Purification of a Recombinant Monoclonal Antibody — a Rituximab Biosimilar — with a Non–Affinity Based Chromatographic Process

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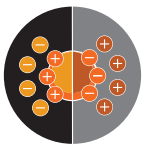

ION EXCHANGE

MIXED-MODE

Abstract

While Protein A resins are capable of producing high selectivity and purity, they also have the disadvantage of high cost and the need to use a low pH for elution, which can cause aggregation and limit resin lifetime. To address these issues, we evaluated the purification of a recombinant monoclonal antibody (mAb), a rituximab biosimilar, using two non–Protein A purification schemes. These were a cation exchange (CEX) resin for capture purification followed by an anion exchange (AEX) resin for intermediate purification and two mixed-mode media (CHT™ Ceramic Hydroxyapatite XT Media; hydrophobic cation exchange, Nuvia cPrime Media) for polish purification. Simultaneous purification was performed with a Protein A process. The CEX–AEX–CHT XT workflow yielded >96% purity and ~86% yield of rituximab, indicating a more economical process. Host cell protein (HCP) levels were reduced by 4 logs and host cell DNA (hcDNA) levels by 5 logs after the final purification step. A pharmacokinetic (PK) bridging assay demonstrated the purified monoclonal antibody behaved in a similar way to a rituximab standard, showing no quantifiable loss of structure or function throughout purification. We therefore propose that a non–affinity based purification workflow for a rituximab biosimilar provides better process economics and has the potential to facilitate uncompromised results.

Introduction

Protein A–based chromatographic processes are often used for the purification of antibodies because Protein A binds selectively and efficiently to antibodies in complex cell culture supernatants and removes the majority of product impurities in a single step. The major disadvantages of chromatographic workflows with Protein A as the capture step include protein aggregation, high cost, poor resin stability, and a long lead time for delivery. An alternative approach, one that considers process economics, is the use of non-affinity resins like cation exchange chemistries. According to Follman and Fahrner (2004), it is feasible to create three-step purification schemes based on non-affinity resins that can compete with three-step chromatographic workflows that start with Protein A. The results of this study impressively indicate that the order of process steps is important for the successful establishment of a high-performance antibody purification scheme.

Here we have evaluated the performance of well-proven orthogonal resin combinations (Figure 1) for the non-affinity purification of a rituximab biosimilar (144 kD), which is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. A high-capacity cation exchanger (Nuvia S Resin) was chosen for the initial purification step because of its high capture performance over a wide range of operating conditions. Additionally, it is well-known to efficiently remove product-related contaminants, such as HCPs and monoclonal antibody (mAb) aggregates and fragments. For intermediate purification, the cation exchange step is commonly followed by an anion exchange step, performed in flow-through mode, for further reduction of remaining impurities such as hcDNA. In the final polish step, mixed-mode resins like CHT XT (a ceramic hydroxyapatite) or Nuvia cPrime (a hydrophobic cation exchanger) offer unique selectivity for the final

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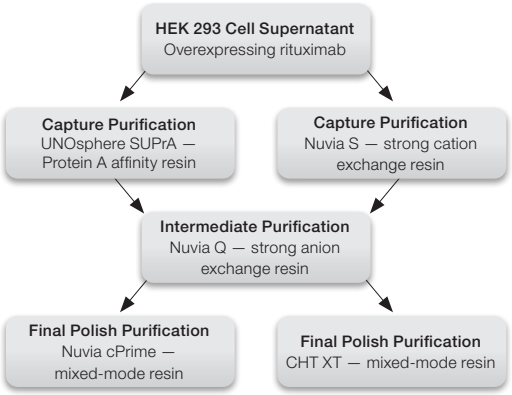


Fig. 1. Different processes using three chromatography steps for the purification of a rituximab biosimilar.

removal of both product- and process-related impurities. In order to compete against a Protein A–based chromatography workflow, it was critical to determine the optimal operating conditions for the resins in key positions, like Nuvia S and Nuvia cPrime. Operating conditions were determined by using simple screening experiments in spin columns. To rate the success of the individual chromatographic steps, important process performance parameters, such as mAb purity and yield, were measured. Antibody integrity was evaluated with an anti-idiotypic antibody pharmacokinetic bridging assay.

Materials and Methods

Biological Material and Sample Preparation

HEK 293 cell culture supernatant containing a rituximab biosimilar (Absolute Antibody, Catalog #Ab00126-10.0) was centrifuged to remove cellular debris and dialyzed (20 kD cutoff membrane) overnight in the corresponding binding buffer of Protein A or Nuvia S Resin (Table 1). Protein concentration after dialysis was about 0.35 mg/ml containing 6–8% of monoclonal antibody. Alternatively, cell culture supernatant containing the rituximab biosimilar was diluted 1:1 (v/v) with phosphate buffered saline (PBS) for Protein A chromatography or the pH was adjusted to 6.0 and further diluted 1:1 (v/v) with 1x binding buffer for Nuvia S chromatography. Buffer exchange of the protein pools between chromatographic runs was performed with ultrafiltration devices (10 kD cutoff) in a spin column format.

Chromatography

All equipment and resins were from Bio-Rad Laboratories, Inc. (Table 1). Chromatography was monitored at 280 nm and performed at room temperature using an NGC 10 Medium-Pressure Chromatography System. The four-tier chromatography system was configured with a sample pump, including sample inlet valve, two buffer inlet valves, column switching valve, multi-wavelength ultraviolet (UV) detector, pH and conductivity meter, outlet valve, and fraction collector with a benchtop Peltier cooling option. The chromatography operating conditions are presented in Table 1. The buffers were degassed and filtered through 0.45 µm membranes before use. Cell culture supernatant (50 ml) was used for chromatography.

Purification Condition Screening for Nuvia S and cPrime Resins in Spin Columns

The chromatographic conditions for Nuvia S Resin were analyzed by a simple pH screening experiment (Table 2) whereas for Nuvia cPrime Resin a more complex approach was appropriate (Table 3). The corresponding step-by-step protocol is described in bulletin 7128. In brief, the spin columns were loaded with 100 µl chromatography resin and equilibrated with the binding buffers to be tested. Five hundred microliters of cell supernatant (0.25 µg/µl), buffer exchanged into its respective binding buffer, were added and incubated with the resin at room temperature for 10 min with agitation (end-over-end mixer). Unbound material was removed by centrifugation for 1 min at 1,000 x g. The resin was washed two times with binding buffer and eluted twice. For yield estimations, the resin was stripped with 2% SDS solution to detect any remaining protein material on the resin. The samples collected from the spin column experiments were qualitatively analyzed by SDS-PAGE under nonreducing conditions.

Table 1. Overview of all columns and their operating conditions for the chromatographic purification of rituximab biosimilar.

Column Type	Resin Type	Mode of Operation	Workflow Position	Buffer A	Buffer B	Flow Rate, cm/hr
Bio-Scale Mini UNOsphere SUPrA (5 ml)	Protein A	Bind and elute	Capture step	10 mM phosphate, 27 mM KCl, 140 mM NaCl, pH 7.4	100 mM citrate, pH 3.3; step elution	240–480
Nuvia S (1 ml)	Strong cation exchanger	Bind and elute	Capture step	25 mM MES, 10 mM NaCl, pH 6.0	Buffer A with 1 M NaCl; 10 column volumes (CV) gradient to 1 M NaCl or step elution	240
Nuvia Q (1 ml)	Strong anion exchanger	Flowthrough	Intermediate step	50 mM Tris, pH 8.5	Buffer A with 1 M NaCl; 10 CV gradient to 1 M NaCl or step elution	120
Nuvia cPrime (1 ml)	Hydrophobic cation exchanger (mixed mode)	Bind and elute	Polishing step	50 mM Tris, pH 8.5	Buffer A with 1 M NaCl; 10 CV gradient to 1 M NaCl	240
CHT XT (1 ml)	Ceramic hydroxyapatite (mixed mode)	Bind and elute	Polishing step	10 mM phosphate, pH 7.0	Buffer A with 2 M NaCl; 20 CV gradient to 2 M NaCl or step elution	240

For Nuvia cPrime Resin, the effects of buffer pH and conductivity were evaluated by JMP Software to predict optimal binding and elution conditions.

Table 2. pH screening experiment in spin columns of Nuvia S Resin for the purification of a rituximab biosimilar. Buffer molarity: 25 mM. Condition #3 was applied in chromatography (capture step).

	Binding		Elution	
Condition	pH	NaCl, mM	pH	NaCl, mM
1	4.5	10	4.5	1,000
2	5.5	10	5.5	1,000
3	**6**	**10**	**6**	**1,000**
4	6.5	10	6.5	1,000
5	7	10	7	1,000

Analytical Assays

Protein concentrations were measured with the *DC* Protein Assay Kit I (Bio-Rad, catalog #5000111).

Antibody aggregate detection was performed using size exclusion chromatography (SEC) with a prepacked high-resolution ENrich SEC 650 Size Exclusion Column (10 x 300 mm, 24 ml; Bio-Rad, #7801650) at a flow rate of 1 ml/min on the NGC Chromatography System. Filtered PBS was used as the equilibration buffer and peaks were recorded at 215 nm. Two hundred microliters of sample (adjusted for protein amount) were injected into the chromatography system and aggregates were defined as the front eluting peak of the SEC profile. Fractions (1 ml) were collected and analyzed with SDS-PAGE under nonreducing conditions.

HCP levels were analyzed with the HEK 293 HCP ELISA Kit (Cygnus Technologies, #F650R). The lower limit of detection of this kit is about 1–2 ng/ml.

Levels of hcDNA in chromatographic fractions were measured with Droplet Digital™ PCR (ddPCR™). Reactions were made using samples diluted 1:25 to 1:200 in TE Buffer and ddPCR Supermix for Residual DNA Quantification (Bio-Rad, #1864038). A proprietary probe assay for a multicopy human gene was used. A standard curve using BamH1-digested human genomic DNA was generated to verify that the samples were analyzed in the linear range of the assay. Droplets were generated using the Automated Droplet Generator (Bio-Rad, #1864101) according to instructions. Following amplification, droplets were read in a QX200 Droplet Reader (Bio-Rad, #1864003) and analyzed using QuantaSoft™ Software (Bio-Rad, #1864011).

A pharmacokinetic bridging enzyme-linked immunoabsorbent assay (ELISA) was carried out according to the provided protocol: bio-rad-antibodies.com/protocol-pk-bridging-elisa-rituximab-antibodies.html.

Electrophoresis

Samples from the purification condition screening in spin columns and the chromatographic purification steps were monitored for purity and yield by SDS-PAGE with Criterion TGX Stain-Free Precast Gels (4–20% midi gels; Bio-Rad, #5678093). HEK 293 cell culture supernatant containing the rituximab biosimilar was characterized with two-dimensional (2-D) electrophoresis (Posch et al. 2013). In brief, after sample preparation according to Ambort et al. (2008), 100 µg protein were loaded onto 11 cm ReadyStrip IPG Strips, pH 3–10 (Bio-Rad, #1632014) and separated according to charge for 40 kVh. For the second dimension, Any kD Criterion TGX Stain-Free Precast Gels (Bio-Rad, #5678121) were used. SDS-PAGE gels and 2-D gels were UV activated after the run for 2.5 and 5 min, respectively. The corresponding Stain-Free images were recorded with a ChemiDoc™ MP Imaging System (Bio-Rad, #12003154). After imaging, the gels were counterstained with Oriole Fluorescent Gel Stain (Bio-Rad, #1610495), followed by QC Colloidal Coomassie Stain (Bio-Rad, #1610803). Gel analysis was performed with Image Lab Software 6.01 and PDQuest 2-D Analysis Software from Bio-Rad Laboratories.

Results and Discussion

Two-Dimensional Electrophoresis of HEK 293 Cell Culture Supernatant Containing the Rituximab Biosimilar

Two-dimensional electrophoresis is a high-resolution technique for the characterization of complex protein mixtures. The technique is usually performed under denaturing and reducing conditions, thus only protein subunits can be displayed on a 2-D gel. Figure 2 shows

Table 3. A wide range custom design for spin column experiments with Nuvia cPrime Resin for the purification of a rituximab biosimilar. Includes data analysis with SDS-PAGE. Buffer molarity: 50 mM. Condition #9 was applied in chromatography (final polish).

	Binding			Elution			
Condition	pH	NaCl, mM	Binding Target, %	pH	NaCl, mM	Target Yield, %	Target Purity, %
1	5.5	0	96	5.5	0	0	N/A
2	5.5	0	99	8.5	0	0	N/A
3	5.5	400	68	5.5	1,000	45	38
4	5.5	400	70	8.5	1,000	42	32
5	7	200	1	7	500	0	N/A
6	7	200	1	7	500	0	N/A
7	7	200	1	7	500	0	N/A
8	8.5	0	99	5.5	1,000	82	65
9	**8.5**	**0**	**98**	**8.5**	**1,000**	**91**	**68**
10	8.5	400	0	5.5	0	0	N/A
11	8.5	400	0	8.5	0	0	N/A
12	8.5	400	0	8.5	0	0	N/A

a typical 2-D gel (pH range 3–10) of cell supernatant containing rituximab, which has a theoretical pI of about 8.6. Light and heavy chains of rituximab were localized on the 2-D map by comparing against cell supernatant sample without rituximab (data not shown). Image analysis was performed with PDQuest Software and the pI values of the heavy and light chains of rituximab were calculated to be about 8.9 and 8.5, respectively. These values are consistent with the theoretical values of 8.7 and 8.4. In general, pI information on target proteins is helpful when developing chromatographic methods for the separation of molecules on the basis of their charge, as in ion exchange chromatography.

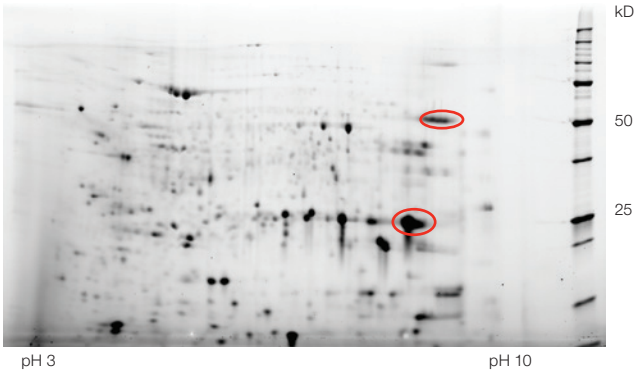


Fig. 2. 2-D electrophoresis of supernatant using a Stain-Free gel. HEK 293 supernatant expressing rituximab biosimilar was analyzed by first separating proteins according to their pI using an 11 cm IPG strip followed by a size separation with SDS-PAGE. Heavy and light chains are circled in red. *Rituximab biosimilar content was about 8%.*

The 2-D map shown in Figure 2 provides an excellent overview of the nature of contaminating proteins. In total, about 550 contaminating protein spots were detected and the majority of them were located in the pH range between 3.5 and 8. Rituximab, in contrast to the majority of contaminating proteins, showed positive net charge characteristics. Therefore, it is possible to successfully apply cation exchange chromatography as a capture step in a three-step purification scheme while a significant amount of HCP remains unbound.

Semi-quantitative 2-D gel analysis is also capable of providing information about the relative abundance of a protein of interest in a complex sample. Here, rituximab biosimilar expression in the cell supernatant was exceptionally low (8% of the total protein content) compared to an industrial mAb-producing cell line. To produce this antibody at production scale, additional upstream work to increase expression would have been required.

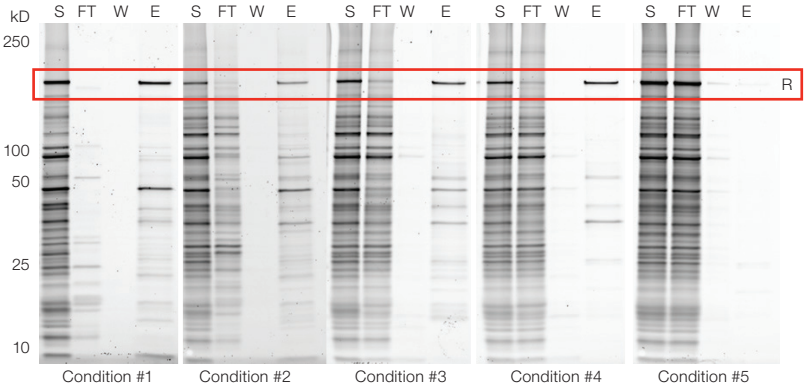
Purification Condition Screening

Nuvia S Strong Cation Exchange Resin

The purpose of this simple pH screening experiment in spin columns was to find chromatographic conditions with good binding of the rituximab biosimilar to the resin and a significant amount of HCPs in the flow-through fractions. The only variable changed was the pH of the binding buffer (pH 4.5–7). The protein samples were analyzed by nonreducing SDS-PAGE (Figure 3), and qualitative gel analysis revealed strong binding of rituximab to the resin between pH 4.5 and about 6.5. At pH 7, binding of the rituximab biosimilar to the resin became negligible and most of the target protein could be found in the flow-through sample. The highest amount of contaminating proteins in the flow-through samples was detected at pH 6 and 6.5. Consequently, all chromatographic runs with Nuvia S Resin as capture step were performed with 25 mM MES, 10 mM NaCl, pH 6.0. Working at pH 6.0 also offers more stability in handling higher conductivity conditions of the starting sample compared to higher pH values.

Nuvia cPrime Resin

Mixed-mode chromatography is becoming an important tool for downstream process purification, owing to the fact that the media have a large design space for binding and elution, allowing for the development of highly robust methods in a commercial manufacturing setting. However, the behavior of an antibody during purification by mixed-mode chromatography is often not predictable due to the joint action of multiple functionalities present on the ligands. To account for the specific characteristics of mixed-mode media, including their high salt tolerance, a wide range custom design was used to elucidate initial binding and elution conditions for the rituximab biosimilar on Nuvia cPrime Resin. The samples of the spin column experiments were analyzed



	Binding		Elution	
Condition	pH	NaCl, mM	pH	NaCl, mM
1	4.5	10	4.5	1,000
2	5.5	10	5.5	1,000
3	6	10	6	1,000
4	6.5	10	6.5	1,000
5	7	10	7	1,000

Fig. 3. Screening experiment using pH. Nuvia S Resin in spin columns was used to find chromatographic conditions with good binding of the rituximab biosimilar **(R)** to the resin and a significant amount of HCPs in the flow-through fraction. Samples were evaluated by SDS-PAGE with Stain-Free technology under nonreducing conditions. **S**, HEK 293 supernatant containing rituximab biosimilar; **FT**, flowthrough; **W**, wash fraction in binding buffer; **E**, eluate.

on nonreducing SDS-PAGE and quantitatively evaluated for binding capacity and yield (Table 3). Then purity calculations were performed as well and the obtained datasets were used to predict binding and elution conditions (Figure 4). The results suggest that the rituximab biosimilar employs very different modes of action with Nuvia cPrime Resin under the conditions explored. Although more extensive design of experiments could be performed to perfect and determine edge of failure of the chromatographic buffer conditions, buffer condition #9 represents an acceptable compromise concerning yield and purity of the rituximab biosimilar.

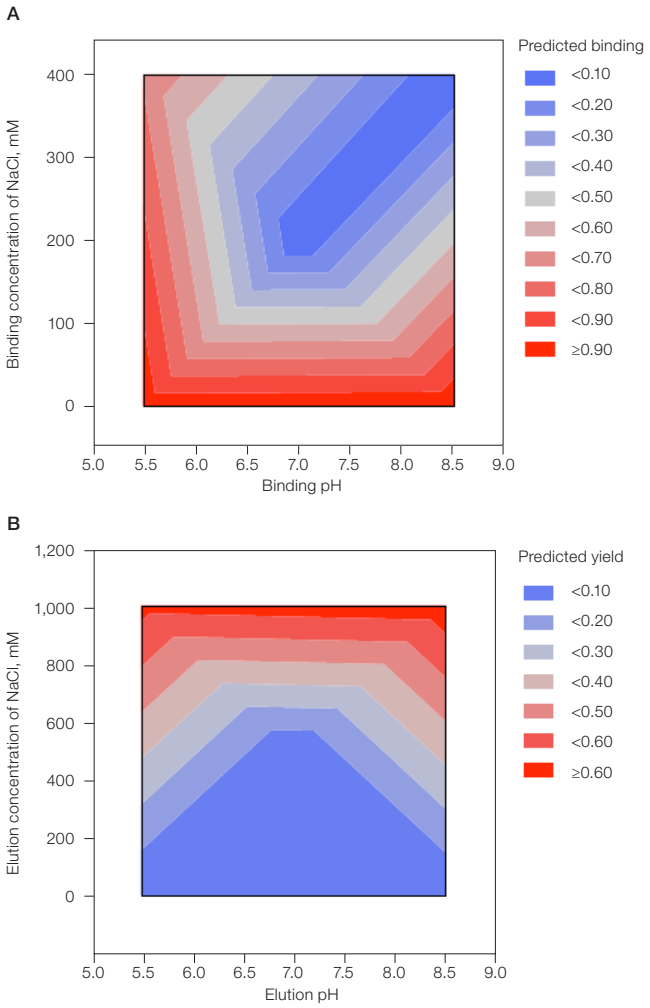


Fig. 4. Predicted purification conditions for a rituximab biosimilar using Nuvia cPrime Resin. A, effect of buffer pH and concentration of NaCl on binding capacity; **B**, effect of buffer pH and concentration of NaCl on yield.

Performance Comparison of Four Different Three-Step Chromatographic Workflows

A purification process composed of Protein A chromatography as the robust capture step followed by two additional purification steps is typical. For the subsequent intermediate and polishing steps, various resin combinations have been described in literature. Among those resins are, for example, cation exchange, anion exchange, mixed-mode ion exchange, ceramic hydroxyapatite, and hydrophobic interaction.

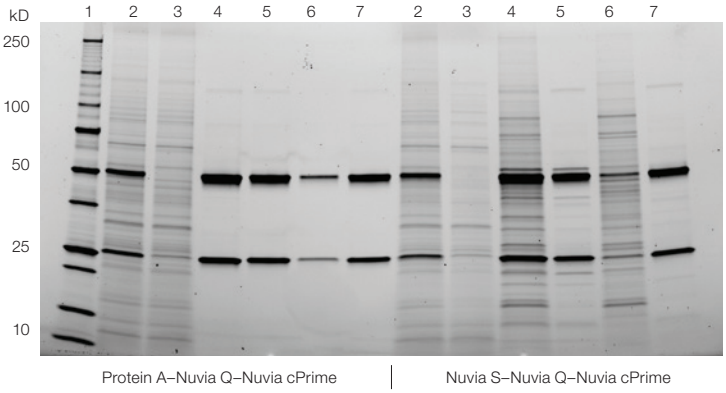
Here we were interested in whether the two non–affinity based processes illustrated in Figure 1 could achieve a similar level of purification compared to a purification scheme starting with Protein A. In the two non–affinity based workflows, Protein A was replaced by Nuvia S Resin as the binding step. Nuvia S is an ultra-high capacity, innovative cation exchange resin built on the industry proven UNOsphere base matrix technology. Nuvia Q Anion Exchange Resin was applied for intermediate polishing in flow-through mode across all workflows. Final polishing was performed with one of two mixed-mode media, either Nuvia cPrime or CHT XT.

Host cell protein levels were measured in all sample pools and served as the main indicator of target protein purity (Table 4). In addition to the HEK 293 HCP ELISA, the protein fractions of all workflows were analyzed by SDS-PAGE with Stain-Free technology to monitor the success and progress of each purification step (Figures 5 and 6), detect contaminating proteins, and measure both purity and yield. The antibody capture step with Protein A affinity chromatography followed by anion exchange chromatography removed more than 99% HEK 293 HCPs. Following the final polishing step, performed with either Nuvia cPrime Resin or CHT XT Media, the host cell protein level was determined to be below the detection limit of the HEK 293 HCP ELISA. SDS-PAGE analysis of the respective sample pools confirmed the high-purity results of the HEK 293 HCP ELISA, since no distinct contaminating protein bands were observed in either final sample.

In both non–affinity based chromatographic workflows, Nuvia S Resin showed acceptable binding affinity of rituximab at pH 6, as indicated by the lane comparison of the feedstream and the flow-through and eluate fractions in Figures 5 and 6. The combination of Nuvia S and Nuvia Q Resins was able to remove a significant portion of contaminating HEK 293 proteins and diminished the HCP content to about 450 ppm. For the final polishing step, the sequence of purification of having Nuvia Q Resin as the intermediate step followed by Nuvia cPrime Resin as the polish step was attractive because no buffer exchange is

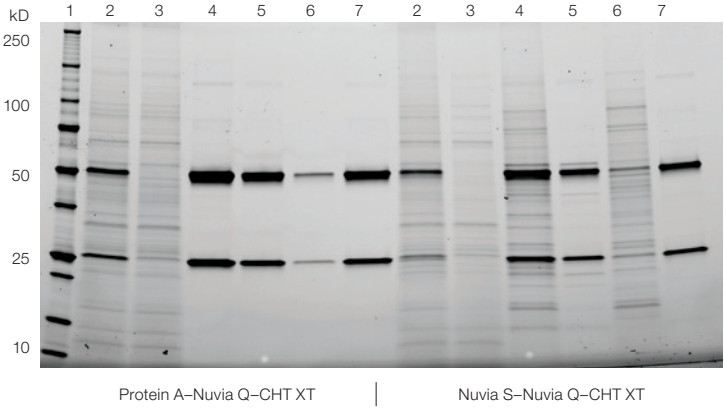
Table 4. HCP removal by different workflows for rituximab biosimilar purification.

Steps	→	Capture	Intermediate	Polish		
Protein A workflow	Cell supernatant	Protein A eluate pool	Nuvia Q flow-through pool	Nuvia cPrime eluate pool	or	CHT XT eluate pool
HCP, ppm	1,100,000	200 40,000	60 450	<2 250		<2 20
Nuvia S workflow	Cell supernatant	Nuvia S eluate pool	Nuvia Q flow-through pool	Nuvia cPrime eluate pool	or	CHT XT eluate pool



Lane	Sample
1	Marker
2	Cell culture feed
3	Flow-through pool
4	Protein A/Nuvia S eluate pool
5	Nuvia Q flow-through pool
6	Nuvia Q eluate pool
7	Nuvia cPrime eluate pool

Fig. 5. SDS-PAGE analysis of the chromatographic samples from Protein A– or Nuvia S–Nuvia Q–Nuvia cPrime workflow.



Lane	Sample
1	Marker
2	Cell culture feed
3	Flow-through pool
4	Protein A/Nuvia S eluate pool
5	Nuvia Q flow-through pool
6	Nuvia Q eluate pool
7	CHT XT eluate pool

Fig. 6. SDS-PAGE analysis of the chromatographic samples from Protein A– or Nuvia S–Nuvia Q–CHT XT workflow.

necessary between the two chromatographic steps. However, a few distinct protein bands are still visible after Nuvia cPrime chromatography, yielding a final HCP value of 250 ppm. On the other hand, the final polishing step for rituximab with CHT XT Media seems to be more effective than Nuvia cPrime Resin, with HCP levels as low as 20 ppm. Typical chromatograms of the workflow Nuvia S–Nuvia Q–CHT XT are displayed in Figures 7A–C. The purity of rituximab was evaluated for the latter workflow by quantitative SDS-PAGE image analysis and was in the range of 96–98%. In addition, hcDNA clearance was measured with Droplet Digital PCR in two chromatographic workflows (Protein A– or Nuvia S–Nuvia Q–CHT XT) and the corresponding levels were reduced by 5 logs (Table 5).

In the course of this project, process performance parameters, such as antibody aggregates and antibody integrity, were measured. However, chromatographic workflows with Nuvia cPrime Resin were not considered for further analysis.

Qualitative, Quantitative, and Functional Assays

Quantitation of Rituximab with Stain-Free Technology

For yield and recovery calculations of antibody concentrations in the cell culture feed and in sample pools generated during chromatography, a Protein A or G high-performance liquid chromatography (HPLC) assay is usually applied. Column selection depends on binding affinity of Protein A and G to different human and mouse IgG subclasses. Those assays have a cycle time of 2–4 minutes and are capable of quantitating mAbs in the range of 0.01–5 mg/ml. Here, we have evaluated the applicability of SDS-PAGE in combination with Stain-Free technology for antibody concentration measurements as a low-cost alternative to Protein A/G HPLC assays. Stain-Free technology employs in-gel chemistry available in precast SDS-PAGE gels. The gel formulation incorporates a trihalo compound, which, when exposed to UV irradiation, catalyzes a covalent reaction to tryptophan residues. The resulting activated protein fluoresces under UV excitation and can be readily detected within the gel by suitable imaging systems.

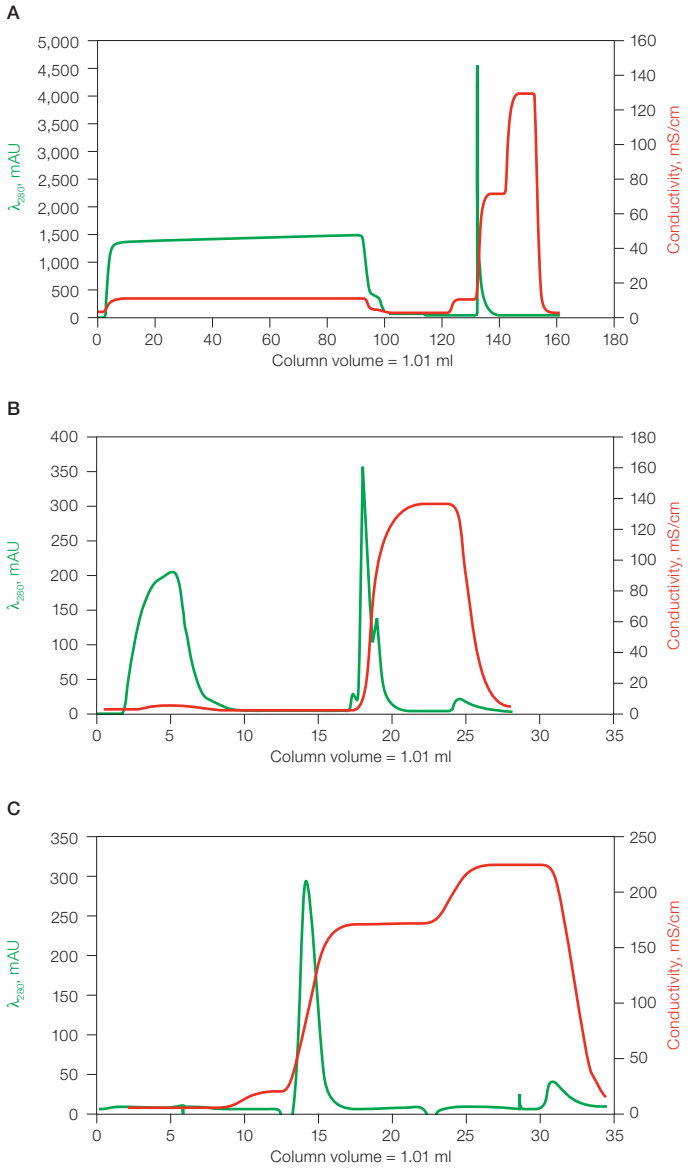


Fig. 7. Chromatograms from the purification of a rituximab biosimilar. **A**, capture step with Nuvia S Resin; **B**, intermediate polish purification with Nuvia Q Resin; **C**, final polish purification with CHT XT Media.

Sensitivity and linearity of the Stain-Free approach was evaluated by running a dilution series of a commercially available rituximab biosimilar (Protein A purified) under nonreducing conditions. Band intensities were automatically calculated with the Auto-Analysis tool in Image Lab Software and the corresponding calibration curve, ranging from 125 to 4,000 ng per lane, is shown in Figure 8. The graph indicates good linearity between the two sample loading extremes with a coefficient of determination of 0.99.

A

Lane Number	Rituximab, ng/lane
1	4,000
2	2,000
3	1,000
4	500
5	250
6	125

B

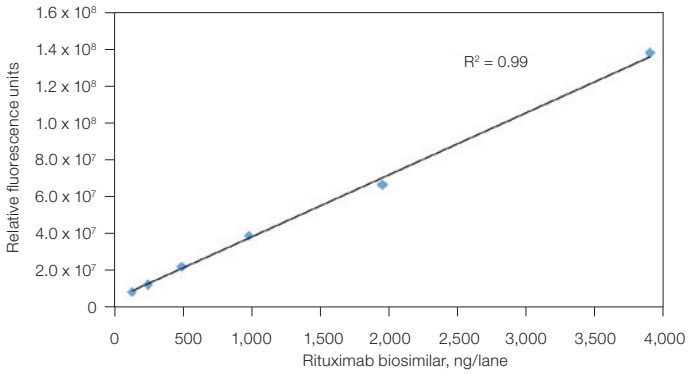


Fig. 8. Electrophoretic Stain-Free assay for the semiquantitative analysis of a rituximab biosimilar in chromatographic samples. A, SDS-PAGE of rituximab biosimilar; **B**, intensity of Stain-Free vs. rituximab biosimilar, ng/lane.

Table 5. hcDNA removal by the two major workflows for rituximab biosimilar purification.

Sample	Protein A Workflow hcDNA Removal, %	Nuvia S Workflow hcDNA Removal, %
Capture eluate pool	99.97	75.06
Nuvia Q flow-through pool	100.00	99.53
CHT XT eluate pool	100.00	100.00

Table 6. Step and process yields calculated for the two major workflows for rituximab biosimilar purification.

	Yield, %			
Chromatographic Process	Capture	Intermediate	Polish	Overall
Protein A → Nuvia Q → CHT XT	97	98	95	90
Nuvia S → Nuvia Q → CHT XT	93	98	95	86

Yields were calculated for all individual chromatography steps and indicated that the step yield for Protein A (97%) is slightly higher compared to that for Nuvia S Resin (93%). Nuvia Q Resin (98%) showed almost no protein losses and the step performance for CHT XT Media was 95%. Thus, we observed an overall yield of 86% for the non-affinity process while the yield for the control Protein A process was 90% (Table 6). Possible protein losses between individual NGC runs due to buffer exchange were not considered in those calculations and may reduce overall yield by 5% in each step. Theoretical recovery of those spin columns is 90–95%, according to the technical specifications.

Rituximab Aggregate Analysis With Size Exclusion Chromatography

Aggregate detection and removal is an essential component of mAb purification (Xu et al. 2012). Elevated levels of antibody aggregates are often associated with the overproduction of mAbs. In addition, the strong acidic elution conditions needed for Protein A chromatography can promote unwanted oligomerization. In this study, possible rituximab aggregates were analyzed in samples obtained after the capture steps with Protein A or Nuvia S Resin and after the final polishing step with CHT XT Media. Figure 9 provides an overview of the corresponding SEC profiles, which were evaluated by quantitative SDS-PAGE analysis. Aggregate measurement data after Protein A purification (Figure 9A, red arrows) show 5.5% aggregate levels while data after Nuvia S purification (Figure 9A, blue arrows) show only 1%. However, in both purification workflows, CHT XT Media reduced the final aggregate content to less than 0.7% (Figure 9B, green arrows, Table 7).

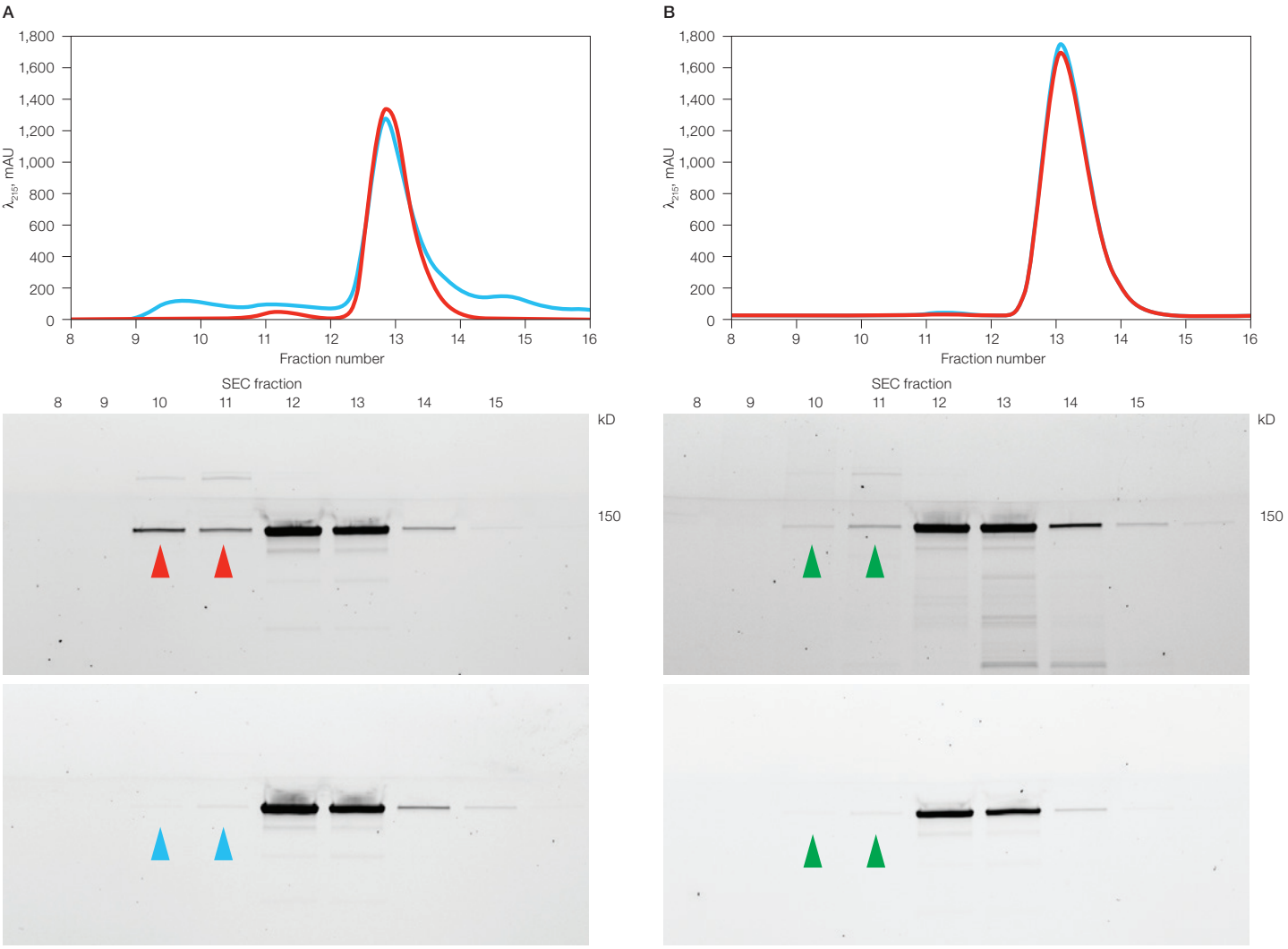


Fig. 9. Rituximab aggregate analysis with SEC and SDS-PAGE with Stain-Free technology. Arrows indicate samples used to evaluate aggregate levels as shown in Table 7. **A**, SEC after Nuvia S (—), SEC after Protein A (—); **B**, SEC after Nuvia S-Nuvia Q–CHT XT (—), SEC after Protein A–Nuvia Q–CHT XT (—).

Table 7. Aggregate percentages for the two major workflows for rituximab biosimilar purification.

	Aggregate, %	
Chromatographic Process	**Postcapture**	**Final**
Protein A → Nuvia Q → CHT XT	5.5	0.5
Nuvia S → Nuvia Q → CHT XT	1	0.7

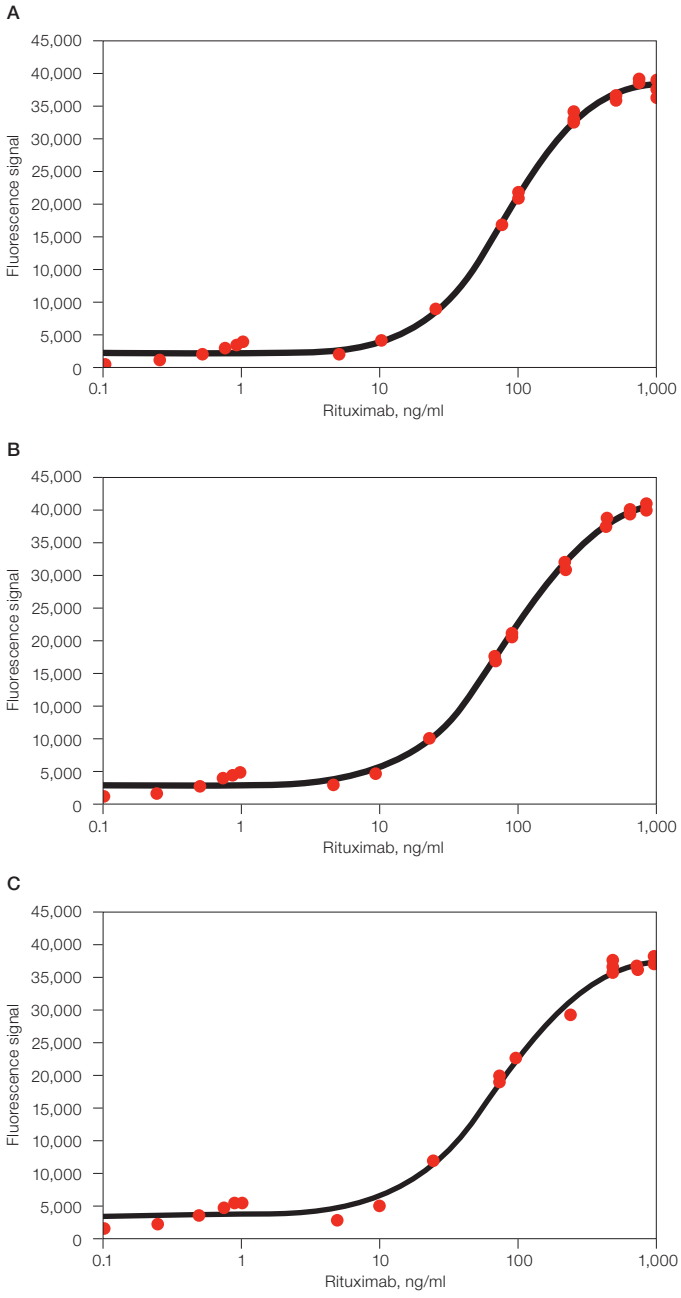


Fig. 10. PK bridging ELISA of standard and purified rituximab biosimilar. **A**, rituximab standard; **B**, Protein A–Nuvia Q–CHT XT workflow; **C**, Nuvia S–Nuvia Q–CHT XT workflow.

PK Bridging ELISA

A PK bridging assay can be used for quantitating the therapeutic protein in biological matrices such as serum. The drug binds to two sites simultaneously, with one binding site to an immobilized anti-idiotypic antibody and with the other binding site to a labeled second anti-idiotypic antibody. Here, the in vitro assay was used as an indicator of the antibody's integrity/intactness after chromatographic purification compared to that of a rituximab biosimilar standard. As can be seen in Figure 10, the two purified mAbs obtained after Nuvia S–Nuvia Q–CHT and Protein A–Nuvia Q–CHT behaved in a similar way to the standard antibody, showing no recognizable loss of structure or function during purification.

Conclusions

The development of an efficient non-affinity purification process for biosimilar production is challenging. But, when successful, it confers benefits such as improved process economics, superior resistance to sodium hydroxide cleaning, and eliminated ligand leaching. Here, we show that a workflow combining two ion exchange resins with a mixed-mode resin can deliver similar results to a Protein A–based workflow in terms of purity, recovery, and viability of rituximab biosimilar monoclonal antibody. Little process development was necessary to establish operating conditions for maximum HCP protein clearance. A cell culture supernatant with low rituximab biosimilar titer (and thus an exceptionally high HCP contamination level) was applied in this study.

The results of the non-affinity process composed of the combination Nuvia S–Nuvia Q–CHT XT are very encouraging for applications where the antibody titer relative to host cell proteins is much more pronounced. Therefore, due to the reduction in manufacturing costs they offer, non-affinity processes should be considered for purifying antibody-mediated therapeutics, specifically biosimilars.

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