Purification of Antibodies with Nuvia™ w Prime 2A Media, a Scalable Hydrophobic Weak Anion Exchange Resin

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Abstract

Chromatographic purification is required to achieve specified purity and safety targets established for drug products such as monoclonal antibodies (mAbs), biosimilars, and other recombinant proteins. After the initial capture step, typically two to four additional orthogonal steps using anion exchange (AEX), cation exchange (CEX), and/or hydrophobic interaction chromatography (HIC) are required to meet the final product specifications. Alternatively, downstream processes often utilize multimodal ligands as a key purification step. These ligands show enhanced selectivity due to their multiple interaction modalities (electrostatic, hydrophobic, aromatic, and/or hydrogen bonding) when compared to the traditional single-mode interaction resin and by reducing the number of steps, may improve process economics and help achieve sustainability goals.

Here, we describe the purification of a mAb with Nuvia wPrime 2A Media, a weak anion exchange hydrophobic interaction chromatography (AEX-HIC) mixed-mode resin. By using a design of experiments (DoE) approach, optimal operating conditions were selected that predicted effective clearance of aggregates or high molecula weight (HMW) species, a product-related impurity, in a flow-through mode. The results predicted by the DoE study were then confirmed on a bench-scale column. Nuvia wPrime 2A Media reduced the HMW species from 7.3% in the load to 0.7% in the purified pool and provided total mAb monomer recovery of 85%. These results demonstrate the effectiveness of using Nuvia wPrime 2A Media in a polishing step during mAb purification, offering a promising process intensification opportunity to streamline downstream operations.

Introduction

The global antibody therapeutics market is estimated to expand to approximately $200 billion between 2023 and 2026.[1,2] This massive growth is fueled by cutting-edge research into various therapeutic aspects of antibodies in drug development. With increasing upstream cell culture titers, additional burdens are placed on making downstream antibody purification and recovery both cost- and time-effective. This, in turn, results in an ever-increasing demand for innovative and advanced chromatography resins.

Antibody purification by IEX supports has a rich history, going back to the 1950s,[3] when it was demonstrated that IgG can bind to and be eluted from an anion exchange matrix. IgG can also be purified by anion exchange in flow-through mode.[4] Similar purification of antibodies in both bind-elute and flow-through modes has been demonstrated using hydrophobic interaction chromatography.[5]

Moreover, recent work by Konrad et al. (2023) and Lorek (2023) demonstrated the capability of multimodal anion exchange adsorbents to remove both process and product-related impurities in a flow-through mode, which has lately been shown to be an imperative part of a continuous manufacturing process.[6,7]

As next-generation biotherapeutics become increasingly sophisticated, ongoing innovation in purification technologies is essential to support downstream process development. Bio-Rad's scalable weak AEX-HIC mixed-mode resin, Nuvia wPrime 2A Media, was specifically designed to deliver the selectivity needed to address persistent challenges in antibody purification.

Regarding the chemistry of the Nuvia wPrime 2A Media ligand, the secondary amine operates as a weak anion exchanger, with a pKa of 7.4 (Figure 1). The charge state of the resin's anion exchange component of the ligand can be modulated by the pH of the mobile phase. This adjustable positive charge state may be used to purify otherwise difficult-to-separate biomolecules from other impurities. Conveniently, Nuvia wPrime 2A Media can be used in either bind-elute or flow-through modes.

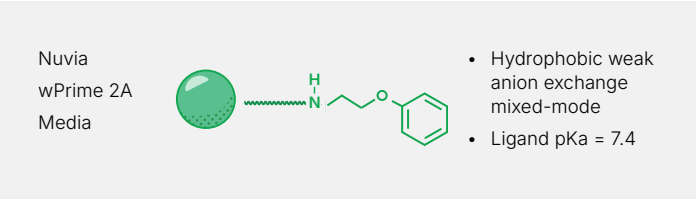


Fig. 1. Mixed-mode ligand for Nuvia wPrime 2A Media. The pKa of the secondary amine is 7.4.

Here, we describe the purification of a mAb with Nuvia wPrime 2A Media. The mAb aggregate/HMW species was present in a post-Protein A pool at 7.3%, which was reduced to 0.7% by Nuvia wPrime 2A Media, with mAb monomer recovery of 85%. These results demonstrate Nuvia wPrime 2A Media as an effective polish purification step for mAbs.

Materials and Methods

A Protein A-purified pool containing custom-human immunoglobulin G1 (IgG1) kappa mAb with a pI of 6.91 was used for this study. The IgG1 mAb was subjected to a viral inactivation (VI) treatment by exposing it to a pH 3.0 condition using 0.1 M hydrochloric acid for 1 hr. The sample was subsequently neutralized to pH 5.0 by drop-wise addition of 0.1 M sodium hydroxide solution. This treated pool was termed Neutralized Viral Inactivated Pool (NVIP). NVIP was analyzed by the size-exclusion chromatography-ultra-performance liquid chromatography (SEC-UPLC) method to ascertain both the mAb's stability as well as estimate its aggregate content. An Acquity UPLC BEH200 SEC column (4.6 x 150 mm, 1.7 µm; Waters, catalog #176003904) was used on a Waters Acquity UPLC unit consisting of a quaternary pump, an autosampler with a cooling unit, a column oven, an ultraviolet (UV) detector, and Waters Empower software. The column was equilibrated with 100 mM phosphate buffer, pH 6.8. Determination of aggregates was performed at 280 nm, and the flow rate was maintained at 0.3 mL/min with a run time of 10 min. A sample volume of 5 µL with appropriate dilution was injected for all chromatographic fractions.

To check the robustness (repeatability) of the SEC-UPLC method, 10 replicate injections of the same IgG1 sample were performed. The percent relative standard deviation (RSD) was calculated for the HMW (1.22%) and IgG monomer peaks (0.09%). RSD results of <2% were considered good.

A Protein A high-performance liquid chromatography (HPLC) column was used to assess mAb monomer recovery. A step-gradient HPLC method was used per the manufacturer's recommendation for mAb monomer estimation in the chromatographic fractions. A Thermo Scientific MAbPac Protein A HPLC Column (35 x 4.0 mm ID, 12 µm; Thermo Fisher Scientific, catalog #082539) was used on an Agilent 1260 Infinity II series HPLC unit consisting of a quaternary pump with degasser, an autosampler with a cooling unit, a column oven, a diode array detector, and Agilent OpenLab CDC Data Analysis software. The column was equilibrated with Buffer A (50 mM sodium phosphate, 150 mM NaCl, 5% acetonitrile, pH 7.5) and eluted with Buffer B (50 mM sodium phosphate, 150 mM NaCl, 5% acetonitrile, pH 2.5) per the manufacturer's instructions. Determination of mAb monomer purity was performed at 280 nm, and the flow rate was maintained at 2.0 mL/min with a run time of 4 min. A sample volume of 20 µL was injected for all chromatographic fractions. The step-gradient method followed is outlined in Table 1.

Table 1. Step-gradient protocol for Protein A HPLC assay.

Time, min	Buffer A, %	Buffer B, %
0.00	100	0
0.75	100	0
0.80	0	100
1.75	0	100
1.80	100	0
4.00	100	0

A 3-point calibration curve was plotted to set the linearity of the assay. Samples were injected in triplicate, and a regression coefficient (R^2) of 0.999 was obtained for a mAb concentration range of 1.0–4.0 mg/mL (data not shown).

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To test the initial screening process conditions, preliminary high-throughput screening (HTS) was carried out using AcroPrep Advance 96-well plates with a 0.45 µm PES membrane bottom plate (Cytiva, catalog #8129). Resin slurries (80 µL; 50% v/v) were distributed into the filter plate, which was vacuum-filtered to remove the fluid. For each screening stage, 200 µL of solution was applied to the 40 µL of resin in each well, resulting in a phase volume ratio of 5:1. For the load preparation, NVIP was buffer-exchanged in the appropriate buffer determined from the DoE study, as described below, using 30 kDa Amicon filters (Millipore Sigma, catalog #UFC9030). Each batch experiment consisted of sequential equilibration, sample loading, washing, and regeneration steps. After each step, the plate was vacuum-filtered at ~700 mbar, and the filtrate was collected in a 2 mL flat-bottom collection plate stacked beneath the membrane bottom plate. All experiments were performed at room temperature.

DoE analysis: To further optimize the process conditions for optimal HMW species clearance and IgG1 recovery, a DoE study with a multifactorial design with two factors (pH and salt concentration) and three levels was created using Minitab software version 21.3.

Based on the optimized outputs obtained from the DoE studies, chromatographic separation of the IgG1 monomer from NVIP containing aggregates was performed using a preparative column with Nuvia wPrime 2A Media and an NGC Chromatographic System (Bio-Rad) at room temperature.

For this, 0.5 mL Nuvia wPrime 2A Media was packed in a 5 mm internal diameter (ID) x 50 mm resin bed height column. The chromatographic steps involved along with the optimized buffer components at each stage are listed in Table 2.

Table 2. Protocol for mAb monomer purification using Nuvia wPrime 2A Media.

Step	Solution	Column Volumes (CVs)
Equilibration	20 mM phosphate + 221.2 mM NaCl, pH 8.0	4
Load	Buffer-exchanged Protein A pool containing IgG1 mAb at 5 mg/mL (though a 5 mL loading loop) at 50 mg/mL of resin	10
Wash	20 mM phosphate + 221.2 mM NaCl, pH 8.0	24
Regeneration	100 mM citrate buffer, pH 3.0	
Re-equilibration	20 mM phosphate + 221.2 mM NaCl, pH 8.0	10

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Results & Discussion

NVIP Analysis

Analysis of Protein A-purified IgG1 and NVIP confirmed that the viral inactivation treatment led to the formation of HMW aggregates (Figure 2).

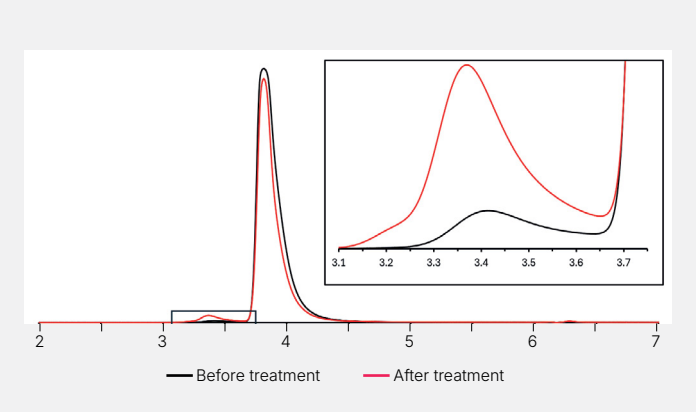


Fig. 2. SEC-UPLC chromatogram of custom-human IgG1 kappa, pI 6.91 NVIP. Shown are IgG1 before VI treatment (---) and after VI treatment with the formation of aggregates (---).

Preliminary Process Condition Screening for Flowthrough Chromatography

Nuvia wPrime 2A Media possesses both charged and hydrophobic groups, and the ligand's charge state can be modulated by adjusting the process conditions (see Figure 1). Initial process condition screening was performed by considering the pKa of the ligand (pKa = 7.4). Since the predicted pI of the mAb was indicated to be 6.91, chromatographic steps including equilibration, loading, and washing steps were performed at pH 5.0. At this condition, it was hypothesized that both Nuvia wPrime 2A Media and the IgG1 mAb monomer would possess a net positive charge and thus would be repelled from the column in a flowthrough fraction but that aggregates would be selectively retained in the column due to their relatively higher hydrophobicity.

The purification protocol is shown in Figure 3.

Equilibration

- 40 µL of resin was equilibrated twice with 200 µL of 20 mM acetate buffer, pH 5.0, in a 96-well plate

Loading

- 200 µL of the conditioned NVIP Protein A pool was added to the equilibtrated resin at 700 rpm for 60 min. Protein load at 50 mg/mL resin
- The flowthrough fraction was collected into a collection plate using a vacuum manifold

Wash

- 200 µL of 20 mM acetate buffer, pH 5.0, was added to the well at 700 rpm for 20 min
- The wash fraction was collected

Regeneration

- The bound proteins were de-adsorbed by adding 200 µL of 0.1 M citrate buffer, pH 3.0
- The regenerated fraction was collected

Fig. 3. Workflow of the process condition screening.

The flowthrough and wash fractions were collected and analyzed by the SEC-UPLC method, and HMW clearance % was calculated by the following equation described by Konrad et al. (2023)[7]:

$$\textit{HMW removal} \text{ in } \% = 100 - \left(\frac{HMW_{FT}}{HMW_{load}} \times 100\%\right)$$

Nuvia wPrime 2A Media resulted in >70% clearance of the HMW species in preliminary testing.

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DoE Analysis for Process Conditions Screening

Next, DoE studies were carried out to further optimize conditions for HMW clearance and recovery. Two factors at three levels were considered, as presented in Figure 4. A multilevel full factorial design was created with two replicates. The total number of runs performed was 18 (2 x 3^2).

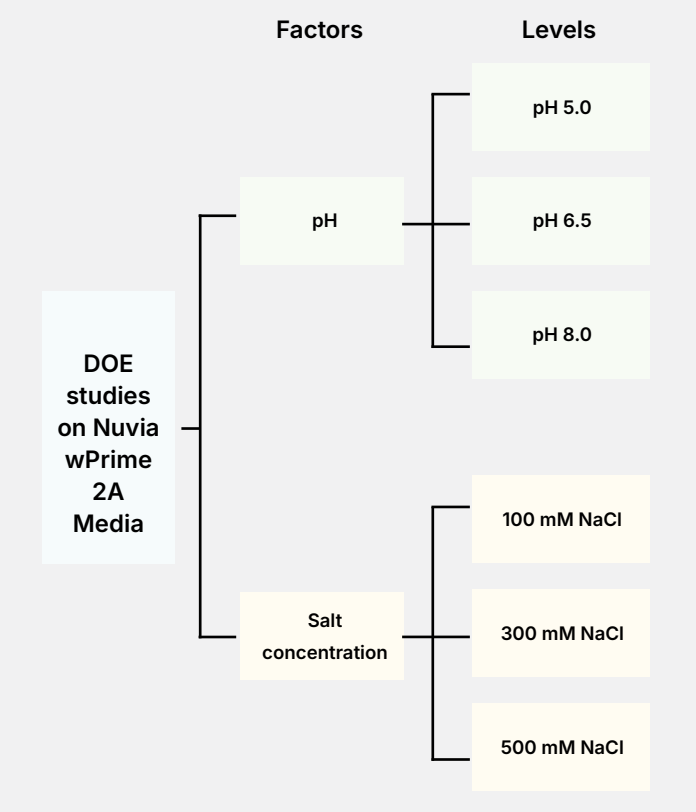


Fig. 4. Factors examined as part of the DoE study.

The output variables analyzed were HMW clearance and mAb monomer recovery. The overlaid contour plots and surface plots generated from the study are shown in Figure 5.

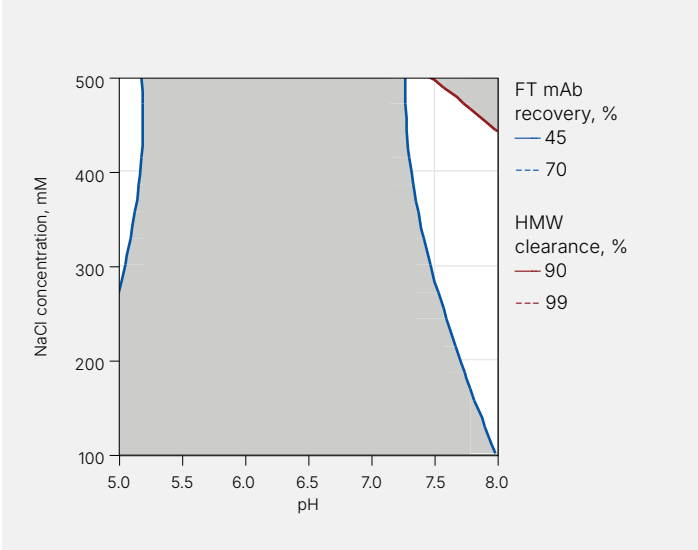


Fig. 5. Overlaid contour plots of mAb recovery and HMW clearance as a function of pH and NaCl concentration (mM). The clear (white) area represents the optimal conditions for recovery and HMW clearance.

The response optimization function in Minitab software predicted that for maximal mAb recovery and HMW clearance, the process must be run at pH 8.0 with 221.2 mM NaCl in the equilibration, load, and wash steps. Hence, these predicted process conditions were implemented in running the on-column final validation.

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Column Validation

The preparative chromatography step was performed as described in the Methods section. The preparative chromatogram obtained during the purification process is provided in Figure 6.

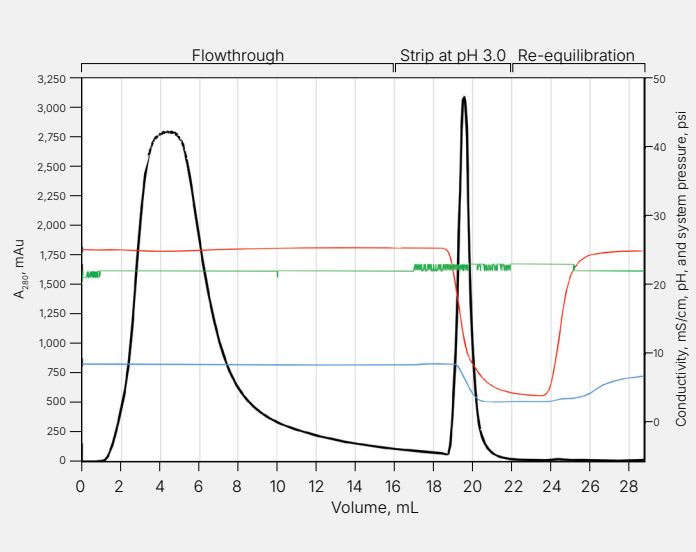


Fig. 6. Preparative chromatogram of on-column validation experiment performed with Nuvia wPrime 2A Media. Flowthrough: 0 mL to 16 mL; strip at pH 3.0: 16 mL to 22 mL; re-equilibration: 22 mL to 28.5 mL.

The process was carried out at a linear flow rate of 76.5 cm/h (0.25 mL/min), which provided a residence time of 2 min. Two CV fractions were collected in a 2 mL deep-well plate using the NGC Fraction Collector. Each collected fraction along with the load was further analyzed by SEC-UPLC to determine the HMW percentage. Subsequently, the appropriate flowthrough and wash fractions were pooled and re-analyzed to determine the final HMW percentage (Figure 7).

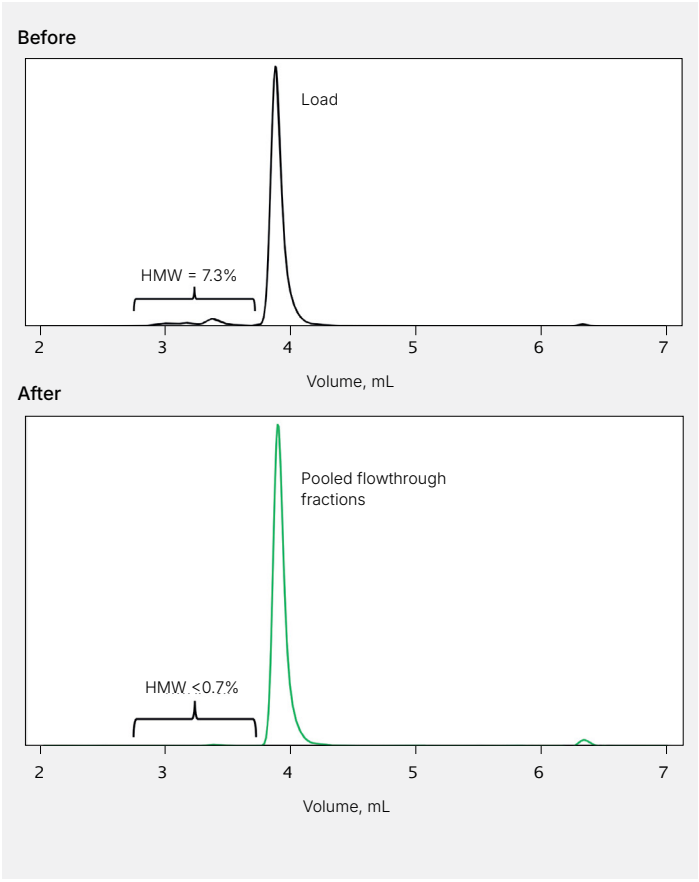


Fig. 7. SEC-UPLC chromatogram of load and pooled flowthrough fractions obtained during the on-column run with Nuvia wPrime 2A Media.

These pooled fractions were also analyzed by the Protein A HPLC assay to determine the final mAb monomer recovery percentage. Nuvia wPrime 2A Media reduced the HMW species from 7.3% in the load to 0.7% in the purified pool while providing total mAb monomer recovery of 85% (Table 3).

Table 3. Overall results from on-column validation performed with Nuvia wPrime 2A Media.

	Load	Final
Monomer content	92.8%	99.3%
Aggregate/HMW	7.3%	0.7% (>1 log-reduction value, LRV)
Recovery		85%

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Conclusion

We performed an initial screen of process conditions for testing a hypothesis based on the ligand pKa and IgG1 pI. This initial screening provided a high level of HMW clearance. We then performed further conditions screening via a DoE approach to achieve maximal HMW clearance and IgG1 monomer recovery. These conditions were validated by column chromatography, achieving >90% HMW clearance and 85% IgG1 monomer recovery.

These results demonstrate the ability of Nuvia wPrime 2A Media to serve as an effective polishing step in mAb purification. Futhermore, the findings highlight the importance of performing a DoE study. Specifically, the study results led us to use the resin in flowthrough mode in a high pH buffer as opposed to in bind-and-elute mode in a lower pH buffer given the mAb's pI. Through a systematic DoE approach, the resin’s adjustable charge feature was leveraged to identify the optimal condition that removed the hydrophobic aggregates while the IgG1 kappa mAb monomer flowed through the column, retaining high monomer recovery. Importantly, the study demonstrates Nuvia wPrime 2A Media’s capability to clear aggregates from a high mAb load concentration (~50 g/L resin), which often is an essential requirement for intensifying processes during continuous manufacturing of biologics.

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