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

Pete Gagnon, Validated Biosystems Inc., 240 Avenida Vista Montana Suite 7F, San Clemente, CA 92672

Paul Ng, Jie He, Julia Zhen, Cheryl Aberin, Heather Mekosh
Bio-Rad Laboratories, 6000 James Watson Drive, Hercules, CA 94547

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Why use CHT for MAb Purification?

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Method</th>
<th>Clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregates</td>
<td>HPSEC</td>
<td>1-2 logs</td>
</tr>
<tr>
<td>Protein A</td>
<td>Cygnus</td>
<td>1-2 logs</td>
</tr>
<tr>
<td>CHOP</td>
<td>ELISA</td>
<td>2 logs</td>
</tr>
<tr>
<td>DNA</td>
<td>Picogreen</td>
<td>&gt; 3 logs</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>LAL (chromo)</td>
<td>&gt; 4 logs</td>
</tr>
<tr>
<td>aMULV</td>
<td>Infectivity</td>
<td>&gt; 4 logs</td>
</tr>
<tr>
<td>xMULV</td>
<td>Infectivity</td>
<td>&gt; 3 logs</td>
</tr>
<tr>
<td>MVM</td>
<td>Infectivity</td>
<td>2 logs</td>
</tr>
<tr>
<td>PPV</td>
<td>Infectivity</td>
<td>&gt; 1 log</td>
</tr>
</tbody>
</table>

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.
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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chloride</th>
<th>Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer recov.</td>
<td>82%</td>
<td>78%</td>
</tr>
<tr>
<td>Aggregate</td>
<td>&lt; 1%</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>Protein A</td>
<td>&lt; 1 ppm</td>
<td>&lt; 1 ppm</td>
</tr>
<tr>
<td>CHOP</td>
<td>&lt;12 ppm</td>
<td>&lt; 72 ppm</td>
</tr>
<tr>
<td>DNA</td>
<td>&lt; 1ppm</td>
<td>&lt; 7 ppm</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>&lt; 0.1 EU/mL</td>
<td>&lt; 5.0 EU/mL</td>
</tr>
</tbody>
</table>

Human/mouse IgG1 chimera

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How CHT works with IgG

protein A purified human IgG1

10 mM NaPO4, pH 6.5
10CV gradient to 2M NaCl (10mM PO4)
Clean 0.5M NaPO4

monomeric IgG

CHT type I, 40 micron, 300 cm/hr

LPA DNA LPS MVM xMuLV
How CHT works with IgG

protein A purified IgG1 chimera

5 mM NaPO4, pH 6.5
40CV gradient to 2M NaCl (5mM PO4)
Clean 0.5M NaPO4

monomeric IgG aggregate

LPA DNA LPS

CHT type I, 20 micron, 300 cm/hr
How CHT works with IgG

protein A purified human IgG1

5 mM NaPO4, pH 6.5
40CV gradient to 2M NaCl (5mM PO4)
Clean 0.5M NaPO4

monomeric IgG

aggregate

LPA DNA LPS

CHT type I, 20 micron, 300 cm/hr
How CHT works with IgG

protein A purified mouse IgG1

10 mM NaPO4, pH 6.5
20CV gradient to 1.5M NaCl (10mM PO4)
Clean 0.5M NaPO4

monomeric IgG

aggregate

CHT type I, 20 micron, 300 cm/hr
How CHT works with IgG

Aggregate clearance with NaCl gradients

**HPSEC of process pools**
- Bio-Silect 400-5
- 50 µL protein A purified
- IgG1 chimera, 0.8 mL/min
- 50mM Hepes, 1.0M NaCl,
- 2.0M urea, pH 7.2

Protein A, IgG pool

CHT, IgG monomer pool
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

<table>
<thead>
<tr>
<th>Phosphate mM</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein A ng/mL</td>
<td>0.03</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>DNA ng/mL</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Endotoxin EU/mL</td>
<td>&lt;0.05</td>
<td>1.0</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Sample: protein A purified chimeric monoclonal IgG1. 22 ng/mL leached protein A, 2.3x10³ ng/mL DNA, 1.9x10⁴ 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 μm, 300cm/hr.
Conversion to steps

Protein A purified human monoclonal IgG1, CHT type I 20 μm

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

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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)

Aggregate% 75

% Recovery native IgG

M NaCl in 5mM NaPO4, pH 6.5
Flow-through Characterization
CHT type I, 40 μm, 300 cm/hr
# 3-Step (CHT bind/elute vs flow-through)

<table>
<thead>
<tr>
<th>CHT-I 40µm</th>
<th>OM</th>
<th>Protein A IgG pool</th>
<th>Platform I native pool</th>
<th>Platform II native pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregate %, HPSEC</td>
<td>n.d.</td>
<td>&gt;40%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Protein A ng, Cygnus</td>
<td>0</td>
<td>162</td>
<td>&lt;0.2*</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>DNA, ng picogreen</td>
<td>9.9 x 10^5</td>
<td>3.8 x 10^4</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Endotoxin EU, LAL</td>
<td>2.8 x 10^3</td>
<td>5.0 x 10^2</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>% Recovery</td>
<td>100</td>
<td>25**</td>
<td>75***</td>
<td>54***</td>
</tr>
</tbody>
</table>

* 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

1 Liter aliquots of 50 μM FeCl₃ were adsorbed with 10 mg CHT type I 40 micron for the indicated time intervals, then filtered through a 0.22μm 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


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.