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Monoclonal antibody purification with hydroxyapatite

Review

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Hydroxyapatite (HA) has been used for IgG purification since its introduction in the 1950s. Applications expanded to include IgA and IgM in the 1980s, along with elucidation of its primary binding mechanisms and the development of ceramic HA media. With the advent of recombinant monoclonal antibodies, HA was demonstrated to be effective for removal of antibody aggregates, as well as host cell proteins and leached protein A. HA's inherent abilities have been enhanced by the development of elution strategies that permit differential control of its primary binding mechanisms: calcium metal affinity and phosphoryl cation exchange. These strategies support reduction of antibody aggregate content from greater than 60% to less than 0.1%, in conjunction with enhanced removal of DNA, endotoxin, and virus. HA also has a history of discriminating various immunological constructs on the basis of differences in their variable regions, or discriminating Fab fragments from Fc contaminants in papain digests of purified monoclonal IgG. Continuing development of novel elution strategies, alternative forms of HA, and application of robotic high throughput screening systems promise to expand HA's utility in the field.

Contents

Composition and retention mechanisms

HA is a naturally occurring mineral of calcium and phosphate with the structural formula $(Ca_5(PO_4)_3OH)_2$. A synthetic form was introduced as a chromatography medium by Tiselius in 1956 and was soon applied to the task of IgG purification [1,2]. The Tiselius method of HA synthesis produced thin flat brittle crystals that were poorly suited for chromatography. HA produced by derivative methods is available commercially, as well as a variant version in which HA crystal fragments are embedded in porous particles of agarose, but most current applications employ porous ceramic microspheres produced by fusing cylindrical HA nanocrystals at elevated temperatures [3].

The adsorptive surface unit of HA exhibits two primary features distributed in a continuously repeating hexagonal pattern: C-sites and P-sites. Each C-site comprises a doublet of positively charged calcium residues [4]. Their dominant interactions are calcium chelation by protein carboxyl clusters and calcium coordination by phosphoryl groups on DNA and other phosphorylated solutes.

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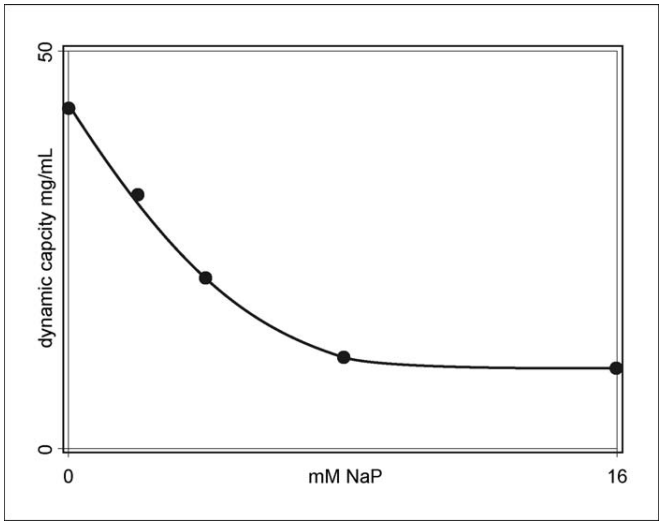

FIGURE 1

Dynamic capacity of monoclonal mouse IgG_{2a} as a function of phosphate concentration. CHT type I, 20 μm, 5 × 50 mm, 1 mL/min (300 cm/h), 0.05 M MES, plus the indicated sodium phosphate concentration, pH 6.5. Refer to ref. [9] for more detailed conditions. Redrawn from the ref. [9], with permission.

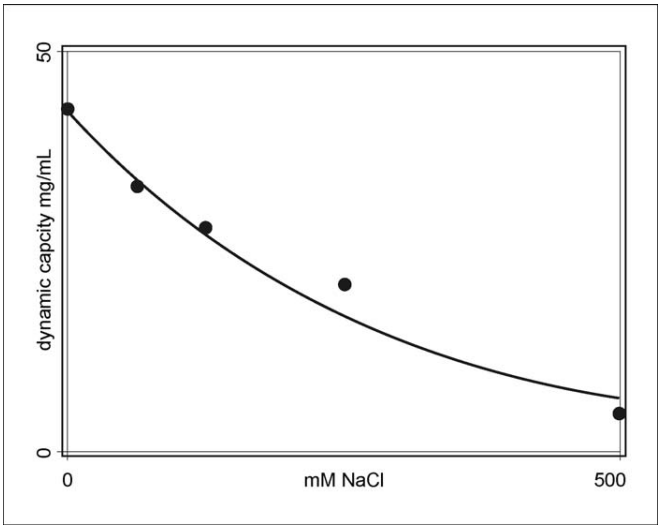

FIGURE 2

Dynamic capacity of monoclonal mouse IgG_{2a} as a function of sodium chloride concentration. CHT type I, 20 μm, 5 × 50 mm, 1 mL/min (300 cm/h), 0.05 M MES, plus the indicated sodium chloride concentration, pH 6.5. Refer to ref. [9] for more detailed conditions. Redrawn from ref. [9], with permission.

C-sites may also be capable of anion exchange interactions with negatively charged solute residues [4–8]. Chelation and coordination bonds can be eluted with phosphate, which has a strong affinity for HA-calcium, but they are stronger than electrostatic interactions and usually survive exposure to 4 M sodium chloride in the absence of phosphate.

The majority of IgG monoclonal antibodies exhibit relatively weak interactions with HA-calcium. Figure 1 illustrates dynamic binding capacity for a mouse monoclonal IgG over a range of 0–16 mM sodium phosphate. Capacity drops by about 75% from 0 to 8 mM phosphate, then remains fairly level up to 16 mM phosphate [9]. The profile suggests that calcium binding for this antibody is largely eliminated by about 10 mM phosphate, and that concentrations up to 16 mM are insufficient to significantly reduce binding by ion exchange. Carboxyl-rich (acidic) proteins such as BSA require up to 60 mM phosphate for elution, even in the presence of 1 M sodium chloride. The fact that BSA retention is little affected by the presence or absence of 1 M sodium chloride suggests that anion exchange does not make a significant contribution to binding. This hypothesis is supported by the fact that proteins on which carboxyls have been chemically substituted with sulfo groups fail to bind HA even though the native protein binds strongly [5–7].

Each P-site comprises a triplet of phosphate groups; each phosphate with a pair of negatively charged oxygen residues [4]. They act as cation exchangers, binding to protein amino groups under appropriate conditions [4–8]. Small strongly alkaline proteins such as lysozyme bind almost exclusively by cation exchange. They can be eluted with any salt, even in the absence of phosphate. Figure 2 illustrates the dynamic binding capacity curve of the antibody from Figure 1, but as a function of sodium chloride concentration. The ability of sodium chloride to reduce capacity by about 90% over the range from 0 to 500 mM salt suggests that phosphoryl cation exchange is the primary retention mechanism for this antibody, but the fact that HA still retains antibody at 500 mM salt, pH 6.5, suggests that a sodium chloride resistant mechanism persists: calcium chelation [9].

These results highlight the important point that C-site and P-site binding are cooperative. With antibodies, they are positively cooperative. For the antibody illustrated in Figures 1 and 2, dynamic binding capacity in the absence of both phosphate and chloride was greater than 40 mg mL, about four times higher than under conditions that selectively eliminated calcium chelation, and nearly 10 times higher than under conditions that selectively eliminated phosphoryl cation exchange. With other solutes, C-site and P-site interactions are negatively cooperative. DNA elutes at about 250 mM phosphate in the absence of sodium chloride, but requires about 500 mM phosphate to elute in the presence of 1 M NaCl. The high concentration of sodium chloride is believed to weaken charge repulsion between DNA phosphates and HA-phosphates, allowing a larger number of DNA phosphates to form coordination bonds with C-sites [10].

Antibody capture from cell culture supernatants

A number of investigators have reported the ability of HA to achieve 1-step IgG purification rivaling affinity methods such as protein A and protein G [11–15]. These applications have significant limitations nevertheless. As discussed above, IgG binding capacity is strongly affected by conductivity. The physiological conductivity of cell culture supernatants is sufficient to reduce capacity. In addition, many cell culture media are buffered with phosphate, which will depress capacity further. Dilution or diafiltration to reduce salt and phosphate concentration may increase capacity, but dilution fails to address two other significant limitations: first, it increases volume. This translates into longer process time, which is contrary to industry goals of increasing throughput efficiency at the capture step. Second, dilution does not eliminate chelating agents and metal ions that commonly populate cell culture supernatants. Chelating agents may remove calcium from the structure of HA, causing its gradual dissolution. Metal ions,

such as iron, bind strongly to HA, causing discoloration. Discoloration has been demonstrated not to affect purification performance but may attract regulatory scrutiny and increase the validation burden [16]. Diafiltration of the sample largely solves the problems of conductivity, phosphate content, chelating agents, and metal contamination, but leaves an important obstacle that likewise limits the utility of dilution: competition for binding substrate. Many of the contaminating proteins in cell culture supernatant bind to HA. Most bind more weakly than IgG and their binding can be prevented by elevating the phosphate and/or sodium chloride concentration of the feedstream, but this also reduces IgG binding capacity.

HA bears one more significant burden as an IgG capture step: interference of the eluting buffer with downstream purification steps. IgGs fractionated with simple phosphate gradients typically elute in the range of 100–200 mM phosphate. This corresponds to conductivity values ranging roughly from 15 to 30 mS/cm, and also imposes strong buffer capacity. Significant dilution or diafiltration may be required to prepare an eluted IgG for a downstream ion exchange step. Antibodies eluted in sodium chloride gradients may elute at 0.5–2.0 M sodium chloride, creating an even more severe conductivity burden.

HA is more feasible as a capture step for IgA and IgM. To begin with, no affinity equivalent to protein A has emerged for either IgA or IgM. Each of the many candidates suffers from some combination of low capacity, poor antibody recovery due to excessively harsh elution conditions, or poor tolerance of pharmaceutical cleaning conditions [17]. Cation exchange can be used effectively for capture of some IgMs, but not all [17,18]. Most IgMs bind well to anion exchangers, but so do most of the contaminants [17,18]. Hydrophobic interaction media suitable for IgGs often denature IgMs [17,18]. Size exclusion chromatography supports good fractionation of IgMs, but suffers from low capacity and slow flow rates. In this discouraging context, the ability of HA to support IgM capture capacities of 10–20 mg/mL without dilution of the feed stream, and to achieve purity of 90%, stands out as a relatively attractive option. It still bears the potential problems of gradual HA degradation by chelating agents, discoloration by metal ion contaminants, and competition by contaminating proteins, but binding capacity of IgA and IgM are both relatively unaffected by conductivity, and are generally less responsive than IgG to low concentrations of phosphate. Diafiltration of the feed stream may be desirable despite the additional cost, even if not strictly necessary, since it eliminates all of these issues except binding competition by contaminating proteins.

Intermediate and polishing purification

HA has been used for intermediate purification of many IgG [14,15,19–36], IgA [37–41], and IgM antibodies [17,37,38,42–44]. The majority of these applications employ simple phosphate gradients. Some of the more recent IgG applications employ chloride gradients [14,15,20–22]. Table 1 compares the ability of phosphate and chloride gradients to remove host cell proteins (HCP), leached protein A, DNA, and endotoxin from a monoclonal IgG_1 chimera [30]. Removal of leached protein A was equivalent in both systems, but the chloride gradient was dramatically more effective for all other contaminants. Chloride gradients at low phosphate concentrations also support good virus reduction: 2 LRV with MVM and 3 LRV with xMuLV [31]. Higher clearance of xMuLV may reflect strong interactions between viral envelope components and HA: viral envelope phosphate with HA calcium, and viral envelope calcium with HA phosphate.

TABLE 1

Removal of host cell proteins, leached protein A, DNA, and endotoxin by phosphate and chloride gradients.

Contaminant	Phosphate gradient	Chloride gradient
HCP	<72 ppm	<12 ppm
LPA	<1 ppm	<1 ppm
DNA	<7 ppm	<1 ppm
ETX	<5.0 EU/mL	<0.1 EU/mL

HCP: host cell protein. LPA: leached protein A. ETX: endotoxin. Original data reported in ref. [30].

Aggregate removal

HA elution with phosphate gradients has been known to support effective aggregate removal from IgG monoclonal antibodies since the 1990s [19]. The simplicity of making all of the process buffers with dilutions of the 500 mM phosphate-cleaning buffer is attractive for antibodies that are accommodated by this approach, but that unfortunately includes only a small proportion of IgGs. Several studies have demonstrated the ability of phosphate gradients to discriminate IgA aggregates and polymers [38–40]. Phosphate gradients also discriminate IgM 'monomers' from fully assembled pentamers [37,38], but have proven less capable for removal of IgM aggregates [17]. Phosphate gradient screening conditions are suggested in Table 2.

Chloride gradients in the presence of low phosphate concentrations have been demonstrated to extend the range of IgG monoclonal antibodies from which HA can remove aggregates [20,21]. This strategy has been recommended for routine selectivity screening with HA since 1991 [45], specifically for antibodies since 1996 [18], and it was demonstrated to be effective for aggregate removal from IgG in 2001 [23]. A constant low level of phosphate weakens calcium affinity interactions but leaves ionic interactions relatively intact (see Figures 1 and 2). A chloride gradient can then dissociate ionic bonds. Native IgG elutes first, followed by aggregates, generally in order of size (Figures 3 and 4). Aggregate levels greater than 60% have been reduced to less than 0.1% [20–22]. Table 3 suggests chloride gradient screening condi-

TABLE 2

Buffers and conditions for phosphate gradient screening.

1 mL (5 × 50 mm), CHT™ Type I 40 μm, 1 mL/min (300 cm/h)
Buffer A: 10 mM sodium phosphate, pH 6.7
Buffer B: 500 mM sodium phosphate, pH 6.7
Equilibrate column with A
Inject 5–10% CV protein A purified IgG
Wash 5 CV buffer A
Elute 30 CV linear gradient to 40% buffer B
Clean 5–10 CV 100% B
Use this protocol for IgG. For IgA, use the same buffers and conditions but use CHT type II, 40 μm. For IgM, use CHT type II 40 μm at a flow rate of 0.50–0.67 mL/min (150–200 cm/h) and continue the elution gradient to 75% B. pH can be varied in subsequent experiments, from 6.5 to 8.5 for HA, from 5.0 to 8.5 for FA.

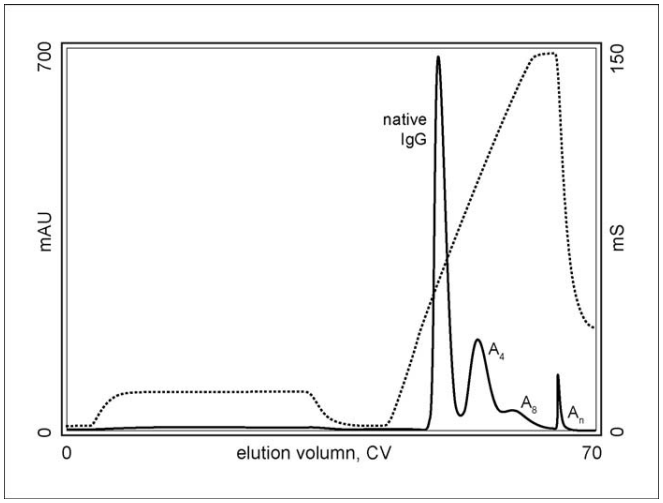

FIGURE 3

Aggregate removal with a chloride gradient. CHT type I, 20 μm, 8 × 50 mm, 2.5 mL/min (300 cm/h). Equilibrate with 5 mM sodium phosphate, pH 6.7. Load protein A purified monoclonal IgG1 chimera (elevated conductivity area from about 5 to 30 CV). Wash with 5 mM phosphate. Elute with a 20 CV liner gradient to 5 mM phosphate, 1.5 M NaCl, pH 6.5. Clean with 500 mM phosphate, pH 6.5 (descending conductivity area from about 65 to 0 CV). The peak marked A_4 indicates aggregates comprising four IgG molecules; A_8, eight molecules; A_n, larger aggregates.

tions. Effective aggregate removal is obtained with most antibodies that can be eluted at 5–15 mM phosphate, but declines in parallel with removal of HCP, DNA, and endotoxin for antibodies that require higher phosphate concentrations [30,32].

More recent work has revealed the ability of polyethylene glycol (PEG) to enhance aggregate removal by imposing secondary size selectivity on HA. PEG thereby disproportionately enhances aggregate retention, enabling effective IgG or IgM aggregate removal

TABLE 3

Buffers and conditions for chloride gradient screening.

1 mL (5 × 50 mm), CHT Type I 40 μm, 1 mL/min (300 cm/h)
Buffer A: 5 mM sodium phosphate, 50 mM MES, pH 6.7
Buffer B: buffer A + 1 M NaCl
Buffer C: 500 mM sodium phosphate, pH 6.7
Equilibrate column with A
Inject 5–10% CV protein A purified IgG
Wash 5 CV buffer A
Elute 30 CV linear gradient to 100% buffer B
Clean 5–10 CV buffer C

Use this protocol for IgG monoclonal antibodies. If the antibody fails to elute within the NaCl gradient at 5 mM phosphate, increase the phosphate concentration to 10 mM in buffers A and B. If it still fails to elute, increase it to 15 mM. In general, the lowest phosphate concentration at which a given antibody elutes will support the best removal of aggregates and other contaminants. pH can be varied in subsequent experiments, from 6.5 to 8.5 for HA, from 5 to 8.5 for FA.

when either phosphate or chloride gradients alone prove insufficient (Figure 5). The mechanism is discussed in detail in ref. [32]. It has been suggested that this approach should be most effective when used in conjunction with chloride gradients because of their superior removal of HCP, leached protein A, DNA, endotoxin, and virus [32]. Screening of either phosphate or chloride gradients may be conducted by adding 10% PEG-400 or PEG-600 to both gradient buffers. If testing reveals the main antibody peak to be sufficiently free of aggregates, then lower PEG concentrations may be evaluated. PEG preparations with average molecular weights up to 600 Da are FDA approved inactive ingredients for intravenous injection [46].

Beyond removal of aggregates and non-antibody contaminants, HA has demonstrated compelling ability to discriminate among antibodies according to differences in their variable regions. This has proven especially useful for purifying IgG idiotypes [33,34] and bispecific antibodies [35,36]. These separations imply that HA should also be effective for purification of Fab and other variable

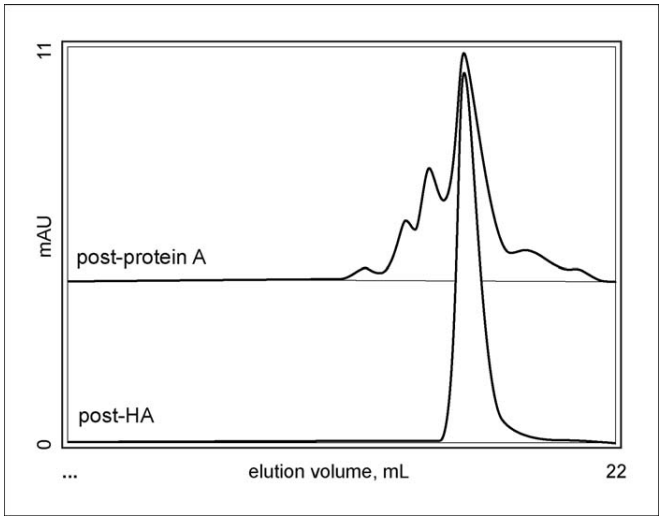

FIGURE 4

Analytical size exclusion chromatography of protein A purified monoclonal IgG_1 chimera, before and after fractionation by HA with a chloride gradient. Bio-Sil™ 400-5 (Bio-Rad), 5 × 300 mm, 0.5 mL/min (150 cm/h) 20 mM MES, 0.2 M arginine, pH 6.5. The content of the protein A purified antibody was estimated at about 40% aggregate, 45% native antibody, and 15% fragment. Both aggregates and fragments were removed by the chloride gradient. Redrawn from ref. [10], with permission.

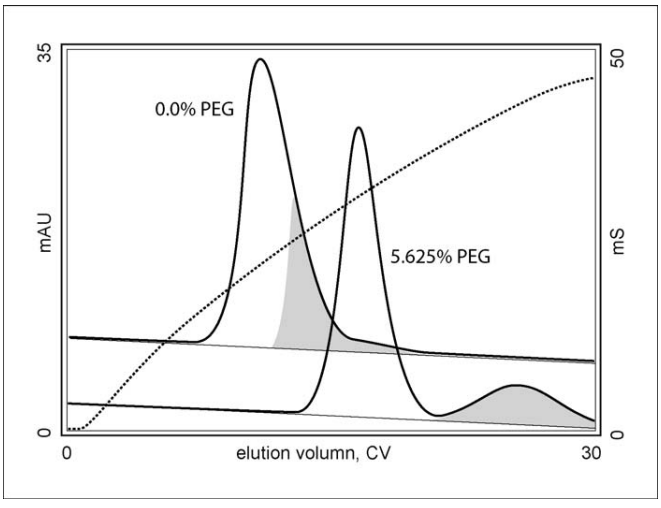

FIGURE 5

The effect of PEG on aggregate separation in a phosphate gradient. CHT Type I, 40 μm, 5 × 50 mm, 2 mL/min (600 cm/h). Equilibrate with 10 mM sodium phosphate pH 7.0. Inject. Wash with 5 CV equilibration buffer. Elute in a 30 CV linear gradient to 500 mM sodium phosphate pH 7.0. Repeat with 5.625% PEG-4600 added to both buffers. Gray areas indicate aggregates. See ref. [32] for more experimental detail and discussion. Redrawn from ref. [32], with permission.

region constructs. Indeed, Fab purification with phosphate gradients has been known for 20 years [47], and has recently been shown to be effective with chloride gradients as well [48]. Fab elutes in advance of Fc fragments with phosphate gradients, and after Fc with chloride gradients. HA has also been used successfully in the purification of diabodies and minibodies [49].

Emerging applications

Recent conference presentations suggest that HA is entering a renaissance. It has been known since the 1980s that HA can be converted to a calcium-derivatized form in the presence of soluble calcium and the absence of phosphate [5–7]. This converts P-sites into secondary C-sites, which blocks phosphoryl cation exchange, leaving calcium affinity as the sole retention mechanism (Figure 6). Ca-HA was not exploited until recently because no elution method had been identified that permitted conservation of the Ca-derivatized form during the separation. Phosphate concentrations as low as 5–10 mM convert Ca-HA back to native HA [50]. Calcium chloride was attempted but did not elute most test analytes [5–7]. Neither do chlorides or acetates. Recent investigations suggest that the Ca-HA form can be conserved during elution with sulfates and borates, both of which produce selectivities reminiscent of but distinct from phosphate gradients [50]. Sulfates and borates can also be used to elute native HA.

Preliminary data indicate that Ca-HA may be especially useful for purification of Fab. Fab binding to HA is heavily dominated by phosphoryl cation exchange, and elimination of that mechanism by conversion of native to Ca-HA can be used to selectively elute Fab from Fc contaminants [51,52]. Alternatively, calcium can be added to the crude Fab preparation and it can be applied to Ca-HA. Fab flows though the column. Fc fragments are retained and elute in the 500 mM phosphate cleaning step (Figure 7). Ca-HA also exhibits up to four times higher IgG binding capacity than native HA, and bears the additional benefit of being minimally affected by high concentrations of sodium chloride [50]. This could make HA more attractive as an IgG capture method.

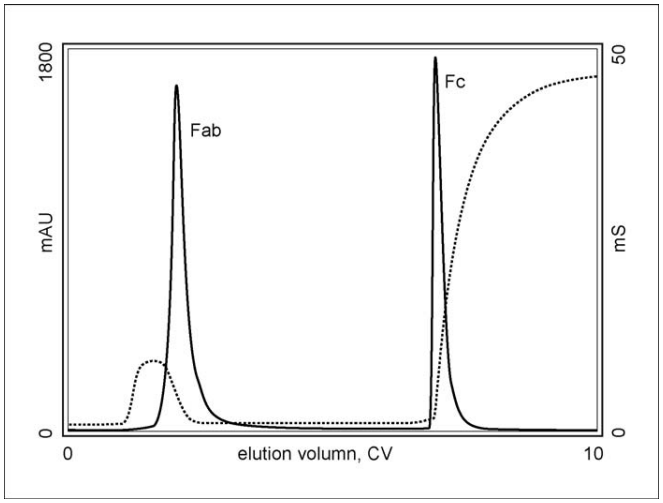

FIGURE 7

Fab purification on Ca-HA. CHT Type I, 20 μm, 8 × 50 mm, 2.5 mL/min (300 cm/h). Equilibrate with 25 mM Hepes, 2.5 mM $CaCl_2$, pH 7.0. Inject 2 mL of papain-digested IgG_1 chimera in 50 mM Tris, 50 mM sodium chloride, 2.5 mM $CaCl_2$, pH 7.5. Wash with 5 CV equilibration buffer. Elute with 500 mM sodium phosphate, pH 7.0. See ref. [51] for more experimental detail and discussion. Redrawn from ref. [51], with permission.

Preliminary data indicate that borate gradients on native HA support more effective aggregate removal than either phosphate or chloride gradients (Figure 8). In addition, the conductivity of 1 M borate at pH 7.0 is only about 9 mS/cm. The conductivities of 1 M

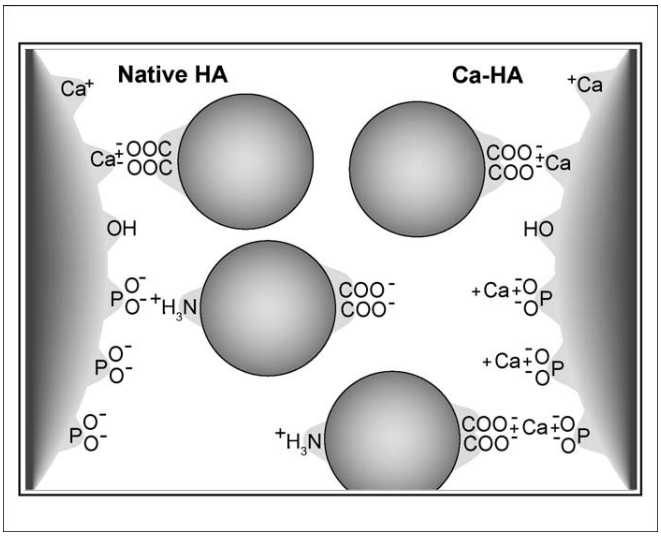

FIGURE 6

Native and Ca-HA. Native HA is illustrated on the left. The di-carboxy protein is retained by a calcium chelate interaction with one of the two calcium residues in the C-site. Protein below is retained by cation exchange with one of the three phosphoryl residues of the P-site. Ca-HA is illustrated on the right. P-sites are converted to secondary C-sites, which have the same ability to form calcium affinity bonds as native C-sites. Phosphoryl cation exchange is abolished.

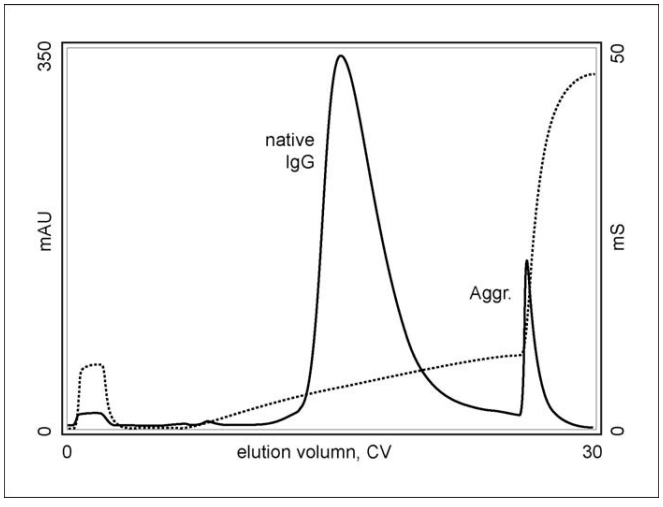

FIGURE 8

IgG aggregate removal in a borate gradient. CHT Type I, 20 μm, 8 × 50 mm, 2.5 mL/min (300 cm/h). Equilibrate with 5 mM sodium phosphate, pH 7.0. Inject 2 mL protein A purified monoclonal IgG_1 chimera. Wash with 5 CV equilibration buffer. Elute in a 20 CV linear gradient to 5 mM phosphate, 1 M borate (boric acid), pH 7.0. Clean with 500 mM sodium phosphate, pH 7.0. The native IgG peak was collected from about 50 mAU on the ascending side to about 50 mAU on the descending side. Aggregate content was reduced from about 6% to less than 0.1%. Average conductivity was about 5.2 mS/cm.

sodium chloride, sulfate, or phosphate are all in the neighborhood of 90 mS/cm. This means that antibodies elute at much lower conductivity in borate gradients, thereby facilitating sample application to a subsequent ion exchange step. This could also make HA more attractive as a capture method.

In addition to an expanding range of elution options, HA is now available commercially in an alternative form; treatment of HA with fluoride converts the hydroxyl groups to fluoride, yielding fluorapatite (FA, $(Ca_5(PO_4)_3F)_2$). Preliminary data show that HA and FA respond in essentially the same way to the same eluting agents, but there are differences between the two [15,52,53]. These results imply that the hydroxyls on HA may contribute more to HA's overall selectivity than has been revealed [52]. Thorough characterization of the differences will hopefully reveal their basis and point out the applications that can benefit most from the use of HA or FA.

Traditional modes of column chromatography still prevail among HA process developers, but the application of robotic high throughput screening has already been shown to offer value. These systems allow numbers and ranges of variables to be evaluated within time intervals that are unthinkable for traditional chromatography formats. High throughput screening was used to develop the conditions for the removal of 60% aggregates from an IgG preparation noted above [21]. The combined impact of these advances; novel buffer systems, new forms of apatite, and high throughput screening seem certain to increase the utility of HA in the coming years.

Acknowledgement

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