A Protocol to Transfer a Fed-Batch Platform Process into Semi-Perfusion Mode: The Benefit of Automated Small-Scale Bioreactors Compared to Shake Flasks as Scale-Down Model

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*Continuous processes such as perfusion processes can offer advantages compared to fed-batch or batch processes in bio-processing: improved product quality (e.g. for labile products), increased product yield, and cost savings. In this work, a semi-perfusion process was established in shake flasks and transferred to an automated small-scale bioreactor by daily media exchange via centrifugation based on an existing fed-batch process platform. At first the development of a suitable medium and feed composition, the glucose concentration required by the cells and the cell-specific perfusion rate were investigated in shake flasks as the conventional scale-down system. This lead to an optimized process with a threefold higher titer of 10 g/L monoclonal antibody compared to the standard fed-batch. To proof the suitability and benefit as a small-scale model, the established semi-perfusion process was transferred to an automated small-scale bioreactor with improved pH and dissolved oxygen control. The average specific productivity improved from 24.16 pg/(c*d) in the fed-batch process and 36.04 pg/c*d in the semi-perfusion shake flask to 38.88 pg/(c*d) in the semi-perfusion process performed in the controlled small-scale bioreactor, thus illustrating the benefits resulting from the applied semi-perfusion approach, especially in combination with controlled DO and pH settings.*
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Introduction

Production of monoclonal antibodies (mAbs) with mammalian cell cultivation processes is an important and challenging step in modern medicine to achieve the final pharmaceutical product. Pharmaceutical companies must balance strong market requirements with the pressure to decrease costs of production. Therefore, improved process efficiency due to implementation of intensified processes such as perfusion has raised attention with the recently improved perfusion equipment. Establishing perfusion processes can offer advantages for specific cell lines in product yield, manufacturing costs, and flexibility compared to fed-batch or batch processes.[1–3] The characteristics of intensified processes are based on a continuous removal of inhibiting metabolites as well as continuous addition of fresh medium. Therefore, high cell concentrations and high productivity can be achieved in relatively small volumes of bioreactors.[1,4] Thus, the adaptation of an industrially relevant fed-batch process to perfusion mode leads to higher product yields and an increase in efficiency.

Viable cell concentrations (VCCs) for CHO cell cultures in batch cultures were reported from 0.13 million cells/mL up to 6 million cells/mL.[5–7] VCCs of CHO fed-batches range between 1 and 26 million cells/mL,[8–10] whereas in perfusion processes constant VCCs from 27 million cells/mL to high concentrations of more than 200 million cells/mL were reported.[6,11,12] Product titers of batch processes were reported for mAbs with titers up to

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0.9 g/L.[5,12] Fed-batch processes showed advantageous antibody concentrations in the range of 1–13 g/L.[10,13] Recently, perfusion processes were established with 10-fold higher mAb production up to 1.9 g/L per day of cultivation, that can lead to cumulative titers up to 25 g/L of mAb and more in one cultivation process.[14,15]

Nevertheless, intensified mammalian cell culture processes have to overcome challenges. To keep the cell culture in a pseudo-steady-state, feed rates and uptake rates need to be well-known and controlled during the complete cultivation time.[16] The cell-specific perfusion rate (CSPR) is one indicator giving the relation of the chosen perfusion rate and existing cell concentration in a process (see Eq. 1). The CSPR optimum depends on each cell line, medium composition and process.[17] For a stable perfusion culture, this value should be monitored and controlled leading to an optimal cell growth and productivity.[18] There are many other influencing factors, like the medium and glucose concentration and the implementation of a cell bleed.[19–21]

$$\text{CSPR} = \frac{\text{perfusion rate}}{\text{viable cell concentration}} \quad (1)$$

One important and critical step in perfusion processes is the cell retention device that keeps the cells inside the bioreactor while the medium with the inhibiting metabolites leaves the reactor. Depending on the cell line and the process characteristics, the product is either kept in the bioreactor or it is part of the medium that leaves the reactor.[22] A cell retention device allows the removal of inhibiting metabolites, like lactate and ammonia, and enables fresh nutrient supply through the medium exchange during cultivation.[23] For this step, different approaches and devices are available that are based on either filtration principles, sedimentation, or centrifugation.[24] One commonly used method in industry is to implement a filtration step during the process. The two common used applications within filtration are crossflow membrane filtration called alternating tangential flow (ATF) filtration and tangential flow filtration (TFF).[11,25,26] Moreover, cell retention can be implemented through devices like spin filters, inclined settlers, continuous centrifugation, or ultrasonic separators.[4,6,22,25]

Besides process development, the transfer of pharmaceutical processes to automated bioreactors and the subsequent scale-up are essential and delicate steps in biopharmaceutical manufacturing. Critical process parameters (e.g. temperature, pH, or the volumetric oxygen transfer coefficient) need to be studied and considered for process transfers. Especially, the cell retention step in a perfusion process needs to be adjusted during process scale-up and automation.

The goal of the reported work was to develop a semi-perfusion protocol and to show an easy transfer of an optimized semi-perfusion process from classical shake flasks to an automated small-scale bioreactor. Thus, fast and efficient process development of a semi-perfusion process based on an established fed-batch process platform was investigated and optimized. Further on, the process transfer and the possibility to equally use the automated small-scale bioreactor as scale-down model with increased process control compared to shake flasks was tested. Due to existing limitations of continuous medium exchange in shake flasks and disposable small-scale bioreactors, the medium exchange was applied on a daily basis by centrifugation resulting in a semi-perfusion process. During the centrifugation step, there is no aeration and process control that can lead to a short period of starving of the cells. Despite this technical restriction of missing process control during the centrifugation step, the developed protocol still provides a good approximation of a genuine perfusion process. Semi-perfusion operation modes in small-scale systems have been successfully investigated to simulate perfusion processes for screening and process development.[17,27,28] Based on these results, a strategy was developed to meet the requirements for a perfusion process.

The standard medium formulation with its proprietary feeds used in fed-batch processes was adapted to the nutritional requirements in perfusion cultivations. Afterward, critical process variables, like CSPR, glucose concentrations, and cell bleed were investigated for the semi-perfusion operation. Finally, the newly established semi-perfusion process was transferred from shake flasks to an automated small-scale, single-use, stirred tank bioreactor system. The small-scale bioreactor offers advantages for future testing of the critical process parameters in early process development of continuous processes and enables conditions close to production scale due to pH and DO control.

Micro-scale bioreactors are commonly used as a scale-down model for fed-batch processes.[29–31] Recently, the additional application of small-scale bioreactors for semi-perfusion processes with a sedimentation approach for media exchange was investigated.[17,32,33] The sedimentation approach showed limitations and is in conflict with the advantages of such controlled micro bioreactors as sedimentation times with no bioreactor control of 40 min are reported (i.e. DO and pH control). [33] Therefore, this study focuses on transferring the centrifugation approach from the shake flasks to the small-scale bioreactors with a maximum of 15 min without bioreactor control. The centrifugation method drastically reduces the time of non-controlled process parameters in the bioreactors. Accordingly, it efficiently supports the advantages of the small-scale bioreactors in contrast to shake flasks or other small-scale models for semi-perfusion processes. The goal of this work was to develop an easy perfusion-based protocol using existing devices to reduce costs and maximize the benefits from process intensification in terms of product increase.

Materials and Methods

Cell lines and medium

A DG44 CHO cell line expressing an IgG1 mAb was used in this study (Sartorius Stedim Cellca GmbH). All medium and feeds used were part of the Sartorius Stedim Cellca medium platform and were chemically defined. In total, stock medium for the seed culture (SM) and production medium as basal medium for production (PM) were used. Moreover, two different feeds, Feed Medium A (FMA) for macro nutrients (e.g., glucose) and Feed Medium B for micro nutrients (e.g., amino acids) (FMB) were used (Sartorius Stedim Cellca GmbH).

Seed culture

A cryo vial containing 1 mL CHO suspension (passage 8) at a concentration of 30 million cells/mL was thawed and transferred in a 15 mL Falcon® tube (Sarstedt) with 10 mL pre-warmed (36.8 °C) seed medium. This suspension was centrifuged (Centrifuge 3–30 K, Sigma) at 190*g* at room temperature for 3 min to remove all components of the freezing medium. After decanting the supernatant, the pellet was resuspended with 10 mL pre-warmed seed medium and transferred

into a 500 mL Erlenmeyer flask (Corning) filled with 150 mL pre-warmed seed medium. The shake flask was incubated in an incubation shaker (Certomat CTplus, SSB) at 36.8 °C and 7.5% pCO_2 with a shaking rate of 120 rpm and 85% humidity. Cells were passaged for five times every 3–4 days until inoculation of the production culture was done.

Semi-perfusion shake flask studies

Semi-perfusion shake flask cultures were inoculated from the seed train with a starting cell concentration of 2.5 million cells/mL. All cultivations were performed in 125 mL baffled shake flasks (Corning) with a vent cap and with a working volume of 25 mL. The experiments were performed in an incubation shaker (Certomat CTplus, SSB) with the same conditions as described for the seed culture. Each medium exchange took place in a 24 h rhythm. For sample analysis, shake flasks were moved to the biological safety cabinet, and 1 mL was removed after sufficient shaking. The remaining cell suspension was transferred into a 50 mL Falcon tube by pipetting, and centrifuged at 500*g* for 5 min at room temperature (3-30K, Sigma). Following centrifugation, 2 mL of the supernatant were taken for IgG quantification, and the remainder was discarded by decanting. The cell pellet was resuspended with 25 mL of pre-warmed, fresh medium by gentle pipetting, transferred into the shake flask, and placed back into the incubator. The total duration of each centrifugation step was not exceeding a total time of 15 min to keep the time as less as possible. The medium composition of each experiment is described in detail in the corresponding paragraphs.

Design of experiment study

The experimental set-up and the analysis of the results were carried out with the design of experiment (DoE) software MODDE 12 (Umetrics, Sartorius Stedim Data Analytics). A full factorial design was used to evaluate the influence of different medium compositions on the VCC as a single and quantitative response using a quadratic regression model, with FMA (three levels) and FMB (three levels) as the controllable and qualitative mixture factors, and PM (nine levels) as the fill factor (to 100%).

Cell bleed in semi-perfusion experiments

The cell bleed in shake flasks was applied based on the defined conditions for each experiment, and was calculated according to Eq. 2 based on the daily VCC and the total volume of the culture (V_c). The calculated volume of cell suspension was removed by manual pipetting and replaced with fresh, pre-warmed medium. Therefore, the cell bleed was applied in a semi-continuous mode.

$$V=\left(1-\frac{VCC\text{target}}{VCC\text{measured}}\right)\cdot V_c \tag{2}$$

Parallel, automated small-scale bioreactor

The automated small-scale bioreactor system ambr® 15 with up to 48 disposable cell culture bioreactor vessels (Sartorius Stedim Biotech GmbH) was used in the experiments. The working volume of each bioreactor can be in the range of 10 mL and 15 mL. Placed in a biological hood, the ambr® 15 can be used sterile and fully automated due to pH and DO control and independent gassing for each bioreactor of O_2, CO_2, and N_2. Each culture station containing up to 24 bioreactor slots can be temperature controlled and run at fixed impeller speeds. Sampling and feeding is applied by the liquid handler inside the hood.

Each bioreactor was inoculated from the seed train with a seeding density of 2.5 million cells/mL. To achieve this higher seeding density, cells from the seed train were centrifuged at 500*g* at room temperature for 5 min (3-30K, Sigma) and resuspended in pre-warmed medium for the semi-perfusion process. The total duration of each centrifugation step was not exceeding a total time of 15 min to keep the time as less as possible. All cultivations were performed in a 15 mL ambr® 15 system with sparged cell culture vessels (Sartorius Stedim Biotech GmbH). The working volume of each bioreactor was 10 mL. The temperature set point was chosen at 36.8 °C and the pH was controlled at 7.1 through CO_2 sparging. Agitation of each bioreactor was set to 1300 rpm and dissolved oxygen was controlled at 60%. A constant N_2 flow was set to 150 μL/min to allow gas transfer. To prevent foaming 20 μL of a 2% solution of Antifoam C Emulsion (30%) was added every 24 h (Sigma). The process lasted 12 days with one vessel volume exchange (VVD) of medium per day. This was carried out by spinning down the cells in the vessel every 24 h (centrifuge 6K15, Sigma), and resuspending the cell pellet afterward in 10 mL fresh medium using the liquid handler of the ambr® 15 system. 2 mL of supernatant before the daily medium exchange were stored at −20 °C for further IgG quantification. In addition, supernatant was taken after the daily media exchange. The volume of the second supernatant sample was variable to keep the working volume at 10 mL in each bioreactor.

Offline analytics

Process specific values for pH, glucose and lactate concentrations were measured in a blood gas analyzer (ABL800 Basic, Radiometer). The osmolality was determined (Osmomat 030, Gonotec) for every sample. The VCC indicating the amount of viable cells in the cultivation and the viability of cells according to the total cell concentration, as well as the cell diameter, were analyzed with the Trypan Blue Assay based Cedex HiRes Cell Counter and Analyzer system (Roche). The CSPR, used to monitor the specific medium supply per cell per day was calculated according to Eq. 1. The integral viable cell concentration (IVCC), also called cumulative cell hours, indicates the total amount of time the cells have produced mAb and is calculated by trapezoidal integration of the VCC over the time (t) (Eq. 3).[34]

$$IVCC_i=\int_{t=0}^{t}VCC(t)dt\approx\sum_{i=1}^{n}\frac{VCC_i+VCC_{i-1}}{2}\cdot(t_i-t_{i-1}) \tag{3}$$

Analysis for IgG quantification

The cell-free supernatants of all samples were stored in the freezer at −20 °C before IgG titer quantification with high-performance liquid chromatography (HPLC) was performed using a Dionex UltiMate 3000 HPLC System (Thermo Scientific) and a Yarra 3 μm SEC 3000 (Phenomenex). As buffer

solution a mixture of 1 M Na_2SO_4, 0.5 M NaH_2PO_4 + 0.5 M Na_2HPO_4 (Sigma) and water (1:1:8) with a pH at 6.6 was used. Before analysis, samples were thawed and diluted with water, and filtered through Minsart RC4 0.2 µm syringe filters (Sartorius) into autosampler cups (Carl Roth) using 1 mL syringes (Optifine-F). The calibration curve was measured based on an IgG stock solution (Cytoglobin 5%, human Immunoglobulin G, Bayer Vital GmbH).

To calculate the indicated cumulative product, first the measured product concentrations c_i were normalized to a volume of 1 L resulting in the product amounts m_i (Eq. 4). The product amount of the supernatant after centrifugation $m_{1,i}$ and the product amount of the resuspended cells after the exchange of medium $m_{2,i-1}$ were considered for the calculation of the cumulative product m_{total} (Eq. 5) considering 1 L working volume. By plotting the cumulative product against the integral viable cells (IVC), which are calculated with Eq. 6, the productivity of each process is indicated.

$$m_i = c_i \cdot 1\,L \quad (4)$$

$$m_{\text{total}} = \sum_{i=1}^{n} (m_{1,i} - m_{2,i-1}) \quad (5)$$

$$IVC = IVCC \cdot 1\,L \quad (6)$$

Calculation of cell-specific productivity and the lactate yield coefficient from glucose

The cell-specific productivity (Q_p) was calculated to compare the key experiments from each bioreactor system, the shake flask and the automated small-scale bioreactor, and the new established semi-perfusion process. The Q_p was calculated according to Eq. 7.[35]

$$Q_P = \frac{V_c(m_i - m_{i-1})}{t_i - t_{i-1}} \left(\frac{V_c(VCC_i + VCC_{i-1})}{2} \right)^{-1} \quad (7)$$

The lactate yield coefficient from glucose ($Y_{\text{Lac/Glc}}$) (Eq. 10) is calculated based on the specific glucose consumption (q_{Glc}) (Eq. 8) and the specific lactate production (q_{Lac}) (Eq. 9), with the glucose concentration (c_{Glc}), the lactate concentration (c_{Lac}) and the geometric mean of the VCC ($\bar{x}$).[35]

$$q_{\text{Glc}} = \frac{V_c\,(c_{\text{Glc},i-1} - c_{\text{Glc},i})}{(t_i - t_{i-1})} \frac{1}{\bar{x}} \quad (8)$$

$$q_{\text{Lac}} = \frac{V_c\,(c_{\text{Lac},i} - c_{\text{Lac},i-1})}{(t_i - t_{i-1})} \frac{1}{\bar{x}} \quad (9)$$

$$Y_{\text{Lac/Glc}} = \frac{q_{\text{Lac}}}{q_{\text{Glc}}} \quad (10)$$

Results and Discussion

An overview of the experiments performed in this study is presented in Figure 1. First of all, the medium composition for the semi-perfusion process was optimized. For this purpose, different blends from a CHO-fed-batch platform-medium and its proprietary feeds were investigated applying DoE. The basal process medium (PM) was used as a basis for the new medium formulation. FMA was added to the DoE as it is known to deliver macro nutrients (e.g., glucose) to the cells. FMB was investigated in the DoE as it is necessary for micro nutrients (e.g., amino acids) in the cell culture. The optimized medium was used afterwards for further process optimization. In particular, the glucose addition to the cell culture and the cell bleed were investigated to improve the final viability. The medium and process optimization was conducted in shake flasks as a scale-down model using centrifugation for media exchange. In a last step, the optimized semi-perfusion process was transferred to an automated small-scale bioreactor system in which a continuous DO and pH control can be achieved. By transferring the process to the automated small-scale bioreactor, the great potential of the bioreactors to replace the classical shake flasks as parallel scale-down system was investigated.

DoE studies for medium optimization

In the first part of the studies, medium optimization based on an existing fed-batch process was performed. Therefore, FMA, FMB, and the PM were combined in different blends to receive one medium that meets the requirements of higher cell concentrations in the semi-perfusion process. Therefore, the PM was enriched with FMA and FMB to receive one media formulation for the semi-perfusion. For optimization and fast progress with as few experiments as necessary a DoE study was implemented in shake flasks as shake flasks are commonly used as scale-down model for process development.[17]

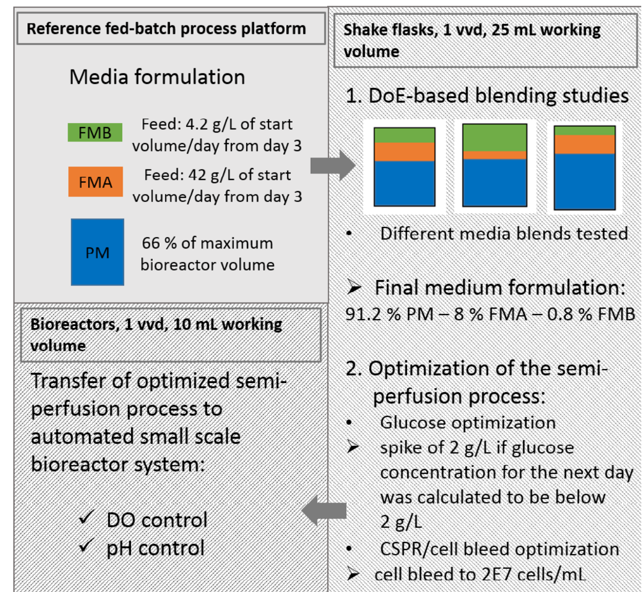

Figure 1. Schematic overview of the experimental structure in this work. The fed-batch process platform was used as a base for further process improvements meeting the requirements for process intensification. The PM and the respective feeds, FMA and FMB, were investigated with DoE to find an optimal medium mixture for semi-perfusion processes. The process strategy was further optimized by adjusting the glucose concentration and the CSPR by implementation of a cell bleed. The optimal conditions of the semi-perfusion process were transferred to a small-scale bioreactor system with DO and pH control. Each VVD of medium per day is displaced for the semi-perfusion processes.

Table 1. DoE Set-up for Medium Development in Shake Flasks Suitable for the Semi-Perfusion Process

Composition ID	PM (%)	FMA (%)	FMB (%)
PF1_DoE1	100.0	0	0
PF1_DoE2	99.2	0	0.8
PF1_DoE3	92.0	8	0
PF1_DoE4	91.2	8	0.8
PF1_DoE5	96.0	4	0
PF1_DoE6	95.2	4	0.8
PF1_DoE7	99.6	0	0.4
PF1_DoE8	91.6	8	0.4
PF1_DoE9	95.6	4	0.4

The variations of each fraction within the DoE were highly beneficial to understand the nutrient requirements of the high cell concentrations. The medium exchange was applied on a daily basis by centrifugation. Therefore, the VVD for each experiment was 1. Conditions of each shake flask run and the center point PF1_DoE9 that was performed in a triplicate is shown in Table 1. The DoE design was based on a full factorial central composite face-centered design (CCF) containing the three factors PM, FMA, and FMB. The VCC was integrated as response factor. The experimental design was based on an optimization response surface methodology with a quadratic process model.

The criteria for optimization were the VCC and viability of the cell population (Figure 2A). VCCs up to 45 million cells/mL were obtained, which is twice the amount of cells compared to the previous fed-batch process (Supporting Information Figure S1). As it can be seen from the VCC and viability data, exclusively in the medium blends PF1_DoE4 and PF1_DoE8, the viability and VCC were high over a period of 250 h. In all other shake flasks, the viability decreased dramatically after 170 h. Therefore, one indication from the DoE is the addition of both feeds, and 8% FMA is necessary for a stable process. This demonstrates that only in the highlighted medium compositions the nutrient supply is sufficient for long productivity.

However, the increase of the FMA concentration is also limited due to higher osmolality with rising nutrient concentration. The glucose profile (Figure 2B) showed a lack of glucose for nearly every cultivation after 150 h. Comparing the cultivation time, the lack of glucose corresponded with a strong decrease in viability and VCC. The correlation of the glucose concentration and the decreased viability and VCC indicated a possible glucose limitation during the cell cycle and other metabolic processes (e.g., nucleotide synthesis). Optimization is necessary in further steps.

The CSPR was analyzed for each semi-perfusion process based on Eq. 1 taking into consideration the VCC after each 24 h interval. The CSPR values for the DoE study (Figure 2B) indicate a lower limit for the CSPR at 50 pL/(c*day). However, the critical CSPR needs to be further investigated in the following experiments. The cumulative product summarizes the product concentrations before and immediately after centrifugation based on the integral of viable cells in the cell culture according to Eqs. 5 and 6. The cumulative product in the semi-perfusion processes showed a clear improvement up to 12 g compared to the normal fed-batch process with an average of 3 g in the corresponding working volume of 1 L (Supporting Information Figure S1).

Besides the results of the beneficial medium compositions PF1_DoE4 and PF1_DoE8 seen from the offline measurements in viability, VCC and CSPR (Figure 2), the DoE response surface plot showed strong tendency that with 8% FMA a local optimum was reached in the presented DoE setup

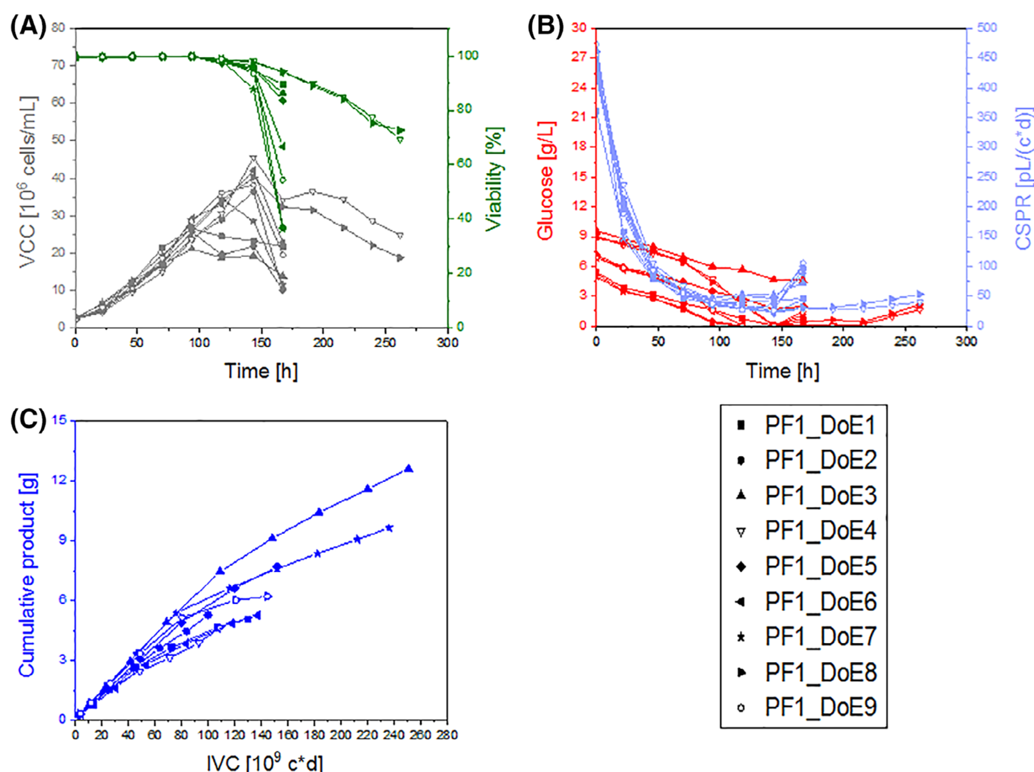

Figure 2. Results of DoE experiments for semi-perfusion medium optimization with a mAb producing DG44 CHO cell line. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) of each shake flask run are shown. Center point PF1_DoE9 based on n = 3.

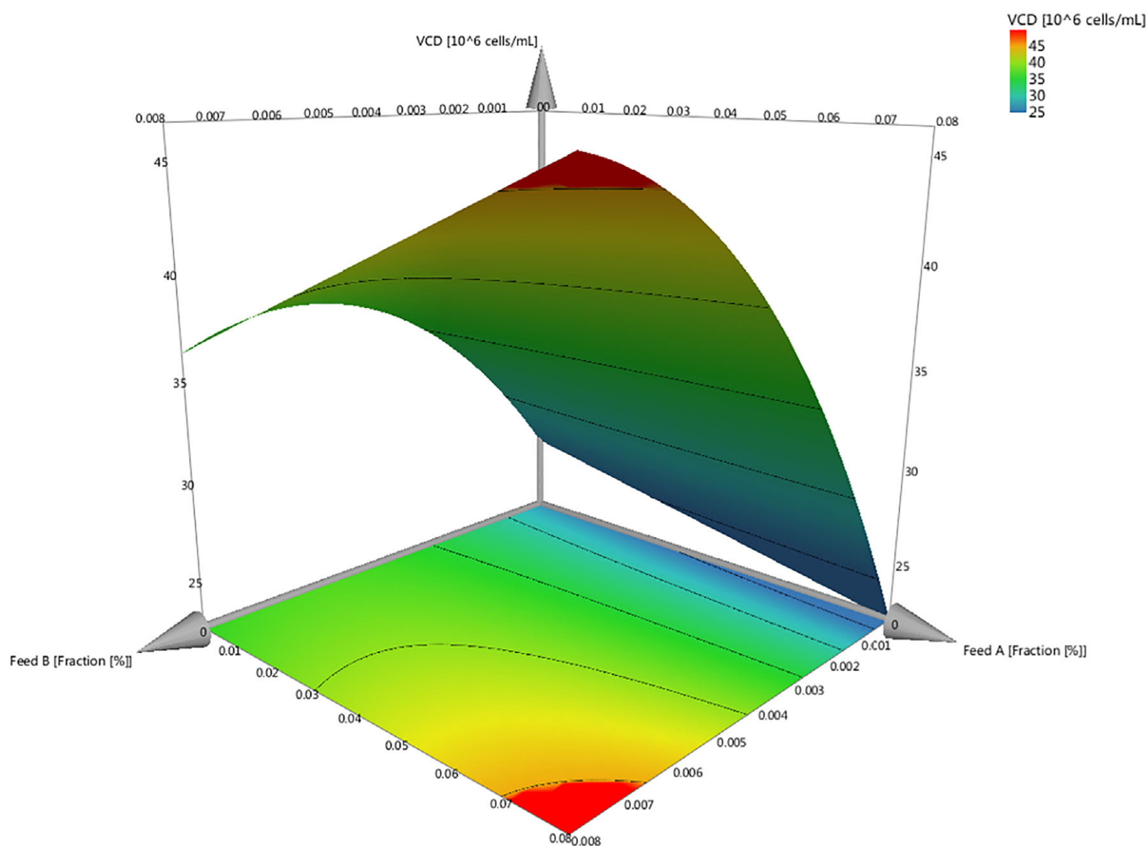

Figure 3. Response surface plot of DoE experiment. The evaluation is based on VCC and different feed fractions (%) in the medium blends. The response of the target variable is color-coded, ranging from blue (low) to red (high).

(Figure 3). However, further increase of the fractions FMA and FMB to optimize the first screening DoE is likely to result in an osmolality exceeding 400 mOsmol/kg. Compared to literature, an elevated osmolality from 320 to 435 mOsmol/kg reduced production rates by 50% for CHO cells.[36] As further refinement of the media formulation requires detailed information on the original recipe, no additional optimization runs were conducted. The resulting local optimum was used as a satisfactory compromise between performance and osmolality.

Optimization of glucose concentrations for stability in the semi-perfusion process

One critical factor during process transfer from fed-batch mode to perfusion processes is the glucose concentration. As it can be seen from the DoE study (see Section "DoE studies for medium optimization"), it is necessary to optimize the amount of glucose in accordance to the higher cell concentrations obtained in the semi-perfusion process. Therefore, two different approaches of glucose bolus were analyzed. In the first part, different compositions of PM, FMA, FMB together with glucose (added directly to the compositions) were analyzed. These compositions were tested to gain a better understanding of the correlation between each component (Supporting Information Figure S2 and Table S1). In a second step, glucose spikes were added directly to the culture after the daily medium exchange (Figure 4).

Both experiments showed similar culture behavior achieving a longer stability and the favored 12 days of cultivation with high cumulative products. The control composition PF2_Glc1 without any additional glucose showed the same glucose depletion after five cultivation days as in previous experiments (Supporting Information Figure S2B). Best results in viability were achieved in PF2_Glc4, PF2_Glc5, and PF3_GlcSpike1. All of those approaches contained 91.2% PM, 8% FMA, and 0.8% FMB. Being aligned with the previous DoE results, this medium composition was used in every following experiment and is now called semi-perfusion medium.

The glucose addition was highly beneficial for the stability of the new established semi-perfusion process with VCCs achieved up to 40 million cells/mL. The results are in good agreement compared to perfusion cultures in the literature.[12] Analyzing the CSPR values of the different approaches, it is indicative, that below a CSPR of 50 pL/(c*day) a decrease in viability can be seen in the following days. Glucose additions starting from 2 g/L up to 8 g/L were beneficial for higher CSPR values. The best glucose concentration in the range between 2 g/L and 8 g/L needs to be further specified in the following experiments.

As no difference between the two tested approaches of how to add glucose to the cell culture was observed, the glucose spike was further tested as it offers advantages in easier handling and better adaption to batch to batch variations in cell growth.

However, the viability in both experimental setups decreased to 60% on the last two cultivation days (Supporting Information Figure S2 and Figure 4). As viabilities below 70% are common harvest criteria,[10,37,38] further improvement of the process stability and higher CSPR values are desirable.

Influence of the cell bleed on cell culture stability

After improving the medium in the DoE (see Section "DoE studies for medium optimization") and the process strategy by

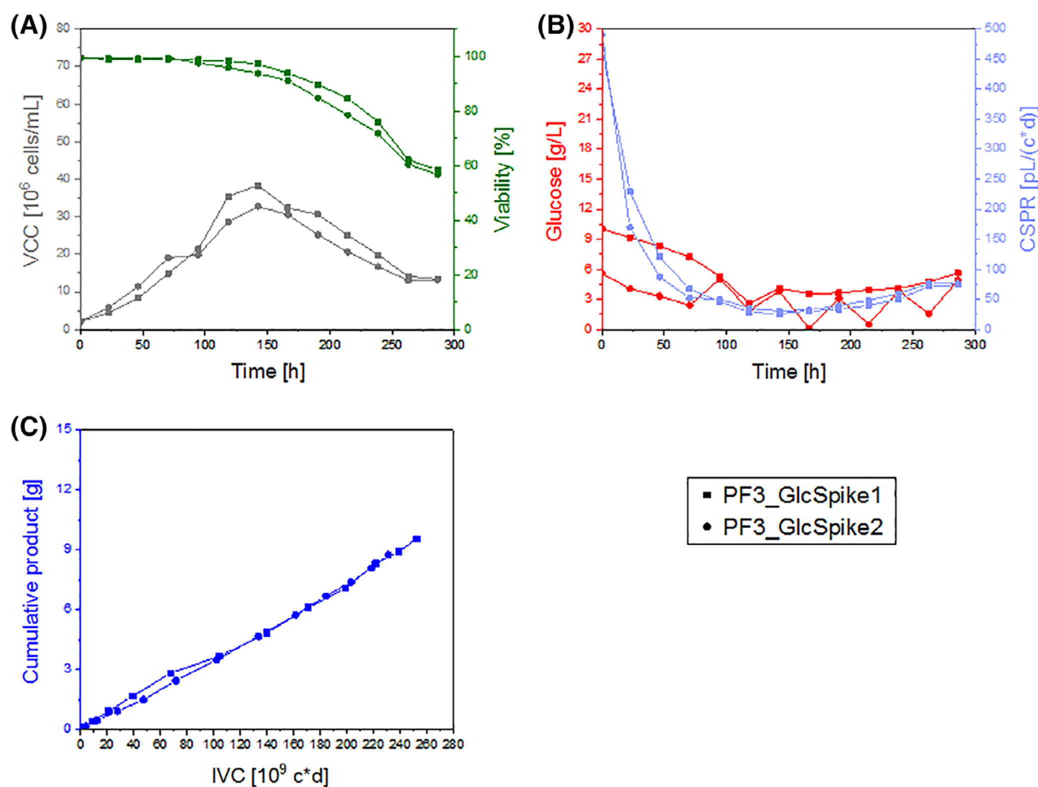

Figure 4. Performance of shake flasks with implementation of additional glucose spikes in the semi-perfusion processes of a mAb producing DG44 CHO cell line. For PF3_GlcSpike1 glucose was spiked with 4 g/L daily after Day 5 of cultivation. The medium composition for PF3_GlcSpike1 was 91.2% PM, 8% FMA, and 0.8% FMB. The glucose spike for PF3_GlcSpike2 was applied on a daily basis starting from Day 1 if the subtraction of the glucose consumption of the last 24 h from the actual glucose concentration was below 2 g/L. In that case, a spike of 4 g/L was carried out. The medium composition for PF3_GlcSpike2 was 100% PM in the first 3 days and 91.2% PM, 8% FMA, and 0.8% FMB in the following days. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the produced antibody in relation to the IVC (C) of each shake flask run are shown. Depicted are the mean values of duplicate measurements (n = 2).

adapting glucose additions (see Section "Optimization of glucose concentrations for stability in the semi-perfusion process") another key factor in the new established semi-perfusion process, the cell bleed, was investigated. During a cell bleed, the VCC is reduced to a fixed target bleed VCC after medium exchange. The cell bleed protects the culture from being limited by nutrients provided with a fixed, daily medium exchange. To move from a fed-batch culture to perfusion cultures, the cell bleed is essential to maintain a stable VCC over prolonged process time provided that the VVD of 1 is kept constant. As the established process is based on semi-perfusion, a requirement of a fixed VVD of 1 was set for the experiments. Therefore, different cell bleed strategies were tested (Table 2) instead of increasing the VVD. In addition, to further improve the glucose concentrations and the correlation of cell bleed and glucose spikes, different combinations of bleed and glucose additions were tested in the cell bleed studies. The cell bleed was combined with the daily media exchange. Therefore, the cell bleed is considered as being applied in semi-continuous mode within the semi-perfusion process.

In all approaches applying the cell bleed to the semi-perfusion shake flask cultures, peak VCCs of 25 million cells/mL or higher were achieved (Figure 5). However, the first bleed condition PF4_Bleed1 with a bleed VCC of 40 million cells/mL and a high glucose addition to 4 g/L from Day 5 on, showed a decrease in viability to 65% on the last cultivation day. This viability is lower than the demanded viability of 70% at the end of the semi-perfusion culture. Therefore, the high bleed VCC is not beneficial for a stable semi-perfusion process with the given VVD of 1. Moreover, the VCC of PF4_Bleed1 was similar to the VCC achieved in PF4_Bleed4 with a bleed threshold of 25 million cells/mL. In both approaches, the CSPR fell below 50 pL/(c*day) after 110 h of cultivation, thus resulting in a decrease of viability and inhibition of further cell growth to higher VCCs. The result indicates that the bleed should be applied at an earlier stage with lower target bleed VCCs. Higher VVDs might support stable cell populations with higher VCCs without the need to bleed, but increase the medium consumption and costs significantly.

Indeed, every bleed condition with a target bleed concentration of 20 million cells/mL (PF4_Bleed2 and PF4_Bleed3) or 25 million cells/mL (PF4_Bleed4) showed viabilities above 70% on the last day of cultivation with similar peak VCCs around 30 million cells/mL. In addition, the glucose concentration was not limited in the semi-perfusion medium over the complete process time (Figure 5B). Interestingly, PF4_Bleed2 with no additional glucose spiking showed no glucose limitation and was characterized by the highest end viability of above 80%. This indicates that the bleed in a semi-perfusion process enables constant CSPR values above the critical CSPR and therefore is fundamental for culture stability. The detected importance of the CSPR for the semi-perfusion culture is in good agreement to results from literature that report controlling, modeling, and optimizations of semi-perfusion processes based on the CSPR.[16] Even more, the highlighted condition

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Table 2. Cell Bleed Strategies Tested During the Semi-Perfusion Processes in Shake Flasks. All Batches with 91.2% PM, 8% FMA, and 0.8% FMB

Composition ID	Glucose Addition	Bleed VCC
PF4_Bleed1	From Day 5 adjusted to 4 g/L	Cell bleed to 4E7 cells/mL
PF4_Bleed2	No addition	Cell bleed to 2E7 cells/mL
PF4_Bleed3	if: glc^{d}-[$glct^{d-1}$-glc^{d}] ≤ 2	Increase bleed level by 0.3E7 cells/mL every third day, starting with 2E7 cells/mL
PF4_Bleed4	if: glc^{d}-[$glct^{d-1}$-glc^{d}] ≤ 2	Cell bleed to 2.5E7 cells/mL

was constantly bled to the smallest investigated bleed level of 20 million cells/mL. According to previous observations, the decrease of the CSPR below 50 pL/(c*day) in each shake flask run, was aligned with a lower viability in the following culture days. Concluding with the cumulative product (Figure 5C), the improvement of the cumulative product was significantly up to 10 g in comparison to the fed-batch process (Supporting Information Figure S1).

As shown, medium optimization and corresponding cell bleed adaptation led to successful semi-perfusion cultures with final viabilities of more than 70% after 12 days of cultivation. Based on these findings, the process was fixed to 91.2% PM, 8% FMA, and 0.8% FMB and a target bleed concentration of 20 million cells/mL. This semi-perfusion process can be transferred into an automated small-scale bioreactor system in the following experiments. Ultimately, this will enable more process control and easier future investigations of the new process. Due to the direct transfer of the optimized process into the small-scale bioreactor, the advantage of the automated bioreactor as scale-down model for process optimization over the classical shake flask approach was investigated.

Use of automated small-scale bioreactor system to control pH and DO

After successful conversion of an existing industrially relevant CHO fed-batch process to a semi-perfusion process in shake flasks, the new process was transferred to an automated small-scale bioreactor system. The transfer into the bioreactors enables higher process control of relevant process parameters (e.g., DO, pH). In addition, a better process understanding can be achieved due to monitoring of the key performance indicators and the future possibility to establish automated medium exchanges. By applying the centrifugation approach in a small-scale bioreactor, the system's advantage in process control was fully used compared to previous literature that were using sedimentation-based approaches to exchange the media.[17,32,33] As mentioned before, the medium composition of 91.2% PM, 8% FMA, and 0.8% FMB and a cell bleed target of 20 million cells/mL were applied. Due to improved process control of the parameter set points and especially the pH control at 7.1 in the automated bioreactor, higher cell growth rates were expected. Therefore, glucose was additionally spiked

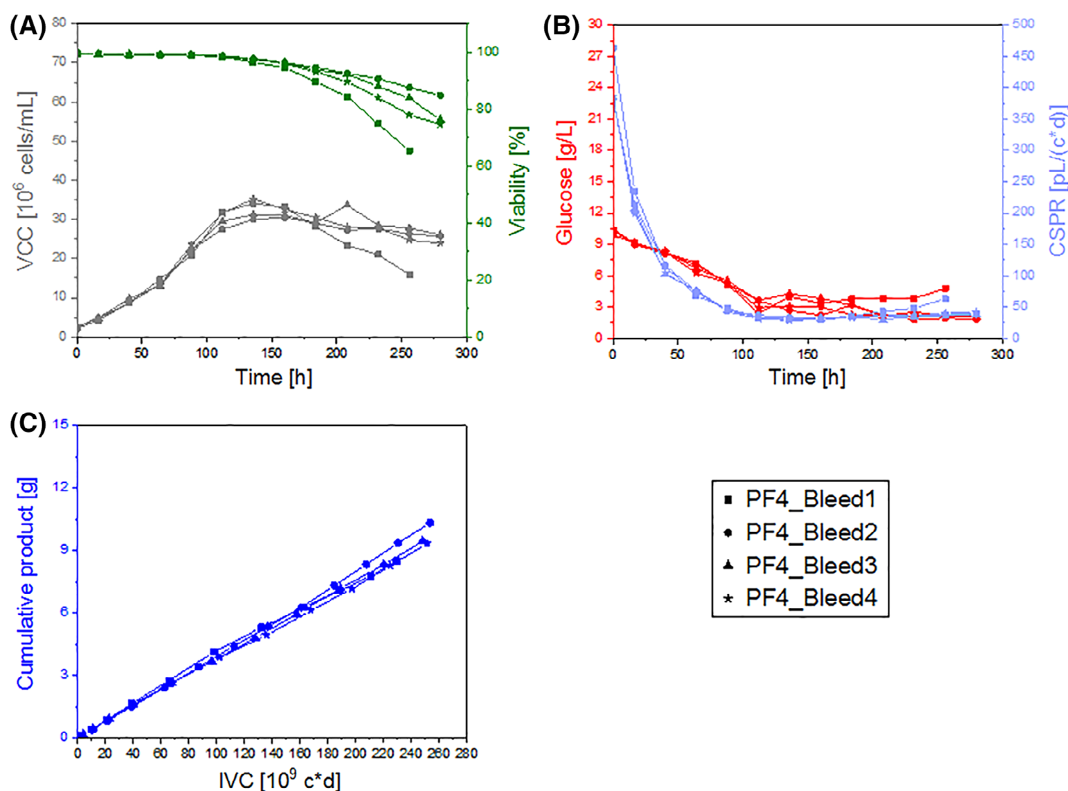

Figure 5. **Implementation of cell bleed in the semi-perfusion processes of a mAb producing DG44 CHO cell line. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) of each shake flask run are shown. Depicted are the mean values of duplicate measurements (*n* = 2).**

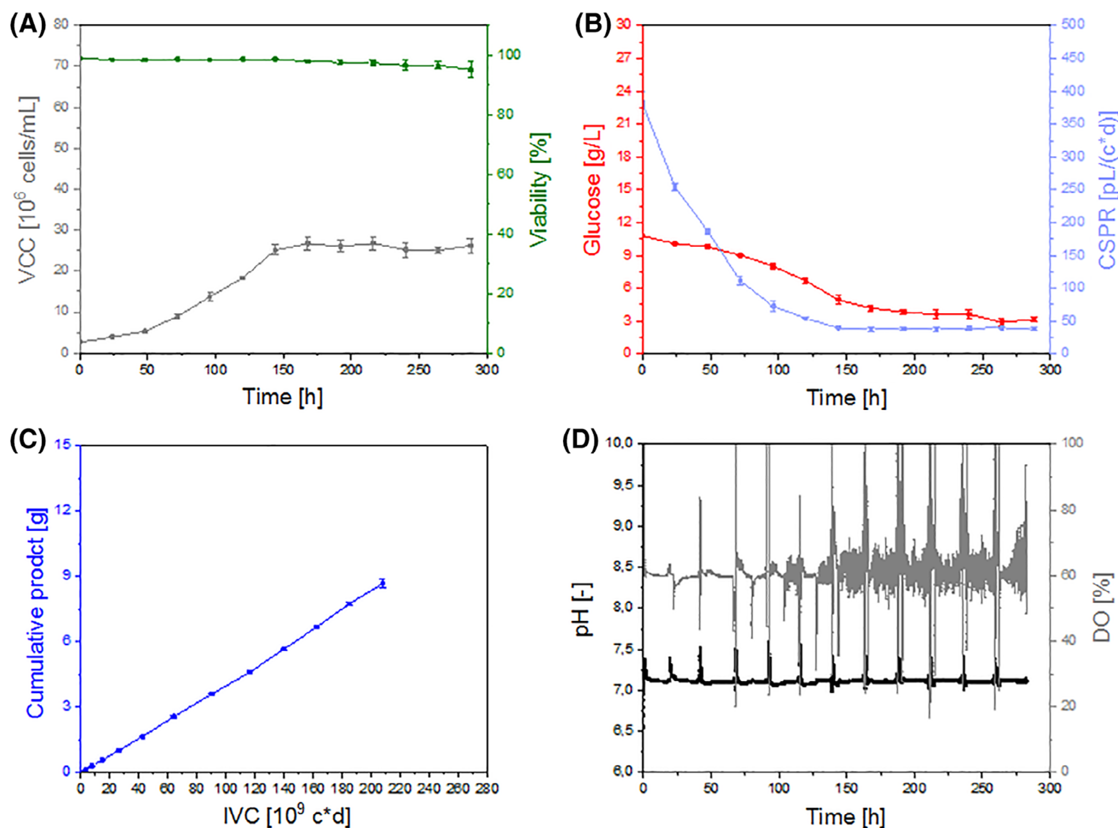

Figure 6. Transfer of the semi-perfusion processes of a mAb producing DG44 CHO cell line to an automated small-scale bioreactor system. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) are shown. Error bars are reported for each value. The controlled parameters, here the pH value and the DO of the process are shown (D). Depicted are the mean values of triplicate measurements (*n* = 3).

with 2 g/L to each bioreactor whenever the resulting glucose concentration for the following day was calculated to be below 2 g/L based on the specific glucose consumption per cell. The glucose spike was implemented to maintain a stable culture over the total cultivation time.

Figure 6 displays the results of the process transfer to the automated system. For all critical parameters shown in the figure, the favored criteria were achieved. The culture was stable over the complete culture time of 12 days. Even more, 20–30 million cells/mL were accomplished and kept as steady-state concentration with the help of precise cell bleeds (Figure 6A). In addition, the viability was above 98% for half of the cultivation time and decreased to 95% once the previous detected critical CSPR of 50 pL/(c*day) (Figure 6B) was reached. Therefore, it was shown that keeping the CSPR above 50 pL/(c*day) results in a stable cell culture with high viabilities. With the help of the bioreactor system, the viability was significantly higher than in any other shake flask experiment and the process was more stable than in uncontrolled shake flasks. The process was controlled to the pH set point of 7.1 and the DO set point of 60% over the complete culture time (Figure 6D). Compared to the pH values in the shake flask experiments (Supporting Information Figures S4–S6), a huge improvement of the pH control was observed. In addition to that an oscillation of DO could be detected in the automated bioreactors over the cultivation time. At later stages, high cell densities led to an increased oxygen demand of the culture that caused a more active control loop of the system and subsequent oscillations in the DO signal. Based on the generated data for DO and pH control experienced users will be able to have a sound understanding of the process's behavior at larger scales. Therefore, the use of small-scale bioreactors

Table 3. Lactate Yield Coefficient from Glucose in the Fed-Batch Process Platform, the semi-perfusion shake flask, and the ambr® 15 Semi-Perfusion Process, *n* = 3

Day of Culture	$Y_{Lac/Glc}$ (Fed-Batch Process Platform)	$Y_{Lac/Glc}$ (Semi-Perfusion Shake Flask)	$Y_{Lac/Glc}$ (ambr® 15 Semi-Perfusion)
1	0.91	0.45	0.5
2	0.58	0.19	0.08
3	0.31	−0.16	0.06
4	0.30	−0.07	−0.06
5	1.58	−0.06	−0.01
6	−0.07	0.02	−0.02
7	−0.03	0.10	−0.00
8	0.03	−0.02	0
9	−0.37	0.03	0.00
10	0.14	0.02	0.00
11	−0.15	−0.02	0.00
12	−0.01	−0.09	0.00

Table 4. Comparison of the Cell Diameter Increase Over Process Time, the Average Specific Productivity, the Medium Consumption Based on the Reactor Volumes (RV) and Other Key Process Parameters of the Fed-Batch and the Semi-Perfusion in Shake Flask and ambr® 15

Parameter	Fed-Batch Process Platform	Semi-Perfusion Shake Flask	ambr® 15 Semi-Perfusion
Cell diameter increase over culture time	3.14 μm	2.33 μm	2.69 μm
Average specific productivity	24.16 pg/(c*d)	36.04 pg/(c*d)	38.88 pg/(c*d)
Cumulative product	3.62 g	10.32 g	8.67 g
Integral of viable cells	180.71 10E9 c*d	253.62 10E9 c*d	208.15 10E9 c*d
Overall process time	12 days	12 days	12 days
Medium consumption	1.4 RV	12 RV	12 RV
End viability of cells	86.0%	83.55%	95.23%

is highly favored to gain deep knowledge for intensified bioprocesses. However, in a large-scale bioreactor the process will be implemented as a fully continuous process with a suitable cell retention device. The change from centrifugation in small-scale bioreactors to cell retention in large-scale bioreactors will enable culture control and process monitoring over the complete process time.

Applying glucose spikes whenever the glucose concentration was below the threshold of 2 g/L led to a sufficient nutrient supply for the semi-perfusion cell culture. The cumulative product of 8.7 g in the semi-perfusion process was three-fold higher than in the corresponding fed-batch process (Supporting Information Figure S1). The resulting standard deviations showed the three bioreactors to perform consistently throughout the process. The product amount over the total culture time can even be improved by cultivating the semi-perfusion culture in the controlled system for longer periods in the future. The possibility of longer cultivations is given by the higher stability and high viability in the controlled bioreactors. Due to the consistent and controlled semi-perfusion process in the small-scale bioreactors, the conclusions can be compared and used to improve process understanding for continuous processes and the implementation of perfusion processes in suitable bioreactors with cell retention devices.[17] Furthermore, the results can be used to predict and optimize the scale-up to production scales.

To conclude the presented study, the fed-batch process, the successful implementation of semi-perfusion processes in shake flasks and the process transfer in the automated small-scale bioreactor can be compared. This comparison can be based on the $Y_{Lac/Glc}$, the specific productivity, the increase in cell diameter, the cumulative product as well as the IVC and the end viability of each process (Tables 2 and 3). $Y_{Lac/Glc}$ allows deeper information about the cell state and the metabolism in the cells.[35] Glucose is used in different pathways based on the metabolic state and the nutrients in the environment. Lactate is a waste product in unfavorable pathways that can inhibit the cell growth. Therefore, a low $Y_{Lac/Glc}$ indicates efficient cell metabolism with low lactate production or lactate consumption by the cells.[35,39] Especially in the beginning of each process, $Y_{Lac/Glc}$ is significantly smaller in the semi-perfusion process indicating a more efficient metabolism than in the corresponding fed-batch cultivation (Table 3). The biggest positive effect was achieved in the automated small-scale bioreactor to values around 0, so that the ratio of glucose consumption and lactate production indicates a highly productive and healthy cell culture. These findings are supported by the lactate production over time in each of the three cultures (Supporting Information Figure S3). Especially in the beginning of the historic fed-batch a significantly higher lactate production was detected before the cells switched into lactate consumption.

Even more, the increase in the cell diameter is much less for the established semi-perfusion process compared to the historic fed-batch indicating a more viable cell culture (Table 4).[40] Another benefit of the semi-perfusion process is the low osmolality around 310 mOsmol/kg at the end of the process in comparison to an osmolality around 360 mOsmol/kg in the fed-batch. Besides the effect that apoptotic cells show larger diameter, the higher osmolality can additionally support the increase in diameter in the fed-batch.

Finally, the average specific productivity was increased to a final productivity of 38.88 pg/(c*day) in the automated small-scale bioreactor. The cumulative product and the IVC increased significantly in the semi-perfusion processes during the same culture time compared to the fed-batch. The automated small-scale bioreactor performed better with regards to the end viability of 95.23% compared to the semi-perfusion in shake flask or the fed-batch process. Therefore, the ambr® 15 performed best in the demonstrated studies regarding product yield and viability of the cell culture. The benefit of the automated small-scale bioreactor as scale-down model was clearly shown compared to the shake flasks.

Conclusion

With this work the conversion of an existing, industrially relevant fed-batch to a semi-perfusion process was successfully demonstrated by using the same platform medium and its proprietary feeds. The product yield normalized to 1 L of the corresponding fed-batch process increased from 3 g up to a cumulated product amount of 10 g in the semi-perfusion process for the same cultivation duration. However, in further experiments the product quality attributes of the newly established semi-perfusion process need to be compared to the product quality from the historic fed-batch. With the presented methodology, a powerful but low resource demanding approach to transfer an existing CHO fed-batch process into a semi-perfusion process implementing DoE studies and perfusion specific handling was established. The developed strategy to establish a semi-perfusion process can be adopted and used for different cell lines. In addition, the suitability of the process for a multiparallel controlled micro-bioreactor system as scale-down model has been proven so that early stage development of a semi-perfusion process can be done in a controlled system in future. Moreover, in a next step, process scalability and transfer will be tested in different bioreactor systems and scales. Changing to intensified cultivations enables high product yields with time and cost savings as well as keeping the same size of biopharmaceutical plant capacities or even reducing them.[3]

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