

Practical Issues in the Industrial use of Hydroxyapatite for Purification of Monoclonal Antibodies

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Why use CHT for MAb Purification?

| Contaminant | Method | Clearance |
|--------------------|---------------|------------------|
| Aggregates | HPSEC | 1-2 logs |
| Protein A | Cygnus | 1-2 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 |
| PPV | Infectivity | > 1 log |

NaCl gradients at constant phosphate concentration

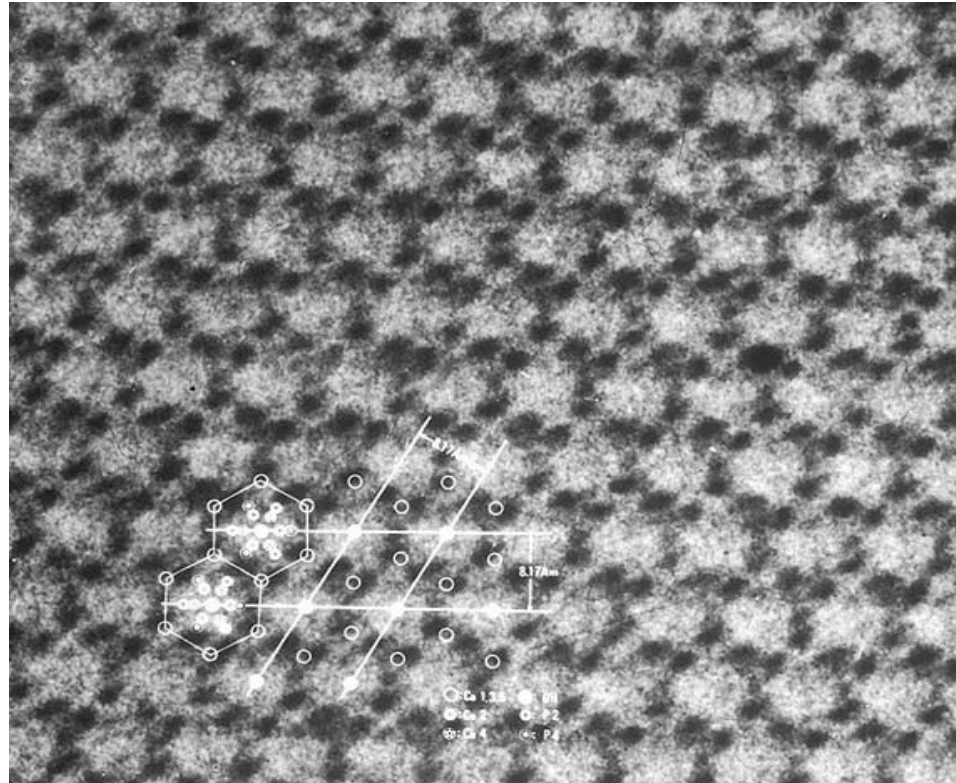


What is CHT?

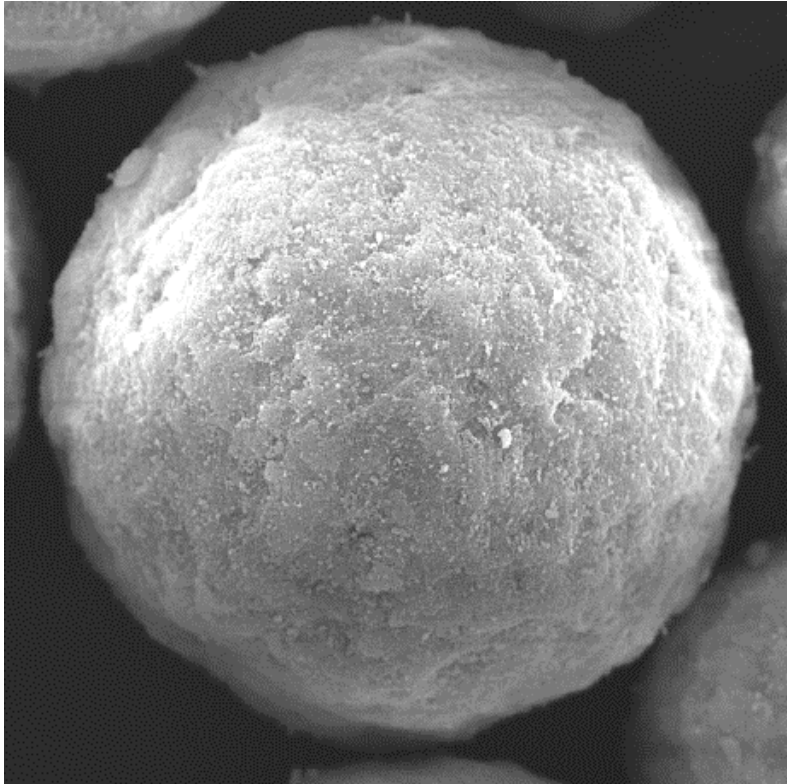
1. Hydroxyapatite is a crystalline mineral of calcium and phosphate with the structural formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$
2. Sets of 5 calcium doublets (C-sites) and pairs of phosphate triplets (P-sites) are arranged in a repeating geometric pattern.
3. Unlike most other chromatography adsorbents, CHT is both the ligand *and* the support matrix.
4. Hexagonal cross section nanocrystals are agglomerated and fused into porous ceramic spheres at high temperature to form a stable chromatography adsorbent.



CHT lattice structure



CHT particle structure



CHT type I, 40 μm

Sinter temp: 400°C

pore diam: ~600–900Å

Surface area: ~40 m²/g

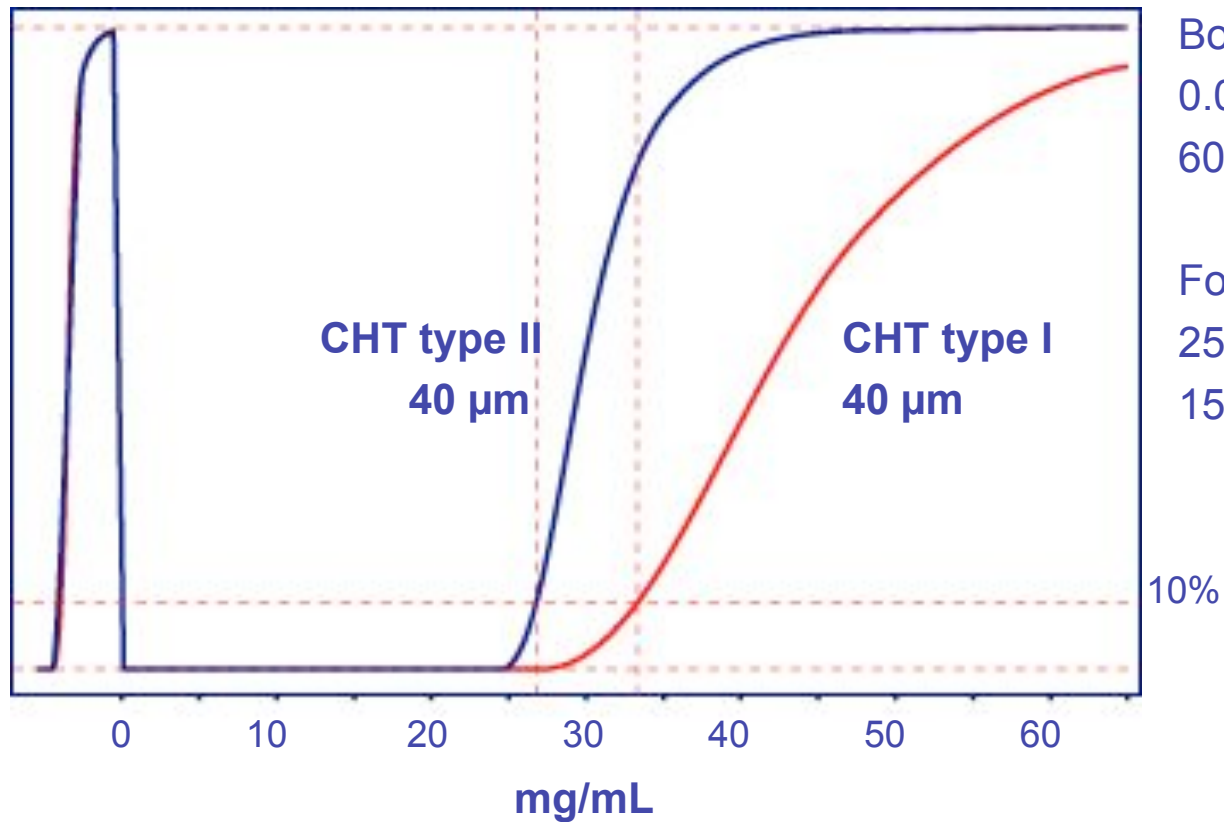
CHT type II, 40 μm

Sinter temp: 700°C

pore diam: ~800–1200Å

Surface area: ~19 m²/g

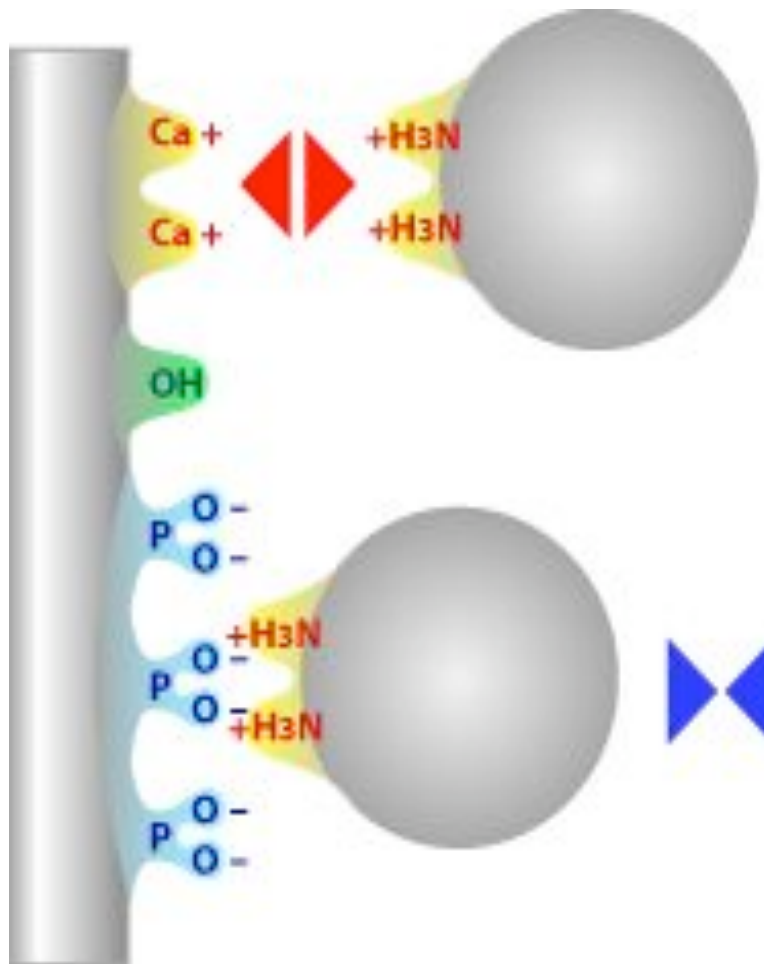
Choice of CHT media



Bovine IgG, 1mg/mL
0.05M MES, pH 6.5
600 cm/hr

For IgG MAbs:
25 - 60mg/mL CHT I
15 - 25 mg/mL CHT II

How CHT works



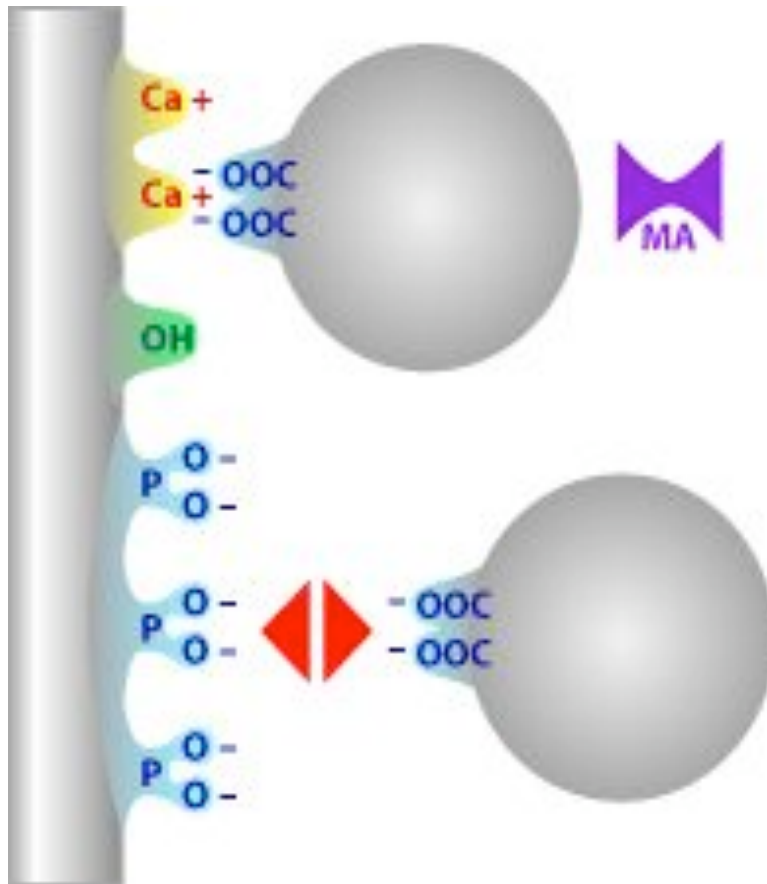
Amino residues

Classical cation exchange

Dissociate with neutral salts like sodium chloride or with buffering salts like phosphate.

Weaken or dissociate with increasing pH

How CHT works



Carboxyl clusters

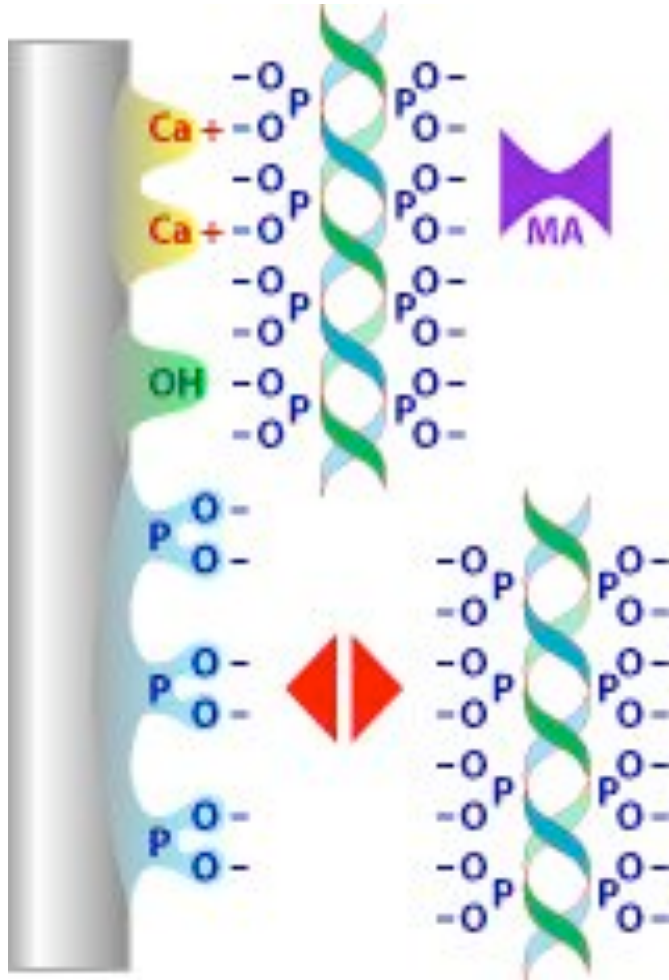
Calcium chelation modulated
by ion exclusion

15–60x stronger than ionic
interactions alone

Will not dissociate at any
concentration of sodium
chloride

Dissociation requires
phosphate

How CHT works



Phosphoryl residues

Calcium coordination
modulated by ion exclusion

15–60x stronger than ionic
interactions alone

NaCl causes *stronger* DNA
binding by suppressing charge
repulsion between phosphates

Dissociate with phosphate

How CHT works with IgG

Most published applications report the use of phosphate gradients for IgG purification.

Phosphate gradients simultaneously dissociate calcium affinity and cation exchange, but do not permit individual control of the two mechanisms.

Recent experience indicates that more effective contaminant clearance can be achieved with sodium chloride gradients at constant low phosphate concentrations.



How CHT works with IgG

Most IgG monoclonals have weak affinity for CHT calcium but fairly strong charge interactions with CHT phosphates.

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. This elutes monomeric IgG. Aggregates elute at higher sodium chloride concentrations.

Contaminants with a strong calcium affinity remain bound to the column until it is cleaned with concentrated phosphate. These include leached protein A-IgG complexes and phosphorylated contaminants such as DNA, endotoxin, and lipid enveloped viruses.



How CHT works with IgG

Chloride versus phosphate gradients

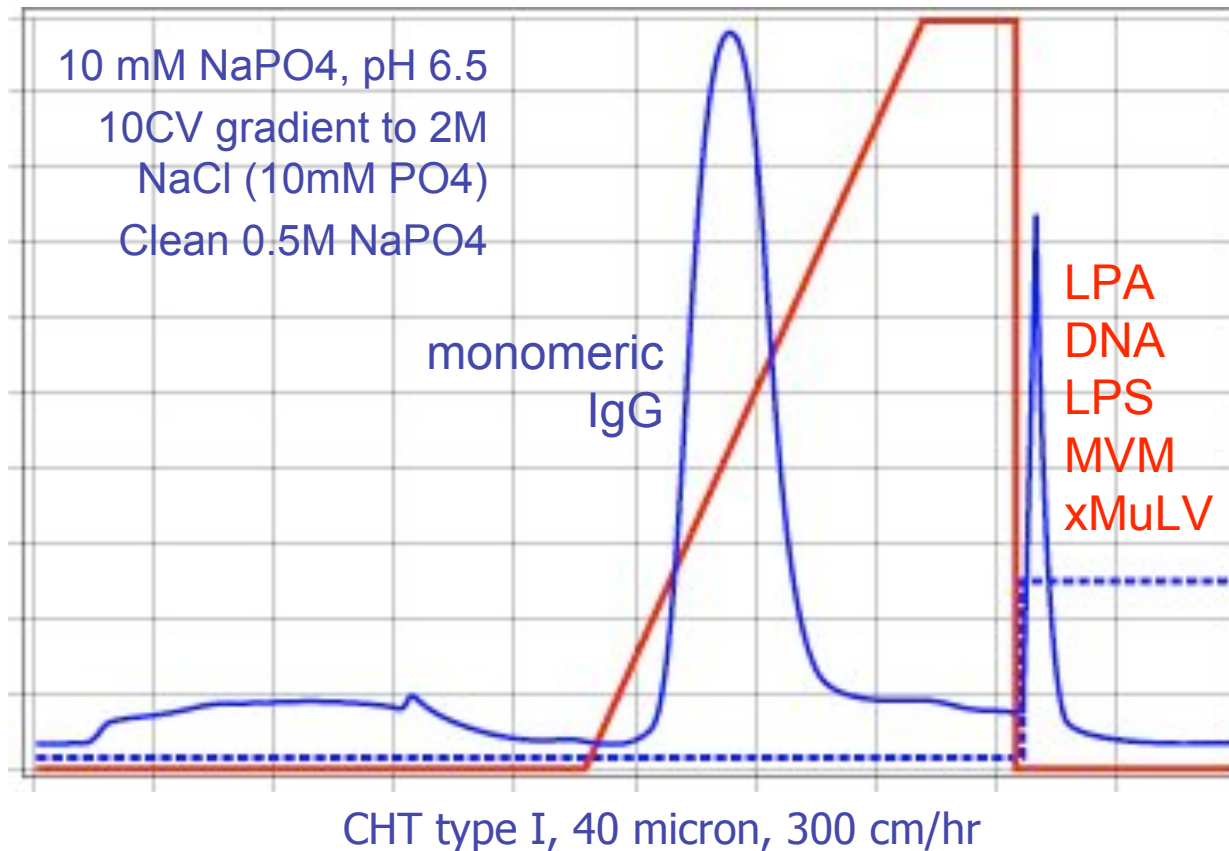
| Parameter | Chloride | Phosphate |
|----------------|-------------|-------------|
| Monomer recov. | 82% | 78% |
| Aggregate | < 1% | < 1% |
| Protein A | < 1 ppm | < 1 ppm |
| CHOP | <12 ppm | < 72 ppm |
| DNA | < 1ppm | < 7 ppm |
| Endotoxin | < 0.1 EU/mL | < 5.0 EU/mL |

Human/mouse IgG1 chimera



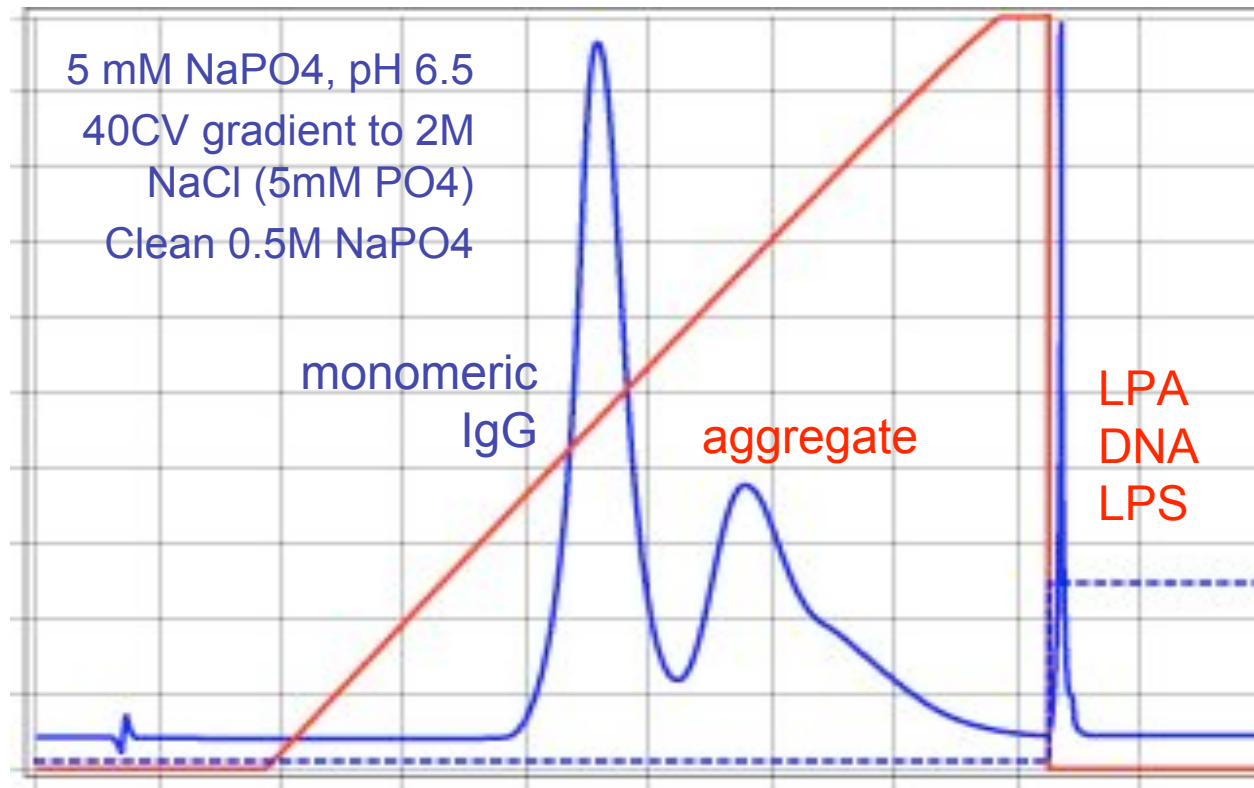
How CHT works with IgG

protein A purified human IgG1



How CHT works with IgG

protein A purified IgG1 chimera

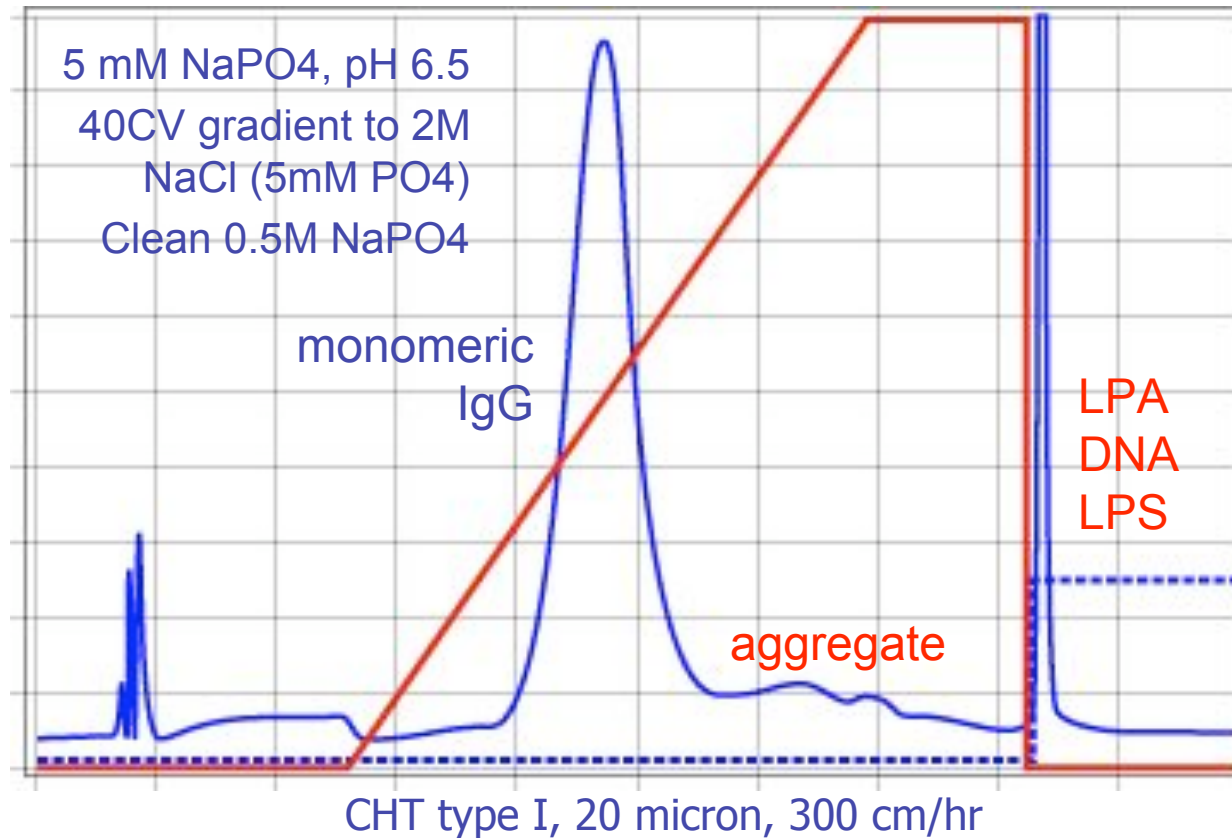


CHT type I, 20 micron, 300 cm/hr



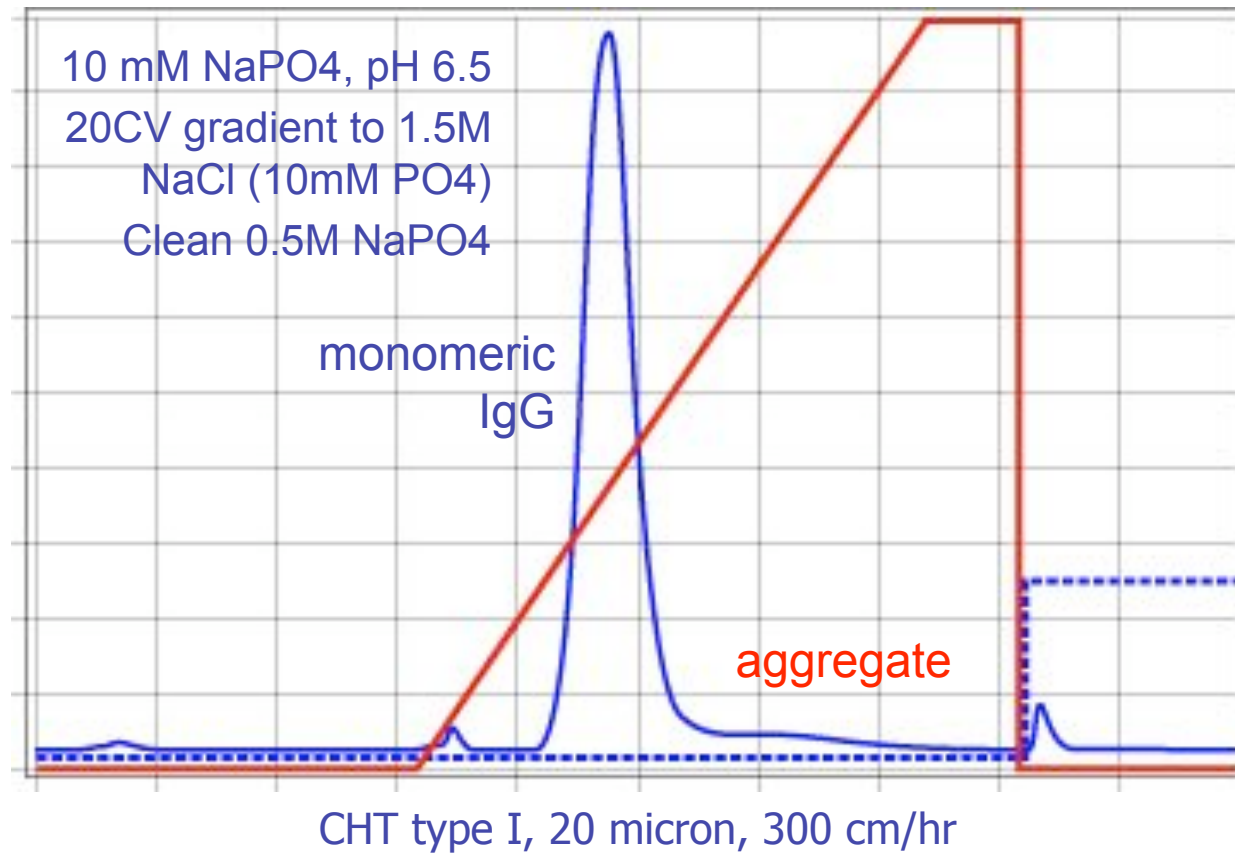
How CHT works with IgG

protein A purified human IgG1



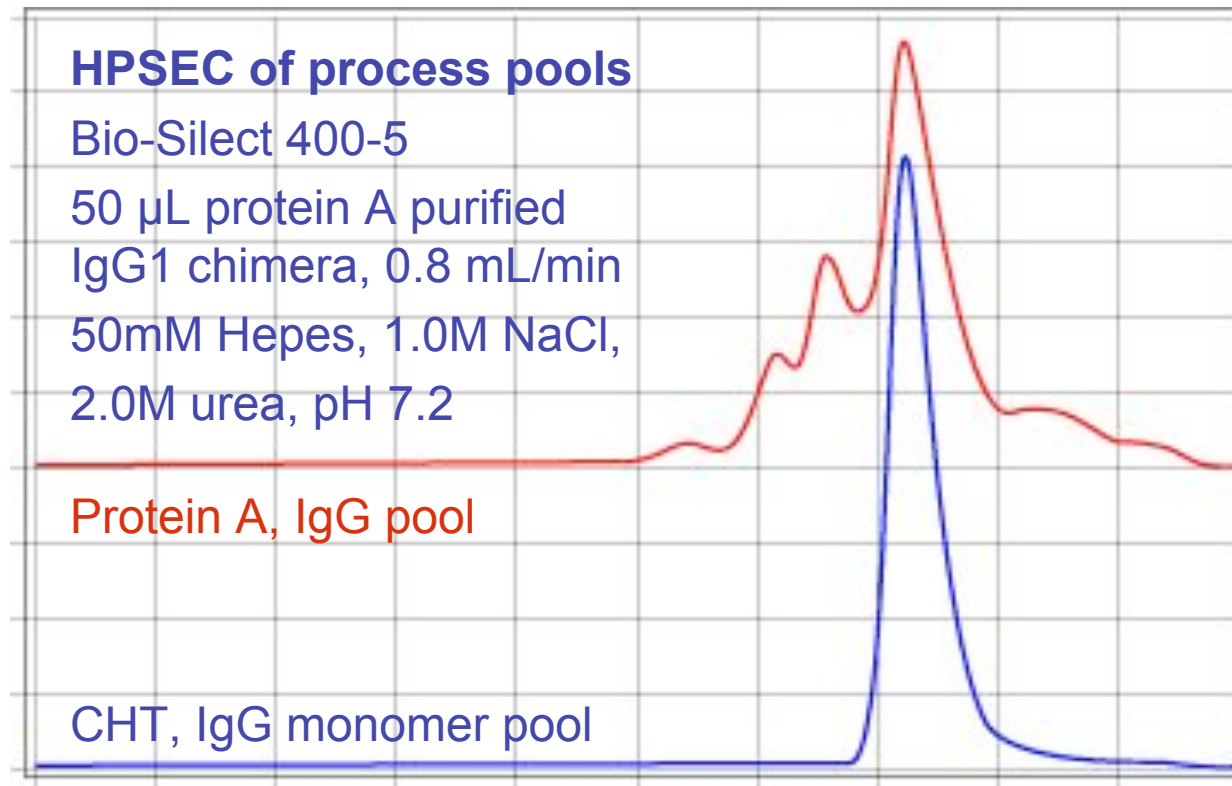
How CHT works with IgG

protein A purified mouse IgG1



How CHT works with IgG

Aggregate clearance with NaCl gradients



CHT method development

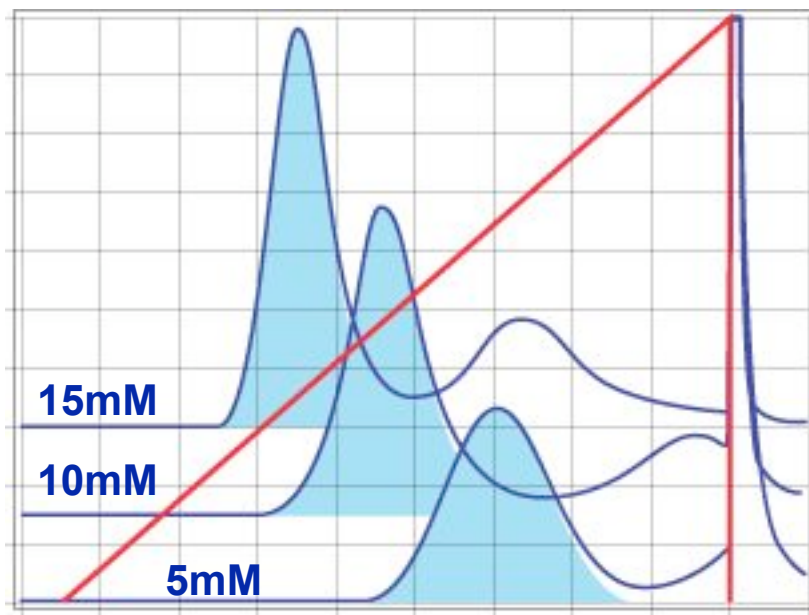
Separation conditions

1. Determine the phosphate concentration at which the antibody will elute in a linear sodium chloride gradient to 2.0 M. 10 mM phosphate is the most common value. Occasional antibodies may elute at 5 mM or require 15 mM phosphate. Rare IgGs may require up to 40 mM phosphate.
2. Select the lowest phosphate concentration that supports NaCl elution (but no lower than 5 mM).
3. Determine the pH that gives the best aggregate separation. This is usually pH 6.5
4. Convert gradient to steps



The influence of phosphate

40 CV linear gradient to 1.0M NaCl at constant phosphate concentrations as indicated



Blue areas indicate monomeric IgG, trailing peak is aggregate

Red line indicates NaCl gradient trace

NaCl gradient followed by cleaning with 0.5M phosphate

All experiments at pH 6.5
300 cm/hr

protein A purified IgG on CHT type I 20 µm

The influence of phosphate

| Phosphate mM | 5 | 10 | 15 |
|-----------------|-------|------|------|
| Protein A ng/mL | 0.03 | 0.03 | 0.01 |
| DNA ng/mL | <1.0 | <1.0 | 3.9 |
| Endotoxin EU/mL | <0.05 | 1.0 | 1.6 |

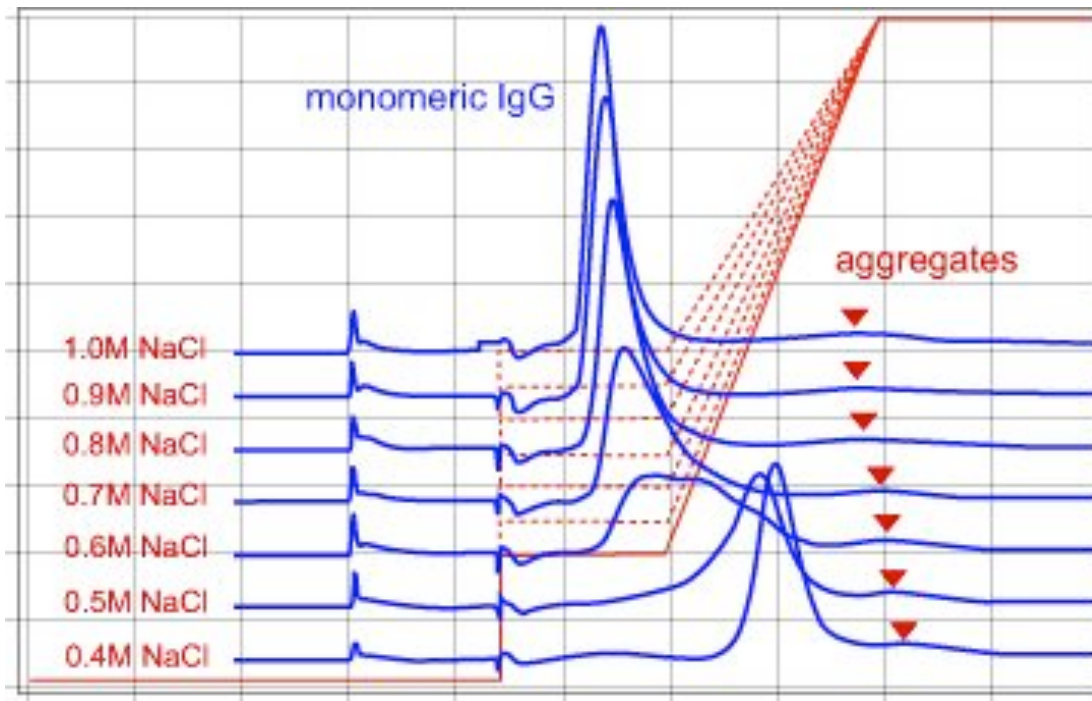
Sample: protein A purified chimeric monoclonal IgG1. 22 ng/mL
leached protein A, 2.3×10^3 ng/mL DNA, 1.9×10^4 EU/mL endotoxin
Linear detection limit of protein A assay: 0.2ng/mL

All results 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 μ m, 300cm/hr.



Conversion to steps

Protein A purified human monoclonal IgG1, CHT type I 20 μ m



All experiments in
5mM NaPO₄ at pH
7.0, 300 cm/hr

Elution gradients
25CV (step + linear)

Red lines indicate
NaCl gradient traces

Red notations indicate
step concentration

CHT method development

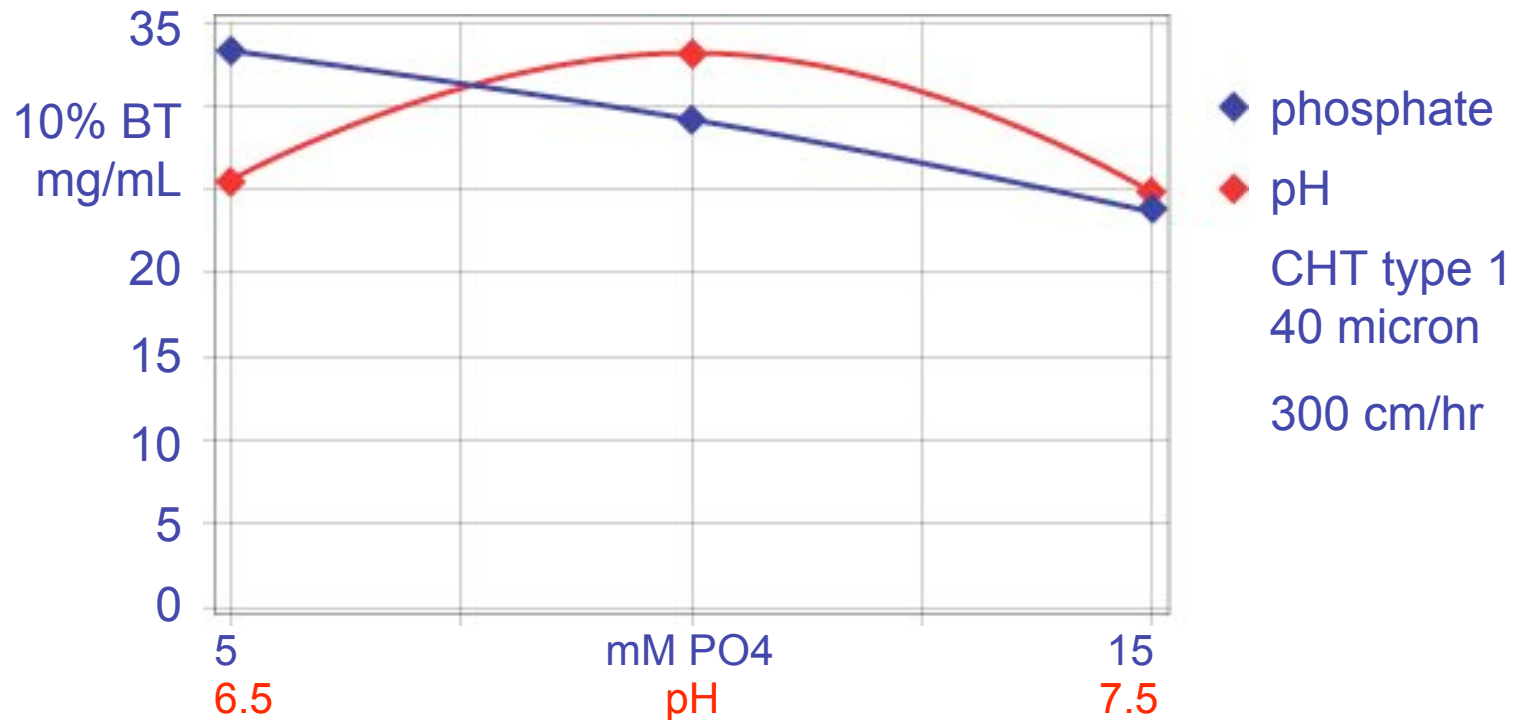
Capacity

1. Determine the pH that gives the highest binding capacity with a phosphate concentration of 5 mM.*
This is usually about pH 7.0, but may be pH 6.5 for some antibodies and 7.5 or higher for others.
 2. Establish capacity tolerance for NaCl. Some antibodies are affected severely, some mildly.
- * 5mM phosphate is required to maintain the stability of CHT at pH 6.5; about 2.5 mM at pH 7.5. Use the minimum phosphate concentration because excess phosphate depresses antibody binding capacity. Operation at pH values below 6.5 is not recommended.



Capacity versus phosphate and pH

Dynamic binding capacity, polyclonal human IgG



phosphate experiments conducted at pH 7.0
pH experiments conducted in 5mM phosphate



3-Step platform (CHT bind/elute)

Elute protein A with 0.1M glycine,* 0.05 M NaCl, pH 3.8**

Hold for virus inactivation

Titrate pH to 7.0** with 1M Tris

EQ strong anion exchanger to 0.05M Tris, 0.05M NaCl, pH 7.0** Apply sample. Collect flow-through

Add 0.5M NaPO₄, pH 7.0** to achieve optimal phosphate concentration (1% v:v yields 5mM)

Conduct virus filtration

Equilibrate CHT with optimal NaPO₄, pH 7.0**

Load, wash, elute under optimized conditions

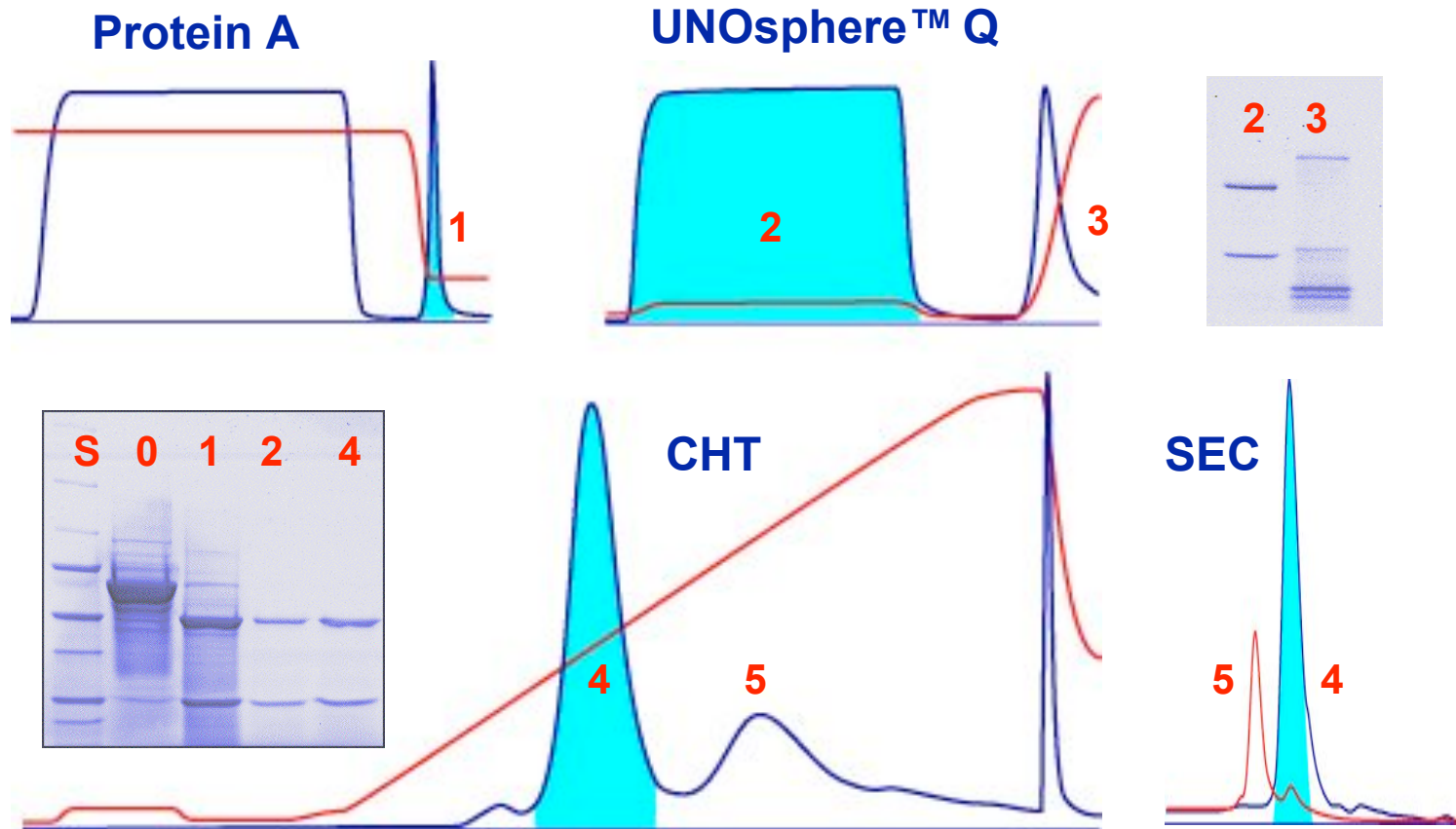
Concentrate/diafilter to final formulation conditions

* Arginine or acetate can also be used for elution. Citrate degrades CHT and has been shown to reduce leached protein A removal efficiency of anion exchange chromatography.

** Or other pH according to scouting results

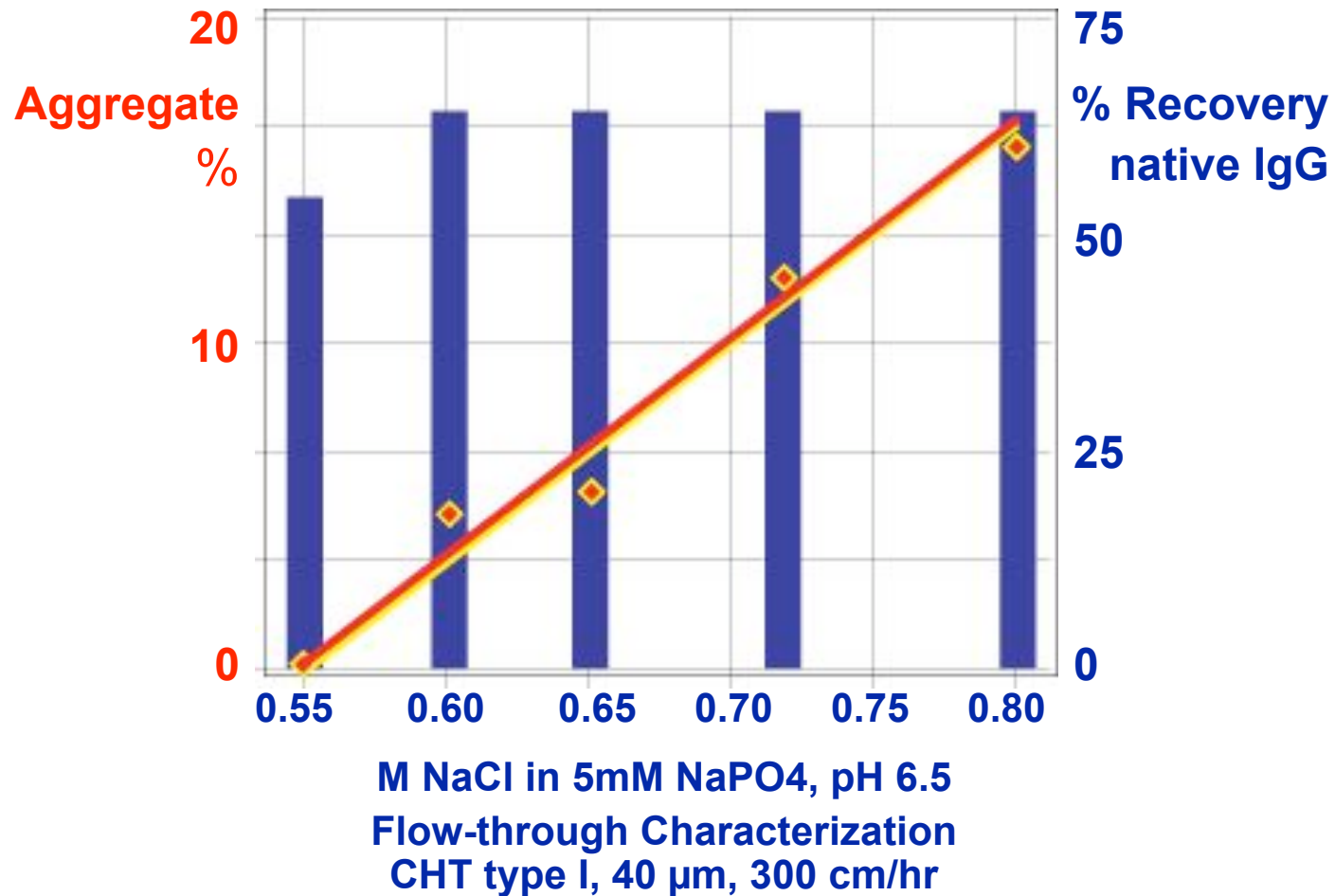


3-Step platform (CHT bind/elute)



Reduced SDS PAGE fractions: S=standards, 0=original material, 1=protein A elution, 2=UNOsphere Q flow-through, 3=UNOsphere Q elution, 4=CHT monomeric IgG pool

3-Step platform (CHT flow-through)



3-Step (CHT bind/elute vs flow-through)

| CHT-I 40µm | OM | Protein A IgG pool | Platform I native pool | Platform II native pool |
|-----------------------------|-------------------|---------------------------|-------------------------------|--------------------------------|
| Aggregate %, HPSEC | n.d. | >40% | <1% | <1% |
| Protein A ng, Cygnus | 0 | 162 | <0.2* | <0.2 |
| DNA, ng picogreen | 9.9×10^5 | 3.8×10^4 | <1 | <1 |
| Endotoxin EU, LAL | 2.8×10^3 | 5.0×10^2 | <0.05 | <0.05 |
| % Recovery | 100 | 25** | 75*** | 54*** |

*detection limit of the assay, **low recovery due to aggregation, ***monomeric IgG



Flexibility of CHT

The ability of CHT to reduce aggregate, leached protein A, CHOP, DNA, endotoxin, and virus makes it a perfect complement to protein A in a variety of platforms.

Protein A / CHT / anion exchange. As shown.

Protein A / cation exchange / CHT. The ability of CHT to achieve efficient contaminant reduction in flow-through mode supports its replacement of anion exchange.

Protein A / HIC / CHT. Protein A / CHT / HIC. The high salt elution of CHT is directly compatible with HIC, or the low salt elution of HIC with CHT.



Applicability of CHT

The strategy of eluting CHT with a sodium chloride gradient at a fixed low concentration of phosphate has provided excellent reduction of aggregates, leached protein A, HCP, DNA, endotoxin, and virus with every monoclonal antibody evaluated to date. *This includes murine, chimeric, and human IgG monoclonals from various subclasses* (see chromatograms on slides 12-17).

The consistency of elution behavior among these diverse samples suggests that the applicability of sodium chloride gradients on CHT may be essentially universal.



CHT buffer tips

Maintain operating pH at 6.5 or above.

Avoid exposure to chelating agents; EDTA, citrate.

Avoid anhydrous phosphates. The process of making them anhydrous creates polyphosphates that can affect performance.

Include phosphate in all samples and buffers

minimum 5 mM phosphate at pH 6.5

minimum 2 mM phosphate at pH 7.5

If the phosphate level required to achieve the best selectivity is too low to provide adequate buffering capacity, co-formulate with MES, Hepes, Tris, etc. 20-50 mM is generally adequate.



CHT column hygiene

Clean: 0.5 M phosphate

Sanitize: 1.0 M NaOH*

4 hours or more at 23°C

Store: 0.1 M NaOH

* >15,000 hours stability in 1.0 M NaOH



CHT column hygiene

CHT binds metals from process solutions, causing discoloration at the top of the column.

These metals may come from production media, buffers and salts, process water, or corroded stainless steel process equipment.

To prevent discoloration, try adding 100 mg CHT (type I, 40µm)* per liter of buffer during formulation.

Incubate 1 hour.*

Microfilter buffers as usual.

* Suggested starting points. Experiment with quantity and time to accommodate your specific process solutions.



CHT column hygiene

Removing metal from buffers to prevent discoloration of CHT



0 15 30 60 120
Minutes buffer exposure to
adsorbent before filtration

1 Liter aliquots of 50 μM FeCl_3 were adsorbed with 10mg CHT type I 40 micron for the indicated time intervals, then filtered through a 0.22 μm membrane to remove the particles. 800 mL of each treated buffer was applied to a fresh 1mL CHT column (Type 1, 40 μm) at 300 cm/hr.

Removal of ferric contamination is indicated by the absence of discoloration from the CHT column after 60 minutes buffer treatment. Discoloration was also prevented by buffer treatment with 100 mg CHT/L after 15 minutes incubation, or with 1 mg CHT/L after 16 hours.

Acknowledgments

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CHT references

P. Ng, A. Cohen, P. Gagnon, 2006, Monoclonal antibody purification with CHT, *Genetic Engineering News*, **26**(14) 60

P. Gagnon, P. Ng, C. Aberrin, J. Zhen, J. He, H. Mekosh, L. Cummings, R. Richieri, S. Zaidi, 2006, [A ceramic hydroxyapatite based purification platform: simultaneous removal of leached protein A, aggregates, DNA, and endotoxins](#), *BioProcess International*, **4**(2) 50-60.

For more information on antibody purification with hydroxyapatite, please visit the downstream processing library at www.validated.com. You are also welcome to contact pete@validated.com.

