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Research review paper

Perfusion mammalian cell culture for recombinant protein manufacturing – A critical review

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ABSTRACT

The manufacturing of recombinant protein is traditionally divided in two main steps: upstream (cell culture and synthesis of the target protein) and downstream (purification and formulation of the protein into a drug substance or drug product). Today, cost pressure, market uncertainty and market growth, challenge the existing manufacturing technologies. Leaders in the field are active in designing the process of the future and continuous manufacturing is recurrently mentioned as a potential solution to address some of the current limitations. This review focuses on the application of continuous processing to the first step of the manufacturing process. Enabling technologies and operation modes are described in the first part. In the second part, recent advances in the field that have the potential to support its successful future development are critically discussed.

1. Introduction

Modern industrial production of recombinant proteins in mammalian hosts is challenged by technology, regulatory and market changes. If the field has been in constant evolution since the first biological drug manufactured in recombinant mammalian cells was commercialized in 1986 (Ecker et al., 2015; Wurm, 2004), a more profound mindset evolution is being discussed for some time among the scientific community: the transition of traditional batch steps into continuous and integrated manufacturing (Farid et al., 2014; Karst et al., 2018; Konstantinov and Cooney, 2015; Langer, 2011; Sawyer et al., 2017a). This transition is encouraged by health authorities such as the FDA (Allison et al., 2015; Chatterjee, 2012; Nasr et al., 2017). If it is clear that interest in continuous cell culture is raising, many questions about the true potential and risks of the technology seem to refrain this decade long trend from booming. This review will focus on the upstream aspects of continuous technologies for biomanufacturing to help to understand the real potential and the remaining challenges for perfusion. This operation mode is, it will be discussed, considered as the primary solution for continuous cell culture.

The most common cultivation modes used in biomanufacturing are fed-batch and perfusion (Fig. 1). The use of one or the other technology depends on different factors linked to the protein or the host (Kadouri and Spier, 1997). Cells are cultivated either attached on carriers or in suspension. The easiest mode to operate is probably the batch bioreactor. After inoculation, cells grow and produce until a limitation due to media consumption is reached and cell density starts to decrease. The second very common process is fed-batch where nutrient limitations are prevented by adding highly concentrated feeds at different time points during the cultivation. The culture duration is therefore longer than in batch mode and the final productivity is increased. A simple way to have a continuous process is the so called chemostat process. Media is added at a constant flowrate and the bioreactor content is removed at the same flowrate, without any cell retention (Henry et al., 2008). This strategy is rather common for microbial fermentation because of the high division rate of bacteria. Finally there is perfusion. As for chemostat, there is a constant in and out flow but the cells are now retained inside the bioreactor. This opens a number of different operation strategies that will be discussed in this review.

Perfusion is not exactly new for cell culture (Croughan et al., 2015). From the early 90s to today, different commercial molecules such as recombinant follicle stimulating hormone (Gonal-*f*©), interferon beta-1a (Rebif©) and factor-VIII (Kogenate-FS©) were launched using perfusion processes (Pollock et al., 2013). Most of these molecules are labile and their indication range is expressed in micrograms. In most cases, a shared feature of their respective manufacturing processes is that cells are adherent. Spin filters, fixed bed or gravity settlers are used for cell retention. These technologies enable an efficient separation because the cells are attached on a carrier. Physical parameters used for cell retention are particle size and density, which are both larger for the carrier than for the single cell in suspension (Pollock et al., 2013; Voisard et al., 2003).

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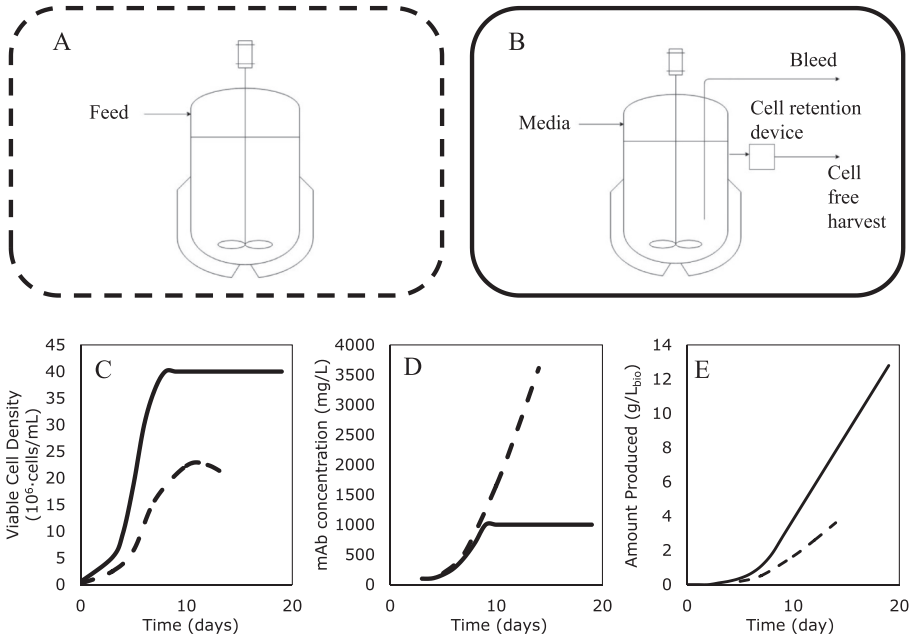

Fig. 1. Schematic of fed-batch (A) and perfusion (B) process of a cell line producing a mAb. In a fed-batch bioreactor feeds are added during the run and the product accumulates in the bioreactor. In perfusion, media is fed continuously and harvest is removed continuously. The cell retention device clarifies the harvest stream and the product is (in this example) not retained and therefore not accumulating. The bleed stream is used to remove excess cells to keep a steady concentration, in this example at 40·106 cells/mL. Cell density (C), protein concentration inside the bioreactor (D) and total productivity (E); broken line represents fed-batch and solid line perfusion.

During the same period, suspension culture technologies (expression system, media, process control) improved tremendously (Chu and Robinson, 2001; Lim et al., 2010). The yield of a batch process was doubling every five years. Therefore industry moved away from perfusion technologies to favor the high yield of batch cultures (Langer and Rader, 2014; Meuwly et al., 2006). Today the industry standard for the production of stable proteins such as monoclonal antibodies (mAbs) is a fed-batch process in stirred tank bioreactors of up to 20 kL (Moyle, 2017). These cultivation vessels were extensively used for bacterial fermentation and can provide very high mixing and mass transfer rates. They also provide high flexibility for the working volume and can be used for different cell types and operation modes (Rodrigues et al., 2010). Merck KGaA on its main production site in Corsier-sur-Vevey (Switzerland) is equipped with 8 × 15 kL stainless steel bioreactors. According to the Biophorum Operations Group (BPOG), this type of facility represents a tremendous capital investment of around 500 million dollars (Sawyer et al., 2017a).

Pressure coming from the payer and the access to developing markets is driving down the manufacturing costs and the capitals for new investments (Sawyer et al., 2017a). Also, the rapidly changing market calls for manufacturing flexibility. Uncertainties due to product success, demand variability, competition, clinical trial failures, growing clinical pipeline including new classes of molecules, time to the market pressure or facilitated market adoption contribute to this trend (Croughan et al., 2015; Sawyer et al., 2017a; Zhang et al., 2017). As an example in the biopharma landscape, the mAbs market is growing at a rapid pace (Kelley, 2009). Between 2010 and 2014, 30% of the approved biopharmaceutical drugs were mAbs and in general the number of approved biologics is still constantly increasing (Walsh, 2014). Manufacturing capacity is not only challenged by new molecules entering the pharmaceutical pipeline but also by the biosimilar market. Patents of key biologics expired recently and will continue to do so. In 2005 first guidelines were published by the European Medicines Agency (EMA) and the first biosimilar was approved one year later (Li et al., 2015; Saenger, 2017). Companies working in the field need rapid development tools but also flexible manufacturing capacity. In 2013 the first mAb biosimilar (Remsima and Inflectra, biosimilar of Remicade) was approved in Europe (Bui et al., 2015; Ecker et al., 2015). Additionally, new product classes (Antibody Drug Conjugates or ADCs. Gene therapy, cell therapy) will significantly impact the demand (Sawyer et al., 2017a).

The already mentioned BPOG is a group of experts from leading biomanufacturing companies and academics that was formed to drive reflections on manufacturing changes in order to answer the mentioned challenges. Recently a technology roadmap was published giving ideas on how the manufacturing of the future could look like (Sawyer et al., 2017b). Different scenarios, to evaluate the impact of different technology changes, were evaluated. As an example, the expected decrease of the initial investment cost in ten years from now is almost a factor 10, and continuous technologies largely contribute in this direction (Sawyer et al., 2017a). To answer the need for high productivity and flexibility, one vision of the future of biomanufacturing is an end-to-end integrated and continuous process (upstream and downstream) in a single use facility (Jacquemart et al., 2016; Karst et al., 2018; Kavanaugh et al., 2017; Kelley, 2009; Kratzer et al., 2017; Moyle, 2017; Swann et al., 2017). Continuous manufacturing offers different advantages that will be discussed throughout the review. In terms of cell culture with cells in suspension, one main advantage is the intensification of the volumetric productivity resulting from the increased biomass concentration. Therefore, the intensification potential of continuous manufacturing would allow to build much smaller plants (with similar or larger productivity output), limit the capital investment and provide manufacturing flexibility. Stable operation reduces product heterogeneity, making it safer and potentially more efficient. This is giving another key argument in favor of continuous manufacturing (Karst et al., 2018, 2016a).

At this point one might ask the legitimate question why this technology, which has been around for such a long time and offers so many advantages, is not yet state of the art. The following points that may complicate the implementation of perfusion were mentioned (Croughan et al., 2015): mixing difficulties due to high cell densities, high cost and time demand for process development and genetic instability of the cells. Others also stress the increased demand for oxygen transfer (Xu et al., 2017b) and the scalability of the cell retention device to the commercial size (Lin et al., 2017). In addition, one has to consider that process development efforts during the past decade were mainly focused on fed-batch processes. Scale-down models, decisional tools, equipment and procedures are currently in place for fed-batch in most companies. For perfusion to be successful, vendors and manufacturers will have to adapt their platforms and capabilities to achieve similar robustness as the current standards. All things considered, establishment of perfusion does not appear to be such a trivial change, although

it may be necessary to move into integrated continuous manufacturing.

In this review, recent advances in the development of perfusion technologies that address these limitations are discussed. First, the challenge of cell retention is discussed briefly to understand how enabling technologies helped to regain interest in continuous cell culture. Then, different applications of perfusion are introduced as this technology can play a role in different stages of manufacturing. The last part focuses on the process development itself. In particular, we review the recent advances in perfusion technology that help to accelerate process development timelines, to optimize production performance in terms of productivity and reliability, to improve the product quality.

2. Perfusion cell culture

Let us first introduce the basic parameters that describe the operation of a perfusion process. The media exchange rate can be defined either by the perfusion rate (P, Eq. 1), expressed in vessel volumes of media per bioreactor volume per day (vvd^{-1}) or by cell specific perfusion rate (CSPR, Eq. 2) expressed in picoliters per cell per day (pL_{media}/cell/day). Note that the perfusion rate represents the operational volume but does not give any information on the cell density, while the cell specific perfusion rate (CSPR) is the media consumption normalized to the cell density, and therefore gives an indication about the performance of the media. A lower CSPR means more cells can be sustained with a certain amount of medium. Another parameter that needs to be mentioned here is the bleed rate. This parameter corresponds to the cell biomass removed from the bioreactor in order to maintain a constant cell density value equal to the desired set-point (Fig. 2). Since this stream is wasted with no recovery of the target protein, in order to maximize the process yield, its rate should be minimized (Deschêne et al., 2006; Lin et al., 2017). The performance and yield of a process depend on the different flowrates. An increased perfusion rate generally enables the generation of more biomass and thus more product. The faster the cells grow, the larger is the bleed rate, and the smaller the yield. Stable operation should therefore be defined in a range were the cell density is large enough to achieve an economically viable productivity, but in a state were cell growth is controlled either by nutrient limitation or other environmental factors to minimize the bleed rate. Of course all these parameters highly depend on the performance of the given cell line that imposes its own consumption and specific productivity constraints.

$$Perfusion\ rate = P = \frac{Q_{in}}{V_R}\left[\frac{L_{media}}{L_R \bullet day}\right] \quad (1)$$

$$Q_{in} = media\ volumetric\ flow\ rate\left[\frac{L_{media}}{day}\right]$$

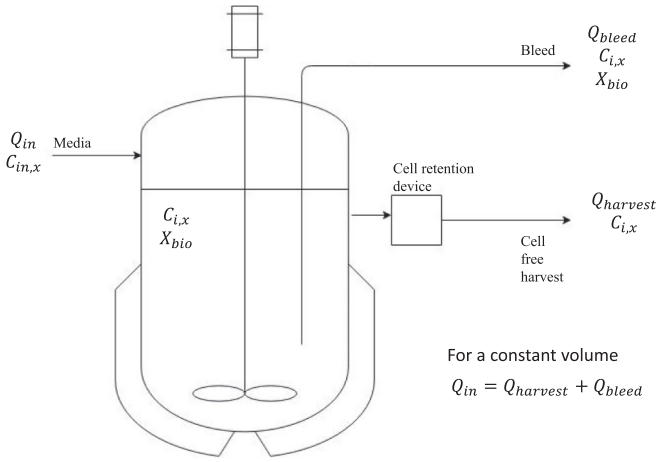

Fig. 2. Perfusion bioreactor set-up with media, harvest and bleed stream.

$$V_R = bioreactor\ working\ volume\ [L_R]$$

$$CSPR = \frac{P}{VCD}\left[\frac{pL_{media}}{cells \bullet day}\right] \quad (2)$$

$$VCD = viable\ cell\ density\left[\frac{10^6 \bullet cells}{L_R}\right]$$

2.1. Cell retention devices for suspended mammalian cells

Existing cell retention devices are based on either cell size or density. They include filters (cross-flow, hollow fibers), centrifuges, gravity settlers of different geometry and acoustic wave separators (Chatterjee, 2012; Konstantinov and Cooney, 2015; Voisard et al., 2003). Each of them has advantages and drawbacks that are well discussed in the cited reviews. The retention devices that receives the most attention today are tangential filtration systems such as the alternating tangential flow (ATF) and the tangential flow filtration (TFF) hollow fiber systems. The ATF benefits from a self-cleaning effect induced by the alternating flow (Bonham-Carter and Shevitz, 2011).

A study from Karst et al. (2016b) compares different features of the commercially available ATF (Repligen, USA) and a homemade tangential flow filtration device using the same hollow fibers. Perfusion runs with three operating set-points at 20, 60 and 40 million cells/mL (in this order) were performed and repeated using both ATF and TFF. A lower maximum shear stress was measured when using the ATF. The quality of the produced mAb was not affected by the retention device but a significantly larger portion of product was retained in the bioreactor with the TFF (up to 50% compared to 10% with the ATF). Both devices were also compared by Clincke et al. (2011) using wave bioreactors. This study showed that the very high cell densities were limited by the vacuum capacity of the ATF device. The maximum cell densities reached before failure for ATF and TFF were respectively $1.32 \cdot 10^8$ cells/mL and $2.14 \cdot 10^8$ cells/mL, which are still lower by a factor 5 compared to the theoretical maximum cell density calculated by Ozturk (1996), based on packed tissue geometries and which was equal to 10^9 cells/mL. Regarding volumetric occupancy, the potential to reach higher cell densities is therefore still significant. Nevertheless, other parameters like mixing capacity, oxygen demand and fluid viscosity might be limiting at some point.

A reliable retention device should not only prevent cells from leaving the reactor in the harvest stream, but also satisfy some important operation requirements. These include avoid product retention, operate for a reasonable range of perfusion rate values, provide sufficient aeration to the cells while preventing harmful shear, operate across scales from process development to manufacturing, and finally guarantee a sufficiently long operation without failure, thus avoiding for example filter fouling (Bonham-Carter and Shevitz, 2011; Voisard et al., 2003). These aspects have been considered in various studies where different cell retention devices have been compared. For example, it was shown that when using an ATF instead of an internal spin filter (ISF), the maximum cell density and the maximum perfusion rate could be increased (Bosco et al., 2017). Wang et al. (2017) showed that replacing a traditional peristaltic pump by a low shear centrifugal pump to operate a TFF, the level of sieving was similar to that of an ATF. Finally, Kelly et al. (2014) recently also developed a model to predict filter fouling depending on cell density and antifoam levels. These examples show the importance of cell retention in the development of perfusion bioreactors and that currently ATF is probably the most interesting cell retention device available on the market.

2.2. N-stage perfusion cell culture

In the production bioreactor, usually referred to as an N-stage bioreactor, cell culture can rapidly reach a biomass exceeding the

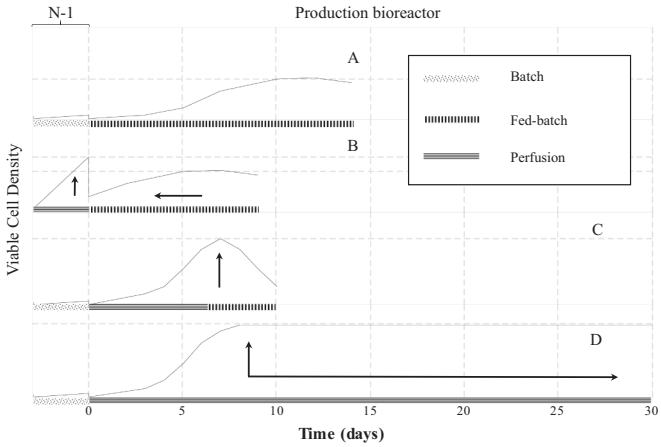

Fig. 3. Schematic of different bioreactor operation strategies for N-1 and production (N-stage) bioreactors (A) batch N-1 followed by fed-batch production bioreactor (B) perfusion N-1 with high density seeding of fed-batch production bioreactor (C) batch N-1 and perfusion used at the beginning of the production stage to increase biomass, followed by fed-batch operation (D) batch N-1 and perfusion production bioreactor with cell density control. Arrows highlight the benefit of using perfusion technology in the various cases: higher biomass for inoculation, shorter production duration (B), higher biomass during production phase (C), higher biomass and longer duration of the production phase (D).

system capacity (due to nutrient or equipment limitations) where the cell viability rapidly drops. To prevent this, a control on the cell density can be implemented. This way, the biomass is kept constant during a longer production phase. When considering both the N-1 and the N phase, different strategies can be adopted, where batch, fed-batch and perfusion modes can be alternated in different ways. A few are illustrated in Fig. 3. In the following we review a non-exhaustive list of recent examples where different approaches have been implemented to control and maintain stable, for a reasonably long time, high density perfusion cell cultures.

Semi-continuous and continuous bleeding were used based on daily cell count or on-line biomass sensors monitoring, respectively. Daily bleed induces a discontinuous saw-tooth behavior, where the cell density increases continuously between two consecutive bleeding points where it then obviously drops. This method can be used if no on-line monitoring and control is available. As an alternative, continuous bleed adjusted on a daily basis based on cell count, can be used and leads to more stable reactor behavior. This strategy differs from the semi-continuous approach because of the continuous cell removal, but is subject to variations if the cell growth rate changes in time for whatever reason. Of course the method of choice is to use an on-line biomass sensor which acts directly on the bleeding flowrate to maintain a predefined set-point value.

Dowd et al. reported 5 day long stable operation states at about $50{\cdot}10^6$ viable cells/mL (CHO DUKX-B11 producing t-PA) using an acoustic filter and a CSPR of 0.05–0.4 nL/cell/day (Dowd et al., 2003). Clincke et al. (2013) reported viable cell densities of 20 to $35{\cdot}10^6$ cells/mL at about 1.4 vvd^{-1} using a TFF. Using an ATF, 90 to $130{\cdot}10^6$ viable cells/mL using a perfusion rate of 4.5 to 5 vvd^{-1} were sustained for 2 weeks, using both: CHO and IgG. Karst et al. (2016b) reported 10 day long stable operation, both with TFF and ATF, at 20, 60 and $40{\cdot}10^6$ viable cells/mL using a perfusion rate between 1.06 and 1.44 vvd^{-1}. The chemically defined perfusion media was enriched with a high density feed with different proportions depending on the targeted perfusion rate. Warikoo et al. (2012) reported stable operation at 50 to $60{\cdot}10^6$ viable cells/mL for about 50 days (CHO, mAb) and at $50{\cdot}10^6$ viable cells/mL (CHO, rhenzyme) using ATF and a CSPR of 0.04 to 0.05 nL/cell/day. Finally, Xu and Chen (2016) reported two stable operations at 42 and $68{\cdot}10^6$ cells/mL respectively, using manual bleed and the lowest reported CSPR of 15 and 23 pL/cell/day. More specific information about the product, the retention device, the experiment duration and the reactor performance are summarized in Table 1.

Xu et al. (2017a) compared the performance of different processes using a very similar media in order to provide a fair comparison in their productivity. In particular batch, fed-batch (low and high seeding concentrations), concentrated fed-batch and perfusion processes were compared. It was found that the specific cell productivity remains in a similar range for all production modes, and therefore the reactor final volumetric productivity is defined by the achieved cell density. For perfusion, high seeding and fed-batch the observed productivity values were 2.29 g/L/day, 2.04 g/L/day and between 0.39 and 0.49 g/L/day respectively. This demonstrates that continuous technologies can lead to process intensification.

2.3. Steady-state or stable operation

The set-up sketched in Fig. 2 which includes on-line monitoring and control of the bleed flowrate allows continuous operation at a constant cell density. As a result, the environment inside the bioreactor reaches a constant state with a fixed biomass or cell density and is usually referred to as a *steady-state* operation. This state is typically reached after about 3 or 4 residence times after starting up the bioreactor. According to its definition, at steady-state there is no product accumulation inside the system that is the sum of any species entering (IN), exiting (OUT) and being produced/consumed is zero. Accordingly all concentrations and physical parameters in the reactor are constant in time and, to the extent that this is a well stirred vessel, these quantities are also uniform through the entire reactor volume. In a system governed by chemical

Table 1
Example of perfusion bioreactor biomass control for stable operations reported in literature with CHO cells.

Ref	Product	Retention device	Biomass control	Cell densities (10^6 viable cells/mL)	Perfusion rate (vvd^{-1})	CSPR (pL/cell/day)	Steady-state duration (days)
(Dowd et al., 2003)	t-PA	Acoustic filter	On-line biomass sensor, dielectric spectroscopy	~ 50	2.5–20	50–400	~5
(Clincke et al., 2011)	IgG1	TFF/ATF	Daily	20–35	~1.4	40–70	5 to 20
				90–130	~4.5–5	38–50	14
(Warikoo et al., 2012)	mAb	ATF	Off-line measurement and/or online capacitance probe	50–60	2–3	40–50	~ 50
	rhEnzyme	*ATF*		*50*			
(Karst et al., 2016b)	IgG	ATF	On-line biomass sensor, dielectric spectroscopy	20	1.44	72	~ 10
				60	1.06	18	
				40	1.28	32	
		TFF		20	1.42	71	~10
				60	1.10	18	
				40	1.28	32	
(Xu and Chen, 2016)	mAb	ATF	Daily	42	1	23	~30
				68		15	

reactions, a steady-state assumption can be well accepted and understood. In a system involving living organisms, this notion becomes more complex and should be taken with care.

Next to the chemical reactions or the component addition/removal due to the different streams mentioned above, in a bioreactor some other processes, linked to the cell biology and usually characterized by slower kinetics, can take place. Biological reaction rates are not constant and might change over time during the process. Because the operation time extends to days, weeks and months, such slow processes become eventually a non-negligible component that typically leads to a slow drift during the run. For example, the specific cell productivity can decrease over time due to some kind of aging process. It is therefore important to use the term *steady-state* cautiously when a perfusion bioreactor system is described, and always keep in mind that the overall system could always be subject to a slower drift away from the described steady-state.

Steady-state operation was demonstrated for example using metabolomics by Karst et al. (2017a, 2017b). Concentrations of metabolites such as nucleotides, nucleotide sugars and lipid precursors were observed at different operation states. Metabolic steady-states could be observed after a period of 3 days in stable operation. Interestingly, in another study it was shown that steady-state conditions for intracellular processes like glycosylation were reached only after a transient of about 6 to 7 days (Karst et al., 2016a). Transcriptomics and proteomics were used by Bertrand (2018) to investigate the dynamics of extra and intra cellular metabolites. Three groups of transcripts were identified. The first group reached steady-state after 3 days (majority), the second after 7 days (medium group), and the last was never stable (minority). This demonstrates that even if a stable operation was reached, during which many or even most metabolites achieve an intra or extra cellular steady-state, there were still some biological events, probably with very minor macroscopic effects, that were varying over the course of the run.

Another common use of the term *stable operation* is to describe an operation that maintains critical process parameters and quality attributes in a well-defined range of values as for example in (Nasr et al., 2017). It is shown that small perturbation can deviate the system from the stable operation set-point but with a well-defined control strategy they can be rapidly corrected to minimize the impact on the product quality. This is clearly a definition of the term stability which indicates the range of the reactor conditions providing product quality attributes within predefined boundaries or specifications.

2.4. Integrated continuous manufacturing

The full potential of perfusion upstream can probably be best appreciated in the context of a fully continuous end-to-end integrated manufacturing unit, where these are directly connected to continuous downstream units (Karst et al., 2018). Although capture and further polishing steps are in fact usually operated in the batch mode using single column units, continuous multi columns units are also available (Pfister et al., 2018; Steinebach et al., 2016b). Although the aim of this review is not to focus on purification technologies, a few comments are needed to clarify some relevant issues about process integration and the role played by perfusion bioreactors. In particular, we consider in the following some case studies referring to the entire process, both up and down-stream, in order to help to identify the critical parameters that impact manufacturing costs.

Continuous operation is advantageous for capture and polishing steps. It was shown for example that the resin capacity utilization could be increased 2.5-fold by using a two column continuous unit instead of a batch single use column one (Steinebach et al., 2016a), and similar performances where confirmed for the same unit but at the pilot scale more recently (Angelo et al., 2018). Modeling techniques can be used to optimize capture in batch and continuous mode and compare the respective performances (Hilbold et al., 2017). The results are of course case dependent but for the specific system considered, Baur et al. (2015) showed that using a two column continuous unit, the productivity at a given capacity utilization was increased by 40%, while for a given productivity the capacity utilization was increased by 25% compared to the batch mode. Increasing the resin utilization for a capture step is of interest especially for mAbs since the protein A resins are very expensive. Modeling studies have also investigated how the number of columns influence the performance of this step. In general, the choice of the number of columns used for continuous operation is not relevant in terms of process performance compared to the huge benefit gained when moving from batch to continuous operation (Baur et al., 2016).

The feasibility of a long term operation connecting continuous cell culture and continuous capture was demonstrated in a few cases (Karst et al., 2016c; Steinebach et al., 2017, 2016a). In particular, Warikoo et al. (2012) produced mAbs and recombinant human enzymes (stable vs less stable molecule) using continuous cell culture and capture. The cell density in the production phase reached $50{\cdot}10^6$ viable cells/mL. The volumetric productivity for cell culture compared to fed-batch was 5-fold and 40-fold higher for the mAb and the enzyme, respectively. For the mAb capture, resin utilization was reported to be 20% higher whereas the buffer consumption was reduced by 25%. Individual column size was decreased 75-fold compared to the batch mode. For the enzyme capture, resin utilization was shown to be 50% higher whereas the buffer consumption was lowered by 46%. Individual column size was reduced 50-fold compared to the batch mode (Warikoo et al., 2012). Godawat et al. (2015) also reported higher productivities in a feasibility study of end-to-end processing including continuous cell culture, capture, viral inactivation and intermediate and polishing step. The volumetric productivity increase was 10-fold for upstream and 6-fold for downstream. Resin utilization was raised by 25% and the buffer consumption was decreased by 20%. Finally, the column size was decreased 20-fold. These examples show the feasibility of integrated continuous manufacturing. For the examined systems, these studies demonstrated significant improvements in terms of bioreactor volumetric productivity (but not media consumption which actually increased), resin capacity utilization, buffer consumption and in both bioreactor and column size compared to batch operation. Finally, the reduction in the number of unit operations and of unnecessary steps such as hold tanks has also to be considered (Godawat et al., 2015).

In general, economic and environmental benefits of continuous biomanufacturing are not so obvious and a careful comparison with the corresponding batch technologies is needed for each system. Different studies try to quantify the difference of capital expenses (CAPEX), operational expenses (OPEX) or cost of goods (COG) between batch and continuous operations. Walther et al. used a Monte Carlo simulation to evaluate CAPEX and OPEX for different scenarios. They modeled the costs for the production of a stable and highly demanded protein such as mAbs (estimated 200 kg/y) and for a more labile and less demanded fusion protein (20 kg/y). Most of the savings attributed to integrated continuous biomanufacturing were due to CAPEX and the main driver of OPEX cost was media consumption. OPEX savings also correlated with the total protein production (Walther et al., 2015). These results indicate that perfusion needs media optimization to maximize the process productivity, minimize the CSPR and eventually decrease media cost. Similar results in terms of cost drivers were reported by Pollock et al. (2013). Two manufacturing strategies were compared: one assuming the use of a spin-filter perfusion and another using an ATF. Cost benefits only appear with the ATF that has the potential to increase the biomass sufficiently to increase the volumetric productivity. The best case scenario for perfusion was again for higher market demands (estimated up to 1000 kg/y). Other aspects of the process such as water usage, energy consumption and solid waste were also considered in a life-cycle and cost of good assessment by Bunnak et al. (2016) to compare fed-batch and perfusion based manufacturing. They found that the COG was similar but that the demand for a perfusion process was higher in energy (17%), water consumption (35%)

and CO_2 emission (17%). The results of the mentioned studies are of course highly dependent on the different assumptions but they give interesting insights into what the key levers are to modulate both cost and environmental impact and consequently select batch or perfusion based manufacturing technologies.

2.5. Other applications of perfusion

Perfusion technologies are also used in many situations which typically come before the production stage. In this case the bioreactor is only used to generate biomass without the need to maintain or control a certain state. For example, highly concentrated cell pools are often used to prepare novel cell banks improving seed train strategies. The biomass accumulation potential of this technology is also used for N-1 bioreactors to seed fed-batch processes at high densities, with cells still in the exponential phase. These types of strategies are often used to intensify current processes in existing facilities.

2.5.1. Cell banking and seed train

Reduction of the seed train time is a relevant aspect of process intensification and optimization. A larger initial cell mass decreases the time required to build the necessary biomass for seeding an N stage bioreactor. To achieve this, there are only two options: increase the volume of the initial inoculum, or increase its concentration. To increase the inoculum volume, high density cryotubes or cryobags and well controlled freezing procedures were used (Table 2). Perfusion technologies have been used for generating high density cell banks. Novel approaches for cell banking and seed train strategy have been described by Heidemann et al. (2002); Seth et al. (2013); Tao et al. (2011) and Wright et al. (2015).

Heidemann et al. (2002) were able to reduce the interval between thawing and inoculation by 25 to 30 days using cryobags. Cells were prepared from perfusion runs at $20{\cdot}10^6$ cells/mL and $40{\cdot}10^6$ cells/mL. A 2 L bioreactor could directly be inoculated at $1{\cdot}10^6$ cells/mL thus reducing the seed train to a single bioreactor followed by a stepwise volume increase. Seth et al. (2013) used 20 L perfusion bioreactors, equipped with an ATF for cell retention, to produce 150 mL bags at densities of $> 70{\cdot}10^6$ cells/mL. By using one or multiple bags they were able to directly seed the N-3 stage of their process thus eliminating the usual prior steps and significantly reducing the needed infrastructure. In both cases, using frozen bags, no open manipulation was required thus reducing the contamination risk (Heidemann et al., 2002; Seth et al., 2013).

Tao et al. (2011) reduced the seed train time by 9 days using a highly concentrated ($100{\cdot}10^6$ cells/mL) 5 mL cryotube. In particular, the usual operation in shake flasks was avoided since the cell density was large enough to directly seed a 20 L wavebag. To reach these high cell densities, perfusion in a single-use wave bag equipped with an internal floating filter was used. Starting from a highly concentrated 4.5 mL cryotube, Wright et al. (2015) also demonstrated a very efficient seed train strategy. In their process, perfusion was used in the N-1 stage (and N stage) to rapidly increase the cell mass.

Yang et al. (2014) published a proof-of-concept study where an N-1 perfusion bioreactor was used to generate $> 40{\cdot}10^6$ cells/mL and subsequently seed fed-batch cultures at $10{\cdot}10^6$ viable cells/mL. In this way the growth phase duration in the production bioreactor was decreased and room for more runs per year was created, resulting in increased manufacturing capacity. Although perfusion increases the complexity of the seed train process, the short operation time and the remarkable cell density reached in these works did not result in filter or oxygen transfer limitations, which are the typical concerns in high cell density perfusion operations.

Pohlscheidt et al. (2013) developed a perfusion N-1 strategy using an inclined settler for cell retention. They report a growth to $15.8{\cdot}10^6$ cells/mL in a 3000 L scale bioreactor. A portion of this biomass was recycled to restart an N-1 run in the same bioreactor to save changeover time.

These examples show the potential of perfusion to rapidly generate cell mass for cell banks or seed train applications. The reduction of process time and the increased safety in terms of reduced contamination risk provide the basis for the main economic advantages (Wright et al., 2015).

Table 2
Freezing container type and corresponding maximum viable cell density values.

Reference	Freezing container	Maximum cell density (10^6 cells/mL)
Heidemann et al. (2002)	50 or 100 mL cryobags	20 or 40 respectively
Tao et al. (2011)	5 mL cryotubes	90–100
Seth et al. (2013)	150 mL cryobags	70
Wright et al. (2015)	4.5 mL cryotubes	100

2.5.2. Concentrated fed-batch process

Concentrated fed-batch is defined by Yang et al. (2016) as a perfusion culture using ultrafiltration, which means that the secreted protein of interest is retained and accumulated inside the bioreactor, because of the lower pore size of ultrafiltration devices. The produced protein is retained inside the bioreactor and therefore it accumulates in time. This study showed that, at least at bench scale, the manufacturing capacity could be increased by 70 to 105% with two different Chinese Hamster Ovary (CHO) cell lines expressing mAbs. The developed feeding strategy allowed the system to reach very high cell densities. Waste removal and an increased total nutrient provision pushed the limit of the system. In some cases oxygen transfer limitation appeared when a cell concentration of about $200{\cdot}10^6$ cells/mL was reached and an ATF was used as cell retention device (Yang et al., 2016).

2.5.3. Hybrid process

In this process perfusion in the production bioreactor was used only during the first 4 days of the culture to increase the biomass rapidly. After this initial period the perfusion was stopped and a continuous feed of highly concentrated nutrients was applied and adjusted daily. The study was performed with 5 CHO-k1 clones and the final productivity was increased up to 2.5 fold with respect to classical fed-batch operation. Cell densities reached between 60 and $80{\cdot}10^6$ viable cells/mL depending on the clone (Hiller et al., 2017). This approach can be used to rapidly improve the performance of an already existing production unit. The final medium consumption was relatively low (between 1 and 1.8 times the final bioreactor volume) which limits the challenges of a potential scale-up. Nevertheless, other limitations might appear with the high cell densities and the control of the bioreactor environment, typically due to oxygen transfer and insufficient mixing (Hiller et al., 2017).

3. Development for perfusion processes

Besides the reliable control of the reactor environment, that is the inlet and outlet flowrates, many engineering challenges appear when operating at high cell densities. Ozturk (1996) discuss in particular mixing, aeration, pH control and cell aggregation. The main limiting factors are well discussed by Konstantinov et al. (2006), including characteristics of the produced molecule. These include the maximum allowable residence time of the protein (which limit the ratio between the reactor volume and the perfusion rate), the maximum harvest rate (limited by the cell retention device), the maximum cell density (limited, among others, by the oxygen supply) and the minimum CSPR (limited by the media formulation and its ability to sustain cells) (Mercille et al., 2000).

In the following parts different key aspects of perfusion process development such as process monitoring and control, media

development, scale-down models, cell line development and other levers for productivity maximization are addressed.

3.1. Process control strategy

To reach a stable operation, media feed, harvest and bleed flow rates must be properly controlled on-line to maintain the desired perfusion rate, keep a constant reactor hold-up (working volume) and a constant cell density. As already discussed, real time monitoring of viable cell concentration can be used to regulate the bleed rate, and by fixing a desired CSPR value, also the perfusion rate. Direct methods such as acoustic resonance densitometry, conductivity, capacitance sensors, optical sensors, mass spectrometry and real time imaging were reported to monitor cell density (Joeris et al., 2002; Konstantinov et al., 1994). Indirect estimates are also possible through measurements of oxygen uptake rate, carbon dioxide evolution rate or the ATP-production rate. Each of these methods has advantages and drawbacks. Although the rigorous measurement of biomass and viability is not a trivial concept, the sensor currently most widely used for online biomass monitoring is based on capacitance measurements (Ducommun et al., 2001a, 2001b; Konstantinov et al., 1994). This technology only detects cells that have an intact membrane, thus giving an indication on the viable cell concentration. The basic principle of this measurement is discussed by Davey et al. (1993) while Carvell and Dowd (2006) reported a number of applications, some of which include cGMP manufacturing.

As already mentioned, semi-continuous (most often daily) bleeding strategies were used when no on-line measurement of biomass or cell density was available. This strategy was used by Clincke et al. (2011) to maintain cells around 20–35·10^6 viable cells/mL in several runs, and operational consistency could be demonstrated. Another stable operation between 90 and 130·10^6 viable cells/mL was achieved and maintained for about 2 weeks using the same set-up strategy. Karst et al. (2016b) used on-line and off-line measurements to control continuous bleed and maintain steady-states at 20, 60 and 40·10^6 cells/mL for about 10 days each. In all cases the desired stable operation was achieved, although the viable cell density profile in the presence of on-line monitoring appeared to be much smoother. These results show that, in principle, it is possible to control a perfusion run without online measurements. However, since the level of control has significant impact on the process performance and the product quality, semi-continuous approaches should be used with caution, and definitely avoided for manufacturing at commercial scale.

On-line monitoring and control of biomass with the capacitance sensor was also used by Dowd et al. (2003). Interestingly they report a drift in the relation between the on-line measurement and the off-line cell count, with the cell count being underestimated. This effect is attributed to a change in the culture dielectric properties, potentially linked to the decreasing or increasing cell volume depending on the process. This effect was also described and discussed in fed-batch studies (Ansorge et al., 2010; Zalai et al., 2015). Most likely, more than the cell volume it is the physiological state of the cell that impacts the relation between the measured capacitance and the viable cell density obtained with a standard cell counter (Opel et al., 2010).

Various online spectroscopy techniques were also used to monitor bioprocesses (Vojinović et al., 2006). These analytical methods are highly appreciated since they are non-invasive, non-destructive and can be used to monitor multiple parameters simultaneously on-line, increasing process understanding and enabling real-time feedback control of the monitored parameters (Whelan et al., 2012). The most promising of such techniques is probably Raman spectroscopy. Process parameters such as glucose, glutamate, glutamine, lactate and ammonium concentrations, total cell density (TCD), and viable cell density were monitored in fed-batch processes (Abu-Absi et al., 2011; Whelan et al., 2012). In both studies, partial least square (PLS) regression was used to fit the raw data to the off-line measurements. More recently, Mehdizadeh et al. (2015) developed a generic model with different cell lines and media and Berry et al. (2016) used Raman to control glucose concentration in their process, leading to a decreased level of protein glycation. The potential of Raman to measure on-line multiple parameters seems very promising to monitor perfusion processes. For example the perfusion rate, the bleed rate and even a separate glucose feed could be controlled by a single probe measuring VCD and glucose concentration, and fixing a desired CSPR. To the authors knowledge there has been no report yet of the use of Raman for perfusion.

3.2. Media for perfusion

The perfusion flowrate is a key parameter for process performance. It is, as already mentioned, generally defined either as perfusion rate (vessel volume per day, vvd^{-1}) or cell specific perfusion rate (CSPR, $nL{\cdot}cell^{-1}{\cdot}day^{-1}$). In general, in order to minimize media consumption, the objective is to operate the perfusion at the minimum value compatible with a stable reactor operation at a given VCD value, thus minimizing the CSPR.

Konstantinov et al. (2006) described the *push-to-low* strategy to assess the performance of a medium for a perfusion process. The idea was to first establish a steady-state and then decrease the CSPR stepwise until the VCD or viability was not sustained anymore, thus determining the minimum value, $CSPR_{min}$. The CSPR can be lowered either by decreasing the media feed flow rate or by increasing the biomass setpoint. To avoid limitations due to high cell densities, such as oxygen limitations or cell retention device fouling, it is preferred to decrease the media flow rate at a constant and sustainable VCD.

Once the ideal CSPR value is known, the perfusion rate can be controlled on-line based on the cell density or biomass. This strategy was used for a perfusion N-1 bioreactor and a concentrated fed-batch process investigated by Yang et al. (2016, 2014). It was also used by Clincke et al. (2013) to compare TFF and ATF at very high cell densities. Although large perfusion flowrates (say, $> 2\ vvd^{-1}$) can indeed support very high cell concentrations, this is generally not convenient since the required media volume can become unpractical. Very high perfusion rates might be feasible for rapid increase of biomass, i.e., cryopreservation and N-1 perfusion, but not to sustain a long term production perfusion bioreactor for economic and operational reasons.

In order to reduce the media consumption for high cell density processes it is necessary to operate on the media composition. Indeed concentrated media can be used to reduce the $CSPR_{min}$ significantly (Ozturk, 1996). In principle, if a medium is concentrated twice, the perfusion rate can be reduced by a factor two to maintain the same VCD, under the assumption that the increased nutrient concentration and the reduced metabolite removal rate will not affect the cell metabolism and in particular its productivity. Yang et al. (2016) have used this strategy for concentrated fed-batch and N-1 perfusion. For the concentrated fed-batch, the two times more concentrated-media was not ideal. The maximum cell density was higher than with other feeding strategies but the cells did not switch from growth to production. In addition, the retention system failed because of the high cell density. Using the two times more concentrated media for an N-1 perfusion bioreactor was more successful: the growth rate was in fact higher with the reduced CSPR, since the increased medium concentration favored the cell growth. The targeted cell density (40·10^6 viable cells/mL) was reached in 5 days instead of 6 or more when using other strategies (Yang et al., 2014). Interestingly, this result is in contradiction with the results of the previous fed-batch case where the cell growth rate was instead higher with the original non-concentrated medium. This is not surprising since these relations between media concentration and cell growth rate are specific to the particular cell line under examination. Waste metabolite or cell signaling might drive the cell to grow more rapidly in one set-up or in the other. It is therefore crucial to understand the characteristic behavior of different cell lines and to define which ones are more relevant for perfusion, before proceeding with the media

optimization.

Media development has been the most important aspect of cell culture development and optimization since the beginning of biomanufacturing. It became a high priority not only for process performance itself but also and more importantly for safety reasons. The first cell culture media were prepared using animal derived products (Yao and Asayama, 2017). The consequence for the patients was the exposure to many hazardous factors such as viruses and prions. Infectious risks for patients were important, especially for chronic diseases because the patients were continuously exposed to the drug (Grillberger et al., 2009). Process inconsistency due to batch heterogeneity was also a driver to suppress animal or even plant derived media compounds. This was not a trivial exercise and even today significant efforts are devoted to the identification of new components in chemically defined media that can enhance cell growth.

For example, yeast lysates were recently fractionated and characterized. Growth factors such as polyamines were identified as having a positive effect on cell culture. Based on these findings, the yeast additives could be replaced by the identified compounds while obtaining similar cell culture performances (Spearman et al., 2016). Chemically defined media are now available commercially and most of the large biomanufacturing companies develop their own formulations. Progress is still on-going in the field. Highly concentrated feed for example can be challenging because of the physical properties of some compounds that cause solubility or stability constraints. A good example was provided by cysteine and tyrosine in the case of an industrial fed-batch platform. Cysteine is not stable at neutral pH and the solubility of tyrosine is very low. Therefore derivatives of both were identified to increase their respective concentration in concentrated feeds (i.e., sulfocysteine and phosphotyrosine sodium salts) and sustain similar cell culture performance (Hecklau et al., 2016; Zimmer et al., 2014).

Concentration limitations become more important for perfusion media as they become more complex and rich. McCoy et al. investigated the stability of concentrated media designed for perfusion culture. A proprietary chemically defined media was concentrated five times and used with a feed supplement consisting of cysteine, tyrosine and folic acid. After evaluating the effect on stability of the different compounds it was concluded that the best option was to use a four part system including the five time concentrate, cysteine/tyrosine/folic acid supplement, glutamine and water. The concentrate was stable for one month (McCoy et al., 2015).

The idea of using decoupled perfusion media, that is different inlet streams so as to be able to have a better control on specific nutrient and perfusion rate needs, opens new development concepts. Yang et al. (2016) used a similar method for one of the feeding strategies of a concentrated fed-batch. The perfusion rate was fixed to 1 vvd^{-1} and was constituted by a glucose feed controlled by daily glucose measurements, a feed concentrate controlled by daily cell counts and a basal media to complete the targeted flow rate. Lin et al. (2017) describe a method were the fed-batch platform media and feeds were mixed to obtain a more concentrated medium. After finding the optimal ratio and adjusting some components concentrations and the osmolality, the depth of the medium was increased by two. This means that to sustain a defined cell concentration, only half of the perfusion rate was required in the second case. As a result, a cell culture at about $30{\cdot}10^6$ viable cells/mL was achieved at a CSPR of 100 pL/cell/day. Another example of media improvement for perfusion was the demonstration that increased pluronic concentration in perfusion media was beneficial. No product retention in the filtering device was observed when increasing pluronic concentration, on the contrary, since the viability of the culture remained unchanged, the product (and host cell proteins) retention was minimized compared to cultures with low viabilities and low pluronic concentration (Xu et al., 2017b).

The examples above show that media development is still a high priority in perfusion cell culture and indeed more work needs to be done in this direction in order to fully exploit the potential of the continuous operating mode. The media currently available on the market have been mostly optimized for fed-batch, and they are therefore expected not to be optimal for perfusion operation. For example, media components which stimulate cell growth are essential in fed-batch operation while they are not needed, if not even deleterious, in the production phase of a perfusion process. The peculiarity of perfusion can require specific media, maybe even different depending on the process phases (i.e., N-1 and N), to optimize process performance and media costs. The decoupling of different media or feed streams would allow more flexibility and maybe a better control of the process. For example, one could independently control several media components based on on-line monitoring of relevant parameters in the bioreactor. There is indeed a lot of room for improvement in all these concepts.

3.3. Scale-down models

Scale down tools are needed to support process development and subsequently to assist the operation of the production process in manufacturing throughout its lifecycle for any kind of trouble shooting or other investigation that may be needed. Lab-scale bioreactors (1 to 10 L) are generally used as scale-down models for the manufacturing scale. Validation activities are also performed in these bioreactors since they currently provide the best compromise between size, throughput and level of control. Parameters such as aeration, mixing energy input, dissolved oxygen and pH control can be well aligned to the manufacturing scale.

Other scale down systems such as deepwell plates, shake flasks, shake tubes or micro-bioreactors are commonly used for different applications in the frame of batch-like cell cultures. In Table 3 their main characteristics are summarized to understand their potential and limitations. Unfortunately there is today no scale down tool in the open literature, other than the lab-scale bioreactors mentioned above, that can reproduce the cell retention capability nor the continuous media exchange that would be required to run a perfusion process. Some examples and ideas of an alternative use of existing scale down system to support perfusion process development are discussed in the following. These are based on semi-continuous operations that can be applied to deepwell plates, shake tubes or micro-scale bioreactors.

Different scales of deepwell plates can be used for cell culture. Mixing and aeration were characterized in these systems and their

Table 3
Equipment commonly used as scale-down model for process development and their characteristics in terms of mixing, pH control, DO control, pCO2 control, aeration strategy, cell retention and operation mode.

Parameters	Deepwell plate	Shake tubes	Micro-scale	Lab-scale
Mixing	Orbital shaking	Orbital shaking	Stirred	Stirred
pH control	No	No	Yes	Yes
DO control	No	No	Yes	Yes
pCO_2 control	No	No	Yes	Yes
Aeration	Passive	Passive	On demand	On demand
Cell retention	Centrifugation sedimentation	Centrifugation sedimentation	Sedimentation	ATF/TFF/other
Operation mode for perfusion	Semi-continuous	Semi-continuous	Semi-continuous	Continuous

applicability to cell cultivation was demonstrated (Barrett et al., 2010; Duetz, 2007). Nevertheless the lack of control, in particular of pH and DO, limits the use of these technologies mostly to screening applications. For example, a method for screening of up to 470 clones in fed-batch mode using 96 deepwell plates was reported (Rouiller et al., 2015). Performance and quality attributes were compared for fed-batch runs performed in deepwell plates, shake tubes, micro-scale bioreactors and 3.5 L bioreactors. It was concluded that the performance assessment across the different scale down systems was aligned to the one obtained in the 3.5 L bioreactors (Rouiller et al., 2015). Media development activities were also reported at this scale. The very high throughput provided by these plates can be used with an appropriate robotic liquid handler to create a large number of different culture conditions, for example by performing media blending. Jordan et al. shuffled the concentration of amino acids by creating 10 media formulation, and blending them into 192 different compositions (Jordan et al., 2012). A similar method was used by Rouiller et al. (2013) to create up to 376 blends: 16 media formulations were blended changing the concentration of 43 out of 47 compounds present in the media. These very high throughput techniques are very powerful for screening experiments either for cell line selection or media and feed development. However, no similar activities related to perfusion cell culture in deepwell plates are known.

Shake flasks were used since the beginning of suspended cell culture. Aeration and mixing were controlled by incubation and orbital shaking agitation. The most commonly used systems are the 50 mL vented shake tubes. This device was first reported in 2004 and rapidly became a hit for cell culture activities in suspension (De Jesus et al., 2004). The shake tube (cylindrical shape) replaced to some extent the use of shake flasks (pyramidal shape) probably because of the smaller surface requirement in the incubator, the ease of operation and their similar cultivation capacity (Villiger-Oberbek et al., 2015). If these systems do not allow to perform a truly continuous cell culture, an alternative is to use, for example, centrifugation to separate the cells from the media. The supernatant can then be replaced with fresh medium to simulate the perfusion process in a semi-continuous operation mode.

Such semi-continuous operating mode has been investigated to simulate perfusion cultures. Spinner flasks were used with hybridoma cells to compare batch, fed-batch, semi-continuous, chemostat and perfusion (with acoustic cell separator) operations. Specific production and consumption rates were well reproduced using the semi-continuous mode for lower media exchange rates (Henry et al., 2008). A similar concept was used in shake tubes for CHO cell lines producing enzymes by Villiger-Oberbek et al. (2015). Various operating parameters such as the tube angle and orbital shaking speed were first optimized for high cell density cultures. Then, with illustrative purposes, data were generated for 4 clones and 3 media. Performance was ranked based on predefined criteria such as enzyme affinity or cell culture parameters. It was concluded that the semi-continuous system can be used to predict some process parameters such as growth and specific productivities in larger scale bench-top bioreactors. In particular, a good alignment between the two systems was found for cell density, product titer and enzyme activity, affinity and purity. It should be noted that in both studies the reactor control, for example the pH and DO, was different in the various devices. This in principle affects the cell metabolism and impacts the consumption or accumulation of some metabolites but this did not significantly alter the overall performance of the culture. It could be then concluded that the increased throughput and the good alignment provided by the semi-continuous strategy proved to be valuable for screening and process development activities (Villiger-Oberbek et al., 2015). Finally, Gomez et al. (2017) investigated 13 clones using semi-continuous shake tube operation and perfusion bioreactor for an intensified fed-batch process. Cell growth, productivity and also some quality attributes such as galactosylation, afucosylation and aggregation correlated between both scales. Other quality attributes that depend more on the product residence time than on the cell line or protein characteristic, like for example deamidation, C-terminal lysine and clipping, were found to be different in the semi-continuous mode.

Micro-scale bioreactor is another option for scale down models. They provide pH and DO online monitoring and control, and have been discussed in various papers (Bareither et al., 2013; Hsu et al., 2012; Ratcliffe et al., 2012). Although until now their application was mostly limited to fed-batch, some trials to do semi-continuous media exchange were performed (Davis et al., 2015; Kreye et al., 2015). A new system was recently commercialized including continuous media exchange and cell retention capacity (Zoro and Tait, 2017). These equipments have proven their efficiency for fed-batch and will probably also plays a significant role for perfusion process development, although more data is still needed to assess their full potential.

Microfluidic devices, are worth at least mentioning here, since they are more and more frequently used for various applications in the field of biology. These devices allow to work with very small volumes, laminar flows and high surface to volume ratios (Mehling and Tay, 2014; Oliveira et al., 2016). Bacteria were cultured in microliter scale bioreactors (batch and fed-batch) with real time monitoring and control of pH, DO and temperature (Bower et al., 2012; Lee et al., 2006). This technology was also used to cultivate bacteria in chemostat for up to 3 weeks, and yeasts in 11 days long perfusion cultures (Lee et al., 2011; Mozdzierz et al., 2015), but no reports using mammalian cell cultures for perfusion process development are available in the open literature. However, a recent study reports an innovative use of microfluidics for mammalian perfusion cell culture. Kwon et al. (2017) used a microfluidic device not as a bioreactor, but as a cell retention device coupled with a 350 mL cell culture tool. The fluid dynamics inside the spiral shaped microchannel generated two separate flows: the cell free permeate and the retentate, and provided a good retention efficiency depending on variables such as the cell density, the channel size etc. Parallelization of this device could be an interesting option scaling this concept to larger scales (Kwon et al., 2017). The recent developments discussed in this section are encouraging and should be further developed, since they have the potential to become the industrial process development tools in the near future.

3.4. Cell line for perfusion

Cells are the factories within our factories and each of them is a complex biological entity. The so valuable large biologics that they secrete could not be reproduced, at least for now, without the high degree of organization provided by a living organism. An infinite number of phenotypes can emerge from a transfection procedure depending on the host, the transfected DNA sequence, the integration location, the cultivation parameters and so on. Because of the sensitivity of these production systems, cell culture for biomanufacturing requires a high level of process understanding and control.

The most common mammalian host for recombinant proteins and more specifically for mAb production are CHO cells (Chu and Robinson, 2001; Kelley, 2009). They were first recovered by Theodore Puck in 1956 when he discovered a spontaneously immortalized population (Jayapal et al., 2007; Puck, 1958). Originally these cells were used because of their robust growth. They were also easily transfected and did not propagate human viruses which gave a significant safety advantage. The use of this cell line in industry started after the introduction of a selection marker, but since then genetic modifications gave rise to many different strains (Wurm and Hacker, 2011). Other cell lines are also used for stable transfection such as Sp2/0, mouse myeloma cells that are easily adaptable to suspension culture and rather good secretors (Geisse et al., 1996). NS0 cells are another example and originate from mouse plasmacytoma cells like the Sp2/0. One drawback of NS0 cells is that they barely grow without the supply of cholesterol in the culture media unlike most of other mammalian host. Cholesterol is poorly soluble in water and therefore requires the addition of undesired

animal serum. Murine hybridomas were also used for some of the first commercialized mAbs since it was the expression system used at the time (Chartrain and Chu, 2008).

History of cell line generation strategies including vector design, transfection, selection pressure and screening could largely provide enough material for an entire review. The areas where the most significant advances have been registered in recent years include: cell metabolism, cell cycle control, protein secretion, apoptosis and glycosylation regulation (Arden and Betenbaugh, 2004; Costa et al., 2009; Fan et al., 2013; Krampe and Al-Rubeai, 2010; Lim et al., 2010). In addition to cell engineering, it is possible to act on the external environment of the cells to affect their growth rate, apoptosis and/or specific productivities. Some of the most relevant ones are reviewed in the next section. Nevertheless, the development of high producing, robust and stable cell clones best suited for the perfusion operation mode remains another open area where we expect to see a lot of work in the near future. Here a crucial role will be played by perfusion scale-down models.

3.5. External environment levers

As mentioned above, under steady state perfusion operation, cell growth defines the bleed flow rate, which is one of the major responsible for product losses and therefore, for the yield of the process. Different levers were reported and used to reduce the cell growth rate. Mild hypothermia is probably the best known environmental factor that can improve the performance of a mammalian cell culture process. A temperature operation between 30 and 34 °C has shown to affect the cell cycle by blocking them in G0/G1 state (Kaufmann et al., 1999; Moore et al., 1997). Cell growth is inhibited and glucose or amino acid consumption rates tend to decrease (Furukawa and Ohsuye, 1999). Specific productivity increase after a temperature decrease was also reported and found to be cell line dependent (Sung et al., 2004). These findings are obviously very interesting for the optimization of perfusion cultures (Vergara et al., 2014). Indeed, controlling the cell cycle could help to control a stable operation and decrease the amount of energy and building blocks that are directed towards biomass generation, thus improving the expression of the desired product while decreasing the media consumption, which is a critical aspect in all perfusion operations. Other environmental factors such as pH and osmolality are also known to impact cell growth and productivity (Monteil et al., 2016; Zhu et al., 2005).

The relation between cell cycle, specific productivity and cell size was investigated in an early study in batch, fed-batch and perfusion mode using hybridoma cells (Al-Rubeai et al., 1992). The cell cycle can also be influenced by chemical compounds that act on enzymes (cyclins) that regulate the different states of the cell division process (Du et al., 2015). There are several compounds known to have this effect such as different carboxylic acids (Coronel et al., 2016; Gorman et al., 1983; Liu et al., 2001; Park et al., 2015). Valproic acid was reported to increase the productivity of transiently transfected CHO DG44 cells (Wulhfard et al., 2010). Valeric acid, which is much cheaper than chemicals used in this context, was reported to increase mAb productivity in a fed-batch process without impacting quality attributes (Park et al., 2015).

It is clear that the proper understanding and control of all these aspects are key points for the design of high performing bioreactors, in terms of yield, productivity and media consumption.

3.6. Critical quality attributes

Probably the main advantage of perfusion operation, which it shares with all the continuous reactors like chemostat, is related to the quality attributes of the produced protein (Karst et al., 2018). Protein related attributes such as post-translational modifications (PTM) are considered as critical quality attributes (CQA) (Brühlmann et al., 2015; Gleixner and Eon-duval, 2012). Glycosylation or charge isoforms are examples of quality attributes that impact pharmacokinetics (PK) and protein physiochemical characteristics (Abès and Teillaud, 2010; Hintersteiner et al., 2016; Khawli et al., 2010; Van Beers and Bardor, 2012; Walsh, 2010). The recent biosimilar developments greatly challenged the way these attributes were controlled since the quality needed to be modulated to match the originator. Accordingly, technologies enabling the strict modulation and control of these attributes are necessary.

In non-continuous systems, such as batch or fed-batch, the product is retained in an environment that is accumulating toxic and reactive by-products that can affect the quality attributes (Godawat et al., 2015; Karst et al., 2016a). The continuously changing environment leads to the expression of different protein isoforms, and the final product is the sum of all proteins produced at different time during the entire run. Therefore it exhibits rather heterogeneous product quality distribution. Many parameters have been studied in fed-batch to identify levers that are able to control and modulate these parameters (Brühlmann et al., 2015; Mason et al., 2014). Villiger et al. (2016) studied the time evolution of N-linked glycosylation in a fed-batch system to observe the changing pattern of the produced glycoforms. Based on this, and using specific complements that are known to affect specific glycosylation forms, they were able to define a specific feeding strategy of these complements so to produce the same glycosylation pattern along the entire fed-batch. This obviously reduces the heterogeneity of the final product leading to narrower distributions.

In a perfusion process, where a constant environment is maintained during the entire production phase, the kinetics of all processes in the reactor do not change in time, including those related to impurities or post-translational modifications (Karst et al., 2018). Accordingly, the reactor produces always the same product, without the accumulation effect characteristic of the fed-batch operation, thus leading to more homogeneous distributions. Karst et al. (2016a) showed that in the constant environment of a perfusion bioreactor, the glycosylation profile of the produced mAb remains in fact constant in time. In the same study, the effect of manganese, galactose and VCD on N-linked glycosylation was investigated and the expected effect on glycosylation was observed. In another study, the effect of temperature decrease on glycosylation of erythropoietin was also measured in a perfusion system (Woo et al., 2008). These studies demonstrate that the potential of perfusions systems is not only to produce more uniform product characterized by narrower isoform distributions, but also to modulate quality attributes to a specific target.

Another fundamental difference between batch and continuous reactors is in the residence time distribution. In batch systems the product remains in the reactor, from the moment of its formation until the end of the operation, while the environment changes and accumulates waste metabolites and cellular content release due to cell death. Accordingly, the proteins are subject to many PTMs including oxidation, deamidation, aggregation and other processes that may lead to non-desired species. In a continuous reactor with reasonably good stirring, the residence time distribution is much narrower, with almost no protein remaining inside the bioreactor for more than two or three times the average residence time. This leads to an obvious decrease in the formation of aggregates and of all the non-desired products mentioned above (Karst et al., 2018).

Another aspect to be considered with respect to process related impurities such as DNA, HCP, raw material derived impurities or even contaminants refers to the presence or not of an outlet stream (Gleixner and Eon-duval, 2012). In fed-batch, most of these impurities accumulate during the run and must be removed during the downstream procedure. The continuous media exchange of a perfusion system prevents accumulation of these undesired compounds giving yet another advantage to this type of process.

4. Regulatory perspectives

Interesting aspects of regulatory considerations for continuous manufacturing were discussed by Allison et al. (2015). As already mentioned, regulatory authorities support the transition towards continuous manufacturing if the technology favors a *cleaner, more flexible and more efficient* manufacturing. Currently, the validation procedure appears to be more complicated for continuous processes, although the general guidelines are of course the same for batch and continuous operations. However, for continuous processes some additional parameters must be considered: operation time, flow rates, control system and some aspects such as the definition of a product batch and the sampling and in-process control (IPC) strategy need careful consideration (Allison et al., 2015). One relevant aspect refers to the fact that in batch operation the product is clearly identified and can be collected and analyzed at the end of the run. In continuous processes, reliable product sampling implies the stability of the process at steady state and the consistency of the corresponding product, which of course needs to be demonstrated. On the other end it must be noted that perfusion is not new in the biopharmaceutical industry, and therefore good examples of validation procedures approved by the regulatory authorities are available.

In this context the emerging importance of techniques based on big data and machine learning tools which allow monitoring and control of these systems far beyond the boundaries of the current technologies should at least be mentioned (Brühlmann et al., 2017; Sokolov et al., 2017). To transform the vision of continuous upstream processes and even more of integrated end-to-end continuous manufacturing processes into reality, optimal control strategies that comply with stringent and fully legitimate regulations must be developed together with regulators.

5. Conclusion

This review discusses contemporary technological challenges in biomanufacturing that are linked to market cost pressure, need for flexibility and increased manufacturing demand. Continuous technologies have the potential to address the limitations of current technologies based on batch operation in many instances. Therefore, they have to be considered with great care when designing the production scenario of a therapeutic protein from the clinical tests to its commercialization. Here the focus is on continuous cell culture in suspension or perfusion. This cultivation technique was successfully used to generate large biomass concentrations for cell banking, high seeding and other intensified fed-batch applications. With proper process control, stable and continuous production operations can be achieved as well as integration with the downstream capture and polishing operations. In this review we discussed the most relevant remaining challenges including scale down models, the optimization of cell line and media, and the scalability issues, but also highlighted the many opportunities that encourage the further development of perfusion technologies for recombinant protein manufacturing.

Indeed perfusion provides significant intensification capacity and it is therefore not any more interesting only for labile proteins, but becomes appealing also for stable protein production such as mAb. Using continuous technologies, the plant footprint can be significantly reduced, which strongly impacts the time between the moment when the decision to build a new facility is taken and its inauguration. The initial investment requirement is also significantly decreased, thus limiting the risks associated with new capacity management. Technology, capacity management and regulatory aspects are also impacted by continuous technologies, but the benefits might be worth the effort to understand and evaluate them to their full potential. The result will obviously be strongly case dependent, but we are convinced that in the coming years the role of continuous technologies in biomanufacturing will increase with respect to what it is today.

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