New Insights into IgG Binding and Aggregate Removal with Hydroxyapatite

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Trends in antibody production

Cell culture productivity of monoclonal antibodies has increased dramatically in recent years.

200 to 500 mg of antibody per liter was formerly considered high productivity, and many commercial products are based on such production levels.

Expression levels of 1-2 g/L are now common, 5-10 g/L is anticipated to be broadly achievable, and productivity to 30 g/L has been documented.

Trends in antibody production

These increases stress the capacity of downstream processing methods, but capacity may not be the biggest challenge.

Aggregate levels greater than 20%, as well as substantial fragment loads, are consistently observed in cell cultures producing antibody in the vicinity of 5 g/L.

Cell cultures producing antibody in the vicinity of 10 g/L contain more than 50% aggregates and fragments.

Higher production levels seem likely to compound the challenge.

Trends in antibody purification

Ion exchange and HIC have a history of effectively removing aggregate levels in the 1-3% range, sometimes modestly more, and are effective for removing similar fragment loads.

Recently introduced charged/hydrophobic mixed modes have exhibited similar capabilities.

Presentations and publications have documented the ability of HA to reduce aggregate loads from 40-60%, to less than 0.1%, even with preparative loads of 20-50 mg IgG/mL HA. HA has also proven effective for removal of fragments, HCP, leached protein A, DNA, endotoxin, and virus.

Trends in antibody purification

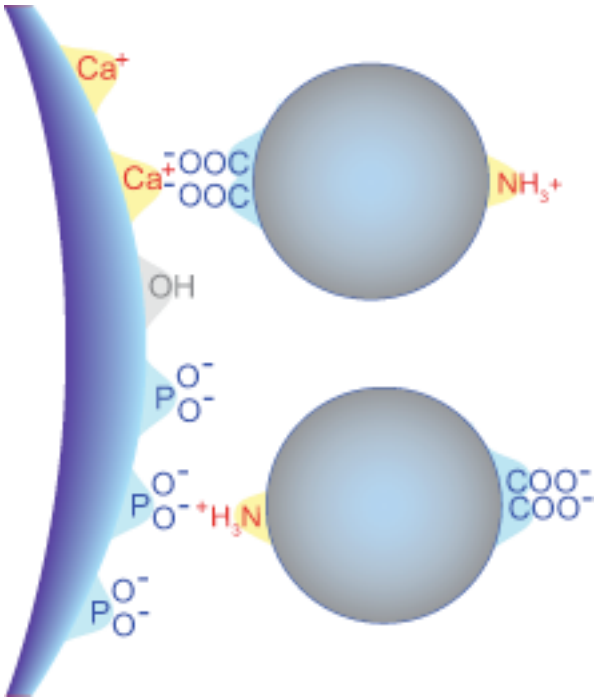
HA's unique capabilities raise the question of how it achieves these results.

IgG binding to HA has been described generally as exploiting a combination of phosphoryl cation exchange and calcium metal affinity, but this does not explain why it is so effective for aggregate removal — nor how it might be improved.

This presentation offers new data that provide more detailed definition of IgG binding to HA. The results suggest a hypothesis that may explain why HA is so effective for aggregate removal, and an elution strategy that may provide better results than presently known methods.

Hydroxyapatite

Primary retention mechanisms on HA



Calcium metal affinity. Protein carboxyl clusters form chelation bonds in the same way as the carboxyl doublets on EDTA. These bonds are stronger than ionic bonds and often survive exposure to high concentrations of 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 with HA phosphate. As with traditional carboxy- or sulfo- cation exchangers, protein binding becomes weaker with increasing pH and/or conductivity.

The positive charge on HA calcium is theoretically capable of anion exchange interactions with single carboxyls but this has not been shown to contribute significantly to protein binding.

Experimental design

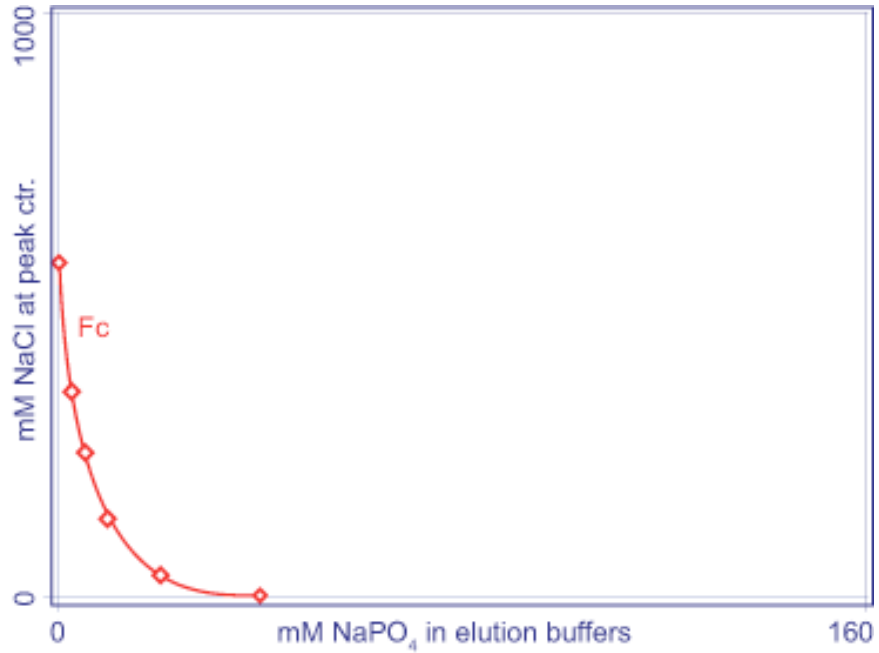
Retention of intact monoclonal IgG, plus Fc and Fab fragments from the same antibody, were mapped on native HA over a range of sodium phosphate and sodium chloride concentrations.

This was done by conducting sodium chloride gradients at level phosphate concentration, over a range of phosphate concentrations.

CHT™ type I, 20 µm, 1.0 mL; MediaScout® 5 x 50 mm; 1.0 mL/min (300 cm/hr).
Equilibrate: 50 mM Hepes, pH 7.0; Inject: 50 µL papain-digested hIgG$_1$ Mab; Wash: 50 mM Hepes, pH 7.0; Elute: 20 CV LG to 50 mM Hepes, 1.0 M NaCl, pH 7.0; Clean: 500 mM NaPO$_4$ pH 7.0.
Repeat with equal increments of phosphate added to the equilibration and eluting buffers .

Retention mapping

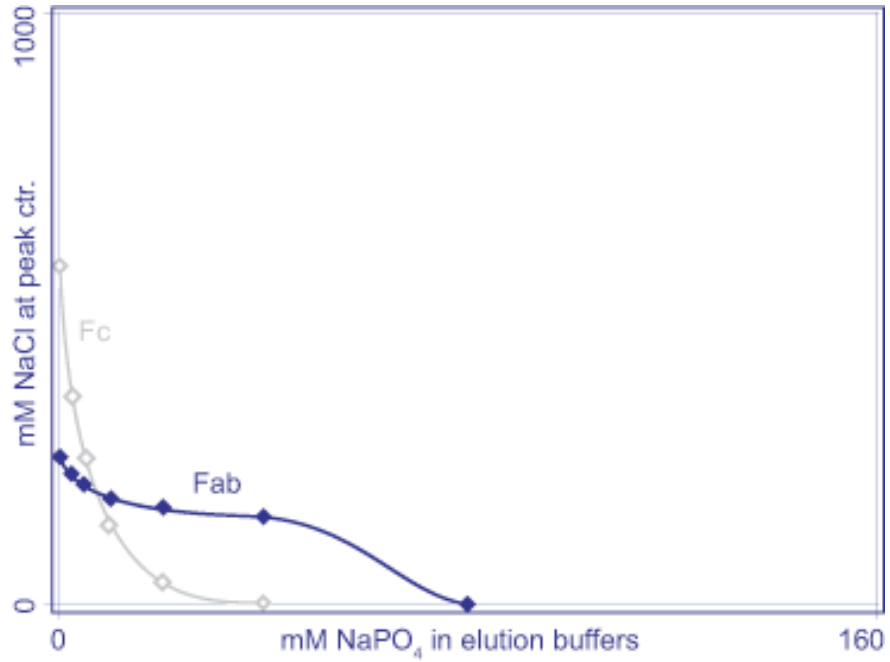
Fc retention in NaCl gradients at level phosphate concentrations



Fc requires more than 500 mM NaCl for elution in the absence of phosphate but loses more than half its binding strength in 10 mM phosphate, and fails to bind in 40 mM phosphate. This indicates that binding is dominated by calcium affinity, but that calcium affinity itself is weak. The shallowing of the slope form 20 to 40 mM may indicate a weak contribution by phosphoryl cation exchange.

Retention mapping

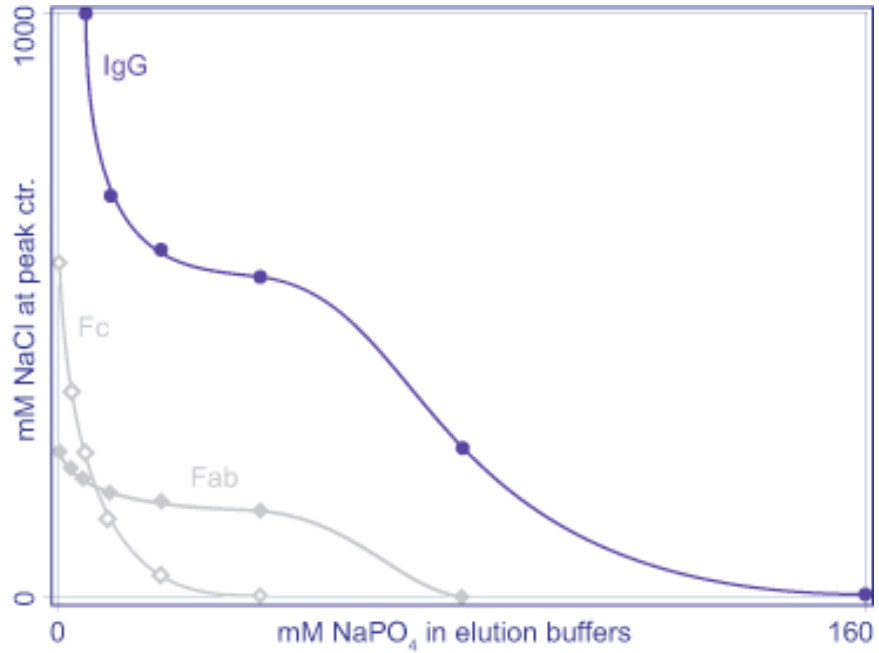
Fab retention in NaCl gradients at level phosphate concentrations



Fab elutes at about 250 mM phosphate in the absence of phosphate, indicating a weaker contribution by calcium affinity than with Fc. In addition, binding is reduced by only about 30% at 40 mM phosphate, demonstrating that it is less responsive to phosphate than Fc. This relative resistance to phosphate indicates the influence of another mechanism which is understood to be phosphoryl cation exchange. The cation exchange component is eventually eluted by the conductivity of phosphate at 80 mM.

Retention mapping

IgG retention in NaCl gradients at level phosphate concentrations



The IgG retention curve is not only biphasic but clearly embodies the retention characteristics of both Fc and Fab. The curves are not merely additive however. IgG is much more strongly retained than either of the fragments.

Cooperative binding

Cooperativity between phosphoryl cation exchange and calcium metal affinity is important in the retention of intact IgG and its fragments.This is apparent in the biphasic retention curves of each solute.

Cooperativity is emphasized by the fact that the curve shapes for both Fc and Fab are apparent in the IgG curve, and by the fact that IgG is retained more strongly than the combined retention values for the fragments.

This suggests that aggregate separation is likewise a result of cooperativity: aggregates have a larger binding footprint than monomers, and therefore require stronger elution conditions.

Cooperative binding

If cooperativity is the key to preferential retention of aggregates, it makes sense that aggregate separation should be enhanced in an elution buffer system that maintains a balance of both binding mechanisms.

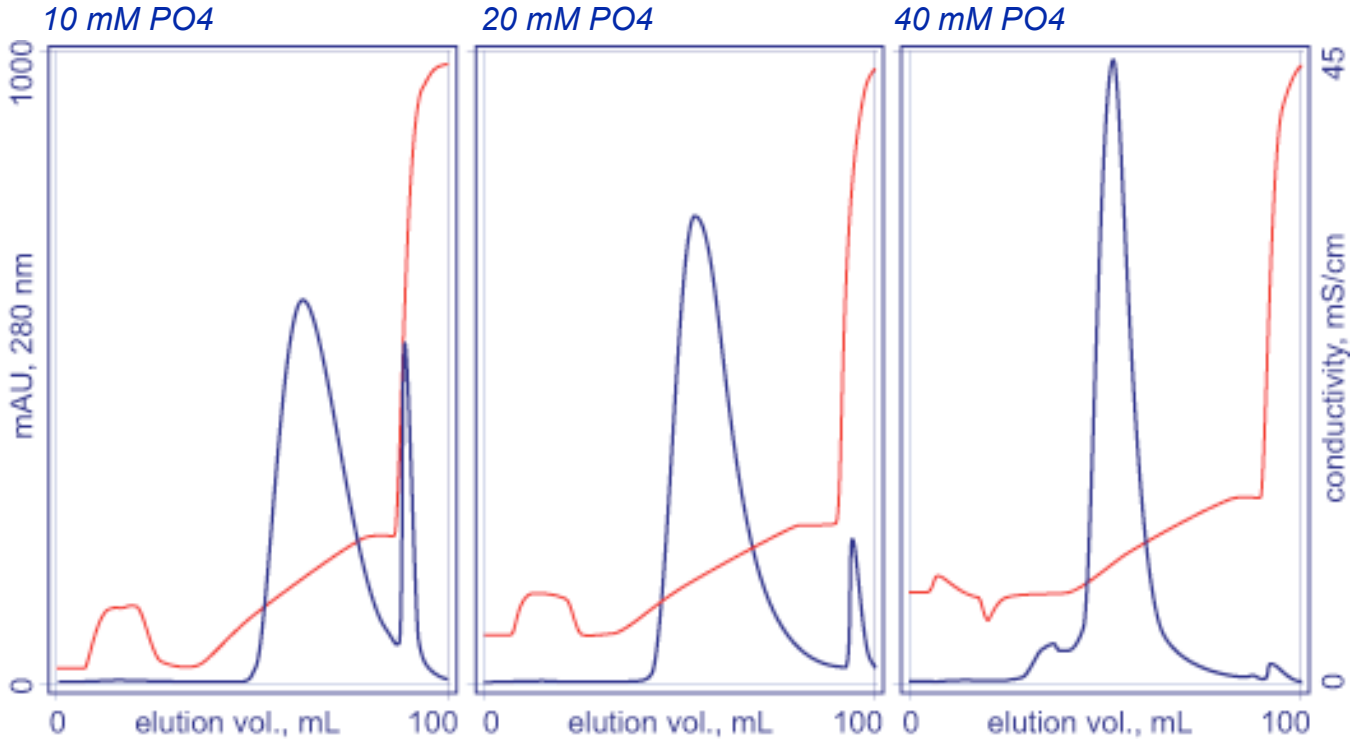
Phosphate has too high a calcium affinity.

Chloride has too high a conductivity, about 90 mS/cm/mole.

Borate offers a possible compromise. Its calcium affinity is substantially less than phosphate, and the conductivity of 1.0 M borate is only about 9 mS/cm at pH 7.0.

Gradient comparison

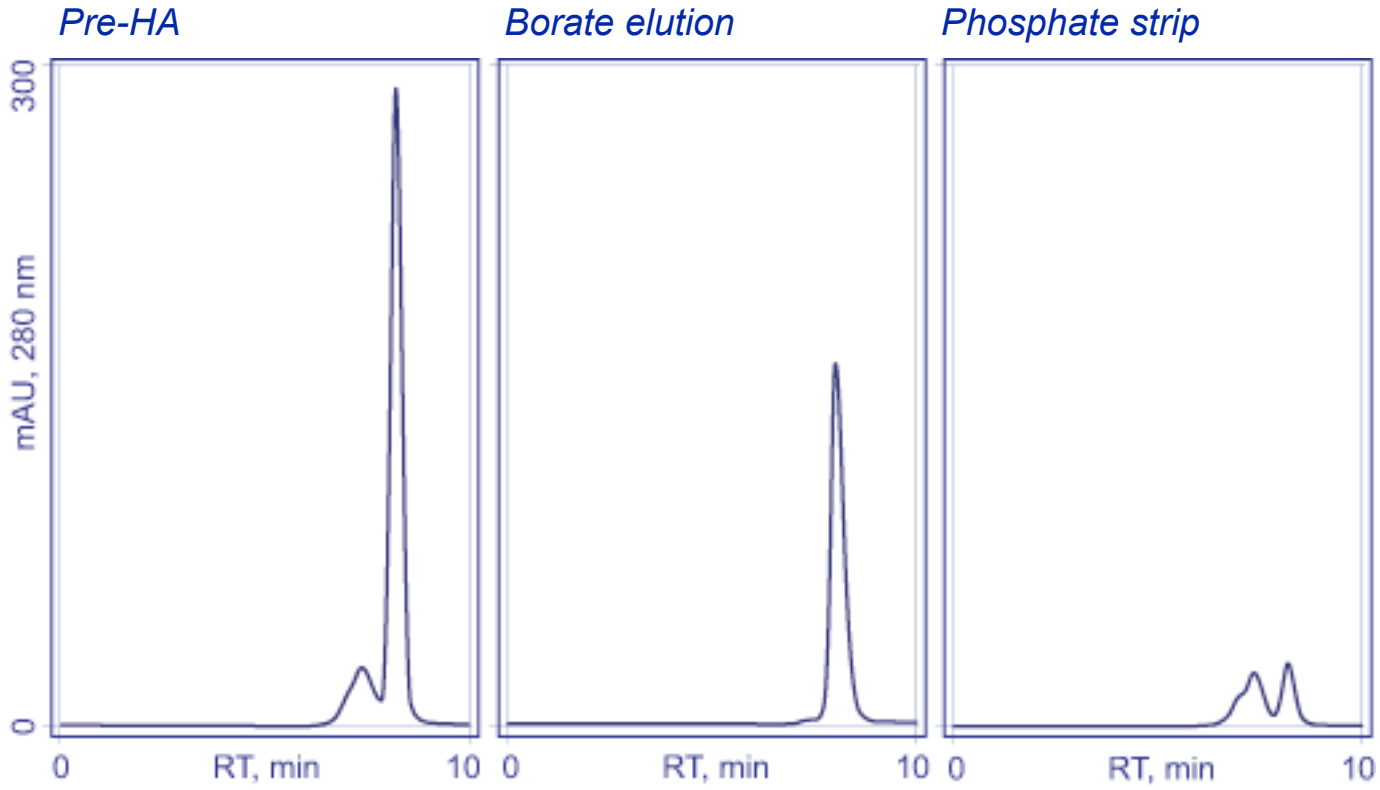
Comparison of borate gradients at different phosphate concentrations



CHT type I, 20 µm, 2.5 mL; MediaScout 8 x 50 mm; 2.5 mL/min (300 cm/hr). Equilibrate: indicated phosphate concentration, pH 7.0; Inject: protein A purified hIgG$_1$ Mab; Wash; Elute: 20 CV LG to indicated phosphate plus 500 mM borate, pH 7.0; Clean: 500 mM NaPO$_4$.

Aggregate removal

Analytical SEC from the borate gradient at 20 mM phosphate



All borate gradients achieved equivalent aggregate removal. A chloride gradient at 5 mM phosphate was also effective. Phosphate gradients were not.

Summary

Phosphate gradients have been shown to be effective for removal of aggregates and fragments from some IgG monoclonal antibodies, but not for most.

Chloride gradients appear to be effective for the majority of IgGs, including preparations that contain up to 60% aggregates. They have also been shown to offer more effective removal of HCP, leached protein A, DNA, endotoxin and virus.

Summary

Preliminary data suggest that borate gradients may represent the most broadly applicable approach.

Aggregates were removed from all clones that were served by phosphate or chloride gradients.

In addition, aggregates were removed from clones that were not accommodated by either phosphate or chloride gradients.

The low conductivity of borate allows the IgG to be applied to a subsequent ion exchange step with only pH titration.

Summary

Experience to date suggests a 3-step approach for developing aggregate removal methods:

1) Run a phosphate gradient to identify the highest concentration at which the antibody does NOT elute.

2) Run a linear gradient to 500 mM borate while holding that phosphate concentration constant.

3) Refine the conditions and convert to a step or flow-through format.

Acknowledgements

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