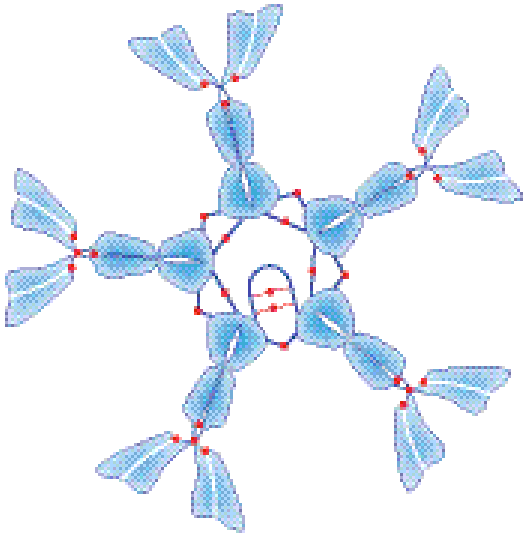


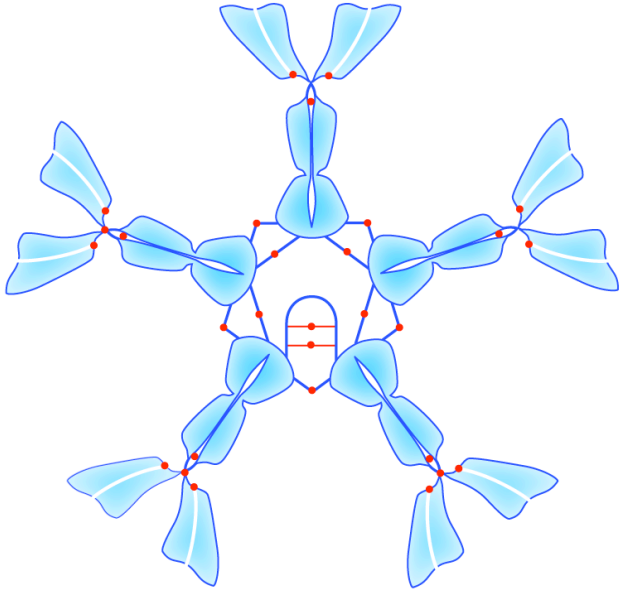
IgM Purification
The Next Generation

Pete Gagnon, Validated Biosystems
Richard Richieri, Avid BioServices, Inc.
Frank Hensel, Patrys, GmbH

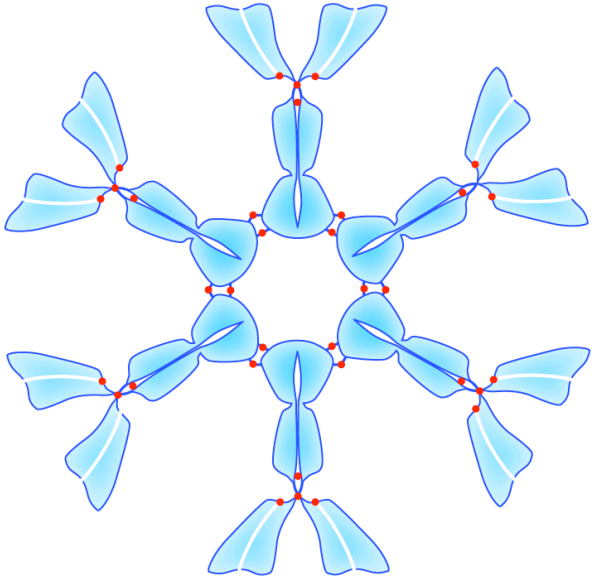
13th Annual Waterside Conference, Miami, February 4-6, 2008

Structure and properties of IgM

Pentameric, 0.96 Md



Hexameric, 1.15 Md



7.5 -12 % carbohydrate, extinction coefficient 1.18

*Randall et al, 1990, The biological effects of IgM hexamer formation, Eur. J. Immunol., **20** 1971-1979*
Johnstone and Thorpe, 1987, Immunochemistry in Practice, 2nd Ed., Blackwell Scientific Publishers, Oxford

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.*

Are IgMs really difficult to purify?

The size of IgMs is also a challenge.

- *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.*

On the positive side…

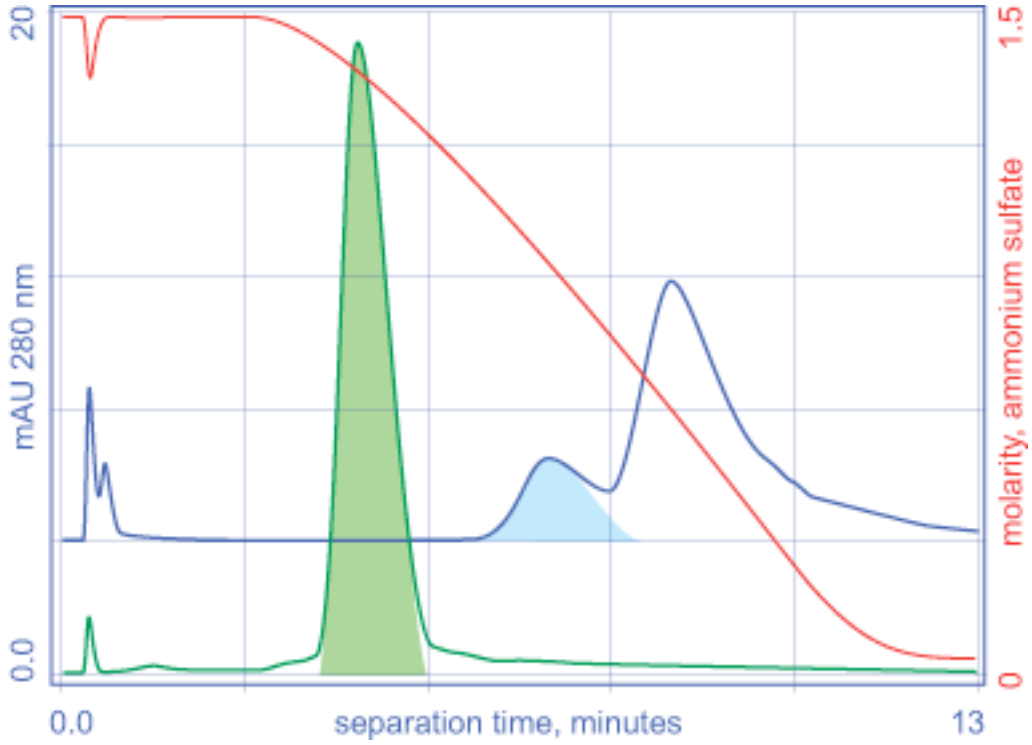
IgMs are typically more charged than IgGs. They bind more strongly than IgG to anion exchangers, cation exchangers, or both.

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.

On the positive side…

HIC on weak and strong hydrophobic media



Blue trace
ReSOURCE PHE, 1 mL

Green trace
ReSOURCE ETH, 1 mL

Buffer A: 10 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.0

Buffer B: 10 mM sodium phosphate, pH 7.0

Highlighted areas indicate native immunoreactive IgM

On the positive side…

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

Membranes and monoliths rely on convection for mass transport.

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.

Diffusion vs convection

Diffusion constants for selected antibody classes and fragments

Protein	Mass	K_{diff} cm^2/sec
IgM	960 kD	2.6×10^{-7}
IgA	335 kD	3.7×10^{-7}
IgG	150 kD	4.9×10^{-7}
Fab	50 kD	7.4×10^{-7}
Light chain	23 kD	9.1×10^{-7}

Diffusion vs convection

A protein's-eye view of porous particle media

- *Fluid flows preferentially between the particles in a packed column, not through the particles.*
- *Most of the binding surface area resides on the walls of the pores within the particles, which the proteins must enter and exit by diffusion.*
- *Pore diameter on most porous particle-based media is less than 1000 Angstroms.*
- *If the protein is large relative to the diameter of the pores, it has access to fewer pores, which reduces the surface area available for binding.*

N. Afeyan, N. Gordon, J. Mazaroff, C. Varaday, S. Fulton, Y. Yang, and F. Regnier, 1990, Flow through particles for the high performance liquid chromatography separation of biomolecules, *J. Chrom.,* **519** 1-29

Diffusion vs convection

A protein's-eye view of porous particle media

- *Good binding capacity requires that the flow rate be slow enough so that proteins can diffuse into the pores as they pass down the column.*
- *Good resolution requires that the flow rate be slow enough to allow proteins to diffuse out of the pores as a group while the buffer flows past.*
- *Fluid movement slows at the particle surface, due to friction. This causes mixing – called eddy dispersion – in the void space between the particles. This erodes much of the separation provided by the chemistry. It also dilutes the protein peak as it passes through the column.*

Diffusion vs convection

A protein's-eye view of monoliths

- *Channel diameters range from 1-5 microns; 10-50 times larger than the average pore diameter on most particles.*
- *Proteins are carried into the channels by the force of the fluid flowing through the channels: convection.*
- *Convection is like a turbulent river. It does not matter if a protein is large or small. It tumbles along with the current, contacting the channel walls at the same rate, no matter how fast or slow the river is flowing.*

R. Hahn, M. Panzer, J. Mollerup, A Jungbauer, 2002, Mass transfer properties of monoliths, *Sep. Sci. Technol.,* **37** (7)1545-1556

Diffusion vs convection

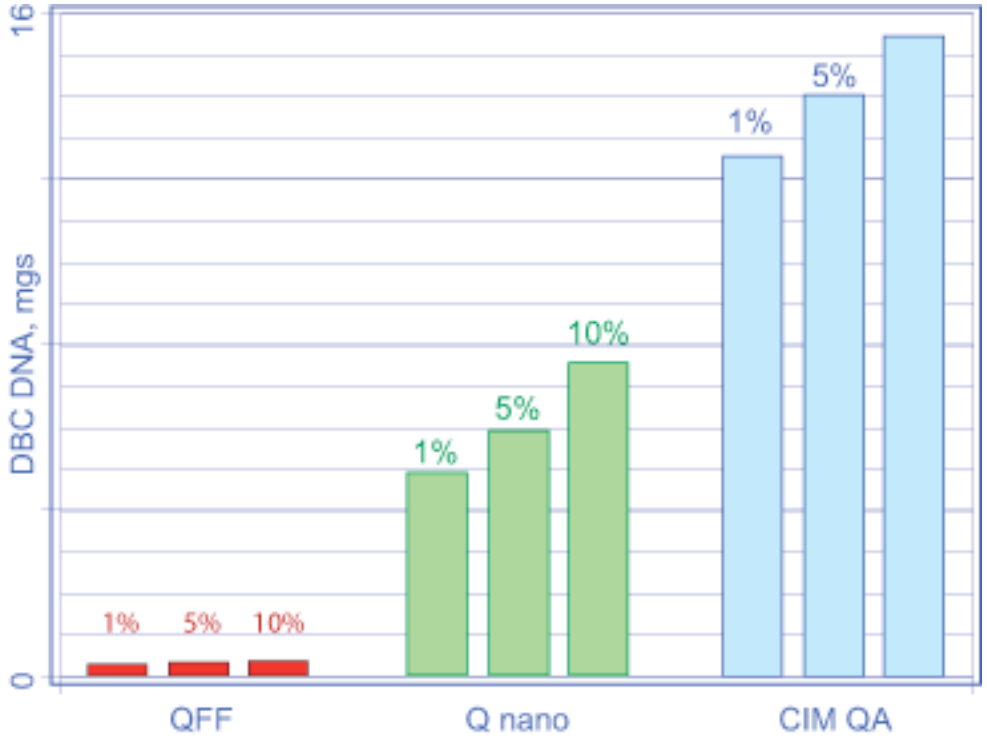
A protein's-eye view of monoliths

- *Since the rate at which proteins encounter the channel walls is directly proportional to flow rate, binding efficiency is limited only by the association kinetics between the ligand and the protein.*
- *Response to elution conditions is instantaneous. There is no time lag for proteins to diffuse out of pores.*
- *Monoliths do not have a void volume, so there is no eddy dispersion. Proteins elute in narrower zones at higher concentrations.*

A. Strancar, A. Podgornik, M. Barut, R. Necina, 2002, Short monolithic columns as stationary phases for biochromatography, *Adv. Biochem. Eng. Biotechnol.*, **76** 50-55

Diffusion vs convection

Comparison of DNA binding by particles, membranes, and monoliths



Red
Q Sepharose Fast Flow
Porous particle media

Green
Q nano
Stacked membrane

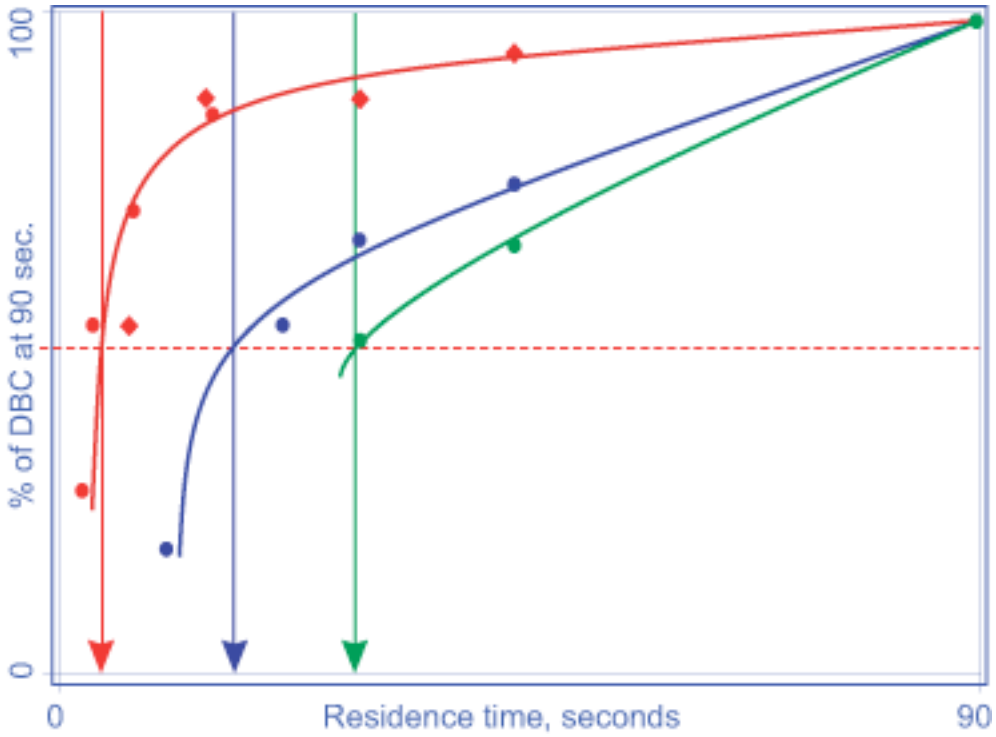
Blue
CIM QA
Monolith

All media: 1 mL
Flow rate: 1 mL/min

P. Gagnon, R. Richieri, S. Zaidi, F. Aolin, 2007, A comparison of microparticulate, membrane, and monolithic anion exchangers for polishing applications in the purification of IgG monoclonal antibodies, *IBC International Conference and Exposition, October 1-4, Boston*

Diffusion vs convection

Affinity binding efficiency as a function of mass transport



Red trace
CIM protein A HLD, 1 mL
Convective, pK_T ~4 sec.

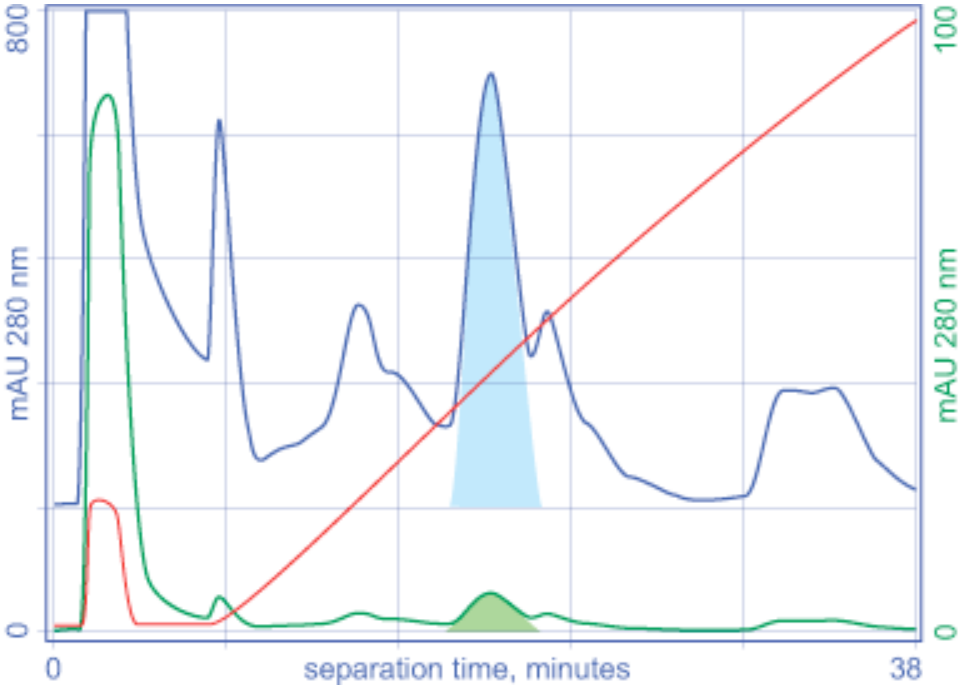
Blue trace
MabCapture A, 1 mL
Mixed convective/diffusive
pK_T ~17 seconds

Green trace
MabSelect Xtra, 1 mL
Diffusive, pK_T ~30 seconds

P. Gagnon, 2007, The protein A paradigm: can it be improved, can it be replaced? 18th International IBC Conference on Antibody Development and Production, La Costa Spa and Resort, Carlsbad, CA, 3/2/07

Method scouting

Initial screening, hydroxyapatite

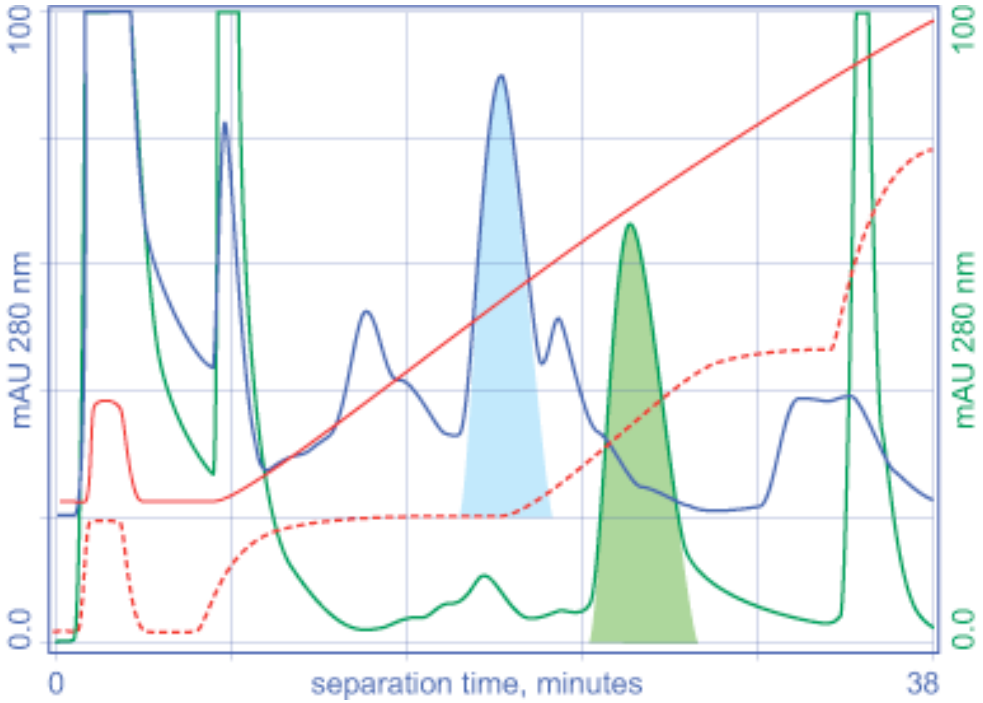


Media: 1 mL CHT™ type II 40 µm
Column: MediaScout® 5 x 50 mm
Flow rate: 0.67 mL/min
Buffer A: 10 mM NaPO4, pH 7.0
Buffer B: 500 mM NaPO4, pH 7.0
Equilibrate: buffer A
Sample: IgM CCS
Load: 2 mL
Wash: 5 CV buffer A
Elute: 20 CV LG to buffer B

Highlighted peak is IgM. This profile is typical of IgMs, most of which elute from hydroxyapatite in a well defined peak between 200 and 300 mM phosphate. Lower trace illustrates same profile at lower sensitivity.

Method scouting

Gradient optimization, hydroxyapatite

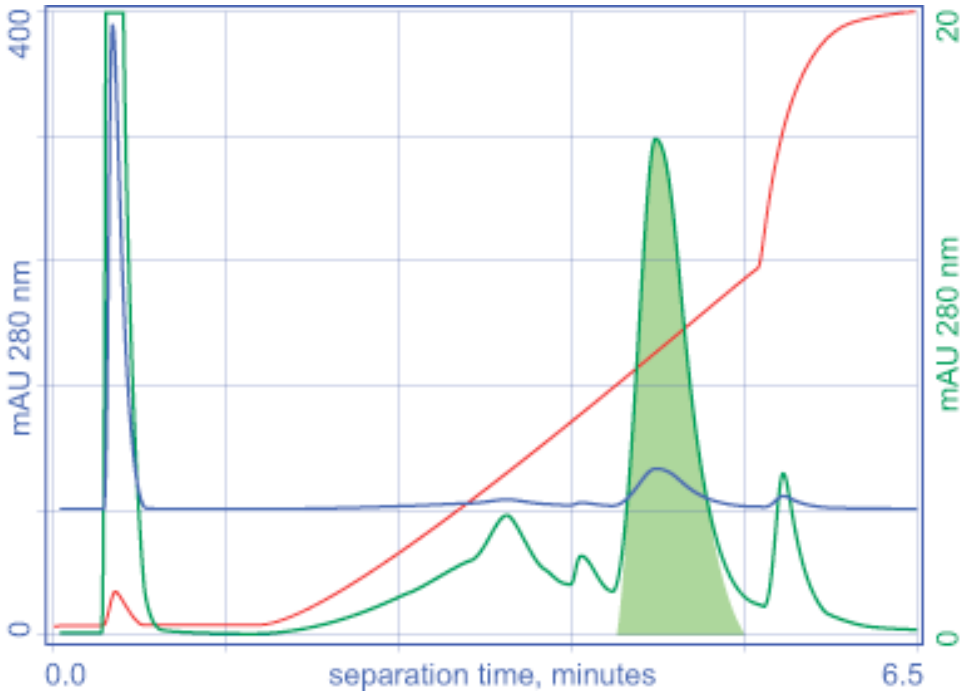


Media: 1 mL CHT™ type II 40 µm
Column: MediaScout® 5 x 50 mm
Flow rate: 0.67 mL/min
Buffer A: 10 mM NaPO4, pH 7.0
Buffer B: 500 mM NaPO4, pH 7.0
Equilibrate: buffer A
Sample: IgM CCS
Load: 2 mL
Wash: 5 CV buffer A
Elution gradients indicated in red

Highlighted peak is IgM. Blue trace reproduces screening results from previous slide. Lower trace illustrates improved purity from optimized gradient set points.

Method scouting

Initial screening, cation exchange, pH 7.0



Media: CIM SO_3, 0.34 mL

Flow rate: 4 mL/min

Buffer A: 10 mM NaPO4, pH 7.0

Buffer B: 500 mM NaPO4, pH 7.0

Equilibrate: buffer A

Load: 100 µL IgM CCS

Wash: buffer A

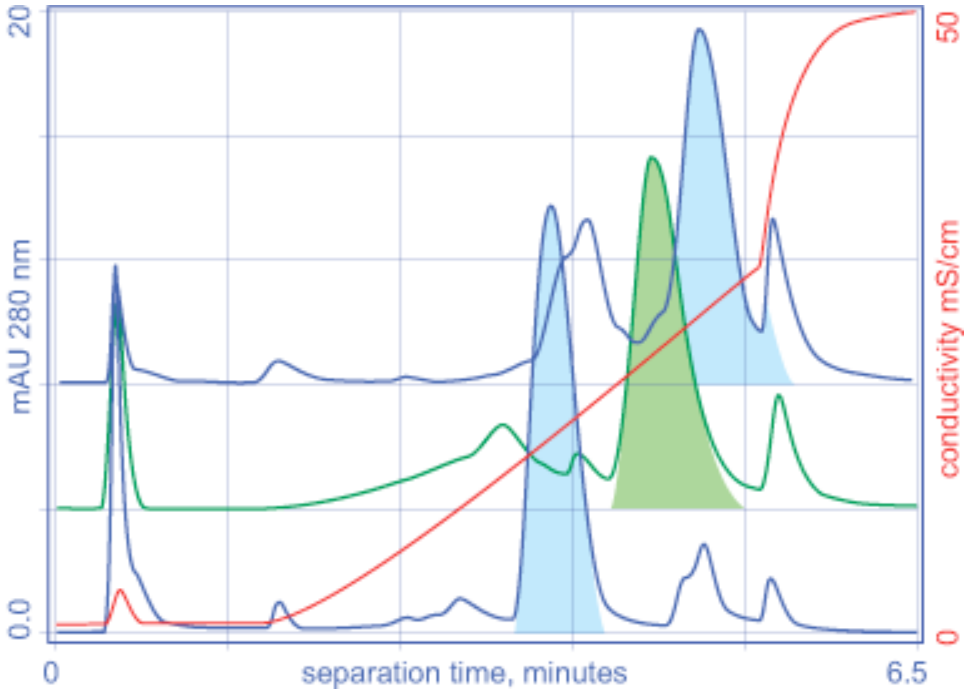
Elute: 34 CV LG to 50% buffer B

Clean with 100% buffer B

Highlighted peak is IgM. This IgM binds more strongly to cation exchange than most. Blue trace illustrates same profile at lower sensitivity.

Method scouting

Initial screening, cation exchange, pH 6.0, 7.0, 8.0



Media: CIM SO_3, 0.34 mL

Flow rate: 4 mL/min

Buffer system A: pH 6.0

Buffer system B: pH 7.0

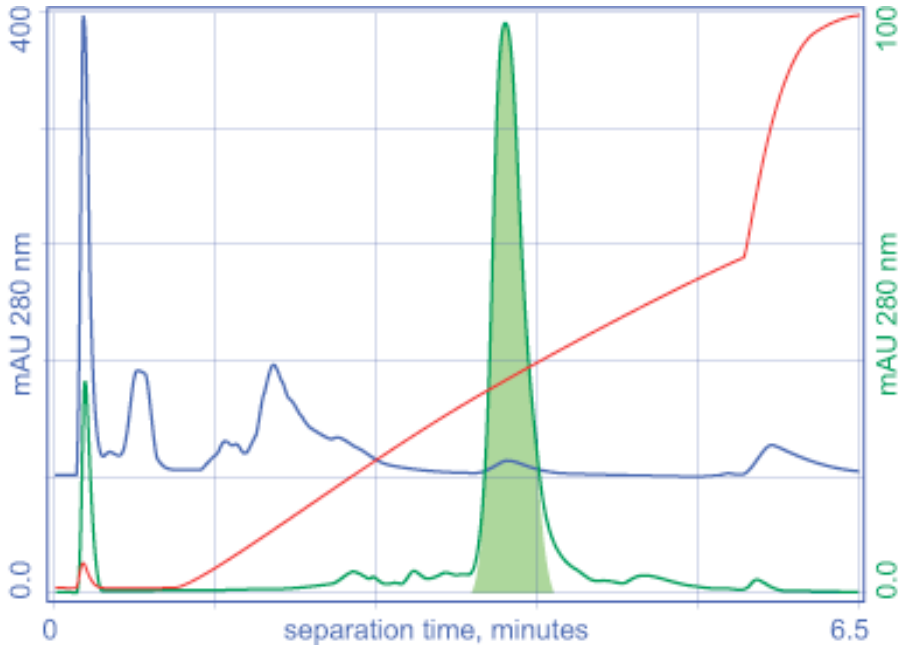
Buffer system C: pH 8.0

Same sample and run configuration as previous slide

Highlighted peak is IgM. Uppermost trace shows results at pH 6.0. Center trace, pH 7.0. Lowermost trace, pH 8. Note stronger binding at pH 6, better purification at 8.0. Further improvement is achievable with gradient optimization.

Method scouting

Initial screening, anion exchange, pH 7.0



Media: CIM QA, 0.34 mL

Flow rate: 4 mL/min

Buffer A: 10 mM NaPO4, pH 7.0

Buffer B: 500 mM NaPO4, pH 7.0

Equilibrate: buffer A

Load: 100 µL

Wash: buffer A

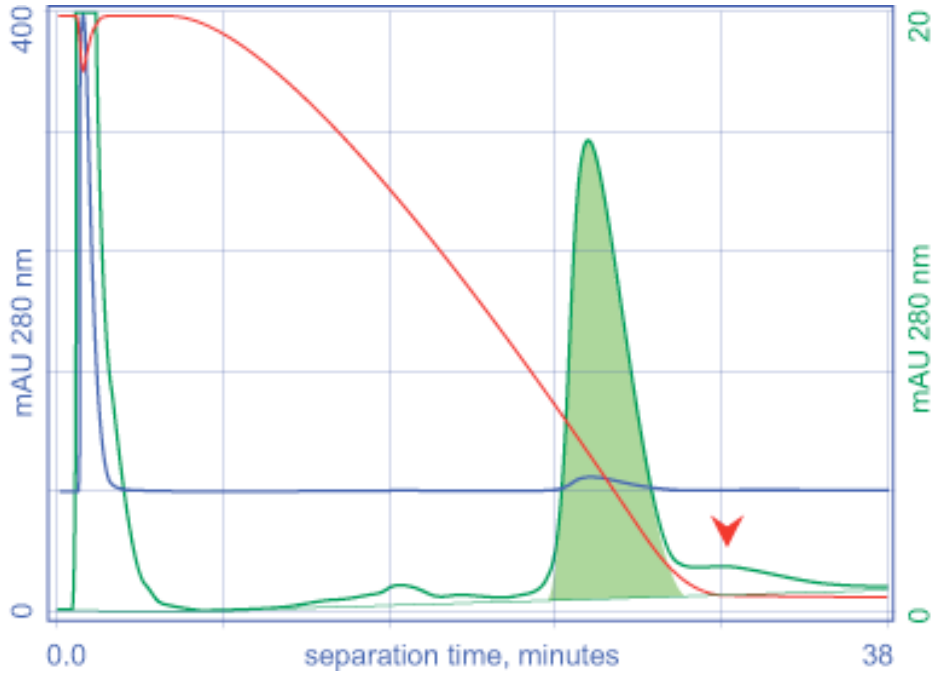
Elute: 34 CV LG to 50% buffer B

Clean with 100% buffer B

Highlighted peak is IgM. Blue trace, IgM CCS. Green trace, CHT-purified IgM. Strong anion exchange retention is typical of IgMs.

Method scouting

Initial screening, HIC, pH 7.0



Media: Expmntl wide pore PPG

Column: 1 mL, 5 x 50 mm

Flow rate: 0.67 mL/min

Buffer A: 10 mM NaPO4, 1.5 M ammonium sulfate, pH 7.0

Buffer B: 10 mM NaPO4, pH 7.0

Equilibrate: buffer A

Sample: 25 µL IgM CCS

Wash: 5 CV buffer A

Elute: 20 CV LG to buffer B

Highlighted peak is IgM. Red arrow indicates aggregates. Blue trace at lower sensitivity to illustrate relative amount of protein bound.

Sample loading and capacity

Hydroxyapatite

- *Directly compatible with CCS, with minor modification.*
- *Filter to 0.2 µm*
- *Titrate pH to 7.0*
- *Add phosphate to 5 mM*
- *Capacity up to 25 mg/mL*

Sample loading and capacity

Ion exchange

- *IgMs often precipitate at low conductivity, progressively over time. This usually disqualifies batch sample equilibration.*
- *Load sample by in-line dilution.*

  Titrate sample pH to target, load through inlet A

  Dilute with buffer, through inlet B

  Dilution factor depends on the charge characteristics of the antibody, the starting conductivity, and the desired capacity. Typical dilution factors range from 1:2 to 1:9
- *Capacity: 30-40 mg/mL at 12 CV/min on monoliths*

Sample loading and capacity

HIC

- *IgMs usually precipitate at the high salt concentrations required to support good capacity on moderately hydrophobic media. This disqualifies batch equilibration.*
- *Load sample by in-line dilution.*

  Titrate sample pH to target, load through inlet A

  Dilute with buffer, through inlet B

  Typical dilution factors range from 1:4 to 1:9
- *Capacity for IgG is frequently in the range of 20-25 mg/mL. Capacity for IgMs has not yet been characterized.*

Sample loading and capacity

Lower in-line dilution factors reduce sample loading time.

Higher in-line dilution factors increase capacity, which reduces column size and buffer volumes. This increases eluted product concentration, which reduces sample volume going into the next step.

In-line dilution suspends the need to employ diafiltration for intermediate sample preparation. This eliminates costly equipment, materials, preparation/operating/maintenance time, validation, and the inevitable losses that occur at each processing step.

Process sequencing

Hydroxyapatite is generally well suited for IgM capture.

- *Mild operating conditions.*
- *Minimal sample conditioning.*
- *Most contaminants flow through.*
- *Most IgMs elute at 200-300 mM phosphate*
- *May discolor from iron in CCS. Chelating agents may reduce column life.*

- *Hydroxyapatite also accommodates high-salt samples from other methods, as long as the phosphate concentration is low.*

Process sequencing

Cation exchangers may bind some IgMs strongly enough to be practical for capture.

- *Dilution will still be required to reduce conductivity, but the flow capacity of monoliths may make the compromise worthwhile.*
- *Cation exchange capture removes metal ions and chelators from CCS, which can be advantageous if hydroxyapatite is the next process step…*
- *However, citrate buffers cannot be used if hydroxyapatite follows cation exchange because citrate is a calcium chelating agent.*

Process sequencing

Quaternary amine anion exchangers are poorly suited for capture because they bind a large proportion of contaminants that compete with IgM for capacity.

- *It is also difficult to remove DNA because strong anion exchangers remain charged even in 1 M NaOH. DNAase is required for quantitative DNA removal.*
- *If you want to use anion exchange for capture, use a weak anion exchanger like DEAE. The same contaminants will compete for capacity but DEAE loses its charge in NaOH. Dilution will still be required but the high flow rate of monoliths will reduce the time factor.*

Process sequencing

HIC is generally not well suited for IgM capture.

- *It requires in-line dilution, usually at least 1:4.*
- *This increases sample volume but in this case, monoliths with appropriate selectivity are not available to reduce loading time.*
- *Dilution also multiples salt requirements. If 1.2 M ammonium sulfate is required to achieve good binding capacity, and this is achieved by 1:4 dilution with 1.5 M ammonium sulfate, each liter of CCS loaded will require 0.99 kg of ammonium sulfate.*

Process modeling

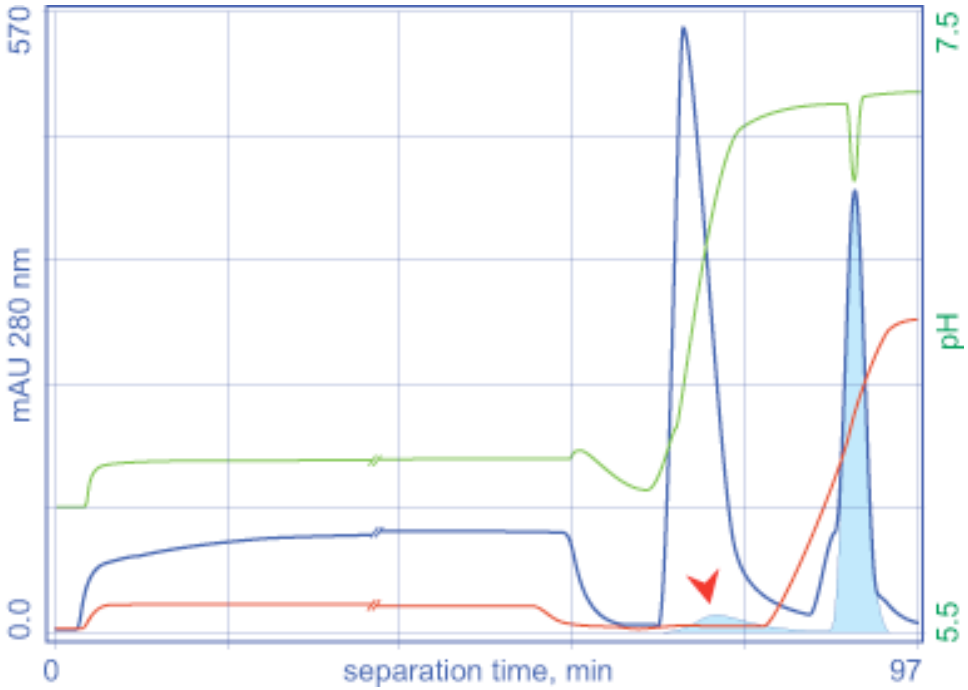
After establishing a preliminary process order, loading conditions, capacity, and separation conditions, it is useful to run the integrated process to provide a benchmark of overall process performance and economics.

A good working process model highlights its own deficiencies, provides an order of priority for addressing them, and allows each one to be evaluated in a meaningful context.

Defer optimization of individual steps until after the benchmark has been run.

Process modeling

Product loss in the pre-elution wash



Media: CIM SO_3, 8.0 mL

Flow rate: 20 mL/min

Buffer A: 20 mM MES, pH 6.0

Buffer B: 10 mM NaPO4, pH 7

Buffer C: 125 mM NaPO4, pH 7.0

Equilibrate: buffer A

Load: 2.5 L diluted CCS

Wash: buffer A, then B

Elute: linear gradient to buffer C

Blue: 280 mm. Green: pH. Red: conductivity. Light blue highlight indicates IgM. Red arrow indicates IgM loss.

Process modeling

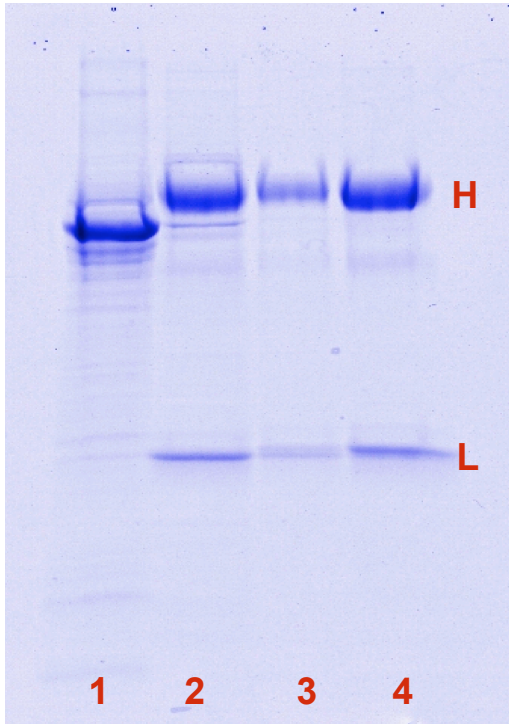
Process summary

	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.

Process modeling

Purification stages



Reduced SDS PAGE

Bio-Rad Criterion gel

10-20% gradient

1. IgM CCS
2. Cation exchange pool
3. Anion exchange pool
4. Hydroxyapatite pool

H: Heavy chain

L: Light chain

Conclusions

IgM purification is not more difficult than purification of IgG, but it is different.

Lower solubility and higher sensitivity to denaturation must be accommodated.

The unique charge characteristics of IgM monoclonals provide purification opportunities that are rarely or never encountered with IgGs.

This permits development of orthogonal processes without exposing the product to unnecessary stress.

Conclusions

Purification of clinical grade IgM can generally be achieved with 3 bind-elute chromatography steps.

Step recoveries are comparable to those achieved with IgG purification.

The lack of an affinity step is a positive contribution to process economy.

The lack of intermediate diafiltration, made possible by in-line dilution, also improves process economy.

Conclusions

Monolithic ion exchangers eliminate the productivity bottleneck.

This is valuable at any point in the process, but especially at capture where sample volume is usually highest.

Rapid flow rates also permit process development to be conducted more rapidly.

This encourages more thorough process development and characterization.

Acknowledgements

The authors sincerely thank Bio-Rad Laboratories for providing hydroxyapatite; ATOLL for packing hydroxyapatite into their MediaScout columns to facilitate this work; BIA Separations, for providing monolithic ion exchangers; GE Healthcare for providing ReSOURCE HIC media; and Tosoh Bioscience for providing experimental wide pore PPG HIC media. Thanks also to the Process Applications group at Bio-Rad for performing early characterization of IgM behavior on hydroxyapatite, for performing PAGE analysis, and for other lab support.

Copies of this presentation can be downloaded at www.validated.com