BIA Separations
CIM Monolithic Columns for Purification and Analytics of Biomolecules

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HIGH PURITY NEW ENGLAND

BIA separations

Outline

- BIA Separations
- Chromatography
  - Monolithic Chromatography
  - Design of monolithic columns
- DSP applications
- PAT columns and applications:
  - Case study: combining DSP and PAT of Adenoviruses
- Conclusions

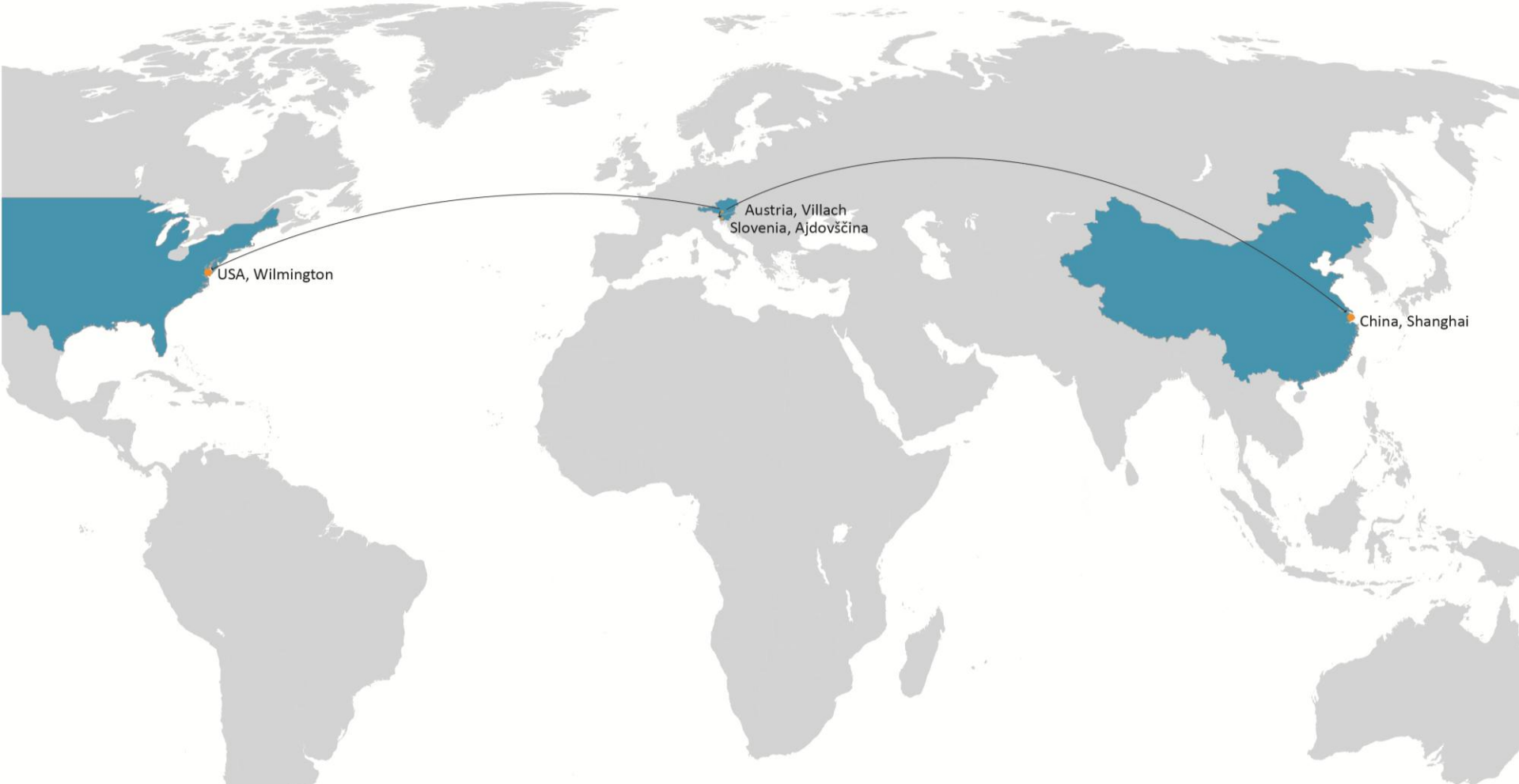

Slovenia


Austria
Hungary
Slovenia
Italy
Ljubljana
Ajdovščina
Croatia
Adriatic sea

Ajdovščina

BIA Separations

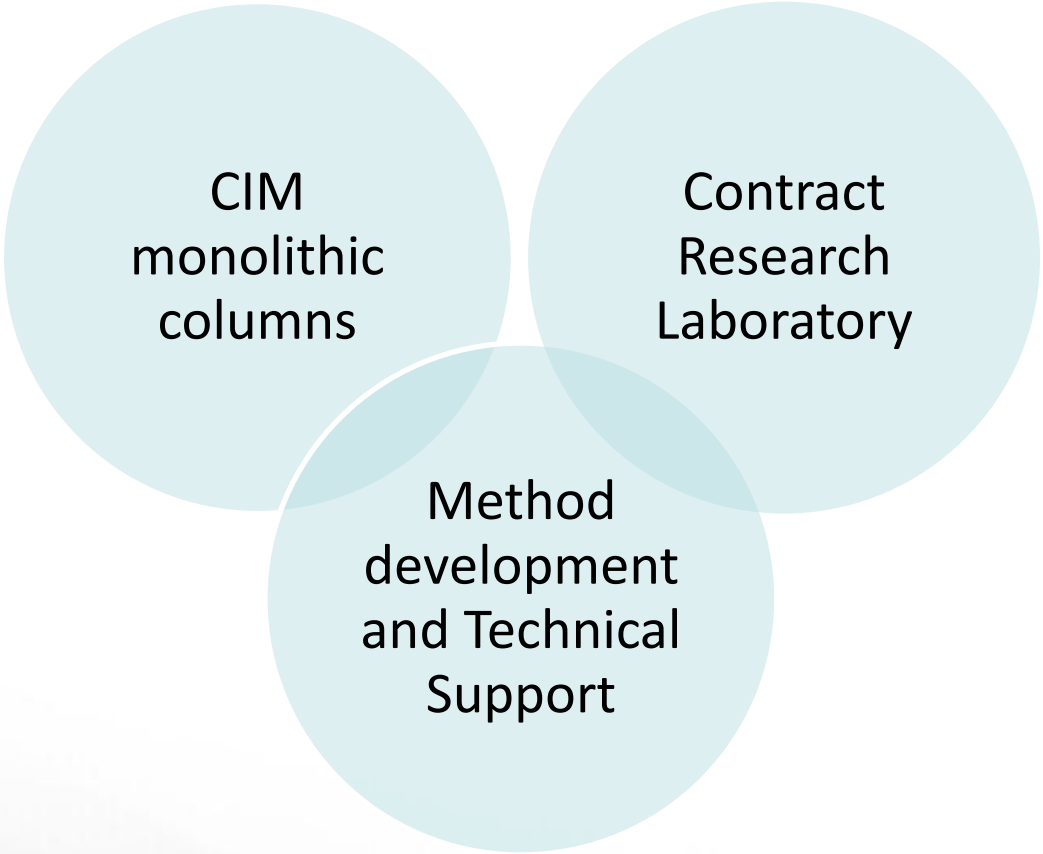
- BIA Separations was founded in September 1998.
- Headquarters in Austria, R&D and Production in Slovenia.



BIA Separations

- BIA Separations was founded in September 1998.
- Headquarters 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.
- 90 employees world wide
- Main focus: To develop and sell methacrylate monolithic columns & develop methods and processes for large biomolecules separation and purification.

BIA Separations – Products and Services



Important Milestones

- 2004: First monolith used for the industrial cGMP purification for plasmid DNA at Boehringer Ingelheim provide 15-fold increase in productivity
- 2006: First cGMP production of a vaccine (influenza) using CIM®
- 2008: **OEM Partnership with Agilent Technologies** – develop and produce analytical monolithic columns for PAT
- In 2011 BIA Separations was awarded by KAPPA-Health as a model SME in the EU Co-funded research projects

- 2012: **co-marketing and co-development agreements with JSR and SDK**
- 2012: Strong R&D partner in EU projects – currently involved in three FP7 projects (http://cordis.europa.eu/)

CERTIFICATIONS & APPROVALS

- DMF for DEAE, QA and SO3 CIM® monoliths were filed
- FDA
- Partners (Novartis, Boehringer Ingelheim, Octapharma,..)
- ISO 9001: 2008

IP

- 4 US patents and their foreign equivalents (more than 50) granted, more pending:
  - CIM® technology and manufacturing
  - Different geometries including scale-up

CIM® 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, Inter-alpha-inhibitors).
- 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).

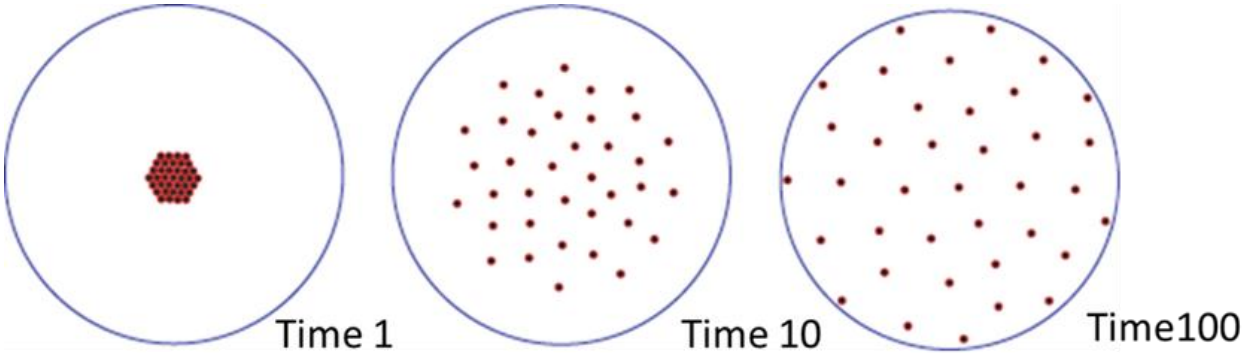
Chromatographic Separations

- Principle of Chromatography:
  - Equilibrium between the molecules in the mobile and stationary phase
  - The movement of the solutes (proteins, DNA, virus particles) between the two phases and through the column - MASS TRANSFER

- MASS TRANSFER
  - Diffusion
  - Convection

Chromatographic separations: mass transfer

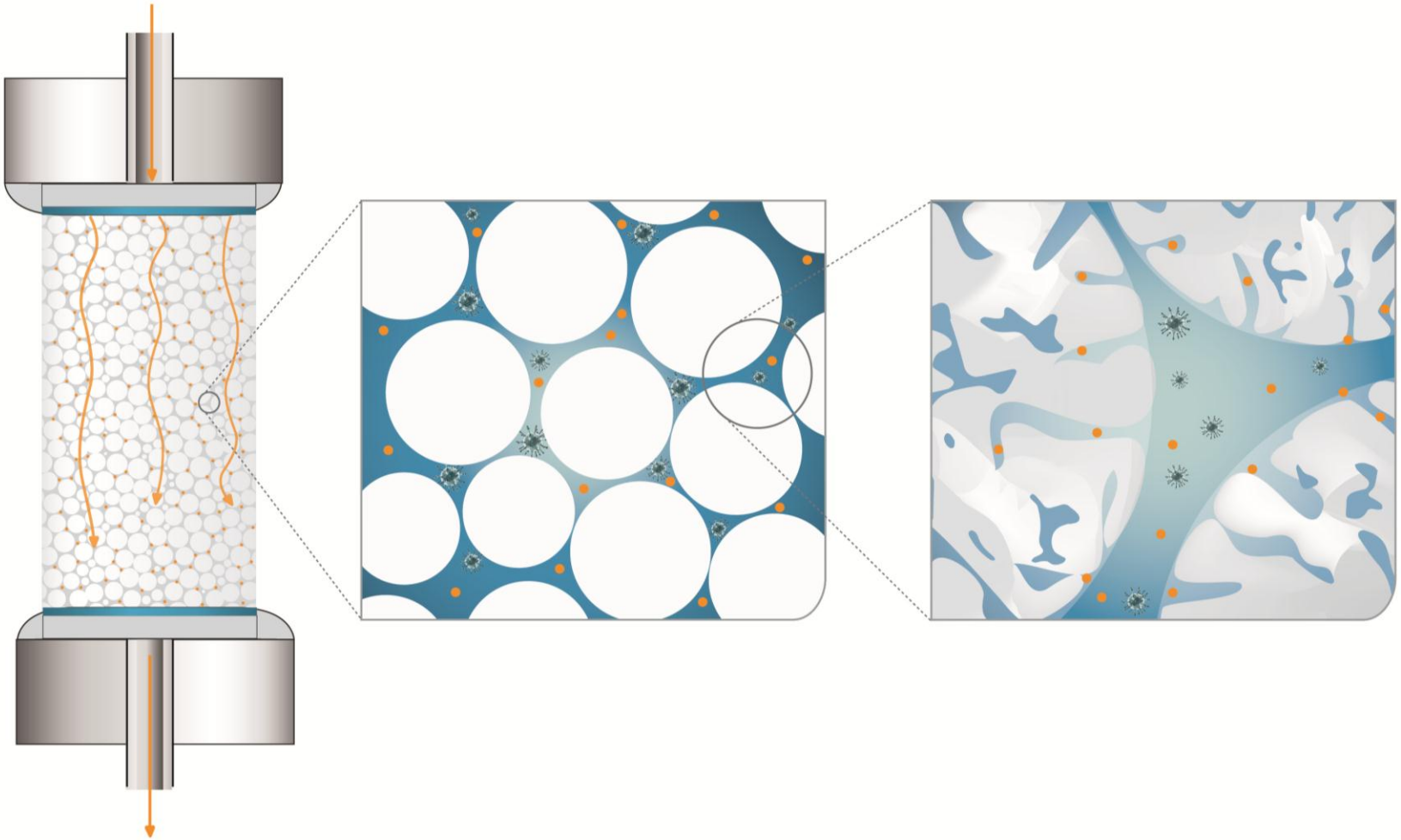
- Diffusion - random thermal movement from an area of high concentration to an area of low concentration



- Convection - movement induced by an external force, such as the flow of buffer, induced by gravity or a pump

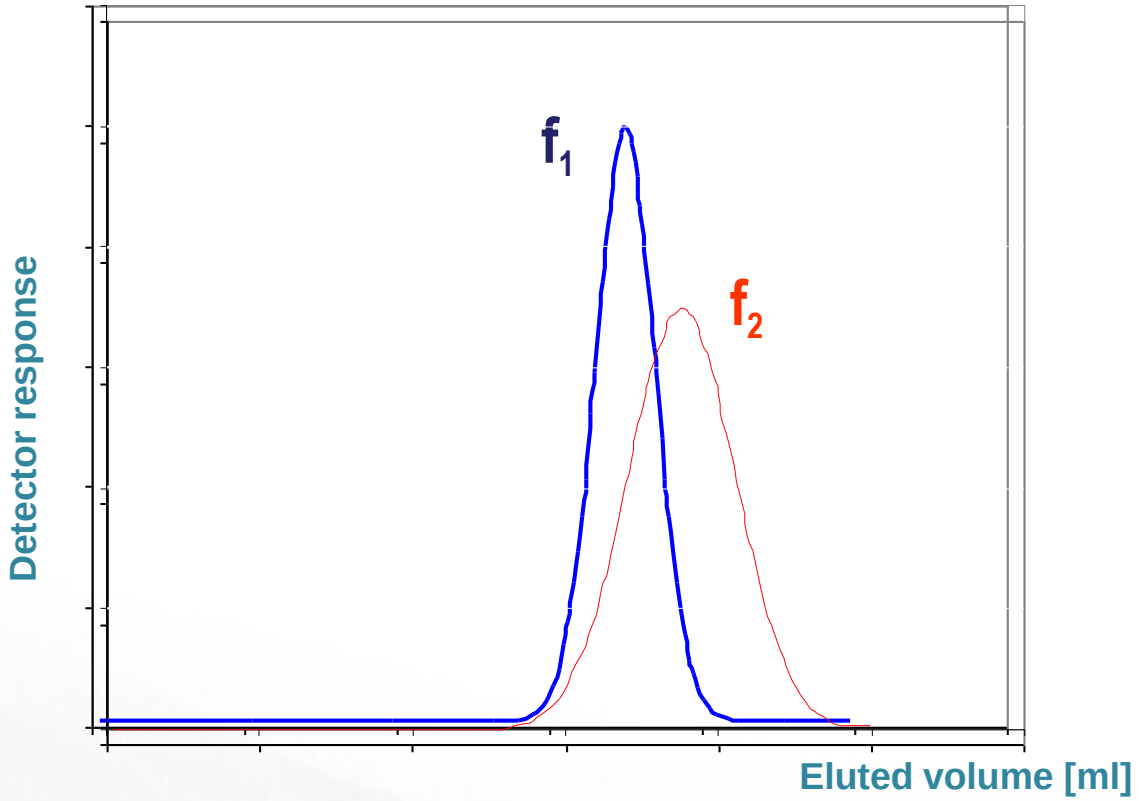
Courtesy of: Pete Gagnon.
www.validated.com

Conventional packed media



Diffusion limitations – compromised resolution

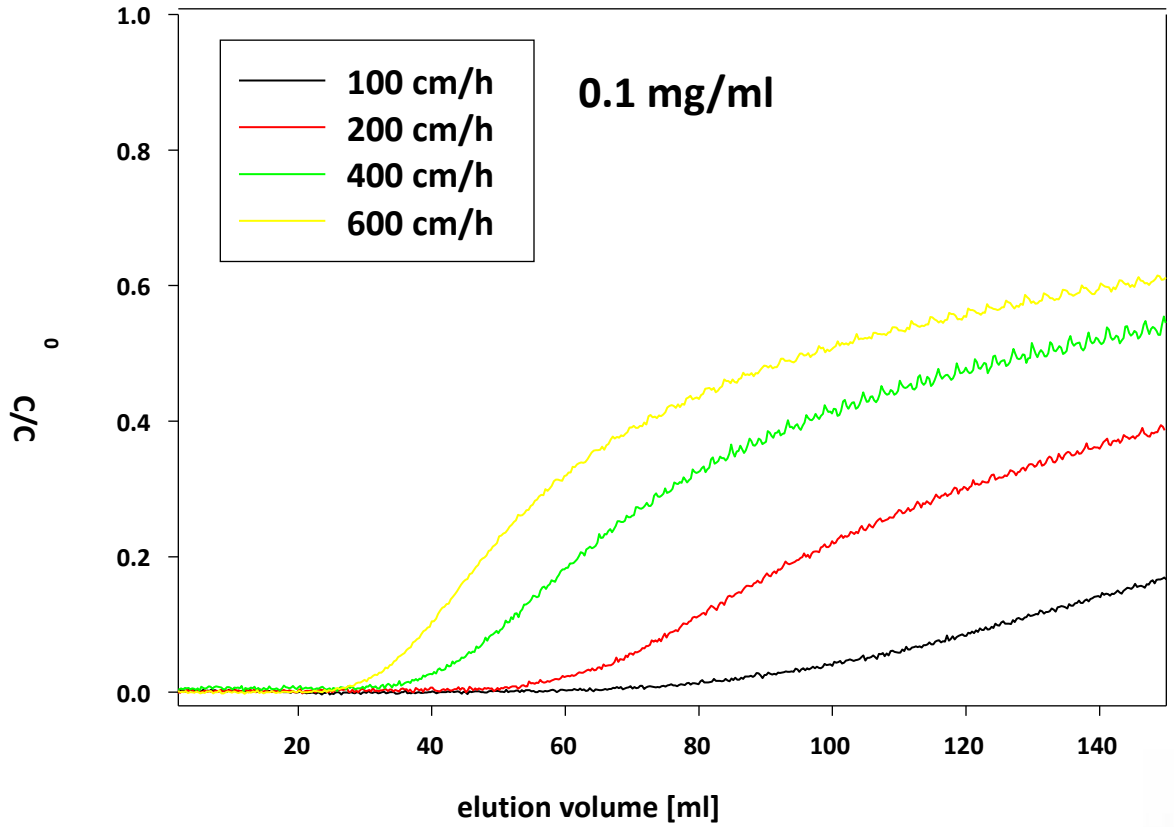
Speed limitation - resolution in linear gradient elution at high flow rate: f2 > f1



Higher the flow rate - wider the peak – and lower the resolution!

Diffusion limitations: compromised binding capacity

Chromatographic material: Source 30 S
Sample: IgG



Courtesy of Prof. A. Jungbauer, IAM, University Vienna, Austria

Diffusion limitations: lower flow rates

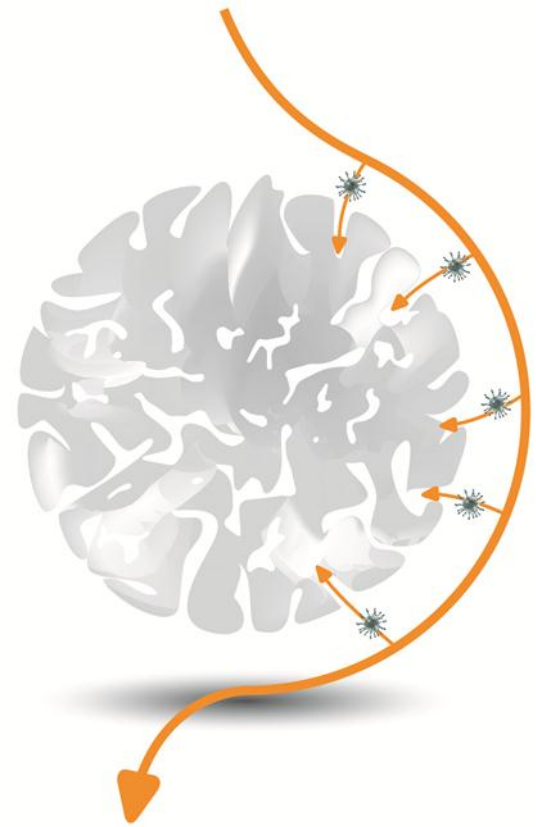
Mass transport within the chromatography column :

- differences in diffusion 'speed'
- low diffusivities - slow flow rates in order for the molecule to reach the binding site

Solute	Size	K_{diff} (cm^2/s)	Delta $_{BSA}$
Sodium	53 Da	1.4 E-5	> 479x
BSA	66 kDa	6.7 E-7	= 1x
IgG	150 kDa	4.9 E-7	< 1.4x
IgM	1 MDa	2.6 E-7	< 2.6x
CMV	5 MDa	1.2 E-7	< 5.6x
TMV	40 MDa	5.0 E-8	< 13.4x
DNA	33 kbp	4.0 E-9	< 167x

Diffusivities of some of the representative molecules (BSA normalized).
BSA = Bovine Serum Albumin, CMV = Cucumber Mosaic Virus, TMV = Tobacco Mosaic Virus.

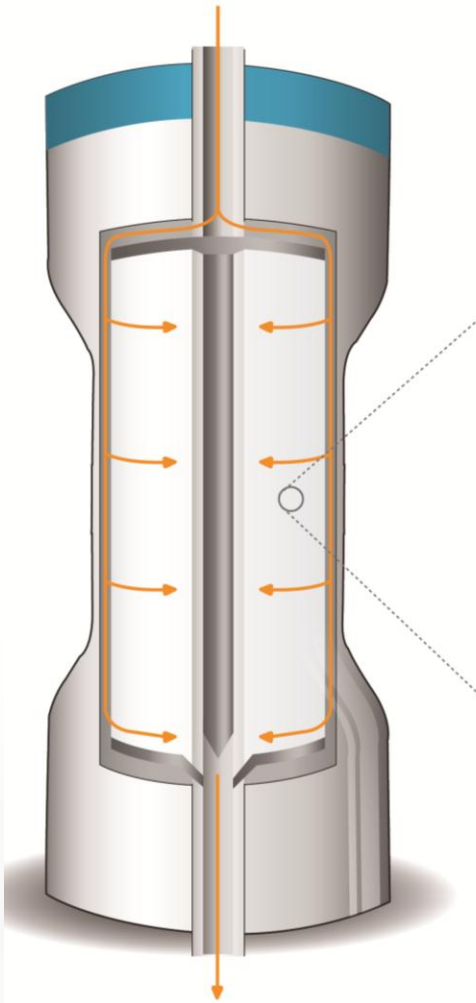
Molecule Size: Surface Accessibility



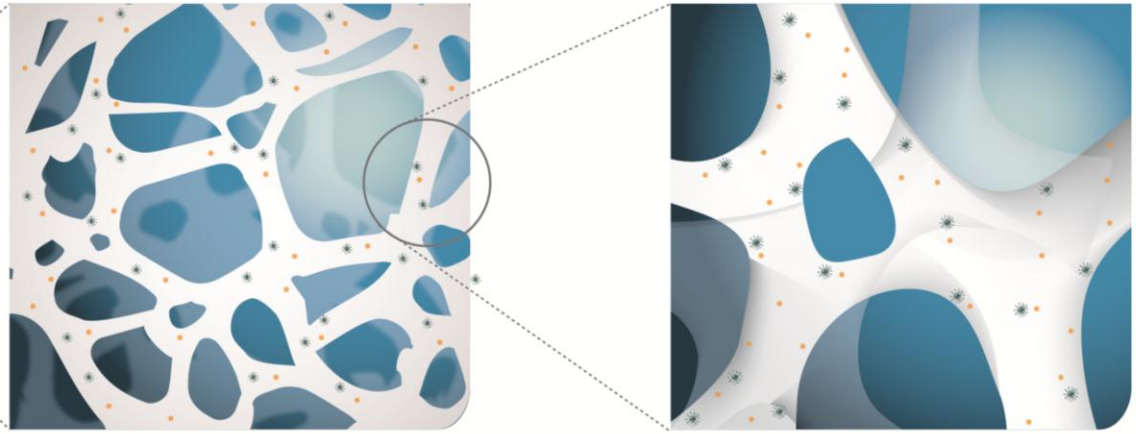
Molecule	nm
Proteins	1-3
IgM	25
Plasmids	150-250
Rotavirus	130
Poxvirus	200 x 500
T4	220 x 85

Many plasmids and viruses are larger then pores, which dramatically reduces the binding capacity.

Monoliths – convection enhanced mass transport



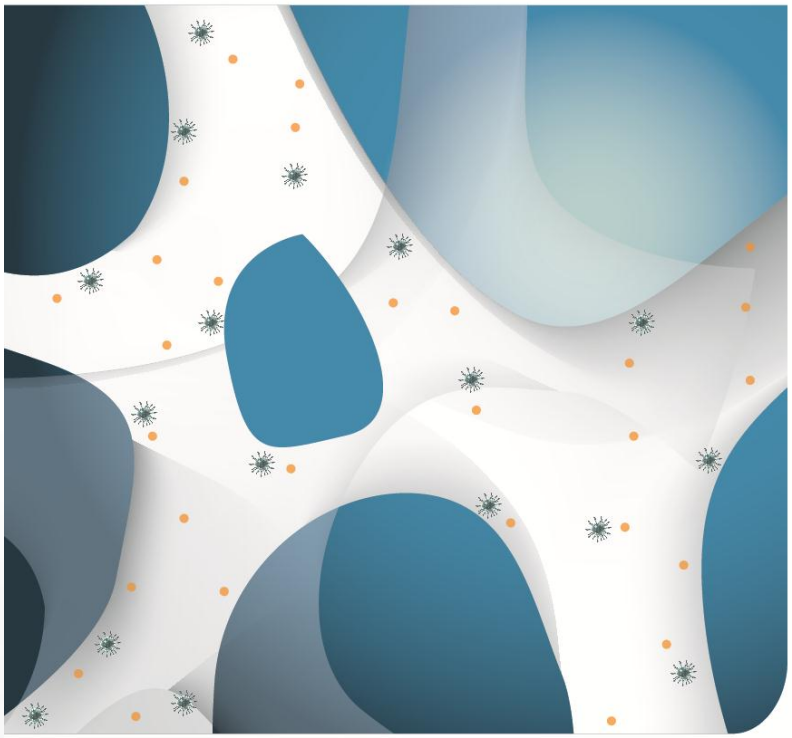
Monoliths are cast as a single block and inserted into a chromatographic housing.



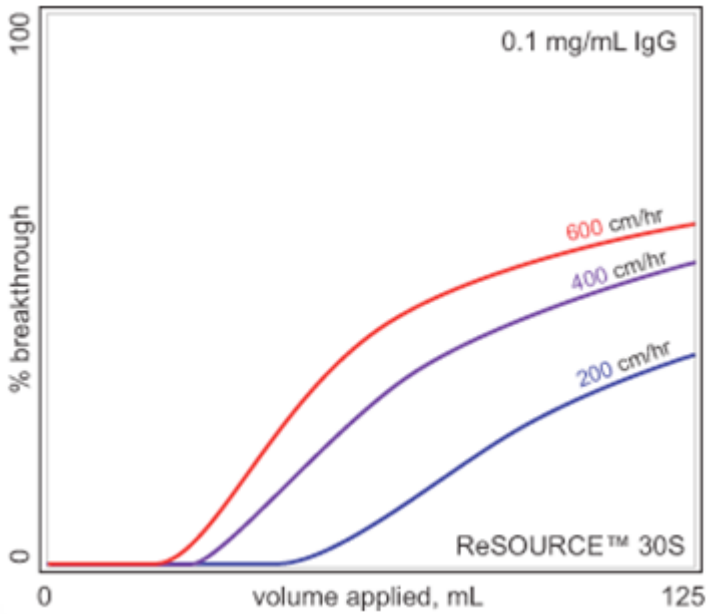
They are characterized by a highly inter-connected network of channels -a sponge.

Monoliths – convection enhanced mass transport

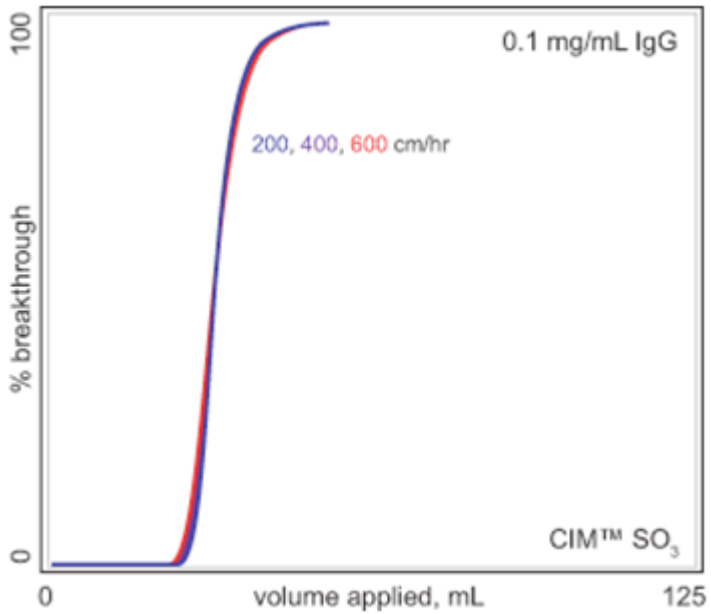
- Binding sites are situated inside the channels – no dead end pores – no diffusion limitations – same performance at lower and at higher flow rates
- Channels are large (1-2 µm) - optimal for molecules like viruses, virus-like particles and DNA to flow through the channels and bind to the binding sites



Mass transport within the chromatography column – consequence of convection



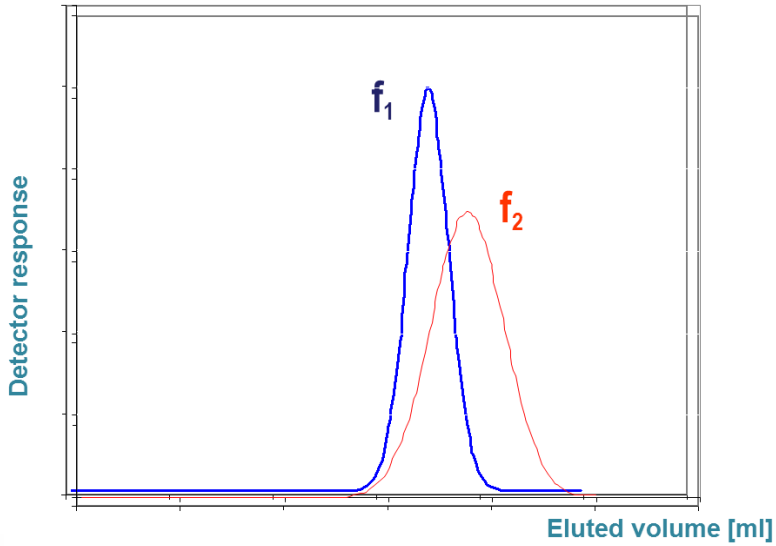
The dominating factor is the low efficiency of diffusive mass transport, which manifests as decreasing capacity with increasing flow rate.

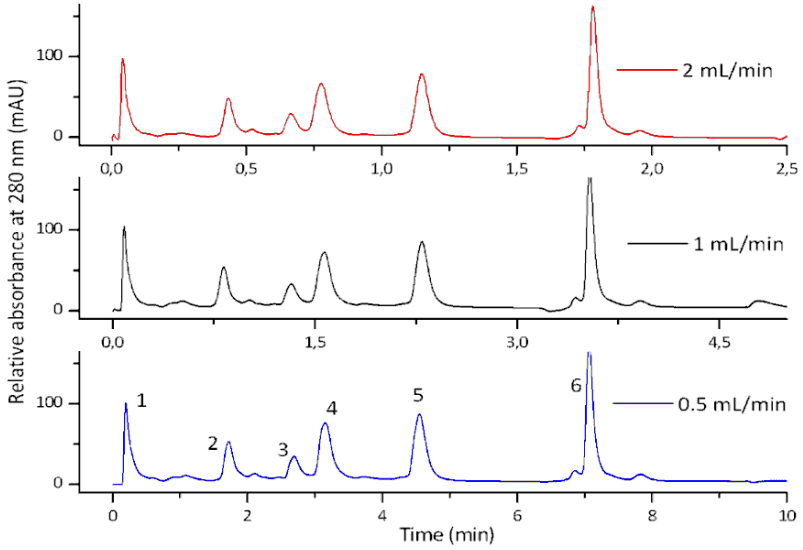


The fact that the curves overlay illustrates independence from flow rate, which translates into better reproducibility across process scales, as well as faster operation.

Courtesy of: Pete Gagnon.
www.validated.com

Mass transport within the chromatography column – consequence of convection





Particle based column – due to diffusion limitations the efficieny of the column is affected by higher flow-rate resulting in peak broadening.

HPLC (with an analytical monolithic column) separation of a mixture of 6 proteins at 3 different flow rates. Demonstrating high efficiency and flow-unaffected resolution.

Size of the biomolecule - influence on the binding capacity

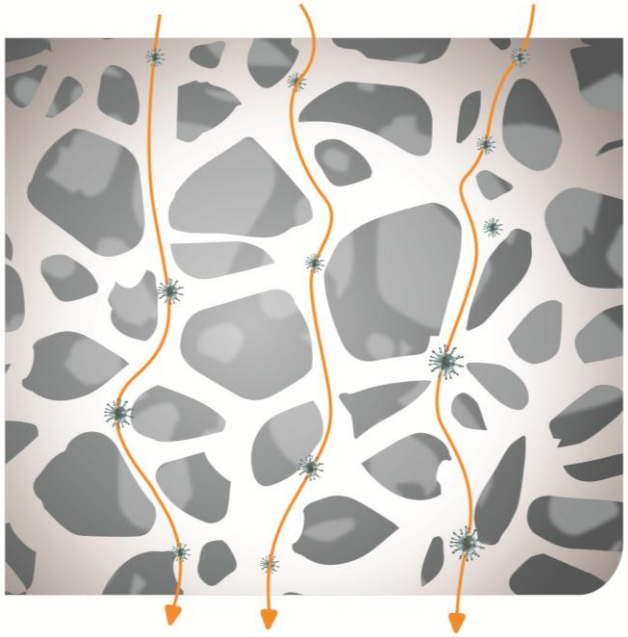
Solute	Method	Monolith	Particle based
BSA	Ion exchange	20 – 30	75 – 300
IgG	Affinity	10 – 15	25 – 60
IgG	Ion exchange	20 – 25	50 – 150
IgM	Ion exchange	20 – 50	10 – 50
DNA	Ion exchange	10 – 15	0.5 – 3
Flu virus	Ion exchange	10 – 100x	1x

Dynamic binding capacities = the amount of molecule that will bind to the column under practical conditions are expressed in mg/ml column (except for the flu virus).

Courtesy of: Pete Gagnon. www.validated.com

What distinguishes monoliths from conventional supports?

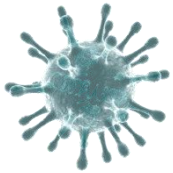
1. Structure of the monolith:
   - Low pressure drop
   - High surface accessibility
   - High dynamic binding capacities for large molecules



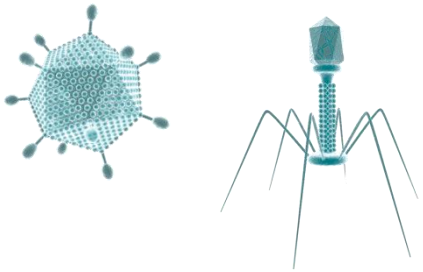
2. Convective transport
   - Flow independent performance – operating at high flow rates

Suitable for the separation and purification of large biomolecules; pDNA, viruses, proteins.

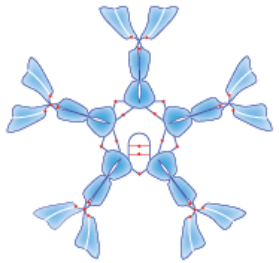
Main Applications – molecule type



Viruses & VLPs



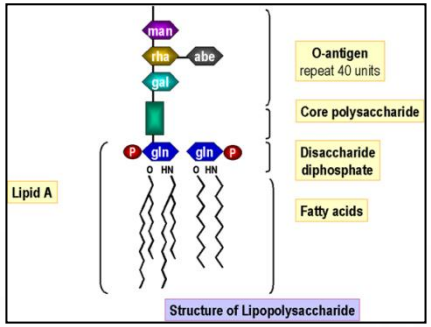
Large proteins

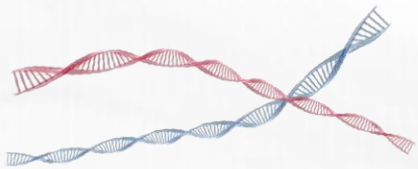


CIM Columns

Plasmid DNA

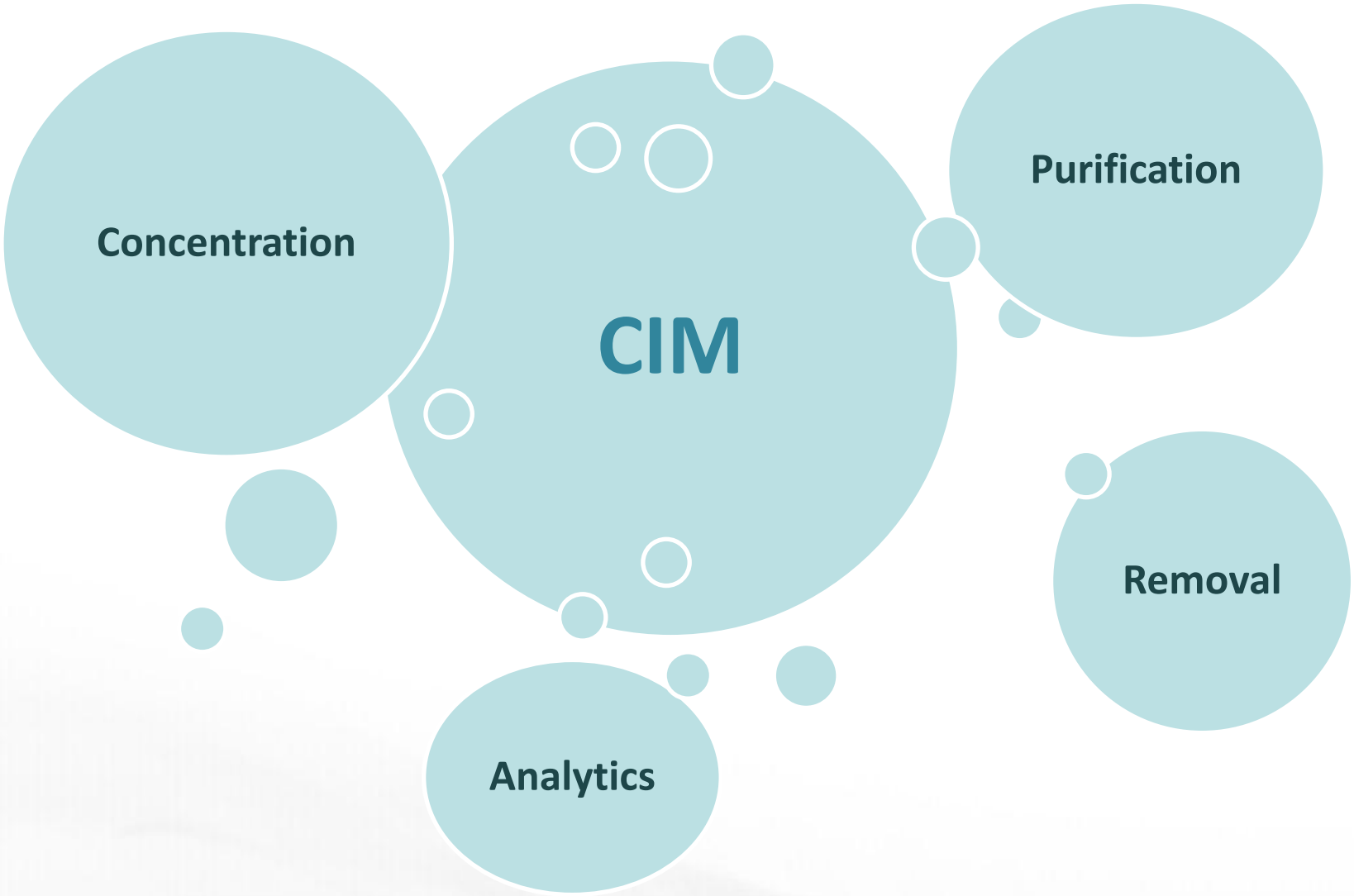
Endotoxins





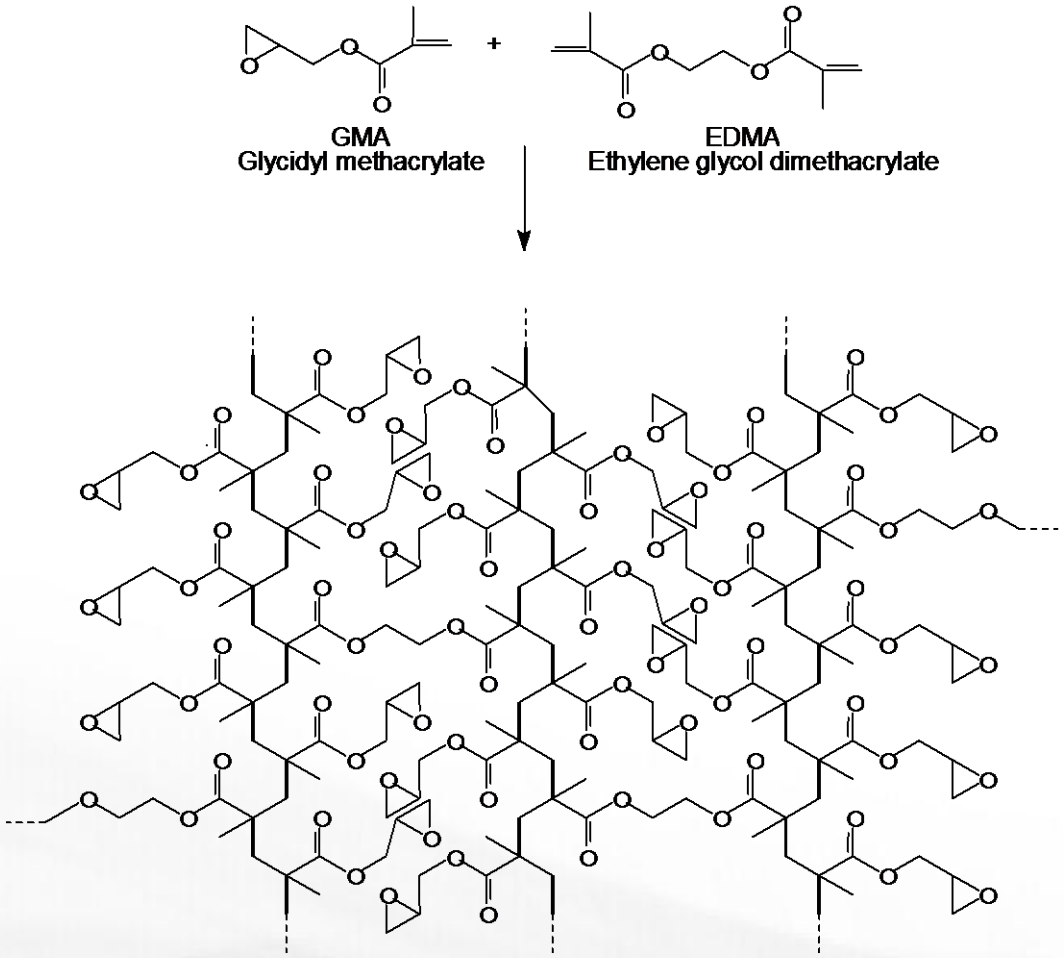
DNA depletion

CIM monoliths application areas

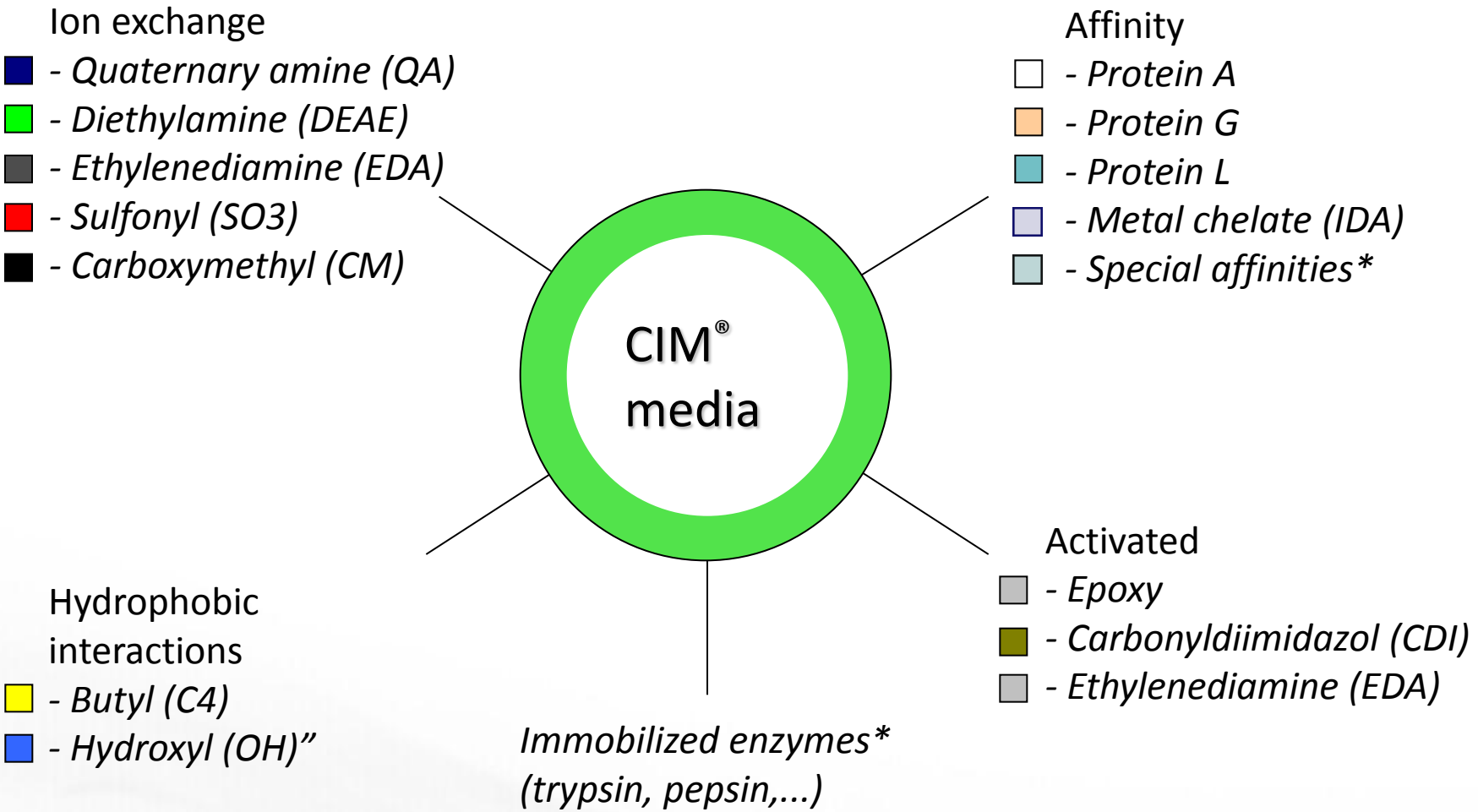


CIM Monoliths

Made of highly cross-linked porous rigid monolithic *poly(glycidyl methacrylate-co-ethylene dimethacrylate)*

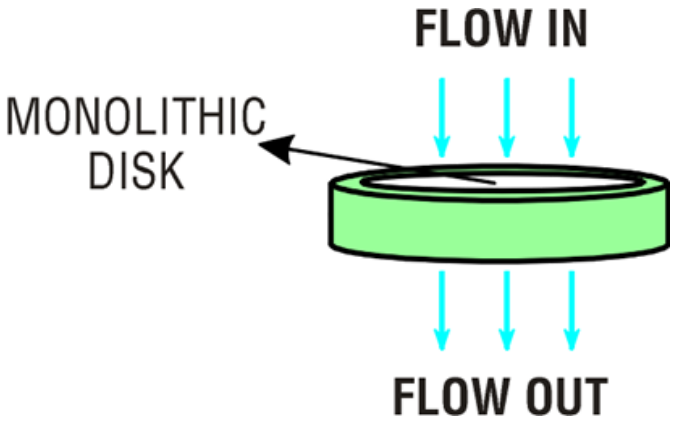


Available Chemistries

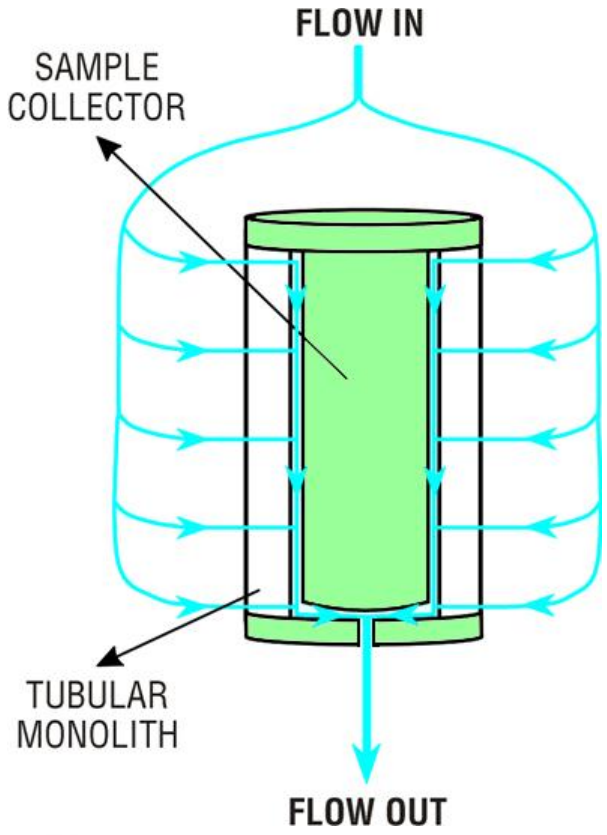


* *on request*

Radial flow geometry

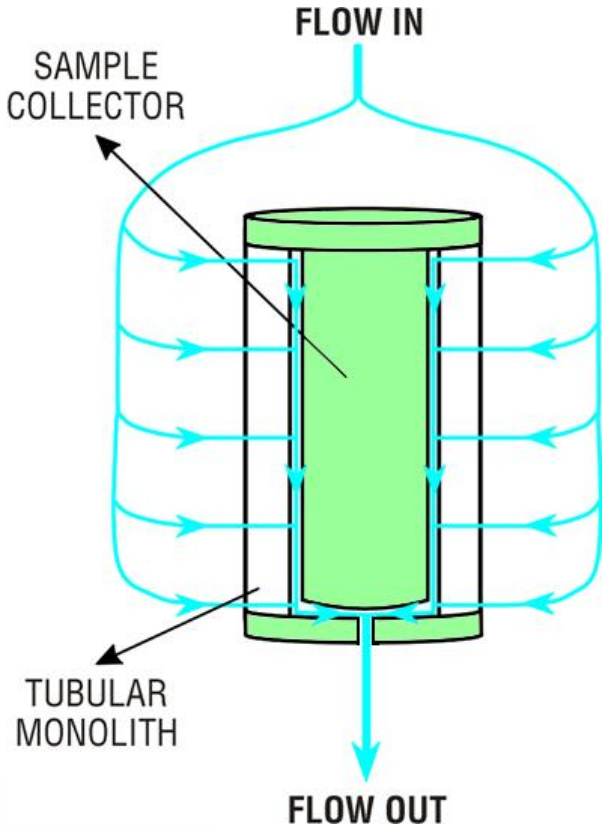


axial flow



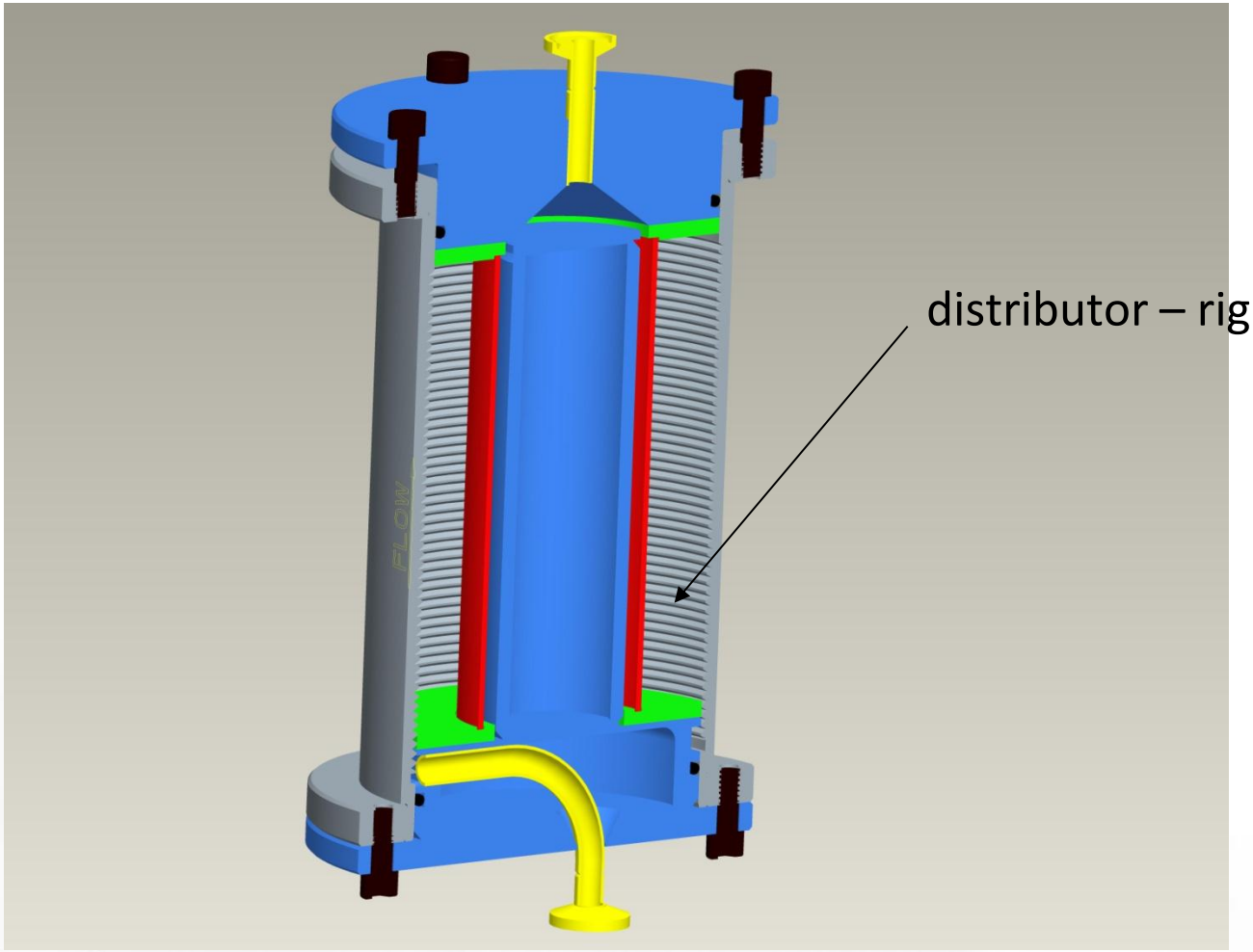
radial flow

Radial flow geometry



radial flow

Distributor – right turning helix



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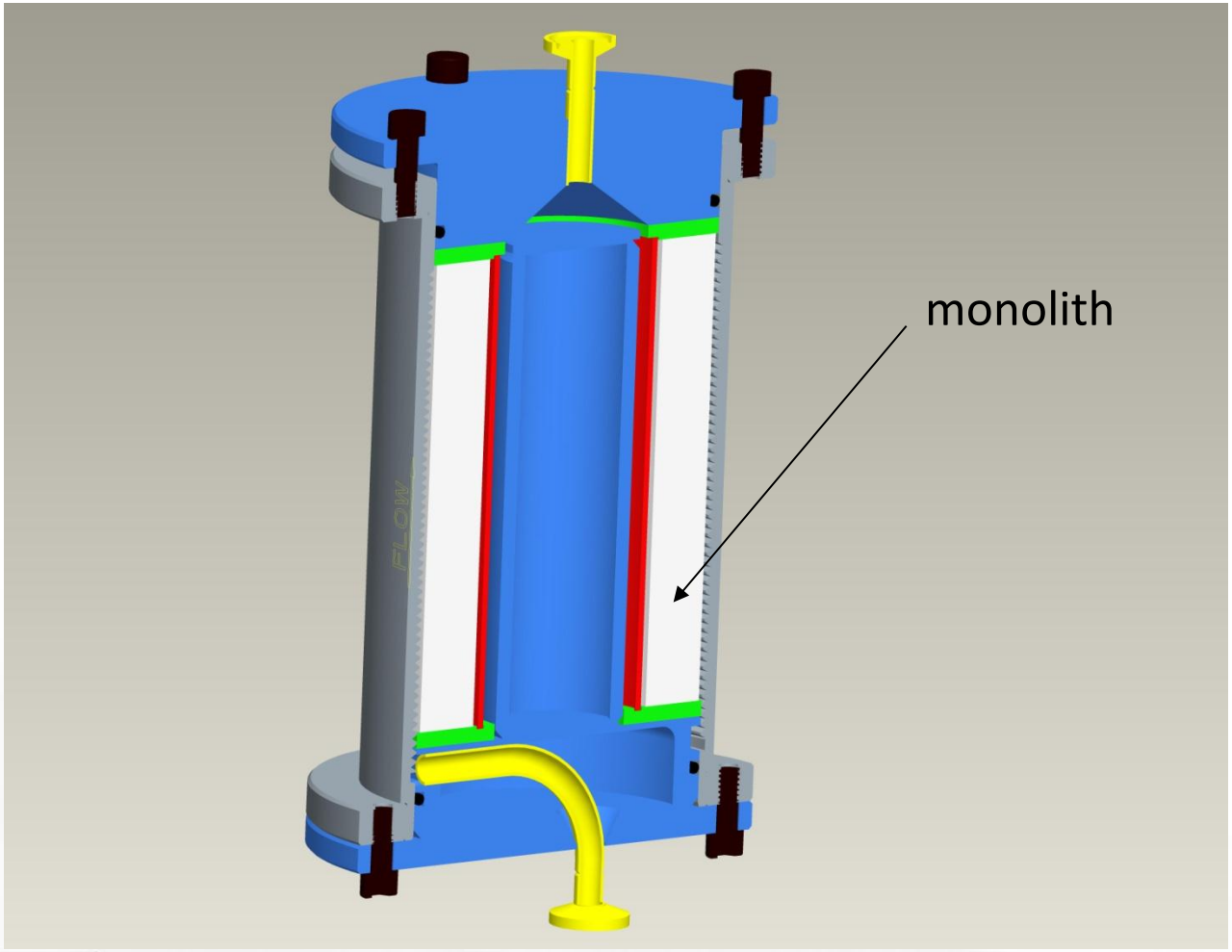
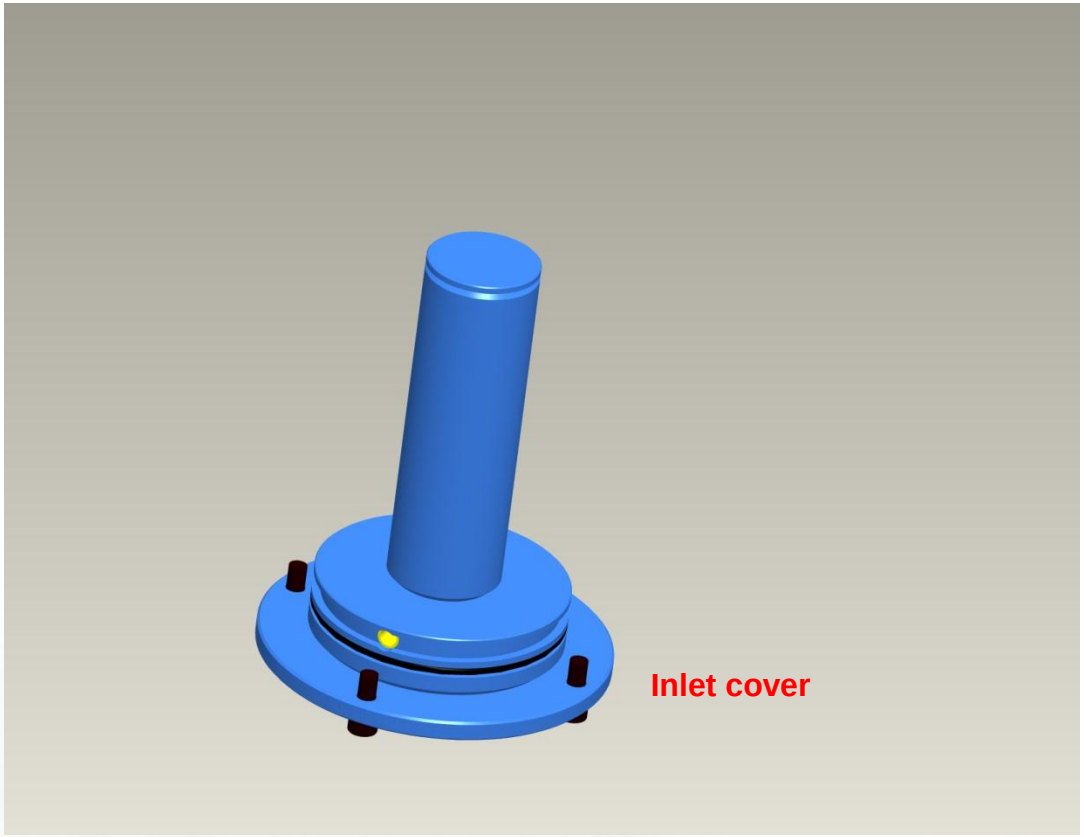


Constructon of radial flow monolithic columns



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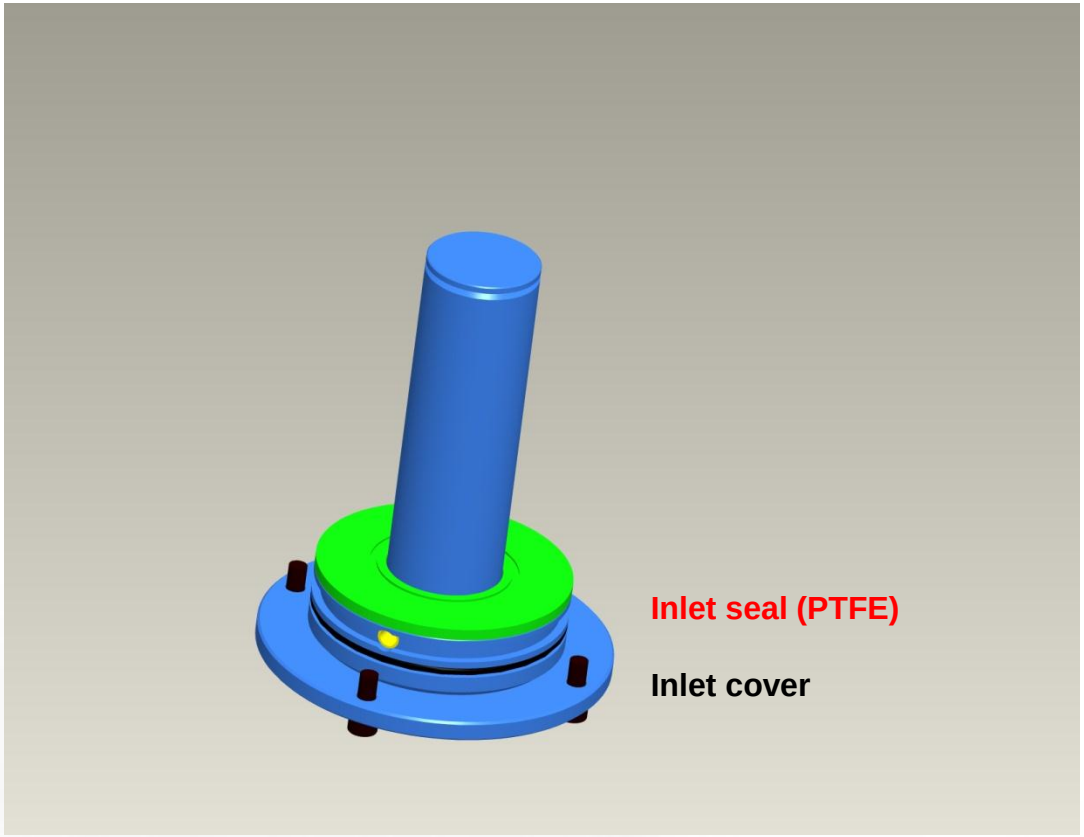

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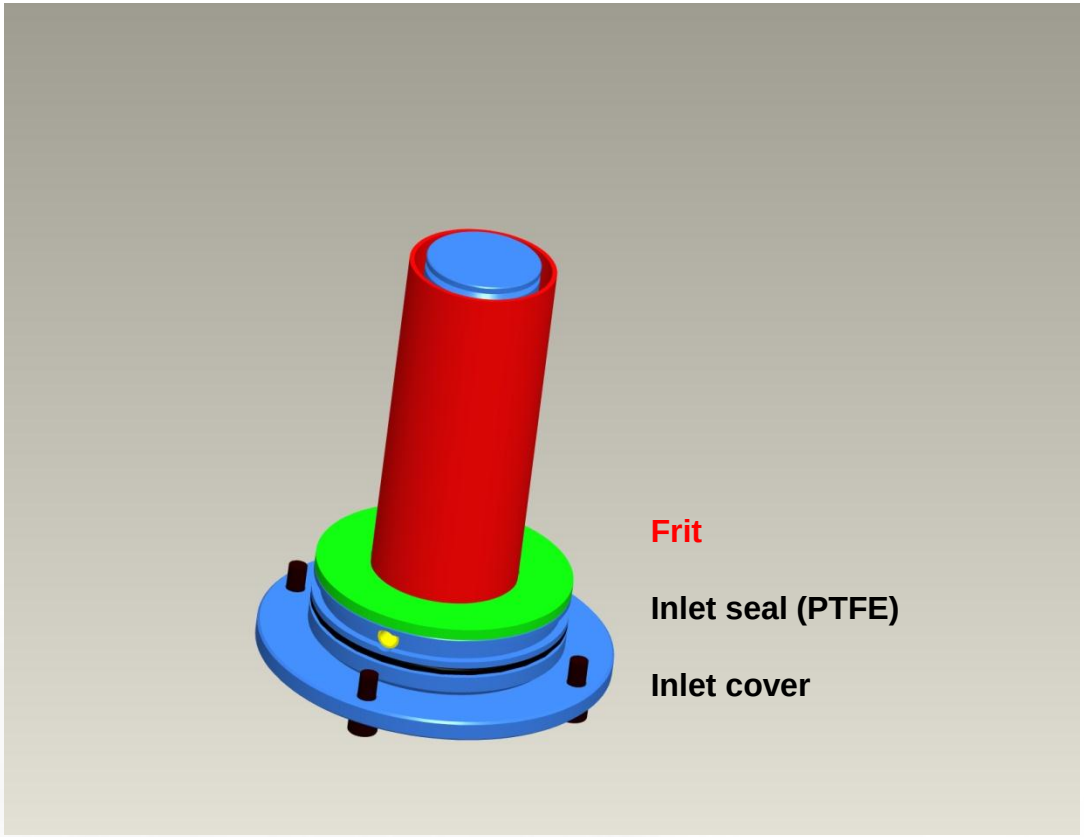

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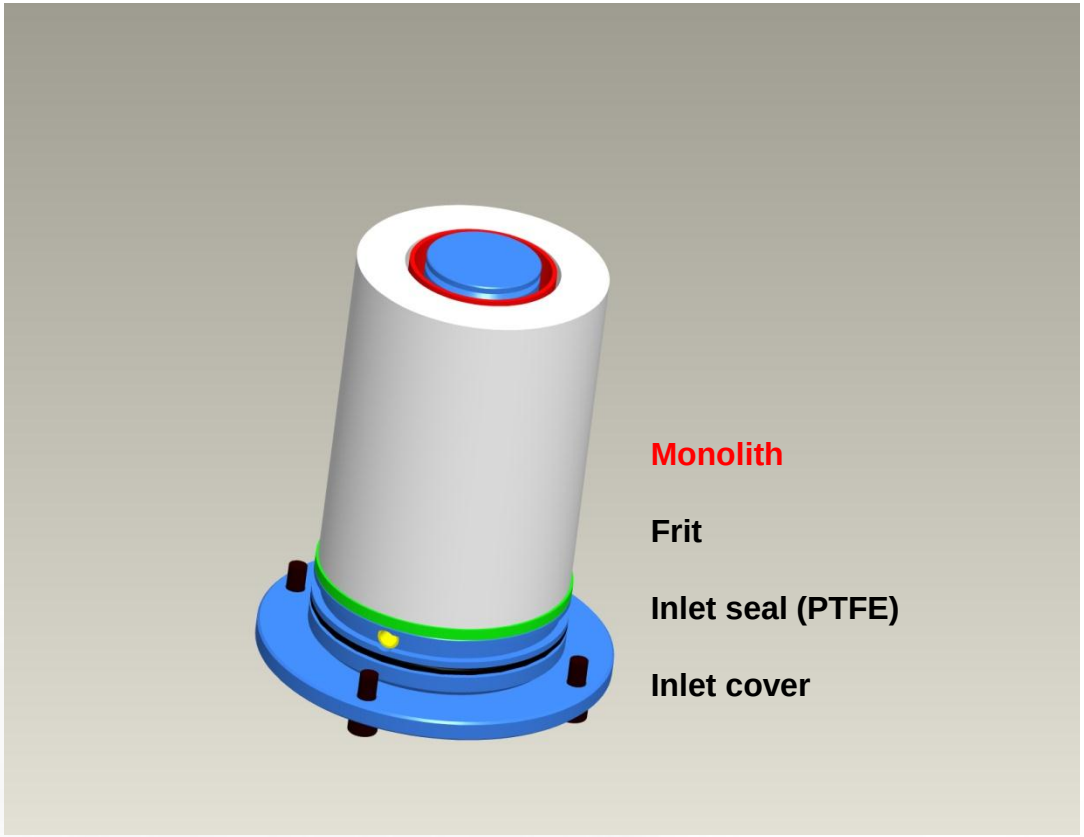

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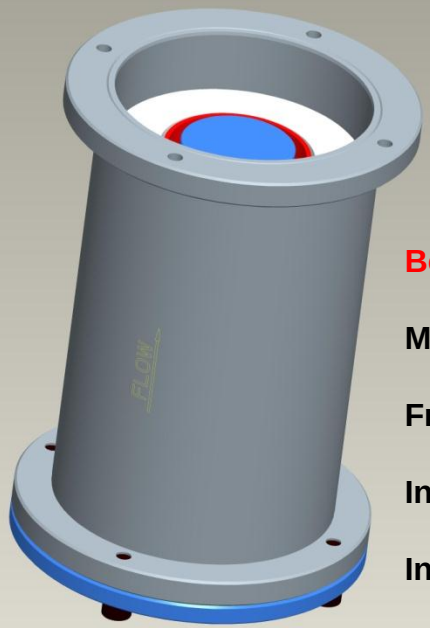


Body with distributor

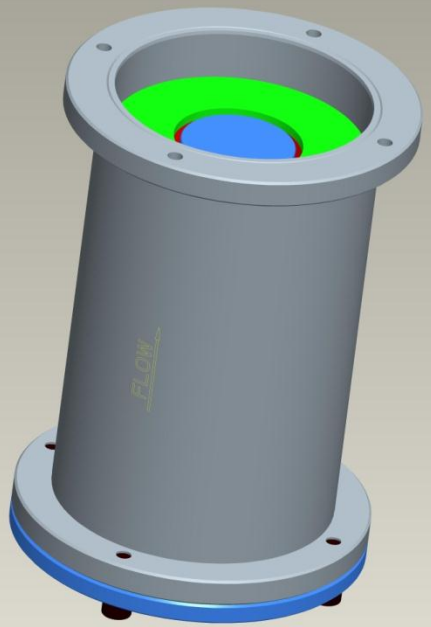
Monolith

Frit

Inlet seal (PTFE)

Inlet cover

Constructon of radial flow monolithic columns



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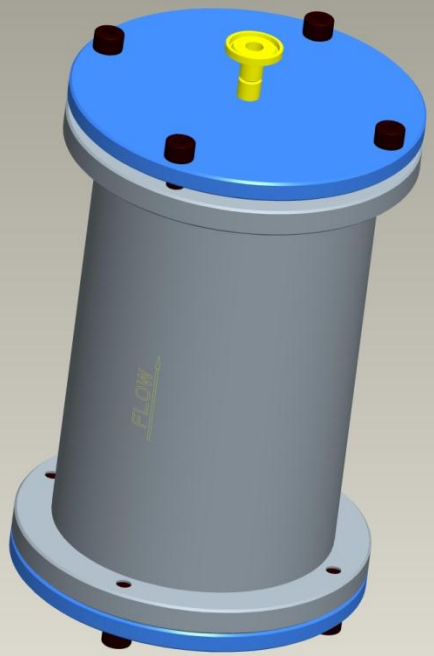


Outlet cover

Outlet seal (PTFE)

Body with distributor

Monolith

Frit

Inlet seal (PTFE)

Inlet cover

Advantages of short column layers

Additional reduction of pressure drop

- Avoid pressure drop becoming limiting

High throughputs possible

- Take advantage of fast convective mass transfer for high speed separations

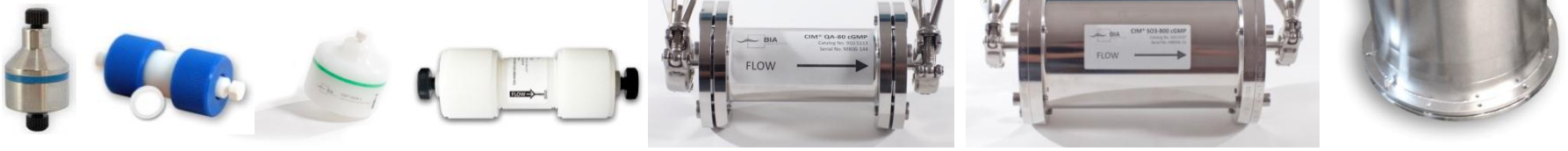
Short residence time

- Avoid unspecific binding, product degradation or minor structural changes of biomolecules

Minimal dispersion

- Sharp peak resolution

Avaliable CIM Monolithic Suppports



0.1 | 0.34 | 1 | 8 | 80 | 800 | 8000 ml

CIMmultus (multi-use disposable columns)

CIMmultus line

Concentration

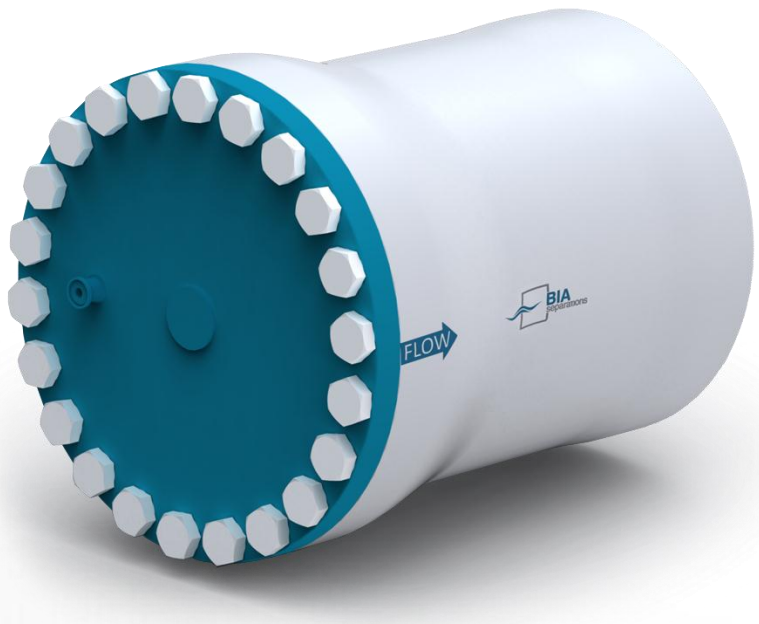
Removal

Purification

Improved monolith technology

in innovative multi use disposable housing.

Housing Composition



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

Disposable

Stainless steel performance characteristics

GMP compliant

Parylene C Coating

- Certifications
  - USP 29 Class VI
  - ISO-10993
  - Applied pin-hole free

- **Parylene**
  - 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

NO LEACHABLES

CIMmultus column volumes

CIMmultus – Matching Stainless Steel Performance

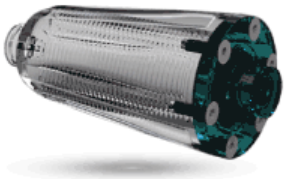
	1 mL		8 mL		80 mL		800 mL		8000 mL	
Type of column	CIM SS	CIMmultus™	CIM SS	CIMmultus™	CIM SS	CIMmultus™	CIM SS	CIMmultus™	CIM SS	CIMmultus™
Max pressure	18 bar	18 bar	20 bar	20 bar	20 bar	20 bar	7 bar	14 bar	7 bar	14 bar
Recommended flow rates (mL/min)	1-5	1-5	8-60	8-60	80-240	80-240	200-1300	200-1300	2000-10000	2000-10000
Max. flow rate (mL/min)	16	16	100	100	400	400	2000	2000	10000	10000
Max. operating temperature	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C
L-t storage conditions	20% ethanol									
Sanitization for IEX, C4 HLD	1 M NaOH for at least an hour									

Stainless Steel
Columns

Single-use
Disposable

Bridging
the Gap Between

CIMmultus™



Multi-use
Disposable

Stainless Steel
Columns

Single-use
Disposable

Reasons to use in...

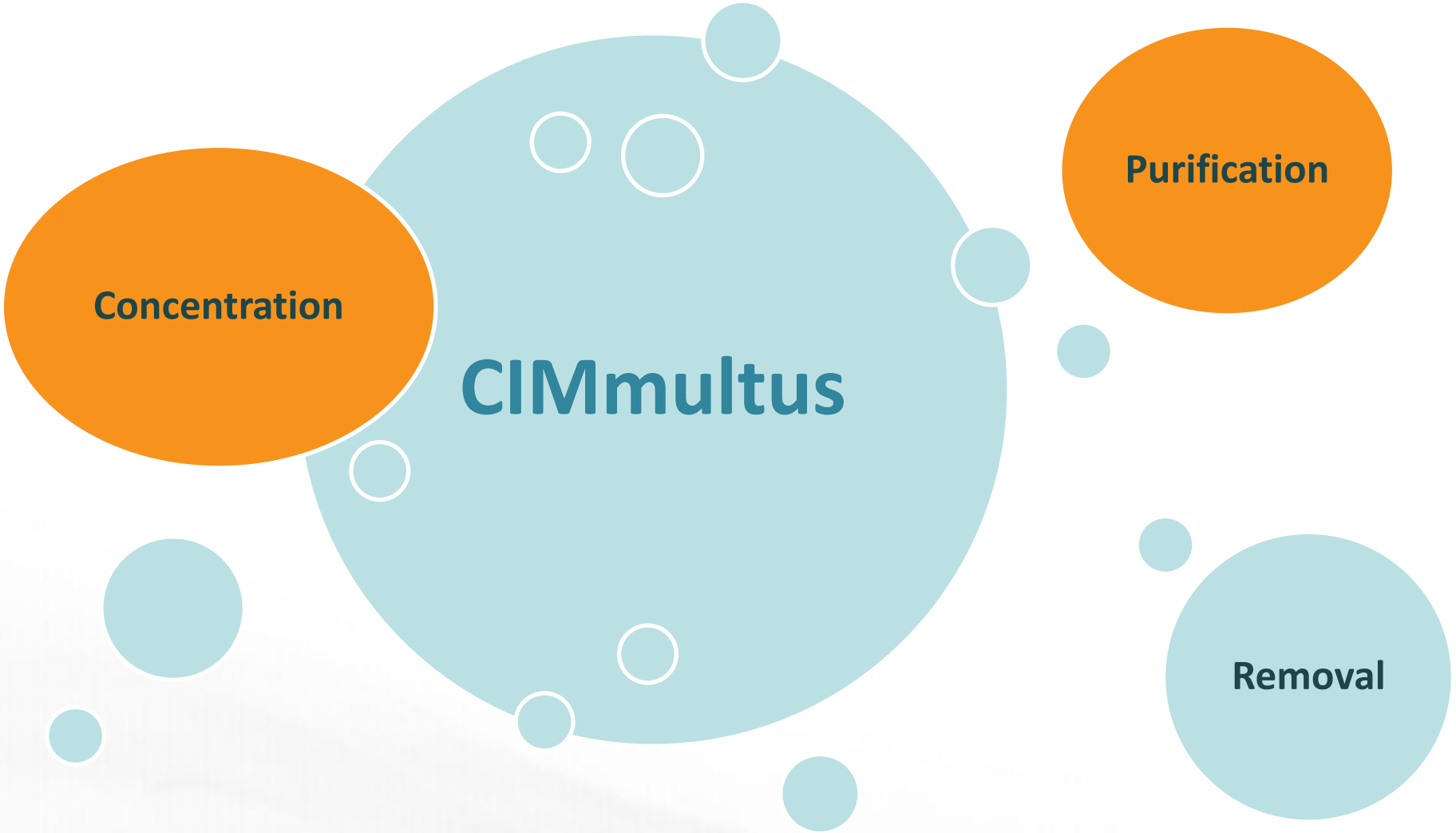
SINGLE-USE MODE	MULTI-USE MODE
- Prevent cross-contamination - Eliminate cleaning and validation - Reduce validation cost	- Application allows multiple use - Longer time between running batches (use-clean-store-reuse) - Reduce manufacturing costs

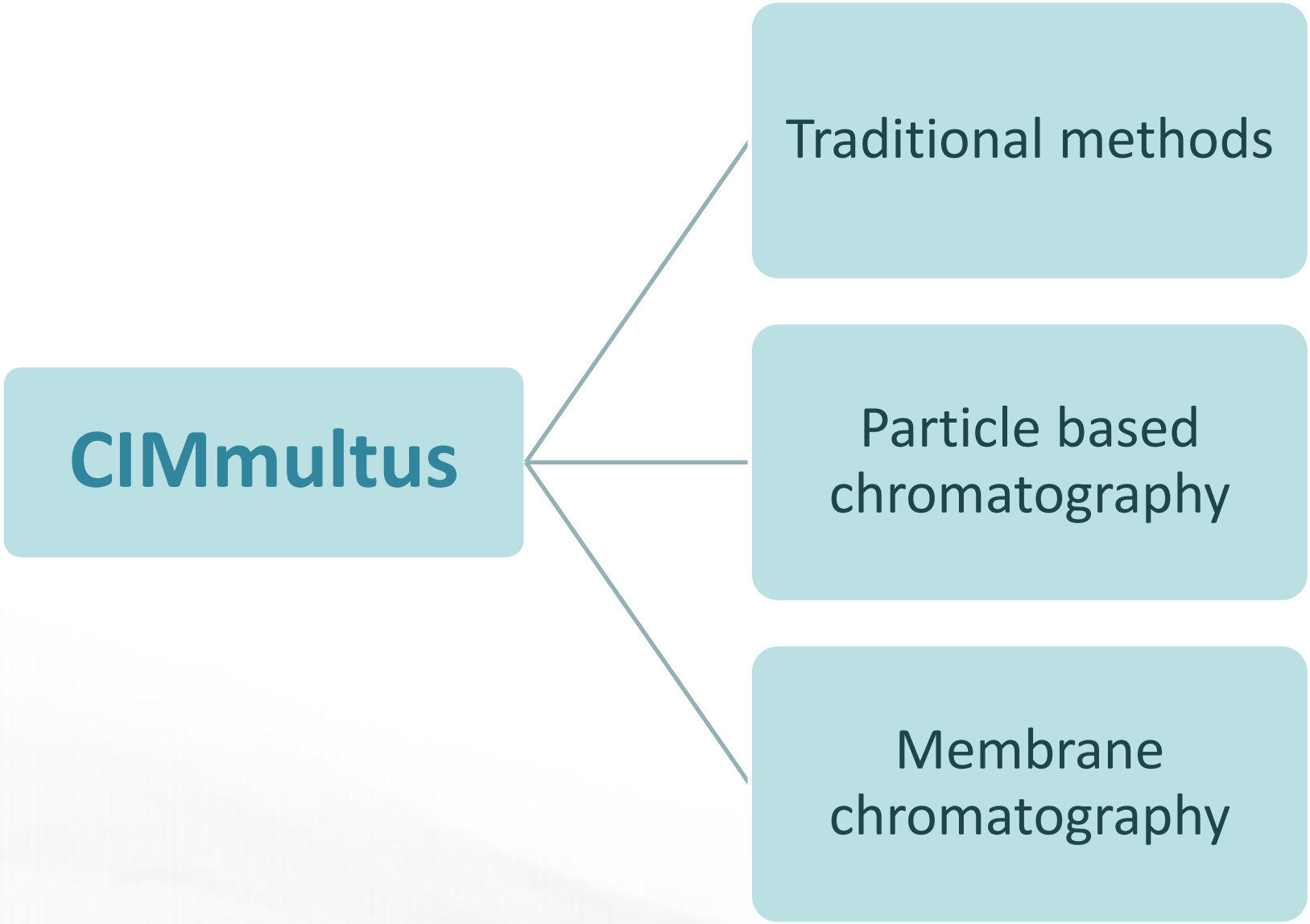
Depends on your application

Currently Available Chemistries

Chemistry	Description
DEAE	Weak anion exchanger
QA	Strong anion exchanger
SO3	Strong cation exchanger
C4 HLD	Hydrophobic
OH	Hydrophobic

Additional chemistries upon request


Concentration
CIMmultus
Purification
Removal


CIMmultus
Traditional methods
Particle based chromatography
Membrane chromatography

Traditional method for virus purification - Ultracentrifugation

- Long process time – 10 to 18 hours per run.
- Expensive equipment.
- Multiple runs may be needed for impurity removal.
- Scalabilty is difficult.
- Shear forces are a problem! Sometimes infectivity of viruses can be lost.

Virology 434 (2012) 265–270

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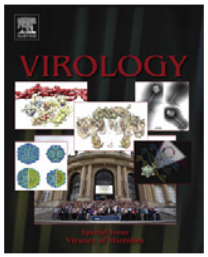
journal homepage: www.elsevier.com/locate/yviro



CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles

Evelien M. Adriaenssens [a,b,c], Susan M. Lehman [d], Katrien Vandersteegen [a], Dieter Vandenheuvel [a], Didier L. Philippe [e], Anneleen Cornelissen [a], Martha R.J. Clokie [e], Andrés J. García [d], Maurice De Proft [b], Martine Maes [c], Rob Lavigne [a,*]

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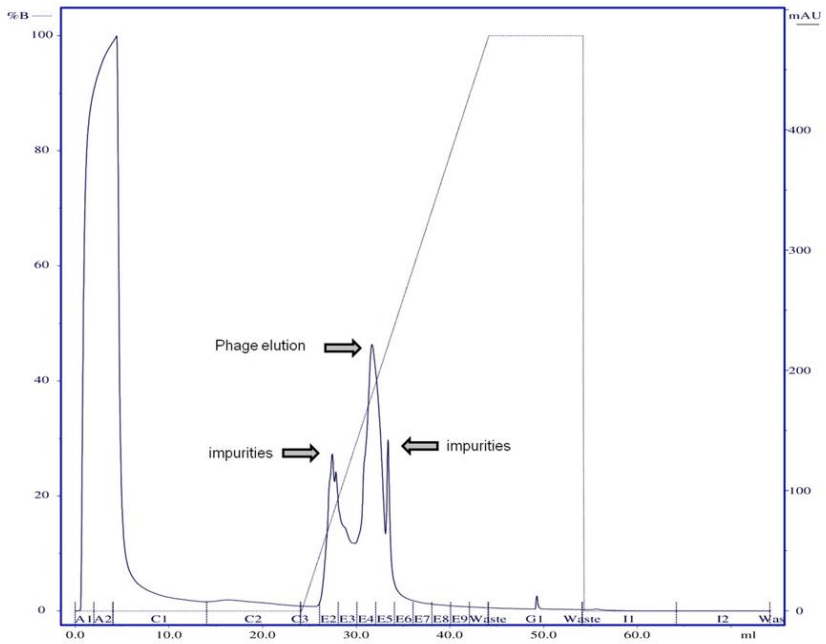
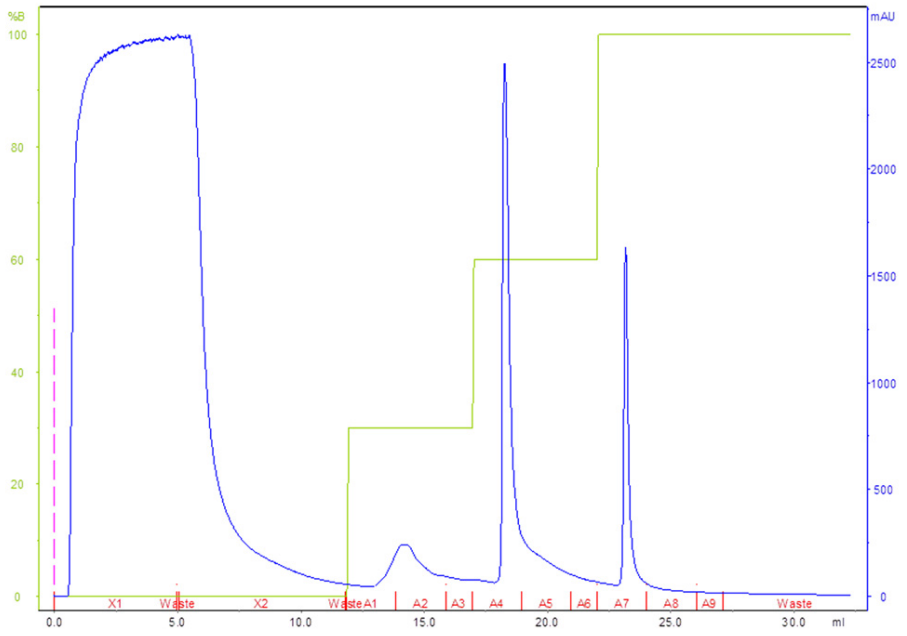
[c] Institute for Agricultural and Fisheries Research, Unit Plant, Crop Protection, Burgemeester Van Gansberghelaan 96-b2, 9820 Merelbeke, Belgium
[d] Georgia Institute of Technology, Petit Institute for Bioengineering and Bioscience & Woodruff School of Mechanical Engineering, Atlanta, GA, USA
[e] Department of Infection, Immunity and Inflammation, Medical Sciences Building, University of Leicester, PO Box 138, Leicester LE1 9HN, UK

Purification method development

- Individual methods developed for a particular phage,
- Linear gradients adapted to step gradients.





Capacity – up to 1.2 × 10^{12} phage particles/ml

Adriaenssens et al./Virology 434 (2012) 265-270

11 morphologically distinct phages infecting different hosts

Bacteriophages purified with CIM® monolithic columns.

Phage	Phage family (morphotype)	Host species	Host strain	Growth medium	Loading suspension[a]	Columns used[b]	Optimized Buffer set[c]	Elution of pure phage fraction	Capacity (pfu/ml column)	Recovery of phage in pure fraction (%)
Dickeya phage LIMEstone1	*Myoviridae* (Vil-4like)	"*D. solani*"	GBBC 2072	LB	Undiluted	CIM® QA/DEAE disk	Tris(a)	0.6 M NaCl	$> 7.4 \times 10^{12}$	99.9
Dickeya phage LIMEstone2	*Myoviridae* (Vil-4like)	"*D. solani*"	GBBC 2072	LB	Diluted (1/2)	CIM® QA/DEAE disk	phosphate	0.6 M NaCl	$> 5.9 \times 10^{11}$	70
Staphylococcus phage ISP	*Myoviridae* (Twort-like)	*S. aureus* subsp *aureus*	Rosenbach ATCC 6538	MH	Undiluted	CIM® QA/DEAE disk	Tris(a)	0.8 M NaCl	$> 2.9 \times 10^{11}$	35–65
Pseudomonas phage ΦE2005-A	*Myoviridae* (PB1-like)	*P. aeruginosa*	EAMS2005-A	25% TSB	Diluted (1/1)	CIM® QA-8 f	Tris(a)	0.25 M NaCl	1.3×10^{11}	40–70
Pseudomonas phage ΦPaer14	*Myoviridae* (PB1-like)	*P. aeruginosa*	Paer14	25% TSB	Diluted (1/1)	CIM® QA-8 f	Tris(a)	0.25 M NaCl	1.3×10^{11}	40–70
Pseudomonas phage ΦE2005-C	*Myoviridae* (PB1-like)	*P. aeruginosa*	EAMS2005-C	25% TSB	Diluted (1/1)	CIM® QA-8 f	Tris(a)	0.25 M NaCl	1.3×10^{11}	40–70
Pseudomonas phage ΦM4	*Myoviridae* (KPP10-like)	*P. aeruginosa*	M4	25% TSB	Diluted (1/1)	CIM® QA-8 f	Tris(a)	0.56 M NaCl	1.3×10^{11}	40–70
Burkholderia phage Phi208	*Podoviridae*	*B. thailandensis*	DW503	LB	Dialyzed	CIM® QA disk	Tris(b)	0.3 M NaCl	5.0×10^{9}	70
Pseudomonas phage Φ15	*Podoviridae* (T7-like)	*P. putida*	PpG1	LB	Diluted (1/2)	CIM® QA/DEAE disk	Tris(a)	0.3 M NaCl	2.4×10^{11}	87
Pseudomonas phage ΦPaer4	*Podoviridae* (LUZ24-like)	*P. aeruginosa*	Paer4	25% TSB	Diluted (1/1)	CIMac™ QA; **CIM® QA-8 f**	Tris(a)	0.3 M NaCl	Ac QA: 5×10^{9} to 1×10^{10} QA-8f: $> 1.3 \times 10^{11}$	40–70
Pseudomonas phage LUZ19	*Podoviridae* (ϕKMV-like)	*P. aeruginosa*	PAO1	LB	Diluted 1/2)	CIM® QA/DEAE disk	Tris(a)	0.6 M NaCl	$> 3.5 \times 10^{12}$	70

[a] Undiluted: lysates were loaded; diluted: lysates were diluted in the corresponding loading buffer; dialyzed: phage suspension dialyzed against corresponding loading buffer.
[b] When multiple columns were tested, the best column for purification is in bold.
[c] Tris(a) and Tris(b) buffers differ in composition as described in the materials and methods section.
[d] Rodney Donlan, CDC Biofilm Lab, Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Atlanta, GA.

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Monoliths vs centrifugaton

Ultracentrifugation with CsCl	Purification with CIM monolith
• Good yields, but the volume of loaded suspension of a bacteriophage is constricted. • Equal purity (SDS determined). • Layering of CsCl gradient is a time consuming process, followed by centrifugation step that last for 1-3 hours, finishing with dialisis that takes several hours. • Equipment is expensive. • Amount of CsCl to purify one sample of phage is cheaper than one column.	• Unlimited volumes of phage can be loaded on each column. • The CIM monoliths scalability would permit higher titers to be reached when using industrial columns. • Equal purity (SDS determined). • Process does not take longer than an hour, and the eluted phage can be stored directly. • Equipment is expensive but has a broader general applicability. • But one column can be reused many times making it cheaper after several uses.

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Centrifugation versus chromatography based Flu purification process

CENTRIFUGATION BASED PURIFICATION PLATFORM

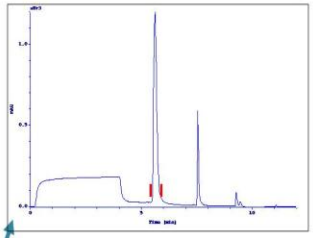
- Expanison of Vero cells
- Infection
- Harvest and Clearance
- Benzonase
- Tangential Flow Filtration (TFF)
- Ultracentrifugation (UCF)
- Adjustment to final formulation

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

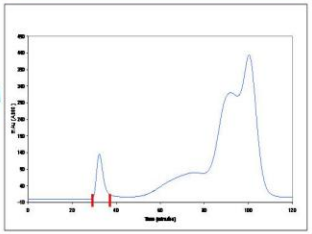
MONOLITH BASED PURIFICATION PLATFORM

- 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



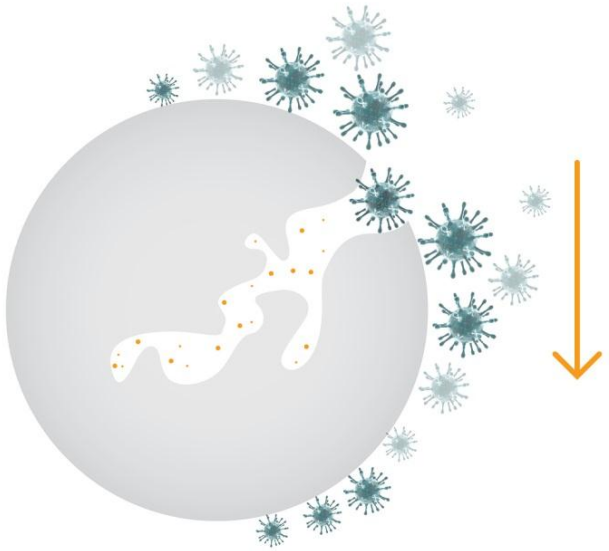
Infectious virus yield	47.3 %
DNA removal	99.96 %
Protein removal	97.8 %

E. Roethl et al., GreenHillsBiotechnology, BioProcess International, Raleigh, NC, 2009

4-times better yield results in e.g. 4 M doses of vaccine instead of 1 M doses for similar costs of the process = 3 M doses are pure profit, = more vaccine for pandemic

Particle based media

- Diffusion limitations:
  - Limited flow rates
  - Long process times
- Low dynamic binding capacities for large biomolecules:
  - Low diffusivity constants
  - Too small pore sizes
- Column packing is an issue



Chromatographic media - membranes

- Dynamic binding capacity (comparable or lower).
- Relatively large void volumes.
- Turbulent mixing between membrane layers in the void volume – eddy dispersion.

Evaluation of different supports for purification of live influenza A

Average values	QA monolith	Q membrane	Q porous particles	semi-affinity porous particles
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

Maurer et al., Purification of Biological Products, Waltham, MA/USA, 2007

50% better recovery results in e.g. 1,5 M doses of vaccine instead of 1 M doses, at the same costs of the process = 0,5 M doses are pure profit

Membrane versus CIM Monolith Canine Adenovirus Type 2

Bioprocess development for canine adenovirus type 2 vectors

P Fernandes[1,2], C Peixoto[2], VM Santiago[2], EJ Kremer[3], AS Coroadinha[1,2] and PM Alves[1,2]

Effect of different purification strategies on ΔE1 CAV-2 yields

Step	*Strategy*	*Recovery (%)*
Clarification		
	Microfiltration	30
	Centrifugation and microfiltration	**90 ± 2[a]**
Purification		
	Membrane adsorber	42 ± 5[a]
	Monolithic column	**82 ± 2[a]**
Polishing		
	Size exclusion chromatography	87 ± 6[a]
	Core bead prototype	**86 ± 9[a]**

Abbreviation: ΔE1, E1-deleted. [a]Standard deviation of triplicate assays. The strategies in bold represent the best options to purify CAV-2 vectors.

Fernandes, P et al_Bioprocess development for canine adenovirus type 2 vectors, Gene Therapy (2012), 1–8

Membrane versus CIM Monolith Lentiviral vector

INFECTIOUS TITERS, CONCENTRATION FACTORS, AND RECOVERIES OBTAINED AT THE END OF EACH DOWNSTREAM PROCESS STEP, BEFORE AND AFTER OPTIMIZATION

	Before optimization			*After optimization*		
	Infectious titer (x 10^7 IP/ml)	*CF*	*Recovery (%)*	*Infectious titer (x 10^7 IP/ml)*	*CF*	*Recovery (%)*
Clarification						
Centrifugation	0.24 ± 0.01	–	71 ± 6			
Depth-filtration	0.25 ± 0.01	–	74 ± 5	0.30 ± 0.02	–	**91 ± 6**[a]
Purification (AEXc)						
Sartobind D MA75	2.3 ± 0.1	12.5	28 ± 4			
CIM DEAE	6.1 ± 0.2	27.1	55 ± 2	8.0 ± 0.4	21.7	**80 ± 5**[b]
Concentration (UF/DF)						
Vivaspin 100 KDa	4.50 ± 0.04	3.4	67 ± 6			
300 KDa	4.5 ± 0.2	1.1	68 ± 9			
Vivaflow 100 KDa	4.8 ± 0.1	1.6	72 ± 1			**72 ± 1**[c]
Polishing (SEC)	0.11 ± 0.02	–	27 ± 2	0.82 ± 0.05	–	**68 ± 7**[d]
Overall Recovery (%)		8				**36**[e]

Results after optimization are shown for the methods presenting higher yields and chosen to be part of the downstream protocol developed herein due to their advantages.

[a–d]Recovery efficiency of total infectious particles, obtained after optimization of several conditions in each downstream processing (DSP) step: [a]increase of the flow rate from 50 to 100 ml min^{-1}; [b]immediate five-fold dilution of viral preparations after elution; [c]no optimization was performed in this step due to the high recoveries obtained; [d]increase of the concentration of the loading material by six-fold; [e]overall recovery obtained after using the techniques that gave the best recoveries in each purification step. The errors correspond to standard deviation (n = 3).

CF, concentration factor (in volume).

V. Bandeira et al., Downstream Processing of Lentiviral Vectors: Releasing Bottlenecks, Human Gene Therapy Methods 23:1-9 (August 2012)

CIM Monolith versus particles and membranes: Adenovirus 5 vector

Resin	Particle number by OPU assay (P/mL)	Volume (mL)	Total Particles	Capacity (P/mL of resin)
Capto Q	3.55E+11	1.7	2.04E+11	2.09E+11
CIM disk	3.75E+11	1.2	2.97E+11	9.19E+11
Fractogel	3.05E+11	1.2	2.72E+11	7.70E+11
Q Sepharose XL	2.21E+11	1.3	1.70E+11	1.70E+11

Reece-Ford et al, Evaluation of different anion-exchange resins for purification of recombinant human Ad5 vectors, poster Cobra

Separation of empty and full AAV capsid – enabling feature

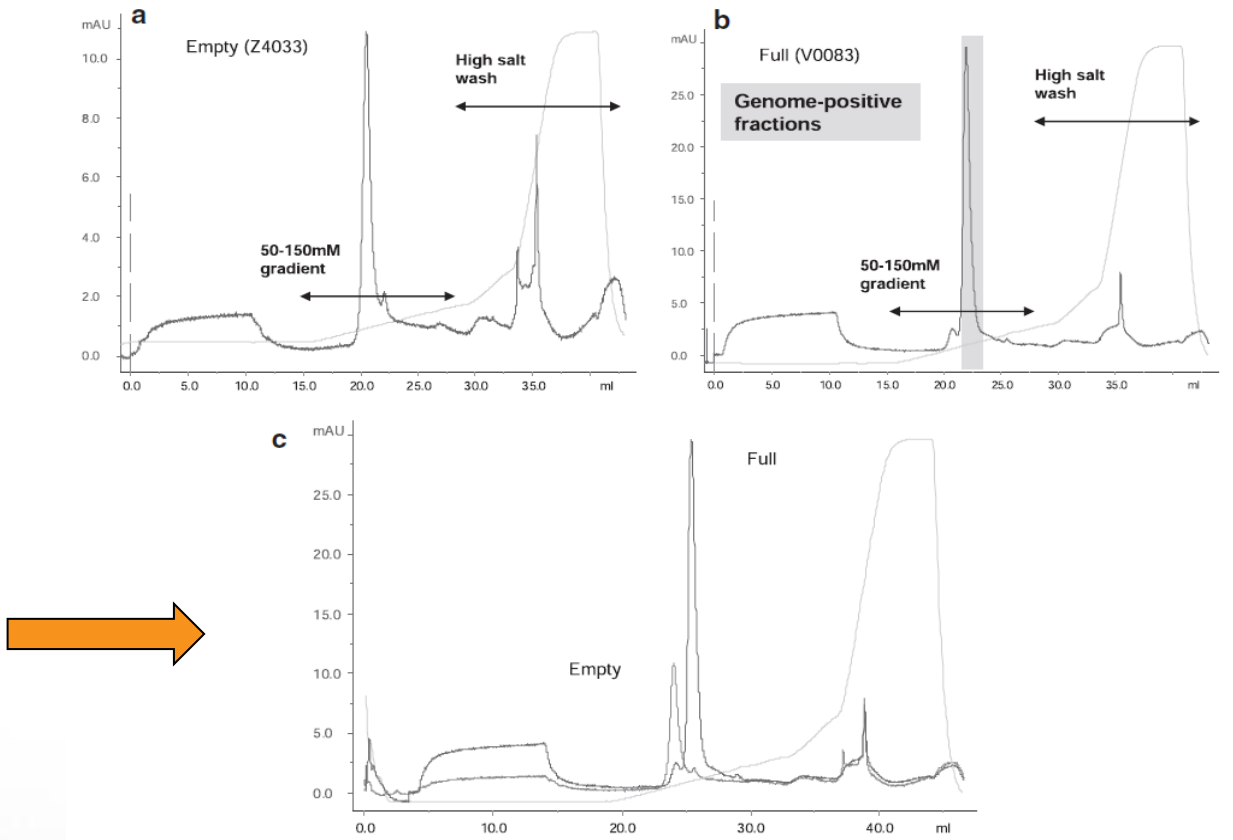


FIG. 3. IEX particle assay. **(a)** Seventy-five microliters of an empty particle AAV8 preparation (lot no. Z4033) was loaded onto a 0.34-ml CIM-QA disk, using FPLC, and eluted with a 50–150 m*M* salt gradient. The y axis shows the absorbance (mAU) at 280 nm and the *x* axis the elution volume (ml). The detected conductivity and absorbance are represented by solid light and dark blue lines, respectively. The vertical dashed pink line represents the point of vector injection. **(b)** A full AAV8 vector preparation (lot no. V0083, 1×10^{12} GC) was run under the same binding/elution conditions as used for the empty particle preparation. Fractions were quantified for vector GC content and those fractions containing >99% of the loaded material are indicated (shaded box). **(c)** An overlay of the elution profiles of the empty and full AAV8 vector preparations is shown.

HUMAN GENE THERAPY METHODS: Part B 23:56–64 (February 2012)

Lock et al., HUMAN GENE THERAPY METHODS: Part B 23:56–64 (February 2012)

Capacities

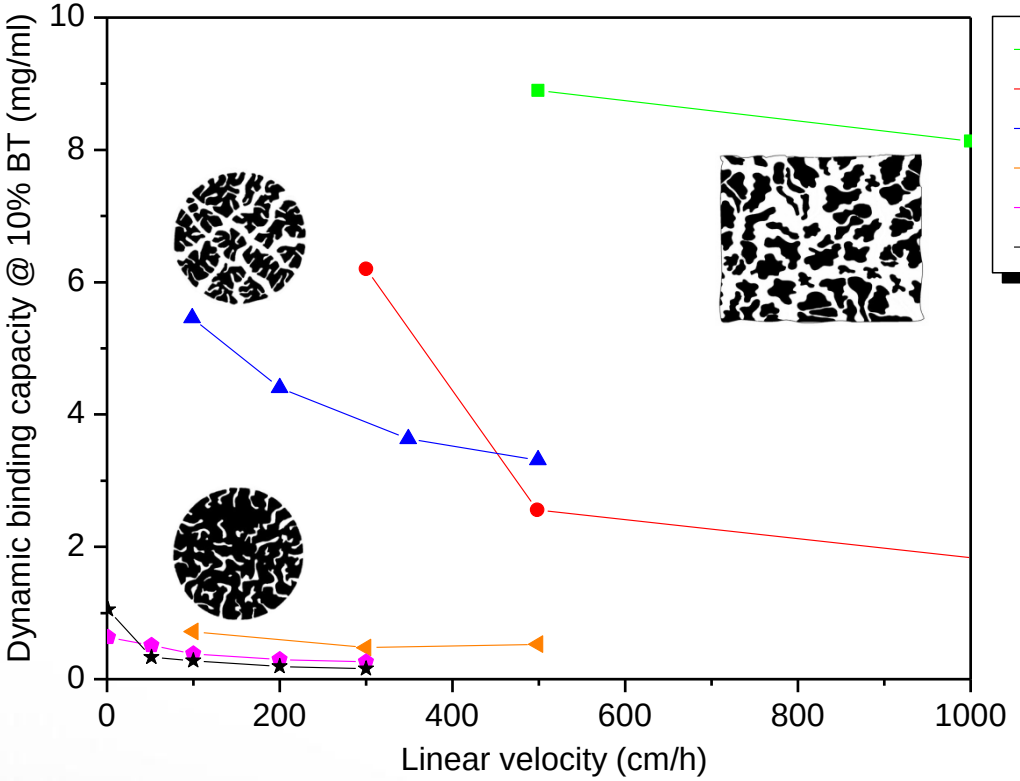
Molecules	Dynamic binding capacity
influenza	2 E+12 vp/mL
T7 phage	1 E+13 pfu/mL
M13 phage	4.5 E+13 pfu/mL
lambda phage	1 E+13 pfu/mL
PRD1 phage	6 E+13 pfu/ml
adenoviruses	2 E+12 vp/mL
baculovirus	2.4 E+11 pfu/ml
pDNA	8 mg/mL
genomic DNA	15 mg/mL
IgM	25 – 50 mg/mL
endotoxins	> 115 mg/mL

Theoretical amount of virus purified per single run

Column	virus purified (vp/mL)
CIMmultus QA 1 mL	1 E+12
CIMmultus QA 8 mL	8 E+12
CIMmultus QA 80 mL	8 E+13
CIMmultus QA 800 mL	8 E+14
CIMmultus QA 8000 mL	8 E+15

BIA separations

Plasmid DNA Binding Capacity Using AEC



CIM DEAE binding capacity = ~8 mg/ml

Used for CP III trials

Boehringer Ingelheim: „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

Plasmid DNA purification process

E. coli culture with plasmid

↓

Cell harvest

↓

Alkaline lysis with adjustment to 0.5 M $CaCl_2$

↓

Clarification

↓

Adjustment to binding conditions

↓

CIM DEAE monolith

↓

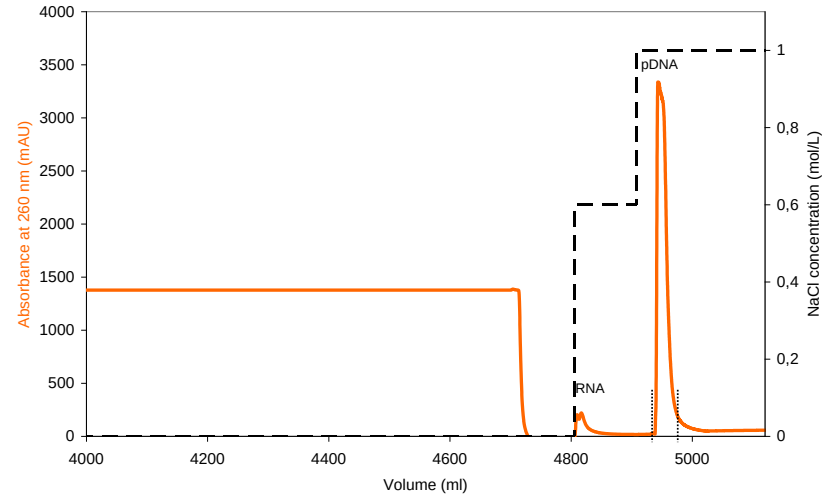
Adjustment with $(NH_4)_2SO_4$

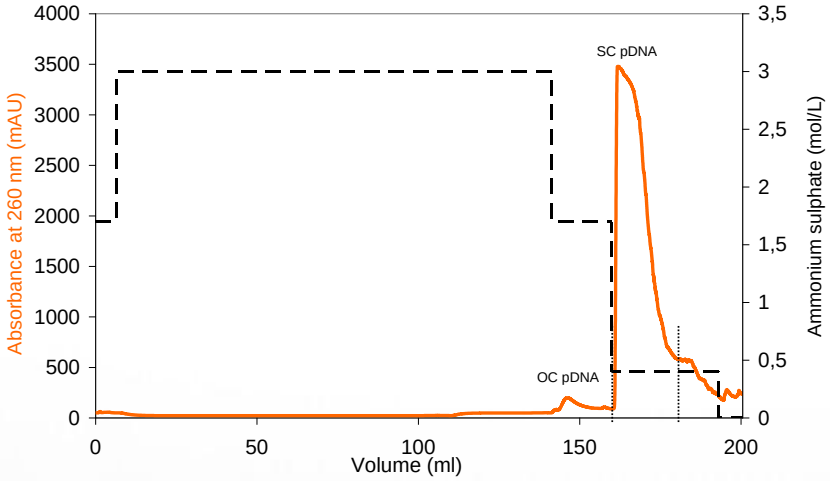
↓

CIM C4 monolith

↓

Buffer exchange





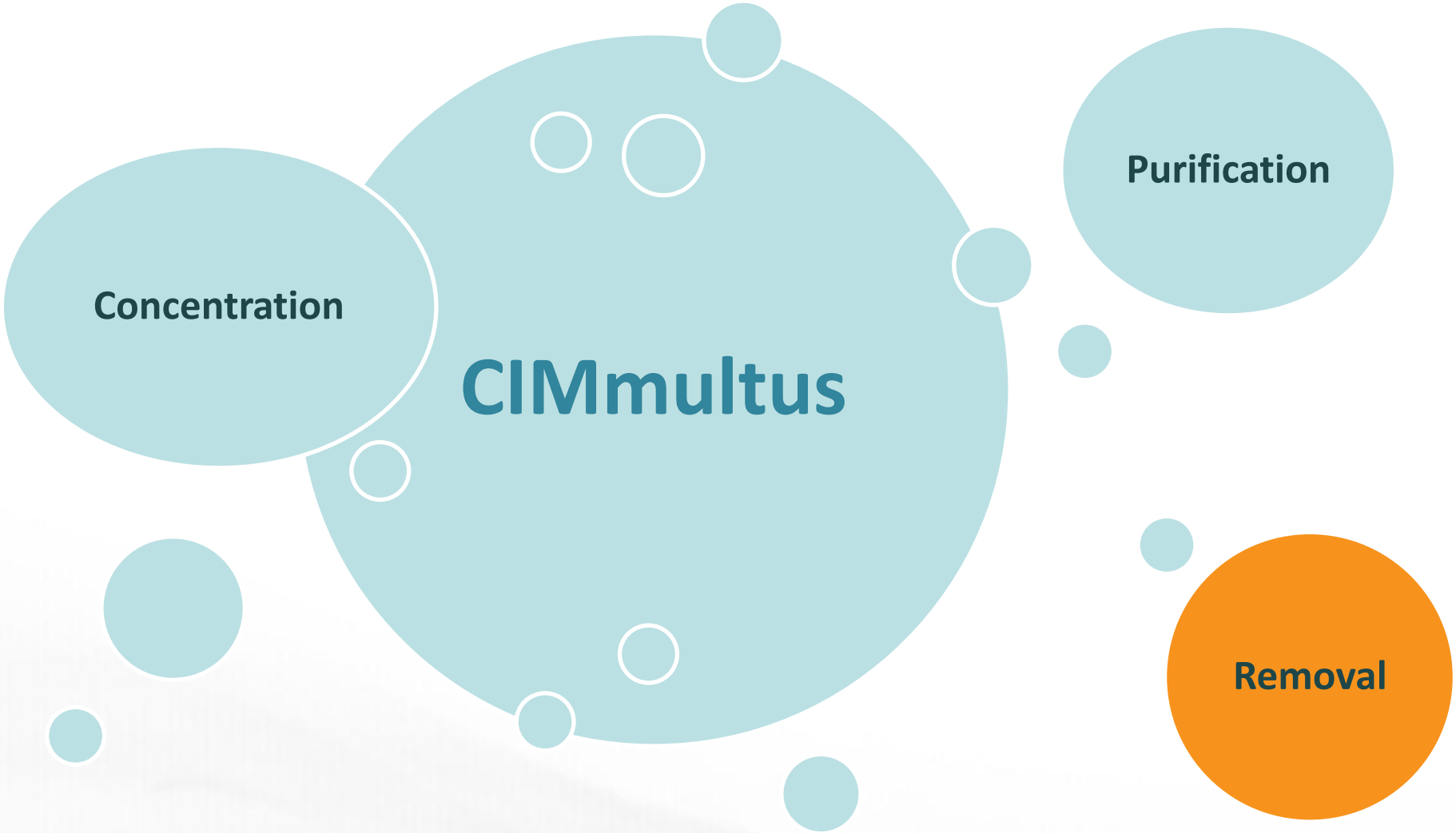
High Quality pDNA

	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)	200	12.4	1.1
Host cell proteins (µg/ml)	190	30	11
gDNA (µg/mg pDNA)	20	10.3	3.4
RNA (µg/ml)	N.D.	0	0
Yield (%)	100%	95%	90%

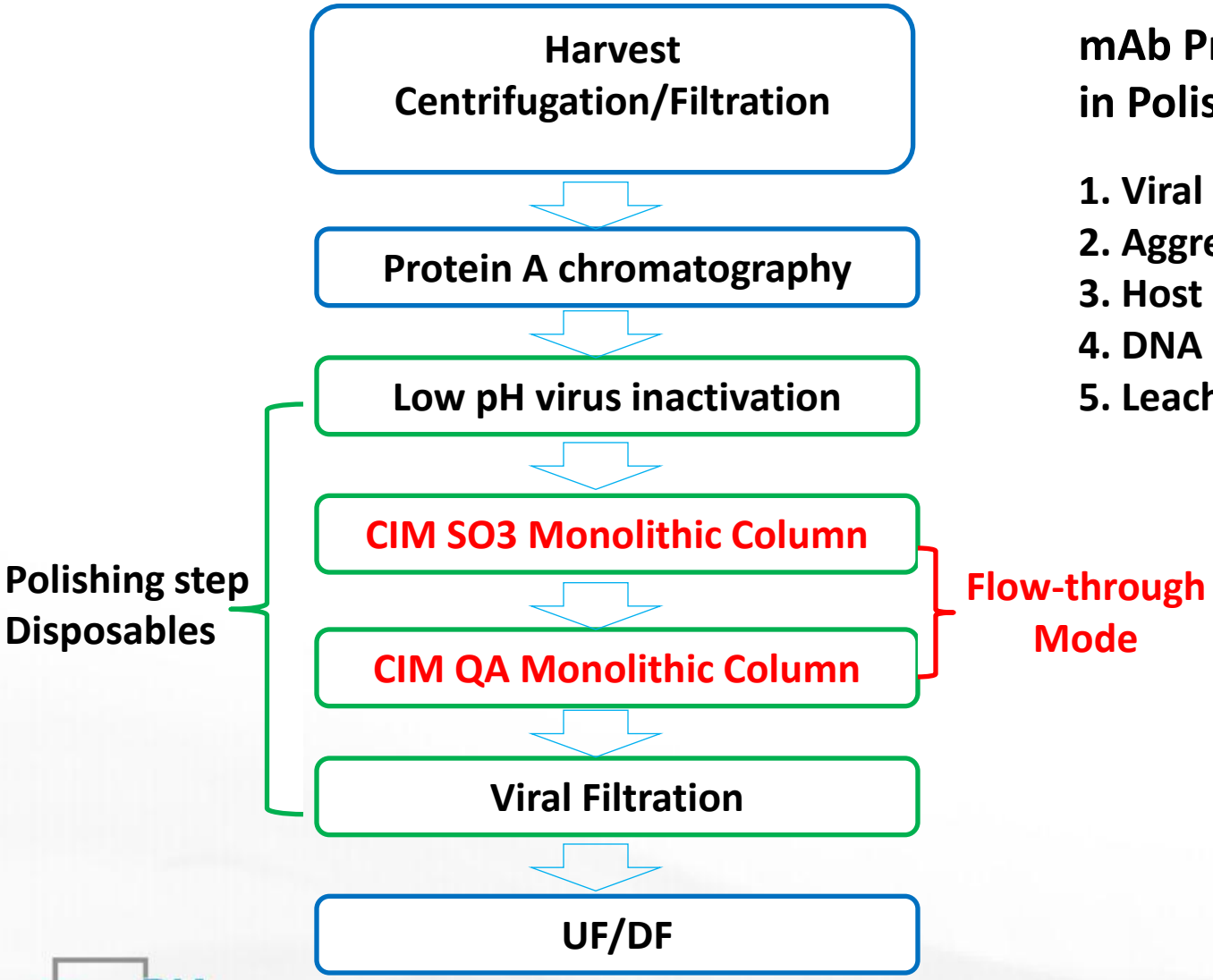
Process Yield	**>80%**
A260/280	**1.93**

Theoretical amount of pDNA purified per single run

Sizes	pDNA (g/run)
1 mL	0.006
8 mL	0.048
80 mL	0.480
800 mL	4.8
8000 mL	48


Concentration
CIMmultus
Purification
Removal

Proposal process of IgG mAb



mAb Production Challenges in Polishing Steps

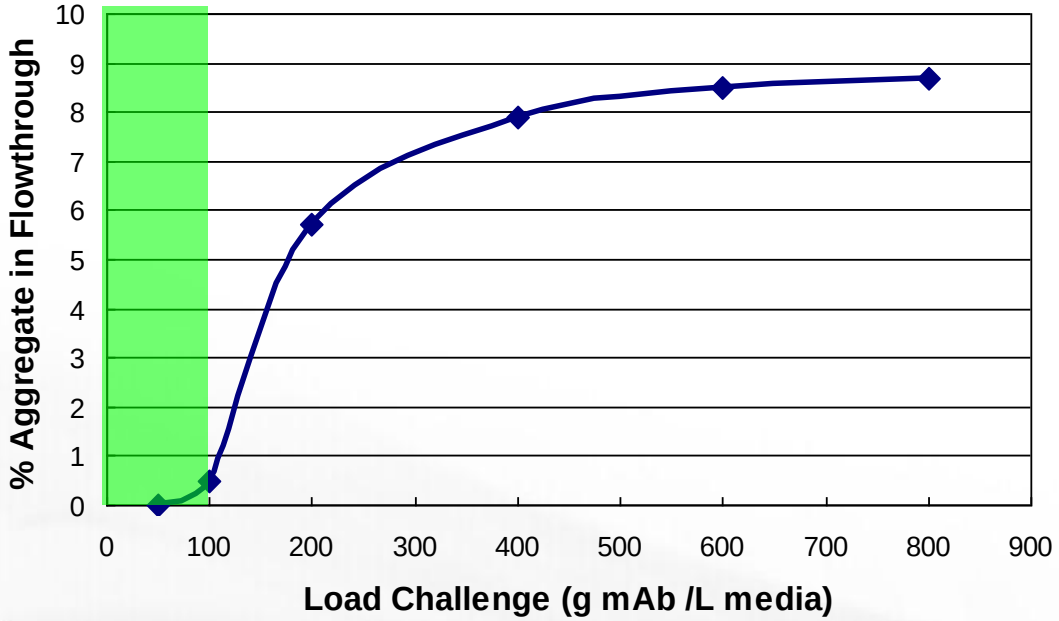
1. Viral Clearance
2. Aggregate Removal
3. Host Cell Protein Removal
4. DNA Removal
5. Leached Protein A Clearance

Immunoglobulin G - monoclonal

- Platform purification process consisting of an affinity step (Protein A) and one to two ion-exchange steps.
- The cation-exchange (SO3) step is used for the removal of aggregates.
- Usually, an anion-exchange (QA) step is used for final polishing – DNA and virus removal.

Aggregates removal on a CIM SO3 column – negative mode

Recent results demonstrate a high efficiency of CIM SO3 monolithic columns for the removal of aggregates in the negative chromatography mode. The same holds for the HCP - recent data.



- High binding capacity for aggregates
- High loading of monoclonal antibodies

HCP and DNA removal on a CIM QA column – negative mode

- HCP and DNA can be efficiently removed by the QA polishing step at various levels of antibody loading – in the negative chromatography step.

Flow rate (CV/min)	Load challenge (g mAb/L monoliths)	HCP (ppm)	DNA (ppb)
3	100	TLTD*	<0.8
15	100	TLTD*	1
3	1000	10.4	0.7
15	1000	10.8	<0.5

***TLTD, too low to be determined**

A Comparison of Microparticulate, Membrane, and Monolithic Anion Exchangers for Polishing Applications in the Purification of Monoclonal Antibodies.

Pete Gagnon, Richard Richieri[1], Simin Zaidi[1], Francis Aolin[1]

Validated Biosystems, 240 Avenida Vista Montana, Suite 7F San Clemente, California 92672; Avid Bioservices Inc[1] 14282 Franklin Avenue, Tustin, California 92780

Introduction

Membrane based anion exchangers are being used increasingly for purification of monoclonal antibodies. The transition from particle-based anion exchangers is driven partly by the convenience of membranes and partly by the cost saving associated with their disposability, however the factors that make them financially superior is more effective mass transport.

Mass transport is a major contributor to anion exchange binding efficiency, especially for large contaminants such as DNA, endotoxins, and viral particles. Fluid flows preferentially through the spaces between particles—the void volume — in traditional packed beds, while binding depends on diffusion of solutes into and out of dead-end pores as the mobile phase passes down the column. The larger the contaminants, the slower their diffusion constants and the slower the flow rate must be to allow them to come in contact with binding sites inside the pores. DNA in particular has a very low diffusion constant, making it a good model for anion exchange efficiency (Table 1, Figure 1). Pore accessibility is another limitation with particle based media. So-called wide-pore media generally have average pore diameter of about 1000 Å, roughly the same as a 100 nm viral particle. Anything larger has access to only the particle surface, which represents a small fraction of the total ion exchange surface.

Convective mass transport operates independently of diffusion and is consequently independent of solute size. It is also independent of flow rate. This allows anion exchange membranes to achieve good capacity at high flow rates, however their mass transport efficiency is offset by the fact that each membrane represents only a single chromatographic plate. Space must be left between layers because the pore distribution between layers is discontinuous. Chromatographic efficiency declines further from turbulent mixing between membrane layers and elsewhere within the housing.

Monoliths are characterized by a network of highly interconnected channels, with diameter ranging from 1-4 μm. This architecture permits convective mass transport, endowing monoliths with the ability to capture large solutes with high efficiency at high flow rates. In addition, monoliths exhibit plate efficiencies rivaling the best microparticulate packings, and they lack the void volume that plagues both membranes and microparticles. [1,2] This last feature is important because turbulent mixing in the void volume (eddy dispersion) is a primary cause of band spreading in chromatographic separations. This combination of attributes suggests that monoliths should offer higher efficiency than either membranes or porous particles. This study challenges that hypothesis with two large, clinically significant contaminants: endotoxin and DNA.

Materials and methods

All experiments were conducted on an AKTA™ Explorer 100 (GE Healthcare). DNA, endotoxin, bovine serum albumin (BSA), buffers, and salts were obtained from Sigma. Q Sepharose™ Fast Flow in 1 mL HiTrap™ columns was obtained from GE Healthcare. Sartobind™ Q nano (1 mL) membranes were obtained from Sartorius. CIM® QA monoliths, 0.34 mL (axial) and 1.3 mL (radial flow), were obtained from BIA Separations.

DNA and endotoxin binding capacities were determined by conducting dynamic breakthrough studies with 0.1mg/mL DNA or endotoxin in 0.02 M Hepes pH 7.0. Solutions were membrane filtered to 0.2μm before chromatography. Q Fast Flow HiTraps were run at 1 mL/minute. Sartobind Q and CIM QA (axial flow) anion exchangers were run at 4 mL/min. Three CIM disks were combined in a single housing to give a 1 mL volume. Fresh media (all types) was used for each experiment.

To confirm the ability of monoliths to remove DNA from IgG solutions, 320 mL of 0.1 mg/mL DNA mixed with 1.0 mg/mL protein A-purified monoclonal IgG, chimeric was applied to a 1.3 mL radial flow QA monolith at 6.0 mL/min. Samples were taken at 10 mL intervals. DNA levels were measured by pico-green testing, conducted by Southern Research Institute, Birmingham, AL DNA (www.southernresearch.org).

Results and Discussion

Breakthrough curves for DNA are shown in Figure 2. Dynamic binding capacities for endotoxin and DNA are given in Table 2. DNA capacities are plotted in Figure 3. Consistent with the combinations of low diffusion constants and narrow pore diameters, capacities for both DNA and endotoxin were lowest on the particle based anion exchanger. At 10% breakthrough, endotoxin capacity per mL of media was more than 4 times higher on the membrane and more than 13 times higher on the monolith, even though both the latter were operated at a 6-fold higher flow rate. A similar but more dramatic pattern was observed with DNA capacity, which was nearly 30 times higher on the membrane and almost 80 times higher on the monolith.

Among convective anion exchangers, monoliths offer not only higher capacity than membranes but also higher binding efficiency. This is illustrated in Figure 4, where the profiles are scaled to 10% breakthrough. The earlier breakthrough and shallower slope of the membrane curve are consistent with lower binding efficiency. The percent differential from the point where breakthrough was virtually detectable to the 10% breakthrough value was calculated. The "no-breakthrough" portion of the monolith curve was 95% of the 10% breakthrough value, compared to only 60% for the membrane. This corresponds to 14.3 mg/mL of no-breakthrough capacity for the monolith versus 4.8 mg/mL for the membrane. The presence of IgG did not impair DNA removal by the monolith. DNA levels in all fractions were beneath the detection level of the assay, about 1 ng/mL, indicating at least 5 logs of DNA removal across the entire sample application.

Table 1. Retarded Diffusion Constants

Solute	Size	K_{diff}
BSA	66 kDa	8.7×10^{-7}
IgG	150 kDa	4.0×10^{-7}
Unase	480 kDa	3.0×10^{-7}
IgM	900 kDa	2.6×10^{-7}
Endotoxin	1 MDa	2.1×10^{-7}
CMV	5 MDa	1.3×10^{-7}
TMV	40 MDa	5.0×10^{-8}
DNA_1	4.4 kbp	1.9×10^{-6}
DNA_2	33.0 kbp	6.0×10^{-8}

CMV Cucumber mosaic virus
TMV Tobacco mosaic virus

Table 2. Dynamic Capacities

Exchanger	Endotoxin	DNA
QFF		
1% bt, mg/mL	8.0	0.3
5% bt	12.1	0.4
10% bt	14.0	0.5
Q nano		
1% bt, mg/mL	30.0	9.0
5% bt	40.4	8.0
10% bt	42.9	7.6
CIM QA		
1% bt, mg/mL	114.7	14.6
5% bt	137.1	19.1
10% bt	147.2	19.4

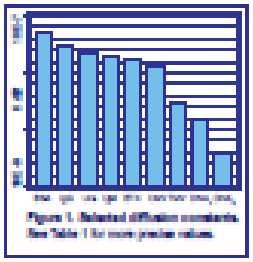


Figure 1. Retarded diffusion constants. See Table 1 for more precise values.

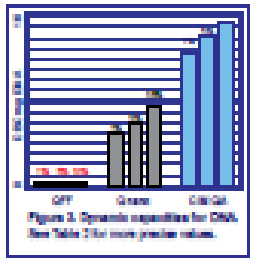


Figure 3. Dynamic capacities for DNA. See Table 2 for more precise values.

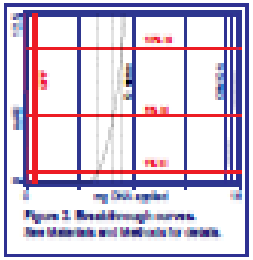


Figure 2. Breakthrough curves. See Materials and Methods for details.

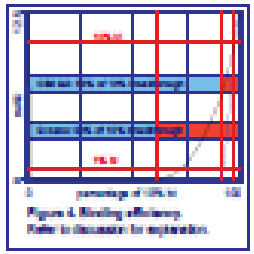


Figure 4. Binding efficiency. Refer to discussion for explanation.

Among convective anion exchangers, monoliths offer not only higher capacity than membranes but also higher binding efficiency. This is illustrated in Figure 4, where the profiles are scaled to 10% breakthrough. The earlier breakthrough and shallower slope of the membrane curve are consistent with lower binding efficiency. The percent differential from the point where breakthrough was virtually detectable to the 10% breakthrough value was calculated. The "no-breakthrough" portion of the monolith curve was 95% of the 10% breakthrough value, compared to only 60% for the membrane. This corresponds to 14.3 mg/mL of no-breakthrough capacity for the monolith versus 4.8 mg/mL for the membrane. The presence of IgG did not impair DNA removal by the monolith. DNA levels in all fractions were beneath the detection level of the assay, about 1 ng/mL, indicating at least 5 logs of DNA removal across the entire sample application.

Conclusions

This study has important implications for manufacture of therapeutic antibodies. Although diffusive particle anion exchangers have proven adequate for reducing DNA and viral contamination to clinically acceptable levels, it is clear that they have done so in spite of their fundamental inappropriateness for the task. The higher capacity and efficiency of convective anion exchangers promise not only better process economics but, more importantly, lower patient risk in the clinic. According to the results of this study, a monolith with a bed volume 10% the size of a conventional anion exchanger could remove 5 times as much DNA in about the same amount of time. A monolith 10% the size of a conventional exchanger could remove 10 times as much DNA in half the time. Given their large size and slow diffusion constants, viral particles should be expected to behave similarly to DNA. Additional studies are required to confirm this, and to characterize the behavior of aggregates, leached protein A, and host cell proteins. This will be of special interest with the weak-partitioning conditions employed in 2-step (protein A /anion exchange) IgG purification procedures, where the low dispersion characteristics of monoliths should enhance contaminant discrimination.

Literature cited

1. Stanton et al, 2001, *Adv. Biochem. Eng. Biotechnol.*, 76:99
2. Hahn et al, 2001, *Dyn. Sol. Biotech.*, 37(7):1264

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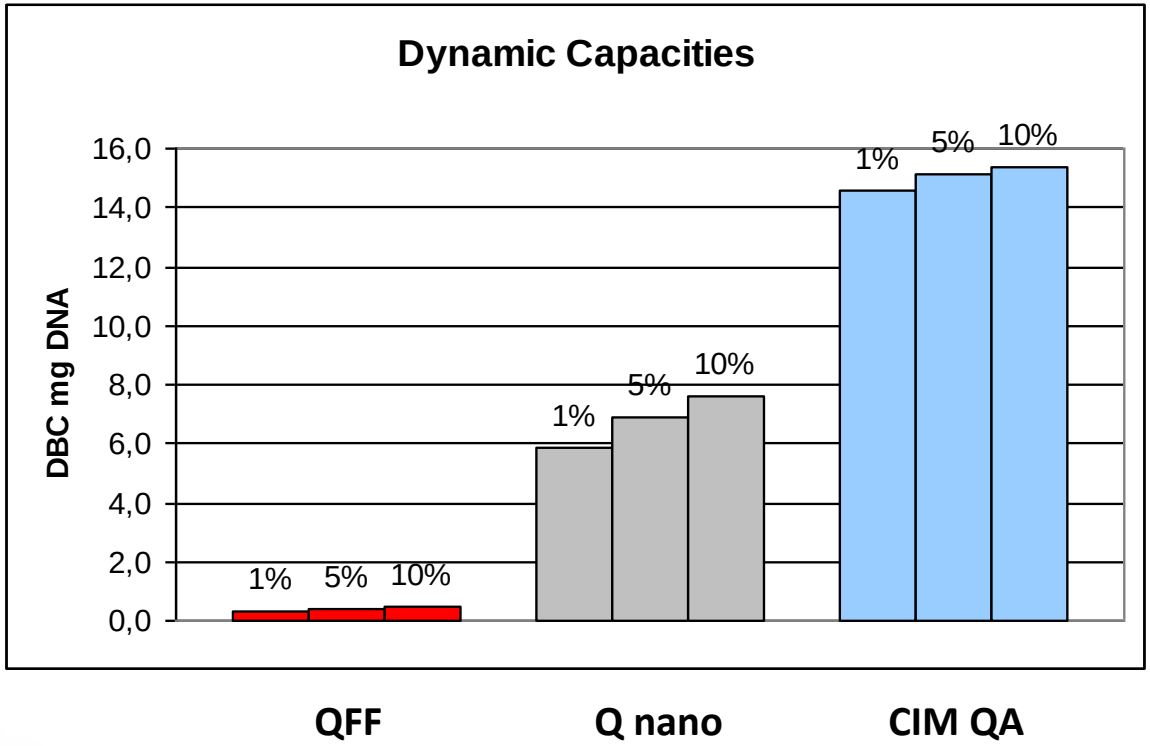
Dynamic Binding Capacity for DNA



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

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CIM monoliths application areas