A Non-Affinity Based Method for Purification of IgG Monoclonal Antibodies

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Abstract
Finding an alternative to protein A based purification of IgG has emerged as a focal interest for the biopharmaceutical industry, the major drivers being procurement cost, media life, and bioburden issues. However, protein A has proven so effective that an entire paradigm —the platform concept—has evolved around it, creating an even greater challenge for potential alternatives. This study will evaluate the utility of a purification procedure consisting of hydroxyapatite, anion exchange, and hydrophobic interaction chromatography.

Introduction
Protein A has achieved overwhelming dominance as the capture method of choice for industrial purification of therapeutic antibodies. This results from a suite of compelling process attributes, including applicability to the majority of IgGs, good dynamic binding capacity without need for substantially modifying sample pH or conductivity, high purification factor in a single step, and good compatibility—through choice of eluting buffer—with a range of polishing methods. Protein A has limitations nevertheless, notably including high procurement cost, limited media life, and bioburden issues, all of which have encouraged users to seek alternatives.

Realistically, only an alternative affinity method could be expected to replace protein A directly, but such a product would likely bear similar burdens. Anion exchange and cation exchange chromatography have occasionally proven to be effective capture methods but both depend on the extreme charge properties of a fortuitous but small subset of antibodies. The large sample dilution factor and pH adjustment that would be required to attain broad applicability are prohibitive. Hydrophobic interaction can also support effective capture but requires either the direct addition of dry sodium chloride to the sample at concentrations in the hundreds of grams per liter, or development of in-line dilution conditions with stronger kosmotropes. All of these limitations can be acceptable for intermediate or polishing steps, but they are an excessive burden with the large sample volumes that must be accommodated at capture.

Hydroxyapatite is more broadly applicable to IgG purification than protein A, since it binds all subclasses, and is equally applicable to IgG from various species. It is typically able to achieve 80-90% IgG purity in a single step and recent publications have shown it to be highly effective for removal of aggregates, DNA, endotoxin, and retrovirus, as well as host cell proteins (Table 1). Binding capacities for monoclonal IgG are commonly in the range of 30–40 mg per mL of hydroxyapatite, inviting its application to capture.

Table 1. Reduction of important contaminants by hydroxyapatite

Contaminant	Method	Reduction
Aggregates	HPSEC	1-2 logs
Protein A	Cygnus	1-2 logs
CHOP	ELISA	2 logs
DNA	Picogreen	> 3 logs
Endotoxin	LAL	> 4 logs
aMULV	Infectivity	> 4 logs
xMULV	Infectivity	> 3 logs
MVM	Infectivity	2 logs
PPV	Infectivity	> 1 log

Table 2. Purification Summary

Capture
Ceramic hydroxyapatite CHT™ type I 20 µm (Bio-Rad Laboratories)
Bind-elute mode
Volume: 5 mL Flow rate: 300 cm/hr
Mab loaded: 42 mg
Recovery: >90%
Estimated purity by protein A affinity: >85%

Intermediate purification
UNOsphere™ Q (Bio-Rad Laboratories)
Flow-through mode
Volume: 1mL Flow rate: 300 cm/hr
Mab loaded: 35 mg
% Recovery: >95%
Estimated purity by protein A affinity: >90%

Polishing
Toyopearl® Butyl-650M (Tosoh Biosciences)
Bind-elute mode
Volume: 2 mL Flow rate: 300 cm/hr
Mab loaded: 30 mg
% Recovery: >90%
Estimated purity by protein A affinity: >95%

Overall recovery: >75%

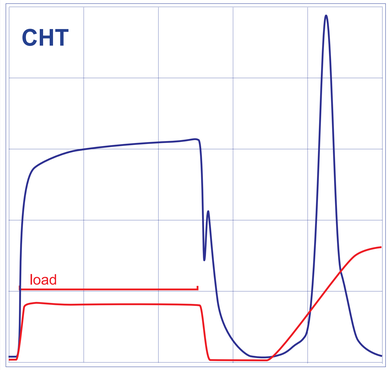

Figure 1. Capture/purification on hydroxyapatite. Human IgG1 cell supernatant was diluted 1:1 with water and the pH adjusted to 6.7 with 0.2M MES, pH 5.0. The column was equilibrated with 5mM sodium phosphate, pH 6.7, loaded with a 25X volumetric excess of sample, washed with equilibration buffer, then eluted with 10 CV linear gradient to 150 mM sodium phosphate.

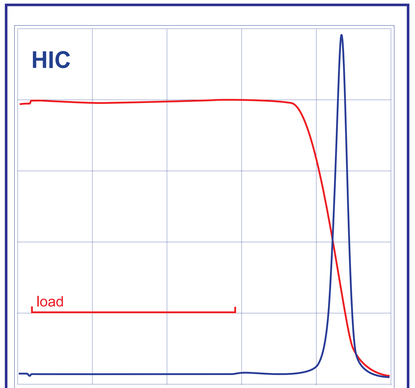

Figure 3. Final purification by hydrophobic interaction on Toyopearl Butyl 650-M. The flow-through from anion exchange was brought to 4M sodium chloride by direct addition of dry salt. The column was equilibrated to 4M sodium chloride, 0.05M sodium phosphate, pH 6.8, loaded, washed with equilibration buffer, then eluted in a 10CV linear gradient to 0.05M phosphate.

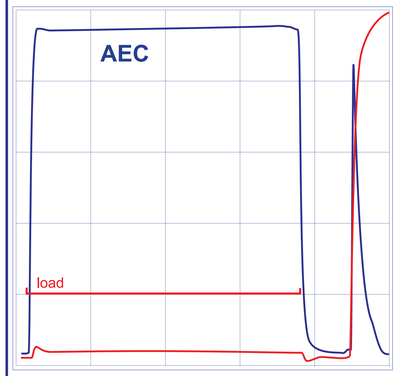

Figure 2. Intermediate purification by anion exchange on UNOsphere Q. The pH of the sample from the capture step was raised to 8.0 by addition of 1M Tris, pH 8.5 and diluted 0.5:1 with WFI. The column was equilibrated to 0.05 M sodium phosphate, pH 8.0, loaded, washed with equilibration buffer, then eluted with a step to 0.5 M sodium phosphate, pH 8.0.

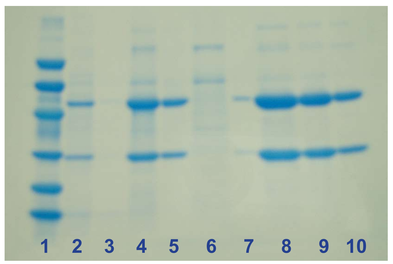

Figure 4. Reduced SDS PAGE of selected samples. 4-20% gradient gel. **1**: Stds. **2**: Supe, diluted 1:1. **3**: CHT flow-through. **4**: CHT elution. **5**: AEC flow-through. **6**: AEC elution. **7**: blank. **8**: HIC elution. **9**: HIC elution diluted 1:1. **10**: Protein A purified reference.

Acknowledgements
Thanks to Bio-Rad Laboratories for supporting this work. Copies of this poster and more information about the use of hydroxyapatite for purification of monoclonal antibodies can be downloaded at **www.validated.com**.

Results
Figure 1 illustrates the chromatogram from capture on CHT Type I, 20 micron. The majority of the contaminants are unretained while the antibody elutes in a well defined peak. Purity was estimated at >85% based on integration of the flow-through and elution peaks from analytical scale protein A affinity chromatography. This estimate is consistent with the anion exchange profile (Figure 2) but more favorable than the PAGE gel (Figure 4) due to deliberate overloading. Recovery was estimated to be in excess of 90% (Table 1).

Previous studies with this antibody indicated a dynamic binding capacity of about 37 mg/mL on CHT type I, 40 micron in 5mM sodium phosphate, pH 6.7 at 300 cm/hr. When the antibody was applied in supernatant however, higher conductivity reduced binding capacity to less than 4 mg/mL. Diluting 1:1 with WFI increased the capacity to about 9 mg/mL. The column was eluted with phosphate to minimize the conductivity of the eluted antibody fraction.

The antibody fraction was applied to UNOsphere Q under the conditions described in Figure 2. No antibody was apparent in the flow-through and a significant amount CHOP was removed. The flow-through fraction from anion exchange was then applied to the Toyopearl butyl 650-M column (Figure 3) and eluted in a single peak at the end of the gradient.

Discussion and Conclusions
The overall process scheme is ideal in every major respect except binding the product from supernatant at the CHT step. It avoids the key limitations of protein A. Neither intermediate buffer exchange nor substantial product dilution were required between steps. Purity was at least equivalent to the protein A purified reference sample.

The low binding capacity of hydroxyapatite for this antibody disqualifies it from being a serious alternative to protein A. In this regard, it is similar to anion and cation exchange chromatography, both of which also require substantial modification of sample conditions to achieve commercial capacity.

Hydroxyapatite nevertheless stands alone as a process tool for removal of aggregates and leached protein A. These two attributes make it an ideal polishing partner for protein A (1). The fact that it further offers highly effective and orthogonal reduction of CHOP, DNA, endotoxin, and virus makes it even more compelling, especially in combination with anion exchange chromatography in flow-through mode.

Literature cited
1. P. Gagnon et al, 2006, BioProcess International, 4(2) 50-60.