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Improving Aggregate Removal to Enhance Clinical Manufacturing of MAbs

Hydrophobic interaction chromatography (HIC) in flow-through mode offers a more efficient and cost-effective polishing/purification process to remove monoclonal antibody aggregates while maintaining purity at ≥99% than a mixed-mode bind/elute procedure.

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Over the past two decades, monoclonal antibodies (mAbs) have been one of the most dominant segments of the biotherapeutics market. Hundreds of mAbs are either under clinical development or commercially available (1). Ensuring mAbs' safety and efficacy is vital, but is complicated by the fact that the antibodies tend to aggregate during production, decreasing efficacy and increasing the risk of immunogenicity. Maintaining aggregate levels of less than 1% of final product is a critical quality attribute (2) for mAb manufacturing. Integrating an aggregate-removal step into downstreaming is crucial to ensuring a safe final product.

Immunoglobulin G (IgG)-type mAbs share a common framework, with an excellent degree of homology and shared physicochemical properties, including a binding site for Protein A on the Fc portion of the molecule. This similarity has enabled the use of a platform approach to mAb purification that centered around Protein A affinity capture as its first step to remove many of the contaminants from the upstream processing, such as host cell protein (HCP), residual DNA, adventitious and endogenous viruses, endotoxins, aggregates, and other contaminating species.

Aggregates can be especially challenging to remove from monomeric mAb product, however, and post-Protein A purification levels can still be as high as 10% for certain mAbs. Therefore, a number of chromatographic polishing tools have been developed to remove excess aggregates.

One of these methods is hydrophobic interaction chromatography (HIC), which leverages differences in hydrophobicity between monomer and aggregate species. Until recently, HIC resins required high salt concentrations to be most effective, but the proteins of interest are often unstable under such conditions. To avoid product instability, the resins must provide the selectivity required at lower salt concentrations and still maintain superior resolution and high capacity.

HIC selectivity between aggregate and monomer can be modulated by adjusting salt type, salt concentration, and by buffer type and pH. Furthermore, if the resin chemistry and properties enable high selectivity towards the impurities then they can be used in flow-through, rather than standard bind-and-elute, mode. Flow-through offers the advantages of reducing the number and volume of buffers required and decreasing operator workload and operation times. Therefore, HIC can enhance flexibility for process operating conditions.

In this study, two HIC resins (POROS Benzyl and POROS Benzyl Ultra, Thermo Scientific) were evaluated in flow-through mode as a polishing step in the purification of mAb-A, an established therapeutic mAb. This article will describe how HIC in flow-through mode can be optimized to develop a scalable and robust polishing procedure that significantly improves the efficiency and cost of aggregate removal, compared with mixed-mode bind-and-elute purification processes (**Figure 1**). Using this method, even under low conductivity conditions and high loading densities, the final product showed effective clearance of dimers and high molecular weight (HMW) aggregates. Furthermore, the process allowed final monomeric product purity to be maintained at ≥99%, while enabling increased resin loading and reducing residence time, resulting in an 8% increase in final product yield.

Materials and Methods

Materials and Equipment

mAb-A , a Chinese hamster ovary (CHO) cell-produced IgG mAb, was used in this study after it had been purified using a Protein A capture step followed by an anion exchange (AEX) chromatography step in flow-through mode. The AEX flow-through pool contained approximately 12% mAb-A aggregate. Materials and equipment used included POROS resins (Thermo Fisher), an automated liquid handler (Thermo Fisher), 96-deep well polypropylene plates (Thermo Fisher), 96-well flat-bottom (Thermo Fisher), clear polystyrene plates (Thermo Fisher), a Novus electronic multichannel pipette (Thermo Fisher), a LUX multimode microplate reader (Thermo Fisher), centrifuge (Thermo Fisher), a standard dual-system vacuum manifold (Thermo Fisher), a size-exclusion column (Thermo Fisher), a magnetic particle processor (Thermo Fisher), a polymerase chain reaction (PCR) system (Thermo Fisher), and a CHO-HCP quantitation kit(Thermo Fisher). In addition, the reagents used included sodium chloride (NaCl) (molecular weight [MW] 58.44) (Fisher Bioreagents), sodium acetate trihydrate (MW 136.08) (Fisher Chemical), ammonium sulfate (MW 132.14) (Fisher Chemical), and sodium citrate dihydrate (MW 294.1) (Fisher Chemical).

Predictive analytics software was also used (JMP Statistical Discovery).

Methods

The workflow for HIC process optimization consisted of the following four steps:

1. Defining the conductivity range for mAb-A (resin interaction)

The POROS HIC resins were packed into individual columns of 0.66 cm (D) x 10 cm (L). Each column was equilibrated with 600 mM sodium acetate in Tris buffer at pH 7.5. The mAb-A AEX pool was then loaded onto each column at 5 mg/mL of resin at a flow rate of 300 cm/hr. To define optimal elution conductivity, a gradient elution over 10 column volumes (CV) was performed, at 300 cm/hr, starting with the high-salt equilibration buffer and gradually moving to a Tris buffer (pH 7.5) containing no salt.

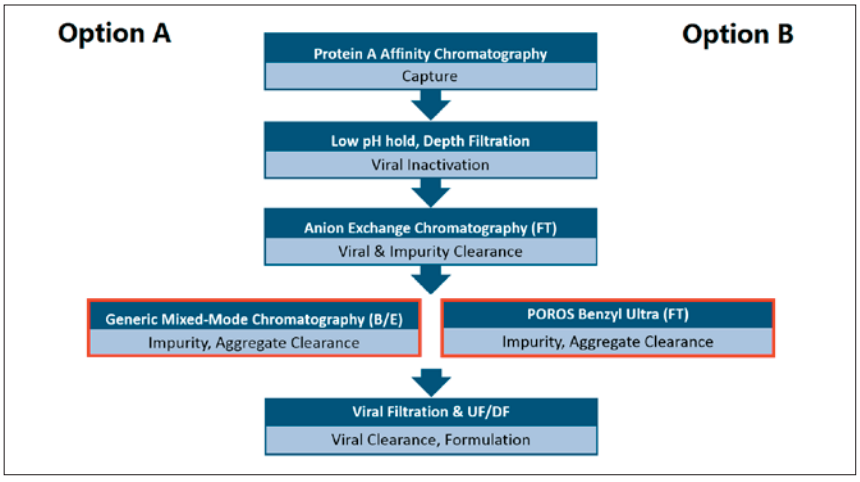

Figure 1. Schematic overview of the purification process of mAb-A. To obtain a more efficient purification process, the final Mixed-Mode polishing step in bind/elute mode is compared with HIC in flow-through mode.

2. High-throughput screening for flow-through mode (resin selection)

A high-throughput screening approach was employed to determine the most suitable resin and corresponding conditions for aggregate removal. To determine the optimal pH, salt type, and salt concentration each well of a 96-well filter plate was loaded with 30µL of either POROS Benzyl or POROS Benzyl Ultra resin. Then 185 µL of buffer containing varying salt types, i.e., sodium chloride, acetate, and citrate, and ammonium sulfate at a pH of either 5.5, 6.5, or 7.5 were pipetted into the plate. Four different salt concentrations were tested for each resin: for POROS Benzyl, the concentration of an individual salt type ranged between 10-300 millimoles (mM)(4–40 milliSiemens[mS])/L. For POROS Benzyl Ultra, the salt concentrations ranged between 5–150 mM (1.5mS–25mS/L). Each well was spiked with 15 µL of concentrated mAb-A solution to give a final phase ratio of 6.6 and loading density of 6mg/mL resin.

After mixing for 30 minutes, the plates were centrifuged at 1000 rpm for 3 minutes and the flow-through pools collected in 96-well ultraviolet (UV) transparent collection plates. The yield of the desired monomeric product was measured using a plate reader at UV280. Purity was determined by high-performance liquid chromatography (HPLC)-size exclusion chromatography (SEC) analysis using an UltiMate 3000 system fitted with a MAbPac-SEC-1 column at 50mM sodium phosphate pH7.0, 200mM NaCl isocratic elution, with a 15 minute run time.

Contour plots for mAb-A monomer recovery, aggregate removal, and selectivity factor (a) were generated using predictive analytics software. Monomer recovery and aggregate removal values were calculated using the combined total concentration and HPLC-SEC purity data. The selectivity factor was calculated as the ratio of aggregate to monomer partition coefficients (*Kp*) as published by Kramarczyk *et. al* (3).

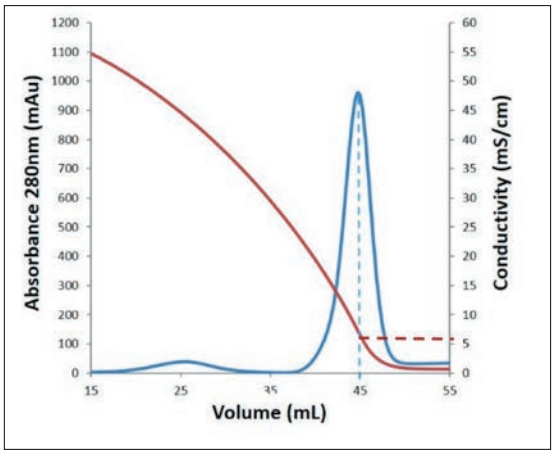

Figure 2. Example screening chromatogram showing the elution profile for monoclonal antibody-A using POROS Benzyl Ultra resin in flow through mode. The gradient was high to low conductivity using sodium citrate.

3. Chromatography optimization using a scaled-down model

POROS Benzyl Ultra resin was packed into 0.66cm (D) x 10 cm (L) (3.4 mL) columns. The columns were equilibrated with 25mM Tris-Acetate pH6.8, 1.8 mS/cm. Each column was then loaded with mAb-A AEX pool to give 2.4mg/mL at conductivity of 1.8 mS/cm and a pH of 6.8.

Two conditions were evaluated to further optimize the process:

1. A flow rate of 300 cm/hr, 2-min residence time, and load density up to 350 g/L
2. A flow rate of 800 cm/hr, 45 sec residence time, and load density up to 145 g/L.

Fifteen-milliliter fractions of both the load and wash steps were collected and analyzed for monomer purity and yield, as described above.

4. Chromatography process verification

A verification run was executed to confirm the selected conditions. A POROS Benzyl Ultra 0.66cm (D) x 10 cm (L) (3.4 mL) column was equilibrated with 25mM Tris-Acetate buffer at pH 6.8, at a conductivity of 1.8mS/cm. The column was loaded with mAb-A AEX pool (2.4 mg/mL) using a 1.2-min residence time at a conductivity of 1.8 mS/cm and pH of 6.8. The final load density tested was 80 g/L resin to ensure a conservative and robust process for aggregate removal. Again, purity and yield were determined as described above.

Residual host cell protein quantitation on both the load and flow-through pool was determined by immuno-qPCR proximity ligation assay using a commercially available assay kit (ProteinSEQ, ThermoFisher). A 96-well automatic magnetic particle processor (KingFisher Flex, ThermoFisher) was used for sample preparation, and PCR was performed (7500 Real-Time PCR System, ThermoFisher). The lower limit of quantitation (LLOQ) for the HCP assay is 0.2ng/mL.

Results and discussion

Defining the conductivity range for mAb-A (resin interaction)

Process optimization first involved a bind elute experiment to define salt concentration ranges for flow-through operation. The elution conductivity at peak maximum was used to determine the highest approximate salt concentration required to remove impurities, but still allow the target molecule to flow through.

Using POROS Benzyl Ultra resin, mAb-A was found to elute at lower salt concentrations that corresponded to a conductivity of around 7mS/cm, **Figure 2**, while for POROS Benzyl resin, elution was at 28mS/cm (data not shown).

Therefore 7mS/cm and 28mS/cm were the salt conductivities used to explore the flow-through parameters for POROS Benzyl Ultra and Benzyl, respectively.

High-throughput screening (HTS) to determine optimal resin and operating conditions for flow-through mode

A HTS approach was used to determine the critical parameters affecting aggregate removal in the flow through mode for both resins. The results for aggregate removal, monomer recovery, and selectivity factor, as a function of pH and salt concentration, were graphed in a series of contour plots, **Figure 3**.

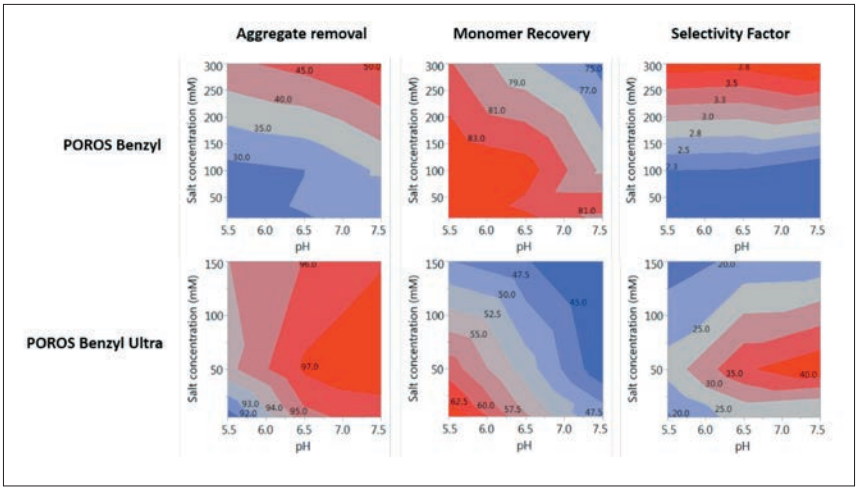

Figure 3. Contour plots showing aggregate removal, monomer recovery, and the selectivity factor for POROS Benzyl and POROS Benzyl Ultra resins tested under a range of pH and salt concentrations. A higher selectivity factor indicates stronger aggregate binding compared to monomer.

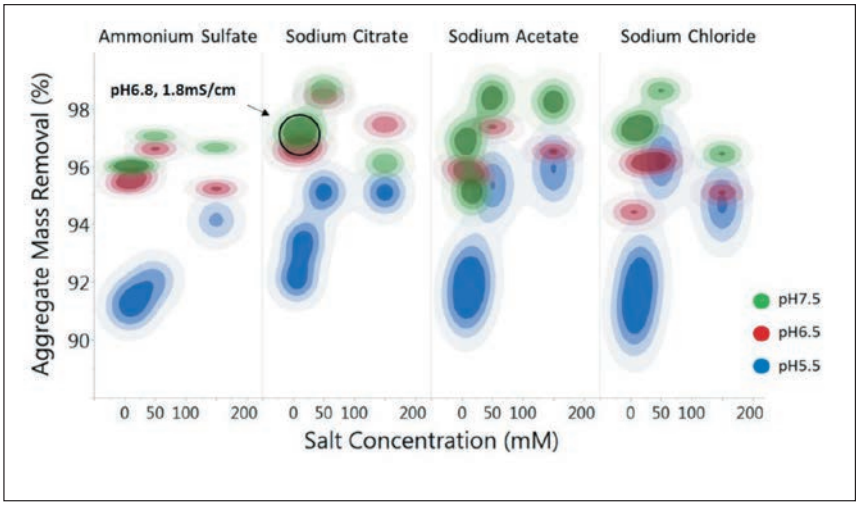

Figure 4. Amalgamated contour plot showing aggregate removal as function of salt type, salt concentration, and pH. Sodium citrate, pH 6.8 at 1.8mS/cm (5mM) was chosen for column scale-up.

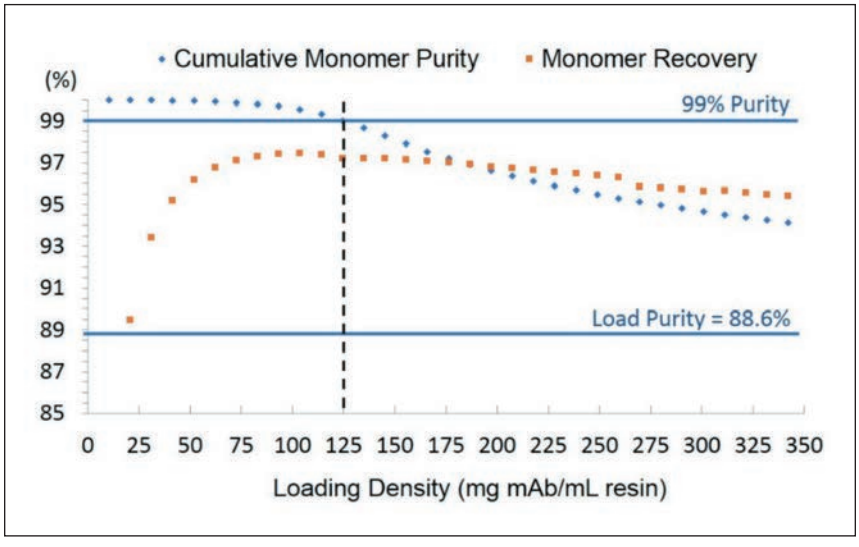

Figure 5. Breakthrough analysis of scale-down model for POROS Benzyl Ultra resin run at a flowrate of 300cm/hr, load density up to 350 g/L.

Compared to POROS Benzyl, POROS Benzyl Ultra resin showed stronger aggregate binding with >90% aggregate mass removal over a broad range of the conditions tested. It also exhibited a greater selectivity factor indicating a better separation between the aggregates and monomer. Although POROS Benzyl resin showed higher monomer recovery, it did not significantly bind and remove aggregates, and the selectivity factor remained low. While a static binding HT model at low protein load is representative of selectivity, the recoveries achieved are not indicative of those seen in a dynamic flow-through process at high protein load. In addition, analytical bind-elute experiments showed mAb-A elution at 7 mS/cm for Benzyl Ultra, which is compatible with the desired flow-through process step. Therefore, POROS Benzyl Ultra was selected as the optimal resin for use in further scale-up mAb-A purification experiments.

Selection of flow-through mode process conditions for POROS Benzyl Ultra resin

An HTS approach was used to determine the conditions suitable for scale-up verification in column format for POROS Benzyl Ultra resin. The resulting data were visualized in an amalgamated contour plot, showing aggregate removal as function of salt type, salt concentration, and pH, **Figure 4**.

From these data, sodium citrate at pH 6.8 at approximately 2mS/cm was chosen as the most suitable. Under these conditions, very high aggregate clearance was observed. In addition, these conditions meant that the overall purification process would be streamlined as the mAb-A AEX pool could be loaded directly onto the HIC column without further buffer conditioning.

Chromatography optimization using a scale-down model

To model what occurs at manufacturing scale in dynamic mode, a scale-down column model was tested to determine the optimal process conditions for flow through mode. Break through analysis demonstrated that monomer purity remained above 99% until a load density of 125g/L was reached. Monomer recovery remained stable at 97% up to a load density of 200g/L and remained above 95% for the course of the experiment, **Figure 5.**

During breakthrough analysis, the aggregate levels in the collected fractions were analyzed by HPLC-SEC. The purity goal of 1% breakthrough of aggregates was achieved up to a loading density of 125 g/L resin, showing significantly high loading capacity for this resin. The results for this resin in flow-through mode showed a significant improvement over the original

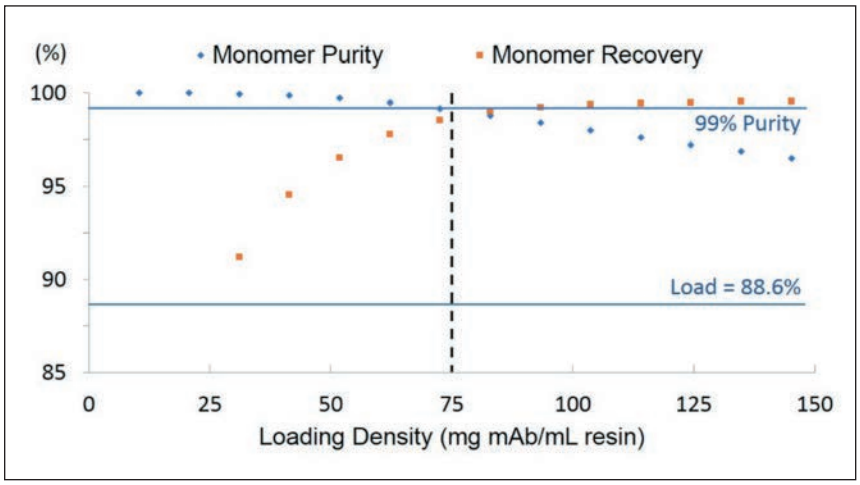

Figure 6. Results from breakthrough analysis at a flow rate of 800cm/hr (load density up to 150 g/L). Even at high flow rate high aggregate clearance is obtained.

	Mixed-Mode B/E	POROS HIC FT
Load Density (g/L resin)	25	80
Monomer Purity FT (%)	99	>99
Mon. Recovery (%)	90	98
HCP* (ppm)	<LLOQ	<LLOQ
Residence time (min)	6	1.2

Table I. Comparative data for mixed-mode bind/elute and POROS Benzyl Ultra hydrophobic interaction chromatography flow-through steps. *ProteinSEQ Immuno-qPCR HCP quantitation assay LLOQ <0.2ng/mL.

aggregate removal mixed-mode process which operated at only 25 g/L loading density with just 90% monomer recovery.

To demonstrate that high linear flows that can also be achieved with this resin, an additional breakthrough experiment was conducted at a flowrate of 800 cm/hr (45 second residence time). At this higher flow rate, a 75-g/L resin-loading density was achieved without compromising monomer purity (99%) and recovery (98%), **Figure 6**.

This experiment illustrated the breadth of flow rate capability designed into these POROS HIC resins.

Based on the data obtained from the POROS Benzyl Ultra resin optimization experiments and considering the existing purification process parameters, the flow-through verification run was performed under more conservative conditions in order to establish a robust HIC flow-through polish step suitable for integrating in the total mAb-A purification process. A flow rate of 500cm/hr (1.2-minute residence time), 80g/L mAb-A loading density was selected, and the resulting fractions collected and pooled.

The final flow through pool had a mAb-A purity of 99.3% vs 85.5% for the load, with a very low level of 0.5% aggregates vs 9.5% for the load, and a reduction from 5% to just 0.1% for high molecular weight proteins.

A direct comparison was made between the existing mAb-A second polishing step, which occurs post anion exchange flow-through chromatography, and the new HIC flow through process.

Data in **Table I** show that the mAb-A polishing step is more efficient when using HIC with POROS Benzyl Ultra resin in flow through mode than existing mixed-mode bind and elute chromatography.

The results showed that the flow properties of the POROS-based resin allowed fast mass transfer and enhanced performance at high flow rates (800 cm/hr, 45-sec residence time) without compromising impurity clearance. A purity of >99% with 98% monomer recovery was achieved at a loading density three times higher than the mixed-mode conditions. In addition, a significant reduction of mAb HMW aggregate and dimer were achieved at low conductivity conditions and a 5-fold reduction in residence time (6 min to 1.2 min).

Conclusion

Overall, results of this study suggest that mAb-A clinical development would benefit from using HIC in flow-through rather than the original mixed-mode in bind and elute as the polishing step in purification. This approach appears to improve both process efficiency and productivity, allowing processes to be simplified and throughput increased, and providing an alternate purification option for mAb development.

References

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