doi:10.2533/chimia.2025.330 Chimia 79 (2025) 330–338

Perfusion-Based Antibody Production in the Ambr® 250 Modular

Vivian Ott*, Jan Ott, Andry D. Mannone, and Regine Eibl

Abstract: The perfusion mode has become increasingly important in biopharmaceutical production in recent years. A bioreactor system used in many laboratories for the development of monoclonal antibodies (mAbs) production processes is the Sartorius' Ambr® 250 system. Vessels designed for perfusion mode are only available for its high throughput version, while the modular version of the Ambr 250 is not designed for perfusion mode. In this study, perfusion processes for the production of a mAb with Chinese Hamster Ovary (CHO) cells were realized in the Ambr 250 Modular in combination with Repligen's ATF 1 single-use device for the first time, to the authors' knowledge. After testing a semi-perfusion setup in well plates and the Ambr 250, an N–1 perfusion process was developed to produce ultra-high cell densities of more than 150 × 10^6 cells mL^{-1} for the inoculation of subsequent mAb production processes. In a second step, continuous mAb production was successfully realized over 23 days in a proof-of-concept experiment, achieving a volumetric productivity of 0.65 g L^{-1} d^{-1}. The results of the N–1 and continuous perfusion processes were comparable to a 3 L HyPerforma™ Glass bioreactor (Thermo Scientific) with an ATF 2 (Repligen).

Keywords: ATF · CHO cells · Continuous perfusion · N–1 perfusion · Scale-down model

Vivian Ott completed her Master of Science in Biotechnology at the Anhalt University of Applied Sciences in Germany. As a research assistant, she works in the Section for Cell Cultivation Techniques at the ZHAW Zurich University of Applied Sciences in Wädenswil, where she is primarily involved in the development, scale-up, and process intensification of antibody production processes.

Dr.-Ing. Jan Ott studied biotechnology at Anhalt University of Applied Sciences in Germany. He then moved to the Section for Cell Cultivation Techniques at the ZHAW Zurich University of Applied Sciences and worked mainly on the development of intensified antibody production processes. He completed his doctorate in this field in 2024 in cooperation with the Technische Universität Berlin.

Andry D. Mannone completed his BSc and MSc degrees at the ZHAW Zurich University of Applied Sciences. During his Bachelor's and Master's theses, he had the opportunity to carry out research in the group for Cell Cultivation Techniques under the guidance of Prof. R. Eibl. As a research assistant, he now continues to work in this group, where he is primarily engaged in the development, optimization, and scale-up of mammalian cell cultivation processes.

Prof. Dr. Ing. Regine Eibl is a Professor at the ZHAW Zurich University of Applied Sciences and heads the Center for Cell Cultivation Techniques, Tissue Engineering and Medical Biology. For more than 30 years, she has been working on the development and scale-up of processes based on plant cells, mammalian cells and stem cells. She is also a pioneer in the field of single-use technologies.

1. Introduction

The perfusion mode for the production of biopharmaceuticals was already being used in the 1990s to produce unstable or toxic products that should have a residence time in the bioreactor that is as short as possible.[1] For stable monoclonal antibodies (mAbs), on the other hand, fed-batch operation in large stainless steel bioreactors in the double-digit cubic meter range remained the standard for a long period of time.[2–4] After the FDA lowered the regulatory barriers for continuous processes in 2002 with the 'Pharmaceutical cGMP Initiative for the 21st Century – a Risk Based Approach',[5,6] the perfusion mode also came back into focus for mAb production processes. Supported by the increasing implementation of single-use bioreactors and the associated trend towards smaller production facilities, so-called process intensification has become the focus of biopharmaceutical manufacturers in recent years, often achieved through the use of perfusion processes.[7–10] The perfusion mode can be used for N–1 processes, the last inoculum production step before inoculating the N production bioreactor, to expand the cells during inoculum production up to cell densities of 10^8 cells mL^{-1} or more,[11–15] as well as for continuous production processes. While N–1 perfusion merges several inoculum production steps in one bioreactor and enables high-seed production processes, the space-time yield of a production facility can be maximized in the continuous perfusion mode, whereby volumetric productivities above 1 g L^{-1} d^{-1} have

Correspondence: V. Ott, E-mail: vivian.ott@zhaw.ch
ZHAW Zurich University of Applied Sciences, School of Life Sciences and Facility Management, CH-8820 Wädenswil, Switzerland.

already been described for mAb production processes with Chinese Hamster Ovary (CHO) cells.[16–19]

Perfusion processes are mainly realized by filtration-based cell retention. Hollow fiber modules used either in tangential flow filtration (TFF) or alternating tangential flow filtration (ATF) have become established in this context.[10,20,21] Repligen's ATF systems (Waltham, MA, USA) are widely used and are available in various sizes, starting at 0.5 L recommended minimum working volume for the smallest system.[22] However, if the aim is to realize perfusion processes on an even smaller scale, the possibilities are limited. Therefore, for the development or scale-down of perfusion processes, semi-perfusion processes, in which part of the working volume is exchanged once or several times a day, are often used rather than real continuous processes. For example, shaken tubes or flasks,[23–30] shaken well plates,[19,27,28,31,32] or automated microbioreactors[25,33–36] can be used for this purpose. Here, however, cell retention is achieved by sedimentation or centrifugation, which means that the conditions for the cells, which are actually consistently good in perfusion mode, cannot be maintained. For real perfusion, highly automated parallelized bioreactor systems can be used. While such systems for batch and fed-batch processes have already enabled high throughput for process development and scale-down experiments for several years, the Ambr 250 High Throughput Perfusion (Sartorius, Göttingen, Germany), which is available with TFF and ATF devices, has also made it possible to implement perfusion processes since 2018.[37,38] With a working volume of only 2 mL, the Mobius® Breez bioreactor, distributed by Merck (Darmstadt, Germany), has also recently become available. In this microbioreactor, successful perfusion cultivations have already been carried out.[39,40]

The Ambr 250 system has meanwhile become an important tool in process development and, according to Zoro and McHugh, was already being used by over 80% of the biopharma industry in 2019.[41] The ATF version of the perfusion vessel was successfully used by Joe *et al*. for Human Embryonic Kidney (HEK) cultivations, but only for a few days and at cell densities $<2 \times 10^7$ cells mL^{-1}.[42] Klimpel *et al.* recently published HEK cell perfusion experiments with acoustic cell retention in the Ambr 250 High Throughput.[43] So far, perfusion experiments using the Ambr 250 with CHO cells and at higher cell densities have not yet been published, to the authors' knowledge, despite the widespread use of the Ambr 250 and despite the fact that CHO cells are the most commonly used mammalian cell line for biopharmaceutical production. Furthermore, the perfusion vessels are only available for the high throughput version, not for the more basic and less expensive modular version of the Ambr 250. Researchers in laboratories that have chosen this version must therefore be creative in order to realize perfusion processes. For example, the previously mentioned perfusion process using acoustic cell retention published by Klimpel *et al*. could also be used for the modular Ambr version.[43] The lentivirus production experiments were successfully transferred to 4.5 L bioreactors.[43] Such scale transfers of perfusion processes are often challenging due to the cell retention systems. For Repligen's ATF systems, our group has already demonstrated good transferability from 2 L to 50 L bioreactors with CHO high-cell density cultivations over 50 days.[16]

In this study, the smallest available ATF system from Repligen, the ATF 1 single-use device, which is recommended by the manufacturer from 500 mL working volume,[22] was connected to the Ambr 250 Modular in order to realize ATF-based perfusion processes with 270 mL working volume. Since the CHO cells in the ultra-high cell density range require large amounts of oxygen, cultivations were carried out both in the mammalian version and in the microbial version of the Ambr 250 vessel. To evaluate the scalability of the process, experiments were also carried out in a 2 L bioreactor with the ATF 2 single-use device. In addition, semi-perfusion experiments were realized in well plates and the Ambr 250 Modular system to explore the possibilities and limitations of this process mode with the combination of cell line and medium used.

2. Material and Methods

2.1 *Cell Line and Medium*

Cultivations were performed with rituximab-producing ExpiCHO-S™ cells (Gibco™, Waltham, MA, USA). High-Intensity Perfusion CHO Medium (Gibco) was used as the cultivation medium, according to the manufacturer's instructions in 0.66x concentration as the basal medium for the perfusion cultivations, the batch experiments and the inoculum production, and 1x concentrated as the feed medium for the perfusion cultivations. The medium was supplemented with 4 mmol L^{-1} L-glutamine (Gibco) and 0.1% Anti-Clumping Agent (Gibco) for both concentrations.

2.2 *Standard Inoculum Production*

Cryopreserved cells were thawed one week before the start of the experiment and transferred to a shake flask (Corning, Corning, NY, USA) with pre-warmed medium. The cells were passaged every second to third day. To maintain selection pressure, 400 nmol L^{-1} methotrexate (Sigma Aldrich, St. Louis, MO, USA) was added to the cell suspension in the first passage. The shake flasks were incubated in a shaking incubator (Adolf Kühner AG, Birsfelden, Switzerland) at a shaking speed of 120 min^{-1}, 25 mm shaking diameter, 8% CO_2, 37 °C and 80% relative humidity.

2.3 *Semi-Perfusion Experiments*

The semi-perfusion experiments were performed either in 48 round well microtiter plates (Beckman Coulter, Brea, CA, USA) in the BioLector (Beckman Coulter) with 1.2 mL or in the Ambr 250 Modular (mammalian vessel, Sartorius, Göttingen, Germany) with 200 mL working volume. The experiments were started with 1×10^6 cells mL^{-1}. After a two-day batch phase, depending on the viable cell density (VCD), part or all of the medium was exchanged one or two times per day. The cell-specific perfusion rate (CSPR) was kept over 55 pL cell^{-1} d^{-1}, but was limited to a maximum of two complete media exchanges per day. Before the media exchange, a sample was taken, and the cell-specific and metabolic parameters were determined according to section 2.6. In the case of the BioLector, three wells were harvested and analyzed at each sampling point. The 48-well plate and the Ambr 250 cell suspension, transferred to 50 mL tubes, were centrifuged at 500 g for 3 min, the calculated volume of supernatant was discarded and the required volume of fresh feed medium, supplemented with a 450 g L^{-1} glucose solution, if necessary, was added to ensure a minimum glucose concentration of 2 g L^{-1} until the next sampling time point. The 48-well plate was incubated in the BioLector at 37 °C, shaking speed of 1,100 min^{-1} and 5–8% CO_2. The process parameters in the Ambr 250 were 37 °C, a stirrer speed of 620 min^{-1}, and 20 mL min^{-1} headspace gassing with process air. Furthermore, the pH was controlled to ≤7.2 with CO_2 and dissolved oxygen (DO) to ≥40% with O_2 addition through the sparger.

2.4 *N−1 and Continuous Perfusion Experiments*

The bioreactors used for the perfusion experiments were the Ambr 250 Modular and the 3 L HyPerforma Glass bioreactor (Thermo Scientific, Waltham, MA, USA). As single-use vessels for the Ambr 250 Modular, the mammalian version with two 30° segment impellers was used for two N−1 perfusion cultivations, and the microbial version with two Rushton impellers was used for a further N−1 perfusion cultivation and a continuous perfusion.

The microbial version was used to check whether the CHO cells might grow better in these vessels at ultra-high cell densities, as the microbial vessels are designed for the high oxygen demand of bacteria. The HyPerforma Glass bioreactor was operated with a G3Lab control unit (Thermo Scientific). ATF 1 and ATF 2 single-use devices were used as cell retention systems for the perfusion cultivations, operated with an XCell® Lab controller (Repligen). The ATF rate selected for the ATF 1 was 0.14 L min^{-1} (shear rate 1,981 s^{-1}) and for the ATF 2 0.9 L min^{-1} (shear rate 1,981 s^{-1}). The Ambr 250 silicone lid was adapted as shown in Fig. 1b. A dip tube was inserted into the silicone lid for the connection to the ATF 1. For the N−1 perfusion processes, a level probe was inserted to control the level (level control realized with a G3Lab control unit) and a bleed tube for continuous perfusion. These were installed in the silicone lid in such a way that they were attached at a filling volume of 260 mL. This represents the maximum filling level during the perfusion process, as the volume in the bioreactor fluctuated between 240 and 260 mL due to the ATF 1. Taking into account the dead volume of the ATF 1 and the connection to the bioreactor, the total volume of cell suspension during the process was 270 mL. The level probe and the dip tube were fixed to the inoculation port with FDA approved and autoclavable silicone adhesive (DOWSIL™ 734 Clear, DOW, Midland, MI, USA). Instead of using the standard ATF-to-bioreactor connector that is already attached on the ATF 1, a piece of 1/8” × 1/4” C-Flex® tubing (Saint-Gobain, Courbevoie, France) was connected directly to the bioreactor dip tube on the one side and to a conical sanitary flange with a hose barb (inner diameter 4 mm) on the ATF side, attached directly on the hollow fiber module (Fig. 1a). This setup ensured that no flow-limiting and shear-intensive components with an inner diameter of less than 3 mm were installed between the ATF and the bioreactor. The silicone lid adapted for perfusion processes was autoclaved for 20 min at 2 bar and 121 °C.

The cultivations were started with a VCD of 1 × 10^6 cells mL^{-1} in batch mode. On day two of the perfusion cultivations, the mode was switched to perfusion mode. The perfusion rate was adjusted twice daily for the N−1 perfusion processes and once a day for continuous perfusion. In both processes, the perfusion rate was increased with the VCD to ensure a CSPR of at least 55 pL cell^{-1} d^{-1} until the maximum perfusion rate of 7 volumes of medium per cultivation volume and day (vvd) for the N−1 perfusions or 2 vvd for the continuous perfusion was reached. For continuous perfusion, a cell-specific perfusion rate of 28 µL mm^{-3} d^{-1} was targeted after the maximum perfusion rate of 2 vvd was reached, in line with the results of a perfusion process published earlier.[44] Additionally to the perfusion medium, a 450 g L^{-1} glucose solution was added to ensure a glucose concentration of approximately 2 g L^{-1}.

In the continuous perfusion processes in the 3 L HyPerforma Glass bioreactor, the cell bleed was automated using a permittivity probe (Incyte® ARC®, Hamilton Bonaduz, Bonaduz, Switzerland), whereas in the Ambr 250 the bleed was set manually based on the current specific growth rate. The most important cultivation parameters can be found in Table 1.

2.5 *Batch Experiments*

For the batch experiments, which were inoculated from the N−1 perfusion bioreactors on different days, a 50 mL sampling tube (OmniTop, VWR, Radnor, PA, USA) was welded to the respective bioreactor and 3–5 mL of cell suspension was removed from the bioreactor. The cell suspension was immediately diluted to <10 × 10^6 cells mL^{-1} in a prepared and already incubated 125 mL shake flask (Corning) with medium, and the cell-specific parameters were determined (see Section 2.6). Subsequently, the batch experiments were inoculated with a working volume of 40 mL and a VCD of 1 × 10^6 cells mL^{-1} in 125 mL shake flasks. The experiments were analyzed daily according to section 2.6 and terminated after reaching a viability of <50%. All shake flasks were incubated at a shaking speed of 120 min^{-1}, 25 mm shaking diameter, 8% CO_2, 37 °C and 80% relative humidity in a shaking incubator (Adolf Kühner AG).

2.6 *Analytics*

During cultivation, the samples were analyzed with the Cedex® HiRes (Roche Diagnostics, Basel, Switzerland) for cell-specific parameters and with the Cedex Bio (Roche) for substrates, metabolites, and the product immunoglobulin G (IgG). Cell-specific parameters included VCD, viability, and cell diameter. For the analysis with the Cedex Bio, the cell suspension was centrifuged at 3,000 g for 2 min and the concentrations of glucose, glutamine,

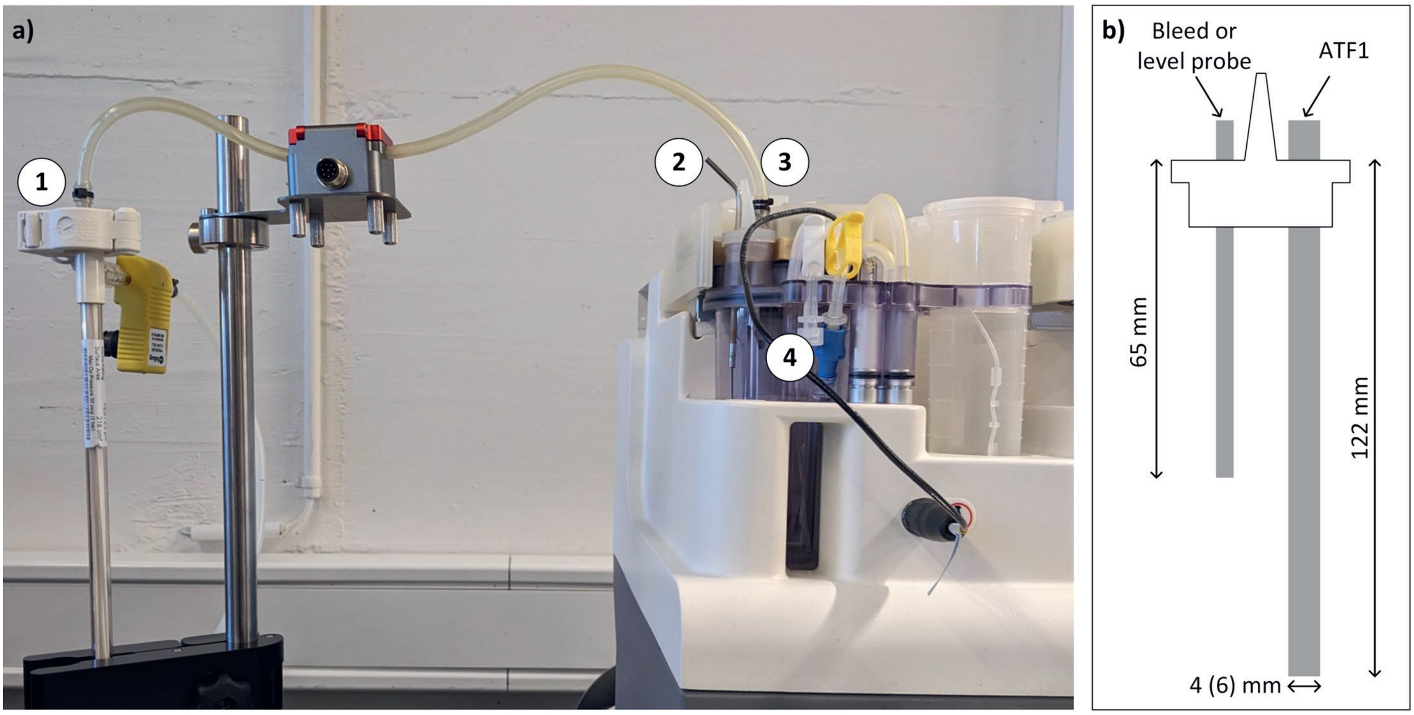

Fig. 1. a) Connection of the ATF 1 to the Ambr 250 Modular single-use vessel; b) schematic representation of the adapted silicone lid for perfusion processes. 1 = ATF 1 with sanitary flange, 2 = level probe or bleed tube; 3 = ATF dip tube in the silicone lid; 4 = port used for feed connection.

Table 1. Cultivation parameters for N–1 and continuous perfusion cultivations.

Parameter	Ambr 250 mammalian	Ambr 250 microbial	3 L HyPerforma Glass
Process mode	N–1	N–1 continuous perfusion	N–1 continuous perfusion
Volume	270 mL	270 mL	2 L
Temperature	37 °C	37 °C	37 °C
Cell retention	XCell ATF 1 single-use	XCell ATF 1 single-use	XCell ATF 2 single-use
Stirrer type	2× pitched blade (3 blades)	2× Rushton impeller	2× pitched blade (3 blades)
Stirrer speed	620–1,400 min^{-1} (N–1)	600–1,200 min^{-1} (N–1) 300–970 min^{-1} (continuous perfusion)	300–435 min^{-1} (N–1) 300–350 min^{-1} (continuous perfusion)
Dissolved oxygen (DO)	≥40% 1. addition of O_2 *via* open pipe sparger, max 82 mL min^{-1} 2. increase stirrer speed	≥40% 1. addition of O_2 *via* open pipe sparger, max 82 mL min^{-1} 2. increase stirrer speed	≥40% 1. addition of O_2 *via* ring sparger, max 1,050 mL min^{-1} 2. manual adjustment of stirrer speed
pH	≤7.2 (CO_2 addition *via* open pipe sparger)	≤7.2 (CO_2 addition *via* open pipe sparger)	≤7.2 (CO_2 addition *via* ring sparger)
Feed	addition when the liquid level drops below the setpoint (pump of the G3Lab control unit)	N–1: addition when the liquid level drops below the set point (pump of the G3Lab control unit); continuous perfusion: fixed flow rate (external pump)	fixed flow rate
Harvest	fixed flow rate (external pump)	fixed flow rate (external pump), daily adjustment according to specific growth rate to adjust the bleed	pump is automatically controlled through the bioreactor weight to ensure a working volume of 2 L
Bleed	-	continuous removal with high flow rate to keep the bioreactor level constant (external pump)	pump is automatically controlled by the permittivity probe to keep a viable cell volume (VCV) of 71 mm^3 mL^{-1} constant

lactate, ammonium, and IgG as well as the lactate dehydrogenase activity were determined in the supernatant. For batch experiments, only glucose and IgG were determined.

3. Results and Discussion

3.1 *Semi-Perfusion Experiments*

In the first part of the study, semi-perfusion experiments were carried out, in which part or all of the medium was exchanged twice a day. The aim of these investigations was to explore the possibilities and limits of the semi-perfusion mode for the cell line-medium combination used. After starting with a VCD of 1 × 10^6 cells mL^{-1} in the BioLector and Ambr 250, 39.0±2.3 × 10^6 cells mL^{-1} were achieved in the BioLector and 44.1±0.9 × 10^6 cells mL^{-1} in the Ambr 250 after 5.8 d (Fig. 2a). Until then, specific growth rates decreased more or less continuously from approximately 0.04 h^{-1} on the first day in both systems until almost no growth occurred after the first 5.8 d (Fig. 2b). Between days 5.8 and 7.8 the VCD in the Ambr 250 still increased slowly but steadily up to a maximum value of 50.5±0.7 × 10^6 cells mL^{-1}. In the BioLector, the cell density stagnated, the maximum measured value was 40.2±4.3 × 10^6 cells mL^{-1} on day 7.8.

The rapidly decreasing growth rate shows that the semi-perfusion mode can only be used to a limited extent as a scale-down model for the cell line-media combination used. In a perfusion process, the conditions would ideally be so good that the cells grow exponentially at a high growth rate throughout the process. It can be assumed that the cells in both the Ambr 250 and the BioLector were provided with good conditions by the control units. The critical conditions for growth, on the other hand, were the media exchanges, during which the cell suspension was centrifuged, then resuspended with fresh medium, and finally the cultivation conditions had to be reached again in the cultivation vessels. During this time, the cells are oxygen-limited and the temperature drops; consequently, a subsequent lag phase is unavoidable. On the other hand, the composition of the medium may also play a role in stagnating growth at around 40–50 × 10^6 cells mL^{-1}. In order to cover the glucose requirement of the cells until the next medium exchange, the perfusion medium had to be supplemented by up to 4 g L^{-1} glucose on the last few days. This meant not only a concentration of 10 g L^{-1} glucose in the medium, but also an increase in osmolality to approximately 400 mOsm kg^{-1}. If the osmolality is too high, this can have a growth-inhibiting effect.[45,46] The growth curves indicate that both the BioLector and the Ambr 250 can be used as a scale-down model for continuous perfusion processes, at least up to a VCD of 30 × 10^6 cells mL^{-1}. With more frequent media exchanges, it could be possible to reach higher cell densities, if the cells can handle the stress from the manual media exchanges. Experiments have been described in the literature in which comparable results were achieved for semi-perfusion processes. For example, Jin *et al*. were able to realize a semi-continuous process at 30 × 10^6 cells mL^{-1} in Ambr 15.[34] Dorn *et al*. achieved the same in 24-well plates with up to 40 × 10^6 cells mL^{-1}.[27] If growth-limiting media changes need to be avoided and cell densities >10^8 cells mL^{-1} are aimed for, however, a continuous supply of fresh medium and removal of used medium must be implemented.

3.2 *N-1 Perfusion and Cell Quality*

To enable real perfusion in the Ambr 250 Modular, Repligen's ATF 1 was connected to an Ambr vessel. To investigate the possibilities and limitations of this setup, N–1 perfusion cultivations were performed in the first step, two cultivations in the mammalian version and one cultivation in the microbial vessel. As the experiments ran similarly in both vessel versions, the three experiments were grouped together for data evaluation. Three cultivations in a 3 L HyPerforma Glass bioreactor with ATF 2 were used as references. Cultivations in Ambr 250 were started with a VCD of 1.07±0.09 × 10^6 cells mL^{-1} and with 1.06±0.07 × 10^6 cells mL^{-1} in the HyPerforma Glass (Fig. 3a). The maximum VCD was reached in the HyPerforma Glass on day 7.2 with 173±26 × 10^6 cells mL^{-1}, growth in Ambr 250 was slightly slower, so a comparable cell number of 167±16 × 10^6 cells mL^{-1} was reached 0.7 d delayed (day 7.9). Viability remained very high at >97% in both systems over the entire cultivation period.

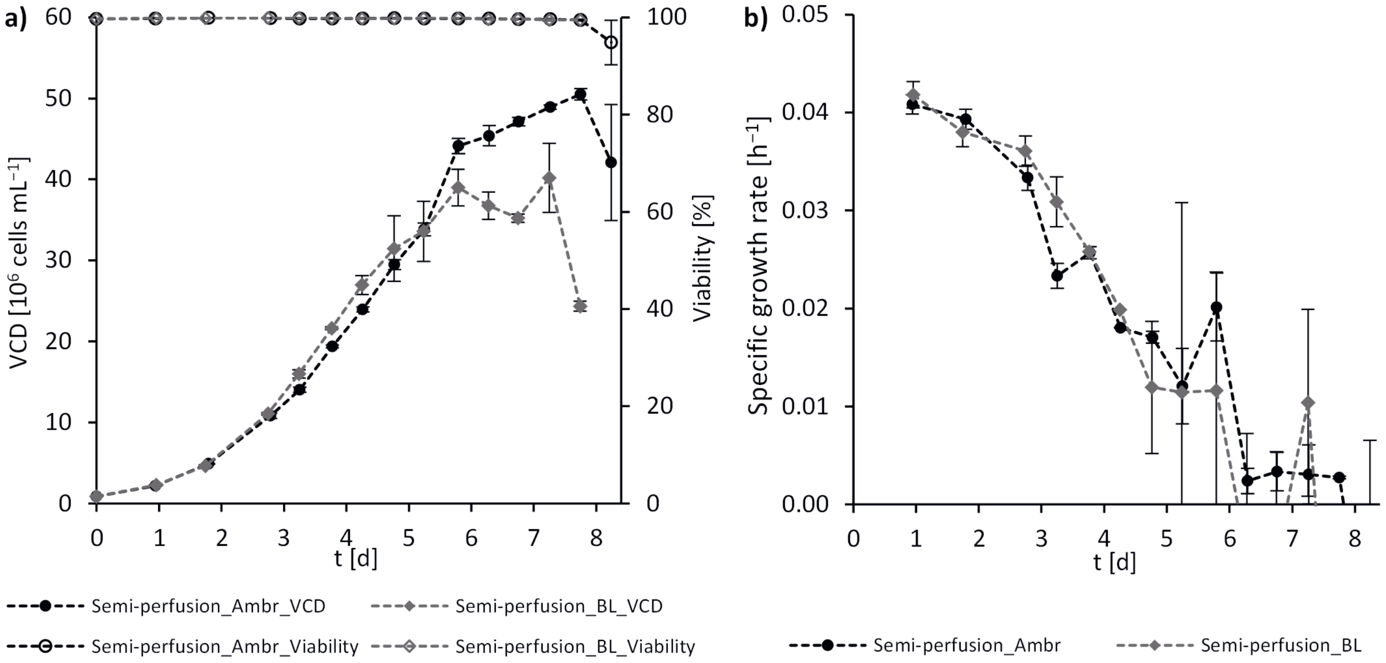

Fig. 2. Course of a) VCD and viability and b) the specific growth rate during the semi-perfusion processes. Ambr = Ambr 250 (N=2) und BL = BioLector (N=3). Error bars represent the data range (N=2) or standard deviation (N=3).

The arithmetic mean specific growth rates over the period in which the perfusion rate was not limited were 0.026±0.008 h^{-1} in the Ambr 250 experiments and 0.029±0.006 h^{-1} in the HyPerforma Glass experiments (Fig. 3b). These specific growth rates are comparable to the value of 0.026–0.030 h^{-1} previously published by our group for a similar perfusion process with the same cell line and the same medium in a 2 L wave-mixed bioreactor.[15] While high specific growth rates of >0.03 h^{-1} were achieved at the beginning of the cultivation, growth rates decreased continuously with increasing VCD. The same effect also occurred in the wave-mixed bioreactors.[15]

The fact that growth is slightly delayed in the Ambr 250 compared to the HyPerforma Glass may potentially indicate excessive shear stress. Looking at the online data for the DO control of an Ambr 250 perfusion process, for example, it can be seen that due to the low k_La values present in Ambr 250, the maximum oxygen gassing rate of 82 mL min^{-1} was already reached after 4.2 d (Fig. 4). The stirrer speed was then increased to further cover the

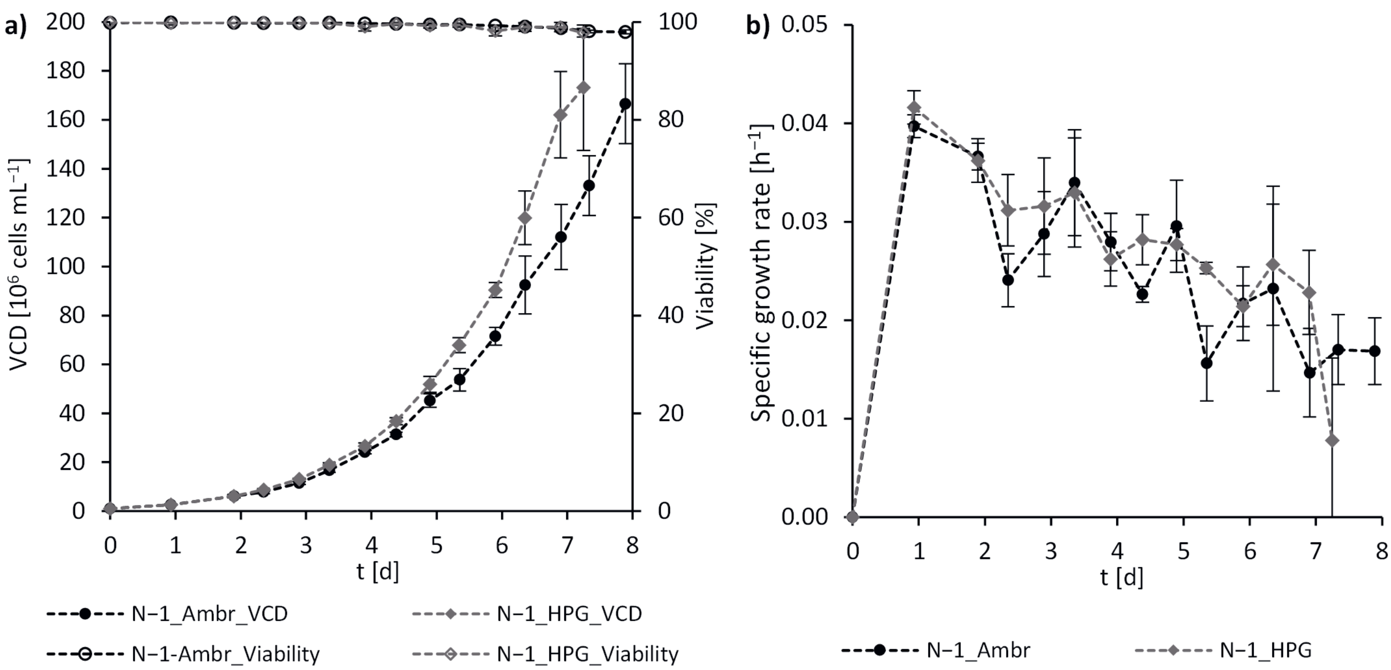

Fig. 3. Course of a) VCD and viability and b) the specific growth rate during the N−1 perfusion processes (N=3). Ambr = Ambr 250 und HPG = HyPerforma Glass bioreactor. Error bars represent the standard deviation.

cells' oxygen requirements. It increased from 620 min^{-1} to up to 1,400 min^{-1} from this point onwards; the jump in speed from 770 to 930 min^{-1} on day 5.4 can be explained by the manual addition of antifoam. As the speed increases, so does the shear stress for the cells. For the mammalian vessel of the Ambr 250, Šrom *et al.* experimentally determined critical threshold values for shear stress for two different CHO cell lines, below which no significant influence on cell behavior was observed; these were on average around 820 min^{-1} for one cell line and 1,040 min^{-1} for the other.[47] In the perfusion process shown in Fig. 4, these speeds were exceeded after 5.4 d and 5.9 d, for example. The fact that these thresholds for two very similar CHO K1 cell lines are already more than 20% apart shows that the shear sensitivity of different CHO cell lines can differ significantly.[47] For the cultivations shown in this study, it is difficult to identify a critical threshold based on the available data. For example, the growth rate decreased linearly during the Ambr 250 experiments; no abrupt drop was observed. Analogous to the investigations by Šrom *et al.,* further investigations at different speeds in batch or fed-batch mode are recommended here.

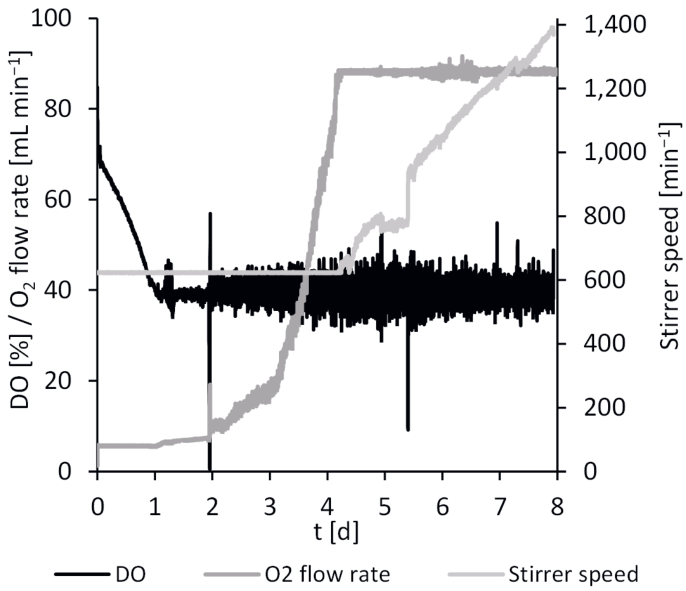

Fig. 4. Course of the DO, the O_2 sparger flow rate and the stirrer speed of an N−1 perfusion cultivation in an Ambr 250 mammalian vessel.

Since the cells of an N−1 perfusion basically serve as an inoculum for the N-bioreactor, it must be ensured that the cells are still of high quality in terms of growth and product formation after the N−1 perfusion process. The cell quality was therefore examined at regular intervals by removing cells from the N−1 bioreactors and inoculating batch experiments in shake flasks with these cells. From the batch experiments, the maximum VCD, the maximum IgG titer and the specific growth rate within the first 24 h were used to determine the cell quality. As can be seen from Fig. 5c, the specific growth rates on the first day of batch cultivation are below the arithmetic mean of the specific growth rates of 0.038±0.002 h^{-1} for cells that have undergone a standard inoculum production procedure in a shake flask. It should be noted that the cells harvested from the perfusion processes at higher VCDs had lower specific growth rates on the first day of the subsequent batch cultivations, so the majority of the first-day growth rates from the experiments inoculated from cell densities >80 × 10^6 cells mL^{-1} were below the tolerance interval of 0.035 to 0.042 h^{-1} of the reference batches (90% probability, 90% confidence, according to Howe[48] and Günther[49]). There are hardly any differences between the two-cultivation systems Ambr 250 and HyPerforma Glass. After the first cultivation day, there were no further differences in the specific growth rate between the standard inoculum production method (0.039±0.001 h^{-1}, day 2) and the cells taken from the N−1 perfusion. Across all harvest time points from the perfusion, the specific growth rate on day 2 was 0.039±0.001 h^{-1} in the Ambr 250 and HyPerforma Glass batches. It can therefore be concluded from these results that the cells removed from the N−1 perfusion bioreactor at a later time, *i.e.* at a higher cell density, have a longer lag phase in the IgG production process, but that the maximum specific growth rate is subsequently comparable. However, the slightly lower growth rate of the cells on day 1 only prolonged the batch cultivations by a few hours, consequently the influence on the final production process is negligible. The maximum VCD achieved in standard inoculum production was 11.6 × 10^6 cells mL^{-1} on average (Fig. 5a). The maximum VCDs achieved in the batch cultivations varied between 10.2 and 12.1 × 10^6 cells mL^{-1}. Up to a cell density of 150 × 10^6 cells mL^{-1}, the maximum VCDs were similar to the reference, above a cell density of 150 × 10^6 cells mL^{-1}, three out of five maximum VCDs were below the tolerance interval, indicating that the cell quality is decreasing at values higher than 150 × 10^6 cells mL^{-1} in the perfusion process. The IgG titers achieved in almost all batch experiments were slightly above the mean value of the batch experiments with standard inoculum production (212 mg L^{-1}, Fig. 5b). However, the IgG titers of 211 to 229 mg L^{-1} for the batch cultivations carried out were within the tolerance interval of the reference cultivations between 183 and 242 mg L^{-1}, irrespective of the N−1 perfusion system used and the VCD at which they were removed from the perfusion process. These data are consistent with previously published experiments in N−1 perfusion wave-mixed bioreactors.[15]

3.3 *Antibody Production in Continuous Perfusion Mode – Proof-of-Concept*

In addition to the N−1 perfusion experiments, a proof-of-concept cultivation was performed in continuous perfusion mode. The cultivation in the Ambr 250 (microbial version) was carried out over a period of 23 d, whereas in the HyPerforma Glass bioreactor the cultivation had to be terminated after 21 d due to a pump problem (Fig. 6). Based on the results of the previously published continuous perfusion with the same cell line and the same medium in the 2 L bioreactor,[44] a target VCV of 71 mm^3 mL^{-1} was aimed for at a perfusion rate of 2 vvd. After reaching the steady-state, the average VCV in the Ambr 250 was 67.7±9.6 mm^3 mL^{-1} and 74.1±5.5 mm^3 mL^{-1} in the HyPerforma Glass process (Fig. 6a). Growth in the Ambr 250 was lower than in the 2 L bioreactor during the steady state, the bleed rate in the Ambr 250 was 0.33±0.15 d^{-1} compared to 0.41±0.12 d^{-1} in the 2 L bioreactor. While the VCV in the HyPerforma Glass experiment was subject to fewer fluctuations due to the use of a permittivity probe and the associated automated regulation of the bleed, the VCV in the Ambr 250 had stronger fluctuations, as the pump rate of the bleed was adjusted manually each day, meaning that slight changes in the specific growth rate or deviations in feeding had a direct influence on the VCV. However, similar VCV-specific IgG production rates of 13.1±1.0 µg mm^{-3} d^{-1} (HyPerforma Glass) and 13.3±2.4 µg mm^{-3} d^{-1} (Ambr 250) were achieved in both bioreactors (Fig. 6b). The same applies to volumetric productivity. An arithmetic mean value of 0.65±0.11 g L^{-1} d^{-1} IgG in the Ambr 250 and 0.68±0.07 g L^{-1} d^{-1} IgG in the HyPerforma Glass was harvested from day 6 onwards (Fig. 6c). The increase in the volumetric productivity in the Ambr cultivation between day 13 and day 16 of the cultivation is the result of the increase in VCV at the same time. The volumetric productivities are comparable to those previously published with this cell line and medium, where the highest productivity was 0.73±0.05

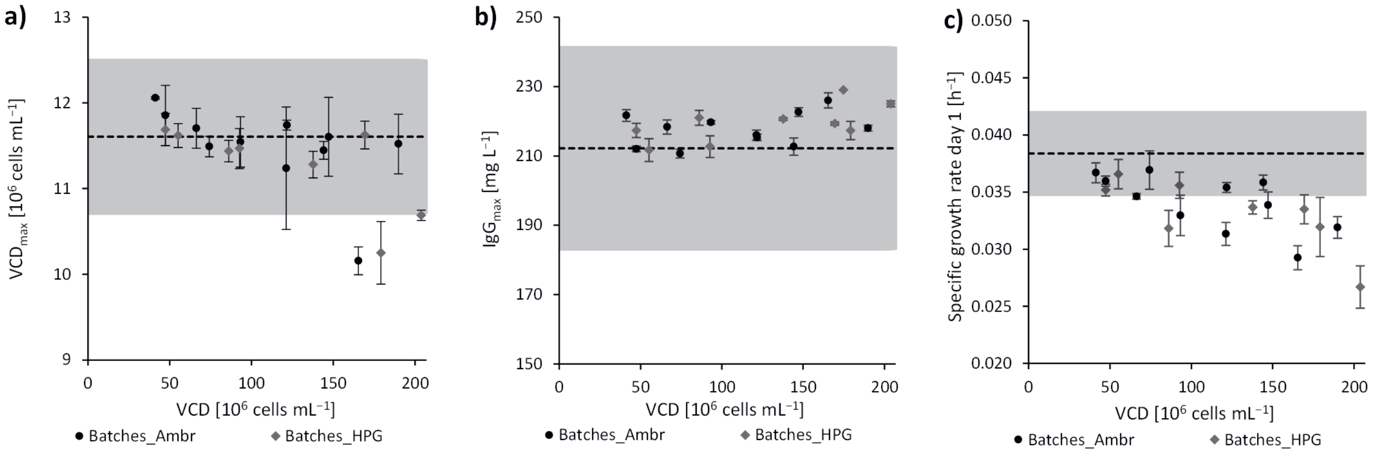

Fig. 5. a) Maximum VCD; b) maximum IgG titer; and c) specific growth rate on the first day of the batch experiments as a function of the VCD in the N–1 perfusion process at the time of harvest (N=3). Ambr = Ambr 250, HPG = HyPerforma Glass bioreactor, black line = arithmetic mean value in the reference batches with standard inoculum production (N=12), grey area = corresponding tolerance interval (p=90% and α=90%). Error bars represent the standard deviation.

g L^{-1} d^{-1}.[44] Viability was consistently high at ≥94% in both processes until day 18, then decreased to 82% in the Ambr 250 until day 22 and stabilized at 84% on day 23 (Fig. 6a). The drop in viability can probably be attributed to a problem with the foam control, although growth and product formation remained stable. Deviations between the two experiments were seen in IgG retention (Fig. 6d), which is determined from the ratio between the IgG concentration in the harvest and the IgG concentration in the bioreactor. From the 5th day of cultivation, IgG retention in the Ambr 250 increased more than in the HyPerforma Glass, and this continued until the end of cultivation. Thus, the IgG retention on day 21 of the cultivation in the HyPerforma Glass (last cultivation day) was only about half as high (30.4%) as in the Ambr 250 (60.5%), although the specific filter area of the hollow fiber module in relation to the working volume was about 25% higher in the Ambr 250 process than in the 2 L process. The higher retention would have to be investigated in more detail in further cultivations. It is possible that the shear stress in the Ambr 250 is significantly higher than in the 2 L bioreactor, resulting in smaller cell debris and higher concentrations of host cell protein (HCP) and DNA being released, which leads to greater fouling. The stirrer speed during the steady state was 811±78 min^{-1}, which may already mean a problematic shear stress for the cells. The critical threshold values for the cell line-media combination would have to be investigated in more detail in further experiments. In addition, the shear exposure in the mammalian and microbial vessel can be investigated in more detail in further experiments.

4. Conclusions and Outlook

In the first part of this study, semi-perfusion experiments were performed in BioLector 48-well plates and in the Ambr 250 Modular. Approximately 40 × 10^6 cells mL^{-1} could be reached within 5.8 d before growth decreased abruptly, which was probably due either to the stress on the cells caused by the manual media changes or to the high osmolality in the glucose-enriched medium. Such issues can be circumvented by real perfusion processes, so in the second part of this study the Ambr 250 Modular bioreactor was successfully adapted with an ATF 1 single-use device to realize N−1 and continuous perfusion processes. In the N−1 perfusion mode in the Ambr 250, a maximum VCD of 167±16 × 10^6 cells mL^{-1} was achieved. No differences were found between the cultivation in mammalian or microbial vessels. Throughout the entire process, the quality of the cells regarding growth and IgG production remained comparable to the 2 L reference system in terms of growth-relevant parameters and IgG titer. Up to 150 × 10^6 cells mL^{-1}, the cells from the perfusion runs reached similar maximum VCDs in the subsequent batches compared to cells from standard inoculum production; at higher perfusion VCDs, the maximum VCDs decreased. It should be noted that the specific growth rate in the Ambr 250 was slightly lower than in the N−1 perfusion cultivations in the 3 L HyPerforma Glass bioreactor. One possible reason for this could be the high stirrer speeds required in the Ambr 250 to supply the cells with sufficient oxygen. The corresponding shear stress can possibly have an influence on the growth of the cells. At what point the shear stress becomes critical for the cell line-medium combination used in this study can be determined in further investigations. In the last part of the study, continuous perfusion over a period of 23 d was successfully realized in the Ambr 250. The average VCV was 68 mm^3 mL^{-1}. Both the VCV-specific IgG production rate (13.3±2.4 µg mm^{-3} d^{-1}) and the volumetric productivity (0.65±0.11 g L^{-1} d^{-1}) were comparable to the reference cultivation in the 3 L HyPerforma Glass bioreactor used in this study and the previously published process.[44] The main difference in the results of the two cultivation systems Ambr 250 and 3 L HyPerforma Glass bioreactor was the IgG retention by the ATF. This was about twice as high in the Ambr 250 as in the 3 L HyPerforma Glass bioreactor, despite the larger specific filter area. Higher IgG retention can be caused by increased fouling of the membrane. The HCP and DNA content, for example, have an influence on the fouling; these concentrations could be higher in the Ambr 250 than in the 3 L HyPerforma Glass bioreactor. Further investigations should be carried out here with regard to product retention and a possible connection to shear stress should be studied.

The modification of the modular Ambr 250 vessels into perfusion bioreactors using the ATF 1 demonstrated great potential. This setup allows perfusion processes to be realized in a small-volume, stirred and fully controllable bioreactor, simplifying process development and enabling scale-down experiments with up to eight parallel bioreactors.

Acknowledgements

We would like to thank Volkan Dönderici for his support in carrying out experiments as part of his Master's thesis. Additionally we would like to thank ZHAW's English Department Language Services team for the English correction of the manuscript. Furthermore we would like to thank Thermo Fisher Scientific's Gibco team for providing the ExpiCHO-S cells.

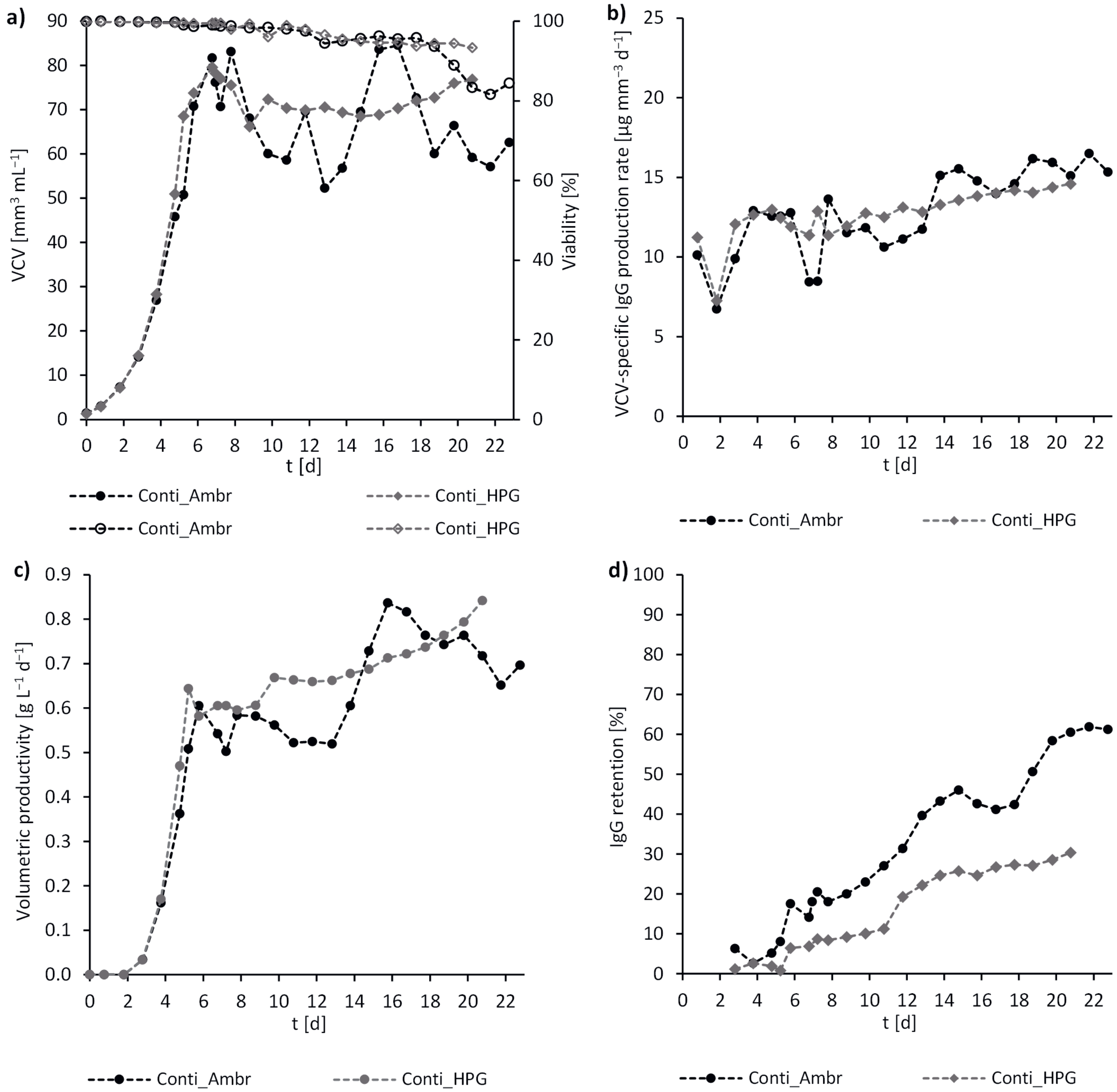

Fig. 6. Course of a) VCV and viability; b) the VCV-specific IgG production rate; c) the volumetric productivity, and d) the IgG retention through the ATF during the continuous perfusion experiments. Conti = continuous perfusion, Ambr = Ambr 250 and HPG = HyPerforma Glass bioreactor.

Author Contributions

Conceptualization, V. O., J. O., and R. E.; methodology, V. O. and J. O.; validation, V. O., J. O., and A. D. M.; formal analysis, V. O. and J. O.; investigation, V. O., J. O., and A. D. M.; resources, R. E.; writing—original draft preparation, V. O. and J. O.; writing—review and editing, V. O., J. O., A. D. M., and R. E.; visualization, V. O., J. O., and A. D. M.; supervision, R. E.; project administration, R. E.

Received: December 12, 2024

[1] S. K. Gupta, in 'Continuous Biomanufacturing - Innovative Technologies and Methods', Ed. G. Subramanian, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, **2017**, Ch. 8, https://doi.org/10.1002/9783527699902.ch8.
[2] H. Meyer, W. Minas, D. Schmidhalter, in 'Industrial Biotechnology', Eds. C. Wittmann, J. C. Liao, Wiley, Weinheim, Germany, **2017**, Ch. 1, https://doi.org/10.1002/9783527807833.ch1.
[3] M. Chartrain, L. Chu, *Curr. Pharm. Biotechnol*. **2008**, *9*, 447, https://doi.org/10.2174/138920108786786367.
[4] B. Kelley, R. Kiss, M. Laird, in 'New Bioprocessing Strategies: Development and Manufacturing of Recombinant Antibodies and Proteins', Eds. B. Kiss, U. Gottschalk, M. Pohlscheidt, Springer, Cham, **2018**, Ch. 14, https://doi.org/10.1007/10_2018_59.
[5] Pharmaceutical CGMPs for the 21st Century - A risk-based Approach. Report **2004**, https://www.fda.gov/files/drugs/published/Pharmaceutical-cGMPs-for-the-21st-Century---Final-Report-%28pdf%29.pdf, accessed May 13, 2024.
[6] Pharmaceutical Quality for the 21st Century - A Risk-Based Approach. Progress Report **2007**, https://www.fda.gov/about-fda/center-drug-evaluation-and-research-cder/pharmaceutical-quality-21st-century-risk-based-approach-progress-report, accessed May 13, 2024.
[7] J. Strube, R. Ditz, M. Kornecki, M. Huter, A. Schmidt, H. Thiess, S. Zobel-Roos, *Chem. Eng. Process.* **2018**, *133*, 278, https://doi.org/10.1016/J.CEP.2018.09.022.

[8] J. Schaub, A. Ankenbauer, T. Habicher, M. Löffler, N. Maguire, D. Monteil, S. Püngel, L. Stepper, F. Stiefel, J. Thoma, A. Unsöld, J. Walther, C. Wayne, T. Wucherpfennig, *Phys. Sci. Rev*. **2023**, *9*, 2989, https://doi.org/10.1515/PSR-2022-0113.

[9] D. Müller, L. Klein, J. Lemke, M. Schulze, T. Kruse, M. Saballus, J. Matuszczyk, M. Kampmann, G. Zijlstra, *Chem. Eng. Process.* **2022**, *171*, 108727, https://doi.org/10.1016/j.cep.2021.108727.

[10] J. Müller, M. Teale, S. Steiner, S. Junne, P. Neubauer, D. Eibl, R. Eibl, in 'Cell Culture Engineering and Technology', Ed. R. Pörtner, Springer, Cham, **2021**, Ch. 13, https://doi.org/10.1007/978-3-030-79871-0_13.

[11] J. Xu, M. S. Rehmann, M. Xu, S. Zheng, C. Hill, Q. He, M. C. Borys, Z. J. Li, *Bioresour. Bioprocess*. **2020**, *7*, 17, https://doi.org/10.1186/s40643-020-00304-y.

[12] B. Wright, M. Bruninghaus, M. Vrabel, J. Walther, N. Shah, S.-A. Bae, T. Johnson, J. Yin, W. Zhou, K. Konstantinov, *Bioprocess Int*. **2015**, *13*, 16.

[13] M. Schulze, J. Lemke, D. Pollard, R. H. Wijffels, J. Matuszczyk, D. E. Martens, *J. Biotechnol*. **2021**, *335*, 65, https://doi.org/10.1016/J.JBIOTEC.2021.06.011.

[14] J. Müller, V. Ott, N. Weiss, P. Neubauer, D. Eibl, R. Eibl, *Chem. Ing. Tech*. **2022**, *94*, 1977, https://doi.org/10.1002/CITE.202200097.

[15] J. Müller, V. Ott, D. Eibl, R. Eibl, *Processes* **2022**, *10*, 911, https://doi.org/10.3390/pr10050911.

[16] V. Ott, J. Ott, D. Eibl, R. Eibl, *Processes* **2024**, *12*, 806, https://doi.org/10.3390/pr12040806.

[17] H. Schwarz, M. E. Mäkinen, A. Castan, V. Chotteau, *Biochem. Eng. J*. **2022**, *182*, 108426, https://doi.org/10.1016/J.BEJ.2022.108426.

[18] P. Romann, J. Kolar, L. Chappuis, C. Herwig, T. K. Villiger, J. M. Bielser, *Biochem. Eng. J*. **2023**, *193*, 108873, https://doi.org/10.1016/J.BEJ.2023.108873.

[19] H. Lin, R. W. Leighty, S. Godfrey, S. B. Wang, *Biotechnol. Prog*. **2017**, *33*, 891, https://doi.org/10.1002/BTPR.2472.

[20] V. Chotteau, H. Schwarz, M. Mäkinen, in 'Biopharmaceutical Manufacturing', Vol. CEEN, volume 11, Springer, Cham, **2023**, Ch. 4, https://doi.org/10.1007/978-3-031-45669-5_4.

[21] H. E. Wong, C. Chen, H. Le, C. T. Goudar, *J. Chem. Technol. Biotechnol*. **2022**, *97*, 2297, https://doi.org/10.1002/JCTB.6841.

[22] XCell ATF® Devices and Controllers, https://www.repligen.com/products/upstream-filtration/xcell-atf/xcell-atf-devices-and-controllers, accessed November 29, 2024.

[23] A. Villiger-Oberbek, Y. Yang, W. Zhou, J. Yang, *J. Biotechnol*. **2015**, *212*, 21, https://doi.org/10.1016/J.JBIOTEC.2015.06.428.

[24] P. Mayrhofer, A. Castan, R. Kunert, *J. Chem. Technol. Biotechnol*. **2021**, *96*, 2930, https://doi.org/10.1002/jctb.6848.

[25] S. Janoschek, M. Schulze, G. Zijlstra, G. Greller, J. Matuszczyk, *Biotechnol. Prog*. **2019**, *35*, e2757, https://doi.org/10.1002/BTPR.2757.

[26] N. Gomez, M. Ambhaikar, L. Zhang, C. J. Huang, H. Barkhordarian, J. Lull, C. Gutierrez, *Biotechnol. Prog*. **2017**, *33*, 490, https://doi.org/10.1002/BTPR.2418.

[27] M. Dorn, K. Klottrup-Rees, K. Lee, M. Micheletti, *Biotechnol. Bioeng*. **2024**, *121*, 1759, https://doi.org/10.1002/BIT.28682.

[28] J. M. Bielser, J. Domaradzki, J. Souquet, H. Broly, M. Morbidelli, *Biotechnol. Prog*. **2019**, *35*, e2790, https://doi.org/10.1002/BTPR.2790.

[29] M. K. F. Wolf, V. Lorenz, D. J. Karst, J. Souquet, H. Broly, M. Morbidelli, *Biotechnol. Bioeng*. **2018**, *115*, 2703, https://doi.org/10.1002/BIT.26804.

[30] S. Rapala, A. Barton, S. W. Harcum, *Biochem. Eng. J*. **2024**, *207*, 109332, https://doi.org/10.1016/J.BEJ.2024.109332.

[31] M. Tregidgo, C. Lucas, M. Dorn, M. Micheletti, *Biochem. Eng. J*. **2023**, *195*, 108906, https://doi.org/10.1016/j.bej.2023.108906.

[32] M. Dorn, C. Lucas, K. Klottrup-Rees, K. Lee, M. Micheletti, *Biotechnol. Bioeng*. **2024**, *121*, 1774, https://doi.org/10.1002/BIT.28685.

[33] S. Kreye, R. Stahn, K. Nawrath, V. Goralczyk, B. Zoro, S. Goletz, *Biotechnol. Prog*. **2019**, *35*, e2832, https://doi.org/10.1002/BTPR.2832.

[34] L. Jin, Z.-S. Wang, Y. Cao, R.-Q. Sun, H. Zhou, R.-Y. Cao, *Biotechnol. Lett*. **2021**, *43*, 423, https://doi.org/10.1007/s10529-020-03026-5.

[35] T. M. Gagliardi, R. Chelikani, Y. Yang, G. Tuozzolo, H. Yuan, *Biotechnol. Prog*, **2019**, *35*, e2811, https://doi.org/10.1002/BTPR.2811.

[36] D. J. Sewell, R. Turner, R. Field, W. Holmes, R. Pradhan, C. Spencer, S. G. Oliver, N. K. H. Slater, D. Dikicioglu, *Biotechnol. Bioeng*. **2019**, *116*, 1315, https://doi.org/10.1002/BIT.26946.

[37] Sartorius Stedim Biotech Launches New ambr® 250 High Throughput Bioreactor System For Perfusion Culture, https://www.bioprocessonline.com/doc/sartorius-stedim-ambr-throughput-bioreactor-perfusion-culture-0001, accessed December 3, 2024.

[38] B. Zoro, A. Tait, M. Carpio, K. McHugh, in 'Cell Culture Engineering XVI', Vol. ECI Symposium Series, Eds. A. Robinson, R. Venkat, E. Schaefer, **2018**.

[39] W. X. Sin, N. S. Jagannathan, D. B. L. Teo, F. Kairi, S. Y. Fong, J. H. L. Tan, D. Sandikin, K. W. Cheung, Y. H. Luah, X. Wu, J. J. Raymond, F. L. W. I. Lim, Y. H. Lee, M. S. F. Seng, S. Y. Soh, Q. Chen, R. J. Ram, L. Tucker-Kellogg, M. E. Birnbaum, *Nat. Biomed. Eng*. **2024**, *8*, 1571, https://doi.org/10.1038/s41551-024-01219-1.

[40] H. Schwarz, K. Lee, A. Castan, V. Chotteau, *Biotechnol. Bioeng*. **2023**, *120*, 2523, https://doi.org/10.1002/BIT.28397.

[41] B. Zoro, K. McHugh, *BioProcess. J.* **2020**, *19*, https://doi.org/10.12665/J19OA.ZORO.

[42] C. C. D. Joe, R. R. Segireddy, C. Oliveira, A. Berg, Y. Li, D. Doultsinos, S. Scholze, A. Ahmad, P. Nestola, J. Niemann, A. D. Douglas, *Biotechnol. Bioeng*. **2024**, *121*, 176, https://doi.org/10.1002/BIT.28553.

[43] M. Klimpel, B. Pflüger-Müller, M. A. Cascallana, S. Schwingal, N. I. Lal, T. Noll, V. Pirzas, H. Laux, *Biotechnol. Bioeng*. **2024**, *122*, 344, https://doi.org/10.1002/bit.28880.

[44] J. Ott, V. Ott, P. Neubauer, D. Eibl, R. Eibl, *Chem. Ing. Tech*. **2024**, *96*, 453, https://doi.org/10.1002/cite.202300189.

[45] N. Romanova, T. Niemann, J. F. W. Greiner, B. Kaltschmidt, C. Kaltschmidt, T. Noll, *Biotechnol. Bioeng*. **2021**, *118*, 2348, https://doi.org/10.1002/bit.27747.

[46] S. Alhuthali, P. Kotidis, C. Kontoravdi, *Int. J. Mol. Sci*. **2021**, *22*, 3290, https://doi.org/10.3390/ijms22073290.

[47] O. Šrom, V. Trávníková, J. Wutz, M. Kuschel, A. Unsoeld, T. Wucherpfennig, M. Šoóš, *Biochem. Eng. J*. **2022**, *177*, 108240, https://doi.org/10.1016/J.BEJ.2021.108240.

License and Terms