Journal of Chromatography A 1664 (2022) 462806

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Design of an integrated continuous downstream process for acid-sensitive monoclonal antibodies based on a calcium-dependent Protein A ligand

Julia Scheffel[a,1], Madelène Isaksson[b,1], Joaquín Gomis-Fons[b,1], Hubert Schwarz[c], Niklas Andersson[b], Björn Norén[d], Anita Solbrand[d], Veronique Chotteau[c], Sophia Hober[a,*], Bernt Nilsson[b,*]

[a] Department of Protein Science, KTH Royal Institute of Technology, SE-106 91 Stockholm, Sweden
[b] Department of Chemical Engineering, Lund University, SE-211 00 Lund, Sweden
[c] Department of Industrial Biotechnology, KTH Royal Institute of Technology, SE-106 91 Stockholm, Sweden
[d] Cytiva, SE-753 23 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 15 November 2021
Revised 2 January 2022
Accepted 4 January 2022
Available online 8 January 2022

Keywords:
Monoclonal antibody
Integrated continuous downstream process
pH-sensitive antibodies
Mild purification
Z_{Ca}
Process design

ABSTRACT

Monoclonal antibodies (mAb) are used as therapeutics and for diagnostics of a variety of diseases, and novel antibodies are continuously being developed to find treatments for new diseases. Therefore, the manufacturing process must accommodate a range of mAb characteristics. Acid-sensitive mAbs can severely compromise product purity and yield in the purification process due to the potential formation of aggregates. To address this problem, we have developed an integrated downstream process for the purification of pH-sensitive mAbs at mild conditions. A calcium-dependent Protein A-based ligand, called Z_{Ca}, was used in the capture step in a 3-column periodic counter-current chromatography operation. The binding of Z_{Ca} to antibodies is regulated by calcium, meaning that acidic conditions are not needed to break the interaction and elute the antibodies. Further, the virus inactivation was achieved by a solvent/detergent method, where the pH could remain unchanged. The polishing steps included a cation and an anion exchange chromatography step, and screening of the capture and polishing steps was performed to allow for a seamless integration of the process steps. The process was implemented at laboratory scale for 9 days obtaining a high yield, and a consistently pure drug substance, including high reduction values of the host cell protein and DNA concentrations, as well as aggregate levels below the detection limit, which is attributed to the mild conditions used in the process.

1. Introduction

Monoclonal antibodies (mAbs) have been used in therapy for 35 years targeting a broad range of diseases, both rare and those afflicting vast patient groups, like cancer and rheumatoid arthritis. Due to their highly specific nature, these tailor-made proteins have been successful in curing or improving the quality of life in patients [1]. Therapeutic antibody engineering has since evolved to pursue upcoming diseases, recently COVID-19, further accelerating the rate of approvals worldwide with the potential of a new record in 2021 [2]. Alongside persistent engineering efforts, the manufacturing of antibodies has been improved upstream, focusing on increased cell densities, mAb titers and productivity. For sensitive proteins susceptible to degradation or aggregation, continuous biomanufacturing has been developed to avoid long residence times [3].

To facilitate the continued expansion of the mAb industry, the techniques used to purify the antibodies should also maximize productivity and accommodate the variety in mAb characteristics that comes with the increasing use of IgG2 and IgG4 isotypes and emerging bispecific antibodies [2]. Currently, however, mAbs like these that are less stable at acidic pH cannot be efficiently purified without significant losses in yield as a consequence of mAb aggregation. This is mainly due to the low pH (3–4) needed to elute the antibody in the first chromatography step, which usually utilizes a resin based on a high-affinity interaction between the antibody and a Protein A ligand [4–7]. Recently, the elution conditions in

* Corresponding authors.
E-mail addresses: sophia@kth.se (S. Hober), bernt.nilsson@chemeng.lth.se (B. Nilsson).
[1] Co-first authors with equal contribution.

https://doi.org/10.1016/j.chroma.2022.462806

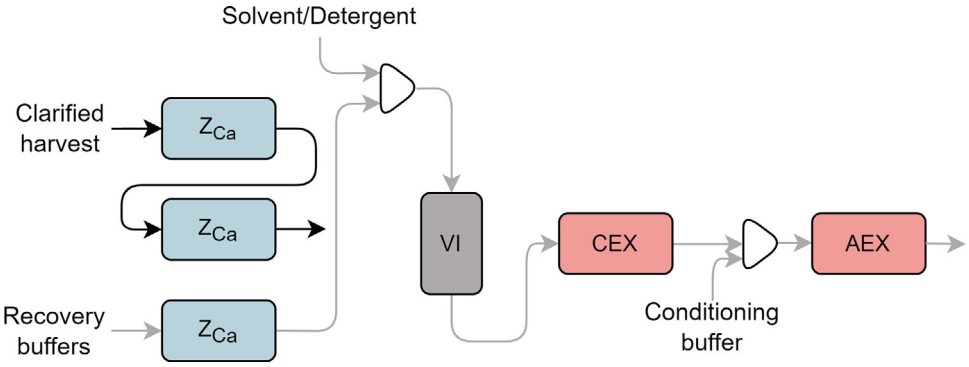

Fig. 1. Downstream process diagram. A 3-column Protein A capture PCC process is followed by solvent/detergent-based virus inactivation (VI), a cation exchange chromatography (CEX) step and an anion exchange chromatography (AEX) step. In the PCC capture process, two columns are interconnected and simultaneously loaded with clarified harvest, while the third column undergoes the recovery phases.

the vital Protein A chromatography step could be modified using a novel engineered calcium-dependent Protein A domain, called Z_{Ca} [8]. The binding of Z_{Ca} to antibodies is regulated by calcium, and the elution pH can be increased to pH 6 for the IgG1 subclass and to neutral pH for IgG2 and IgG4 through sodium chloride-mediated depletion of calcium. The addition of calcium post elution restores the ability of Z_{Ca} to bind to antibodies and it has been shown to repeatedly bind and release antibodies for numerous cycles [9]. Z_{Ca} in its tetrameric version coupled to a Sepharose resin provides a very high antibody recovery as well as a dynamic binding capacity (DBC) of ca 35 mg IgG/ml resin, comparable to commercial Protein A resins [9,10]. In contrast to capture by conventional Protein A resins, where the yield is compromised by aggregated antibodies, the highly selective Z_{Ca} and the mild conditions that this ligand allows for leads to both high purity and yield, which is especially relevant for the new emerging more aggregation-prone antibodies.

Further downstream in the purification process, a virus inactivation (VI) step is employed to reduce the amount of active enveloped viruses. In a typical mAb process, the viruses are inactivated through exposure to acidic pH following the low-pH Protein A elution [11]. In this work, we instead propose the use of a solvent/detergent inactivation step following elution from the Z_{Ca} resin, to consistently avoid subjecting the antibodies to low pH throughout the mAb production process. The solvent/detergent virus inactivation is a well-known method that has been used for decades in the blood product industry [12] and has proved to be a robust method for the inactivation of enveloped viruses [13]. The method includes the addition of a solvent/detergent mixture to the product, followed by an incubation time, usually 60 min at production scale, without the need for a reduction in pH [13]. Several examples of the implementation of solvent/detergent VI in the purification process of a biopharmaceutical can be found elsewhere [14,15].

Here, we demonstrate the integration of the Z_{Ca} resin in the capture step, applied in a periodic counter-current chromatography (PCC) setup, with a solvent/detergent-based VI step and subsequent cation and anion exchange chromatography (CEX and AEX, respectively) steps for polishing (Fig. 1). To allow for a seamless integration of this mild downstream process, the process conditions for the capture and the polishing steps were evaluated and selected accordingly. The integrated process was assessed in terms of yield in each step, productivity, and product purity, including the presence of mAb aggregates, host cell protein (HCP), DNA, and solvent in the final drug substance. We show the applicability of Z_{Ca} in a continuous downstream process for mild purification suitable for acid-sensitive antibodies aiming at maximizing the antibody yield and supporting the production of all antibodies with promising therapeutic potential.

2. Materials and methods

2.1. Production and coupling of engineered Protein A ligand

The tetrameric version of the affinity ligand Z_{Ca}, used for the antibody capture, was expressed in *E.coli* using the construct and culture protocol as reported in a previous publication [10], except that ampicillin was replaced by carbenicillin (100 ug/ml) and the fermentations were scaled up to 500 ml. The ligand was purified from the *E. coli* cell lysate by affinity chromatography, using an XK-50/20 column (Cytiva) packed with 175 ml IgG Sepharose 6 Fast Flow (Cytiva). The purification was performed according to Scheffel et al., [10] besides the following adjustments. It was carried out on an ÄKTA^{TM} pure 150 chromatography system (Cytiva) at a flow rate of 10 ml/min. The column was loaded with ca 500 ml cell lysate in each purification cycle at a flow rate of 5 ml/min and the column was washed with 1xTBST-C (50 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween20, 1 mM $CaCl_2$, pH 7.5), as previously described, followed by 2 CV of 5 mM NH_4Ac with 1 mM $CaCl_2$ (pH 5.5–6). The eluted protein was fractionated into 10 ml aliquots and freeze dried using a ScanVac CoolSafe freeze dryer (LaboGene). It was dissolved in a coupling buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$ at pH 8. The purity of the protein was analyzed by SDS-PAGE according to a previous protocol [10]. The ligand was coupled to a chromatography resin via a unique C-terminal cysteine through thiol-based chemistry and packed into 1 ml HiTrap^{TM} columns (Cytiva).

2.2. Screening of the loading density and buffers in the capture step

Two different ligand densities (3.5 mg/ml and 5.4 mg/ml) were evaluated on an ÄKTA start chromatography system (Cytiva) to identify the optimal column performance in the downstream process. The capacity of both columns was evaluated in a standard purification setup by saturating the columns with human polyclonal IgG, according to the ÄKTA purification method earlier described [10]. The antibody recovery and elution profiles were also assessed through capture and elution of pure IgG1 (rituximab, Roche), according to Scheffel et al. [9].

The effect of the calcium concentration in the sample and wash buffer (1xTBST-C) was evaluated with regards to the antibody recovery and elution peak width. Pure IgG1 was captured on a Z_{Ca} column (ligand density: 3.5 mg/ml) through the purification procedure referenced above [9], with either 1 mM or 10 mM $CaCl_2$ in both the IgG1 sample and wash buffer. Each step of the purification was fractionated separately and the absorbance at 280 nm was measured to determine the recovery.

Table 1
Experimental conditions used in the CEX trials.

Experiment	pH	NaCl in equilibration buffer (mM)	NaCl in wash2 buffer (mM)	NaCl gradient (mM)
1	5.0	50	125	125–500
2	5.0	50	n/a	125–500
3	5.0	50	n/a	50–425
4	5.5	50	n/a	50–425
5	5.0	75	n/a	75–450
6	5.5	75	125 mM	125–500

Elution of IgG1 captured on Z_{Ca} was studied at pH 5.5 with different NaCl concentrations (300 mM, 150 mM, 50 mM). These experiments were conducted in the same manner as the purifications used to test the recovery above, capturing and eluting IgG1 with 1 mM $CaCl_2$ in the sample and wash buffer.

2.3. Screening of the buffers in the CEX step

The Capto™ S Impact (Cytiva) chromatography resin was used for the CEX step in bind-elute mode for the removal of mAb aggregates. Different values of pH and NaCl concentration in the purification buffers were screened to find suitable process conditions. The benchmark process conditions for the screening (experiment 1 in Table 1) were based on a previous integrated process [16].

A series of experiments with a 1 ml HiTrap column were performed with varying pH and NaCl concentration in the buffers. In each experiment, the column was loaded with 64 mg of mAb per ml resin in equilibration buffer (based on a study by the manufacturer of this resin [17]), with aggregate content varying from 4 to 13%. The mAb had previously been purified on MabSelect SuRe™, a commercial Protein A resin using low-pH elution. The tested experimental conditions are summarized in Table 1. All buffers used in each experiment had the same pH (5.0 or 5.5) and a concentration of 50 mM sodium acetate. In experiments 2–5 the second wash phase was not performed. The remaining process conditions that are not shown in the table are the same as the benchmark conditions.

During the experiments, fractions of 1 ml were collected during the load, the first wash, the second wash and regeneration phases. During the gradient elution, fractions of 0.6 ml were collected. All collected fractions were analyzed by analytical SEC to check the concentration of mAb aggregates.

2.4. Solvent removal trials

The removal of the solvent added in the VI step (tri-n–butyl–phosphate, TnBP) in the CEX and AEX steps was evaluated through a solvent removal trial where the complete DSP process was run in a non-integrated setup. 34 ml of a mAb sample in 50 mM sodium acetate buffer, pH 5.5, 50 mM NaCl at a mAb concentration of 0.56 g/L was incubated for 1 hour with 0.3 g/L Tnbp and 1 g/L Tween 20 in a stirred flask. After VI, the sample was loaded onto a 1 ml CEX column and purified according to the conditions determined during the CEX screening. The load flowthrough (FT) and wash pools, as well as the CEX elution pool were collected for analysis. The CEX elution pool was diluted 1:1 with AEX dilution buffer (50 mM sodium phosphate, pH 6.35, 104 mM NaCl), and 1.5 ml of the diluted CEX elution pool were loaded onto a 1 ml AEX column, the column was washed with AEX separation buffer, and the product pool was collected for analysis. The AEX dilution buffer had been adapted so that it would result in the required AEX separation conditions after inline dilution 1:1 with the CEX pool. The samples collected during the solvent removal trial for the non-integrated process were analyzed by RP-chromatography to determine the Tnbp concentrations according to the analytical method described below.

2.5. Integrated downstream process

2.5.1. Process conditions and buffers

In this section, the process conditions and the buffers used in the integrated downstream process are described, including the ones resulting from the aforementioned screening studies.

In the integrated downstream run, three identical columns were used for the capture step in a PCC setup. Each capture column was equilibrated with 5 column volumes (CV) of 50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5. The capture columns were continuously loaded with cell-free supernatant at a mAb concentration of 0.76 mg/ml, produced in house in a perfusion process using Chinese Hamster Ovary cells (CHO-GS), after filtration with a 0.2 µm filter. After the loading, the columns were washed with 10 CV of equilibration buffer followed by 1 CV of 50 mM sodium acetate and 1 mM $CaCl_2$, pH 5.5. The elution was done with 10 CV of 50 mM sodium acetate, 50 mM NaCl, pH 5.5. After elution, the columns were regenerated with 1 CV of elution buffer. Finally, a cleaning-in-place (CIP) phase was performed with 3 CV of 0.3 M acetic acid, pH 3.3. All capture phases were done at a flow rate of 1 ml/min, except the loading and CIP phases that were done at 0.2 ml/min.

For the VI step, a solvent/detergent VI method was derived from a classically adopted procedure with Tnbp and Tween 20 as reviewed by Dichtelmüller et al. [13]. An aqueous stock solution of 3 g/L Tnbp and 10 g/L Tween 20 was mixed with the capture elution pool, followed by 60 min of incubation in a 50 ml stirred bottle. The stock solution was added in the volume ratio 1:9, thus resulting in a concentration of 0.3 g/L Tnbp and 1 g/L Tween 20 [13].

The CEX column was equilibrated with 5 CV of 50 mM sodium acetate, 50 mM NaCl, pH 5.5. After the loading, it was washed with 4 CV of equilibration buffer. A 20 CV gradient elution from 50 to 425 mM NaCl, 50 mM sodium acetate, pH 5.5 was performed. The column was regenerated with 4 CV of 50 mM sodium acetate, 500 mM NaCl, pH 5.5. Finally, a CIP phase was performed with 3 CV of 1 M NaOH. The flow rate was 2 ml/min in all the phases, except in the elution phase, where it was 0.37 ml/min, and in the CIP phase, where it was 0.4 ml/min.

For the AEX step, the Capto Adhere resin (Cytiva) was used in flow-through mode. The column was equilibrated with 5 CV of 25 mM sodium phosphate, 25 mM sodium acetate, 120 mM NaCl, pH 6.2 at a flow rate of 1 ml/min. A sample conditioning was performed to obtain the right loading pH and salt concentration (which was the same as the equilibration buffer), as the eluate from the CEX column had lower pH and higher salt concentration than the desired loading condition for the AEX column. The conditioning was done with an inline dilution, where the dilution buffer was 50 mM sodium phosphate, 104 mM NaCl, pH 6.35, and the dilution ratio was 1:1, leading to a loading flow rate of 0.74 ml/min. The AEX column was regenerated with 4 CV of 0.1 M acetic acid at a flow rate of 1 ml/min, and a CIP was performed with 3 CV of 1 M NaOH at a flow rate of 0.2 ml/min.

2.5.2. Process scheduling in the PCC process

In a 3-column PCC, two columns are simultaneously loaded: the breakthrough from the first column is applied directly onto the second column. Meanwhile, a third column undergoes the recovery phases, which include the wash, elution, regeneration and equilibration phases, meaning that a synchronization between the loading and the recovery phases is needed [18]. In every PCC cycle, 9 ml of CHO cell supernatant (6.84 mg) was loaded on the capture columns at a flow rate of 0.2 ml/min, resulting in a PCC cycle time of 45 min. This is the same time it took for the third capture column to undergo the recovery phases, and therefore it was set as the cycle time. The polishing steps were approximately 97 min long. To ensure continuity in the feed of the capture step while maintaining the PCC cycle in 45 min, the polishing steps were run with the eluted product from three PCC cycles, meaning that the amount loaded on the polishing columns was 20.52 mg, assuming no product loss in the process.

2.5.3. Column volumes

The column volumes were calculated considering the protein load for each column. In the capture column, the DBC at 50% breakthrough from a previous study with this resin was used [10]. The DBC at 50% breakthrough was estimated to be 37 mg/ml. Therefore, a column volume of 0.18 ml was needed in the capture step. The protein loads of the CEX and AEX columns were obtained from the manufacturer [17]. They were 43 and 87 mg/ml, respectively. Therefore, the column volumes were 0.47 and 0.24 ml for the CEX and AEX columns, respectively. The prepacked HiTrap columns were used for the three chromatography steps, and as the minimum volume for the HiTrap columns is 1 ml, it was decided to run with 1 ml columns for all steps, meaning that the columns were oversized.

2.5.4. Experimental setup

The implementation of the integrated downstream process was carried out using the same setup as in a previous implementation of PCC in an integrated purification process [16]. The capture step was implemented as a 3-column PCC process in an ÄKTA pcc 75 chromatography system (Cytiva). The two polishing steps were implemented on an ÄKTA pure 150 system, while the VI bottle was placed between the two systems. Product collection was performed with the use of outlet valves in the ÄKTA systems. A sampling point was located after each step (capture, VI, CEX and AEX). Since the whole product pool was taken for collection, the samples from the different steps could not be taken in the same cycle, and therefore the product collection was done in different cycles in the process at steady state.

2.5.5. Process control

Both chromatography systems were simultaneously controlled with the research software Orbit, which is a supervisory control and data acquisition system developed at Lund University (Lund, Sweden). Details on how Orbit is used to control downstream processes can be found in some of our previous research publications [19,20]. In particular, the version of Orbit that allows for the control and synchronization of multiple systems was used in this work [16].

2.6. Analytical methods

The concentration of mAb was measured with a CEDEX analyzer in the output from each purification step of the downstream process, as well as in the CHO supernatant. The presence of antibody aggregates in the purification samples was measured using a Superdex 200 Increase 5/150 column (Cytiva) on an ÄKTA pure chromatography system (Cytiva) at 280 nm. The column was equilibrated with 2 CV of running buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7) at a flow rate of 0.35 ml/min. The samples were injected at a volume of 25 µl, followed by 2 CV of running buffer. The raw data was imported into Python to determine the aggregate content by comparing the peak areas for monomeric and aggregated species at 280 nm.

The HCP content in the CHO cell supernatant and after each purification step was analyzed using a Gyrolab® CHO–HCP E3G Kit on a Gyrolab system (Gyros Protein Technologies). The supernatant was diluted 1:1600, the capture eluate 1:10, and the remaining samples 1:2 prior to analysis. Each sample was run in triplicates according to the instructions of the manufacturer. The DNA content was determined as previously published [10]. The amount of DNA or HCP was normalized to the antibody content in the sample, and the log reduction value (LRV) was determined for each purification output by the common logarithm ratio of the DNA/HCP in the supernatant to the DNA/HCP in the purified samples.

The Tnbp sample concentration was determined by analytical Reverse Phase (RP) chromatography, based on an analytical method used in a previous publication [21]. The instrument setup was an Agilent 1260 HPLC with connected autosampler and Refractive Index (RI) detector. The column used was a Kromasil C18 with 10 µm particle size and the dimensions 4.6 × 250 mm, and the mobile phase was 70 vol% acetonitrile in 30 vol% dH_2O. After sample injection, the flow rate was set to 0.5 ml/min for 30 min. Tnbp amounts ranging from 0.0025 µL to 0.3 µL were injected on the RP-column to create a Tnbp standard curve. The Agilent raw data (RI signal vs. time) was imported into Python where all calculations were performed. The Tnbp peak areas were determined and plotted against the injected Tnbp amount in µL (Figure S1, in supplementary material). To determine the Tnbp concentration in samples, aliquots of 100 µl were loaded onto the RP-column. After separation and detection, the area of the Tnbp peaks was calculated and the amount of Tnbp in each injected sample could be derived from the Tnbp standard curve.

3. Results and discussion

3.1. Selection of the downstream process conditions

3.1.1. Capture step

The effect of the ligand density of the Protein A capture column on the elution was evaluated (Figure S2). The capacity was shown to be equal for the two columns with different Z_{Ca} ligand densities when saturated with human IgG, despite approximately a 50% difference in ligand density (Figure S2A). This could have been caused by steric hindrance introduced on the more crowded, high-ligand density column. Additionally, the recovery of IgG1 was similar for both columns, with no antibody released in the flow through nor during washing. However, for the high-ligand density column, 3% of the captured antibody was retained after sodium-chloride mediated elution for 13 CV. Explanations for this could be avidity effects and mass transfer limitations on the high-ligand density column, with possibility for improvement of the latter in case of prolongation of the elution. Consequently, when comparing the width of the elution peaks, the low-ligand density column demonstrated a considerably narrower peak by 30%, outperforming the high-ligand density column since a small elution pool volume was desired downstream of this step (Figure S2B). As a result, a capture column with a Z_{Ca} ligand density of 3.5 mg/ml was used.

In order to minimize the capture elution pool volume that is applied on the CEX column, both the wash buffer and elution buffer used in the capture step were screened. It was shown that the concentration of calcium in the sample and wash buffer had a large effect on the elution profile from the calcium-dependent Z_{Ca}

column (Fig. 3A). The elution occurred considerably earlier when using only 1 mM $CaCl_2$ in the sample and wash buffer as compared to 10 mM $CaCl_2$. The width of the elution peak was also affected, with a more concentrated elution pool when using a lower calcium concentration in the wash buffer. However, the antibody recovery did not depend on the calcium concentration, and equally high recoveries were obtained as previously demonstrated when purifying IgG1 [9]. The shift in elution profile when the Z_{Ca} column had been washed with 10 mM $CaCl_2$ could be explained by a slower sodium chloride-mediated depletion of calcium ions from the Z_{Ca} ligand, and thus also a slower release of the bound antibodies, in comparison with when the column was loaded with 10 times fewer calcium ions. Consequently, the lower concentration of calcium was applied when integrating the capture step in the process.

The NaCl concentration in the elution buffer was screened with the aim to achieve the smallest possible capture elution pool volume with minimal need for sample conditioning for the loading of the CEX column (Fig. 3B). Although complete elution of captured IgG1 from Z_{Ca} using sodium chloride as elution agent can be accomplished at pH 6.5 [9], the pH used in this case was 5.5 since the subsequent CEX step in the downstream process required a lower pH (5.0–5.5) during sample loading. As observed in Fig. 3B, the elution pool volume decreases as the NaCl concentration increases, with a maximum difference of 50% in the elution pool volume when using 300 mM NaCl compared to 50 mM. However, if the capture pool has a higher NaCl concentration than what is suitable for the loading of the CEX column, which ranges from 50 mM to 75 mM NaCl, it must be diluted to adjust the NaCl concentration, with the consequent increase in volume. Dilution of the 300 mM elution pool to 50 mM NaCl would lead to a six times larger volume, and dilution of a 150 mM NaCl elution pool to 50 mM NaCl would give a three times larger volume. Consequently, elution at 50 mM NaCl and pH 5.5 was selected.

3.1.2. Cation exchange chromatography step

Although aggregates are not expected in the integrated run due to the mild conditions used in the capture and the VI steps, a CEX step capable of removing aggregates is important to ensure product quality in a robust way, as aggregates could also be formed in the perfusion culture. mAb binding capacity and aggregate removal are highly dependent on the pH and NaCl concentration of the purification buffer. The objective was to find suitable NaCl concentration and pH that could facilitate the integration with the upstream capture step, while maintaining a good removal of antibody aggregates.

In the screening trials of the CEX step, the process conditions used in experiment 6 (75 mM NaCl, pH 5.5 during loading and in the first wash phase, 125 mM NaCl in the second wash phase, and a 125–500 mM NaCl gradient) resulted in the elution of the protein already during the end of the loading phase and first wash phase. Therefore, this experiment was not further analyzed. During all other experiments, good binding was achieved during the loading phase and no mAb was detected in the fractions collected during the loading and wash phases. The benchmark conditions (experiment 1: 50 mM NaCl, pH 5.0 during loading and in the first wash phase, 125 mM NaCl in the second wash phase, and a 125–500 mM NaCl gradient) resulted in the best removal of aggregates compared to monomer yield, which can be seen in Fig. 2. Nevertheless, there were small differences in aggregate removal between the tested process conditions and overall, all experiments resulted in high aggregate clearance. The effect of the second wash phase on the aggregate removal (which can evaluate by comparing experiments 1 and 2 in Fig. 2) is negligent, which means that this phase can be excluded.

The experimental trials showed that when increasing the pH from 5.0 to 5.5 during the loading phase, the protein would bind at

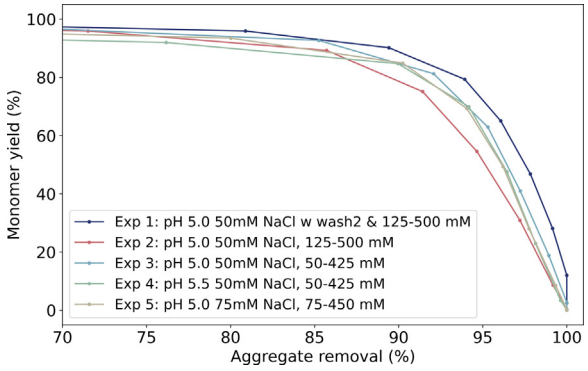

Fig. 2. Monomer yield vs aggregate removal in fractions collected during the gradient elution phase in the screening trials of the CEX step for experiments 1-5.

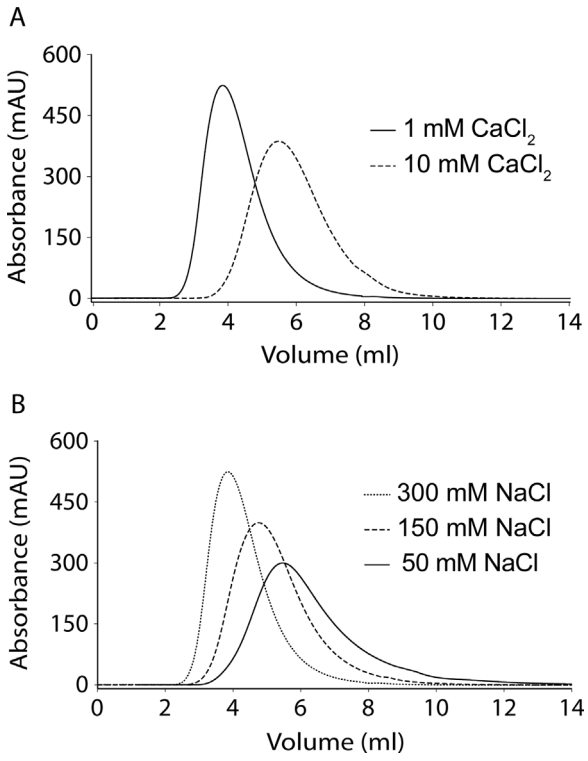

Fig. 3. Optimization of buffers used in the capture step with the Z_{Ca} resin. (A) Overlay of elution chromatograms when using either 1 mM or 10 mM $CaCl_2$ in the wash buffer and eluting with 300 mM NaCl, pH 5.5. (B) Overlaid elution peaks using three different NaCl concentrations (50 mM, 150 mM, 300 mM) for elution at pH 5.5, and with 1 mM $CaCl_2$ in the wash buffer.

50 mM NaCl (experiment 4), but not at 75 mM NaCl (experiment 6). As a more neutral pH is ideal for the antibodies and elution in the capture step can be done at pH 5.5, it was decided to use the process conditions of experiment 4 (pH 5.5, 50 mM NaCl in loading and the first wash phase, no second wash phase, and a gradient of 50–425 mM NaCl in the elution phase). This way, the pH and the NaCl concentration would be the same in both the capture and the CEX steps, thus enabling the complete removal of the conditioning step, while still having an aggregate removal similar to that of the benchmark case. Moreover, as a consequence of the mild conditions used in the capture step and virus inactivation, aggregation during the downstream process is minimized and the CEX column only has to remove potential aggregates formed in the upstream process.

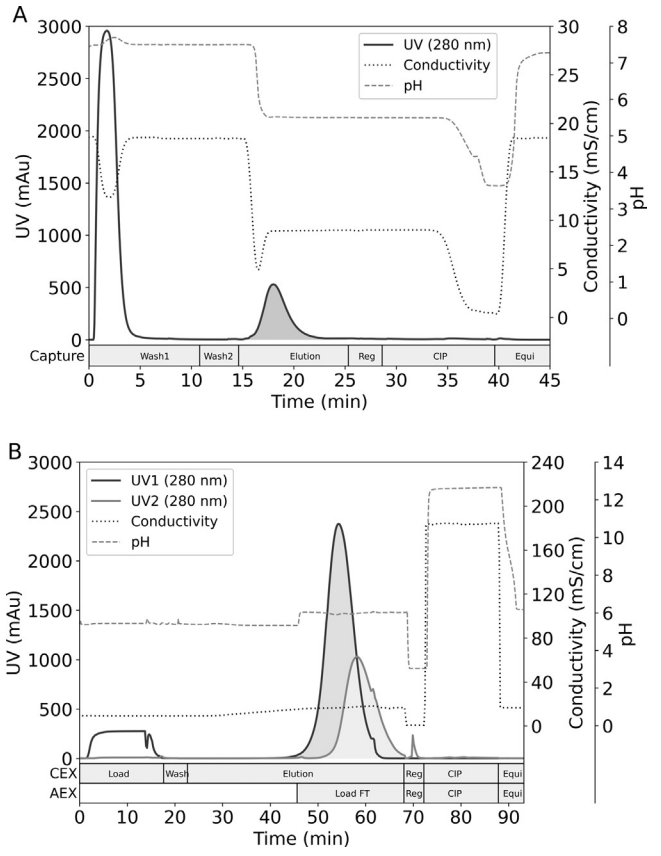

Fig. 4. Chromatographic profiles for the affinity capture step (A) and the polishing steps (B). The shaded areas correspond to the product pools in each step. UV1 corresponds to the UV signal after the CEX column/before the AEX column, and UV2 is the signal after the AEX column.

3.2. Implementation of the integrated downstream process

The complete downstream process was implemented in an integrated setup during a run that was in operation for 9 days to ensure steady state. In Fig. 4, the chromatograms from the different steps of the process are shown. In the chromatogram from the capture step (Fig. 4A), the largest absorbance peak corresponds to the impurities and unbound antibodies that were washed away in the first wash phase. As a result of the PCC operation, this wash pool is loaded onto a second column, so that the antibodies that do not adsorb on the first column can bind to the second column, while the impurities flow through both columns and finally go to waste. After the two wash phases, the product was eluted at pH 5.5 in a pool that was approximately 9 ml. The VI bottle was filled with the eluted pool and the solvent/detergent stock solution was dosed at a ratio of 1:9. After 60 min of incubation time, the product was loaded on the CEX column.

In Fig. 4B, the plateau that can be seen in the UV1 signal during the first 15 min corresponds to the UV signal before the CEX column during the loading phase, while the UV2 signal is the absorbance after the CEX column during the same phase. Since no absorbance for the UV2 signal was observed, it can be derived that all product was adsorbed on the CEX column during the loading phase. Additionally, nothing was detected by the UV monitors during the wash phase. In the gradient elution of the CEX column, the UV1 signal is the absorbance after the CEX column, and the UV2 signal is the absorbance after the AEX column. When the product started to elute from the CEX column, the eluate was directly loaded on the AEX column after conditioning to pH 6.2 by inline dilution 1:1. After a delay time, the product was detected by the UV2 monitor, since the AEX column was run in flow-through mode. Due to the inline dilution, the AEX peak is approximately half the size of the CEX peak. The regeneration, CIP and equilibration phases were run simultaneously for both the CEX and the AEX columns, but with different pumps. In these phases, the absorbance after the AEX column was measured by the UV2 detector, while the absorbance after the CEX column was not measured and is therefore not shown.

Table 2
Recovery yield for each step of the downstream process, including the total yield, and the percentage of aggregates, HCP levels and DNA log reduction at one time point during the process run. Numbers within parenthesis are references from other, traditional three-step mAb processes.

Process step	Yield (%)	Aggregates (%)	HCP (ppm)	DNA (LRV)
Z_{Ca} capture	89 (95)[1]	ND (2–3)[1]	230 (300–2000)[1]	5.2 (3.0)[2]
VI	96	–	–	–
CEX	91 (90)[1]	ND (<1)[1]	20 (<100)[1]	6.5
AEX	100 (99)[1]	ND (<1)[1]	5 (<5)[1]	7.5
Total	78 (86)[1]	ND (<1)[1]	5 (<5)[1]	7.5

ND (could not be detected).
[1] Cytiva (2015B) [24].
[2] Butler et al. [25].

Table 2 shows the recovery yield for each process step as well as the total yield of the downstream process at steady state. Yields of around 90% or above are obtained in all the steps, and the total yield was 78%. These values are similar to previously reported yields in state-of-the-art works, with integrated mAb downstream processes where conventional Protein A resins were used [16,22]. The main difference between these processes and the current one, is that, in the present work, the elution phase in the capture step and the virus inactivation step are carried out at a pH of 5.5, instead of 3.5, which is the common pH for these steps, thus allowing the application of this purification process at mild conditions for pH-sensitive monoclonal antibodies with a competitive yield.

The present work is the first proof of concept of this novel process, aiming at demonstrating the feasibility of a downstream process for the purification of monoclonal antibodies at mild conditions, with focus on yield rather than productivity. For this reason, the column volumes have not been optimized and therefore the downstream process is oversized. Consequently, the resulting productivity at steady state (73.0 mg/$ml_{Protein\ A\ resin}$/day) is lower than that of state-of-the-art processes (e.g., 123.8 mg/$ml_{Protein\ A\ resin}$/day with MabSelect SuRe™ [23]), but very promising this early on in the development and with large potential to be increased after optimization of the column sizes. Further, the Z_{Ca} resin used in the present work has been proven to have a high DBC, comparable to MabSelect SuRe which shares the same base matrix, indicating the potential for high productivities with Z_{Ca} [10]. Therefore, it is expected that the process presented here can reach productivities close to those obtained in processes with conventional Protein A resins, after an optimization of the column sizes.

3.3. Product quality

The objective of the downstream process is to remove HCPs, host cell DNA as well as aggregates formed both upstream and downstream, in addition to the removal of viruses and potential process-derived impurities. In this process, aggregates which commonly arise in the capture step and during viral inactivation are non-existent, because of the mild pH used in these process steps, as seen in Table 2. No aggregates could be detected in any step of the downstream process, thus not compromising the yield. In a traditional mAb process, which is most often designed to produce a stable antibody, these would constitute around 1% [24], increasing drastically if manufacturing a pH-sensitive antibody. This was

observed in a previous study where an IgG4 antibody was purified on Z_{Ca} and MabSelect SuRe, and the proportion of aggregates in the MabSelect SuRe eluate was as much as a third of all eluted antibody and less than 1% for Z_{Ca} [9]. Furthermore, the capture step resulted in HCP levels below those observed for other mAb processes, followed by a further reduction in both the CEX and AEX steps. The DNA log reduction was also efficient in all the steps, demonstrating superior reduction by Z_{Ca} compared to other Protein A resins [25].

The virus concentration was not measured, but the efficiency and robustness of the solvent/detergent VI method has been demonstrated previously [13,14]. Dichtelmüller et al. performed a very comprehensive study of the virus inactivation process conditions and showed that a Tnbp concentration of 3 g/L and a detergent concentration of 10 g/L with an incubation time of 60 min, which are the same conditions used in this work, are standard conditions in the industry. Further, a >5-log virus inactivation was achieved [13].

The solvent used in the VI step, Tnbp, is harmful to aquatic life and may have cancerogenic effects [26], hence it must be removed during the subsequent downstream steps (CEX and AEX). In the solvent removal trial, only 1.8% of the original Tnbp amount in the sample remained in the collected CEX product pool, when comparing the Tnbp concentration before and after the CEX step (Table S1, in supplementary material). Subsequently, the removal obtained with the AEX step was 50%. Overall, a reduction in Tnbp concentration of 34 times and removal of 99.1% of the Tnbp was achieved through the polishing steps during the solvent removal trial (non-integrated downstream process). In the integrated continuous purification process, a Tnbp concentration reduction factor of 63 and a Tnbp removal of 99.4% was obtained, hence comparable results. The detected concentration in the AEX pool was close to the limit of detection of the analytical method (as seen in Figure S3), thus a higher removal degree of Tnbp could not have been detected with this method. A final ultrafiltration-diafiltration step for formulation would further reduce the Tnbp concentration as the buffer exchange degree reached in the diafiltration step is usually higher than 99% [27,28], thus obtaining a total logaritmic reduction value of 4.2.

4. Conclusions

This study is a proof of concept of an integrated continuous downstream process at mild conditions, appropriate for the purification of pH-sensitive monoclonal antibodies. A novel calcium-dependent Protein A-derived ligand, Z_{Ca}, was used for the capture step, and suitable conditions in the downstream process were found, which allowed for a seamless integration of the different steps. It was possible to eliminate the conditioning step before the CEX loading by slightly adjusting the CEX process conditions (pH and NaCl concentration), while keeping a good aggregate removal. To completely avoid the use of low pH, and thus prevent the formation of aggregates, a virus inactivation method based on solvent/detergent was used as an alternative to the traditional acidic virus inactivation approach. The minimum pH in the process was 5.5, which is significantly higher than the pH values used in a traditional mAb purification process, ranging between 3.2 and 3.5 [20,23].

The downstream process was run continuously for 9 days in two ÄKTA chromatography systems, where the capture step in a 3-column PCC operation and the virus inactivation were implemented in one ÄKTA system, and the polishing steps (CEX and AEX columns) were implemented in the other ÄKTA system. The integrated continuous process was implemented at laboratory scale obtaining a high recovery yield. The downstream process could deliver a consistently pure monoclonal antibody, with HCP and DNA concentrations similar to those obtained in state-of-the-art processes [23–25], and aggregate levels below the detection limit, thanks to the use of mild conditions. In addition, this has been the first time, to our knowledge, that the removal of solvent after a solvent/detergent-based VI has been addressed for the purification process of mAbs.

The rising promise of bispecific antibodies and other antibody-based chimeric proteins brings new hope for therapies; however, these novel molecules are more challenging for the manufacturing, both upstream and downstream. An important problem is mAb aggregation. Owing to its mild pH operation range, the process presented here can be applied to a much larger number of molecules compared to methods based on legacy Protein A, and, together with an integrated continuous process, brings an elegant, efficacious and thus more cost-effective solution for tomorrow's challenging mAbs. Therefore, this proof of concept can be used as a reference for future implementations of integrated continuous processes for the purification of pH-sensitive monoclonal antibodies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

SH has filed a patent application regarding the novel Z_{Ca} domain.

CRediT authorship contribution statement

Julia Scheffel: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Madelène Isaksson:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Joaquín Gomis-Fons:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Hubert Schwarz:** Investigation, Writing – review & editing. **Niklas Andersson:** Software, Writing – review & editing, Supervision. **Björn Norén:** Resources, Investigation. **Anita Solbrand:** Resources, Writing – review & editing. **Veronique Chotteau:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Sophia Hober:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Bernt Nilsson:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgements

This work is financially supported by Vinnova (through the Competence centre for Advanced BioProduction by Continuous Processing, AdBIOPRO) under Grant 2016–05181, the Swedish research council under Grant 2017–04664, the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 812909 CODOBIO, and the industrial partner Cytiva.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.462806.

References

[1] R.-.M. Lu, Y.-.C. Hwang, I.J. Liu, C.-.C. Lee, H.-.Z. Tsai, H.-.J. Li, H.-.C. Wu, Development of therapeutic antibodies for the treatment of diseases, J. Biomed. Sci. 27 (1) (2020) 1-1, doi:10.1186/s12929-019-0592-z.

[2] H. Kaplon, J.M. Reichert, Antibodies to watch in 2021, mAbs 13 (1) (2021) 1860476–1860476, doi:10.1080/19420862.2020.1860476.

[3] P. Gronemeyer, R. Ditz, J. Strube, Trends in upstream and downstream process development for antibody manufacturing, Bioengineering 1 (4) (2014) 188–212, doi:10.3390/bioengineering1040188.

[4] A.A. Shukla, P. Gupta, X. Han, Protein aggregation kinetics during Protein A chromatography: case study for an Fc fusion protein, J. Chromatogr. A 1171 (1) (2007) 22–28, doi:10.1016/j.chroma.2007.09.040.

[5] B. Liu, H. Guo, J. Xu, T. Qin, L. Xu, J. Zhang, Q. Guo, D. Zhang, W. Qian, B. Li, J. Dai, S. Hou, Y. Guo, H. Wang, Acid-induced aggregation propensity of nivolumab is dependent on the Fc, mAbs 8 (6) (2016) 1107–1117, doi:10.1080/19420862.2016.1197443.

[6] A.J. Paul, K. Schwab, F. Hesse, Direct analysis of mAb aggregates in mammalian cell culture supernatant, BMC Biotechnol. 14 (2014) 99, doi:10.1186/s12896-014-0099-3.

[7] M. Vázquez-Rey, D.A. Lang, Aggregates in monoclonal antibody manufacturing processes, Biotechnol. Bioeng. 108 (7) (2011) 1494–1508, doi:10.1002/bit.23155.

[8] S. Kanje, R. Venskutonytė, J. Scheffel, J. Nilvebrant, K. Lindkvist-Petersson, S. Hober, Protein engineering allows for mild affinity-based elution of therapeutic antibodies, J. Mol. Biol. 430 (18) (2018) 3427–3438 Part B), doi:10.1016/j.jmb.2018.06.004.

[9] J. Scheffel, S. Hober, Highly selective Protein A resin allows for mild sodium chloride-mediated elution of antibodies, J. Chromatogr. A 1637 (2021) 461843, doi:10.1016/j.chroma.2020.461843.

[10] J. Scheffel, S. Kanje, J. Borin, S. Hober, Optimization of a calcium-dependent Protein A-derived domain for mild antibody purification, MAbs 11 (8) (2019) 1492–1501, doi:10.1080/19420862.2019.1662690.

[11] C. Gillespie, M. Holstein, L. Mullin, K. Cotoni, R. Tuccelli, J. Caulmare, P. Greenhalgh, Continuous In-line virus inactivation for next generation bioprocessing, Biotechnol J 14 (2) (2019), doi:10.1002/biot.201700718.

[12] G.M. Liumbruno, M. Franchini, Solvent/detergent plasma: pharmaceutical characteristics and clinical experience, J. Thromb. Thrombolysis 39 (1) (2015) 118–128, doi:10.1007/s11239-014-1086-1.

[13] H.O. Dichtelmüller, L. Biesert, F. Fabbrizzi, R. Gajardo, A. Gröner, I. von Hoegen, J.I. Jorquera, C. Kempf, T.R. Kreil, D. Pifat, W. Osheroff, G. Poelsler, Robustness of solvent/detergent treatment of plasma derivatives: a data collection from Plasma Protein Therapeutics Association member companies, Transfusion (Paris) 49 (9) (2009) 1931–1943, doi:10.1111/j.1537-2995.2009.02222.x.

[14] D.L. Martins, J. Sencar, N. Hammerschmidt, B. Tille, J. Kinderman, T.R. Kreil, A. Jungbauer, Continuous solvent/detergent virus inactivation using a packed-bed reactor, Biotechnol. J. 14 (8) (2019) 1800646, doi:10.1002/biot.201800646.

[15] A. Löfgren, J. Gomis-Fons, N. Andersson, B. Nilsson, L. Berghard C, Lagerquist Hägglund, An integrated continuous downstream process with real-time control: a case study with periodic countercurrent chromatography and continuous virus inactivation, Biotechnol. Bioeng. 118 (4) (2021) 1645–1657, doi:10.1002/bit.27681.

[16] J. Gomis-Fons, N. Andersson, B. Nilsson, Optimization study on periodic counter-current chromatography integrated in a monoclonal antibody downstream process, J. Chromatogr. A 1621 (2020) 461055, doi:10.1016/j.chroma.2020.461055.

[17] Continuous chromatography in downstream processing of a monoclonal antibody, Application Note 29170800 AA, Cytiva, Uppsala, Sweden, 2015.

[18] R. Godawat, K. Brower, S. Jain, K. Konstantinov, F. Riske, V. Warikoo, Periodic counter-current chromatography – design and operational considerations for integrated and continuous purification of proteins, Biotechnol. J. 7 (2012) 1496–1508, doi:10.1002/biot.201200068.

[19] J. Gomis-Fons, A. Löfgren, N. Andersson, B. Nilsson, L. Berghard, S. Wood, Integration of a complete downstream process for the automated lab-scale production of a recombinant protein, J. Biotechnol. 301 (2019) 45–51, doi:10.1016/j.jbiotec.2019.05.013.

[20] J. Gomis-Fons, H. Schwarz, L. Zhang, N. Andersson, B. Nilsson, A. Castan, A. Solbrand, J. Stevenson, V. Chotteau, Model-based design and control of a small-scale integrated continuous end-to-end mAb platform, Biotechnol. Progr. 36 (4) (2020) e2995, doi:10.1002/btpr.2995.

[21] D.D. Dicholkar, L.K. Patil, V.G. Gaikar, S. Kumar, U. Kamachi Mudali, R. Natarajan, Direct determination of tri-n-butyl phosphate by HPLC and GC methods, J. Radioanal. Nucl. Chem. 291 (3) (2012) 739–743, doi:10.1007/s10967-011-1445-8.

[22] R. Godawat, K. Konstantinov, M. Rohani, V. Warikoo, End-to-end integrated fully continuous production of recombinant monoclonal antibodies, J. Biotechnol. 213 (Supplement C) (2015) 13–19, doi:10.1016/j.jbiotec.2015.06.393.

[23] F. Steinebach, N. Ulmer, M. Wolf, L. Decker, V. Schneider, R. Wälchli, D. Karst, J. Souquet, M. Morbidelli, Design and operation of a continuous integrated monoclonal antibody production process, Biotechnol. Prog. 33 (5) (2017) 1303–1313, doi:10.1002/btpr.2522.

[24] Purification of monoclonal antibodies using modern chromatography media and membranes, Application Note 29094443 AB, Cytiva, Uppsala, Sweden, 2015.

[25] M.D. Butler, B. Kluck, T. Bentley, DNA spike studies for demonstrating improved clearance on chromatographic media, J. Chromatogr. A 1216 (41) (2009) 6938–6945, doi:10.1016/j.chroma.2009.08.049.

[26] ECHA, Tributyl phosphate, 2021. Retrieved from https://echa.europa.eu/registration-dossier/-/registered-dossier/13548/7/8. (Accessed September 2021).

[27] L. Schwartz, Diafiltration: a Fast, Efficient Method for Desalting, or Buffer Exchange of Biological Samples, Pall Life Sciences, Ann Arbor, MI, U.S.A., 2003.

[28] J. Rucker-Pezzini, L. Arnold, K. Hill-Byrne, T. Sharp, M. Avazhanskiy, C. Forespring, Single pass Diafiltration integrated into a Fully Continuous mAb purification process, Biotechnol. Bioeng. (2017), doi:10.1002/bit.26608.