



# ***Nonionic Polymer Enhancement of Aggregate Removal in Ion Exchange and Hydroxyapatite Chromatography***

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# *Aggregate removal*

Aggregate removal is a key determinant of product safety.

It is also an important contributor to process economy.

It may increase the difficulty of process development.

It may limit the selection of polishing methods for a given antibody.

It may increase purification costs.

# Aggregate removal

The simplest and most direct method of aggregate removal is size exclusion chromatography (SEC). It offers the additional benefit of exchanging the product into a buffer ready for chromatography by a subsequent method.

*But*, SEC is slow, provides poor capacity, requires disproportionately large columns that require superior packing skills, and requires large buffer volumes.

This has created strong interest in adsorptive chromatography methods for aggregate removal.



# Aggregate removal

The limitation with adsorptive methods is that their selectivity is not directly related to protein size.

Aggregates tend to be retained more strongly by adsorptive methods than non-aggregated proteins, presumably because they participate in a larger number of interactions with the solid phase.

*But*, variations in charge distribution from one clone to another, and between aggregated and non-aggregated forms of the product, make the degree of separation unpredictable.



# Aggregate removal

## What if...

there was a buffer additive with an effect proportional to protein size?

Would it be compatible with adsorptive chromatography methods?

Would it enhance the ability of adsorptive methods to separate aggregates from non-aggregated antibody?

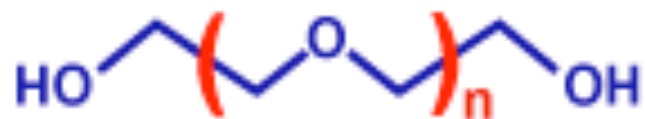
Would it meet regulatory requirements for processing human-injectable products?



# Nonionic polymers and proteins

Nonionic organic polymers are often used as antibody precipitating agents.

Polyethylene glycol (PEG) is one example.



PEG is nontoxic (conjugated to therapeutic proteins), USP, protein-stabilizing, and cheap.

# *Nonionic polymers and proteins*

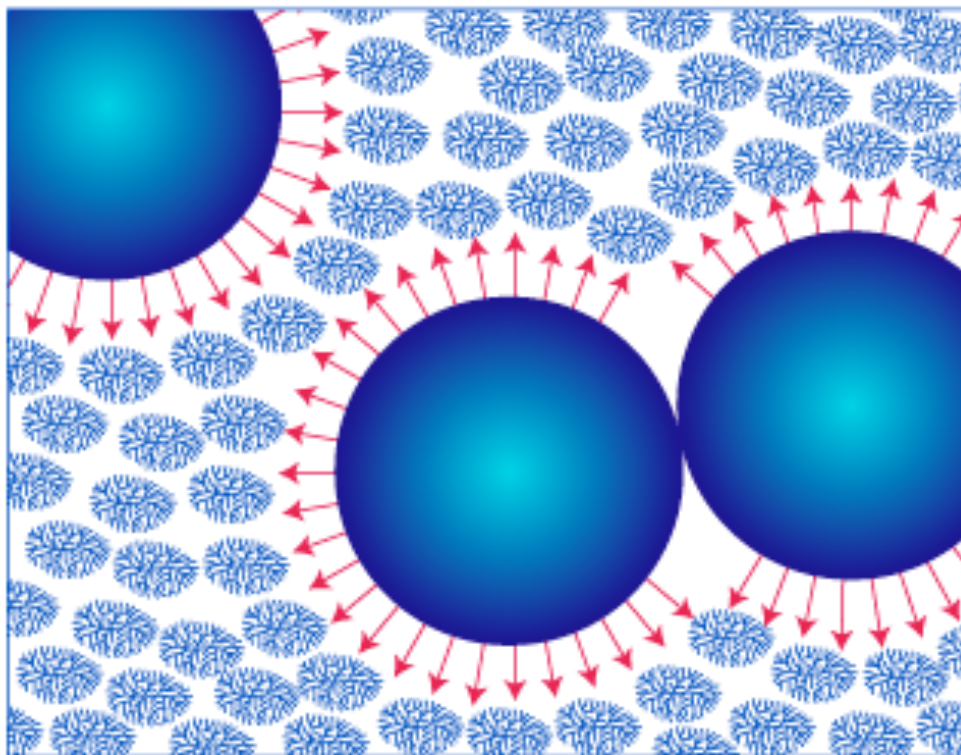
PEG is preferentially excluded from protein surfaces. This creates a pure water hydration sheath around the protein. The discontinuity between the pure water sheath and the PEG-concentrated bulk solvent is thermodynamically unfavorable.

When proteins come into contact in a solution of PEG, they share some hydration water with each other, thereby releasing some back to the bulk solvent. They also present a smaller surface than the combined surface area of the individual proteins. Both phenomena are thermodynamically favorable, and tend to stabilize the association between the proteins.



# *Nonionic polymers and proteins*

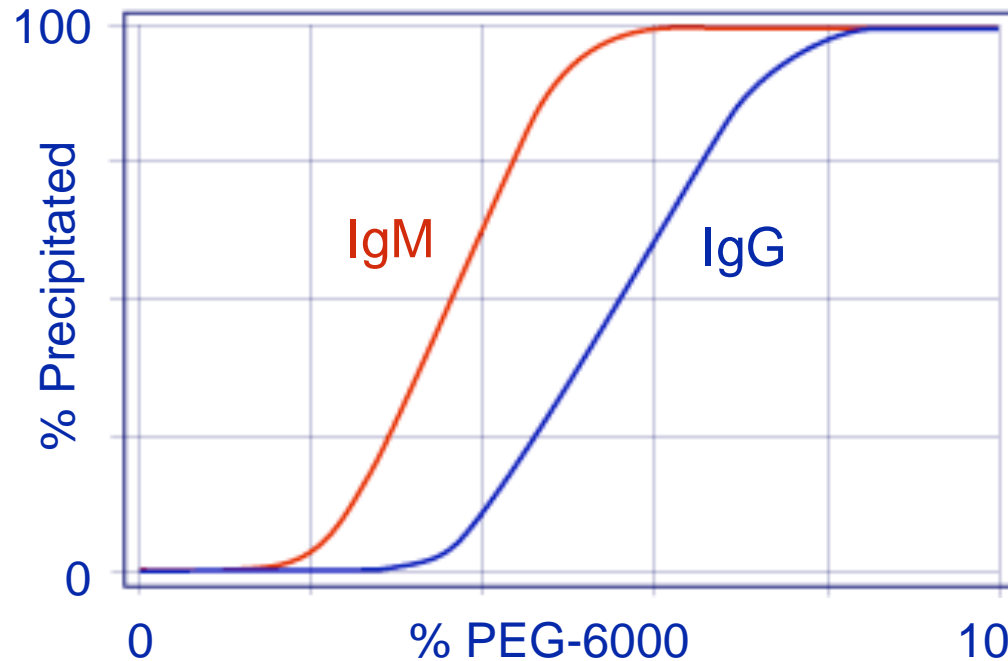
## Preferential exclusion of PEG





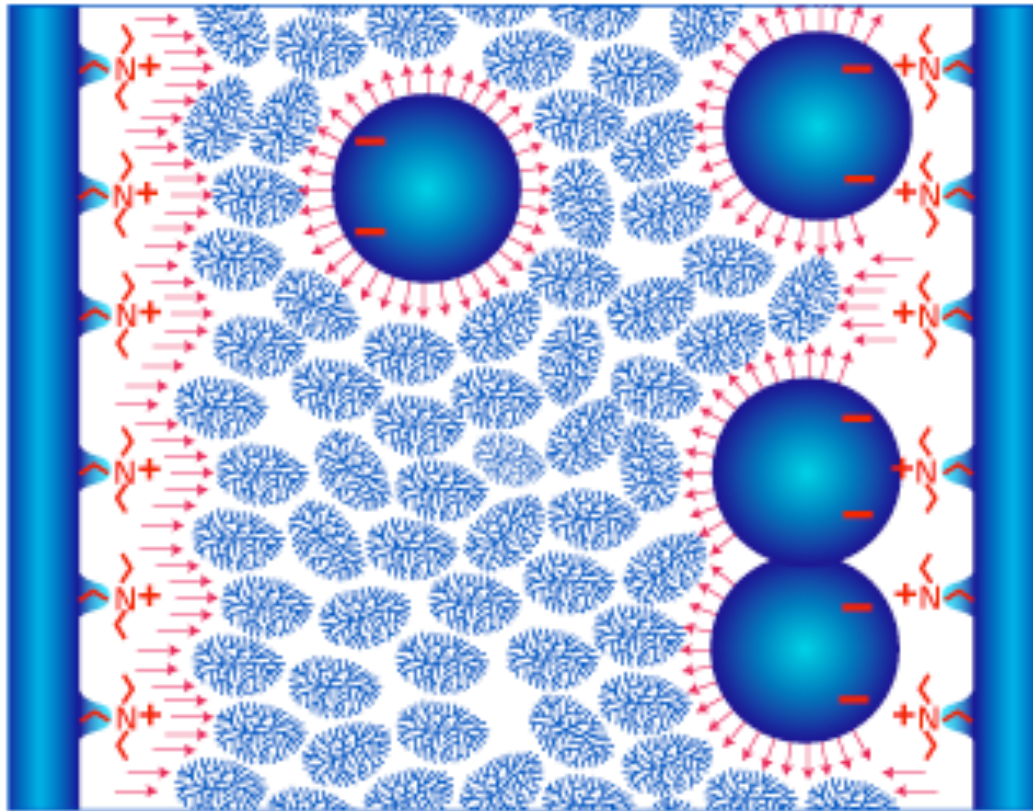
# Nonionic polymers and proteins

Since protein surface area is proportional to protein size, the magnitude of the effect of nonionic organic polymers is proportional to protein size.



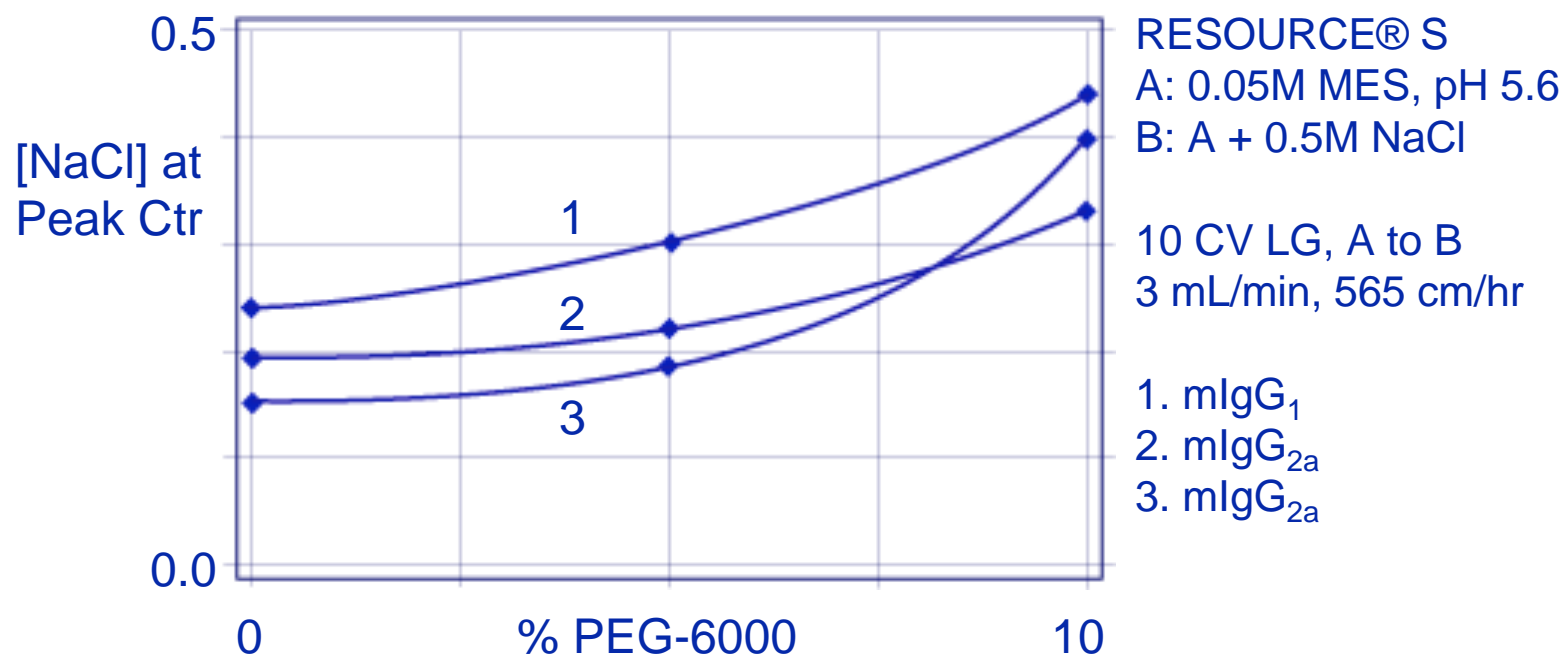
# PEG and ion exchange

Does the exclusionary effect carry over to ion exchange?



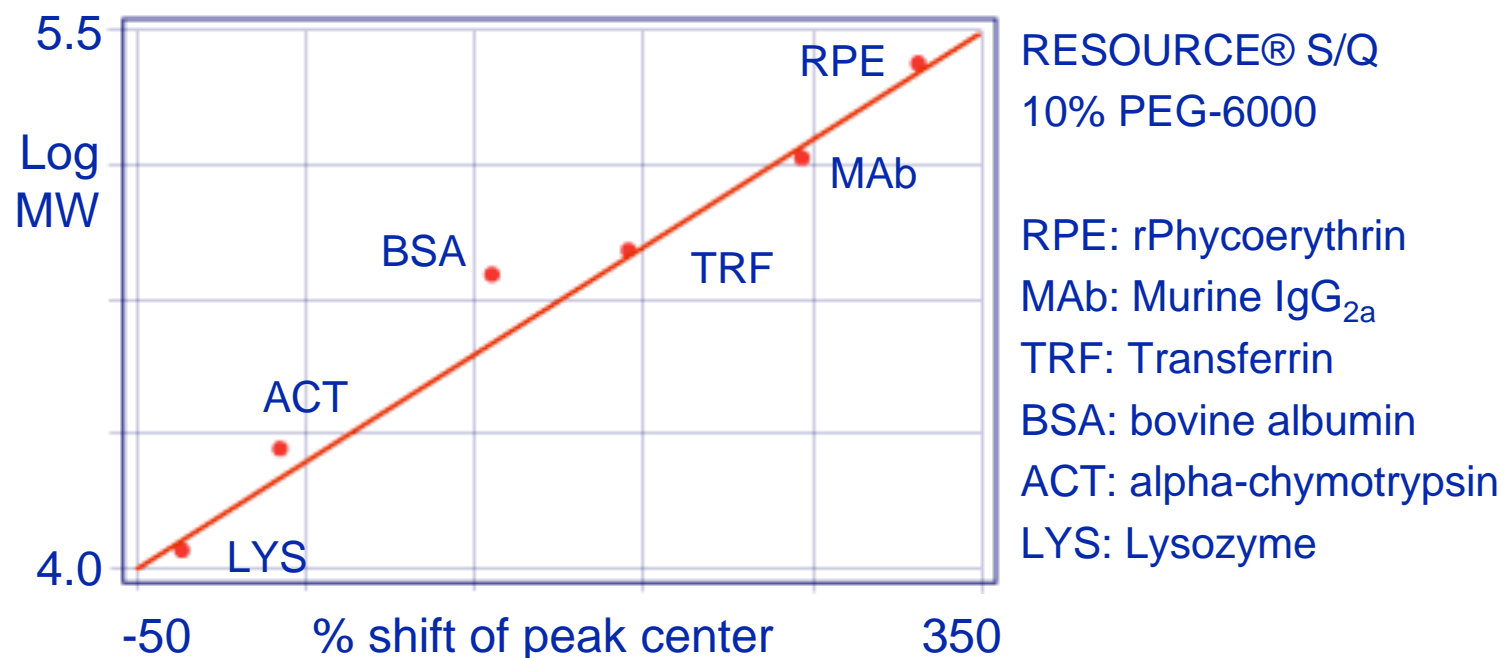
# PEG and ion exchange

Does the exclusionary effect carry over to ion exchange?



# PEG and ion exchange

Does the size selectivity carry over to ion exchange?

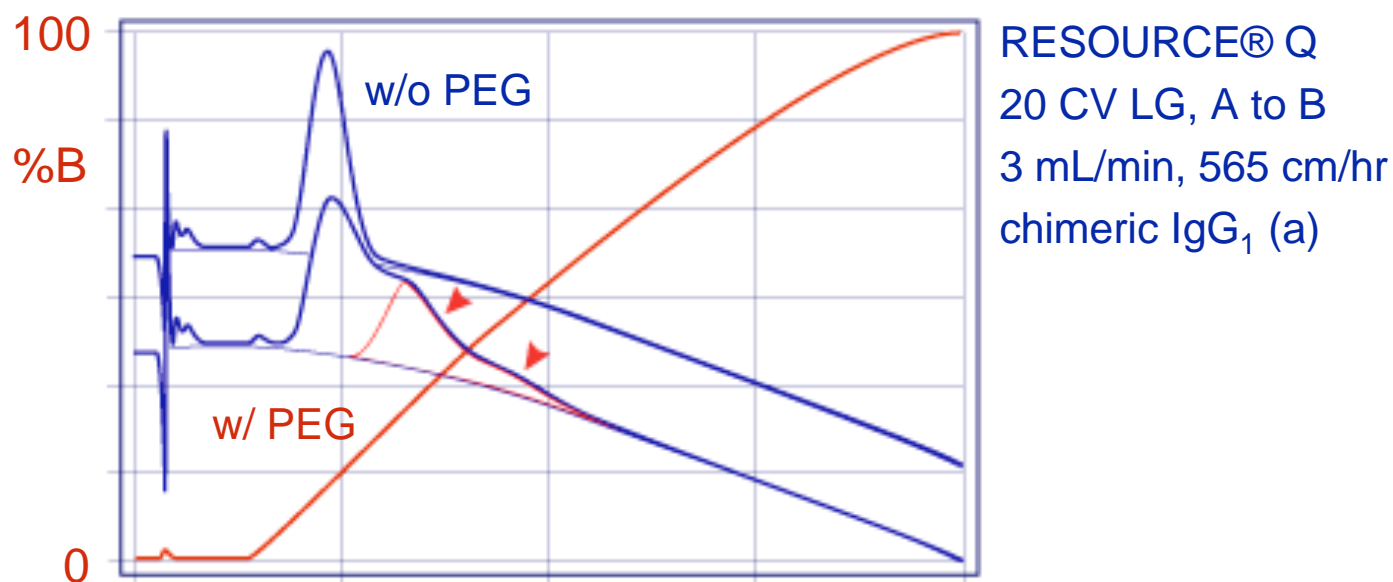


Gagnon et al, 1996, *J. Chromatogr.* **743** 51



# PEG and ion exchange

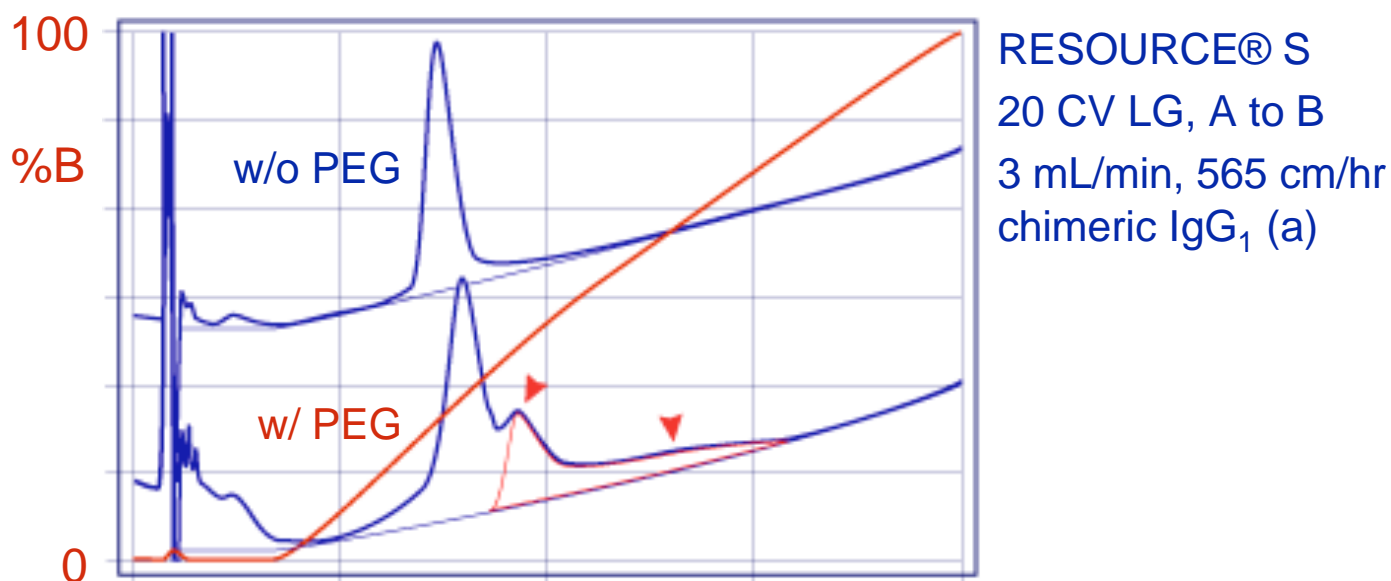
## Aggregate separation by anion exchange



Buffers: A1: 10 mM NaPO<sub>4</sub>, pH 7.5 B1: 500 mM NaPO<sub>4</sub>, pH 7.5  
A2,B2: same as above but both containing 7.5% PEG-4600.

# PEG and ion exchange

## Aggregate separation by cation exchange



Buffers: A1: 10 mM Na citrate, pH 6.0 B1: 200 mM Na citrate, pH 6.0  
A2,B2: same as above but both containing 7.5% PEG-4600.

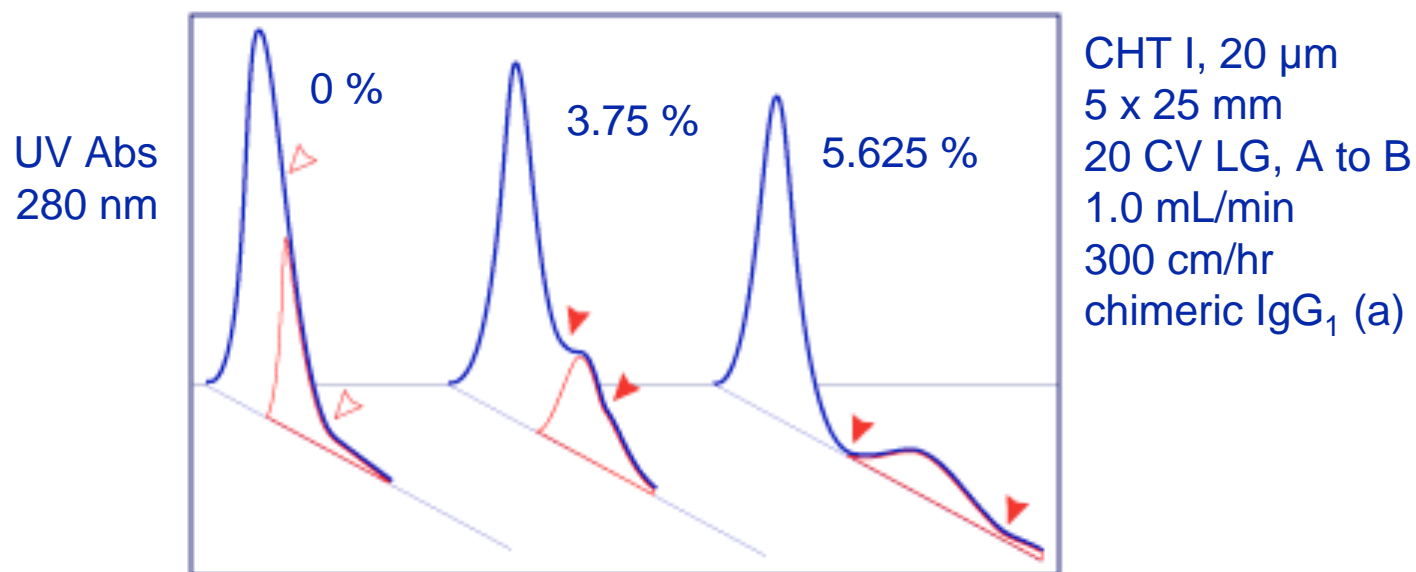
# PEG and hydroxyapatite

Does the exclusionary effect carry over to hydroxyapatite?



# PEG and hydroxyapatite

## Aggregate separation on hydroxyapatite

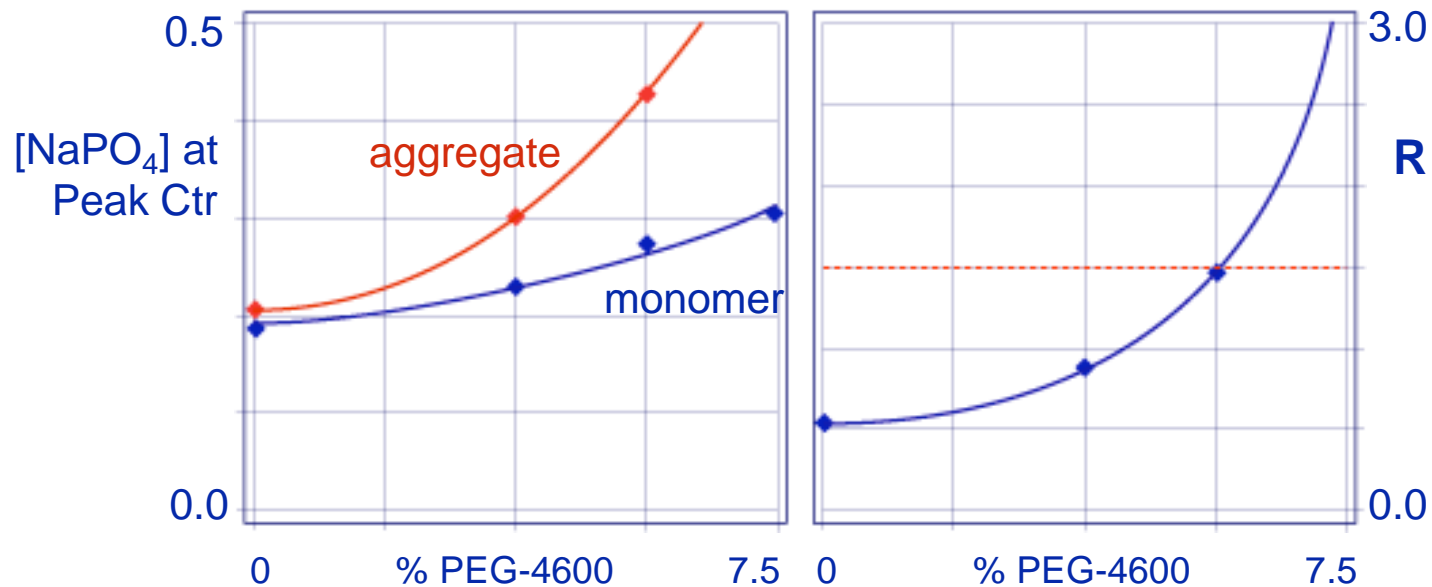


Buffers: A1: 10 mM NaPO<sub>4</sub>, pH 7.0 B1: 500 mM NaPO<sub>4</sub>, pH 7.0  
A2,B2: same as above but both containing 3.75% PEG-4600.  
A3,B3: same as above but both containing 5.625% PEG-4600.



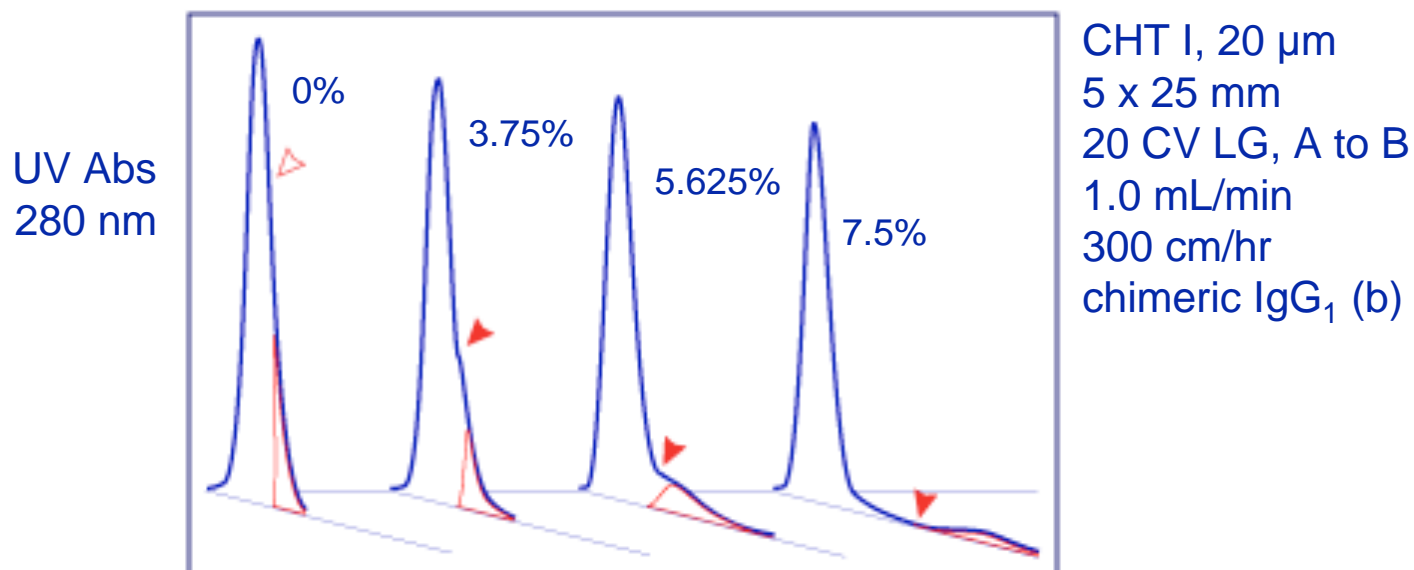
# PEG and hydroxyapatite

Aggregate separation on hydroxyapatite, chimeric IgG<sub>1</sub> (a)



# PEG and hydroxyapatite

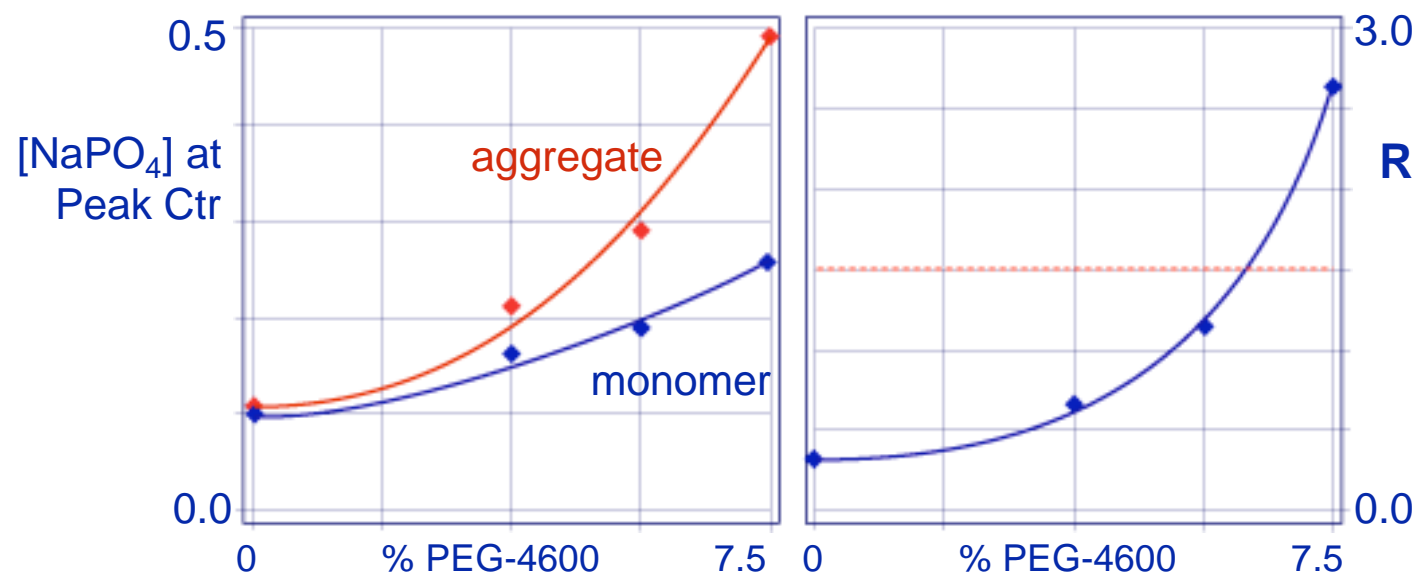
## Aggregate separation on hydroxyapatite



Buffers: A1: 10 mM NaPO<sub>4</sub>, pH 7.0 B1: 500 mM NaPO<sub>4</sub>, pH 7.0  
A2,B2: same as above but both containing 3.75% PEG-4600.  
A3,B3: same as above but both containing 5.625% PEG-4600.  
A4,B4: same as above but both containing 7.5% PEG-4600.

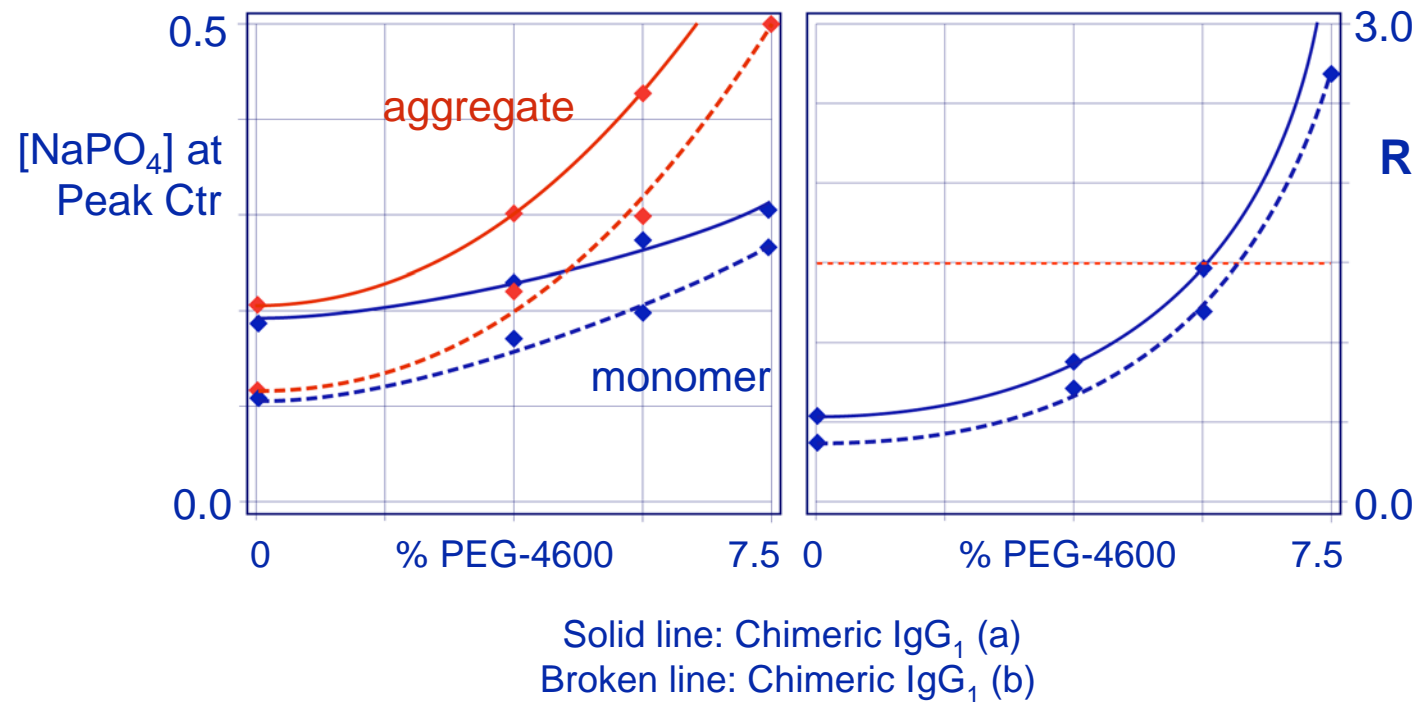
# PEG and hydroxyapatite

Aggregate separation on hydroxyapatite, chimeric IgG<sub>1</sub> (b)



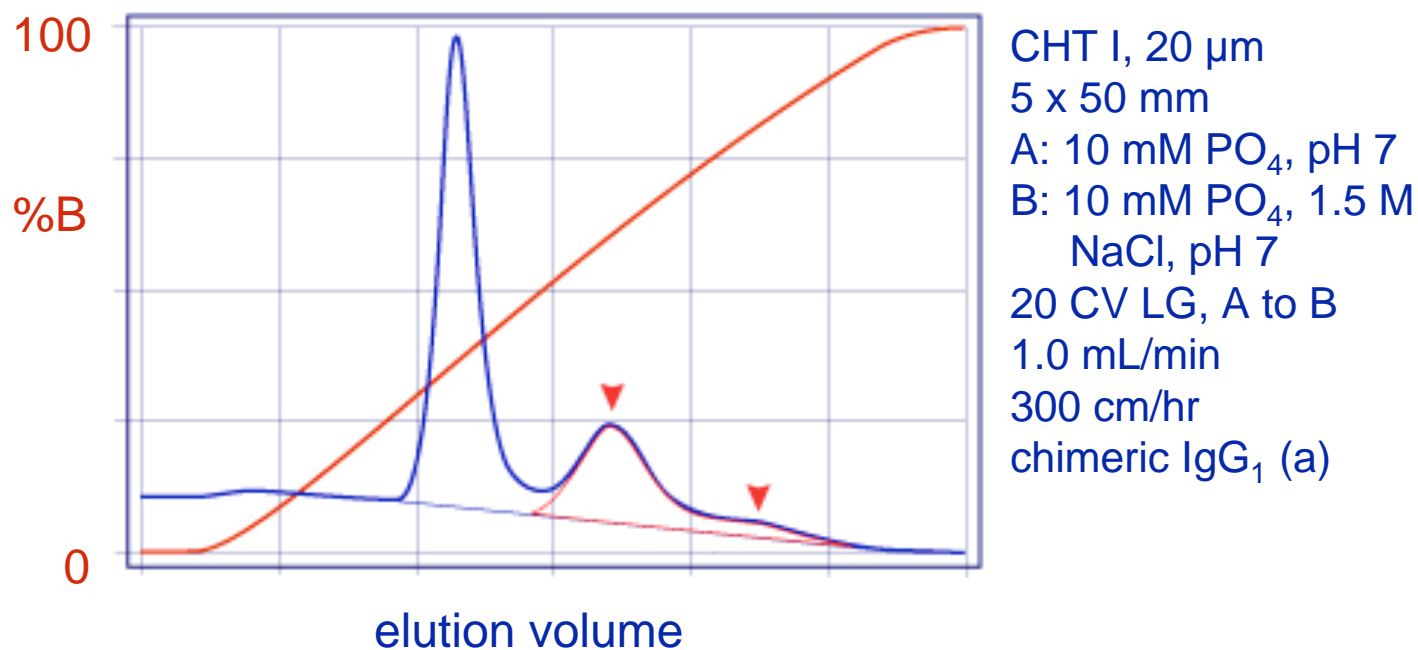
# PEG and hydroxyapatite

Aggregate separation on hydroxyapatite, chimera (a) vs (b)



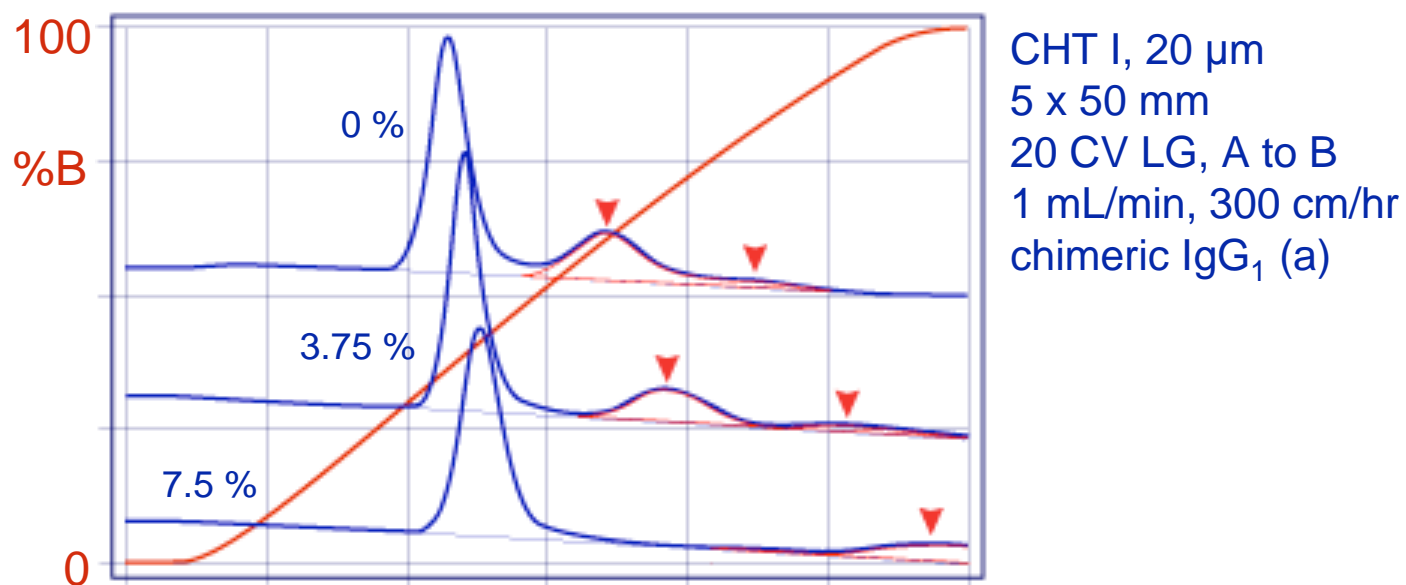
# NaCl and hydroxyapatite

Aggregate separation on hydroxyapatite, NaCl gradient



# PEG, NaCl, and hydroxyapatite

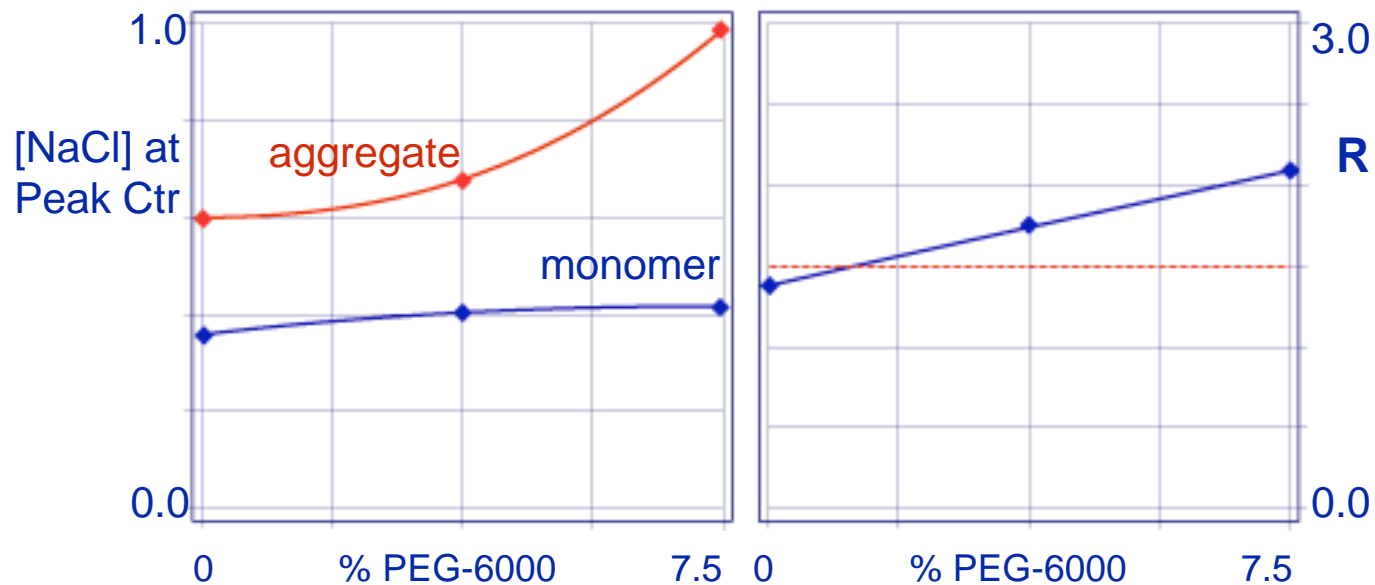
## Aggregate separation on hydroxyapatite



Buffers: A1: 10 mM NaPO<sub>4</sub>, pH 7.0 B1: 500 mM NaPO<sub>4</sub>, pH 7.0  
A2 = A1. B2: same as B1 except containing 3.75% PEG-6000.  
A3 = A1. B3: same as B1 except containing 7.5% PEG-6000.

# PEG, NaCl, and hydroxyapatite

Aggregate separation on hydroxyapatite, chimeric IgG<sub>1</sub> (a)



# *What to expect with other contaminants*

Host cell proteins are generally smaller than IgG. PEG should increase their retention to a lesser degree. This should give better clearance in cases where they normally elute before IgG.

DNA, endotoxin, and virus are generally larger than IgG. PEG should increase their retention to a greater degree. This should give better clearance in cases where they normally elute after IgG.





# *Method development*

Including PEG in only the gradient endpoint buffer simplifies screening and method development.

It is also more “generic” in the sense of accommodating a wider diversity of antibodies.

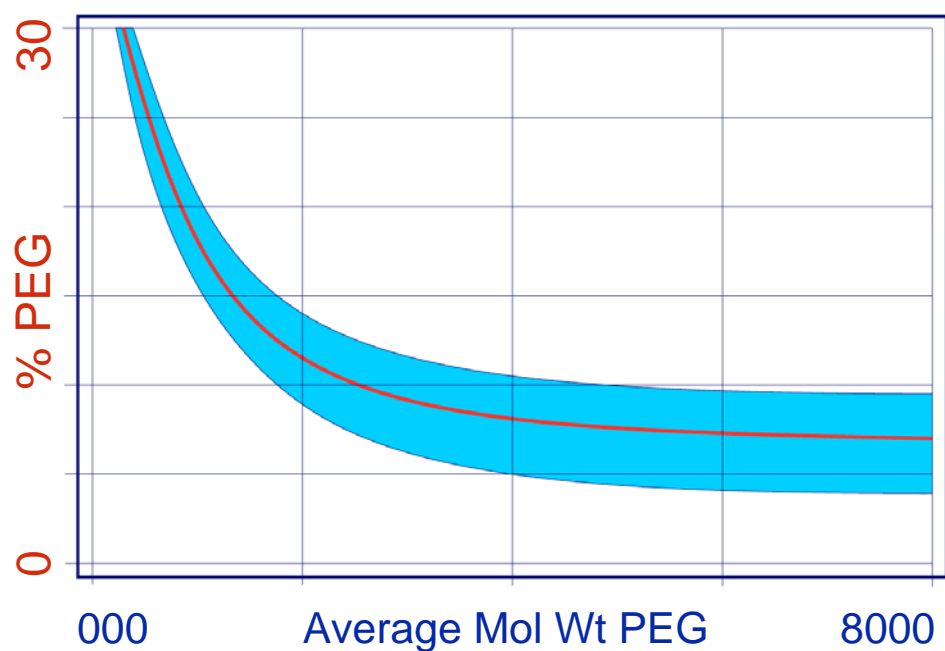
It also appears to give better results. The aggregate experiences a higher concentration of PEG than the earlier eluting monomer, thereby compounding aggregate retention and the degree of separation.

This approach also leaves the monomer fraction with a lower concentration of PEG to remove.



# Method development

## Effectivity of different PEG sizes



Approximate range of PEG concentration vs PEG molecular weight for enhancement of aggregate separation. The red trace indicates recommended starting concentrations where PEG is applied as described in the previous slide.



# Method development

## Ion exchange

At the chosen pH, run a 10 CV linear gradient to 0.5M NaCl, 7.5% PEG-6000.\*

Determine salt concentration at the point where antibody monomer is completely eluted.

Rerun the gradient to end at that salt concentration + 20%, 7.5% PEG-6000 (no need to add PEG to the equilibration buffer).

Adjust conditions as desired and convert to step gradient or flow-through format.

\* or higher percentage of lower MW PEG



# Method development

## Hydroxyapatite

At the chosen pH, run a 10 CV linear gradient to 0.5M NaPO<sub>4</sub>, 7.5% PEG-6000.\*

Determine phosphate concentration at the point where antibody monomer is completely eluted.

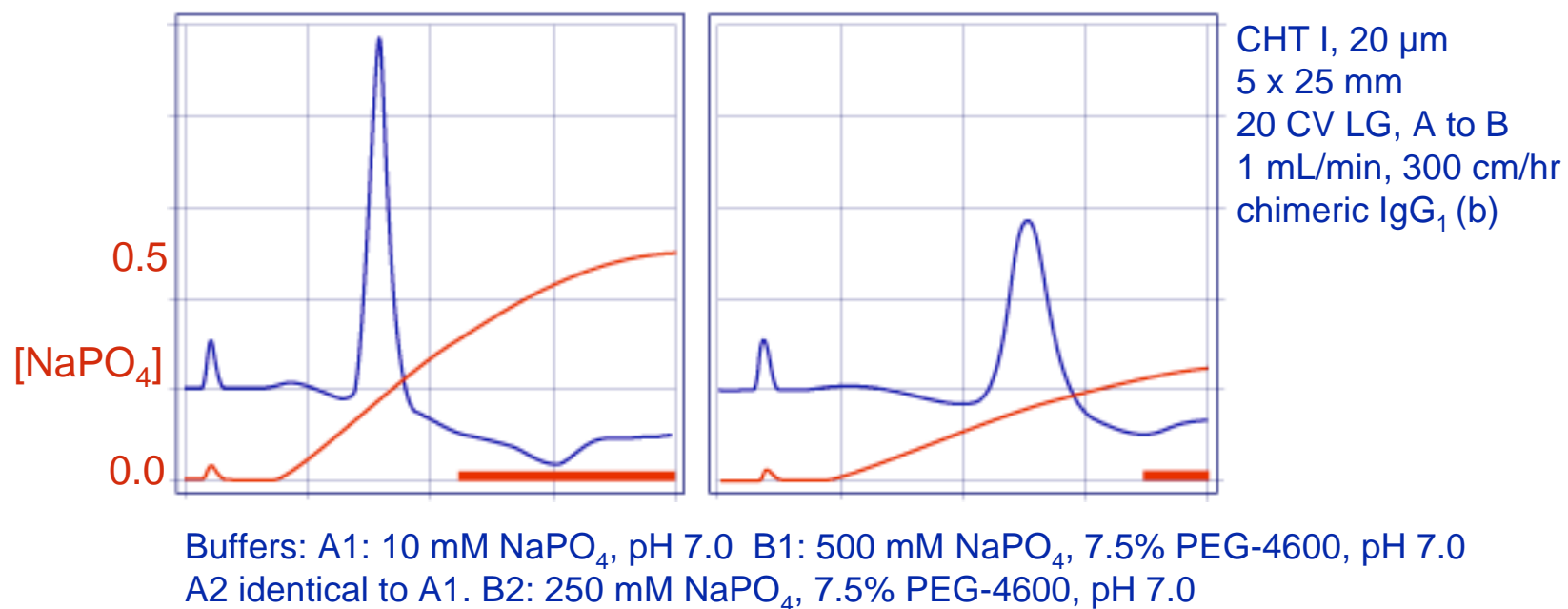
Rerun the gradient to end at that phosphate concentration + 20%, 7.5% PEG-6000 (no need to add PEG to the equilibration buffer).

Adjust conditions as desired and convert to step gradient or flow-through format.

\*or higher percentage of lower MW PEG

# Method development

## Scouting chromatograms, hydroxyapatite



# Method development

## Hydroxyapatite (optional)

If a sodium chloride gradient has been used to enhance aggregate removal, measure the NaCl concentration at the point where antibody monomer is completely eluted.

Run the gradient to end at that sodium chloride concentration + 20%, 7.5% PEG-6000\* (no need to add PEG to the equilibration buffer).

Adjust conditions as desired and convert to step gradient or flow-through format.

\* or higher percentage of lower MW PEG



# *How to remove PEG*

PEG is inert to most adsorption mechanisms.

It is easily removed by binding the protein of interest to the solid phase of choice and letting the PEG flow through.

Low molecular weight PEG may be removed by diafiltration or size exclusion chromatography.



# *Negative features of PEG*

PEG depresses antibody solubility.

PEG increases buffer viscosity:

Higher backpressure

Lower protein diffusivity

Residual high molecular weight PEG  
may alter selectivity of analytical  
HPSEC.



# Conclusions

PEG imposes a “size discriminating” selectivity on ion exchange and hydroxyapatite chromatography.

This dramatically enhances aggregate removal efficiency and should enhance removal of other important contaminants classes, especially including viral particles.



# Conclusions

While PEG's size discrimination ability is technically secondary to the primary selectivity of the solid phase, it is functionally dominant.

Thus it produces roughly equivalent enhancement on both anion and cation exchangers. The magnitude of enhancement is greater on hydroxyapatite, but the sizing factor is still apparent.

These results demonstrate that PEG functions similarly over a wide range of pH and conductivity, and suggest that it will probably improve aggregate clearance with other mixed mode methods.



# Conclusions

On hydroxyapatite, PEG/phosphate gradients offer better aggregate clearance than NaCl gradients at fixed low phosphate concentration.

Combination PEG/NaCl gradients at fixed phosphate offer better clearance than NaCl gradients at fixed phosphate, but NaCl depresses the magnitude of enhancement compared with PEG/phosphate gradients.

These results suggest that PEG provides *double enhancement* on hydroxyapatite by acting cooperatively with both its phosphoryl cation exchange *and* calcium metal affinity binding mechanisms.



# Acknowledgements

Thanks to GE Healthcare for providing RESOURCE® ion exchangers, Bio-Rad Laboratories for providing ceramic hydroxyapatite CHT™, and Atoll for providing pre-packed columns of CHT.

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

