NOVEL HYDROPHOBIC INTERACTION CHROMATOGRAPHY RESINS FOR NEXT GENERATION BIOTHERAPEUTIC CHALLENGES

Easier implementation of hydrophobic interaction chromatography in your downstream process

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Designed to solve complex downstream separation and purification challenges for many biotherapeutic modalities, a suite of hydrophobic chromatography resins (POROS HIC) has been developed that cover a wide range of hydrophobicity. Suitable for bind-elute and flow through applications the resins can be utilized at lower salt concentrations and overcome the typical limitations associated with current HIC separations such as resolution, capacity and product recovery. Using the POROS resins, a HIC step can be easily implemented resulting in increased yields and reduced processing times through effective and efficient removal of impurities.

INTRODUCTION

Advances in mAbs and other biotherapeutics are generating a wider range of biomolecules. However, with this molecular diversity, unique separation and purification challenges arise that are often difficult to overcome. Typical product- and process-related impurities include nucleic acids (DNA, RNA), host cell proteins, aggregates and glycosylation and charge variants as well as potential viral contamination. These impurities can prove problematic to separate, but need to be cleared to acceptable levels in accordance to guidelines established by regulatory agencies. Since several polishing steps are usually required to reduce the levels of impurities, process developers are constantly looking to expand their repertoire of purification tools. While ion-exchange resins are often examined as a first choice to accomplish these separations, hydrophobic interaction chromatography (HIC) offers a powerful alternative that has now become easier to implement with the recent arrival of new resin formats.

Traditionally, the use of HIC in biomolecule purification processes has presented challenges due to the properties of the resins. Classic HIC resins often require high molarity kosmotropic salt buffers for optimal resin capacity and to promote target molecule binding. Elution is then enabled by decreasing salt concentrations. However, these kosmotropic salts are associated with concerns regarding product stability, hardware corrosion, as well as proper waste disposal at manufacturing. Additionally, compared to ion-exchange resins, resolution and binding capacity is in generally lower. The narrow range of hydrophobicity covered by classic HIC resins also reduces the selectivity available toward the specific characteristics of the target molecule and decreases the chance of being able to purify difficult molecules.

To allow the advantages of HIC to be realized without the compromises that classic HIC resins bring, a new set of HIC resins has been developed.

DIFFERENTIATED PROPERTIES OF HIC RESINS

The three novel HIC resins (POROS Ethyl, POROS Benzyl and POROS Benzyl Ultra) are based on the 50 μm POROS polystyrene divinylbenzene base bead that was designed utilizing a novel coating procedure to enable functionalization with the unique hydrophobic ligands, thereby creating a portfolio of resins covering a very broad range of hydrophobicity. This wide and varying range of hydrophobicity enables unique selectivity towards the target molecule and provide flexibility around the process operating conditions.

These resins are suitable for bind-elute and flow through applications at lower salt concentrations than classical HIC resins and in addition have higher capacity, superior resolution and a broad range of hydrophobicity to enable differentiating selectivity for a variety of biomolecules. Resin performance is independent of flow rate thereby supporting higher productivity and process intensification.

SUPPORTING PROCESS INTENSIFICATION

POROS HIC resins have a high capacity for a range of molecules, which results in reduced column size, smaller footprint as well as reducing the process volume significantly for subsequent steps. Dynamic binding capacity and resolution are not impacted by flow rate and thus columns can be run faster while maintaining excellent separation of proteins without compromising capacity (**Figure 1**).

INCREASING PROCESS FLEXIBILITY

POROS HIC resins were designed for high performance in a variety of buffered salts and salt concentrations. Loading salt conditions can be optimized based on purification needs while maintaining the resolving char-

Figure 1A – Dynamic binding capacity of POROS Benzyl measured at 10% breakthrough
Experimental details: Protein sample IgG at 7 mg/mL. Load buffer, 1 M NaCl, 50 mM sodium phosphate, pH 7
Column format: 0.5 cmD x 5 cmL

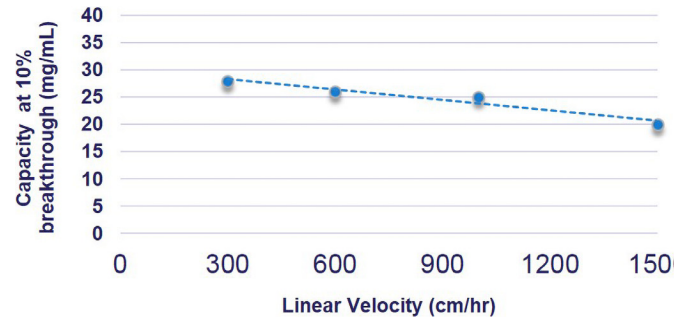


Figure 1B – Capacity comparison with competitor resins
Experimental details: Protein sample 1.5 mg/mL lysozyme; Buffer: 1.5 M ammonium sulfate, 50 mM sodium phosphate pH 7.0; Linear velocity: 300 cm/hr
Column format: 0.66 cmD x 20 cmL; Protein loaded until breakthrough, measurement taken at 5% breakthrough

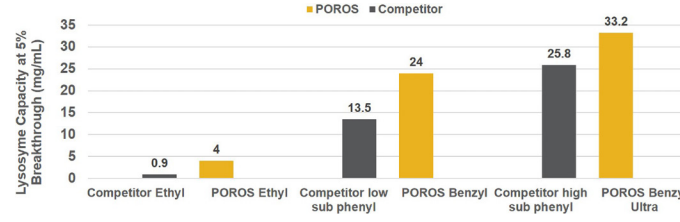
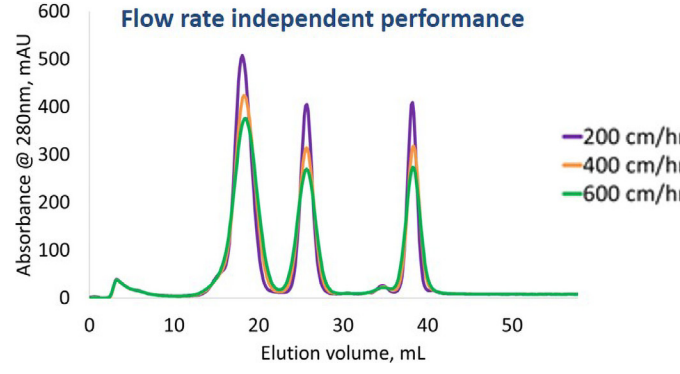


Figure 1C – Separation comparison of POROS Benzyl at different flow rates
Experimental details: Protein mixture: ribonuclease A, lysozyme, chymotrypsin, chymotrypsinogen A
Load buffer: 1.7 M ammonium sulfate in 50 mM sodium phosphate, pH 7, Buffer gradient: Load buffer to 50 mM sodium phosphate, pH 7 in 10 CVs Format: 0.46 cm D x 20 cm L; Flow rate: 200, 400, 600 cm/hr



acteristics of the resin. Using HIC Benzyl Ultra resin in bind-elute mode, **Figure 2** shows the separation of four proteins in three different salt concentrations (gradient slope constant). These data show that even at low salt concentrations, excellent resolution is maintained. The ability to use HIC resins in lower salt or weaker kosmotropic salts (i.e., low conductivity buffers increases process flexibility).

This article will illustrate the properties of POROS HIC resins with the use of three case studies, demonstrating how the resins can be optimized and utilized to solve process developer's challenges for biotherapeutic polishing. These case studies include a mAb with a high level of aggregates, an enzyme with a dominant process-related impurity and an antibody–drug conjugate (ADC) with the correct drug-antibody ratio (DAR) from a heterogeneous mixture of other conjugates.

Figure 2 – Separation comparison in different salt concentrations
Experimental details: Protein mixture - ribonuclease A, lysozyme, chymotrypsin and chymotrypsinogen A; Eluent A: 1.7M, 1.0M or 0.5M ammonium sulfate/50 mM sodium phosphate pH 7.0; Eluent B: 50 mM sodium phosphate pH 7.0; Gradient: 0 to 100% B at 0.17M ammonium sulfate change per CV; Linear velocity: 100 cm/h; Detection: UV at 280 nm; Column format: 0.66 cmD x 20 cmL.

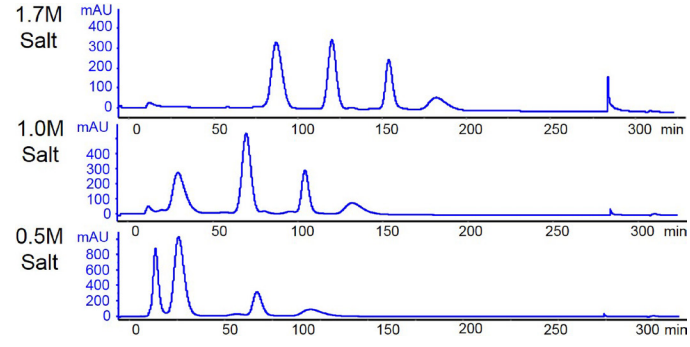
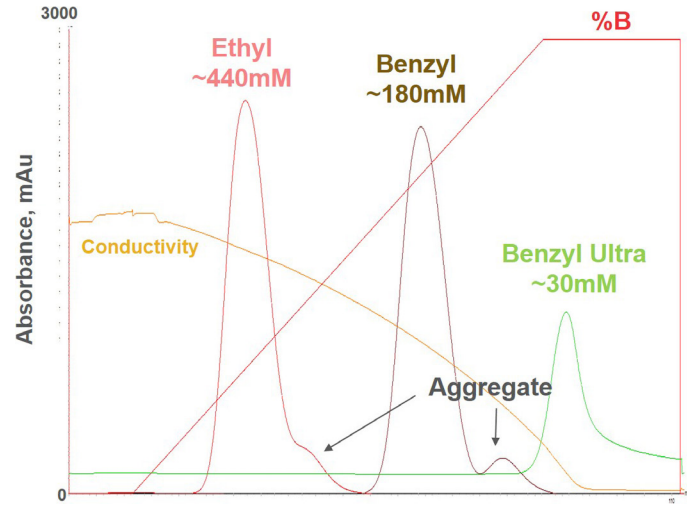


Figure 3 – Retention analysis of mAb-A with sodium citrate gradient elution
Column: 0.5 cmD x 5 cmL, CV=1 mL. Residence time: 2 min. Buffer A: 600 mM sodium citrate, tris-acetate pH 7.0; Buffer B: tris-acetate pH 6.9. Linear gradient: 15 CV.



CASE STUDY 1: CLINICAL mAb-A REQUIRING AN IMPROVED STRATEGY FOR AGGREGATE CLEARANCE

A critical quality attribute in the final formulation of mAbs is to maintain the aggregate levels at less than 1% of the final soluble product. From a downstream perspective, mAb-A is a clinical stage antibody that is challenging to work with as it has a high level of aggregates in the feed stock (>12%). The original purification process involved an initial protein-A affinity step followed by anion exchange in flow-through mode. However, a significant percentage of aggregates was still remaining in the flow through pool which resulted in the implementation of an additional second polishing step that utilized a mixed-mode chromatography was implemented in bind-elute mode. However, the second polishing step was deemed as being inefficient.

In a previous study, we showed how POROS Benzyl Ultra was optimized in flow through mode, under low conductivity conditions, to efficiently remove aggregates in the downstream process of mAb-A (1). In addition to the flow through study, the performance of POROS Benzyl was demonstrated in bind-elute mode as an efficient polish step for mAb-A. The general approach outlined can be used to solve similar downstream process purification challenges for other biomolecules.

OPTIMIZING THE BIND-ELUTE PROCESS

To determine the aggregate selectivity of the three POROS HIC resins in bind-elute mode, a retention study was performed using a gradient elution from high to low salt concentrations. Yield and purity were measured by collecting fractions across the eluting peaks and analyzing UV280 absorbance and HPLC-SEC. As shown, POROS Benzyl demonstrated the highest selectivity for mAb-A aggregates with aggregate shoulder peak showing almost baseline separation from the main monomer peak. POROS Ethyl was slightly less selective, while POROS Benzyl Ultra showed incomplete recovery. From these experiments, it was concluded that POROS Benzyl performed best, having the highest yield and purity (data not shown). Combined with the lower salt concentration needed, POROS Benzyl was selected as the best option for aggregate removal in bind-elute mode.

After the resin selection screening was completed, the bind-elute process was further optimized in a scale-down model, followed by a process verification run. In the scale-down model, load optimization and elution optimization was carried out. Dynamic binding capacity was determined at different residence times (**Figure 4**). The data shows that dynamic binding capacity approaches static binding capacity beyond a 5-minute residence time, reflecting the fast mass transfer properties of the POROS bead. The final load concentration and residence time chosen for the verification run are 32 mg/mL at 2 min residence time.

After the load optimization was completed, elution optimization was performed. By decreasing the salt concentration for elution, purity decreased and recovery increased (data not shown). In addition, recovery was also improved by increasing elution collection (CV). The final process verification run was performed at the combined optimum of all three process parameters (**Figure 5**).

Figure 4 – Load optimization: Dynamic Binding Capacity at 1% breakthrough
Loading buffer: 575 mM sodium citrate in tris-acetate pH 7

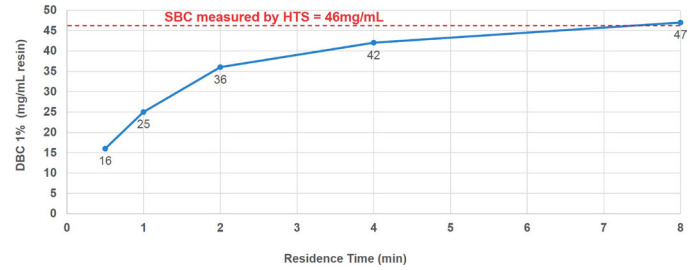
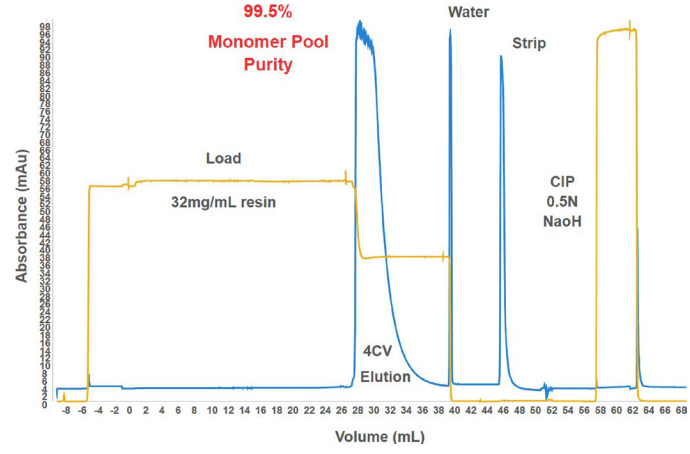


Figure 5 – Final verification run with POROS Benzyl Ultra in bind-elute mode
Experimental details: Load purity 88% monomer; Column format 0.8 mD x 10 cmL (CV = 5 mL); residence time 2 min; load density 32 mg/mL; load buffer 575 mM citrate, tris pH7; elution buffer 265 mM citrate pH 7; elution pool 4 CV.



SUMMARY RESULTS

Using the POROS HIC resins in flow through mode (1) as well as bind-elute mode, resulted in all the process performance metrics being improved and therefore a higher productivity process (i.e., higher loading, lower residence time, better monomer recovery, and smaller pool volume) (**Table I**). This equates to a 4–12 times higher process step productivity for the HIC processes compared to the clinical mixed mode chromatography process. The flow-through process has the added benefit that it can be directly coupled to the upstream anion exchange process. This avoids any hold ups between these steps as buffer manipulation is not required, and so it is possible to achieve a higher productivity, more intensified process.

Table I: Overall comparison of the two developed HIC processes (flow-through and bind-elute) compared to the "old" Mixed-Mode process used for the production of mAb-A.

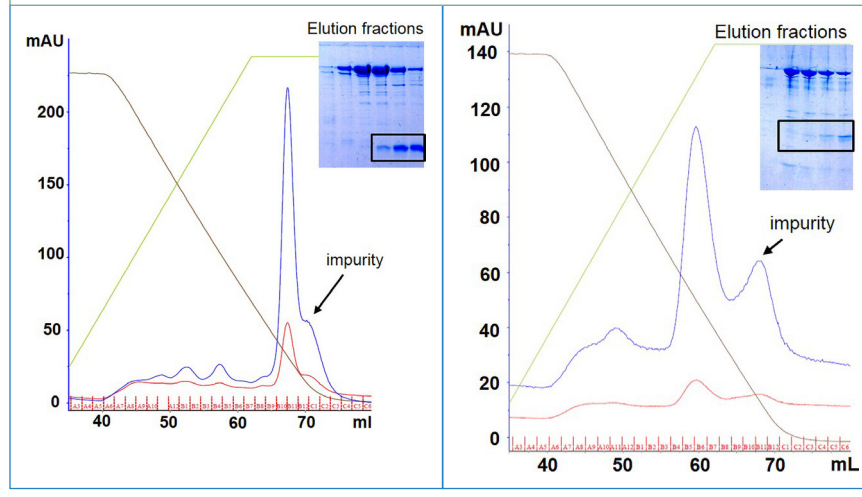
mAb-A Process	Generic Mixed-Mode (bind-elute)	POROS Benzyl (bind-elute)	POROS Benzyl Ultra* (flow through)
Load monomer purity (%)	90	88	85.5
Load density (g/L resin)	25	32	80–100
Monomer purity pool (%)	99	99	>99
Monomer recovery (%)	90	>99	98
Residence time (min)	6	2	1.2
Pool volume (50-50 mAu)	5 CV	4 CV	NA
Productivity (g/L/hr)	7	27	89

* http://www.biopharminternational.com/improving-aggregate-removal-enhance-clinical-maJ.J. Li, O. Terova, and J. De Rooij, BioPharm International, Sept. 28 2018, www.biopharminternational.com/improving-aggregate-removal-enhance-clinical-manufacturing-mabsnufacturing-mabs

CASE STUDY 2: THERAPEUTIC ENZYME REQUIRING SEPARATION FROM A DOMINANT PROCESS-RELATED IMPURITY

During the manufacture of a 94kD therapeutic enzyme a dominant process-related impurity needed to be removed at the final polishing step. In addition to this impurity, it was desirable to have the DNA removed during the same step. The goal of this study was to optimize the current polishing step (HIC), more specifically to improve resolution.

Figures 6A and 6B – Impurity removal with the butyl 34 µm HIC resin (left) and POROS Ethyl (right), demonstrating increased impurity clearance due to the superior resolution of the POROS resin.
Process details: ~7 mg total protein separated with a 5 CV gradient from buffered 1.5 M ammonium sulfate containing 2 mM EDTA pH 7.4 to buffer containing 2 mM EDTA pH 7.4; Detection: A280 & A254nm; Column format: 1.0cmD x 7.5cmL; Flow rate: 100 cm/hr (6A) and 230 cm/hr (6B)



A comparative study of two commercially available HIC resins and POROS Ethyl was carried out. The first HIC resins tested a 34 µm agarose bead with butyl and phenyl chemistry. In addition to these resins, POROS Ethyl was tested. All resins were used in flow through mode.

Under the same operating conditions, the butyl 34 µm HIC resin only demonstrated partial separation of the process-related impurity (**Figure 6A**). Further testing of this resin showed that product fractionation could be obtained, however this was only achievable at a low flow rate due to the limited flow properties of the bead. By contrast, the phenyl 34 µm HIC resin proved too hydrophobic for this application and did not result into separation of the enzyme from the impurity (data not shown).

In comparison to the other resins tested, the POROS Ethyl resin allowed fractionation of the product from the impurity at a high flow rate (4.5 min residence time) while maintaining satisfactory operating pressures (**Figure 6B**). DNA was also removed in the flow-through (data not shown).
In summary, high productivity was achieved with POROS Ethyl resulting from the superior resolution at 2.5x higher flow rates.

CASE STUDY 3:
ADC PURIFICATION–DAR SEPARATION

ADCs are emerging as a promising targeted therapies for the treatment of cancers.

However, developing purification processes for these therapeutics can often prove to be challenging when it comes to separating the molecules with the required DAR from the unconjugated antibody and those that have the incorrect ratio of drug to antibody.

In this initial screening study, two alternative HIC resins (phenyl and butyl chemistry) were compared with all three POROS HIC resins in bind-elute mode (**Figures 7A and 7B**). The crude material was purified using a salt gradient of 100% HIC buffer A to 100% HIC buffer B over 10 column volumes at 1mL/min. The alternative resins are high-performing resins known for their resolving capabilities, however due to small bead size, they are also known for poor flow properties when scaled up in manufacturing. DAR 1 (30%) is the target ADC while DAR 2 (1%) is the impurity. DAR 0 (69%) refers to the antibody without a conjugated drug.

As shown by the data outlined in **Figure 7A**, the alternative HIC resins are not able to separate the target ADC from the other conjugates. The butyl HIC resin (left) shows very poor resolution between the unconjugated antibody (DAR 0), DAR 1 and DAR 2, while the phenyl HIC resin (right) is showing no resolution at all.

Figure 7A – DAR separation by alternative HIC resins, showing little to no resolution
Experimental details: 1ml HiTrap™ (7x25 mm) column; HIC loading buffer: 15 µL HEPES (0.1 M), NaCl (0.1 M), pH 7.5 + 200 µL ammonium sulphate (3 M), HEPES (0.1 M), NaCl (0.1 M), pH = 7.5; HIC buffer A (ammonium sulphate (1 M), HEPES (0.1 M), NaCl (0.1 M), pH = 7.5); HIC buffer B (HEPES (0.1 M), NaCl (0.1 M), 20 % isopropyl alcohol v/v, pH = 7.5); flowrate 1 mL/min

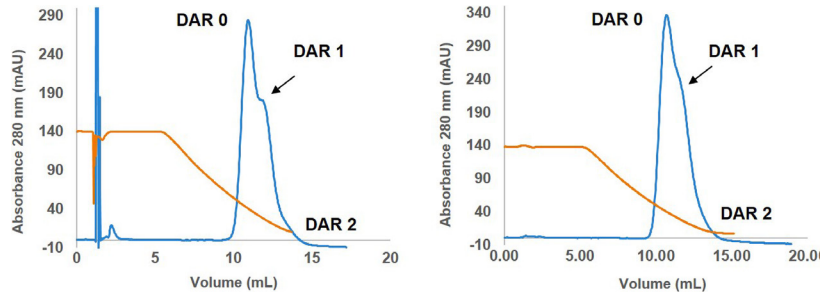
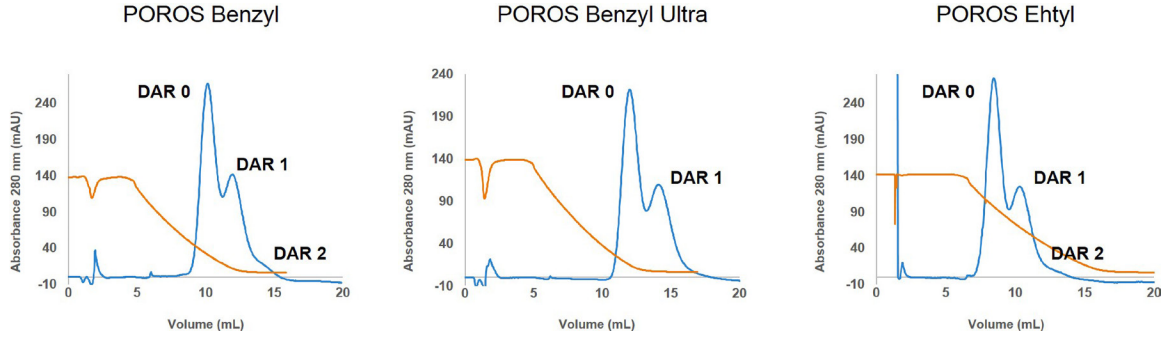


Figure 7B – DAR separation by POROS HIC resins, showing superior resolution over the butyl and phenyl HIC resins
Experimental details: Tricorn (5x50mm) column; HIC loading buffer: 15µL HEPES (0.1 M), NaCl (0.1 M), pH7.5 + 200 µL ammonium sulphate (3 M), HEPES (0.1 M), NaCl (0.1 M), pH = 7.5; HIC buffer A (ammonium sulphate (1 M), HEPES (0.1 M), NaCl (0.1 M), pH = 7.5); HIC buffer B (HEPES (0.1 M), NaCl (0.1 M), 20 % isopropyl alcohol v/v, pH = 7.5); 300 cm/hr (1 min residence time)



> "All case studies clearly show the excellent performance of POROS HIC resins and how they address the challenges process developers are facing when using HIC as a chromatography step in their purification platform."

In contrast to the butyl and phenyl resins tested, all three POROS resins are selective enough to reach separation, with ethyl being the strongest performing resin (**Figure 7B**). These initial but promising screening results could be optimized leading to adequate separation of the DAR species. This case study highlights the unique selectivity and high resolution of the POROS HIC resins in ADC DAR polishing.

CONCLUSION

All case studies clearly show the excellent performance of POROS HIC resins and how they address the challenges process developers are facing when using HIC as a chromatography step in their purification platform.

Through the differentiated selectivity of the resins, a wide range of polishing applications for diverse classes of biotherapeutic molecules is addressed. POROS HIC provide excellent resolution, capacity and product recovery, even at lower salt concentrations, offering greater process design flexibility. Using these resins, a HIC step can be easily implemented resulting in increased yields and reduced processing times through effective and efficient removal of impurities.

REFERENCES

1. J.J. Li, O. Terova, and J. De Rooij, *BioPharm International*, Sept. 28 2018, www.biopharminternational.com/improving-aggregate-removal-enhance-clinical-manufacturing-mabs