Model-based design and control of a small-scale integrated continuous end-to-end mAb platform

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Abstract
A continuous integrated bioprocess available from the earliest stages of process development allows for an easier, more efficient and faster development and characterization of an integrated process as well as production of small-scale drug candidates. The process presented in this article is a proof-of-concept of a continuous end-to-end monoclonal antibody production platform at a very small scale based on a 200 ml alternating tangential flow filtration perfusion bioreactor, integrated with the purification process with a model-based design and control. The downstream process, consisting of a periodic twin-column protein A capture, a virus inactivation, a CEX column and an AEX column, was compactly implemented in a single chromatography system, with a purification time of less than 4 hr. Monoclonal antibodies were produced for 17 days in a high cell density perfusion culture of CHO cells with titers up to 1.0 mg/ml. A digital twin of the downstream process was created by modelling all the chromatography steps. These models were used for real-time decision making by the implementation of control strategies to automatize and optimize the operation of the process. A consistent glycosylation pattern of the purified product was ensured by the steady state operation of the process. Regarding the removal of impurities, at least a 4-log reduction in the HCP levels was achieved. The recovery yield was up to 60%, and a maximum productivity of 0.8 mg/ml/day of purified product was obtained.

KEYWORDS
end-to-end continuous bioprocessing, monoclonal antibody, perfusion bioreactor, process integration, process modelling

1 | INTRODUCTION

Monoclonal antibodies (mAbs) and related products are used to treat many diverse diseases. The revenues for mAbs were around $90 billion by 2015, representing over 60% of the global biopharmaceutical...
industry revenues. The annual cost for a treatment with these biologics can however be up to $35,000 per patient, due to the high doses needed for medication. Most of the processes for mAb production are based on batch operation: a batch or fed-batch bioreactor run followed by a series of batch purification steps, including several chromatography steps, a virus inactivation step and filtration steps. These robust and well-characterized legacy processes are nevertheless inefficient and inflexible with regards to the constantly changing market demands and upcoming competition from biosimilars.

The increasing cost pressure has led the biopharmaceutical industry to search for alternative approaches, such as continuous bioprocessing. Although higher product titers can be obtained in fed-batch operations, the obtained volumetric productivities are generally lower in comparison to intensified perfusion processes operated under high cell density. An improved process efficiency is also obtained in continuous purification by minimization of chromatography column volumes and improved resins capacity utilization. The integration of upstream and downstream processes, so called integrated continuous bioprocesses (ICB), enable productivity increase, decrease of the equipment size, and overall a reduction of the manufacturing costs. In addition, product hold-up steps are completely eliminated in a fully continuous operated and automated end-to-end platform.

The lack of scale-down models and the long duration for the development of continuous processes is considered as a limitation for the implementation of industrial continuous processes. ICBs from the very first stages of process development can accelerate the transfer of a new drug candidate to commercial manufacturing. These offer an easier and safer scalability of the process. Furthermore, they considerably alleviate the small-scale production of drug candidates, which is currently a highly labor demanding task, requiring many manual and inefficient steps. Several studies have demonstrated the potential of ICBs. The size of these lab-scale ICBs is based nowadays on bioreactor volumes from 1.2 up to 12 L, and extensive equipment with separate control systems is required to perform all the purification steps. These are unnecessarily too large for the early process development and characterization of candidate drugs or their production for preclinical testing.

Process control is needed for the robust operation of bioprocesses. This particularly important for highly complex processes with a large set of parameters and several connected unit operations, such as an ICB. However, some control strategies need process information that cannot be obtained in real-time from analytical techniques. This issue can be solved by modelling. Modelling is a powerful tool, not only to obtain a better understanding of the process and the effect of certain parameters on process outputs, but also to assist the process control with real-time information continuously used for a robust and automated operation. For example, model-based simulations of an ICB can be used to determine how certain process parameters such as the loading time should be tuned to yield the desired output in the purification steps, for example, increasing the resin utilization, based on real-time estimations of parameters like the harvest concentration. In the present study, mathematical modelling of chromatography provided a digital twin of the complete downstream process. The simulated responses from this digital twin were used to design the downstream process and enabled the implementation of control strategies.

A proof-of-concept of an end-to-end ICB at small scale for the production of purified mAb was demonstrated. The objective of this small-scale system was to obtain a compact design with minimal use of equipment, based on a 200 ml working volume bioreactor with alternating tangential flow filtration (ATF) perfusion, and a single lab-scale chromatography station (ÅkTA™ pure) for all the downstream steps. To avoid any manual interaction, the whole purification process was automated and controlled by a software that allowed for advanced control strategies assisted by modelling.

# MATERIALS AND METHODS

## 2.1 Upstream system

Two mAb producing Chinese Hamster Ovary cell lines were cultivated in a Dasbox Mini Bioreactor System (Eppendorf, Germany) with 200 ml working volume. The cell lines were CHO-K1 cells, kindly provided by GE Healthcare Life Sciences, and CHO-S cells, kindly provided by Cobra Biologics. The cells were inoculated from a shake flask culture at around $5 \times 10^6$ cells/ml into the bioreactor and were grown in chemically defined HyClone™ ActiPro medium (GE Healthcare Life Sciences, Sweden). The temperature was set to 37°C, and the pH was maintained at 6.8 by addition of CO2 into the headspace of the bioreactor. The dissolved oxygen concentration (DO) was kept at 40% by sparging of oxygen. Cultivations were performed in a bioreactor in perfusion mode using an ATF2 system (Repligen, USA) with a hollow fiber cartridge of 0.45 μm pore size and 110 cm² membrane area (model CFP-4-E-3MA, GE Healthcare Life Sciences, Sweden). Details on the perfusion bioreactor can be found in a previous publication. The alternating tangential flow rate was set to 0.7 L/min. Cell-free harvest was removed with a pump through the hollow fiber and transferred to a hold-up flask prior to loading on the chromatography system. The hold-up flask was a sterile 250 ml glass bottle with three ports for harvest inlet, harvest outlet and a 0.2 μm air filter. It was placed on an external balance, model PCB 2000-1, provided by KERN (Balingen, Germany). The feed medium consisted of a mixture of 92% HyClone ActiPro medium (without glucose), 7% HyClone Cell Boost 7a and 1% HyClone Cell Boost 7b (GE Healthcare Life Sciences, Sweden). Glucose was added to the feed medium according to the cells' need, at up to 60 mM final concentration. The perfusion, that is, continuous addition of feed medium and harvest removal, was initiated immediately after the inoculation. The cells were grown to a target cell density of $80 \times 10^6$ cells/ml with a stepwise increase of the perfusion rate before cell bleeding was applied to keep the cell density constant. At steady state, a cell specific perfusion rate (CSPR, given as the perfusion rate divided by the cell density) of 25 pl/cell/day was applied.
2.2 | Downstream system

2.2.1 | Downstream materials

For the present work a typical mAb purification process was used. It included the following steps: (a) capture step by a protein A resin mAb Select Sure™ with an isotropic elution; (b) virus inactivation step at pH 3.5 for 60 min; (c) cation exchange chromatography (CEX) step in blind-and-elute mode with a gradient elution, using the resin Capto™ S ImpAct; and (d) anion exchange chromatography (AEX) step in flow-through mode, using Capto adhere resin. Usually, a final ultrafiltration step is included to formulate the product in the final buffer at desired concentration. However, this process step was left out of the scope of this article since a larger process scale would have been needed to run an ultrafiltration process, as shown in previous work. Notice that the omission of this step does not affect the present results focusing on the ICB and the control strategies since these are intended for the chromatography steps.

Prepacked HiTrap™ columns with 1 ml column volume, provided by GE Healthcare Life Sciences (Uppsala, Sweden), were used in the chromatography steps. The small column size made it possible to adjust the required column volume by coupling several columns in series. Therefore, the minimum column volume was 1 ml. The run conditions, including flow rates and buffers, were based on the vendor information about the mAb Select Sure resin, for the capture step, and the Capto S ImpAct and Capto adhere resins, for the ion exchange chromatography steps. Two FEP (Fluorinated Ethylene Propylene) tubes were used for product hold-up during the virus inactivation (VI loop, for later references) and between the CEX and the AEX step (hold-up loop). During the hold-up time of the virus inactivation, strip, CIP and equilibration of the columns were performed, and after that, the virus-inactivated product was loaded on the CEX column.

The system used to run the purification was the ÄKTA™ pure 150 unit, controlled by the software UNICORN™ 7, from GE Healthcare Life Sciences (Uppsala, Sweden). The system included the following modules: four versatile valves (VVs) to lead the flow in several directions, two column valves, two UV monitors, a pH sensor, a conductivity sensor, two valves functioning as 3-way valves, a sample pump to load the capture column, and the gradient pumps, consisting of a Pump A and a Pump B, to wash and elute the columns. The experimental process set-up can be found in Figure S1.

2.2.2 | Process control

The downstream process was controlled by Orbit, a research software developed in the department of Chemical Engineering at Lund University, Sweden, which is deeply discussed by Andersson et al. and Gomis-Fons et al. This software communicates with UNICORN and sends instructions sequentially in an order determined by the process and selected by the user. For this case, the software was modified so that it could be continuously run without the need of manual labor, for which certain control strategies, discussed in detail in Section 3.1.3, were developed. Orbit was also adapted to record the data from the simulation and use them to carry out the control strategies. The ÄKTA pure unit was installed at KTH in Stockholm, Sweden, and the downstream process was supervised from Lund University, Sweden. Therefore, the software was adapted so that it could be run remotely.

2.2.3 | Process modelling

All the downstream unit operations were modelled and simulated to predict process information that was used to make process-related decisions based on the predicted output. For the capture step, a General Rate model developed by Perez-Almodovar and Carta, with two adsorption rates (one quick, corresponding to the index $i = 1$, and the other one slow, $i = 2$) was implemented. The model is described with the differential equations Equations (1–3) together with the boundary conditions (1a), (1b), (2a), and (2b):

\[
\frac{\partial c}{\partial t} = D_{\text{eff}} \frac{\partial^2 c}{\partial z^2} \frac{v}{c_c} \frac{1 - c_c}{c_c} \frac{3}{r_p} \left( c - c_{p,i} \right) \quad (1)
\]

\[
\frac{\partial c}{\partial t} = \frac{v}{c_c} \frac{1 - c_c}{c_c} \left( c - c_{p,i} \right) \quad \text{at} \quad z = 0 \quad (1a)
\]

\[
\frac{\partial c}{\partial t} = 0 \quad \text{at} \quad z = L \quad (1b)
\]

\[
\frac{\partial c_p}{\partial t} = \frac{1}{r_p} \left( r^2 \frac{\partial c_p}{\partial r} \right) - \frac{1}{r_p} \frac{\partial (q_1 + q_2)}{\partial t} \quad (2)
\]

\[
\frac{\partial c_p}{\partial t} = 0 \quad \text{at} \quad r = 0 \quad (2a)
\]

\[
\frac{\partial c_p}{\partial t} = k_f \frac{1}{r_p} \left( c - c_{p,i} \right) \quad \text{at} \quad r = r_p \quad (2b)
\]

\[
\frac{\partial q_i}{\partial t} = k_i \left[ (q_{\text{max}} - q_i) c_p - q_i / K \right] \quad (3)
\]

Several parameters were obtained from frontal analyses of breakthrough curves on the protein-A resin, and calibration of Langmuir isotherms ($D_{\text{eff}}, q_{\text{max}}, L, K, k_1, k_2$). The axial dispersion and the mass transfer coefficient were determined by empirical correlations. The column void was obtained by fitting the Kozeny equation to pressure drop experiments, as performed by Pabst et al. An estimation of the harvest concentration and the measured flow rate from the bioreactor were used to model the breakthrough curve. This model was used for the loading of the capture step, and it was essential to determine the switching of the cycle based on the saturation of the capture column, as discussed in Section 3.1.3.

The loops were modelled as a one-dimensional dispersive–convective transport (Equation 4), with the same boundary conditions as the ones shown in Equations (1a) and (1b), where the axial...
dispersion was obtained from an empirical correlation.\textsuperscript{22} Note that no void was used in this equation because the loops are not packed.

\[
\frac{\partial c}{\partial t} = D_{av} \frac{\partial^2 c}{\partial z^2} - v \frac{\partial c}{\partial z}
\]  

(4)

The CEX and AEX chromatography steps, as well as the elution of the protein-A step, were modelled with a kinetic model with salt-dependent (pH-dependent in the case of the capture elution) equilibrium constant\textsuperscript{25} (Equations (5–7)), with the boundary conditions shown in Equations (1a) and (1b). The parameters of these models (H\textsubscript{0}, D\textsubscript{app}, q\textsubscript{max}, k, and b) were obtained by fitting them to experimental elution peaks and breakthrough curves.

\[
\frac{\partial c}{\partial t} = D_{av} \frac{\partial^2 c}{\partial z^2} - e \frac{\partial c}{\partial z} + \frac{1 - e_c}{e} \frac{\partial q}{\partial t}
\]  

(5)

\[
\frac{\partial q}{\partial t} = k \left[ H_{0} c_{eq}^{-2} \left( 1 - q \right) c_{eq} \right]
\]  

(6)

\[
\frac{\partial c_S}{\partial t} = D_{av} \frac{\partial^2 c_S}{\partial z^2} - e \frac{\partial c_S}{\partial z}
\]  

(7)

All units were put together and simulated to create a digital twin of the downstream process. Both the loops and the ion exchange columns were discretized in space using the Finite Volume Method with 50 grid points for the axial coordinate, and in the General Rate model, the column was discretized in 30 axial grid points, and the particle in 10 grid points. The downstream models were solved by simultaneously integrating the ordinary differential equations (ODE) with the built-in MATLAB ODE solver ode15s, suitable for stiff problems. In Table S1, all the parameters of the models are shown.

### 2.3 Estimation of the harvest concentration

The estimation of the harvest concentration in real time was necessary in order to model the downstream process, which, in turn, was used to carry out control strategies that allowed an automated operation of the process, and are discussed in more detail in the subsequent sections. The concentration was calculated as the loaded mass of mAb divided by the loaded volume. The mass was estimated from the area of the absorbance peak of the CEX inlet stream, that is, the loading stream onto this column. With an extinction coefficient of 1.4 m\textsuperscript{L}/(mg cm), taken from Maity et al.,\textsuperscript{26} the amount of mAb could be estimated, and it was assumed to be the same as the loaded amount onto the capture column. In addition, the volume loaded onto the capture column was calculated based on the flow rate from the bioreactor, which was continuously measured online. The harvest concentration could then be estimated from the volume and the amount of mAb. This concentration corresponded to the product loaded onto the capture column in the previous cycle, in accordance with the operation of a twin-column capture process. It was assumed that the harvest concentration remained unchanged during a cycle, hence this value was used to model the downstream process in the next cycle. In the first downstream cycle, where no information of a previous cycle was available, the harvest concentration was determined by an offline concentration measurement before starting the downstream process.

### 2.4 Analytical methods

The viable cell density (VCD), total cell density (TCD), and viability were measured with a BioProfile FLEX Analyzer (Nova Biomedical, USA) in samples daily taken from the bioreactor. From the same sample, the concentration of glucose, lactate, ammonium and mAb was determined using a Cedex Bio Analyzer (Roche, Switzerland). Additionally, samples from the harvest line were taken to measure the mAb titers. Online pH was daily compared with offline pH measurements using an external pH meter and the calibration was adjusted if necessary.

Purified mAb from each cycle of the last anion exchange chromatography step was pooled in a storage flask at 4°C and once a day collected and aliquoted (−20°C) for later analysis. MAb was detected by a Photodiode Array detector (Waters, USA) at 280 nm. MAb aggregation was analyzed by size-exclusion chromatography with a TSKgel G3000SW column on a HPLC system (Waters, USA) at 280 nm wavelength. 100 mM Na\textsubscript{2}SO\textsubscript{4} and 25 mM Na\textsubscript{2}HPO\textsubscript{4} solution (pH 7.0) was used as mobile phase with a flow rate of 0.3 ml/min. N-glycosylation analysis of mAb was performed with a GlycoWorks™ Rapifleur-M5™ N-Glycan Kit (Waters, USA). The N-glycans were first cleaved from mAb and labeled with a fluorophore and a basic tertiary amine tag. The labeled N-glycans were then measured on an UPLC system with an ACQUITY UPLC Glycan BEH Amide column (Waters, USA). 50 mM ammonium formate solution (pH 4.4) for binding and 100% acetonitrile for elution were used as mobile phases for this method. A fluorescence detector was used for the measurement of the labeled N-glycans at a FLR wavelength of E\textsubscript{m} 425 nm/E\textsubscript{r} 425 nm. A CHO HCP third Generation ELISA kit (Cygnus Technologies, USA) was used to detect host cell proteins (HCPs).

For the chromatography columns, online absorbance measurements at 280 nm at the outlet of the columns were performed. For the capture column, these measurements were not sufficient to detect product breakthrough during the column loading, due to the high absorbance level of the impurities going through the column. For that reason, offline measurements of the mAb concentration at the outlet of the capture step were done with a Cedex Bio Analyzer to confirm the absorption of all the loaded antibodies on the column.

### 3 RESULTS AND DISCUSSION

#### 3.1 Process design

The high cell density perfusion process in 200 ml working volume was integrated to a continuous purification process. In this continuous
process, the upstream and downstream had to be integrated in a way that the flow from the bioreactor would be continuously processed by the downstream process, where the challenge was the very small scale and the usage of a single ÄKTA instrument. The purification of antibodies based on chromatography is of discontinuous nature, while the upstream is a fully continuous process. For this reason, the focus was put on adapting and automating the downstream process to purify the continuous stream from the bioreactor using only one chromatography unit.

3.1.1 | Continuous and integrated downstream in a single chromatography unit

To overcome the discontinuous nature of the chromatographic steps, one option is to perform the process periodically. In this case, the twin-column capture process, consisting of two columns in the capture step as presented by Tiainen et al,11 was used. While one of the columns is loaded, the other one is eluted, and the rest of the downstream steps is performed. For the next cycle, the capture columns switch positions, and the regenerated column is loaded. Consequently, the load flow rate for the capture is continuous. The different purification steps were integrated with each other in a process based on the concept of integrated column sequence (ICS).

Regarding the process setup, the columns were alternately connected to two column valves, in such a way that what was eluted from a column placed in one of the column valves, went through the sensor package, got preconditioned if needed, and got loaded onto the next column or loop, placed in the other column valve, as shown by the blue line in Figure 1. The switch between the two capture columns was possible thanks to two VVs placed before and after the column. Pump B from the gradient pumps was used both for the gradient elution and to condition the sample for the next step, and for this switch
3.1.2 Design of the column volumes

For the design of the column volumes, two constraints needed to be fulfilled: (C1) the cycle time had to be shorter than the necessary time to exceed the DBC of the capture column; and (C2) the loaded amount of product in the polishing steps had to be lower than the dynamic binding capacity (DBC) of those columns. These constraints are represented in Equations (C1) and (C2):

\[ V_i \cdot DBC_i \geq t_{\text{cycle}} \cdot F_F \cdot C_F \]  
\[ V \cdot DBC \geq V_1 \cdot DBC_i \sum V_i \]  

where \( V \) is the column volume, \( DBC \) is the DBC at 1% breakthrough, \( t_{\text{cycle}} \) is the cycle time, \( i \) refers to the number of column from 1 (capture column) to 3 (AEX column), \( F_F \) and \( C_F \) are the harvest flow rate and concentration, respectively, and \( y \) is the yield of a chromatography step. Seventy percent of DBC is considered to leave a safety margin to avoid product loss even after a certain number of cycles. According to available resin capacity data from the manufacturer, it can be affirmed that at least 70% of the capacity is kept after 100 CIP cycles.

The first constraint (C1) is used to calculate the volume of the capture column, based on the amount of mAb loaded in a cycle. To obtain this value, the cycle time must be calculated. The cycle time is the sum of the times for all downstream steps, and these depend on the residence times, which were taken as recommended by the column manufacturer. With the determined residence times, a downstream cycle was simulated and the process time for each step was obtained (Table 1), with a total cycle time of 3.6 hr. For the design of the capture column, nominal values for \( C_F \) and \( F_F \) were considered: 1 mg/ml and 2 RV/day (i.e., 16.7 ml/hr), respectively. With the cycle time and the nominal values, a loaded amount of 60 mg mAb per cycle was calculated. Then, assuming the volume for the capture column was 1 ml (the minimum column volume), the residence time for the loading step was 3.6 min, for which a DBC of 54.1 mg/ml resin was obtained from the simulation. With a column void of 0.32 and by considering 70% DBC, the limit for the mAb loading is 25.8 mg, which would not fulfill the requirement of Equation (C1). If the volume was 2 ml, the DBC would be 65.5 mg/ml (for a residence time of 7.2 min), and therefore the load limit would be 62.4 mg, which is higher than the 60 mg that are loaded per cycle.

Once the volume of the capture column was known, the other volumes could be obtained using Equation (C2). In this constraint, the yields were assumed to be 1, thus obtaining feasible column volumes regardless of the actual yield. Therefore, the ratio between the volume of any column and the volume of the capture column could be obtained as the ratio of the column capacities. The DBC of the CEX column was 135 mg/ml resin, which was obtained from the simulation (for a residence time of 5.4 min) and experimentally validated. Leaving a 30% safety margin in the column capacity and with a column void of 0.32, the resulting CEX column volume was calculated to be a minimum of 0.97 ml, so a volume of 1 ml was chosen for that column. Since the AEX column was used in FT mode, the binding sites were occupied only by the impurities. Consequently, the amount of product that can be loaded on the column was much higher than for the other columns due to the low impurity concentration of the loaded sample in that step. Thus, the volume for the AEX column was also set to the minimum of 1 ml. Table 1 shows a summary of the design of the columns.

The volumes of the loops were calculated according to the expected column volumes of the eluates obtained from a downstream simulation. The virus inactivation loop was placed after the capture column. The elution pool was around 2.5 column volumes (CV), thus resulting in 5 ml since the capture column volume is 2 ml. A loop volume of 10 ml was selected to account for the axial concentration dispersion. For the hold-up loop after the CEX column, the eluate was expected to be approximately 4.5 column volumes, that is, 4.5 ml since the CEX column volume is 1 ml. In this case, the loop was also set to 10 ml. The filling of the loops was simulated to confirm the suitability of the selected volumes.

### 3.1.3 Control strategies

To ensure an automated operation of the process during long periods and to handle changes in the harvest concentration and flow rate, it was necessary to implement several control strategies.

| Parameter          | Units | Capture | CEX | AEX |
|--------------------|-------|---------|-----|-----|
| Column volume      | ml    | 2.0     | 1.0 | 1.0 |
| Process time       | hr    | 0.7     | 1.8 | 1.1 |
| Load flow rate     | ml/hr | 16.7    | 11.1| 22.2|
| Elution flow rate  | ml/hr | 60.0    | 5.6 | NA  |
**Capture flow rate control**

Since the filtrate pump after the bioreactor was a peristaltic pump that could not provide the pressure that is required to load the capture column, a hold-up flask between the bioreactor and the capture column was used prior to loading the column with the sample pump in the ÄKTA system. The weight of the flask was monitored with a balance, and the objective was to keep it constant. However, the value of the weight was actually irrelevant, since the aim was that the flow rate of the sample pump was equal to the harvest rate. This was achieved with a proportional controller implemented in Orbit.

With this strategy, the harvest rate was precisely measured using the high accuracy P9H pumps from the ÄKTA pure 150 system. In addition, it made the process more robust, since the flask functioned as a surge vessel, that is, a sudden stop in the chromatography system did not force a stop of the whole process, which would take much longer time to re-start.

**Adaptive pooling**

Variable mAb concentrations and flow rates from the bioreactor lead to different amounts to be treated in every downstream cycle, therefore the peaks move, enlarge, or shrink accordingly. An automatic way of pooling the eluates based on the absorbance has been already implemented in Orbit in previous work. In this process, the absorbance values at which the pooling begins/ends (absorbance cut-off limits) were constant. In the present work, a feed-forward control feature was introduced. The adaptive pooling was based on the loaded amount of product, which was obtained online from the simulation of the downstream process. The elution peak was simulated in real time and the absorbance cut-off limits, determined from the simulated peak, were then applied to the real peak. This strategy was applied to the eluate of the CEX and the AEX column. It was especially relevant to have adaptive cut-off limits in the case of the cation exchange step, since it contained significant impurities that needed to be cut off at different absorbance levels depending on the size of the peak. In Figure 2, showing a chromatogram of the CEX column extracted from the continuous runs, it can be seen that the elution peak gets higher, wider and shifted to the left when the loaded amount, related to the peaks on the left, is also higher. The absorbance cut-off limits (cross signs in Figure 2) adapt so that the impurities eluting before and after the product are always removed regardless of the peak size.

**Loading factor control in the capture step**

Nominal values for the concentration and the flow rate from the bioreactor were used for the design of the columns. However, these values were variable, and the columns would be oversized if the mAb concentration or harvest flow rate from the bioreactor were lower. For that reason, a control strategy was implemented in Orbit to maximize the resin utilization in the capture columns and avoid product loss in case the concentration or the flow rate would become larger than the nominal values.

The mAb concentration in the harvest, the harvest flow rate and the DBC of the capture column (taking the 70% to account for eventual capacity loss during the run) were used to calculate the required volume for the total load of the column. The harvest concentration was estimated as described in Section 2.3, and the DBC was predicted using the General Rate model for the capture column (see Section 2.2.3). The DBC was defined as the amount of product that can be loaded before reaching 1% of the breakthrough curve. Since the breakthrough curve changes with residence time, the DBC depends on the loading flow rate. For that reason, a constant value could not be used, and a model providing the DBC for a particular flow rate was necessary. As the harvest flow rate was measured continuously, the Orbit software could determine whether an extra time was needed after a downstream cycle was completed.

Figure 3 shows a simulation of the whole integrated downstream process, illustrating how the downstream process reacts to an initial increase and final decrease of mAb concentration in the harvest, in cases where the loading factor control is applied or not (Figure 3c,b). When this control is applied (Figure 3c), the downstream cycles are longer for lower concentrations and lower harvest rates, and they become increasingly shorter with time. In the first cycles the harvest flow rate is lower, thus leading to a higher DBC. That implies that in those cycles more mAb is loaded on the capture columns, and as a result the peaks are higher. From around Day 7, the harvest rate approaches a steady state (Figure 3a), and the DBC of the capture column remains approximately constant. At this stage, a stable amount of protein is loaded in every cycle, and thus the peaks height, and consequently the final product concentration, are constant (as long as the DBC is constant). For this reason, the purified mAb concentration is independent of the mAb concentration in the harvest when the harvest rate becomes constant, if the loading control is applied, as it can be seen in Figure 3c. On the contrary, when the loading factor control is omitted, the cycle time is always the same, and the peak height corresponds to the harvest concentration (Figure 3b).

Another key difference is that, with the loading factor control strategy, the number of cycles is reduced by 2.4 times, which
positively impacts the expected life of the columns and allows maintaining a higher column capacity through time, as the columns get regenerated fewer times. A direct consequence of the longer cycles is that the product concentration is higher. As can be seen in Figure 3, the concentration of the purified product in the process using the loading factor control is up to twice the concentration obtained without control strategy, which would decrease the process time of a subsequent concentration step.

3.2 | Integrated continuous process run

3.2.1 | Perfusion culture

MAb was continuously produced in a high cell density perfusion culture of CHO cells. The culture data of the CHO-K1 cell line are shown in Figure 4. After inoculation, the cell density in the bioreactor was increased, accompanied by a daily increase of the perfusion rate to keep the average CSPR constant. A VCD of approximately $80 \times 10^6$ cells/ml was achieved 6 days after the inoculation, with a perfusion rate of 2 RV/day. At that day, a cell bleed was initiated to keep the VCD constant between 70 and $90 \times 10^6$ cells/ml. The VCD was successfully maintained at the set point for a period of 10 days with a viability mostly above 95% (Figure 4a). The metabolic consumption and production rates of glucose, lactate, and ammonium displayed a constant profile, thus suggesting that the culture reached a steady state, particularly due to the maintenance of process parameters such as the CSPR (Figure S3). Note that a partial loss of product occurred due to the automatic cell bleed applied in the culture. When the VCD reached $80 \times 10^6$ cells/ml, the mAb concentration in the bioreactor raised to 1 mg/ml and remained stable until Day 13 (Figure 4b). The titer decreased on Days 13–17 due to slightly lower cell densities. The cell specific productivity (Figure 4a) remained fairly constant throughout the whole process with 17.1–21.7 pg/cell/day. The mAbs were partially retained in the bioreactor by the hollow fiber membrane, and the mAb concentration in the harvest was slightly lower.

**FIGURE 3** Simulation of the complete downstream process with and without the loading control: (a) experimental harvest mAb concentration and flow rate. (b) simulated concentration profile for the downstream steps in cyclical operation without the loading control strategy. (c) simulated concentration profile for the downstream steps with the loading control strategy. The concentration peaks correspond to the eluates from the different downstream steps in each cycle.
compared to the bioreactor with titers between 0.6 and 0.9 mg/ml (Figure 4b). The continuous purification was initiated on Day 4 and run for 2 weeks.

3.2.2 | Downstream process

The product was continuously purified in a periodic process, where the purification of the mAb took around 3.6 hr. When necessary, the cycle length was extended by up to 4.4 additional hours to further load the capture column based on the estimated mAb concentration and the measured harvest flow rate, which was possible thanks to the aforementioned loading factor control strategy. Therefore, the maximum cycle time was 8 hr. In Figure 5a, a downstream operation for 43 hr (corresponding approximately to Days 6 and 7) is shown. It can be seen that the process time is different for every cycle. The first cycles are around 8 hr long, whereas for the following cycles the process time is continually diminishing, due to an increase of the harvest mAb concentration and harvest rate.

Regarding the residence time of the mAbs in the downstream process, it was determined as the sum of the capture loading time (corresponding to the cycle time, 3.6–8 hr) and the purification process time (3.6 hr), as the mAbs were loaded during a cycle and purified in the next cycle. The mAbs loaded on the capture column at the beginning of the cycle had a higher residence time than the ones loaded at the end of the cycle, because the former stayed adsorbed on the column during the whole loading period, whereas the latter were eluted right after being adsorbed. For that reason, the product residence time ranged from a minimum of 3.6 hr, for those molecules being loaded at the end of the cycle, and a maximum of 7.2 hr (for 3.6 hr-long cycles) or 11.6 hr (for 8 hr-long cycles).

A zoomed view of a cycle is shown in Figure 5b. After washing the capture column, it was eluted and pooled into the VI loop (first red peak). Then, during the 60 min of virus inactivation, the regeneration, CIP, and equilibration steps were performed, and afterward the virus inactivated product was loaded on the CEX column (second red peak). The abrupt end of this second peak reveals that some product remained in the VI loop, as a result of back mixing with the stream that was used to push the virus-inactivated product out of the loop. This is confirmed by the simulated outlet stream from the VI loop.

**FIGURE 4** Culture data of CHO cell perfusion process. (a) Viable cell density, viability and cell specific productivity profile. (b) mAb concentration in the bioreactor and harvest

**FIGURE 5** (a) Long-term downstream operation for Days 6 and 7. The dark and light shaded areas correspond to the periodic cycles. (b) Zoomed view of a downstream cycle. The red peaks represent the pooled fractions from every column/loop
The dispersion coefficient for the VI loop is very high (Table S1) as a result of a relatively wide loop compared to the chromatography columns. The residence time in the loop is also very high (a total of around 100 min, counting the capture column elution, the virus inactivation and the CEX column loading), thus leading to a very broad peak coming out the VI loop. The CEX column was washed and eluted, and the pool (third red peak) was held in a hold-up loop, and finally loaded on the AEX column (fourth red peak). This stream flowed through the AEX column, where the impurities adsorbed, and the outlet was stored at 4°C. The concentration of the purified product ranged from 0.7 to 1.2 mg/ml, and it was fairly regular, as a result of the loading factor control strategy, which made the final product concentration independent of the harvest concentration, as explained previously.

The simulation of the product recovery enabled the supervision of the process in real time. For example, it was used to predict at which time the product was being processed in each step, and how much product was obtained. Additionally, the simulation was helpful to detect anomalies in the process, especially during the initial runs, by comparing the simulated response with the actual chromatograms.

### 3.2.3 Recovery yield and productivity

In this first proof-of-concept, the overall recovery yield (ratio of purified product to harvested mAb) was relatively low for a mAb purification, as shown in Figure 6a. Low yields can be a result of underdimensioned columns, which would lead to product breakthrough during column loading. However, the mAb concentration was measured at the outlet of the columns when these were loaded, and these measurements showed no significant amount of mAbs being lost when loading the columns (data not shown). Furthermore, the downstream simulation revealed no product breakthrough during the loading of the columns.

On the other hand, product can be lost during the pooling. In this process, the pooling was very conservative to remove as much impurities as possible, and consequently clipping out a large proportion of the product. This was in particular the case during the elution of the CEX column, as it can be seen in Figure 5b. Also, during the loading of the virus-inactivated product onto the CEX column, some product remained in the VI loop, as previously explained. To confirm these assumptions, yields for each step in Figure 5b were calculated based on the online absorbance measurements. The results show that a major loss of product happens during the loading and the elution of the CEX column (with a yield of 78 and 77%, respectively), whereas the yield for the capture step is around 93%, and the yield for the AEX step is almost 100%. These yield measurements are not accurate since the absorbance is, in some cases, above the linear range of the UV detector. However, they provide an idea of which steps are critical in terms of product loss. Thus, the conservative pooling and the VI loop were the main factors responsible for the relatively low yields.

An alternative VI design with a lower diameter to decrease back mixing of the virus-inactivated product as well as an optimized pooling would improve the yield. In addition, the implementation of a product recycling strategy, like the one used in a MCSGP process, could result in a better yield/purity compromise. In addition, the yield had an irregular profile. This was due to repeated problems of the network connection of the chromatographic system with the computer,
causing the downstream process to stop, with the consequent loss of product in some cases where the connection problem happened. Data from preliminary experiments, where no connection problem occurred, showed a more regular yield profile (Figure S4).

The downstream productivity, which is based on the amount of purified product per bioreactor volume and day, should remain constant because the same amount of product was loaded at every cycle thanks to the loading factor control. However, as a result of the fluctuating yield, the downstream productivity follows the same irregular pattern (Figure 6a). It is expected that with a more regular yield, the downstream productivity would be constant. On the other hand, the upstream productivity, based on the amount of product in the harvest stream, follows a profile similar to the VCD (Figure 4a), and reaches a value of up to 1.5 mg/ml/day.

3.2.4 | Product quality

N-glycosylation

N-linked glycosylation is one of the most important quality attributes of mAbs and primarily affected by the cultivation parameters and media. A consistent glycan profile was observed from Day 9 (Figure 6b), i.e., 3 days after reaching the target cell density in the culture. In this study, the ratio of galactosylated glycoforms (G1F, G2F) initially increased over GO and GOF species until the culture reached a steady state at Day 9. According to these data, the purified product obtained until Day 9 of the process, showing different product quality, would have to be excluded in a real production campaign. In order to obtain similar glycosylation patterns from the beginning of the process, the upstream process would need to be optimized by changing the culture conditions or modifying the media. Alternatively, in a less optimized system, the purification process can be started when the target VCD is reached and a steady state established. Besides, some samples from the harvest were purified by Protein A magnetic beads, and it was observed that no significant difference in the glycosylation profile compared to the purified product was obtained (Figure S5). The results confirm that this product quality attribute is entirely dependent on the culture conditions and not affected nor selected by the subsequent downstream train.

Host cell proteins

HCPs represent one of the major process related impurities in biopharmaceutical production. Continuous purification resulted in a >4-log reduction of the HCP in the purified protein fraction compared to the harvest stream from the bioreactor, as shown in Figure 6c. The HCP concentration in the AEX eluate was below the limit of quantification (1 ng/ml) of the ELISA assay, thus meaning that it was lower than this value during the whole run.

MAb aggregates

There are several factors that affect the formation of mAb aggregates. Temperature, protein concentration, pH, and abrupt changes of these parameters are some of the aspects that are considered of most importance in protein aggregation.31 In Figure S4, data from a similar run with another CHO-S cell line, show low levels of aggregation in the purified product during the whole run, independently of the aggregate level in the harvest. That means that the aggregates were satisfactorily removed in the downstream process. In particular, they were removed in the CEX step, as shown in Figure 2. The aggregate levels were also measured for the CHO-K1 cell line (data not shown), but due to the aforementioned connection problems of the chromatographic system with the computer, the profile of aggregate concentration was more irregular than the one shown in Figure S4.

| Reference | VCD (10⁶ cells/ml) | Working volume (L) | CSPR (pl/cell/day) | Integrated continuous unit operations | Volumetric productivity (g/L/day) | Process duration (days) |
|-----------|-------------------|-------------------|-------------------|-------------------------------------|-------------------------------|------------------------|
| Godawat et al¹² | 60<sup>a</sup> | 12 | 40–50<sup>a</sup> | PCC1 (capture), VI, PCC2 (CEX), FT membrane (AEX) with two chromatography stations and a reservoir for VI | ~0.67 | 31 |
| Steinebach et al³⁰ | 40 | 1.5 | ~25 | Cont. 2-column capture, VI, MCSGP (CEX), FT column (AEX) with two chromatography stations and a VI loop | ~0.33 | 3.5 |
| Arnold et al¹³ | 100 | 1.2, 30 | N/A | Multicolumn capture, VI, TFF, FT membrane (AEX), VF, UFDF with one chromatography station, a SEC column for VI and a series of filtration elements | N/A | 30, 17<sup>b</sup> |
| Present study | 80 | 0.2 | ~25 | ICS: Cont. 2-column capture, VI, bind-and-elute column (CEX), FT column (AEX) in a single chromatography station and a loop for VI | Max. 0.79 | 13 |

<sup>a</sup>Warikoo et al.⁶
<sup>b</sup>Process duration of perfusion culture, operation time of integrated continuous system not given.
3.3 | Process comparison with other ICB platforms

The integration of continuous downstream operations with perfusion bioreactors have been reported in previous studies. However, until now only a few end-to-end ICBs have been presented (Table 2). The examples from the literature are ATF perfusion bioreactors of various sizes with mAb expressing CHO cells and different combinations of integrated continuous downstream operations. The first successful implementation of a fully continuous mAb production platform was published by Godawat et al. In this process, a 12 L perfusion bioreactor was integrated with a series connection of two periodic countercurrent chromatography (PCC) stations for capture and polishing and a membrane adsorber in FT mode as final polishing step. A similar sequence of downstream operations was implemented in the present study with the advantage of reducing the footprint by eliminating one of the chromatography stations and running all the operations in a single system. Steinebach et al. presented an ICB utilizing a multicolumn countercurrent solvent gradient purification (MCSGP) station after a 2-column capture for the removal of process-related impurities. In a perfusion process that was operated for 18 days, the integrated purification process was carried through the last 3.5 days with a yield of 92%. Arnold et al. showed the first high cell density ICB in lab scale (1.2 L) as well as in pilot scale (30 L). After a chromatographic multicolumn capture and VI the product was processed by a series of filtration steps. This filter train carried out the product concentration, removal of particles and virus, and diafiltration for buffer exchange. Although a successful run at pilot scale was shown with a significant size reduction of the purification steps, the process still relied on a lot of downstream equipment similar to their fed-batch platform. In the present study, the processed volumes could further be reduced by of at least sixfold reduction of the working volume in the bioreactor compared to the other studies. Furthermore, the ICB presented in this work was the first one that included a comprehensive model of the whole downstream process, which was used for the design of the columns and the development of strategies for automatizing and optimizing the product recovery. The implemented platform could potentially be further extended for pilot/commercial production with harvest flow rates of up to 150 ml/min (216 L/day), for example by integrating a 100–200 L perfusion bioreactor with the purification unit. Scale-up of both upstream and downstream units do not suppose a big challenge in principle, since they are well established technologies that have already been implemented at different scales.

4 | CONCLUDING REMARKS

This work presents an ICB at small-scale for the production of biological therapeutics. In a 200 ml ATF perfusion bioreactor with CHO cells operated at steady state with high cell density, mAbs were produced and harvested continuously. The harvest was purified in a single lab-scale chromatography system, in an integrated and automated downstream process with three chromatography steps and a virus inactivation. The continuous operation of the purification process was possible thanks to a periodic twin-column capture based on the process presented by Tiainen et al. and the downstream process was externally controlled by the research software Orbit, also used in other integrated chromatography implementations. A series of control strategies were implemented in the software to cope with the variations in harvest flow rates and mAb titers. A comprehensive downstream model was developed and used for the design of the column volumes and the automatization of the process by using simulated data, like the breakthrough curves or the elution peaks, in the implemented control strategies. The downstream model included a General Rate model for the protein A capture step, a kinetic chromatography model for the polishing steps, and a convective–dispersive model for the hold-up loops. As a result of the automatic control strategies, a long-term continuous production process was obtained with minimized manual intervention by the operator. Stable product quality attributes, such as conserved N-glycosylation patterns, consistently low HCP levels and low aggregate levels were obtained through the steady state operation of the whole bioprocess, with an overall productivity of up to 0.8 mg/ml/day and maximum recovery yields of 60%.

With this proof-of-concept, we have demonstrated the technical feasibility of a continuous process with a compact design, integrating several unit operations in a single device and using small-size equipment for upstream and downstream operations.

NOTATION

- $\beta$: equilibrium modifier-dependence parameter (-)
- $\epsilon$: total column void (-)
- $\epsilon_C$: extra-particle column void (-)
- $\epsilon_p$: particle porosity (-)
- $C$: MAb concentration in the mobile phase (mg/ml)
- $C_F$: harvest MAb concentration (mg/ml)
- $C_s$: modifier concentration (M)
- $C_p$: MAb concentration inside the particle (mg/ml)
- $D_{ax}$: axial dispersion coefficient (cm$^2$/min)
- $D_{app}$: apparent dispersion coefficient (cm$^2$/min)
- $DBC$: dynamic binding capacity (mg/ml column)
- $D_{eff}$: effective pore diffusivity (cm$^2$/min)
- $F_F$: harvest flow rate (ml/hr)
- $H_0$: Henry equilibrium constant (M)$^{-1}$
- $K$: Langmuir equilibrium constant (ml/mg)
- $k$: kinetic constant (ml/(mg min))
- $k_t$: mass transfer coefficient (cm/min)
- $L$: column length (cm)
- $q$: adsorbed MAb concentration (mg/ml)
- $q_{max}$: column capacity (mg/ml)
- $r_p$: particle radius (dm)
- $t_{cycle}$: downstream cycle time (hr)
- $V$: column volume (ml)
- $v$: superficial fluid velocity (cm/min)
- $V_L$: loop volume (ml)
- $y$: yield of chromatography step (-)
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AUTHOR CONTRIBUTIONS
Joaquín Gomis Fons contributed with the following: design and implementation of the downstream process, process modelling, code writing to implement the extensions in Orbit to be able to run the downstream process in an automated and continuous way, planning of the experimental work, and writing of the manuscript. Hubert Schwarz contributed with the following: design and implementation of the upstream process, off-line analyses for culture data, product quality data and mAb concentration, planning of the experimental work, and writing of the manuscript. Liang Zhang contributed with the execution of analyses and the interpretation of the experimental data. Niklas Andersson is the main developer of the Orbit software, and his role was to provide support in the further development of the software, apart from technical support about the process. He also reviewed the article. Bernt Nilsson was the co-supervisor of the project, and he provided support and knowledge during all the stages of the project, including the reviewing of the article. Veronique Chotteau was the co-supervisor of the project, and she provided support and knowledge during all the stages of the project, including the reviewing of the article.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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