographic Layers

### Disk dimensions

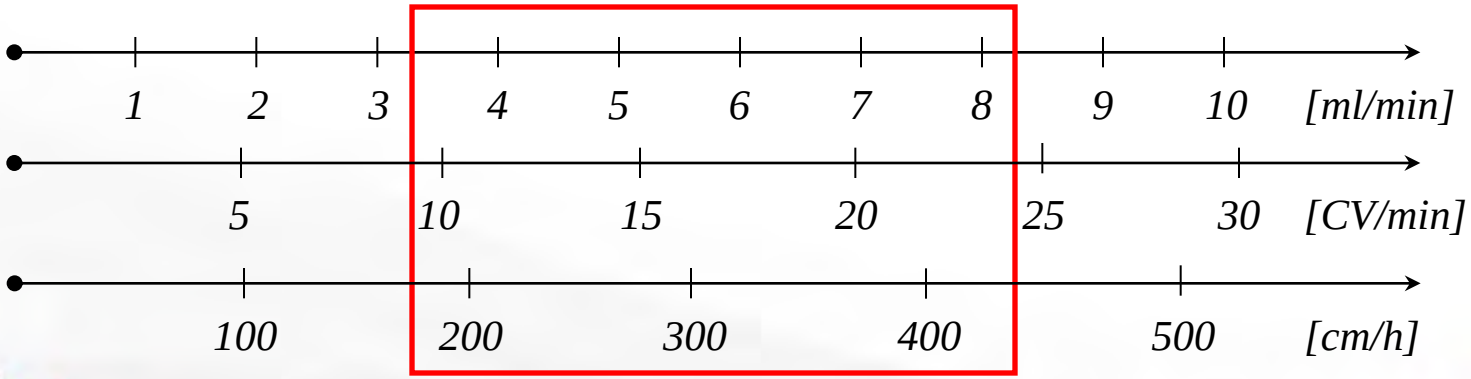



V = 0.34 ml

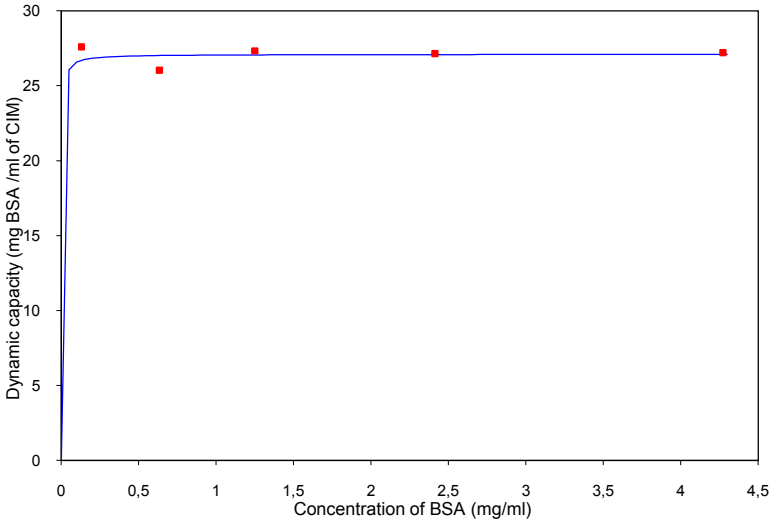
### Disks used with housing



### Typical flow rates



# What About the Separation on Short Chromatographic Layers?



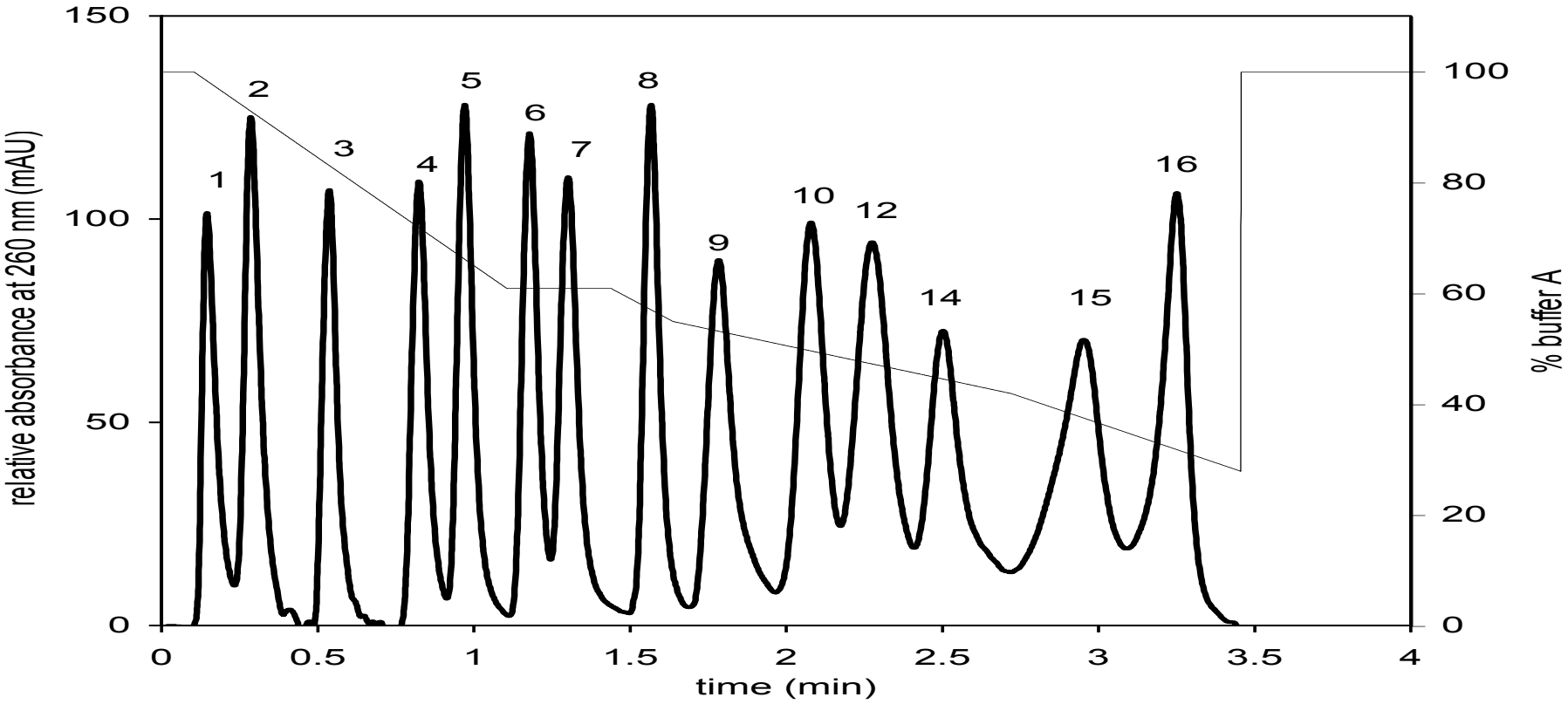

Due to an almost rectangular adsorption isotherm, macromolecules remain adsorbed on the column almost irreversibly.

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

**"Theory of short chromatographic layers":**

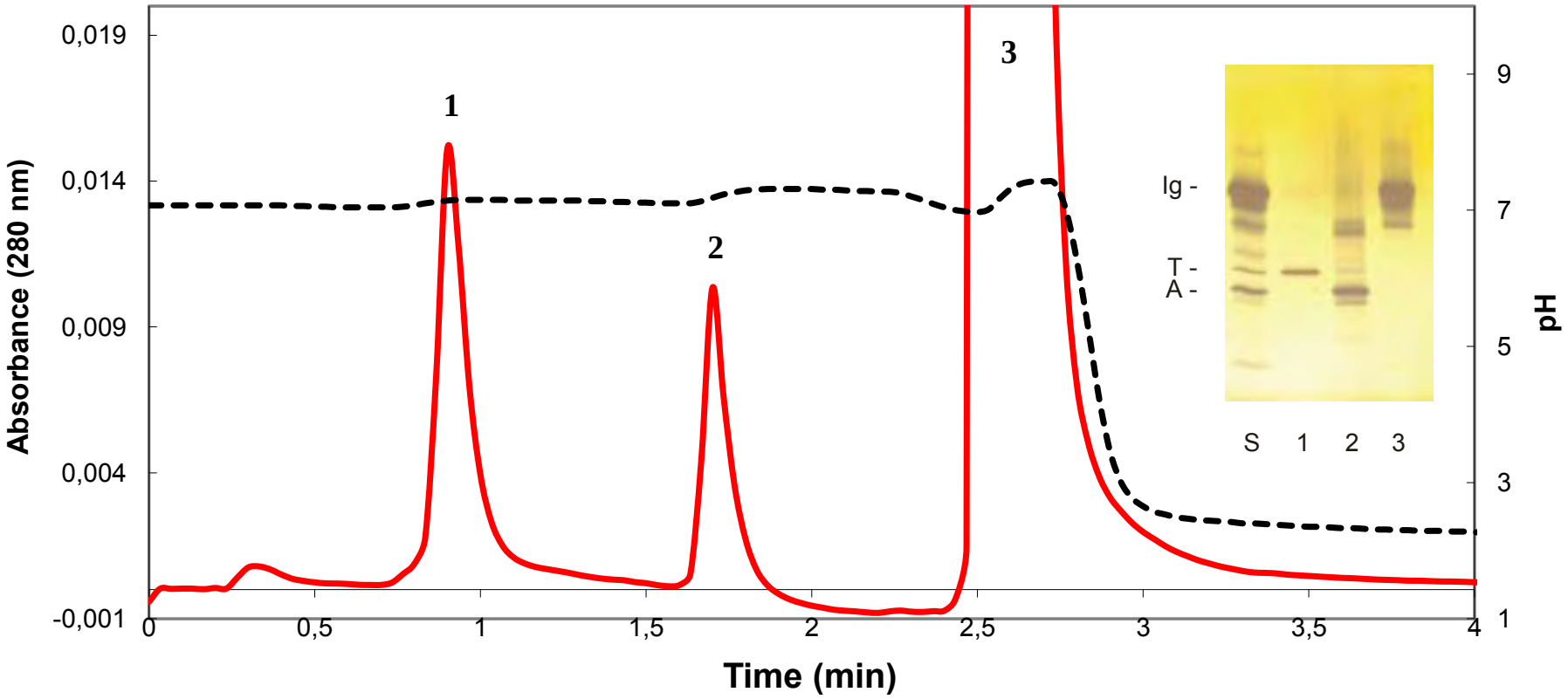
Protein remains adsorbed at the top of the column until the eluting power of the mobile phase reaches the point at which a small change in the composition of the mobile phase causes the movement of the protein without any retention (Yamamoto, 1988). As a result, **even very short columns can provide very good separations.**

# Very Short CIM® Monolithic Columns Offer Outstanding Resolution




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)

# CIM® CLC - Multidimensional chromatography




Separation of IgG (8 mg), Transferrin (within range of 1mg/mL) and Albumin (within range of 1mg/mL) on monolithic column consisting of **two Protein G** and **one CIM® QA** Disks.

**Buffer A**: 20 mM Tris-HCl, pH 7.4
**Buffer B**: 20 mM Tris-HCl, 1 M NaCl, pH 7.4
**Buffer C**: 0.1 M Gly-HCl, pH 2.6
**Flow**: 4 ml/min
**Injection volume**: 250 µl

Order of elution: 1- Transferrin
2- Albumin
3- IgG

*Courtesy of Dr. A. Buchacher, Octapharma, Vienna, Austria*

# Fast Method Development

| | Short Layer Monolith (3x12 mm ID) | Porous Particles |
|---|---|---|
| Column volume | 0.34 ml | 1 ml |
| Flow rate applied | 4 ml/min | 1 ml/min |
| Flow rate applied | 12 CV/min | 1 CV/min |
| Time – loading (5 CV) | 0.4 min | 5 min |
| Time – elution (10 CV) | 0.9 min | 10 min |
| Time – equilibration (5 CV) | 0.4 min | 5 min |
| Time – total per run | 1.7 min | 20 min |
| Time for 20 runs | 0.6 h | 6.7 h |
| Time for 100 runs | 2.8 h | 33.3 h |

# Lab Scale Columns – CIM® Disks

- Smaller units in disk format - intended for media screening, method development, laboratory purification

**CIM Disk Monoliths with housing**

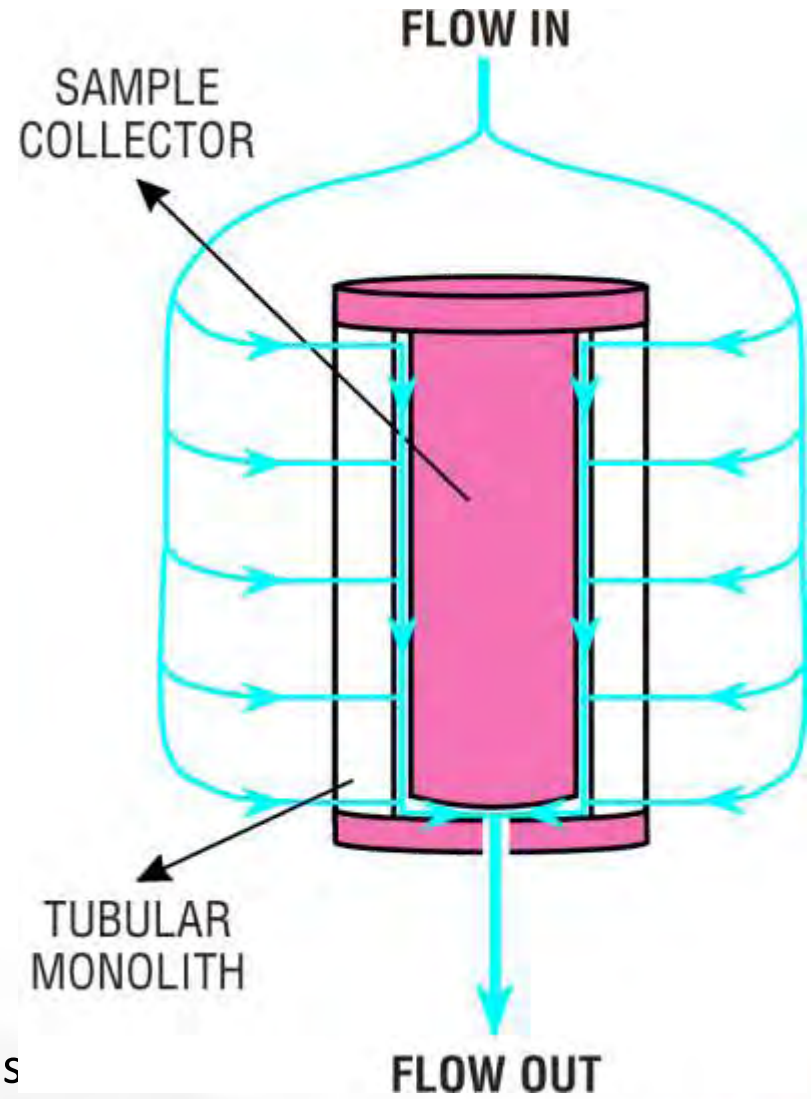
**CIM Disk housing parts**

**Color of ring denotes the disk chemistry**

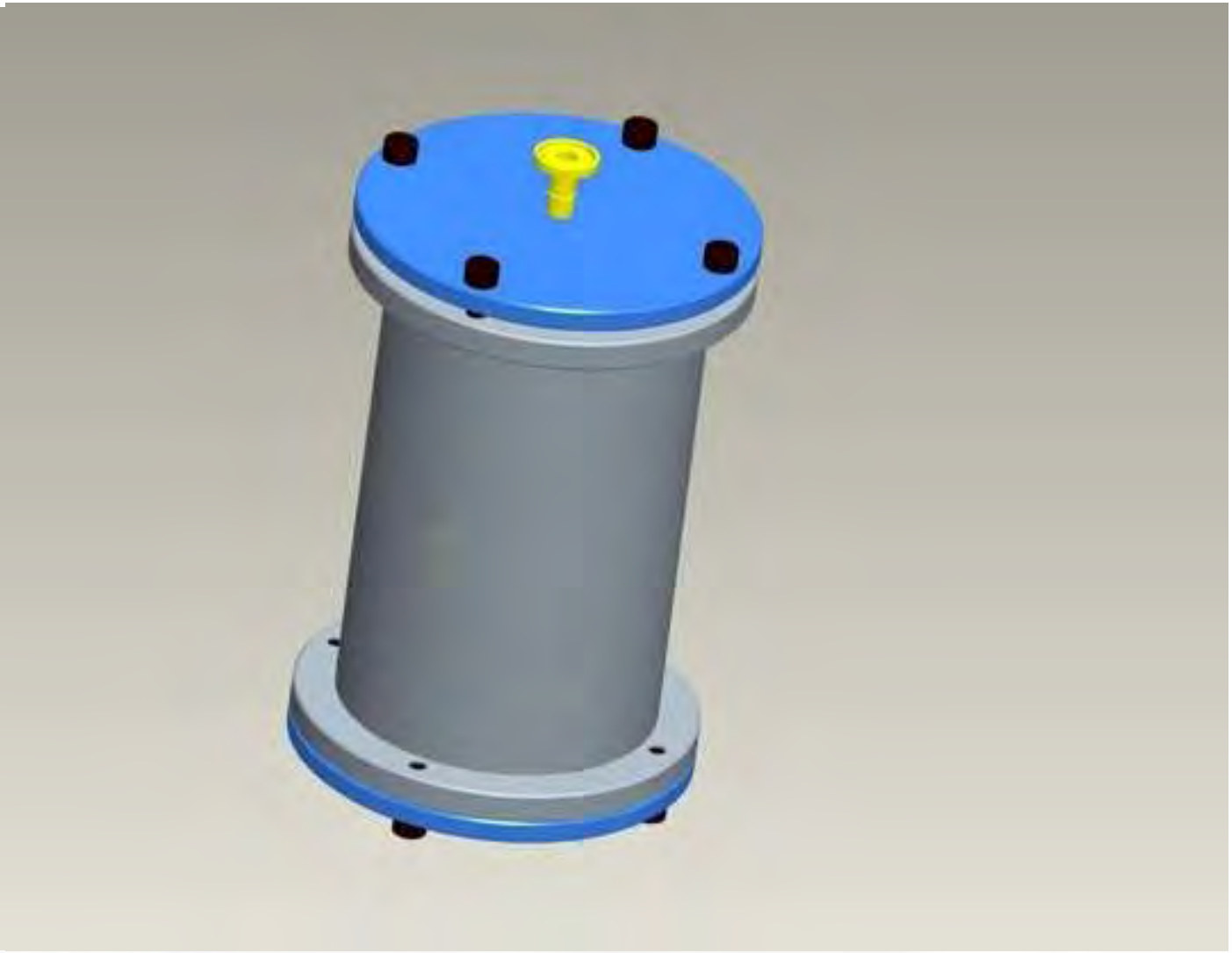
- Strong AEx (QA)
- Weak AEx (DEAE)
- Strong CEx (SO3)
- Weak CEx (CM)

# Industrial Scale Units – CIM® Tubes

80, 800, and 8.000 ml CIM Monoliths



# CIM® Tube Column Structure



1. Piston – Collector with flow-out
2. Seal
3. Frit
4. Monolith
5. Housing - Distributor
6. Upper plate with flow-in

# Currently Available Sizes

**Performance and process time maintained!**

***0.34 ml disk***

***3-8 ml/min***

***8 ml column***

***10-40 ml/min***

***80 ml column***

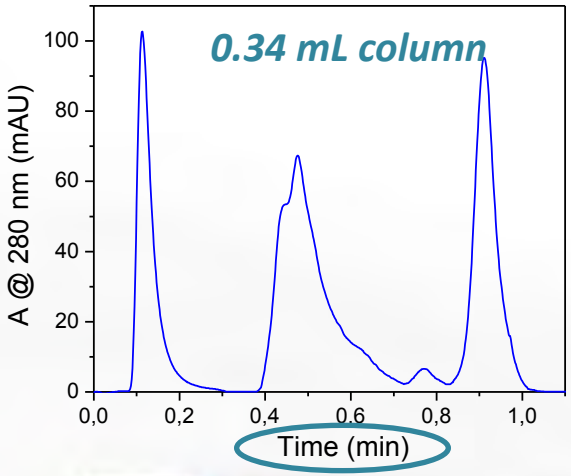
***40-250 ml/min***

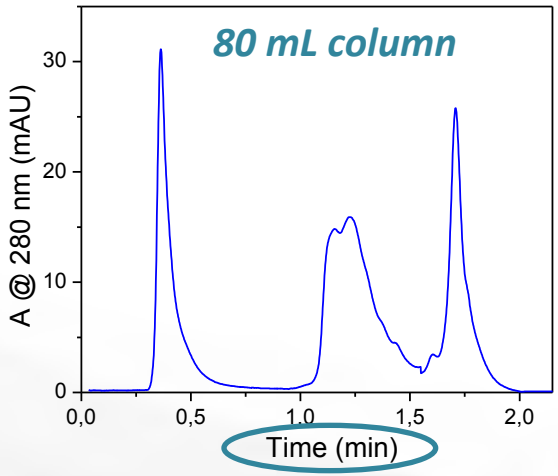
***800 ml column***

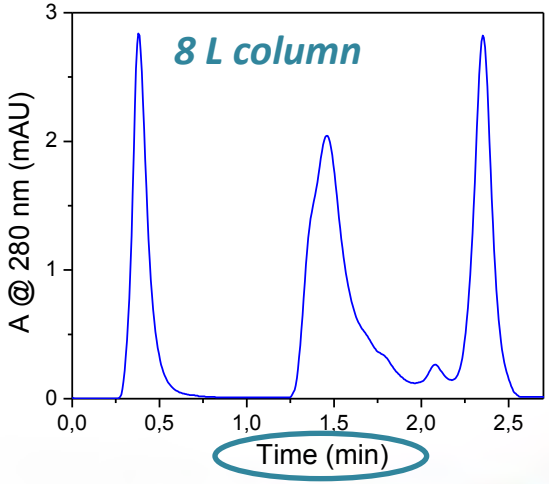
***400-2000 ml/min***

***8000 ml column***

***2000-10000 ml/min***



# Applications

# Clotting Factor VIII/von Willebrand Factor Complex

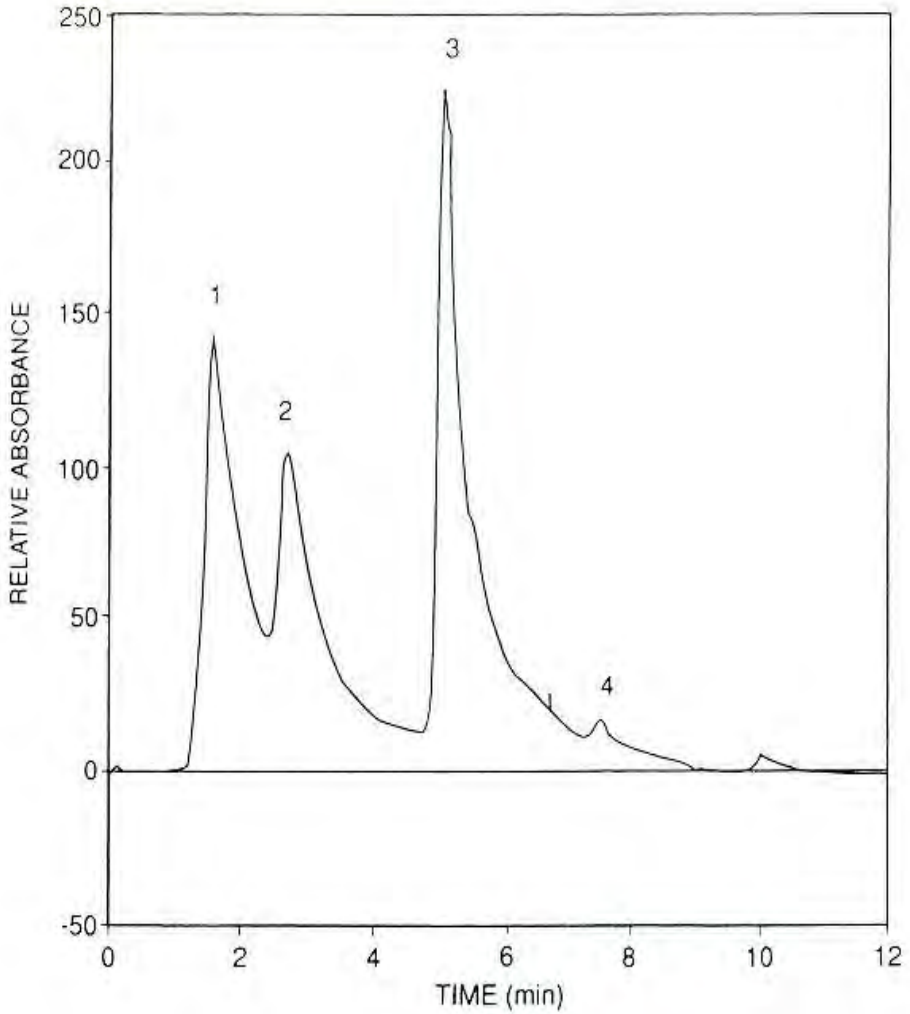
# Separation of Clotting Factor VIII on a CIM® QA Disk Monolithic Column

Separation of clotting factor VIII/von Willebrand factor complex from contaminating (model) proteins

Peaks 1 & 2 - Transferrin and IgG

Peak 3 – Human Serum Albumin

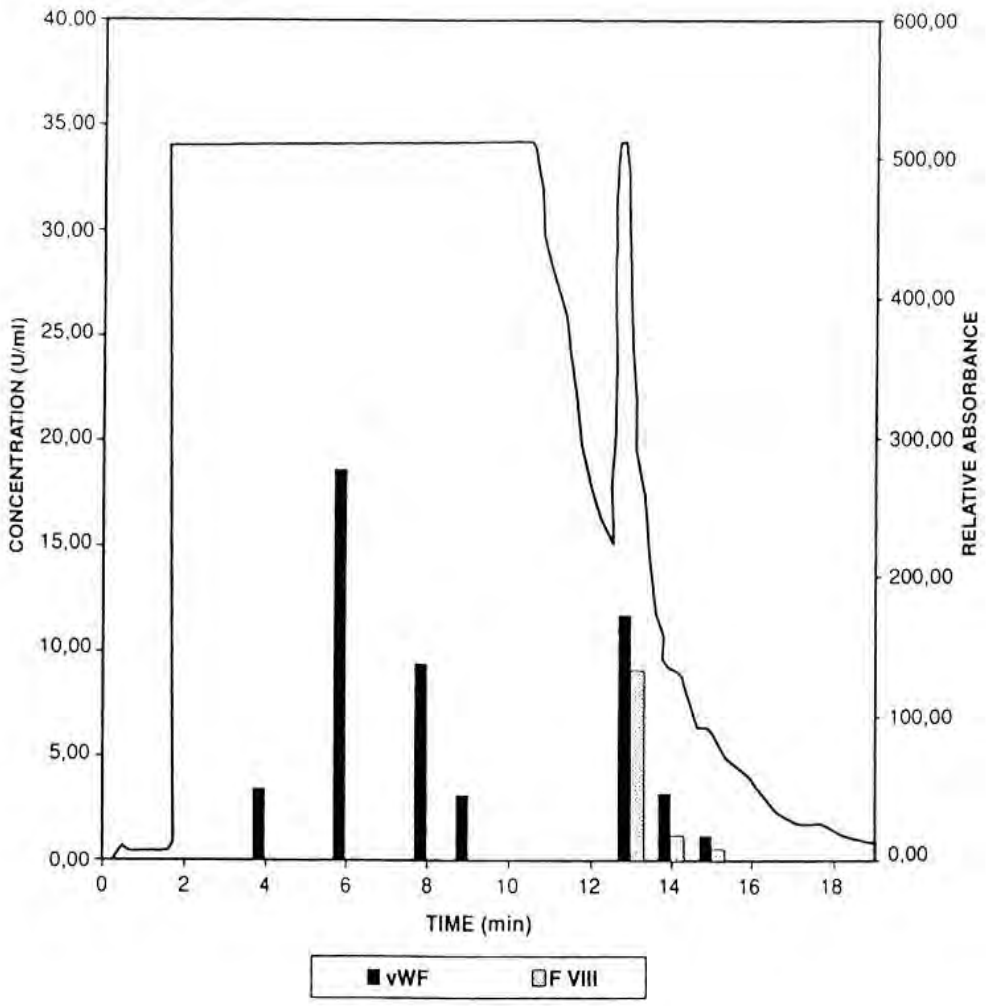
Peak 4 – FVIII/vWF




(Štrancar A. et al. J. Chromatogr. A 1997; 760: 117-123)

# Semi-Preparative Isolation of Clotting Factor VIII/von Willebrand Factor Complex

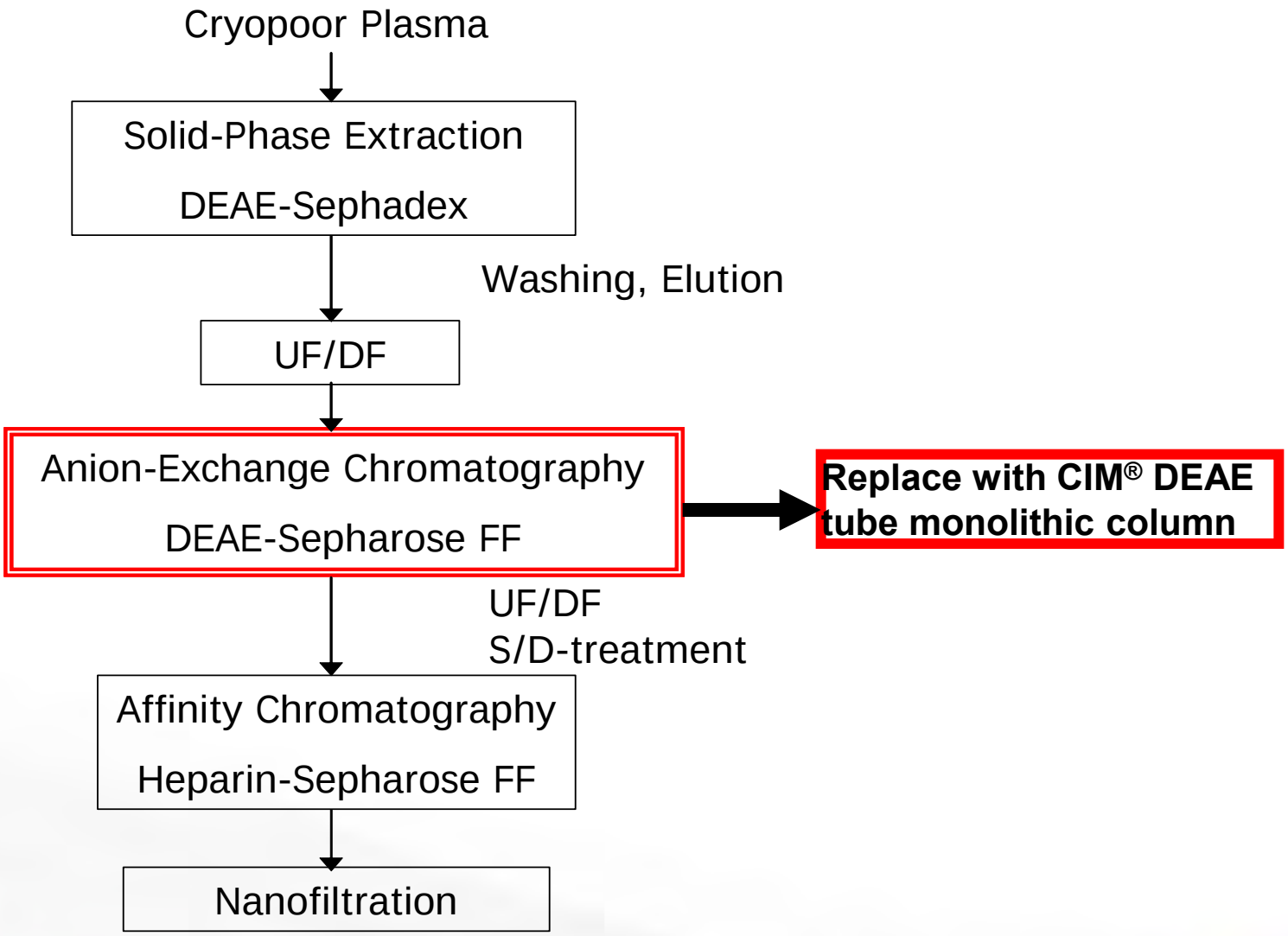
Separation of sample No. 3 from FVIII production resolved cryoprecipitate after $Al(OH)_3$ precipitation and S/D virus inactivation, obtained on a QA anion-exchange compact porous tube (53 mm long, 23 mm diameter and with a 1-mm inner hole). Conditions: Buffer A, 10 mM sodium citrate, 120 mM glycine, 1 mM $CaCl_2$, pH 7.0; buffer B, buffer A containing 1 M NaCl; injection volume, 20 ml.




(Štrancar A. et al. J. Chromatogr. A 1997; 760: 117-123)

# Clotting Factor IX

# FIX – Production Process

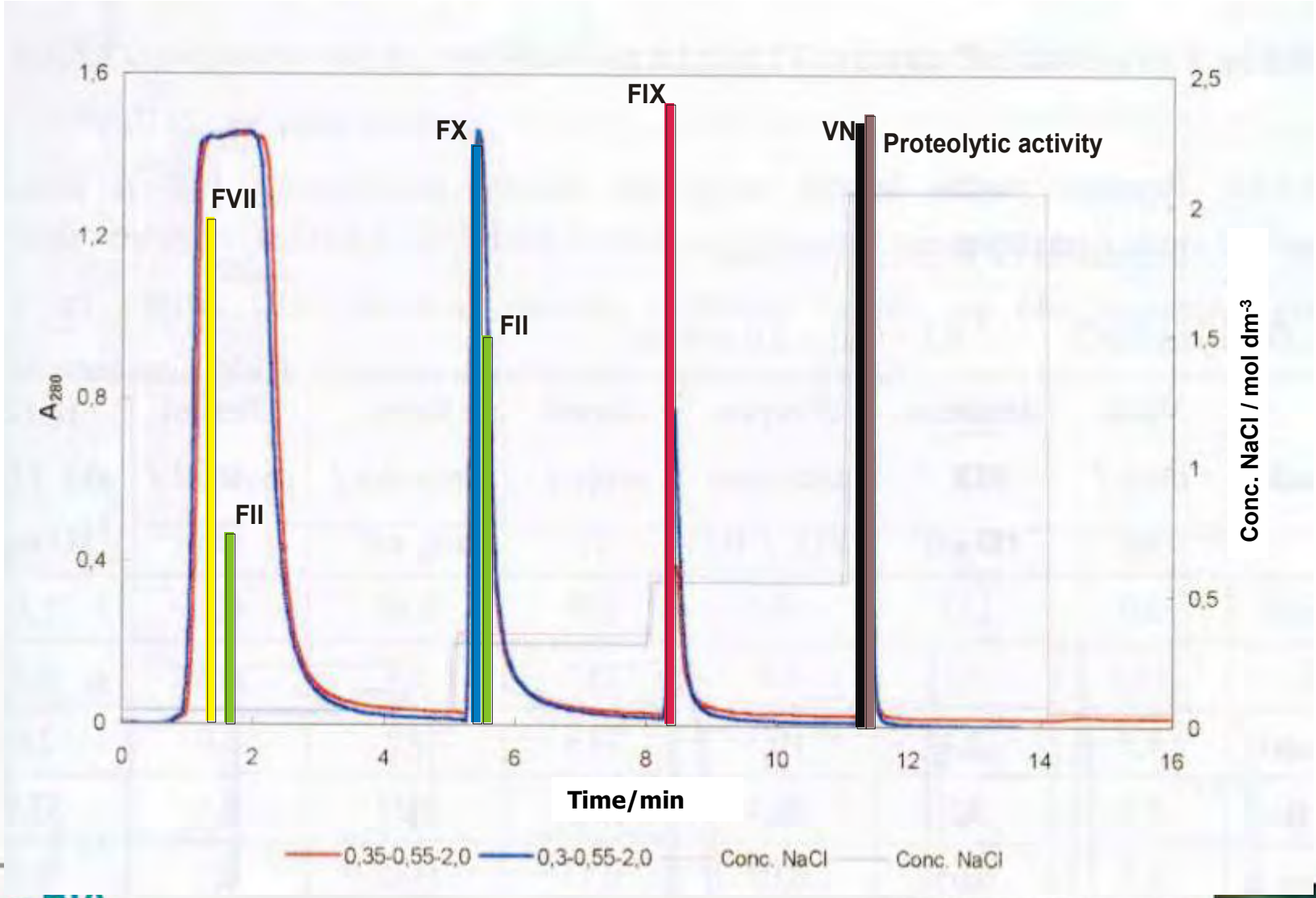


# Anion exchange chromatography: Purification of FIX out of the Sephadex eluate

| Column | Buffer | Step gradient | Dynamic capacity | Recovery (%) | Specific activity |
|---|---|---|---|---|---|
| **CIM® DEAE tube** | **Citrate** | **10-50-100** | **109.4** | **70.1** | **12.5** |
| | **Phosphate** | **10-50-100** | **187.5** | **76.2** | **10.6** |
| **DEAE conventional column** | **Citrate** | **10-50-100** | **93.4** | **100** | **3.4** |
| **CIM® QA tube** | **Citrate** | **5-30-100** | **156.1** | **86.3** | **14.5** |
| | **Phosphate** | **5-30-100** | **177.1** | **97.5** | **12.8** |

*Courtesy of Prof. Dj. Josic, Octapharma, Vienna, Austria*

# Process Design: Use of Step Gradient Disk-Shaped Monolithic Column (340 µl)

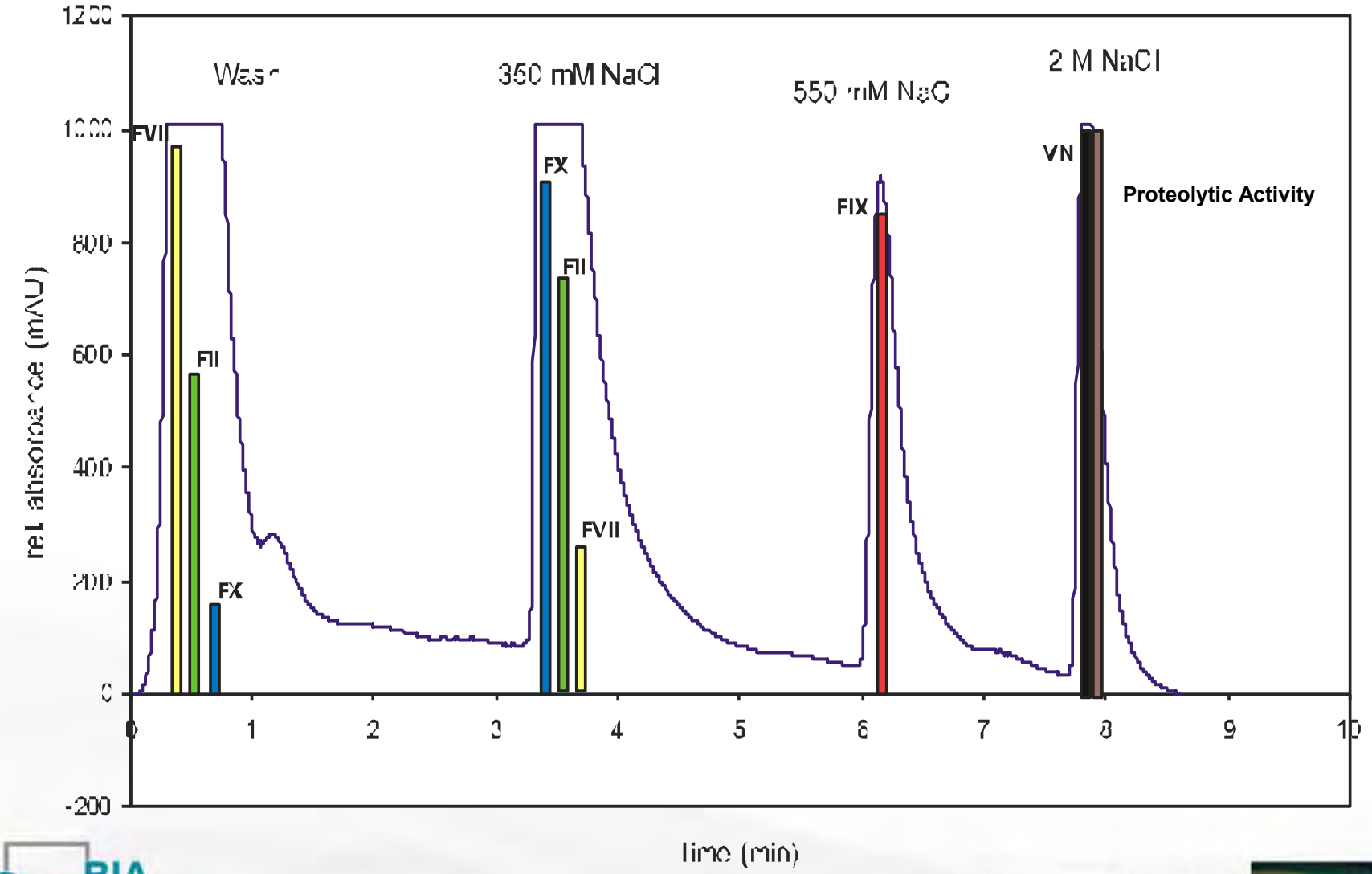


# Process Design
# Disk-Shaped Monolithic Column (340 µl)

| | | |
|---|---|---|
| **FIX-yield** | **–** | **77.1 %** |
| **FIX-specific activity Protein** | **–** | **44.4 IU/mg** |

*Courtesy of Prof. Dj. Josic, Octapharma, Vienna, Austria*

# Scaling-up
# 8 ml Tube Monolithic Column

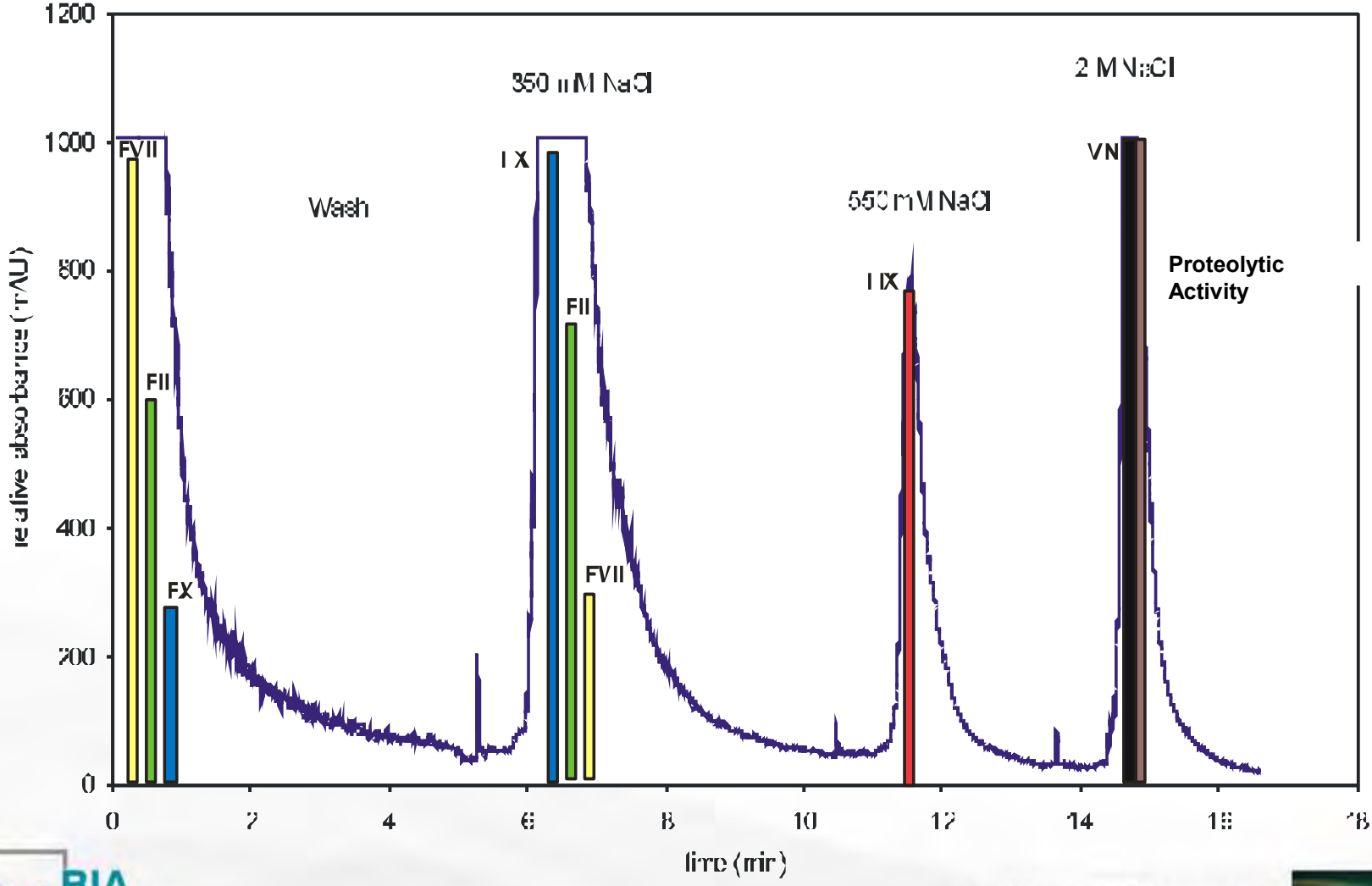


# Optimization

## Scaling-up, 8 ml tube

| | FVII (IU) | FIX (IU) | FX (IU) | PA x10³ (U) | VN (mg) | Protein (g) | Vol. (ml) | Spec. act. (IU FIX/mg) |
|---|---|---|---|---|---|---|---|---|
| Load | 240 | 500 | 485 | 19.85 | 12.50 | 0.423 | 10 | 1.02 |
| F/T | 4.32 | 0.24 | 12.7 | 0.47 | 0.48 | 0.157 | 12 | - |
| W | 27.50 | 0.9 | 25.2 | 0.12 | 2.30 | 0.167 | 45 | - |
| $E_1$ | 292 | 80 | 372 | 0.08 | 1.98 | 0.124 | 40 | - |
| $E_2$ | 1.5 | 487.5 | 15 | 0.13 | 1.28 | 0.018 | 25 | 27.05 |
| $E_3$ | 0.25 | 15 | 1 | 25.2 | 9.0 | 0.165 | 25 | - |
| Σ | | | | | | | | |

# Optimization Separation with 800 ml CIM® DEAE Tube Monolithic Column



# Optimization Separation with 800 ml CIM® DEAE Tube Monolithic Column

**Scaling-up, 800 ml tube sephadex eluate**

| | FVII (IU) | FIX (IU) | FX (IU) | PA x$10^3$ (U) | VN (mg) | Protein (g) | Vol. (ml) | Spec. act. (IU FIX/mg) |
|---|---|---|---|---|---|---|---|---|
| Load | 28800 | 40680 | 58200 | 2382 | 1500 | 39 | 1200 | 1.02 |
| F/T | 853 | 1114 | 1651 | 132 | 152.7 | 8.05 | 1376 | - |
| W | 6173 | - | 4079 | 21.2 | 747.8 | 22.5 | 4249 | - |
| $E_1$ | 23233 | 3319 | 33927 | 17.5 | 545.8 | 8.85 | 1843.9 | - |
| $E_2$ | 40 | 40172 | 331 | 9.33 | 86.4 | 1.2 | 1004.3 | 33.3 |
| $E_3$ | 30 | 1719 | 69 | 1806 | 288 | 2.0 | 955.9 | - |
| Σ | 30329 | 46324 | 40057 | 1986 | 1819 | 42.6 | - | - |
| % | 105 | 113 | 68.8 | 83.3 | 121 | 109 | - | - |

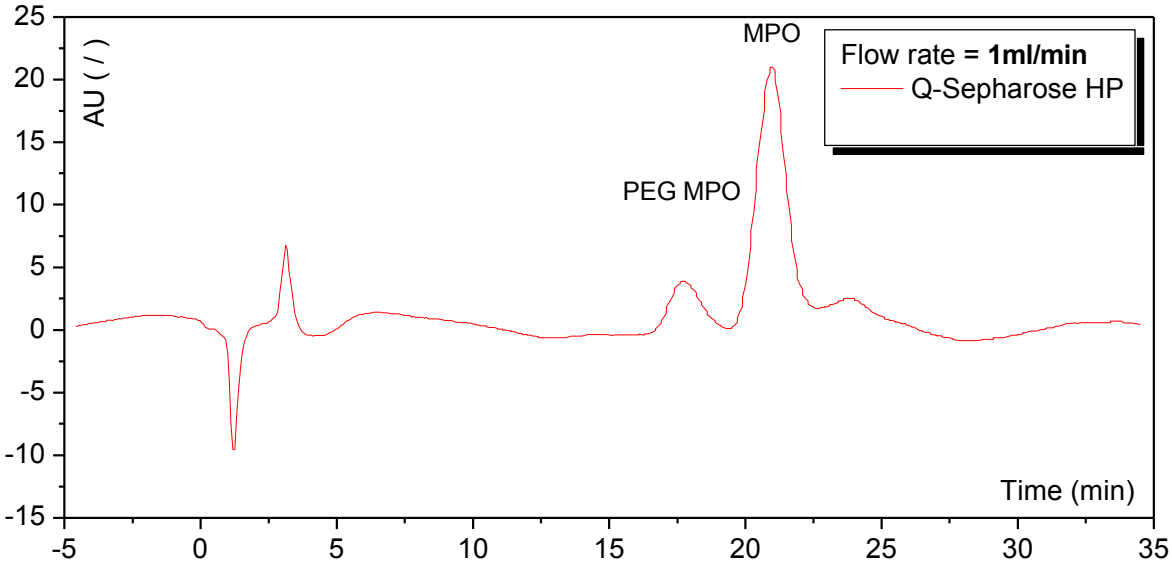
# Major advantages using CIM® columns for FIX purification

- Very fast method development.
- Very fast scale-up to industrial scale.
- Highly reproducible results regarding speed, specific activity and yield from the disk to industrial columns.
- About 20 times faster purification than using column packed with bulk support.
- Specific activity in the eluate 5 times higher than in corresponding purification step using column packed with bulk support.

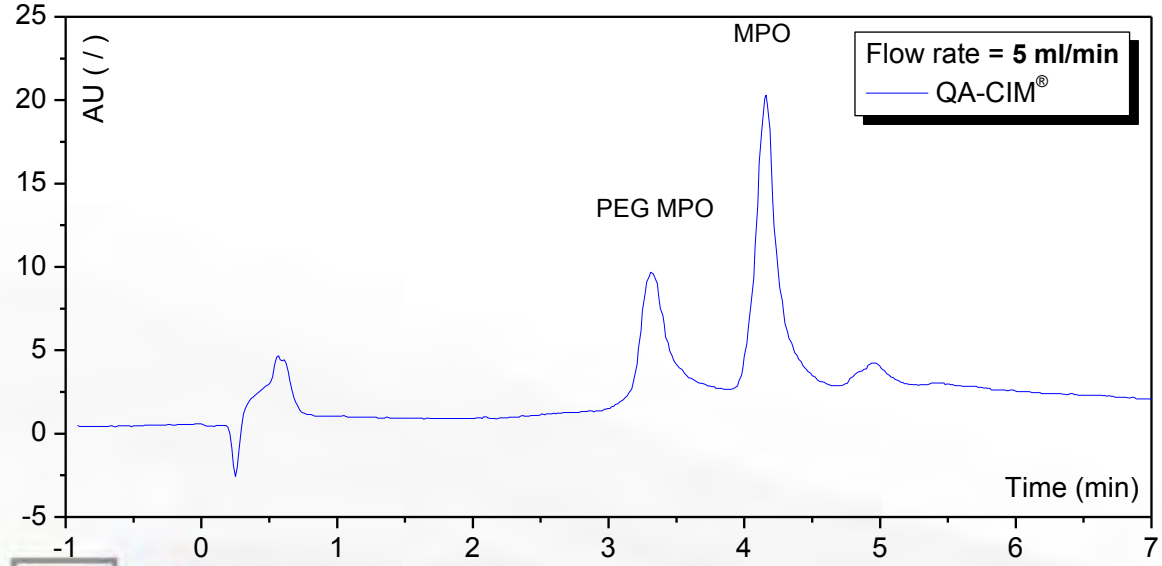
*Josic, PPB 03, Curacao*

# Pegilated proteins

# Faster Separation of PEGylated Proteins



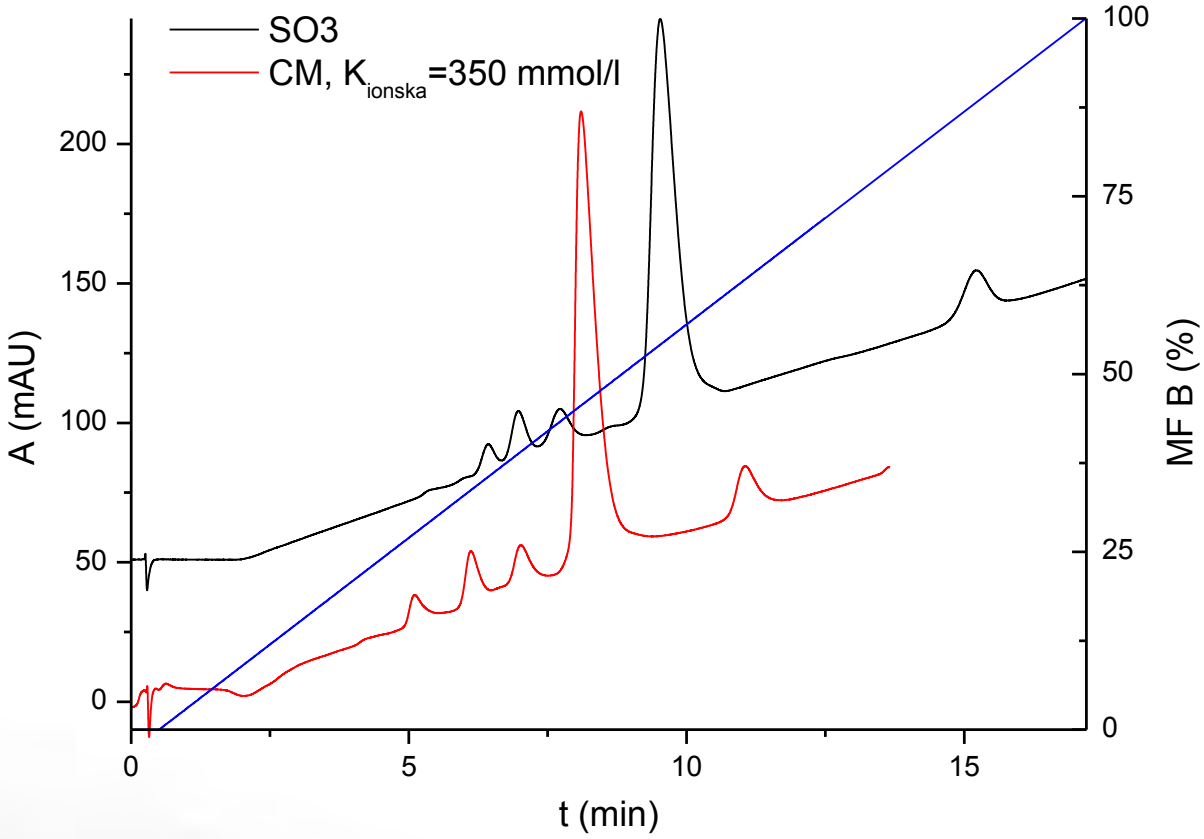

Q-Sepharose HiTrap
25mm x 7mm (1ml)
Amersham/GE




***QA-CIM®***
3 disks (1ml)
BIA Separations

***- 5-fold faster run!***
***- Sharper peaks!***

# Separation of PEGylated Proteins on cation exchange CIMac™ columns

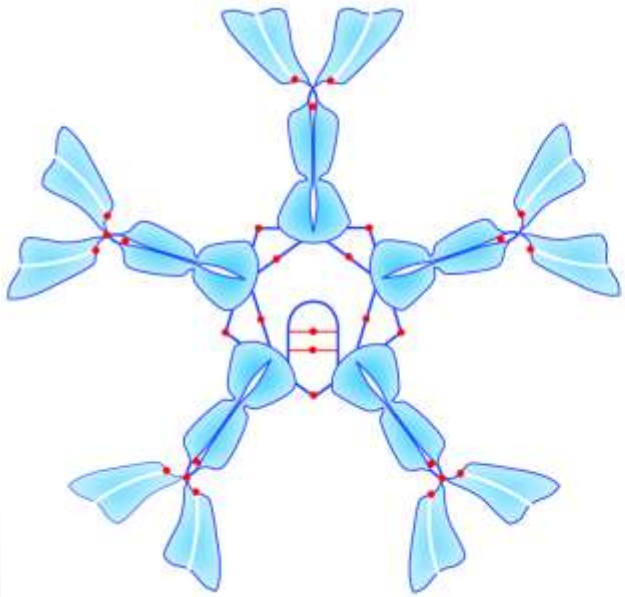



CIMac™ SO3 and CM columns, 15 mm lenght; Gradient: 0- 0,5 min 100 % Buffer A, than 0-100 % Buffer B in 52 Column Volumes; Buffer A: 20 mM phoshate, pH 6,5, Buffer B: Buffer A + 0,3 M NaCl

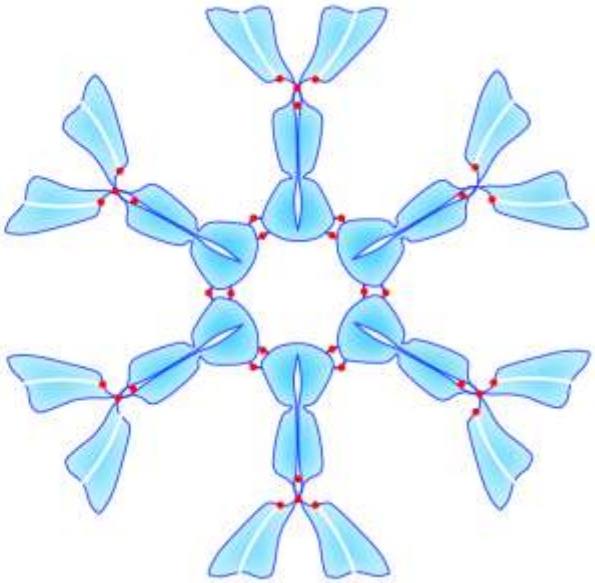
# Platform IgM purification processes

# Immunoglobulin - IgM

Pentameric, 0.96 Md



Hexameric, 1.15 Md



# Are IgMs really difficult to purify?

IgMs have some characteristics that can limit the application of standard purification tools:

- They tend to be less soluble than IgGs and more susceptible to denaturation at extremes of pH. This can limit application of affinity chromatography.

- Low solubility is compounded by low conductivity. This can limit ion exchange chromatography.

-They are generally tolerant of high salt concentrations, but susceptible to denaturation from exposure to strongly hydrophobic surfaces. This can limit hydrophobic interaction chromatography.

- Large size corresponds with slow diffusion constants.

- Porous particle based chromatography media depend on diffusion for mass transport.

- Slow diffusion constants translate into lower capacity and lower resolution, and/or lower flow rates.

- This is a particular limitation for size exclusion chromatography because it already suffers from low capacity and low flow rate.

# Are IgMs really difficult to purify?

On the other hand:

- IgMs are typically more charged than IgGs. They bind more strongly than IgG to anion exchangers or cation exchangers.

- They also bind more strongly than IgG to hydroxyapatite, and much more strongly than most contaminants.

- HIC on moderately hydrophobic supports usually elutes IgM in a well defined peak at reasonably low salt concentration.

- A new generation of industrial ion exchangers is available that does not rely on diffusion.

- Convection is independent of size and flow rate, so capacity and resolution are not affected by the large size of IgM, nor does flow rate need to be reduced.

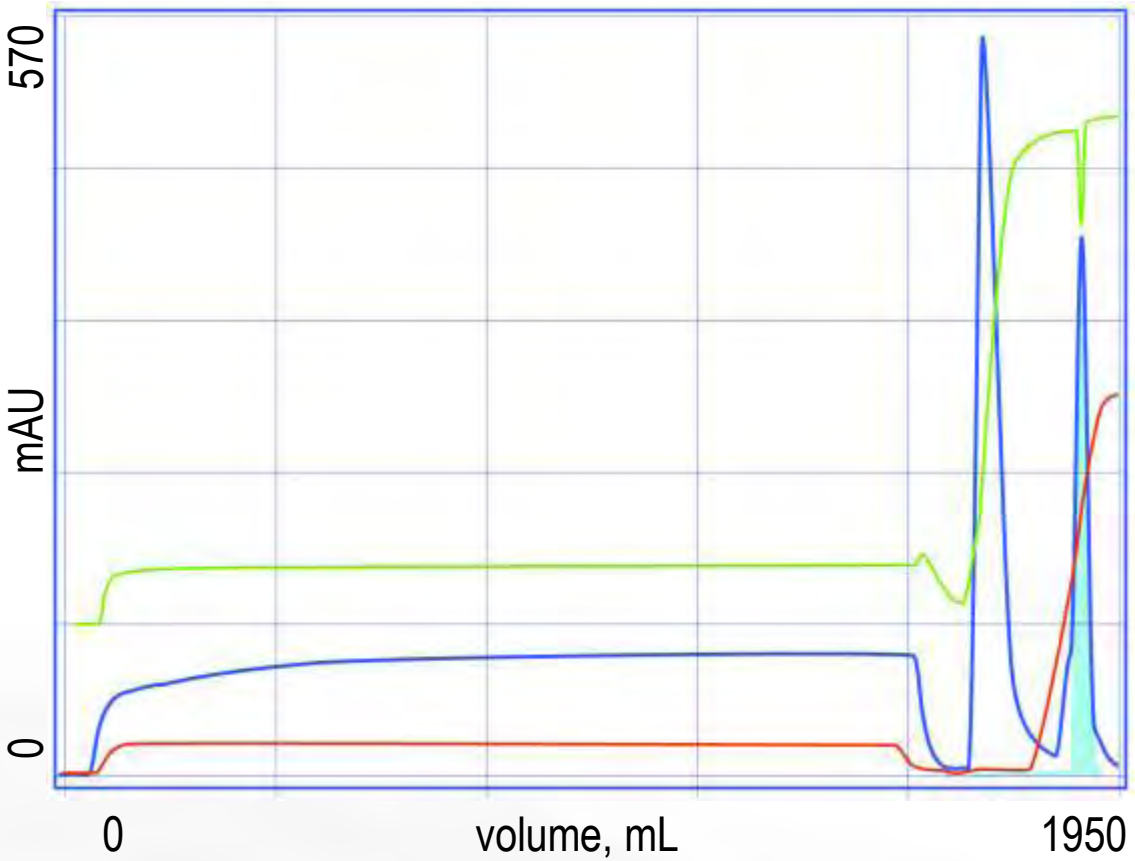
For details visit validated.com

# Purification of Clinical Grade Human IgM from Cell Culture Supernatant

| | |
|---|---|
| STEP 1 | Cation exchange chromatography on a **CIM® SO3 Tube Monolithic Column** |
| STEP 2 | Anion Exchange Chromatography on a **CIM® QA Tube Monolithic Column** |
| STEP 3 | Polishing on a hydroxyapatite column |

For details visit [validated.com](validated.com)

# STEP 1:
# Cation Exchange Chromatograpy

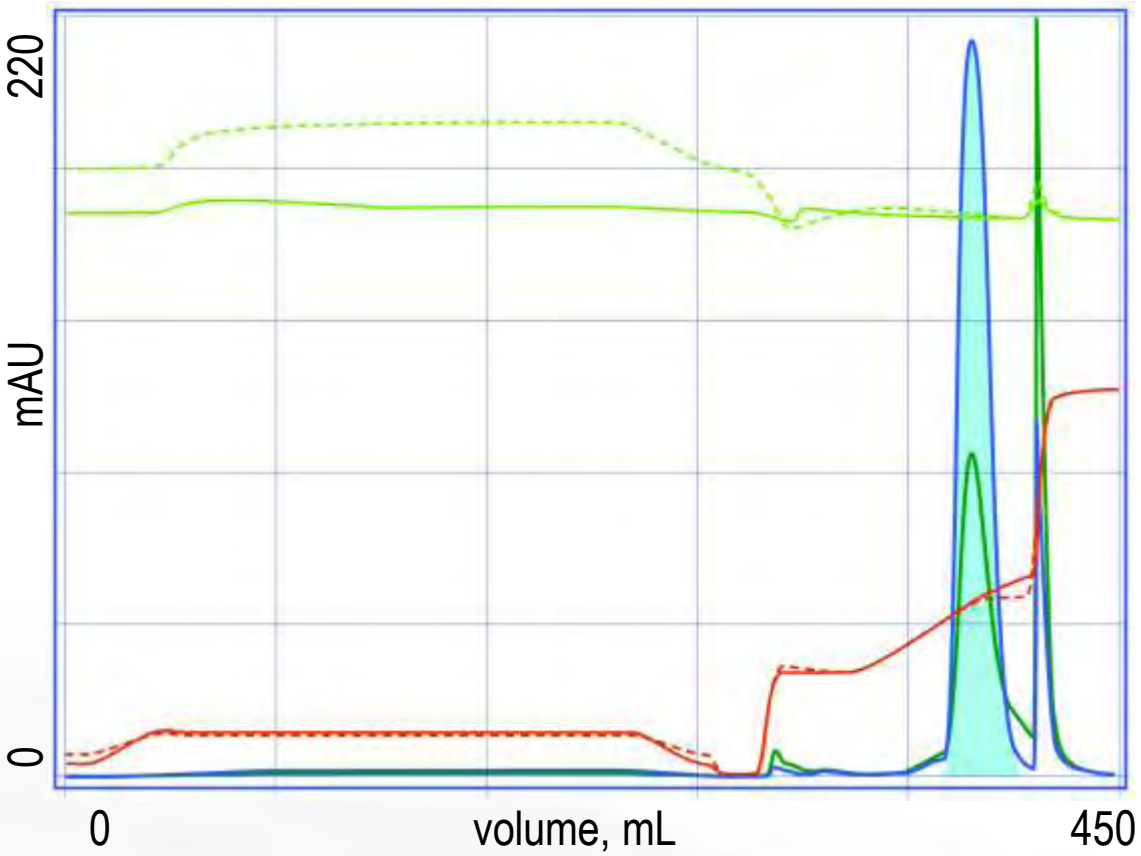



**Column:** CIM® $SO_3$ Tube Monolithic Column (V=8 mL)
**Flow rate:** 20 mL/min

Equilibrate: 50 mM MES, pH 6.0
Titrate dilute load: 1500 mL
Wash: 50 mM MES, pH 6.0
Wash: 25 mM NaPO4 pH 7.0
Elute: LG to 225 mM NaPO4
Clean: 500 mM NaPO4 pH 7.0
Sanitize/store: 1.0/0.01 M NaOH

For details visit validated.com

# STEP 2:
# Anion Exchange Chromatograpy

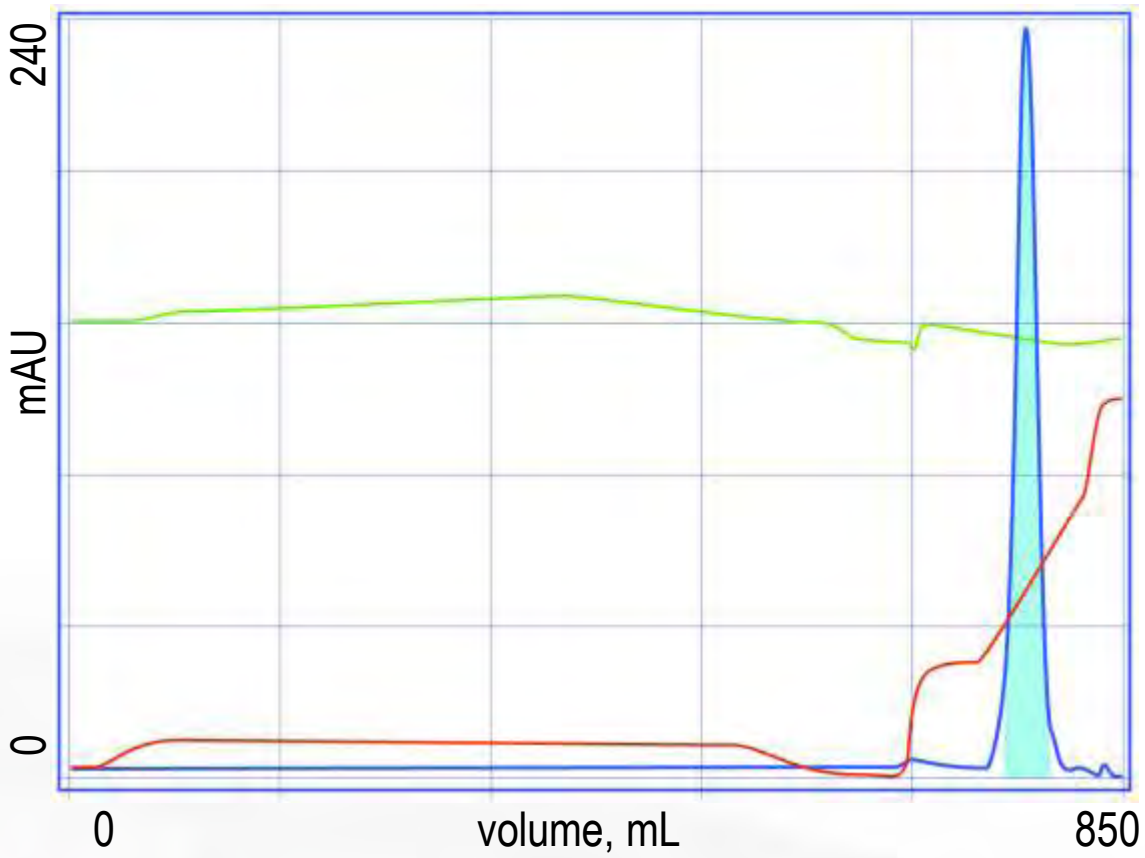



Column: CIM® QA Tube Monolithic Column (V=8 mL)
Flow rate: 20 mL/min

Equilibrate: 10 mM NaPO4 pH 7.0
Load 1: pH 7.0 Load 2: pH 8.0
Wash: 75 mM NaPO4 pH 7.0
Elute: LG to 225 mM NaPO4
Clean: 500 mM NaPO4 pH 7.0
Sanitize/store: 1.0/0.01 M NaOH

For details visit validated.com

# STEP 3:
# Hydrophobic Interaction using Hydroxyapatite




Media: 10 mL CHT type II 40µm
Column: Hydroxyapatite column
Flow rate: 3.4 mL/min

Equilibrate: 10 mM NaPO4 pH 7
Load: Adjusted eluate
from STEP 2
Wash: 10 mM NaPO4 pH 7.0
Wash: 75 mM NaPO4 pH 7.0
Elute: LG to 225 mM NaPO4 pH 7
Clean: 500 mM NaPO4 pH 7.0
Sanitize/store: 1.0/0.1 M NaOH

For details visit validated.com

# IgM Purification Process Summary

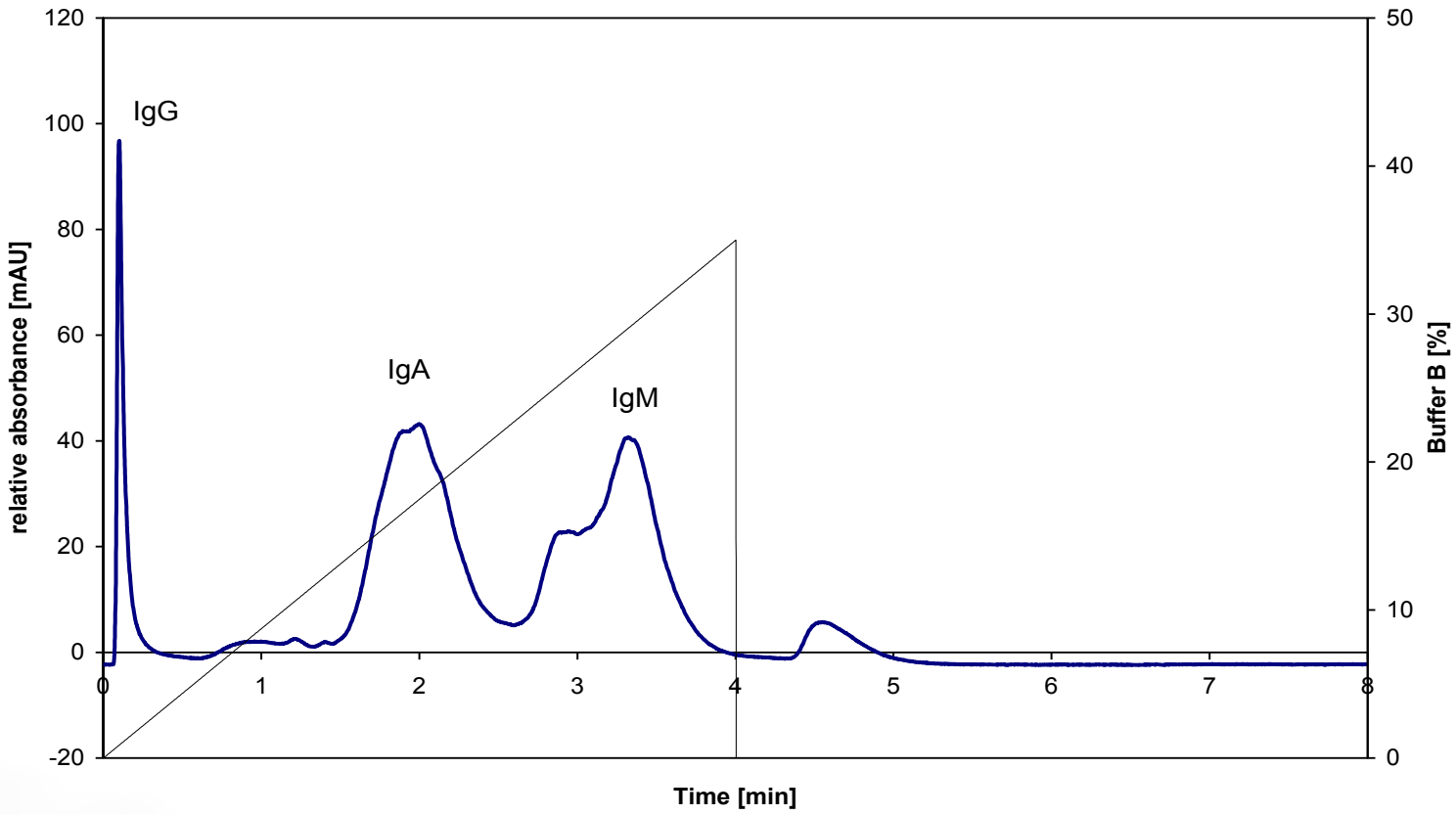
## *Process summary*

| | *Cation exchange 8 mL monolith* | *Anion exchange 8 mL monolith* | *Hydroxyapatite 10 mL column* |
|---|---|---|---|
| *Sample volume, mL* | *250* | *25* | *25* |
| *Diluted Sample, mL* | *1250* | *250* | *250* |
| *Diluted Sample, CV* | *156* | *31* | *25* |
| *Flow rate, mL/min* | *20* | *20* | *3.34* |
| *Flow rate, CV/min* | *2.5* | *2.5* | *0.67* |
| *Application time* | *62.5* | *12.5* | *75.0* |
| *Total volume[1]* | *1950* | *950* | *650* |
| *Total time, min* | *98* | *48* | *195* |
| *Recovery %* | *78(86)[2]* | *84* | *88* |
| *Purity %* | *~90* | *~95* | *~99* |

*[1]Includes equilibration, sample application, wash, elution, cleaning.*
*[2]Includes the IgM that eluted prematurely in the wash.*

*Courtesy of Pete Gagnon, Validated Biosystems, USA – details at [www.validated.com](http://www.validated.com)*

# Separation of IgG, IgA and IgM using CIM® QA




Column: **CIM® QA (strong anion exchanger)** column (5,2 mm I.D. x 4,95 mm L; V = 100 ul)
Sample: A mixture of human IgG (Octapharma, Md = 150 kDa), IgA (Sigma, xxxx, Md = 160 kDa) and IgM (Sigma,yyy, Md = 950 kDa) 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).
Detection: UV at 280 nm Column pressure: 15 bar (1,5 MPa)

# IgG impurity removal processes

# IgG Impurity Removal

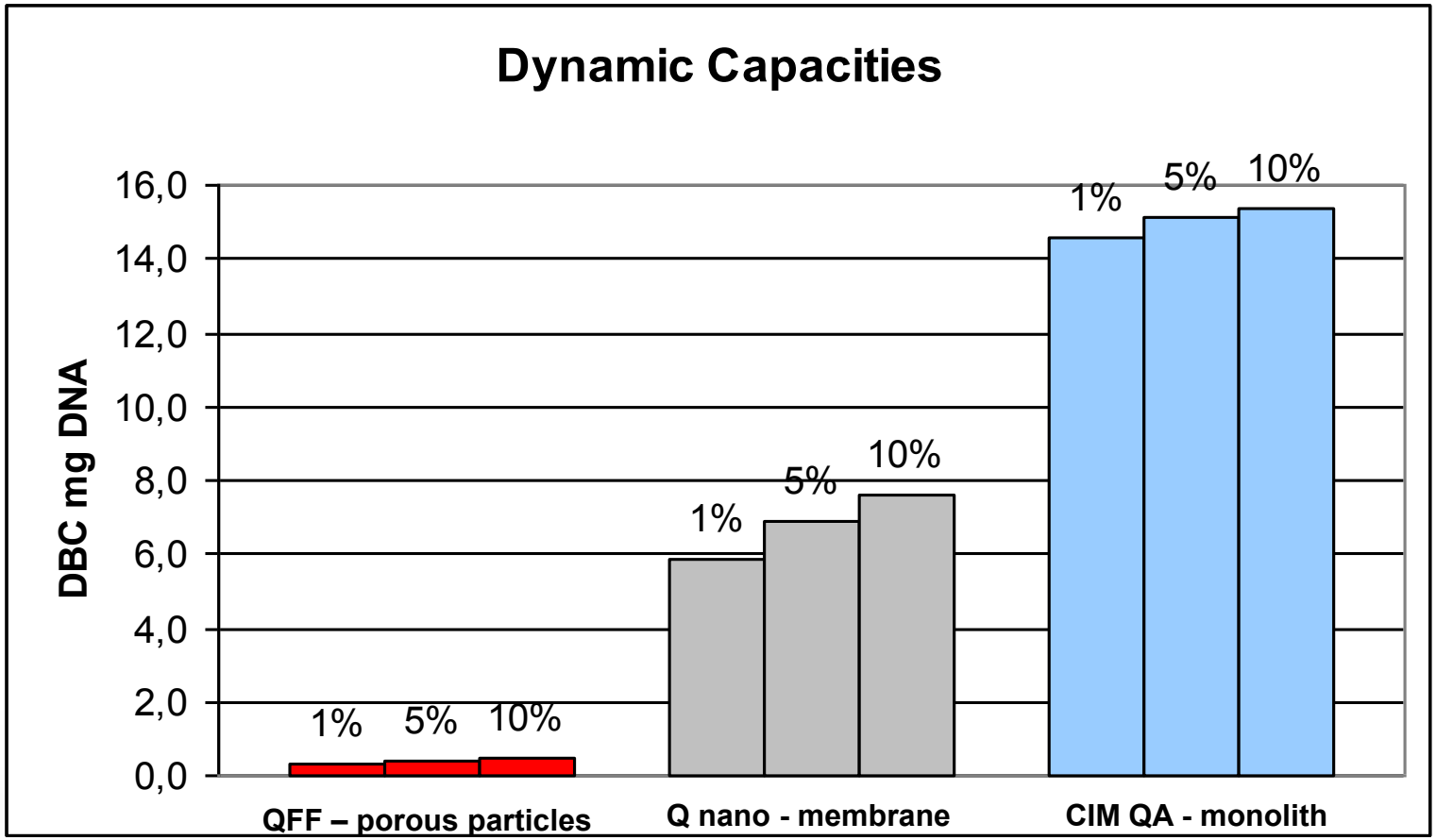
- Sample:
  - Chimera
    - 1 mg/mL purified monoclonal IgG + 350 ml of 0.1 mg/mL DNA
- Comparison of polishing applications of 3 different resins:
  - Microparticulate

    Q Sepharose™ Fast Flow (1mL HiTrap™ column)
  - Membranes

    Sartobind™ Q nano (1mL)
  - Monoliths

    CIM® QA Disk Monolithic Columns (1 mL - 3 disks)

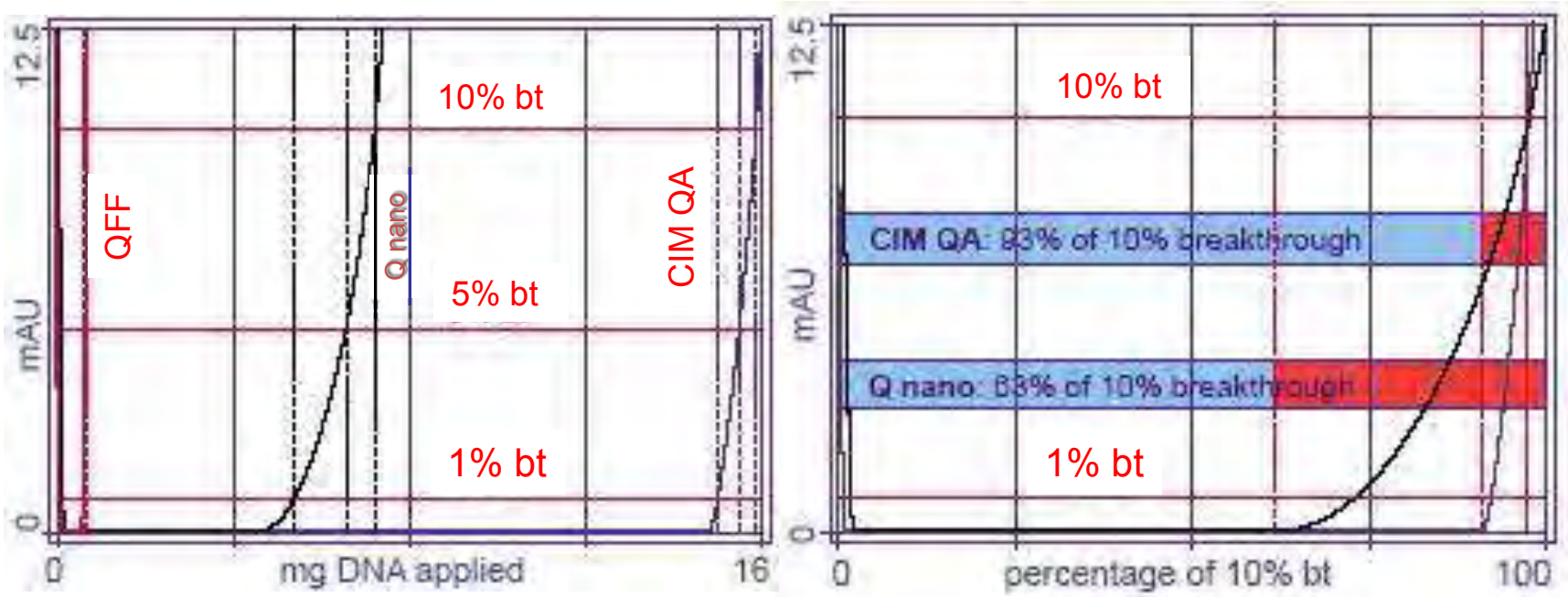
# Dynamic Binding Capacity for DNA




Note 50 times higher dynamic binding capacity than particle based resin while operating at 4-fold higher flow rate!

*Courtesy of Pete Gagnon, for details visit [validated.com](validated.com)*

# DNA Binding Efficiency




**Membrane:** earlier breakthrough, shallower slope

**CIM® QA:** later breakthrough, steeper slope

Lower Binding Capacity
4.8 mg/mL no-bt capacity = Membrane

Higher Binding Capacity
14.3 mg/mL no-bt capacity = CIM® QA

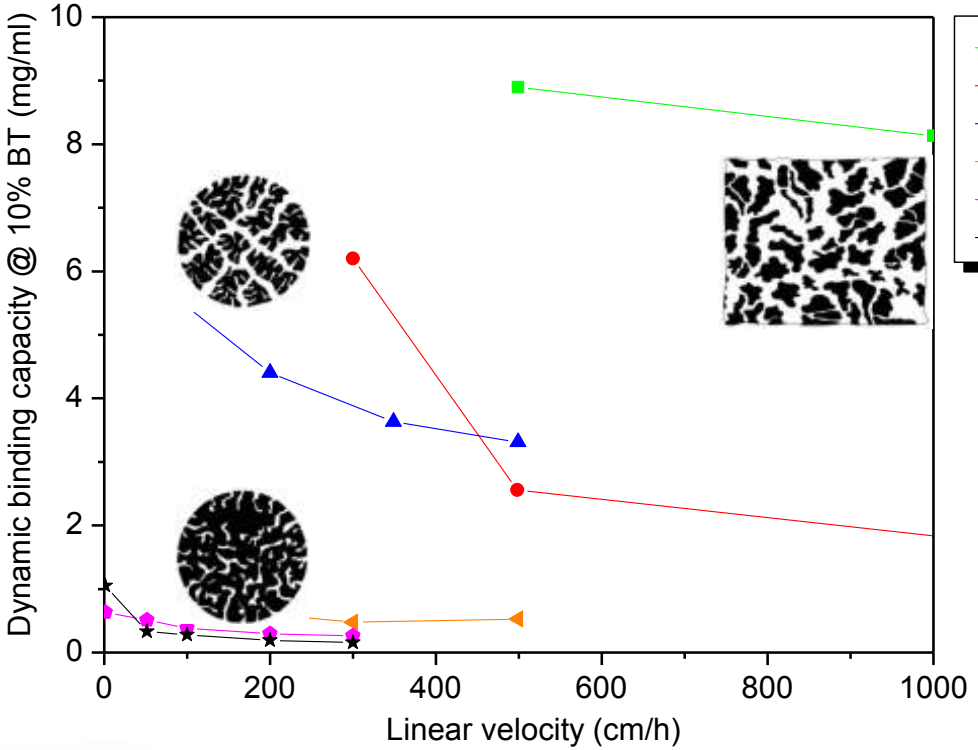
**Important implications for manufacturing of therapeutic antibodies**

*Courtesy of Pete Gagnon, for details visit [validated.com](validated.com)*

# Platform plasmid DNA purification proces

# Plasmid DNA Process

# Speed + Capacity = Productivity = < Costs




**CIM DEAE binding capacity**
**~10 mg pDNA/ml**

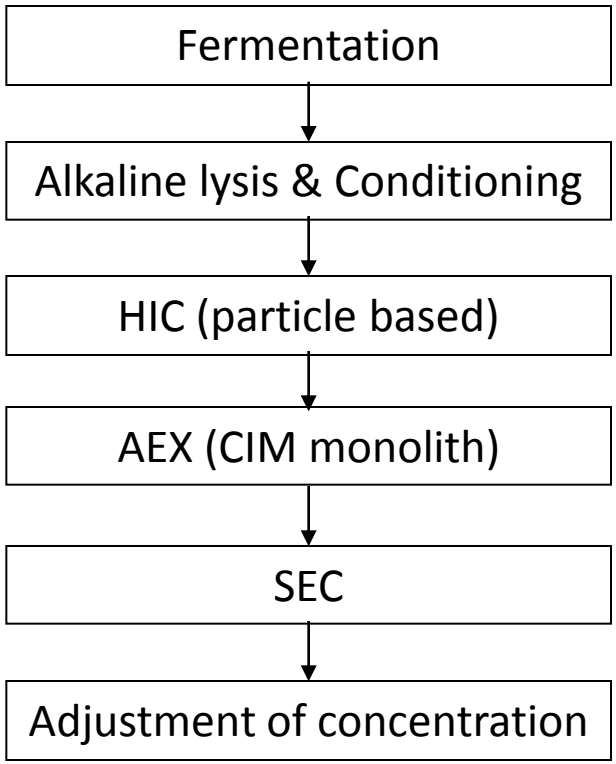
**Passed CP III trials**

15-fold increase in productivity reported by Boehringer Ingelheim. Used for gene therapy, **DNA vaccines**.

- High binding capacity at relevant flow rates
- High elution concentration - pDNA eluted in lower volume (important for SEC!)
- Fast process (no product loss due to oxidative degradation or enzymatic attack)

*Urthaler et al., Boehringer Ingelheim, J.Chrom. A, 1065 (2005), 93-106*

# RNA-se Free pDNA Purification Process




*Urthaler et al., Chem.Eng.Technol., 28 (2005), 1408-1420*

HIC is a bottleneck due to low capacity and slow process – larger columns are needed, more buffers are consumed.

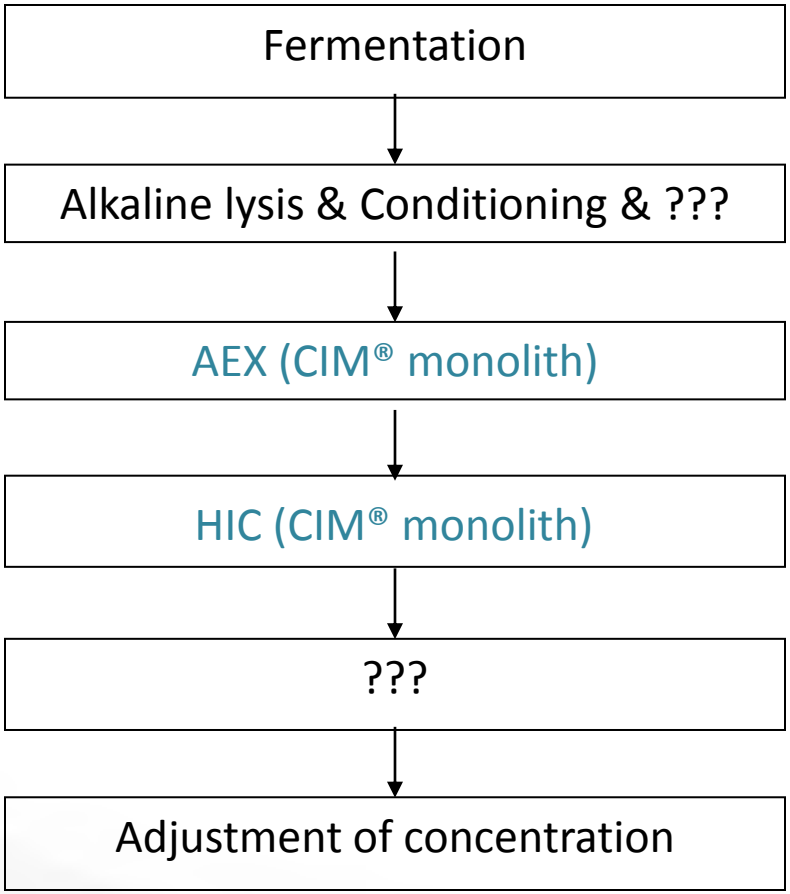
# Novel plasmid DNA purification process

The goal:

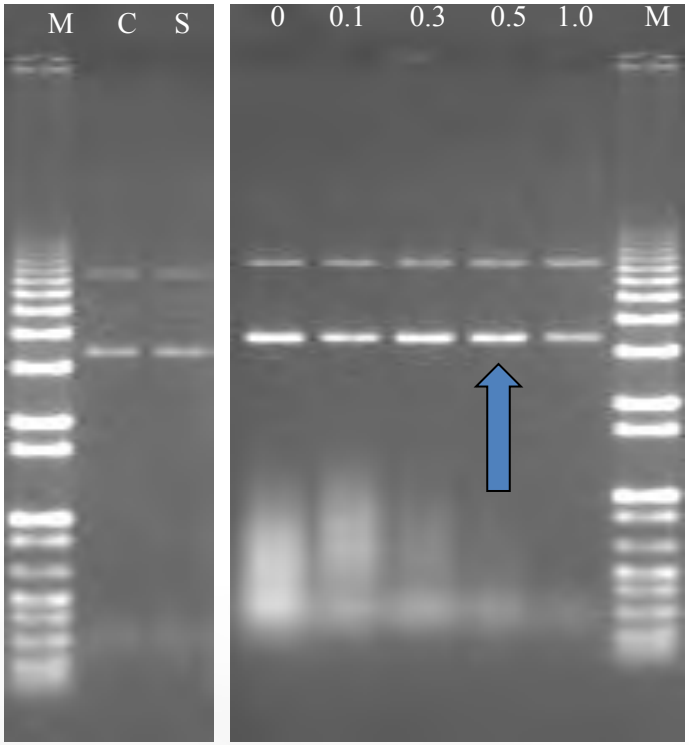
to introduce a resin with higher capacity and better flow propertis as a capturing step –

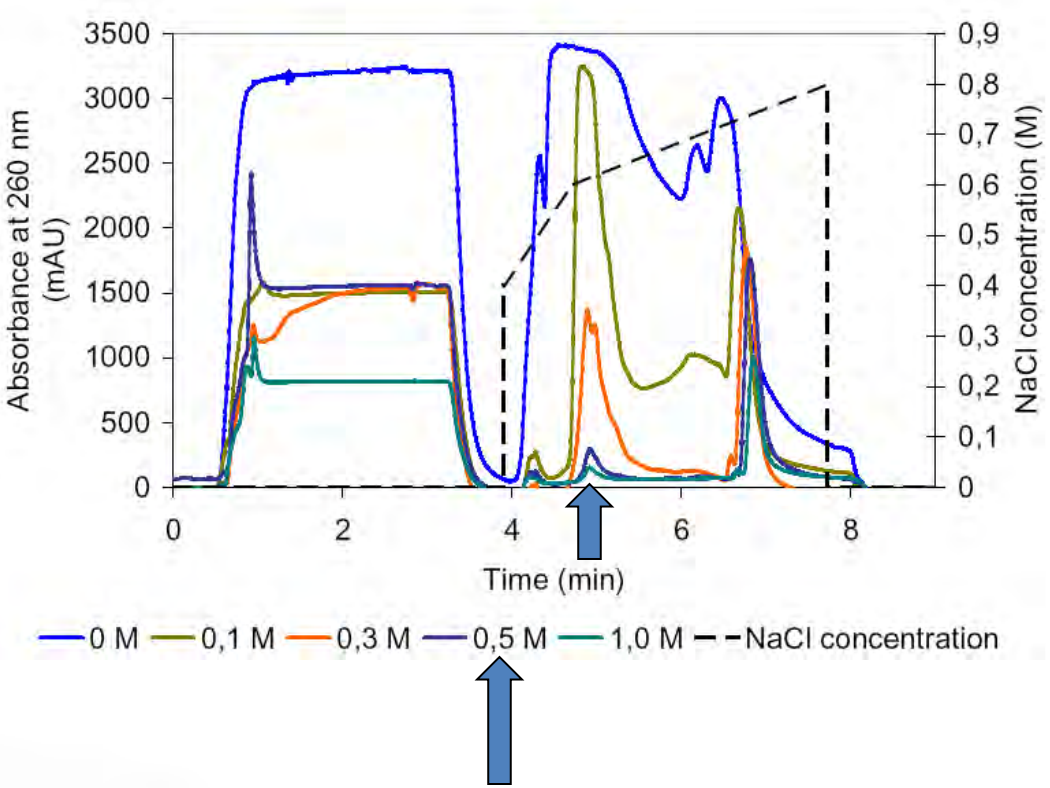
smaller columns and less buffer consuption

# Novel pDNA purification process design



# RNA Removal - Precipitation with $CaCl_2$

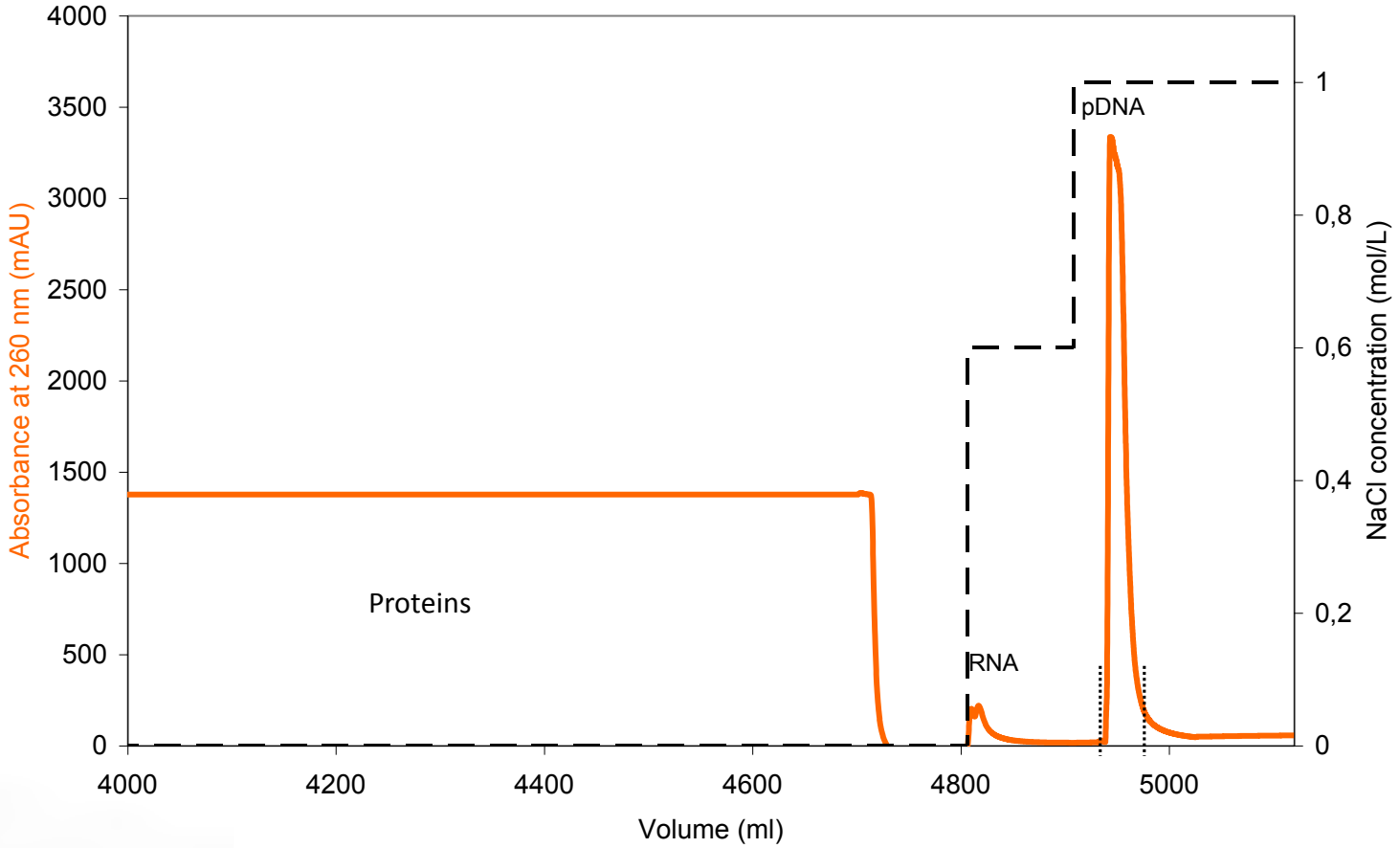


# Selective Precipitation with $CaCl_2$

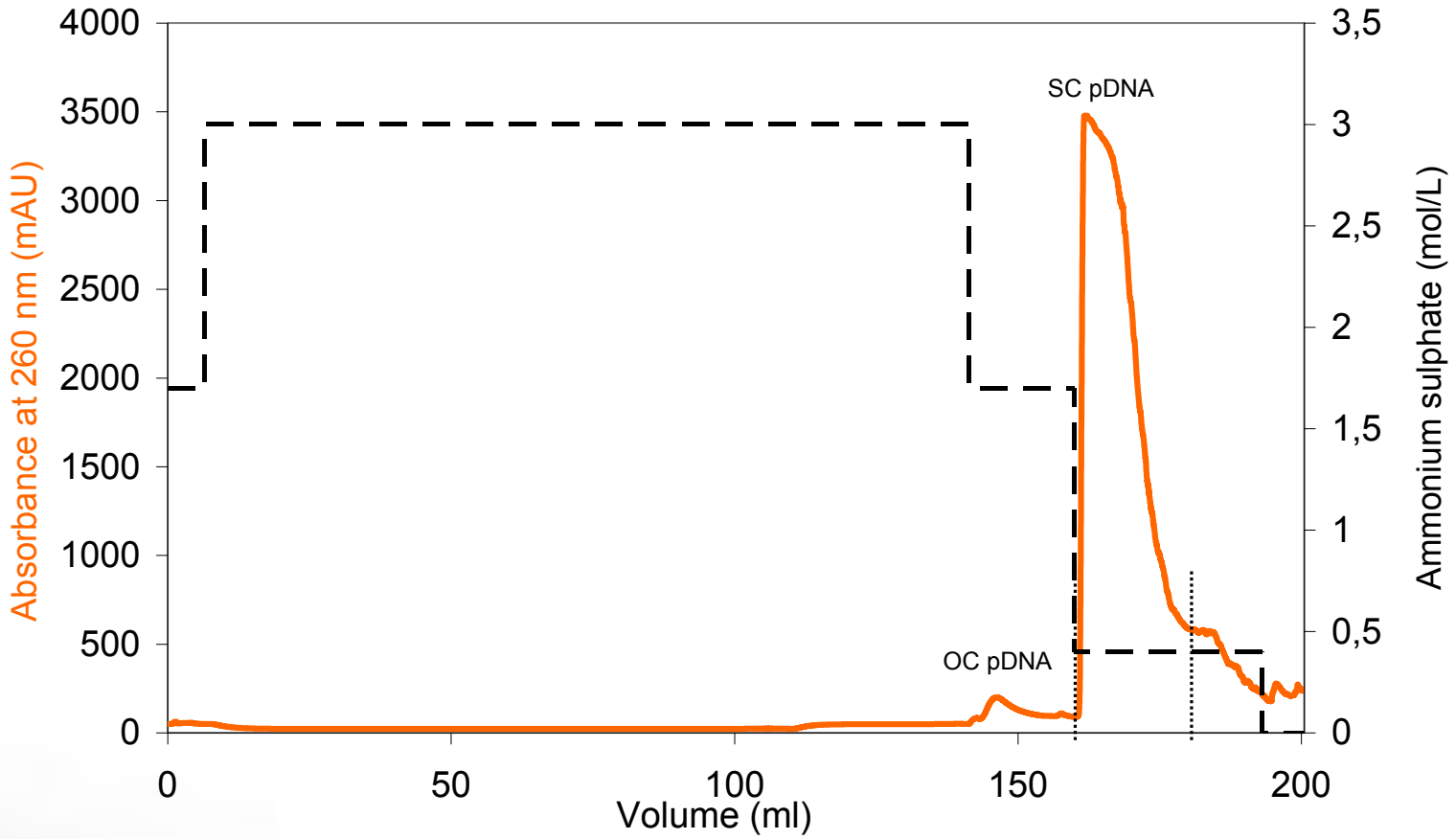
- Alkaline lysis
  - 50 mM Tris pH 8.0/ 10 mM EDTA
  - 0.2 M NaOH/ 1% SDS
  - 3 M potassium acetate, pH 5.0
- Adjustment to proper $CaCl_2$ concentration (0,3-0,8 M)
- Incubation for 15 minutes at 4°C
- Centrifugation/Filtration

# Anion Exchange Step




Equilibration buffer: 50 mM Tris, 10 mM EDTA, pH 7.2
Washing buffer: 50 mM Tris, 10 mM EDTA, 0.6 M NaCl, pH 7.2
Elution buffer: 50 mM Tris, 10 mM EDTA, 1 M NaCl, pH 7.2
Regeneration buffer: 50 mM Tris, 10 mM EDTA, 2 M NaCl, pH 7.2
**Working capacity: 6 mg/ml DEAE monolith**

# Hydrophobic Interaction Step

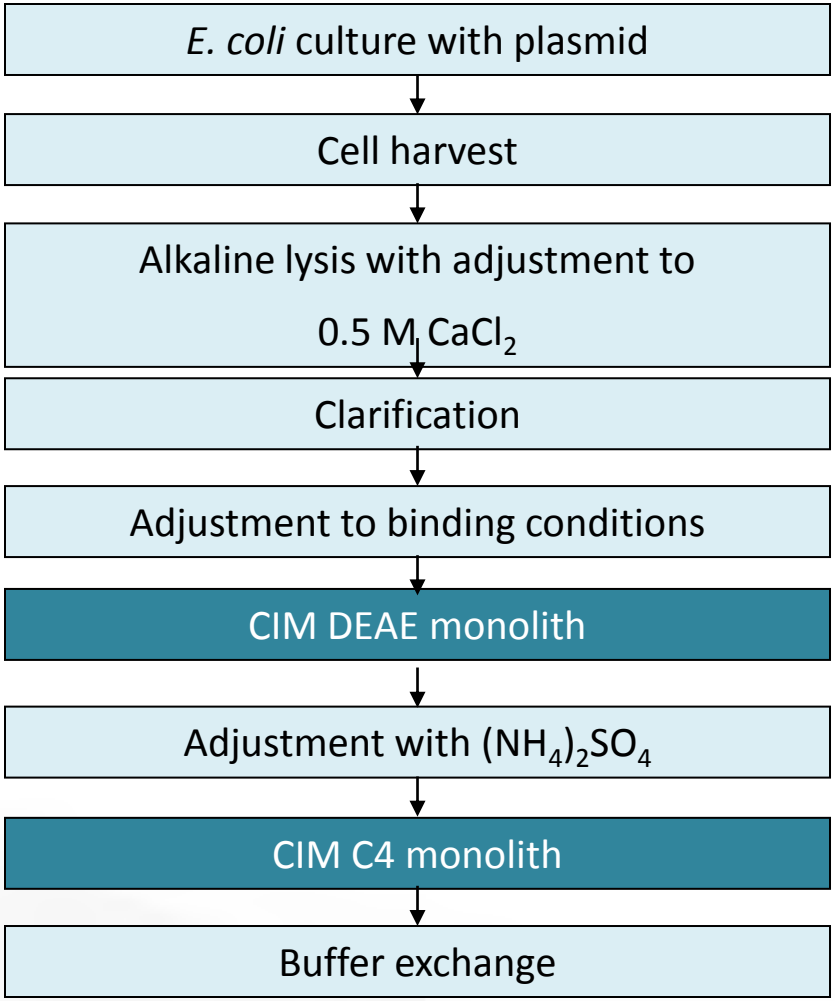



Equilibration and washing buffer: 50 mM Tris, 10 mM EDTA, 1.7 M (NH4)2SO4, pH 7.2
Elution buffer: 50 mM Tris, 10 mM EDTA, 0.4 M (NH4)2SO4, pH 7.2
Regeneration buffer: 50 mM Tris, 10 mM EDTA, pH 7.2
**Working capacity: 3 mg/ml C4 HLD monolith**

# Yield and Purity

| | Alkaline lysate | CIM® DEAE-8 | CIM® C4-8 |
|---|---|---|---|
| **pDNA (µg/ml)** | 28 | 630 | 300 |
| **pDNA (mg)** | 40 | 38 | 34 |
| **Homogeneity (%SC)** | 94 | 95 | 98 |
| **Endotoxins (EU/mg pDNA)** | 80000 | 12,4 | 1,1 |
| **Host cell proteins (µg/ml)** | 5000 | 20 | 1,1 |
| **gDNA (µg/mg pDNA)** | 30 | 74,3 | 3,4 |
| **RNA (µg/ml)** | N.D. | 0 | 0 |
| **Step yield (%)** | 100% | 95% | 90% |

# Novel Plasmid DNA Purification Process



# Consistent Scale-up

| | Monolith Bed Volume (AEX and HIC) | pDNA produced per batch |
|---|---|---|
| **CIM-1** | 1 ml | 6 mg |
| **CIM-8** | 8 ml | 48 mg |
| **CIM-80** | 80 ml | 480 mg |
| **CIM-800** | 800 ml | 4,8 g |
| **CIM-8000** | 8.000 ml | 48 g |

# Costs Comparison of Monolith and Particle Based Plasmid DNA Purification Processes

## 1 ml CIM® monolith

| Calculations | |
|---|---|
| Buffer | 76,3 ml buffer/mg pDNA |
| Time | 23,6 min/mg pDNA |
| Recovery | 85% |
| Purity | cGMP grade |

| Costs using columns for 1 Run | |
|---|---|
| Quantity of purified pDNA | 5,10 mg pDNA |
| € (Column costs) | 114 €/mg pDNA |
| € (column+buffer) | 114 €/mg pDNA |
| €(column+buffer+work) | 123 €/mg pDNA |

| Costs using columns for 10 Runs | |
|---|---|
| Quantity of purified pDNA | 51 mg pDNA |
| € (Column costs) | 11,4 €/mg pDNA |
| € (column+buffer) | 11,8 €/mg pDNA |
| €(column+buffer+work) | 21,1 €/mg pDNA |

| Costs using columns for 20 Runs* | |
|---|---|
| Quantity of purified pDNA | 102 mg pDNA |
| € (Column costs) | 5,7 €/mg pDNA |
| € (column+buffer) | 6,1 €/mg pDNA |
| €(column+buffer+work) | 15,4 €/mg pDNA |

## Particle based

| Calculations | |
|---|---|
| Buffer | 108,0 ml buffer/mg pDNA |
| Time | 70,0 min/mg pDNA |
| Recovery | 79% |
| Purity | cGMP grade |

| Costs using columns for 1 Run | |
|---|---|
| Quantity of purified pDNA | 4 mg pDNA |
| € (Column costs) | 227 €/mg pDNA |
| € (column+buffer) | 228 €/mg pDNA |
| €(column+buffer+work) | 257 €/mg pDNA |

| Costs using columns for 10 Runs | |
|---|---|
| Quantity of purified pDNA | 40 mg pDNA |
| € (Column costs) | 23 €/mg pDNA |
| € (column+buffer) | 24 €/mg pDNA |
| €(column+buffer+work) | 53 €/mg pDNA |

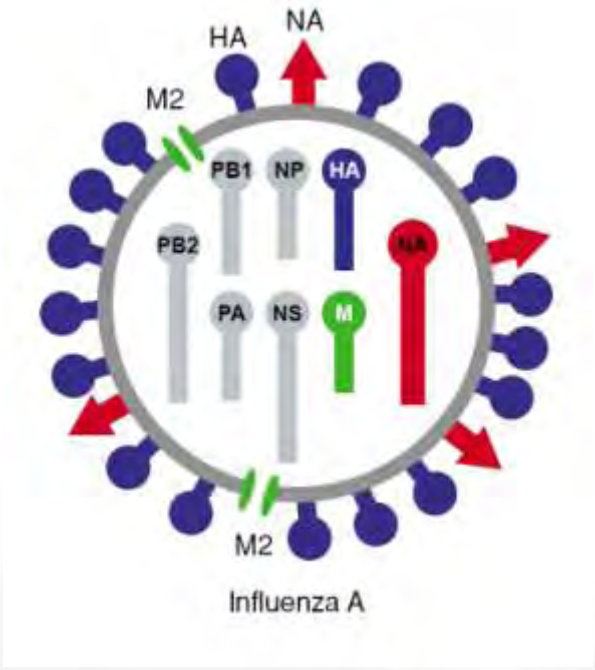
| Costs using columns for 20 Runs | |
|---|---|
| Quantity of purified pDNA | 79 mg pDNA |
| € (Column costs) | 11 €/mg pDNA |
| € (column+buffer) | 12 €/mg pDNA |
| €(column+buffer+work) | 42 €/mg pDNA |

# Platform Flu Vaccine Purification Proces

# Uhhhh, how do we Scale-up our vaccine production process?

# Influenza Viruses

- *Orthomyxoviridae*: enveloped, negative strand RNA virus
- Segmented genome: eight segments encode 11 viral proteins
- Host range
  - Influenza A: humans, swine, horses, domestic and wild avian species
  - Influenza B: humans
- Size: 80 – 120 nm



Influenza A

# Conventional purification based on Continuous Flow Ultracentrifugation

- 40 years of reliable operation carrying out runs every day for much of the year
- The units of choice for 85% of current Influenza vaccine market
- Already used with cell culture-based virus production
- Combines Concentration and Purification
  replacing multiple steps by alternative technologies
- Nevertheless:
  - Long processing time (10-18hours per run)
  - Expensive equipment / potential safety hazards
  - Multiple runs may be needed for impurity removal

*Dr. Charles Lutsch, Process Development, Sanofi Pasteur Inc., USA, MSS2008, Portorož*

# Conventional Vaccine Purification is Based on Continuous Flow Ultracentrifugation

- In addition:
  - Expensive service contracts and maintenance of centrifugation equipment.
  - Footprint of the equipment (size of the facility) might be an issue, as well the completely closed processing loop.
  - How to address in-process control (PAT)?
  - Can centrifugation address requests for “Disposable factory”?
  - What about the yield when life virus particle is in question (Conventional methods of virus purification using ultracentrifugation frequently result in distorted particles with low levels of biological activity)?
  - Can centrifugation address purification of non-viral particle candidates (proteins, pDNA)?

# Evaluation of Different Supports for Purification of Influenza A

| Average values | CIM QA | Mustang® Coin Q | Q Sepharose™ XL | Celufine Sulfate |
|---|---|---|---|---|
| Virus Recovery | 54% | 35% | 35% | 27% |
| DNA Depletion | 96% | 95% | 95% | 91% |
| Protein Depletion | 95% | 94% | 98% | 99% |
| Dynamic Binding Capacity | **10.3** $\log_{10}$ TCID50/mL Support | **10.3** $\log_{10}$ TCID50/mL Support | **9.0** $\log_{10}$ TCID50/mL Support | **8.4** $\log_{10}$ TCID50/mL Support |

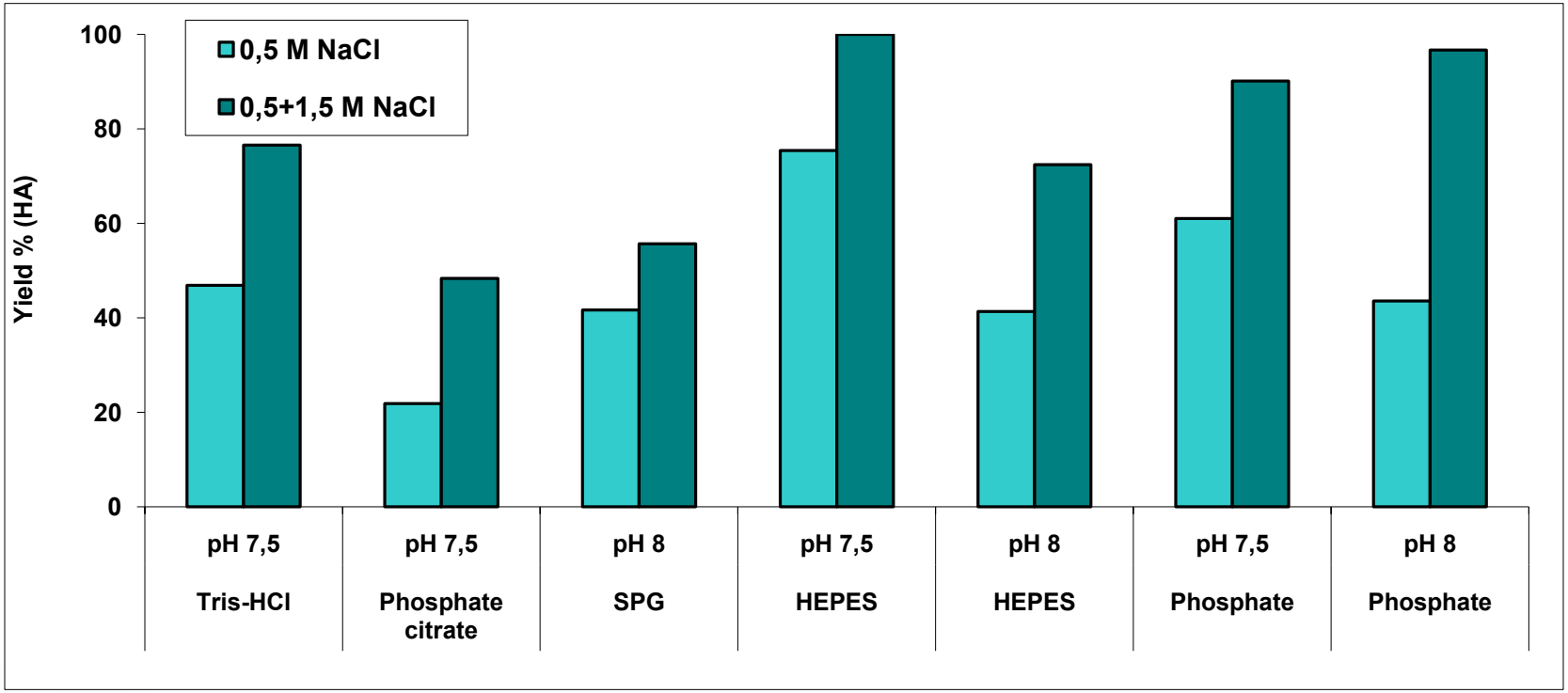
# To Sum Up: Novel Chromatography Supports Shall be Used for Vaccine Purification Processes

- Monolithic and membrane adsorbers offer an order or two higher capacity to particle based supports (1 L can replace 10 to 100 L column).
- Much smaller footprint of the facility.
- Much lower buffer consumption when using monoliths or membranes.
- Monolithic and membrane adsorbers offer much shorter process time.
- As a result; lower production costs, lower COGs of the vaccine.
- Ease of use (easier to handle smaller units).
- No column packing needed.
- Purity of the product is the same or better.
- Membranes (Stack of very thin Monoliths) may offer lower yield and/or lower purity than single piece Monoliths.

# IEX Monoliths Screening

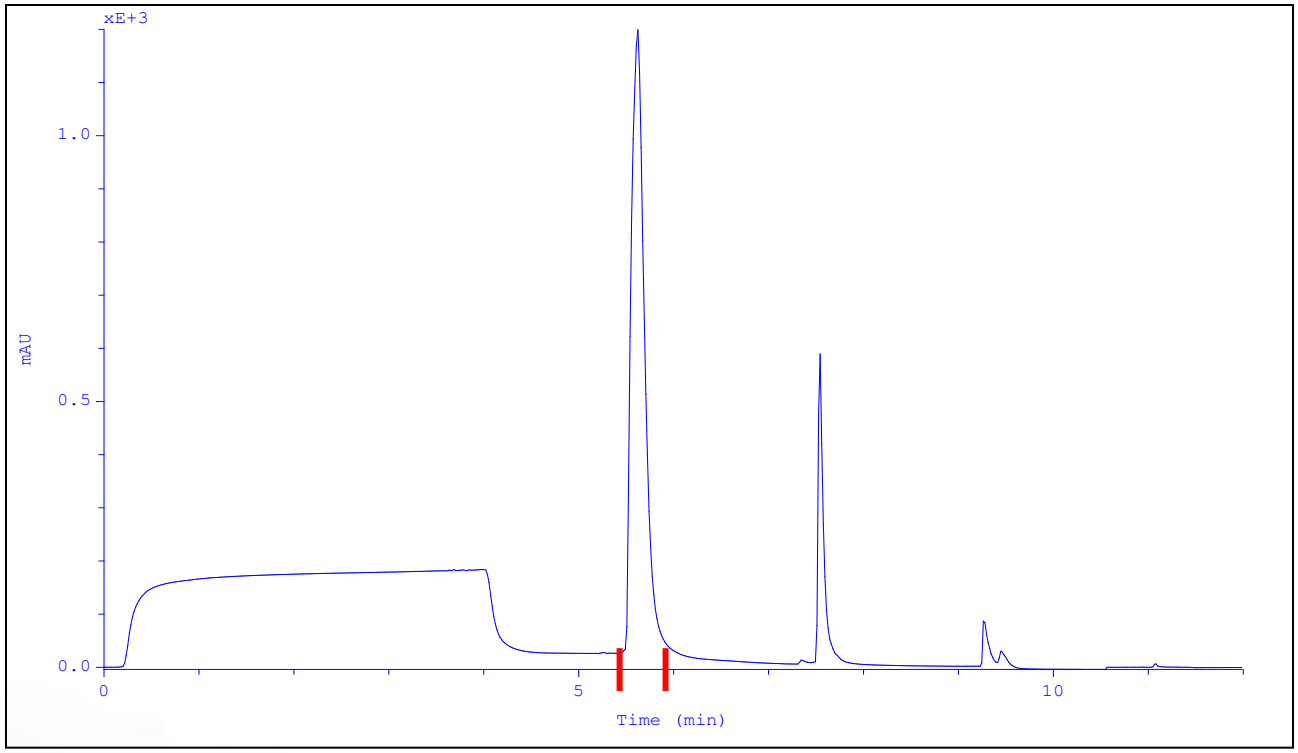
| Virus | IEX | HA Yield (%) Flowthrough | HA Yield (%) Elution |
|---|---|---|---|
| **H1N1** | CIM QA | 0 | 43.7 |
| | CIM DEAE | 0 | 47.3 |
| | CIM SO3 | 0 | 49.6 |
| **H3N2** | CIM QA | 0 | 50.0 |
| | CIM DEAE | 0 | 37.1 |
| | CIM SO3 | 0 | 62.1 |
| **H5N1** | CIM QA | 0 | 84.0 |
| | CIM DEAE | - | - |
| | CIM SO3 | **3.6** | 114.6 |
| **FLUB** | CIM QA | 0 | 51.2 |
| | CIM DEAE | 0 | 35.4 |
| | CIM SO3 | **37.1** | 30.2 |

# Mobile Phase Screening using CIM QA Columns




Zwitterionic buffers stand out for two important reasons:
- first that that they have no inherent conductivity,
- are immune from binding to charged groups which results in more robust process.

# Purification of Clinical Grade Flu Vaccine Using CIM QA Columns

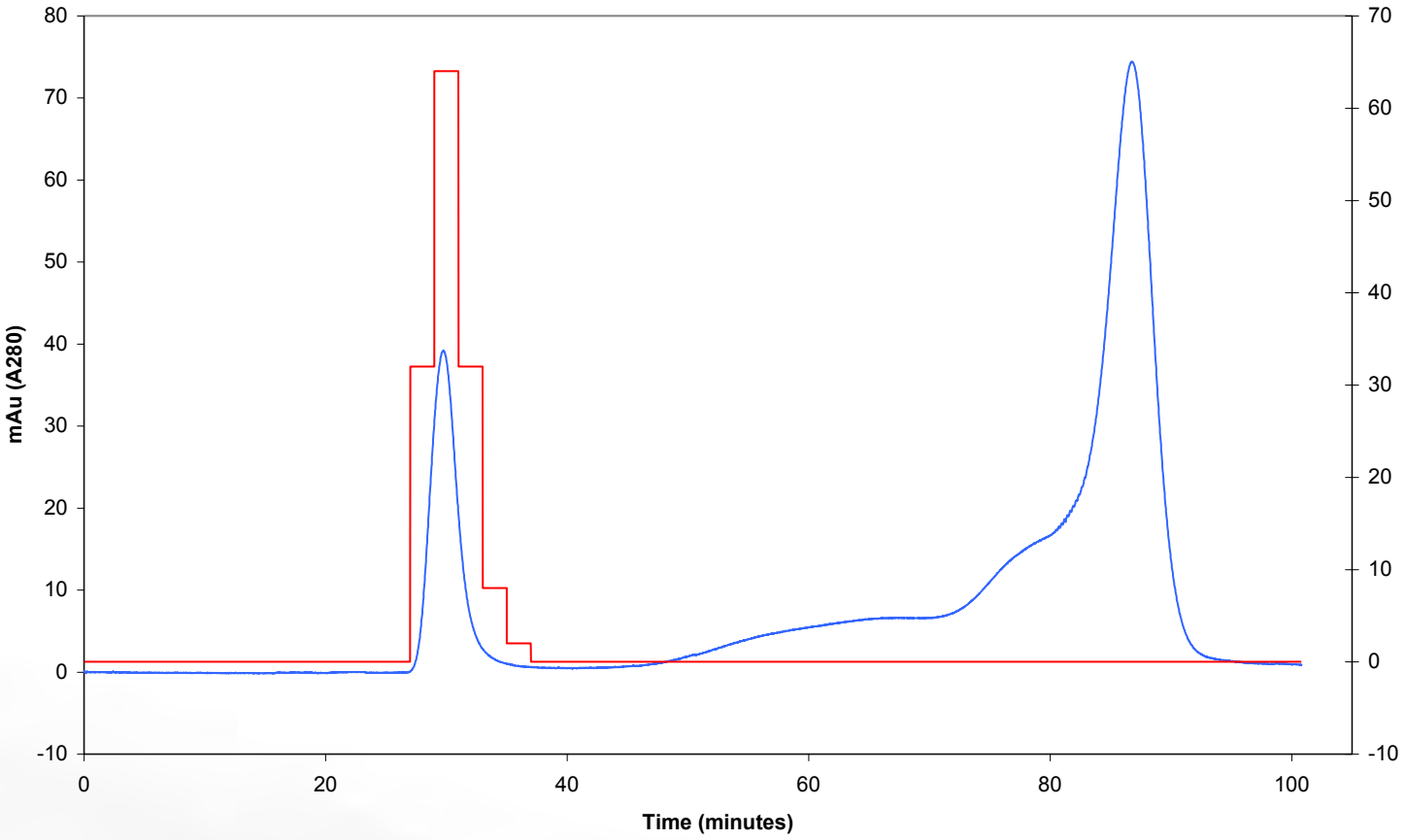



CIM QA (strong anion exchange column), 8 mL tube

Flow rate: 45 ml/min (150 cm/h)

Load: 140 ml of H1N1 TUF concentrate

# Final Polishing and Buffer Exchange using SEC



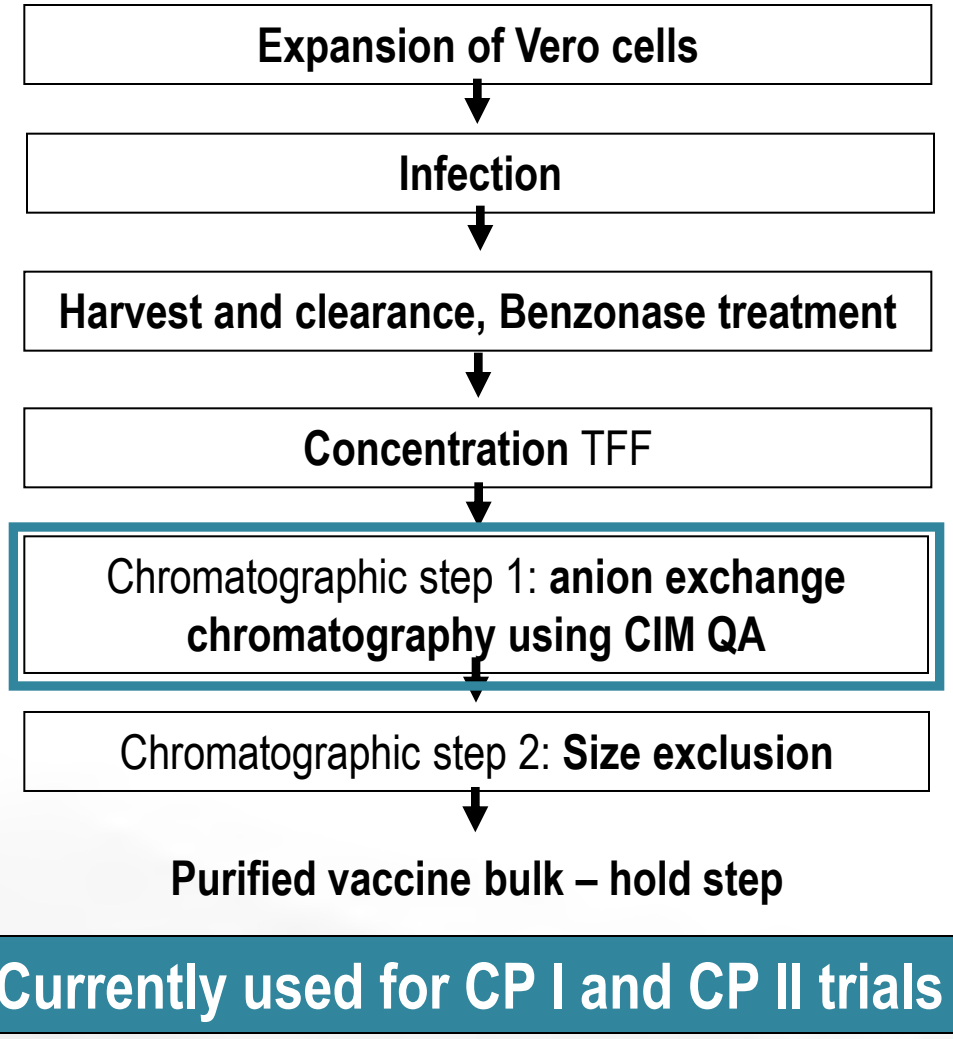
# Mobile Phase Optimisation for SEC Step

| | SPG | $SPG_{modified}$ |
|---|---|---|
| Virus recovery* | 59% | 100% |
| DNA depletion | 54% | 48% |
| Protein depletion | 79% | 81% |

*TCID50

| | |
|---|---|
| SPG | Sucrose-phosphate-glutamate buffer |
| $SPG_{modified}$ | Sucrose-phosphate-glutamate buffer modified |

# Chart of the Clinical Grade Flu Vaccine Manufacturing Process



# Process Yields and Purity Obtained

| Step | Aim | Virus yield* |
|---|---|---|
| Clarification | DNA depletion, cell debris clearance | 50-100% |
| TUF | Protein and DNA depletion, virus concentration | 80-100% |
| CIM QA | Protein and DNA depletion, virus concentration | 50-100% |
| SEC | Protein and DNA depletion, buffer exchange | 100% |
| Overall | From harvest to purified vaccine bulk | **≥ 25%** |

*TCID50

| | H1N1 | H5N1 |
|---|---|---|
| DNA depletion | ≥ 99.9% | ≥ 99.9% |
| Protein depletion | ≥ 99.0% | ≥ 99.5% |

# Centrifugation versus Chromatography Based Flu Purification Process

## CENTRIFUGATION BASED PURIFICATION PLATFORM

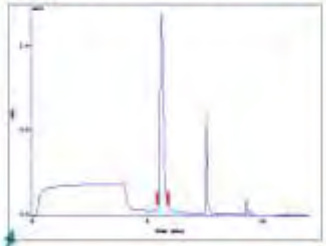
- Expanison of Vero cells
- Infection
- Harvest and Clearance
- Benzonase
- Tangential Flow Filtration (TFF)
- Ultracentrifugation (UCF)
- Adjustment to final formulation

| | |
|---|---|
| Infectious virus yield | 11.4 % |
| DNA removal | 99.50 % |
| Protein removal | 97.4 % |

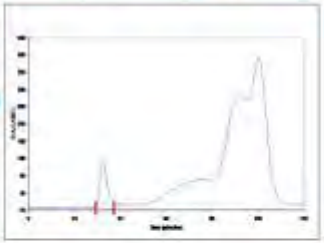
## MONOLITH BASED PURIFICATION PLATFORM

- Expanison of Vero cells
- Infection
- Harvest and Clearance
- Benzonase
- TFF
- CIM QA Monolith: Anion exchange chromatography (AIEX)
- Size Exclusion (SEC)

AEX of Influenza A H1N1



SEC of Influenza A H1N1



| | |
|---|---|
| Infectious virus yield | 47.3 % |
| DNA removal | 99.96 % |
| Protein removal | 97.8 % |

# Centrifugation versus Chromatography Based Phage Purification Process

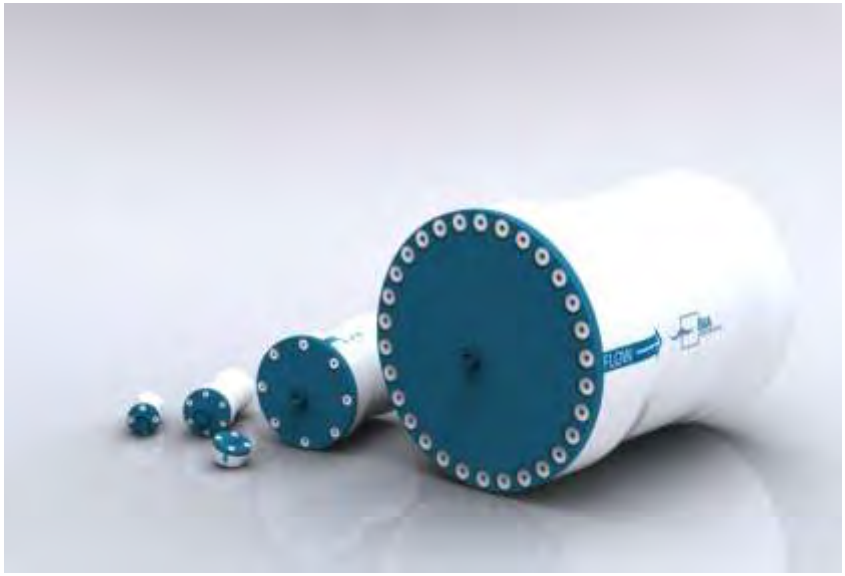
| CsCl-ULTRACENTRIFUGATION | | CIM MONOLITHIC COLUMNS |
|---|---|---|
| ▪ None | OPTIMIZATION | ▪ 10 days (pH-stability & cond.) |
| ▪ 5 days | TIME | ▪ 3 days |
| ▪ costs & time | UP-SCALING | ▪ Multiple runs on the same column |
| ▪ $10^{10}$-$10^{11}$ phages | CAPACITY | ▪ $10^{11}$-$10^{14}$ phages |
| ▪ not for CsCl-sensitive phages | APPLICABILITY | ▪ all phages |
| ▪ 0,01 – 0,1 % | RECOVERY (P. Putida phage ϕ15) | ▪ about 71% |

*A. Cornelissen et al., K.U.Leuven, MSS 2010, Portoroz, Slovenia, 2010*

# To Sum Up: Chromatography Using Novel Supports can Better Address the Needs of Novel Vaccine Purification Platforms than Ultracentrifugation

- Yield of life virus particle is (much) higher (lower product degradation).
- Much smaller footprint of the facility and more flexible equipment.
- Lower production and equipment maintenance costs.
- Purity of the product is the same or better.
- Faster and easier scale-up, scale-down.

# Multiuse Disposable Units - “Plug and Play”



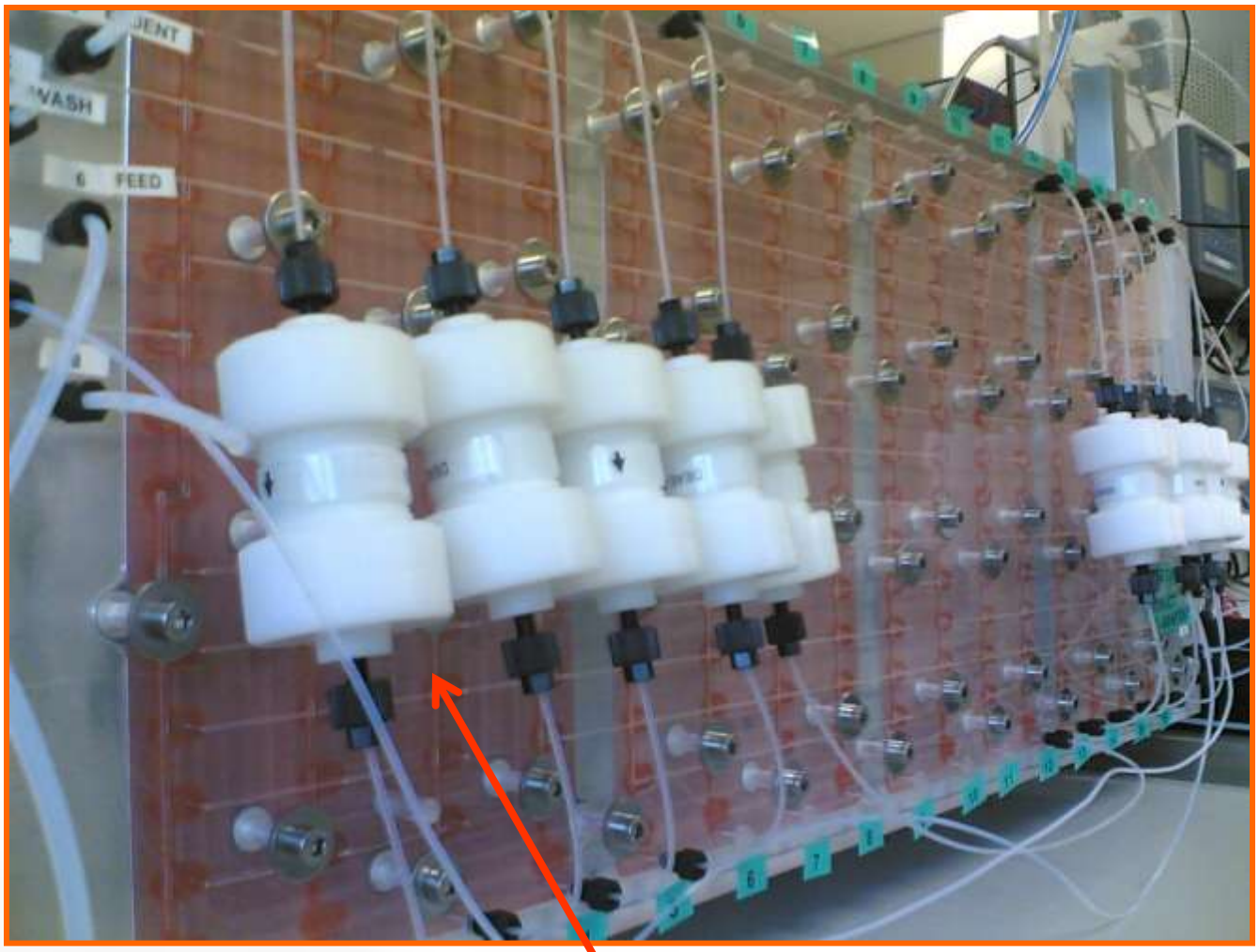
CIMmultus™ from BIA Separations (1 mL – 8 L)

Carbon fibre reinforcement embedded into epoxy thermoset resin (carbon fibre composite); tough, light material; 5-times lower density than stainless-steel; operate at 20 bar (291 psi).

**Replace the SS housings.**

**More inert than polypropylene, less leachables, less unspecific binding.**

# Introduction of Continuous LC (BioSMB™)

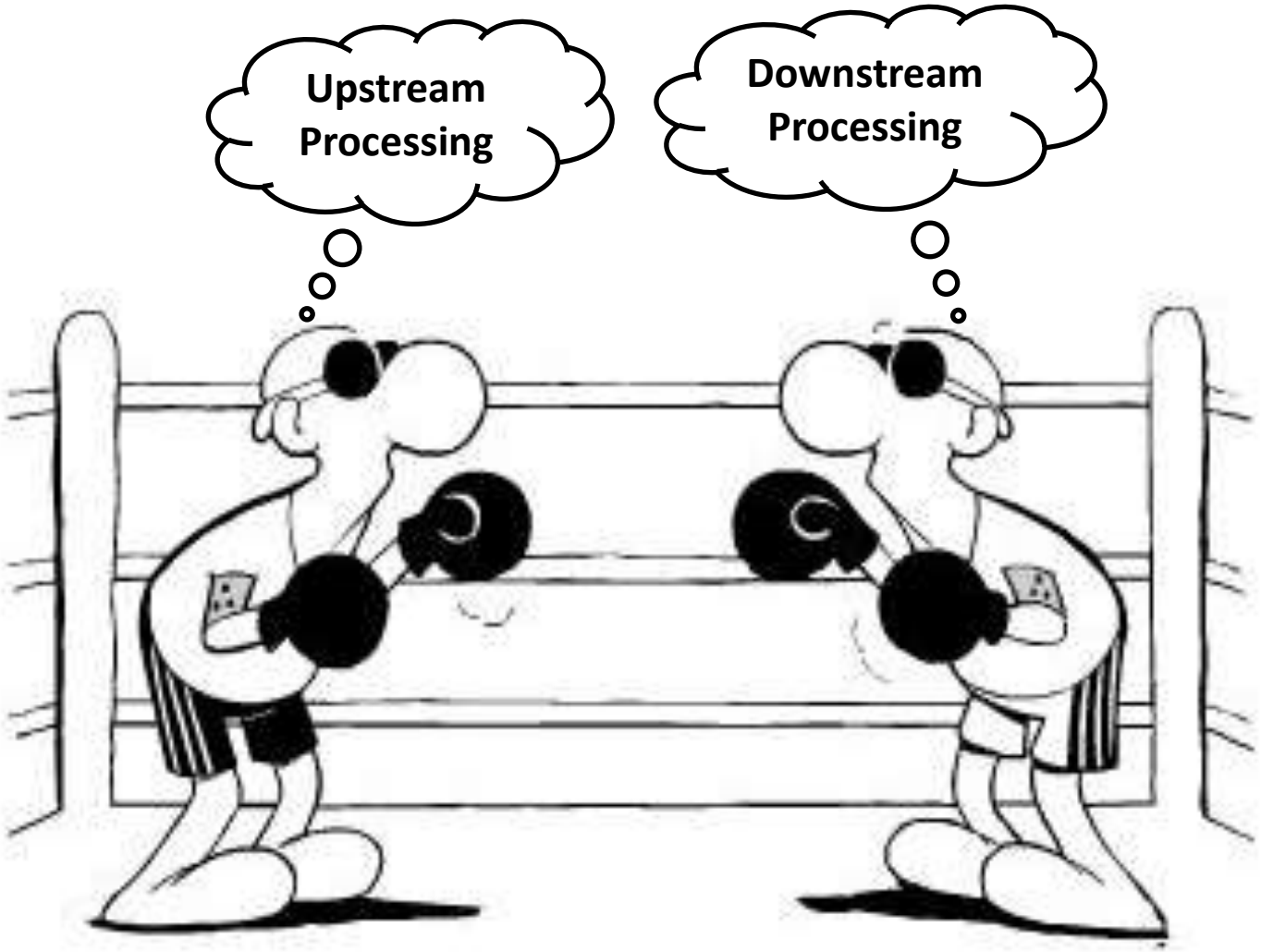



**Disposable monolithic or membrane columns**

# Disposable and Continuous Liquid Chromatography Systems Fit to "Single use" Vaccine Production Facility


*Courtesy of Xcellerex*

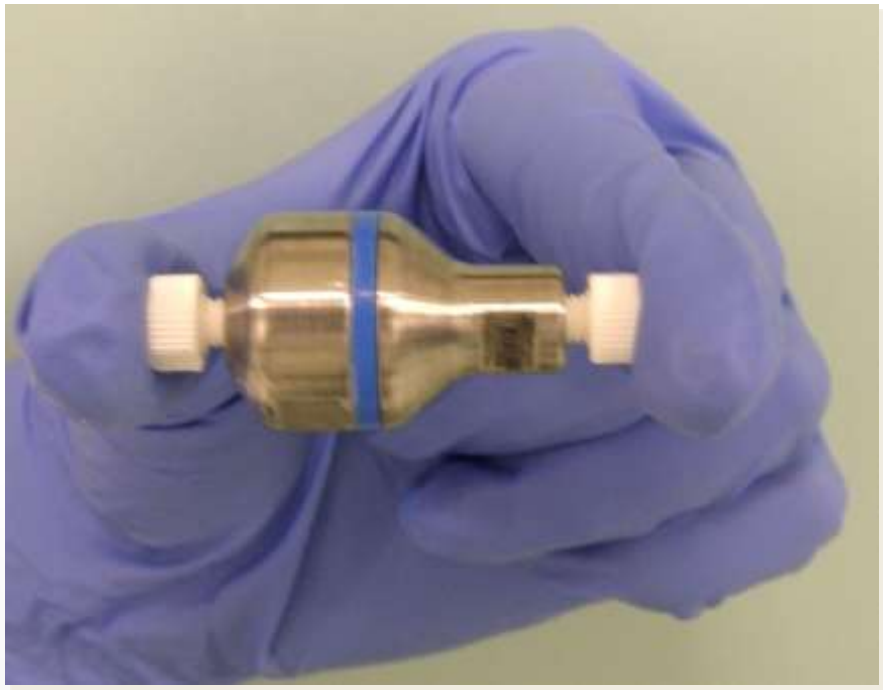
# Do we know enough about our complex molecule samples to develop safe product and/or run the production process?!


Upstream Processing
Downstream Processing

# In-process control (PAT) using CIMac™ monolithic HPLC columns

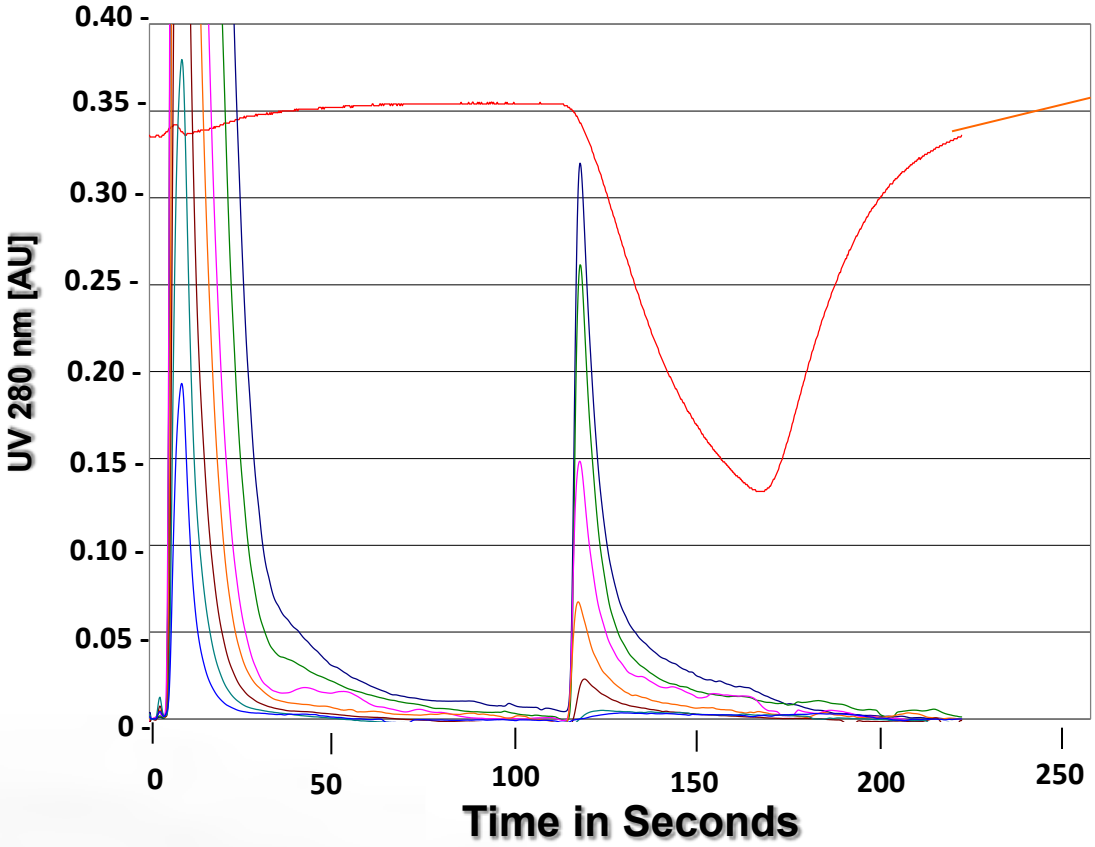
# Monolithic Analytical Columns for In-process Control (PAT)

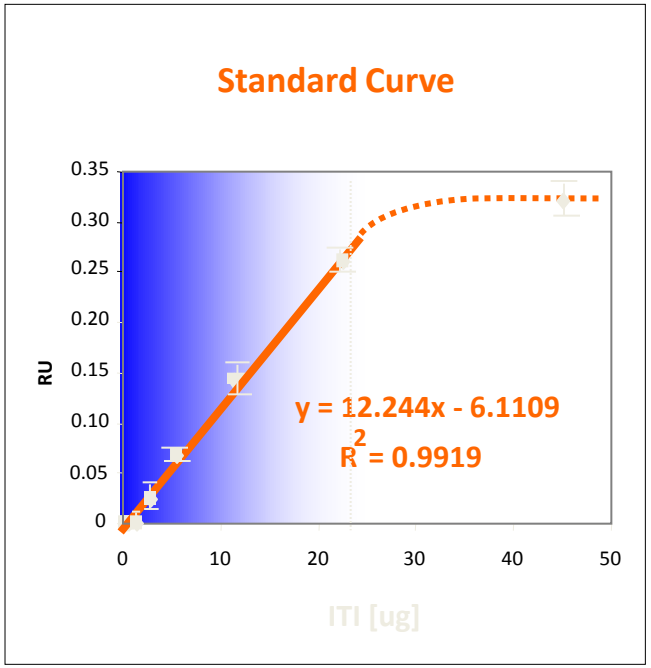
**CIMac™ HPLC Columns**



10 ml/min = 4500 cm/h = 360 CV/min (res. time: 0,1 s) = faster than biosensor

# CIM® ImmunoDisk allows rapid quantification of biomarkers

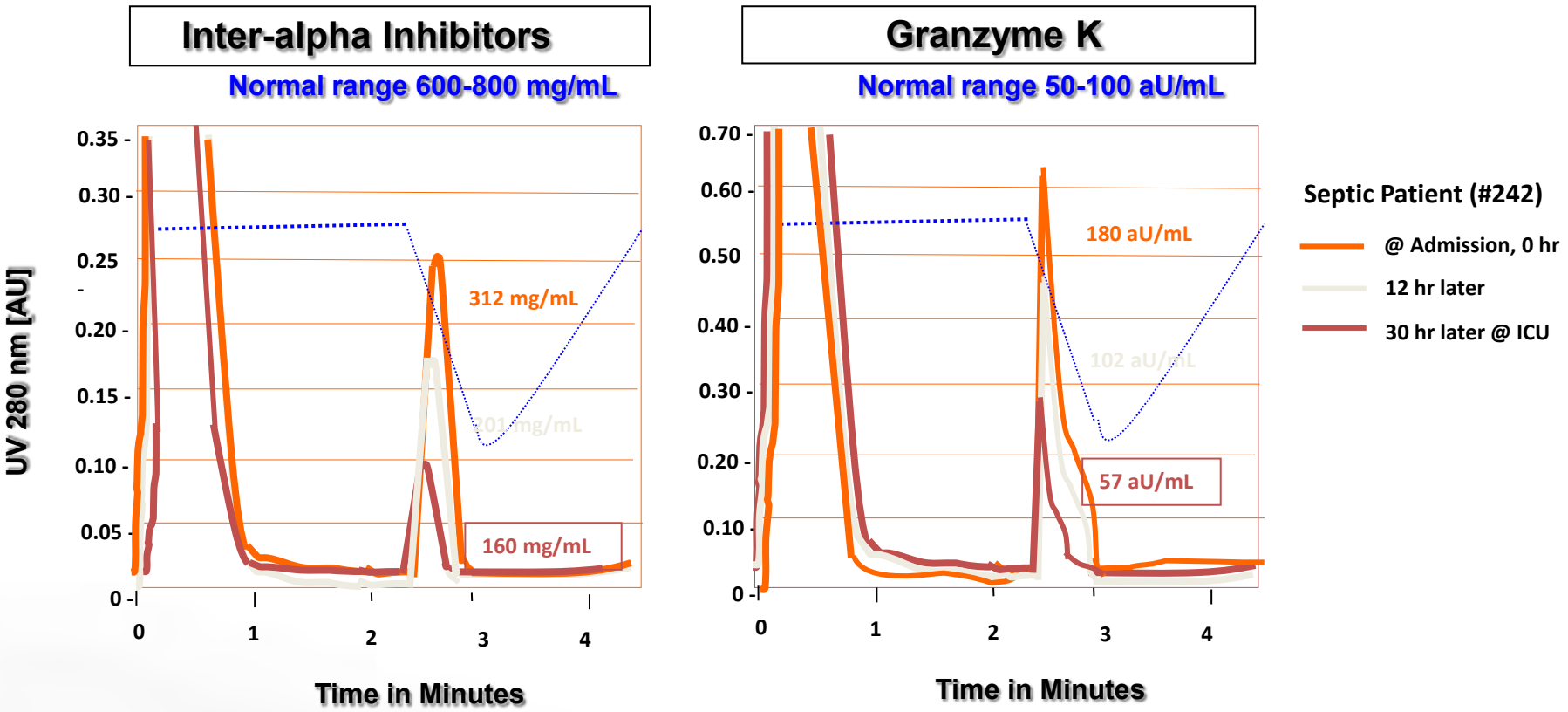



**MAb 69.31 ImmunoDisk standard curve**
**Fresh Frozen Human Plasma - 250 mg/L of INTER-ALPHA INHIBITOR**

*Courtesy of Prof. Yow-Pin Lim, ProThera Biologics, Providence, USA*

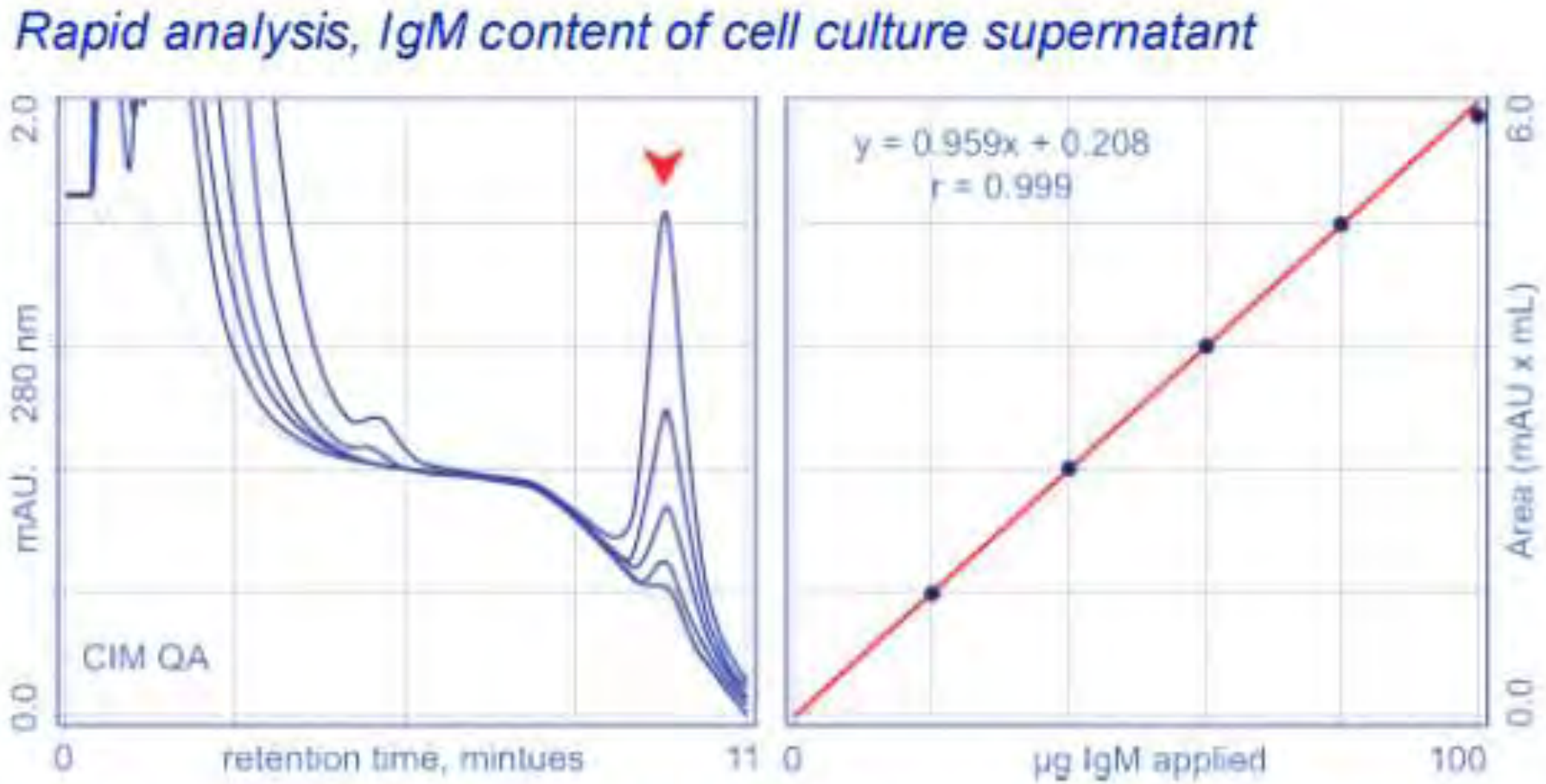
# Results on progres of Sepsis within few minutes CIM® ImmunoDisk




**SERIAL PLASMA STUDY OF SEVERE SEPTIC PATIENT**

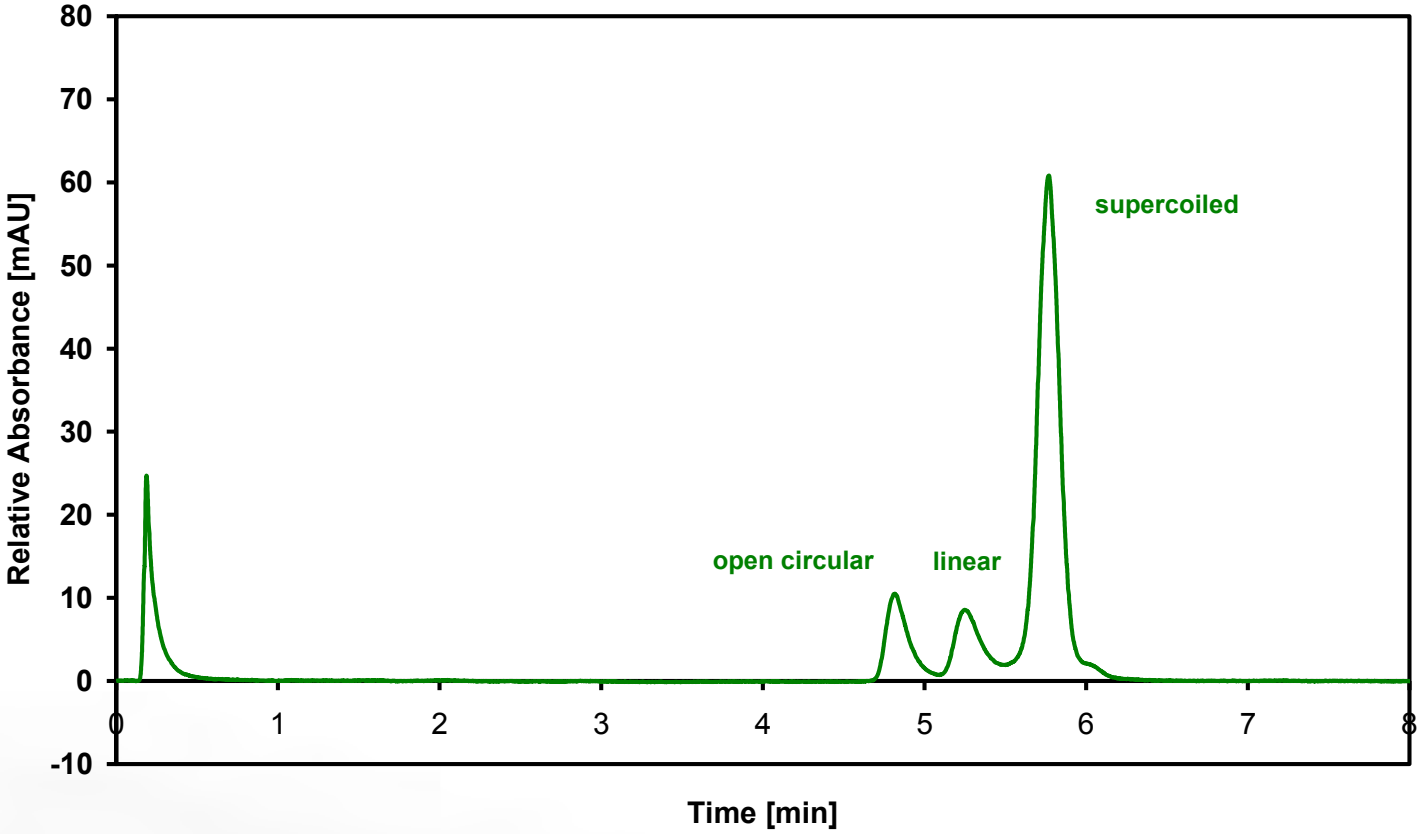
*Courtesy of Prof. Yow-Pin Lim, ProThera Biologics, Providence, USA*

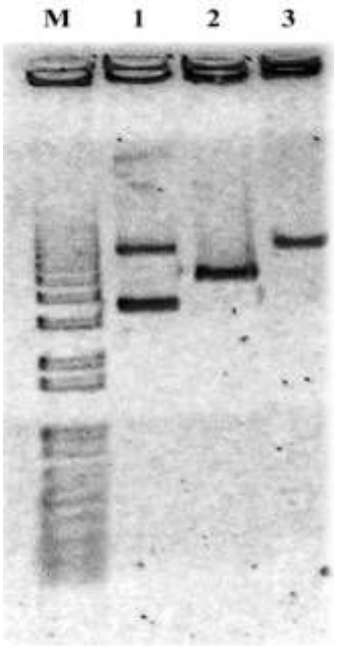
# Rapid IgM Content Analysis




Reproduced from A high speed monolithic assay for IgM quantitation in cell culture production and purification process monitoring: Pete Gagnon, Richard Richieri, Simin Zaidi, Roy Sevilla, Alexander Brinkman, 3rd Wilbio Conference on Purification of Biological Products, September 24-26, 2007, Waltham, MA USA

# Separation of Plasmid DNA Isoforms Using Monolith Columns – PAT of pDNA Production



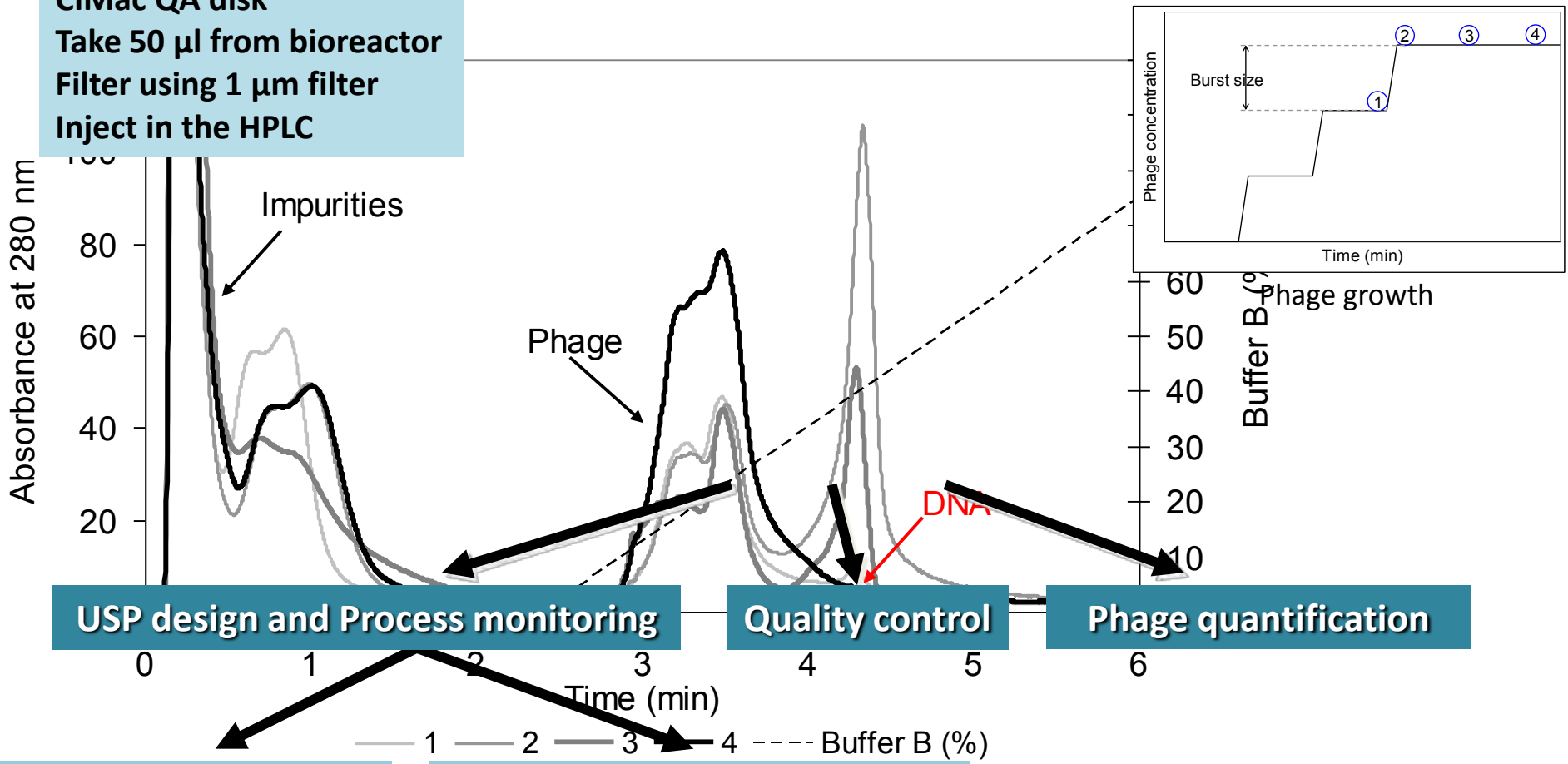

**AGE of different isomers**
1 Initial pDNA sample
2 Linear form
3 OC form

Column: CIMas DEAE, 5.2 mm x 4.95 mm, max. pressure: 150 bars
Buffer A: 20 mM Tris-HCl; pH 8.5, Buffer B: Buffer A + 1 M NaCl, Flow rate: 1 ml/min, Gradient: 60 to 75 % buffer B within 100 CV, Injection volume: 2 µl, Detection: UV at 260 nm.

*B. Gabor et al., MSS 2010, Portoroz, Slovenia, 2010*

# PAT - Phage and Impurity Growth Monitoring in Pilot Scale Bioreactor

**CIMac QA disk**
**Take 50 µl from bioreactor**
**Filter using 1 µm filter**
**Inject in the HPLC**




**USP design and Process monitoring**

**Quality control**

**Phage quantification**

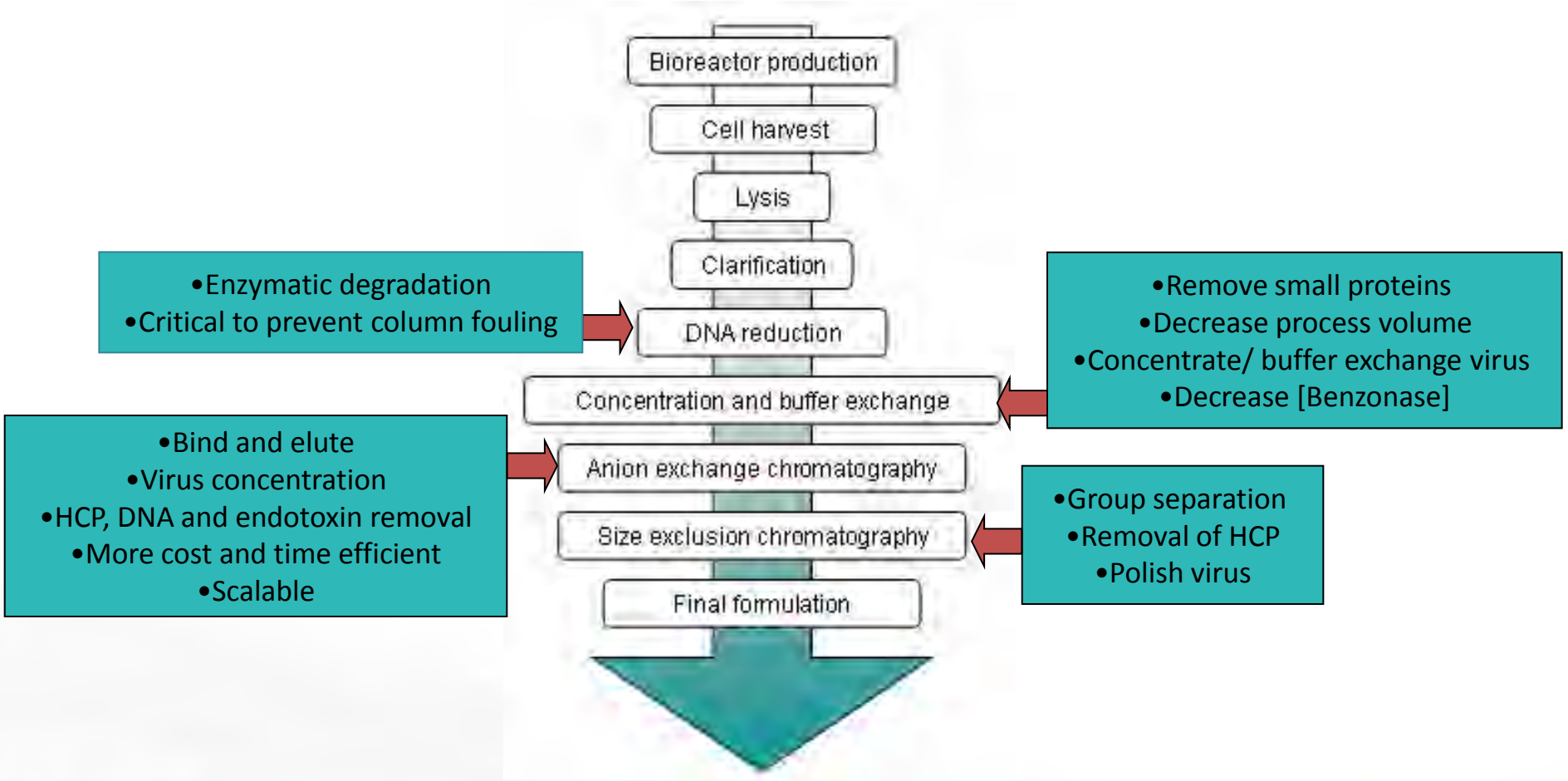
**Growth monitoring**

**Monitoring impurity profile**

*F. Smrekar et al., J Chromatogr B 861, 2008, 77*

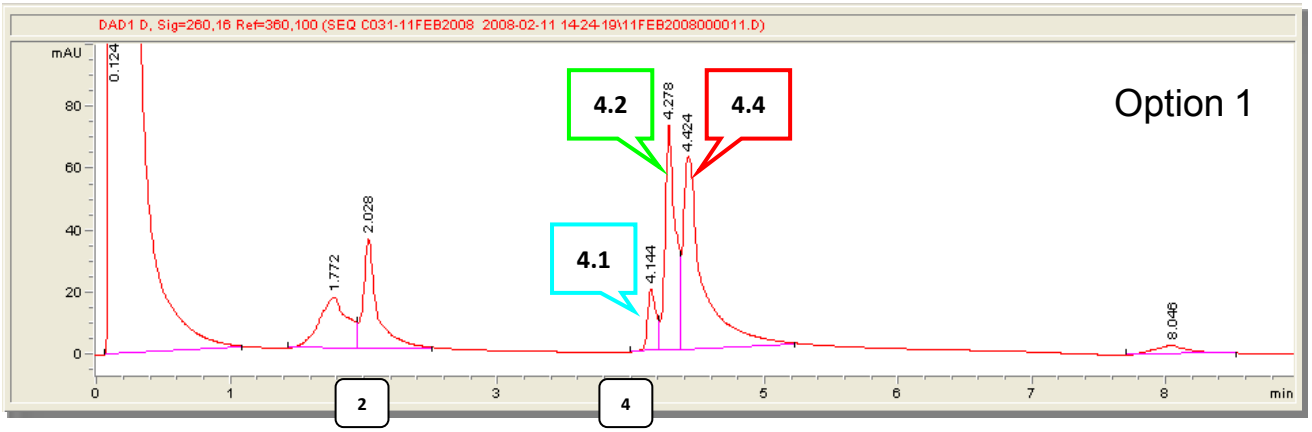
# PAT of Ad5 Production Using Monolithic HPLC

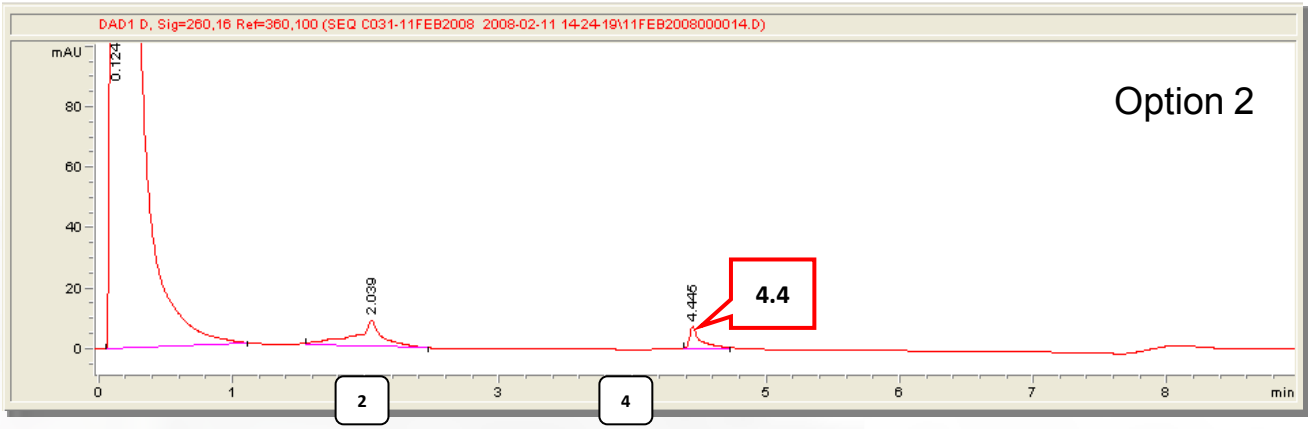
## Eden Biodesign Platform Ad5 Process

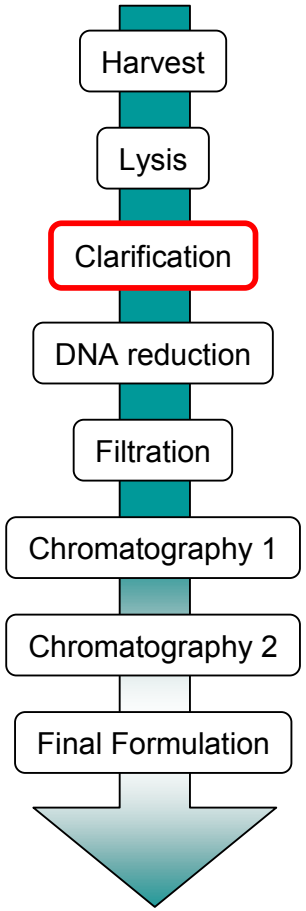



*C. Sims et al., Eden Biodesign, MSS 2010, Portoroz, Slovenia, 2010*

# Monolithic HPLC used for Ad5 Production Process Development – Basis for the PAT



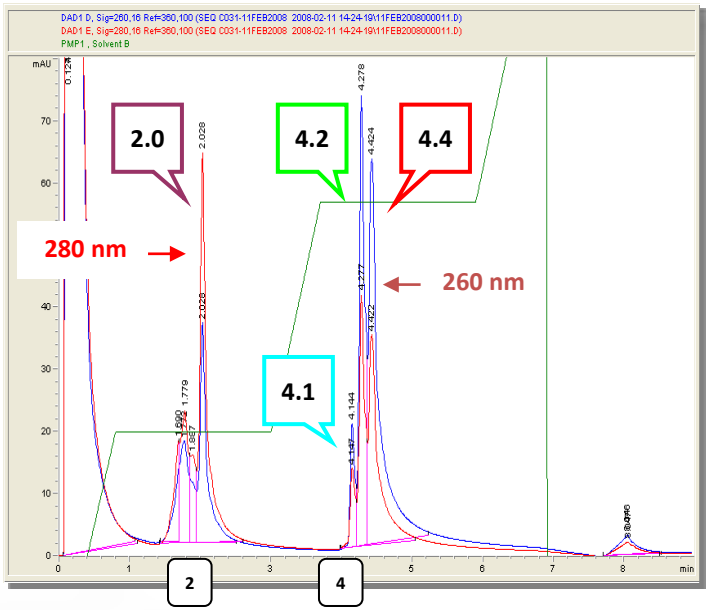



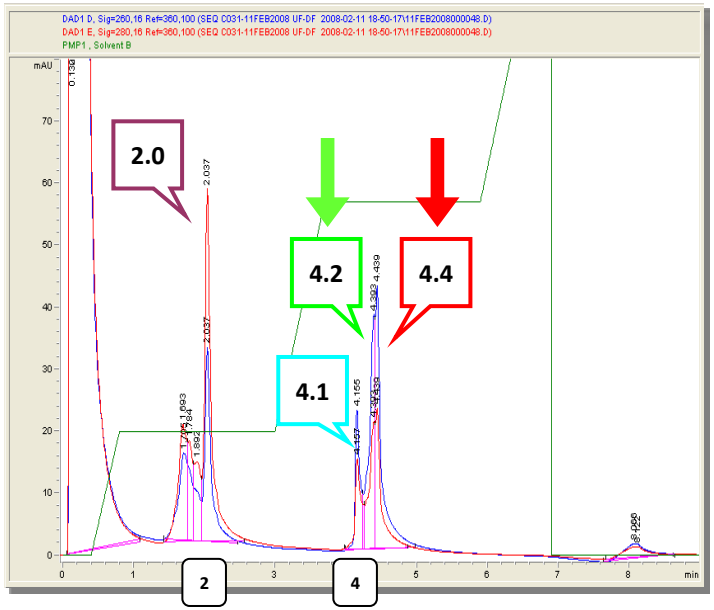

*P. Ball et al., Eden Biodesign, MSS 2008, Portoroz, Slovenia, 2008*

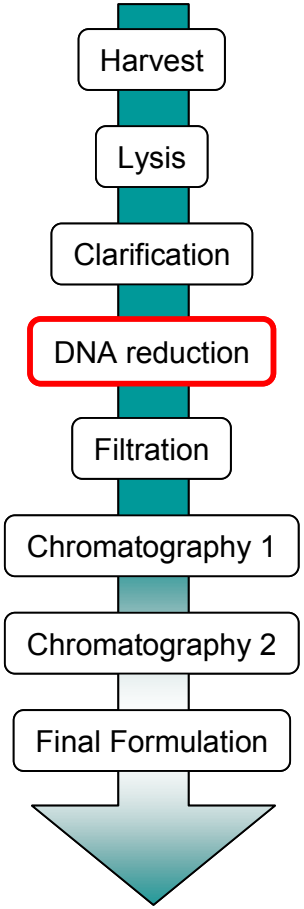
# Monolithic HPLC used for Ad5 Production Process Development – Basis for the PAT

DNAse Pre-treatment




DNAse Post-treatment

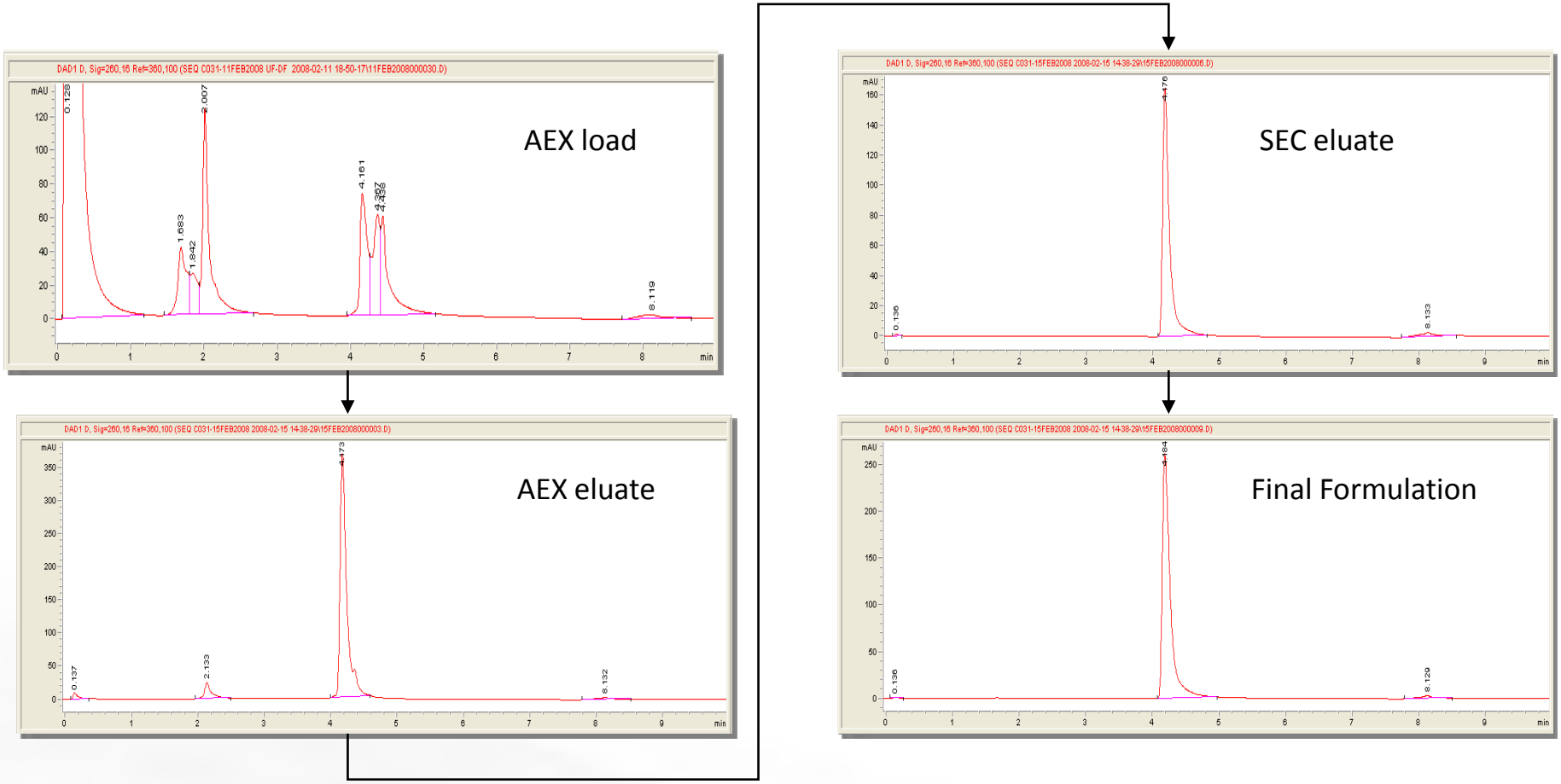



- Species eluting at 2 minutes are proteinaceous (280 nm > 260 nm). Likely to be HCP or Ad5 proteins.
- Species eluting at 4 minutes a mixture of Ad5 particles, possibly free DNA.

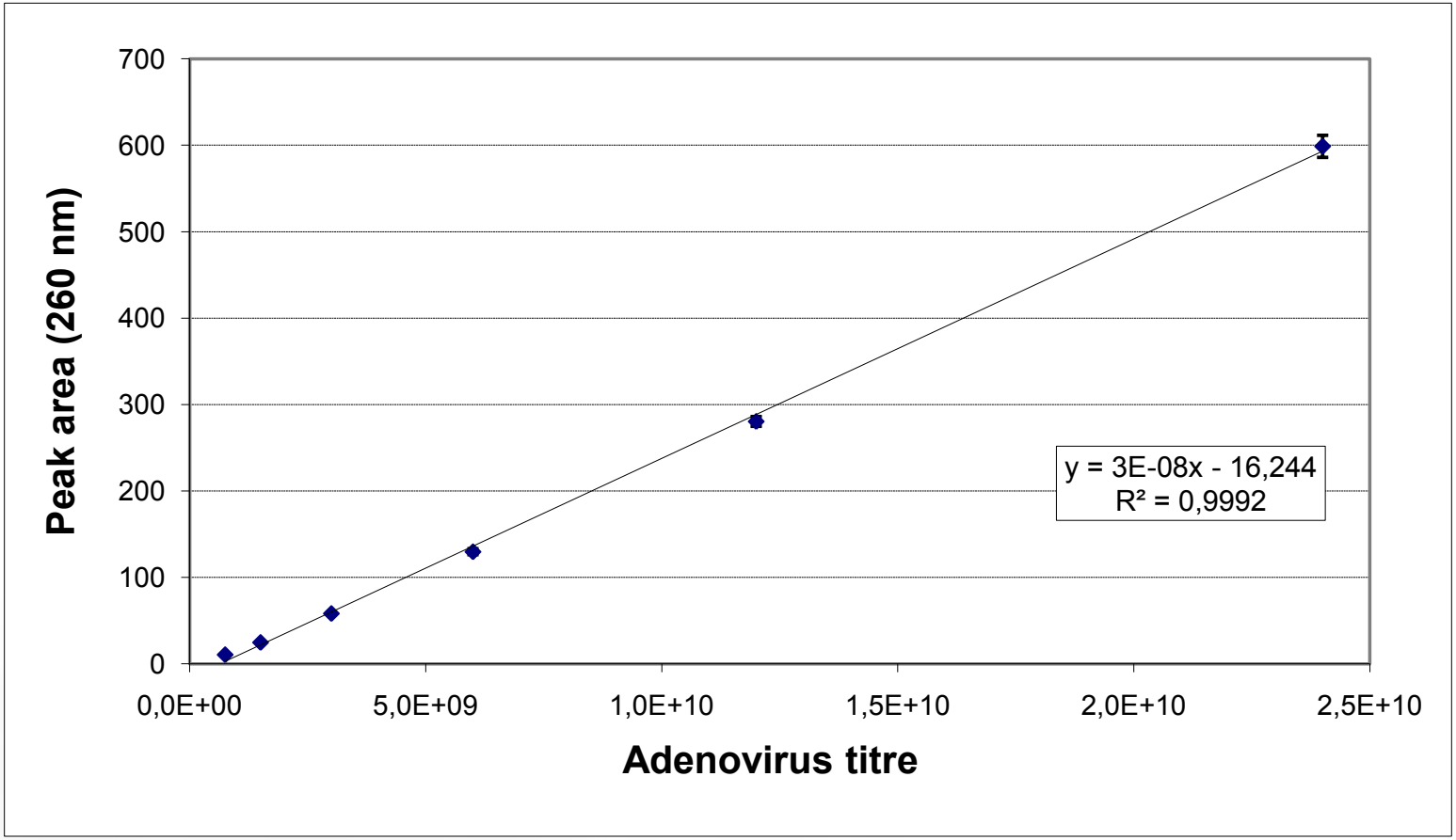
*P. Ball et al., Eden Biodesign, MSS 2008, Portoroz, Slovenia, 2008*

# Monolithic HPLC used for the Ad5 Production Process PAT



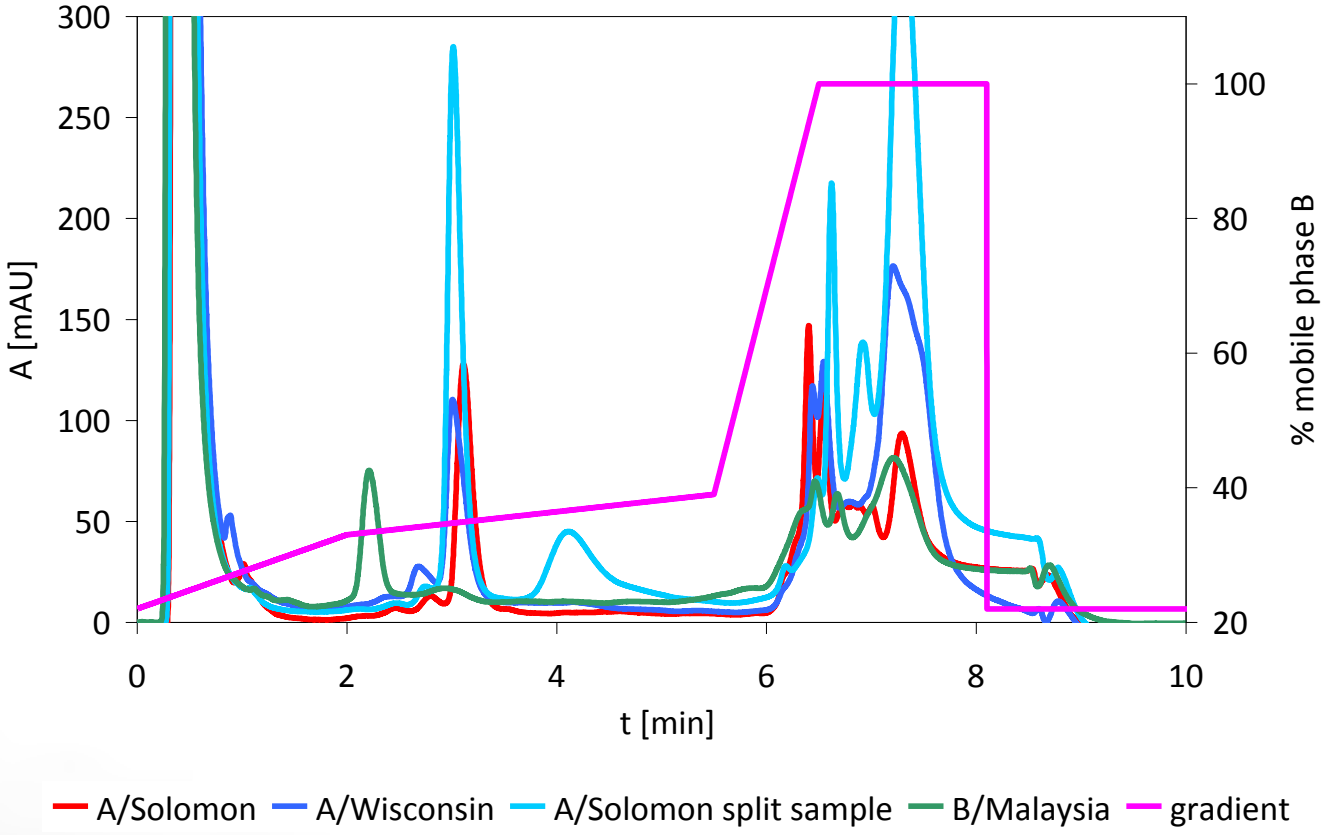

*C. Sims et al., Eden Biodesign, MSS 2010, Portoroz, Slovenia, 2010*

# Monolithic HPLC Used for the Ad5 QC/QA of the Final Product




*P. Ball et al., Eden Biodesign, MSS 2008, Portoroz, Slovenia, 2008*

# Monolithic RP HPLC used for the Recognition of Different Flu Strains




**Virus disruption with detergent (Zwittergent) followed by Trypsin treatment and Injection to CIMac SDVB.** Conditions: mobile phase A = 5% AcCN, 0.1 % TFA, mobile phase B = 90% AcCN, 0.1% TFA, Injection volume = 30 µL, λ = 215 nm; flow rate = 0.8 mL/min.

*L. Urbas et al., MSS 2010, Portoroz, Slovenia, 2010*

# Take Home Message

Vaccine development and manufacturing can be supported by many new innovative technologies and materials but chemistry and biochemistry are staying the same.

Without understanding the basic of the technology and investment in R&D one cannot expect successful adoption. Miracles rarely happen - Good analytical methods are the key.

Unspecific binding, column clogging, product degradation and/or inactivation are NOT caused by innovation or new technology but materials used.

Even perfect DSP cannot solve problems of badly designed, not reproducible USP. PAT is a key to understand and design robust USP.

# BIA Separations CIM® Monolithic Columns are Becoming Industry Standard for Production of Complex Biomolecules

- Drug Master Files (DMF) for CIM® DEAE, QA and SO3 columns in place, HIC in preparation.
- First drug purified using CIM monoliths passed CPIII trial (pDNA for gene therapy).
- More than 15 projects in CPI – CPIII trials (various Influenza, various Adenovirus, bacteriophages, various IgMs, Inter-alpha-inhibitors).
- More than 200 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).

mss monolith summer school

mss monolith summer symposium