

Ca⁺⁺Pure-HA[®] Instruction Manual

Ca⁺⁺Pure-HA Media

Hydroxyapatite Chromatography

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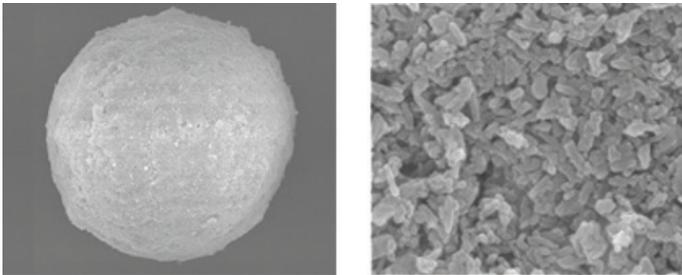
1. INTRODUCTION

Hydroxyapatite (HA) has become one of the best-characterized multimodal chromatography packings, with valuable research and industrial applications throughout the field of bioprocessing. Its combination of ion exchange and metal affinity have given it the ability to achieve separations that no other chromatography media can match, and it continues to provide unique solutions for emerging challenges in the field.

This handbook provides you with the practical knowledge to leverage the unique abilities of Ca⁺⁺Pure-HA media to fulfill your most demanding separation requirements. Ca⁺⁺Pure-HA is manufactured by a proprietary process that produces ultrapure 10 nm × 100 nm hexagonal cross section crystals (Figure 1). They are agglomerated into particles and then heated to create stable welds at the crystal contact points. Particle size and porosity are tightly controlled, the particles are easy to pack, and they support outstanding chromatographic performance.

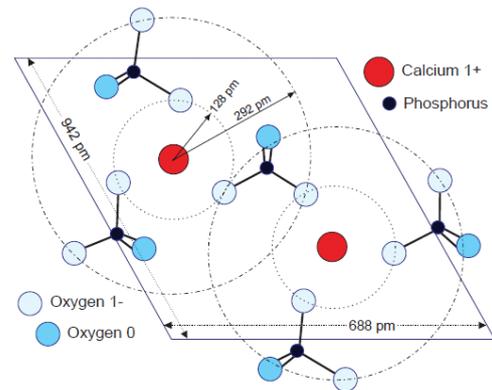
➤ **FIGURE 1**

CA⁺⁺PURE-HA PARTICLE PHOTOMICROGRAPH



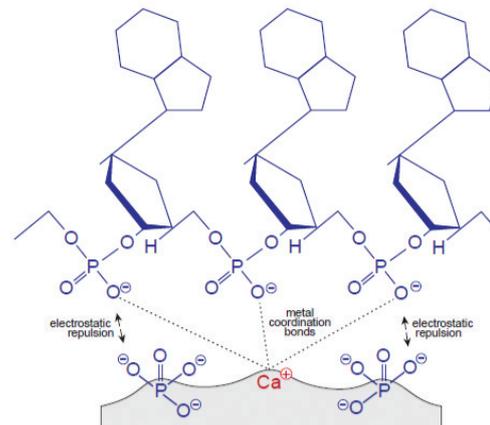
➤ **FIGURE 2**

CA⁺⁺PURE-HA SURFACE CHEMISTRY



➤ **FIGURE 3**

INTERACTIONS OF CA⁺⁺PURE-HA MEDIA WITH POLYNUCLEOTIDES



2. SURFACE CHEMISTRY AND INTERACTION

Ca⁺⁺Pure-HA is a naturally occurring crystalline compound with the structural formula of Ca₅(PO₄)₃OH. Its formula creates an impression that calcium residues are the dominant feature of the crystal surfaces, but most of them are involved in maintaining subsurface structure. This leaves phosphate residues as the dominant surface feature. Instead of the 5:3 Ca:P ratio that might be expected, surface ratios average closer to 1:6 (Figure 2).

2.1 Ca⁺⁺Pure-HA Calcium Interactions

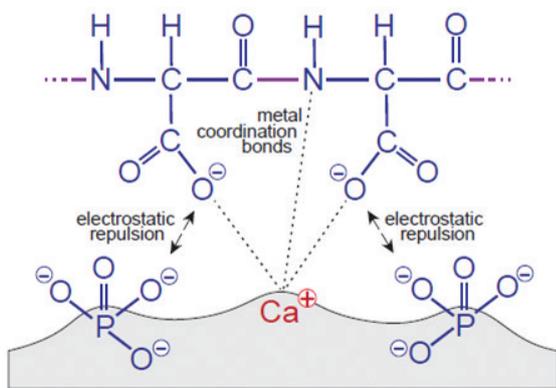
Ca⁺⁺Pure-HA surface calcium residues are positively charged. This invites an assumption that calcium acts like an anion exchanger¹. This seems to be supported by DNA and acidic proteins binding more strongly with increasing pH, but their response to increasing conductivity points to a different explanation.

In the absence of phosphate ions, DNA binds and remains bound to Ca⁺⁺Pure-HA even in 5 mol/L NaCl, roughly 10 times the concentration at which it elutes from strong anion exchangers. Acidic proteins do the same. This does not mean that anion exchange does not occur, but it does mean that whatever influence it may have is overwhelmed by a different retention mechanism.

As shown in Figure 3, nucleotide phosphates bind Ca⁺⁺Pure-HA calcium by formation of metal coordination bonds with their phosphate-associated oxygen atoms. Such interactions may range from 15 to 50 times stronger than electrostatic interactions. Mono-, di- and trinucleotides bind weakly. Polynucleotides bind more strongly; double-stranded DNA more strongly than single-stranded, single-stranded DNA more strongly than RNA, and all generally in proportion with the number of bases. Free phosphate ions elute nucleotides by competing directly with the nucleotide phosphates for Ca⁺⁺Pure-HA-calcium.

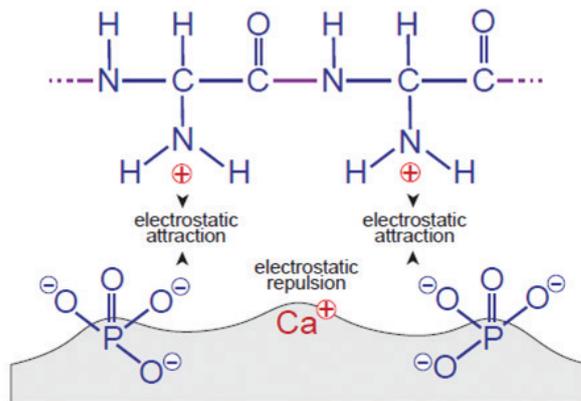
➤ **FIGURE 4**

INTERACTIONS OF CA⁺⁺PURE-HA MEDIA WITH PROTEIN CARBOXYLS



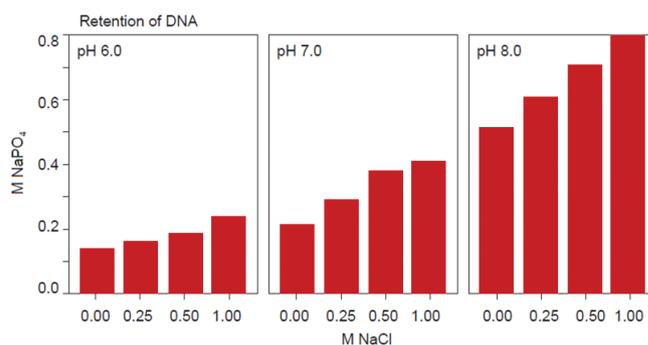
➤ **FIGURE 5**

INTERACTIONS OF CA⁺⁺PURE-HA MEDIA WITH PROTEIN AMINO RESIDUES



➤ **FIGURE 6**

PHOSPHATE ELUTION MOLARITY OF DNA AT DIFFERENT SALT CONCENTRATIONS AND pH VALUES



SURFACE CHEMISTRY AND INTERACTION

Ca⁺⁺Pure-HA-calcium forms coordination bonds with phosphate residues on other phosphorylated biomolecules as well, including phosphoproteins, endotoxins, and lipid enveloped virus particles among others. This is a major reason why Ca⁺⁺Pure-HA supports outstanding reduction of endotoxins and lipid enveloped viruses.

Ca⁺⁺Pure-HA-calcium residues also form coordination bonds with the carboxyl oxygen atoms of glutamyl and aspartyl residues on proteins (Figure 4). Interactions with a single carboxyl residue are not strong enough to mediate protein retention on Ca⁺⁺Pure-HA²⁻⁴. Doublets, triplets, or clusters are required. Coordination bonds between Ca⁺⁺Pure-HA-calcium and peptide backbone nitrogen atoms stabilize retention through carboxyl residues but they do not contribute sufficient binding energy by themselves to achieve retention.

2.2. Ca⁺⁺Pure-HA Phosphate Interactions

Ca⁺⁺Pure-HA-phosphates are negatively charged. Like sulfo- and carboxy- cation exchangers, Ca⁺⁺Pure-HA-phosphates bind strongly alkaline species through electrostatic interactions with positively charged amino residues²⁻⁴. They repel negatively charged residues like carboxyl and phosphoryl groups, as shown in Figure 5.

Ca⁺⁺Pure-HA-phosphates have potential to form stable hydrogen bonds with biomolecules but this has not been shown to contribute significantly to biomolecule retention on Ca⁺⁺Pure-HA²⁻⁴. That does not mean hydrogen bonding doesn't occur. More likely its influence is dominated by stronger contributions to binding energy from phosphoryl cation exchange and calcium metal affinity.

Ca⁺⁺Pure-HA-phosphates also have potential to form coordination bonds with metals. This creates a pathway for binding a variety of metalloproteins. It also endows Ca⁺⁺Pure-HA with the ability to scavenge contaminating metal ions from applied buffers and samples.

2.3. Ca⁺⁺Pure-HA Hydroxyl Interactions

Hydroxyl groups have potential to participate in hydrogen bonding²⁻⁴, but this pathway has not been demonstrated to contribute significantly to biomolecule retention. Hydroxyl groups are much less abundant than phosphate groups so even to the extent they may participate in hydrogen bonding, they likely contribute less binding energy than phosphate oxygen mediated hydrogen bonding.

2.4. HA Multimodal Interactions

Individual surface calcium residues of the Ca⁺⁺Pure-HA media are surrounded by a double ring of phosphate triplets (Figure 2). There are no sites represented exclusively by phosphate or by calcium.

Reactivity of Ca⁺⁺Pure-HA-calcium is heavily modulated by the influence of its surrounding Ca⁺⁺Pure-HA-phosphate groups. This ultimately explains why DNA and other acidic biomolecules bind more strongly with increasing pH and NaCl concentration. Increasing pH and/or salt reduces the magnitude of charge repulsion between DNA phosphates and Ca⁺⁺Pure-HA-phosphates (see Figure 6); also between protein carboxyl residues and Ca⁺⁺Pure-HA-phosphates⁵. Reducing the magnitude of repulsion enables acidic biomolecules to interact more fully with Ca⁺⁺Pure-HA-calcium residues. The more fully they interact with Ca⁺⁺Pure-HA-calcium, the more strongly they are bound.

Reactivity of Ca⁺⁺Pure-HA-phosphates is likewise modulated by the influence of adjacent Ca⁺⁺Pure-HA-calcium, but in the opposite direction. Retention of alkaline proteins is enhanced by simultaneous metal affinity interactions with Ca⁺⁺Pure-HA-calcium. This highlights the important point protein alkalinity does not mean carboxyl groups are absent from a protein, just that they represent a relative minority. Lysozyme (pI ~10.6) binds to and elutes from Ca⁺⁺Pure-HA at significantly higher pH and NaCl concentrations than on cation exchangers. The same pattern occurs with IgG⁵. Such behavior is typical in head-to-head comparisons between Ca⁺⁺Pure-HA and cation exchangers.

Multimodality also explains how Ca⁺⁺Pure-HA provides useful binding capacity under conditions where ion exchangers cannot. Most antibodies bind cation exchangers but they require low pH and conductivity to do so. Ca⁺⁺Pure-HA provides good capacity at neutral pH and sometimes even at physiological conductivity. This is a good example of cooperativity between Ca⁺⁺Pure-HA surface chemistries. Metal affinity overcomes the weakness of cation exchange retention, and the two weak interactions together create enough binding energy to provide real practical value.

3. COLUMN PACKING

Ca⁺⁺Pure-HA particles are denser and settle more rapidly than polymer-based chromatography particle. Moreover, in contrast to polymer-based chromatography particles, Ca⁺⁺Pure-HA should never be compressed.

One way to avoid compression is to leave minimal headspace at the top of a packed Ca⁺⁺Pure-HA column. The ideal is to kiss-fit the adaptor, so it is barely in contact with the top of the bed.

NOTE: A frit mesh size < 20 μm is recommended.

3.1. Slurry-Packing for laboratory- and process-scale columns

A slurry of Ca⁺⁺Pure-HA should be prepared in a buffer in which the media is stable. Phosphate-containing solutions at pH 6.5 or higher (e.g., 0.02 mol/L phosphate, 0.15 mol/L NaCl, pH 6.8), or a high-pH solution (e.g., 0.1 mol/L NaOH) are convenient for this purpose. Care must be taken to keep Ca⁺⁺Pure-HA in suspension as it will settle rapidly. Do not use a magnetic stir bar to keep media in suspension as this will generate fines.

For laboratory-scale columns, Ca⁺⁺Pure-HA slurry should be added to a column containing 2 - 3 cm of packing buffer, ensuring that no air bubbles are introduced to the bed. If a packing adaptor is used, bed may be consolidated into column by packing buffer flow, or by gravity/assisted gravity settling. Use a flow velocity of at least 2x the intended run velocity. A flow rate of 600-1000 cm/hr is usually appropriate.

For process-scale columns, after adding the packing buffer to the media, use a paddle or overhead impeller to mix and keep the media in suspension. Introduce media to the column by pouring or by transfer with a diaphragm pump. It is often advisable to run the column for an extended time in upflow mode at a slow rate (ca. 60 cm/hr) to remove any air that may have become trapped in the bed. For flow packing, the flow velocity should be at least 1.5x the intended process velocity. Gravity or assisted gravity packing may also give acceptable results and are often convenient due to the fast settling rate of Ca⁺⁺Pure-HA.

NOTE: Before packing your process-scale column, contact our Technical Specialists for individual recommendations.

Once in the column, flow pack Ca⁺⁺Pure-HA for approximately 10 CV to ensure a stable bed formation. Column adaptor should be lowered to just touch the top of the bed. DO NOT attempt to compress Ca⁺⁺Pure-HA bed.

3.2. Dry-Packing for laboratory-scale columns

The dry-packing method has been successfully used in columns up to 44 mm in diameter.

To dry-pack Ca⁺⁺Pure-HA, determine the amount of dry media needed for the given column volume based on the provided tap density for given lot. For instance, for a 10-mL column with a media with a tap density of 0.63 g/mL, the amount of media needed would be:

$$10 \text{ mL} \times 0.63 \frac{\text{g}}{\text{mL}} = 6.3 \text{ g}$$

Carefully add media to the column and tap column bottom on a hard surface to settle or vibrate column. A tapping / vibration duration of 1 - 2 minutes (approx. 250 - 500 taps) is usually appropriate. After the media has been added to the column, place the top adaptor to just touch the top of the bed surface. DO NOT attempt to compress Ca⁺⁺Pure-HA bed.

Column should then be hydrated with an appropriate packing buffer, such as 0.02 mol/L phosphate, 0.15 mol/L NaCl, pH 6.8, or 0.1 mol/L NaOH, in upflow at a flow rate of 40 - 60 cm/hr for at least 1.5 CV to ensure that the media is thoroughly hydrated and that any air has been removed from the column bed. Column should then be flow packed in down flow for 10 CV at 600 - 1000 cm/hr to ensure an efficient pack. Adjust column adaptor if necessary to reduce the gap between piston and bed top.

3.3. Packing a process-scale Verdot InPlace™ column

Verdot InPlace columns are especially well suited for the gentle packing of Ca⁺⁺Pure-HA.

The slurry preparation is similar to §3.1. The slurry is then introduced in the column by raising the piston at 200 cm/h. Due to the fast sedimentation of the medium, a re-homogenization after slurry introduction is recommended. This can be easily done via air sparging for 15 - 30 minutes. After this procedure, deflating the seal and tilting the column will allow fast removal of trapped air bubbles.

Subsequently, the bed is formed by lowering the piston at 200 - 400 cm/h (depending of the process flow rate) with the outlet valve open. Please note that the bed is consolidated from bottom to top (usually slightly whiter gel on the consolidated part).

When the piston reaches 1 cm above the consolidated gel, lower the speed to 60 cm/h and continue to lower the piston to 1 mm above the bed top. DO NOT attempt to compress Ca⁺⁺Pure-HA bed.

COLUMN PACKING

Equilibrate the column with 2 – 3 CV of buffer before performing the performance test.

A detailed Standard Operating Procedure can be provided on-request. Contact your Tosoh Sales Representative or the Technical Support at techsupport.tbg@tosoh.com.

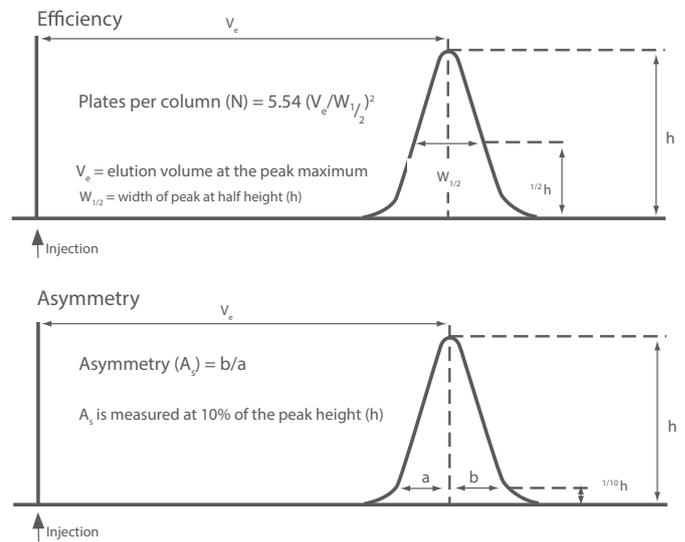
3.4 Performance test

Evaluation of packing quality can be tested and documented by the standard measures used for polymer-particle columns: efficiency and asymmetry (Figure 7).

Choice of test substances is important. They should not include substances that interact directly with Ca⁺⁺Pure-HA. Acetone is recommended. It can be added at a concentration of 0.1-1.0% to the running buffer. Acetone absorbs UV at 280 nm.

It is most common to use the flow rate at which the column will be run during the intended application for the performance evaluation.

FIGURE 7 HOW TO CALCULATE EFFICIENCY AND ASYMMETRY FACTOR



4. ELUTION METHODS

Diverse elution strategies for Ca⁺⁺Pure-HA have evolved over the decades, and like Ca⁺⁺Pure-HA-biomolecule interactions, all elution strategies are multimodal. Knowing that in advance helps to understand how Ca⁺⁺Pure-HA works and ultimately leads to development of more effective purification methods.

4.1. Phosphate Gradients

The elution method for Ca⁺⁺Pure-HA is a simple phosphate gradient at neutral pH. Potassium phosphate is more convenient because it remains fully soluble at concentrations up to at least 2 mol/L. Sodium phosphate tends to be slightly less costly but saturates at about 0.8 mol/L. It also solubilizes more slowly than potassium phosphate. In either case a gradient to about 0.3 mol/L phosphate elutes most biomolecules, but there are exceptions. Elution of some lipid enveloped virus species will require a double buffer concentration.

Phosphate buffers affect Ca⁺⁺Pure-HA-biomolecule interactions in two major ways. First, their conductivity competes with cation exchange interactions, weakening attractive interactions between Ca⁺⁺Pure-HA phosphates and protein amino residues, while weakening repulsion between Ca⁺⁺Pure-HA phosphates and protein carboxyl or biomolecule phosphoryl groups. In short, phosphates mediate the same influences with Ca⁺⁺Pure-HA that sulfate and carboxyl groups mediate with traditional cation exchangers.

The second way phosphate elution buffers operate is to compete with metal affinity interactions. These interactions fall into two groups. Phosphate buffers compete for coordination bonds between Ca⁺⁺Pure-HA calcium and biomolecule carboxyl or phosphate groups. In addition, phosphate buffers compete for coordination bonds between Ca⁺⁺Pure-HA phosphates and the metal moieties on metalloproteins.

Despite their simplicity and convenience, phosphate gradients impose two compromises. The first is that there is no opportunity for individual control of Ca⁺⁺Pure-HA's two dominant retention mechanism. The second is that acidic proteins, polynucleotides, and other phosphorylated contaminants elute across the same phosphate gradient intervals as neutral and alkaline proteins. This means phosphate elution provides less discrimination between highly differentiated biomolecule classes than is possible with other Ca⁺⁺Pure-HA elution strategies.

4.2. Calcium Gradients

Calcium gradients in the absence of phosphate were evaluated briefly in the 1980's but proved to elute only small strongly alkaline proteins with extremely low calcium affinity²⁻⁴. They are not routinely included in development of Ca⁺⁺Pure-HA methods.

4.3. Chloride Gradients

Routine Ca⁺⁺Pure-HA screening with chloride gradients can sometimes elute alkaline biomolecules in the absence of phosphate but require a higher salt concentration than would ever be considered for ion exchange chromatography. This highlights the cooperative contribution of calcium affinity to protein binding by phosphoryl cation exchange. Gradients up to 2 mol/L chloride are common and can extend up to twice that or more.

It was emphasized at the same time that sodium and potassium salts can be used interchangeably¹, though not within a run. Use exclusively potassium salts within a given run or exclusively sodium salts. Combinations will work and probably not cause an overt process failure. However, potassium and sodium salts impose subtle differences on Ca⁺⁺Pure-HA selectivity. Mixing—and especially mixing them inconsistently—will lead to diminished performance and reproducibility.

Experience has shown that chloride gradients promote strong retention of acidic proteins, polynucleotides, viruses, and endotoxins at the same time they elute many neutral to alkaline proteins, including IgG. Their most serious limitation is that they induce uncontrolled pH excursions that reduce Ca⁺⁺Pure-HA column lifetime (see section 7.1)^{6,7}. Another disadvantage of chlorides is that they are highly corrosive, including to stainless steel containers and plumbing.

The limitations of chloride salts can be reduced by substituting acetate salts for a typical recombinant protein separation. They have lower molar conductivity than chlorides and are also less corrosive. Conductivity of protein elution will be about the same as with chlorides but there are subtle differences in selectivity and resolution.

ELUTION METHODS

4.4. Multicomponent Gradients

The main value of multicomponent gradients is that they allow Ca⁺⁺Pure-HA's individual retention mechanisms to be controlled with a degree of independence^{1,5}. Chloride gradients at low constant phosphate concentrations were recognized by 2000⁸, phosphate gradients at constant chloride concentrations by 2005⁹, and application of both to routine selectivity screening by 2006⁵.

At a practical level, all of these variations can be classified into two groups.

First: acetate or chloride gradients at constant phosphate concentration. During initial screening, a baseline run may be conducted with no phosphate present, then subsequent runs at increments of phosphate, such as 0.005 mol/L, 0.01 mol/L, and 0.015 mol/L or higher. The incremental increase of phosphate gradually reduces the contribution of Ca⁺⁺Pure-HA metal affinity, thereby increasing dependence of retention on phosphoryl cation exchange. Neutral-to-alkaline proteins tend to be well served by this approach. The lowest increment of phosphate that permits elution of the target solute within the acetate or chloride gradient typically supports the highest degree of purification.

Second: phosphate gradients at constant levels of acetate or chloride. During initial screening a baseline gradient is normally run in the absence of acetate or chloride, then subsequent runs at increments of acetate or chloride, such as 0.1 mol/L, 0.5 mol/L, and 1.0 mol/L. The incremental increase of acetate or chloride leaves Ca⁺⁺Pure-HA metal affinity almost unaffected while it weakens the intensity of phosphoryl cation exchange interactions. This favors earlier elution of neutral-to-alkaline proteins. Acidic solutes including proteins and polynucleotides tend to be well served by this approach. The highest increment of acetate or chloride that conserves binding of the target solute typically supports the highest degree of purification.

4.5. Pre-elution Washes

As with all adsorptive chromatography media, pre-gradient washes can improve the degree of purification achieved by the gradient. Ca⁺⁺Pure-HA's multimodal selectivity however offers much more opportunity than single-mode packings like ion exchangers and hydrophobic interaction chromatography media.

There are two fundamental approaches, coordinated with the two multicomponent gradient systems described above. If a given target molecule is to be eluted in a chloride or acetate gradient, a pre-elution wash conducted exclusively with phosphate will pre-clear many impurities bound by weak-to-moderate calcium affinity.

If a given molecule is to be eluted in a phosphate gradient, the pre-elution wash should be conducted with a low concentration of phosphate and a high concentration of acetate or chloride to pre-clear cation exchange-bound impurities.

This ability to bind solutes by both chemistries, dominantly target one chemistry during the wash, then target the other chemistry during elution is unique in the field of adsorption chromatography, and it is uniquely powerful. It essentially allows a single run on Ca⁺⁺Pure-HA to achieve the impurity removal of two distinct chromatography steps. This can be a decisive advantage in situations where the objective is to achieve clinical product quality with a capture step and a single polishing step.

4.6. Selectivity Modifiers

Ca⁺⁺Pure-HA tolerates a wide range of surfactants, chaotropes, reducing agents, organic solvents and polymers, and other modifiers that extend its utility. These include arginine, DTT, DTE, mercaptoethanol, urea, guanidine, polyethylene glycol, zwitterionic and nonionic detergents, among others. Tolerance of guanidine and other high conductivity modifiers applies only to biomolecules that bind Ca⁺⁺Pure-HA by calcium metal affinity, but that includes most biomolecules to some degree. For example, most IgG antibodies remain bound to Ca⁺⁺Pure-HA in 1 mol/L guanidine so long as phosphate is absent.

Modifiers can have valuable utility in various contexts. Guanidine can be used in unfolding and refolding. An improperly folded protein can be bound, washed with guanidine, then the guanidine removed, gradually if helpful, and the refolded protein eluted. Combinations of urea, reducing agents, and/or detergents and various salts can be applied in the same way. Modifiers can otherwise be used to promote or maintain protein solubility or stability, perform detergent exchange or removal, dissociate aggregates, enhance retention of aggregates, or fine tune selectivity to achieve better purification.

5. CLEANING, SANITIZATION AND STORAGE

The unique surface chemistry of Ca⁺⁺Pure-HA has important ramifications for column maintenance.

5.1. Cleaning

Ca⁺⁺Pure-HA calcium affinity renders chloride salts ineffective as cleaning agents. Retention of DNA, lipid enveloped viruses, endotoxins, and many acidic proteins persists even in saturated NaCl. However, they are all removed effectively by high concentrations of phosphate. Phosphate also has sufficient conductivity to remove materials bound by electrostatic interactions with Ca⁺⁺Pure-HA-phosphates.

0.5 mol/L phosphate has been most commonly recommended for cleaning Ca⁺⁺Pure-HA. Sodium phosphate can be used for this purpose but bears a solubility limitation that can be inconvenient and limiting in some circumstances. It saturates at about 0.8 mol/L. Cleaning concentrations can be achieved but slow solubilization during buffer preparation is undesirable and there is virtually no latitude to produce concentrated stocks. Potassium phosphate transcends these limitations. It is easily soluble to at least 2 mol/L.

Cleaning-phosphate concentrations higher than 0.5 mol/L should be approached with awareness. Phosphate is a strong precipitating ion, immediately adjacent to sulfate in the Hofmeister series. It is known to promote hydrophobic interactions. The highly charged surface of Ca⁺⁺Pure-HA is generally not considered to be hydrophobic, and certainly not in comparison to chromatography media intended for hydrophobic interaction chromatography. However, strong precipitating ions have been shown to cause protein retention on ion exchangers and could potentially do so on Ca⁺⁺Pure-HA surfaces as well.

It can be tempting to combine chlorides with phosphate to clean Ca⁺⁺Pure-HA but this too should be approached with awareness. Chlorides increase the retention of highly phosphorylated solutes like DNA and lipid enveloped viruses, requiring higher phosphate concentrations for elution than in the absence of chloride.

5.2. Sanitization

Ca⁺⁺Pure-HA becomes increasingly stable at elevated pH. This invites application of hydroxide as a sanitizing agent; either NaOH or KOH. As with polymer based chromatography media, 1.0 mol/L NaOH is used widely. Contact duration can be the same as with polymer based media, with one hour contact time being common. Unlike many polymer-based media however, stability of HA in high concentrations of hydroxide is essentially indefinite.

Ca⁺⁺Pure-HA exposure to concentrated hydroxide should not immediately follow highly concentrated phosphate. It is recommended to rinse the column before introduction of hydroxide with 1–2 column volumes of buffer containing less than 0.02 mol/L phosphate. In most cases, the column equilibration buffer serves this purpose. Some sources recommend water for this purpose but it risks loss of pH control and adds another solvent to the overall process.

Hydroxide sanitization also gives a boost to cleaning, and especially if it is conducted in dynamic mode. Static mode describes a situation where hydroxide is pumped onto the column then flow is paused during sanitization. With dynamic sanitization, hydroxide is pumped onto the column then flow rate is maintained but reduced so at least 1 column volume of hydroxide passes through the column over the duration of sanitization.

5.3. Storage

Ca⁺⁺Pure-HA can be stored indefinitely in hydroxide solutions even at 1.0 mol/L but they are more commonly stored at 0.1 mol/L. Lesser concentrations could prove adequate but should be validated experimentally.

Storage in the presence of antimicrobial agents such as chlorhexidine is not recommended. Their multiple positive charges may cause them to be retained by Ca⁺⁺Pure-HA-phosphates, leaving the bulk solvent unprotected and allowing microbial growth between the particles.

The use of azides is not recommended. They are known to interact with various metal ions and might interact with Ca⁺⁺Pure-HA calcium, potentially leaving the bulk solvent unprotected and allowing microbial growth between the particles.

6. APPLICATIONS AND METHOD DEVELOPMENT

Routine screening of phosphate and chloride gradients as a starting point in developing Ca⁺⁺Pure-HA fractionation methods is recommended¹. This remains good advice. Alkaline products overall seem best served by chloride or acetate gradients at low phosphate concentrations. Acidic products overall seem best served by phosphate gradients. Products with intermediate charge characteristics are frequently served well by both approaches, though screening usually reveals one or the other as superior.

Like all adsorptive chromatography media, Ca⁺⁺Pure-HA can be used effectively in bind-elute and flow-through modes. Flow-through is more convenient, requires less mechanical sophistication, and uses fewer buffers. Fractionation performance and reproducibility may be sacrificed to a degree; sometimes recovery as well, but no more than flow-through methods on other types of adsorbents.

Bind-elute applications can be performed in either step or linear gradient mode. Linear gradients are often preferred for lab scale operation because they provide higher resolution and reproducibility. These benefits carry over to industrial separations and can help enable two-step purifications for facilities equipped to perform them at scale. Step gradients are nevertheless more common at industrial scale.

The following sections provides practical tips for Ca⁺⁺Pure-HA method development with several popular product classes. They are written to be performed on columns but the conditions may be adapted to multi-column or plate-based robotic formats, and both can be used in conjunction with DoE systems to reduce the experimental workload.

6.1. Purification of IgG

Purification of IgG monoclonal antibodies has proven to be one of Ca⁺⁺Pure-HA's most popular applications. Phosphate gradients provide fair fractionation from crude cell culture harvests, and sometimes outstanding fractionation from aggregates and IgG fragments in polishing mode^{10,11}. Non-phosphate gradients provide outstanding polishing for the majority of protein A-purified IgG monoclonal antibodies. In addition to removing aggregates, they support extraordinary reduction of leached protein A, DNA, endotoxins, and lipid enveloped viruses.

The template for non-phosphate gradient fractionation of IgG monoclonal antibodies was developed in early 2000 by Guerrier et al^{18,12}. A linear chloride gradient at a level concentration of 0.01 mol/L phosphate achieved good impurity reduction. Interchangeability of sodium and potassium phosphate salts was already well established¹, and the Guerrier template method was soon extended to include both.

Development of Phosphate Gradient Systems

Most IgG monoclonal antibodies elute in the range of 0.075–0.15 mol/L phosphate at pH 7.0. A screening gradient beginning at 0.005 mol/L phosphate and continuing to 0.2 mol/L phosphate is a good place to start. Since this phosphate interval is quite broad it makes sense to run a long gradient, such as 20 CV. For a second run, reset the gradient endpoint to the phosphate concentration at the center of the IgG peak and adjust gradient length as desired. The lower the end-point phosphate concentration, the more effective the removal of DNA and other acidic impurities. Wash the column with 0.5 mol/L phosphate before re-use to clear DNA and other acidic impurities.

Note that 0.005 mol/L phosphate is not sufficient to maintain adequate buffer control during column equilibration or in the early region of a phosphate gradient. It is recommended that the equilibration formulation be augmented with 0.02–0.05 mol/L HEPES or other non-phosphate buffer of choice. There is no need to provide secondary buffering for the gradient-end buffer because the high concentration of phosphate provides excess buffer capacity.

Impurity removal may be improved if the overall run pH is reduced to 6.5, but pH control will be compromised to a greater degree coincident with introduction of chlorides. Consider replacing HEPES with 0.05 mol/L MES. MES has a pKa of 6.0. If pH drops below 6.5, buffer capacity becomes stronger because it is approaching the pKa. HEPES has a pKa of 7.0. Buffer capacity becomes weaker at pH 6.5 because it is moving away from its pKa.

Development of Pre-Gradient Wash Conditions for Phosphate Gradients

Where the antibody is intended to be eluted in a phosphate gradient, the pre-gradient wash should be conducted with a high concentration of non-phosphate salt in combination with a low concentration of phosphate. The basic rationale is to apply a high non-phosphate salt wash at the highest phosphate concentration that does not elute the antibody, then return to equilibration conditions and apply the phosphate gradient. This wash will pre-clear impurities bound dominantly by Ca⁺⁺Pure-HA cation exchange.

This tactic works only with antibodies that do not elute in a non-phosphate gradient at 0.01 mol/L phosphate or higher. For example, with an antibody that elutes in a potassium chloride gradient at 0.015 mol/L phosphate, load purified IgG, wash with equilibration buffer, then conduct a wash with 1.0 mol/L chloride, 0.005 mol/L phosphate. Collect the wash. Wash again with 1.0 mol/L chloride 0.01 mol/L phosphate. Check the washes for the presence of IgG and select the wash with the highest phosphate and non-phosphate salt concentration that does not elute IgG. If no IgG is present, then phosphate concentration can be increased

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modestly and/or non-phosphate can be increased dramatically, such as to 2.0 mol/L. DoE can be used to optimize the conditions.

Note that it is important to return to the equilibration conditions before beginning the phosphate gradient. Not doing so will risk loss of IgG at the beginning of the phosphate gradient.

Development of Non-Phosphate Gradient Systems

Most proteins with a pI greater than 7 will elute in an acetate or chloride screening gradient to 2.0 mol/L at a constant phosphate concentration of 0.01 mol/L and a pH of 7.0. As with phosphate gradients, operating at pH 6.5 will increase protein retention and capacity but may compromise pH control.

Reducing phosphate concentration to 0.005 mol/L will increase protein capacity and improve contaminant removal, but many proteins will not elute, even at 2.0 mol/L chloride or acetate. Rarely, some proteins can be eluted in the complete absence of phosphate but a minimum of 0.005 mol/L phosphate is still recommended to maintain stability of Ca⁺⁺Pure-HA. Increasing phosphate concentration above 0.01 mol/L will be required to enable elution of some molecules. Proteins that require more than 0.015 mol/L phosphate to elute in an acetate or chloride gradient represent a minority and may be served better by phosphate gradient elution.

These variations and limitations make it logical to begin screening with a potassium phosphate-acetate elution system as described below:

EQ: 0.01 mol/L potassium phosphate, 0.05 mol/L HEPES, pH 7.0.

Load: Sample protein titrated to pH 7.0.

Wash: 0.01 mol/L potassium phosphate, 0.05 mol/L HEPES, pH 7.0.

Elute: 20 CV linear gradient to 0.01 mol/L potassium phosphate, 2.0 mol/L potassium acetate, pH 7.0

Clean: 0.5 mol/L potassium phosphate, pH 7.0

Elution within the first half of the gradient indicates that gradient phosphate concentration can probably be reduced to 0.005 mol/L. Elution in the second half of the gradient generally supports better impurity reduction. If the protein fails to elute at 0.01 mol/L phosphate, increase it. DoE can be used to optimize the conditions.

Reducing pH to 6.5 may also be considered at this point. It enhances binding of proteins with a pI greater than 7 by phosphoryl cation exchange. This tends to improve impurity removal. It also implies that there should be increase binding capacity but that is not necessarily the case.

Development of Pre-Gradient Wash Conditions for Non-Phosphate Gradients

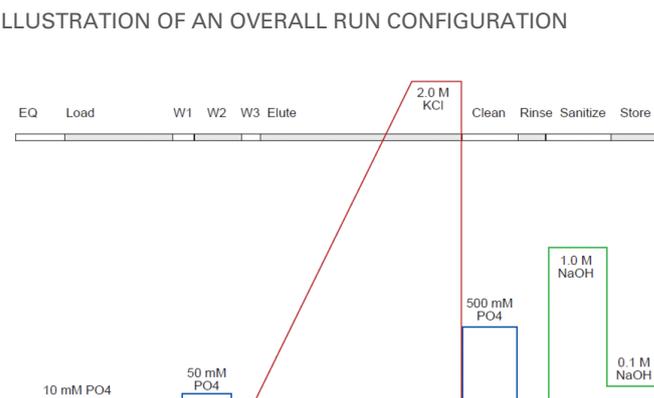
Where a protein is intended to be eluted by a non-phosphate gradient, such as with acetate or chloride, Ca⁺⁺Pure-HA should be washed in advance with a simple phosphate step, and then washed back to equilibration buffer before initiating the gradient. Washing the column with a higher concentration of phosphate than will be experienced during elution in the non-phosphate gradient will remove a subset of impurities that bind Ca⁺⁺Pure-HA with a higher proportion of calcium affinity than the protein. The objective is to determine the highest phosphate concentration that does not compromise recovery (Figure 8).

Measure the phosphate concentration at protein peak center from in a run where elution is performed in an exclusively phosphate gradient. In a separate run, after loading purified protein and washing with equilibration buffer, apply a 5 CV phosphate wash at half the phosphate concentration measured in the phosphate-only screening run. Collect the wash. Apply another wash at 0.01 mol/L higher phosphate, then another, and potentially another, etc., until the protein begins to appear in the wash. Set the final wash about 0.01 mol/L phosphate below the point at which protein losses in the wash first become apparent.

6.2. Purification of Fab, F(ab')₂, VHH

Published studies show that IgG Fab regions tend to be positive charge-rich while the Fc region tends to be carboxyl rich. This means that that VHH, Fab, and F(ab')₂ tend to be bound dominantly by cation exchange with Ca⁺⁺Pure-HA phosphates, while Fc fragments and intact IgG tend to be bound more strongly by metal affinity with Ca⁺⁺Pure-HA calcium¹³. Chloride gradients at low phosphate concentrations therefore provide outstanding fractionation. Phosphate gradients often provide adequate resolution. The best choice depends on the antibody. It is recommended to screen both, following the guidelines suggested for IgG.

FIGURE 8 ILLUSTRATION OF AN OVERALL RUN CONFIGURATION



APPLICATIONS AND METHOD DEVELOPMENT

6.3. Purification of Fc-fusion Proteins

Fc-fusion proteins are wild-cards, with their purification behavior heavily influenced and sometimes dominated by the fusion partner. Screen the same conditions recommended for IgG but be prepared to increase end-point phosphate concentration in phosphate gradients to 0.3 mol/L. For non-phosphate gradients, be prepared to increase the baseline phosphate concentration.

6.4. Purification of IgM

Ca⁺⁺Pure-HA is an extraordinary tool for IgM purification. Most IgM monoclonal antibodies are well served by phosphate gradient elution^{11,14}. Most elute in the range of 0.1-0.25 mol/L phosphate at pH 7.0. This makes an initial screening gradient to 0.3 mol/L a good place to start. It can be increased if necessary to accommodate a particular antibody. Some IgMs can be eluted in acetate or chloride gradients but typically require a fairly high phosphate baseline, such as 0.02–0.05 mol/L or more.

As with IgG, pre-elution washes can contribute substantially to purification. Follow the same guidelines described for IgG.

6.5. Purification of IgA

IgA monoclonal antibodies tend to be more acidic than IgGs, more highly glycosylated, and they often exist as dimers linked by a j-chain. They have been purified effectively with phosphate gradients^{15,16}. Whether they might be better purified by non-phosphate gradients remains to be seen. The extent to which purification performance might be improved by coordinated washes also has yet to be determined.

6.6. Purification of Phosphoproteins

Phosphate moieties on phosphoproteins can form strong coordination bonds with Ca⁺⁺Pure-HA calcium but it is impossible to make generalizations about preferred elution strategy because the overall properties of the protein will have a strong influence. Both phosphate and non-phosphate gradients should be screened.

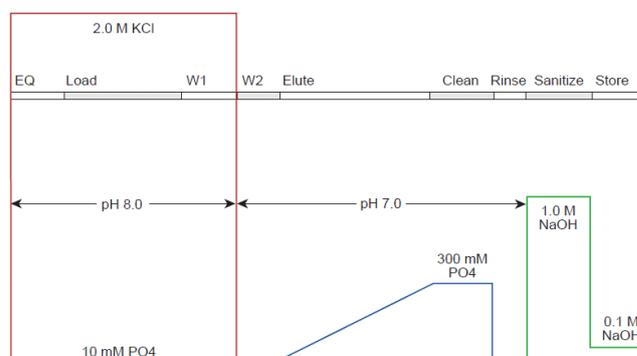
If a given phosphoprotein is alkaline, the phosphate moiety or moieties will cause it to elute later in an acetate or chloride gradient than a non-phosphorylated version of the same protein. The phosphorylated version may even fail to elute in an acetate or chloride gradient that elutes the non-phosphorylated version. Phosphate moieties on acidic proteins will have less influence because the protein will already have a tendency to bind Ca⁺⁺Pure-HA calcium residues through the many carboxyl domains that make the protein acidic. However, they may still increase net binding energy sufficiently to enable good separation from non-phosphorylated species.

6.7. Purification of DNA and RNA

Ca⁺⁺Pure-HA tends to be used more often to remove DNA and RNA than purify them, but it offers unique purification capabilities that no other chromatography method can. DNA retention on Ca⁺⁺Pure-HA increases with the number of base pairs. RNA retention increases with number of bases. Anion exchangers do an outstanding job with purifying both compounds but they lack the ability to fractionate on the basis of size, so the two methods together offer extraordinary purification (Figure 9).

FIGURE 9

INTEGRATED MODEL FOR PURIFICATION OF POLYNUCLEOTIDES



APPLICATIONS AND METHOD DEVELOPMENT

RNA elutes from Ca⁺⁺Pure-HA at substantially lower phosphate concentrations than DNA. Otherwise the same platform template can be applied to both. Begin scouting with a simple phosphate gradient. Equilibrate with 0.01 mol/L phosphate, 0.05 mol/L Tris, pH 8.0–8.5, load, wash, and elute with a linear gradient to 0.5 mol/L phosphate, 0.05 mol/L Tris, pH 8.0–8.5.

In a second run, equilibrate the column at 2.0 mol/L chloride in 0.010 mol/L phosphate, 0.05 mol/L Tris, pH 8.0–8.5. Equilibrate the sample to the same conditions and load it. Wash with equilibration buffer and follow with a linear gradient to 0.5 mol/L phosphate in 2.0 mol/L chloride at pH 8.0–8.5. The RNA and DNA will elute much later.

Three important benefits are achieved by the alkaline chloride run. Its strong suppression of cation exchange effects provides the polynucleotide with much better access to Ca⁺⁺Pure-HA calcium residues, resulting in higher capacity. It will also clear a large proportion of contaminants that are bound dominantly by cation exchange with Ca⁺⁺Pure-HA phosphate groups. Finally, it harmonizes very well with a previous anion exchange step in which the polynucleotide elutes at a high chloride concentration at alkaline pH.

Hybridizing the two retention formats provides the best of both worlds:

Loading and washing in high chloride at alkaline pH gives the best capacity and pre-gradient impurity clearance. Follow with a wash in 0.01 mol/L phosphate, pH 7.0 then elute with a linear gradient to 0.3 mol/L phosphate, pH 7.0. The simpler elution formulation will save expense in materials and cause the DNA to elute earlier due to restoration electrostatic interactions; specifically electrostatic repulsion between HA phosphate and polynucleotide phosphates.

There are three key points where the conditions may be optimized:

- 1) The higher the phosphate concentration at which initial polynucleotide binding can be conserved, the higher the impurity reduction during loading and the initial post-load wash. However, be aware that increasing phosphate will also reduce polynucleotide binding capacity.
- 2) If there is a desire to enhance size discrimination within the bound polynucleotide population, increase chloride concentration in conjunction with the phosphate gradient. The higher the chloride concentration at endpoint, the better the discrimination. Alternatively increase pH during the gradient. However, be aware that controlling conductivity is usually easier to achieve with precision than controlling pH.
- 3) Adjust the gradient slope and length.

DoE can be used to reduce the experimental workload during method development. For manufacturing operations, the gradients can be converted to a step format if desired.

6.8. Purification of Viruses

Lipid enveloped viruses exhibit extraordinary affinity for Ca⁺⁺Pure-HA because the phosphate groups in the envelope form strong coordination bonds with HA calcium, and the calcium groups in the envelope form strong coordination bonds with Ca⁺⁺Pure-HA phosphate. Some require as much as 0.6 mol/L phosphate for elution¹⁷. A correlation of virus size with the amount of phosphate required for elution has not been reported but seems likely given that this pattern occurs with most solutes applied to Ca⁺⁺Pure-HA. Eluting lipid enveloped viruses in non-phosphate gradients should be a practical impossibility. However, high salt washes at substantial phosphate levels should be very effective for contaminant reduction ahead of a phosphate-only elution gradient.

Ca⁺⁺Pure-HA behavior of non-lipid enveloped viruses cannot be predicted for the same reason that Ca⁺⁺Pure-HA behavior of proteins as a whole cannot be predicted: the range of diversity is too broad. Screen both phosphate and non-phosphate gradients.

7. PRACTICAL CONCERNS

7.1. Stability as Function of pH

Stability of Ca⁺⁺Pure-HA is pH dependent. Current consensus is that pH 6.5 is the minimum value at which Ca⁺⁺Pure-HA should be operated. This implies Ca⁺⁺Pure-HA columns should be stable so long as all the buffers are pH 6.5 or above, but that neglects a phenomenon that occurs with all charged media: so-called pH excursions. pH excursions are uncontrolled pH variations that occur in conjunction with changes in conductivity.

The dominance of Ca⁺⁺Pure-HA -phosphates on the crystal surfaces makes Ca⁺⁺Pure-HA net negative. During equilibration, positively charged hydronium ions accumulate in the region of Ca⁺⁺Pure-HA phosphates. Any individual hydronium ion may bind briefly to an Ca⁺⁺Pure-HA-phosphate but it is rapidly displaced by competition from others so its net residence time is extremely short. However, they do form a stable condensate cloud in the region of the chromatography surface where the ionic concentration is many times higher than in the bulk solution. Simulations in the field of fuel cell research have indicated counterion density at a charged surface can be 100 times the concentration in the bulk solution.

Increasing conductivity displaces these hydronium clouds to varying degrees into the bulk solution^{6,7}. This has the same effect as adding acid to the system: pH drops. During elution with phosphate gradients, the effect is buffered to some extent because phosphate itself is a buffer and its increasing concentration moderates the effect. During elution with chloride or acetate gradients, there is no increase in buffer capacity and pH excursions are more severe; commonly reducing pH briefly by a full unit, sometimes by 2 units or more. Duration can range up to 5 CV or more.

Various tactics have been considered to moderate these excursions but none has solved the problem. One approach concerns the addition of calcium to Ca⁺⁺Pure-HA buffers with the idea that their continuous presence in the buffer will discourage calcium leaching from the crystal surface. The most common difficulty with this approach is that calcium and phosphate together in solution spontaneously form stable associations, including formation of Ca⁺⁺Pure-HA. Turbidity becomes elevated and visible particles are sometimes evident.

Another approach involves using zwitterionic buffers such as MES and HEPES that do not form associations with Ca⁺⁺Pure-HA phosphates, and to titrate phosphate buffers with cationic buffering compounds like Tris, instead with hydroxides. The idea is that the positively charged buffering compounds become incorporated into the counterion condensate cloud so that an increase in conductivity increases buffer capacity at the same time it releases hydronium ions. The limitation in this case is that the buffering counterions have narrow pKas that limit their ability to counteract deep reductions in pH.

A third tactic is to use linear elution gradients rather than steps. The magnitude and duration of pH excursions is proportional to the rate of conductivity increase. The lower molar conductivity of acetates produces pH excursions of lesser magnitude in comparison to chlorides.

Also consider using a higher operating pH.

7.2. Stability as a Function of Phosphate Concentration

Consensus in the field presently indicates that a minimum phosphate buffer concentration of 0.005 mol/L must be maintained to conserve stability of Ca⁺⁺Pure-HA at pH 6.5. Otherwise, phosphate leaches from the crystal structure and the particles gradually degrade. Lower phosphate concentrations are required to maintain stability at higher pH values but no quantitative guidance has been provided as to what those minimums might be. This has led to the common practice of setting 0.005 mol/L as a minimum phosphate concentration even at higher pH values, such as 7.0, 7.5, and 8.0. Phosphate is routinely omitted from hydroxide solutions used to sanitize and store Ca⁺⁺Pure-HA.

7.3. Stability as a Function of Other Factors

Stability of Ca⁺⁺Pure-HA is also compromised by exposure to chelating agents. Their effects can be compounded by exposure at low pH, but even alkaline pH does not protect Ca⁺⁺Pure-HA from chelators. Compounds of particular concern include citrate, which is a well-known calcium chelator, frequently used to elute affinity columns or buffer cation exchangers. This can be an issue when protein A is eluted with citrate buffers. Protein A elution with acetate avoids this limitation.

EDTA and EGTA are also obvious concerns, as well as aspartic acid, glutamic acid, and other compounds with known ability to solubilize and transport metals. The heavy presence of chelating agents in cell culture harvests may explain in part why Ca⁺⁺Pure-HA is more commonly used as a polishing method rather than for initial product capture. Stability of Ca⁺⁺Pure-HA is not affected by autoclaving. The temperatures achieved in autoclaves are more than 200°C below the temperatures used to create the intercrystalline welds that stabilize Ca⁺⁺Pure-HA particles.

PRACTICAL CONCERNS

7.4. Symptoms of Destabilization

Symptoms of excessive loss of Ca⁺⁺Pure-HA mass include changes increases in operating pressure and eventual bed collapse. Both effects are believed to be caused by individual particle collapse, caused in turn by loss of Ca⁺⁺Pure-HA in the regions of the intercrystalline welds. These effects are most evident in large diameter columns because small diameter columns provide more wall support.

Chemically speaking, loss of Ca⁺⁺Pure-HA from the surface of Ca⁺⁺Pure-HA exposes more Ca⁺⁺Pure-HA. This implies that selectivity should remain unchanged. However, loss of surface Ca⁺⁺Pure-HA still translates into loss of surface area. That translates into loss of capacity, and separation performance will eventually be affected. When and how much depends on the buffers, gradient configuration, and column diameter. This makes column lifetime studies a necessary part of process validation.

7.5. Other Buffer Restrictions

Phosphate buffers are a necessary part of Ca⁺⁺Pure-HA chromatography but they can impose hidden limitations. It is recommended that anhydrous phosphate buffers be avoided. The intense heat required to eliminate hydration water during their processing causes formation of polyphosphates that can strongly bind Ca⁺⁺Pure-HA-calcium residues. Such polyphosphates potentially compete with sample components for binding substrate, potentially altering selectivity and capacity. A high phosphate cleaning step might adequately eliminate them after a run but cannot prevent them from interfering at earlier method steps.

7.6. Discoloration

Ca⁺⁺Pure-HA columns sometimes accumulate discoloration at the top of the column from binding metal ion contaminants. The discoloration is typically yellow–reddish–to brown and derives largely from ferric or ferrous leachates from stainless steel surfaces during the production of Water for Injection (WFI) and its subsequent storage. Cell cultures are also heavily laden with added metal ions, iron being the most prevalent. Metal contamination may also carry over from previous purification methods.

The mechanism of metal accumulation on Ca⁺⁺Pure-HA involves exchange of the contaminating metals for calcium residues in the crystal structure. They initially bind to Ca⁺⁺Pure-HA phosphates then displace calcium and become permanently incorporated into the crystal structure. They cannot be washed away or selectively eluted.

Studies have shown that non-calcium metal accumulations do not alter fractionation performance by Ca⁺⁺Pure-HA¹⁸. In fact, discoloration of Ca⁺⁺Pure-HA documents that Ca⁺⁺Pure-HA is providing a service that no other commonly used industrial resin can: it scavenges metal ions from contaminated feed streams. Beyond its high affinity for iron, Ca⁺⁺Pure-HA scavenges a broad spectrum of heavy metals including chromium, lead and cadmium. It also scavenges aluminum ions, which leach heavily from depth filtration media employing diatomaceous earth.

7.7. Residual Calcium in Eluted Product

Proteins eluted from Ca⁺⁺Pure-HA typically contain about 0.001 mol/L calcium. Human body fluids contain about 0.001 mol/L calcium. No adverse effects have been reported.

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9. TECHNICAL ASSISTANCE

Tosoh Bioscience GmbH has developed a staff of dedicated professionals to assist you with developing and scaling up Ca⁺⁺Pure-HA methods. Their knowledge of other Tosoh Bioscience GmbH products and how they work together also enables them to help you integrate Ca⁺⁺Pure-HA into multistep processes to achieve the best overall results. In addition, Tosoh Bioscience GmbH professionals can assist you with Ca⁺⁺Pure-HA testing procedures to evaluate lot consistency and compare HA products from various sources.

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