Multiple Modes of Aggregate Removal by Hydroxyapatite

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Aggregate removal by hydroxyapatite

Antibody purification with HA has been dominated historically by simple phosphate gradients for elution. These have been adequate for aggregate removal from IgA, some IgMs, and a minority of IgGs.

Compound gradients of phosphate and chloride salts have increased the effectiveness of aggregate removal from IgG monoclonal antibodies.

Aggregate removal by hydroxyapatite

Polyethylene glycol (PEG) has enabled effective aggregate removal from IgGs that are not adequately served by phosphate-chloride gradients, and increased effectiveness for those that are. It also supports effective aggregate removal from IgM monoclonals.

Most recently, sulfate gradients have been revealed to offer an alternative to elution with phosphate, and potentially offer an additional family of selectivities for aggregate removal.

Aggregate removal by hydroxyapatite

This presentation will:

- *Place currently known HA binding and elution mechanisms in a rational hierarchy.*

- *Give examples of their aggregate removal capabilities.*

- *Make practical recommendations for selecting the most promising approach for aggregate removal from a particular monoclonal antibody.*

Antibody interactions with HA

Antibodies bind to native HA by a combination of calcium metal affinity, calcium anion exchange, and phosphoryl cation exchange.

For the majority of IgA and IgM antibodies, calcium affinity is the dominant retention mechanism.

For most IgG antibodies, ion exchange is dominant.

Antibody interactions with Ca-HA

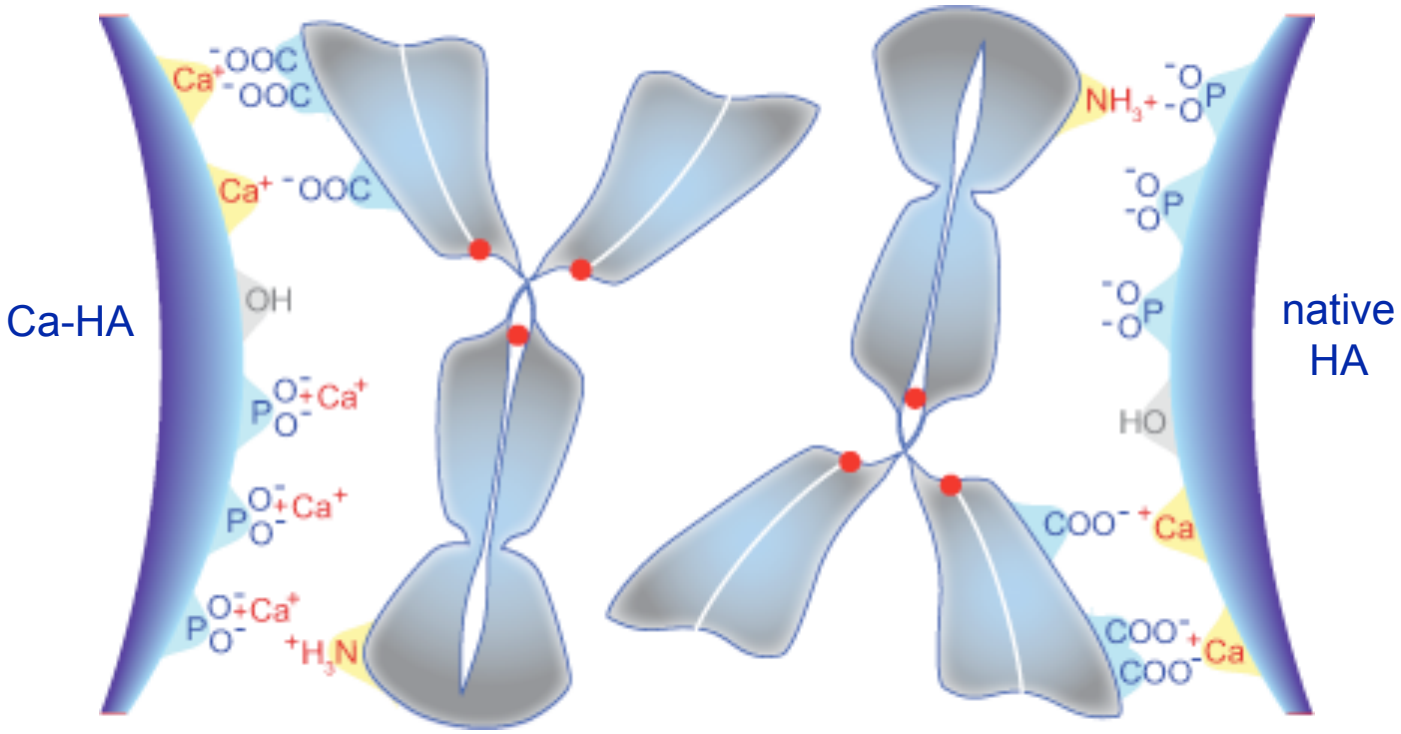
Soluble calcium converts primary HA phosphates into secondary calcium ligands. This abolishes phosphoryl cation exchange.

Antibodies bind to Ca-HA by a combination of calcium metal affinity and calcium anion exchange.

Calcium affinity appears to be the dominant retention mechanism for all antibodies on Ca-HA.

Antibody interactions with HA

IgG retention on native and Ca-hydroxyapatite



Phosphate gradients

The strong affinity of phosphate for calcium makes it an effective agent for controlling the calcium affinity component of antibody retention.

Its ionic character makes it an effective agent for controlling the ion exchange components.

However, it provides no opportunity for independent control of the respective mechanisms.

Removal of aggregates and polymers from IgA and IgM antibodies is often good.

Effective aggregate removal from IgG MAbs is infrequent.

Phosphate-chloride gradients

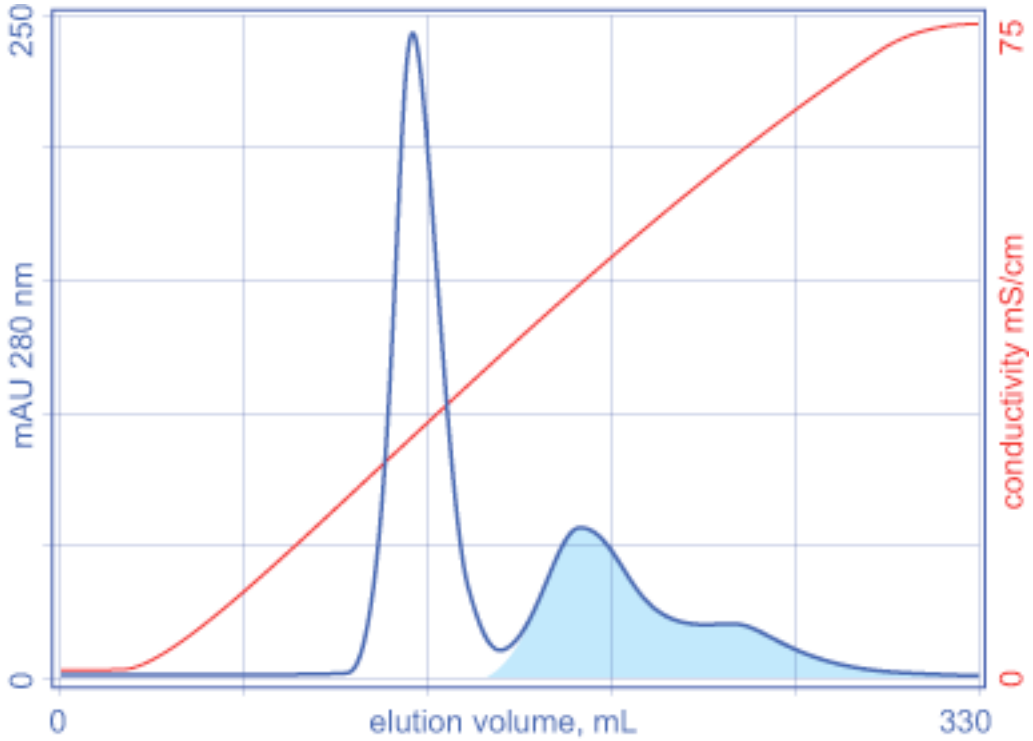
The ability of chloride salts to elute the calcium affinity component of HA retention is nil. This permits their use to selectively control HA ion exchange interactions.

For IgG monoclonals, low phosphate levels suspend weak calcium affinity interactions but leave strong ion exchange interactions largely intact. A sodium chloride gradient weakens ionic bonds, eluting non-aggregated IgG first, then aggregates at higher salt concentrations.

This approach works well with IgG antibodies that elute in sodium chloride at less than 20 mM phosphate. It does not work well with IgM antibodies evaluated to date.

Phosphate-chloride gradients

Aggregate removal with a phosphate-chloride gradient



Chimeric IgG_1 MAb

CHT™ Type I, 40 µm

ATOLL MediaScout™

11.3 x 100 mm (10 mL)

5.0 mL/min 300 cm/hr

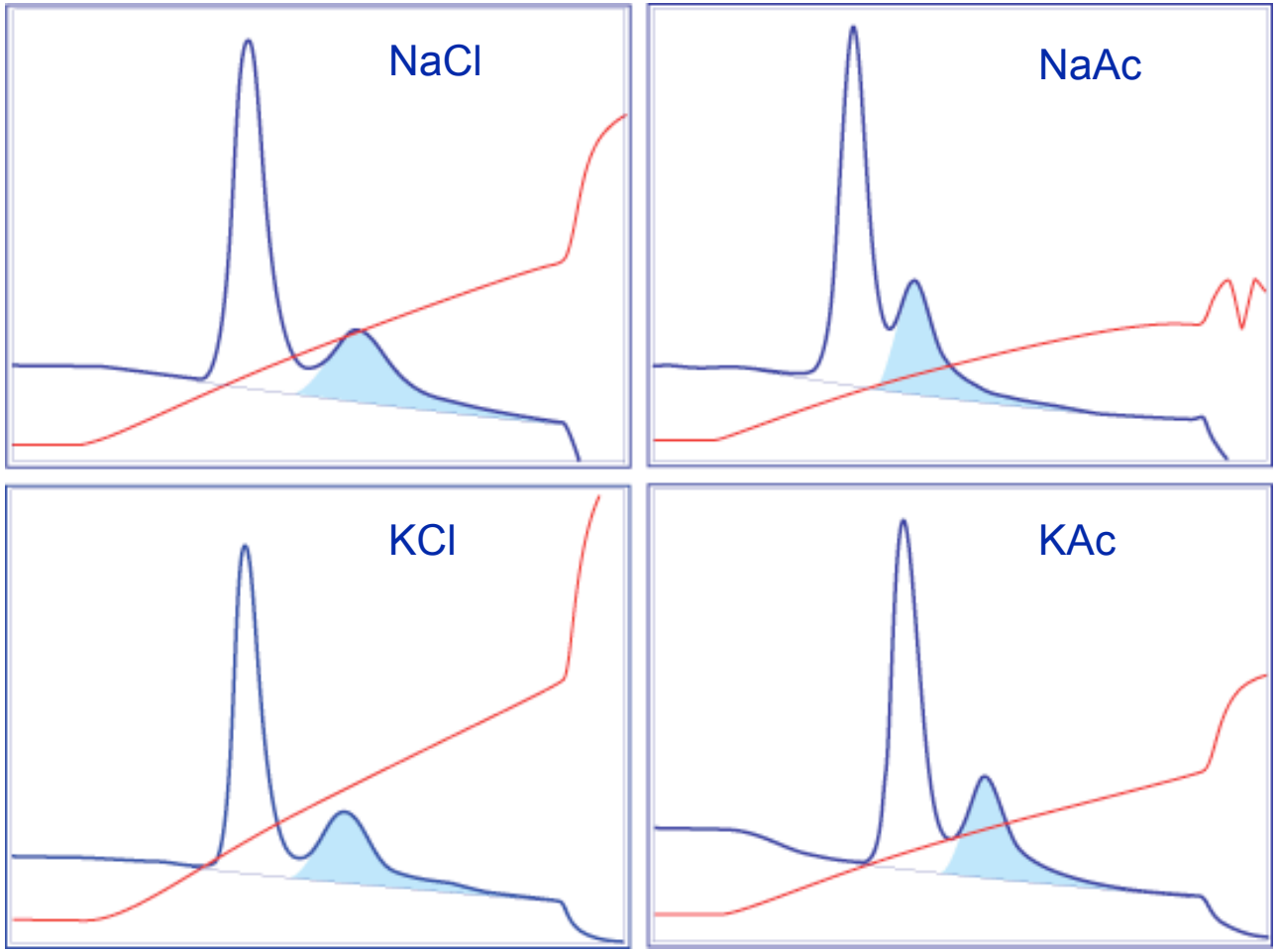
A: 10 mM PO_4, pH 7

B: 10 mM PO_4, 1 M NaCl, pH 7

Elute: 30 CVLG, A > B

The first aggregate peak contains tetramers, the second peak octamers. Larger aggregates were eluted in a 500 mM phosphate cleaning step.

Phosphate-chloride/acetate gradients



Chimeric IgG_1 MAb

CHT Type I, 40 µm

MediaScout 5 x 50 mm

1.0 mL/min (300 cm/hr)

All gradients 30 CV

10 mM Na or KPO4 to

10 mM Na or KPO4 +

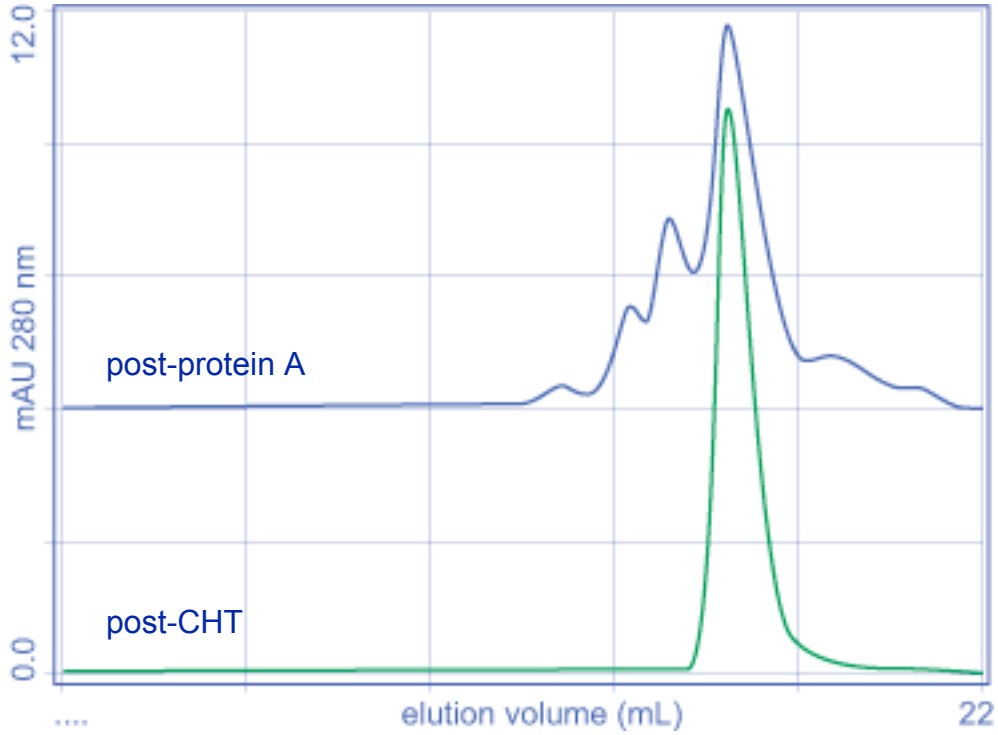
1.0 M indicated salt,

pH 6.5

Note the difference in conductivity profiles.

Phosphate-chloride gradients

SEC before and after HA with a phosphate-chloride gradient



Chimeric IgG_1 MAb

Bio-Sil™ 400-5

5 x 300 mm

0.50 mL/min, 150 cm/hr

20 mM MES, 200 mM arginine, pH 6.5

Note that fragments are also removed.

Phosphate-chloride: +/-

+ *All USP components*
+ *Easy development with minimal secondary testing*
+ *Outstanding removal of:*
  - *host cell protein*
  - *leached protein A*
  - *DNA, endotoxin, virus*

– *Not applicable to IgA or IgM*
– *Many IgGs not adequately accommodated*
– *pH excursions caused by NaCl require additional buffering*
– *IgG elutes at high conductivity > constrains process sequence*

Phosphate-chloride: +/-

Contaminant	Method	Clearance
Aggregates	HPSEC	1-3 logs
Protein A	Cygnus	1-3 logs
CHOP	ELISA	2 logs
DNA	Picogreen	> 3 logs
Endotoxin	LAL (chromo)	> 4 logs
aMULV	Infectivity	> 4 logs
xMULV	Infectivity	> 3 logs
MVM	Infectivity	2 logs

The effects of PEG

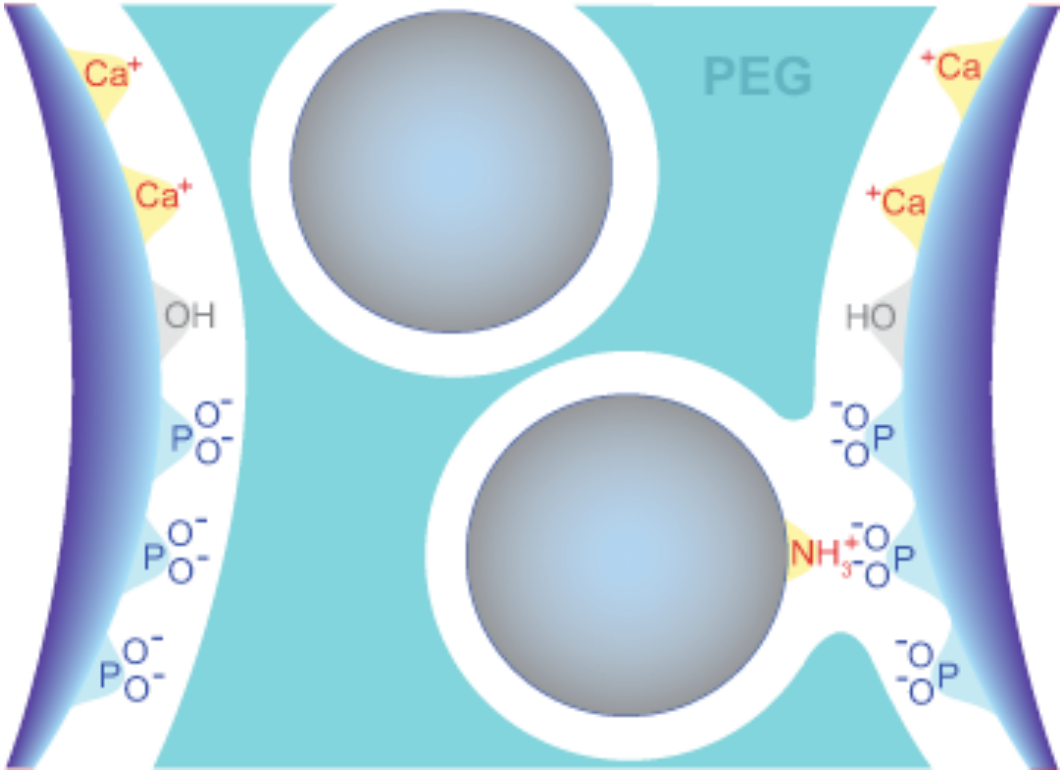
PEG is a nonionic organic polymer. It is assumed to have no direct effect on HA binding mechanisms.

It is also assumed that PEG does not perturb water structure.

PEG's effects are believed to result from preferential exclusion of PEG from protein and stationary phase surfaces.

The effect of PEG

Preferential exclusion of PEG



The effect of PEG

The discontinuity between the PEG-free exclusion zone and the high-PEG mobile phase is thermodynamically unfavorable.

When a protein binds to the stationary phase, the two share hydration water, allowing some water to transfer to the mobile phase, thereby lowering the bulk PEG concentration. This reduces the discontinuity between the PEG-free exclusion zone and the high-PEG mobile phase. This is thermodynamically favorable.

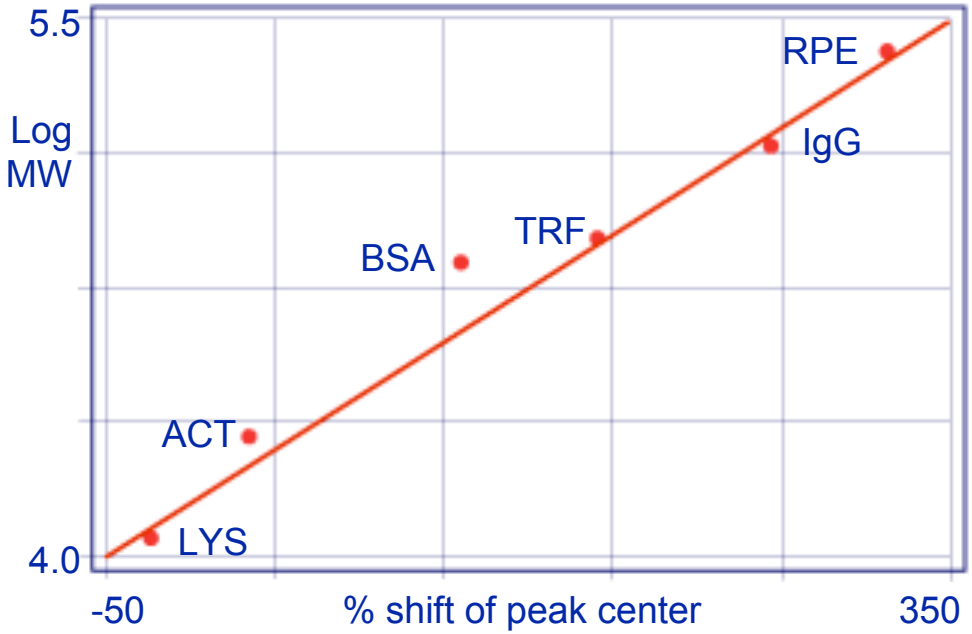
The effect of PEG

In addition, the PEG-free surface area of the bound protein is lower than the additive PEG-free surface areas of the protein and stationary phase separately. This is also thermodynamically favorable.

The combination of these effects tends to stabilize the association of the protein with the stationary phase. Proteins consequently elute at higher concentrations of the primary eluting agent than in the absence of PEG.

The effects of PEG

PEG enhances retention in proportion to solute size

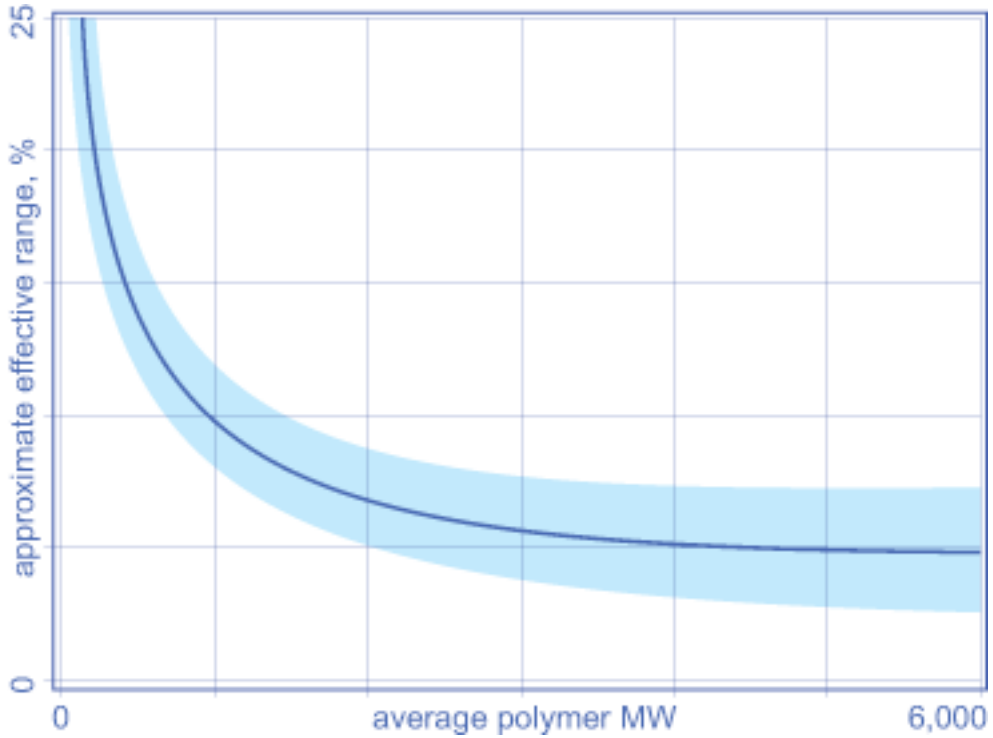


RESOURCE® S/Q
0% PEG versus
10% PEG-6000

RPE: 240 kD
IgG: 155 kD
TRF: 80 kD
BSA: 67 kD
ACT: 23 kD
LYS: 11 kD

The effects of PEG

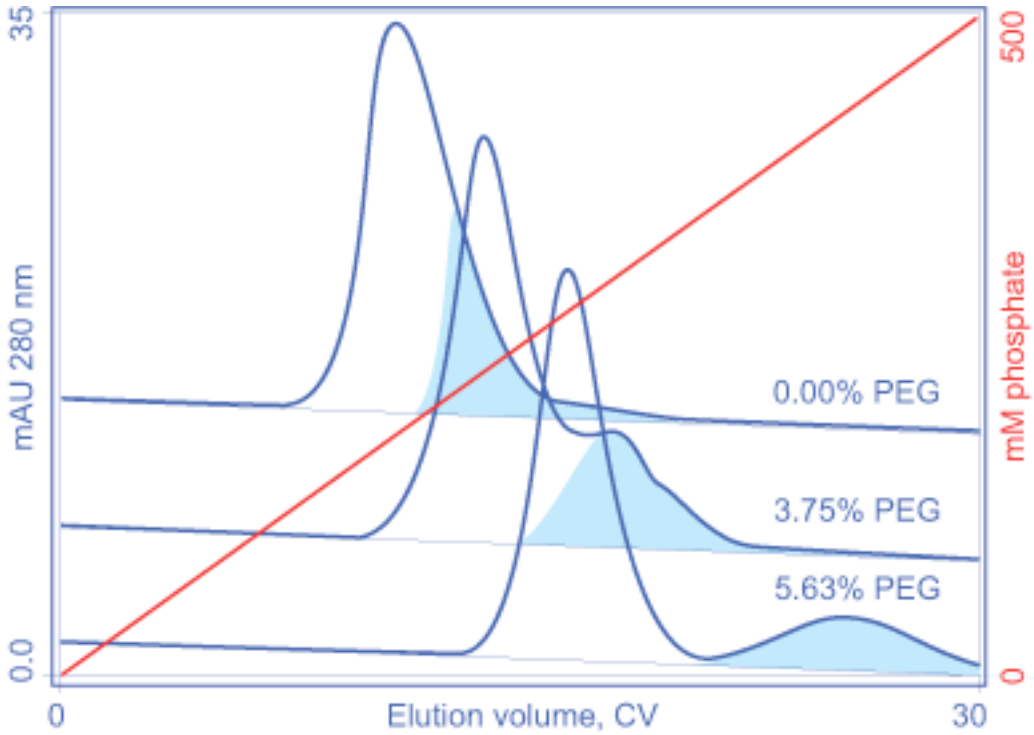
PEG enhances retention in proportion to polymer molecular weight



Polymer molecular weight is highly influential up to about 3000 Daltons. Larger polymers increase viscosity without benefit and complicate removal of residual PEG.

PEG-phosphate gradients

The effect of PEG on phosphate gradients: IgG



Chimeric IgG_1 MAb

CHT Type I, 20 µm

MediaScout 5 x 50 mm

1.0 mL/min, 300 cm/hr

A: 10 mM PO_4, pH 7

B: 500 mM PO_4, pH 7

Elute: 30 CV LG, A to B

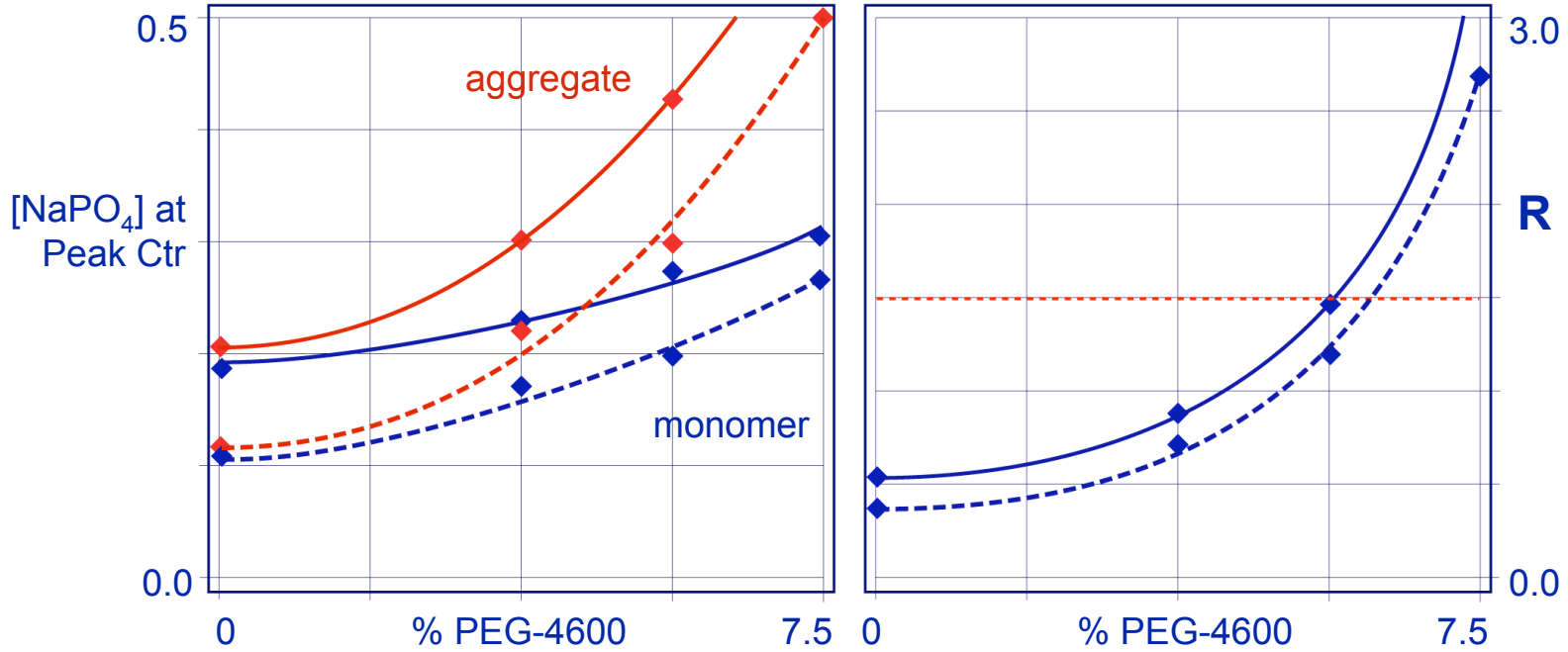
Same + 3.75% PEG-4600

Same + 5.63% PEG-4600

This is the same antibody used to illustrate aggregate removal with a phosphate-chloride gradient. 5.63% PEG supports more effective removal without recourse to NaCl.

PEG-phosphate gradients

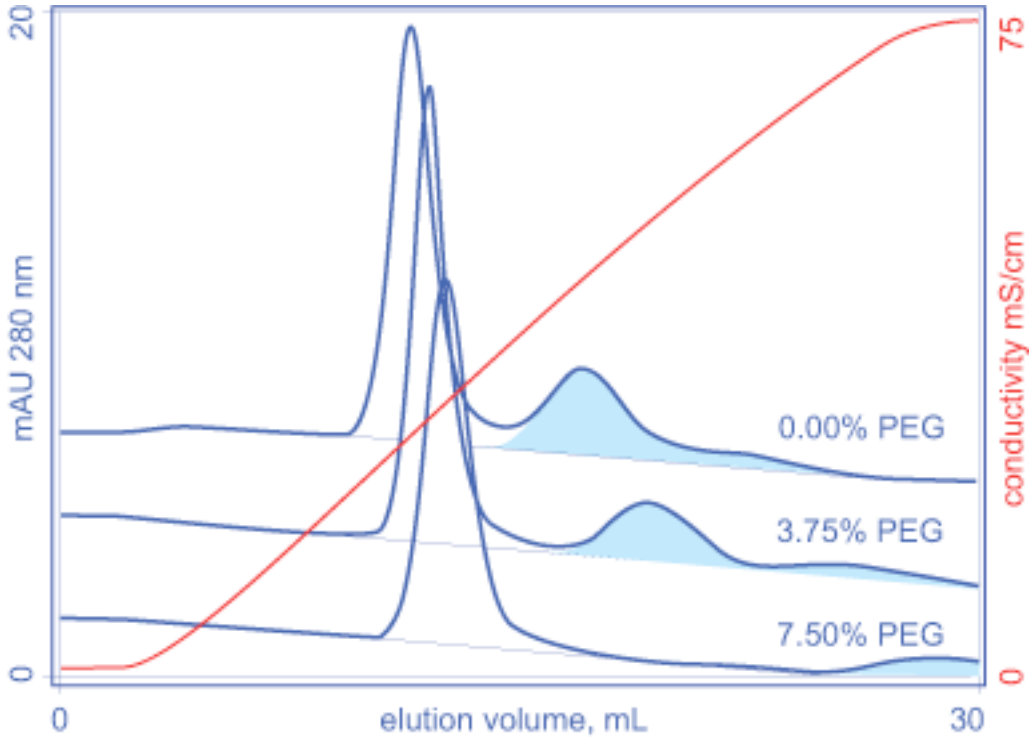
The effect of PEG for 2 different IgG monoclonals



The solid lines represent the results shown in the previous slide. The broken lines represent results for a different IgG monoclonal treated under the same experimental conditions. The vertical offset corresponds to the difference in their respective elution properties. The consistency of the curve shapes shows that PEG dominates the native selectivity of HA and suggests that its effects should be fairly uniform for all IgGs; stronger for IgAs and IgMs.

PEG-phosphate-chloride gradients

The effect of PEG on NaCl gradients: IgG



Chimeric IgG_1 MAb

CHT Type I, 20 µm

MediaScout 5 x 50 mm

1.0 mL/min, 300 cm/hr

A: 10 mM PO_4, pH 7

B: A + 1M NaCl, pH 7

Elute: 30 CV LG, A to B

Same + 3.75% PEG-4600

Same + 7.50% PEG-4600

These results show that the effects of PEG and other aggregate separation enhancing methods are cooperative. This has practical value because it shows that the size and/or concentration of PEG can be reduced below levels required with phosphate gradients.

PEG: +/-

+ *Applicable to all antibody classes*
+ *Enhances aggregate removal with all HA base gradients*
+ *Approved inactive ingredient in many parenteral formulations*
+ *Easy development with minimal secondary testing*
+ *Easy to remove*
+ *Enhances virus removal*

– *Increases viscosity > backpressure, peak width*
– *Risk of product precipitation at high PEG concentration*
– *Needs to be removed from final product.*
– *Removal needs to be validated.*

Sulfate gradients

Like phosphate, sulfate is able to suspend both ion exchange and calcium metal affinity, but its calcium affinity is relatively weak.

Weak calcium affinity means that a higher sulfate concentration is required to elute a given antibody.

This means that a given antibody will elute at higher conductivity, which means in turn that sulfate gradients suppress the relative contribution of ion exchange to a greater extent than phosphate elution. This creates a unique window of selectivity.

Sulfate gradients

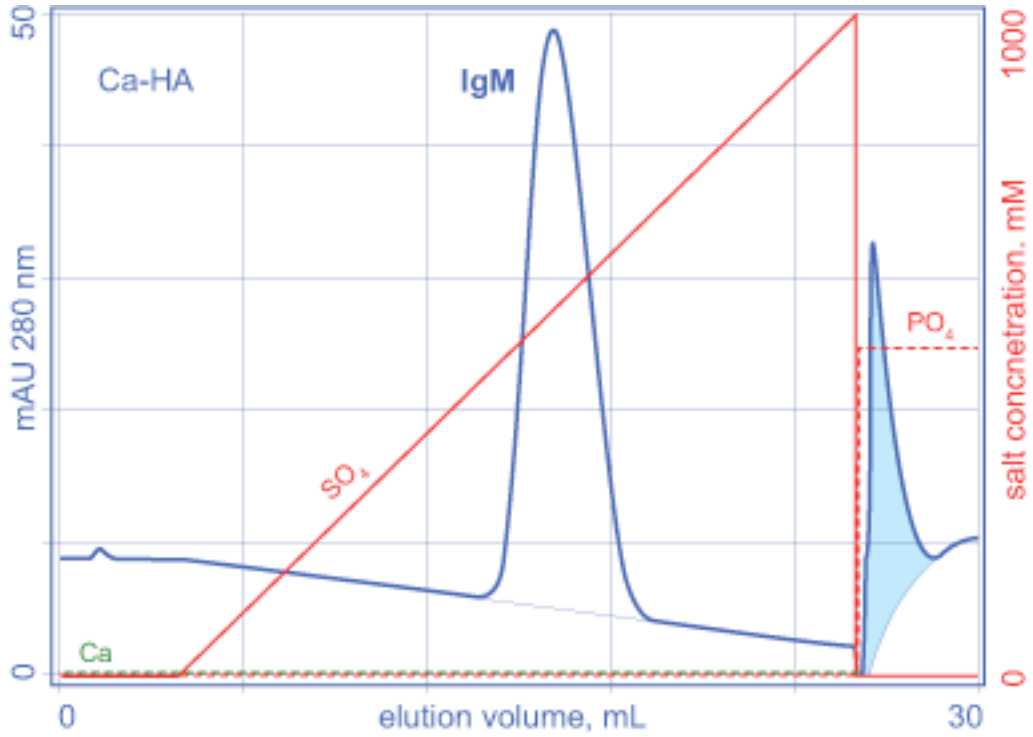
Like PEG, sulfate is strongly excluded from protein and stationary phase surfaces, causing proteins to be more strongly retained.

Unlike PEG, sulfate directly affects HA's primary retention mechanisms, and also affects water structure.

Note: Phosphates are also strongly excluded from protein and stationary phase surfaces, but since most antibodies elute from HA at fairly low phosphate concentrations, exclusionary effects on retention are usually negligible.

Sulfate gradients

IgM, Ca-HA, sulfate gradient



CHT Type II, 40 µm

MediaScout 5 x 100 (2 mL)

0.67 mL/min, 200 cm/hr

A: 50 mM MES, 3 mM $CaCl_2$
pH 6.7

B: A + 1M Na_2SO_4, pH 6.7

EQ: 100% A

Inject: 100 µL purified IgM

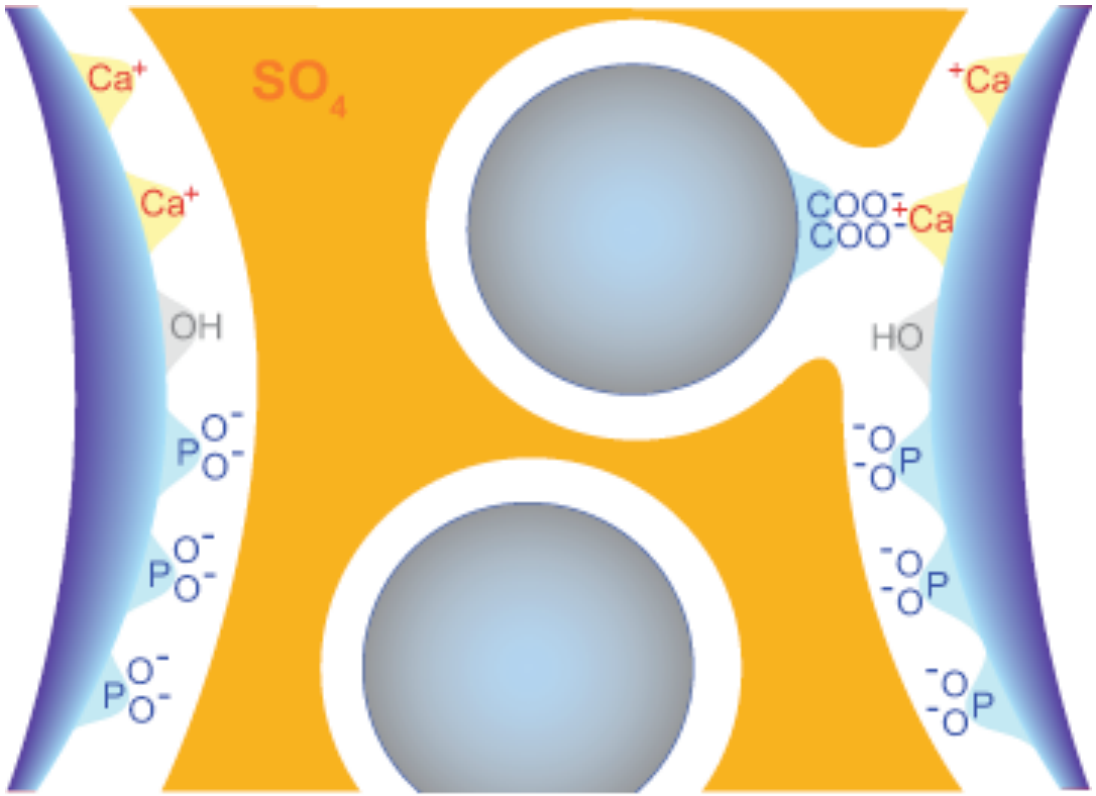
Elute: 10 CVLG to 100% B

Clean: 500 mM $NaPO_4$, pH 7

This application works because IgMs elute at a fairly high concentration of sulfate, where its exclusionary effects are able to preferentially enhance aggregate retention. IgGs tested to date elute at too low a sulfate concentration to enhance aggregate separation to a useful degree.

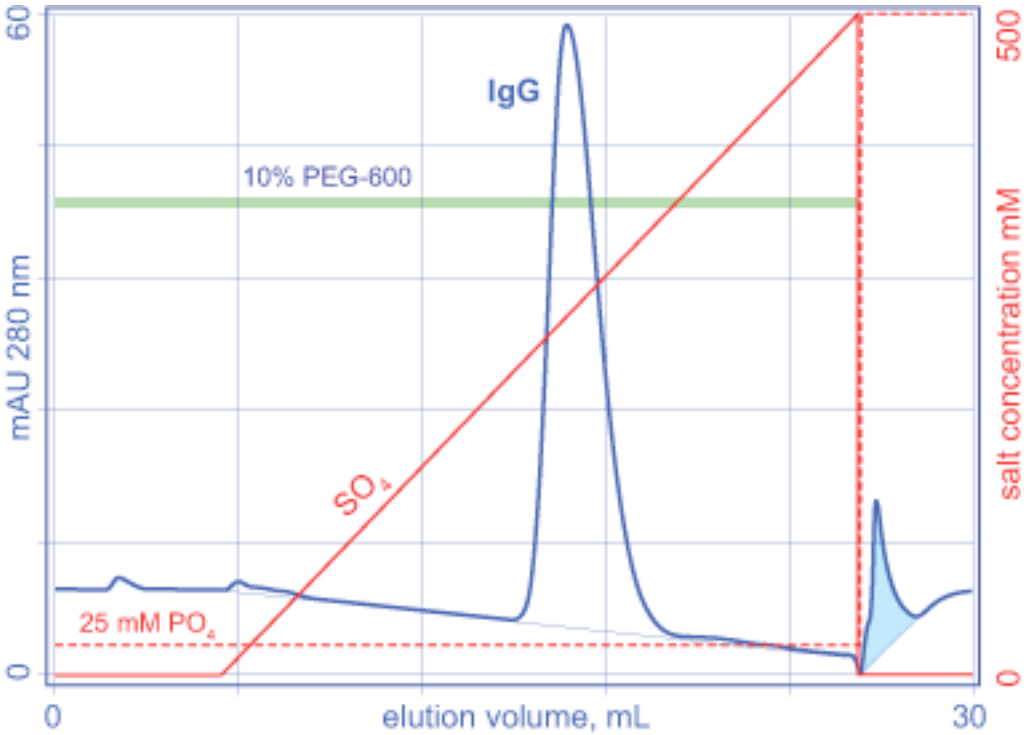
Sulfate gradients

Preferential exclusion of sulfate



Sulfate/PEG gradients

IgG, native HA, PEG/SO_4 gradient



Protein A-purified MAb

CHT Type I, 40 µm

MediaScout 5 x 100 mm

1 mL/min, 300 cm/hr

A: 10 mM $NaPO_4$, 10% PEG-600, pH 7

B: A + 0.5 M Na_2SO_4, 10% PEG-600, pH 7

EQ: 100% A, Inject 100 µL

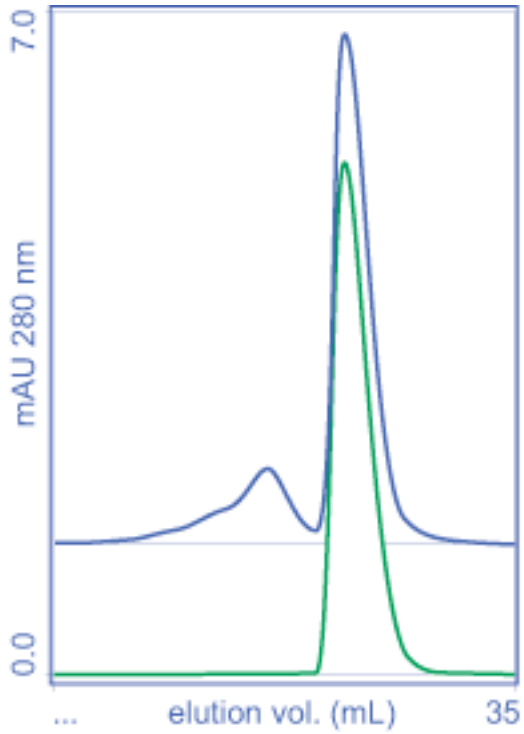
Wash: A. Elute: 10 CVLG to B

Clean: 500 mM $NaPO_4$, pH 7

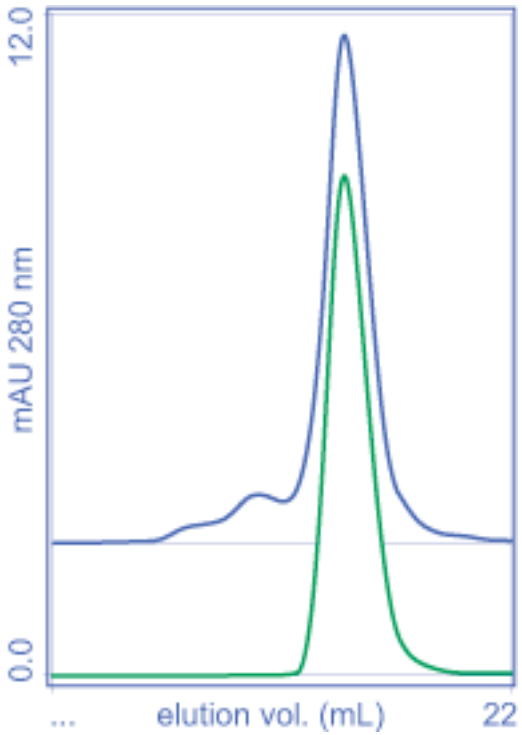
Inclusion of PEG in the equilibration buffer reduces the number of buffers and simplifies the overall process. Pressure increase is negligible. PEG addition to the sample may risk precipitating the protein before adsorption to the column.

Aggregate removal with sulfate

Analytical size exclusion



IgM MAb
HA/PO4 > CX
Ca-HA/SO4 > CX



IgG MAb
Protein A
HA SO4/PEG

IgM
TSK-gel® G4000SW$_{XL}$
7.8 x 300 mm
0.50 mL/min, 62 cm/hr

IgG
Bio-Sil™ 400-5
5 x 300 mm
0.50 mL/min, 150 cm/hr

20 mM MES, 200 mM arginine, pH 6.5

Sulfate gradients: +/-

+ *All USP components*
+ *Unique selectivities with native and Ca-HA*
  - *Outstanding removal of DNA*
  - *Improved protein fractionation*

– *Applicable to IgM only (except in combination with PEG)*
– *Antibodies elute at high conductivity > constrains process sequence*
– *Risk of product precipitation at high sulfate concentration*
– *Requires presence of phosphate or calcium to stabilize HA*
– *Presently the least characterized approach*

Conclusions

HA in the presence of PEG is the closest candidate to a universal method for aggregate removal.

If the objective is to quickly obtain aggregate-free antibody for investigational use, a phosphate gradient in 10% PEG-1000 is a good place to start. Use CHT type I 40µm for IgG; type II 40µm for IgA or IgM.

Remove residual PEG by dialysis, diafiltration, buffer exchange chromatography, or by binding the antibody to an ion exchanger and allowing the PEG to flow through.

Conclusions

Aggregate removal in PEG-phosphate gradients

Equilibrate: 50 mM Hepes, 10 mM $NaPO_4$ 10% PEG-1000, pH 7.0

Apply sample

Wash: 50 mM Hepes, 10 mM $NaPO_4$ 10% PEG-1000, pH 7.0

Elute IgA,IgG: 20 CV LG to 250 mM $NaPO_4$ 10% PEG-1000, pH 7.0

Elute IgM: 20 CV LG to 500 mM $NaPO_4$ 10% PEG-1000, pH 7.0

Clean: 600 mM $NaPO_4$ pH 7.0

Sanitize: 1.0 M NaOH

Store: 0.1 M NaOH, or 20% ethanol plus 5 mM $NaPO_4$ pH 7.0

Conclusions

If the objective is to develop a fully integrated commercial purification process, then choose the elution format that supports the most effective removal of host cell proteins, leached protein A, DNA, endotoxin, and virus.

Phosphate-chloride gradients offer better aggregate removal and overall IgG purification than simple phosphate gradients. Sulfate gradients should support equivalent benefits but remain to be fully characterized.

Sulfate gradients offer better aggregate removal and overall IgM purification than either phosphate or phosphate-chloride gradients.

Conclusions

If the base gradient fails to support adequate aggregate removal, its effectiveness can be augmented with PEG.

Begin by adding 10% PEG-1000 to the wash and elution buffer of the method that provides the best overall purification.

It will be possible in most cases to reduce the concentration and/or use a lower molecular weight PEG. Experience to date indicates that some antibodies can be accommodated with as little as 3% PEG-600.

Acknowledgements

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

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