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Small-scale perfusion mimic cultures in the ambr250 HT bioreactor system

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ABSTRACT

Perfusion cell cultures generate higher viable cell densities (VCD) and volumetric productivity compared to fed-batch cultures. However, due to the limited availability of small-scale perfusion models, perfusion systems are seldom used to produce licensed biotherapeutics. This study evaluated two small-scale perfusion mimic protocols to bridge the research-to-production gap. Shake flasks and the ambr250 HT were used to compare centrifugation and *in situ* gravity settling protocols. The centrifugation protocol achieved a peak VCD >50 million cells/mL and is well-suited to media formulation and feeding strategy comparisons. The *in situ* gravity settling protocol achieved ~20 million cells/mL and is well-suited to cell productivity and stability studies. The cell retention steps resulted in temporary DO and pH changes but did not affect the overall culture's health. Further, both protocols were able to recover from an imposed long-duration DO stress, although the centrifugation protocol cultures had higher cell specific oxygen consumption rates indicative of higher culture stress. Both protocols sustained the cultures for 39 days with stable cell specific productivities (~27 pg/cell·day). Overall, this study demonstrated the feasibility of two economic small-scale perfusion mimic protocols in a standard small-scale bioreactor system, which could be translated to other multi-unit small-scale bioreactor systems.

1. Introduction

The past few decades have witnessed a significant increase in the production and use of monoclonal antibodies (mAbs) as therapeutic agents [1]. Most mAbs are commercially manufactured in either batch or fed-batch mode, with fed-batch currently being the workhorse for most manufacturing processes [2]. However, with the growing emphasis on continuous biomanufacturing and increased demand for recombinant proteins, newer cell cultivation modalities, like perfusion, are being employed to increase throughputs and improve product quality [3–6]. In perfusion cultures, fresh media is added continuously, and spent media is removed while the cells are returned to the vessel via a cell retention device [7]. Due to the cell retention and media exchange, perfusion cultures will have higher viable cell densities (VCD), which results in higher volumetric productivity compared to fed-batch cultures [8]. Additionally, perfusion cultures characteristically have stable operation and low product residence times, which makes this method preferred for producing hard-to-express and unstable proteins [9–11]. The switch from fed-batch to perfusion cultures has been termed process intensification, which has garnered a significant amount of new research [12].

Despite these apparent advantages of perfusion cultures, these culture modes are still rarely used to produce licensed products [13]. A significant barrier to implementation is the limited availability of small-scale perfusion systems for media design, cell line stability evaluation, process development, and process characterization [7]. There are several reliable small-scale batch and fed-batch culture systems available, such as the 2-L Applikon bioreactor, 5-L Sartorius UniVessel bioreactor, DASGIP parallel bioreactor system, DASbox mini bioreactor system, ambr15 cell culture system, and ambr250 HT bioreactor system; yet there are only a few small-scale culture systems for perfusion processes available [7,14–20].

Currently, there are five small-scale perfusion systems reported in the literature. The simplest systems are microwell plates, vented 50 mL conical tubes, and shake flasks [21–26]. These three simple systems use centrifugation or gravity settling for cell retention and are operated as perfusion mimic models. These perfusion mimic models are routinely

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conducted in CO_2 and temperature-controlled incubators, and agitation is used to enhance the gas exchange; however, these models lack the capability to monitor and control dissolved oxygen (DO) and pH. To bridge this gap in DO and pH monitoring and control, the ambr15 system has been adapted to be a perfusion-mimic using gravity settling or centrifugation [27–30]. The ambr15 system can monitor and control DO and pH, which allowed for higher viable cell density (VCD) compared to the simpler systems [31]. Further, the smaller height (38 mm) for a 15 mL working volume of the ambr15 vessels allowed for cell separation via gravity settling in approximately 20–45 minutes [27,28,30,32,33].

One drawback of the ambr15 vessel is its non-standard, rectangular cuboid geometry, which does not mimic the stirring and sparging dynamics of most current commercial bioreactors with 2:1–3:1 height-to-diameter ratios [32,34–36]. In contrast, the ambr250 HT bioreactor system, which has a geometry similar to large-scale commercial bioreactors, has been modified to operate in perfusion mode (ambr250 HT Perfusion system) [37]. The ambr250 HT Perfusion system is equipped with either alternating tangential flow filtration (ATF) or tangential flow filtration (TFF) capability; however, it increases the capital costs by roughly 50% over an ambr250 HT bioreactor system. This cost limits the number of ambr250 HT Perfusion systems in academic settings. Thus, there is a need to have small-scale models that could be used in academic or industrial settings with the ambr250 HT bioreactor systems, the ambr250 modular system, or other multi-unit small-scale bioreactor systems. The small volume of these multi-unit systems significantly decreases media consumption.

The objective of this study was to develop two perfusion mimic protocols that could be conducted in the ambr250 HT bioreactor system. One perfusion mimic protocol used centrifugation to concentrate the cells. The second perfusion mimic protocol used *in situ* gravity settling (hereby referred to as gravity settling) to concentrate the cells. Initially, the perfusion mimic protocols were conducted in shake flasks with real-time DO and pH monitoring to select the perfusion media formulation to be used in the ambr250 HT bioreactors. Then, the two perfusion mimic protocols were conducted in the ambr250 HT system. In addition to normal perfusion operation, the ambr250 HT perfusion mimic protocols were used to examine the effects of DO depletion and recovery. For both the shake flask and standard ambr250 HT perfusion mimic protocols, VCD, cell viability, and titer were evaluated. Additionally, volumetric and cell specific productivities (q_p) for the perfusion mimics were compared to standard fed-batch protocols conducted in the ambr250 HT bioreactor system. And, the cell specific oxygen consumption rates (q_{O2}) were compared to each other for the perfusion mimic protocols.

2. Materials and methods

2.1. Cell line, media, and pre-cultures

The CHOZN GS23 cell line (MilliporeSigma) was used for all studies. This cell line was developed using the CHOZN platform and expresses a monoclonal antibody against an undisclosed target. For the shake flask experiments, ActiPro media (Cytiva, SH31037.01) and Cell Boost 7a/7b feed media (Cytiva, SH31026.07/SH31027.01) were used. For the ambr250 HT bioreactor experiments, EX-CELL Advanced CHO Fed-batch Media (MilliporeSigma, 14366 C), EX-CELL CD CHO Fusion media (MilliporeSigma, 14365 C), EX-CELL Advanced CHO Feed 1 (without glucose) (MilliporeSigma, 24368 C), and Cell Boost 7a/7b feed media (Cytiva, SH31026.07/SH31027.01) were used.

Shake flasks of 125- or 250-mL with 30- or 70-mL working volumes, respectively, were used. The shake flask cultures were maintained in a humidified incubator at 37°C with 5% CO_2 at 180 rpm with a 19 mm throw. A 1 mL working cell bank vial, stored in liquid nitrogen, was thawed into a 125 mL shake flask containing EX-CELL CD CHO Fusion media and expanded. Cells were seeded at 0.5 million cells/mL for all pre-cultures. For scale-up, cells were passaged when the VCD was between 3 and 6 million cells/mL. Cells were passaged at least three times in the ActiPro media prior to conducting the shake flask perfusion mimic protocols. Cells were passaged two times in EX-CELL CD CHO Fusion media and three times in EX-CELL Advanced CHO Fed-batch media prior to conducting the ambr250 HT perfusion mimic protocols.

2.2. Shake flask perfusion mimic protocols

Shake flask perfusion mimic protocols used flasks equipped with optical DO and pH sensors (SBI Scientific Bioprocessing, USA) to monitor real-time DO and pH. Feeding for the perfusion mimic protocols and parallel fed-batch protocols began on day 3. For the perfusion mimic protocols, the media exchanges were 0.3 VVD (vessel volumes exchanged per day). Three perfusion media formulations were used: 0% feed (100:0:0), 10% feed (90:9.1:0.9), 30% feed (70: 27.3: 2.7), ActiPro media: Cell Boost 7a: Cell Boost 7b (v:v:v), respectively. For the fed-batch protocols, the feeding schedule was: Cell Boost7a/7b (v/v) – day 3 to day 5: 3%/0.3%; day 6 to day 7: 4%/0.4%; day 8 to day 9: 5%/0.5%; day 10 to day 11: 4%/0.4%. Glucose was added daily to bring the culture concentration up to 5 g/L when the concentration dropped below 3 g/L. A 450 g/L (45 w/v%) glucose solution was used.

For the shake flask centrifugation perfusion mimic protocol, 30% of the total culture volume was removed daily and centrifuged at 800 g for 10 minutes at 4°C. Then, the cell-free supernatant was removed and discarded. The cell pellet was resuspended in an equal volume of fresh perfusion media. The resuspended cells were transferred back to the shake flask. For the shake flask gravity settling perfusion mimic protocol, cells were settled for 45 minutes in the temperature and CO_2-controlled incubator. The top layer of the clarified cell broth (30% of the total culture volume) was removed outside the incubator. An equal volume of fresh perfusion media was added, and the cultures were returned to the incubator.

2.2.1. ambr250 HT perfusion mimic protocols

A 12-way ambr250 HT bioreactor system (Sartorius Stedim, Göttingen, Germany) was used for the two perfusion mimic protocols (centrifugation and gravity settling) and the control fed-batch protocol. Mammalian vessels with dual-pitched blade impellers and an open pipe sparger (Sartorius Stedim, 001–5G25) were used. The centrifugation and gravity settling perfusion mimic protocols and fed-batch protocol had initial volumes of 210 mL. During operation of the perfusion mimics, the gravity settling culture volumes were reduced to 200 mL to decrease settling times. The pH was maintained between 6.9 and 7.3 by sparging CO_2 and providing base (1 M sodium carbonate). The culture temperature was maintained at 37°C. The DO set point was 30% air saturation. The control algorithm settings used for pH and DO are provided in Tables 1 and 2, respectively. The proportional, integral, and derivative (PID) settings are based on the tuning experiments for the fed-batch cultures with modifications (stir speed maximums increased) for the perfusion cultures to maintain the DO [38]. EX-CELL Advanced CHO Fed-batch Media was used as the basal media for the two ambr250 HT perfusion mimic protocols and the fed-batch protocol. Media exchange for the perfusion mimic protocols began on day 3. Initially, for both perfusion mimic protocols, a perfusion media comprising 90:9.1:0.9 EX-CELL Advanced CHO Fed-batch Media: Cell Boost 7a: Cell Boost 7b

Table 1
Control algorithm settings for pH in the ambr250 HT bioreactor system. The initial pH was pH 7.3, as per recommendations from MilliporeSigma, the cell line originator.

pH limit setpoint	Variable (Range)		Control Parameters		
	Lower - Base flow rate (mL/h)	Upper - CO_2 flow rate (mL/min)	k_P	t_I (s)	t_D (s)
6.9	0–5	na	10	100	0.02
7.3	na	0.1–10	10	200	0

na – not applicable

Table 2
Control algorithm settings for DO in the ambr250 HT bioreactor system. The initial stir speed was 300 rpm. The initial gas flow rate was 2 mL/min for perfusion mimics and 5 mL/min for fed-batch cultures.

Level	Variable	Range	k_P	t_I (s)	t_D (s)
1	Gas flow (Air/mix) (mL/min)	2[a] to 20	0.1	100	0
2	O_2 mix (%)	0–50	0.15	100	0
3	Stir speed (rpm)	300–400[b]	2	100	0
4	O_2 mix (%)	50–100	0.15	100	0
5	Stir speed (rpm)	400–800[c]	1	100	0
6	O_2 added flow (mL/min)	0–20[d]	0.5	100	0

[a] Initial gas flow rate was 5 mL/min for the fed-batch protocol
[b] Upper stir speed set point was 800 rpm for the centrifugation perfusion mimic protocol
[c] Stir speed range was 800–1000 rpm for the centrifugation perfusion mimic protocol
[d] Upper O_2 added flow setpoint was 80 mL/min for the centrifugation perfusion mimic protocol

(v:v:v) was used. Feeding for the fed-batch protocol also began on day 3. The feeding schedule for the fed-batch protocol was: 5% (v/v) EX-CELL Advanced CHO Feed 1 (without glucose) every other day. Glucose was added to bring up to 5 g/L when dropped below 3 g/L using a 450 g/L (45 w/v%) glucose solution. Antifoam solution (Cytiva, SH30897.01) was added as needed.

The ambr250 HT centrifugation perfusion mimic protocol removed the culture broth and centrifuged at 800 g for 10 minutes at 4°C then, the cell-free supernatant was discarded, and the cell pellet was resuspended in an equal volume of fresh perfusion media. The resuspended cells were transferred back to the bioreactor. The ambr250 HT gravity settling perfusion mimic protocol stopped PID controls, agitation, and aeration for 45 minutes. The clarified supernatant was removed, and then an equal volume of fresh perfusion media was added. Then, the PID controls, agitation, and aeration resumed. Fig. 1 outlines the steps for the perfusion mimic centrifugation and gravity settling protocols.

2.3. Off-line analytical methods

VCD, cell viability, and metabolites were measured daily. VCD and cell viability were measured using a Vi-Cell XR cell viability analyzer (Beckman Coulter, Brea, CA). Extracellular glucose, lactate, glutamine, glutamate and IgG concentrations were measured using the Cedex Bio-analyzer (Roche Diagnostics, Mannheim, Germany). Osmolality was measured using the OsmoTech Pro Multi-Sample Micro-osmometer (Advanced Instruments, Norwood, MA). Amino acids were measured using the REBEL Cell Culture Analyzer (908 Devices, Boston, MA).

2.4. Calculations

The integral viable cell density (IVCD; cells·days/mL) for the cultures was determined using Eq. (1) [39]:

$$IVCD_i = IVCD_{i-1} + \left(\frac{VCD_i + VCD_{i-1}}{2}\right) \bullet (t_i - t_{i-1}) \quad (1)$$

Where $IVCD_i$ and $IVCD_{i-1}$ represent the IVCD (cells·days/mL), VCD_i and VCD_{i-1} represent the VCD (cells/mL) at consecutive timepoints t_i and t_{i-1}. To calculate $IVCD$ for the gravity settling perfusion model, Eq. (1) was modified to account for the percent cell bleed as shown in Eq. (2).

$$IVCD_i = IVCD_{i-1} + \left(\frac{VCD_i + \left(\left(1 - \left(\frac{B_i}{100}\right)\right) \bullet VCD_{i-1}\right)}{2}\right) \bullet (t_i - t_{i-1}) \quad (2)$$

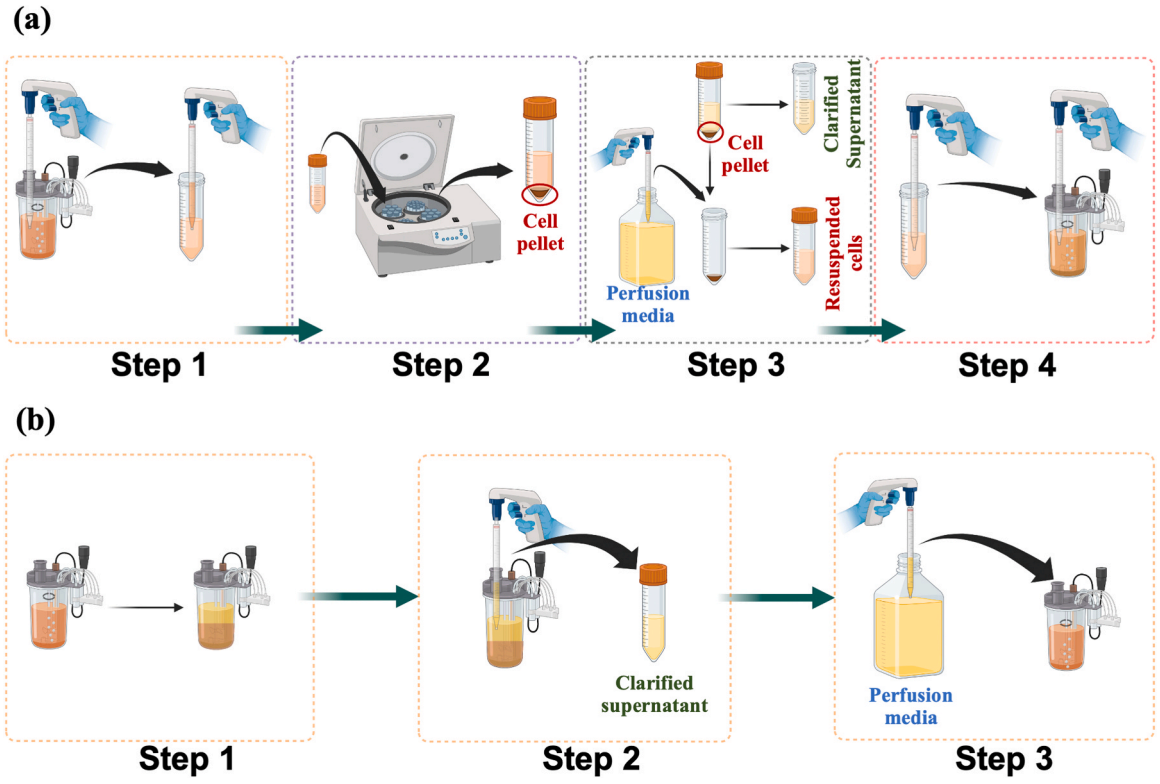

Fig. 1. Protocols steps for the perfusion mimic centrifugation and gravity settling methods in the ambr250 HT bioreactor system. a) Centrifugation method. **Step 1:** Cell culture broth removed from bioreactor. **Step 2:** Cell culture broth centrifuged. **Step 3:** Clarified supernatant removed, and the cell pellet resuspended in perfusion media. **Step 4:** Resuspended cells are transferred back to the bioreactor. b) Gravity settling methods. **Step 1:** Agitation and aeration stopped, and cells settled. **Step 2:** Clarified supernatant removed from bioreactor. **Step 3:** Media added to the bioreactor, agitation, and aeration resumed.

Where the cell bleed (B_i) is defined in Eq. (3)

$$B_i(\%) = \left(\frac{P_i \bullet sVCD_i}{VCD_i}\right) \bullet 100 \quad (3)$$

Where $sVCD_i$ represents the VCD of the clarified supernatant (cells/mL), VCD_i represents the culture VCD (cells/mL) and P_i represents the perfusion rate (VVD), all at time t_i. The cell bleed due to sampling was neglected since the bleed amount due to sampling were 1.1% and 0.75% for the shake flask and bioreactor perfusion mimic protocols, respectively. The cumulative titer ($T_i; g$) was determined using Eq. (4):

$$T_i = (c_i \bullet V_i) + \sum_{k=3}^{i-1} (c_k \bullet P_k \bullet V_k) \quad (4)$$

Where c_i and V_i represent the titer (g/L) and bioreactor volume (L), respectively, at the time t_i. c_k, P_k, and V_k represent titer, bioreactor volume, and perfusion rate beginning at the time, day 3 ($k = 3$) until t_{i-1}. The cell-specific productivity (q_p; 10^9 pg/cell·day) over time was determined using Eq. (5):

$$q_{p,i} = \left(\frac{T_i}{V_i \bullet IVCD_i}\right) \quad (5)$$

The oxygen uptake rate (OUR; 10^{-3} g/L·h) was determined using the stationary liquid mass balance method using the Eq. (6) [40]:

$$OUR_i = (k_L a)_i \bullet (C^* - C_i) \quad (6)$$

Where $(k_L a)_i$ is the volumetric mass transfer coefficient (h^{-1}), C^* is the oxygen saturation concentration (mg/L) and, C_i is the dissolved oxygen concentration (mg/L), all at time t_i. The ambr250 DO sensor spots are calibrated to 100% DO at 37°C at 1013 mbar in Merck pH 7 buffer (catalog number 1.09439.1000). Thus, C^* in air $C^*(air)$ was estimated to be 6.40 mg/L [41]. Henry's Law was used to adjust C^* to account for oxygen enrichment of the air. C_i was obtained from the actual DO reading converted to [mg/L], see Supplementary Material for more details. $k_L a$ at time t_i was calculated using the van't Riet equation [42]. The constants 'K', 'a', and 'b' in the van't Riet equation were determined based on the estimations from ambr250 HT characterization data from [35]. In the Supplementary Materials, the method to obtain the van't Riet constants is outlined.

The cell specific oxygen consumption rates (q_{O_2}; 10^3 pg/cell·h) were determined by Eq. (7):

$$q_{O_{2,i}} = \frac{OUR_i}{VCD_i} \quad (7)$$

The cell specific perfusion rates (CSPR; 10^3 pL/ cell·d) were determined using Eq. (8):

$$CSPR_i = \frac{P_i}{VCD_i} \quad (8)$$

2.5. Statistical analysis

Statistical analysis was performed using JMP Pro 17 (SAS Institute Inc., Cary, NC) for the replicate data. VCDs, titers, cumulative titers, cell specific productivity and cell specific oxygen consumption were analyzed using the Generalized Linear Model ($p \leq 0.05$) to determine if the culture method or culture phase were an effector. Error bars on graphs represent standard deviations. All averages are reported as mean ± SD.

3. Results and discussion

3.1. Shake flask perfusion mimic protocols

Initially, only the centrifugation perfusion mimic protocol was conducted in shake flasks to compare perfusion media formulations. Once the media was selected, the two perfusion mimic protocols, centrifugation and gravity settling, were compared to each other and the standard fed-batch protocol. The perfusion media formulations examined were: 0% feed (100:0:0), 10% feed (90:9.1:0.9), and 30% feed (70: 27.3: 2.7) (v:v:v) ActiPro media: Cell Boost 7a: Cell Boost 7b, respectively. The growth and productivity characteristics of these initial centrifugation perfusion mimic cultures are shown in Fig. 2. Beginning on day 4, the 30% feed formulation showed an increased osmolality relative to other formulations, and by day 5, the osmolality had reached ~440 mOsm/kg. Osmolality measurements exceeding 400 mOsm/kg in CHO cell cultures were previously reported to substantially hinder cell growth and protein production [43–45]. Hence, the 30% feed formulation was discontinued on day 5. The 10% feed formulation resulted in slightly higher VCDs, titers, and cumulative titers compared to the 0% feed formulation. Thus, the 10% feed formulation was selected for the subsequent gravity setting perfusion mimic experiment.

The centrifugation and gravity settling shake flask perfusion mimics are compared to each other and to a fed-batch culture, also shown in Fig. 2**.** The cell bleed for the gravity settling perfusion mimic culture is shown, since it was significant (Fig. 2**f**). In comparison, the cell bleed for the centrifugation perfusion mimic culture was only due to sampling and negligible. Both perfusion mimic cultures in the 10% feed formulation performed similarly in the shake flasks and had higher VCD and cell viability compared to the fed-batch cultures; however, the titers were higher for the fed-batch cultures, as expected [8]. The cumulative titers for both perfusion mimic cultures were higher than the fed-batch cultures, also as expected [8].

Since the VCDs for the perfusion mimics in the shake flasks were higher than standard fed-batch cultures in shake flasks, non-invasive DO and pH spot sensors were used to monitor DO and pH throughout the perfusion mimic cultures. The DO and pH profiles are shown in Fig. 3. As expected, the higher VCDs in the shake flask perfusion mimic cultures resulted in oxygen depletion. For both perfusion protocols, the DO was rapidly depleted soon after each media addition (Fig. 3**a, c**). These DO profiles indicate that the standard shake flask agitation rate cannot support the oxygen demands for these high VCD perfusion cultures. Additionally, the pH was non-optimal for most of the culture duration, also due to the higher VCD (Fig. 3**b, d**). Essentially, the common bicarbonate buffer and 5% CO_2 incubator conditions could not provide sufficient buffering capacity under perfusion operation. Since DO and pH are critical process parameters that affect cell growth, protein productivity, and protein quality, these DO and pH observations suggested sub-optimal perfusion culture conditions in shake flasks and other uncontrolled systems [46,47]. Thus, for the perfusion mimics protocols to be predictive, these perfusion mimics models needed to be examined in controlled bioreactors.

3.2. ambr250 HT perfusion mimic protocols

Due to the significant oxygenation and pH control issues encountered in the shake flask perfusion mimic experiments, the two perfusion mimic protocols were adapted to the standard ambr250 HT bioreactor system, where DO and pH control capabilities exist. A 10% feed media formulation was used throughout even if the basal media changed. Further, the two perfusion mimic protocols were conducted in the ambr250 HT bioreactor system using three phases to evaluate normal growth, DO stress, and recovery. Again, the perfusion mimics were compared to the standard fed-batch cultures conducted in the ambr250 HT bioreactor system. Specifically, Phase I evaluated the two perfusion mimic protocols using 0.3–0.5 VVD medium exchange rates. In the ambr250, if the perfusion rate is greater than 0.9 VVD, the discrete centrifugation or gravity settling operations would need to occur more often than once daily. Phase II evaluated the effects of a long-duration DO stress on cell growth and productivity. Phase III evaluated the capability of these perfusion mimic protocols to enable cell recovery

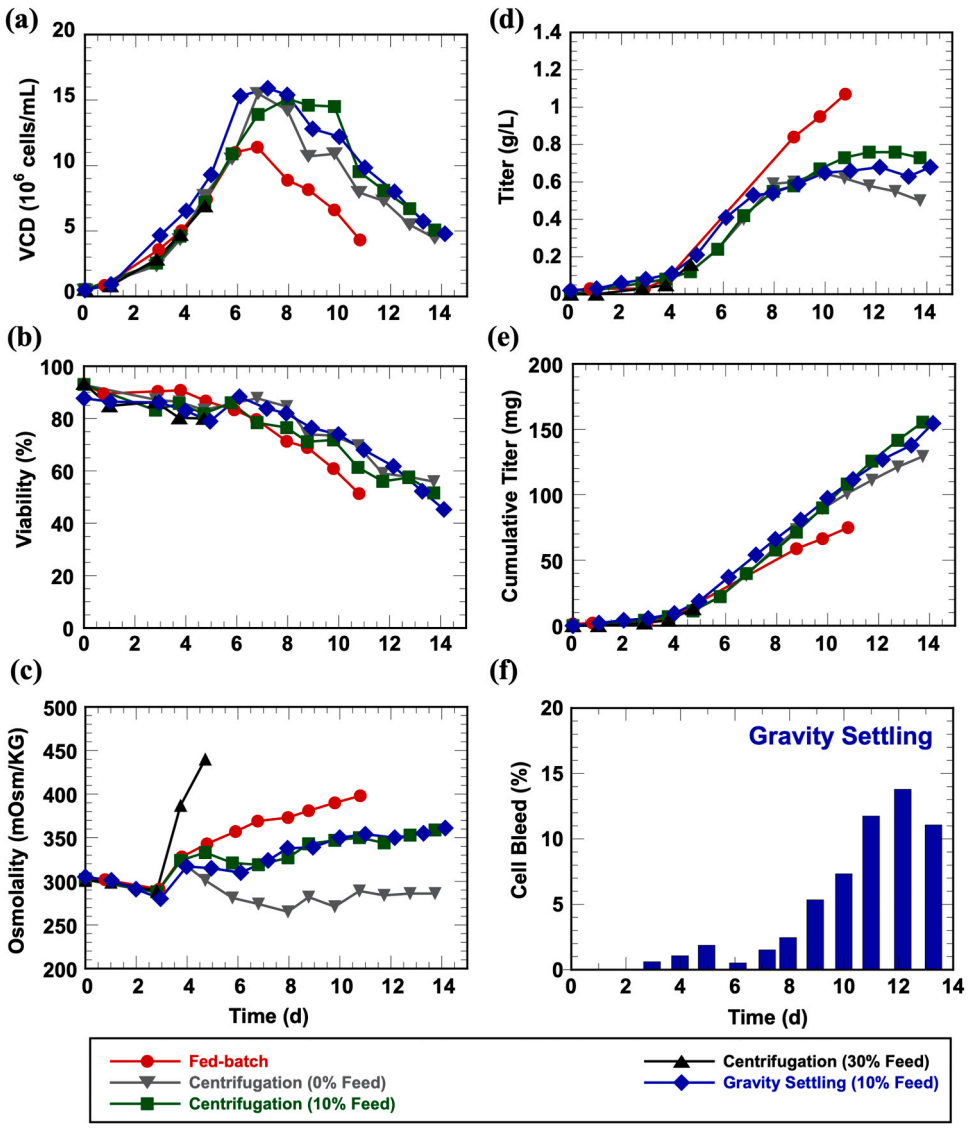

Fig. 2. Growth and productivity characteristics of the perfusion mimic and fed-batch cultures conducted in shake flasks. (a) VCD; (b) cell viability; (c) osmolality; (d) titer; (e) cumulative titer; (f) gravity settling cell bleeds. Centrifugation perfusion mimics: Perfusion media formulations with 0% feed media are shown as upside down triangles (N = 1), 10% feed media as green squares (N = 1), and 30% feed media as black triangles (N = 1). Gravity settling perfusion mimic with 10% feed media is shown as blue diamonds (N = 1). Fed-batch culture is shown as red circles (N = 1).

from a long-duration DO stress.

3.2.1. *Phase I: Comparison of two perfusion mimic protocols*

In Phase I, the two perfusion mimic protocols were benchmarked against a standard fed-batch protocol using EX-CELL Advanced CHO Fed-batch Media for the batch phases. The fed-batch cultures had 5% every other day feeding starting on day 3. The perfusion mimic protocols began perfusion on day 3 at 0.3 VVD using the 10% feed formulation media. The cell culture profiles are shown in Fig. 4 for all three phases for both perfusion mimic and the standard fed-batch cultures. Fig. 4**a** and **b** show the VCD and cell viability, respectively. Fig. 4**c** and **d** show the daily perfusion rates for the centrifugation and gravity perfusion mimic cultures, respectively. By day 6, the centrifugation perfusion mimic cultures had the highest VCD, and all cultures had similar cell viabilities. Since both perfusion mimic cultures consumed glucose well and had well-controlled lactate levels (Fig. 5), on day 7, the perfusion rate was increased to 0.5 VVD in both protocols. Note: The glucose and lactate concentrations shown are pre-media exchange. By day 10, both perfusion mimic cultures had reached VCDs higher than the fed-batch cultures. The centrifugation perfusion mimic cultures reached ~50 million cells/mL, whereas the gravity settling perfusion mimic cultures reached ~20 million cells/mL. Similar cell densities have been reported in other perfusion mimics; however, these other studies utilized perfusion rates higher than 1 VVD [28].

By day 12, both perfusion mimic cultures appeared to have reached steady-state with respect to VCD and titer (Fig. 6). During this steady-state, the cell specific perfusion rate (CSPR) for the centrifugation perfusion mimic culture was ~10 pL/cell·day, and for the gravity settling perfusion mimic cultures it was ~24 pL/ cell·day (Fig. 7**a**). CSPR describes the media consumption rate on a per-cell basis and is often considered a key perfusion process performance criterion [48]. Notably, the observed CSPR in both ambr250 perfusion mimics is consistent with the previously reported ranges for standard perfusion-based CHO cell cultures that employ cell retention devices [7,49]. This indicates that both the ambr250 perfusion mimic protocols produced cell cultures with similar media consumption per cell as traditional perfusion cultures.

The cell-specific oxygen uptake rates (q_{O_2}) for both perfusion mimics were determined. To determine q_{O_2}, the stationary liquid mass balance method was used to obtain the oxygen uptake rate (OUR) using the van't Riet equation [40,42,50], where the van't Riet equation constants for

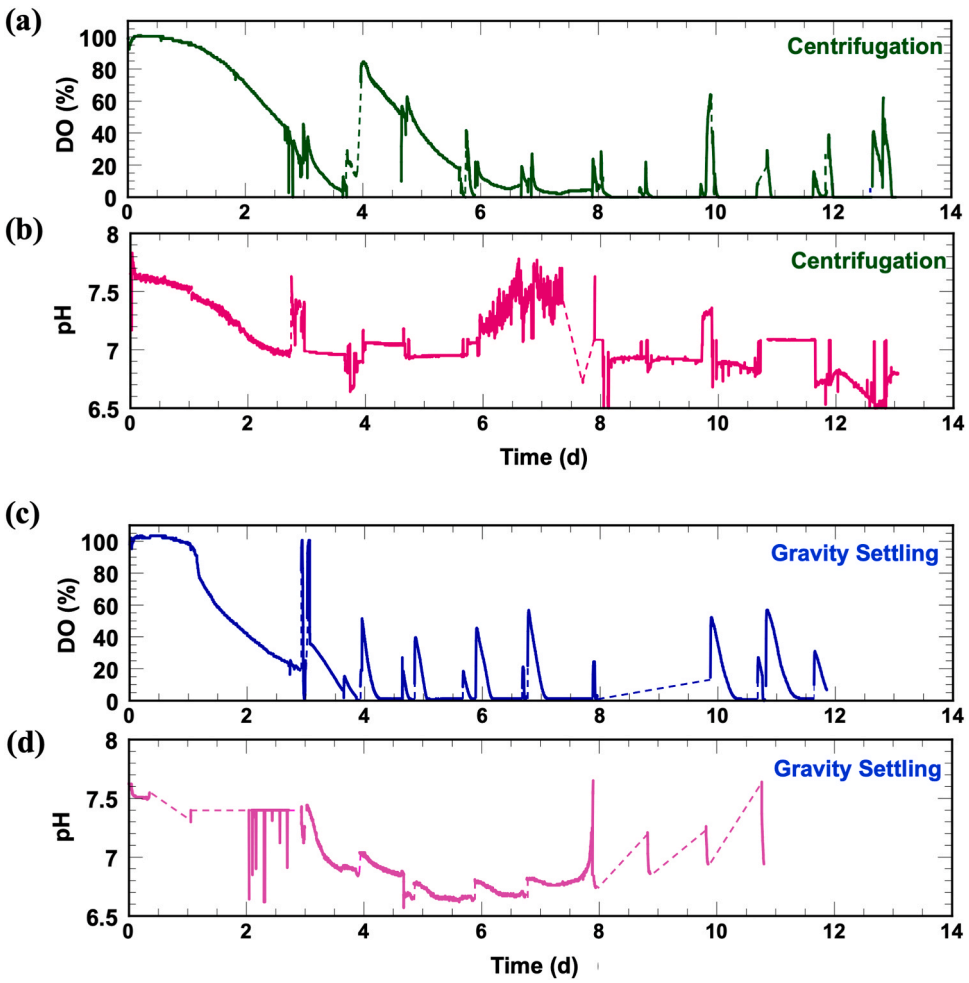

Fig. 3. DO and pH profiles for the perfusion mimic protocols conducted in shake flasks. Centrifugation perfusion mimic: (a) DO; (b) pH. Gravity settling perfusion mimic: (c) DO; (d) pH. Note: Dotted lines in the graphs represent missing data. DO and pH data from day 13 for the centrifugation perfusion mimic and from day 12 for the gravity settling perfusion mimic were not recorded due to detachment of optical probes in the shake flasks. The fed-batch cultures were not equipped with DO or pH probes.

the ambr250 vessels were extrapolated from the k_La data provided in Xu et al., 2017. The cell-specific oxygen uptake rate is an indicator of the metabolic state for cells and is routinely used to identify changes in cell respiration [51]. The cell specific oxygen consumption rates were the same for the centrifugation and gravity settling perfusion mimic culture thru day 12 ($p > 0.05$), Fig. 7**b**.

And, as expected, the fed-batch cultures only had 14-day durations as defined by the cell viability below ~70%. The final cell densities for the fed-batch cultures were approximately 5 million cells/mL with ~2.0 g/L titer. For the perfusion mimics, the daily titers at the steady-state phase were ~2 g/L and ~0.9 g/L for the centrifugation and gravity settling cultures, respectively, and are statistically different ($p \leq 0.05$). This resulted in cumulative titers for the perfusion mimic cultures to be significantly higher than the cumulative titers for the fed-batch cultures ($p \leq 0.05$) (Fig. 6**b**).

The daily cell bleed for the gravity settling perfusion mimic cultures is shown in Figs. 4**d** and 4**e**. The gravity settling perfusion mimic cultures averaged a daily cell bleed of 18.6 ± 2.5%. The cell bleeds in the gravity settling perfusion mimics are mainly the result of the incomplete cell separation in the settling phase, which has also been observed for the ambr15 gravity settling protocols [27,28,30]. Cell bleeds for the ambr15 gravity settling perfusion mimic cultures were observed to be lower at approximately 9% [27,28,30]. The ambr250 gravity settling cell bleeds were higher due to increased settling time compared to the ambr15 vessels [32,35,36]. The settling velocities (terminal velocities) would be comparable between the ambr15 and ambr250 with only minimal differences due to media and cell size [52]; however, the distance required in the ambr250 to result in half the volume as clarified supernatant is higher (~50 mm vs ~19 mm). In contrast, the only significant cell bleeds for the ambr250 centrifugation perfusion mimic cultures were due to the sample volumes, as was also observed for the ambr15 centrifugation perfusion mimic [29]; the cell bleeds due to sampling for the ambr250 centrifugation perfusion mimic cultures are not shown graphically, as it only represented an average cell bleed of 0.75% per day.

3.2.2. *Effects of daily perfusion mimic operation on DO and pH*

Cell retention and media exchanges for the centrifugation and gravity settling perfusion mimic protocols caused short-term disruptions, which in turn impacted the DO and pH profiles, as shown in Fig. 8. These short-term DO and pH have been observed previously and are common during media manipulations for perfusion mimic cultures [27,28]. For the centrifugation perfusion mimic cultures, a rapid increase in DO occurred when the culture broth was removed (Fig. 8**a**). This increase was due to slow control responses caused by the sudden decrease in culture volume. In parallel, the pH gradually increased due to the higher gas flow rate relative to the culture volume, which resulted in CO_2 stripping (Fig. 8**b**). Nonetheless, the DO returned to the setpoint, and the pH value stabilized within ~16 minutes of the volume decrease. And when the cell suspension in fresh perfusion media was returned to

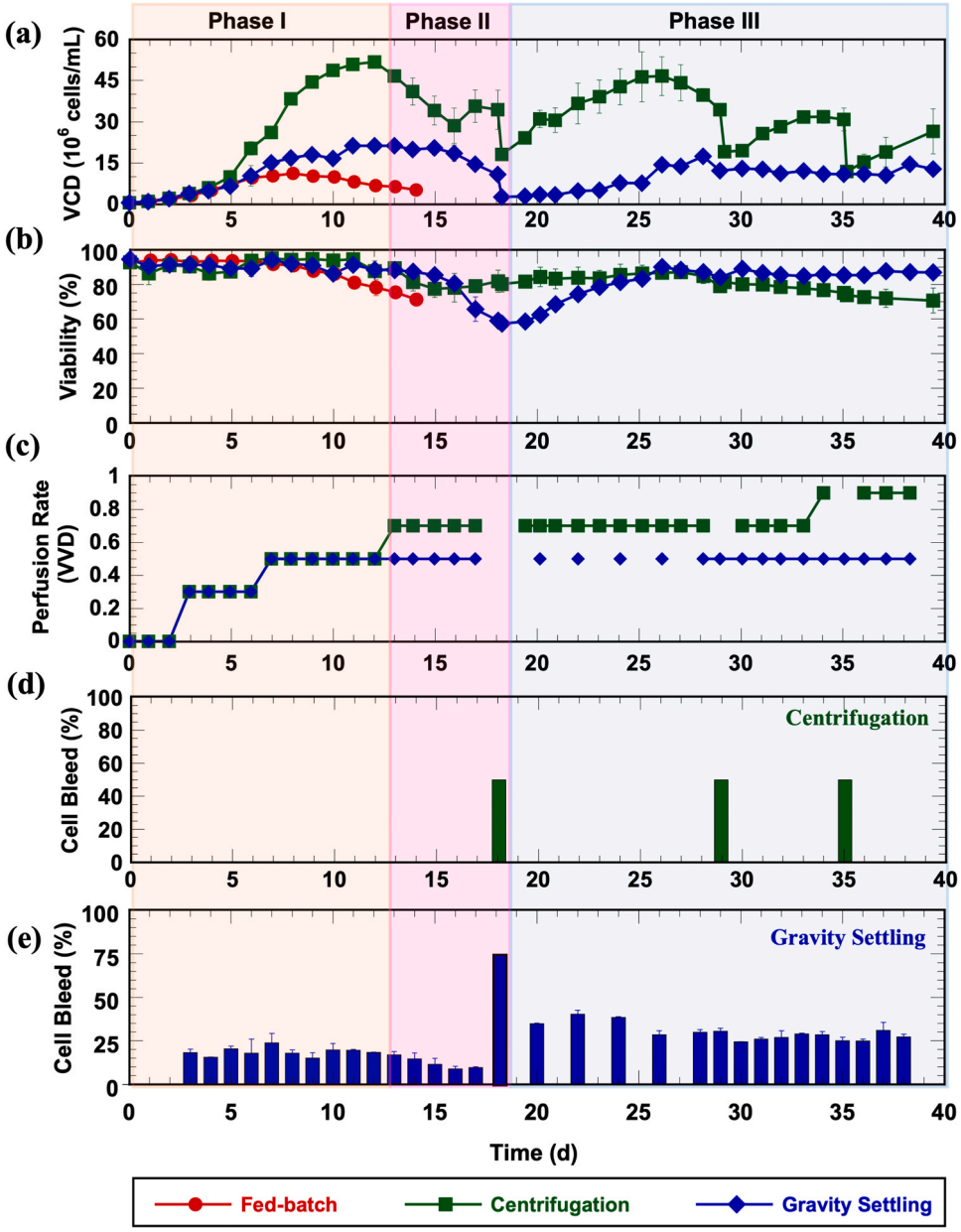

Fig. 4. Growth profiles for the perfusion mimic and fed-batch protocols conducted in the ambr250 HT bioreactor system. (a) VCD; (b) cell viability; (c) perfusion rate; (d) centrifugation cell bleed; (e) gravity settling cell bleed. Phase I: Batch and Perfusion; Phase II: DO Stress; Phase III: Recovery. The centrifugation perfusion mimic cultures (N = 2) are shown as green squares. The gravity settling perfusion mimic cultures (N = 2) are shown as blue diamonds. Fed-batch cultures (N = 5) are shown as red circles. Error bars represent the standard deviation.

the bioreactor, there was a sudden increase in both cell numbers and culture volume, which resulted in a DO decrease to ~0% for approximately 25 minutes while the controller responded. The pH decreased after the cells were reintroduced, possibly from some anaerobic metabolism.

For the gravity settling perfusion mimic protocol, the DO decreased to 0% during the settling period as both agitation and sparging were stopped (Fig. 8**c**). Additionally, the pH gradually decreased during the settling period. After the settling period, the clarified broth was removed and replaced with fresh perfusion media. The agitation and sparging control were resumed, and the pH increased immediately (Fig. 8**d**). The DO required about 5 minutes to return to the setpoint and took an additional 60 minutes to stabilize. The fed-batch cultures did not experience these short-term DO and pH, yet the cell specific productivities were higher for the perfusion mimic cultures compared to the fed-batch cultures (Fig. 6**c**), indicating these short-term DO and pH disturbances did not negatively impact the cells.

3.2.3. *Phase II: Effects of a long-duration DO stress on perfusion mimics*

As both perfusion mimic cultures experienced daily DO stress and appeared unaffected, so a long-duration DO stress was investigated as Phase II. To create this long-duration DO stress, on day 12, the air and oxygen supply to both perfusion mimics was stopped for 5 hours. As expected, the DO for both perfusion mimics dropped to ~0%. Anaerobic metabolism occurred during the DO stress, as indicated by the continued glucose consumption and high lactate production (Fig. 5). Since the centrifugation cultures had higher VCDs prior to the DO stress, lactate production was also observed to be higher. This increased lactate production resulted in higher base additions to the centrifugation perfusion cultures to control pH, and subsequently increased culture osmolality

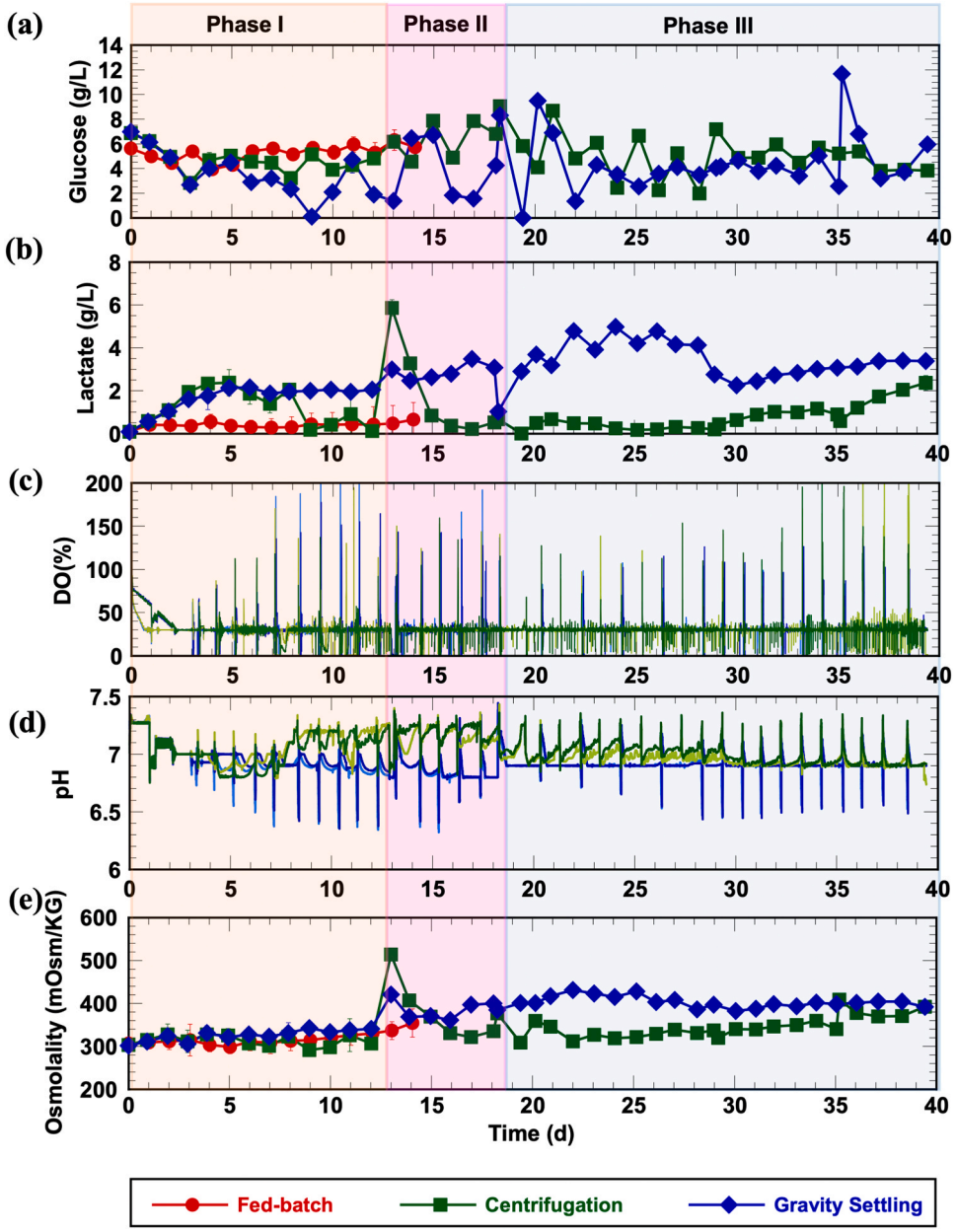

Fig. 5. Metabolite profiles for the perfusion mimic and fed-batch protocols conducted in the ambr250 HT bioreactor system. (a) Glucose; (b) Lactate; (c) DO; (d) pH; (e) Osmolality. Phase I: Batch and Perfusion; Phase II: DO Stress; Phase III: Recovery. The centrifugation perfusion mimic cultures (N = 2) are shown as green squares or as green lines for DO and pH. The gravity settling perfusion mimic cultures (N = 2) are shown as blue diamonds or as blue lines for DO and pH. Fed-batch cultures (N = 5) are shown as red circles. Error bars represent the standard deviation.

compared to the gravity settling perfusion cultures (Fig. 5**c**). Consequently, the VCD and cell viability decreased for the centrifugation perfusion mimic cultures soon after the DO stress; however, in comparison, the gravity settling perfusion mimic cultures only had a slight decrease in VCD and cell viability.

To aid in the recovery of the centrifugation perfusion mimic cultures, and reduce the lactate and osmolality, on day 13, the perfusion rate was increased from 0.5 VVD to 0.7 VVD. For the gravity settling perfusion cultures, perfusion rate continued at 0.5 VVD. Recall that the cell specific perfusion rates for the gravity settling perfusion cultures were already higher than the centrifugation cultures. Likewise, the lactate and osmolality changes for the gravity cultures due to the DO stress were lower; hence, increasing the perfusion rate higher than 0.5VVD was not necessary for the gravity settling perfusion cultures to reduce lactate and osmolarity.

After the imposed DO stress, the q_{O_2} values for the centrifugation perfusion cultures increased sharply, while the q_{O_2} values for the gravity settling perfusion cultures remained relatively stable (Fig. 8**b**). The increased q_{O_2} for the centrifugation perfusion mimic cultures indicates the culture was adversely affected by the long-duration DO stress [53,54]. In contrast, q_{O_2} was fairly constant for the gravity settling perfusion mimic cultures, indicating that the lower VCD and resulting lower lactate and osmolarity increases helped these cultures withstand the long-duration DO stress. By day 17, both perfusion cultures had not fully recovered to pre-DO stress VCD levels, such that more extreme recovery approaches were investigated.

3.2.4. *Phase III: Recovery and Robustness of Perfusion Mimics*

The long-duration DO stress negatively affected VCD and cell viability in both perfusion mimic cultures. Phase III was used to examine

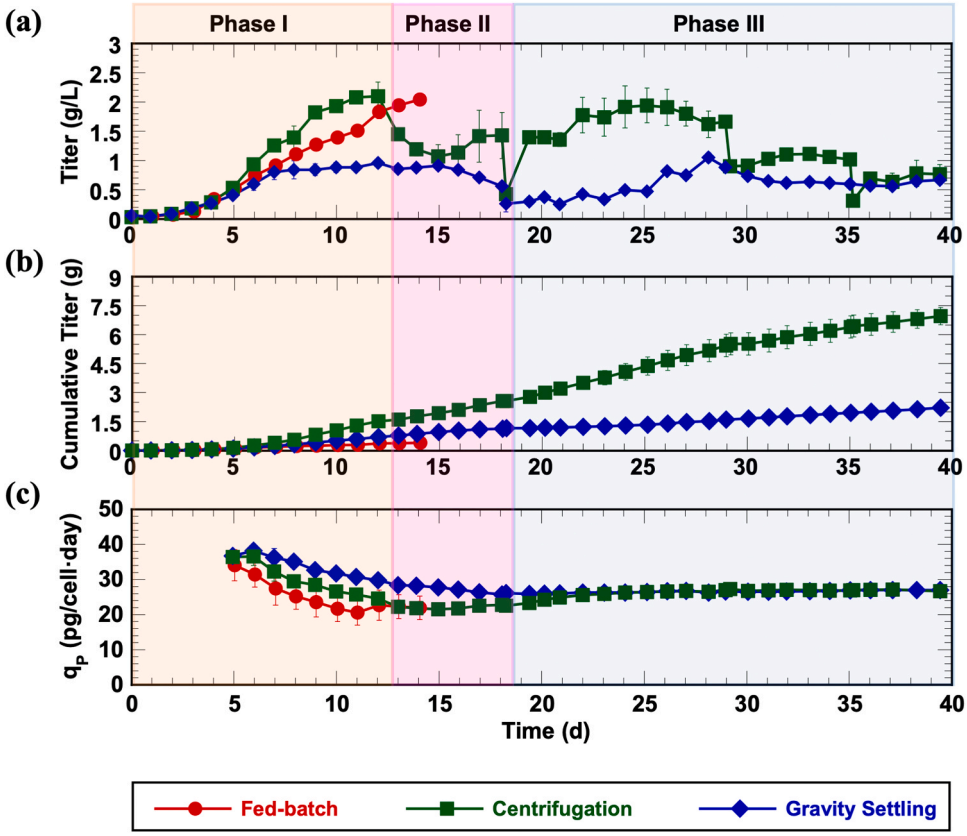

Fig. 6. Process outcomes for the perfusion mimic and fed-batch protocols conducted in the ambr250 HT bioreactor system. Phase I: Batch and Perfusion; Phase II: DO Stress; Phase III: Recovery. (a) Titer; (b) Cumulative Titer; (c) Cell Specific Productivity (q_P). The centrifugation perfusion mimic cultures (N = 2) are shown as green squares. The gravity settling perfusion mimic cultures (N = 2) are shown as blue diamonds. Fed-batch cultures (N = 5) are shown as red circles. Error bars represent the standard deviation.

strategies to aid recovery. The first strategy was a large cell bleed to reduce the VCD. This strategy had been used previously with good success [55]. Therefore, on day 18, 50% (v/v) culture broth from the centrifugation perfusion mimic cultures and 75% (v/v) culture broth from the gravity settling perfusion mimic cultures were exchanged with fresh perfusion media (Fig. 4**d** and **e**). After the large cell bleeds, the perfusion rate was continued at 0.7 VVD for the centrifugation perfusion mimic cultures. Since the gravity settling cultures had regular cell bleed due to the media exchanges, the perfusion rate was continued at 0.5 VVD, but only every other day until day 28. Additionally, at day 18, the EX-CELL Advanced CHO Fed-batch Media in the perfusion media formulation was replaced with EX-CELL CD CHO Fusion media. Unlike the EX-CELL Advanced CHO Fed-batch Media, the EX-CELL CD CHO Fusion media is specifically formulated for long-term CHO cell culture; hence, it was thought this media might better support cell recovery. And, to prevent a long adaptation, from day 18 to day 22, the basal media were mixed 50–50 (v-v%), such that the perfusion media was EX-CELL Advanced CHO Fed-batch Media: EX-CELL CD CHO Fusion Media: Cell Boost 7a: Cell Boost 7b (45:45:9.1:0.9; v:v:v:v). On day 23, the perfusion media was switched entirely to EX-CELL CD CHO Fusion Media (90:9.1:0.9; v:v:v) (EX-CELL CD CHO Media: Cell Boost 7a: Cell Boost 7b).

As was anticipated, by day 25, both perfusion mimic cultures had increased VCD and cell viability (Fig. 4**a**), as the large cell bleed allowed for exponential growth. For the centrifugation cultures, this is observed as a significant increase in VCD to 45 million cells/mL. For the gravity settling cultures, cell viability returned to pre-long-duration DO stress levels by day 25 and remained stable for the duration of the cultures. However, the centrifugation cultures after 25 days had decreased growth, as shown by declining VCDs and titers, while the cell viabilities were relatively stable (Figs. 4**a** and 6**a**). Therefore to aid recovery again, a second large cell bleed was implemented on day 28 (50% v/v), and then the perfusion rate was increased to 0.9 VVD on day 34. Finally, a third large cell bleed was implemented on day 35 (50% v/v) (Fig. 4**d**). After each large cell bleed, the cultures reached new steady-state VCDs; however, these VCDs were lower than the VCDs observed prior to the long-duration DO stress. Despite the slow recovery from the long-duration DO stress, the cell specific productivity (q_P) for the centrifuge perfusion mimic cultures stabilized after day 22 at ~27 pg/cell·day for the remainder of the cultures (Fig. 6**c**). Interestingly, the same q_P was observed from day 22 onward for the gravity settling perfusion mimic cultures ($p > 0.05$), yet the q_{O_2} values were significantly higher for the centrifugation cultures compared to the gravity settling cultures in the recovery phase ($p \leq 0.05$). Further, the gravity settling q_{O_2} values were similar between recovery and the pre-long-duration DO stress phases.

For the gravity settling cultures, the large cell bleeds allowed sufficient time to recover from the DO stress, and the gravity setting cultures maintained ~15 million cells/mL and had cell viabilities close to 90% until harvest. Further, the cell specific productivity for the gravity settling and centrifugation perfusion mimic cultures were the same, but were both higher than the fed-batch cultures (~22 pg/cell·day) ($p \leq 0.05$). These high cell specific productivities indicate that both perfusion mimics maintained a more favorable cell culture environment, that promoted higher protein production per cell, even following a significant long-duration DO stress [8]. These q_P and cell viability results support the hypothesis that large cell bleeds can be used to aid culture recovery for perfusion cultures after a moderate DO stress [25].

3.2.5. *Amino acid profiles*

Amino acid concentrations for both perfusion mimic cultures were

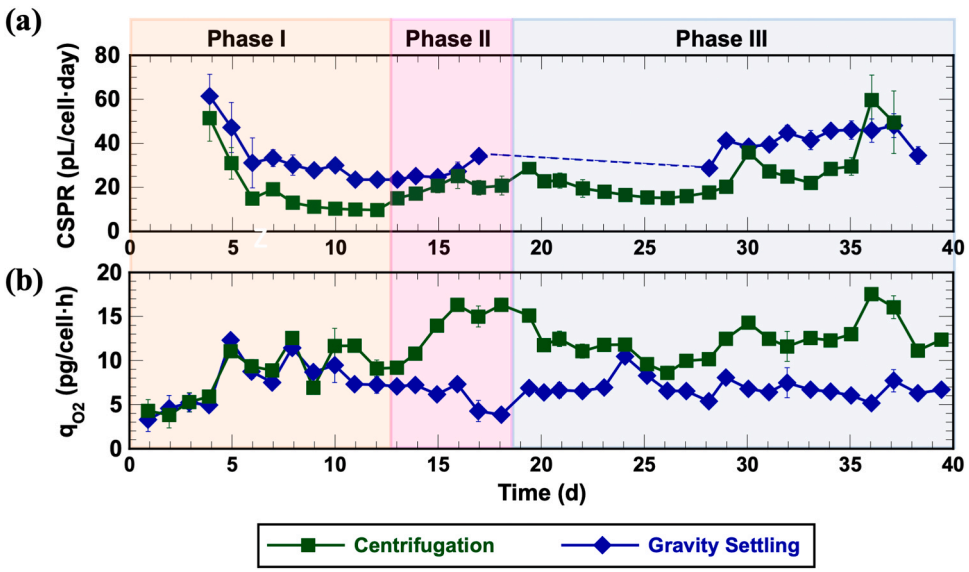

Fig. 7. Cell specific rates profiles for the two perfusion mimic protocols conducted in the ambr250 HT bioreactor system. (a) Cell specific perfusion rate (CSPR); (b) Cell specific oxygen uptake rate (q_{O_2}). Phase I: Batch and Perfusion; Phase II: DO Stress; Phase III: Recovery. The centrifugation perfusion mimic cultures (N = 2) are shown as green squares. The gravity settling perfusion mimic cultures (N = 2) are shown as blue diamonds. Error bars represent the standard deviation.

measured daily to monitor consumption patterns (Figure S3). For the centrifugation perfusion mimic cell cultures, aspartic acid, asparagine and cystine were rapidly consumed by day 3. The levels for these three amino acids briefly increased when fresh media was added on day 3, but were subsequently rapidly consumed. Furthermore, glutamate was also found to be entirely consumed from day 20 through day 35. In contrast, for the gravity settling perfusion mimic cultures, only asparagine was found to be consumed entirely after day 7. All other amino acids had adequate concentrations in both perfusion mimic cell cultures. As expected, in the centrifugation perfusion cell cultures, due to the higher cell densities and the associated higher metabolic demand, the levels of most other amino acids were lower than that of gravity settling perfusion cell cultures. Only glutamine levels were found to be considerably higher in the centrifugation perfusion mimic cell cultures, also as expected, since generated from glutamate, which is fed, and has been previously observed for this and other CHO cell strains [56–58].

3.3. Comparison of perfusion mimics in ambr250 with fed-batch and ambr15

The final cumulative titers on day 39 for the centrifugation perfusion mimic cultures and the gravity settling perfusion mimic cultures were 6.9 g ± 0.5 and 2.2 g ± 0.04, respectively ($p \leq 0.05$) (Fig. 6**b**), which were significantly higher than obtained for the fed-batch cultures cumulative titers of 0.5 g/L ($p \leq 0.05$). As expected the average titers for the perfusion mimics day 5 to day 39 for the centrifugation (1.29 ± 0.48 g/L) and the gravity settling (0.66 ± 0.21 g/L) were lower than the final fed-batch culture titers (2.04 ± 0.05 g/L). Also recall, these average titers for the perfusion mimics include the long-duration DO stress, but the fed-batch cultures were not subjected to the DO stress. The average titers observed agree with literature reports where perfusion and fed-batch were directly compared by cell line and media [8,24,48,59].

The first reported ambr15 semi-continuous study did not use gravity settling or centrifugation, but simply removed a fraction of the culture broth and replaced the volume with fresh media [60]. Yet, they were able to demonstrate that the use of the ambr15 could aid high throughput comparisons to evaluate media and different cell lines [60]. Gagliardi et al. used a centrifugation method to screen clones in a perfusion mimic ambr15. Even though stable VCD were only maintained for 4 days, they demonstrated the ambr15 as a perfusion mimic could provide a high throughput clone screening tool [29]. Sewell et al. and Kreye et al. used gravity settling to conduct perfusion mimics in the ambr15 [27,28]. Both were able to reach stable VCD and noted pH and DO disturbances, much like was observed for the ambr250 perfusion mimics in this study. Sewell et al. also characterized cell bleed and settling times, where the cell bleed increased for shorter settling times [27]. More recently, Jin et al. used the ambr15 with gravity settling to conduct media screens and obtained results similar to their in-house benchtop bioreactors. They also confirmed that the ambr15 gave better results; long stable VCDs, compared to the vented 50 mL conical tube model [30]. All of these perfusion mimics in the ambr15 noted lactate between 1 and 2.5 g/L [27–30], similar to what was observed in the ambr250 perfusion mimics.

Direct comparison of VCDs and titers between the ambr15 and ambr250 perfusion mimics is difficult, as the cell lines and media were not always described other than as in-house cell lines and media [27–30]. However, each of the studies did compare their results to benchtop or other fed-batch systems, with acceptable outcomes to adopt the ambr15 perfusion mimics. As was noted in this study and the ambr15 perfusion mimic studies, the gravity settling perfusion mimic is less labor intensive, as the media removed can be programmed to be automatic. In all cases, they concluded the ambr15 provided good alternatives to benchtop bioreactors for clone or media screening.

As the proof-of-concept ambr250 perfusion mimics were being developed, labor had not been considered in the design and manual media removal was used due to faster speed to remove and replace media in 50 mL aliquots with a vacuum-driven battery-powered pipettor. In future work, the robotic Liquid Handling (LH) arm could be used for media removal for both perfusion mimics. For the centrifugation system, the LH arm can remove 10 mL in 2.5 min cycles, thus to remove 100 mL once a day (~0.5 vvm), the process would take about 25 min. The vessels can be staggered to allow each vessel to be completed prior to the addition of fresh media from the pump. After the centrifugation step has been completed, the resuspended cell can be returned to the vessel by the LH arm This approach take about 1.25 hours, however, the labor is reduced to transporting the cells to the centrifuge and resuspending the cells in fresh media. As the gravity settling perfusion mimics also require the agitation and aeration to be stopped to allow settling, by using calculated settling times, the LH arm could be used to remove

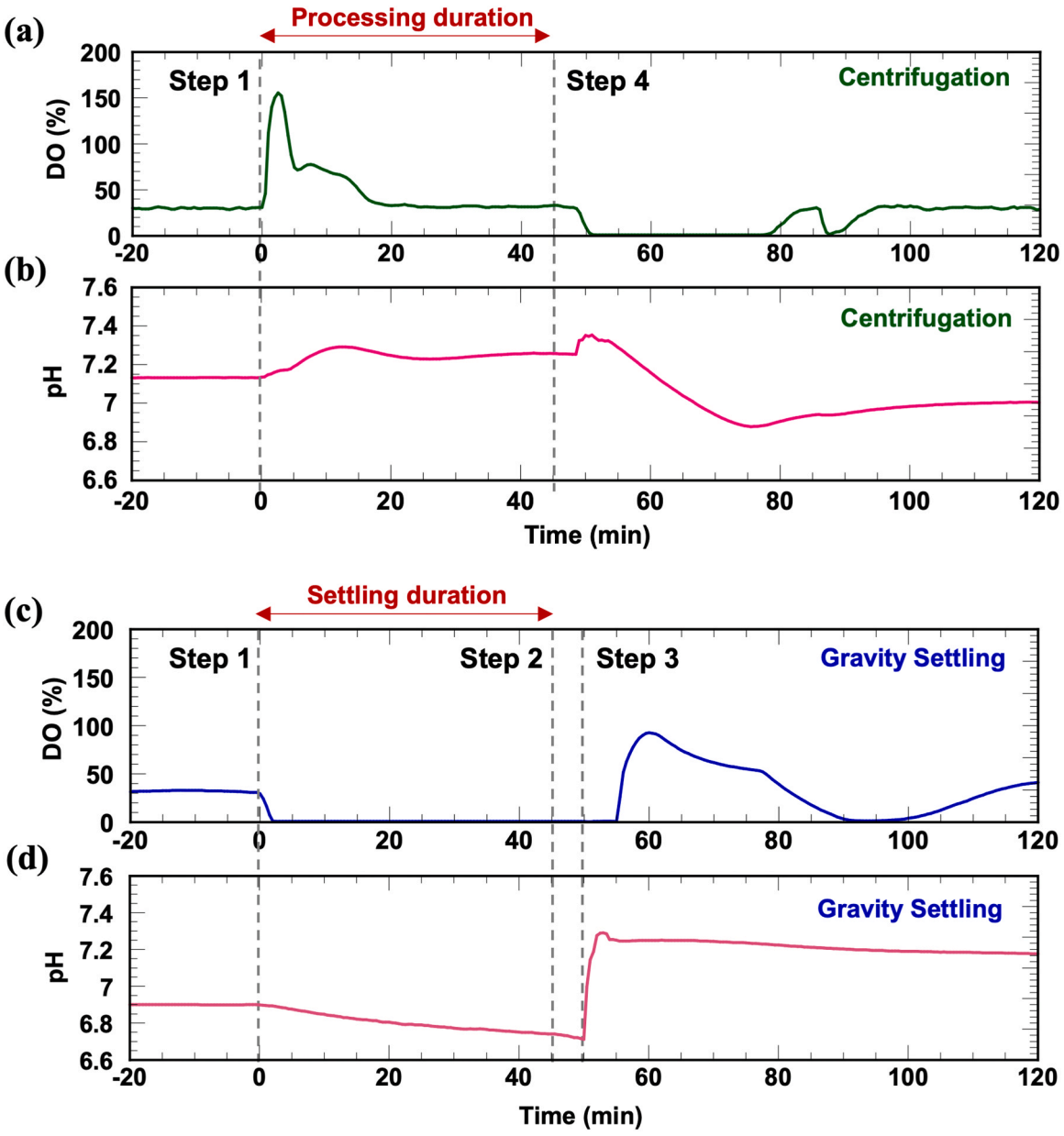

Fig. 8. Example DO and pH profiles for the perfusion mimic operations in the ambr250 HT bioreactor system. Centrifugation perfusion mimic culture: (a) DO and (b) pH. Gravity settling perfusion mimic culture: (c) DO and (d) pH. Centrifugation (Step 1: Cells removed. Step 4: Resuspended cells transferred back to the bioreactor). Gravity (Step 1: Agitation/aeration stopped; cells settled. Step 2: Fresh media added to bioreactor). Step 3: Agitation/aeration resumed). Step numbers correspond to the steps shown in Fig. 1 for the centrifugation and gravity perfusion mimic protocols. The DO and pH profiles shown correspond to day 22. Times are aligned to the beginning of each process.

10 mL from each vessel at predetermined depths over the 40 min settling time [27]. Each round of removed would increase the tip depth to follow the clarified supernatant level. Once the last volume has been removed, agitation and aeration can resume and the pump or LH can be used to add fresh media. The automated feed pumps can operate up to 100 mL/h, which is longer than would be needed by the LH arm, but would free up the LH to move to the next vessels. Therefore, utilizing the ambr250 LH arm and internal pumps could be used to reduce hands-on labor, but might extend the time needed per media exchange steps.

Overall, this study has demonstrated that ambr250 centrifugation and gravity settling perfusion mimics can obtain high VCDs, maintain cell viability, result in high cumulative titers, and sustain high cell specific productivities. Additionally, the perfusion mimics demonstrated that large cell bleeds can aid recovery after a DO stress to enable a period of exponential growth. As the ambr20 centrifugation perfusion mimic protocol reached higher VCDs, this protocol could be used to evaluate media and compare feeding strategies. Since the ambr250 has the capability to provide feeds via pumps, and the ambr15 relies on pipetting to deliver feeds, the ambr250 would be more amiable to non-bolus feeding studies than the ambr15. The more stable gravity settling perfusion mimic protocol could be used to assess genome and product stability during cell line development. Additionally, these small-scale perfusion mimic protocols could be used in other non-perfusion outfitted systems, such as the ambr250 modular bioreactor system and DASGIP parallel bioreactor systems. These standard bioreactor configurations are more commonly available, enabling perfusion experiments to be examined in a wider range of laboratories.

4. Conclusions

Small-scale models in CHO cell cultivation play a vital role in the development and characterization of biopharmaceutical production processes. However, unlike batch or fed-batch processes, economic DO and pH controlled small-scale perfusion mimics are less frequently used. In this proof-of-concept study, two small-scale perfusion mimic protocols were examined, first in shake flasks and then standard bioreactors, both without cell retention devices. Similar to the vented 50 mL conical tube and the ambr15 perfusion mimic models, centrifugation and gravity settling perfusion mimic protocols were examined, respectively. The centrifugation perfusion mimic protocol was first conducted in shake flasks to select a media formulation that could support high VCDs. Then, using the 10% feed media formulation, the centrifugation, and gravity settling cultures were compared. These shake flasks were also configured with optical DO and pH sensors for monitoring only. These uncontrolled shake flasks clearly demonstrated that these perfusion mimics resulted in cultures with high VCDs and good titers; however, the system was unable to support the oxygenation and pH control needed. To address DO and pH control, standard bioreactors were then examined.

Once the media formulation was selected, the standard ambr250 HT bioreactors without a cell retention device were used to compare the centrifugation and gravity settling protocols with each other and a standard fed-batch protocol. The perfusion mimic protocols conducted in the ambr250 HT bioreactor system were able to attain higher VCDs relative to the fed-batch protocols and higher cumulative titers. The centrifugation perfusion mimic cultures reached higher VCDs and cumulative titers overall, while the gravity settling perfusion mimic cultures had more stable VCDs and cell viabilities overall. Both perfusion mimic cultures were relatively unaffected by the DO and pH disturbances due to the protocols.

In order to examine culture responses to a long-duration DO stress, the perfusion mimic cultures were subjected to a 5-hour DO stress. Despite the initial negative impact on culture VCD and cell viability, the perfusion mimic cultures recovered, where recovery was aided by large cell bleeds, and these cultures were maintained for 39 days. While the centrifugation perfusion mimic protocol resulted in a higher cumulative titer, the gravity settling perfusion mimic protocol resulted in a more stable process. These results indicate that a centrifugation perfusion protocol in standard bioreactors could be used to compare media formulations and feeding strategies, while the gravity settling perfusion mimic protocol is well-suited to study cell productivity stability. The elimination of a cell retention device enables these perfusion strategies to be conducted on small-scale unmodified bioreactors, thus providing alternate and economic small-scale platforms to study perfusion cultures.

Ethical Compliance

The manuscript does not include any data or description of human or animal patient data.

CRediT authorship contribution statement

Abiageal Barton: Investigation, Conceptualization. **Srikanth Rapala:** Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Sarah Waterman Harcum:** Writing – review & editing, Supervision, Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests Sarah W. Harcum reports financial support was provided by National Science Foundation. Sarah W. Harcum reports financial support was provided by Advanced Mammalian Biomanufacturing Innovation Center. Sarah W. Harcum reports financial support was provided by Amgen Inc. 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.

Acknowledgments

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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.2024.109332.

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