



# ***Multiple Options for Removal of Antibody Aggregates by Apatite Chromatography***

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# *Aggregate removal by hydroxyapatite*

*Hydroxyapatite has become the pre-eminent method for removal of antibody aggregates.*

*It has been shown to be effective with IgA, IgM, and IgG monoclonal antibodies of all subclasses.*

*Aggregate levels are often reduced below 0.1%, even when initial aggregate levels range from 40–60%.*



# Aggregate removal by hydroxyapatite

*The ability of hydroxyapatite to remove other important contaminants has also contributed to its growth.*

<b>Contaminant</b>	<b>Method</b>	<b>Clearance</b>
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



# *Aggregate removal by hydroxyapatite*

*The multimodal character of hydroxyapatite supports a variety of different elution strategies.*

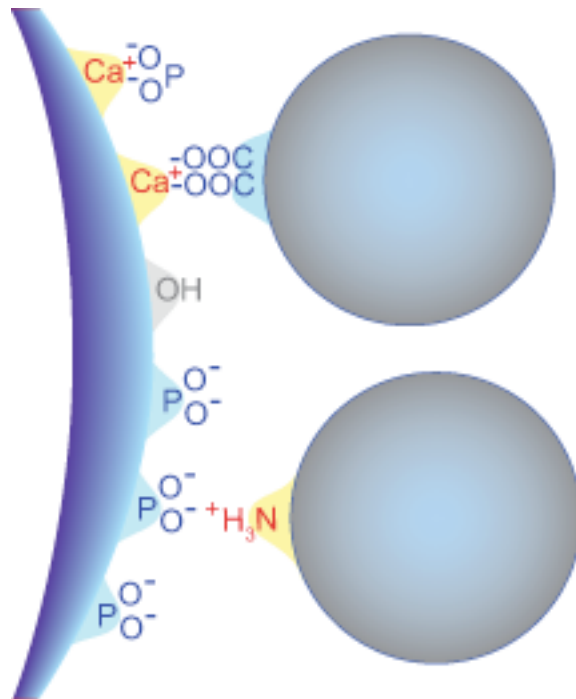
*No single elution strategy accommodates all antibodies.*

*But, aggregates can be removed from any antibody by at least one of these strategies.*



# Retention mechanisms

## Primary retention mechanisms



*Calcium metal affinity. Protein carboxyl clusters form coordination bonds with HA calcium in a manner similar to EDTA. These bonds are 15–60 times stronger than ionic bonds and typically survive exposure to NaCl. Elution normally requires an agent with strong calcium affinity, such as phosphate.*

*Phosphoryl cation exchange. Protein amino residues can participate in cation exchange interactions occurs with HA phosphate. As with other cation cation exchangers, protein binding becomes weaker with increasing pH and/or conductivity.*

*Recent experimental evidence indicates that the potential for anion exchange between protein carboxyl groups and HA calcium is probably eliminated by strong complexes formed between HA calcium and mobile phase phosphate ions.*

# Phosphate gradients

*Most of the historical literature describes antibody elution with simple phosphate gradients.*

*Phosphate gradients support effective aggregate removal from IgA monoclonal antibodies.*

*They are also effective for a minority of IgG monoclonal antibodies.*



# Phosphate gradients

*Phosphate gradients offer valuable practical benefits:*

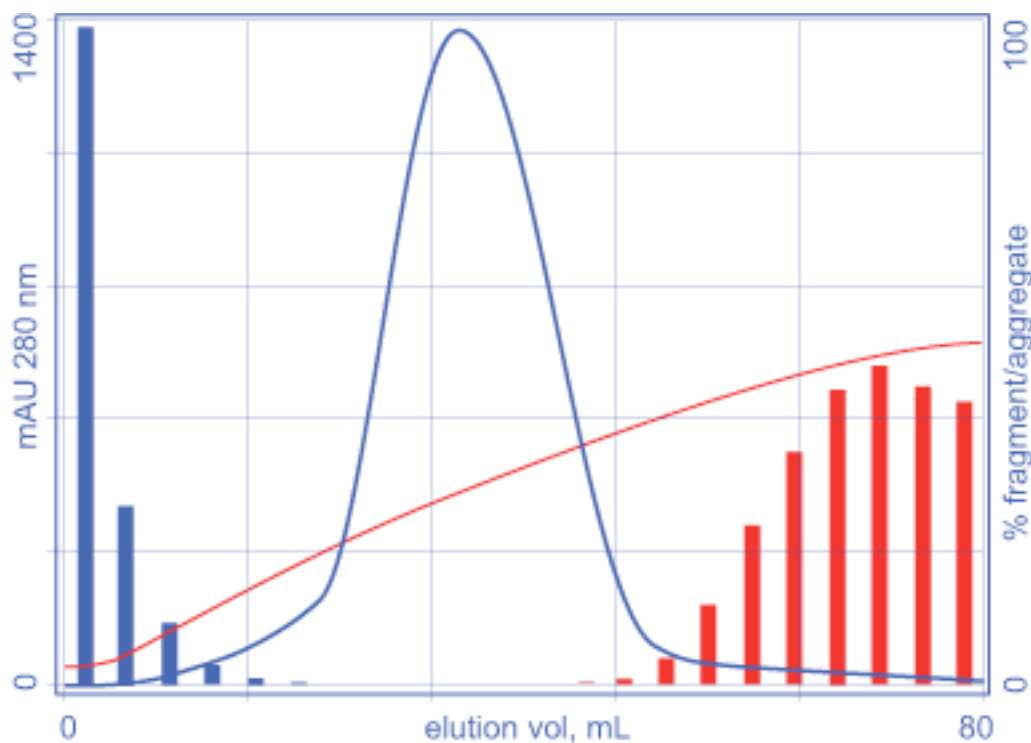
- *Process development is limited to identifying the most effective gradient configuration.*
- *The antibody usually elutes at fairly low conductivity, which facilitates a final anion exchange step.*
- *All process buffers can be made as dilutions from the cleaning buffer (500 mM sodium phosphate, pH ~7).*
- *Hydroxyapatite eluted exclusively with phosphate gradients has a longer usage lifetime than chloride gradients.*

*But, they are less effective than chloride gradients for removal DNA and endotoxin. This is not a concern if the process contains an anion exchange step.*



# Phosphate gradients

## Fragment and aggregate removal with a phosphate gradient



CHT Type I, 40  $\mu$ m, 200 cm/hr  
A: 10 mM NaPO<sub>4</sub>, pH 7  
B: 500mM NaPO<sub>4</sub>, pH 7  
EQ: A  
Inject:  
Wash: A  
Elute: 10 CVLG to 40% B

Modified from Gagnon, P., Beam, K., Antibody aggregate removal with hydroxyapatite, *Curr. Pharm. Biotechnol.*, 2009, 25 287-293



# Phosphate gradients

*Phosphate vs chloride gradient performance, IgG<sub>1</sub> chimera*

<b>Parameter</b>	<b>Phosphate</b>	<b>Chloride</b>
Monomer recovery	78%	82%
Aggregate level	< 1%	< 1%
Leached protein A	< 1 ppm	< 1 ppm
CHO proteins	< 72 ppm	< 12 ppm
DNA	< 7 ppm	< 1 ppm
Endotoxin	< 5.0 EU/mL	<0.1 EU/mL



# Chloride gradients

*Chloride gradients at constant low levels of phosphate have proven to be the most effective elution strategy for aggregate removal from IgG monoclonals.*

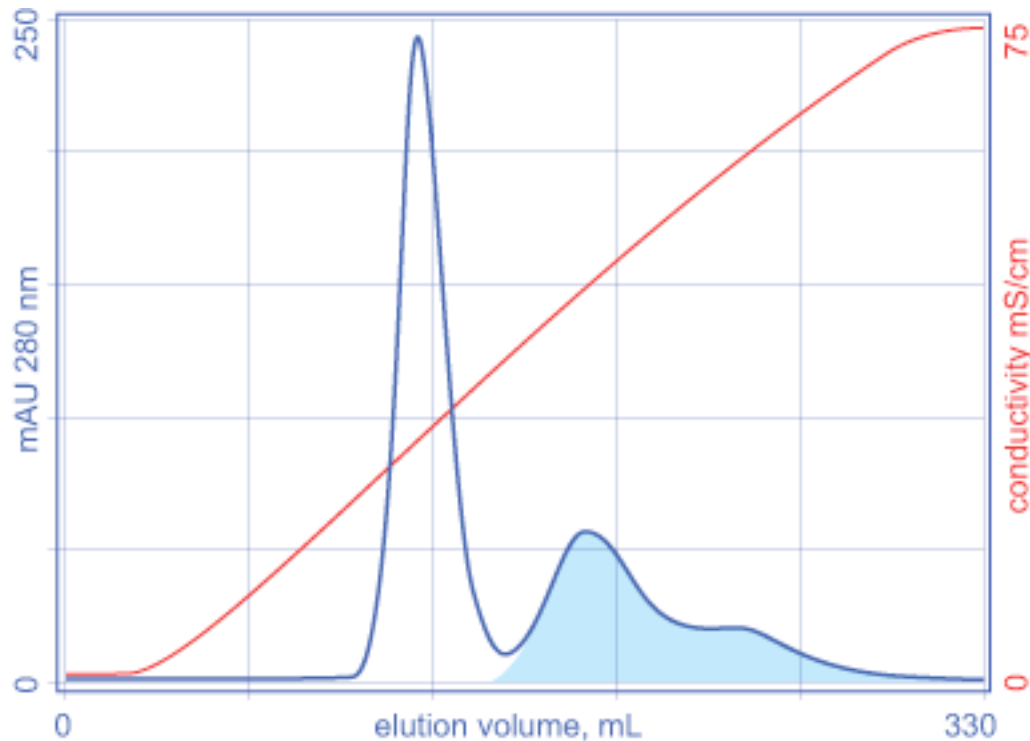
*Setting a constant low level of phosphate suspends weak calcium affinity interactions but leaves strong ionic interactions intact. A sodium chloride gradient can then dissociate ionic bonds, eluting monomeric IgG. Aggregates elute later.*

*Contaminants with strong calcium affinity remain bound until the column is cleaned with 500 mM phosphate. These include leached protein A-IgG complexes, DNA, endotoxin, and lipid enveloped viruses.*



# Chloride gradients

## Aggregate removal with a chloride gradient

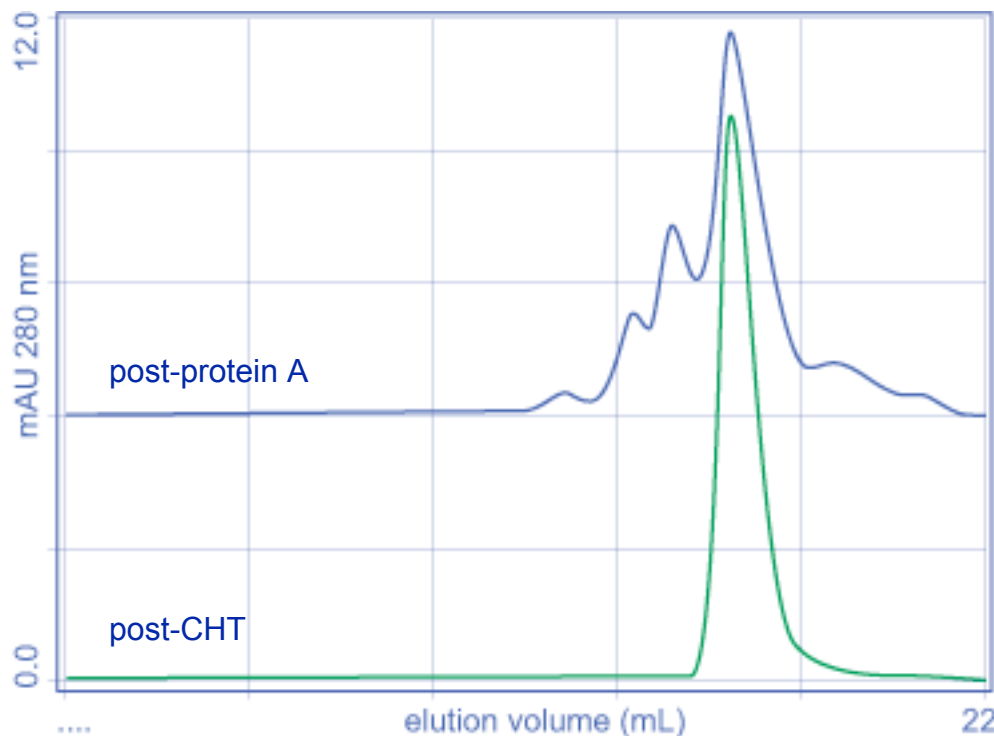


Chimeric IgG<sub>1</sub> MAb  
CHT™ Type I, 40 μm  
ATOLL MediaScout™  
11.3 x 100 mm (10 mL)  
5.0 mL/min 300 cm/hr  
A: 10 mM NaPO<sub>4</sub>, pH 7  
B: 10 mM NaPO<sub>4</sub>, 1 M  
NaCl, pH 7  
Elute: 30 CVLG, A > B

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

# Chloride gradients

*SEC before and after HA eluted with a chloride gradient*

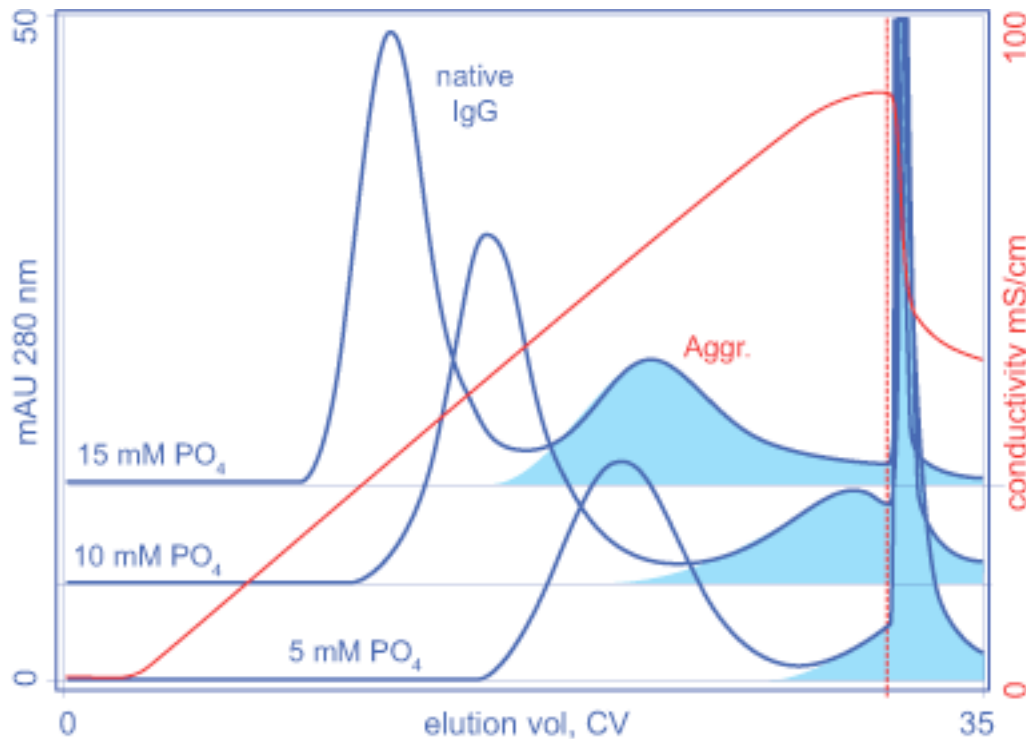


Chimeric IgG<sub>1</sub> MAb  
Bio-Sil™ 400-5  
5 x 300 mm  
0.50 mL/min, 150 cm/hr  
20 mM MES, 200 mM  
arginine, pH 6.5

Note that fragments are also removed.

# Chloride gradients

## Development of chloride gradients



Chimeric IgG<sub>1</sub> MAb  
CHT type I, 20 μm, 300 cm/hr  
EQ: indicated [NaPO<sub>4</sub>] pH 6.5  
Inj: IgG<sub>1</sub> chimera  
Wash: EQ buffer  
EL: 30 CVLG to 1M NaCl at  
indicated [NaPO<sub>4</sub>] pH 6.5  
Clean: 500 mM NaPO<sub>4</sub>, pH 7

# Chloride gradients

*In general, use the lowest phosphate concentration (> 5 mM) that allows non-aggregated antibody to elute in a gradient to 1 M NaCl.*

<b>[PO4]</b>	<b>5 mM</b>	<b>10 mM</b>	<b>15 mM</b>
Protein A, ng/mL	< 0.2	< 0.2	< 0.2
DNA ng/mL	< 1.0	< 1.0	4.9
ETX, EU/mL	< 0.05	1.0	1.6

Sample: protein A purified chimeric monoclonal IgG<sub>1</sub>.

Initial contaminant levels: 22 ng/mL leached protein A, 2.3x10<sup>3</sup> ng/mL DNA, 1.9x10<sup>4</sup> EU/mL endotoxin.

Limits of detection: 0.2 ng/mL for protein A. 1.0 ng/mL for DNA. 0.05 EU/mL for endotoxin.

All results are for the monomeric IgG pool from a sodium chloride gradient to 1.5 M at pH 6.5 with phosphate concentration held at the indicated level. CHT Type I, 40 µm, 300cm/hr.



# Chloride gradients

*There are two disadvantages associated with chloride gradients:*

- *Most antibodies elute at high conductivity (0.5 - 1.0 M NaCl). This makes it necessary to place the HA step at the end of the process (after anion exchange), or perform buffer exchange after HA to accommodate the lower conductivity needs of a final anion exchange step.*
- *Chloride steps displace  $H^+$  ions from HA phosphate groups. This reduces pH. HA becomes weakly soluble below pH 6.5, so this may have the effect of decreasing the number of cycles for which a packed column can be used. These pH reductions can be moderated, but not eliminated, by including MES in the process buffers and/or by running separations at pH 7 (or higher) instead of pH 6.5.*



# *Enhancement with PEG*

*Polyethylene glycol (PEG) enables effective aggregate removal from IgGs that are not adequately served by other gradient formats. It also supports effective aggregate removal from IgM monoclonals.*





# *Enhancement with PEG*

*PEG is a nonionic organic polymer.*

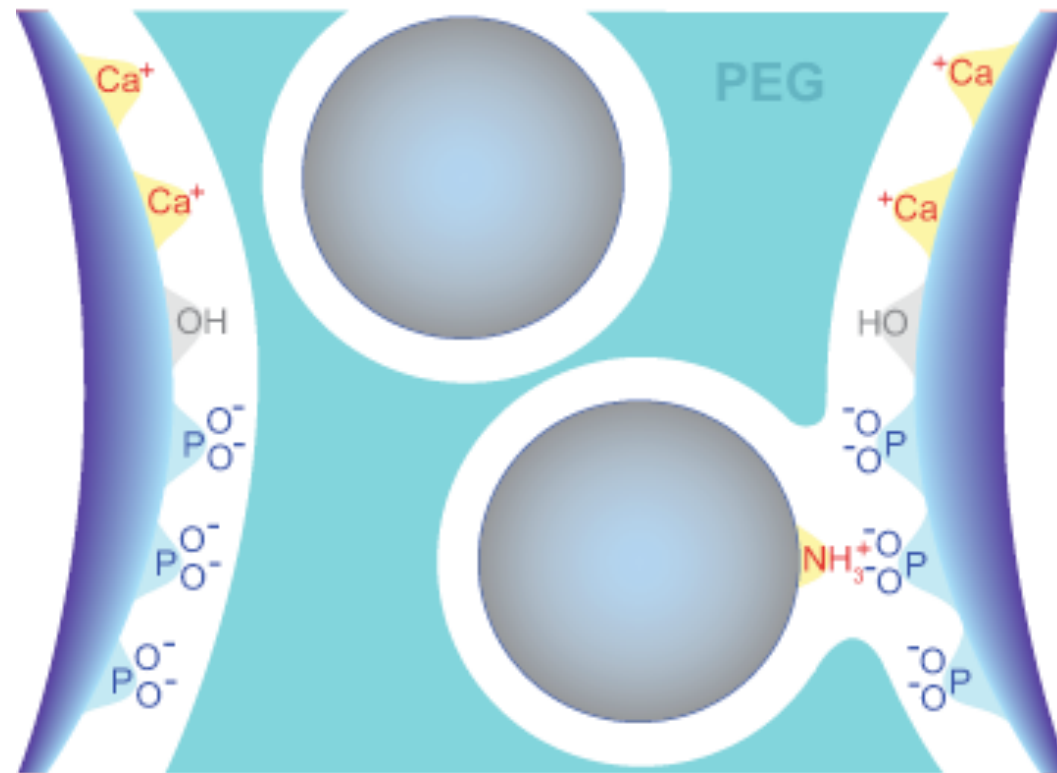
*It does not interact directly with either proteins or hydroxyapatite.*

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



# Enhancement with PEG

*Preferential exclusion of PEG*



# Enhancement with 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.*



# Enhancement with PEG

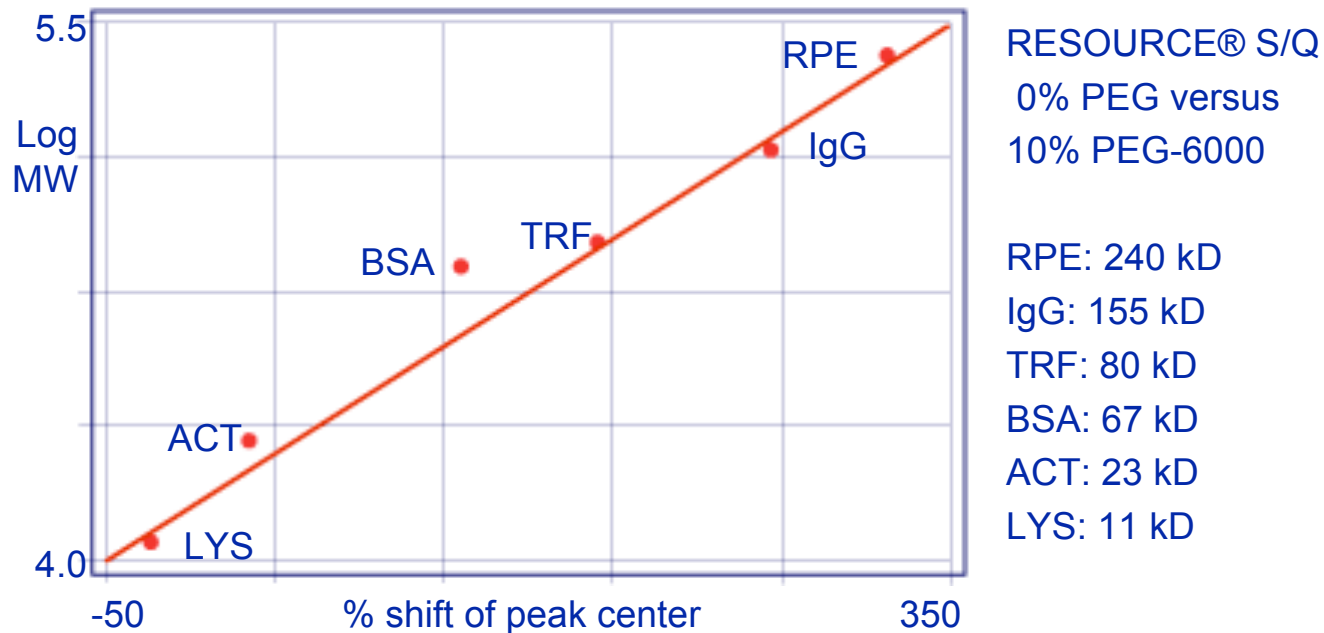
*In addition, the PEG-free surface area of the bound protein is lower than the additive PEG-free surface areas of the protein and stationary phase separately. This is also thermodynamically favorable.*

*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.*



# Enhancement with PEG

*PEG enhances retention in proportion to solute size*

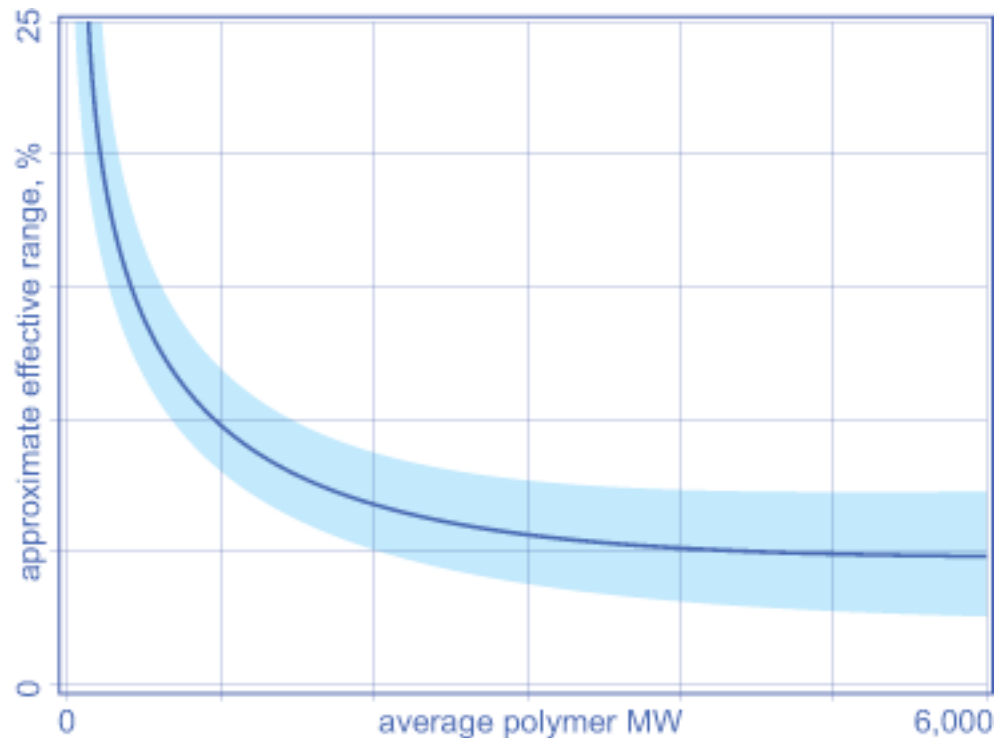


LYS: lysozyme. ACT: antichymotryosin. BSA: bovine serum albumin.  
TRF: bovine transferrin. RPE: R-phycoerythrin



# Enhancement with PEG

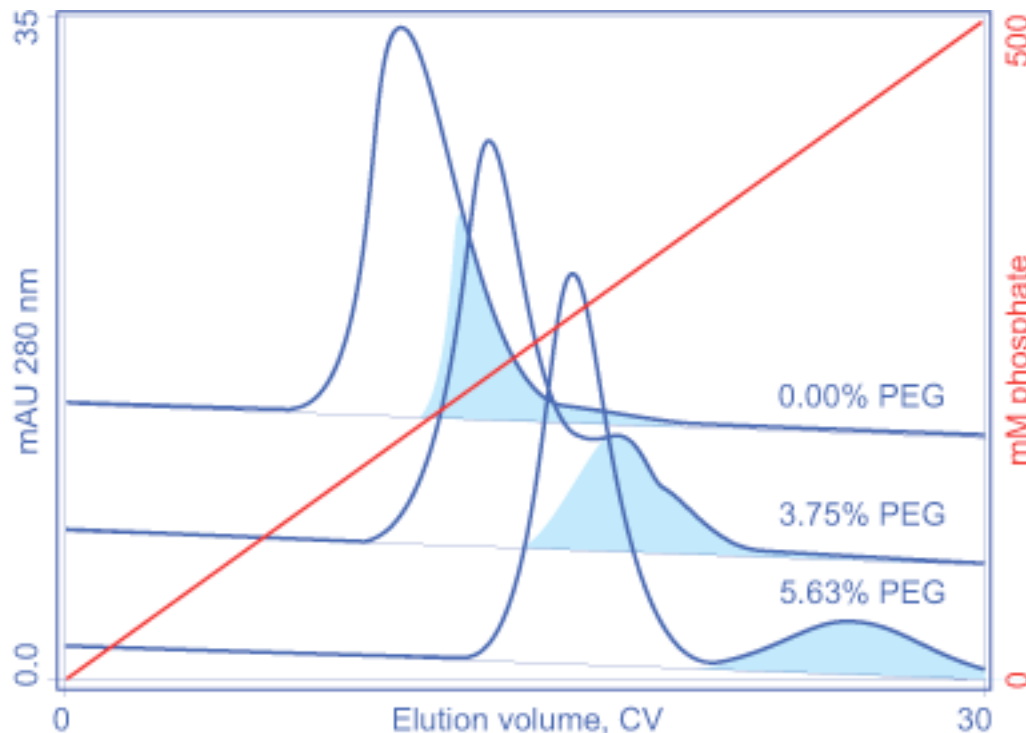
*PEG enhances retention in proportion to polymer molecular weight*



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

# Phosphate gradients in PEG

## The effect of PEG on phosphate gradients: IgG

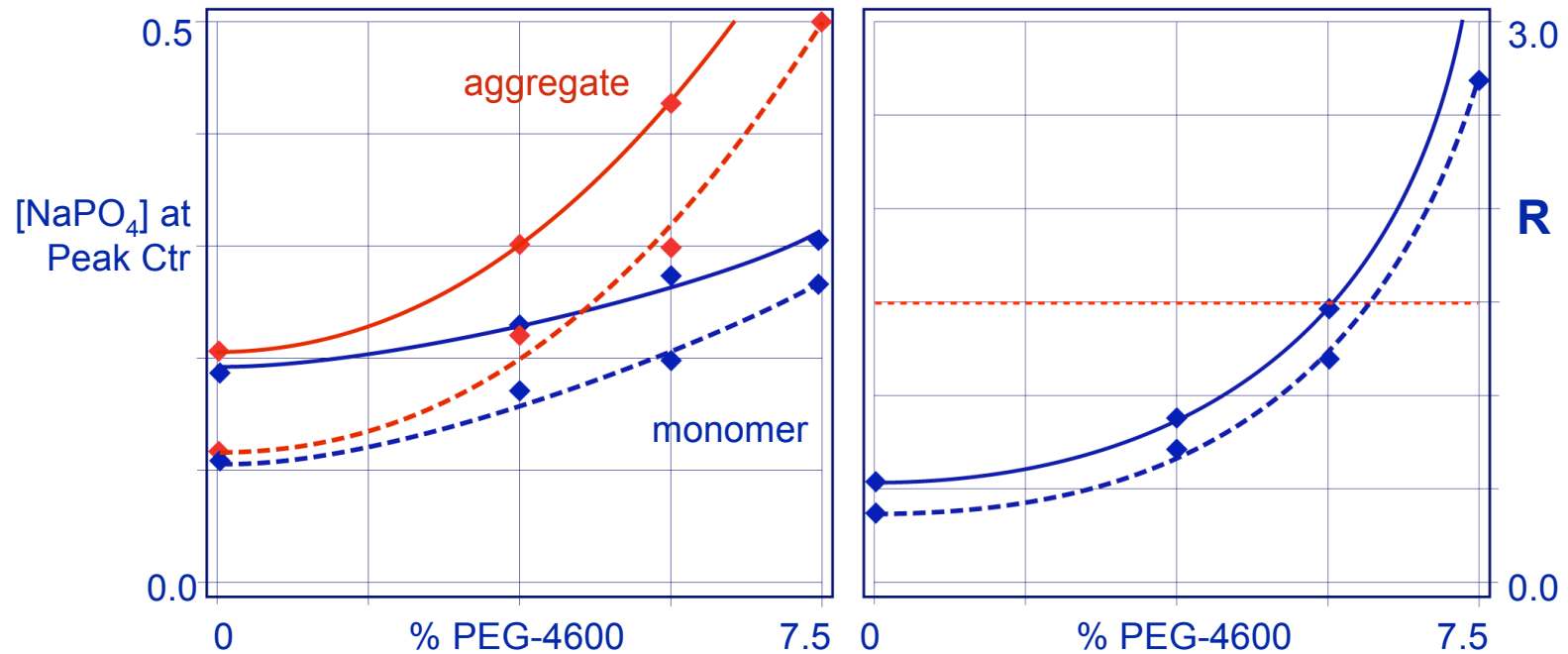


Chimeric IgG<sub>1</sub> MAb  
CHT Type I, 20  $\mu$ m  
MediaScout 5 x 50 mm  
1.0 mL/min, 300 cm/hr  
A: 10 mM PO<sub>4</sub>, pH 7  
B: 500 mM PO<sub>4</sub>, pH 7  
Elute: 30 CV LG, A to B  
Same + 3.75% PEG-4600  
Same + 5.63% PEG-4600

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.

# Phosphate gradients in PEG

The effect of PEG for 2 different IgG monoclonals

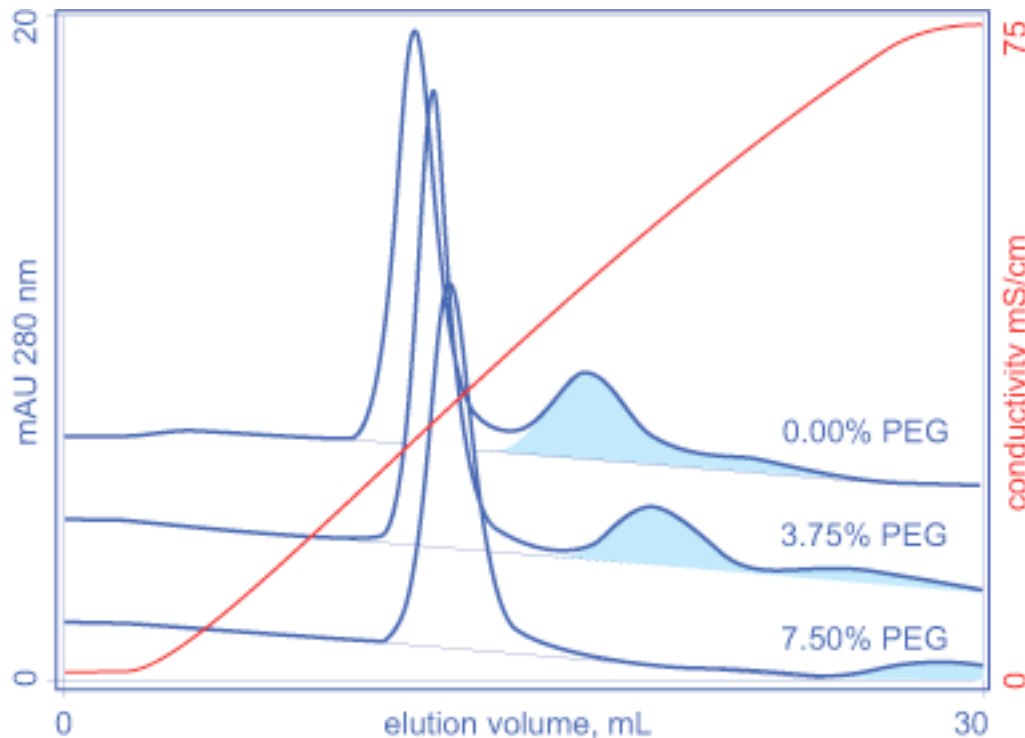


The solid lines represent the results shown in the previous slide. The broken lines represent results for a different IgG monoclonal treated under the same experimental conditions. The vertical offset corresponds to the difference in their respective elution properties. The consistency of the curve shapes 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.



# Chloride gradients in PEG

## The effect of PEG on NaCl gradients: IgG



Chimeric IgG<sub>1</sub> MAb  
CHT Type I, 20  $\mu$ m  
MediaScout 5 x 50 mm  
1.0 mL/min, 300 cm/hr  
A: 10 mM PO<sub>4</sub>, 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 method development*

*Choose the gradient system that best serves your needs without PEG: phosphate or chloride.*

*Conduct the same gradient with 10% PEG-600 included in both the gradient-start, and gradient-end buffers.*

*If enhancement is not sufficient, consider increasing the concentration of PEG or using a larger molecular weight PEG polymer.*



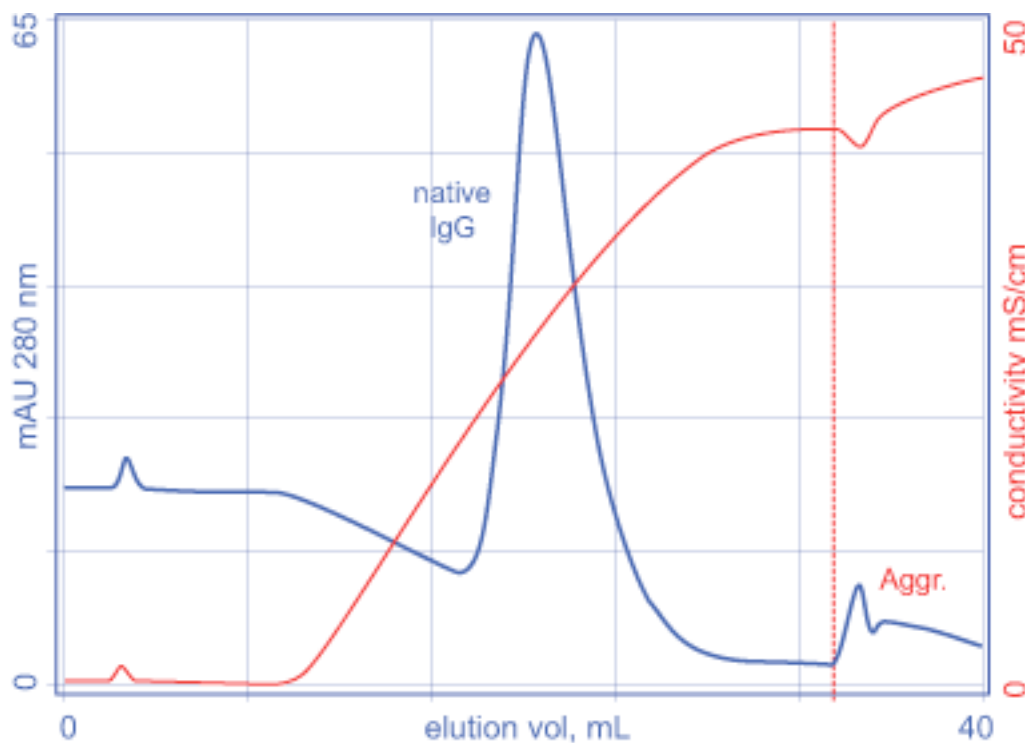
# PEG: +/-

- + *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 about 5x*
  
- *Increases viscosity > backpressure, peak width*
- *Risk of product precipitation at high PEG concentration*
- *Needs to be removed from final product.*



# Emerging areas of investigation

## *IgG aggregate removal in a lactate gradient*

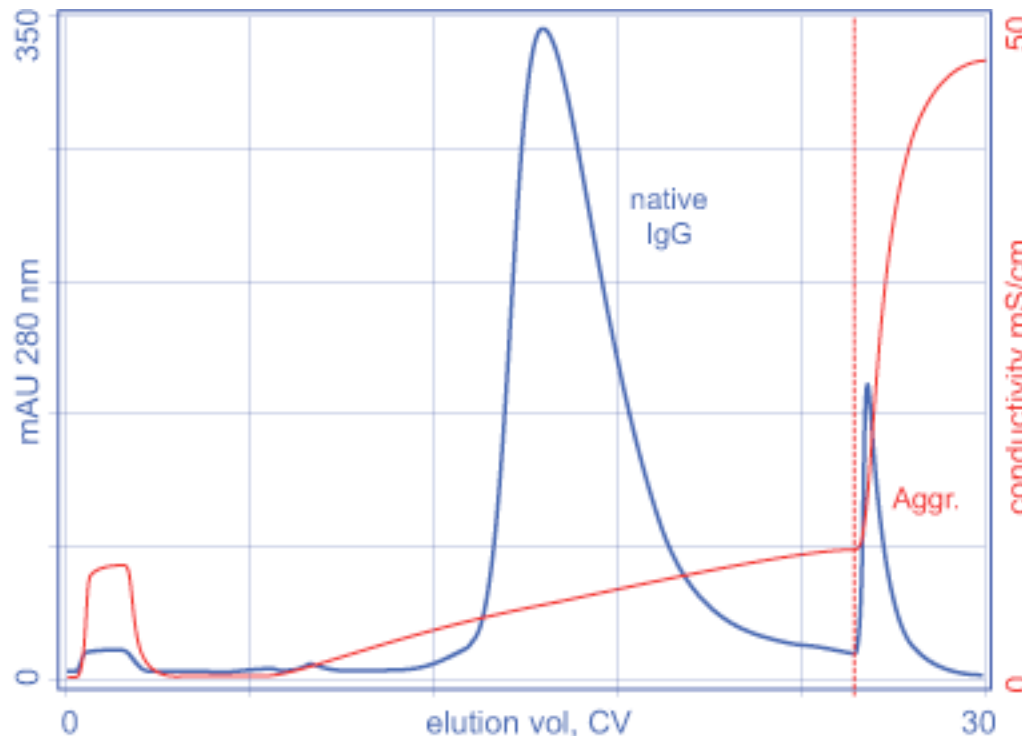


CHT Type I, 40  $\mu$ m  
MediaScout 5 x 50 (1 mL)  
1 mL/min, 200 cm/hr  
A: 20 mM Hepes, pH 7  
B: 20 mM Hepes, 1 M lactic acid, pH 7  
EQ: 100% A  
Inject: 100  $\mu$ L purified IgG containing about 5% aggr.  
Elute: 20 CVLG to 100% B  
Clean: 500 mM NaPO<sub>4</sub>, pH 7

Note that the absence of phosphate may be damaging to HA at pH 7. Note also that the conductivity of 1 M lactate is only about half the conductivity of 1M NaCl.

# Emerging areas of investigation

## IgG aggregate removal in a borate gradient

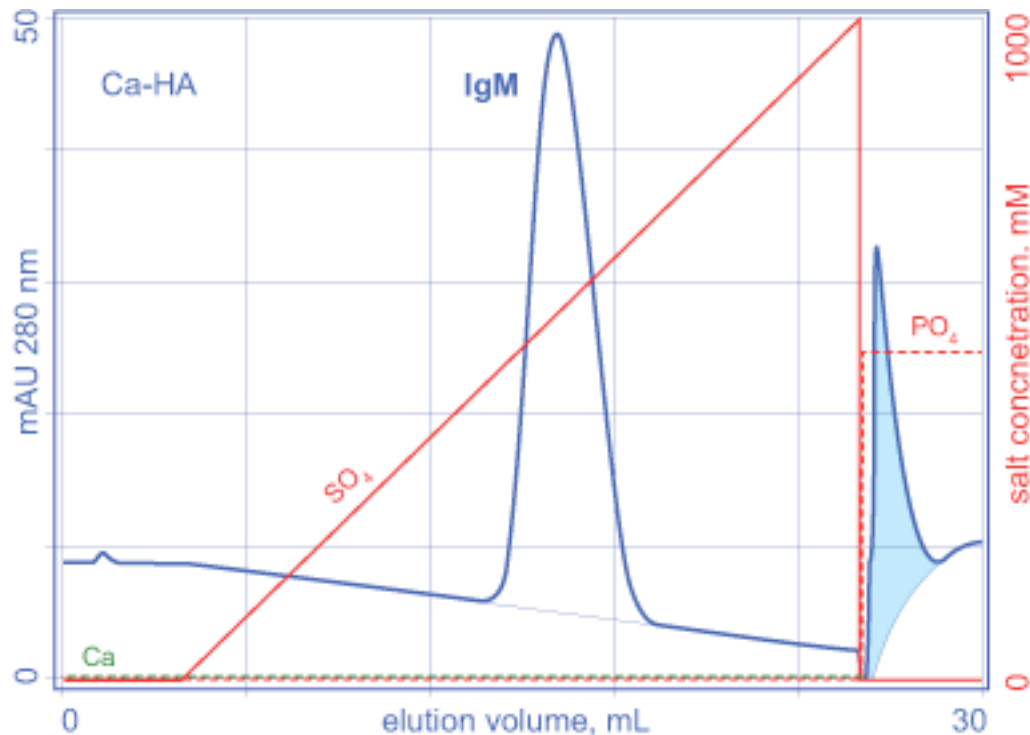


CHT Type I, 40  $\mu$ m  
MediaScout 5 x 50 (1 mL)  
1 mL/min, 200 cm/hr  
A: 20 mM Hepes, pH 7  
B: 20 mM Hepes, 1 M boric acid, pH 7  
EQ: 100% A  
Inject: 100  $\mu$ L purified IgG  
containing about 5% aggr.  
Elute: 20 CVLG to 100% B  
Clean: 500 mM NaPO<sub>4</sub>, pH 7

Note that the absence of phosphate may be damaging to HA at pH 7. Note also that the conductivity of 1 M borate is only about 10% the conductivity of 1M NaCl.

# Emerging areas of investigation

## IgM aggregate removal in a sulfate gradient



CHT Type II, 40  $\mu\text{m}$   
MediaScout 5 x 100 (2 mL)  
0.67 mL/min, 200 cm/hr  
A: 50 mM MES, 3 mM  $\text{CaCl}_2$   
pH 6.7  
B: A + 1M  $\text{Na}_2\text{SO}_4$ , pH 6.7  
EQ: 100% A  
Inject: 100  $\mu\text{L}$  purified IgM  
containing about 15% aggr.  
Elute: 10 CVLG to 100% B  
Clean: 500 mM  $\text{NaPO}_4$ , 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.

# Recommended reading

- Josics, Dj., Loster, K., Kuhl, R., Noll, F., Reusch, J., Purification of monoclonal antibodies by hydroxyapatite HPLC and size exclusion HPLC, *Biol. Chem. Hoppe-Seylars*, 1991 372(3) 149-156
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- Schubert, S., Freitag, R., Investigation of the interaction mechanism of the recombinant human antibody MDJ8 and its fragments with chromatographic apatite phases, *J. Chromatogr. A.*, 2009 1216 3831-3840
- Gagnon, P., Beam, K., Antibody aggregate removal with hydroxyapatite, *Curr. Pharm. Biotechnol.*, 2009, 25 287-293



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*Copies of this presentation can be downloaded at [www.validated.com](http://www.validated.com)*

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