Efficient removal of aggregates from monoclonal antibodies by hydrophobic interaction chromatography in flow-through mode

Abstract

Monoclonal antibody (mAb)–based therapeutics have been very successful in treating various diseases, including cancer. The manufacturing of these therapeutic antibodies is a complex operation since it needs to ensure quality, safety, and efficacy of the products. Low levels of aggregates are a critical quality attribute of the final product, as this will impact the biological activity. Therefore, efficient removal of aggregates from the final therapeutic product is of great importance. Hydrophobic interaction chromatography (HIC) is a versatile purification technique that can be utilized to remove aggregates in the downstream processing of mAbs. Here we illustrate an effective solution for aggregate removal in therapeutic mAb production utilizing HIC in flow-through mode, under low-conductivity conditions. Analysis of the final product revealed efficient clearance of dimers and high molecular weight (HMW) aggregates. The final process resulted in a more productive and cost-effective technique compared to the established mixed-mode bind/elute process originally designed for a challenging mAb.

Important: Thermo Scientific™ POROS™ Benzyl and Benzyl Ultra HIC Resins are designed for use with lower salt concentrations than the traditional HIC resins. With some molecules, high salt concentration can cause poor recovery due to a strong interaction between the target and the ligand.

Introduction

Since levels of aggregates impact the biological activity of a biopharmaceutical, monitoring these levels in the final mAb therapeutic is critical [1]. In general, the lowest possible concentration of aggregates is desired in the final formulation of mAbs, typically less than 1% [2].

HIC can be used as an aggregate removal tool in the downstream processing of mAbs. Because the selectivity of HIC can be affected by different factors (i.e., type of salt and concentration, buffer pH, and temperature), a well-designed process together with a robust resin are the keys to successful and highly efficient purification for aggregate removal.

Anion exchange (AEX) chromatography in flow-through mode is often utilized as the first polishing step in mAb purification after affinity chromatography. Flow-through chromatography is a mode of operation that has the benefit of increased productivity and throughput; however, it requires the resin chemistry and properties to be highly selective towards the impurities to be removed. As AEX chromatography in flow-through mode is not highly selective towards mAb aggregates, the process generally requires an additional polish step involving an orthogonal chemistry in bind/elute mode. A final polishing step in flow-through mode would be beneficial to productivity of the purification process.

Summary of the study

For the purpose of aggregate removal, the highly hydrophobic POROS Benzyl Ultra HIC Resin was optimized by high-throughput screening, for use in flow-through mode as an alternative to a generic mixed-mode bind/elute step in a customer's original purification process (Figure 1). The goal of the study was to design a more efficient and cost-effective process than the original one, but to maintain the purity at ≥99%. Introduction of an HIC flow-through (FT) step resulted in a more productive polishing process with an 8% increase in yield, greatly improved resin loading, and a reduction in residence time. Even under low-conductivity conditions and high load densities, the final product showed efficient clearance of dimers and HMW aggregates after the HIC FT step. The data show that the POROS Benzyl Ultra resin can be utilized for efficient aggregate removal in the downstream process of therapeutic monoclonal antibody production.

Goal of the study

Design a more simple and cost-effective polish step utilizing POROS Benzyl Ultra resin in flow-through (FT) mode as an alternative to the mixed-mode bind/elute (B/E) step in the original purification process (Figure 1).

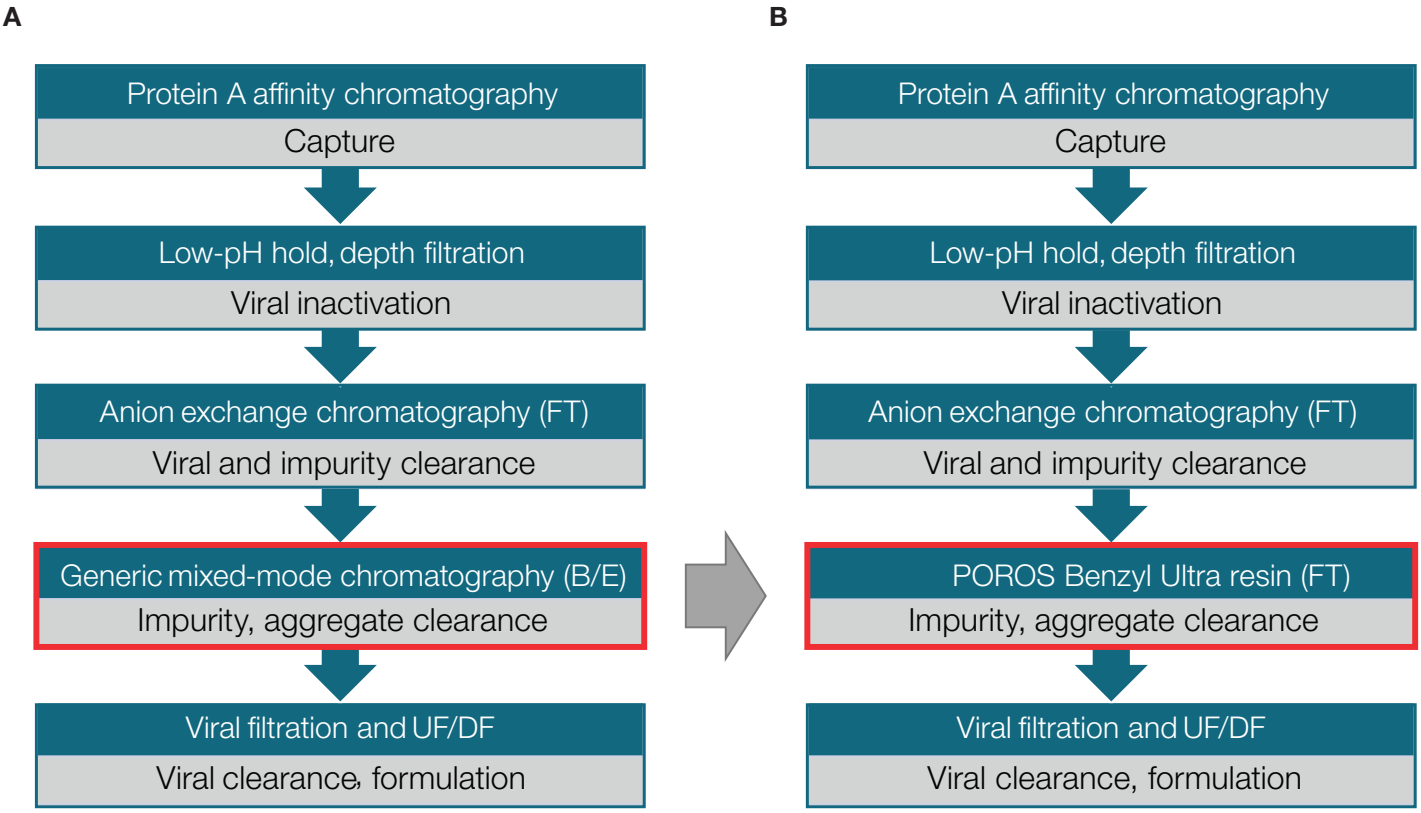

Figure 1. Comparison of (A) the original purification process with (B) the newly optimized process. The goal of this study was to design a more simple and cost-effective polish step utilizing POROS Benzyl Ultra resin in a flow-through (FT) mode as an alternative to a mixed-mode bind/elute (B/E) step in the original purification process. UF/DF = ultrafiltration/diafiltration.

Materials

Antibody

- mAb-A: a CHO-produced monoclonal antibody, purified using a protein A capture step followed by an AEX chromatography step in flow-through mode. The AEX flow-through pool contained approximately 12% aggregate.

Consumables

- POROS Benzyl HIC Resin, Thermo Fisher Scientific (Cat. No. A32558)
- POROS Benzyl Ultra HIC Resin, Thermo Fisher Scientific (Cat. No. A32565)
- Fisherbrand™ 96-Well DeepWell™ Polypropylene Microplates, Fisher Scientific (Cat. No. 12-566-121)
- Thermo Scientific™ Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Polymer Base, Thermo Fisher Scientific (Cat. No. 152028)
- Thermo Scientific™ MAbPac™ SEC-1 Size Exclusion LC Columns, Thermo Fisher Scientific (Cat. No. 088789)
- Sodium Chloride (MW 58.44), Fisher Bioreagents (Cat. No. BP358-1)
- Sodium Acetate Trihydrate (MW 136.08), Fisher Chemical (Cat. No. S209-500)
- Ammonium Sulfate (MW 132.14), Fisher Chemical (Cat. No. A702-500)
- Sodium Citrate Dihydrate (MW 294.1), Fisher Chemical (Cat. No. S279-50)

Equipment and software

- Thermo Scientific™ Versette™ Automated Liquid Handler, Thermo Fisher Scientific (Cat. No. 650-INSTR)
- Thermo Scientific™ Finnpipette™ Novus Electronic Multichannel Pipette, Thermo Fisher Scientific (Cat. No. 46300800)
- Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader, Thermo Fisher Scientific (Cat. No. VLBLATD1)
- Thermo Scientific™ Sorvall™ Legend™ XT/XF Centrifuge Series, Thermo Fisher Scientific (Cat. No. 75004521)
- Thermo Scientific™ Ultimate™ 3000 Standard Dual System, Thermo Fisher Scientific (contact our sales support team for purchase)
- Thermo Scientific™ HyperSep™ Universal Vacuum Manifold, Thermo Fisher Scientific (Cat. No. 60104-231)
- Thermo Scientific™ Pharma KingFisher™ Flex 96 Deep-Well Magnetic Particle Processor, Thermo Fisher Scientific (Cat. No. A31508)
- Applied Biosystems™ 7500 Real-Time PCR System, Thermo Fisher Scientific (Cat. No. 4351104)
- Applied Biosystems™ ProteinSEQ™ CHO HCP Quantitation Kit, Thermo Fisher Scientific (Cat. No. A27601)
- JMP™ Pro predictive analytics software, JMP Statistical Discovery
- ÄKTA™ Pure Chromatography System, GE Healthcare Life Sciences

Methods

The workflow used for process optimization can be broken down into the steps shown in Figure 2.

Defining conductivity range for mAb-A and resin interaction

The POROS HIC resins were packed into a 0.66 cm (D) x 10 cm (L) column. Each column was equilibrated with 600 mM sodium acetate in Tris buffer, pH 7.5. The mAb-A AEX pool was then loaded onto each column at 5 mg of mAb per milliliter of resin (mg/mL) 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 2. Breakdown of steps for process optimization.

High-throughput screening for flow-through mode

To explore the critical parameters affecting resin selectivity towards aggregate removal in the flow-through mode, high-throughput screening was used (Figure 3). Yield was measured by A_{280} on the Varioskan LUX Multimode Microplate Reader. Purity was determined by high-performance liquid chromatography (HPLC)–size-exclusion chromatography (SEC) analysis (UltiMate 3000 HPLC, MAbPac SEC-1 LC column, 50 mM sodium phosphate, pH 7.0, 200 mM NaCl isocratic elution, 15 min).

To determine the optimal pH, salt type, and salt concentration of the resins, each well of a 96-well filter plate was filled with 30 µL of POROS Benzyl or POROS Benzyl Ultra resin. Then 185 µL of buffers with various salt types, salt concentrations, and pH were pipetted into the plate, followed by a 15 µL concentrated mAb spike to achieve a final phase ratio of 6.6 and load density of 6 mg/mL resin. After mixing for 30 min, the plate was centrifuged at 1,000 rpm for 3 min. The flow-through pools were collected in a 96-well UV-transparent collection plate. Protein concentration was determined by A_{280} on the Varioskan plate reader, and monomer purity was analyzed by HPLC-SEC on the UltiMate 3000 system with a MAbPac-SEC-1 column.

Conditions tested

- **Salt types:** sodium chloride, sodium acetate, ammonium sulfate, and sodium citrate
- **Salt concentrations:**
  - For POROS Benzyl resin: 10–300 mM (4–40 mS/cm)
  - For POROS Benzyl Ultra resin: 5–150 mM (1.5–25 mS/cm)
- **pH:** 5.5, 6.5, and 7.5

Contour plots for monomer recovery, aggregate removal, and selectivity factor (α) were generated using the JMP software. Monomer recovery and aggregate removal values were calculated based on a mass balance equation using the combined total concentration and HPLC-SEC purity data. The selectivity factor was calculated as the ratio of aggregate to monomer partition coefficients (K_P) as published by Kramarczyk et al. [3].

Chromatography optimization in scale-down model

POROS Benzyl Ultra resin was packed into a 0.66 cm (D) x 10 cm (L) (3.4 mL) column. Each column was equilibrated with 25 mM Tris-acetate at pH 6.8 and conductivity of 1.8 mS/cm. Each column was loaded with the Mab-A AEX pool (2.4 mg of mAb per mL of resin) at conductivity of 1.8 mS/cm and pH 6.8.

The following conditions were evaluated to further optimize the process:

- Flow rate 300 cm/hr, residence time 2 min, load density up to 350 g/L
- Flow rate 800 cm/hr, residence time 45 sec, load density up to 145 g/L

Fractions of the load (15 mL) and wash steps were collected and analyzed for monomer purity and recovery as described in Figure 3.

Chromatography process verification

After scaled-down model optimization, a verification run was executed to confirm the conditions. POROS Benzyl Ultra resin packed in a 0.66 cm (D) x 10 cm (L) (3.4 mL) column was equilibrated with 25 mM Tris-acetate at pH 6.8 and conductivity of 1.8 mS/cm. The column was loaded at 500 cm/hr (1.2 min residence time) with Mab-A AEX pool (2.4 mg/mL of resin) at conductivity of 1.8 mS/cm and pH 6.8. The final load density tested was 80 g/L resin to ensure a conservative and robust process for aggregate removal.

Figure 3. The general workflow for aggregate removal consists of filter-plate screening followed by A_{280} concentration determination and purity analysis by HPLC-SEC; statistical analysis is used to generate trends and to select process conditions.

CHO host-cell protein (HCP) of the load and flow-through pools was quantitated by an immuno-qPCR proximity ligation assay using the ProteinSEQ assay kit. Sample preparation and qPCR were performed on the KingFisher Flex 96-well automatic magnetic particle processor and the 7500 Real-Time PCR System, respectively. The lower limit of quantitation (LLOQ) for the assay is 0.2 ng/mL.

At the end of the run, the flow-through pool was collected and analyzed for monomer purity and recovery as described in Figure 3.

Results and discussion

Defining conductivity range for interaction between mAb and resin

Process optimization was started with a bind/elute experiment to define salt concentration ranges for flow-through operation. The conductivity at the maximum of the elution peak is used to determine the highest approximate salt concentration that is required to remove impurities but that also allows the target molecule to flow through. Using the POROS Benzyl Ultra resin, mAb-A elutes at the lower salt concentration range corresponding to conductivity of around 7 mS/cm (Figure 4), whereas the POROS Benzyl resin showed an elution profile at 28 mS/cm (data not shown).

The salt conductivity values of 7 mS/cm and 28 mS/cm were used to explore the flow-through parameters on POROS Benzyl Ultra and Benzyl resins, respectively.

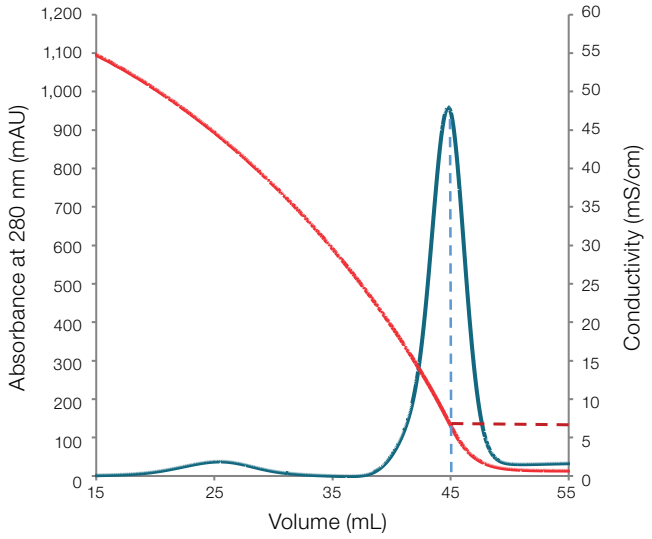

Figure 4. An example of the screening chromatogram to obtain the ideal low-salt condition for the flow-through steps. Process screening for a monoclonal antibody using the POROS Benzyl Ultra resin in flow-through mode. Gradient: high conductivity to low conductivity using sodium citrate. Based on this chromatogram, the resin was further optimized in flow-through mode under low-salt conditions starting at 7 mS/cm. Dashed lines: the maximum of the elution peak corresponds to a salt conductivity of 7 mS/cm).

High-throughput screening for flow-through mode: resin selection

High-throughput screening was conducted to determine the critical parameters affecting resin selectivity towards aggregate removal in the flow-through mode. Figure 5 shows the aggregate removal by total mass, and the selectivity factor of both resins.

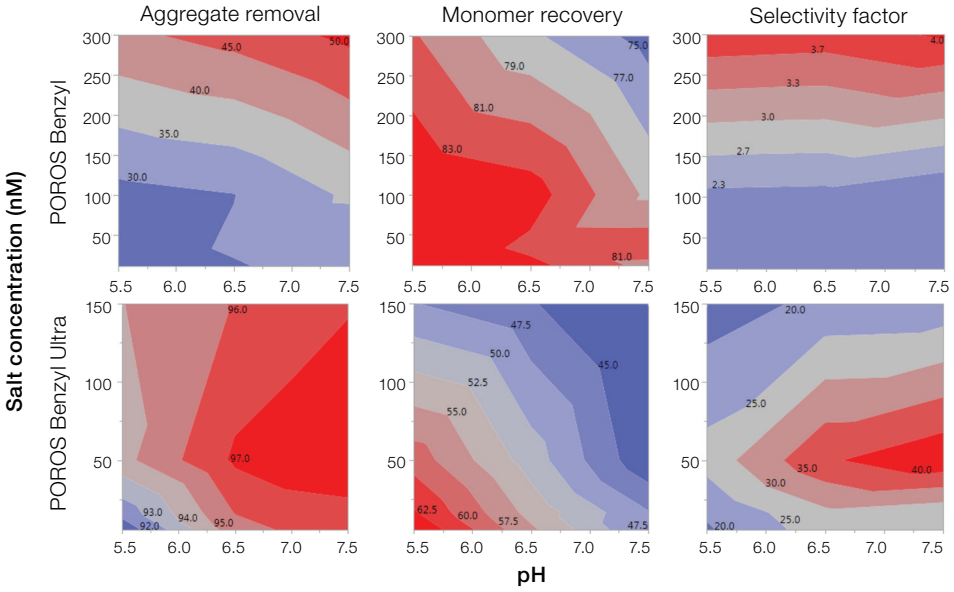

Figure 5. Contour plots showing aggregate removal, monomer recovery, and the selectivity factor for POROS Benzyl and POROS Benzyl Ultra resins, tested under a range of pH and salt concentrations. The selectivity factor is calculated as the ratio of aggregate to monomer partition coefficients (K_p) [3]. A higher selectivity factor indicates stronger aggregate binding compared to the monomers.

The POROS Benzyl Ultra resin shows strong selectivity for aggregate binding, with >90% aggregate mass removal over a broad range of conditions tested. It also exhibits a greater selectivity factor than the POROS Benzyl resin, indicating a better separation between the aggregates and monomers. Although the POROS Benzyl resin showed high monomer recovery, it did not significantly bind and remove the aggregates. The selectivity factor remained low, indicating that both the aggregates and monomers were not partitioned by the resin. In addition, the bind/elute experiments on Benzyl Ultra resin showed mAb elution at 7 mS/cm, which is compatible with the desired flow-through process step (data not shown).

While a high-throughput model for static binding of mAb at low protein load is representative of selectivity, recoveries are not indicative of a dynamic process at high protein load. Due to its high aggregate selectivity combined with bind/elute data that showed mAb elution at 7 mS/cm, the POROS Benzyl Ultra resin was selected as the optimal resin for further scale-up experiments.

High-throughput screening for flow-through mode: selection of process conditions for POROS Benzyl Ultra resin

To determine the conditions suitable for scale-up verification in column format, the raw data are visualized in an amalgamated contour plot showing aggregate removal as a function of salt type, salt concentration, and pH (Figure 6).

For scale-up to column format, the most suitable process condition chosen was pH 6.8 at conductivity of approximately 2 mS/cm. Data showed very high aggregate clearance under these conditions and also allowed for a streamlined process by directly loading onto the HIC column from the AEX flow-through step without the need for further buffer conditioning.

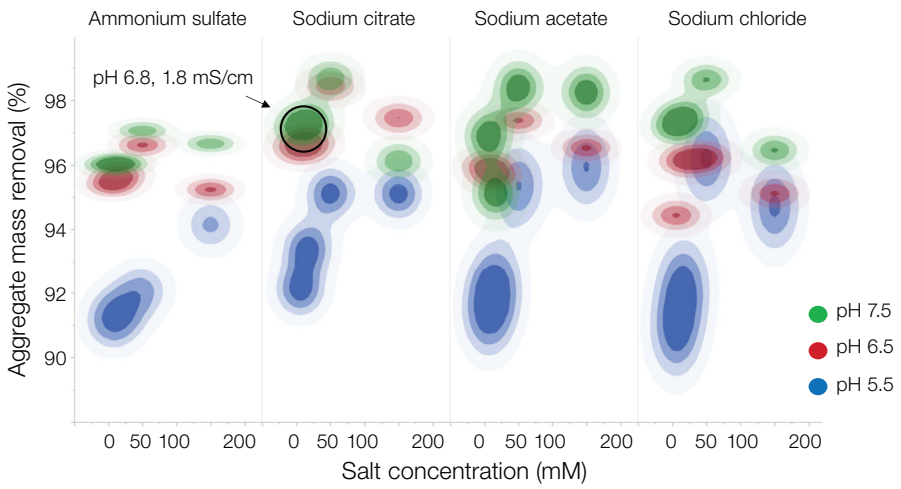

Figure 6. Amalgamated contour plot showing aggregate removal as a function of salt type, salt concentration, and pH. Sodium citrate at pH 6.8 and conductivity of 1.8 mS/cm (5 mM) was chosen for column scale-up.

Chromatography optimization in scale-down model

To simulate the manufacturing scale in a dynamic mode, a column scale-down model was tested to determine the optimal process conditions for flow-through chromatography. Breakthrough analysis demonstrated that monomer purity remained above 99% until a load density of 125 g/L resin was reached. Monomer recovery remained stable at 97% up to a load density of 200 g/L and remained above 95% for the course of the experiment (Figure 7). HPLC-SEC analysis was conducted to analyze aggregate levels in the collected fractions during the breakthrough analysis. The purity goal of 1% breakthrough of aggregates was achieved up to a load density of 125 g/L resin, showing significantly high loading capacity. This shows a critical improvement over the original aggregate removal by mixed-mode process, which was operated at a load density of 25 g/L resin with only 90% monomer recovery.

Next to high resolution and capacity, another main design goal of the POROS HIC resins was to achieve excellent linear flow rate capability. To demonstrate the ability to run at high flow rates, a similar breakthrough experiment was conducted at a flow rate of 800 cm/hr (45 sec residence time). Even at this high flow rate, a load density of 75 g/L resin was achieved without compromising monomer purity (99%) and recovery (98%) (Figure 8).

Chromatography process verification

The flow-through verification run was performed under more conservative conditions to establish a robust HIC flow-through polish step suitable for integrating with the total purification process. At a flow rate of 500 cm/hr (1.2 min residence time) and a load density of 80 g/L, the flow through pool was collected and analyzed.The final flow-through pool showed 99.3% purity, with very low levels of aggregates and high molecular weight (HMW) and low molecular weight (LMW) proteins, a significant improvement compared to the initial material with 85.5% purity (Figure 9).

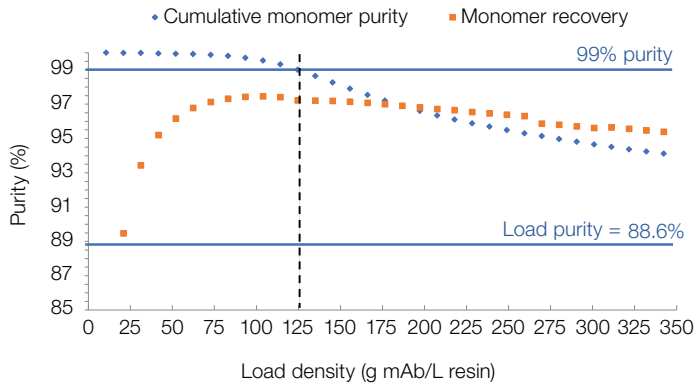

Figure 7. Breakthrough analysis of the column scale-down model, run at a flow rate of 300 cm/hr (load density up to 350 g/L resin). Monomer purity and recovery were analyzed by HLPC-SEC from 15 mL fractions. Monomer purity remained above 95% up to the maximum load density.

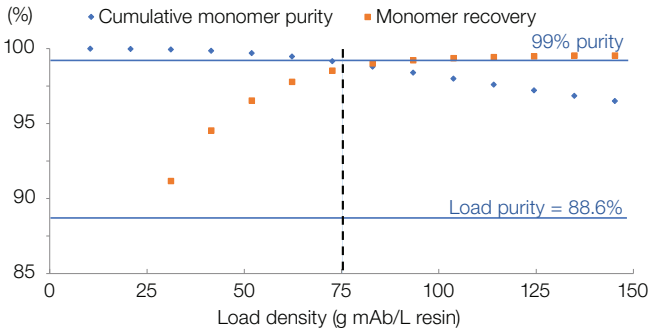

Figure 8. Breakthrough analysis of the column scale-down model, run at a flow rate of 800 cm/hr (load density up to 150 g/L). Even at the high flow rate, high aggregate clearance was obtained.

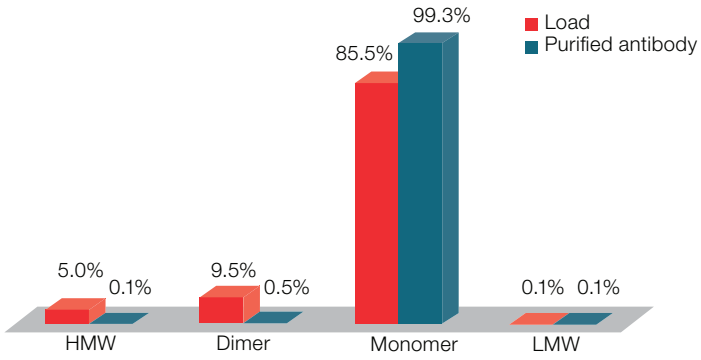

Figure 9. POROS Benzyl Ultra flow-through resin verification results. Very efficient removal of HMW aggregates and dimers was demonstrated using a load density of 80 g/L.

Conclusions

This study shows a successful and structured approach for process optimization—all the way from high-throughput screening to process scale-up and verification. The findings of this study demonstrate a more efficient and cost-effective process by performing HIC using the POROS Benzyl Ultra resin in flow-through mode, compared to the original mixed-mode bind/elute chromatography step (Table 1).

Results showed:

- Significant reduction of mAb HMW aggregate and dimer, achieved under low-conductivity conditions
- A 5-fold residence time improvement (from 6 min to 1.2 min)
- >99% purity, and 98% monomer recovery at a load density 3 times higher than the mixed-mode conditions
- Fast mass transfer and high performance at high flow rates (800 cm/hr, 45 sec residence time) without compromising efficient impurity clearance

In this study, important process parameters have been optimized, leading to a more efficient process and improved productivity. The study further demonstrates that POROS HIC resins can be utilized as a powerful tool to simplify mAb purification schemes and improve process throughput.

Supporting information to demonstrate improved process efficiency and productivity

The illustrative cost model shown in Table 2, which was developed for a 200 L harvest with a titer of 5 g/L, summarizes the cost of buffer, labor, and resin, and the total cost to process a batch of mAb-A through either the mixed-mode bind/elute step or POROS Benzyl Ultra resin in flow-through mode. By using the POROS Benzyl Ultra resin, a total time reduction of 78% could be achieved and total cost savings of >50% could be realized, demonstrating the improved process efficiency and productivity.

Table 1. Comparison between mixed-mode bind/elute and POROS Benzyl Ultra HIC Resin flow-through steps.

	Mixed-mode B/E	POROS HIC FT
Load density (g/L resin)	25	80
Monomer purity FT (%)	99	>99
Monomer recovery (%)	90	98
HCP assay* (ppm)	<LLOQ	<LLOQ
Residence time (min)	6	1.2

* ProteinSEQ Immuno-qPCR HCP quantitation assay, LLOQ <0.2 ng/mL.

Table 2. Cost model to illustrate time and cost savings from improved process efficiency and productivity.

Resin	Residence time (min)	Flow rate (cm/hr)	Loading (mg/mL)	No. of cycles required	Volume of load/cycle (L)	Total buffer volume (L)	Cumulative process time (hr)	Buffer cost (USD)	Process labor cost (USD)	Cost of resin/ batch (USD)	Total cost/ batch (USD)	Time reduction (%)	Buffer cost reduction (%)	Total cost reduction (%)
Mixed-mode (B/E)	6	200	25	3	67	794.5	5.6	$3,972.3	$1,686.8	$353.3	$6,012.3			
Benzyl Ultra (FT)	1.2	500	80	2	100	440.2	1.2	$ 2,201.1	$373.9	$335.6	$2,910.5	78	45	52

thermo scientific

Ordering information

Resin	Volume or column size	Cat. No.
POROS Ethyl HIC Resin	10,000 mL	A32552*
	5,000 mL	A32553*
	1,000 mL	A32554*
	250 mL	A32555
	50 mL	A32556
	25 mL	A32557
POROS Ethyl Pre-packed Columns	0.5 x 5 cm, 1 mL	A34983
	RoboColumn, 200 µL	A34810
	RoboColumn, 600 µL	A34812
POROS Benzyl HIC Resin	10,000 mL	A32558*
	5,000 mL	A32559*
	1,000 mL	A32560*
	250 mL	A32561
	50 mL	A32562
	25 mL	A32563
POROS Benzyl Pre-packed Columns	0.5 x 5 cm, 1 mL	A34984
	RoboColumn, 200 µL	A34813
	RoboColumn, 600 µL	A34814
POROS Benzyl Ultra HIC Resin	10,000 mL	A32564
	5,000 mL	A32565
	1,000 mL	A32566
	250 mL	A32567
	50 mL	A32568
	25 mL	A32569
POROS Benzyl Ultra Pre-packed Columns	0.5 x 5 cm, 1 mL	A34985
	RoboColumn, 200 µL	A34815
	RoboColumn, 600 µL	A34816

* Pharmaceutical Grade Reagent. For Manufacturing and Laboratory Use Only.

References

1. Moussa EM, Panchal JP, Moorthy BS et al. (2016) Immunogenicity of therapeutic protein aggregates. *J Pharm Sci* 105: 417-430.
2. Vazquez-Rey M, Lang DA (2011) Aggregates in monoclonal antibody manufacturing processes. *Biotechnol Bioeng* 108:1494-1508.
3. Kramarczyk JF, Kelley BD, and Coffman JL (2008) High-throughput screening of chromatographic separations: II. Hydrophobic interaction. *Biotechnol Bioeng* 100: 707-720.

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