CHT™ Ceramic Hydroxyapatite — A New Dimension in Chromatography of Biological Molecules

Pete Gagnon,[1] Russ Frost,[2] Peter Tunón,[2] Tetsuro Ogawa;[3]
Validated Biosystems, Inc., 5800 N Kolb Road, Suite 5127, Tucson, AZ 85750 USA; [2]Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules CA 94547 USA; [3]Asahi Optical, Co., Ltd., 2-36-9, Maeno-Cho, Itabashi-Ku, Tokyo 174, Japan

Introduction

Hydroxyapatite chromatography was introduced in 1956, but despite numerous publications describing its merits as a unique chromatography tool, it has failed to attain the popularity of ion exchange (Tiselius et al. 1956). A number of reasons account for its neglect, most of which are artificial and the remainder obsolete. In practice, it offers a unique assemblage of process characteristics that merit serious evaluation.

Structure and Composition

The formula of hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$ (Kawasaki et al. 1985). The functional groups comprise positively charged pairs of crystal calcium ions (C-sites) and clusters of six negatively charged oxygen atoms associated with triplets of crystal phosphates (P-sites). C-sites, P-sites, and hydroxyls are distributed in a fixed pattern on the crystal surface (Kawasaki et al. 1978a, b, 1985).

Hydroxyapatite made by the Tiselius method or adjustments thereto often have excess phosphate in the structure (Tiselius et al. 1956, Atkinson et al. 1973, Bernardi 1971b, Spencer 1978, Spencer and Grynpas 1978). This leads to the formation of unstable rectangular plate-shaped crystals with poor flow, pressure, and stability characteristics. Recently developed synthesis methods yield hexagonal cross-section columnar crystals with the ideal Ca:P ratio of 1.67. They can be agglomerated to form particles, and sintered at high temperatures to fuse the particles into a stable porous “ceramic” mass. This yields media that possess flow properties, capacity, and scale-up attributes that are competitive with other popular methods (Kadoya et al. 1986, Kawasaki et al. 1986a, b, Kato et al. 1988). The available surface area and pore size are affected by the sintering process used during manufacturing, and this will affect the selectivity, capacity, and separation characteristics.

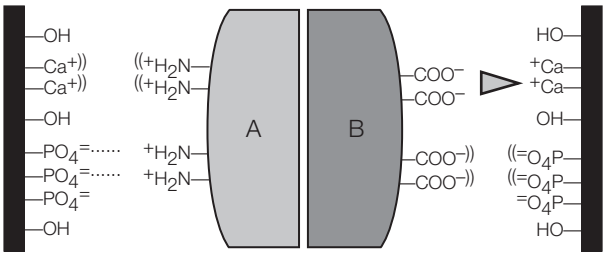

Fig. 1. Protein binding to hydroxyapatite. A is a basic protein. B is an acidic protein. Double parentheses indicate repulsion. Dotted lines indicate ionic bonds. Triangular linkages indicate coordination bonds.

Separation Mechanism

The hydroxyapatite:biomolecule interactions are complex. The following simplified discussion is an attempt to clarify the most significant features. Amino groups are attracted to P-sites but repelled by C-sites. The situation is reversed for carboxyls (Figure 1) (Gorbunoff 1984a, b, Gorbunoff and Timasheff 1984, Kawasaki 1991).

Although amine-binding to P-sites and the initial attraction of carboxyls to C-sites are electrostatic, the actual binding of carboxyls to C-sites involves formation of much stronger coordination complexes between C-sites and clusters of protein carboxyls. This has been proven experimentally by evaluating the retention of proteins on which the carboxyls have been replaced by sulfo groups (Gorbunoff 1984b). Binding is reduced dramatically even though net charge is unaltered (Gorbunoff 1984b). Further proof that carboxyl/C-site binding does not reflect a classical anion exchange interaction is found in the fact that binding capacity diminishes for acidic proteins with increasing pH (Bernardi and Kawasaki 1972, Gorbunoff 1984a, b, Ogawa and Hiraide 1996).

Phosphoryl groups on proteins and other solutes interact even more strongly with C-sites than do carboxyls (Kawasaki 1991). This is reflected in extremely strong binding by phosphoproteins (Bernardi and Cook 1960). DNA does not bind as strongly as

expected for a phosphoryl-rich solute. The spacing of the phosphoryl groups along the backbone apparently prevents an ideal match with the steric distribution of C-sites (Bernardi 1971a, Bernardi et al. 1972, Kawasaki 1991, Martinson 1973, Martinson and Wagenaar 1974). DNA binds well nonetheless and the strength of the interaction increases with its size. Endotoxins bind by the numerous phosphoryl groups on their core polysaccharide and lipid-A moieties (Homma 1984).

Applications

The initial development of a buffer system for use with ceramic hydroxyapatite is a simple and straightforward process. The sample is normally loaded in low ionic strength phosphate buffer (1–10 mM sodium or potassium phosphate) at or near neutral pH. Higher loading concentrations can be advantageous (Figure 2). Elution is normally done with a gradient of phosphate buffer (100–400 mM sodium phosphate) of the same pH, but as will be seen many different combinations are possible.

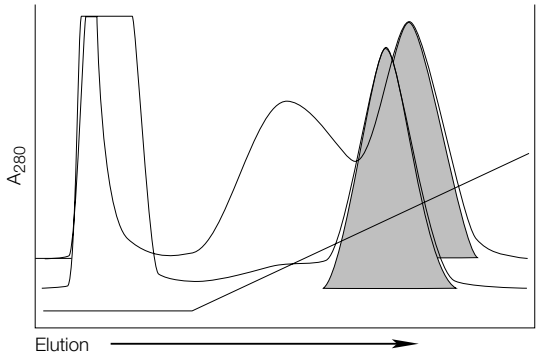

Fig. 2. The effect of phosphate concentration on protein binding. The upper profile was loaded with 1 mM phosphate. The binding buffer for the lower profile included 50 mM phosphate. IgG binding capacity (shown in gray) was improved by reduced substrate competition with contaminants.

Binding of basic proteins becomes stronger with reducing pH, due to increasing positive charge on the protein. As a rule of thumb, the lower the pH of the buffer the stronger the binding to the support is, and the higher the molarity of the sodium phosphate buffer required to desorb the protein will be. This reflects the dominant cation exchange component of the interaction, but the selectivity is distinct from classical cation exchange. Concurrent repellence of amines by C-sites, and the geometric distribution of charges, impart a unique stereochemical element that sometimes endows hydroxyapatite chromatography with the ability to discriminate among closely related protein variants. Examples include fractionation of light chain idiotypes from monoclonal mixtures with common heavy chains and fractionation of bifunctional antibodies from complex parent/sibling mixtures (Brooks and Stevens 1985, Bukovsky and Kennett 1987, Josic et al., 1991, Juarez-Salinas et al. 1984, 1986, Mariani et al. 1989a, b, Stanker et al. 1985).

Antibody Purification

Antibodies constitute the most widely purified molecule class of all. Hydroxyapatite chromatography is very suitable for antibody work and antibodies can be used to illustrate the versatility of the technique. With the proper buffer scheme most antibodies can be purified with hydroxyapatite chromatography, whether they are acidic, neutral, or basic.

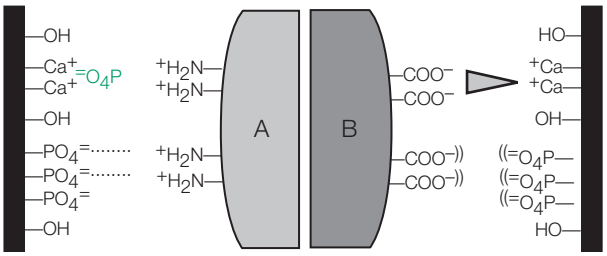

Fig. 3. Enhanced amine-binding by blocking C-site repulsion. A is a basic protein. B is an acidic protein. Double parentheses indicate repulsion. Dotted lines indicate ionic bonds. Triangular linkages indicate coordination bonds.

Antibodies that bind predominantly as acidic proteins can be applied to the column in a sufficient concentration of sodium chloride to maintain their solubility during loading (Josic et al. 1991). Tolerance of high sodium chloride allows dissociation of ionic complexes between antibodies and acidic contaminants like DNA, thereby increasing purification performance and product binding capacity. High sodium chloride tolerance also allows sample to be loaded with no equilibration other than pH titration.

Binding of weakly interacting basic proteins can be strengthened by inclusion of 1 mM phosphate in the buffer (Gorbunoff 1984a). The free phosphate ions pair with C-sites and suppress their ability to repel amines (Figure 3). This low concentration does not interfere with ionic binding between amines and P-sites. Basic proteins can be eluted with chloride or phosphate ions in a gradient from 50–500 mM (Gorbunoff 1984a, b, Gorbunoff and Timasheff 1984, Kawasaki 1991).

Acidic solutes will not elute in sodium chloride, even at concentrations >3.0 M (Figure 4). Elution requires displacers with stronger affinity for C-sites, such as phosphate, citrate, or fluoride ions. This has important ramifications, for example for antibodies that behave as basic proteins. It means that elution can be achieved with sodium chloride, completely avoiding the risk of contamination from the bulk of acidic sample components (Figure 5) (Kawasaki 1986, 1991). This specifically includes the ability to elute IgG under conditions where DNA and endotoxin remain quantitatively bound.

Solubility of a protein in a weak phosphate solution can be an issue, especially for antibodies. Many hydroxyapatite chromatography protocols for antibody production prescribe a 10 mM phosphate loading buffer. Virtually all IgMs and many IgGs are either partially or wholly insoluble under these conditions. This is easily remedied. Most antibodies tolerate significant

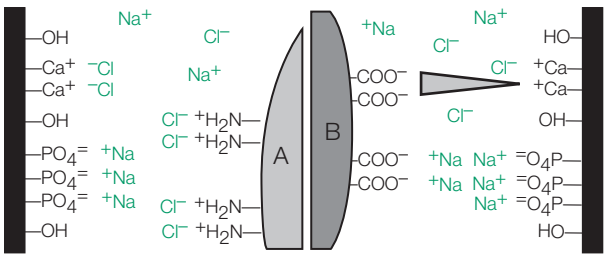

Fig. 4. Selective dissociation of amine binding with sodium chloride. A is a basic protein. B is an acidic protein. Triangular linkages indicate coordination bonds. Note that coordination bonds are unaffected.

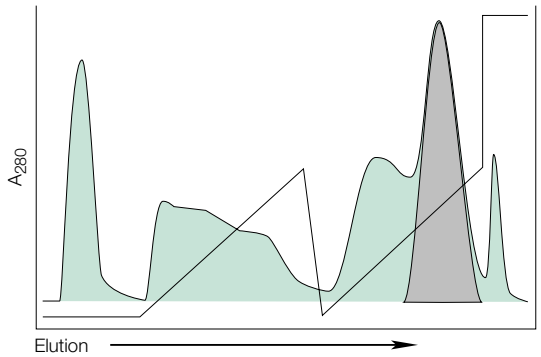

Fig. 5. Dual-gradient screening. The first gradient goes from 0 to 500 mM sodium chloride. The second goes from 0 to 500 mM potassium phosphate. The base buffer (A) was 0.5 M MES, 1 mM potassium phosphate, pH 6.0. The column was equilibrated in 10 column volumes (CV) of buffer A, then 2% CV of unequilibrated sample was injected and the column was washed with 2 CV of buffer A. Elution was with 10 CV of the first linear gradient, a 2 CV wash with a reverse gradient, then 10 CV of the second gradient. IgG elution shown in gray.

concentrations of sodium chloride without loss of binding efficiency. Inclusion of 100 mM NaCl in the loading buffer has been observed to suspend solubility problems with IgMs (Figure 6) (Hansson and Nilsson 1973, Josic 1991, Porath and Ui 1964).

Viral Clearance

Hydroxyapatite chromatography supports viral clearance of 0.6–3.5 logs (Dove et al. 1990, Grun et al. 1992). In one study it provided less average clearance than other purification methods (Grun et al. 1992). In another, it outperformed size exclusion chromatography by a factor of 15, ion exchange chromatography by a factor of 75, and precipitation by a factor >150 (Dove et al. 1990). Differential clearance abilities of the chloride and phosphate elution modes have not been characterized.

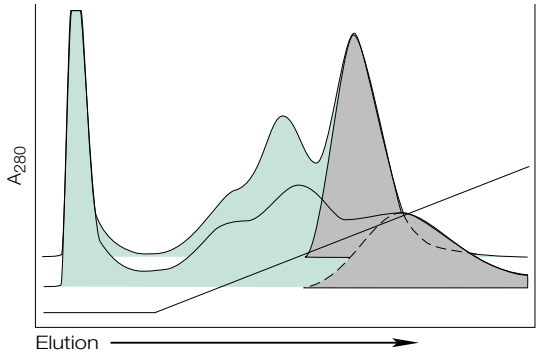

Fig. 6. The effect of solubilizing sodium chloride in the sample buffer. Sample was mouse IgM (shown in gray) in ascites. The column equilibration and sample buffer for the upper profile was 5 mM sodium phosphate, 100 mM sodium chloride, pH 6.8. IgM recovery was >90%. Buffers for the lower trace were the same except lacking sodium chloride. Recovery was ~75%.

Endotoxin Removal

Endotoxin removal can exceed 3 logs for antibodies that elute in sodium chloride. Clearance for antibodies that require phosphate elution is variable but seldom as good as 10-fold. As shown in Figure 7, endotoxin elutes continuously from 0.0–1.0 M phosphate. As with DNA, clearance efficiency depends on the relative elution position of the antibody. Removal of Phenol Red is similarly antibody dependent (Bukovsky and Kennett 1987).

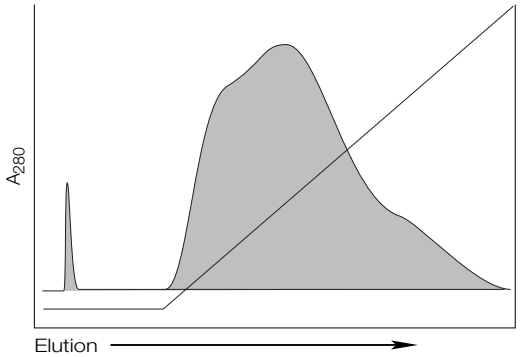

Fig. 7. Endotoxin elution from hydroxyapatite. A commercial endotoxin preparation was injected onto a column equilibrated with 50 mM MES, pH 5.6, then eluted with a linear gradient from 0.0–1.0 M potassium phosphate.

Recoveries

Mass recoveries of 90% are typical. Recoveries below 80% usually reflect inadequate method development. Frequent causes include inadequate binding conditions, inadequate column equilibration, poor antibody solubility under the loading conditions, and excessively narrow pooling.

Activity recovery per mass unit of antibody is typically quantitative but may be reduced with IgMs that are exposed to low ionic strength for prolonged periods awaiting chromatography (Josic et al. 1991).

Conclusions

Hydroxyapatite has not been exploited to its potential as a process chromatography tool mainly for lack of suitable media and specific application guidelines, with antibodies being a prime example. With proper maintenance and method development customized to its unique characteristics, hydroxyapatite chromatography offers process capabilities on par with the best alternatives. CHT ceramic hydroxyapatite provides all the performance characteristics needed for effective scale-up.

Hydroxyapatite supports a variety of two-step procedures for purification of in vitro products. Hydroxyapatite combined with size exclusion chromatography is effective for IgMs; infrequently for IgGs. Two-step combinations with hydrophobic interaction chromatography can be applied to both classes, generally supporting better purification, higher capacities, and better overall process economy. Combinations with immobilized metal affinity chromatography is worth evaluation. Combinations with ion exchange chromatography may provide adequate performance but combinations of charge-based methods are generally less effective than partnerships between dissimilar separation mechanisms.

For in vivo applications, hydroxyapatite may provide a useful selectivity alternative to ion exchange. Its nucleic acid clearance capabilities take on special value for antibodies that elute in chloride. Processes that combine either high-salt immobilized metal affinity or hydrophobic interaction chromatography, with chloride-hydroxyapatite chromatography, especially in that order, although not necessarily in immediate sequence, promise better DNA and endotoxin removal than any other combination.

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Additional Information

This work was originally presented at Recovery of Biological Products VIII, in Tucson, AZ, 1996. The material is adapted from Gagnon (1996). Figures 1 and 3–8 and portions of the text are reproduced here with the permission of the author and publisher. This book contains a hands-on approach to monoclonal antibody purification, with sufficient theory to guide and support the reader through a variety of different separation scenarios. Chapter 5 is dedicated to the use of hydroxyapatite. Along with a range of specific method development and manufacturing recommendations, it includes an extensive list of references to earlier work. More information about this book can be obtained on the Internet at **www.validated.com**

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