An Enigma Unmasked: How Hydroxyapatite Works, and How to Make It Work For You

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Despite the diversity of chromatography products on the market, the number of fundamentally unique selectivities is small. Differences among anion exchangers, for example, represent differences of degree, not of kind. Those differences are certainly significant but not to the extent that it would normally be considered beneficial to develop a purification procedure with two or more anion exchange steps. The same is individually true for cation exchange, hydrophobic interaction, size exclusion, and even affinity chromatography. This limitation restricts opportunities for maximizing orthogonal complementarity in multistep purification schemes. In this context, ceramic Hydroxyapatite (cHA) represents an important tool for process developers: a truly unique selectivity with chromatographic performance features on a par with the best of more widely used chemistries. The objective of this article is to highlight cHA's features and describe how to exploit them to your best advantage.

Composition and mechanisms of adsorption. Hydroxyapatite (HA) is a crystalline mineral of calcium phosphate. It has been available for purification of proteins and nucleotides since 1956, but mostly in the physical form of easily fractured rectanglular plates, generally unsuitable for industrial column applications. In the late 1980s, new synthesis procedures yielded HA in hexagonal-cross section microrods. These rods could be agglomerated into spheres of similar diameter, then sintered at high temperature to bond their structures. This "ceramic" HA is the subject of this article.

The functional groups of HA consist of positively charged pairs of crystal calcium ions (C-sites) and the six negatively charged oxygen atoms associated with triplets of crystal phosphates (P-sites). C-sites, P-sites, and hydroxyl groups are distributed in a fixed topogeographic pattern on the crystal surface. This combination of active groups supports retention by at least three distinct mechanisms: cation exchange with P-sites, calcium coordination with C-sites, and anion exchange with C-sites. Hydrogen bonding has been noted as being theoretically possible, but hasn't been described. Which mechanism or combination of mechanisms dominates in a given application depends on the operating pH, buffer composition, and the surface properties of the protein (or other solute) applied to the column.

Strongly alkaline proteins tend to adsorb mostly by phosphoryl cation exchange of positively charged amino acid residues with P-sites (Figure 1). Adsorption becomes stronger with reduced operating pH due to the increasing charge on these residues. As with classical cation exchange, there is a loose and limited correlation between elution order and isoelectric point (pI). The most alkaline proteins generally elute last in a linear gradient of NaCl or other salts. Although alkaline protein interactions with HA are dominated by cation exchange with P-sites, concurrent amine repellance by C-sites, and the crystal surface distribution of both P- and C-sites impart a unique stereochemical influence that renders the selectivity of HA wholly distinct from traditional-cation exchangers.

Binding of strongly acidic proteins at acidic and neutral pH is heavily dominated by formation of metal coordination complexes between C-sites and carboxyl clusters on protein surfaces (Figure 2). That this mechanism is distinct from classical anion exchange has been proven experimentally by evaluating retention of proteins on which the carboxyls have been replaced by sulfo groups. Binding is reduced dra-

matically even though net charge is unaltered. Further proof comes from the fact that binding capacity for acidic proteins decreases with increasing pH. This is consistent with calcium's coordination pKa of about 6.0, but in direct opposition to the usual pattern observed with anion exchange. A final point removes any margin for doubt. Acidic proteins that elute from anion exchangers at about 0.3M NaCl remain bound to HA even at concentrations more than 10 times higher. BSA is a good example of this. At acidic or neutral pH, it can't be eluted at any concentration of NaCl (Figure 3). This is not to say that anion exchange doesn't contribute to retention of acidic solutes; rather that coordination of carboxyls with C-sites is dominant at acidic and neutral pH.

Elution of acidic proteins under these conditions requires a displacer with a strong affinity for C-sites, such as phosphate. As with strongly alkaline proteins, there is a loose and limited correlation between pI and elution order, but the order is reversed. In an ascending gradient of phosphate, neutral and acidic proteins elute more or less in order of descending pI. Despite this superficial correlation with anion exchange, it's important to keep in mind that the actual correlation is not with pI but with the relative preponderance of carboxyl doublets and triplets that are surface-available for formation of coordination complexes with C-sites.

At alkaline pH, with the decreasing contribution of metal coordination, anion exchange effects become significant (Figure 4). Calcium-based anion exchange can be eluted with NaCl. Elution order of acidic proteins still correlates loosely with pI, but binding is weaker. This highlights the metal coordination component of the retention mechanism being stronger than the ion exchange component (Figure 4). As with phosphoryl cation exchange at low pH, calcium-based anion exchange exhibits a selectivity wholly distinct from traditional anion exchange. This is partly because of carboxyl repulsion from P-sites, and partly because of the crystalline distribution of C-sites on the matrix.

Figure 1. HA Interactions With Protein Amino Residues.

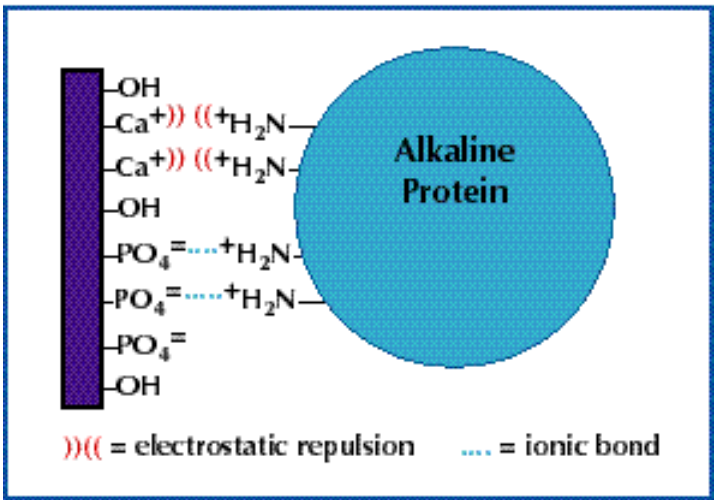
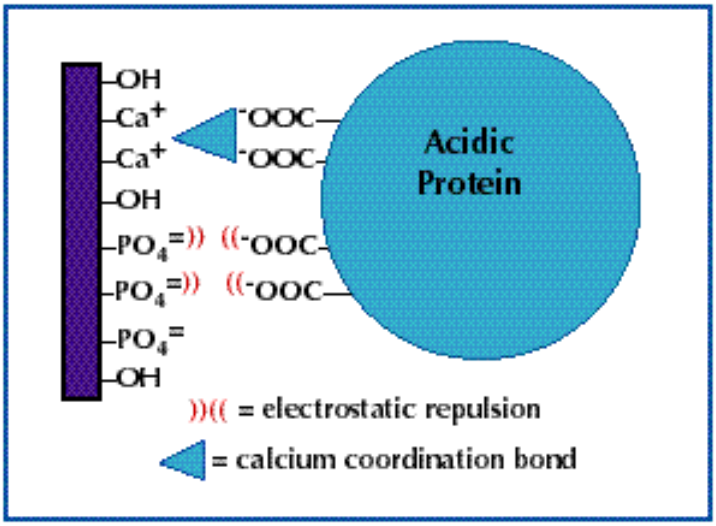


Figure 2. HA Interactions With Protein Carboxyl Clusters. Calcium coordination requires doublets or triplets of carboxyl groups. Singlets do not support good binding. Phosphoproteins and other phorylated solutes are retained by the same chemistry, but retention requires only a single phosphate.



Proteins of intermediate charge character exhibit mixed-mode adsorption, binding by both phosphoryl cation exchange and calcium coordination at neutral and subneutral pH. Large proteins are also likely to exhibit mixed-mode binding, regardless of their net charge character. IgG is a good example of this. It's basic pI makes phophoryl cation exchange the expected retention mechanism, but while NaCl reduces its binding capacity, it can't abolish it (Figure 5). This indicates that IgG carboxyls are coordinating with C-sites.

Phosphoproteins and other phosphorylated solutes — notably including DNA, endotoxin, and phospholipids — bind via coordination of their phosphoryl groups with C-sites. Lipoproteins bind strongly via their phospholipid exteriors. Phosphoryl binding to C-sites is very strong and requires free phosphate ions for displacement. When applied at acidic or neutral pH, NaCl will not elute these materials at any concentration. DNA binding is something of an anomaly nevertheless, since it does not bind as strongly as would be expected for such a phosphoryl-rich polymer. Cytoplasmic DNA requires about 0.3M phosphate for elution, with smaller fragments eluting earlier. The reason for it not being stronger is apparently that the spacing of the phosphoryl groups along the backbone is out of phase with the spacing of C-sites on the HA. Endotoxins elute over a wide zone from trace to 1.0M phosphate. The range of phosphate concentration for elution of phospholipids has not been characterized.

By the same mechanism as phophorylated solutes binding with C-sites, expect calcium-

Figure 3. Alteration of phosphate elution concentration by NaCl for BSA at pH 6.8. BSA elutes at about 0.11M PO4 in the absence of NaCl, but still requires nearly 0.10M PO4 even in the presence of 0.5M NaCl . This demonstrates the inertness of calcium coordination to NaCl. The small differential produced by NaCl corresponds to the combined contributions of anion and cation exchange interactions.

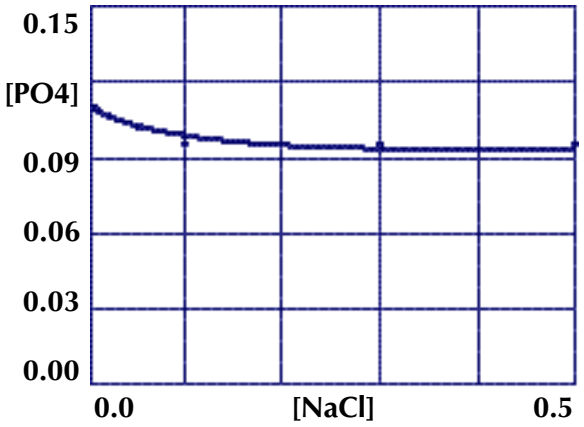
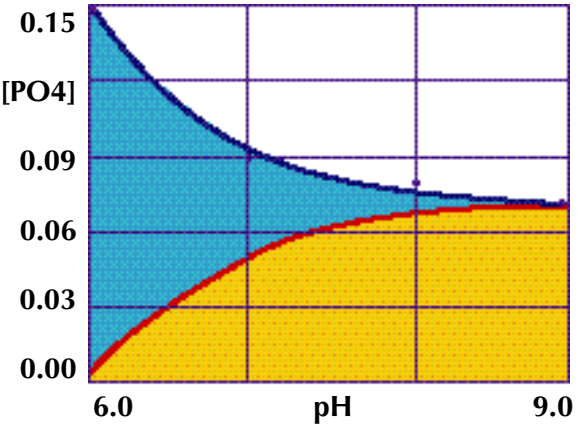


Figure 4. Alteration of phosphate elution concentration as a function of pH. At low pH, retention is dominated by the calcium coordination of protein carboxyls (blue area). As pH increases above its pKa, the interaction weakens and less phosphate is required for elution. However, binding persists even at an alkaline operating pH where coordination is essentially suspended. This reflects the contribution of anion exchange interactions (gold area).



proteins to interact strongly with P-sites. Prothrombin, C-reactive protein, and amyloid P-component are all strongly retained. On the other hand, be wary. Whereas phosphoryl groups are covalently bonded into a solute's structure, calcium moieteies are not. The interaction with HA may be strong enough to strip calcium out of its natural association with a protein.

HA is compatible with a wide variety of solvents and detergents. Consequently, membrane proteins, or proteins with limited solubility in general, can be run in urea or nonionic detergents. Anionic detergents have little effect on binding at low pH since carboxyl doublets or triplets are required to coordinate with calcium. However, such detergents may affect anion exchange retention at high operating pH. Cationic detergents may attenuate binding of amino groups to P-sites, but shouldn't affect low pH binding of carboxyls to C-sites.

Small peptides bind poorly for the most part. However, for those that do bind, and for larger peptides, HA's inertness to organic solvents may be useful. You can employ virtually any solvent necessary to solubilize a particular peptide, without concern for the matrix.

Method development. Although HA's mechanism of adsorption is more complicated than ion exchange, it does have a compensatory feature that keeps method screening and optimization to a reasonable level of effort: both the cation exchange and the metal coordination components of adsorption are optimized at about pH 6.0 . Lowering pH has very little effect on selectivity, and it can't safely be reduced below pH 5.0 in any case since it will destabilize the crystal structure. Raising

Figure 5. Effect of sodium chloride on dynamic binding capacity of IgG. Bio-Rad cHA, Type I, 20 micron. Binding buffer: 0.05M MES, pH 6.5, plus the levels of NaCl indicated. Flow rate: 600cm/hr. Note that dynamic capacity is still above 30mg/mL of gel at 0.1M NaCl. The maximum capacity of about 43mg/mL is equivalent or superior to the best ion exchangers capable of suporting this flow rate.

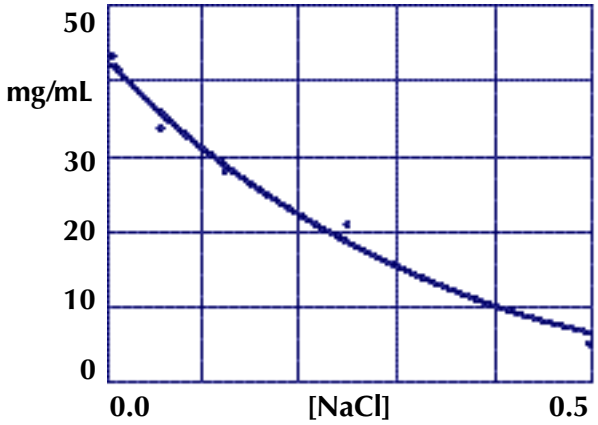
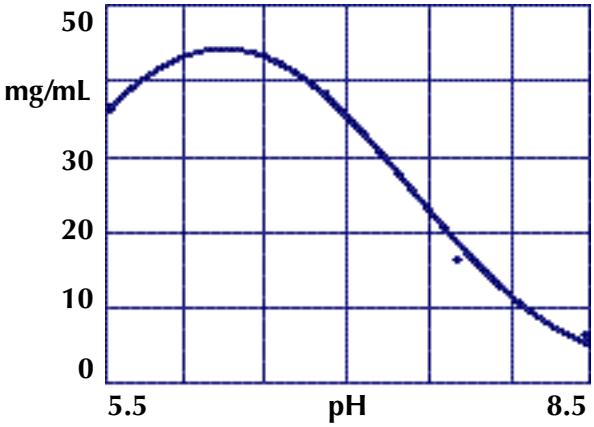


Figure 6. Dynamic Capacity of IgG as a Function of pH. Bio-Rad cHA, Type I, 20 micron, 600cm/hr. Buffers: 0.05M MES, Hepes, or Bicine. The loss of capacity at alkaline pH results from titration of amino groups, thereby weakening cation exchange interactions; and from weakening of calcium coordination. pH dependent loss of dynamic capacity for acidic solutes at high pH is proportionately somewhat less, but then it's usually lower to begin with.



the pH will alter selectivity, but at the direct cost of capacity (Figure 6). Nevertheless, the uniqueness of HA's selectivity, may make a brief look at pH 8.5 worthwhile.

A comment about the choice of buffers is also in order. The HA literature is dominated by the use of phosphate buffers, mostly between pH 6.5 and 7.0. Where nonphosphate buffers are employed, some references directly suggest that amine binding can be strengthened by inclusion of 1 - 2mM phosphate. The rationale is that free phosphate ions will bond with C-sites, neutralize their charge, and suspend amino repulsion. That free phosphate ions interact strongly with C-sites is incontestable, but experimental evidence indicates that this tactic, and the use of phosphate buffers in general, actually weakens protein adsorption (Figure 7). Free phosphate ions evidently compete directly with the P-sites for amino groups, so that even trace concentrations dramatically reduce binding capacity. Phosphate also interferes with carboxyl coordination. When HA is operated in a dominantly anion exchange mode at high pH, phosphate ions still suppress binding, but in this case by simple electrostatic competition.

For neutral and acidic pH applications, the obvious conclusion is that phosphate is best omitted from sample and binding buffers, except to the extent that it is used deliberately to control selectivity. Otherwise, you are just throwing away capacity. The zwitterionic buffer morpholinoethanesulfonic acid (MES) has a pKa of 6.0 and is perfectly suited for use with HA. Citrate also has an ideal pKa but is disqualified by virtue of its chelating ability — it destabilizes the crystal structure.

Figure 7. Effect of Phosphate on Dynamic Capacity of IgG. Bio-Rad cHA, Type I, 20 micron, 600cm/hr, 0.05M MES, pH 6.5., plus phosphate as indicated. Note that even 1mM PO4 causes about a 15% loss of dynamic capacity. 5mM cuts it by more than half and 10mM by over 80%. This explains — in the phosphate buffer-dominated HA literature — why HA has been mislabeled as a low-capacity technique.

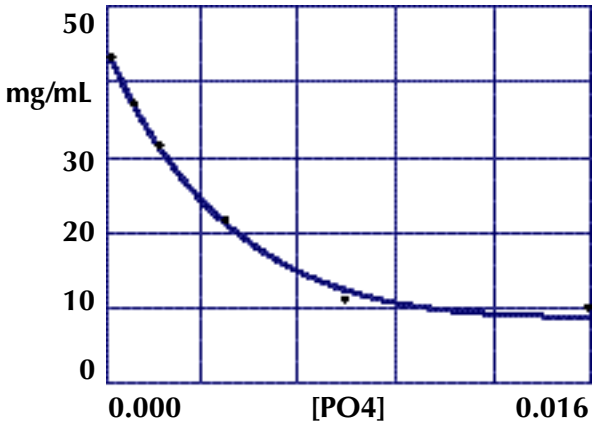
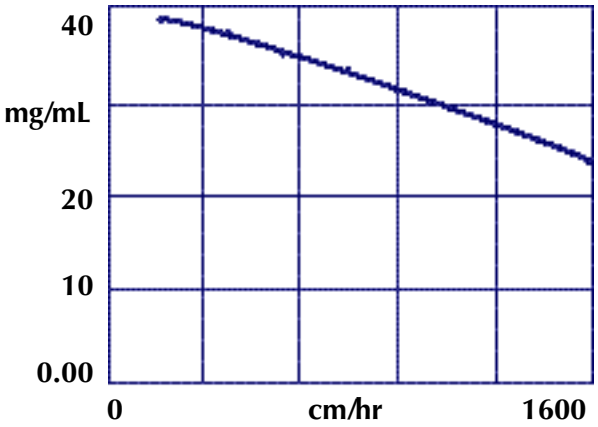


Figure 8. Flow Rate Versus Dynamic Capacity of IgG. Bio-Rad cHA, Type I, 20 micron, 0.05M MES, pH 6.5. Note that even at 1600cm/hr, dynamic capacity dramatically exceeds that observed with so-called perfusive supports.



The pKa of acetate is too low. Maleic acid provides an option if the expense of MES is prohibitive. However, maleate buffers have higher conductivity than MES and will suppress amine binding to P-sites. Depending on your separation, this may or may not be disadvantageous, but you should be specifically aware of it. At pH 8.0 - 8.5, where the contribution of calcium coordination becomes negligible, phosphate acts as a simple ionic competitor, and will reduce capacity by virtue of its high conductivity. A low conductivity buffer like Tris is a better choice.

Choosing the appropriate HA is critical to the performance and longterm consistency of your results. Be very careful to use only ceramic HA — not the traditional HA. Also be careful to purchase it from a supplier who can provide data demonstrating good lot-to-lot reproducibility. No matter how uniform the Ca:P ratio of the crystals, their relative availability on the crystal surface can be altered by the degree of sintering during manufacture. Oversintering reduces the relative availability of C-sites, and grossly reduces the capacity of solutes for which C-site adsorption is involved. Manufacturers often publish dynamic capacity for lysozyme (a P-site adsorbed protein) and BSA (a C-site adsorbed protein). Dynamic capacities for the two proteins should be within 20% of one another, with BSA capacity being the higher of the two. Conspicuously lower BSA capacity is a warning sign. Avoid media exhibiting that characteristic.

Initial screening does not require that you pre-equilibrate your samples to the chromatography starting conditions — so long as you keep the sample injection volume to 2.5% or less of the column volume (CV). This assumes that the sample contains no more than 0.05M phosphate. If phosphate concentration is 0.1M, then you can still get away with 1%CV. Higher phosphate concentrations will require sample dilution or re-equilibration. As long as you stay within these limits there will be sufficient in-column dilution of the phosphate to prevent its interference with binding.

Essentially the full scope of process opportunities can be identified with five sets of screening conditions (Table 1 and 2). It is very useful to have on hand a sample of at least partially purified product to serve as a reference. For each set of conditions, conduct one run with your raw sample, then another with the reference. This enables immediate identification of your product from complex chromatograms. Screening can be done quickly and effectively on 1 - 5mL columns packed with 20 micron media. A linear

Table 1. Screening Buffers for Hydroxyapatite.

Buffer A: 0.05M MES, pH 6.0

Buffer B: 0.50M KPO4, pH 6.0

Buffer C: 0.05M MES, 0.50M NaCl, pH 6.0

Buffer D: 0.50M KPO4, 0.50M NaCL, pH 6.0

Buffer E: 0.05M Tris, pH 8.5

Buffer F: E + 0.50M NaCl

Table 2. Screening Protocols for Hydroxyapatite

Step/protocol	1	2	3	4	5
equilibrate column	10CV, A	10CV,A	10CV, A	10CV, C	10CV, E
inject sample	2.5%CV	2.5%CV	2.5%CV	2.5%CV	2.5%CV
wash.1	5CV, A	5CV, A	1CV, A	5CV, C	5CV, E
wash.2	—	—	5CV, C	—	—
wash.3	—	—	1CV, A	—	—
elute, linear grad.	20CV A to B	20CV A to C	20CV A to B	20CV C to D	20CV E to F
strip	5CV, B	10CV, B	5CV, B	5CV, D	10CV, B

flow rate of 600cm/hr supports excellent separation performance with only about a 10% loss of dynamic capacity from 200cm/hr (Figure 8).

The first screening protocol is the simplest, and gives selectivity most like the majority of separations in the HA literature. It relies on a simple phosphate gradient. Its weakness is that it coelutes acidic with alkaline proteins. The advantage of the second protocol is that it allows selective gradient desorption of weakly carboxylated alkaline proteins. Other solutes are eluted indiscriminantly in the phosphate strip.

The third and fourth protocols remove most alkaline proteins in the post-sample injection wash, allowing selective gradient desorption of strongly carboxylated and/or phophorylated solutes. Solutes that bind exclusively by carboxyl coordination with C-sites behave identically in the two protocols, but those that bind via a mixed mode (with phosphoryl cation exchange) will elute earlier in the fourth protocol.

The fifth protocol exploits HA almost exclusively as an anion exchanger, however as with conducting cation exchange on HA (protocol 2), expect its selectivity to be very different from traditional anion exchange. Once you've chosen the mode of selectivity you wish to pursue, you can proceed to optimize sample loading, elution, and other parameters as you do with other types of adsorption chromatography.

Scale-up. Ceramic hydroxyapatite is available in a variety of particle diameters; most commonly 10, 20, 40, and 80 micron. Their existence implies that scale-up should automatically follow a progression of particle size. However, capacity and selectivity of cHA vary

Figure 9. Dynamic Capacity of IgG Versus Particle Size . Bio-Rad cHA, Type I, 600cm/hr, 0.05M MES, pH 6.5

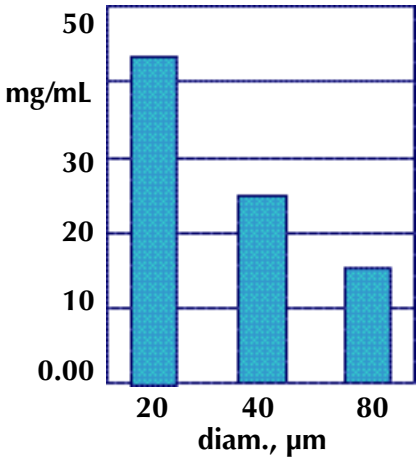
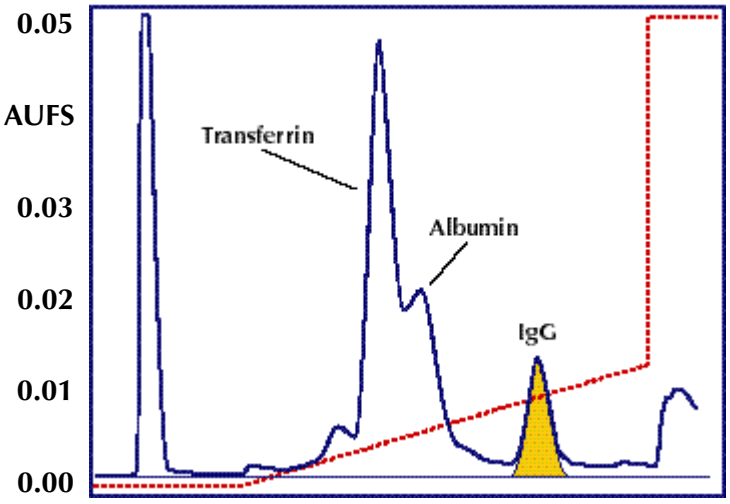


Figure 10. HA purification of monoclonal mouse IgG1 from ascites. Bio-Rad cHA, Type I, 10 micron, 600cm/hr, loaded in 0.05M MES, pH 6.5, eluted in a 15CV linear gradient to 0.25M KPO4, then stripped with 1.0M KPO4. On average, IgGs elute closer to the albumin peak, sometimes partially overlapping it. The illustrated profile is typical for IgMs. Relative elution behavior for other immunoglobulin classes and host species has not been described.



with particle sizes (Figure 9). This apparently reflects differences in the level of sintering required to make a stable particle which, as discussed above, alters the proportionality of surface-available P- and C-sites. Given that the flow properties of the 20 micron material are so good, it makes sense to start with 20 micron media and stay at 20 micron media. If you have specific reasons to use a larger particle at process scale, then do your method development with that particle as well. 10 micron media supports excellent resolution and capacity, but requires high pressure columns and a high pressure chromatography system.

Prime applications. One of the unique features of HA is the ability to selectively elute alkaline proteins with NaCl, while more acidic contaminants— especially including endotoxins and DNA—remain strongly retained. This can be used to support outstanding removal of these contaminants.

Another useful feature is the ability of HA to retain acidic solutes strongly in the presence of high concentrations of sodium chloride (see Figure 3). This makes it possible to proceed from most other separation chemistries directly to HA with little or no intermediate sample equilibration. HA can be followed directly by either size exclusion or hydrophobic interaction chromatography. Following it with ion exchange will usually require at least some sample dilution along with pH titration, but it's usually possible to avoid an outright buffer exchange step.

Hydroxyapatite provides a uniquely valuable selectivity for large proteins. Immunoglobulins, especially IgMs, are a good example (Figure 10). Mixed-mode binding causes them to be retained more strongly than most of their contaminants. It is sometimes possible to obtain monoclonals at greater than 90% purity in a single step. Even with acidic proteins, for which binding is dominated by the calcium coordination mechanism, retention of larger proteins is usually stronger than proteins of similar charge but smaller size. Because of the superficial correlation of size and retention, HA sometimes provides a faster, higher capacity alternative to size exclusion for removal of aggregates.

Limitations. The tolerance of acidic protein retention to NaCl makes it tempting to consider HA as a first process step. However, it's important to keep in mind that most biological media contain phosphates. As illustrated in Figure 7, even trace levels of phosphate severely reduce binding capacity and can prevent adsorption of otherwise weakly retained proteins. If your product is strongly retained and can tolerate this, it may be advantageous since it will prevent binding of more weakly retained contaminants. Otherwise, it will be necessary to dilute or remove the phosphate in advance. Note that residual citrate and other chelating agents in a sample will have the same effect as phosphate.

Another issue with using HA as a first separation step is that its strong binding of acidic solutes — especially phosphorylated solutes like DNA, phospholipids, and lipoproteins — may consume enough substrate that binding capacity is reduced prohibitively for the product of interest. This liability can be overcome by using more media in a larger column, but this is never an attractive proposition. Even then, there is still the problem that phospholipids and lipoproteins are strong foulants. They can potentially affect separation performance within a single preparative run. They will certainly affect cumulative performance and reduce column life. If your product is weakly retained, you may be able to pretreat your raw sample by passing it through or batch treating it with an anion exchanger or soluble polycation. This will remove most of the phosphorylated contaminants and some of the more acidic ones, but you'll still have to deal with the phosphate issue.

HA will not tolerate acid washing. Acid dissolves the crystal structure. pH must be maintained above 5.0, preferably above 5.5. Chelating agents must also be avoided since they too will degrade the crystal structure.

Column packing and unpacking: cHA packs like sand, and packing consistency is excellent across process scale. In fact the bed is so stable that if air is accidentally introduced — even throughout the entire bed — you can re-

move it simply with an upward flow of buffer. Degassed buffer is best for this application. Any air that it's not able to displace will eventually be resolubilized. If your column is cursed with polyethylene frits, add some alcohol to the purging buffer to rewet their surfaces.

Unpacking columns requires a different a proach than agarose and other polymer media because of HA's relatively high density and brittleness. Don't use hard tools to dig it out of a column. Simply flow the column from the bottom, increasing the flow rate until it suspends the bed, then suction, dip, or pour it out.

Cleaning. Of the three major mechanisms contibuting to retention, cation exchange and calcium coordination are strongest at low pH, and are essentially suspended at highly alkaline pH. This makes base treatment attractive as a cleaning method, the moreso since HA tolerates even saturated NaOH without adverse affect. However, it's important to remember that both P- and C-sites retain their charge even at high pH. This makes it important to raise conductivity to outcompete ion exchange effects. If a column is heavily fouled with lipid, it is often helpful to include either a nonionic detergent such as TritonX-100 or ethanol in the washing solution. HA is also tolerant of concentrated urea and guanidine (as long as the pH is maintained above 5.5). As with ion exchangers and other adsorptive media, reverse-flow cleaning is more effective and supports longer column life.

Storage and preservatives. Hydroxyapatite is susceptible to an important degradation pathway that doesn't afflict other types of chromatography media. Just like the HA in your teeth, HA in a column is quickly degraded by attack from acids arising from bacterial metabolism. This is the main source of the "folklore" that HA gives unreproducible separations. Bacterial contamination can and does cause significant loss of resolution and reproducibility, even within a 24 hour period under 4°C storage. Filter your feed solutions and use storage preservatives that either suspend or terminate bacterial metabolism. Unfortunately the selection of preservatives is complicated because HA is both positively and negatively charged. It will bind charged preservatives, removing them from solution, and making them ineffective. This effect can sometimes be overcome by adding salt to the storage formulation, but it requires careful evaluation. Alcohols and sodium hydroxide work well, as do combinations of the two.

Recommended reading. Parts of this article are adapted from the book PurificationTools for Monoclonal Antibodies (ISBN 0-9653515-9-9), which includes an entire chapter on application of HA. It contains citations for most of the points raised in this discussion. Other recommended reading includes a series of three articles published in Analytical Biochemistry by Marina Gorbunoff in 1984: **36** 425, **136** 433, and **136** 440. All three deal with mechanisms of adsorption by HA. A 1991 review by Kawasaki in the Journal of Chromatography (**544** 147) oddly neglects the role of calcium coordination while overemphasizing anion exchange as a retention mechanism, but is otherwise an excellent overview, and contains a wealth of references to interesting applications.

Acknowledgements. Thanks to Bio-Rad for providing ceramic hydroxyapatite (Type I) and support for the work required to support Figures 5–10.