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Performance benchmark of different cell clones in discontinuous and continuous bioprocesses reveals critical impact of cellular diameter

Lucas Nik Reger [a,b,*], Rene H. Wijffels [b], Dirk E. Martens [b], Julia Niemann [a]

[a] Corporate Research, Sartorius, Göttingen, Germany
[b] Bioprocess Engineering, Wageningen University, Wageningen, the Netherlands

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ABSTRACT

Therapeutic proteins such as monoclonal antibodies are usually manufactured either in continuous processes such as steady-state perfusion or in discontinuous processes like fed-batch (FB). Thereby, both process formats have fundamentally different requirements for the utilized cell factories. This poses a problem as common cell line development programs are designed to select cell clones to perform well in FB cultivation. The aim of this study was to identify critical and easy to access cell line attributes for each process format and with that for the transfer of clones from fed-batch to perfusion. As a result, increased cell-volume specific productivity could be identified as beneficial within the FB not only impacting performance but as well diminishing media utilization and host-cell-proteins level. Within the perfusion, a stable cell diameter was identified as major beneficial characteristic, positively influencing cell viability, metabolite and glycan profile, as well as equipment utilization. Consistent with this, our data suggest that the inclusion of cell volume in the screening parameters is important for clone selection for both process formats and can lead to improved process design and robust cell line process transfer. Overall, this work gives valuable new insights in cell behaviour across discontinuous and continuous process formats to improve clone cell selection for both process strategies and streamline the process transfer from discontinuous towards continuous.

1. Introduction

The manufacturing process of therapeutic proteins, like monoclonal antibodies (mAb), is commonly separated in two distinct sequential phases, the upstream process comprising the expression of the desired therapeutic compound in producer cells and subsequently, the downstream process for purification of the drug product. Both process parts consist of multiple unit operations that need to be well synchronized and efficient to ensure high productivity, product recovery and quality [1]. Each unit operation has specific factors impacting the overall yields of the manufacturing process and the quality of the end product [2]. Thereby, the major influencing factors of the upstream process are the bioprocessing strategy alongside with the chosen expression platform [3]. As cell factories for therapeutic proteins commonly mammalian cells like Chinese hamster ovary (CHO) are utilized. These cells are generally considered safe expression hosts and allow for complex post translational modifications e.g. glycosylation or protein folding, necessary for proper protein function and low toxicity in humans [4,5]. Production clones of CHO cells are commonly made by non-targeted integration of the gene of interest (GOI) alongside with an vector system, resulting in multiple clones with distinct geno- and phenotypes. To select clones with suitable properties, such as high productivity, stable cellular properties, and uniformity in product quality, a consecutive screening as part of the cell line development (CLD) is required [6]. Selection of suitable cell clones is commonly done using a small-scale standard fed-batch (FB) process [7]. Hereby, an initial basic data set is generated, comprising cell growth and viability characteristics, productivity and product quality attributes as well as impurity levels.

For industrial production of mAB with CHO cells both the discontinuous FB process and continuous-perfusion process is used. In general, the FB process consists of short batchwise operations, while continuous processes can be run over an elongated duration, often in a steady-state-like fashion.[8,9]. Thereby, the discontinuous, FB operation is state-of-the art for the production of mAb, due to its simplicity, robustness, efficient media utilization and low need for process monitoring [9,10]. The process usually starts with a defined batch phase for 3–4 days. Subsequently, defined feeds of nutrients, such as glucose and amino acids are added daily to elongate the growth phase and sustain

* Corresponding author at: Corporate Research, Sartorius, Göttingen, Germany.
E-mail address: Lucas.Reger@sartorius.com (L.N. Reger).

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cell viability. Thereby, higher viable cell counts and longer process durations are obtained leading to significant increased final product yields compared to simple batch processes. However, due to the accumulation of inhibitory molecules and limitations in overall amount of volume that can be added, and therefore limitations in nutrient supply, the FB concept is limited in cell density as well as duration. As consequence, the FB faces limitations in plant utilization and overall volumetric productivities in comparison to continuous cultivations.

Continuous process types were developed over the latest decades to increase volumetric productivity in comparison to discontinuous operation [3]. In this process a continuous media exchange is applied, enabling a constant supply with fresh nutrients as well as removal of produced mAb and inhibiting molecules [11]. Due to the steady media exchange the cells can maintain their proliferative state and grow up to much higher cell densities compared to FB operations. By implementing a cell bleed to maintain cell densities at a certain threshold, e.g. 50 $\times 10^6$ cells/mL, the system can be operated in a pseudo-steady mode, with cells maintained in the exponential growth phase for up to several weeks. [12,13]. These kinds of processes reach higher volumetric productivities, by maintaining high cell densities and viabilities over time, leading to more efficient use of the production plant and consequently smaller reactors for production can be used [3,14]. The continuous media exchange is enabled by a cell retention device to separate the cells from the outflowing spent media (permeate) containing metabolic byproducts as well as secreted product [15]. Commonly filter-based cell retention devices are used like tangential flow filtration (TFF) or alternating tangential flow filtration (ATF) with integrated hollow-fiber structure for compactness [16]. However, these cell retention devices add additional complexity to the process due to an increase in peripheral devices that require close monitoring. Further, the new distinct characteristics, such as continuous media exchange, elongated cultivation duration, increased cell counts, and presence of cell separation devices may change the requirements for the cell line in comparison to a discontinuous process and could affect the product quality profile [17, 18]. Therefore, the choice of the best performing clones may be different for the discontinuous fed-batch and the continuous perfusion operation. The modification of properties between FB and perfusion process was investigated within several studies either focusing on the impact of cell line parental origin (CHO-K1; CHO-DG44, CHO-S) revealing specific preferences in either mAB production or biomass synthesis for each parental cell line [17]. Other studies focused on the impact of process transfer in regard to product specific properties for either a common mAb constructs or bispecific antibodies, revealing overall increase of productivity as well as antibody quality related attributes within perfusion for both constructs [18,19]. In a common non-targeted cell line development, clones are generated from the same parental cell line, vector system and GOI, but they are different in geno- and phenotype as discussed previously. To our knowledge, no studies have been published that compare the performance of such cell clones in fed-batch and perfusion processes.

The aim of this study was to investigate the performance of different cell clones generated in the same cell line development with similar parental cell line, vector system and gene of interest in discontinuous (fed-batch) and continuous (perfusion) operation. Thereby, a focus was set on easy to access parameters, that are commonly screened within a (limited) small-scale cultivation, to further improve predictability of performance in both operations. Further, special attention will be paid to better understand the relation between specific cell characteristics and observed differences in performance in both systems. This aims to further streamline and improve the transfer of clones between discontinuous and continuous operations. In detail, four non-targeted integrated CHO-DG44 cell lines were cultivated within a platform FB and perfusion process and essential process parameters, such as cellular behavior, productivity, impurity level, and glycan profile were evaluated.

2. Material and methods

2.1. Cell culture system

Four Chinese hamster ovary (CHO-DG44) cell clones generated by non-targeted integration with a similar vector system and GOI, stable expressing an IgG were utilized as model system within this work (Sartorius Stedim Cellca). Each cell culture step (e.g. Cell thaw, split etc.) was conducted in similar manner. All seed train steps were conducted with 4Cell® SmartCHO Selection and Adaption media (Sartorius Stedim Biotech).

2.2. Fed-batch cultivation

All four cell lines were cultivated in triplicate in a platform fed-batch process. The process was conducted in a multi-parallel-bioreactor system ambr250HT (Sartorius Stedim Biotech). As media system the 4Cell® SmartCHO was utilized comprising a basal media (PM) and a two-solution feed (FMA and FMB). The bioreactors were inoculated with 0.3 $\times 10^6$ cells/mL at a working volume of 200 mL. Consecutive to the 72 h batch phase a daily bolus feed was applied starting at 4% (FMA) and 0.4% (FMB) calculated based on the initial vessel volume. Additionally, starting from day 6 a glucose feed was applied with a 400 g/L stock solution to the setpoint of 4.5 g/L. The DO was controlled at 40% by addition of O_2 (maximum 0.1 vvm), pH was set to 7.0 $\pm$ 0.1 and controlled by either CO_2 sparging or base addition (1 M Sodium bicarbonate). For mixing the stirrer speed was held at 600 rpm. To control foaming a daily bolus of Antifoam (C-Emulsion, Thermo Fisher Scientific) was applied. Samples were taken before each daily bolus feed. The threshold for end of the cultivation was set to a viability below 70% for one of the triplicates. Additional data can be found in supplementary Figure SF2.

2.3. Perfusion cultivation

All four cell lines were cultivated within a platform perfusion process. To conduct the perfusion process an ambr250Perfusion (Sartorius Stedim Biotech) system was utilized. The four non-targeted integrated cell lines were cultivated in duplicate within the ATF ambr250 Vessel comprising a 0.2 µm hollow fiber filter with 88 cm^2. Each vessel was inoculated with 0.2 $\times 10^6$ cells/mL and consecutive to the 3 days batch phase a continuous media exchange was applied. The media exchange was slowly increased over the growth phase and set to 2.5 vol exchange per day (VVD) after obtaining the perfusion target of 50 $\times 10^6$ cells/mL. Further, a bleed was applied to hold the cell count at the designated perfusion target. The VVD setpoint was increased up to 3.0 VVD within the process if the glucose concentration dropped below 2 g/L. As media system for media exchange the 4Cell® SmartCHO was utilized [12]. The pH was set to 7$\pm$ 0.1 and controlled by CO_2 sparging or base addition (1 M Sodium bicarbonate). The stirrer speed was set to 850 rpm, DO setpoint was controlled at 40% by O_2 sparging (maximum 0.12 vvm) and consecutive increase of the stirrer speed up to 1300 rpm. Samples were taken once daily from the bioreactor and from the permeate line for further analysis. The process was terminated either by failure of one of the two duplicate vessel or on day 17. Additional information can be found in supplementary Figure S2.

2.4. Analytic

Viable cell count (VCC), viability and cellular diameter were analyzed once daily with the Cedex HiRes Cell Counter (Roche). Metabolite concentration (glucose, lactate, NH_4^+), pH and Osmolality were measured with the NovaBiomedical BioProfile Flex 2. Remaining cell containing samples were centrifuged at 6600 x g for 5 min at room temperature (Sartorius Centrisart A-14; rotor YCSR-A1C) and the supernatant was stored at -20°C for further analysis.

The antibody concentration was analyzed with a size exclusion chromatography (SEC) via a high-performance liquid chromatography system (HPLC; Dionex Ultimate 3000; Thermo Fisher Scientific). The samples were separated by the gel column (Yarra 3 µm SEC-3000, Phenomenex) with a buffer solution containing 100 mM Na_2SO_4, 50 mM NaH_2PO_4 and 50 mM Na_2HPO_4 with 1 mL/min. For quantification a standard curve for each measurement was applied.

N-glycan determination was conducted utilizing the LabCHIP GXII Touch24 (Perkin Elmer). For preparation the samples were purified by Protein A 96-well-plates (Cytivia) and consecutive desalted with Vivaspin (Sartorius) with a 50 kDa molecular weight cut off. Subsequent, the samples were prepared according to the manufacturer protocol with Glycan release and Labeling Kit and analyzed by the LabCHIP GXII Touch.

The host cell impurities were determined by the commercially available ELISA Kit following the manufacturer instructions (CYG-F550–1, Cygnus Technologies).

Values for integral of viable cell count (IVCC) and cell specific productivity (Qp) values were calculated according to [12], cell volume was calculated as described in [20]. Integral of viable cell volume (IVCV) and cell volume specific productivities (Qp_c) values were calculated similar to the IVCC and Qp values. Specific cellular turnover rates were calculated as shown in [18]. Product sieving in perfusion cultivation was calculated as stated in [21]. All equations are attached in the supplementary.

3. Results

In this work, four non-targeted integrated CHO-DG44 cell clones generated with similar vector system and GOI were compared for their performance in both standard process types, fed-batch and perfusion. The aim of the study was thereby to better understand the relation between differences in performance which could further facilitate the selection of optimal clones from a standard cell line development for both processes as well as the transfer from discontinuous process formats towards continuous bioprocessing.

3.1. Cellular characteristics

Firstly, cell growth and viability of the different cell lines were monitored over the duration of each process (Fig. 1 A and B). During the FB process, the different clones exhibited a wide spectrum of different growth behaviors, reaching peak cell densities between day 6 and 7 ranging from 14.2 $\times 10^6$ cells/mL up to 25.7 $\times 10^6$ cells/mL (C2<C4<C1<C3; Fig. 1A). High cell viabilities could be detected for all clones up to day 9 of the process. However, afterwards the decline in cell viability varied strongly between the clones, leading to a drop below the process termination threshold of 70% between day 10 (C4) and day 13 (C2). Fig. 1 B shows the same clones cultivated in a perfusion system with a respective bleed target at 50 $\times 10^6$ cells/mL for each clone. Due to distinct differences in the cell growth profile, the bleed target was reached at different days for the individual cell lines. While the first 2 clones (Clone 1 and 3, respectively) reached the 50 $\times 10^6$ cells/mL on day 7, it took 9 days for C4 and 11 days for C2. This growth behavior resembled the observations made during the FB cultivation, where clones C2 and C4 showed slower growth, with lower peak cell densities. As a steady-state target, we aimed to keep each clone in a stable perfusion process for at least 5 days once the bleed target was reached. As can be seen from the data (Fig. 1B) this could be achieved for all clones except C2. The shorter duration at perfusion target for C2 was mainly due to a high transmembrane pressure that was also seen to some extend for C4 (data not shown). Interestingly, the increase in transmembrane pressure correlated with decreased viabilities for both mentioned clones to around 93% (C2) and 90% (C4). The viabilities for C1 and C3 remained high for the entire cultivation (≈97%).

As an additional cellular parameter, the cell diameter was traced over time and is shown for the fed-batch process in Fig. 1 C. Up to day 4 a very similar pattern and diameter could be detected for all clones. After day 4 all clones started to increase in cell size, however, a significantly stronger increase could be seen for clones C2 and C4. The clone C4 showed a mean off set of +1 µm, while for the clone C2 this was even +3.3 µm on average. Again, similarities in the dynamics of the cell diameter could be observed for the perfusion cultivation (Fig. 1D). In agreement with the fed-batch data set, the diameter of all clones showed similar values over the first 4 days of the perfusion cultivation.

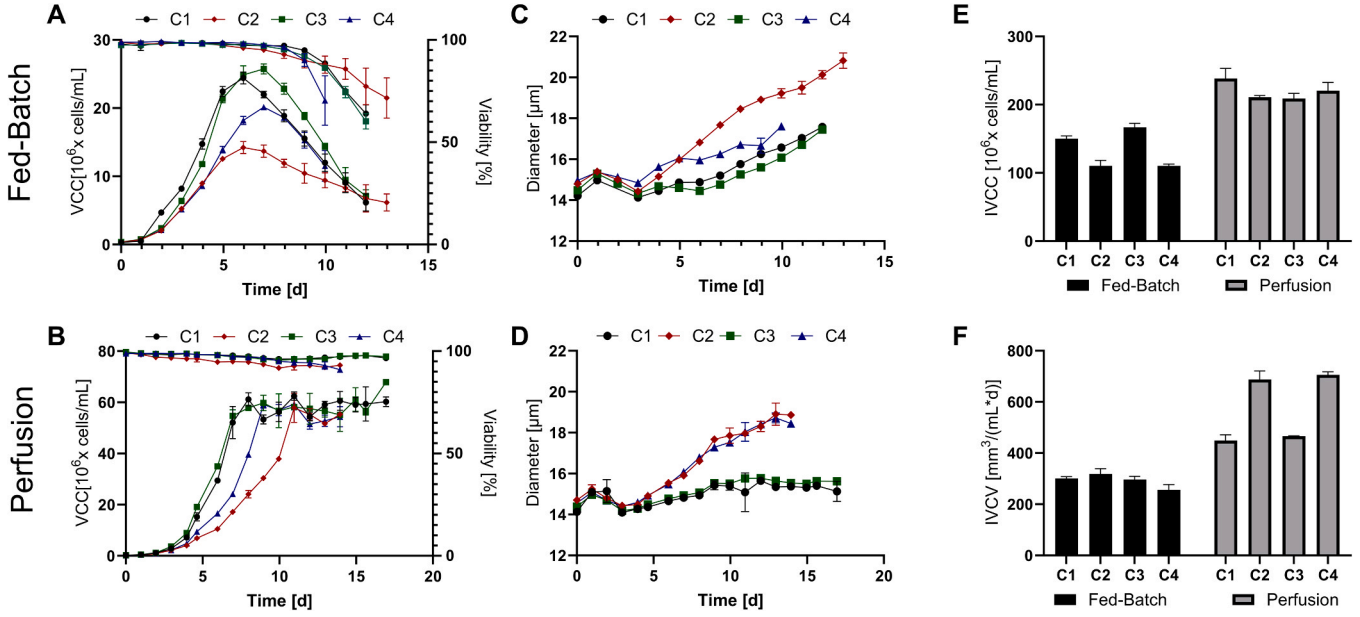

Fig. 1. Different cellular parameters for all four non-targeted integrated cell lines. (A) Viable cell count (VCC) and Viability over time for the fed-batch cultivations. (B) VCC and Viability over time for the perfusion cultivations. (C) Cellular diameter over time for the FB cultivations. (D) Cellular diameter over time for the perfusion cultivations. (E) Integral of Viable Cell Count (IVCC) for FB (day 0 to end) and perfusion cultivation (last 4 days of cultivation). (F) Integral of Cell Volume (IVCV) for all tested cell lines with similar timeframe as for the IVCC.

Subsequently, a strong and steady increase of the cellular diameter could be seen for the clones C2 and C4 up to around 18.7 μm, while the diameter of the clones C1 and C3 stabilized during the second half of the cultivation (from day 9) at around 15.5 μm. As the cell diameter is a mean value and always a function of distribution, we also investigated, if the measured values were representing the most abundant value amongst all cells or were a result of different distinct populations that result in the overall mean value. In order to access this, we extracted the cell diameter histograms, displaying the distribution of different cell sizes over time for both processes and all four clones (see supplementary figure SF3). This analysis revealed an overall comparable distribution of cell diameter, with mean values close to the peak of the distribution curve. Therefore, no split in multiple sub-populations could be detected.

To further assess variations between clones, the Integral of viable cell counts (IVCC) was calculated. For the FB process, the respective calculations were based on the complete cultivation duration (Fig. 1 E). For perfusion the last 4 days of cultivation on perfusion target were used for the calculation to represent the stable process operation, not influenced by the differences in runtimes of the cell clones. Since perfusion cultures can be operated for longer times, the growth phase was not included in the IVCC calculation. The IVCC values for the FB process showed clear differences between the clones dependent on the duration of the process (determined by the viability, see above) and cell growth profiles of the individual clones. Accordingly, the clones with high peak VCCs and comparably long process durations (12 days), C1 and C3, respectively, exhibit the highest IVCC values. Meanwhile, the IVCC for the perfusion process shows comparable values for all clones, which can be expected if an adequate bleed target control is applied. However, due to the various cell diameters amongst the clones, the cell count and accordingly the IVCC, does not give an adequate representation of the biomass present, which is better represented by the total cell volume. Therefore, to account for the observed diameter variations the integral of the viable cell volume (IVCV) was determined and is shown in Fig. 1 F. Interestingly, the IVCV shows an opposite picture compared to the IVCC. In this case, clones cultivated within the FB process showed very comparable IVCV values, indicating a shift from proliferation towards cell size increase for clones with rather low overall cell densities, compared to clones growing to high cell densities. In contrast, clear differences in the IVCV can be observed between the individual clones for the perfusion cultivation. While IVCC values were similar for the different clones due to the constant cell bleed target, a strong variation in the IVCV could be identified with the clones C2 and C4 showing elevated IVCV values due to the strong increase of cell volume (Fig. 1D).

3.2. Productivity

To further assess the performance of the four clones in the fed-batch and perfusion system, the productivity in regard to titer expression was analyzed. Fig. 2 A shows the titer over time for each of the clones in the fed-batch process. As can be seen from the data, a similar titer increase is visible for C1 and C4 as well as for C2 and C3, with the titers of clone C1 and C4 (3.6 and 4.2 g/l, respectively) being higher than those of C2 and C3 (2.7 and 3.0 g/l, respectively). Interestingly, no obvious correlation between peak cell density and overall titer at the end of the process could be observed. To compare the mAb titers in the perfusion process without technical impact (e.g. different bleed rates and sieving effects), the overall titer per day was calculated by taken into account: (i) the concentration in the culture broth (supernatant from bioreactor samples), (ii) the permeate and (iii) the bleed (Fig. 2B). Once the perfusion target was reached, comparably stable values could be observed for C1 (≈ 1.8–2.4 g/L) and C3 (≈ 1–1.7 g/L), while the other 2 clones, C2 and C4 respectively, did not reach a steady-state. Both clones seem to continue to increase in titer formation, possibly caused by the increase in diameter over time which will be discussed in Section 4.1.

To compare clones, cell specific productivities (Qp) for each clone were calculated comprising the complete cultivation for the FB process and an average of the last 4 days in daily productivities for the perfusion set up (Fig. 2C). The Qp data of the fed batch revealed the highest expression for the clone C4 (33.7 pg/c/d) followed by C1 and C2 with around 28 pg/c/d and the lowest for C3 with 16.3 pg/c/d. Interestingly, in perfusion all clones except for C2 showed increased cell specific productivities with C1 = +45.1%, C3 = +63.1% and C4 = +35.6% compared to the FB data. Further, to account for the observed changes in diameter over the course of the cultivation (Fig. 2D) the cell volume specific productivity (Qp_c) was calculated. When taking into account the cell volume over the complete duration of the process, C2 and C3 showed lower productivities (≈0.009 mg/mm^3/d) compared to C1 and C4 (≈0.013 mg/mm^3/d). Meanwhile, the cell volume-based specific productivity calculated in the perfusion operation on a daily basis displays a high increase for C1 (+57.9%) and C3 (+44.7%) and no significant increase for C4 and C2 compared to the FB.

To compare the cell specific productivity with the overall output of

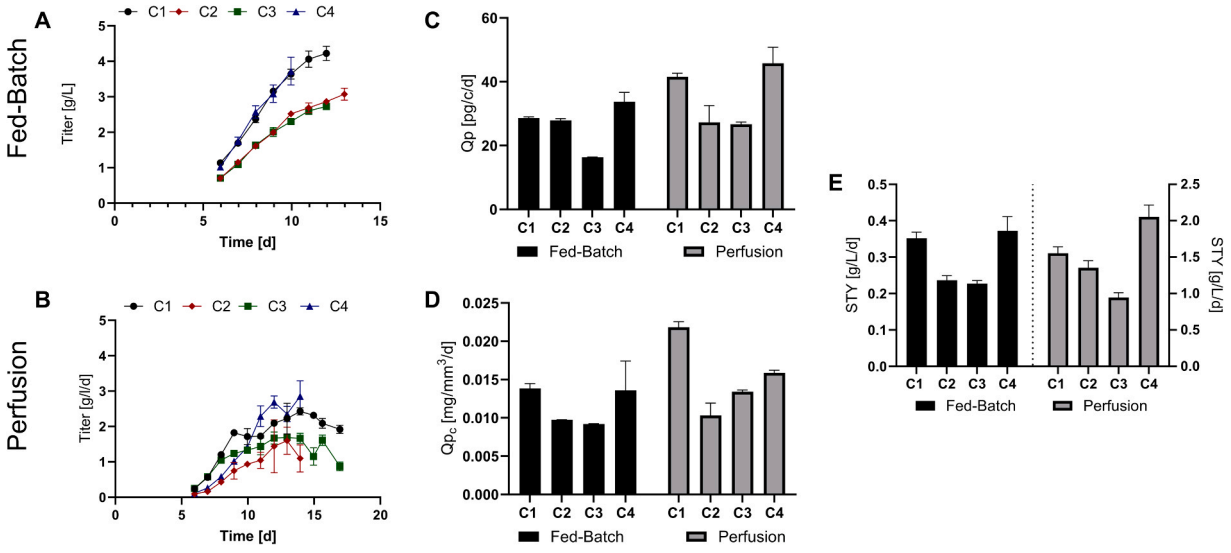

Fig. 2. Titer and cell expression parameter for all cultivated cell lines. (A) Titer over time for the fed-batch cultivations. (B) Perfusion titer from permeate, bioreactor supernatant and bleed cumulated daily. (C) Cell specific productivity (Qp) within FB as well as perfusion cultivation. (D) Cell volume-based productivity for perfusion and FB cultivation. E) Space-time-yield for both process formats.

the process the space-time-yield (STY) for both process types was calculated and is shown in Fig. 2 E. Of note, only the permeate was taken into account when calculating the STY for the perfusion operation, as it is the defining output variable for the process. Within the FB Clone C1 and C4 showed the highest STY overtime with around 0.35–0.37 g/l/d, meanwhile C2 and C3 showed a decreased STY of ~0.22 g/l/d. In perfusion, a similar trend could be observed between the different clones, with higher STY values for clone C4 and C1. However, productivities are significantly higher and individual differences between all clones could be observed. The highest productivity could be observed for clone C4, reaching an STY of 2 g/l/d, followed by C1 (1.5 g/l/d), C2 (1.3 g/l/d) and C3 (0.9 g/l/d). By comparison of the cell specific productivities limited overlap between Qp and STY is visible. Noteworthy, the Qp_c data showed better indication for the actual titer values for the FB cultivation, but not for the perfusion, possibly due to further impacting parameters, such as bleed rates and filter sieving effects additionally impacting the perfusion cultivation.

3.3. Impurities, glycans and metabolites

Besides the cell specific productivity, impurity levels, metabolites and antibody's quality profile are essential parameters that need to be considered during development of the upstream process. Therefore, process related impurities such as NH_4^+ lactate and host cell protein (HCP) content, metabolite profiles for glucose and glutamine alongside with the mAB glycan distribution profiles were determined for both process operation types. Fig. 3A shows the measured NH_4^+ concentrations for the fed-batch cultivation, revealing a comparable trend over time for all clones with different final concentrations of C2 (18.7 mM), C3 (15.6 mM), C1 (11.7 mM), and C4 (9.52 mM), mainly due to variations in cultivation duration. Of note, a slightly delayed increase of NH_4^+ over the course of cultivation could be observed for clone C1, resulting in lower final ammonia concentrations compared to clone C3, despite similar cultivation duration. Interestingly, the increased NH_4^+ concentrations negatively correlate with the titer (compare Fig. 2A). In contrast the NH_4^+ values for the perfusion process were lower and fairly stable over time in comparison to the FB data (Fig. 3B). This can be attributed to the continuous media exchange during perfusion culture. However, different levels of ammonia could be detected between the clones ranging from low levels from 2 mM (C1) to 3 mM (C3) towards increased NH_4^+ concentrations of 6 mM (C2) up to 8 mM (C4). To further facilitate the insight in the NH_4^+ secretion the cell specific and cell volume specific turnover rates were calculated and are visible within the supplementary file (Figure SF7). The data shows an overall comparable picture for the cell lines with increased rates for C2 and C3 in the FB and C2 and C4 within the perfusion system. Interestingly, smaller differences in production rates could be seen for the cell volume specific rates. No obvious correlation could be identified between clone specific NH4+ production in perfusion versus FB cultivation, indicating different impacting factors for the formation of this byproduct. Besides these, specific lactate concentrations and turnover rates were measured and calculated for all cultures, shown in supplementary Figure SF5. Thereby, smaller difference were detected for lactate production with maximum concentration of ≈2 g/L for C3 in FB and ≈ 1.4 g/L for C1 in perfusion. Further, little deviation of the respective cell or cell volume specific turnover rates could be detected.

Another form of process impurities, secreted or released, are HCPs, which mainly impact downstream purification operations due to the high similarity of some of these HCPs to the desired mAB product. HCPs were measured at the respective time points they would be subjected to downstream processing, which translates to the end of the cultivation for the FB and the steady-state phase for the perfusion cultivation, days 13–17 for clones C1 & C3, days 11–14 for clone C2 and days 9–14 for clone C4, respectively. All HCP levels are expressed as relative values in relation to expressed mAb in parts per million (Impurity: Product level) and were measured before purification and should be as low as possible. All clones showed comparable HCP levels for both process styles with levels ranging from ≈1 ×10^5 ppm (C1) over ≈1.5 ×10^5 ppm (C3 and C4)

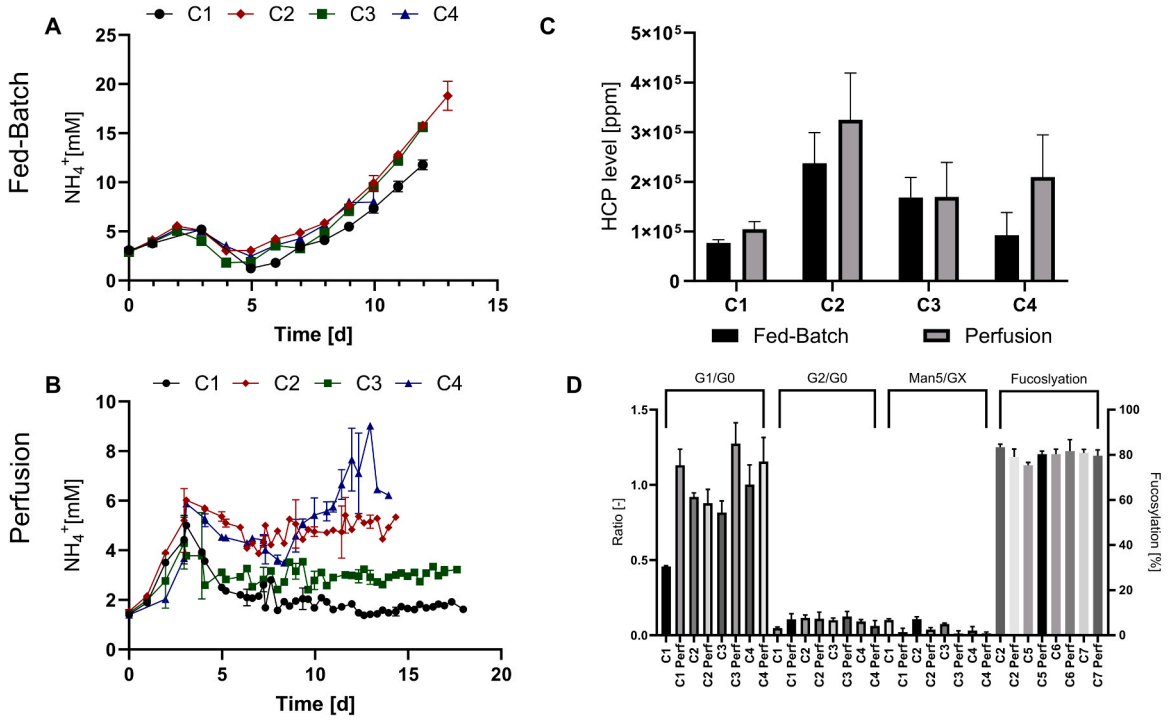

Fig. 3. Impurity level and critical quality attributes for both conducted process operations (Fed-Batch and Perfusion). (A) NH_4^+ concentration over the course of cultivation for FB and all utilized cell lines. (B) Impurity concentration of NH_4^+ for the perfusion cultivation over time. (C) Host cell protein (HCP) level equalized to expressed antibody in parts per million for both conducted process types. (D) Critical quality attributes in form of glycan ratio for G1/G0, G2/GO, Man5/GX and overall fucosylation for FB and perfusion operation.

to moderately higher levels of ≈2.8 $\times 10^5$ ppm (C2). No major differences in the HCP level could be detected between FB and perfusion, indicating a good transferability between both process types.

In addition to the impurity levels, concentrations of glucose and glutamine alongside with specific uptake rates were calculated and can be found in the supplementary file (Figure SF4 and 6). Interestingly, for glucose a nearly similar picture for all cultures in FB vs. perfusion can be seen, further supported by very similar overall uptake rates. Thereby, it can be assumed that the overall cell specific consumption rate is increased for cell clones with larger cell diameter (e.g. C2 and C4). This difference is neglected within the cell volume specific rates, further indicate an shift in uptake rate for the cells in respect to their cell size. This is further supported by the concentration and cell specific uptake rates of glutamine showing partially increased rates for C2 and C4 (Figure SF6 E and F). However, to further facilitate this hypothesis further investigations would be necessary in regard to consumption rates which was not the aim of this work.

Further, the glycan distribution for the produced antibody was analyzed with similar time schedule as for the HCP analysis, however, for the perfusion cultures only three points over time were measured (Fig. 3D). By intra process comparison between the clones in perfusion no clear deviation between the clones can be seen, however for the FB a clear differentiation of C1 is visible for the G1/G0 ration as well as G2/G0 ratio from the other clones. Besides these comparison of the glycan between the processes shows a clear increase in the G1/G0 levels for C1 (+67.3%) and C3 (+46.0%) within the perfusion cultivation. A similar trend could be observed for C1 with regard to the G2/G0 ratio that increased in favor of G2 with around +5.7%. Notably, the relative overall ratio of G2/G0 remained low for all compared clones and processes. Interestingly, the shift for the specific ratio's was not visible for the C2 and C4 with overall stable glycan distribution. Alongside the galactosylated glycans, the share of Man5 was analyzed as an important precursor. The ratio of Man5 to galactosylated forms showed an increased trend for the fed-batch cultivation for all clones with the highest share for C1 (+8%) and C2(+3.7%). The fucosylation percentage of all analyzed glycans was equally distributed at around 80%.

Overall, the impurity level showed interesting differences for the NH_4^+ levels, such as increased values for C2 and C4 within the perfusion cultivation, meanwhile the HCP values revealed constant behavior over time for both investigated process types. The analyzed glycan distribution displayed inconsistent behavior between the process types with shifting glycans for C1 and C3 and stable behavior for C2 and C4. Furthermore, cell volume specific metabolic rates for lactate, glutamin and glucose showed low deviation between the cell lines, showing highly comparable cell volume specific turnover rates.

3.4. Perfusion and media consumption characteristics

One distinct difference between both process types is the necessity for continuous media exchange as well as cell bleed in the perfusion cultivation to enable a steady-state. Thereby, the media exchange by the filter system and bleed function, further related as perfusion parameters, not only add complexity to the process but are also considered one of the main limitations of process duration, which could potentially be impacted by the characteristics of the utilized cell clone. Therefore, the perfusion related parameter such as the volume exchange per day (VVD) in reactor volumes per day (RV) and the daily cell bleed were taken into account to further explain differences between the process types for the four clones. The respective data is displayed in Fig. 4A. The VVD values were adjusted according to the cell density and, therefore, were increased in proportion to the cell growth up to 2.5 VVD at cell bleed target (50 $\times 10^6$ cells/mL). Additionally, as can be seen from the

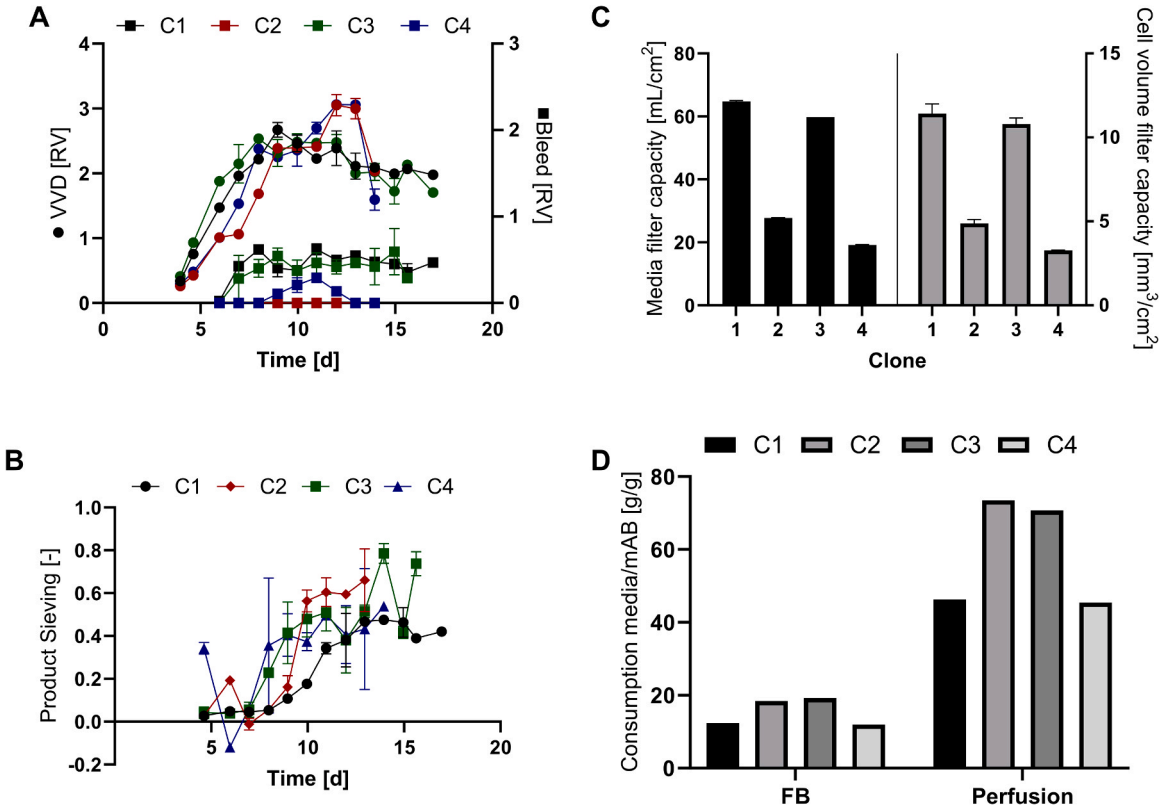

Fig. 4. Perfusion specific and performance indications for the conducted study. (A) Volume exchange per day based on reactor volume (VVD; circles) alongside with daily reactor volume (RV) of bleed (squares) for all utilized cell lines. (B) Product sieving as ratio over the course of cultivation. (C) Filter capacity for the first filter expressed in media volume (mL) per filter size (cm^2) (left) and processed cell volume (mm3) per filter size (cm^2) (right). (D) Consumption of media amount in g per one gram produced mAb for both conducted operations.

respective graphs, the perfusion rate was further increased for the clones C2 and C4 up to 3 VVD after day 10 due to low glucose levels (data not shown) reached with the standard 2.5 VVD. Furthermore, these two clones required only very minor or no bleed over the duration of the cultivation, which indicates a reduced proliferation rate. Both clones had substantial higher diameters than clone C1 and C3. Moreover, the diameter of the clones C2 and C4 was still increasing at the end of the run (compare Fig. 1D) in contrast to clone C1 and C3 that reached steady cell diameters and were run at a rather stable bleed level of ~0.5 VVD after reaching the VCD target. Furthermore, C1 and C3 showed a steady behavior at the targeted 2.5 VVD for 5 days and even tolerated a reduction in media exchange rate to 2 VVD at day 12. In summary this shows that observed differences in cell diameter increase between clones could give a good indication of differences in feed and bleed rate in the perfusion process.

In perfusion, the cell retention device represents an essential element, which performance is ultimately impacted by the cell culture components, including cells, cell viability and cell-related products. Therefore, we determined 2 important performance parameters related to the cell retention device, product sieving and filter capacity, in the context of the four cultivated clones (Fig. 4B and C). Product sieving or product retention, represents the amount of product unable to pass the filter towards downstream and was calculated for all clones over time (Fig. 4 B). A similar trend could be observed amongst the different cell lines with increasing product sieving towards the end of the process. In detail, the C1 cultivation resulted in the least product sieving (up to 42%), while the cultivation with C3 showed the strongest product sieving behavior (up to 78%). Of note, these values are commonly impacted by clogging or exchange of the filter modules, which occurred at different time points and frequencies for the 4 clones (data not shown). The filter utilization or capacity represents another important factor for perfusion processes since filter clogging can result in termination of the process. Accordingly, the impact of the different cell lines on filter clogging was analyzed, by looking at the processed media per cm^2 filter area [mL/ cm^2] was calculated. For all clones this parameter was determined up to the first filter change and therefore represents the capacity of the initially integrated filter, which usually determines the process duration. The media filter capacity (left side) reveals differences between all clones ranging from 64.7 mL/ cm^2 for C1 down to around 27.7 mL/ cm^2 for C2. Thereby, a clear distinction between clones with stable cell volume (C1 and C3) clones with increasing cell diameters over time (C2 and 4) could be identified, indicating a negative impact on filter capacity with increasing cell volumes. To further investigate this factor the cell volume (VCV) capacity processed per cm^2 was calculated. Interestingly, similar results for the cell volume filter capacity are visible showing duplicated capacity for C1 and C3 compared to C2 and C4. These results indicate further underlying changes within these high cell volume cultures impacting the filter capacity.

Finally, since media is one of the main cost drivers during the upstream the respective amounts of media powder utilized to produce a defined antibody amount [g/g] were calculated for all clones and process types (Fig. 4 D). Thereby, a clear and anticipated increase in media consumption towards the perfusion process for each clone could be observed. This enhanced demand is triggered by the constant feed of media to sustain proliferating cells. However, evaluating cell specific characteristics in both processes, it can be highlighted, that the clones C1 and C4 show reduced media consumption rates for both processes compared to the other 2 clones. This can be explained by the higher specific productivities of these clones. Overall, the relative media consumption between different clones seems to be generally transferable between both processes.

In summary, we've collected a comprehensive data set, on four non-targeted integrated cell lines with similar vector system and GOI in fed-batch and perfusion cell culture. Our data shows interesting varieties for the four clones, further impacting clone performance and robustness in the tested process formats FB and steady-state perfusion.

4. Discussion

In this work four different CHO-DG44 clones were evaluated in a discontinuous (fed-batch) and a continuous (perfusion) process. All clones were derived through non-targeted gene integration from a common parental cell line using the same expression vector and GOI. A specified set of analytical parameters, usually available for late-stage candidates during clone selection, was analyzed to identify parameters impacting the performance of the cell clones within both process types.

4.1. Performance evaluation fed-batch

Within the cell line development a high range of different cell phenotypes are typically obtained caused by genotype differences introduced by random gene integration, plasticity of the mammalian genome, and stochastic nature of gene regulation [5,7,22–24]. This variation in cell phenotype between clones is also clearly observed in this study, showing distinct growth & viability profiles as well as titer variations for the different clones in fed batch operation. In detail, VCCs were ranging from 14 $\times 10^6$ cells/mL up to 25 $\times 10^6$ cells/mL, process duration was ranging from 9 to 13 days based on the threshold viability of 70%, and titer values spread from 2.7 to 4.2 g/L (Fig. 1 A,C and Fig. 2 A). These results are exemplary for the results of a common non-targeted integrated cell line development as also observed in other studies [7,25, 26]. However, our data highlight another interesting parameter that is not always included, being the cell diameter and its progression over process time. Our data show an increase in cell diameter over process time from 14 to maximally 21 μm, which means about a factor three increase in cell volume. Moreover, this increase is very different for the four clones resulting in cell diameters at the end of the process that range from around 17–21 μm between the different clones, which is about a factor 2 difference in cell volume. This effect is visible when looking at the IVCC and IVCV measurements. For the four clones distinct differences in IVCC values are present, as could be expected from the variation in peak cell densities. However, for the IVCV values only small differences could be observed between the clones (Fig. 1F). The observation indicates a shift for some cell cultures from cell division towards cell volume increase, commonly reported in the context of hyperosmolality studies and first studies investigating this effect in the FB format [27–30]. This leads to the hypothesis that the overall amount of biovolume obtained over time remains constant for a defined amount of media being supplied to the cells. Further, based on these results, the data suggest benefits in including the cell volume in regular performance parameters, such as the IVCV in addition to the IVCC and cell-volume specific productivity (Qp_c), in addition to the cell specific productivity (Qp). This new cell-volume-specific productivity showed enhanced predictivity towards the final titer as well as the STY as can be seen for the Fig. 2 (compare C1 and C2) possibly by eliminating cell volume related differences between these clones. Furthermore, this cell volume-based calculation was extended towards the specific uptake or secretion rates, confirming better predictability of cell volume specific parameters and giving first insights into metabolic flux changes of the cell. However, meaningful analysis of complex intracellular metabolic pathway changes will need further in-depth investigation.

Besides these, using a volume corrected parameter such as the Qp_c further indicates whether there are other factors impacting the productivity of the investigated clones except cell counts and cell volume. These other factors could be favorable integration of the vector system in so called genome 'hotspot regions', increased metabolic activity or decreased bioburden of the cell [31–33]. Interestingly, clones with a high cell volume specific productivity (C1 and C4) showed a decreased media utilization (Fig. 4D) and HCP level (Fig. 3C) per amount of product formed, possibly connected to the increased product titer. This further makes clear the beneficial characteristics of highly productive cell lines. Besides the impurity and media utilization the glycan-profile was determined showing differences especially for the galactosylation.

However, to further elaborate on possible impacts of the glycan structure further studies would be necessary, including functional assays. Overall, the main beneficial property for a cell line to perform superior in a FB culture could be identified in this study as high cell volume productivity further beneficially impacting other parameters like HCP and media utilization. This would result in C1 being the best performing cell line within the discontinuous FB amongst the 4 evaluated clones.

4.2. Performance evaluation perfusion

Similar to the discontinuous process the cell clones showed a broad spectrum of phenotypes within the perfusion cultivation resulting in different process performances. As visible from the Fig. 1B the different phenotypes impacted the growth phase resulting in elongated growth phase times for C2 (day 11) and C4 (day 9) in comparison to C1 and C3 (day 6). Interestingly, this elongated growth phase was accompanied by an increase of the cell diameter for these cell lines (Fig. 1D) in contrast to the clones C1 and C3 which showed a more stable diameter over time. This diameter increase for clone C2 and C4 can also be seen in the IVCV values showing an increase up to 1.5-fold over the process time for these clones (Fig. 1F). Further, a correlation of increasing cell diameter and reduction in cell viability is visible (Fig. 1B). Since the viability is one of the key factors for a stable perfusion process, this has an impact on the total process duration and subsequently the productivity of the process. Therefore, decreasing viabilities over time are a limiting factor for continuous processes. The high increase of diameter up to +4.4 µm towards the end of the cultivation translates into a 2.1-fold increase in single cell volume and thus a comparable higher biomass concentration and could have led to increased physical stress and disruption of cells, resulting in lower viabilities [34]. This increase in physical stress could be attributed to the enhanced stirrer rates needed for oxygen supply for the higher biomass concentrations (data not shown). Another factor impacting the viability for C2 and C4 in perfusion could be the higher accumulation of inhibitory substances, as shown for the accumulation of NH_4^+ (Fig. 3B) or nutrient limitations possibly also caused by the higher biomass concentrations within these cultures. Both factors have been shown to be related to cell death in other studies [35–37]. Furthermore, the data for the bleed (Fig. 4 A) reveals an absence or very low bleed rates applied to the cultivations with the high diameter clones. Since dead cells are no longer removed this results in a decrease in viability. Moreover, the lower bleed rates for C2 and C4, potentially lead to an increase of inhibiting substances that can not pass the cell retention device and are usually removed by the cell bleed. Examples for these 'trapped' substances are cell debris resulting from dying cells, large secreted HCPs, extracellular vesicles as well as additions like antifoam which can be retained by the 0.2 µm filter system [21,38–40]. Therefore, our data suggests a distinct disadvantage of clones, in the current set-up, with high and constantly increasing diameter for perfusion processes.

Further, the low bleed values for the two cell lines (C2 and C4) are likely connected to the technical issues of the filter system by accumulation of substances like antifoam, extracellular vesicles and cell debris on the filter surface [21,40]. Accordingly, our data shows a clear correlation of reduced filter capacities with increased cell volumes and low bleed rates (Fig. 4C), most likely resulting from accelerated filter fouling. Thereby, the filter lifetime within a perfusion cultivation represents one of the major limiting factors due to high costs associated with this peripheral device and limited feasibility to change the filter during a production run [41]. To further enhance the perfusion longevity, changes in the perfusion set-up and control could be possible (discussed in Section 4.3) to support a broader range of clones. However, based on our data, selection of suitable clones for a straightforward process transfer, should essentially include factors impacting filter capacities to achieve a longer cultivation time with a given filter set. However, common screening for perfusion performance is conducted in small-scale-systems like spin tubes or generally semi-perfusion systems based on cell separation via centrifugation [12,42]. Unfortunately, these screening tools lack the incorporation of a filter system which could give a prospect for the filter utilization of the single cell lines. Therefore, filter based small-scale screening systems would be extremely helpful to support early identification of clone candidates with low impact on filter performance. Overall, the current work shows a clear trend to test the filter comparability of the clones either within a filter-based perfusion system, or by consideration of major inflicting parameter like the unsteady behavior of the cellular diameter.

Another key parameter to quantify process performance of the perfusion cultivation is the space-time-yield (STY) defining the overall process productivity, visible for all four CHO-DG44 cell clones in Fig. 2E. The STY is mainly defined by the cell-specific productivity due to the fixed cell counts by perfusion target. However, as can be seen by Fig. 2C and D, comprising Qp and Qp_c, no perfect alignment towards the STY for both parameters can be seen. In detail, the Qp values do not reflect the overall increase in volume for the single cell as discussed beforehand and therefore can only give limited indication for the overall productivity. Further, Qp and Qp_c values comprise by definition all produced mAb, in contrast to the STY, which only contains the produced mAb that is accessible for downstream purification in the permeate. Therefore, retained antibody within the vessels (sieving effect) will be flushed out by bleed and result in an off-set between the STY and cell specific productivity. This effect can be seen for C1 showing a high Qp_c but a lower STY compared to clone C4, possibly impacted by the increased bleed rate for this clone. A similar effect is visible comparing C3 and C2. However, by comparing clones with similar bleed volumes, C1;C3 or C2;C4, the Qp_c still represent a good prediction method for the overall productivity.

In summary, the current work highlights several key factors for cell line performance in perfusion, including an overall high cell-volume-specific-productivity (Qp_c) combined with a rapid growing cell line to reach the perfusion target. Further, a steady cell-volume on the perfusion cell concentration target showed favorable characteristics due to an overall stabilized operation, improved cell viability profile as well as increased filter capacity possibly impacted by a substantial bleed rate. All of these characteristics could be seen for clone C1, which would be the clone of choice within this work, due to a simultaneous superior performance in the discontinuous process format.

4.3. Cell line transfer

Another aim, besides the performance analysis of the cell lines within each operation, was the identification of cellular characteristics that changed or remained constant between the discontinuous fed-batch and continuous perfusion system. Stable cellular behavior between both process types was visible for cell growth, as well as the HCP level between each clone. Besides these, the cell diameter was found to be a characteristic with major impact for different issues related to the transferability between continuous and discontinuous process types. This parameter showed low impact within the FB process, possibly due to the fact that all clones reached a similar integral of viable cell volume due to the single (initial) supply of fresh media within this concept. However, the difference in cell volume changes had a big impact for the process transfer to perfusion format with application of a fixed cell-concentration perfusion target. Thus, the increase of diameter led to a rise in overall biovolume within the vessels, which can be seen by the increase in IVCV values (Fig. 1F). This possibly had a direct effect on other parameters, such as the increase in by-product concentration due to an elevated biomass within these two cultures (C2 and C4). Further, the increased biomass could have led to decreased nutrient concentrations within the bioreactor, likely reaching levels of nutrient limitation even with the increase in VVD for these cultures. Therefore, the cell volume needs to be considered as critical parameter for transfer to perfusion cultivation.

In order to account for cell volume related effects, we've incorporated the cell volume into process related quality parameters, such as the specific productivity (Qp_c). Interestingly, we did not observe a uniform

transfer trend for all clones between the formats, while Qp_c levels for clones C2 and C4 remained fairly constant, C1 and C3 exhibit increased Qp_c values in perfusion culture. Due to the cell volume specific productivity parameter used, a change in cell size as sole source for the productivity increase can be neglected, indicating other causes for this change. The increase in Qp_c for these cultures (C1 and C3) could be caused by the decreased byproduct level as well as higher nutrient supply per biomass (cell volume) in comparison to the high cell volume clones (C2 and C4). Further, C1 and C3 perfusion processes required a substantial amount of daily bleed, possibly removing high weight impurities like cell aggregates, antifoam and large proteins. Both factors could lead to the increase in cell volume specific productivity. Interestingly, other studies show an inconsistent picture, with some reporting similar or even decreasing cell specific productivities [43–45], while other studies found increasing values for some clones in comparison to a discontinuous process type [18,19,44]. However, most of these studies investigated the productivity independent of cell size and therefore a cell size change impacting the productivity of the cells within these reports cannot be excluded.

The second property which showed major differences for the process transfer towards the perfusion format is the antibody glycosylation structures. In detail, we could identify an increase of the G1/G0 ratio for the clones C1 and C3, and a stable ratio for the C2 and C4 (Fig. 3D). A major impact factor for the galactosylation of the antibody are inhibitory substances within the culture, such as NH_4^+ [46,47]. Since C1 and C3 showed a particular increase in the NH_4^+ concentration, it can be assumed that this has impacted the galactosylation as described. Recent studies indicated a similar effect of improved antibody quality profile as a result of perfusion cultivation in comparison to an FB process, expressed by an increase in terminal galactosylation of N-glycans, resulting in higher potency of the complement-dependent cytotoxicity (CDC) effector function [18,48]. This finding could be partially confirmed by our study for the C1 and C3. Nevertheless, for all perfusion cultures a decrease of the Man5 share (Man5/GX) could be determined. This reduction could be contributed to the overall stable nutrient supply in the perfusion cultivation, in comparison to a FB, which was shown to affect the Man5 structures [49]. Further, increased osmolality values within the cultivation can impact the Man5 share as shown by other studies [50]. Both factors could have altered the glycosylation profile between the both process formats but further investigations would be necessary which was not the goal of this study.

In summary, the different behavior for cell size, observed for 2 (C2 and C4) of the investigated clones, significantly destabilized the perfusion process and prevented the process from reaching steady-state. It can be hypothesized, that the negative impact of a changing cell-diameter over time can be possibly balanced by implementing a VCV bleed target instead of a VCC target. This would possibly compensate for the increase in diameter over time and a steady-state perfusion cultivation could be achieved, also including a stable product titer, viabilities, and nutrient demand as well as introduce a healthy bleed within these cultures. Thereby, it would be interesting to further understand how the cellular uptake rate for various nutrients is impacted by the different cell sizes. If an uptake rate is proportional to the cell size this would not only help to better control the perfusion process by employing a VCV target but could be the key to easily transfer different cell clones and possibly even different expressed proteins by the same cell line across platform processes. However, the actual applicability of this approach would need further investigation as well as more detailed analysis of dynamics influencing metabolic rates to define the proportional demand for nutrients. A simpler solution to bypass the unpredictable behavior and non-existent bleed would be the omission of these clones and selection of fast-growing cell lines. These fast-growing cell lines showed a superior behavior when transferred to the standard perfusion process by reaching a steady-state, overall stabilizing the process and resulting in a need for constant bleed rates, decreasing inhibiting supernatant components and therefore pressure on the filters. As note, the increased bleed volume led to a decrease in productivity due to the transfer of product towards the cell waste, however this fact may be unavoidable in our view to stabilize the process for an elongated duration.

Overall, the transfer of clones from a discontinuous fed-batch process towards a continuous perfusion mode highlighted changes in cell diameter as a key factor. While the fed-batch process was not obviously impacted by changes in cell size, the perfusion process showed strong sensitivity, resulting in filter dependent issues and increased release of metabolic byproducts like NH_4^+, associated with product expression, modified product quality and increased risk for cultivation failure. Therefore, the cellular diameter could be identified as key impacting factor within this work and should be further incorporated in key performance parameters, such as the Qp_c.

5. Summary

In this work the performance of four non-targeted integrated CHO-DG44 clones with similar GOI was investigated in both discontinuous (FB) and continuous (perfusion) cultivation aiming to identify beneficial characteristics within both formats and to streamline process transfer. The results revealed different cellular characteristics, such as growth profile and cell diameter have relatively low impact on process robustness in fed-batch, which is mainly impacted by the cell-volume specific productivity but are increasingly impacting the perfusion cultivation. Especially, the cellular diameter could be identified as one of the most critical parameters in continuous cultivation influencing process feasibility by affecting the viability and metabolite profile. As a consequence, this lead to shortened cultivation durations, impacted glycan profile as well as decreased filter capacities within these cultures. The increase in cellular diameter further challenged the common cell specific productivity parameter leading to a switch within this work towards the cell-volume productivity which showed better predictability for both process modes. Overall, our data suggest easy, straight forward transferability of clones with a stable cell diameter, while process adjustments, such as switching to perfusion VCV target, could improve the transferability of other clones with changing cell diameters. Furthermore, due to the complexity of continuous processes our data highlights the unmet need for implementing filter-based small-scale screening platforms for the development of novel perfusion processes and respective clone selection. Overall, the obtained data in this work could ease the transition between the two process modes as well as increase the predictability of cell line performance within a given process set up. Further, within this work, a set of clones of similar origin were utilized to eliminate possible effects resulting from differences in parental cell line or mAB product. To further enhance insight into this interesting topic different parental cell lines, mAB constructs and vectors systems could be investigated to show possible differences triggered by these factors. Furthermore, the interesting change of metabolic flux within cell lines with different diameter, or the increase of cell volume within one culture could be investigated further to show possible triggers for this.

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CRediT authorship contribution statement

Dirk E. Martens: Writing – review & editing, Supervision, Methodology. **Rene H. Wijffels:** Writing – review & editing. **Julia Niemann:** Writing – review & editing, Supervision, Methodology. **Lucas Reger:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lucas Nik Reger reports a relationship with Sartorius Stedim Biotech GmbH that includes: employment. Julia Niemann reports a relationship with Sartorius Stedim Biotech GmbH that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2024.109359.

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