Viral clearance in end-to-end continuous process for mAb purification: Total flow-through integrated polishing on two columns connected to virus filtration

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Abstract

There are few reports of the adoption of continuous processes in bioproduction, particularly the implementation of end-to-end continuous processes, due to difficulties such as feed adjustment, production batch demarcation, and incorporating virus filtration. Here, we propose an end-to-end continuous process for a monoclonal antibody (mAb) with three integrated process segments: upstream production processes with pool-less direct connection, pooled low pH virus inactivation with automated pH control and a total flow-through integrated polishing process in which two columns were directly connected with a virus filter. The pooled virus inactivation step demarcates the batch, and high impurities reduction and mAb recovery were achieved for batches conducted in succession. Viral clearance tests also confirmed robust virus reduction for the flow-through two column chromatography and the virus filtration steps. Additionally, viral clearance tests with two different hollow fiber virus filters operated at flux ranging from 1.5 to 40 LMH confirmed robust virus reduction over these ranges. Complete clearance with LRV [?] 4 was achieved even with a process pause at the lowest flux. The end-to-end continuous process proposed in this study is highly applicable to production processes, and the investigated virus filters have excellent applicability to continuous processes conducted at constant flux.

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Abstract

There are few reports of the adoption of continuous processes in bioproduction, particularly the implementation of end-to-end continuous processes, due to difficulties such as feed adjustment, production batch demarcation, and incorporating virus filtration. Here, we propose an end-to-end continuous process for a monoclonal antibody (mAb) with three integrated process segments: upstream production processes with pool-less direct connection, pooled low pH virus inactivation with automated pH control and a total flow-through integrated polishing process in which two columns were directly connected with a virus filter. The pooled virus inactivation step demarcates the batch, and high impurities reduction and mAb recovery were achieved for batches conducted in succession. Viral clearance tests also confirmed robust virus reduction for the flow-through two column chromatography and the virus filtration steps. Additionally, viral clearance tests with two different hollow fiber virus filters operated at flux ranging from 1.5 to 40 LMH confirmed robust virus reduction over these ranges. Complete clearance with LRV [?] 4 was achieved even with a process pause at the lowest flux. The end-to-end continuous process proposed in this study is highly applicable to production processes, and the investigated virus filters have excellent applicability to continuous processes conducted at constant flux.

Keywords : continuous process, batch low-pH inactivation, batch definition, feed adjustment, virus filter, inline spike test, process pause

1. Introduction

Following the proposal to use continuous processes for monoclonal antibody (mAb) production (Konstantinov and Cooney, 2015), many studies had demonstrated benefits such as reduced footprint or cost reduction. However, even now, there are few examples of its adoption into actual production. The reasons raised for not adopting this technology include that downstream processes are not yet developed enough to support continuous processes compared to the upstream process or that it is very complicated and difficult to operate each step of the production process while conducting inline quality control and feed adjustment (Somasundaram et al., 2018; Gerstweiler et al., 2021; Kumar et al., 2020). It has been reported that most pharmaceutical manufacturers adopting continuous processes incorporate batch processes into the overall process instead of conducting end-to-end continuous processes (Coffman et al., 2021), and there are also proposals to utilize existing fed-batch process as part of continuous GMP processes (Ferreira et al., 2022). There are also reports of concrete proposals for making continuous processes robust, controllable and practical (David et al., 2020; Rathore et al., 2021; Coffiman et al., 2021).

One of the greatest expected benefits of the continuous process is its cost advantage over batch process, and it was shown that combining continuous processes and single-use technology has a clear cost advantage, especially for processes with relatively small annual production volumes (Pollard et al., 2016; Arnold et al., 2018; Hummel et al., 2019; Farid et al., 2020; Mahal et al., 2021). Furthermore, the benefits of continuous process contribute not only to cost reduction but also to PMI (process mass intensity) reduction (Catalda et al., 2020).

While the benefits and challenges of continuous processes have been extensively examined, there are few reports discussing virus filtration processes to prevent virus contamination, which is a critical step in the production process. Virus filtration is not only a critical step in product safety, but also one of the most costly steps along with media consumption and Protein A chromatography step (Pollard et al., 2016). Compared to batch processes with flux of around 50 to 60 LMH (liters per effective surface area of filter in square meters per hour), David et al. (2019) reported that flux for a virus filter incorporated into a continuous process is extremely small at 0.3 LMH but virus logarithmic reduction value (LRV) of > 4 was still achieved even in these ultra-low flux conditions and the processing volume per unit membrane area became larger with lower flux. To verify the robustness of virus filtration in a long-term continuous process, Lute et al. (2019) performed continuous filtration over 4 days and showed that virus LRV > 4 was achieved even at a throughput of 6900 L/m^2. Bohonak et al. (2021) investigated a continuous mAb process with a connected column process and virus filter operated in constant pressure mode, and they reported that the intensified process in which the mAb solution was concentrated by single-pass tangential flow filtration (TFF) before the column process showed improved purification efficiency of mAb and reduced virus filter membrane area usage. Coolbaugh et al. (2021) achieved continuous operation for 25 days for a virus filtration process operating in TFF mode incorporated into an end-to-end integrated and continuous process.

Validation of the virus filter incorporated into a continuous process cannot be evaluated individually, unlike in a batch process; rather, it is necessary to consider an appropriate virus validation method for continuous process. Proposals for various inline virus spiking methods adapted for virus validation in a continuous process and confirmation of robust virus removal by these methods has been reported by Lute et al. (2011), Bohonak et al. (2021), Shirataki et al. (2021a) and Malakian et al. (2022).

One of the major issues in adopting continuous processes for commercial production is the definition of the product batch, and ICH Q13 Guideline (2021) describe that the product batch should be clearly defined as quantity of output material, quantity of input material and run time at a defined mass flow rate. Use of the greatest common divisor (GCD) of the time required for each step of the process has been reported to be an appropriate definition of batch that meets this requirement (Lali et al., 2021).

In this study, we present an end-to-end continuous process incorporating a constant flux filtration process for the virus filter with feed adjustment between each step and a clear batch concept of the product. Here, the batch is set as the unit of product pooled in the low pH inactivation step. In this process, the solution harvested from the perfusion cell culture is continuously supplied to the affinity process with Protein A column, and column eluate is pooled for the virus inactivation step of low pH treatment for 1 h in an automated manner. After low pH inactivation, the pH-adjusted mAb solution is supplied to a total flow-through two column polishing process including direct connection to a virus filter. The combination of the AEX and CEX columns used here with the virus filter has already been reported to show very high impurity removal, recovery rate and filterability of the virus filter in a total flow-through process (Shirataki et al., 2021a). Though activated carbon is reported to be effective for impurity and virus clearance by flow-through (Ichikawa et al., 2019; Kikuchi et al., 2022), we adopted flow-through two column chromatography in this study.

The total flow-through integrated polishing process has been reported to achieve high mAb purification and robust virus reduction (Shirataki et al., 2021a). In this report, we verify the effectiveness of this integrated process when incorporated into a practical scaled-down end-to-end continuous process. In our previous report (Shirataki et al. 2021a), robust reduction of minute virus of mice (MVM) and xenotropic murine leukemia virus (X-MuLV) using inline spiking was confirmed for a mAb process with throughput to about 100 L/m^2 and low flux levels expected in the continuous process (5 LMH lowest). In this report, we examined MVM removal in a mAb solution on two different hollow fiber virus filters (Planov BioEX and S20N, Asahi Kasei Medical) to a throughput of about 1000 L/m^2 and flux of 5 LMH down to 1.5 LMH with and without process pause to especially test the effect of process pause in low flux filtration. To test a process with reduced filterability, we conducted spiking with X-MuLV at increased titer and compared the performance of two types of hollow fiber virus filters.

2. Materials and Methods

2.1. End-to-end continuous processing

The schematic and process diagram of the end-to-end continuous process from cell culture to purification that was used in this study is shown in Figure 1. In the process diagram, washing, equilibration, regeneration and replacement of columns and filters before and after each step are shown in gray. Starting from day 12 of cell culture, mAb expressed by the perfusion cell culture was harvested and purified by successively performing Protein A column capture, pooled low pH virus inactivation, and flow-through two column chromatography and virus filtration (total flow-through integrated polishing). In the end-to-end continuous process used in this study, the process stream was only pooled for the low pH virus inactivation process, and this production unit is used to demarcate the batch. In this study, a total of three batches were processed in succession. Impurities and mAb concentration at each step were evaluated off-line (Figure 2).

For the end-to-end continuous process trials, mAb (pI 8.5) expressed by a CHO-MK cell line in perfusion cell culture developed by the Manufacturing Technology Association of Biologics (MAB) at its GMP facility was used. The proprietary process consists of harvest of the perfusion cell culture process by XCell ATF-2 (Repligen) at 1.1 vvd (vessel volume exchanges per day), followed by a continuous capture process on two 1-mL Protein A columns (KANEKA KanCapA 3G, Kaneka Corporation) in periodic counter current (PCC) mode on a Contichrom HPLC 30 (YMC Co., Ltd.) with continuous elution of purified mAb with 60 mM acetate (pH 3.6) buffer in cycles of total 20-22 h, with pooling of the eluate for each cycle and column regeneration between total cycles. Each batch of pooled eluate was automatically adjusted to pH 3.4, held for 60 min, then automatically adjusted to pH 6.5, 10 mS/cm with 1 M Tris buffer and 1 M NaCl, and filtered with a micro filter with a nominal pore size of 0.1 μm (USP-043, Asahi Kasei Co., Ltd.) to remove precipitates. The process solution was supplied to the polishing process with two columns directly connected to a virus filter at a constant flow rate.

The total flow-through integrated polishing process consisted of two prepacked mini-columns of AEX (Cellufine MAX IB) and CEX (Cellufine MAX DexS-HbP; both Mini-Column 5LM; 14.6 mm ID, 30 mm L; CV of 5 mL; JNC Corporation) and a Planova BioEX virus filter (0.0003 m^2; Asahi Kasei Medical) without pooling between each step, and the final purified mAb solution was collected. For the polishing chromatography steps, the AEX and CEX columns were directly connected in series to column valves of the AKTA pure 25 (Cytiva), equilibrated with 20 mM Tris-acetate, pH 6.5, 10 mS/cm, which was used for purification of mAb. The virus filter was connected to the outlet valve of the AKTA pure 25 and prepared for use independently of the columns. The AKTA pure 25 sample pump was operated at constant flow rate, and the virus filter inlet pressure (Figure 3) was recorded with a pressure sensor PREPS-N-000 (PendoTECH). Host cell proteins (HCP), host cell DNA and mAb concentrations and aggregates ratio were analyzed at each step of each batch by the following methods.

mAb concentration

mAb concentration (mg/mL) was determined in cell culture by analytical protein A-HPLC performed on a chromatography system consisting of MU701 UV-VIS detector and a PU7710 pump (GL Sciences, Inc.) using a silica monolith Protein A column (Kyoto Monotech, Co., Ltd.) and for process solutions by bind and elute chromatography mode using 50 mM phosphate buffer with 1 M NaCl, pH 7.0 as the mobile wash phase and 100 mM sodium citrate, pH 3.5 as the mobile elution phase followed by quantification of mAb in purified solution by absorption measurement at 280 nm on a UV-VIS spectrophotometer V-750 (JASCO Corporation).

HCP concentration

CHO host cell protein (HCP) concentration (ng/mg-mAb) was determined using the CHO HCP 3rd generation ELISA kit (F550) (Cygnus Technologies) according to manufacturer instructions.

Host cell DNA concentration

Host cell DNA concentration (pg/mg-mAb) was determined by quantitative PCR (qPCR) performed using the PrepSEQ Residual DNA Sample Preparation Kit and resDNASEQ Quantitative CHO DNA Kit (Thermo Fisher Scientific, Inc.) according to manufacturer protocol.

mAb aggregates concentration

mAb aggregate concentration (% in mAb) was determined by analytical size exclusion chromatography (SEC-HPLC) performed on a chromatography system Prominence LC-20 (Shimadzu Corporation) using a TSKgel G3000SWxl 5 µm column (Tosoh Bioscience, LLC) with a mobile phase of 100 mM potassium phosphate with 200 mM NaCl, pH 7.0.

2.2. Viral clearance studies

Viral clearance studies on various components of the flow-through two column chromatography (AEX and CEX) and virus removal filter were conducted at ViruSure (Vienna, Austria) using purified mAb produced from the same cell line but by fed-batch production and purification with Protein A and IEX columns. The mAb used in these studies was 10 mg/mL mAb in 20 mM Tris-acetate, 10 mS/cm, pH 6.5. MVM and X-MuLV virus titers were determined by $TCID_{50}$ method.

2.2.1. Flow-through two column chromatography

The virus removal capability of the Cellufine MAX IB (AEX) and Cellufine DexS HbP (CEX) mini columns (CV of 5 mL) operated in flow-through mode without virus filtration (Figure 4) was evaluated. Following column equilibration, 300 mL of 10 mg/mL mAb (600 mg mAb/mL-resin for each column) spiked with MVM or X-MuLV at 2% and passed through a 0.45 µm PES prefilter was loaded onto the columns at a flow rate of 0.25 mL/min (residence time 20 min) using an AKTA pure 25. Following a 60 min process pause, the columns were flushed with a 30 mL buffer wash. Virus titer assay was conducted on both the 300 mL flow-through fraction and the combined flow-through fraction with the 30 mL buffer wash (Table 1).

2.2.2. Virus filter in total flow-through integrated polishing process (inline spike test)

To confirm the virus removability of the virus filter in the total flow-through integrated polishing process shown in Figure 5, the inline spiking viral clearance study method designed by ViruSure as reported by Shirataki et. al. (2021a) was conducted. For this study, 10 mg/mL mAb spiked at 10% with one of two viruses, MVM or X-MuLV, was passed through a 0.1 µm PES prefilter or 0.2 µm PES prefilter, respectively before use. The flow-through two column chromatography process described in Section 2.2.1 was directly connected without pooling to a 0.0003 m^2 Planova BioEX filter on an AKTA pure 25. Un-spiked 10 mg/mL mAb was supplied to the two column system by system pump at a flow rate of 0.18 mL/min while the 10% virus (MVM or X-MuLV) spiked mAb was supplied by sample pump at a flow rate of 0.02 mL/min to the feed stream after the two chromatography columns and before the virus filter, resulting in mAb with 1% virus spike being applied to the filter at a constant flow rate of 0.2 mL/min, corresponding a flux of 40 LMH. After loading 300 mL of mAb solution, both pumps were stopped for a 60 min process pause. Then, only the system pump was turned on to supply 30 mL of buffer at a flow rate of 0.2 mL/min to wash out feed from the chromatography columns. Using this procedure, viral clearance was conducted at a constant flow rate and the effect of a process pause was evaluated (Table 2). The total mAb solution loaded to virus filter was 300 mL and loading of mAb on the filter by effective surface area was about 10 kg/m^2 and 1000 L/m^2.

2.2.3. MVM spike of low flux filtration on Planova BioEX and S20N filters

Viral clearance studies of the mAb were conducted on 0.001 and 0.0003 m^2 Planova BioEX filters, which has a hydrophilic modified polyvinyldene flouride (PVDF) hollow fiber, and on the 0.001 m^2 Planova S20N filter (Asahi Kasei Medical), which is a newly launched filter with hollow fibers made from regenerated cellulose. In this study, all runs were conducted at low flux of 1.5, 5 and 10 LMH for Planova BioEX and 1.5 LMH for Planova S20N as shown in Table 3. For all runs, 10 mg/mL mAb spiked with 1% MVM (0.1 µm prefiltered) was filtered for 24 h. The spike solution was loaded to the filter using the sample pump of AKTA pure 25. Following mAb load filtration and a 60 min process pause, 10 mL or 5 mL of equilibration buffer wash was

conducted for the 0.001 m^2 or 0.0003 m^2 filters, respectively. Viral clearance was measured for the mAb load permeate and total permeate with buffer wash.

2.2.4. Higher spike X-MuLV on Planova BioEX and S20N filters

To conduct viral clearance with a higher load titer of X-MuLV, 10 mg/mL mAB spiked with 2% X-MuLV (0.2 μm prefiltered) was filtered on 0.0003 m^2 Planova BioEX and 0.001 m^2 S20N filters with a load volume of 300 and 1000 mL, respectively, corresponding to a throughput of 1000 L/m^2, and a flow rate of 0.2 and 0.667 mL/min, respectively, corresponding to a flux of 40 LMH for each filter (Table 4). The spike solution was loaded to the filter using the sample pump of AKTA pure 25. The log titer of load solution in this test is effectively higher than the inline spike test described previously because of higher spike concentration.

3. Results and Discussion

3.1. End-to-end continuous process with total flow-through integrated polishing process

In this end-to-end continuous process, the mAb solution produced by perfusion cell culture was clarified by ATF and purified by Protein A chromatography all with pool-less direct connection. The obtained material was pooled for low pH virus inactivation, and this volume was taken as the batch. Finally, total flow-through integrated polishing was conducted in a pool-less direct connection as shown in Figure 1.

3.1.1. Performance at each step of end-to-end continuous process for mAb purification

Process performance was examined for the end-to-end continuous process incorporating upstream production processes with pool-less direct connection, pooled low pH virus inactivation with automated pH control and a total flow-through integrated polishing process in which two column chromatography was directly connected to a virus filter. The flow rates through the process were set to accommodate completion of batches in succession to allow continuously utilizing the setups (Figure 1). Batch 1 had a smaller volume but higher mAb concentration supplied to the polishing step of 116.14 mL and 7.6 mg/mL (Figure 3), respectively, due to air bubbles impeding the supply of harvest solution to the capture process. For subsequent batches, the load volume and mAb concentration were 315.29 mL and 5.1 mg/mL for Batch 2, and 317.97 mL and 4.4 mg/mL for Batch 3, respectively. The pH- and conductivity-adjusted mAb solution after the low pH virus inactivation step was supplied to the flow-through two column chromatography consisting of AEX and CEX columns directly connected to the virus filter at a flow rate of 0.14 mL/min for Batch 1 and 0.38 mL/min for Batches 2 and 3. The flow rate were determined to adjust the batch cycle time as about 24 hours. At these flow rates, the process feed had a residence time for each column of 35.7 min for Batch 1 and 13.2 min for Batches 2 and 3. The mAb throughput for each column was 176.5 mg/mL-resin for Batch 1, 321.6 mg/mL-resin for Batch 2, and 280.7 mg/mL-resin for Batch 3. At the end of applying the mAb to the flow-through two column process, equilibration buffer (15 mL for Batch 1 and 75 mL for Batches 2 and 3) was applied to wash out residual mAb in the columns.

As the flow-through from the columns was directly loaded to the virus filter, the filtration was conducted at the same constant flow rate as the polishing chromatography step and the filtrate fraction was consisting of the flow-through fraction followed by the buffer wash fraction. At first, the filtration pressure of the Planova BioEX plotted against filtration time (min) and throughput (L/m^2) shows pressure of about 0.073 MPa for Batch 1 and about 0.160 MPa for Batches 2 and 3 for the filtration of equilibration buffer (Figure 3). The pressure increased as mAb entered the filter, remained stable and higher (about 0.077 MPa for Batch 1, about 0.175 MPa for Batch 2 and about 0.170 MPa for Batch 3) during the flow-through mAb filtration, then following a partial depressurization lasting several seconds due to flow path switching, the filtration pressure decreased again. For Batches 1 and 3, pressure fluctuation occurred during filtration of the wash buffer, and this may be due to unstable supply of buffer from the pump and introduction of air during path switching. Batch 2 showed a slightly higher filtration pressure than Batch 3 due to a slightly higher mAb concentration of 5.10 mg/mL compared to 4.41 mg/mL for Batch 3. For all batches, the filtration pressure remained almost constant with almost no increase, even for throughput of more than 1000 L/m^2 for Batches 2 and 3. The flow-through two column chromatography process effectively removes impurities such as HCP

and aggregates (Figure 2), resulting in a very highly purified mAb solution with high filterability. This demonstrates that the total flow-through integrated polishing process adopted in the end-to-end continuous process of this study is extremely effective for purification and for filterability for virus removal.

Focusing on the virus filter performance, the flow rate through the filter matches the flow rate through the columns and was 28 LMH for Batch 1 and 76 LMH for Batches 2 and 3, and by volume-based throughput (L/m^2) and mass-based throughput (kg/m^2), the mAb throughput was 387.1 L/m^2 and 2.9 kg/m^2 for Batch 1, 1051 L/m^2 and 5.4 kg/m^2 for Batch 2, and 1059.9 L/m^2 and 4.7 kg/m^2 for Batch 3. In addition, the total filtrate volume including the wash from the column, was 131.1 mL for Batch 1, 391.8 mL for Batch 2, and 393.9 mL for Batch 3, and throughput for the filter including wash was 437.1 L/m^2, 1301.0 L/m^2 and 1309.9 L/m^2, respectively for each batch.

3.1.2. mAb purification with impurity removal

The impurity levels and mAb concentration are shown in Figure 2. For all three batches, the concentrations of mAb and impurities showed a similar trend for all steps. The mAb concentration increased in the capture step and decreased in the subsequent low pH virus inactivation and polishing steps due to volume increases accompanying pH and conductivity adjustment and the addition of wash buffer, resulting in 3.4 - 5.2 mg/mL mAb after the polishing step including virus filter. The mAb recovery for the total flow-through integrated polishing step was 77%, 93% and 95% for Batches 1, 2 and 3, respectively. The lower recovery rate for Batch 1 was likely due to using less buffer volume for wash and increasing the volume from 15 to 75 mL produced a recovery rate of more than 90%.

Impurities were overall reduced by the process. The HCP concentration exponentially decreased at each process step, especially in the capture and polishing steps, decreasing to 12.0 ng/mg-mAb or less. The DNA concentration also decreased exponentially, particularly in the capture and low pH virus inactivation steps, reaching 1 pg/mg-mAb or less after the polishing step. The aggregate concentration was around 1% in the harvest step, increased to around 3% in the capture and low pH virus inactivation steps, and then decreasing to less than 1% after the polishing step. High aggregate content was produced by the long-term (20-22 h) exposure of mAb to low pH in the capture elution pool, although the KANEKA KanCapA™ 3G Protein A columns can efficiently reduce aggregates (Pabst et al., 2018). SEC analysis of the process solution confirmed that the aggregates were primarily dimers, and trimers or higher aggregates were below the detection limit. Since the virus filter removes virus particles by size exclusion, it is thought to not remove small-sized HCP and dimers. The HCP and aggregate contents were reduced by the polishing step as shown in Figure 2, corroborating that the flow-through two column chromatography shows excellent HCP and aggregate removability as reported previously (Shirataki et. al., 2021a; 2021b).

3.2. Virus removability of total flow-through integrated polishing process

In the end-to-end continuous process for mAb purification, virus removal is expected for the Protein A capture, low pH virus inactivation, two column chromatography and virus filter steps, with the most robust virus removability at the virus filtration step. Here, viral clearance testes were carried out for specific segments of the total flow-through integrated polishing process.

3.2.1. Viral clearance for flow-through two column chromatography

Previously, the virus clearance test for individual column (AEX and CEX) and the inline virus spiking test for virus filter directly connected to a column, showed excellent virus removability as reported (Shirataki et al., 2021a). However, in that report, the residence time for the column was as short as 2 min compared to 13 to 35 min in this study, and each column was evaluated individually with throughput of 300 mg mAb /mL-resin. In this study, virus removability was examined for the flow-through two column chromatography with a residence time of 20 min and a mAb throughput of 600 mg/mL-resin for each column.

For the flow-through two column chromatography system with AEX and CEX, the UV absorbance profile of the MVM-spiked mAb solution run shows that buffer in the columns is rapidly replaced by mAb solution as indicated by the UV absorbance sharply rising and remaining at the maximum level throughout the run

(Figure 4). Following application of the specified volume of solution to the two columns and a 60 min process pause, the buffer wash cleared the mAb from the columns as shown by the gradually decreasing UV absorbance. As shown in Table 1 for mAb spiked with MVM or X-MuLV, complete clearance was achieved for both the flow-through fraction and the flow-through plus wash with LRV of [?] 4 for MVM and X-MuLV. These results confirm that the two column chromatography has high virus removability for both X-MuLV and MVM for a process with residence time of 20 min in the columns and that process pause did not affect virus removal.

3.2.2. Inline spiking viral clearance test for integrated flow-through two column and virus filter

To separately examine the virus removability of the virus filter, an inline spiking viral clearance test for the Planova BioEX filter implemented in the flow-through two column chromatography was carried out. Due to clogging from impurities in the virus spike, the flow rate was reduced to produce a flux of 40 LMH to the filter (compared to 76 LMH for the end-to-end continuous process) to avoid exceeding the operating pressure limit of Planova BioEX (0.35 MPa) before reaching the target throughput. It should be noted that viral clearance determined for the lower flux process (40 LMH) can be taken as confirming viral clearance at 76 LMH, as lower flux is a worse case condition for virus filtration.

As shown in Figure 5, a feed solution of 10 mg/mL mAb without virus spike was supplied to the two columns and the same mAb spiked at 10% MVM or X-MuLV was supplied through inline spiking. The pressure increases on the filter were observed until the peak after the process pause for the loading of mAb solution spiked with MVM or X-MuLV compared to the minimal pressure increase observed for loading of mAb solution without spike. The pressure profile of the virus-spiked mAb filtration shows fluctuations that are considered to be due to pulsation of the pump due to air bubbles. The pressure increases with increasing load volume are greater for the filtration of the X-MuLV spiked solution and approach the upper operating pressure limit of the filter at the target throughput. The more stable filtration pressure of the un-spiked mAb is similar to the pressure profile for the filter incorporated in the end-to-end continuous process shown in Figure 3.

Due to the concern that virus breakthrough to permeate may occur due to the pressure drop during a process pause as previously described (Ajayi et al., 2022; Fan et al., 2021; Johnson et al., 2021), a 60 min process pause was included after loading the virus-spiked mAb, and virus removability for inline spiking of MVM or X-MuLV was evaluated for both the flow-through fraction of the mAb and the flow-through fraction with the buffer wash. As shown in Table 2 for mAb spiked with MVM or X-MuLV, complete clearance was achieved for both the flow-through fraction and the flow-through plus wash with MVM LRV of [?] 5 and X-MuLV LRV of [?] 3. The relatively lower X-MuLV LRV for the filter than the two column process can be attributed to the lower X-MuLV spike titer. These results confirm that the Planova BioEX filter has high virus removability for both MVM and X-MuLV and that the process pause did not affect virus removal.

3.2.3. Viral clearance at low flux for Planova BioEX and Planova S20N

For 24 h filtrations of 10 mg/mL mAb spiked with MVM on two sizes of Planova BioEX and one size of Planova S20N to test low flux (1.5 - 10 LMH) conditions, complete MVM removal was achieved for all load and load plus wash buffer as shown in Table 3. For the load fraction as well as for the total load fraction plus buffer wash after the 60 min process pause, MVM LRV of >5 was confirmed for all runs. Under these low flux conditions, filtration pressure was extremely low, about 0.04 MPa at 5 LMH for Planova BioEX, and at 1.5 LMH, pressure was less than 0.01 MPa for both Planova BioEX and S20N. These results confirm that both Planova BioEX and S20N filters have robust virus removal properties, even for very low flux filtrations with process pause, such as in continuous process applications.

3.2.4. Viral clearance with higher spike concentration

As shown in the viral clearance study in the integrated process (Table 2), the load titer of X-MuLV is sometimes lower than that for MVM even with the same spike level. Lower load titer may cause a lower

virus LRV than expected, and in this case, X-MuLV LRV of > 4 could not be achieved, despite that no virus was detected in the permeate, indicating complete clearance. The lower spike titer for X-MuLV may have been due to the instability of X-MuLV, and similar results have been reported previously (Shirataki et al., 2021a; Gefroh et al., 2014).

To test virus removal for a higher spike level, 10 mg/mL mAb spiked at 2% with X-MuLV was processed to virus filter at 40 LMH with a 60 min process pause. As shown in Table 4, much higher X-MuLV titer was achieved by increasing the virus spike to 2% (6.69 and 7.27 log $TCID_{50}$/mL for Planova BioEX and S20N, respectively) compared to 5.84 log $TCID_{50}$/mL for the 1% spike (Table 2). An X-MuLV LRV > 4 was achieved for both Planova BioEX and S20N filters with complete clearance for both the flow-through fraction and the flow-through fraction plus wash. Thus, the reason X-MuLV LRV did not exceed 4 in Table 2 and in the previous report (Shirataki et al., 2021a) was that the load titer was not sufficient.

4. Conclusions

The end-to-end continuous process for mAb purification reported in this study has upstream and downstream steps with pool-less direct connections and a low pH inactivation step with automated pH adjustment performed in batch mode, which serves as a batch demarcation and also allows for feed adjustment before and after the low pH inactivation step. These characteristic properties make the process amenable for continuous mAb production. Furthermore, the mAb production experiment by the scale-down model of this report confirmed that this continuous process results in high productivity and high purity of mAb. The mAb was highly purified by the total flow-through integrated polishing process in which two columns (AEX and CEX) were directly connected to the virus filter. As a result, even with a filtration throughput of about 1000 L/m^2, filtration pressure increases on the virus filter were extremely small, demonstrating that high purification and extremely stable virus filtration can be realized in the end-to-end continuous process for mAb purification.

Regarding virus reduction, both the flow-through two column chromatography and the virus filter showed robust virus removability for the total flow-through integrated polishing process. While virus filtration is usually carried out by constant pressure filtration and is not easily incorporated into a continuous process, the hollow fiber type virus filters tested in this study showed high virus reduction even at very low flux and with a process pause that may be observed in continuous processing. For very low flux of 1.5 LMH and a condition with process pause of 60 min, both Planova BioEX and S20N showed virus LRV of > 4, indicating that these hollow fiber type virus filters are highly applicable for the continuous process with high filterability and robust virus removability.

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Conflict of Interests

The authors (HS, HM and HK) are employees of their affiliate companies. None have received any compensation for the research reported in this article beyond that from their affiliate employee.

Author Contribution

Hironobu Shirataki: Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - Review & Editing. Yoshihiro Matsumoto: Data curation, Formal analysis. Fuminori Konoike: Data curation, Formal analysis. Shuichi Yamamoto: Conceptualization, Supervision, Writing - original draft.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Figure legends

Figure 1 . Schematic and process diagram representing end-to-end continuous process for mAb production with implementation of total flow-through integrated polishing process

Figure 2 . Changes throughout mAb harvest, capture, low pH virus inactivation and polishing processes for a) HCP (ng/mg-mAb), b) DNA (pg/mg-mAb), c) mAb (mg/mL) and d) aggregate (%).

Figure 3 . Transmembrane pressure of mAb virus filtration flow-through fraction and buffer wash implemented in the end-to-end continuous process shown in Figure 1 for three batches with a) pressure plotted against time (min) and b) pressure plotted against throughput (L/m^2).

Figure 4 . Setup of viral clearance test for flow-through two column chromatography (upper) and 280 nm UV absorbance profile of flow-through output for MVM-spiked mAb run (lower).

Figure 5 . Setup of inline spiking viral clearance test for integrated polishing process with virus filter connected to flow-through two columns chromatography (upper figure) and transmembrane pressure profile of virus filter loaded with virus-spiked mAb flow-through from the two column step (lower figure).

Tables

Table 1 . Viral clearance test results for flow-through two column chromatography consisting of Cellufine MAX IB and Cellufine DexS HbP with 10 mg/mL mAb spiked with MVM and X-MuLV.

Parameter	MVM	X-MuLV
Load titer (log $TCID_{50}$/mL)	8.93	6.29
Flow-through fraction virus LRV	[?]6.34 ± 0.18	[?]4.34 ± 0.25

Parameter	MVM	X-MuLV
Flow-through + Wash virus LRV	[?]6.07 ± 0.18	[?]4.06 ± 0.25

Table 2 . Virus reduction of inline spike virus clearance test for Planova BioEX implemented in the total flow-through integrated polishing process with 10 mg/mL mAb. Spiked virus; MVM and X-MuLV.

Parameter	MVM	X-MuLV
Load virus spike (log $TCID_{50}$/mL)	7.54	5.84
Flow-through fraction virus LRV (log $TCID_{50}$/mL)	[?]5.47 ± 0.25	[?]3.78 ± 0.25
Flow-through + Wash virus LRV (log $TCID_{50}$/mL)	[?]5.07 ± 0.24	[?]3.37 ± 0.25

Table 3 . Viral clearance test results with low flux loading on Planova BioEX and S20N for 10 mg/mL mAb spiked with MVM.

Parameter	Filtration runs	Filtration runs	Filtration runs	Filtration runs
Filter	Planova BioEX 0.001 m^2	Planova S20N 0.001 m^2	Planova BioEX 0.0003 m^2	Planova BioEX 0.0003 m^2
Flow rate (mL/min)	0.025	0.025	0.025	0.05
Flux (LMH)	1.5	1.5	5	10
Load (mL)	36	36	36	72
Throughput (L/m^2)	36	36	120	240
MVM spike (log $TCID_{50}$/mL)	7.71	7.86	7.41	7.96
Flow-through fraction MVM LRV (log $TCID_{50}$/mL)	[?]6.04 ± 0.26	[?]6.30 ± 0.28	[?]5.79 ± 0.28	[?]6.03 ± 0.27
Flow-through fraction + Wash MVM LRV (log $TCID_{50}$/mL)	5.43 ± 0.45	[?]5.91 ± 0.28	[?]5.64 ± 0.28	[?]6.15 ± 0.27

Table 4 . Viral clearance test results for Planova BioEX and S20N with 10 mg/mL mAb spiked with 2% X-MuLV.

Parameter	Filtration runs	Filtration runs
Filter	BioEX 0.0003 m^2	S20N 0.001 m^2
Flow rate (mL/min)	0.2	0.667
Flux (LMH)	40	40
Load (mL)	300	1000
Throughput (L/m^2)	1000	1000
X-MuLV load (log $TCID_{50}$/mL)	6.69	7.27
Flow-through fraction X-MuLV LRV (log $TCID_{50}$/mL)	[?]4.59 ± 0.28	[?]4.65 ± 0.27
Flow-through fraction + Wash X-MuLV LRV (log $TCID_{50}$/mL)	[?]4.53 ± 0.28	[?]4.63 ± 0.27

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