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SPECIAL REPORT

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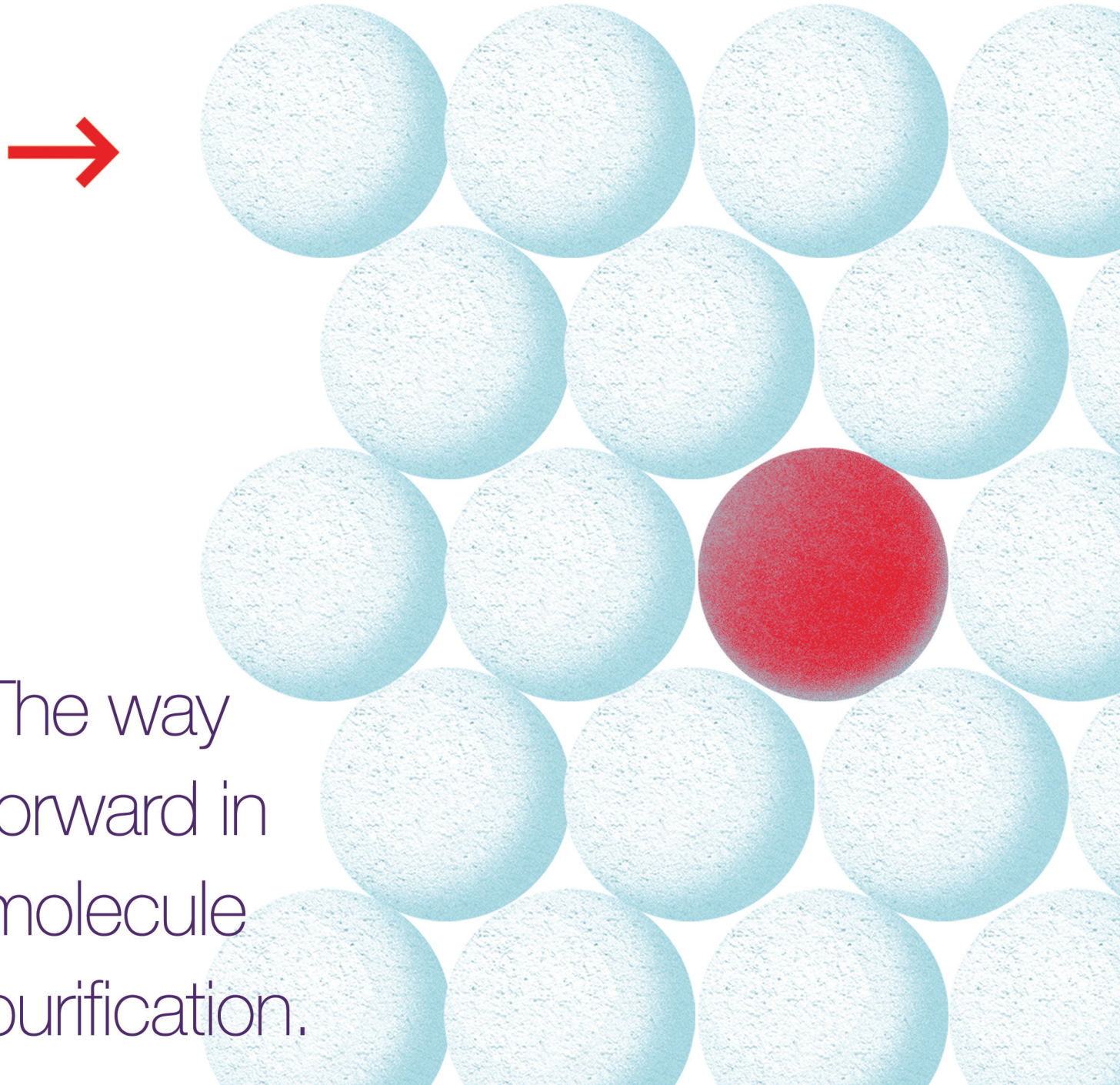
Antibody Therapeutic Polishing

Utility of Hydrophobic-Interaction Chromatography



The way forward in molecule purification.

Antibody Therapeutic Polishing

Utility of Hydrophobic-Interaction Chromatography

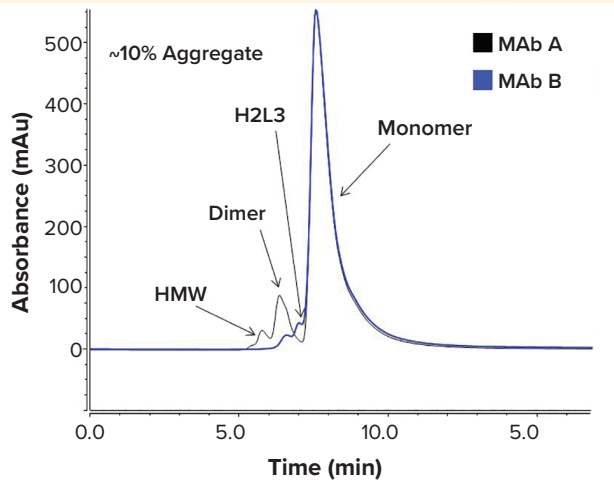
John J. Li, Moira Lynch, and David Cetlin

Therapeutic monoclonal antibodies (MAbs) have played an important part in the personalized medicine revolution, making significant clinical impact in fields such as oncology, immunology, neurology, and infectious diseases. To date, approximately 100 MAbs have been designated as drugs, and the rate of approvals is increasing rapidly. MAbs currently are a $100 billion industry, and their demand is likely to be high and continue to dominate the biologics market for years to come.

As a result of such increasing demand, the past three decades have seen significant improvements to the productivity of large-scale MAb biomanufacturing. Resin manufacturers invested greatly in developing better polishing chromatography solutions to clear residual impurities, provide additional virus clearance, and ultimately meet critical quality attributes (CQAs) required for antibody drug products. For MAb processes, at least one anion-exchange (AEX) polish step typically is required. Depending on a specific residual impurity profile and process challenges being addressed, an AEX step can be preceded or followed by a second polish step, typically using either cation-exchange (CEX) or hydrophobic-interaction chromatography (HIC).

The ultimate goal of downstream purification is to reliably and predictably produce a safe drug product suitable for therapeutic use in humans. To this end, biomanufacturing process- and product-related impurities such as host cell proteins (HCPs) from upstream cell culture, residual DNA, leached protein A from the affinity step, process leachables and extractables, adventitious and endogenous viruses, endotoxins, antibody aggregates, and other antibody variants all must be removed to acceptable levels in conformance with regulatory guidelines. Among all the impurities mentioned, aggregate removal can be especially challenging if levels are high. The FDA generally recommends that MAb aggregates be reduced to <1% for later phase clinical campaigns. For reference, a typical MAb process will start from 1–5% aggregates after protein A capture, but this percentage can be >10% for certain challenging MAbs. To address the need for aggregate clearance in MAb downstream processing, Thermo Fisher Scientific offers a wide range of polish purification tools based on POROS through-pore resin technology. POROS HQ and XQ are AEX resins and POROS HS and XS are CEX resins with different characteristics and selectivity that can be leveraged to remove low-to-moderate levels of MAb aggregates (**1, 2**). Herein, we discuss a family of POROS HIC resins with novel ethyl and benzyl chemistries for the successful polish of two challenging drug products: MAbs A and B, both with high levels of aggregates of >10% (Figure 1). In addition to aggregate clearance, viral clearance strategy on POROS HIC for MAb A and MAb B processes is discussed extensively, including a novel prediction technique that uses parvovirus surrogate mock virus particles (MVPs) from MockV Solutions as well as live viral clearance data using xenotropic murine leukemia virus (XmuLV) and minute virus of mice (MVM).

Figure 1: MAb A and MAb B were run on the UltiMate 3000 HPLC system using MAbPac SEC-1 LC column, with run conditions of isocratic elution using 50 mM sodium phosphate pH 6.8, 250 mM NaCl. Aggregate and monomer peaks are highlighted. Both MAb A and MAb B contain high aggregate levels ~7–12%. The clinical processes developed for MAb A and MAb B aggregate polish are highlighted in the box below.



POROS HIC Case Study: Two Clinical MAbs Requiring Aggressive Strategy for Aggregate Clearance

MAb A and MAb B are two clinical-stage MAbs with difficult downstream processes. Significant aggregates remained after protein A capture and AEX flowthrough (FT) polish, requiring an additional polishing step. For this second polish step, MAb A uses a mixed-mode bind–elute (BE) process, with low resin load density, slow flow, and high yield, whereas MAb B uses a cation-exchange BE process, with high load density, slow flow, and low yield (Figure 1).

We asked the biologics company whether we could design and develop more productive processes for both MAb A and MAb B using POROS HIC resins and compared our approaches with existing, optimized clinical processes. For MAb A, we designed and verified two strategies: one using POROS Benzyl resin in BE mode and the other using POROS Benzyl Ultra resin

Figure 2: POROS Benzyl Ultra FT viral clearance (MAb A); complete XmuLV and minimal MVM clearance

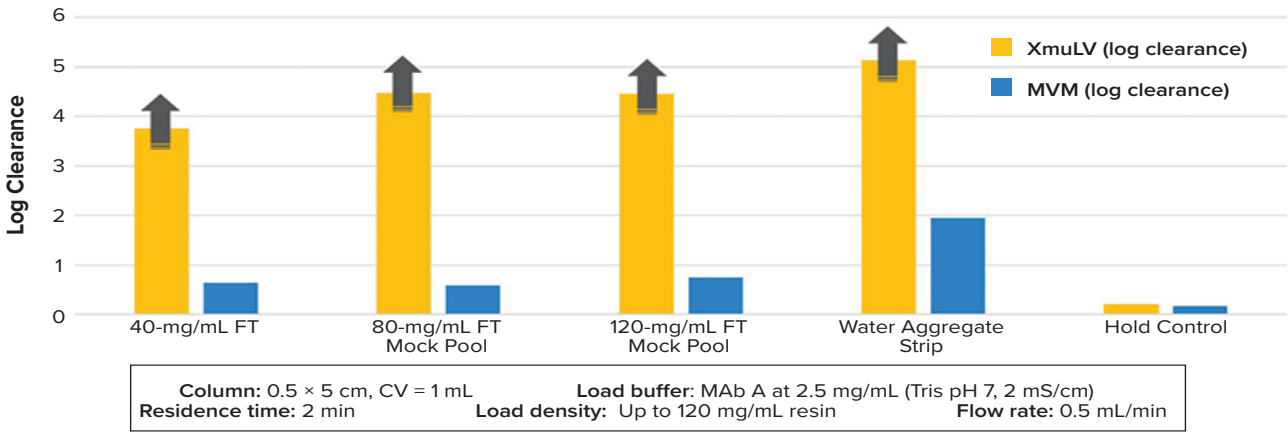


Table 1: Overall comparison of the clinical mixed-mode process for MAb A compared with the two POROS HIC processes; process performance metrics have been improved, resulting in ~4i and ~12i higher productivity.

MAb A Process	Mixed-Mode BE (Clinical)	POROS Benzyl BE Resin	POROS Benzyl Ultra FT
Load monomer purity (%)	90	89	89
Load density (g/L resin)	25	32	120
Monomer purity pool (%)	99	99	>99
Monomer recovery (%)	90	>99	98
HCP (ppm)	NA	120–12 ppm	100–35 ppm
Residence time (min)	6	2	1.2
Pool volume (50–50 mAu)	5 CV	4 CV	NA
Productivity (g/L/h)	7	27	89

Table 2: Comparing the clinical cation-exchange bind–elute process for MAb B with the POROS Benzyl bind–elute process; POROS Benzyl resin achieved complete clearance of a difficult H2L3 aggregate species while improving yield by 20% and decreasing residence time threefold.

MAb B Process	Cation-Exchange BE (Clinical)	POROS Benzyl Ultra BE Resin	POROS Ethyl BE Resin
Load monomer purity (%)	90	90	NA
Load density (g/L resin)	40	40	18
Monomer purity pool (%)	>98	>98	NA
H2L3 (%)	<1	<1	NA
Monomer recovery (%)	>65	>85	NA
Residence time (min)	6	2	NA
Pool volume (50–50 mAu)	4 CV	4 CV	NA

in FT mode. Our processes were four times and 12 times more productive than the original mixed-mode process, respectively (Table 1). For full process development details, please refer to our previous publications (**3**). For MAb B, we optimized a POROS Benzyl Ultra BE process that improved yield significantly and shortened residence time compared with the existing clinical CEX process (Table 2).

MAb A Impurity Clearance and Viral Clearance Study: In a separate study, we performed high-throughput screening for MAb A and demonstrated enhanced selectivity of POROS Benzyl and POROS Benzyl Ultra resins for clearing MAb A aggregates using sodium citrate (**3**). Compared with results from the clinical mixed-mode process, POROS Benzyl resin in BE mode demonstrated higher load capacity and lower residence time (from six to two minutes) with 10% higher monomer recovery, while maintaining aggregate and HCP clearance performance (Table 1). Overall productivity of the process increased about fourfold while achieving product CQAs.

We then optimized an even higher productivity process using POROS Benzyl Ultra resin in FT mode with similar impurity clearance ability (Table 1). A <2-minute residence time combined with a resin load density >100 g/L resulted in a 12-fold increase in productivity. Moreover, using a HIC FT process has the added benefit of a robust AEX-HIC straight-through process design with no additional conductivity or pH adjustments and no intermediate hold up required between AEX and HIC (data not shown).

In addition to the impurity clearance study, we tested the ability of the optimized MAb A POROS HIC BE and FT processes to clear retrovirus XmuLV and parvovirus MVM. In the POROS Benzyl Ultra FT process, we observed complete clearance of XmuLV (>4 log) and minimal clearance of MVM (<1 log) up to 120 g/L_r load at 2-minute residence time (Figure 2). XmuLV is more hydrophobic than MVM and thus bound tightly to the resin in FT mode. MVM is less hydrophobic and did not bind. It is interesting that a water strip was insufficient to remove bound XmuLV from POROS Benzyl Ultra resin.

In the POROS Benzyl BE process, MAb A load was bound at high salt levels, and we performed specific monomer elution with 260 mM sodium citrate. We observed complete clearance of XmuLV (>4 log) and partial clearance of MVM (1.5 log) in the elution fraction. We then performed sequential washes of

decreasing salt concentrations to 130 mM sodium citrate, then buffer alone where bound aggregates eluted, followed by a water strip and finally a 1M arginine strip. It is interesting to note that water and arginine but not buffer alone were sufficient to remove the highly hydrophobic XmuLV from POROS Benzyl resin. The differential stripping behavior for XmuLV is consistent with the relatively less hydrophobic character of POROS Benzyl resin compared with POROS Benzyl Ultra resin.

MAb B Impurity Clearance and Viral Clearance Study: MAb B also was difficult to polish from an aggregation standpoint, with >5% aggregation consistently post AEX-FT and a highly unique cysteine-mediated H2L3 aggregate species (one light chain more than the monomer) that did not behave similarly to dimer or higher molecular-weight aggregate species. To clear H2L3 and dimer species completely, we developed MAb B's clinical process as a CEX-BE step that tolerated a 30–40% yield loss. Using POROS Benzyl Ultra resin in BE mode, we optimized a process for MAb B that decreased residence time from six to two minutes, while yielding a 20% increase in monomer recovery (Table 2).

In preliminary studies, we also observed complete clearance of MAb B H2L3 and other aggregate species in FT mode on POROS Benzyl Ultra resin, though further salt type and concentration optimization was required (results not shown). Finally, we tested stability of MAb B in high-salt buffers and did not observe de novo aggregate formation over a seven-day time course at 4 °C and 25 °C (results not shown).

We tested the ability of the POROS Benzyl Ultra BE process for MAb B to clear retrovirus XmuLV and parvovirus MVM (Figure 4). MAb B was loaded with 700 mM citrate, and specific monomer elution was performed using buffer with no salt added. Note that both XMuLV and MVM bound POROS Benzyl Ultra resin under those load conditions, but buffer alone removed only MVM — not XMuLV. For the MAb B monomer elution fraction, we again observed complete XMuLV clearance and minimal MVM clearance. Water strip did not remove XmuLV from POROS Benzyl Ultra resin, a finding consistent

Figure 3: MAb A POROS Benzyl bind–elute process viral clearance study; complete XmuLV and modest MVM clearance

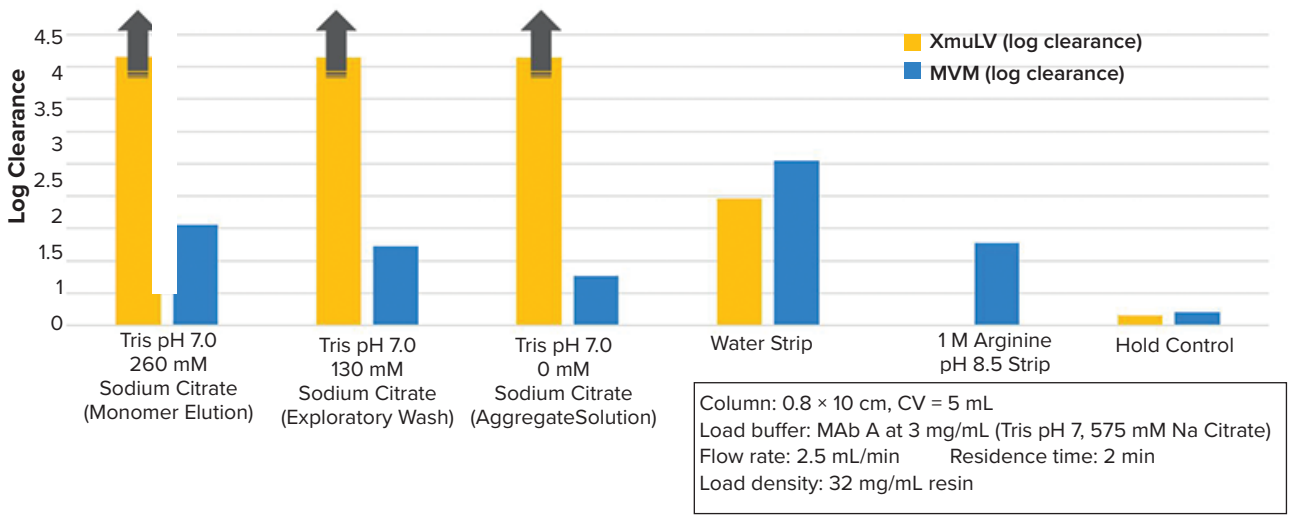


Figure 4: MAb B POROS Benzyl Ultra bind–elute process viral clearance study; complete XmuLV and minimal MVM clearance

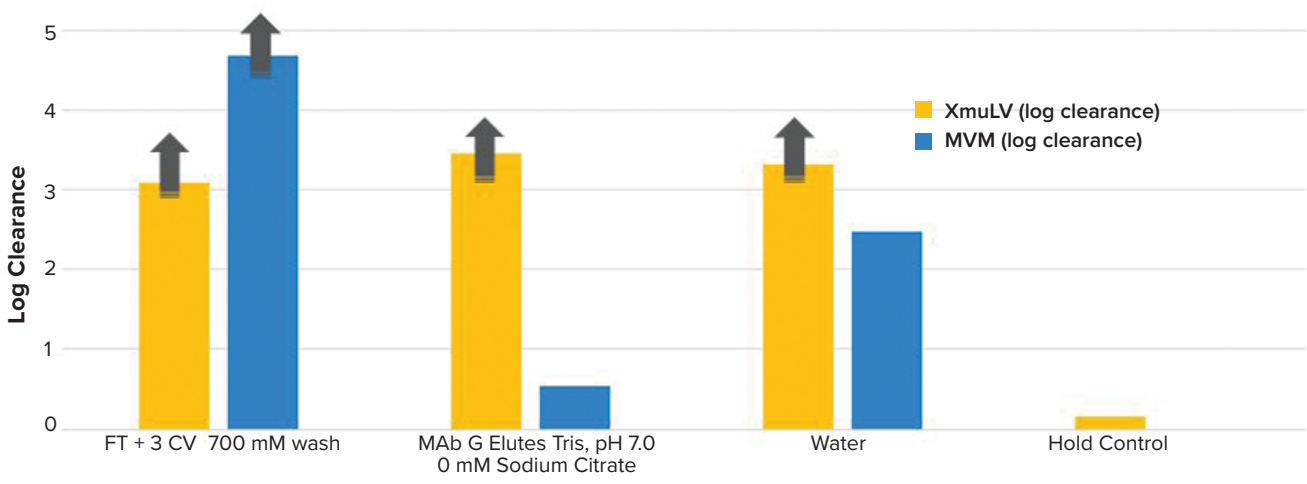
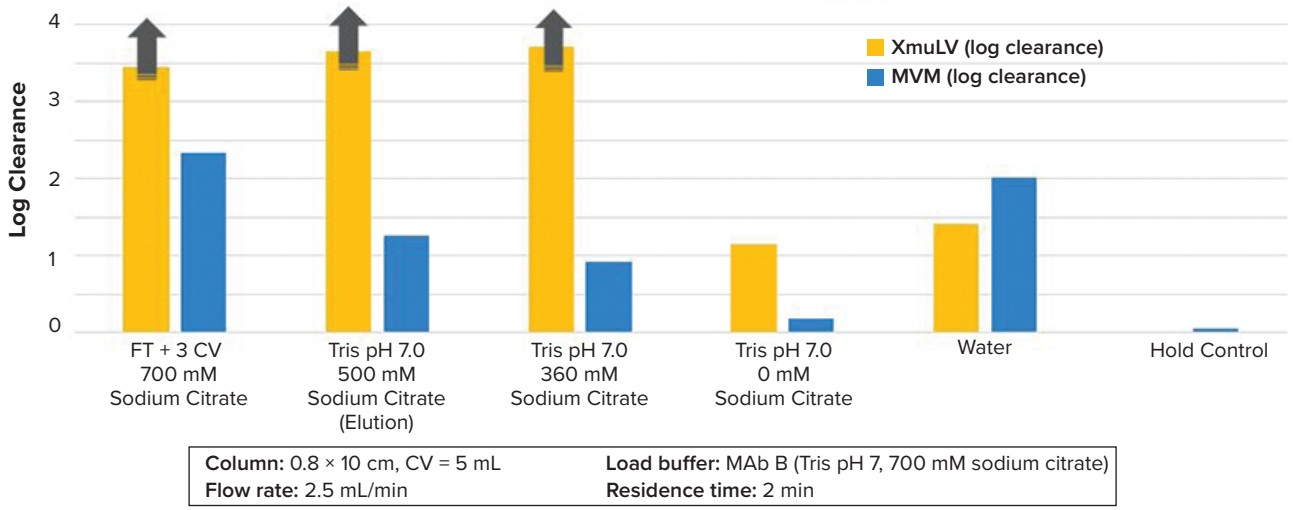


Figure 5: MAb B POROS Ethyl bind–elute process viral clearance study; complete XmuLV and modest MVM clearance



Column: 0.8 × 10 cm, CV = 5 mL
Flow rate: 2.5 mL/min
Load buffer: MAb B (Tris pH 7, 700 mM sodium citrate)
Residence time: 2 min

with results obtained for the MAb A FT process on the same resin.

To test virus-resin hydrophobic interaction, we performed MAb B BE experiments using POROS Ethyl resin without further co-optimizing for H2L3 or aggregate clearance. At 700 mM sodium citrate load phase, we saw both strong XmuLV and moderate MVM binding to POROS Ethyl resin. Using sequential salt drops of 500 mM and 360 mM, then buffer alone, both XmuLV and MVM were stripped from the resin using the minimal buffer wash with no salt added. The relative ease of elution for even the highly hydrophobic XmuLV virus is consistent with POROS Ethyl resin being the least hydrophobic member of the POROS HIC resin family.

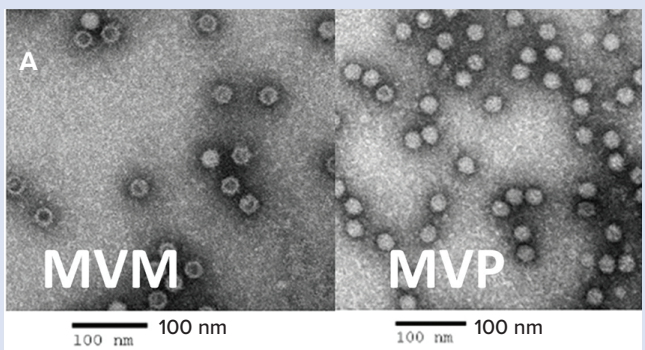
Viral Clearance Prediction and Strategy Using MVPs by MockV Solutions

In the HIC virus clearance studies discussed above, we observed minimal to modest clearance of MVM in all POROS HIC processes conducted in BE and FT modes. To better understand MVM hydrophobicity, binding, and clearance for the three POROS HIC resins, we used MockV's MVM MVP in high-throughput screening (HTS) format. Binding was tested under increasing concentrations of sodium citrate (Figure 6).

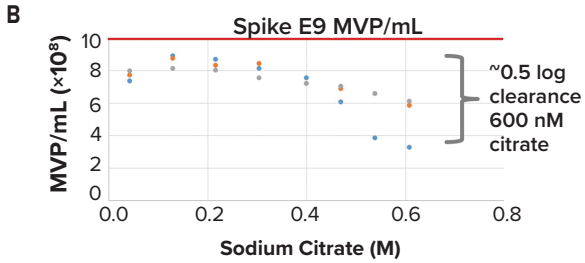
Clearance of the MVP on all three HIC resins was <0.5 log, even under the highest salt concentrations (600 mM sodium citrate for MAb A). Overall, our observations point to the low hydrophobicity of MVP and (by extension) MVM — a finding supported by Johnson et al. (**8**). Our finding also is consistent with our live viral clearance observations above in which minimal binding of MVM was detected for POROS Benzyl Ultra resin in FT mode polish of MAb A.

Similarly, for the POROS Benzyl BE operation, negligible MVM binding was observed during MAb A loading at 600 mM citrate. Taking advantage of this result, the addition of a high-salt chase in the BE process could improve the MVM clearance achieved in the elution pool. We also performed parallel column studies using MVP and the optimized POROS HIC processes for MAb A (Figure 7). We showed that for both Benzyl Ultra FT and Benzyl BE processes, MVP clearance mirrored MVM clearance. Taken together, our results highlight the utility of MockV's MVM MVP kit as a process development and prediction tool for live MVM clearance.

Figure 6: MVP mimics MVM, with transmission electron microscopy (A). High-throughput screening of MVP binding to POROS Ethyl, Benzyl, and Benzyl Ultra resins under increasing sodium citrate concentrations (B) for a load spike of 1 × 10^9 MVP/mL MVP did not bind to any of the HIC resins under all conditions tested.



MVP biochemically and physically resemble MVM
Assay = immuno-qPCR readout MVP are BSL-1 safe (empty)

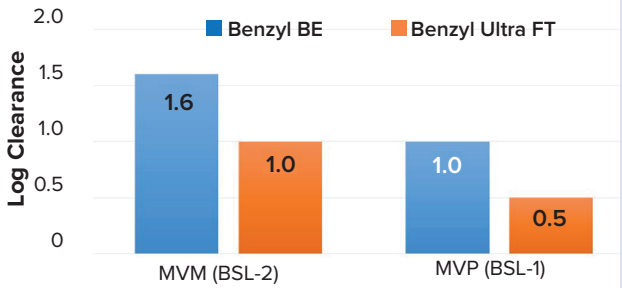


MVP does not significantly bind to POROS HIC resins, consistent with lack of MVM binding to POROS HIC resins

Discussion

HIC is an excellent tool for the downstream purification of biotherapeutics. Thermo Fisher Scientfic has designed a family

Figure 7: MVM and MVP clearance for MAb A BE and FT processes on POROS HIC resins; MVM was spiked as a single virus, and clearance was measured by infectivity assay. MVP was spiked as a single agent and tracked by immuno-qPCR using the MockV analytical kit. MockV MVP clearance at mirrored MVM live virus clearance for all processes tested.



MVP clearance is comparable to MVM clearance in both MAb A FT and bind–elute polish processes.

of POROS HIC resins to confer high selectivity for impurity removal. They are highly efficient for aggregate polish and offer orthogonal HCP and viral clearance capabilities. Here we showed highly productive POROS HIC polishing processes in both BE and FT modes that outcompeted legacy clinical processes designed for two highly challenging case studies (MAbs A and B).

We demonstrated complete XmuLV and partial MVM viral clearance for both MAb A and MAb B processes. Finally, we used MockV Solutions MVM MVP viral surrogate to better understand MVM virus and HIC interactions. Because these MVPs physiochemically resemble live MVM used for spiking studies, we also presented data highlighting the utility of MVP for predicting MVM clearance both in high-throughput screening and column studies using optimized POROS HIC polish processes. As predicted, the clearance of MockV MVPs closely paralleled MVM clearance in all POROS HIC studies. In conclusion, POROS HIC is a highly productive and platform-ready purification step that confer orthogonal selectivity for aggregate and impurity removal as well as viral clearance potential.

Acknowledgments

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References

1 Welsh J, et al. A Practical Strategy for Using Miniature Chromatography Columns in a Standardized High-Throughput Workflow for Purification Development of Monoclonal Antibodies. *Biotechnol Prog.* 30(3) 2014: 626–635; doi:10.1002/btpr.1905.

2 Kang K, et al. Development of an Acidic/Neutral Antibody Flow-Through Polishing Step Using Salt Tolerant Anion Exchange Chromatography. *Pharm. Bioprocess.* 3(8) 2015: 477–487.

3 Li JJ, et al. Improving Aggregate Removal to Enhance Clinical Manufacturing of MAbs. *BioPharm Int.* 28 September 2018.

4 Aranha-Creado H, Brandwein H. Application of Bacteriophages as Surrogates for Mammalian Viruses: A Case for Use in Filter Validation Based on Precedents and Current Practices in Medical and Environmental Virology. *PDA J. Pharm. Sci. Technol.* 53(2) 1999: 75–82.

MVM Mock Virus Particle (MVP) Kit

Commercial kits for analyzing the removal of impurities such as host cell proteins and residual host DNA are used routinely to ensure a quality-by-design (QbD) approach to biologics manufacturing. The information amassed from these types of kits feeds decision-making processes throughout the evolution of a downstream purification process — from early stage process development to late-stage commercial manufacturing. However, today's analytical toolbox lacks an easy-to-implement kit that can provide information about process capability for viral clearance. That is mainly because viral clearance studies require propagation of live mammalian viruses. The cost, time, logistics, and environmental challenges demand specialized laboratories, personnel, and expertise for such studies, leading many drug developers to outsource them or postpone that work until late in process validation or just before filing a biologics license application (BLA).

Viral surrogates such as bacteriophages have been used for over a decade in virus filtration applications because they do a satisfactory job at mimicking the size and physical properties of mammalian viruses (**4**). However, they fail to mimic the overall physiochemical and surface properties of the live mammalian viruses used in viral clearance spiking studies. Moreover, off-the-shelf quantification assays and consistent sources of bacteriophage spiking material are not readily available in standardized kit formats.

MockV Solutions commercialized the MVM mock virus particle (MVP) kit, which uses an immuno-qPCR assay to quantify a noninfectious MVM-surrogate viral particle. These MVPs have similar morphology and physiochemical characteristics to MVM (**4**). The ability of MVM MVPs to predict MVM clearance has been studied for virus filters (**5**) and ion-exchange chromatography (**6**). POROS AEX resins have shown superior salt tolerance in FT mode retention of MVP for a viral vaccine polish application (**7**).

5 Cetlin D, et al. Use of a Noninfectious Surrogate to Predict Minute Virus of Mice Removal During Nanofiltration. *Biotechnol. Progress* 34(5) 2018: 1213–1220.

6 Herbig K, et al. Modeling Virus Clearance: Use of a Noninfectious Surrogate of Mouse Minute Virus As a Tool for Evaluating an Anion-Exchange Chromatography Method. BioProcess Int. 17(5) 2019: 34–41.

7 Clearing the Way for Viral Clearance. *BioProcess Int.* Ask the Expert Webinar; https://bioprocessintl.com/sponsored-content/clearing-the-way-for-viral-clearance.

8 Johnson S, et al. A Step-Wise Framework to Design a Chromatography-Based Hydrophobicity Assay for Viral Particles. *J. Chromatog. B* 2017; doi:10.1016/j.jchromb.2017.08.002.

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This article is based on an "Ask the Expert" webinar for BioProcess International. *Access the entire presentation at https://bioprocessintl.com/sponsored-content/clearing-the-way-for-viral-clearance.*

Efficient Removal of MAb Aggregates

Using Flow-Through Hydrophobic-Interaction Chromatography

Moira Lynch

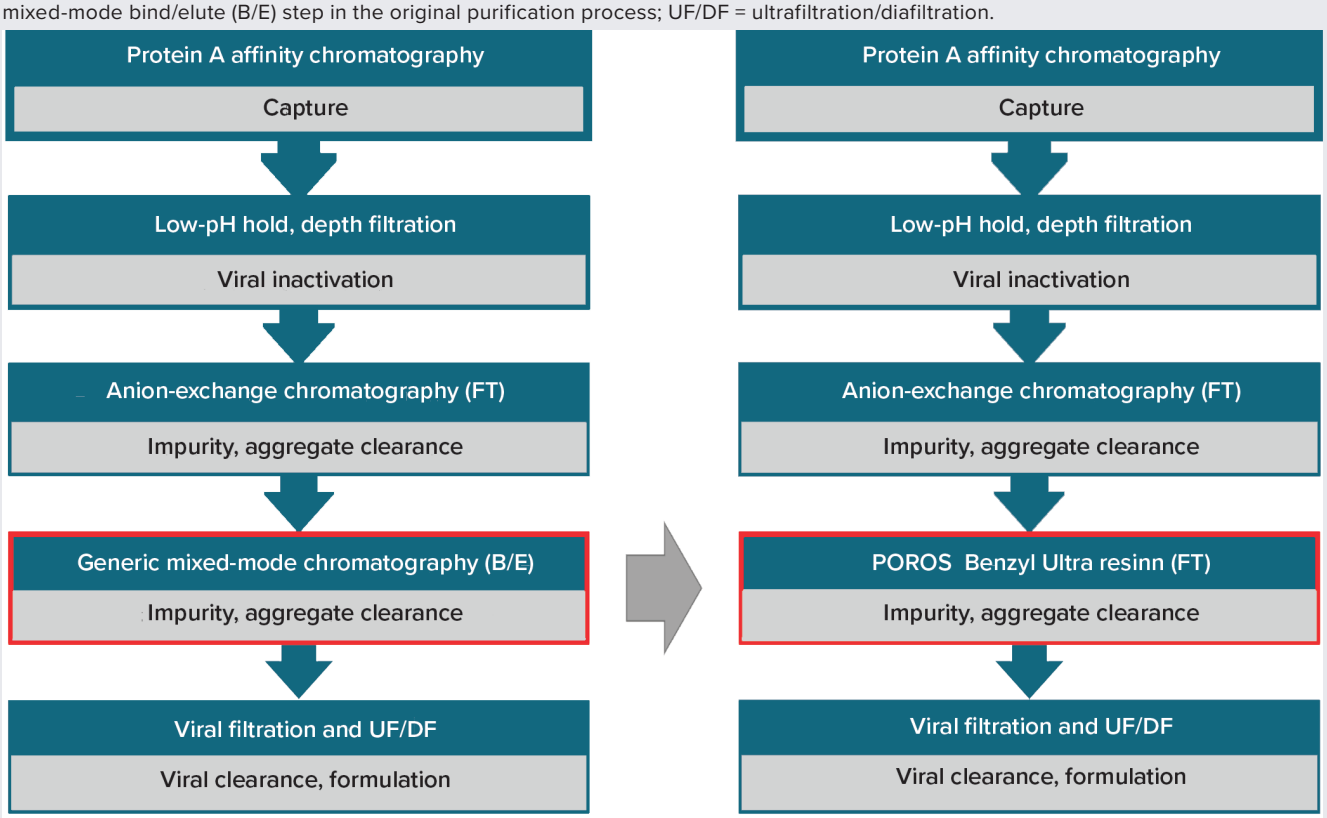
Because levels of aggregates can compromise the biological activity of a biopharmaceutical, monitoring those levels in a final MAb therapeutic is critical (**1**). In general, the lowest possible concentration of aggregates is desired in a final MAb formulation, typically less than 1% (**2**). Hydrophobic-interaction chromatography (HIC) can be used as to remove aggregates in downstream MAb processing. Because the selectivity of HIC can be affected by factors such as 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 used as the first polishing step in MAb purification following affinity chromatography. Flow-through chromatography is a mode of operation that offers the benefit of increased productivity and throughput; however, it requires the resin chemistry and properties to be highly selective toward impurities to be removed. Because AEX chromatography in flow-through mode is not highly selective toward MAb aggregates, the process generally requires an additional polish step involving orthogonal chemistry in bind/elute mode. A final polishing step in flow-through mode would be beneficial to the 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 high molecular weight (HMW) aggregates after the HIC FT step. The data show that the POROS Benzyl Ultra resin can be used for efficient aggregate removal in the downstream process of therapeutic MAb production.

Figure 1: Comparison of (left) the original purification process with (right) the newly optimized process. The goal of this study was to design a more simple and cost-effective polish step using 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.



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) × 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 of more than 10 column volumes (CV) was performed, at 300 cm/hr, with the high-salt equilibration buffer and gradually moving to a Tris buffer (pH 7.5) containing no salt.

High-Throughput Screening for Flow-Through Mode: To explore the critical parameters affecting resin selectivity toward 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, and 200 mM NaCl isocratic elution, for 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 three minutes. 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.

The following conditions were 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 (KP) 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 optimize the process further:

- flow rate 300 cm/hr, residence time 2 minutes, load density up to 350 g/L
- flow rate 800 cm/hr, residence time 45 seconds, 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 (Figure 3).

Materials

The MAb-A antibody is 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
- POROS Benzyl Ultra HIC resin, Thermo Fisher Scientific
- Fisherbrand 96-Well DeepWell polypropylene microplates, Fisher Scientific
- Thermo Scientific Nunc MicroWell 96-well optical-bottom plates with polymer base, Thermo Fisher Scientific
- Thermo Scientific MAbPac SEC-1 size-exclusion LC columns, Thermo Fisher Scientific
- Sodium chloride (MW 58.44), Fisher bioreagents
- Sodium acetate trihydrate (MW 136.08), Fisher Chemical
- Ammonium sulfate (MW 132.14), Fisher Chemical
- Sodium citrate dihydrate (MW 294.1), Fisher Chemical

Equipment and Software

- Thermo Scientific Versette automated liquid landler, Thermo Fisher Scientific
- Thermo Scientific Finnpipette Novus electronic multichannel pipette, Thermo Fisher Scientific
- Thermo Scientific Varioskan LUX multimode microplate reader, Thermo Fisher Scientific
- Thermo Scientific Sorvall Legend XT/XF centrifuge series, Thermo Fisher Scientific
- Thermo Scientific Ultimate 3000 standard dual system, Thermo Fisher Scientific
- Thermo Scientific HyperSep universal vacuum manifold, Thermo Fisher Scientific
- Thermo Scientific Pharma KingFisher Flex 96 deep-well magnetic particle processor, Thermo Fisher Scientific
- Applied Biosystems 7500 real-time PCR system, Thermo Fisher Scientific
- Applied Biosystems ProteinSEQ CHO HCP quantitation kit, Thermo Fisher Scientific
- JMP Pro predictive analytics software, JMP Statistical Discovery
- ÄKTA™ Pure Chromatography System, GE Healthcare Life Sciences

Figure 2: Breakdown of steps for process optimization.

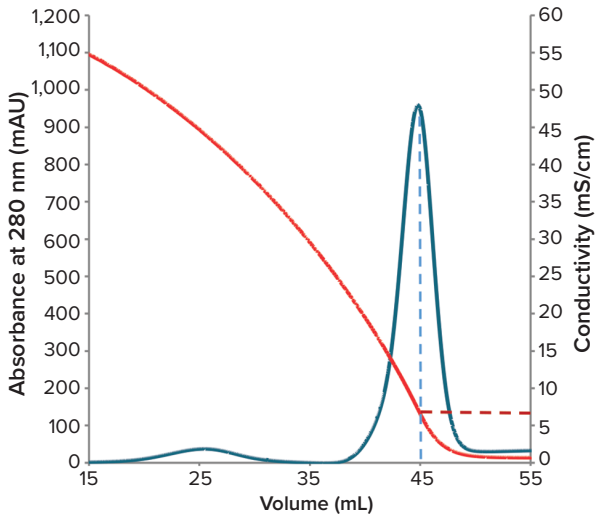
Figure 3: The general workflow for aggregate removal consists of filter-plate screening followed by A_{280} concentration determination and purity analysis by HPLC-SEC; statistical analysis is used to generate trends and to select process conditions.

Table 1: Comparison of 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 quantitative assay, LLoQ <0.2 ng/mL

Figure 4: In an example screening chromatogram to obtain the ideal low-salt condition for a flow-through steps, process screening for a monoclonal antibody used 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 optimized further in flow-through mode under low-salt conditions starting at 7 mS/cm; dashed lines = the maximum of the elution peak corresponding to a salt conductivity of 7 mS/cm.



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) × 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.

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 required to remove impurities but also to allow a 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).

High-Throughput Screening for Flow-Through Mode — Resin Selection: High-throughput screening was conducted to determine the critical parameters affecting resin selectivity toward aggregate removal in the flow-through mode. Figure 5 shows the aggregate removal by total mass and the selectivity factor of both resins.

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 does 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).

Althouh a high-throughput model for static binding of a MAb at low protein load is representative of selectivity, recoveries are not indicative of a dynamic process at a high protein load. Because its high aggregate selectivity combined with bind/elute data 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 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.

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). Aggregate levels in the collected fractions during the breakthrough analysis were analyzed by HPLC-SEC. 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

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 (KP) (**3**). A higher selectivity factor indicates stronger aggregate binding compared to that of the monomers.

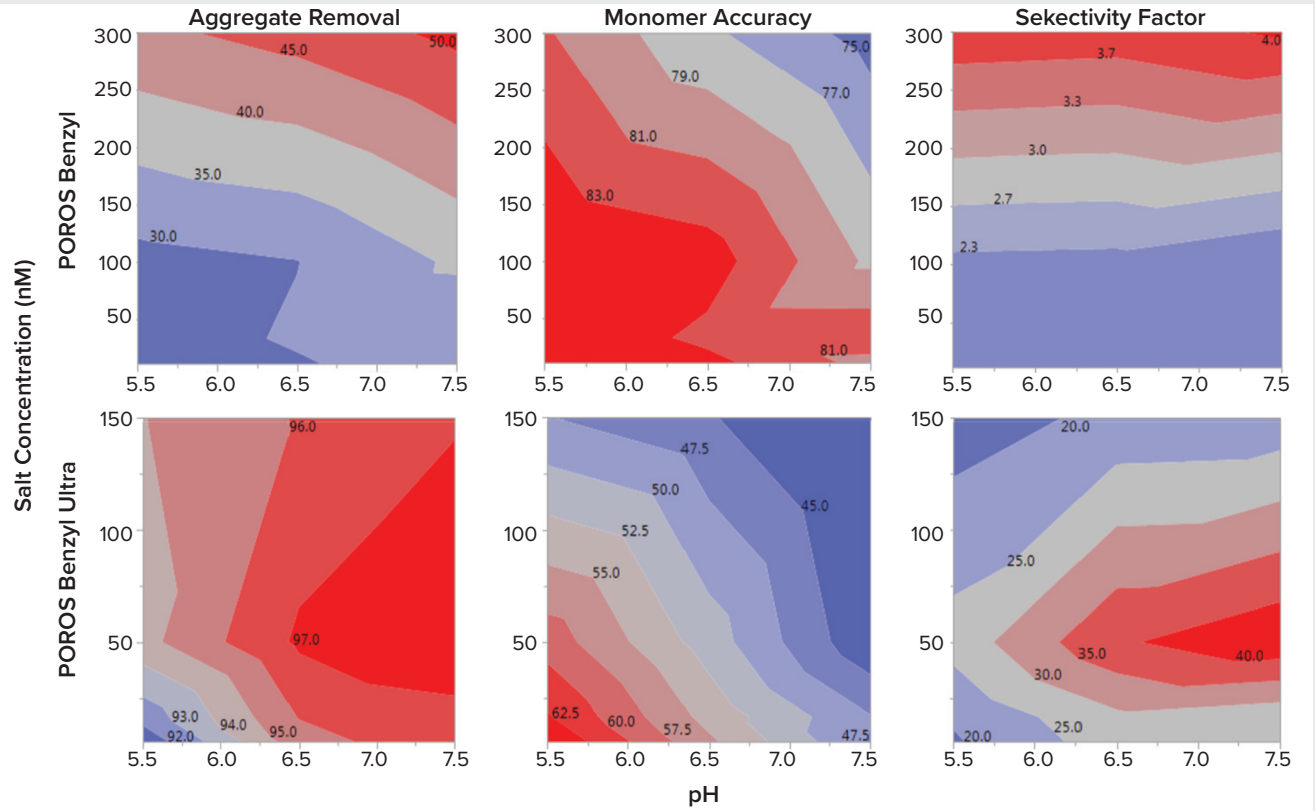


Figure 6: Amalgamated contour plot showing aggregate removal as a function of salt type, salt concentration, and pH. Sodium citrate at pH 6.8 and conductivity of 1.8 mS/cm (5 mM) was chosen for column scale-up.

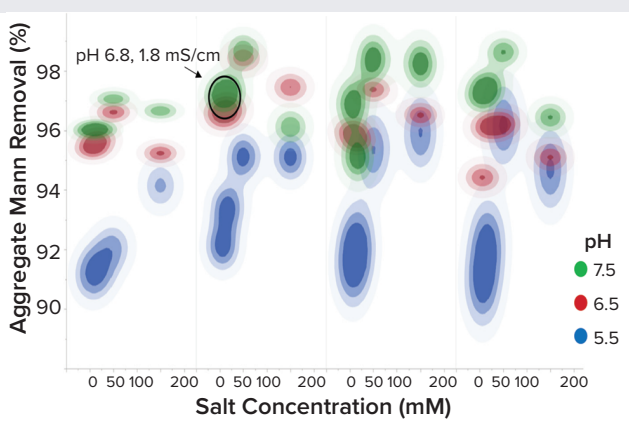


Figure 7: Breakthrough analysis of the column scale-down model run at a flow rate of 300 cm/hr (load density up to 350 g/L resin); monomer purity ad recovery was analyzed by HPLC SEC from 15 mL fractions. Monomer purity remained above 95% up to the maximum load density.

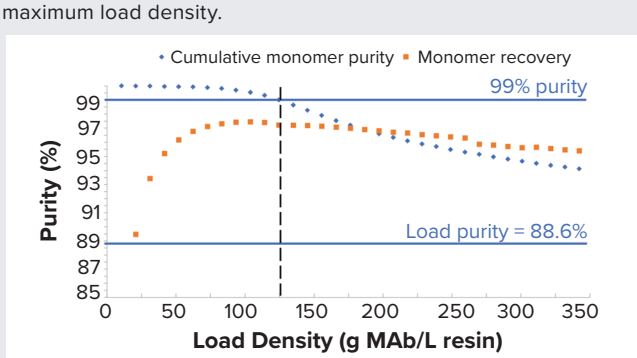


Table 2: Cost model to illustrate time and cost savings from improved process efficiency and productivity

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	26	3	67	794.5	5.8	$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

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.

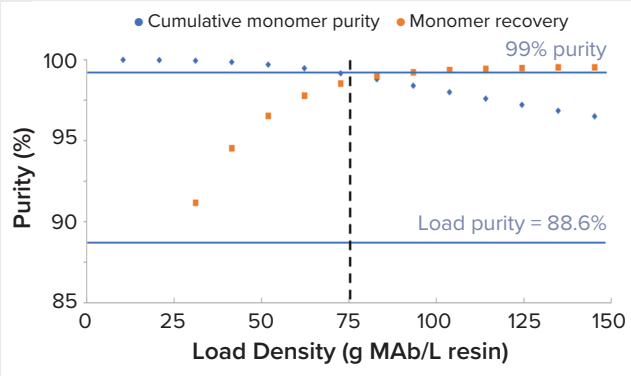
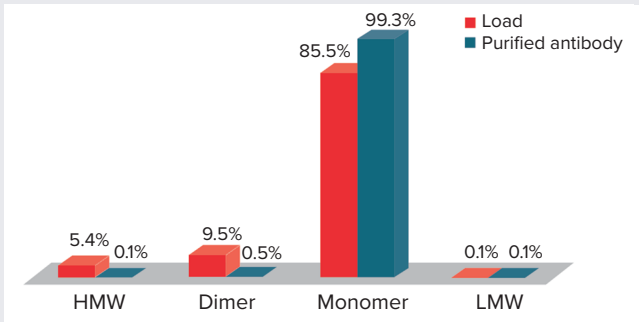


Figure 9: Poros Benzyl Ultra flow-through resun verification results; efficient removal of HMW aggregates and dimers was demonstrated using a load density of 80/g/L.



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 that 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

Optimizing Process Scale-Up and Productivity

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 demonstrate a more efficient and cost-effective process by performing HIC using the POROS Benzyl Ultra resin in flow-through mode, compared with the original mixed-mode bind/elute chromatography step (Table 1).

Results showed

- significant reduction of MAb HMW aggregates and dimers, achieved under low-conductivity conditions
- A five-fold residence time improvement (from 6 to 1.2 minutes)
- >99% purity and 98% monomer recovery at a load density three 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, improving process efficiency and productivity. The study further demonstrates that POROS HIC resins can be used 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. With 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.

References

1 Moussa EM, et al. Immunogenicity of Therapeutic Protein Aggregates. *J Pharm. Sci.* 105(2) 2016: 417–430; https://doi.org/10.1016/j.xphs.2015.11.002.

2 Vazquez-Rey M, Lang DA. Aggregates in Monoclonal Antibody Manufacturing Processes. *Biotechnol. Bioeng.* 108(7) 2011: 1494–1508; https://doi.org/10.1002/bit.23155.

3 Kramarczyk JF, Kelley BD, and Coffman JL. High-Throughput Screening of Chromatographic Separations: II, Hydrophobic Interaction. *Biotechnol. Bioeng.* 100(4), 2008: 707–720; https://doi.org/10.1002/bit.21907.

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EFFICIENT MONOCLONAL ANTIBODY AGGREGATE REMOVAL BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

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Thermo Fisher SCIENTIFIC

Bioprocessing

INTRODUCTION

This case study shows the optimization of POROS™ HIC resin-use in high-throughput screening (HTS) and subsequently upscaling in both Bind-Elute (B/E) and Flow-Through (FT) mode. Our study shows that a well-designed process together with a robust resin are key to a successful and efficient mAb polishing process.

GOAL OF THE STUDY

Design a more efficient, robust and cost-effective polish step utilizing POROS™ HIC resins as an alternative to the mixed-mode step in the original purification process of a clinical mAb containing >12% aggregates.

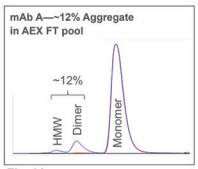


Fig. 1 A. Size Exclusion Chromatogram of mAb A, a clinical mAb containing >12% aggregates.

Fig. 1A

Fig. 1 B. Original purification process of mAb A

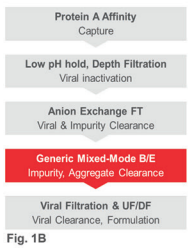
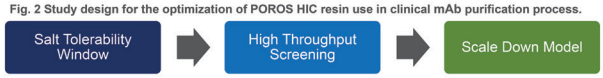


Fig. 1B

Fig. 2 Study design for the optimization of POROS HIC resin use in clinical mAb purification process.



HIGH TRHOUGHPUT SCREENING – RESIN SELECTION FOR SCALE DOWN

Screening variables used to predict conditions for scale down model (FT & B/E):
- resin type
- salt type
- salt concentration

Partition selectivity ratio is used to determine to level of separation.

Resin type	POROS Ethyl				POROS Benzyl				POROS Benzyl Ultra			
Salt type	Sodium Chloride	Sodium Acetate	Ammonium Sulfate	Sodium Citrate	Sodium Chloride	Sodium Acetate	Ammonium Sulfate	Sodium Citrate	Sodium Chloride	Sodium Acetate	Ammonium Sulfate	Sodium Citrate
Partition Selectivity				2.43		1.84						
				3.12	2.74	2.18						
			2.48	4.19	2.59	2.41		2.35	2.38	2.07		
					2.84	2.52	2.70	2.63	2.20	2.27	2.03	
					2.43		2.66	3.53	2.45	3.12	2.02	2.19
							2.54	3.79	2.39	3.73	2.55	2.68
									3.12	3.46	4.34	3.76
									5.36	4.23	7.17	8.16

Salt

Fig. 3 Overview HTS results; aggregate partitioning as a function of resin type, salt and salt concentration. Highly selective monomer/aggregates partitioning observed at low to no salt conditions (red boxes)

Higher the number = greater separation

- ✓ **POROS Benzyl chosen for Bind-Elute optimization**
- ✓ **POROS Benzyl Ultra chosen for Flow-Through optimization**

POROS HIC RESINS

- ✓ **Designed for use with lower salt concentrations**
- ✓ **Differentiated selectivity and ligand chemistry**
- ✓ **Novel 50 µm base bead**
- ✓ **Improved recovery, resolution and capacity**
- ✓ **Superior pressure-flow characteristics**
- ✓ **Consistent lot-to-lot performance**
- ✓ **Robust chemical and base stability**

OPTIMIZATION SCALE DOWN MODEL – POROS BENZYL IN BIND / ELUTE MODE

DYNAMIC BINDING CAPACITY AT 1% BREAKTHROUGH

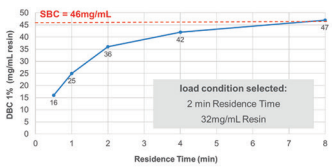


Fig. 4 DBC at 1% breakthrough as function of residence time. Maximum static capacity is reached at ~5min. In order to obtain a faster loading process than the original process, the final load conditions selected were: 32 mg/mL at 2 min residence time.

ELUTION OPTIMIZATION

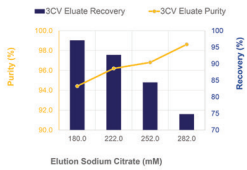
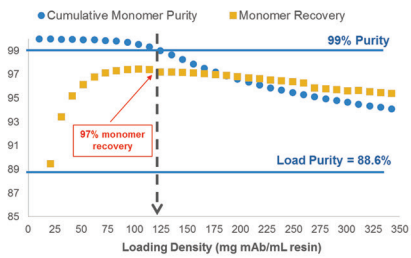


Fig. 5 Elution optimization results. Optimal recovery and purity as function of salt concentration and column volumes.

- ✓ **High Dynamic Binding Capacity at short residence time**
- ✓ **High recovery and purity from a fast elution profile**

OPTIMIZATION SCALE DOWN MODEL – POROS BENZYL ULTRA IN FLOWTHROUGH MODE



Study Format

- 0.8cmx10cmL (CV 5mL)
- **Load 350 mg/mL resin**
- Flow rate: 300 cm/hr
- 2 min residence time
- Load monomer 89%
- 2 mS/cm conductivity
- pH6.8 chosen based on optimized pH of previous AEX polish step

Fig. 6 Flow through purification using Benzyl Ultra. Along the loading step fractions were collected and analyzed for monomer recovery and purity. No additional buffer manipulation was needed after the AEX step.

- ✓ **Aggregate breakthrough after 125mg/mL resin loading**
- ✓ **Complete Aggregate clearance achieved with POROS Benzyl Ultra at low salt and flexible pH**

RESULTS SUMMARY AND CONCLUSIONS

Process Summary	Mixed-Mode (Clinical Process)	POROS Benzyl Bind-Elute Mode	POROS Benzyl Ultra Flow-through Mode
Load Monomer Purity (%)	90	89	85.5
Load Density (g/L resin)	25	32	100
Monomer Purity Pool (%)	99	99	>99
Monomer Recovery (%)	90	>99	98
HCP (ppm)	NA	120 to 12ppm	100 to 35 ppm
Residence time (min)	6	2	1.2
Pool Volume (50-50mAu)	5CV	4CV	NA
Productivity (g/L/hr)	7	27	89

POROS HIC resins drastically improve mAb A polishing step:
- ✓ **Increased load density**
- ✓ **Improved monomer recovery**
- ✓ **Shorter residence time**
- ✓ **4-12 times higher process step efficiency**