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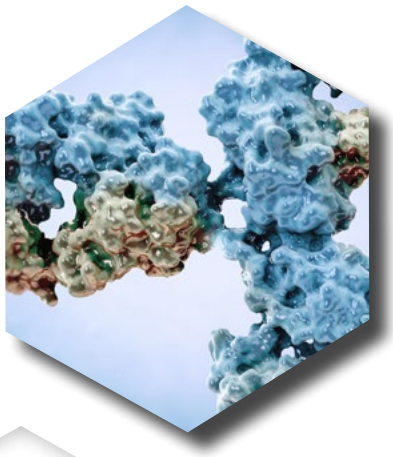
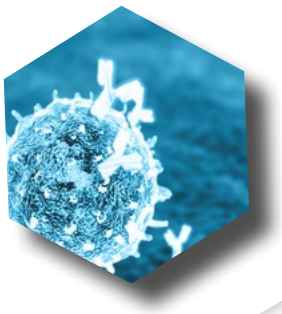
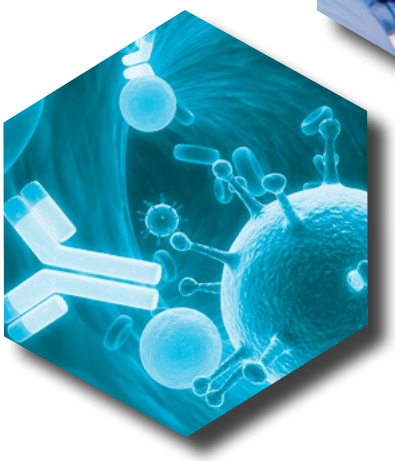
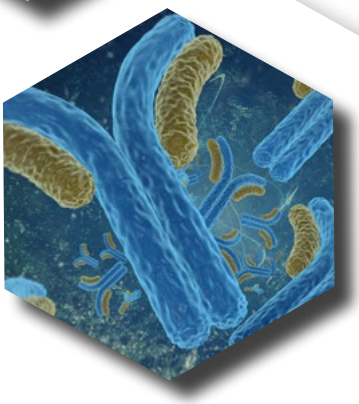


Novel Selectivities and Workflows to Streamline mAb Purification Processes







Novel Selectivities and Workflows to Streamline mAb Purification Processes — A Preview

The development of monoclonal antibody (mAb) technology over the past 25 years has fundamentally changed the questions we ask and led to many innovative discoveries. These highly specific biological products have significantly influenced the direction and progress of research and therapeutics. In research, mAbs are primarily produced and used to isolate, identify, and characterize a specific protein. On the therapeutic front, they have shown promise in treating diseases such as cancer, chronic inflammation, and infection. These applications make mAb purification one of the largest and fastest growing areas of the pharmaceutical industry.

Purification of mAbs relies heavily on column chromatography. However, not all chromatography resins are created equal. Since each resin works within a set range of technical parameters, mAb purification involves multiple consecutive steps using two or more resins. Frequently, these purification steps are referred to as capture, intermediate, and polish. The ideal purification strategy for each mAb is usually customized based on multiple criteria, including its final anticipated use and various purification challenges such as costs, harsh elution conditions, aggregate formation, maximization of monomer recovery, and the desired purity, among other things.

This book presents solutions that offer novel selectivities and approaches for mAb purification addressing the challenges in the quest to develop selective and robust purification processes. These workflows allow you to quickly find a path to the optimal design space with significantly less experimentation that will guide to simplifying method development.

CONTENTS

"Our commitment extends beyond the lab to more practical matters such as faster product delivery time and being as physically close to our customers as possible to serve them better."

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A Purification Strategy
for Clinical-Grade Monoclonal Antibody

Monoclonal antibodies (mAbs) are currently the most important class of therapeutic proteins. Advances in upstream process technologies have led to tremendous improvement of mAb titers in mammalian cell culture. Increases in fermentation volume and the protein mass produced has made the timely processing of harvested material extremely challenging. This is further compounded by the elevated levels of process- and/or product-related impurities resulting from prolonged fermentation and substantially higher cell density in the expression culture. Chromatographic media with high capacity and improved chromatographic and operational performance offer the latest productivity tools to address downstream process challenges. We have employed two next generation ultra high-capacity ion exchangers, Nuvia™ S and Nuvia Q Resins, and Nuvia™ cPrime™ Hydrophobic Cation Exchange Resin, to effectively purify a monoclonal antibody from Chinese hamster ovary (CHO) cell culture harvest. The results demonstrate that this three-step nonaffinity workflow can effectively deliver highly purified monoclonal antibodies with minimal feed conditioning.

CAPTURE

Table 1: Capture on High Capacity Cation Exchange Resin, Nuvia S Resin
Column: 1 ml, 0.56 x 4 cm

Step	Buffer	CV	Flow Rate
Equilibration	20 mM sodium acetate, 20 mM NaCl, pH 4.7 (Buffer A)	15	300 cm/h
Sample Loading	CHO cell culture supernatent diluted 1:4 with dH_2O; adjusted to pH 4.7 with 1 M phosphoric acid; clarified with 0.2 µm filter	–	300 cm/hr
Wash	Buffer A	15	300 cm/hr
Elution	20 mM sodium acetate, 200 mM NaCl, pH 4.9 (Buffer B)	15	300 cm/hr
Sanitization	1N NaOH	5	300 cm/hr

Eluate was adjusted to pH 7.0 with 1 N NaOH.

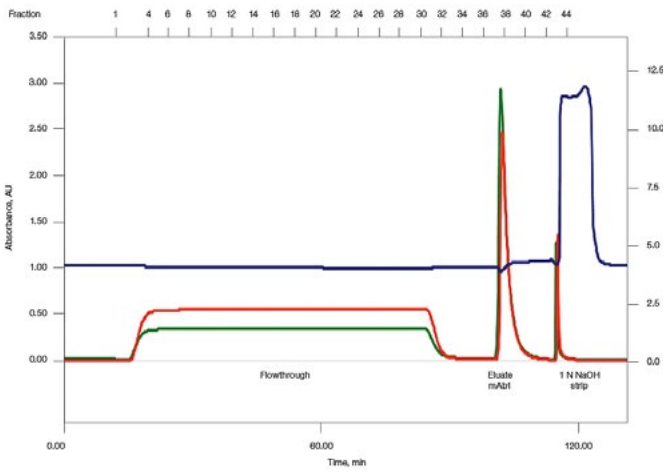


Figure 1: Capturing mAb1 from CHO cell culture harvest using Nuvia S Resin. mAb1 eluted from the column (fractions 38-40) was subjected to further purification and purity analysis. OD 260 (–);OD 260 (–); pH (–).

INTERMEDIATE POLISHING

Table 2: Intermediate Polishing on High Capacity Anion Exchange Resin, Nuvia Q Resin *Column: 1 ml, 0.56 x 4 cm*

Step	Buffer	CV	Flow Rate
Equilibration	10 mM sodium phosphate, 10 mM NaCl, pH 7.0 (Buffer A)	10	300 cm/h
Sample Loading	Eluate from Nuvia S Column adjusted to pH 7.0	–	300 cm/hr
Wash/ Flowthrough	Buffer A	25	300 cm/hr
Strip	100 mM sodium phosphate, 1.5 M NaCl, pH 7.2 (Buffer B)	10	300 cm/hr
Sanitization	1N NaOH	5	300 cm/hr

The mAb in the flowthrough was collected and the pooled fractions were adjusted to pH 5.0 with 100 mM sodium acetate, 500 mM NaCl, pH 4.5.

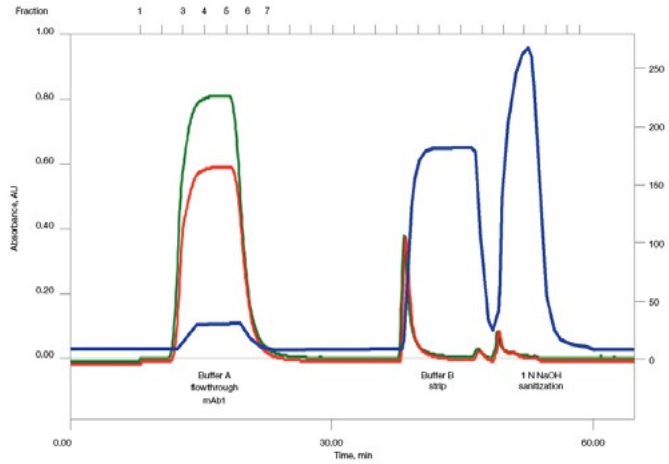


Figure 2: Intermediate polishing purification of mAb1 in flow-through mode using Nuvia Q Resin. mAb1 in column flowthrough (fractions 3-6) was subjected to further purification and purity analysis OD 260 (–);OD 260 (–); conductivity (–).

Table 3: Final Polishing on Hydrophobic Cation Exchange Resin, Nuvia cPrime *Column: 1 ml, 0.56 x 4 cm*

Step	Buffer	CV	Flow Rate
Equilibration	50 mM sodium acetate, 125 mM NaCl, pH 5.0 (Buffer A)	10	300 cm/h
Sample Loading	Pooled fractions from Nuvia Q Column adjusted to pH 5.0	–	300 cm/hr
Wash	Buffer A	15	300 cm/hr
Elution 1	Salt gradient formed between Buffer A and 50 mM sodium phosphate, 50 mM NaCl, pH 6.2 (Buffer B)	15	300 cm/hr
Elution 2	Buffer B	15	300 cm/hr
Strip	200 mM sodium phosphate, 700 mM NaCl, pH 7.5	10	300 cm/hr
Sanitization	1N NaOH	5	300 cm/hr

Method Development

A monoclonal antibody from Chinese hamster ovary (CHO) was purified using a workflow consisting of three steps: capturing the antibody with Nuvia S, intermediate polishing with Nuvia Q, and final polishing using Nuvia cPrime Resins.

RESULTS

Nuvia S is a high-capacity cation exchange resin with readily available negatively charged groups. Meanwhile, host-cell DNA contaminants present in the clarified cell culture harvest are repelled by these ligands. Consequently, dsDNAs mostly presented in the flow-through fractions during the capture step as revealed by the absorbance trace at 260 nm (Figure 1), resulting in a remarkable, more than 3-log reduction of dsDNA contamination level (Table 4).

Further clearance of host-cell DNAs and proteins was accomplished by intermediate polishing on the Nuvia Q column. The primary concern for this purification step was to maximize the recovery of mAb1 in the flow-through fractions, while having the highest level of contaminants bound to the Nuvia Q column. Results from our previous work have indicated that Nuvia Q media has good tolerance of feed conductivity when used in a flow-through mode. Therefore, adjustment of feed conductivity is not needed prior to this chromatographic step. Rather, the impurity removal efficiency and product recovery are

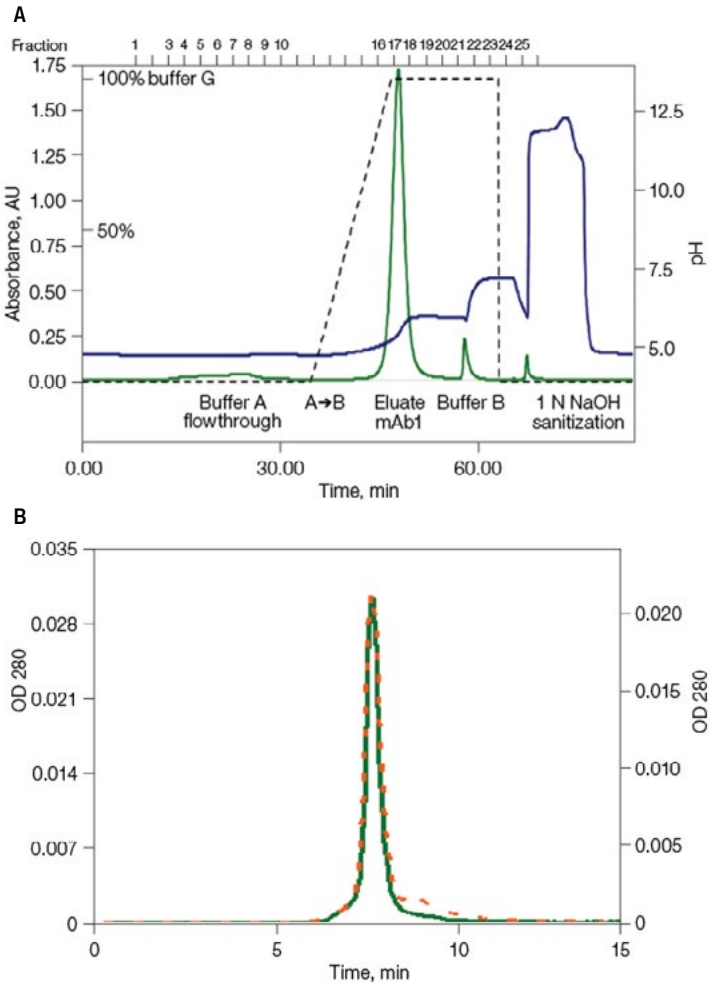


Figure 3: Final polishing purification of mAb1 using Nuvia cPrime Resin. A, chromatogram of mAb1 purification on a Nuvia cPrime Column: OD 280 (—); pH (—); percentage of buffer G (--). mAb1 eluted from the column (fractions 17-19) was collected and subjected to purity analysis. **B**, HPLC-SEC comparison of pooled Nuvia Q fractions (--) and pooled Nuvia cPrime fractions (—).

mainly dependent on the operation pH (data not shown). A flow-through purification of mAb1 purification at pH 7.0 was conducted, slightly below mAb1's determined pI value of 7.2 (Figure 2). Both protein and DNA contaminants were dramatically reduced under this optimized condition.

For final polishing, Nuvia cPrime was employed. Nuvia cPrime is a hydrophobic cation exchange resin (mixed-mode). Its functional ligand (Figure 4) contains structural elements that can interact with biological molecules via electrostatic and hydrophobic forces as well as through hydrogen bonding simultaneously (Figure 5).

The level of these interactions is dependent on protein surface properties and the purification conditions. Unlike traditional cation exchange resins, Nuvia cPrime is tolerant to salts in the feedstream because of its hydrophobic character.

Partially purified mAb1 from the Nuvia Q polishing step could be loaded onto the Nuvia cPrime column following a simple pH adjustment to positively charge mAb1, without extensive dilution or buffer exchange for conductivity reduction. A high concentration of salts, such as $(NH_4)_2SO_4$, was not needed for promoting the binding of target protein either. Such behavior makes Nuvia cPrime distinctive from conventional hydrophobic interaction resins, which require high salt levels for effective protein binding. Under the selected condition, only the desired full-length mAb1 was retained by the column. The bound intact mAb1 was eluted by a buffer with slightly higher pH and lower conductivity, which suggests that mAb1 was likely bound by Nuvia cPrime selectively via a combination of ionic and hydrophobic interactions (Figure 3). This chromatographic step was also very effective at eliminating host cell impurities (Table 4).

The final mAb1 preparation was essentially nucleic acid free because nucleic acids were unretained due to electrostatic charge repulsion between the negative hydrophilic DNAs and the negatively charged hydrophobic ligand of Nuvia cPrime. The purity of mAb1 after this three-step purification was assessed by HPLC–SEC analysis (Figure 6).

Nuvia cPrime Resin's higher affinity for the full-length mAb, compared to process impurities and by-products, gives this final polishing step its powerful purification benefit. It can be thought of as an orthogonal approach, combining cation exchange and hydrophobic interaction chromatography in a single step to achieve unique selectivity for a target protein molecule. Both the binding and elution conditions used for this chromatographic step were effective yet gentle, which is crucial for maintaining the integrity of monomeric mAb.

SUMMARY

Two ion exchange resins, Nuvia S and Nuvia Q, were used in the first two steps to efficiently capture the target mAb from cell culture harvest and to effectively remove impurities produced during the fermentation process. The extraordinarily high binding capacities of these chromatography resins make it possible to process increasing volumes of high-titer feed with existing production facilities and minimal buffer consumption,

Table 4: Impurity Clearance

Sample	Host Cell Proteins, ng/mg	Host Cell dsDNAs, ng/mg	Aggregate Content, %
Cell culture supernatant	6.3×10^4	9.3×10^4	not determined
Nuvia S fraction	2.6×10^3	17	not determined
Nuvia Q fraction	59	4.1	not determined
Nuvia cPrime fraction	5.5	not detected (<0.008)	<0.9

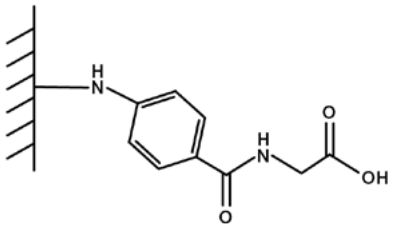


Figure 4: Nuvia cPrime ligand

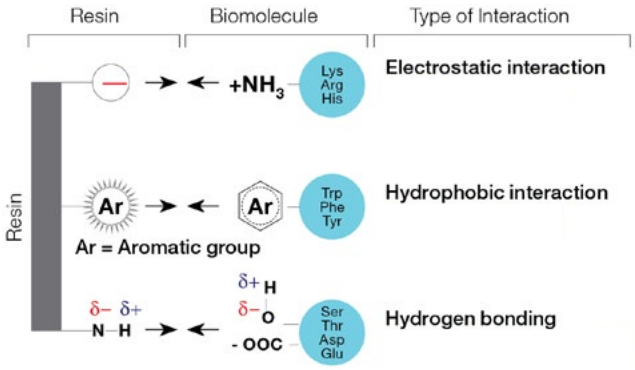


Figure 5: Interaction permutations of ligand and biomolecules

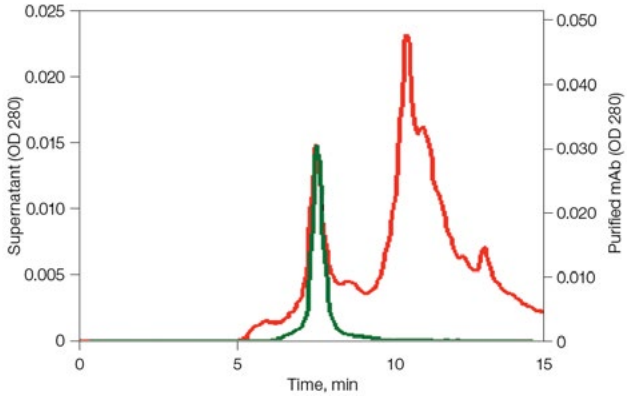


Figure 6: mAb1 purified using three-column purification workflow.
HPLC-SEC comparison of cell culture supernatant (–) and purified mAb1 (–).

easing the pressure on capital investment and process development timelines. In the final polishing step, the orthogonal interaction modes presented by Nuvia cPrime Resin offer unique selectivity for the full-length monomeric mAb molecules. This resin is a powerful tool for the removal of both product-related impurities and host-cell contaminants. The versatility of these resins has allowed us to arrange the order of these three chromatography columns in a sequence that requires no buffer exchange and minimal handling (Figure 7). Transitions between process steps are easy and straightforward. This process is designed to shorten cycle time and to reduce buffer consumption, thus improving overall productivity. All chromatography resins are base stable and mechanically resilient. They can be operated at high flow rates with low backpressures, providing the productivity, robustness, and process economics demanded by today's downstream manufacture of biopharmaceuticals.

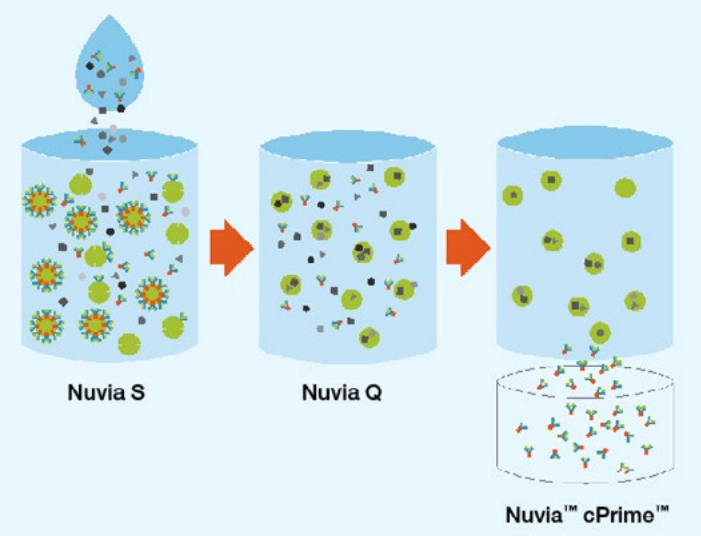
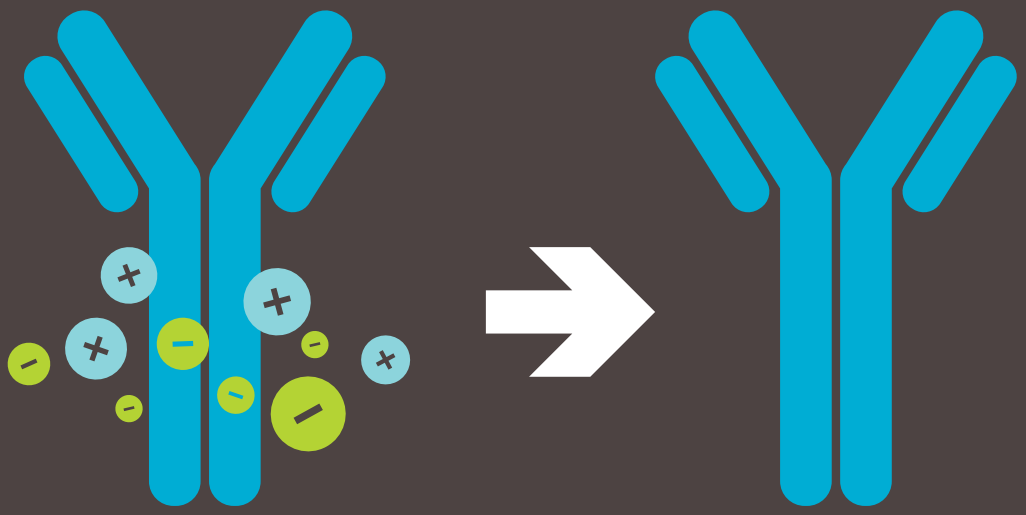


Figure 7: A non-affinity mAb purification workflow. Nuvia S as the capture resin, Nuvia Q as the intermediate purification resin, and Nuvia cPrime as the polish purification resin.



Purification of Polyclonal IgG Antibodies

Bio-Rad offers a wide range of chromatography resins for each process purification phase of polyclonal IgG antibodies, at scales ranging from nanograms to kilograms.

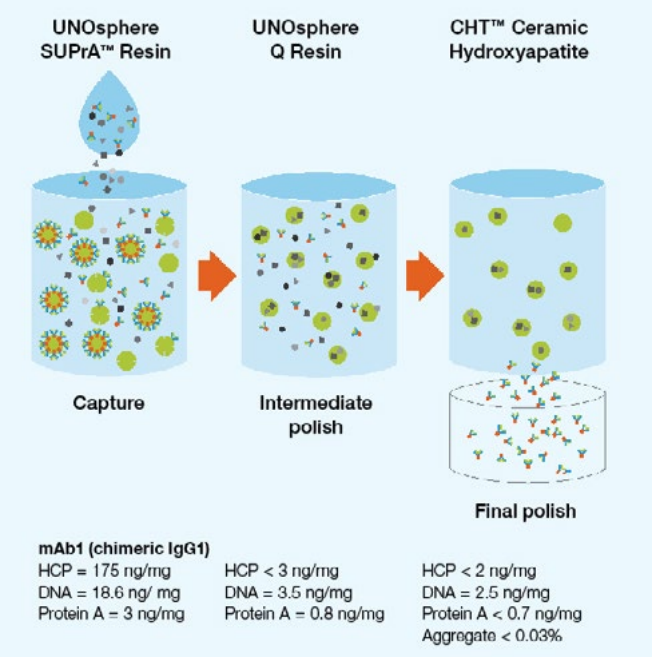
Visit bio-rad.com/resin-library for more information and to request a resin sample.

Monoclonal Antibody

Purification Platform Using High-Capacity Protein A and Mixed-Mode Chromatography

The first step in purification of an important class of therapeutic proteins, the polyclonal or monoclonal antibodies (mAbs), is their capture from plasma or tissue culture supernatants. Protein A–based media are by far the most common class of affinity products used for this purpose. They bind with high affinity to the Fc region of most subclasses of antibodies and are one of the standard tools used in antibody capture and purification. In combination with ion exchange and ceramic hydroxyapatite chromatography, protein A–based resins have been successfully used in the large-scale purification of numerous licensed mAb drugs.

Purification Workflow



UNOsphere SUPrA™ Resin can be integrated in a workflow of mAb purification in combination with UNOsphere™ Q Resin and CHT™ Ceramic Hydroxyapatite to effectively purify mAbs. A unique benefit of the UNOsphere base bead is rapid mass transfer for capture and subsequent release of the target molecule. UNOsphere SUPrA

CAPTURE

Table 1: Capture on High Capacity Protein A Resin, UNOsphere SUPrA Resin *Column: 1 ml, 0.56 x 4 cm*

Step	Buffer	CV	Flow Rate
Equilibration	1xPBS	10	300 cm/h
Sample Loading	CHO cell culture supernatent	–	300 cm/hr
Wash	1xPBS	20	300 cm/hr
Elution	100 mM glycine, pH 3.0	–	300 cm/hr

Pooled mAb fractions were incubated for 1 hr at pH 3.0 (to mimic a virus deactivation step) and then adjusted to pH 7.8 using 10 mM sodium phosphate, pH 8.9.

INTERMEDIATE POLISHING

Table 2: Intermediate Polishing on High Capacity Anion Exchange Resin, UNOsphere Q Resin *Column: 1 ml, 0.56 x 4 cm*

Step	Buffer	CV	Flow Rate
Equilibration	10 mM sodium phosphate, pH 7.8 (Buffer A)	10	300 cm/hr
Sample Loading	UNOsphere SUPrA eluate adjusted to pH 7.8	–	300 cm/hr
Wash	Buffer A	10	300 cm/hr
Strip/Sanitization	1N NaOH	10	300 cm/hr

The mAb in the flowthrough was collected and the pooled fractions were adjusted to pH 6.8 using 20 mM sodium phosphate, pH 4.0.

FINAL POLISHING

Table 3: Final Polishing on Ceramic Hydroxyapatite Media, CHT Type 1, 40 µm *Column: 1 ml, 0.56 x 4 cm*

Step	Buffer	CV	Flow Rate
Equilibration	10 mM sodium phosphate, pH 6.8 (Buffer A)	10	300 cm/hr
Sample Loading	Pooled fractions from UNOsphere Q Column adjusted to pH 6.8	–	300 cm/hr
Wash	Buffer A	15	300 cm/hr
Elution 1	Salt gradient formed between Buffer A and 10 mM sodium phosphate, 1 M NaCl, pH 6.8 (Buffer B)	25	300 cm/hr
Strip	500 mM sodium phosphate, pH 6.5 (Buffer C)	15	300 cm/hr

(Protein A ligand) employs this rapid mass transfer to provide less extreme pH conditions with good recovery in low total volumes. The results demonstrate that UNOsphere SUPrA, in combination with the subsequent polishing steps, delivers a highly pure mAb.

Method

Two antibodies were purified using a workflow consisting of three steps: Capturing the antibodies with UNOsphere SUPrA Resin, intermediate polishing with UNOsphere Q IEX Resin, and final polishing using CHT Ceramic Hydroxyapatite.

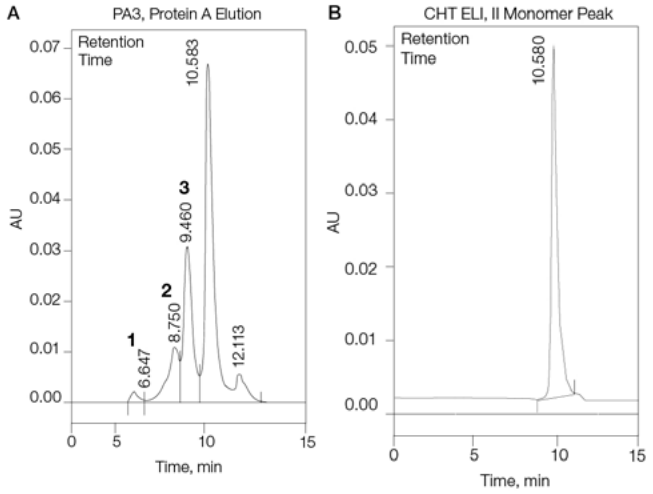


Figure 1: Removal of impurities by CHT chromatography. Elution fractions from the capture (**A**) and final polishing (**B**) chromatographic steps were analyzed by HPLC-SEC using a Bio-Sil® SEC 400-5 to evaluate levels of contamination. The peaks corresponding to aggregates (peaks 1 and 2) and dimers (peak 3) are removed by CHT Ceramic Hydroxyapatite to yield a clean sample containing mAb monomers only.

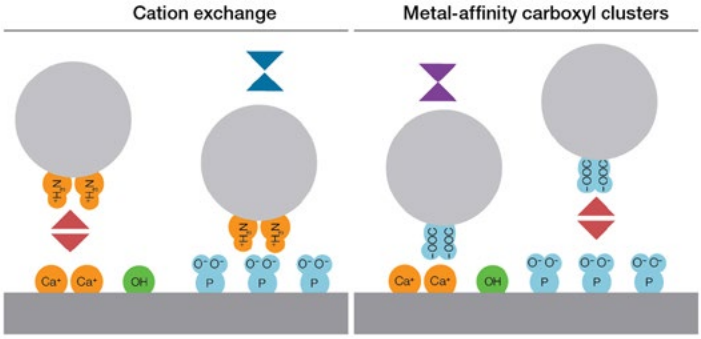
RESULTS

Although there were substantial levels of aggregates in mAb2 eluted from the capturing step (Figure 1A) the final CHT Ceramic Hydroxyapatite pool appears to be completely devoid of these unwanted materials (Figure 1B). Interestingly, one consistent feature of combining CHT Ceramic Hydroxyapatite chromatography with Protein A capture is the remarkable ability to clear these higher-order structures.

UNOsphere SUPrA Resins show an excellent ability to clear contaminating host cell proteins and DNA. CHT Ceramic Hydroxyapatite was capable of clearing Protein A to the level below detection limit in all cases (Table 1).

Advantages of Using Ceramic Hydroxyapatite

A variety of advantages of mixed-mode interactions have been observed beyond simply finer discrimination between product and impurities.



Schematic representation of CHT binding mechanism. Biomolecule (●); metal affinity (⧗); electrostatic repulsion (⬍); electrostatic attraction (⧗).

Figure 2: Schematic representation of CHT binding mechanism.

Table 1: mAb1 and mAb2 impurity clearance data.

	Protein A, ng/mg		HCP, ng/mg		DNA, ng/mg		Aggregate/Dimer Content, %	
Sample	**mAb1**	**mAb2**	**mAb1**	**mAb2**	**mAb1**	**mAb2**	**mAb1**	**mAb2***
Cell culture supernatant	–	–	3.5 x 10	1.4 x 10^6	>5 x 10^3	>1.6 x 10^5	ND	ND
UNOsphere SUPrA fraction	3	ND	175	197	18.6	19	ND	42
UNOsphere Q fraction	0.8	112	<3	86	3.5	1.9	ND	40
CHT ceramic hydroxyapatite fraction	<0.7	<0.4	<2	48	2.5	3	<0.03/<0.03	<0.1

–, Not applicable. ND, not determined.
* Dimer content was not determined for mAb2.

They include:

- Greater resolution compared to unimodal interactions
- Improved yield and activity via advantageous use of charge-charge repulsion between ligand and protein
- Large design space for protein binding
- Minimal feed manipulation prior to binding
- Mild operating conditions preserve activity

CHT is a spherical, macroporous form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which is formed from the chemical combination of calcium and phosphate salts. Unlike most other chromatography media, hydroxyapatite is both the matrix and the ligand, providing multiple modes of interaction. Proteins interact at the negatively charged phosphate (P) site via cation exchange and at the positively charged calcium (C) site via metal affinity (Figure 2).

Multiple interactions between stationary and mobile phases can lead to unique selectivity and facilitate separation of closely related proteins and contaminants.

SUMMARY

UNOsphere SUPrA Affinity Resin can be used to remove impurities to extremely low levels. It is designed for process-scale purification of monoclonal and polyclonal antibodies. This affinity resin is built on the proven UNOsphere matrix for predictable performance over a wide range of antibody concentrations. Higher bed heights can be used to increase residence time without excessive pressure increases, providing developers with a large window of operational freedom. UNOsphere SUPrA typically provide >95% recovery of target antibodies.

In a workflow comprising a capture step and two polishing steps, UNOsphere SUPrA, UNOsphere Q, and CHT Ceramic Hydroxyapatite were able to provide mAbs of high quality and purity. •

Comprehensive Solutions
for Aggregate Issues

The success of any biologic, such as monoclonal antibodies (mAbs), recombinant proteins, or biosimilars, depends greatly on downstream molecule purification. Process scientists often face multiple challenges during this process, including the formation of aggregates. Aggregate molecules are essentially multimers of the monomers. Although they are mostly physically and chemically similar to monomers, their presence in the final purified product, especially a therapeutic mAb, is undesirable due to multiple reasons. First, aggregate formation can lead to a domino effect wherein other impurities like host cell proteins (HCPs) and DNA can bind to the multimers leading to the formation of complex contaminants. These complexes can lower the final monomer concentration, thereby decreasing the efficacy of the therapeutic product. Since the complexes have a multitude of impurities, they can also increase the risk of anaphylaxis or other immunogenic responses in patients. Second, aggregates of therapeutic mAbs often demonstrate different bioactivity/potency profiles, storage stability, immunogenicity, and pharmacodynamic/pharmacokinetic properties than their monomeric counterparts (Lang et al. 2011). For these reasons, aggregate removal has become one of the major focuses of downstream processing.

Aggregate formation can be catalyzed by many processes: (1) overproduction of mAbs due to advancements in upstream technologies leads to the mispairing of disulfide bonds and the unfolding or denaturation of drug molecules at optimal cell growth temperatures (≥25°C) (Rathore et al. 2013); (2) the initial Protein A affinity chromatography employed in the clearance of bulk impurities present in the feedstock, which requires strong acidic elution conditions that can trigger structural changes and promote oligomerization of pH sensitive molecules; and (3) the Protein A eluate is often maintained at low pH for 30–60 minutes as a viral inactivation measure, which has the potential to exacerbate aggregate formation.

Bio-Rad has provided a progressive selection of chromatography resins for process-scale purification of biologics for over 50 years. Despite the high quality of chromatographically-purified therapeutic biotech products, protein immunogenicity remains an important concern. The presence of aggregates is considered an important product-related factor that may increase the risk of an immune response. As the biopharmaceutical industry has grown, the presence of aggregates has been the subject of intense and increasing scrutiny. This book provides a brief snapshot of the different resins that can be used for aggregate removal/minimization in process-scale purification workflows. •

Optimized Purity and Recovery
of a Monoclonal Antibody Using Mixed-Mode Chromatography Media

INTRODUCTION

Monoclonal antibodies (mAbs) remain a predominant class of therapeutic protein products on the market because of their wide range of applications in disease treatment and diagnosis. Over the years, upstream technology advancements have helped improve the titer of target antibodies, from merely 1 g/L two decades ago to 10–13 g/L in fed-batch processes and up to 25 g/L in perfusion cultures today (Gronemeyer et al. 2014). However, these advancements have often adversely affected impurity composition and concentration upon harvest. This has had a significant impact on downstream processing. Elevated levels of antibody aggregates are often associated with the overproduction of mAbs due to the mispairing of disulfide bonds and the unfolding or denaturation of drug molecules at cell growth temperatures (25°C or above) (Rathore et al. 2013).

The strong acidic elution conditions needed for Protein A affinity chromatography, commonly used for the capture of mAbs from clarified tissue culture fluid, can trigger structural changes and promote the oligomerization of pH-sensitive molecules. Additional column chromatography steps are necessary for the clearance of such product-related impurities. The separation of mAb monomers and aggregates has been a major challenge in downstream manufacturing as these molecules exhibit very similar physical and chemical properties and sometimes identical chromatographic behavior. Baseline separation of these mAb species is often difficult to achieve, and extensive process development is required to balance the purity and yield of the monomeric mAbs.

The molecule of interest in this present study, mAb S, was harvested from Chinese hamster ovary (CHO) cell culture.

Typically, a Protein A affinity chromatography eluate contains a mixture of mAb S monomers (~75%) and aggregates (dimers and other oligomers, ~25%). We separated the mAb S monomers from the aggregates using three mixed-mode chromatography media, CHT, Capto™ adhere, and Capto™ adhere ImpRes, which have all been extensively researched for their applications in mAb aggregate clearance (Gagnon 2009). We compared the purity and recovery of monomeric mAb S purified with these chromatography media. Our results underscore the importance of screening multiple media to develop an efficient downstream production process with the highest possible target purity and recovery.

MATERIALS AND METHODS

Chromatography Media and Columns

Bio-Scale™ Mini Cartridges (0.56 x 4 cm) were packed with CHT Ceramic Hydroxyapatite Type I Media, 40 µm (Bio-Rad Laboratories), Capto adhere Media (GE Healthcare Life Sciences), or Capto adhere ImpRes Media (GE Healthcare Life Sciences) to give a final bed volume of 1 ml. The monomer content in eluate fractions or pools was determined using an ENrich™ SEC 650 Column (Bio-Rad Laboratories) with 1x phosphate buffered saline (PBS) as the mobile phase.

Chromatography System

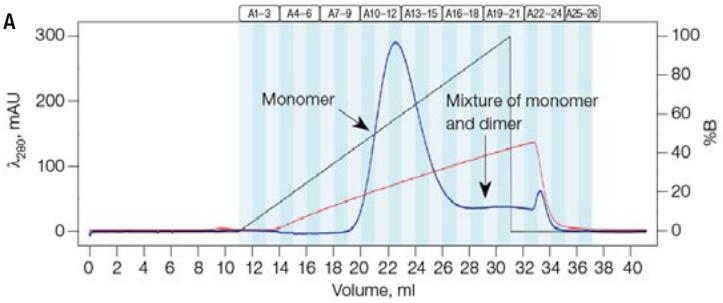
Chromatographic separations and analyses were performed on an NGC™ 10 Chromatography System (Bio-Rad Laboratories), which provides simultaneous monitoring of absorbance at 280 nm, conductivity, and pH.

Test Protein Solution

mAb S was purified from a CHO cell culture harvest by Protein A affinity chromatography and used immediately. Prior to loading onto the columns, the mAb S solution was adjusted to the desired pH with 1 M Tris HCl and filtered through a 0.22 µm membrane. The total mAb S concentration in the test solution was 2.8 mg/ml with monomer content of 74.12%.

$$\text{Monomer recovery (\%)} = 100 \frac{\sum_{i=1}^{n} m_i A_i}{m_t A_t}$$

m_i: Monomer content in an elution fraction; determined by SEC analysis with an ENrich SEC 650 Column
m_t: Monomer content in the starting material; determined to be 0.7412 in this study
A_i: Integration area of individual elution fraction (ml * AU)
A_t: Integration area of total mAb loaded (ml* AU)



Column: 1 ml CHT packed in a Bio-Scale Mini Cartridge (0.56 x 4 cm)
Sample: 2.8 mg affinity-purified mAb S in 1 ml 10 mM sodium phosphate, pH 7.0
Flow Rate: 300 cm/hr
Equilibration: 10 mM sodium phosphate, pH 7.0, 10 column volumes (CVs)
Post-loading Wash: 10 mM sodium phosphate, pH 7.0, 5 CVs
Elution: Buffer A, 10 mM sodium phosphate, pH 7.0; Buffer B, 10 mM sodium phosphate, 1 M sodium chloride, pH 7.0; A to B linear gradient, 0-100% B, 20 CVs

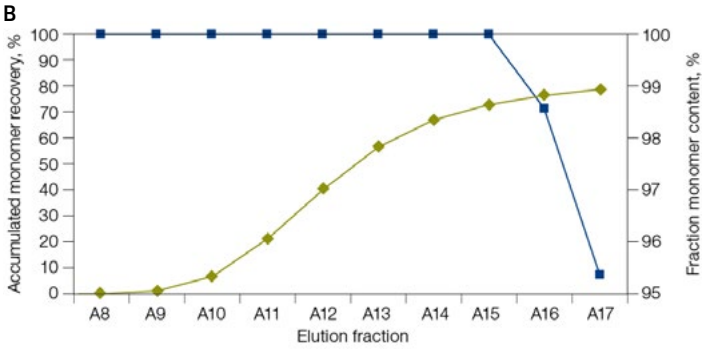


Figure 1: Purification of mAb S on a CHT Column. A, removal of mAb S aggregates using a 0-1,000 mM sodium chloride gradient. OD 280 (—); conductivity (—); %B (—). The blue vertical bands represent where fractions were collected. **B,** mAb monomer content in individual elution fractions (-■-) and accumulated monomer recovery in pooled elution fractions (-◆-).

Calculation of Monomer Recovery

mAb S eluted from the mixed-mode columns was quantified using the Peak Integration feature within the NGC System's ChromLab™ Software. The peak area of each individual elution fraction was measured in ml * AU. Total mAb S loaded onto the column was determined by injecting 1 ml of purified mAb S into the NGC System, in column bypass mode, and measuring the peak area of the flow-through mAb S.

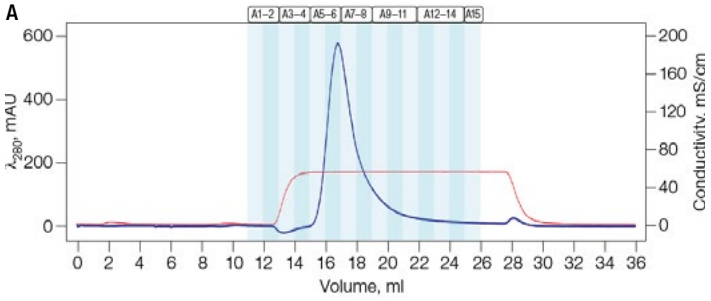
Results

The mAb S aggregates were well separated from the monomer using the CHT Column with a 0–1,000 mM sodium chloride gradient. The bulk of the mAb S monomer was eluted at ~50% B, which is equivalent to ~500 mM sodium chloride (Figure 1A). Elution of the monomer was complete at ~75% B or 750 mM sodium chloride, and increasing the salt concentration resulted in leaching of the undesired mAb S oligomers from the

column (Figure 1B). Step elution of mAb S was then performed using a sodium phosphate buffer containing 550 mM sodium chloride (Figure 2). Fractions A5 to A9 were pooled, and the monomer content in this eluate was determined to be 99.5% with an overall mAb S monomer recovery of 82.7%.

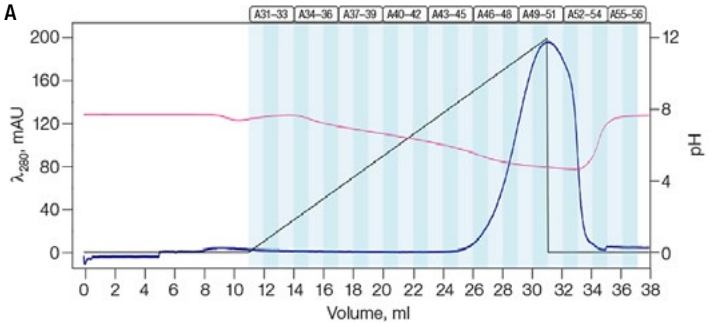
Preliminary design of experiments (DoE) studies were performed to screen for mAb S purification conditions with Capto adhere Media. Binding was maximal with buffers containing ≤50 mM sodium chloride at pH above 8.0. mAb S was only partially eluted from the column under a modest acidic condition (pH 5.0). At pH 4.0, all mAb S species were eluted from the Capto adhere Column without differentiation of monomer and higher molecular weight species. The addition of sodium chloride to the elution buffer further hampered target protein recovery, which indicates a strong hydrophobic interaction between target molecules and this hydrophobic anion exchange media (data not shown). A gradient of pH8–5 was therefore employed in an effort to elute mAb S monomers selectively from the Capto adhere Column (Figure 3A). Dependency of monomer content and elution pH was observed, with the later fractions containing significantly more high molecular weight species (Figure 3B) and exhibiting a plateau of mAb S monomer recovery (Figure 3C).

To further assess the effect of the elution pH on final mAb S monomer purity and recovery, step elutions under various pH conditions were performed. The results are summarized in Table 1. The chromatography of mAb S elution at pH 5.5 is shown in Figure 4. The purity of the product was found to be very sensitive to the pH of the elution buffer. A minor pH drop, from 5.5 to 5.4, resulted in a tripling of the aggregate content in the eluate. The pooled product from a pH 5.4 step



Column: 1 ml CHT packed in a Bio-Scale Mini Cartridge (0.56 x 4 cm)
Sample: 2.8 mg affinity-purified mAb S in 1 ml 10 mM sodium phosphate, pH 7.0
Flow Rate: 300 cm/hr
Equilibration: 10 mM sodium phosphate, pH 7.0, 10 CVs
Post-loading Wash: 10 mM sodium phosphate, pH 7.0, 5 CVs
Elution: 10 mM sodium phosphate, 550 mM sodium chloride, pH 7.0, 15 CVs

Figure 2: Elution of mAb S monomers from a CHT Column. Elution was carried out using 10 mM sodium phosphate and 550 mM sodium chloride, pH 7.0. Fractions A5 to A9 were pooled. OD 280 (–); conductivity (–). The blue vertical bands represent where fractions were collected.



Column: 1 ml Capto adhere packed in a Bio-Scale Mini Cartridge (0.56 x 4 cm)
Sample: 2.8 mg affinity-purified mAb S in 1 ml 10 mM sodium phosphate, pH 7.0
Flow Rate: 300 cm/hr
Equilibration: 50 mM sodium phosphate, pH 8.0, 10 CVs
Post-loading Wash: 50 mM sodium phosphate, pH 8.0, 5 CVs
Elution: Buffer A, 50 mM sodium phosphate, pH 8.0; Buffer B, 50 mM sodium phosphate, pH 5.0; A to B linear gradient, 0-100% B, 20 CVs

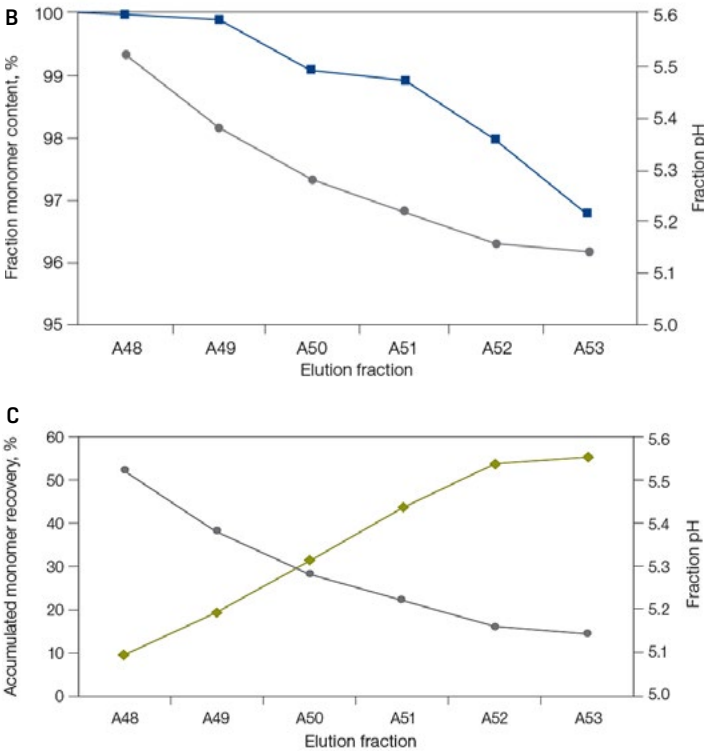
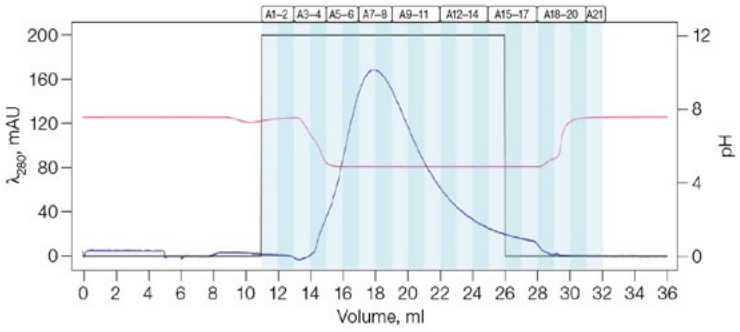


Figure 3: Purification of mAb S on Capto adhere Column. A, removal of mAb S aggregates using a pH 8-5 gradient. OD 280 (–); pH (–); %B (–). The blue vertical bands represent where fractions were collected. **B,** mAb S monomer content in individual elution fractions (-■-) and its correlation with fraction pH (-●-). **C,** accumulated mAb S monomer recovery in pooled elution fractions (-◆-) and its correlation with fraction pH (-●-).

elution failed the quality control (QC) specification of ≥99.5% monomer content. Furthermore, despite the fact that 14 CVs of eluate were collected, monomer recovery was only ~53%.

Table 1: Pooled elution fractions from the Capto adhere Column using 50 mM sodium acetate buffer at different pH.

Elution pH	mAb S Monomer Content, %	mAb S Monomer Recovery, %	Eluate Volume, CV
5.2	96.9	76.9	14
5.3	98.1	64.9	14
5.4	98.8	52.5	14
5.5	99.7	48.8	14

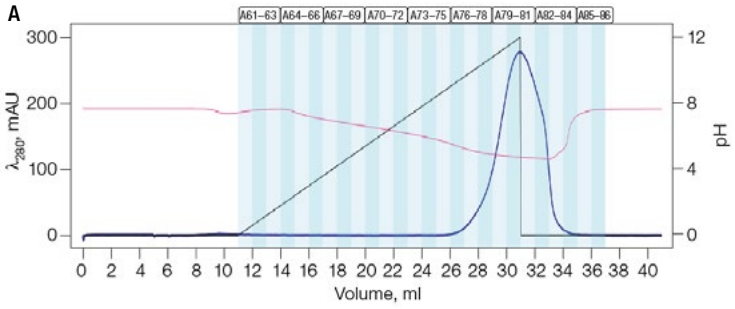


Column: 1 ml Capto adhere packed in a Bio-Scale Mini Cartridge (0.56 x 4cm)
Sample: 2.8 mg affinity-purified mAb S in 1 ml 50 mM sodium phosphate, pH 8.0
Flow Rate: 300 cm/hr
Equilibration: 50 mM sodium phosphate, pH 8.0, 10 CVs
Post-loading Wash: 50 mM sodium phosphate, pH 8.0, 5 CVs
Elution: 50 mM sodium acetate, pH 5.5, 15 CVs

Figure 4: Elution of mAb S monomers from a Capto adhere Column. Elution was carried out using 50mM sodium acetate, pH 5.5. Fractions A35 to A48 were pooled. OD 280 (–); pH (–); %B (–). The blue vertical bands represent where fractions were collected.

Compared with Capto adhere, the binding of mAb S by Capto adhere ImpRes was more sensitive to the concentration of sodium chloride in the buffer. However, the elution of mAb S from this mixed-mode media seemed to be solely dependent on the pH of the buffer, as revealed by DoE screening (data not shown). Again, a pH gradient was used to resolve mAb S monomers from aggregates (Figure 5A). The pH of the elution fractions, monomer content, and monomer recovery were analyzed and the results are shown in Figures 5B and 5C.

Buffers at different pH were used to elute mAb S from the Capto adhere ImpRes Column in step mode (Table 2). Figure 6 depicts the chromatogram resulting from the elution of mAb S with 50 mM sodium acetate (pH 5.4). Overall, Capto adhere ImpRes offered better recovery of mAb S monomer than Capto adhere. The purity of mAb S monomer in the eluate met the QC specification of ≥99.5% monomer content and was consistent at pH 5.4 and higher; however, monomer recovery was only 50–62% after 14 CVs of eluate were collected under these conditions.



Column: 1 ml Capto adhere ImpRes packed in a Bio-Scale Mini Cartridge (0.56 x 4 cm)
Sample: 2.8 mg affinity-purified mAb S in 1 ml 50 mM sodium phosphate, pH 8.0
Flow Rate: 300 cm/hr
Equilibration: 50 mM sodium phosphate, pH 8.0, 10 CVs
Post-loading Wash: 50 mM sodium phosphate, pH 8.0, 5 CVs
Elution: Buffer A, 50 mM sodium phosphate, pH 8.0; Buffer B, 50 mM sodium acetate, pH 5.0; A to B linear gradient, 0-100% B, 20 CVs

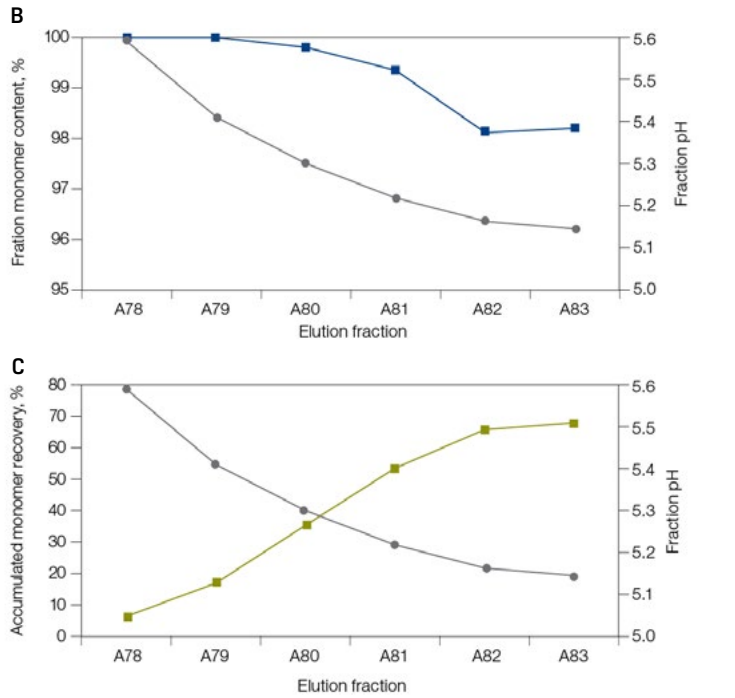
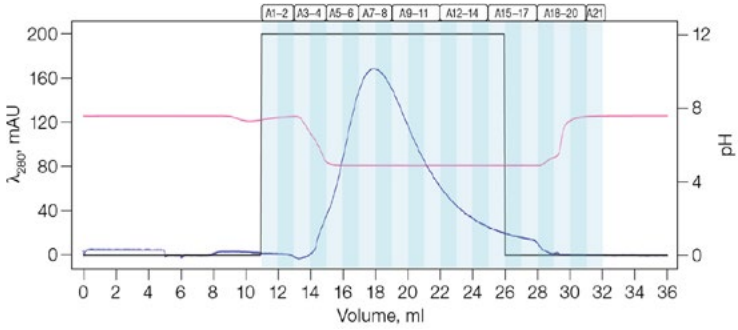


Figure 5: Purification of mAb S on Capto adhere ImpRes Column. A, removal of mAb S aggregates using a pH 8-5 gradient. OD 280 (–); pH (–); %B (–). The blue vertical bands represent where fractions were collected. **B,** mAb S monomer content in individual elution fractions (-■-) and its correlation with fraction pH (-●--). **C,** accumulated mAb S monomer recovery in pooled elution fractions (-■-) and its correlation with fraction pH (-●--).

Table 2: Pooled elution fractions from the Capto adhere ImpRes Column using 50 mM sodium acetate buffer at different pH.

Elution pH	mAb S Monomer Content, %	mAb S Monomer Recovery, %	Eluate Volume, CV
5.3	98.6	68.3	14
5.4	99.6	61.7	14
5.5	99.6	50.5	14



Column: 1 ml Capto adhere ImpRes packed in a Bio-Scale Mini Cartridge (0.56 x 4 cm)
Sample: 2.8 mg affinity-purified mAb S in 1 ml 50 mM sodium phosphate, pH 8.0
Flow Rate: 300 cm/hr
Equilibration: 50 mM sodium phosphate, pH 8.0, 10 CVs
Post-loading Wash: 50 mM sodium phosphate, pH 8.0, 5 CVs
Elution: 50 mM sodium acetate, pH 5.4, 15 CVs

Figure 6: Elution of mAb S monomers from a Capto adhere ImpRes Column. Elution was carried out using 50 mM sodium acetate, pH 5.4. Fractions A4 to A18 were pooled. OD 280 (–); pH (–); %B (–). The blue vertical bands represent where fractions were collected.

Discussion

Aggregates of therapeutic mAbs often demonstrate different bioactivity/potency profiles, storage stability, immunogenicity, and pharmacodynamic/pharmacokinetic properties than their monomeric counterparts (Vázquez-Rey and Lang 2011). These product-related impurities must be effectively removed during the manufacturing process to ensure the safety and efficacy of the final drug formulation. A high level of aggregates was present in the mAb S harvest, which could have been an indication of the intrinsic hydrophobicity of mAb S or the exposure of its hydrophobic amino acid residues due to misfolding, unfolding, or denaturation. The mixture of monomers and aggregates of mAb S could not be separated by traditional cation exchangers (data not shown). Three mixed-mode media, CHT, Capto adhere, and Capto adhere ImpRes, were tested for their efficiency in clearing mAb S aggregates; their optimal performances are summarized in Table 3.

The contamination level of mAb S aggregates in the recovered mAb S monomer fractions was effectively reduced to below 0.5% by all three media. However, CHT offered the best monomer recovery in the smallest eluate volume (Table 3). Since the isoelectric point (pI) of mAb S was estimated at 6.9 (data not shown), it should bind to Capto adhere–based media through strong electrostatic interaction. The difficult elution of mAb S from these two hydrophobic anion exchangers suggests that a significant hydrophobic interaction was also involved in mAb S binding to these columns. A more stringent acidic condition was necessary for protonating mAb S molecules in order to promote their dissociation from these chromatography media; however, this was carried out at the expense of selectivity between monomers and aggregates. To satisfy the QC specification of ≥99.5% monomer in the final product, elution had to be terminated at pH 5.5 and pH 5.4 for Capto adhere and Capto adhere ImpRes, respectively. As a result, the recovery of mAb S monomer from these chromatography media was compromised. Tailing was observed and elution was incomplete even after 15 CVs of elution buffer were applied.

Table 3: Comparison of CHT, Capto adhere, and Capto adhere ImpRes in the clearance of mAb S aggregates.

Media	mAb S Monomer Content, %	mAb S Monomer Recovery, %	Eluate Volume, CV
CHT*	99.5	82.7	5
Capto adhere**	99.5	48.8	14
Capto adhere ImpRes***	99.5	61.7	14

* Step elution with 10 mM sodium phosphate and 550 mM sodium chloride, pH 7.0
** Step elution with 50 mM sodium acetate, pH 5.5
*** Step elution with 50 mM sodium acetate, pH 5.4

CONCLUSIONS

In the commercial production of therapeutic proteins, both purity and yield are essential to product quality and process efficiency and economics. For the hydrophobic and mildly acidic mAb S molecules, this data demonstrates that CHT is the chromatography media of choice for a robust production process with minimal loss of monomeric target molecules. •

References
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-Gronemeyer P et al. (2014). Trends in upstream and downstream process development for antibody manufacturing. Bioengineering 1, 188–212.
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—Vázquez-Rey M & Lang DA (2011). Aggregates in monoclonal antibody manufacturing processes. Biotechnol Bioeng 108, 1,494–1,508.

Improving Aggregate Removal

from a Monoclonal Antibody Feedstream Using High-Resolution Cation Exchange Chromatography

Ever increasing performance demands in protein purification require more selective chromatography methods to effectively remove aggregates and other impurities. At high titer levels, aggregates often present a unique purification challenge. One emerging approach to address this challenge is the use of new, smaller-particle chromatography resins that are optimized for high resolution and capacity. Such resins can be particularly productive in challenging situations and during final polishing steps (He et al. 2010). Nuvia™ HR-S is a high-resolution resin. It features a hydrophilic polymer matrix with an open-pore structure designed for fast and efficient mass transfer and superior flow properties at high flow rates. In this case study, the performance of Nuvia HR-S was compared to that of a high-resolution agarose-based resin.

RESULTS

Preparation of Aggregate and Monomer Test Solution

The in vitro generation of aggregates was designed to obtain a final composition of ~10% aggregates. Several initial attempts were made with varying ratios of monomeric to aggregated antibody. A final ratio of 2.5 volumes of monomeric antibody solution to 1 volume of aggregated antibody solution was chosen to yield a composition with the following properties:

A280 = 6.3 or 4.56 mg/ml
pH = 4.91
Conductivity = 2.6 mS/cm
Aggregate = 8.9%

A plot of log MW of six protein standards vs. their retention times yielded a straight line (R2 = 0.96). Using this plot and the retention times of the aggregates, the apparent MW of the aggregate was calculated to be 386 kD.

Initial Separation of Aggregate from Monomer Using High Resolution CEX Resin

As shown in Figure 1, there was considerable overlap of the monomer and aggregate peaks (fractions (Fr) 17–22). Depending on the fraction pooling method, monomer recovery of 66–81% was achieved with an aggregate content of 0.46–0.85%. This observation is consistent with the premise that greater recovery results in higher aggregate content. The quality of the recovered pools (Figure 2) is shown in the overlay of the enlarged front shoulders of each SEC profile. The results demonstrated the feasibility of using Nuvia HR-S to separate aggregates from the monomer and showed that the extent of residual aggregates depended on the fractions included in the pool.

METHOD

Table 1: Initial Separation of Aggregate from Monomer Using Nuvia HR-S Resin, *Column: 2.1 ml, 0.7 x 5.6 cm*

Step	Buffer	CV	Flow Rate
Equilibration	40 mM sodium acetate, pH 5 (Buffer A)	3	310 cm/hr
Sample Loading	Treated mAb at pH 5	—	310 cm/hr
Elution 1	Gradient of Buffer A to Buffer A + 1.0 M NaCl	20	310 cm/hr
Elution 2	Buffer A + 1.0 M NaCl	2	310 cm/hr
Sanitization	1N NaOH	5	310 cm/hr

This method applies to Figure 1.

METHOD

Table 2: Comparison of Nuvia HR-S Resin with a Commercial Small Particle Size CEX Resin, *Column: 2.1 ml, 0.7 x 5.6 cm*

Step	Buffer	CV	Flow Rate
Equilibration	40 mM sodium acetate, pH 5 (Buffer A)	3	150 cm/h
Sample Loading	Treated mAb at pH 5	—	150 cm/hr
Elution 1	Gradient of Buffer A to Buffer A + 0.4 M NaCl	20	150 cm/hr
Elution 2	Buffer A + 0.4 M NaCl	2	150 cm/hr
Sanitization	1N NaOH	5	150 cm/hr

This method applies to Figure 3.

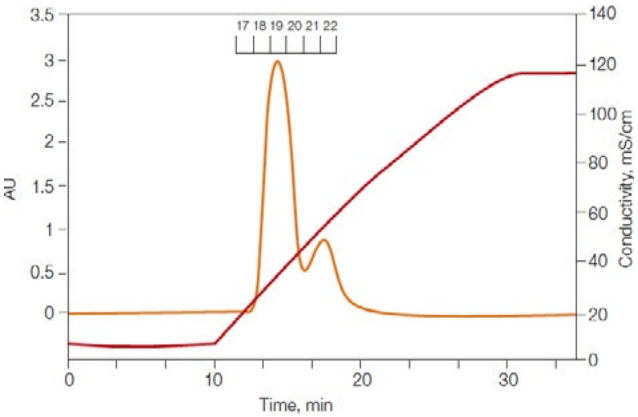

Figure 1: Separation of aggregates from monomer by Nuvia HR-S before optimization. Absorbance (—); conductivity (—).

Additionally, the overlap of monomer and aggregates in the Nuvia HR-S elution profile suggested that a lower flow rate and a shallower gradient (0.1–0.4 M NaCl) could be beneficial.

Comparison of Commercial Small Particle Size CEX Resin

A loading level of 46 mg IgG/ml resin was employed to challenge the binding capacity of Nuvia HR-S and Resin 1, and aggregate clearance by each resin was evaluated. A comparison of the elution profiles obtained from each chromatography run using the method conditions listed is shown in Figure 3. As indicated by the absorbance profile, the chromatography conditions resulted in a low yield for Resin 1 due to incomplete binding during the loading phase. In contrast, Nuvia HR-S was able to quantitatively retain the antibody before elution.

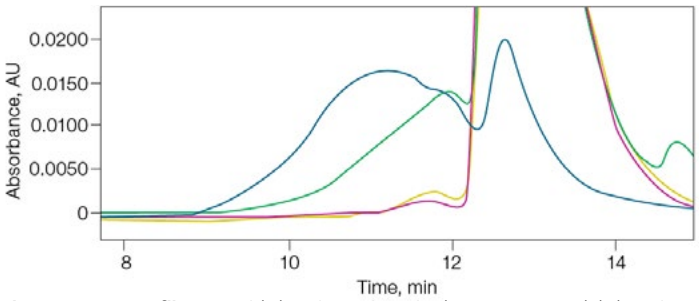

Figure 2: SEC profiles. Load (—); selected pools (Fr 17+18+19+20) (—) and (Fr17+18+19)(—) ; aggregated pool (Fr 21+22) (—) .

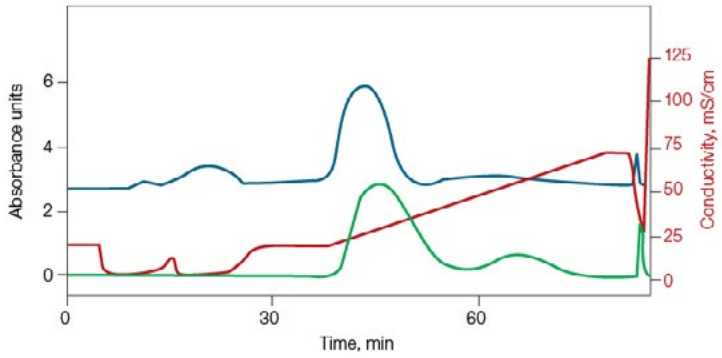

Figure 3: Elution profiles after optimization. Nuvia HR-S (—); Resin 1 (—); conductivity (—) .

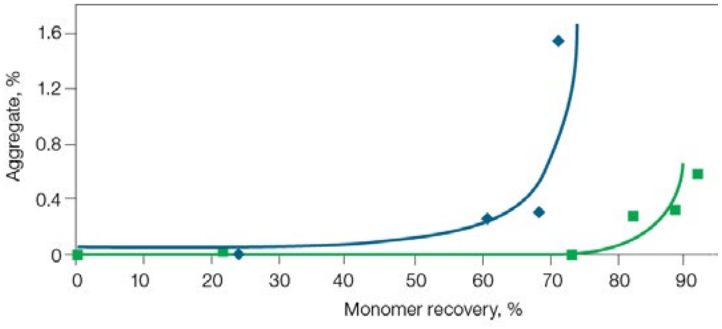

Figure 4: Performance of Nuvia HR-S (■) vs Resin 1 (◆) .

Rather than subjectively determining the pertinent fractions, a strategy was designed to measure the aggregate content and the monomer recovery as fractions were progressively pooled during elution. As shown in Figure 4, both resins show an increase in aggregates as the pooling was extended to increase monomer recovery. While Nuvia HR-S was able to deliver a final aggregate content of ⟨0.3% and a recovery of ⟩80%, Resin 1 recovered less than 70% of total monomer at the same target aggregate content. This is due to the lower binding capacity of Resin 1, as confirmed by the increase in its absorbance during loading (Figure 3).

Correlation between Monomer Recovery, Aggregate Content, and Target Conductivity

As depicted in Figure 4, aggregate recovery increases with increasing monomer recovery. In the Nuvia HR-S gradient elution, conductivity of the last fraction added to the pool was determined as the cutoff target of the pool. As

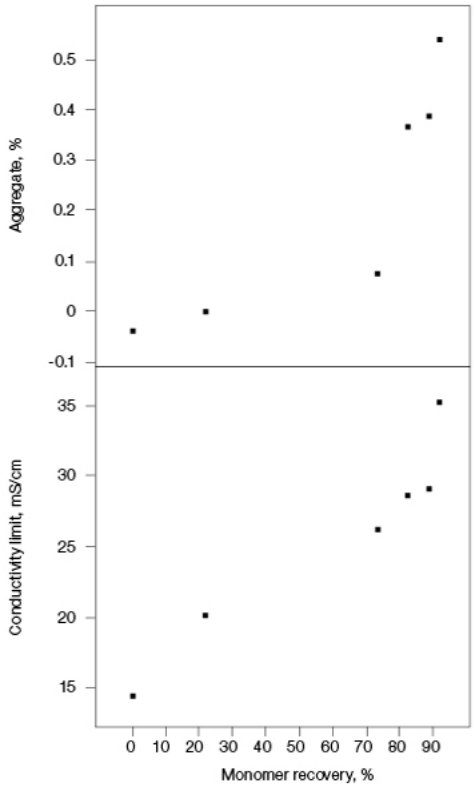

Figure 5: Scatter plot matrix of aggregate content or conductivity vs. monomer recovery on Nuvia HR-S.

shown, recovery is a function of the acceptable aggregate percentage and the target conductivity (Figure 5). Using the Fit Model function in JMP Software (SAS Institute Inc.), recovery could be correlated to those two measurements. A summary of actual and predicted data is shown in Figure 6.

The model yielded excellent correlation (R2 = 0.94). This finding confirms the need to determine conductivity for elution so that both recovery and aggregate levels are within acceptable limits.

SUMMARY

The data presented here demonstrate that Nuvia HR-S High-Resolution Cation Exchange Resin reduced aggregate content to 〈0.3% with 〉80% monomer recovery from a mAb feedstream containing 8.9% aggregates. In addition, Nuvia HR-S provides significantly higher recovery than a comparable agarose-based small-particle CEX resin. Finally, aggregate removal and final recovery are shown to be functions of buffer conductivity measured at the end of eluate collection.

References
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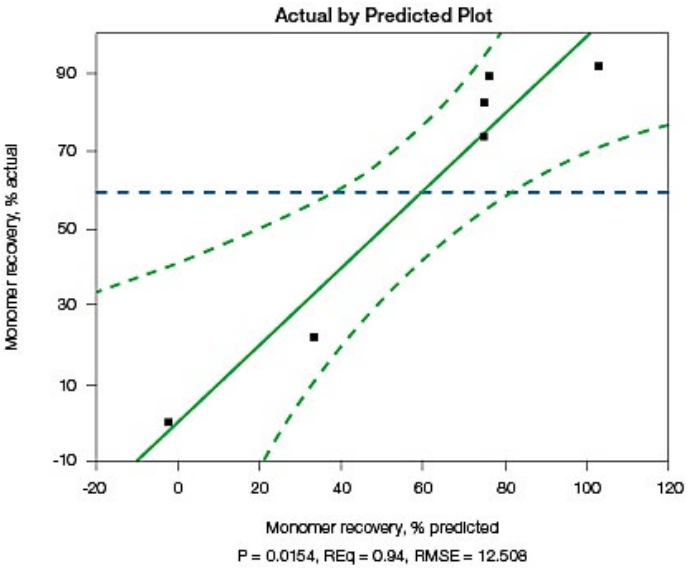
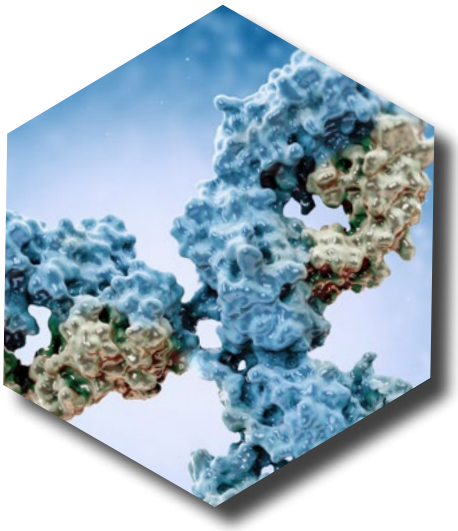


Summary of Fit	
R^2	0.938098
R^2 adj	0.89683
Root mean square error	12.50828
Mean of response	59.78333
Observations (or sum wgts)	6

Figure 6: Fit model of monomer recovery on Nuvia HR-S .



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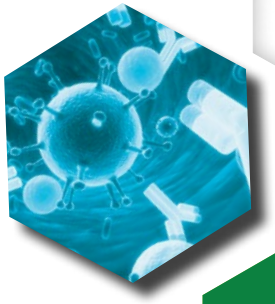

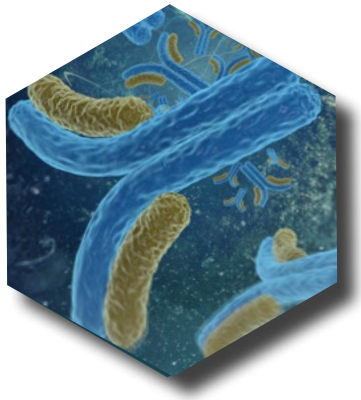

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