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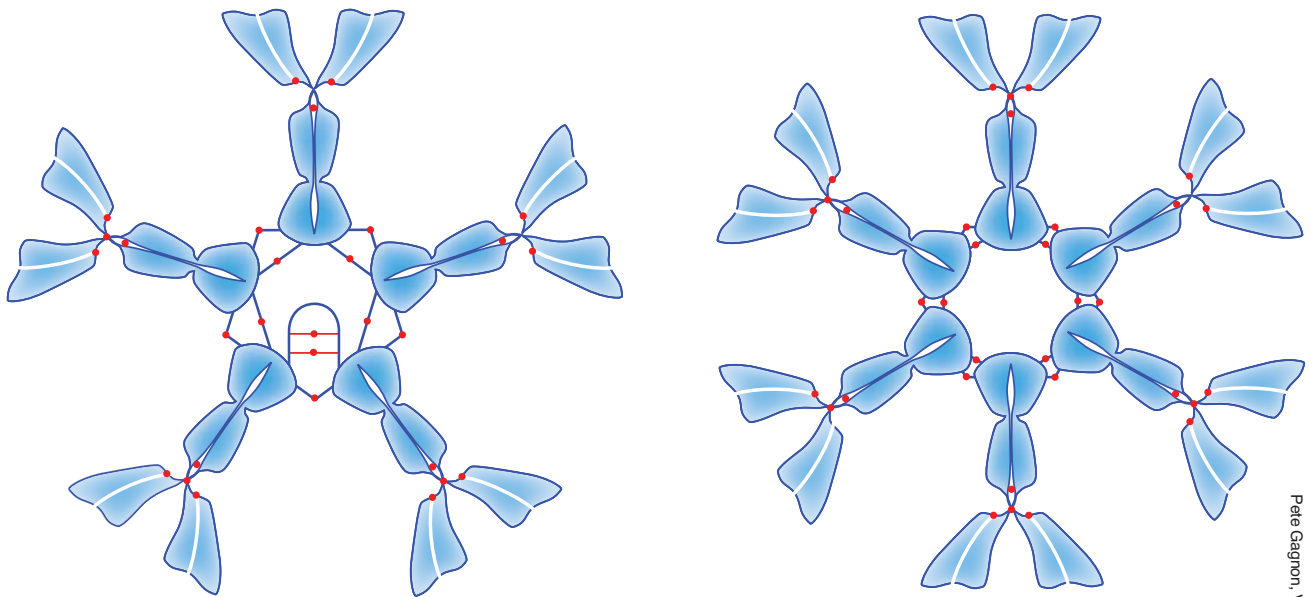
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**Purifying  
Monoclonal Antibodies**



Pete Gagnon, Validated Biosystems

# Purification of IgM Monoclonal Antibodies

Manufacturing challenges surround the use of IgM monoclonal antibodies, but these can be overcome with current technology.

PETE GAGNON, FRANK HENSEL, RICHARD RICHIERI

## ABSTRACT

The affinity capture paradigm that dominates industrial IgG purification has proven unsuitable for IgMs because, in most cases, they are affected adversely by harsh elution conditions. The large size of IgMs is also a challenge because it limits the operating conditions and performance of traditional porous-particle-based chromatography media. This article describes how these challenges can be overcome with available technology to develop effective manufacturing procedures for IgM monoclonal antibodies.

**R**ecent reports that IgM monoclonal antibodies offer promising anticancer activity have created a strong interest in their therapeutic potential.<sup>1–4</sup> IgMs occur naturally in a variety of forms, represented dominantly by cyclic pen-

tamers with a molecular weight of about 960 kDa, and cyclic hexamers with a molecular weight of about 1.15 MDa.<sup>5</sup> IgMs are more heavily glycosylated than are IgGs, with a range of 8–12% carbohydrate. The extinction coefficient for polyclonal IgM is 1.18, but as with IgG, that value can vary from one monoclonal IgM to another.<sup>6</sup>

The primary challenge to purification process development is that IgMs tend to be soluble in a narrower range of conditions than IgGs, and they are more susceptible than IgGs to denaturation.<sup>7–9</sup> Turbidity is the usual consequence of exposure to unsuitable conditions. Light turbidity is often

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**Table 1.** Diffusion constants for antibody classes and fragments

Protein	Mass	$\kappa_{diff}$ cm <sup>2</sup> /sec
Light chain	23 kD	$9.1 \times 10^{-7}$
Fab	50 kD	$7.4 \times 10^{-7}$
IgG	150 kD	$4.9 \times 10^{-7}$
IgA	335 kD	$3.7 \times 10^{-7}$
IgM	960 kD	$2.6 \times 10^{-7}$

(Data from Gagnon.<sup>7</sup>)

reversible on restoration of more moderate conditions, but heavy or persistent turbidity may precede aggregate formation or precipitation. The extreme pH values used to elute immunoaffinity and mixed-mode affinity columns sometimes appear tolerable at laboratory scale, but these frequently cause recovery problems at process scale.<sup>7,10–11</sup> Protein-based affinity ligands such as mannan-binding protein and C1q can be eluted under more moderate conditions, but they are susceptible to proteolysis and cannot withstand sanitization by sodium hydroxide.<sup>12–14</sup>

Low conductivity tends to compound the sensitivity of IgMs to pH. Conditions routinely used for ion exchange purification of IgG, such as pH 8.5 or 4.5, may result in precipitation. IgMs are generally tolerant of high salt concentrations but are sensitive to denaturation on exposure to strongly hydrophobic surfaces. Hydrophobic interaction chromatography (HIC) media commonly used for purification of IgG often denature IgMs.<sup>7</sup> Size exclusion chromatography (SEC) is gentle and provides good fractionation, but it is undesirable for manufacturing applications because of its low productivity.

The large size of IgMs poses another challenge. Large size is associated with slow diffusion constants, and slow diffusion constants limit both capacity and resolution on traditional porous-particle-based chromatography media. Table 1 lists diffusion constants

for several antibody classes and fragments. The diffusion constant for IgM is approximately twice as slow as for IgG; this means that the flow rate would have to be twice as slow to achieve similar capacity and separation performance, assuming that equivalent surface area was accessible. But equivalent surface area is not accessible; pore diameters for a given gel type span a characteristic distribution of values, and the larger the protein, the lower the proportion of pores accessible to it. This compounds the capacity limitation already imposed by the slow diffusion constant.

These limitations have collectively engendered the misconception that IgMs are difficult to purify. Indeed, their chemical sensitivities must be accommodated, but IgMs manifest a range of chemical characteristics that enable development of effective orthogonal purification procedures under conditions that avoid unnecessary stress.<sup>15</sup> Most IgM monoclonals are highly charged and retained strongly enough by ion exchangers to support high binding capacities at moderate pH values.<sup>7,15</sup> Hydroxyapatite binds IgM strongly at physiological pH and conductivity.<sup>7,15–18</sup> HIC on weakly hydrophobic ligands provides good retention without risk of denaturation.<sup>7</sup> Screening may reveal aggregate separation on any of these methods.

In addition, the emerging generation of industrial ion exchangers includes solid phase supports that do not rely on diffusion. Both membranes and monoliths exploit convection for mass transport, and both have been applied effectively to IgM purification.<sup>15,19–21</sup> Convection is independent of molecular size and flow rate, so dynamic binding capacity and resolution are unaffected by either parameter.<sup>22,23</sup> Convective supports thereby offer a solution to the productivity bottleneck that afflicts traditional chromatography media. This solu-

tion has already had a positive impact with industrial purification of IgG, where anion exchange membranes are increasingly exploited for flow-through removal of host cell protein, DNA, endotoxin, and virus.<sup>24,25</sup>

Membranes support less effective peak separation in bind–elute applications because of dispersion in membrane housings and between membrane layers. This dispersion produces a high degree of peak spreading, which erodes resolution and dilutes the eluted proteins. Monoliths lack these dispersion zones, and they further lack the void volume that truncates separation efficiency on porous particle media. These efficiencies result in sharper elution peaks, with the practical benefits of higher resolution and higher eluted product concentration. Capacities for large molecules are very high: dynamic binding capacity for endotoxin is more than 10 times higher on monoliths than on porous particle anion exchangers, and DNA capacity is nearly 50 times higher.<sup>26</sup> These features all favor highly effective purification of IgM.

## Materials and Methods

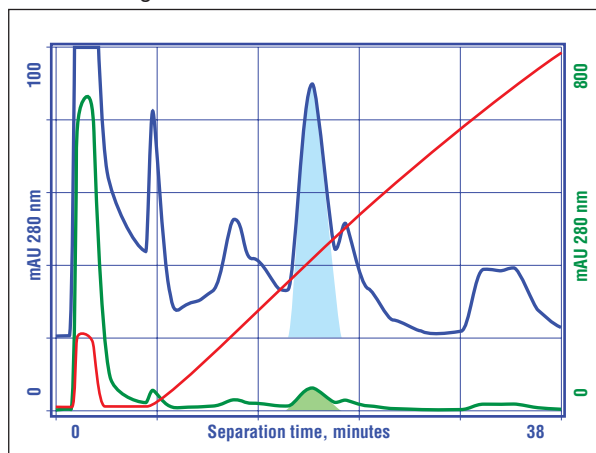
IgM cell culture supernatants (CCS) were filtered to 0.22  $\mu$ m and stored at 4 °C. Buffer components were obtained from Sigma-Aldrich (St. Louis, MO). Buffers were made with water for injection (WFI) and filtered to 0.22  $\mu$ m before use. Ceramic hydroxyapatite (CHT) Type II, 40  $\mu$ m, was obtained from Bio-Rad Laboratories (Hercules, CA) and packed by Atoll GmbH (Weingarten, Germany) into 1 mL (5 x 50 mm) and 10 mL (11.3 x 100 mm) MediaScout columns. Monolithic convection interaction media (CIM) QA (quaternary amino) anion exchangers and CIM SO<sub>3</sub> cation exchangers were obtained from BIA Separations GmbH (Klagenfurt, Austria). For initial screening and method development, 0.34 mL monolithic disks were used; 8 mL

**Table 2.** Initial screening conditions for hydroxyapatite

Ceramic hydroxyapatite (CHT) Type II, 40  $\mu\text{m}$   
 Column: 1 mL (5 x 50 mm) 0.67 mL/min (200 cm/hr)  
 Buffer A: 10 mM sodium phosphate, pH 7.0  
 Buffer B: 500 mM sodium phosphate, pH 7.0  
 Buffer C: 1.0 M NaOH  
 Buffer D: 0.1 M NaOH or 20% ethanol, 5 mM sodium phosphate, pH 7.0  
 Equilibrate column with buffer A.  
 Inject 1–5 mL filtered CCS or 100  $\mu\text{L}$  purified reference.  
 Wash 5 Cv with buffer A.  
 Elute with a 20 Cv linear gradient to buffer B.  
 Clean with 100% buffer B.  
 Sanitize with buffer C.  
 Store in buffer D.

radial flow monoliths were used for process modeling. One mL RESOURCE ETH (ether) and PHE (phenyl) columns for HIC were obtained from GE Healthcare (Piscataway, NJ).

An experimental large-pore polypropylene glycol (PPG) HIC resin from the Tosoh Resin Innovation Program (TRIP) was provided by Tosoh Bioscience (Montgomeryville, PA). It was packed in a 1 mL (5 x 50 mm) column. Screening buffers and conditions for hydroxyapatite are described in Table 2, for anion exchange and cation exchange in Table 3, and for HIC in Table 4. Dynamic binding capacity was

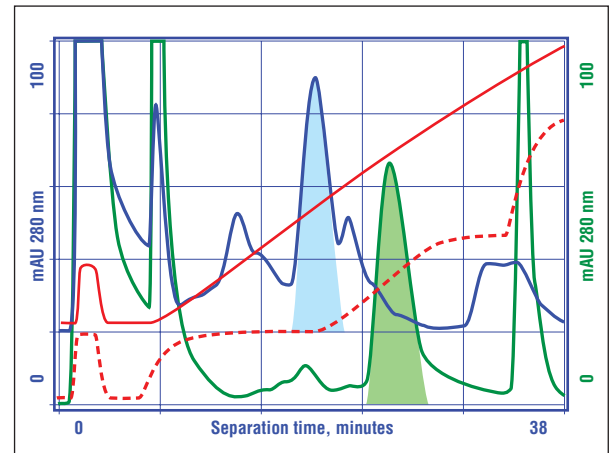
**Figure 1.** Hydroxyapatite screening profiles at different monitor settings. Conditions as in Table 2

determined as described in Gagnon.<sup>7</sup> IgM breakthrough was detected with the monolith-based anion exchange assay described in Gagnon, Richieri, Zaidi, et al.<sup>27</sup> All chromatography experiments were performed on an ÄKTAexplorer 100 from GE Healthcare. Polyacrylamide gel electrophoresis (PAGE) was performed on Bio-Rad Criterion gels (10–20% gradient).

## Results and Discussion

### Initial Screening

A few milligrams of highly enriched product are a valuable asset in early method development because they allow product and contaminant behavior to be evaluated visually from chromatograms. This evaluation permits initial screening and process optimization to be done without secondary testing, and it accelerates development. Enriched product is easily obtained by Protein A affinity chromatography for human IgG monoclonals. Hydroxyapatite provides a useful alternative for IgM. Figure 1 illustrates screening results for an IgM on hydroxyapatite. The IgM peak is clearly identifiable, and the majority of contaminants flow through the column during sample application. Elution between 200 and 300 mM phosphate is the norm.<sup>7</sup> Collecting the IgM peak from an

**Figure 2.** Comparison of screening and optimized profiles on hydroxyapatite. Conditions as in Table 2

initial hydroxyapatite screening run typically provides antibody of 65–80% purity. This purity is sufficient to proceed with initial screening of anion and cation exchange, but only a few more experiments are required to produce a reference sample that better reflects antibody purity under process

**Table 3.** Initial screening conditions for ion exchange

Anion exchange monolith: convection interaction media (CIM) QA (quaternary amino), 0.34 mL, 4 mL/min  
 Cation exchange monolith: CIM SO<sub>3</sub>, 0.34 mL, 4 mL/min  
 Buffer A1: 20 mM MES, pH 6.0  
 Buffer B1: 20 mM MES, 1.0 M NaCl, pH 6.0  
 Buffer A2: 20 mM Hepes, pH 7.0  
 Buffer B2: 20 mM Hepes, 1.0 M NaCl, pH 7.0  
 Buffer A3: 20 mM Tris, pH 8.0  
 Buffer B3: 20 mM Tris, 1.0 M NaCl, pH 8.0  
 Buffer C: 1.0 M NaOH  
 Buffer D: 0.01 M NaOH or 20% ethanol  
 Equilibrate column with buffer A.  
 Inject 50–100  $\mu\text{L}$  ceramic hydroxyapatite (CHT)—purified reference or filtered cell culture supernatants (CCS).  
 Wash 1 minute or less with buffer A.  
 Elute with a 36 Cv (3 min) linear gradient to 50% B; hold 1 minute.  
 Clean with 12 Cv (1 min) linear gradient to 100% B, or step.  
 Sanitize with buffer C.  
 Store in buffer D.

**Table 4.** Initial screening conditions for hydrophobic interaction chromatography

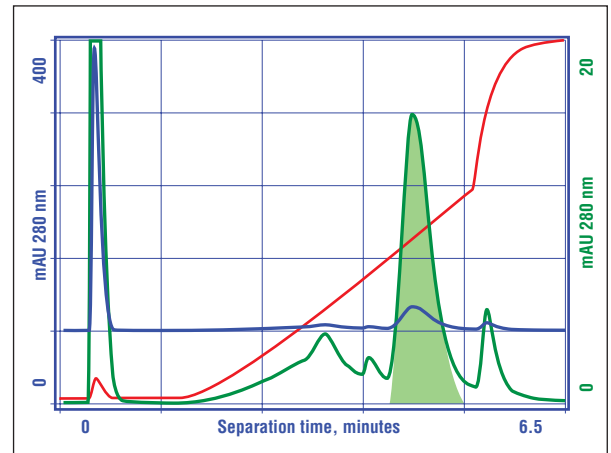
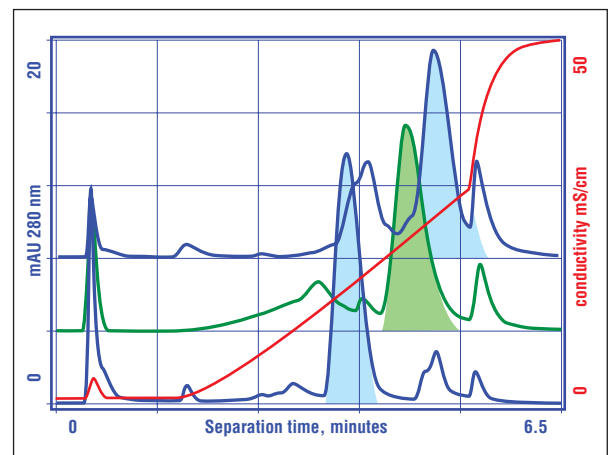
Moderately hydrophobic media with wide pores (>1,000 Å) if available; otherwise, moderately hydrophobic media with an average pore size distribution of 600–1,000 Å
Column: 1 mL (5 x 50 mm) 0.67 mL/min (200 cm/hr)
Buffer A: 10 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.0
Buffer B: 10 mM sodium phosphate, pH 7.0
Buffer C: 1.0 M NaOH
Buffer D: 0.01 M NaOH or 20% ethanol
Equilibrate column with buffer A.
Inject 25–50 µL purified reference or filtered CCS; use multiple injections if sample is dilute.
Wash 2–5 Cv with buffer A.
Elute with a 10–20 Cv linear gradient to buffer B.
Clean with 100% buffer B.
Sanitize with buffer C.
Store in buffer D.

conditions, which is typically approximately 90%. Figure 2 compares the initial screening profile with the profile from an optimized gradient. Conditions were set so that most contaminants were either eliminated in the pre-elution wash, or were left on the column after the antibody had eluted. The 1-mL column was subsequently loaded with 50 mL of CCS containing approximately 12.5 mg of IgM, then eluted with the optimized gradient. IgM from this run was used to evaluate other methods.

Figure 3 illustrates screening results for an IgM on cation exchange at pH 7.0. The profile is reminiscent of hydroxyapatite in the sense that the IgM binds much more strongly than is usual for IgG antibodies, and the majority of host cell proteins fail to bind. Figure 4 compares retention characteristics at pH 6.0, 7.0, and 8.0. Binding is weaker at pH 8.0, but purification is improved remarkably. These results suggest a process strategy where the IgM can be bound at pH 6.0 to maximize capacity, then washed and eluted at pH 8.0 to maximize contaminant removal.

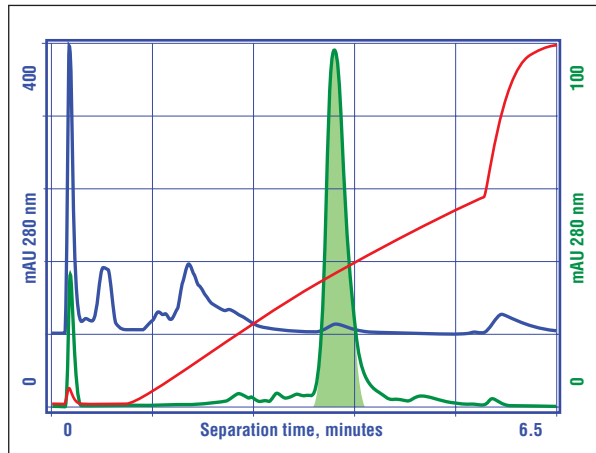
Figure 5 illustrates screening results for an IgM on anion exchange at pH 7.0. The upper trace was produced from hydroxyapatite-purified reference, the lower trace from CCS. Again, the IgM binds much more strongly than is typical for IgG monoclonals, and the degree of purification appears to be excellent; however, a substantial proportion of contaminants also bind. Strong binding by IgMs may support a process strategy such as that discussed above for cation exchange: Bind at the pH that supports the highest capacity, and wash and elute at the pH that supports the most effective contaminant removal.

IgM typically elutes from weakly hydrophobic columns in a single well-defined peak, as shown by the ETH profile in Figure 6, but this antibody was severely denatured by the more strongly hydrophobic PHE column. The small first peak was the only remnant of native IgM. The later eluting dominant peak and shoulders represent denatured forms. They were turbid on elution and precipitated overnight at 4 °C. Although weakly hydrophobic columns avoid the denaturation problem, they leave another challenge in its place: The IgM elutes at very high salt concentrations, which can be difficult to accommodate in later process steps.

**Figure 3.** Cation exchange screening profile at pH 7.0 with different monitor settings. Conditions as in Table 3**Figure 4.** Cation exchange screening profiles at pH 6.0, 7.0, and 8.0. Conditions as in Table 3

HIC media with intermediate hydrophobicity offer a practical solution. As shown in Figure 7, the IgM elutes from the PPG column as a well-defined peak near the end of the gradient. Conductivity of the eluted IgM pool is sufficiently low to support binding to an ion exchanger with moderate dilution. Another benefit of HIC on moderately hydrophobic supports is that the high salt required for binding can dissociate ionic complexes that may exist between IgM and contaminants of opposite charge.<sup>7</sup> This fact can be important, because the same positive charges that cause IgM to bind strongly to cation exchangers can just as easily bind

**Figure 5.** Anion exchange screening profiles of CCS and hydroxyapatite-purified reference. Conditions as in Table 3



DNA fragments. This is highlighted by the use of immobilized DNA for affinity purification of IgM.<sup>28</sup> As with hydroxyapatite and cation exchange, the majority of contaminants flow through HIC columns on sample application (Figure 7).<sup>7</sup>

#### Sample Application and Binding Capacity

Loading sufficient IgM to obtain antibody for characterization is simple on hydroxyapatite because it accommodates filtered CCS with only minor adjustments. If the CCS contains less than 5 mM phosphate, then phosphate should be added to that concentration to stabilize the hydroxyapatite during large-volume sample applications. Titration may also be required to bring the sample to operating pH. Dynamic binding capacity (5% breakthrough at 200 cm/hr with a 10 cm bed height) was approximately 23 mg/mL for one IgM, and approximately 19 mg/mL for another. Dilution to reduce conductivity increases binding capacity for purified IgM. For CCS, however, it may allow more contaminants to bind, and so it may potentially reduce net product binding capacity. Dilution also increases preparative sample application time, which already accounts for several hours at 200 cm/hr. IgM capacity increases at slower flow rates, or with longer resi-

dence time at the same flow rate on a taller bed.

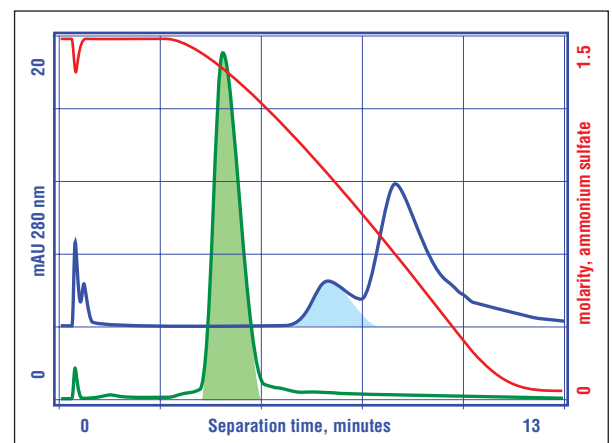
Sample loading is more complicated for ion exchangers because many IgMs begin to develop turbidity soon after they are equilibrated to the low conductivity conditions required to support high binding capacities. Turbidity may be absent initially, but it tends to form

progressively over time. This fact not only risks product integrity, it also creates a source of process variation, because sample composition varies over the duration of the load. Turbidity can be measured conveniently on a spectrophotometer at 600 nm. Solubility limitations generally disqualify bulk offline sample equilibration of IgMs for ion exchange. Sample application by inline dilution provides an alternative.<sup>7</sup> Sample is titrated to target pH and loaded through one inlet line. Diluent buffer at target pH is loaded through another. Residence time of the IgM at dilution, defined as the elapsed time from the point of mixing to column contact, represents seconds or fractions of a second, depending on flow rate and configuration of the chromatography system. The short duration is generally insufficient time for turbidity to develop. An equally important benefit is that sample composition is uniform for

the duration of the load, no matter how long that duration might be.<sup>7</sup>

Inline dilution factors vary according to the salt concentration of the sample, the charge characteristics of the individual antibody, and the desired binding capacity. A dilution ratio of 1 part sample to 2 parts diluent is a reasonable starting point with feedstreams at roughly physiological conductivity, although higher dilution is likely to support higher binding capacity. Samples with higher conductivities may require still higher dilution factors. Any dilution invites criticism for water or buffer consumption and increased column loading time, but it is important to consider that inline dilution eliminates the need for diafiltration with its attendant equipment, equilibration, and chase buffers; preparation, process, and maintenance time; cleaning validation; and inevitable product losses. Moreover, monoliths support such high volumetric flow rates that loading time ceases to be a consideration. Capacities for hydroxyapatite-purified reference IgM on monoliths ranged from 30 to 40 mg/mL for both anion and cation

**Figure 6.** HIC profiles on weak and strong hydrophobic media. Blue profile: RESOURCE PHE (GE Healthcare). Green profile: RESOURCE ETH, 1 mL, 2 mL/min (approximately 600 cm/hr). Equilibrate with 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0. Inject 20  $\mu$ L (200  $\mu$ g) hydroxyapatite-purified mouse IgM. Wash 2.5 Cv with equilibration buffer. Elute with a 10 Cv linear gradient to 50 mM sodium phosphate, pH 7.0. The colored areas represent native fully active IgM.



exchangers at flow rates of 12 Cv.

Inline dilution is necessary for HIC as well, because the concentration of salt required to support good binding capacity on moderately hydrophobic columns is typically sufficient to precipitate the antibody.<sup>7,29</sup> Development of inline dilution conditions for HIC on such ligands is discussed in depth by Gagnon, Grund and Lindback.<sup>29</sup>

### Aggregate Removal

SEC supports effective aggregate removal for IgMs, but its low productivity is unattractive for manufacturing operations.<sup>30,31</sup> The primary handicap is the sample volume, typically in the range of 2–5% Cv. This limitation is compounded by flow rates potentially as low as 20 cm/hr.<sup>7</sup> A theoretical benefit of SEC is that it simultaneously equilibrates the sample for the next step, but this benefit is moot for most IgMs because equilibrating a sample to ion exchange conditions on an SEC column is likely to result in formation of turbidity and to jeopardize product quality. It may result in the IgM precipitating on the column. This is the basis of an IgM purification technique called euglobulin partitioning chromatography, in which the antibody is retained by SEC media at conductivity values too low to support solubility.<sup>7,32–34</sup> Soluble contaminants flow through on sample application. The IgM is subsequently eluted by increasing the salt concentration.

Many high-capacity alternatives are available for aggregate removal. Although it is impossible to predict which will best serve a particular IgM, it is likely that the screening conditions suggested above will reveal one or more methods that offer useful levels of aggregate reduction. Hydroxyapatite has frequently proven effective for removal of aggregates and polymers from IgG, IgA, and IgM monoclonal antibodies.<sup>16–18,35–37</sup> HIC and ion exchange frequently provide worthy aggregate fractionation of IgG aggre-

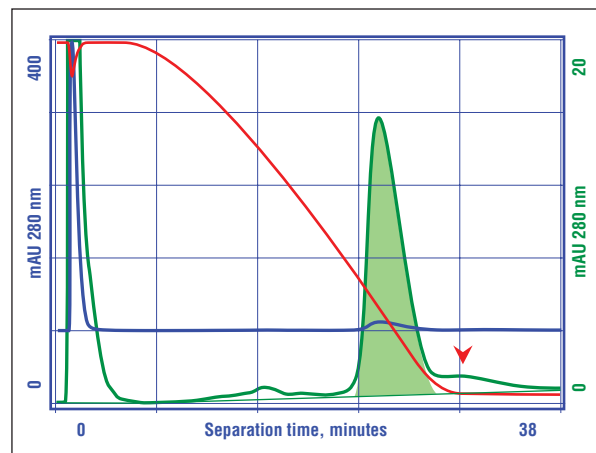
gates, and can be reasonably expected to do so with IgMs as well. Figure 7 illustrates promising separation of IgM aggregates on a PPG HIC column.

If the native abilities of the methods in a process are not sufficient to achieve adequate aggregate reduction, it may be possible to enhance them. Recent investigations have demonstrated the ability of 3.75–7.5% polyethylene glycol (PEG) to promote effective IgG aggregate removal on ion exchangers and hydroxyapatite, even when no separation is apparent in the absence of PEG.<sup>38</sup> Larger proteins are more responsive than IgG to the effects of PEG, suggesting that this treatment should be even more effective for IgMs.<sup>39</sup> PEG is economical, is protein-stabilizing, and is an approved inactive ingredient in a large number of parenteral formulations.<sup>40,41</sup>

### Process Sequencing

The combination of good capacity, excellent contaminant removal, minimal sample preparation, and consistent applicability for most IgMs makes hydroxyapatite a good default candidate for capture. Typically, the eluting salt concentration is sufficiently low, with dilution, to make ion exchange practical as a second step. The high salt tolerance of HIC makes it a good candidate for a second step, but HIC is poorly suited to capture because of the large amounts of salt that are required. This would be an impediment even if the salt could be added directly to the sample, but the usual need for inline dilution multiplies salt requirements to a prohibitive level. Assuming that a concentration of 1.2 M ammonium

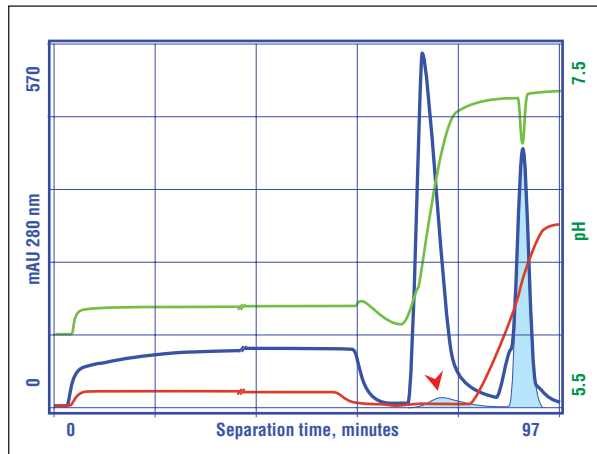
**Figure 7.** HIC screening profiles on PPG at different monitor settings. Conditions as in Table 4. Red arrow indicates aggregates.



sulfate was required for binding, and that this was achieved by inline dilution of 1 part sample with 4 parts 1.5 M ammonium sulfate, 0.99 kg ammonium sulfate would be required for every liter of CCS. Column loading time would be an issue, since the dilution factor would quintuple sample volume.

The strong binding of the IgM shown in Figure 4 suggests the feasibility of cation exchange capture. Published data, however, indicate that cation exchange retention is highly variable for IgMs, so it should not be relied on as a default method.<sup>7</sup> Under the best of circumstances, mild reduction of pH and 3–5 fold dilution of the CCS will probably be necessary to obtain good binding capacity. Another positive feature of cation exchange capture is that it removes CCS contaminants that can affect hydroxyapatite, principally including metal ions and chelators. Iron binds to and discolors hydroxyapatite, but published studies indicate that separation performance is unaffected.<sup>42</sup> Continuous presence of at least 5 mM phosphate and a minimum pH of 6.5 generally stabilizes hydroxyapatite, but how well it does so in the presence of chelators in CCS remains to be evaluated. One important restriction with cation

**Figure 8.** Preparative capture profile on cation exchange. Equilibrate column with 50 mM MES, pH 6.0. Load sample by inline dilution, 1 part CCS, 4 parts equilibration buffer. Wash with equilibration buffer. Wash with 10 mM sodium phosphate, pH 7.0. Elute with a 10 mM linear gradient to 250 mM sodium phosphate pH 7. Clean with 500 mM sodium phosphate (not shown).



exchange as a capture method is that it cannot use citrate buffers if hydroxyapatite is the next step: citrate is a calcium chelator.

Most IgM monoclonals bind strongly to anion exchangers. Anion exchange capture is handicapped, however, because contaminating proteins consume a significant portion of the binding capacity.<sup>7</sup> Stronger binding contaminants are an even greater liability for strong anion exchangers, such as Q, quaternary amino (QA), quaternary amino ethyl (QAE), and trimethylaminoethyl (TMAE), because they maintain sufficient positive charge in 1.0 M NaOH to retain DNA through multipoint binding of numerous phosphoryl residues. Combinations of 1–2 M NaCl with NaOH provide more effective cleaning than NaOH alone, but only column treatment with DNase has proven adequate to achieve quantitative removal.<sup>43,44</sup> If capture by anion exchange is desired, it is prudent to use a weak anion exchanger, such as diethylaminoethyl (DEAE). The liability of binding competition by contaminating proteins is still present, but DEAE loses its charge in NaOH, thereby releasing DNA and other strongly bound contaminants.

### Process Modeling

After establishing a preliminary process order, loading conditions, dynamic binding capacity, and separation conditions at each step, it is useful to run the integrated process to provide a benchmark of overall process performance and economics. In-depth optimization of the individual steps before running the integrated process is premature. For example, one

might expend considerable resources developing conditions to remove a difficult contaminant with one particular method, only to learn that it is removed effortlessly by another method. A good working process model highlights its own deficiencies, helps provide an order of priority for addressing them, and allows each to be evaluated in a meaningful context.

Figure 8 provides an example of the sort of deficiency that benchmark evaluation can reveal. In this case, the wash and gradient conditions had been determined with small

injections of CCS, and dynamic binding capacity had been estimated with hydroxyapatite-purified reference IgM. CCS loading volume was set at 80% of that capacity, with the expectation that all of the IgM would bind and elute solely in the gradient. No antibody was lost during sample application, but approximately 8% of the IgM eluted prematurely in the pH 7.0 wash. This fact indicated that the column was saturated, apparently due to competition from binding contaminants, and it suggested two possible corrections: reduce the sample volume, or reduce the wash and elution pH. Figure 9 illustrates product purity at various process stages.

Table 5 summarizes process metrics. The impact of monoliths on throughput is striking throughout the process but is especially so at the capture step. A 1,250 mL sample was loaded on an 8 mL monolith in 62.5 min. The same load would have required 375 min on a 10 mL conventional cation exchange column (11.3 x 100 mm, 200 cm/hr). This fact highlights but under-expresses the ability of monoliths to relieve the much-discussed bottleneck in downstream processing. The ÄKTA used in these experiments was configured for a maximum flow rate of 20 mL/min, limiting the 8 mL monoliths to a maximum flow rate of 2.5

**Table 5.** IgM benchmark purification process summary

	Cation exchange	Anion exchange	Hydroxyapatite
	8 mL monolith	8 mL monolith	10 mL column
Sample volume, mL	250	25	2
Diluted volume, mL	1,250	250	250
Flow rate mL/min	20	20	3.34
Application time, min	62.5	12.5	75
Total buffer volume	1,950	950	650
Total step time, min	98	48	195
Recovery %	78 (86*)	84	88
Purity %	~90	~95	~99

\*Includes the IgM that eluted prematurely in the wash. Conditions as described in Figure 8



Cv/min. Separation conditions and capacity determinations during early development were conducted at 12 Cv/min. Had the ÄKTA been configured to accommodate its maximum flow rate, the cation exchange sample loading time would have been 13 min.

After fundamental deficiencies in the basic process have been addressed, individual variables can be optimized. For all methods, inline dilution factors are key determinants of overall productivity. Higher dilution factors support higher binding capacities, which support the use of smaller columns and elute more concentrated product in a smaller volume. Lower dilution factors consume less buffer and less process time.

With hydroxyapatite, one should consider evaluating binding and elution pH values as low as 6.5 and as high as 8.0. As in the previous discussion concerning ion exchangers, it may be useful to bind at one pH to maximize capacity, and to elute at another to improve contaminant removal. CHT Type II, 40  $\mu\text{m}$ , is typically the best option for process development and manufacturing. On most industrial-scale columns, 20  $\mu\text{m}$  is too small for the frits, and 80  $\mu\text{m}$  provides less backpressure but has lower capacity. CHT Type I (for all particle diameters) has a substantially lower range of pore diameters and will likely support lower binding capacity for IgMs.<sup>45</sup> Selectivity is slightly different, however, and could be useful in a given situation. Ceramic fluorapatite (CFT) has yet to be characterized for IgM purification.

For cation exchange, one should consider evaluating more common manufacturing buffers such as citrate and phosphate. Using citrate or phosphate as eluting ions in place of NaCl may also improve pH control during the separation.<sup>46</sup> Citrate and phosphate are generally considered inappropriate for anion exchangers, but they may still be effective. Many IgMs

tolerate pH values above 8.0 or below 6.0, but these should be evaluated with care. Weak anion exchange monoliths (DEAE; EDA, or ethylenediamine) or weak cation exchange monoliths may be evaluated. Conventional ion exchangers may also be considered.

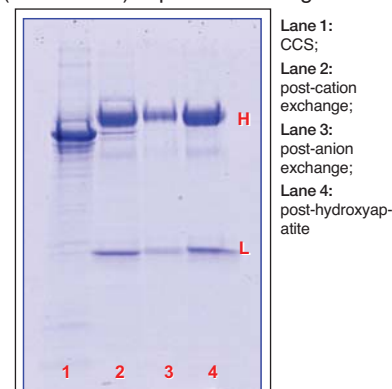
For HIC on weakly hydrophobic media, different binding salts can give remarkably different selectivities.<sup>47</sup> A good starting point for such media is 1.2–1.5 M ammonium sulfate. Potassium phosphate supports similar average binding strength at the same concentrations. Also of possible interest is 1.0 M sodium sulfate or sodium citrate. Sometimes 4.0 M sodium chloride is used for IgG on phenyl columns, but it generally does not support adequate IgM binding on moderately hydrophobic columns. Different pH values occasionally produce worthwhile selectivities.

#### Scale-up Issues

The low diffusion constant of IgM imposes slow flow rates on porous particle media such as hydroxyapatite, HIC, and conventional ion exchangers. A practical maximum is 200 cm/hr; 100 cm/hr provides better performance. Slow flow rates favor scale-up on columns with shallow bed heights, ideally no greater than 20 cm. Consistently good quality packing at industrial scale can be obtained with 15 cm, or even 10 cm bed height, in columns that permit packing by dynamic axial compression. Such columns are especially effective for hydroxyapatite because they can accommodate its high density and rapid settling rate. These columns also overcome the primary cause of performance loss by hydroxyapatite: particle damage coincident with repacking. When repacking is required, these columns resuspend the hydroxyapatite by upward flow, thereby avoiding the use of tools that might damage the particles.

Experimental results from cycling

**Figure 9.** Reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purification stages



studies indicate that hydroxyapatite can be used for at least 50 cycles without detectable change in performance; however, these studies have mostly been performed using purified IgG as a model.<sup>48</sup> As noted above, CCS contains components that can interact directly with hydroxyapatite and may reduce its effective lifetime. Hydroxyapatite has been shown to withstand more than 15,000 hours of exposure to 1.0 M NaOH.<sup>45</sup>

Scale-up with monoliths is simpler because column packing is eliminated. If air is introduced into a monolith, it can be displaced quickly and efficiently, and without loss of column performance, by restoration of buffer flow. Industrial monoliths are available at 8, 80, 800, and 8,000 mL volumes. For the antibodies in this study, an 8 L monolith represents an IgM capacity of approximately 250–300 g per cycle. Larger scale requirements can be accommodated by plumbing multiple units in parallel, or by configuring multiple units to create a simulated moving bed. The current generation of preparative monoliths is synthesized from polymethacrylate, which is the same polymer found in many porous-particle ion exchangers and HIC media used for industrial purification of antibodies and other injectable products. Likewise, monoliths withstand

repeated exposure to sodium hydroxide, and they can be expected to support reusability similar to other poly-methacrylate media.

## CONCLUSION

Industry experience is insufficient at present to identify a standard platform for IgM purification, but the tools available to process developers provide an abundance of effective options. Either hydroxyapatite or cation exchange may support effective capture, depending on the properties of a particular IgM. The high capacity and resolution of monolithic ion exchangers at high volumetric flow rates—despite the large size of IgM—make them a compelling process option at any point in a purification process. All methods discussed in this study are potential candidates for aggregate removal, and their capabilities can be enhanced, if necessary, by the presence of PEG. Together, these tools provide a level of purification, recovery, and throughput that rival the best commercial IgG purification procedures. Far from being a liability, the lack of an affinity step makes a positive contribution to overall process economy. **BP**

## References

- Bieber M, Twist C, Bhat N, Teng N. Effects of human monoclonal antibody 216 on B-progenitor acute lymphoblastic leukemia in vitro. *Pediatr Blood Cancer*. 2007;48:380–6.
- Irie R, Ollila D, O'Day S, Morton D. Phase I pilot clinical trial of human IgM monoclonal antibody to ganglioside GM3 in patients with metastatic melanoma. *Cancer Immunol Immunother*. 2004;53:110–17.
- Azuma Y, Ishikawa Y, Kawai S, Tsunenari T, Tsunoda H, Igawa T, et al. Recombinant human hexamer-dominant IgM monoclonal antibody to ganglioside GM3 for treatment of melanoma. *Clin Cancer Res*. 2004;13:2745–50.
- Vollmers H, Zimmermann U, Krenn V, Timmermann W, Illert B, Hensel F, et al. Adjuvant therapy for gastric adenocarcinoma with the apoptosis-inducing human monoclonal antibody SC-1: first clinical and histopathological results. *Oncol Rep*. 1998;5:549–52.
- Randall T, King L, Corley R. The biological effects of IgM hexamer formation. *Eur J Immunol*. 1990;20:1971–9.
- Johnstone A, Thorpe R. *Immunochemistry in practice*. 2nd ed. Oxford: Blackwell Scientific Publications; 1987.
- Gagnon P. Purification tools for monoclonal antibodies. Tucson: Validated Biosystems; 1996.
- Garcia-Gonzalez M, Bettinger S, Ott S, Oliver P, Kadouche J, Poulety P. Purification of a murine IgG3 and IgM monoclonal antibodies by euglobulin precipitation. *J Immunol Met*. 1988;111:17–23.
- Middaugh C, Litman G. Effects of solutes on the cold-induced solubility of monoclonal cryoglobulins. *J Biol Chem*. 1977;252:8002–6.
- Palombo G, Vedoliva A, Fassina G. Affinity purification of immunoglobulin M using a novel synthetic ligand. *J Chromatogr B*. 1998;715:137–45.
- Fassina G, Palombo G, Verdoliva A, Ruvo M. Affinity purification of immunoglobulins using Protein A mimetic (PAM). In: Walker J, editor. *The protein protocols handbook*. 2nd ed. Totowa, NJ: Humana Press; 2002. p. 1013–24.
- Nethery A, Raison R, Easterbrook-Smith S. Single-step purification of immunoglobulin M on C1q Sepharose. *J Immunol Met*. 1990;126:57–60.
- Ohta M, Okada M, Yamashina I, Kawasaki T. The mechanism of carbohydrate-mediated complement activation by the serum mannan-binding protein. *J Biol Chem*. 1990;265:1980–4.
- Nevens J, Mallia A, Wendt M, Smith P. Affinity chromatographic purification of immunoglobulin M antibodies utilizing immobilized mannan binding protein. *J Chromatogr*. 1992;597:247–56.
- Gagnon P, Hensel F, Andrews P, Richieri R. Recent advances in the purification of IgM monoclonal antibodies [presentation]. Third Wilbio Conference on Purification of Biological Products; 2007 Sep 24–26 [cited 2008 Feb 3]; Waltham, MA.
- Yamakawa Y, Chiba J. High performance liquid chromatography of mouse monoclonal antibodies on spherical hydroxyapatite beads. *J Liq Chromatogr*. 1988;11(3):665–81.
- Aoyama K, Chiba J. Separation of molecular forms of mouse IgA and IgM monoclonal antibodies by high performance liquid chromatography on spherical hydroxyapatite beads. *J Immunol Met*. 1993;162(2):201–10.
- Coppola G, Underwood J, Cartwright G, Hearn M. High performance liquid chromatography of amino acids, peptides, and proteins. XCIII. Comparison of methods for the purification of mouse monoclonal immunoglobulin M autoantibodies. *J Chromatogr A*. 1989;476:269–90.
- Jacobina M, Santarellia X, Laroche-Trineau J, Clouet-Sanchez G. Production of a human monoclonal IgM directed against human cardiac myosin in a hollow-fiber bioreactor for membrane anion exchange chromatography purification in one-step. *Human Antibodies*. 2004;13:69–79.
- Santarellia X, Domerqieb F, Clouet-Sanchez G, Dabadied M, Grisselye R, Cassagne C. Characterization and application of new macroporous membrane ion exchangers. *J Chromatogr B*. 1998;706:13–22.
- Brne P, Podgornik A, Benčina K, Gabor B, Trancar A, Peterka M. Fast and efficient separation of immunoglobulin M from immunoglobulin G using short monolithic columns. *J Chromatogr A*. 2007;1144:120–5.
- Hahn R, Panzer M, Hansen E, Mollerup J, Jungbauer A. Mass transfer properties of monoliths. *Sep Sci Technol*. 2002;37(7):1545–56.
- Strancar A, Podgornik A, Barut M, Necina R. Short monolithic columns as stationary phases for biochromatography. *Adv Biochem Eng Biotechnol*. 2002;76:50–5.
- Zhou J, Tressel T. Basic concepts in Q membrane chromatography for large-scale antibody production. *Biotechnol Prog*. 2006;22(2):341–9.
- Zhou J, Tressel T, Gottschalk U, Solamo F, Pastor A., Dermawan S, et al. New Q membrane scale-down model for process-scale antibody purification. *J Chromatogr A*. 2006;1134:66–73.
- Gagnon P, Richieri R, Zaidi S, Aolin F. A comparison of microparticulate, membrane, and monolithic anion exchangers for polishing applications in the purification of monoclonal antibodies [poster]. IBC International Conference and Exposition; 2007 Oct 1–4; Boston, MA.
- Gagnon P, Richieri R, Zaidi S, Sevilla R, Brinckman A. A high speed monolithic assay for IgM quantitation in cell culture production and purification process monitoring [poster]. Third Wilbio Conference on Purification of Biological Products, 2007 Sep 24–26; Waltham, MA.
- Abdullah M, Davies R, Hill J. The application of DNA-cellulose chromatography in the isolation of immunoglobulin M and C4b-binding protein. *J Chromatogr*. 1985;347:129–36.
- Gagnon P, Grund E, Linbäck T. Large scale process development for hydrophobic interaction chromatography, Part 1: Gel selection and development of binding conditions. *BioPharm*. 1995;8(3):21–7.
- Chen F, Naeve G, Epstein A. Comparison of Mono-Q, Superose 6, and ABx fast protein liquid chromatography for the purification of IgM monoclonal antibodies. *J Chromatogr*. 1988;444:153–64.
- Henniker A, Brastock K. Purification of two monoclonal antibodies of the IgM class by hydroxyapatite and gel filtration. *Biomed Chromatogr*. 1993;7:121–5.
- Bouvet J, Pires R, Pilot J. A modified gel filtration technique producing an unusual exclusion volume of IgM: a simple way of preparing monoclonal IgM. *J Immunol Met*. 1984;66:299–305.
- Clezardin P, Bougro G, MacGregor J. Tandem purification of IgM monoclonal antibodies by ion exchange and gel fast protein liquid chromatography. *J Chromatogr*. 1986;354:425–33.
- Clezardin P, Hunter N, MacGregor J, MacGregor J, Pepper D, Dawes J. Tandem purification of mouse IgM monoclonal antibodies produced in vitro using anion exchange and gel fast liquid protein chromatography. *J Chromatogr*. 1986;358:209–18.
- Luellau E, Marison W, von Stockar U. Ceramic hydroxyapatite: a new tool for separation and analysis of IgA monoclonal antibodies. In: Carondo MJT, Griffiths B, Moreira JLP, editors. *Animal Cell Technology*. Dordrecht, the Netherlands; Kluwer Academic Publishers; 1997. p. 265–69.
- Luellau E, von Stockar U, Vogt S, Freitag R. Development of a downstream process for isolation and separation of immunoglobulin monomers, dimers, and polymers from cell culture supernatant. *J Chromatogr A*. 1998;796:165–75.
- Gagnon P, Ng P, Aberrin C, Zhen J, He J, Mekosh H, et al. A ceramic hydroxyapatite based purification platform: simultaneous removal of leached Protein A, aggregates, DNA, and endotoxins. *BioProcess Int*. 2006;4(2):50–60.
- Gagnon P, Richieri R, Zaidi S. Nonionic polymer enhancement of aggregate removal by ion exchange and hydroxyapatite chromatography [presentation]. Twelfth Waterside Conference; 2007 Apr 23–25; San Juan, Puerto Rico.
- Gagnon P, Godfrey B, Ladd D. Method for obtaining unique selectivities in ion exchange chromatography by addition of organic polymers to the mobile phase. *J Chromatogr A*. 1996;743:51–5.
- US Food and Drug Administration. Center for Drug Evaluation and Research. Inactive ingredient search for approved drug products [database]. Rockville, MD; 2007 Oct 31 [cited 2008 Feb 3].
- Arakawa T, Timasheff S. Mechanism of polyethylene glycol interaction with proteins. *Biochemistry*. 1985;24:6756–62.
- Shepard S, Brickman-Stone C, Schrimsher L, Koch G. Discoloration of ceramic hydroxy-apatite used for protein chromatography. *J Chromatogr A*. 2000;891:93–8.
- Dasarathy Y. A validatable cleaning-in-place protocol for total DNA clearance from an anion exchange resin. *BioPharm*. 1996;9(8):41–4.
- Ng P, McLaughlin V. Regeneration studies of anion exchange chromatography resins. *BioProcess Int*. 2007;5(5):52–6.
- CHT ceramic hydroxyapatite instruction manual. Hercules, CA: Bio-Rad Laboratories; 2007.
- Ghose S, McNery T, Hubbard B. pH transitions in ion exchange systems: role in the development of a cation exchange process for a recombinant protein. *Biotechnol Prog*. 2002;18:530–7.
- Gagnon P, Grund E. Large scale process development for hydrophobic interaction chromatography, Part 4: Controlling selectivity. *BioPharm*. 1996;9(5):54–64.
- Zhen J, Gagnon P, Snyder M. Buffers and conditions for use and maintenance of CHT ceramic hydroxyapatite [poster]. Eighteenth International IBC Conference on Antibody Development and Production; 2007 Feb 28–Mar 2; Carlsbad, CA.

