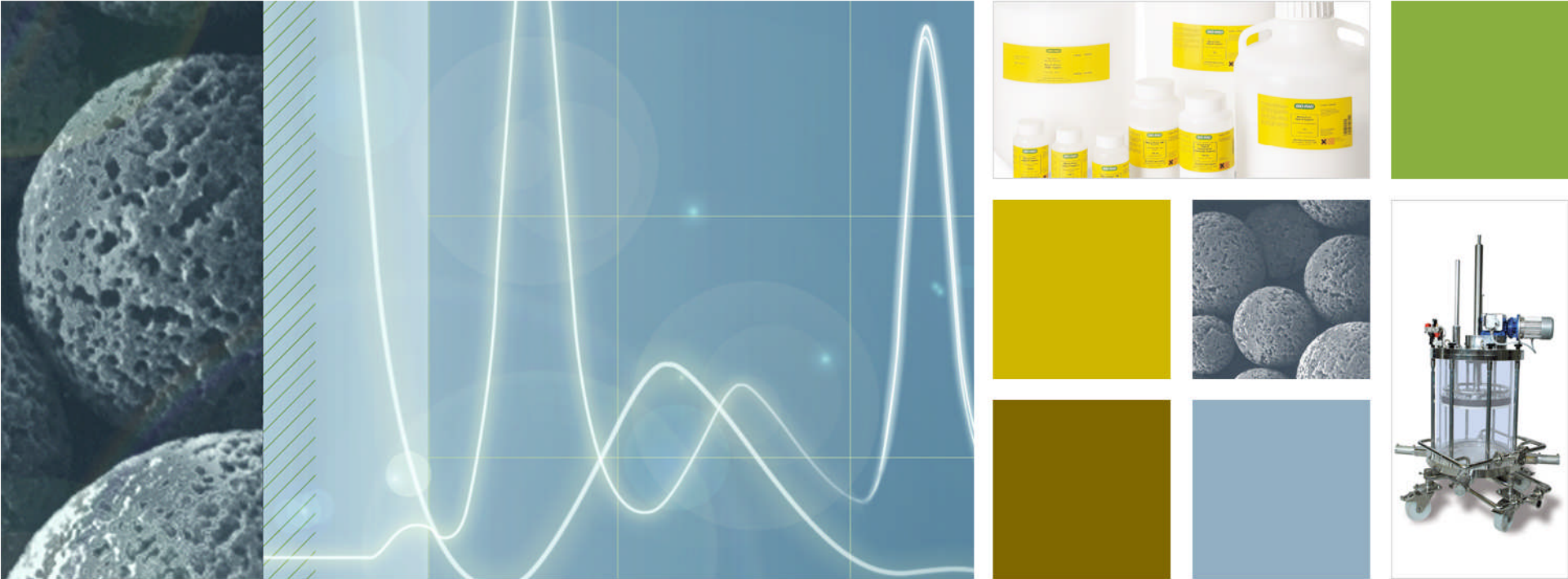


New, Robust Methods for Protein Purification from Ceramic Hydroxyapatite

Recent Updates





Topics to be covered

- Use of ceramic hydroxyapatite, CHT, in process purification applications
- CHT, surface chemistry and pH
- Optimizing the use of CHT
 - Use of co-buffer and calcium
 - Use of surface neutralization methods
 - Use of calcium phosphate
- Your questions



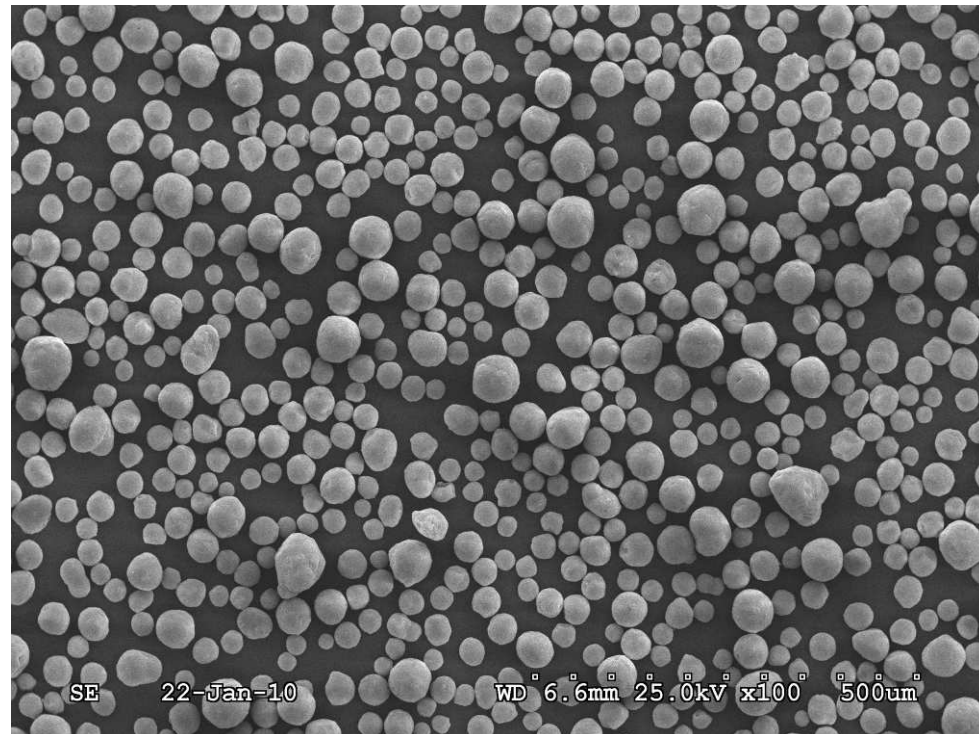
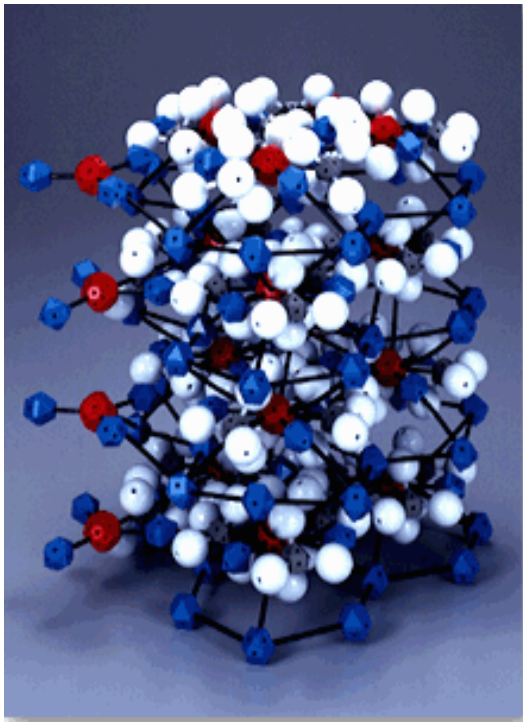
Topics to be covered

- Use of CHT in process purification applications
- CHT, surface chemistry and pH
- Optimizing the use of CHT
 - Use of co-buffer and calcium
 - Use of surface neutralization methods
 - Use of calcium phosphate
- Your questions



What is CHT?

- It is a macroporous inorganic media consisting of calcium and phosphate ions in the form of $\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2$





Why use CHT for MAb Purification?

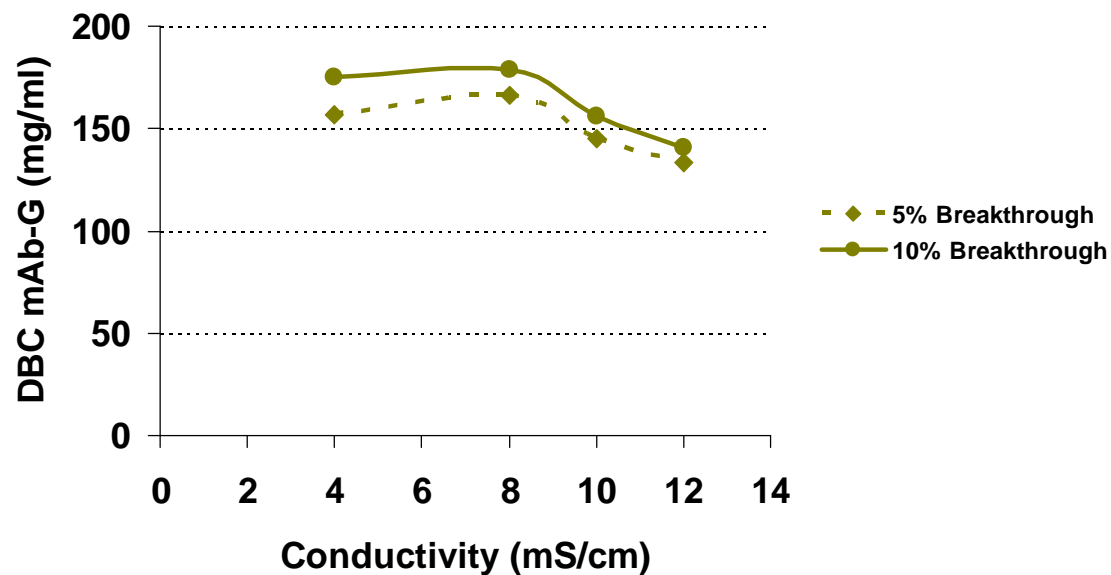
Impurity	Method	Observed Clearance*
Aggregates	HP-SEC	1-2 logs
Protein A	Cygnus	1-2 logs
CHO protein	ELISA	2 logs
DNA	PicoGreen®	> 3 logs
Endotoxin	LAL (chromo)	> 4 logs
xMULV	Infectivity/PCR	> 4 logs
MVM	Infectivity	2 logs (generally; up to 5 validated)

*NaCl gradients at constant phosphate concentration



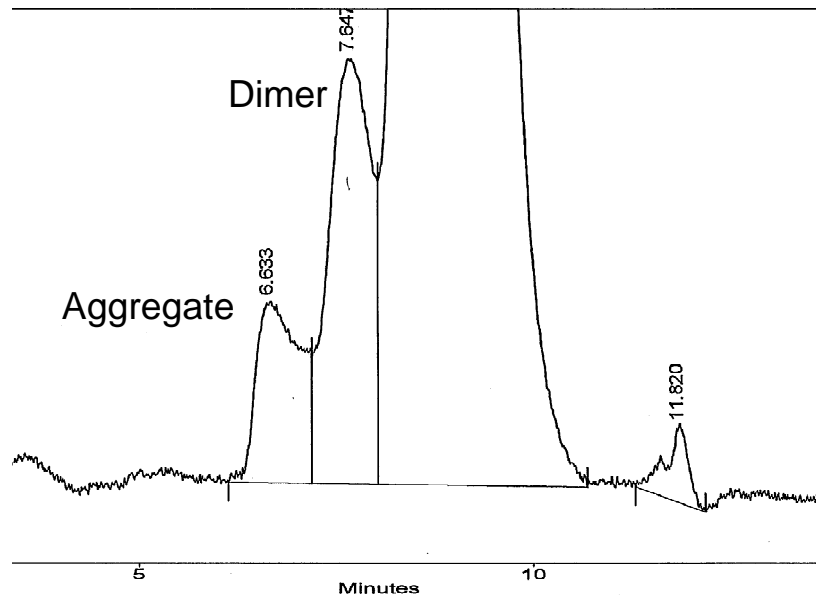
Potential capture steps prior to loading on to CHT Columns

- Protein A, UNOsphere SUPrA
 - Anion exchange, UNOsphere Q
- Cation Exchange, Nuvia™ S: Dynamic Binding Capacity for mAb relative to conductivity of load buffer; 300 cm/hr, 1.1 cm x 10 cm column



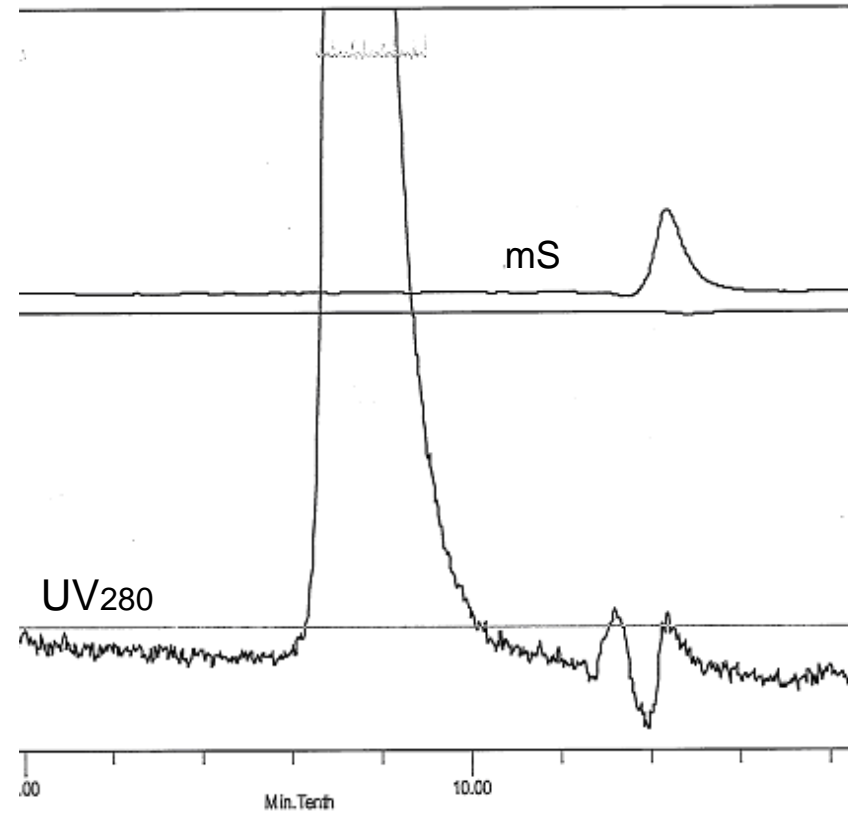


SEC: Effective removal of aggregates by CHT for mAb 1



Area	Area %	Height	Height %
101032	0.635	2602	0.541
227596	1.431	6129	1.274
15558353	97.793	471397	97.968
22424	0.141	1048	0.218

Protein A pool: 0.6% aggregate, 1.4% dimer

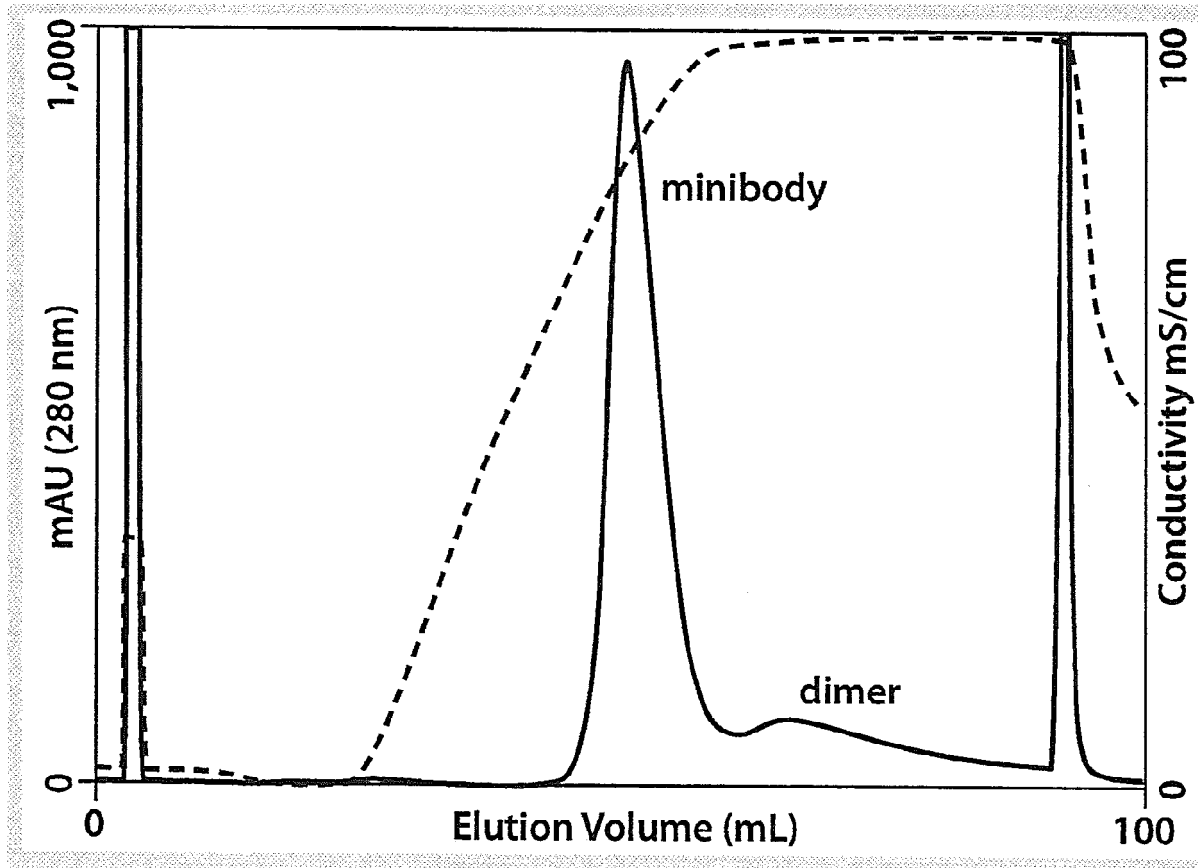


CHT pool: <0.03% dimer, <0.03% aggregate

CHT removes high and low levels of higher-order mAb structures



Aggregate separation is not limited to mAbs



Gagnon *et al.*, *BioProcess Int.* 8, 2-9 (2010)



Use of CHT for process applications

- CHT is the most effective way to remove process impurities and has therefore become part of many established platforms
- CHT is used in more than 15 commercial FDA approved processes as well as dozens of late stage development projects
- CHT is very easy to pack in any column
- CHT is supported by a global network of experts and community of users in most therapeutic applications
- **The utility of CHT has been further extended for use with the advent of simple, yet effective buffer systems.**



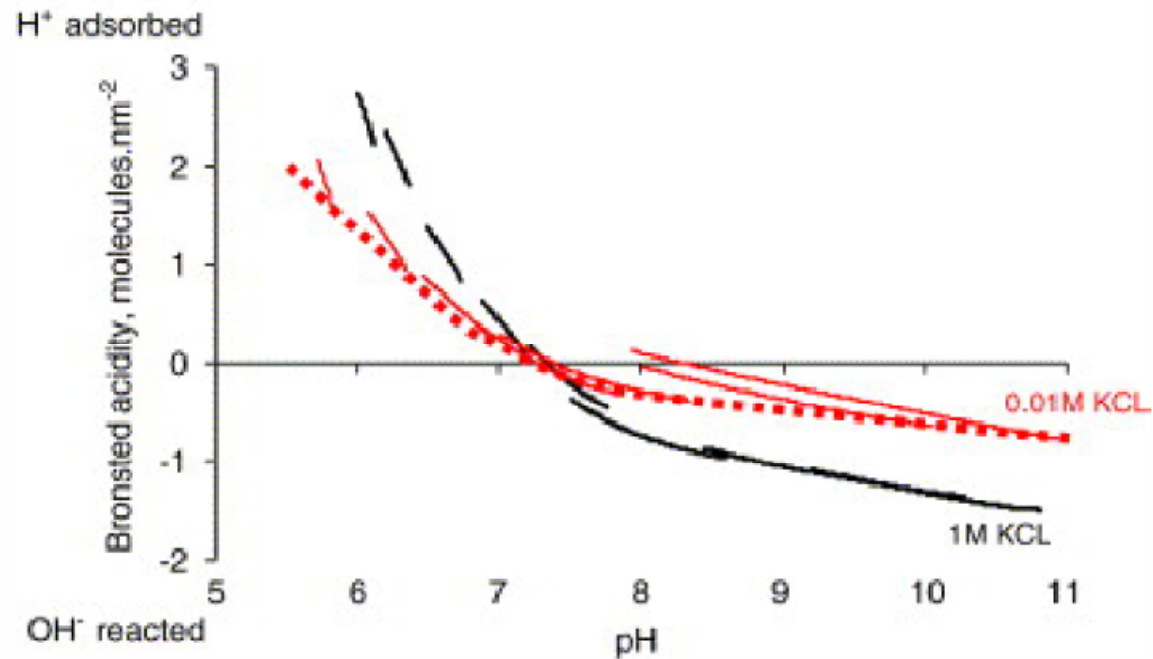
Topics to be covered

- Use of CHT in process purification applications
- CHT, surface chemistry
- Optimizing the use of CHT
 - Use of co-buffer and calcium
 - Use of surface neutralization methods
 - Use of calcium phosphate
- Your questions



Surface Chemistry

The surface of CHT easily adsorbs hydrogen ion (H^+) from aqueous phases (buffers) especially when they are below pH 7.5 and poorly buffered. The zero point charge of HAP is 7.3 ± 0.1 . *Biomaterials*, 26:34 (2004), 6818





Surface Chemistry

NaCl enhances the strength of calcium-oxygen bonds in HAP due to the competition between Na^+ and H^+ ions for the surface protonation sites. The hydrogen ion capacities of ceramic HAP are:

CHT type I: 0.079 meq/ml

CHT type II: 0.043 meq/ml

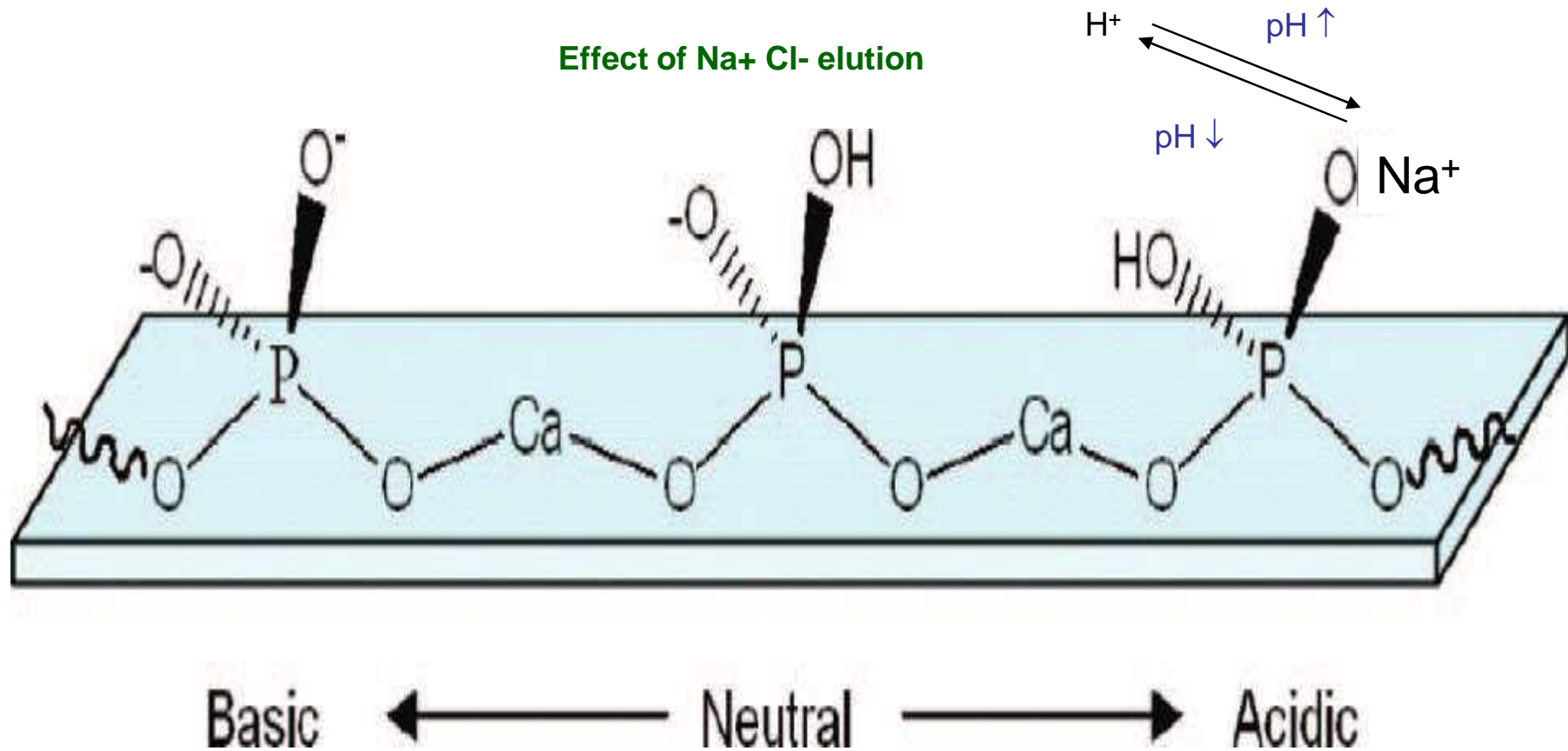
0.05 to 0.10 M **NaCl** provides a substantial amount of Na^+ to compete for HAP's surface protonation sites.

The hydrogen ion concentration is approximately 3×10^{-4} mM at pH 6.5, hence the competitive binding factor for Na^+ to H^+ for 0.05 to 0.1 M NaCl is between 170 – 340.



Surface Chemistry

Effect of Na⁺ Cl⁻ elution



Kwon et al., J. Phys. Chem. C, 113, 3369–3372 (2009).



Topics to be covered

- Use of CHT in process purification applications
- CHT, surface chemistry
- Optimizing the use of CHT
 - Use of co-buffer and calcium
 - Use of surface neutralization methods
 - Use of calcium phosphate
- Your questions



Controlling impact of surface chemistry

- The “common ion effect” to decrease the solubility of HA:

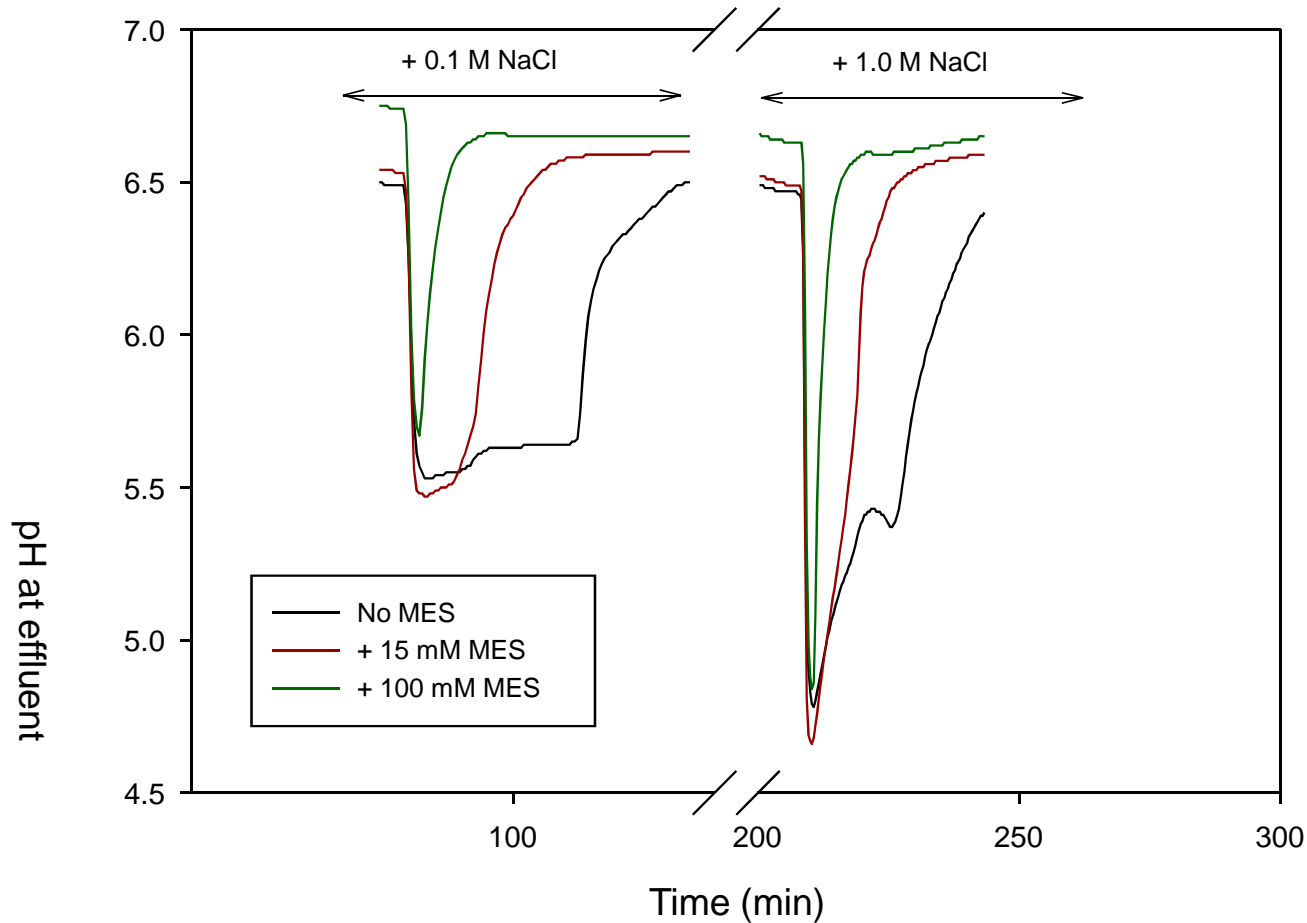


- Include at least 5 mM phosphate in all process solutions
- Note that phosphate is *not* a good buffering agent at pH 6.5
- pH impacts the common ion effect. Generally, use CHT only at pH \geq 6.5
- Co-Buffer, MES, HEPES, MOPS histidine, etc., can be used to increase eluent buffering capacity.
 - Add co-buffer and calcium to steps where $[\text{Na}^+]_2 > [\text{Na}^+]_1$ to adsorb released protons
- Add Ca^{+2} to elution buffer (and others)
 - Prevents Ca^{++} from dissociating from CHT via common ion effect
 - Amount added depends on elution buffer phosphate/co-buffer level/pH
 - Add Ca^{+2} to other buffers which also show calcium in the effluent
- Calcium measurement is extremely simple:
 - <10 min/sample
 - Procedure well-established and standardized (EDTA colorimetric endpoint titration); available from Bio-Rad



Effect of MES: Stabilization & reduction of Ca^{+2} in eluate (NaCl step elution)

10 mM phosphate/NaCl, pH 6.8 elution with and without MES Co-Buffer. Preceding step 10 mM phosphate, pH 6.8.



ppm Ca^{++} in first elution CV

MES	0.1M NaCl	1.0M NaCl
0 mM	11.2	92.3
15 mM	8.2	69.4
100 mM	3.9	30.9



Effect of calcium: phosphate/NaCl step elution

- The preceding buffer is 10 mM sodium phosphate, pH 6.5
- For 20 cycles in 0.1 M sodium phosphate/0.1 M NaCl, pH 6.5 elution system the mass changed from intact particles was -3.54%.
 - Reference 5073-84; 8_25_2009
 - The mass loss is calculated from the recoverable intact particles:
 - $(\text{Starting mass of CHT}) - (\text{Ending mass of CHT})$
- Addition of 10 ppm calcium (in this case, no co-buffer) in the elution step reduced mass change to -0.73%. A significant reduction.
 - Reference 5073-86b; 9_2_2009



Effect of Ca²⁺ + MES: NaCl step elution

	Original	Improved
MES	0	75 mM
Ca ⁺⁺	0	15 ppm
Regen pH	6.5	7.0
50 cycle mass loss	13.2%	0.27%
20 x 20 cm pilot scale	19 cycles	42 cycles

Original - 50 cycles, 0.55M NaCl, 5 mM sodium phosphate elution at **pH 6.5**

Improved - 50 cycles with 75 mM MES and 15ppm Ca²⁺ in elution buffer;

3ppm Ca²⁺ in equilibration/wash buffers, (0.1 M NaCl, 5 mM sodium phosphate, **pH 6.5**)



Effect of Ca²⁺ + MES: NaCl elution

	Original	Better	Best
MES	15 mM	100 mM	100 mM
Ca ⁺⁺	0	0	+10 ppm
50 cycle mass loss	15%	5%	0.89%

- monoclonal Antibody included in each cycle
- 1.1 x 22 cm 190 cm/hr
- Elution 0.35M NaCl, 10 mM Phosphate **pH 6.8**, equilibration and load without NaCl
 - Original
 - 100 mM MES
 - 100 mM MES + 10 ppm Ca⁺⁺



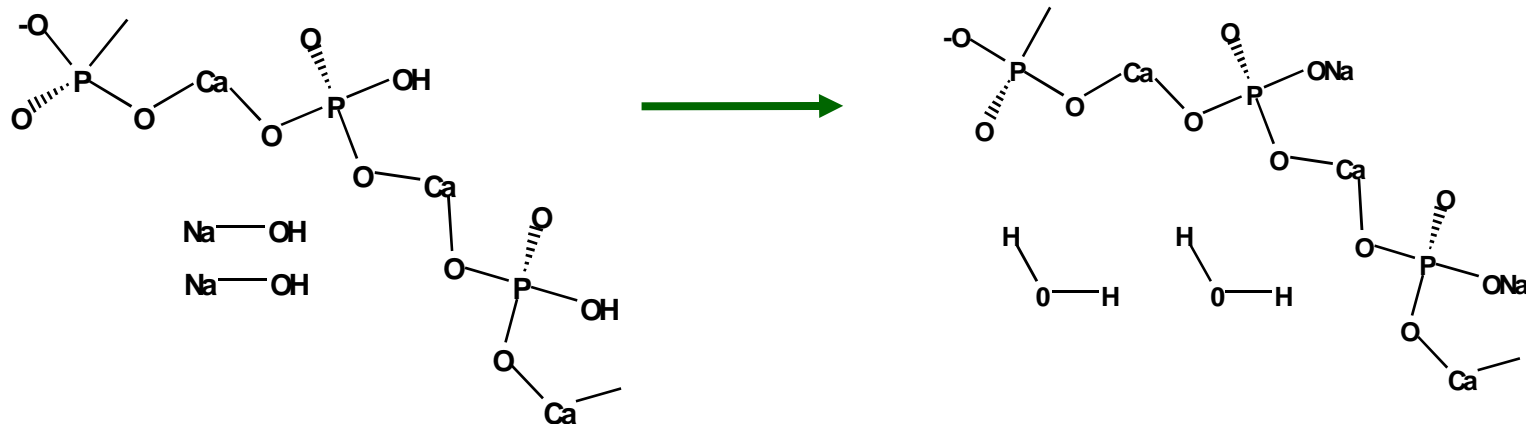
Topics to be covered

- Use of CHT in process purification applications
- CHT, surface chemistry
- Optimizing the use of CHT
 - Use of co-buffer and calcium
 - Use of surface neutralization methods
 - Use of calcium phosphate
- Your questions



Surface neutralization

- Hydroxyapatite adsorbs hydronium (protons) from aqueous solutions below pH 7.3.
 - The amount varies but is usually significant with low ionic strength solutions typical of equilibration and load buffers.
- Step gradients from low ionic strength to high ionic strength desorb hydronium ion..
- One way of removing hydronium ions after protein is bound but prior to elution would be to wash with NaOH—clearly not feasible.





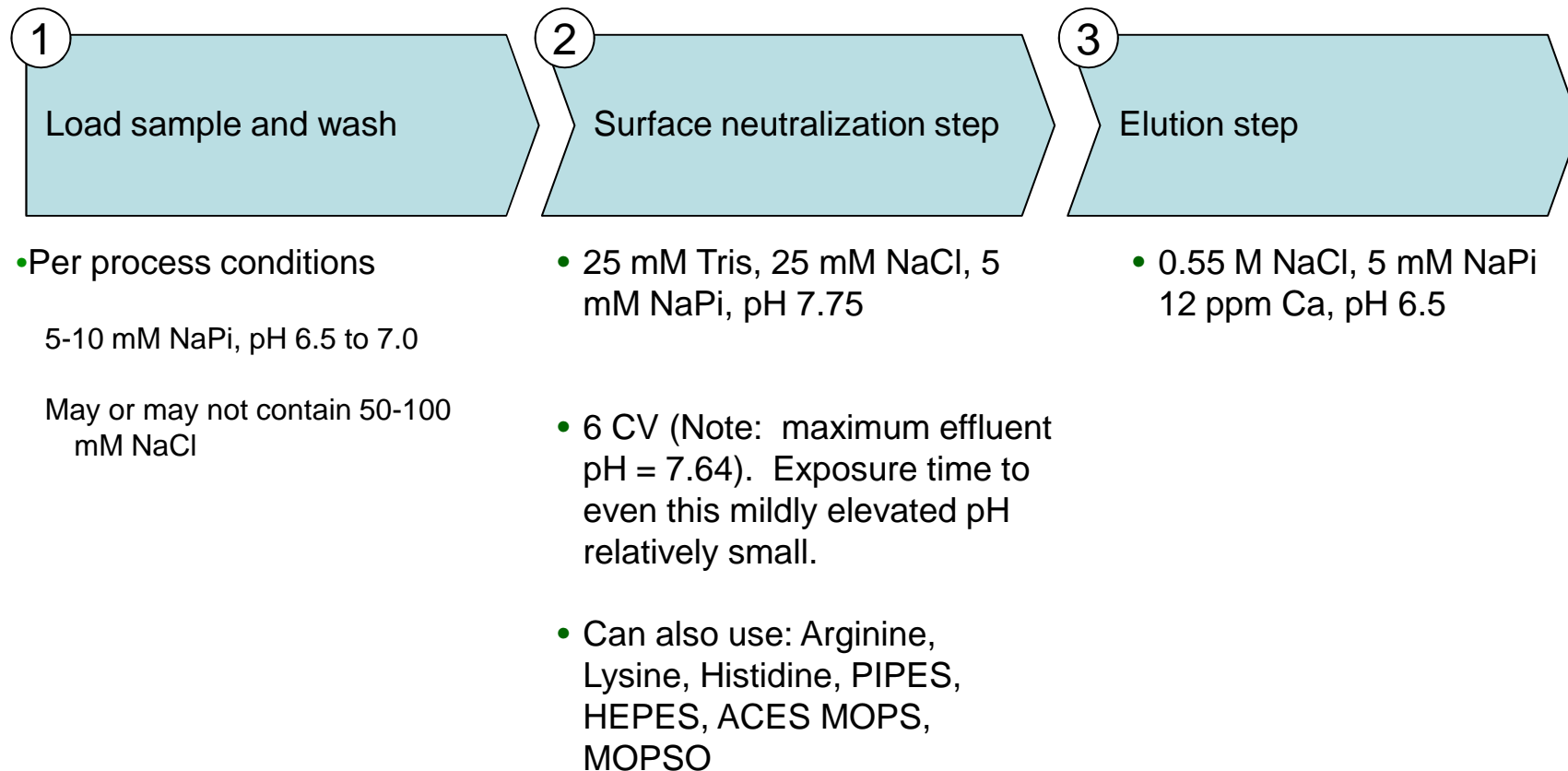
Method rational and supporting concepts

- What if we could desorb hydronium ion, convert it to water, yet keep the pH of the column at a favorable condition for the adsorbed protein?
- The advantage is that there is very little hydronium in the elution solution.
- Bio-Rad has developed a unique technology called the surface neutralization system (SNS; patent pending) to exploit this.
- Method involves a special, gentle wash immediately prior to elution.
- Verification of SNS method complete at 3.2 cm scale
- **Under unusually degrading conditions, 46 cycles obtained at 20 x 20 cm scale.**



Process with surface neutralization step

A straight forward way to ensure best results

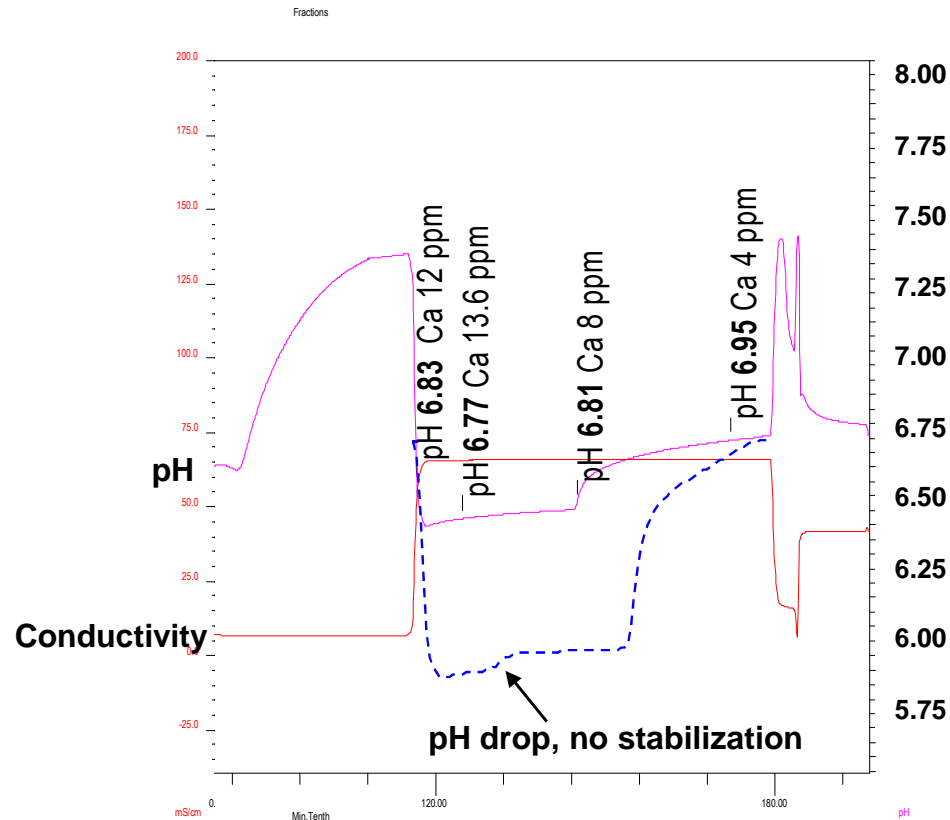




Surface neutralization eliminates the protons that previously caused the pH drop

12.5 mM PIPES buffered SNS, pH 7.75, (offline pH, Ca²⁺ shown)

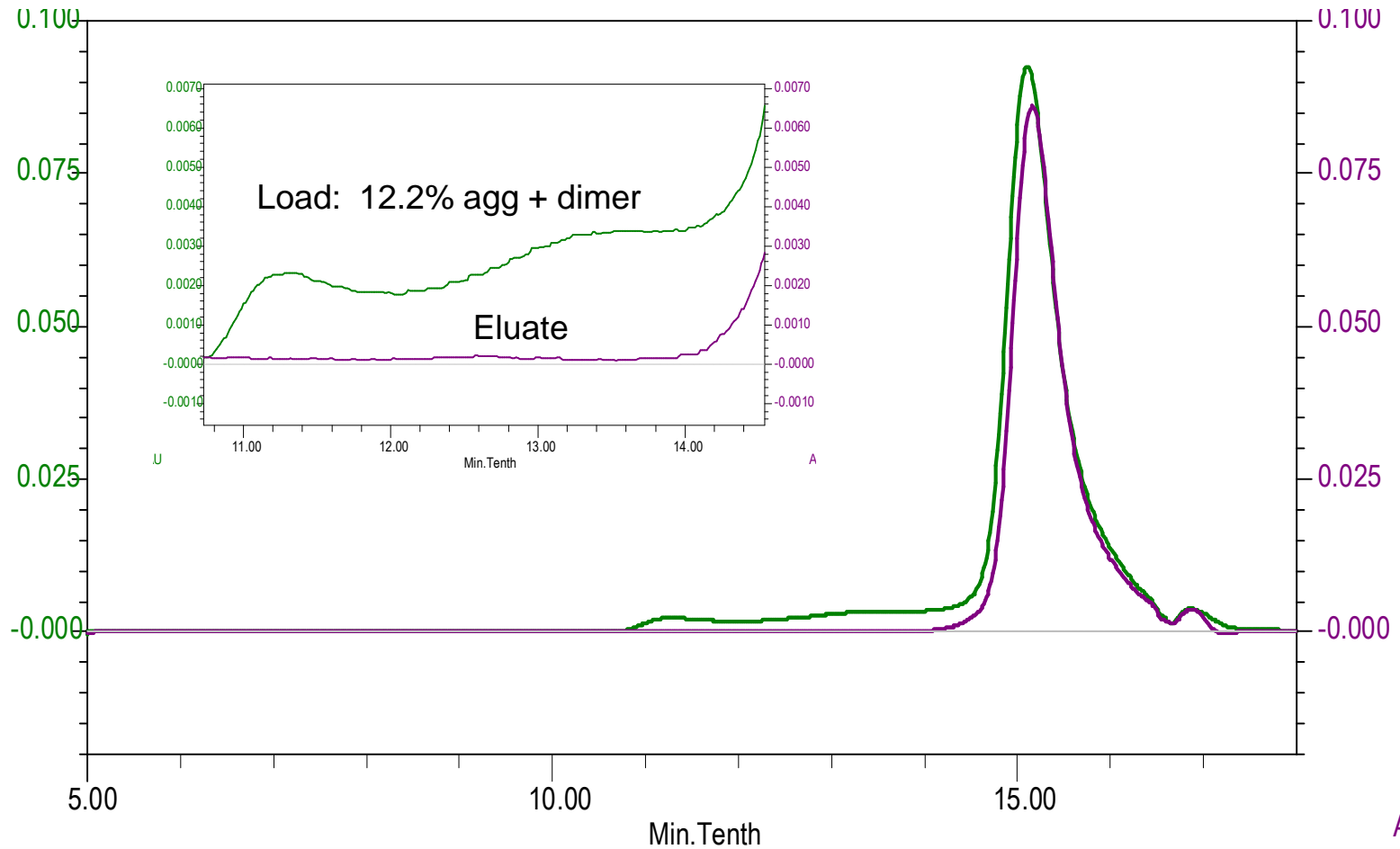
- Elution buffer contained 12 ppm Ca²⁺
 - Effluent Ca²⁺ levels equivalent to input level
- pH drop has been eliminated
Stability achieved





No effect on aggregate clearance performance

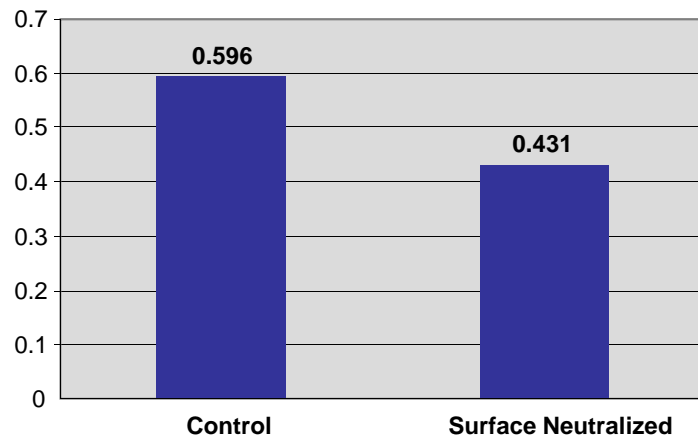
(HPLC-SEC)



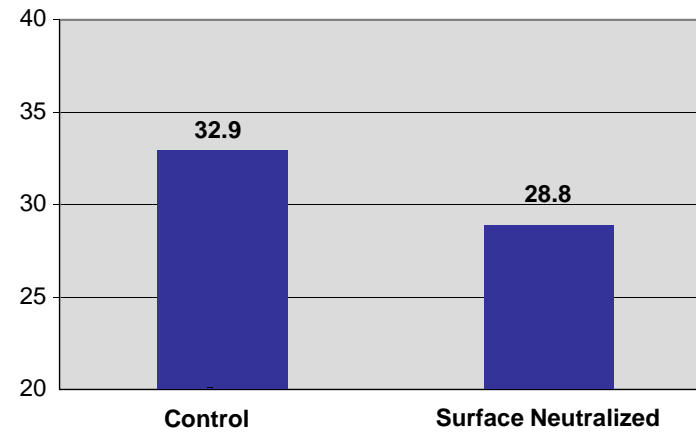


No effect HCP or DNA clearance for mAb R

CHO DNA Clearance (ng/mg)



CHO HCP Clearance (ng/mg)





Topics to be covered

- Use of CHT in process purification applications
- CHT, surface chemistry
- Optimizing the use of CHT
 - Use of co-buffer and calcium
 - Use of surface neutralization methods
 - Use of calcium phosphate
- Your questions



Calcium/Phosphate-Fortified (C/PF) Buffers, pH 5.3-5.8

- Calcium/Phosphate Fortified (C/PF) Buffers are eluants at pH 5.3 – 5.8 that provide **controlled pH and improved CHT stability at acidic conditions**.
- Why should C/PF be used?
 - C/PF provides elution at or near the same chemical composition (pH 5.3 - 5.8, ~1.3 - 5.8 mM calcium as CaCl₂) observed in the column effluent of the more widely-used NaCl, pH 6.5 – 7.0 elution buffer. The objectives are equivalent purity of the rPro and improved column robustness (patent pending)
- When should C/PF be used?
 - When the surface neutralization system, SNS (which operates at a slightly alkaline pH) elutes weakly adsorbed proteins and when SNS provides no benefit to the purification step.
- Benefit: achieving target rPro attributes reproducibly over a larger number of cycles (6 versus 35)



20 X 20 cm pilot scale column

Step	Description	Alternative
Cushion	Ultrapure water (0.5 CV)	
Regeneration	0.4 M NaPi, pH 7.0 (2 CV)	
Equil/Load/Wash	5 mM NaPi, 100 mM NaCl, 0.25 mM CaCl ₂ , pH 6.5 (6, 6, 3 CV)	
Elution	23 mM NaPi, 2.74 mM CaCl ₂ , 20 mM MES, 0.55 M NaCl, pH 5.6 (6 CV)	5 mM NaPi, 1.30 mM CaCl ₂ , 20 mM MES, 0.55 M NaCl, pH 6.5 – 6.8 (6 CV)
Cushion	Ultrapure water (0.5 CV)	
Neutralization	1 N NaOH (0.25 CV)	
Cushion	Ultrapure water (0.5 CV)	
Regeneration	0.4 M NaPi, pH 7.0 (3 CV)	
Cushion	Ultrapure water (0.5 CV)	
Sanitization	1 N NaOH (3 CV – hold)	
Flow rate	175 cm/hr	
Cycles	39 vs 9 C/PF, CS, NaCl	CS, NaCl



Conclusions

- **The utility of CHT was further extended with simple, yet effective buffer systems at pilot scale**

- Simple analytical methods applied to lab scale column eluates can guide method development to robust buffer systems.

- Robust buffer systems include

- **A) Calcium supplementation** - the common ion effect
- **B) NaCl supplementation** – surface protonation sites
- **C) a combination of A and B**
- **D) Surface Neutralization Systems** in combination with **A**
- **E) C/PF** in combination with **A and B**



Thank you.

**Enjoy the total benefits of CHT chromatography
for recombinant protein purification using our
new robust methods.**