A Purification Strategy for Clinical-Grade Monoclonal Antibody Using Hydrophobic Cation Exchange Chromatography

Xuemei He and Mark Snyder, Bio-Rad Laboratories, Inc., 6000 James Watson Drive, Hercules, CA 94547

Introduction

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 media, and Nuvia™ cPrime™ hydrophobic cation exchange media, to effectively purify a monoclonal antibody from Chinese hamster ovary (CHO) cell culture harvest. Our results demonstrate that this three-step nonaffinity workflow can effectively deliver highly purified monoclonal antibodies with minimal feed conditioning.

Material and Methods

General

Monoclonal antibody mAb1 was produced in CHO cell culture. Protein fractions were analyzed by SDS-PAGE using Criterion™ Tris-HCl 4–20% linear gradient gels (Bio-Rad Laboratories, Inc.) stained with Bio-Safe™ Coomassie stain (Bio-Rad). Mass spectrometry analysis of gel-purified proteins was performed on an autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics). Size exclusion (SEC) high performance liquid chromatography (HPLC) was carried out with a Bio-Sil® SEC 250 column (Bio-Rad), and the content of high molecular weight antibody aggregates was quantified by integration using EZLogic™ integration software (Bio-Rad). The clearance of host cell proteins (HCP) and double-stranded DNAs (dsDNA) was determined by a CHO-CM HCP ELISA kit (Cygnus Technologies) and Quant-iT dsDNA high-sensitivity assay kit (Life Technologies Corporation), respectively. mAb1 concentration was determined by UV absorption at 280 nm using a coefficient of 1.4 OD at 1 mg/ml.

Capture by Nuvia S Media

Chromatographic purification was conducted on a BioLogic DuoFlow QuadTech™ 10 system (Bio-Rad). A 1-ml column (0.56 x 4 cm) packed with Nuvia S media (Bio-Rad) was equilibrated with 15 column volumes (CV) of buffer A (20 mM sodium acetate and 20 mM sodium chloride, pH 4.7). CHO cell culture supernatant containing mAb1 was diluted four times with distilled water and adjusted to pH 4.7 with 1 M phosphoric acid. This material was clarified with a 0.2 µm filter, loaded onto the pre-equilibrated column at 300 cm/hr, and washed with 15 CV of buffer A. The bound mAb1 was eluted in 15 CV of buffer B (20 mM sodium acetate and 200 mM sodium chloride, pH 4.9). The used column was stripped with 5 CV of 1 N NaOH.

Intermediate Polishing by Nuvia Q Anion Exchange Chromatography

A 1-ml column (0.56 x 4 cm) packed with Nuvia Q media (Bio-Rad) was equilibrated with 10 CV of buffer C (10 mM sodium phosphate and 10 mM sodium chloride, pH 7.0). mAb1 eluted from Nuvia S media was adjusted to pH 7.0 with 1 N NaOH and loaded onto the pre-equilibrated column at a linear velocity of 300 cm/hr. This column was then washed with 25 CV of buffer C. mAb1 in the flow-through fractions was collected. The bound protein components were removed by 10 CV of buffer D (100 mM sodium phosphate and 1.5 N sodium chloride, pH 7.2), followed by stripping with 5 CV of 1 N NaOH.

Final Polishing by Nuvia cPrime Chromatography

Further chromatographic separation of mAb1 was conducted on a 1-ml column (0.56 x 4 cm) packed with Nuvia cPrime media (Bio-Rad). This column was equilibrated with 10 CV of buffer E (50 mM sodium acetate and 125 mM sodium chloride, pH 5.0). Pooled mAb1 fractions from the previous purification step were adjusted to pH 5.0 with buffer F (100 mM sodium acetate and 500 mM sodium chloride, pH 4.5). It was then applied to the Nuvia cPrime column at a linear velocity of 300 cm/hr, then washed with 15 CV of buffer E. Elution of mAb1 was performed with a 15-CV linear gradient formed between buffer E and buffer G (50 mM sodium phosphate and 50 mM sodium chloride, pH 6.2), followed by a 5-CV hold in buffer G. The column was then cleaned by 10 CV of buffer H (200 mM sodium phosphate and 700 mM sodium chloride, pH 7.5) and followed by 5 CV of 1 N NaOH at the end of the run. Samples were taken from fractions collected during the entire procedure and analyzed by SDS-PAGE under reducing conditions. A 25-kD protein band appearing in the flow-through fractions was extracted from the gel and identified by mass spectrometry.

Results

Nuvia S is a high-capacity cation exchange media 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 1). 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 mainly dependent on the operation pH (data not shown). We eventually conducted a flow-through purification of mAb1 purification at pH 7.0, slightly below mAb1's determined pI value of 7.2 (Figure 2). Both protein and DNA contaminants were dramatically reduced under this optimized condition (Table 1).

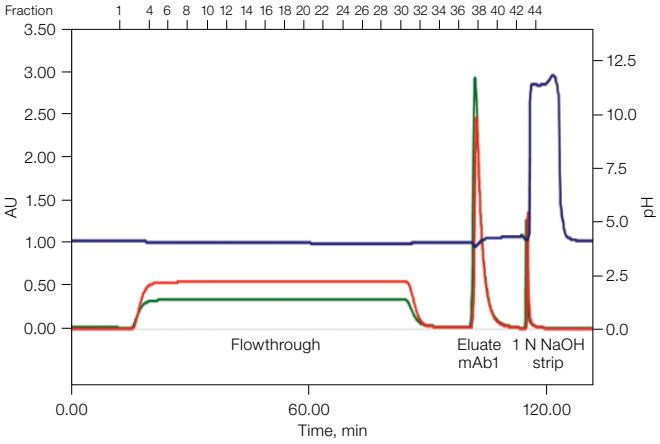


Fig. 1. Capturing mAb1 from CHO cell culture harvest using Nuvia S media. OD 280 (—); OD 260 (—); pH (—). mAb1 eluted from the column (fractions 38–40) was subjected to further purification and purity analysis. AU, absorbance units.

Table 1. Impurity clearance.

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

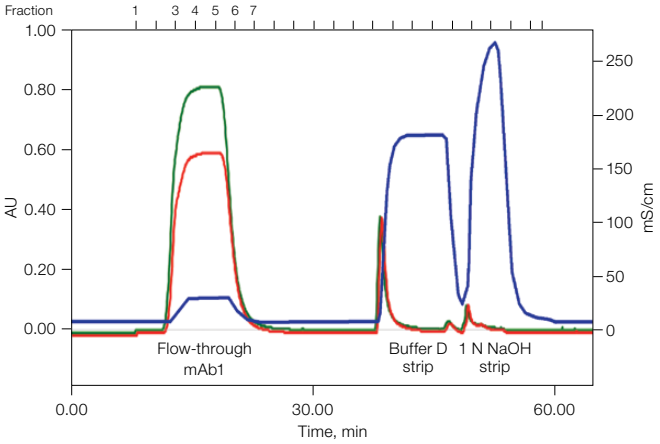
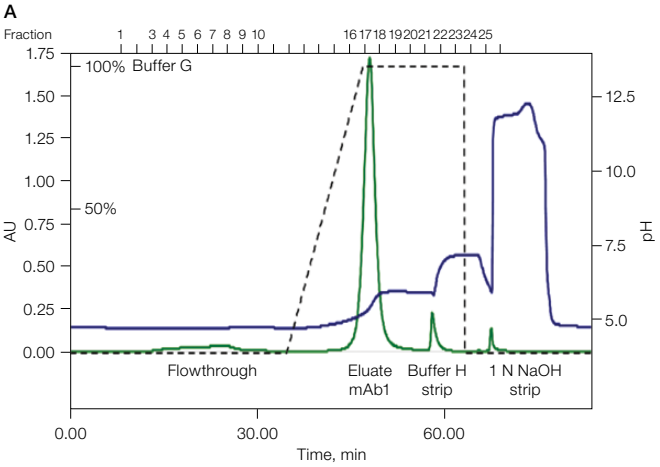
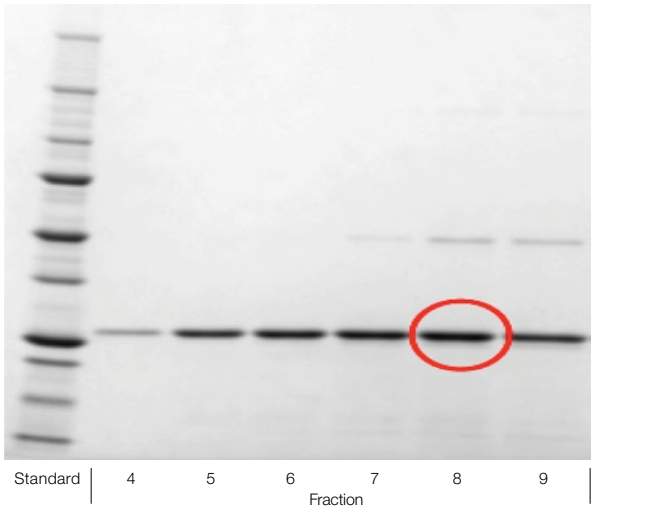


Fig. 2. Intermediate polishing purification of mAb1 in flow-through mode using Nuvia Q media. OD 280 (—); OD 260 (—); pH (—). mAb1 in column flowthrough (fractions 3–6) was subjected to further purification and purity analysis. AU, absorbance units.





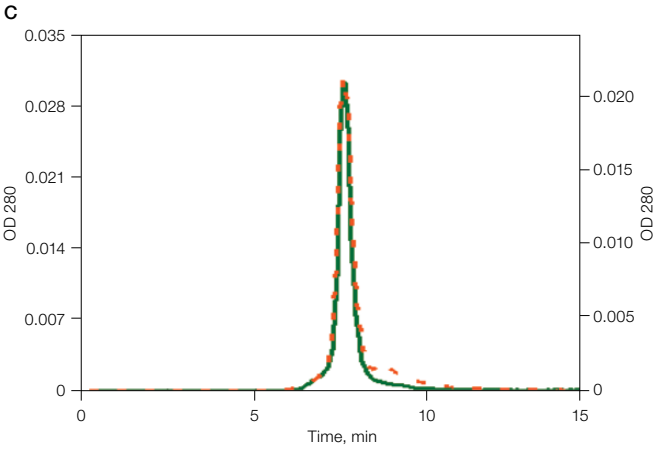


Fig. 3. Final polishing purification of mAb1 using Nuvia cPrime media. **A**, chromatogram of mAb1 purification on a Nuvia cPrime column: OD 280 (—); pH (—); percentage of buffer G (– – –). mAb1 eluted from the column (fractions 17–19) was collected and subjected to purity analysis. **B**, SDS-PAGE analysis of column flowthrough (fractions 4–9); Precision Plus Protein™ unstained standards (Bio-Rad) were used. A major protein impurity was excised from the gel (circled in red) and analyzed by MALDI-TOF mass spectrometry. **C**, SEC-HPLC comparison of pooled Nuvia Q fractions (– – –) and pooled Nuvia cPrime fractions (—). AU, absorbance units.

Nuvia cPrime is a hydrophobic cation exchange media (mixed-mode). Its functional ligand contains structural elements that can interact with biological molecules via electrostatic and hydrophobic forces as well as through hydrogen bonding simultaneously. The level of these interactions is dependent on protein surface properties and the purification conditions. Unlike traditional cation exchange media, Nuvia cPrime media 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 media distinctive from conventional hydrophobic interaction media, which require high salt levels for effective protein binding. Under the selected condition, only the desired full-length mAb1 was retained by the column. A 25-kD L-chain fragment of this antibody appeared in the column flow-through fractions, as visualized on SDS-PAGE and identified by mass spectrometry (Figure 3). 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 media selectively via a combination of ionic and hydrophobic interactions. This chromatographic step was also very effective at eliminating host cell impurities (Table 1).

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 both reducing SDS-PAGE and SEC-HPLC analysis (Figure 4). Nuvia cPrime media's higher affinity for full-length mAb1, 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 mAb1.

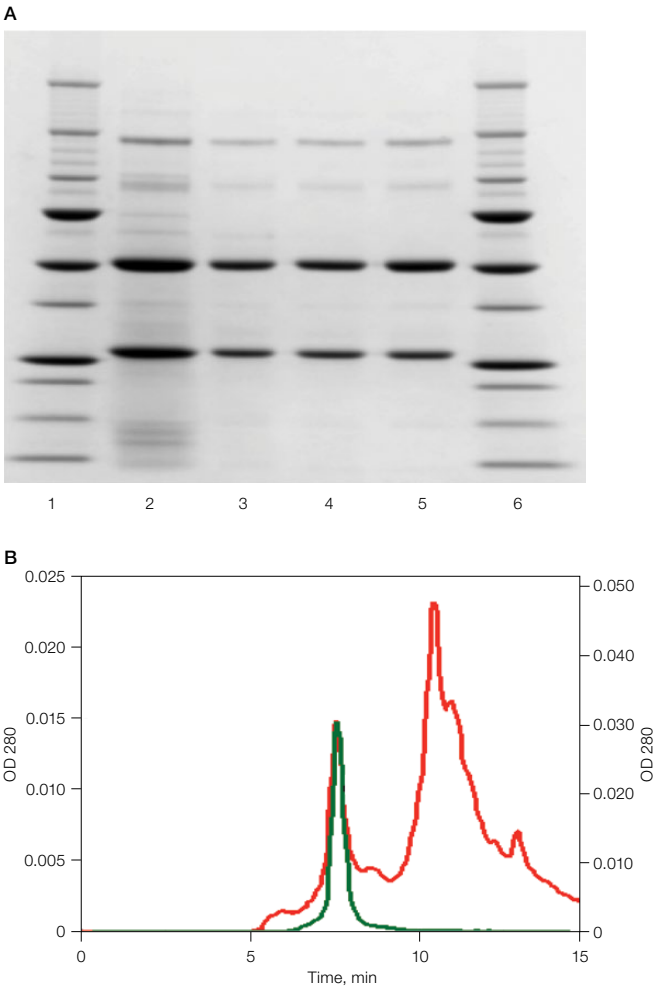


Fig. 4. Three-step purification of mAb1 using Nuvia cPrime media. **A**, SDS-PAGE analysis of mAb1 chromatographic fractions. Lanes 1 and 6, Precision Plus Protein unstained standards; lane 2, cell culture supernatant; lanes 3, 4, and 5, Nuvia S, Nuvia Q, and Nuvia cPrime media chromatographic pools, respectively. **B**, SEC-HPLC comparison of cell culture supernatant (—) and purified mAb1 (—).

Conclusions

Two ion exchange media, Nuvia S and Nuvia Q, were used in the first two steps to efficiently capture target mAb1 from cell culture harvest and to effectively remove impurities produced during the fermentation process. The extraordinarily high binding capacities of these chromatography media make it possible to process increasing volumes of high titer feed with existing production facilities and minimal buffer consumption, easing the pressure on capital investment and process development timelines. In the final polishing step, the orthogonal interaction modes afforded by Nuvia cPrime media offer unique selectivity for full-length monomeric mAb1 molecules. This media is a powerful tool for the removal of both product-related impurities and host cell contaminants. The versatility of these media has allowed us to arrange the order of these three chromatography steps in a sequence that requires no buffer exchange and minimal handling. Transitions between process steps are easy and straightforward. This process is designed to shorten cycle time and reduce buffer consumption, thus improving overall productivity. All chromatography media are base stable and mechanically resilient (Bio-Rad bulletins 5987, 6129, and 6242). They can be operated at high flow rate with low backpressure, providing the productivity, robustness, and process economics demanded by today's downstream manufacture of biopharmaceuticals.

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