# CONTINUOUS BIOPROCESSING

## INDUSTRY BEST PRACTICES

COMPILED BY REPLIGEN

**Four years ago,** I was proud to kick off the first easy-to-access book about continuous Bioprocessing summarizing the technical challenges, existing solutions and the promised opportunities.

Since that time, it is clear that the change in our industry has been rapid and that continuous processing, along with its upstart sibling process intensification, is no longer niche. It was rewarding to hear a scientist from a top 10 biopharma tell me he credits their first move (and subsequent major investment) into this area from that modest publication.

It is unfortunately not as well-known as you would think that first generation, low productivity, perfusion has been producing dozens of therapeutics for the commercial market for over 20 years. This decade started with only two molecules (from Janssen) produced in a modern, productive, perfusion process. By the end of 2018, these two antibodies will have been joined by at least 8 further products: a mixture of antibodies, enzymes and other labile molecules manufactured commercially in the USA, Europe and Asia.

A really positive outcome has been that the growth of skilled and experienced people in the area has been matched by their expansion across the globe into companies of all sizes and types including, importantly, at CMOs such as Patheon and CMC Biologics. Wuxi Biologics, for example, become a world class player in the upstream continuous market in these short years.

Downstream continuous processing is only now starting to take off however: to my knowledge there are no published accounts of clinically manufactured drugs using this mode of manufacture. Yet, in the last year or so, several vendors have sold large scale GMP multicolumn chromatographic systems. Despite being behind the curve until now, the technological barriers have largely been solved and continuous purification processes are set to multiply quickly.

Some of these are to be used in integrated continuous processes, linking the perfusion reactor through a surge tank to the multicolumn system. Considered too complex just a few years ago, two companies have already committed investment to build these integrated continuous plants to manufacture clinical and commercial grade material during 2018. Read that again and remember back to 2013: even the concept was academic. From near zero, conferences abound, articles and papers on these subjects are everywhere, and molecular therapies are manufactured: that's a success.

This book takes one small step more, with chapters looking at high level topics such as facility designs and single use implications, to the detailed challenges of product quality and impurity monitoring. And, not forgetting the popular "get your toes wet" N-1 Perfusion Intensification strategy.

Enjoy. With best wishes,

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(Note, all views expressed are those of the authors and not of Repligen)

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## 1. Introduction

The biopharmaceutical industry is rapidly maturing manufacturing, with sales on the order of $200 billion/year for recombinant biotherapeutics. Yet, in contrast with other manufacturing industries, both low and high-tech, many fundamental aspects of biomanufacturing have remained relatively unchanged for decades. Biopharmaceuticals are arguably the most complex and profitable of all high-technology products, yet nearly all commercial manufacturing today involves legacy "batch" processing, where unit operations are performed, and completed before the process stream moves on to the next step.

In comparison, continuous processing*, with constant flow of the process stream from one unit process to the next, can provide a number of benefits over batch processing[1,2,3,4]. Facilities and process lines running in continuous mode are generally more cost-effective, with the same or more product manufactured in the same, or shorter time period. In addition, continuous Bioprocessing can require less infrastructure, utilities, space, investment, and staff. Most other manufacturing industries have adopted continuous manufacturing, exemplified by the automobile assembly lines Henry Ford introduced over 100 years ago, petroleum refineries and newer steel mills, which essentially run non-stop.

Most every other mature industry has figured out how to adopt continuous manufacturing, realizing this is more efficient and cost-effective. Consider, for example, a steel mill that runs as a single batch, with 100 tons of ore melted and processed in bulk. Each batch can take a week to process, equivalent to 5,000 tons/year. Compare that with a much smaller mill operating continuously – where 1 ton processed per hour would produce 8,500 tons/year. Among the many advantages with continuous processing, the in-process materials need not be moved into and out of storage, meaning that labor and space needs are lower.

*Continuous processing, simply defined, refers to the processed materials/products being moved on to the next unit process as each unit process is completed. It does not literally mean that the processing runs 24/7, non-stop. In Bioprocessing, upstream perfusion is an example, with the product and spent culture media continuously removed from the bioreactor and moved on to downstream processing, while the culture process continues, with fresh culture media added to replace that withdrawn from the bioreactor. Such processing can be done for weeks or even months. In contrast, batch processing involves the material/product undergoing one process, such as product formation in a bioreactor, with all the resulting material/product moved on to the next unit process at one time as a single lot/batch. This is exemplified by fed-batch Bioprocessing, with upstream cell culture performed for a few days, at most a few weeks, and once this unit process is over, e.g., sufficient product expressed in the bioreactor fluid, the bioreactor is emptied and its full contents moved on the next step.

The same holds true for biopharmaceutical manufacturing. A continuous process at smaller-scale not only saves resources, space and labor, but also allows less stressful manufacturing (mixing, temperatures, pressure, etc.) with better product quality and continuous process monitoring and control – a factor vitally important to the biopharmaceutical industry. If something does go wrong, only the already-processed affected material need be collected and discarded. This is unlike batch processing where, after all that work, the entire batch must be discarded and manufacturing restarted. For example, in classic batch commercial-scale monoclonal antibody manufacturing, antibodies are commonly manufactured using fixed stainless steel bioreactors and other vessels holding 10,000L or more, with each cycle of process preparation (cleaning, sterilization) and processing taking weeks. In contrast, using a 500L perfusion bioreactor, the same or even greater amount of product can be produced in the same or even shorter time – the weeks or month(s) it takes for batch processing and preparation for the next batch. There is much less down-time and perpetual waiting for unit processes to be completed, and storage/holding of in-process materials, as is common with batch processing.

Comparative studies at largest scales, such as blockbuster antibody manufacture in fixed stainless steel facilities, continue to show better cost–benefit using fed-batch at the largest scales, with perfusion bioreactors only becoming more cost-effective at below 500kg/year capacity. However, building a batch facility can easily cost more than $120 million, while a much smaller CBP facility with the same overall output could cost $25–$35 million. Also, operating expenses will be lower for the continuous facility. This is because the continuous operating facility will be smaller and operate with higher productivity (more production over time, less down time), compared to a batch Bioprocessing facility making the same product. Another major benefit of continuous Bioprocessing is that its smaller scale and continuous processing at less extremes (of mixing, temperature, pH, pressure, etc.) make it much less stressful. This often results in improved product quality as well as better use of single-use equipment, which brings further benefits and cost-savings. Also, perfusion operating conditions can often be more precisely controlled vs. fed-batch, which can be an advantage for some products – cell densities tend to be much higher and, with milder continuous, shorter culture periods, product quality is often better.

## 2. Industry perceptions are lagging

Continuous Bioprocessing (CPB) is not new. A few commercial recombinant products have been manufactured using upstream perfusion technologies for several decades. For example, adherent fiber-based bioreactors have been in common use for monoclonal antibody (mAb) manufacturing using fused-cell hybridomas since the 1980s, before recombinant mAb manufacture became dominant; perfusion is increasingly being adopted for bioreactor operation; and continuous centrifugation has long been used, including for commercial manufacture of most plasma-derived protein and immunoglobulin products. However, in the big picture, continuous bioprocesses remain the exception. Much current adoption often involves perfusion, continuous centrifugation or other currently-available continuous unit bioprocesses being added or "bolted on" to existing Bioprocessing systems.

Despite the benefits of continuous vs. batch Bioprocessing, its adoption has lagged, with perfusion processes being somewhat of an exception. Batch Bioprocessing has remained the dominant industry manufacturing paradigm, and there are good reasons for this. The main issue the industry has with adopting continuous Bioprocessing is that batch processing is familiar and works very well, while continuous Bioprocessing is new and more complex or, at least, is perceived as inherently more complex vs. batch Bioprocessing. However, exemplified by perfusion, this industry-held perception of continuous Bioprocessing complexity is rapidly becoming outdated, with technologies and products constantly improving and new ones entering the market.

The key areas where industry respondents have reported perfusion as presenting more concerns (vs. fed-batch) included:

1) Process operational complexity
2) Process development control challenges
3) Contamination risks
4) Process development general challenges
5) Validation challenges
6) Upstream development and characterization time
7) Ability to scale-up process
8) Need for greater process control
9) Cell line stability problems.

These data are among the findings from our 12th Annual Report and Survey of Biopharmaceutical Manufacturing. Among the 238 biopharmaceutical manufacturing professionals surveyed about issues concerning perfusion vs. fed-batch upstream Bioprocessing, every aspect surveyed was perceived as more of a concern with perfusion. The top three concerns cited by a majority of respondents with perfusion were:

1) **Process operational complexity** – Increased complexity with continuous processing was perceived as increasing risks in manufacturing that can create very costly, sometimes disastrous operator errors. But in actual implementation, most or all of continuous Bioprocessing is likely to be rather fully automated (another advantage of continuous Bioprocessing), with errors and operator involvement minimized.

2) **Process development control challenges** – If a batch process is changed or improved at small scale, with most having more experience with batch processing and this more established, extrapolation to larger scale is relatively routine. With continuous Bioprocessing, process changes were perceived as more challenging. But in actual current implementation, scale-up and control of perfusion and other continuous Bioprocessing can be much simpler, e.g., with no bulk removal and interim storage of processed material/product, and with much less concern about product degradation as the material is more quickly removed from bioreactors and moved on to the next unit process.

3) **Contamination risks** – Risks of contamination of a process were perceived as higher with continuous Bioprocessing. But in actual implementation, there may be fewer contamination risks with continuous Bioprocessing, including the smaller-scale equipment used more easily and better isolated, including equipment enclosed within isolator cabinets, and connections/disconnections are made only once vs. as each batch is processed.

Perfusion and other continuous processing are increasingly becoming significantly less complex, less prone to contamination, more regulatory-friendly, and more readily scalable than fed-batch methods. However, as the BioPlan survey shows, industry perceptions of perfusion/continuous vs. fed-batch are lagging, and likely reflect a lack of direct exposure or experience with these technologies. But there is incremental progress in terms of perceptions. When those surveyed were asked what types of bioreactor they would implement

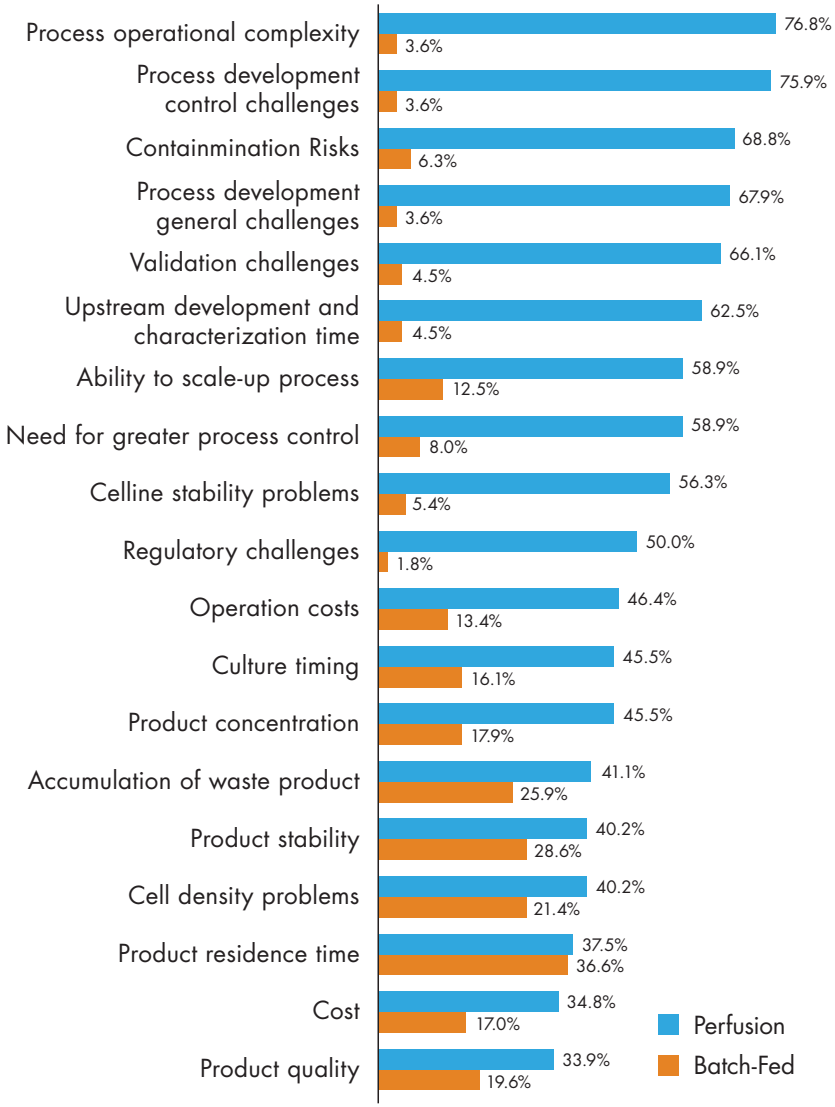


**Fig. 1** The industry-pervasive views of continuous Bioprocessing being too complex are exemplified in results from the BioPlan Associates 2015 12th Annual Survey[5].

for a new facility coming online in two years, as expected, over two-thirds cited batch-fed single use bioreactors, but 24% cited planned adoption of single use perfusion bioreactors at commercial scales and 32% at clinical scales. So, currently, essentially a third of Bioprocessing professionals foresee benefits from future adoption of continuous Bioprocessing. Similarly, 22.5% of Bioprocessing professionals reported having some experience with continuous purification, such as simulated moving bed (SMB) or alternating or tangential flow filtration (ATF or TFF) technologies, with this familiarity doubling since 2012 (11.8%). Continuous chromatography was also cited among major development efforts reported by surveyed industry suppliers.

## 3. Industry adoption

Basic changes in biomanufacturing paradigms take time – decades. This is a highly regulated industry, and regulators must be convinced that manufacturing changes do not compromise drug quality or patient safety. For example, as reported above, industry perceptions of problems and risks with continuous Bioprocessing often lag behind their current implementations. Despite clear advantages, it took over 50 years for continuous steel casting to dominate that industry. The Bioprocessing industry, with its highly regulated operations, is even more conservative. For example, it has taken over a decade for single-use systems to become dominant for routine pre-commercial-scale operations, and it will require perhaps another decade before these processes are substantially adopted for large commercial-scale biopharmaceutical manufacturing.

Regulatory agencies, e.g., EMA and FDA, or rather industry concerns about their acceptance of continuous Bioprocessing, are often cited as reasons for not adopting continuous Bioprocessing. But FDA and other major market regulatory agencies are generally very open and, in fact, welcome continuous Bioprocessing, with this seen as improving process control, product quality, inherently allowing simpler application of PAT, etc. Aspects such as how to define a 'batch' have long been cited as hurdles, but have now largely been resolved. Although regulatory agencies are increasingly familiar and competent in continuous Bioprocessing, few biomanufacturers want to be the test case, when they can simply select a tried (but tired) and trusted batch manufacturing strategy. Again, industry perceptions lag behind current reality, and this is part of what is holding back adoption. The industry prefers to stay with what is familiar, because it is quicker and easier to get very familiar but a dated (and possibly inefficient manufacturing) operations through the regulatory process than it is to introduce robust new technologies. So even though continuous Bioprocessing can be less expensive, and can make better quality products, it will take more time for the technology to replace conventional fed-batch manufacturing.

## 4. Current trends in adoption

In the biopharmaceutical industry, adoption of continuous Bioprocessing at any decent scale has so far been restricted to relatively few unit processes implemented at a minority of facilities. By far, continuous Bioprocessing is currently dominated by smaller-scale perfusion, increasingly single-use, bioreactors. Currently, about a dozen, less than 10% of biopharmaceuticals are reported as manufactured using perfusion bioreactors. Some biomanufacturers are leaders in continuous Bioprocessing implementation, including Genzyme and Bayer, with both companies having considerable experience using perfusion for commercial products manufacture. Amgen recently opened an integrated $200 million continuous Bioprocessing manufacturing facility in Singapore, the first such fully (or maximally) continuous commercial-scale Bioprocessing facility. Hemispherx Biopharma is developing a cell cultured immune modulator, and adoption of continuous Bioprocessing has allowed an 80% reduction in manufacturing staff. Besides recombinant protein and antibody manufacture, continuous Bioprocessing also fits well with cellular, gene therapy and other newer classes of products in development.

Despite the ongoing technology developments and the new products becoming available, continuous Bioprocessing is not yet being broadly adopted for purification and other downstream processing operations, especially at commercial scale. Unlike upstream, where perfusion equipment is becoming more accepted, most consider the related technologies and equipment just not advanced enough yet. There are many variations of continuous purification, including chromatography and centrifugation, increasingly becoming available but they are not yet seeing wider adoption. Some technologies, such as simulated moving bed (SMB), cell retention and periodic countercurrent (PCC), chromatography are projected to provide 20%–30% cost savings compared with current batch methods. This is in addition to the advantages of smaller scale and of generally being single-use. But even such substantial savings and benefits have not been enough to motivate the industry to adopt these systems.

BioPlan Associates expects gradual adoption of continuous Bioprocessing at all scales, including commercial manufacture as products initially developed using these methods enter the market. The imperatives of cost-savings, flexibility and product quality will increasingly drive the industry to explore and adopt continuous processing, much as it has with single-use systems. This, in turn, will expand the industry's current knowledge and experience and ease adoption when making major changes in manufacturing platforms. Particularly, as perfusion, various purification technologies, and other continuous Bioprocessing technologies are further improved and increasingly adapted for single-use equipment and modular systems, adoption will further accelerate. But a major

challenge with single-use equipment for continuous Bioprocessing is the plastic materials tend to be insufficiently robust: Equipment needs to be used for weeks or months vs. days. As time goes on, however, case studies and other reports of cost and performance improvements will promote more rapid adoption.

Many upcoming continuous Bioprocessing technologies are actually relatively novel and will significantly impact Bioprocessing. Eventually, over the next decade, capillary fiber perfusion and other revolutionary bioreactors and other new continuous Bioprocessing technologies, including those for downstream purification, will see increased adoption. For example, a 50L single-use capillary fiber continuous bioreactor setup, such as being developed by FiberCell, can match or beat the overall output of a 5,000L fed-batch bioreactor[1,3]. Similar to single-use systems, once their availability reaches a critical mass, adoption of continuous Bioprocessing systems will rapidly increase, including at commercial manufacture. A Bioprocessing suite being designed a decade in the future may bear little physical resemblance to conventional facilities, with everything at much smaller scale.

BioPlan Associates projects industry adoption of continuous Bioprocessing, particularly perfusion for upstream processing, to follow a trajectory similar to that experienced with single-use systems. That is, a cautious but consistent adoption rate, starting in research and process development labs, then moving to clinical scales as technical, and regulatory issues are worked out. Many of the technologies required to drive a fully continuous Bioprocessing facility are still on the drawing board. So the timeframe for actual clinical scale adoption may be relatively long. And commercial scale will follow once operational and logistical complexities are worked out. Despite this, many in the industry expect continuous Bioprocessing eventually to become a dominant approach over time. Interestingly, the widespread adoption of single-use systems may have reduced the imperatives and critical needs for the cost-savings, flexibility and other benefits derived from continuous Bioprocessing. In the near-term, much increased use of continuous Bioprocessing products is likely to involve bolt-on retrofitting of existing systems, including at commercial scales, such as adding a perfusion pump or swapping-in a continuous chromatography step; and otherwise, adoption, as with single-use systems, will start with initial bioprocess design and follow products as they enter commercial manufacturing.

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# How to Develop a Perfusion Process

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Véronique Chotteau (M.Sc.Elec.Eng. Univ. Libre Bruxelles, M.Sc. Univ. Libre Bruxelles, Ph.D Louvain Univ., Belgium) has more than 25 years of experience in mammalian cell culture cultivation (suspension and adherent) and more than 10 years of experience in biopharmaceutical industry including different responsibilities as project manager, pilot plant head, expert in animal cell culture development (small, pilot and commercial size/GMP), business development support. VÈronique joined Pharmacia Upjohn, Stockholm, (nowadays Swedish Orphan Biovitrum) in 1996. She worked with process development of factor VIII Refacto, other perfusion processes as well as fed-batch processes. She left the industry in 2008 when she was offered to take over the group of Cell Technology, Dept. Industrial Biotechnology/Health, School of Technology, Royal Institute of Technology (KTH), Sweden. The activities of her group are focused on established cell-based processes (perfusion, metabolic flux analysis modeling) and stem cell bioprocessing.

## 1. Introduction

In a continuous process, the culture medium is continuously renewed by removal of conditioned medium and feeding of fresh medium while the cells are totally or partially retained in the bioreactor by a cell separation device. The volume of fresh medium is identical to the one of spent medium, which is cell-free or possibly containing cells.

The process development can be divided in two main parts, the selection/ **optimization** of the parameters and features of the **perfusion process** (excluding the cell separation device) and the **development of the cell separation process** it-self. The selection of the culture length is made from information of both parts supplemented with COGS and failure risk considerations.

The efforts of perfusion process development may vary depending on the purpose of the process: in several cases where the production yield is not critical, the efforts can be limited while more efforts might be needed when developing the production process, it-self where the production yield and the product quality are highly important, and likely even more efforts will be needed if the product of interest (POI) is unstable or sensitive.

Limited efforts are required in the case of production for research purpose (i.e. case where the production yield is less important than the time needed to obtain the target product), for the production of cells aimed at cell banking or for inoculation of a larger bioreactor. In these cases, the accent is put on cell healthiness, i.e. high viability, and increased cell density while the cell stability is maintained. In the case of cell banking or cell seed manufacturing, high viability is required both for the success of the next operation step, respectively cell cryopreservation and seeding of larger bioreactor, and to avoid cell selection by cell death, which could result in cell population shift.

For the case of production for research purpose, often the cell lines are rapidly obtained but sub-optimal, i.e. with low cellular productivity. In that case working with a high cell density can compensate the low cell specific productivity while a high viability gives some insurance that the product quality is sufficient – however this is of course depending on the produced molecule.

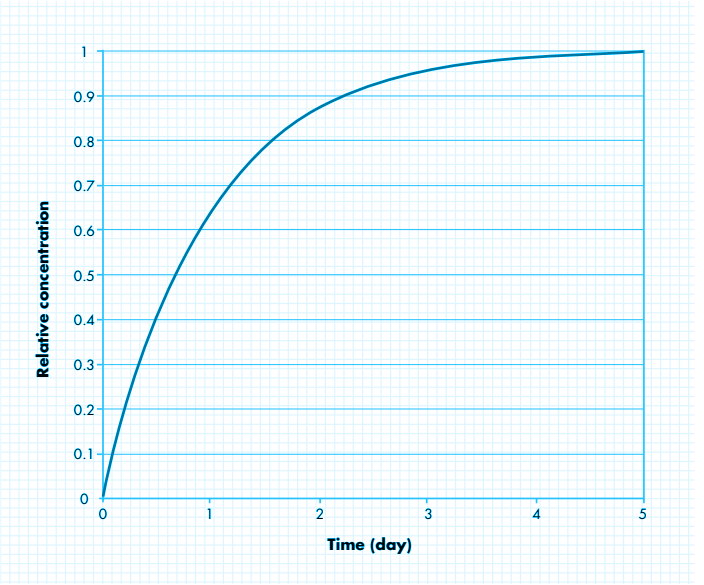


**Fig. 1** Evolution with time of the relative concentration of a new component present at concentration 1 in the fresh medium of a perfusion process operated in bioreactor at 1 RV/day perfusion rate.

## 2. Systems for development of perfusion processes

Generally speaking, the main trends observed in batch culture will remain true in perfusion culture, e.g. a favorable effect of a plant hydrolysate on the POI production observed in batch culture will most likely be confirmed when applied in perfusion process. It is advisable to confirm (and possibly refine) the observations made in batch culture in perfusion system before their application in a process.

### Screening model

Small vessel cultivation systems aimed at screening larger numbers of conditions can be used for a pre-determination of parameters, followed by confirmation or refining in bioreactor scale. Typically, medium selection and effect of medium components can be screened saving labor and time.

As mentioned above, batch culture can give good indications of trends valid in perfusion but perfusion can also be simulated using systems like shake flasks, spinners or 50 mL tubes with vented cap in so-called pseudo-perfusion process (also called semi-perfusion or quasi-perfusion).

In pseudo-perfusion, daily medium renewal is operated manually: the culture is centrifuged, the supernatant is discarded partially or totally and the cells are re-suspended in fresh medium. A main difference between the pseudo-perfusion system and perfusion is the residence time of the components, which is asymptotically evolving to the input value in the latter case. For instance, in a perfusion it takes 3 days at perfusion rate 1 reactor volume/day (RV/day) for a complete medium renewal since the fresh medium is constantly diluted in the culture (see Figure 1). Contrary, in a pseudo-perfusion, the entire medium volume is renewed at once for the same apparent rate of 1 RV/day. Due to this difference, a partial medium renewal is sometimes adopted instead of complete medium renewal. Another difference is that pseudo-perfusion is literally speaking a repeated batch regime although with small variations. For this reason, daily medium change gives better mimic of the perfusion than e.g. medium renewals every second day however this latter option is sometimes adopted to reduce the labor burden. In pseudo-perfusion, it can be chosen to either retain the cells partially or totally depending if a stable or expanding cell density is desired.

### Bioreactor and scale-down model

Bioreactor systems are used for the development of the perfusion processes and most of the parameters can easily be studied in scaled-down models. Exceptions are parameters such as the shear stress and the deleterious effect of bubble/gassing, for which the scaled-down study is more challenging. The study of the cell separation device if-self has to take into account the limitations of the targeted large scale for the parameters like the liquid flows or the power.

## 3. Development of the perfusion process

### Medium selection

A culture medium needs in first hand to include all the necessary components to sustain the cell growth and production of POI, e.g. in case metal(s) or a vitamin are crucial for the POI activity. Nowadays serum-free and chemically defined media provide not only these necessary components but moreover give enhanced cell growth, cell survival and/or production from additional components and optimized formulation.

Most often a medium, which is beneficial for the cell growth and POI production in batch process, will also be favorable for these parameters in perfusion regime. Consequently, selecting a medium resulting in good growth and POI production in batch mode will be satisfying for perfusion operation at first hand. A base medium can be advantageously supplemented with a feed concentrate, which has been developed for fed-batch process, to improve the POI production for instance. Starting the development of a perfusion can for instance be initiated by the evaluation of 5 to 10 commercial media in a batch shake flask productivity test study leading to the selection of 2 to 3 base media. Supplementation of these base media with different feed concentrates can then be studied in the same system at the low value of the concentration indicated by the manufacturer. From this study a base medium, potentially supplemented with feed concentrate, can be selected and tested in perfusion mode.

Subsequent to this, the medium can be further refined/optimized if it is necessary to improve the POI cell specific productivity for the goal of the process. Another aspect is that since perfusion mode requires large volumes of liquid handling, minimizing the perfusion rate (D) without compromising the process performances can be desirable. This can be achieved by tuning the medium composition and use of concentrated media (Konstantinov et al. 2006; Ozturk 1996; Runstadler 1992).

### Perfusion rate strategy

Two main strategies can be distinguished to determine the perfusion rate: either based on the cell density or based on the availability of a main substrate in the culture. Sometimes, it is even desirable to increase the perfusion rate to reduce the by-product accumulation.

### Perfusion rate measurement

The perfusion rate is monitored by daily measuring the volume of harvested spent medium, which is equal to the volume of added fresh medium.

### Perfusion rate strategy based on CSPR

An established strategy is to adjust the perfusion rate as a linear function of the cell density (Ozturk 1996; Konstantinov et al. 2006; Clincke et al. 2013b), i.e. to apply a cell specific perfusion rate, CSPR, where CSPR = perfusion rate/cell density or D/cell density

This allows avoiding the depletion of component(s) in the culture and has been demonstrated to sustain up to 200 x $10^6$ cells/mL (Clincke et al. 2013b). In order to save the medium, identifying the minimal CSPR (CSPR_min) is critical. A method to select CSPR_min is:

- Inoculate the bioreactor at cell density 0.3 to 1 x $10^6$ cells/mL, initiate the culture in batch mode and start the perfusion at D = 1 RV/day when the cell density has reached 2 to 3 x $10^6$ cells/mL – importantly start the perfusion while the cells are still in exponential growth phase
- Allow the cells to grow exponentially until e.g. 20 x $10^6$ cells/mL, by daily monitoring the growth rate, while increasing D to 2 RV/day (or higher) in case the growth would slow down
- Establish then a culture around 20 x $10^6$ cells/mL of exponentially growing cells by performing daily cell bleeds compensating for the cell growth – this culture is an excellent system to test various parameters like CSPR, pH, etc.
- In the culture stabilized at 20 x $10^6$ cells/mL, identify the CSPR_min for the given cell line and medium with the following steps applied at 1 to 3 days intervals (1 to 3 (or more) days are necessary to observe the effects of an implemented modification):

  Increase D of 0.5 RV/day step and go to either i) or ii) depending of the outcome

  i) if the growth is increased (by increasing D), the actual CSPR is too low and D has to be increased (of e.g. 0.5 RV/day step). Repeat increasing D by 0.5 RV/day steps until further increase of D does not result in improved growth. The next to last D gives CSPR_min.

  ii) if the growth is not increased (by increasing D), CSPR_min is not higher than CSPR in use and is possibly lower. Test to reduce D by 0.2 RV/day step and observe if the growth remains (or not) unchanged. In the positive, continue to decrease D; in the negative the next to last D gives CSPR_min.

Notice that after a slower growth has been observed, it requires some time (at least 3 days) for the system to recover from depletion (depending how sever the depletion was).

It is recommended to control the feed of glucose and glutamine separately, at stoichiometric rate and to maintain their concentration at low levels when applying a CSPR during the process development phase. As a matter of fact, the need of these substrates can be different from the need of the other medium components. When the process is established, these substrates can advantageously be delivered together in the fresh medium formulation.

### Perfusion rate strategy based on main substrate

Control of the perfusion rate can be based on a main substrate like glucose (Dowd et al. 2001). Glucose is present at a selected concentration in the medium. From daily glucose concentration measurement, the perfusion rate is increased or decreased in order to maintain the glucose concentration constant in the culture. This can be based on daily manual glucose concentration measurement or on a more sophisticated on-line measurement of glucose.

### Removal of toxic by-products

Ammonia and lactate are known for their negative effect on the cell growth and productivity. High ammonia concentrations are also reported to affect the glycosylation profile (Goochee et al. 1991; Jenkins et al. 1996). Using a dialysis system with 10 kDa cut-off (Buntemeyer et al. 1992) showed that spent medium could be re-used however this is not today an industrial practice. They also showed that other (un-identified) low molecular weight components than lactate and ammonia had a toxic effect.

In case the lactate or ammonia concentrations are reaching unfavorable levels, the perfusion rate can be increased to remove these by-products. A graphical representation of the effect of lactate or ammonia concentrations on the growth rate or the cell specific production rate can provide guidelines for the selection of limits of these by-products in the process.

### Cell density – target, monitoring and control

Several strategies to perform a perfusion process can be adopted.

- *Stable cell density with growing cells:* Maintaining the cell viability as high as possible and the cells in growing stage is one of the main strategies used in perfusion field. After a culture period of increasing the cell density to a target level, the cell density is main-

tained stable at this level in a system where cell removal (automatically or manually operated) is performed at a rate compensating the cell growth (Konstantinov et al. 2006). This is the best way to prolong the culture as long as possible. Industrial processes are operated on this principle for months. The only limitations in time are the cell stability or practicalities like cell and cell debris accumulation in the equipment.

- *Increased cell density:* Another strategy based on growing cells is to increase the cell density until a physical limitation of the cell density it-self or the equipment is reached – or closed to be reached - (Clincke et al. 2013a; Clincke et al. 2013b). This is advantageous in the case of cell separation by ultra-filtration (UF) where the POI is retained in the bioreactor. This process is shorter than the previous one – some 2 weeks – by essence but also due to the risk of POI degradation.

- *Stable cell density with arrested cells:* A third strategy consists in a first culture period of increasing the cell density to a target level, then to slow down or completely arrest the cell growth, which is known to be potentially associated with a higher cell specific productivity in a cell specific way. In the case of cell arrest, the cells are not renewed as often as in growing phase and can be more sensitive to shear, which can lead to increased cell damage in perfusion process potentially involving more mechanical stress.

### Inoculation cell density

Thanks to the medium renewal applied as soon as the cells have reached a couple of millions cells/mL (see Section "Perfusion rate strategy based on CSPR"), the inoculation cell density has not the same major impact as in a fed-batch process. The culture can be initiated as a batch culture in the conditions mimicking shake flask scale. The perfusion is then started when the cells are still in exponential growth phase. A higher inoculation cell density allows shorting down the time required to achieve the target cell density. An option in this latter case is to start the perfusion the same day as the inoculation.

### Selection/optimization of the cell density

Two decades ago, the cell density in perfusion reached a few millions cells/mL in many cases. A standard in industry today is to target around 20 x $10^6$ cells/mL but there is a trend towards much higher cell densities where the benefit of perfusion can be fully exploited. It is probable that today many industrial processes aiming at producing cells like cell bank manufacturing or seed bioreactor are targeting 50 to 80 x $10^6$ cells/mL (Clincke et al. 2013a). Several companies are working with these cell densities or above 100 x $10^6$ cells/mL like DSM, CMC-ICOS, Genzyme – however it is not clear which cell densities are really used a production scale.

### On-line cell measurement

The cell density and viability are usually monitored daily by manual sampling. Besides this, the cell density can also be measured on-line based on the dielectric properties of the cell, i.e. permittivity and/or capacitance, by commercial probes. Recently in-situ microscope technology has also been developed.

Another way is a cell density evaluation obtained by monitoring the consumption of oxygen or glucose (Kyung et al. 1994; Meuwly et al. 2006). To exploit this approach, it is necessary that the consumption rates of these molecules are constant. In particular, the glucose consumption rate varies with the glucose concentration in the culture so this indirect measurement should only be exploited in a process where the glucose concentration is stable in the bioreactor.

### Cell bleeding

Cell bleeding is operated to partially remove the cells from the bioreactor, typically by pumping out the cell broth from the bioreactor. This operation is systematically included in a strategy where the cells are maintained at a stable cell density (see paragraph 'Stable cell density with growing cells'). Three methods can be used:

- The more accurate method is to use a continuous pump automatically controlled based on the on-line cell density measurement in order to track the cell density set point.
- The continuous pump can also be manually tuned based on daily off-line cell density measurement achieving satisfying results.

- Daily manual cell removal can also be used during the process development phase: the perfusion is momentarily stopped; cell broth is removed and then new fresh medium is added to compensate the removed culture volume before re-starting the perfusion. This is an advantageous tool to precisely measure the cell growth or to study and tune a new process. As a matter of fact, since the continuous pumping removes cells whole the time, the cell growth rate calculation is not straightforward in this latter case. Then based on information the cell growth rate and the volume of daily removed cell broth, a continuous pumping can be implemented. A few adjustments are then applied to finally tune the continuous pumping.

Manual cell removals are also operated ad hoc to reduce the cell density, e.g. to respect given cell density limits of a registered process. During the process development, the studied conditions can result in degraded cell population with low viability and/or absence of growth; a manual cell removal is then applied to help the cell recovery. This can be accompanied (or not) of a momentarily increase of the perfusion rate in order to speed up the medium renewal, providing more favorable environmental conditions.

### Cell arrest

Likewise in fed-batch processes cell arrest by physical or chemical means can be used, given that the cell specific productivity is increased and that the protein quality is correct – or even improved – (Angepat et al. 2005; Chotteau 2001; Oh et al. 2005). Cell arrest in GO/G1 phase can be reversibly obtained for instance by reducing the temperature (Angepat et al. 2005; Zhang et al. 2013) or adding a chemical like butyrate (Oh et al. 2005), see Section "Cell density – target, monitoring and control", paragraph 'Stable cell density with arrested cells'. The (more abundant) knowledge reported for batch and fed-batch processes like the ranges of temperatures or butyrate concentrations can be applied in perfusion processes. These parameters have to be optimised on a cell line specific basis. Typically a lot of cell lines are still growing at 34°C or 35°C but slower than at 37°C and are barely growing at 31°C. Butyrate is toxic to the cells so it can severelly affect the viability and should be used at an optimized concentration, compromise between the increased cell specific productivity benefit and potential faster deterioration of the vialibity. As previsiously mentioned, the cells are not renewed as often as in growing phase

and can be more sensitive to shear, which can lead to increased cell damage in perfusion process potentially involving more mechanical stress (see also the impact of the cell viability below).

### Cell viability

Low cell viability can affect the POI quality due to the associated proteolytic activity released by the lysed cells. Another important effect of the presence of dead cells is the release of nucleic acid and cell debris, reported to play a major role in filter clogging (Esclade et al. 1991; Mercille et al. 1994). It is therefore highly advantageous to maintain the cell viability as high as possible. Finally, a supplementary practical aspect of the presence of dead cells is the accumulation in the culture of cell debris, which are not removed in the harvest line in filter-based cell separation perfusion while other cell separation technologies based on acceleration allow their removal.

### Protein quality

Except for the case of UF perfusion, the POI present in the harvest is stored in a cooled harvest tank during the culture, allowing a good preservation of the POI quality. For instance, the proteolytic activity is highly reduced. During the process development, the evolution with time of the POI quality in the cooled tank is studied according to the quality attributes important for the POI, i.e. analyses/characterization. This study together with logistics and COGS factors will contribute to the decision of the harvest frequency for the process. The constant environment of the perfusion greatly contributes to the stability of the quality attributes with time. Another factor of attribute profile variation with time is the application of cell arrest ( see Section "Cell arrest"). If small variations in the POI quality like minor variations in the distribution of species, a common procedure in industry is to pool different POI batches issued from different harvests from different culture runs in order to reconstitute the POI according to the specifications. In practical, after a harvest has been proceeded to the capture step to reduce the liquid volume and possibly provide a first contaminant removal step, it is analyzed and stored. Then the material from several harvests is pooled based on the analyzed POI profile to proceed with the next purification step.

### Parameter optimization

During the process development, the effect of the parameters on the process can be preliminary studied in batch mode or in pseudo-perfusion (see Section "Screening model"). This is then confirmed/further studied in bioreactor scale. During a bioreactor run, several parameter values can be tested sequentially in time (Miller et al. 2000; Hiller et al. 1993):

- A culture at a given constant cell density is established ( see Section "Cell density – target, monitoring and control")
- The effect of a given parameter value is tested during several days, e.g. 4 to 7 days, by monitoring the cell growth, viability, metabolism, POI production and quality (if relevant)
- Then this parameter is changed to a new value to be tested
- After a transition period, e.g. 2 to 3 days, the effect of this new value can be monitored as described above. Notice that in case the previous parameter value was extreme and damageable for the cells, the transition period has to be longer until the cell growth and viability are back to their normal values.

A factorial analysis, also called Design of Experiment, approach can advantageously be adopted to study the effect of several parameters on the process as commonly used in the whole culture process development field (Pinto et al. 2008; Bollin et al. 2011; Sandadi et al. 2006).

### Environmental parameters

Different physical parameters have large or moderate influence on the process.

- *pH:* The pH has a major effect on the cell growth, POI production, glucose/lactate metabolism and therefore alkali addition, osmolality and $pCO_2$ level. pH can also influence the POI quality. Most often, different pH values are optimal for the cell growth and the POI production, leading in some cases to the selection of pH shift between the cell growth phase and a second phase devoted to POI production. In absence of a priori knowledge, a first valid selection of pH value is 7.0 for an antibody producing CHO cell line.
- Dissolved oxygen concentration (DO): The DO has often a much minor effect on the POI production given that DO is between

some 20 and 80% of air saturation (Link et al. 2004). Higher cell specific glutamine and glucose uptake rates have been reported for hybridoma with increasing DO (Jan et al. 1997; Thommes et al. 1993). Below 20% DO, the cells are submitted to stress affecting the metabolic and production rates as well as the growth rate. Above 100% DO, a higher oxidation is taking place. Another factor is the DO signal oscillation during the culture. Large oscillations should be avoided. However oscillations of up to 15% DO have been reported without negative influence on a CHO-based perfusion process (Clincke et al. 2013b).

- *Partial pressure of carbon dioxide, $pCO_2$:* $pCO_2$ values in the range of 15 to 105 mmHg (2 to 14 kPa) have no major influence on the process. Larger values can affect the cell growth, the POI production and quality. A lot of processes tolerate well 150 mmHg (20 kPa) and some processes even 195 mmHg (26 kPa) or higher. An advantage of perfusion compared to fed-batch is lower values of $pCO_2$ provided by the medium renewal.

- *Temperature:* see Section "Cell arrest"

- *Scale-up factors, i.e. shear rate/shear stress, gassing-bubble damage:* see 'Scaling-up and Tech transfer' SS Ozturk

### Substrate concentration optimization

It is recommended to maintain the glucose and glutamine at stable values during a production process to maintain the cell metabolism constant. Low levels of glucose, i.e. ≈ 5 mM, and glutamine, i.e. ≈ 0.5 mM, result in low production of lactate and ammonia so this is valuable strategy for perfusion processes.

## 4. Development of cell separation operation process

When no prior information is available, it can advantageous to test several cell separation devices to select one best fitting the process goals.

The cell separation process generates mechanical force, and therefore potential stress on the cells. This is due to the separation device it-self but also from the cell broth circulation in tubing system and from pumping if applied. The tubing and pump system need to be dimensioned to reduce the mechanical stress and the residence time of the cell broth outside the bioreactor. Using larger

tube diameter reduces the mechanical stress but unfortunately increases the residence time. Shortening the tube length reduces the residence time. Ideally, the residence time should not be longer than 1 or 2 minutes. A long residence time outside the controlled environment of the bioreactor is unfavorable for the cells since oxygen shortage is rapidly appearing. Very long residence time can result in substrate shortage and metabolic oscillations. The optimization of the cell separation should be performed independently of the optimization of the perfusion process it-self when possible.

### Filter

A major drawback of filtration is the retention of the POI upstream the filter – except for UF where it is desired. If the POI concentration is similar in the bioreactor and in the harvest line (directly down-stream of the hollow fiber filter), there is no retention (filter yield = 100%). If the POI concentration is lower in the harvest line than in the bioreactor, there is retention. A major advantage of filter-based cell separation is the potentiality of sustaining very high cell density in comparison with other systems. Filter fouling is due to nucleic acid and cell debris adsorption. In filter-based perfusion processes, the filter properties influence the process. There is no general consensus about the optimal pore size of a hollow fiber filter, often selected 0.2 um, sometimes 0.45 um. The presence of detergent, like pluronic, has some influence on the filter retention properties.

### Renewal of filter during process

Filter renewal during a process can be decided based on known profile of POI retention with time, cell viability and pressure in the harvest line. In case of filter fouling the pressure in the harvest line is dropping. During a process, it can be advantageous for the process performance and for the logistics/planning to replace a filter unit as soon as the first signs of filtration degradation appear before a more sever failure is taking place.

### Re-circulation rate

The re-circulation rate is dictated by the values of the real-scale (large-scale) process. A higher re-circulation reduces the filter fouling and is adopted sometimes for studies in small-scale where filter fouling is not studied.

### 5. Selection of the process timing

The process timing (i.e. culture length, harvest frequency) has been touched on throughout the previous sections. An important aspect is the stability of the cell specific productivity and cell population. The harvest frequency was already discussed in Section 3.5. The COGS and logistics of harvesting include factors like the resources to operate the capture step and the size of the harvest tank.

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# Strategies for development of perfusion media

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## 1. Introduction

Fed-Batch has been the predominant mammalian cell cultivation mode for manufacturing of biopharmaceuticals for the last decades. Perfusion continued to be applied as well, but primarily if product instability demanded short bioreactor residence times. Recently there is increasing interest in perfusion as a means to increase upstream yields and reduce manufacturing costs. Another driver is to leverage the advantages of continuous Bioprocessing in terms of product quality. In combination with disposable bioreactors, perfusion facilitates great increases in flexibility and reduced plant footprints, compared to conventional biopharmaceutical manufacturing facilities[1,2].

However, since pharmaceutical industries have been focused mainly on fed-batch process development, perfusion medium development knowledge is sometimes mentioned as a key gap for the wider adoption of perfusion processing in biologics manufacturing. Compared to fed-batch cultivation, perfusion processes have fundamentally different requirements and challenges in terms of medium composition. The main challenge in perfusion medium development is to determine the cellular nutrient demands to reach a fully stable steady state and to optimally balance the nutrient concentrations. A well-balanced medium should allow perfusion at cell specific perfusion rates (CSPR) well below 50 pL/cell/d. This variable indicates the volume of medium per cell that is being perfused. The lowest possible CSPR for a given combination of medium, cell line and operating conditions, referred to as CSPRmin, can be determined using the "push-to-low" approach[3] and is a valuable indicator for the 'medium depth'. The latter is the reciprocal of CSPRmin and indicates the maximum viable cell density (Xmax) that can be supported by a given flow rate of medium.

## 2. Types of perfusion processes

The term 'perfusion' is widely used for a variety of continuous cultivation processes providing cell retention. Before starting medium development, it is crucial to understand the impact of the specific process on the medium requirements. Different manifestations of perfusion processes require different medium characteristics. During process development, it is crucial to remember that changes in the process specifications (e.g. target VCD, target perfusion

rate, bleeding or steady-state strategy and bioreactor parameters) will change the medium requirements. For this reason, medium development is recommended to either be fully integrated into process development, or to be performed subsequently.

Perfusion processes can be classified in different categories. In a first category, 'open' perfusion systems can be distinguished from 'closed' perfusion systems. 'Closed' perfusion systems provide a physical barrier between harvest and cell culture, typically a filter membrane. This implies a cell retention efficiency of 100%, which can be problematic once the cell concentration is higher than the medium depth capacity at a given perfusion rate. Higher cell concentrations than what the medium can sustain will result in decreased viability. The accumulation of dead cells in the bioreactor can subsequently cause fouling of the filter membranes. Also, a partial retention of the product in filtration based retention devices has been reported ('sieving')[4]. This can result in an increasing product concentration in the bioreactor over time with higher retention time and potential product quality deviations. To avoid overgrowth and to regenerate the culture by removing dead cells from the bioreactor, a strategy called 'cell discard' or 'bleeding' is often applied. On a regular basis (e.g. daily or continuously), cells are withdrawn from the bioreactor and remaining cells are diluted with fresh medium. It is also possible to automate this bleeding procedure by using for example online biomass monitoring. The bleed rate or cell discard rate (CDR) can be an important online parameter for monitoring the culture state[3].

In contrast to 'closed' systems, in 'open' perfusion cell retention the efficiency is generally or temporarily lower than 100%, indicating a loss of cells from the bioreactor through the harvest stream. This inherent cell bleed can be positive to maintain high cell viability. However, cell retention efficiency comes as an additional factor that needs to be optimized during process development, and the inherent cell loss will reduce the cell concentrations at which such devices can be operated effectively.

In a second category, perfusion processes can be classified based on the product characteristics. It is well known that for unstable molecules like blood

clotting factors, a short bioreactor residence time is critical for the activity of the product. For such molecules, perfusion has been the established process of choice. In order to reduce residence time, the perfusion rate may be relatively high (e.g. 5-10 bioreactor volumes per day). In such cases, the required medium depth is low, and by-product accumulation is alleviated by the high dilution rate. However, the medium cost is still of major importance.

On the other hand, if the product quality is relatively robust in the bioreactor environment, residence time has a lower priority. However, it has been reported that also for relatively robust molecules like monoclonal antibodies, a reduction in residence time compared to fed-batch can be beneficial for the product quality in terms of reduced product heterogeneity, e.g. degradation and charge variants[5]. Instead of a short residence time, lower perfusion rates are typically targeted for the production of robust molecules to reduce the cost of goods and operational complexity of handling relatively large medium and harvest volumes, compared to a relatively small bioreactor size.

And in a third category, perfusion processes can be classified based on the steady state characteristics. The desired length of a steady state is on the one hand driven by the balance between the amount of drug needed to supply the market, the plant capacity and the volumetric productivity. In addition, it is driven by product quality requirements and characteristics. For high volume market drugs and/or for proteins that will benefit from a less heterogeneous quality profile, longer steady state processes will be beneficial. On the contrary, for proteins with low volume requirements and less quality restraints, an intensified and short perfusion process could be the most relevant, potentially omitting a full steady-state to shorten development time and maximize volumetric productivity and space-time-yield.

## 3. Perfusion processes and their medium requirements

As discussed above, the requirements for perfusion medium are strongly dependent on the product and process characteristics.

**a.** Impact of product stability

If the product is stable in the bioreactor environment, the target is usually to

perform perfusion at low perfusion rates of 1-3 VVD with the highest possible cell density. This requires highly concentrated medium of great medium depth. If the product is not stable, elevated perfusion rates are required in order to remove the product out of the bioreactor environment at higher rates. Determination of minimum perfusion rate to maintain product quality is a typical aspect of process development with such molecules. To reduce costs, the optimal perfusion rate will be set as low as possible whilst maintaining the highest possible target cell density. The effective control of specific medium cost is a subsequent target for process optimization.

**b.** Cell line specific requirements

The medium requirements also depend on the production organism. Even within the CHO cell line family, collectively originating from a single Cricetulus griseus ovary, there are significant differences in metabolism. This can be explained, for example, by the differences in lineage (CHO-S, CHO K1, CHO DG44)[6], transfection method (e.g. DHFR system, GS system), clonal selection procedure, or the molecule being produced. A common strategy many companies rely on when optimizing medium for a given clone is to begin with a medium platform, such as Cellvento™ CHO-110 or Cellvento™ CHO-220 media (specifically designed for CHO-DG44 and CHO K1, respectively) or EX-CELL® Advanced™ CHO Fed-batch medium (designed for CHOZN-GS, CHO-S, CHO K1 and other DHFR- varieties). A subsequent customization of the medium allowing for improved performance (e.g. VCD, productivity or quality characteristics) for the specific process is then relatively straightforward.

**c.** Targeted process

As explained above, the general term 'perfusion' includes processes that are very different from each other. The reasons to choose a specific manufacturing process is dependent on a lot of factors, and even the same team may choose different processes for different products. These different processes will have different media requirements for a fully optimized performance.

## 4. Medium Development and optimization strategies

As mentioned in section 2, process specifications will have a strong influence on the required medium characteristics. Perfusion process development and

optimization is hence linked to medium development and optimization. For example, a common method to optimize perfusion processes includes the growth arrest of cells or in general a reduction of cellular growth rate. The target with such approaches would be to achieve a steady-state at low bleed rate, which can minimize yield loss due to bleeding. This can be achieved by modifying bioreactor parameters such as temperature[7], or through medium modification itself. Before starting medium optimization, one needs to have some understanding on what the final process is going to be and plan medium and process optimization steps accordingly.

To start medium development, the first step will be to screen commercial or in-house available media. In the absence of a suitable medium specifically designed for the type of perfusion to be run, a good starting point would be a batch medium or a fed-batch medium/feed blend. For example, Cellvento™ CHO-110 Medium or EX-CELL® CD Fusion Medium were designed as medium for CHO batch processes and are comparably rich when compared to typical fed-batch media. Batch media have to contain all of the compounds required for growth and production at concentrations high enough for the whole process, which helps explain why they proved to be a suitable starting point for process and medium development, without feed additions or other modifications[8,9].

In the subsequent medium development step, the goal is to optimally balance the nutrients to sustain very high cell densities with the lowest possible perfusion rates. This will depend on medium depth and cellular requirements as well as on the inherent characteristics of the product. To achieve this goal, a combination of spent media analysis and statistical tools such as multivariate analysis and design of experiments can be applied. The final goal of this optimization is a medium that provides the required nutrients in balanced amounts, while maintaining all other components at the lowest possible concentrations to provide optimum metabolic conditions for the cells. At the same time, this medium needs to minimize the build-up of by-products. Ideally, cell specific productivity should be independent of cell density, culture duration and perfusion rate. Increases in qP through medium optimization including the application of new raw materials are also possible[10], especially if the starting medium before this development step had not been optimized for the specific clone.

**a.** Scale-down models for perfusion medium development

A critical tool for medium development is a robust and highly representative scale-down model of the perfusion process as screening system. Currently, there is a gap between practical aspects and theoretical requirements of scale-down models for perfusion processes. At this time, there is no commercially available cell retention device that can work with working volumes small enough to allow high throughput screening, as required for the generation of robust data suitable for statistical analysis tools such as MVA. Thus, it is considered acceptable to use scale-down models only partly representative if an increase in throughput can be achieved. These models have to be qualified early in the medium development process in order to guarantee the applicability in perfusion for that specific clone and process.

There are a number of strategies commonly used to model perfusion systems in somewhat high throughput systems. Some of the most commonly used strategies include repeated batch, semi-continuous chemostat or semi-continuous perfusion[11,12,13,14]. In these models, a fraction or all the culture with or without cells will be discarded and refilled with fresh medium at a given frequency. One critical aspect with regards to scale down models for perfusion processes is the difference between continuous and semi-continuous operation. Perfusion processes in bioreactors are run as continuous processes, while small scale models are typically run as semi-continuous or fully discontinuous processes. In semi-continuous operation, there will necessarily be fluctuations in the nutrient concentrations, potentially reaching limiting levels for some critical components. Since some nutrient consumption rates depend on their availability[15], the cellular metabolic state of the cells might be different from continuous steady-state behavior when compared to the semi-continuous model. The closer to CSPRmin a process is operated, the more likely semi-continuous operation will result in temporary limiting conditions. If experiments to determine CSPRmin for a certain medium/clone combination are performed semi-continuously, CSPRmin might be systematically overestimated. The higher the frequency of medium exchanges that can be performed, the closer the nutrient concentrations will be to a true steady-state and the more representative the results will be to fully continuous operation. If a sufficient safety margin from CSPRmin is maintained,

semi- or quasi-continuous operation has advantages in terms of experiment throughput as this can be automated[16].

Based on the CSPR concept[17], medium development can theoretically be performed at any cell density if the medium supply is adjusted to result in a similar CSPR. Using this principle, some scientists are using a continuous or semi-continuous culture without a cell retention device, a chemostat, to model perfusion media development. In this process cells are being removed continuously or semi-continuously with the harvest. A consequence of the continuous removal of cells is that the cell concentration at steady state will be inherently lower than in a perfusion process. The nutrients that limit the VCD in a chemostat are most commonly the same ones that will become limiting in a perfusion process and will hence determine the CSPRmin. As a result, optimizing a medium for a chemostat should result in a lower CSPRmin for a perfusion process, given that the metabolic profile of the cells for these critical components is maintained at extremely high cell densities reached during an intensified perfusion process. In addition, it is possible to design media for perfusion such that one specific substrate is limiting. In such cases, the nutrient limitation can be used to control the process in a narrow range. This limiting substrate should allow to transition between perfusion and chemostat and achieve meaningful results in both modes. In other cases, the limiting substrate is not well characterized ('natural limitation')[3], and perfusion is then operated at controlled VCD using automated or manual cell bleeding well below the maximum cell density that can be maintained at a steady state at a given perfusion rate. The comparability of chemostat and VCD-controlled perfusion is not necessarily a given and thus has to be studied empirically case by case.

**b.** Cell culture medium development strategies

Once an appropriate scale-down model has been defined, it can be used to screen different in-house or commercial media for their performance in the specific perfusion process to be developed. For example, MilliporeSigma uses a preselected media library that allows fast screening for desired medium characteristics. Typical attributes screened for are Xmax and CSPRmin of the medium/clone combination as well as specific growth rate μ [1/day] and specific productivity qP [pg/cell/day]. While a high μ is beneficial to achieve

high VCDs faster in the ramp-up of a perfusion process, it may be of advantage to control μ at a lower value during steady-state to avoid excess product loss due to bleeding. Specific productivity should be independent of CSPR and close to the qP max for this particular clone. Already in the screening phase, an analysis of nutrient consumption and metabolite production can yield valuable information about the needs of the particular clone and help understanding as to why certain media will perform better compared to others. Furthermore, with the use of potent statistical tools, one can identify critical components for specific responses of interest and use it as the platform for follow-up optimization. The identification of critical components, together with spent media analysis of those components that can be easily quantified, allows one to fully understand the best pathway for subsequent media optimization.

After the starting medium composition and initial critical components have been defined, there are two main paths, statistical and mechanistic, that one can follow for medium development and optimization. Statistical methods require the most intense throughput in order to obtain the most robust data. On the other hand, metabolic-based methods require less intense throughput but are more complex. Best results are obtained when both optimization paths are used in combination.

In statistical methods, concentrations of single medium components or groups of components are varied and the results evaluated mathematically[18,19,20,21]. Such methods typically require robust, high-throughput, automated scale-down systems as well as capable software for data evaluation. In return, improvements can be achieved rapidly and without in-depth understanding of cellular metabolism.

Metabolism-based methods require analytical capacities to study the concentrations of individual medium components. A basic method is spent medium analysis, where the perfusion harvest is compared with the feed medium to quantify the consumption of components and to detect nutrient depletions. Another simple method is the stepwise reduction of single or groups of nutrient concentrations to determine the minimum concentration to sustain

the desired process condition. In addition, if extensive modeling and –omics are to be applied, the analysis of intracellular content is typically required.

## 5. Conclusion

Perfusion media, particularly for intensified processes, have different requirements than fed-batch media. Cell retention system, bleed strategy, product stability and clone will determine the required medium characteristics for the specific process. Different strategies are available for perfusion medium development, but a crucial tool that is required is a representative, high-throughput scale-down model. Here, two aspects have to be considered or experimentally verified to make sure that the system is representative: if a semi-continuous cultivation is sufficient, and if a simplified cultivation mode can be used, e.g. chemostat instead of perfusion.

The first step is the application of the scale-down model to screen for a starting medium formulation. For this, commercially available batch media (e.g. EX-CELL® CD Fusion or Cellvento™ CHO-110 Medium) or fed-batch media (e.g. EX-CELL® Advanced™ CHO Fed-batch or Cellvento™ CHO-210/-220 media), fortified by their respective feeds can be screened. After a starting medium composition has been identified, classical techniques for medium optimization can be applied, e.g. spent medium analysis. However, so as to develop a truly optimized medium to use in intensified perfusion, more sophisticated methods such as MVA can dramatically shorten development timelines. Typical optimization parameters are Xmax, CSPRmin and high qP.

Using these state of the art methods for cell culture media development, EX-CELL® Advanced™ HD Perfusion Medium was developed. This medium was designed specifically for intensified steady state perfusion processes, targeting high volumetric productivity at low CSPRs. To ensure the broad spectrum of this medium, the development included seven different CHO cell lines (including CHOZN GS, CHO-S and DG44 producing different proteins) using high-throughput scale-down models specifically optimized for perfusion media development applications. Results were confirmed in perfusion bioreactors using the ATF cell separation device with four of the cell lines. In addition, applicability of this medium to other intensified perfusion applications such as N-1 perfusion was verified.

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# Perfusion Application in Mobius® Single-use Bioreactors

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## 1. Abstract

The Mobius® portfolio of Single-use Bioreactors was developed to facilitate the bioproduction of recombinant protein products in the single-use biomanufacturing space. While typically designed for use in conventional batch and fed-batch modes of operation, the goal of this study was to determine if these single-use systems could support perfusion-based bioproduction applications.

The Mobius® 3L and 50L Single-use Bioreactors were connected to the Alternating Tangential Flow, XCell™ ATF, cell retention device as per instructions from Repligen, and evaluated for their performance in generating a proprietary in-house monoclonal antibody. The results of this study demonstrated that the bioreactors could be aseptically connected to XCell™ ATF devices and support the perfusion application.

In addition, it was demonstrated that Cellvento® CHO-100, general-use media, was an appropriate media to support perfusion-based generation of IgG in a CHO cell-based expression platform. These results support the feasibility for evaluating our Mobius® Single-use Bioreactor portfolio and Cellvento® media offering in the upstream perfusion Bioprocessing space.

## 2. Introduction

Manufacturing of biotherapeutics is evolving to encompass widespread use of single-use technologies in order to control costs and optimize operational flexibility. Single-use bioreactors are commonly used for both process development and commercial production of monoclonal antibodies (MAbs) and recombinant proteins. While utilization of these systems have been routinely employed for batch and fed-batch modes of operation for the generation of recombinant products from mammalian expression systems, the use of the Mobius® Single-use Bioreactors for perfusion in this operational space has yet to be sufficiently characterized and implemented.

The Mobius® Single-use Bioreactor platform was developed to support the upstream single-use production space (Figure 1). The Mobius® 3L and 50L Single-use Bioreactors were designed to specifically support cell line expansion, process development and small scale recombinant protein production.

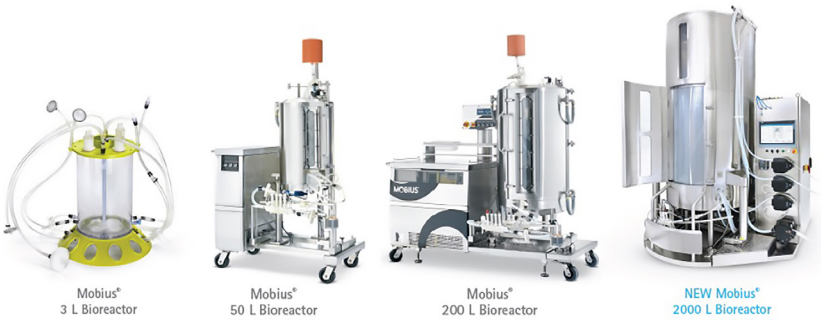


**Fig. 1** Mobius® Single-use Bioreactor options.

## 3. Application

The Mobius® Single-use Bioreactors were originally designed designed for use in batch and fed-batch modes of operation and a large dossier of collateral and applications data support their use in this space. While effective in these applications, there has been no collateral demonstrating their sustainability for use in the perfusion application space.

Perfusion in upstream processing relies on the constant addition of fresh media and concomitant removal of spent media containing IgG product from an operating bioreactor, while utilizing a cell retention device to maintain actively growing cells within the bioreactor under controlled conditions. A filtration-based cell retention device is a central control mechanism by which spent media may be removed from the bioreactor after separation from cells and large cell debris. This spent media, called perfusate, is subsequently processed through a purification scheme where the recombinant product of interest is ultimately enriched. Several different filtration technologies exist to support the perfusion application. A schematic diagram of the perfusion principle is shown in Figure 2.

In this study, the ATF™ hollow fiber-based filtration device was used to evaluate the perfusion application in Mobius® 3L and 50L Single-use Bioreactors. Within the ATF™ device, the appropriate Repligen hollow fiber cartridge was used (1mm lumen, 0.2µm PES).

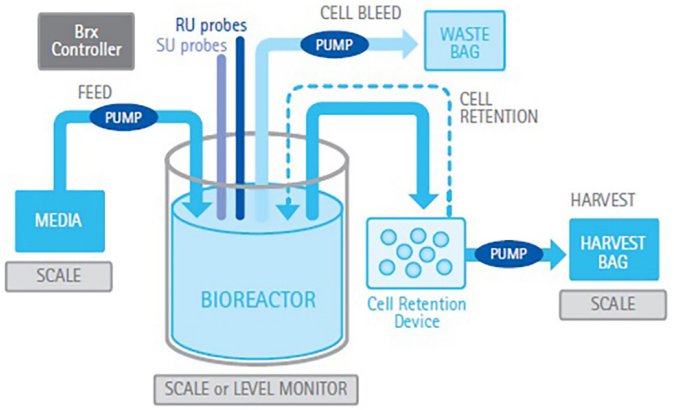


**Fig. 2** Schematic depiction of a perfusion set up supporting bioproduction in bioreactors. The bioreactor is fed with fresh media at the same rate at which spent media is removed. A cell retention device is used to separate spent media from cells in the bioreactor generating the recombinant product. In some instances, cell bleeds are periodically conducted to maintain operational control within the bioreactor.

The ATF™ devices available from Repligen are widely used in the Bioprocessing space. In addition, the principle by which it is used (alternating flow of cell culture media via diaphragm pump) is logistically less complicated than implementing a more conventional TFF-based media exchange in that minimal process development and/or optimization was required to evaluate ATF™ device performance. Given that this study was designed to answer basic questions about the feasibility of using the Mobius® Single-use Bioreactors in perfusion-based applications, it was important to utilize an apparatus that maximized the probability of technical success.

Briefly, a hollow fiber filter (or series of fibers) is contained within an autoclavable housing, and is connected to both a bioreactor as well as a pump apparatus. The pump facilitates the flow of fluid to and from the bioreactor (feed and retentate fluid) as well as the flow of fluid through the hollow fiber device (permeate fluid) to a collection/harvest container.

The pump flow rates, as well as the mechanism by which the ATF™ device is connected to the bioreactor contribute to the performance of the perfusion process.

The purpose of this study was to evaluate the feasibility of implementing a perfusion process using the Mobius® 3L and 50L Single-use Bioreactor using the Repligen ATF™ system and hollow fiber cell retention filter. The study evaluated the ability to maintain sterility of the devices, as well as appropriate assembly procedures to ensure effective operation of both the bioreactors and ATF™ unit. In addition, cell culture performance was evaluated and compared to that of the conventional upstream processing modes of operation.

## 4. Study Design

Studies executed to evaluate the feasibility of employing a perfusion mode of operation were completed in the Mobius® 3L and 50L Single-use Bioreactors. The 3L and 50L bioreactors were utilized with a working volume of 2L and 20L, respectively. An in-house CHO-S GS cell line expressing a proprietary monoclonal IgG (designated mAb05) was used for bioreactor experiments conducted in this study. Cellvento® CH0-100 chemically-defined media was used as the test cell culture media throughout the study, and was also the base media used for cell maintenance and expansion. This media was prepared as per instructions. For the 20L bioreactor run, approximately 800L was prepared using a Mobius® MIX1000 system and sterile filtered using a 10-inch SHRP filter into 200L drum bags. Media prepared for use in the 3L bioreactor studies (30L) was prepared in a Mobius® MIX100 system and sterile filtered using a sterile Opticap® XL600 capsules with Millipore Express® SHC 05/0.2µm filtration device. Prepared media was stored in a 50L pillow bag in the dark at 4°C until point of use.

Repligen provided the ATF™ device and corresponding pump apparatus in order to perform perfusion feasibility studies using the Mobius® 3L and 50L Single-use Bioreactor products. The ATF2 and ATF4 devices from Repligen were used with the 3L and 50L Mobius® Single-use Bioreactors, respectively. These two ATF™ devices differ in filter surface area, as well as media volume exchanged during

operation, and were chosen for this study based on recommendations provided by Repligen. Repligen hollow filter cartridges were used (1mm lumen, 0.2µm PES) in the ATF™ devices in both sets of studies. Specifically, cartridges of 75 and 830 fibers were used for the ATF2 and ATF4 devices, respectively. They corresponded to filtration areas of 1300cm$^2$ and 7700cm$^2$ for the ATF2 and ATF4 devices, respectively.

Inside a biological safety cabinet, a sterile dip tube was inserted into the third probe port on the lid of the Mobius® 3L Single-use Bioreactor, using C-Flex® tubing and a Lynx® S2S connector (Figure 3). Alternatively, a tube welder was used to aseptically connect the ATF2 device via the bioreactor harvest line. These connections served as the retentate lines. Repligen provided guidance to minimize the length of tubing connecting the ATF™ device to the bioreactor, specifying that this tubing length should be less than 20cm. From a logistical perspective, the method of ATF™ apparatus connection to the bioreactor

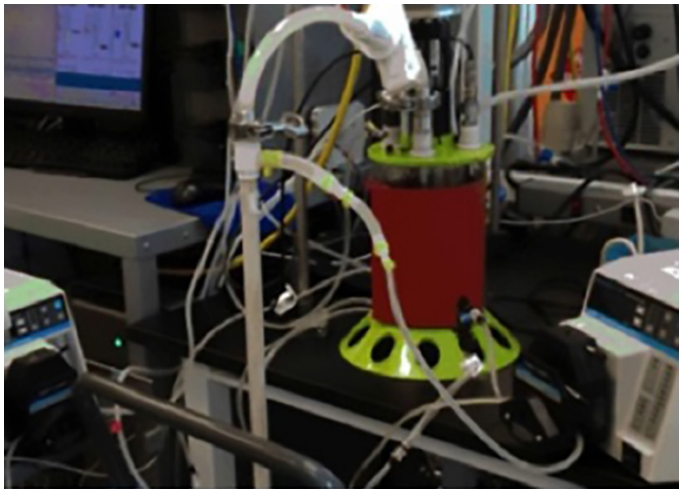

**Fig. 3** Connection of the ATF2 device to the Mobius® 3L Single-use Bioreactor via dip tube, using C-Flex tubing and a Lynx® 525 connector.

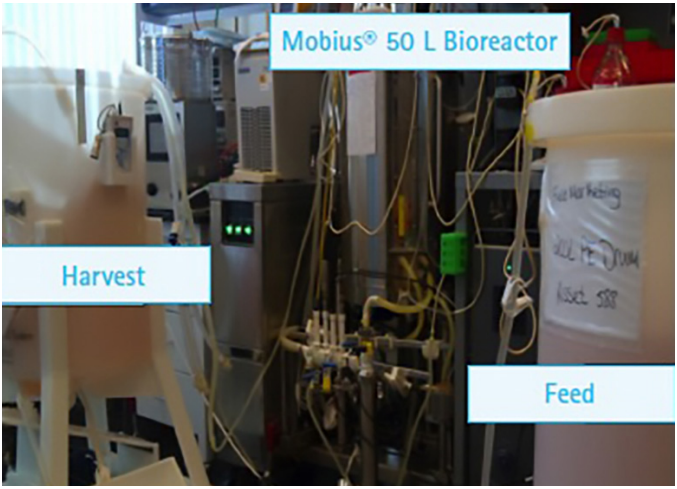


**Fig. 4** Connection of the ATF4 cell retention device to the Mobius® 50L Single-use Bioreactor. The connection was made to the upper harvest line in the Flexware® assembly, and feed and perfusate lines connected as per instructions from Repligen.

vessel was dependent on the capabilities of the tube welder (with respect to accommodating ¼ inch tubing as well as having access to the tubing to facilitate the physical cut and weld). In other words, connection of the ATF™ device to the bioreactor head plate was possible using a Lynx® S2S connector when tube welding to the bioreactor harvest line was not possible. In addition to the ATF™ device connection, a media feed line was tube welded to tubing on the bioreactor head plate, with media addition independently controlled using a peristaltic pump. In both 3L perfusion scenarios, the media feed line was aseptically connected between the media bag and a feed line on the top of the bioreactor vessel, and its rate of addition was controlled by a peristaltic pump calibrated to operate at a constant flow rate (Table 1). The perfusate line was aseptically connected to gamma- irradiated 2D pillow bags, via tube welding, to collect spent media and IgG product. The tubing connecting the ATF2 device and collection bag was run through a peristaltic pump set at a constant flow, which matched that of the feed pump. This was established to ensure that the

rate of media addition matched that of perfusion, creating a steady sate media environment within the bioreactor.
The ATF4 perfusion device was connected to the Mobius® 50L Single-use Bioreactor in order to perform a perfusion run at 20L working volume. The assembly is shown in Figure 4.

The ATF4 was connected to the Mobius® 50L Single-use Bioreactor through the upper harvest line, which served as the retentate line. Similarly, to that described for the set-up at 3L scale, the ATF4 perfusate line was connected aseptically to a Mobius® 200L drum bag contained within a plastic drum and placed on a floor scale to monitor the rate of perfusate accumulation. The feed line was connected aseptically to a feed line at the top of the Flexware® single-use assembly and the rate of media addition was controlled by a peristaltic pump.

The Mobius® 3L and 50L Single-use Bioreactors were filled with media and inoculated using standard procedures. Specifically, CHO-S GS cells expressing a proprietary human IgG molecule were thawed and expanded in shaker flasks containing Cellvento® CHO-100 supplemented with 50 µM MSX. Cells were propagated via passaging at 3-4 day intervals until sufficient cell mass had been generated to inoculate the Mobius® 3L and 50L Single-use Bioreactor. The operating conditions of the bioreactors are summarized in Table 1.

The bioreactors were inoculated at a cell density target of 0.3 – 0.5 x106 cells/mL using cell stocks in mid- logarithmic growth phase expanded in shaker flasks from a 1mL working cell bank. The ATF™ devices were attached on study day 3, at which point the three pumps (feed line, ATF™ diaphragm and perfusate line) were activated to initiate perfusion mode of operation. The perfusion rate was initially set at one vessel volume per day and this rate was increased daily by 25% (v/v) vessel volume increments until a media exchange of 2 vessel volumes per day was achieved. The perfusion rate was then kept constant at this rate until study day 16.

Beginning on study day 6, a small volume of bioreactor contents was siphoned aseptically from the bioreactors such that a target cell density of 30x106 cells/

mL was achieved (henceforth, this activity is referred to as "cell bleeding"). Daily cell counts and viability determinations were conducted using a Vi-CELL® automated cell counter. In addition, process metabolites were measured daily using the NovaFlex™ and Siemens RAPIDLab® 248 blood gas analyzer. Volumetric cell titers were determined daily, starting on study day 5, using the ForteBio Octet™ protein A affinity assay.

## 5. Results

The Mobius® 3L and 50L Single-use Bioreactors were inoculated at working volumes of 1.7L and 20L, respectively. They were assembled and operational parameters were confirmed to match those summarized in Table 1.

At both bioreactor scales, cells were used to inoculate the vessels to a final concentration of 0.3-0.5x106 cells/mL.

The bioreactors were operating under control conditions described in Table 1. Perfusion of the bioreactors started on study day 3 at 1.0 vvd (vessel volume per day), which was progressively increased in 25% (v/v) increments to 2.0 vvd by study day 7. The perfusion rate was subsequently kept constant at 2.0 vvd through study day 17 at both bioreactor scales. Cell growth was monitored daily using an automated cell counter, the results are shown in Figure 5.

Growth curves for both 2L and 20L scale bioreactor were comparable throughout the study. Exponential growth was observed until approximately study day 6, at which point cell bleeds were initiated. Daily cell bleeds were conducted in order to maintain cell densities in this perfusion application within a range consistent with that typically observed in commercial bioprocesses. In addition, once cell bleeds ceased at study day 17 in the 20L bioreactor, cell densities increased significantly through study day 21, when the study was terminated.

In addition to cell counts, viability determinations for each bioreactor scale were determined daily using an automated cell counter. As shown in Figure 6, culture viabilities at 2 and 20L scales were comparable throughout the duration of the study vessel harvest.

| Process Variable | Value | |
|---|---|---|
| Mobius® Single-use Bioreactor | 3 | 50 |
| Cell line | CHO-S-GS | |
| Expressed protein | mAb05 | |
| Cell culture media | Cellvento™ CH0-100, SOμM MSX antifoamC (as needed) | |
| Working volume | 1.7L | 20L |
| Agitation P/V | 30W/m3(250rpm) | 30W/m3(140rpm) |
| Temperature | 36.8° C | |
| pH | 7.0 ± 0.05 (microsparge $CO_2$, 1M sodium carbonate) | |
| Dissolved oxygen | 45% | |
| Perfusion device | ATF2 System | ATF4 System |
| Bioreactor connection | Diptube (3/8" ID) or Harvest line (1/4'' ID) | Harvest line (1/2" ID) |
| ATF™ flow rate* | 0.8L/min | 4.7L/min |
| Perfusion start | Day 3 | |
| Media addition rate | 1 vvd on D3, increasing 0.25 WD daily until 2vvd | |
| Cell bleeds | Daily bleeds to 30E6 vc/ml after VCD> 40E6 vc/ml | |

**Table 1** Mobius® Single-use Bioreactor and Repligen ATP Device operation conditions at 3L and 50L Scale.

* Scaled using equivalent flow per fiber length.

In addition to evaluating a perfusion process performance at two volumetric scales, the media exchange in the context of cell densities that were achieved at the two bioreactor scales evaluated, was analyzed. As shown in Figure 7, the cell specific productivity rates (CSPR) were compatible at 2L and 20L scales.

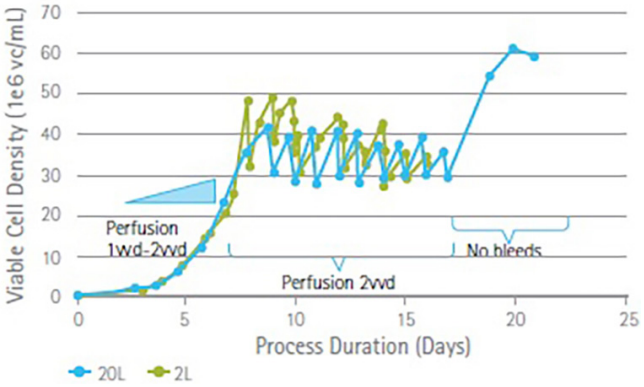


**Fig. 5** Cell growth curves for 2L (green) and 20L (blue) working volume Mobius® Single-use Bioreactors. The perfusion rate was progressively increased from 1 to 2 vessel volumes per day (wd) through study day 6, where a 2 wd media exchange rate was maintained until study day 17. Daily cell bleeds were also performed in order to target a bioreactor cell density of $30x10^6$ cells/ml.

In addition to cell growth and viability, cellular production was also analyzed in the two bioreactor scales evaluated. Perfusate samples were collected daily throughout the study and IgG levels determined using the protein A affinity assay. In the perfusion studies, the daily volumetric titer was approximately 0.5 g/L (data not shown) and this expression level was consistent through the duration of the study. Unlike batch and fed-batch processes where volumetric titers progressively accumulate during a campaign, the titer in perfusion processes typically did not accumulate appreciably, due to the significant volume of media perfusate collected over the process duration. However, evaluating the mass balance of total IgG generation revealed that cellular productivity was similar to other bioreactor process modes of operation. As a representative demonstration of total recombinant protein generated in various modes of bioreactor operation, Figure 8 shows the total amounts of IgG generated at 20L scale in batch, fed-batch or perfusion modes of operation using the same mAb05 cell line. The figure demonstrates that for this 50L bioreactor (20L working volume), operating in a perfusion mode of operation generates significantly more recombinant product than other modes (i.e., batch

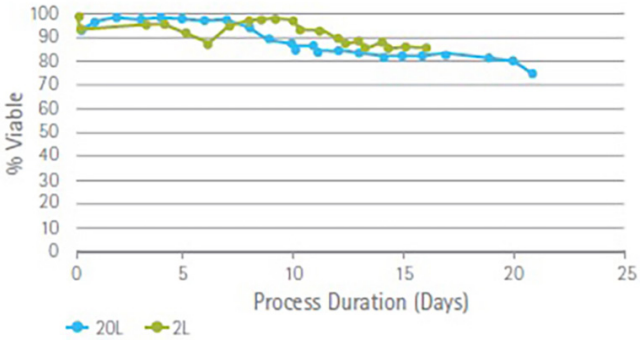


**Fig. 6** Cell viability curves for 2L (green) and 20L (blue) working volume Mobius® Single-use Bioreactors. The 2L and 20L bioreactors were run for 17 and 21 days, respectively, prior to vessel harvest.

or fed-batch) using the same processing footprint.

In order to compare IgG productivity between different bioreactor modes of operation, the specific IgG productivity was determined for batch and perfusion modes of operation employed to produce the mAb05 IgG. As shown in Figure 9, the specific productivity of IgG production was consistent between batch and perfusion bioreactor processes. In addition to comparing cell growth and productivity, the major metabolites glucose and lactate were monitored at both 2L and 20L perfusion scales. As shown in Figure 10, both the consumption of glucose, and the transient accumulation of lactate were consistent between both perfusion scales.

In this study, two methods of conducting an ATF2- facilitated perfusion process in the Mobius® 3L Single-use Bioreactor were evaluated; the ATF2 device was connected to the bioreactor either through the harvest line provided with the Mobius® Single-use Bioreactor, or through incorporation of a sterile dip tube into one of the probe ports provided on the vessel lid. Both methods of ATF2 attachment had advantages and disadvantages. Use of the harvest line allowed for sterile connection of the Mobius® Single-use Bioreactor and ATF2 device through tube welding, although this necessitated incorporation of a reduction in tubing diameter to accomplish the connection, and required use of a tube

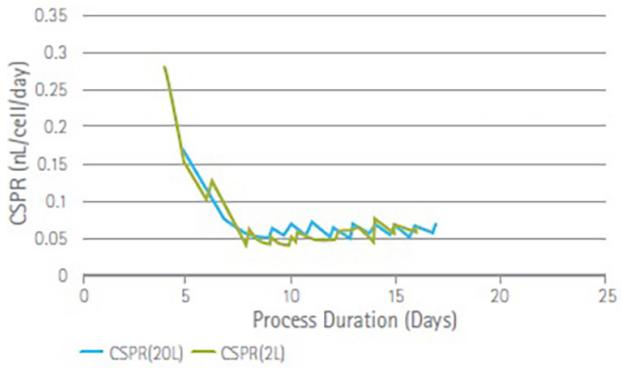


**Fig. 7** Curves of cell specific perfusion rate (CSPR) at 2L and 20L bioreactor scales. Media exchange rates were normalized versus cell density per day and plotted daily throughout the culture duration. Plotting these curves provides an indication of perfusion process comparability between study scales.

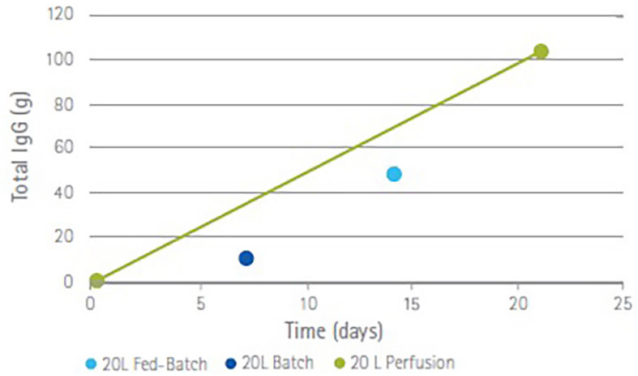


**Fig. 8** Total IgG production in a 20L working volume Mobius® Single-use Bioreactor. Comparison of total IgG generation in batch, fed-batch and perfusion modes of bioreactor operation at 20L (working volume) scale using the Mobius® so L Single-use Bioreactor platform.

welder capable of accessing the tubing in a restricted space. The impact of this tubing diameter reduction on cell viability was not evaluated per se, however no impact on cell growth or performance was observed when compared to a

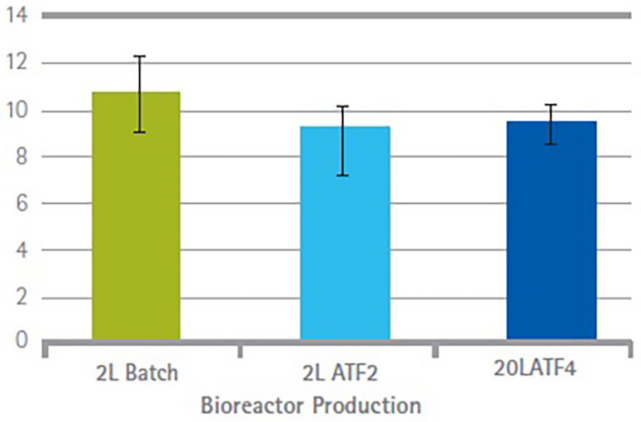


**Fig. 9** Comparison of specific IgG productivity of a CHO cell line expressing the mAbOS IgG in batch and two perfusion bioreactor runs. Specific productivity values are shown as pg protein per cell per day, and error bars represent the range of average values from duplicate samples.

bioreactor employing the ATF™ device connected via a dip tube, Figure 11. Use of a dip tube into one of the probe ports on the lid of the Mobius® Single-use Bioreactor vessel (and connection to the ATF™ device via a Lynx® S2S connector) did not result in any changes to tubing diameter providing media and cells to and from the vessel, although it did require installation by the operator in a biological safety cabinet.

The impact of the method of ATF2 assembly to the Mobius® 3L Single-use Bioreactor on cell growth was evaluated, and the results are shown in Figure 11. The minor differences in growth characteristics appear to be attributable to intrinsic biological variability of the CHO cells used in the study. As mentioned in the study design section above, the ATF2 and ATF4 cell retention devices housed hollow fiber filters with surface areas of 0.13$m^2$ and 0.77$m^2$, respectively. Use of these cell retention devices in the studies described resulted in filter throughputs of 166L/$m^2$ and 790L/$m^2$ for the ATF2 and ATF4 devices, respectively (based on the media volumes utilized in these studies), although hollow fiber filter capacity was not specifically evaluated in this feasibility

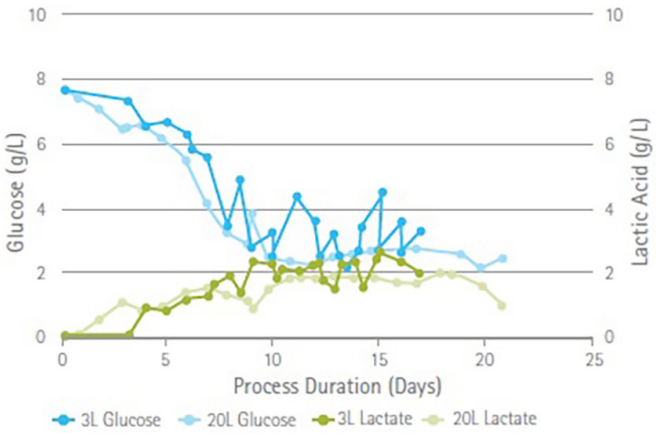


**Fig. 10** Comparison of glucose and lactate profiles at both 2L and 20L perfusion bioreactor scales. Glucose and lactate levels were determined daily using a Nova BioProfile® 400 analyzer.

study. Additional studies are warranted to more fully characterize the filters used in these cell retention devices.

## 6. Discussion

This study was conducted in order to demonstrate the feasibility of operating the Mobius® 3L and 50L Single- use Bioreactors in a perfusion-based mode of operation. The ATF™ technology from Repligen was employed to facilitate effective media exchange by utilizing connections available on the Mobius® 3L and 50L Single-use Bioreactors. In addition, Cellvento® CHO-100 was utilized as the media of choice to evaluate whether it could be used as an effective media for perfusion applications.

This study demonstrated that the ATF™ device could be aseptically connected to both a Mobius® 3L and 50L Single-use Bioreactor in a standard cell culture laboratory using standard tools and equipment typically found in such a lab. In addition, the utilization of single-use systems in this process was shown to be appropriate and convenient. Specifically, single-use bags were used to

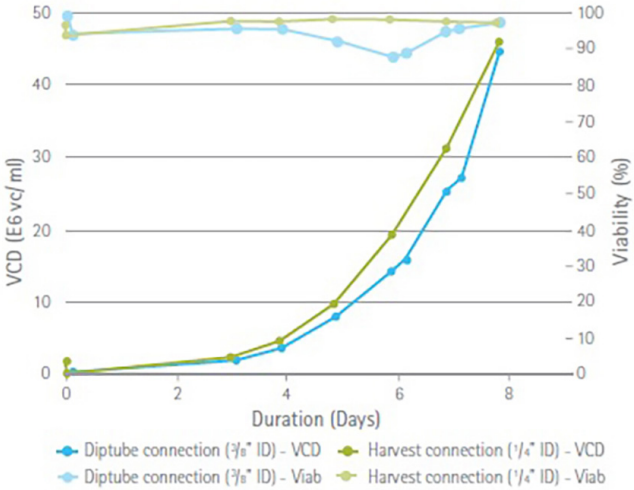


**Fig. 11** Comparison of CHO cell growth curves between differing perfusion set up assemblies with the Mobius® 3L Single-use Bioreactor. The ATF2 perfusion device was connected to the bioreactor either through the supplied harvest line or through an aseptic dip tube manually inserted into a probe port on the vessel lid. Cell counts and viabilities were determined daily using a Vi -CELL® automated cell counter.

hold and dispense feed media, as well as to collect perfusate from the ATF™ device. The Mobius® 3L and 50L Single- use Bioreactors are single-use systems that effectively supported cell growth and production in a perfusion mode of operation.

The results from this study also demonstrated that cell growth, viability and productivity could be maintained in a perfusion mode of operation, and yield recombinant protein product (IgG in this study) consistently in a perfusate continuously collected during the study.

Moreover, the specific cellular productivity using perfusion at 2L and 20L scale mimicked that typically observed in the more conventional batch mode of operation. This observation supports that introduction of the ATF™ device into the cellular environment has negligible impact on overall cell performance, and additionally demonstrates that Cellvento® CHO-100 media can be an effective media choice in this bioreactor application.

Cell bleeds were performed at regular intervals throughout the study, which facilitated operating the bioreactors with minimal biological variability, without extensive process optimization, and demonstrated proof of feasibility of employing a perfusion mode of operation with the Mobius® 3L and 50L Single-use Bioreactors. While these Mobius® Single-use Bioreactors were not originally designed during product development to support extended duration perfusion applications, this study provides guidance on how this mode of operation could be utilized with these devices. The Mobius® 3L Single-use Bioreactor, in particular, was validated for continuous operation for up to 30 days at 37°C, but an extended operational duration claim has yet to be validated. Despite the low risk of vessel failure is low when operated past 30 days (potentially due to loss of vessel integrity at the head plate/vessel interface, or to loss of durability of the impeller shaft), at present it would be incumbent upon the user to evaluate appropriate extended study durations.

While not specifically addressed in the present study, utilization of perfusion in bioreactor operation has a potentially significant impact on reducing the cost of capital investment of upstream Bioprocessing capabilities[1]. Interestingly, it has also been reported that cost of manufacturing per gram of product, however, is not significantly different between fed-batch and perfusion modes of bioreactor operation1. One potential value of employing the perfusion mode of operation is the generation of a larger amount of recombinant protein or mAb generated as a consequence of longer bioreactor processes compared to typical fed-batch applications. In other words, generation of larger amounts of recombinant product is potentially possible using a smaller facility footprint.

In summary, the results of this study demonstrate the feasibility of employing a perfusion mode of operation with the Mobius® 3L and 50L Single-use Bioreactors. CHO cell growth and productivity profile mimic those observed in batch and fed-batch modes of operation, and implementation of the perfusion apparatus can be successfully accomplished with low process contamination risk. The impact on bioreactor integrity, particularly with the Mobius® Single-use Bioreactor of operating this process past 30 days duration, was not specifically evaluated but the operational risk is perceived to be low.

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# XCell™ Perfusion Technology Delivers High Throughput:

## Efficient Seed Train Process with Improved Fed-Batch Production Output

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W. Roy Lin, PhD, Senior Director of Cell Culture Development and Manufacturing at Kinkisa Pharmaceuticals, has 20 years of industrial experience in microbial and mammalian derived biologics process development, commercial manufacturing support, project management, and operational eXCellence. Dr. Lin was formerly Sr. Director of Upstream Bioprocess at Repligen, Head of Fermentation R&D at Lonza and Director of Bioengineering at Shire HGT responsible for bioreactor process development, tech transfer and large-scale manufacturing support. Prior, he was Associate Director of Cell Culture Science at Human Genome Sciences, and Associate Director of Fermentation Development at Covance/Diosynth Biotechnology where he was responsible for upstream development and operations of a small-scale clinical cGMP production facility. Dr. Lin received his PhD in Chemical Engineering from Tufts University and was a Postdoctoral Associate in Biology at M.I.T. Dr. Lin also serves as a peer-reviewer for Biotechnology and Bioengineering and Applied Microbiology and Biotechnology.

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Mario Sinani is a Field Application Scientist at Repligen responsible for supporting the XCell™ ATF product portfolio. Mario was instrumental in the development of the XCell™ ATF N-1 application providing technical and scientific expertise. Mario brings years of industrial pharmaceutical experience to his role including 7 years in process development and cGMP manufacturing obtaining knowledge across range of biological products and upstream technologies. Mario has held key positions at multiple companies, including Novartis and Acceleron Pharma. Mario holds a BS from the Northeastern University in Chemical Engineering.

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## Dr. James Rusche

Dr. James Rusche has 30 years experience leading teams in Drug Development and Life Sciences projects. James co-founded a company in drug discovery technologies in 1993 and was senior VP of Research and Development from 1996 to 2016 at Repligen. During the last five years he helped build a development team to create and launch biomanufacturing tools suitable for single use and continuous manufacturing processes.

## 1. Abstract

The intensification of cell culture processes using media exchange through perfusion has dramatically increased the productivity of upstream processes. While conventionally used for continuous product harvest, perfusion can also be used to drastically alter the bioreactor turnaround and thus productivity in a fed-batch process. A conventional fed-batch process requires multiple stages of seed expansion and scale-up to inoculate a large scale fed-batch production bioreactor. This entire process can last 30 days or longer. However, if high density cell inocula are created from small scale Alternating Tangential Flow (ATF) perfusion bioreactors and then used in the pre-production bioreactor (n-1) with an XCell™ ATF perfusion system, the cycle time for conventional fed-batch production can be cut by as much as half.

We have modeled a production process with perfusion in the cell expansion phase using an XCell™ device. As illustrated in Figure 1, the XCell™ system enables us to prepare High Density Cryo-Seed-Intermediates (HDCSI) CHO cells in 0.1L and 1L cryo-bags, as opposed to small vials at low densities in traditional cell culture process. Integrating the 200L (n-1) seed train bioreactor with a pilot scale ATF-6 perfusion system allows us to generate a large seed inoculum for a 2,000L production bioreactor. The seeding density (10E6 cells/mL) is 30 times greater, and the entire process is 14 days shorter than the conventional seeding process. In addition, because the same cell inoculum from HDCSI is used each time the variability and contamination risk in seed expansion is reduced. In this manner fed-batch production processes can be modified to dramatically increase facility output often with only limited changes to existing capital equipment and facilities.

## 2. Introduction

In a traditional seed expansion, a cryopreserved cell-bank vial is thawed, followed by a series of expansions into larger culture vessels such as shake flasks, wave bags, and stirred bioreactors. Additionally, the time required from thawing a vial to inoculating the first bioreactor is critical as it involves significant human handling and poses a risk of contamination, variation and exposure to various hazards. Multiple cryo-vials need to be thawed to maintain uninterrupted availability of cells in order to inoculate seed expansion

bioreactors. However, each new thaw might bring variation and thereby additional delays to the overall process schedule.

In general conventional methods contain series of shake flask expansion followed by subsequent seed bioreactor stages; e.g., 20L (n-2) and a 200L (n-1) seed train bioreactors that generate enough cells to inoculate the production bioreactor at <1E6 cells/mL. When seeding the production bioreactor at low cell densities, the initial 5-8 days do not contribute much to overall protein production as the cells are still in a growth phase.

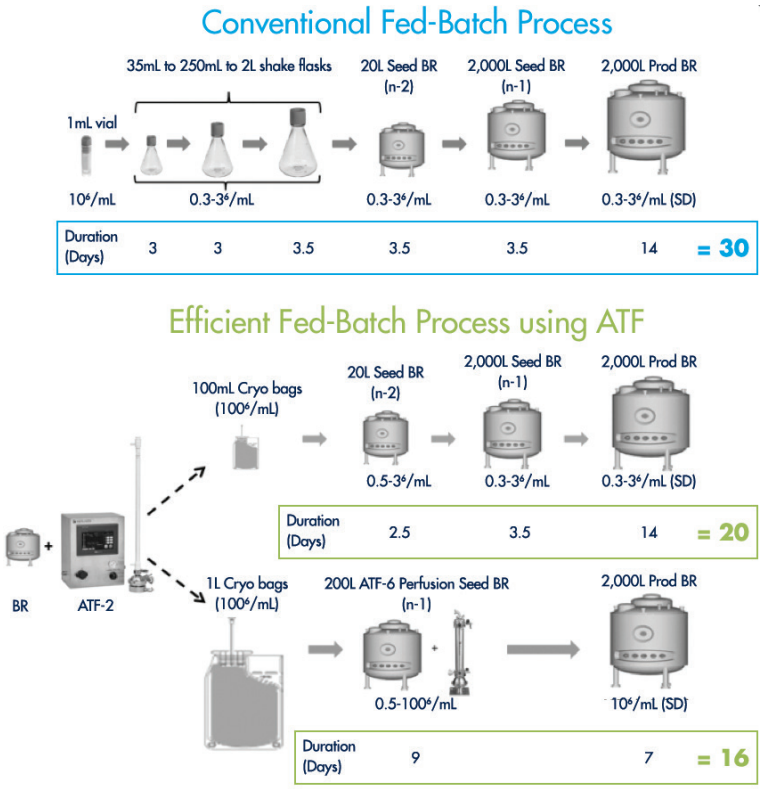


**Fig. 1** Efficient Seed Train Process with Improved Fed-Batch Production Output.

This chapter describes a simplified and speedy approach to seed culture expansion by generating HDCSI in cryo-bags at volume of 0.1L and 1L. The seed expansion process can be made efficient by inoculating the resultant HDCSI directly into an 200L (n-1) ATF perfusion bioreactor, allowing the seeding density in production bioreactor to increase by 30 times, and thus shortens the fed-batch production time from 14 days to 7 days. The use of HDCSI for direct seeding of n-1 ATF perfusion bioreactors and high density seeding of the production bioreactor preserves the fed-batch nature of final product production while maintaining the quality and quantity with a greatly reduced cycle time. While high density cryo bags (Gargi Seth et al. 2013) and n-1 (Benjamin Wright et al. 2015) applications have been reported, this is one of the first reports of combining these steps with CHO cell line to achieve such dramatic improvements in production cycle time.

## 3. Materials & Methods

### Cell line and Media

An industrially relevant mammalian CHO DP12 cell line (ATCC# CRL-12445™) was selected to evaluate the seed train process using an XCell™ ATF perfusion system. This cell line was adapted in-house to grow as a suspension culture in CD OptiCHO medium (Thermo Fisher) supplemented with 100ng/mL LONG®R$^3$IGF-1 (Repligen), and 4mM Glutamax (Thermo Fisher). All the following experiments were conducted using the adapted CHO DP12 cell line which expresses recombinant human anti-IL-8.

### XCell™ ATF-2 Perfusion system and Conditions

The XCell™ ATF-2 system (Repligen) consists of a C24 controller (version 2.5), 0.2-µm polyethersulfone (PES) filters (Repligen, 1mm ID × 60cm L, 0.13 m$^2$) linked to a diaphragm pump and a vacuum system. The ATF rates were maintained at 1 LPM for both pressure and exhaust throughout the runs.

### Bioreactor Conditions

- HD Banking (Perfusion mode)

A 3L glass bioreactor (Applikon) with 1.5L w.v connected to an ATF-2 perfusion system was used for HD banking. The inoculum for this bioreactor was

prepared from a frozen vial of LONG®R³IGF-1 adapted CHO DP12 cells and the seeding density was listed at 0.5E6 cells/mL.

HDCSI were prepared in 0.1L or 1L cryo-bags (Charter Medical) with conditioned medium at a cell density of 100E6 cells/mL. Conditioned medium contains 5% (v/v) DMSO and 0.11% (w/v) carboxymethylcellulose (CMC) (Sigma# C4888) as cryo-protectants. The filled cryo-bags were then placed in freezer cassettes (Custom Biogenics Systems) and the resultant cassettes were positioned in appropriate racks. These racks were kept in -80°C freezer for 24 hours, prior to storing them in liquid nitrogen.

• n-1 Culture (Perfusion mode)
To mimic the 200L (n–1) seed-train stage bioreactor, a 3L glass bioreactor (Applikon) with 1.5L w.v is connected to an ATF-2 perfusion system. The inoculum for this run was prepared from HDCSI and the seeding density was listed at 0.5E6 cells/mL.

• Production Bioreactor (Fed-Batch mode)
To mimic the 2000L production bioreactor, we operated 3L glass bioreactor (Applikon) with 1.5L w.v in a fed-batch mode. This bioreactor was inoculated at a seed density of 10E6 cells/mL directly from the above (n-1) perfusion bioreactor at VCD 100E6 cells/mL. Two shake flasks (40mL w.v) were also inoculated at 0.5E6 cells/mL from (n-1) perfusion culture to compare protein production at high (10E6 cells/mL) and low (0.5E6 cells/mL) seeding densities.

In HD banking and (n-1) perfusion cultures, the cell specific perfusion rate (CSPR) was maintained at 0.05 nL/cell/day with stepwise increments of perfusion rate based on daily cell densities. The fed-batch production bioreactor was fed with Feed A and Feed B (Thermo Fisher).

In all aforementioned bioreactors, a 1mm drilled hole sparger or 15µm sintered micro sparger were used for oxygen to control DO at 40%. The pH was maintained between 6.8 to 7.2 by adding 0.1N NaOH or sparging with CO2 and foam levels were controlled by adding Antifoam-C (Sigma) solution through peristaltic dosing pump.

### Analytical Methods

The cell density and viability measurements were analyzed using Vi Cell Counter (Beckman Coulter). IgG, glucose, lactate and ammonia were measured using Cedex Bio (Roche).

- Protein Quality

For protein quality analysis, small volumes of samples are collected and eluted using Protein A: Purified samples were run on SEC column (Zorbax GF-250) for aggregation studies. Glycan analysis was evaluated using Waters RapiFluor-MS Kit and samples were analyzed on Agilent 1290 Infinity II using ACQUITY UPLC Glycan BEH Amide Column.

## 4. Results & Discussion

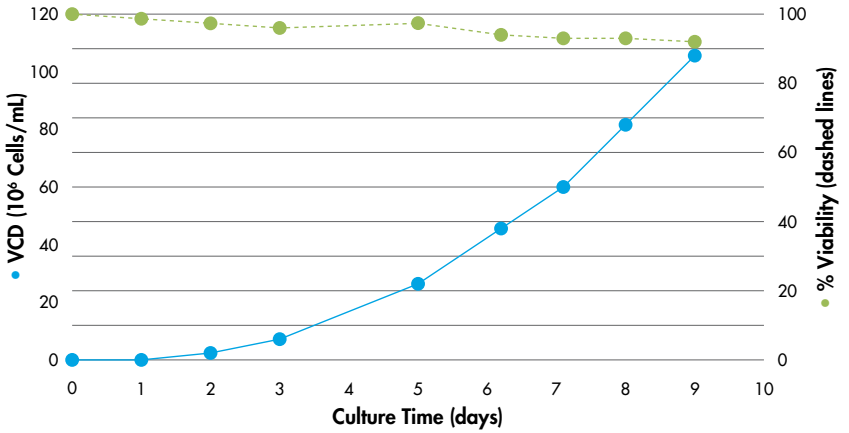


**Fig. 2** VCD and Viability profiles of HD banking in cryo bags (HDCSI).

### High Density (HD) Banking

HDCSIs are generated using an ATF2 connected to a 3L bioreactor. The process lasted for 9 days for the cells to reach 100E6 cells/mL density at a CSPR of 0.05 nL/cell/day. On the 9th day, bioreactor was spiked with final

concentrations of 5% DMSO and 0.11% (w/v) CMC. Right after, a desired number of cryo bags (0.1L & 1L) were welded to the bioreactor in an aseptic manner and transferred the cells along with the conditioned media spiked with aforementioned cryo protectants.

### (n-1) ATF Perfusion Culture

A 3L bioreactor (1.5L w.v) connected to an ATF-2 cell retention system was used to mimic 200L (n-1) perfusion bioreactor. The inoculum was prepared from HDCSI. As demonstrated in Figure 3 the n-1 perfusion bioreactor was operated until a VCD of 100E6 cells/mL is attained, which is the density required to inoculate a 2,000L fed-batch production bioreactor at seeding density of 10E6 cells/mL [from a 200L (n-1) perfusion bioreactor]. On Day 1, an initial drop in the cell viability from 74% to 60% was expected, as these cells were directly inoculated from a high density cryo bag. Similar post thaw performances of high density cryo bags were observed in the article by Gargi et al. 2012.

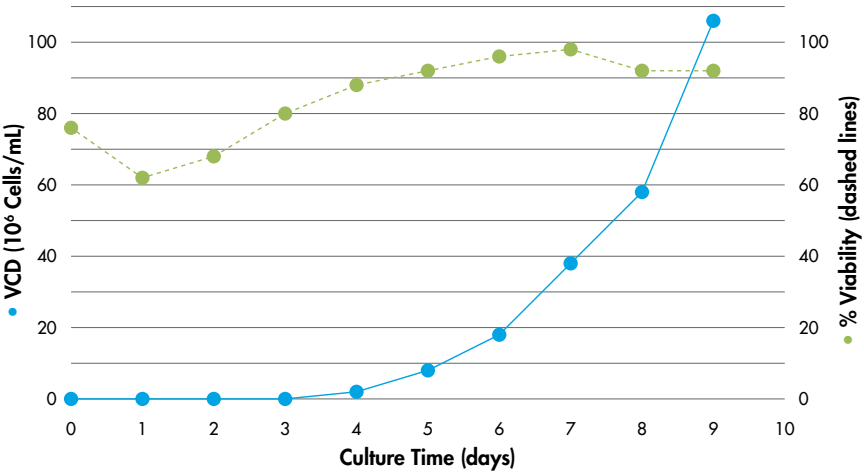


**Fig. 3** VCD and Viability profiles of (n-1) perfusion culture.

On Day 3, perfusion on (n-1) bioreactor was initiated at 1 VVD, and the CSPR was maintained at an approximate value of 0.05 nL/cell/day with manual stepwise increments of perfusion rate. (n-1) perfusion is a short and non-steady state process and is less complex than the continuous, steady-state production perfusion process.

The critical seed train equipment and fed-batch production bioreactor needed for this process remain the same as that of a conventional process, which results in simplifying the overall manufacturing process. According to our results, the total media used for the full (unoptimized) process would rise from 2222L to 5100L.

### Fed-Batch Production (Low seed and High seed)

Once the cell density equaled 100E6 cells/mL in (n-1) perfusion bioreactor, the cells were inoculated into low seeding density (0.5E6 cells/mL) and high seeding density (10E6 cells/mL) fed-batch production cultures. Low seeding density fed-batch was evaluated in shake flasks (historically proven to be

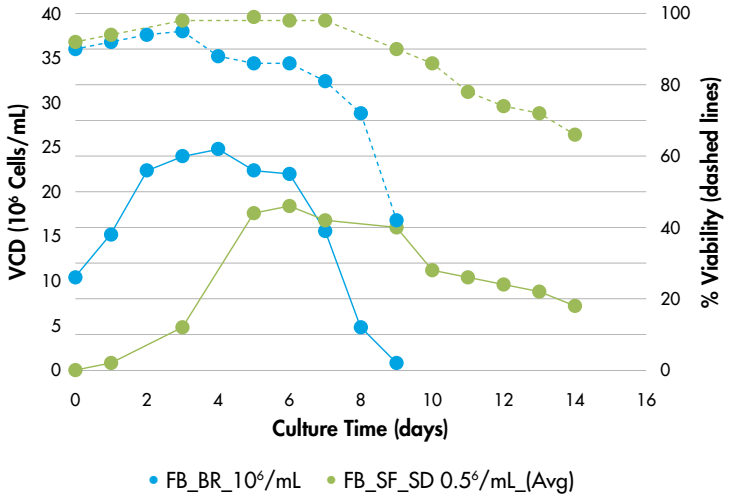


**Fig. 4** VCD and Viability profiles of fed-batch production cultures.(Low seed and High seed).

similar to reactor performance) and the high seeding fed-batch was evaluated in a bioreactor.

On Day 4, the high seeding density fed-batch condition reached a maximum VCD of 25E6 cells/mL, while the maximum VCD for low seed was only 19E6 cells/mL (Figure 4). The high seed culture lasted only 7 days compared to the low seed culture which ran for 14 days. Interestingly, the high seed was able to harvest the same amount of protein titer as the low seed (~0.75 g/L of antibody), in just half the time (see Figure 5). Using this process, the fed-batch production can be processed only in 7 days and produce same amount of protein as the low seed method, which is tedious and longer. Figure 6 plots cumulative productivity against integrated VCC and indicates a similar specific production rate between high seed and low seed fed-batch conditions.

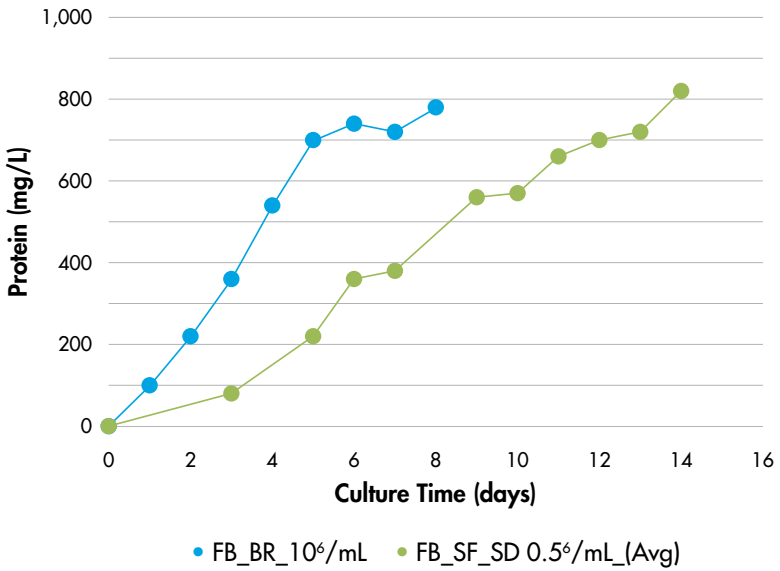


**Fig. 5** Protein profiles of production fed-batch cultures. (Low seed and High seed).

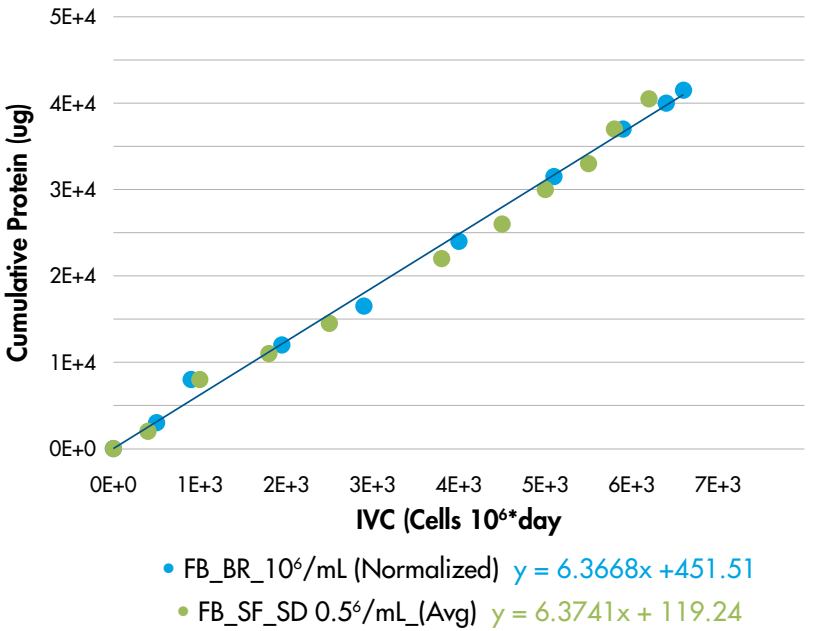


**Fig. 6** VCD and Viability profiles of fed-batch production cultures.(Low seed and High seed).

| Conditions | Glycan Analysis | | Size Distribution |
|---|---|---|---|
| (Seeding Density) | %G0F | %G1F | % Aggregation |
| Low Seed (0.5E6 cells/mL) | 79.70% | 15.80% | 2.3 |
| High Seed (10E6 cells/mL) | 82.20% | 14.40% | 1.9 |

**Table 1** Protein quality from fed-batch production cultures

**Glycan analysis**: Glycan distribution is represented by G0F and G1F levels: No significant difference was observed in glycan distribution between low seed to high seed. Small percentages of G0 and G2F were also observed in the protein samples (data not shown).

**Aggregation**: Total percentage of aggregates compared to the monomer.

In terms of product quality, no significant changes in protein aggregation and glycosylation were noted between both the conditions (see Table 1) (Note: Protein quality data for low seed culture was a separate low seed fed-batch study and cells were not from n-1 perfusion culture).

As the product quality profiles are comparable for both low seed and high seed cultures, it greatly increases the attractiveness of implementing (n-1) ATF perfusion process. Overall, (n-1) perfusion process increases productivity, while reducing the complexity involved in conventional seed-train process by eliminating the small scale expansions and labor intensive operations.

## 4. Summary/Conclusions

- Cell culture intensification using XCell™ ATF perfusion can create an efficient manufacturing process by significantly minimizing seed train stages and reducing the overall duration of fed-batch production process by half. The improved process involves:
  - Preparation of High Density Cryo-Seed-Intermediates (HDCSI)
  - (n-1) seed expansion using ATF
  - High seed fed-batch production bioreactor

- Process reproducibility should improve due to reduced process steps and more consistent n-1 bioreactor seeding.

- Conventional facility and production designs creating discreet fed-batch process lots can be intensified to achieve improved product quality and productivity.

- Similar product titer, cell specific productivity and protein quality were achieved in just half the time (7 days vs. 14 days) for a high seeding density fed-batch bioreactor compared to a typical process with low seeding density.

- Facilities considering implementing this intensified upstream approach will obviously place higher demands on the downstream process. Certain variations of the upstream schedule should be considered in order to optimize the overall facility output.

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# Product Quality Considerations of Perfusion Processes

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## 1. Introduction

Continuous mammalian cell culture processes are not new to industry and have always been the operation mode of choice for unstable proteins such as blood coagulation factors and various recombinant enzymes[1]. In contrast, modern humanized monoclonal antibodies (mAb) are relatively stable over several days in the culture broth. The enormous therapeutic success of mAbs combined with the simplicity and reliability of batch-wise processing has fostered the dominance of fed-batch platforms in biopharmaceutical industry. The optimization of host cells, bioprocess conditions and media formulations have drastically increased peak cell density and longevity, resulting in an increase of titer beyond 10 g/L[2]. Modern fed-batch platforms can considerably speed up process development, offering the possibility to culture several different cell lines by utilizing the same or similar media formulation. Given the change in cellular metabolism during fed-batch processes, unbalanced feeding can cause nutrient limitation or accumulation, particularly towards the end of the cultures. Furthermore, high by-product levels including lactate and ammonia are frequently observed disturbances for long lasting cultures of up to 21 days[3]. These changing environmental conditions and the longer residence time of proteins in fed-batch processes have been reported to significantly affect product quality including N-linked glycosylation[3], charge variants[4] or even structural properties[5].

In perfusion cultures, the continuous exchange of fresh medium and the retention of cells enable even higher cell densities and viability throughout the process. The defined cellular environment with smaller metabolite and nutrient variation are the basis for steady-state or quasi steady-state operation which is essential to keep product heterogeneity to a minimum. Whereas product quality and productivity might be a trade-off in batch-wise processing, continuous operation combines better economics with favourable protein characteristics[6].

However, the impact of protein modifications on therapeutic potency are not always well understood and thus have to be frequently reassessed in a case-by-case scenario[7]. The identification of product attributes that are of significant importance to therapeutic efficacy and safety is a crucial element of the Quality by Design (QbD) initiative. QbD is a proactive approach in pharmaceutical

development and manufacturing to implement and control critical quality attributes based on scientific knowledge, rather than empiricism[8]. In this regard, novel analytical technologies have pathed the way for a more in-depth analysis of various protein modifications ranging from glycosylation, glycation, charge variants, aggregates, LMW to amino acid misincorporation. Most of these attributes can be affected by the host cell itself, process conditions or the cell culture media[9]. Given the short residence time and a constant cellular environment, perfusion processes provide a better basis to obtain a more uniform and controlled product regarding the above mentioned protein characteristics. This shall be highlighted in the following sections where the impacts of perfusion mode on quality attributes are shortly discussed.

## 2. Glycosylation

Glycosylation describes the enzymatic linkage of oligosaccharides structures to amino acids such as asparagine (N-linked glycosylation) or serine/threonine (O-linked glycosylation) in specific sequons[10]. This post-translational modification is not only important for protein folding and homeostasis in the endoplasmic reticulum (ER), but has been reported to influence several product-specific properties. Stability, immunogenicity, circulatory lifetime and therapeutic efficacy have been related to specific glycan structures[11]. The glycosylation machinery of eukaryotic cells is highly influenced by environmental factors including trace elements, nucleotide sugar precursors, enzymatic inhibitors, bioreactor operation (pH, temperature, osmolality, dissolved oxygen) and accumulation of by-products such as ammonia[12]. Extensive effort in genetic engineering, media design and bioprocess optimization are therefore simultaneously applied to control glycosylation patterns during the manufacturing process. The changing environment and proteolytic degradation during fed-batch cultures have been reported to change glycan patterns with time, potentially leading to undesired forms[3, 13]. In contrast, the stable operation in continuous mode offers a consistent production throughout the entire process and allows further adjustments towards a desired glycan pattern[6]. Therefore, continuous processes are the method of choice to circumvent changes in glycosylation patterns, which are in particular important to reduce protein heterogeneities in complex glycosylated proteins with several N-linked and O-linked glycosylation sites.

## 3. Glycation

Glycation is linked to the pathogenesis of several chronic diseases[14]. Unlike glycosylation, glycation refers to the non-enzymatic attachment of sugar residues to the protein backbone and has been reported to predominantly occur in the cell culture medium[15]. The residence time of the protein plays a key role assuming the proposed pseudo first-order kinetics with respect to glucose and product concentration.

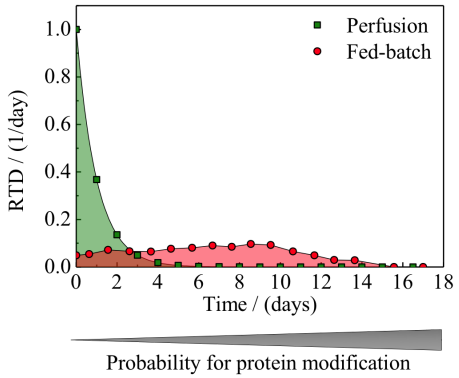


**Figure 1** Comparison of the product residence time of a perfusion process with an exchange flowrate of 1 reactor volume per day (green) and a 17 day fed-batch process (red). The probability and the frequency of many protein modification increases with the duration of the protein inside the reactor.

As shown in Figure 1, the product residence time in perfusion with a dilution rate of 1 reactor volume per day is considerably shorter compared to a 17 day fed-batch process. Therefore, the kinetics suggest that the reduction of the protein residence time in the bioreactor of approximately one order of magnitude decreases the overall glycation by the same extent. The long product residence time in a fed-batch implies not only higher overall glycation but favours a broader distribution: proteins produced early in the process are more likely to be modified, whereas proteins from later stages have less time to undergo such changes. Moreover, the tight control of glucose at low concentration in continuous mode potentially leads to even lower levels of glycation compared to currently applied feeding strategies in fed-batch processes[16].

## 4. Charge variants

IA vast amount of possible chemical degradation and enzymatic modifications on amino acid residues in the protein backbone affect the charge related heterogeneity of the protein product. Deamidation, oxidation and terminal modifications result in a shift of the protein net charge and lead to basic or acidic variants. In addition, charged sugar residues from the above mentioned glycosylation and glycation may also impact the charge distribution of proteins. Although the purification or enrichment of specific isoforms in chromatographic steps even at larger scales is feasible, a tight control in the upstream process is favourable due to the heterogeneous nature of charge variants and their large number of modifications[17]. Chemical modifications that occur predominantly in the cell culture media can be controlled to a certain extent with media supplements such as vitamins (free-radical-scavengers) or trace elements (cofactors)[18]. After all, it is again the substantially shorter product residence time of continuous processes that significantly reduces the degree of most degradation pathways by design.

## 5. Aggregates

The formation of protein aggregates is a major concern in biopharmaceutical production, not only due to product loss during several manufacturing steps but also regarding drug safety. Aggregates are formed by either covalent pairing or non-covalent interactions. The latter may be induced by adverse conditions such as high protein concentration and ionic strength that are particularly present at the end of a fed-batch process. Covalent aggregation is a mechanism commonly originating from disulphide bond formation of unpaired thiols. Reducing or oxidizing agents, such as cysteine or copper, can be added as media supplements to control these inter-protein sulphide bridges[19]. Independent of the aggregation mechanism and the used media additives, the shorter residence time of perfusion processes reduces the probability of aggregation events. Taking into account the proposed kinetics, the lower product concentration in perfusion bioreactors further slows down aggregation[20].

## 6. Low molecular weight (LMW) species

Low molecular weight forms represent a common degradation product of

proteolytic and spontaneous cleavage of covalent peptide bonds[21]. Despite the exceptional resistance of the backbone towards non-enzymatic hydrolysis, fragmentation can occur in more flexible local structures, such as the hinge region of mAbs. Proteases that are released to the culture may not only be active in the culture broth but also during the consequent purification train[22]. Moreover, the presence of metals and radicals can catalyse further degradation steps that lead to protein fragments. In all cases, a lower residence time favours less protein fragments, thereby increasing yields and simplifying downstream processing.

### 7. Amino acid misincorporation

In general, amino acid sequence misincorporation is thought to occur when the host cell is exposed to physiological stress in the production environment. For instance, the depletion of particular amino acids has been reported to significantly contribute to amino acid misincorporation[23]. The replacement of asparagine by serine residues has been the most frequent observed misincorporation in mammalian production cells[24]. Although the elimination of this mistranslation by additional asparagine has been shown, the resulting higher ammonia levels from asparagine metabolism can affect glycosylation[23]. Given the metabolic changes during fed-batch processes, the development of well-balanced feeding strategies is challenging[25]. In perfusion mode, the more stable cellular behaviour in steady-state simplifies the supply of low but sufficient levels of amino acids and other components.

### 8. Product quality optimization in perfusion bioreactors

When considering the numerous advantages of a short product residence time and controlled bioreactor environment, a high perfusion rate is key to obtain superior product quality. In fact, exchange flowrates of several reactor volumes per day might be the operation condition of choice for the production of unstable proteins. However, while keeping the cell specific perfusion rate constant, increasing the perfusion is limited by practical consideration (liquid handling, media, filter capacity, equipment restrictions)[26]. More importantly, safe storage conditions of the cell culture harvest or an immediate capture step is required to take full advantage of the lower residence time in the bioreactor.

The effect of media components, bioprocess conditions and operating strategies on product quality follow the same principles independent of the operation

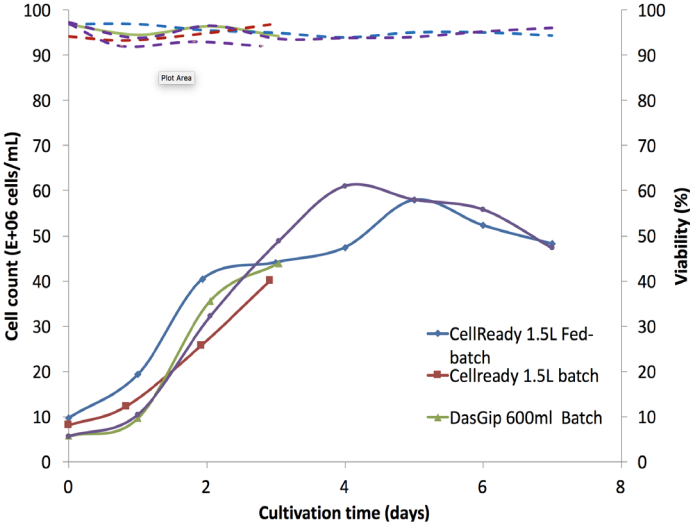


Figure 2 Viable cell density and viability of different steady-state conditions during one perfusion campaign. The different steady-states are indicated with striped surfaces whereas the transition is marked with light grey colour [27]. protein modification increases with the duration of the protein inside the reactor.

mode. Whereas miniaturized and parallelized culture systems support high-throughput screening of process conditions and media compositions in fed-batch, comparable scale-down solutions for continuous processes are still in development.

In order to investigate the effect of multiple conditions on product quality, a sequential screening can be applied within the same culture[28]. This is exemplarily shown for changing viable cell densities in Figure 2, where a sequence of different steady-states of a CHO cell perfusion culture was carried out. At an exchange flowrate of approximately 1 reactor per volume, the three steady-states represent conditions with different cell specific perfusion rates.

The corresponding mAb Fc glycosylation is presented in Figure 3 with a clear difference between the 20 and 60x106 cells/mL steady-state. A lower cell specific perfusion rate requires not only enriched media but simultaneously caused higher ammonia levels inside the culture which has been reported to decrease galactosyltransferase and other Golgi resident enzyme activities[29]. The duration until steady-state of quality attributes has to be determined in a case-by-case study, considering the residence time of the reactor and the adaption of cells to the new condition. Nonetheless, the sequentially steady-state investigation represents a fast way to screen the effect of operating conditions and media components on N-linked glycosylation and other protein modifications.

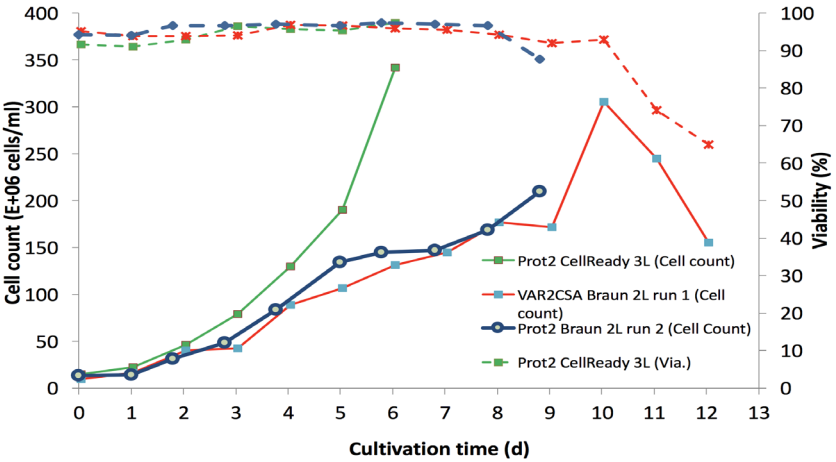


Figure 3 N-linked glycosylation profiles of IgGs produced at different viable cell density with comparable medium composition but different cell specific perfusion rate. rotein modification increases with the duration of the protein inside the reactor.

## 9. Conclusion

Although automated small-scale high-throughput perfusion systems are not yet available, the sequential steady-state screening approach in benchtop bioreactors enables to speed up product quality investigations at an already scalable size. Independent of the applied conditions, continuous upstream processing favours the enhancement of product quality because of three main reasons:

First, the continuous exchange of medium enables a constant physiological environment and thus intracellular post-translational processes function in a steady and physiological way.

Second, the constant cellular behaviour and the adaption of bioreactor media composition at defined C-source and amino acid concentrations may help to further control protein composition and modifications.

Third, the continuous removal of the product considerably shortens the time for protein modification and degradation, including glycation, fragmentation or aggregation inside the bioreactor. Most notably, the advantages of shorter residence time during the cell culture process can only be fully exploited by prompt or integrated purification steps.

Consequently, product quality considerations are expected to further push the increasing interest of continuous processes for the production of therapeutic proteins.

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# Impurity Monitoring as Novel PAT Tool for Continuous Biopharmaceutical Processes

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Oliver Spadiut, has studied Food Science and Biotechnology at the University of Natural Resources and Life Sciences (Vienna). During his studies, he spent several months in other research labs abroad to gain more knowledge and to broaden his horizon. After 18 months of successful post-doctoral research at the Royal University of Technology KTH in Stockholm, Sweden, he became University Assistant at the TU Wien, Austria. Currently, O. Spadiut is group leader of the research group "Integrated Bioprocess Development" and successfully finalized his Habilitation in March 2015. Since December 2016 he started as an Assistant Professor in TU Wien.

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Christoph Herwig, bioprocess engineer, graduated from the process engineering department, university of technology RWTH Aachen in 1994. He worked in industry in the design and commissioning of large chemical facilities prior to enter his interdisciplinary PhD studies at the University of Technology EPFL, Lausanne Switzerland in the field of bioprocess identification. Subsequently he positioned himself at the interface between bioprocess development and facility design of biopharmaceutical facilities working in different companies from process development over engineering services to biopharmaceutical production. Since 2008, he is full professor for biochemical engineering at the TU Wien. The research area focuses on the development of method of science based and efficient bioprocess development for biopharmaceuticals along PAT and QbD principles in upstream and downstream within industry driven projects using data science approaches.

## 1. Introduction

In the last decades, the Quality by Design (QbD) and process analytical technology (PAT) initiatives changed the manufacturer's perspective on biopharmaceutical process development and manufacturing. Slowly, but steadily, the manufacturer's focus shifted from final product testing to a detailed investigation and risk-based lifecycle management of the manufacturing process. QbD aims at achieving a high degree of scientific product and process understanding, which is developed in course of science-based process development using PAT[1]. The maturity of this development was underlined by the success of Genentech (Roche) in submitting the cancer drug Gazyvaas first full QbD process which also gained regulatory approval.

Today, the next biopharmaceutical manufacturing innovation is in the starting blocks. The shift from batch processing to continuous manufacturing, aiming at increased productivities by efficient use of assets and more consistent and predictable product quality is ongoing. Often the terms 'continuous unit operations' and 'continuous processing' are misleading, hence we want to provide clear definitions. A unit operation is defined other continuous when it can endure continuous inflow and subsequent outflow. A process is defined continuous when it is (physically) integrated to different continuous unit operations with no, or minimal, hold volumes in between[2]. If there are hold volumes between unit operations the process is termed as a batch process as shown in Figure 1A. A schematic diagram of a typical continuous process is depicted in Figure 1B.

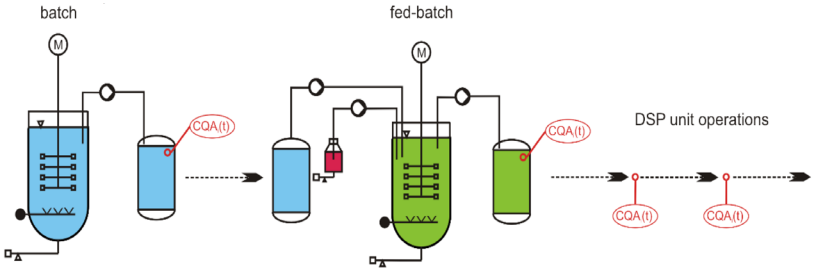


**Fig. 1A** Schematic diagram of a batch processing with hold steps, enabling ease of critical product quality attributes (CQA) measurements offline.

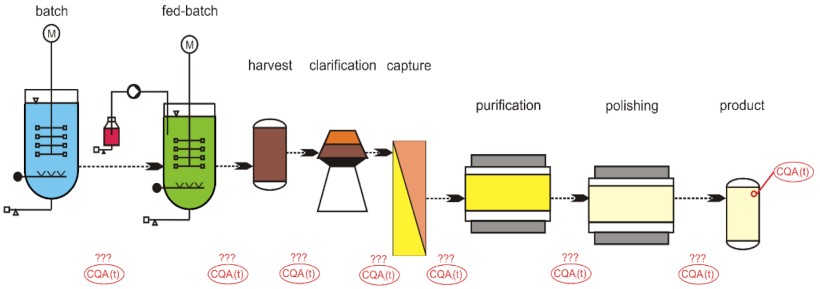


**Fig. 1B** Schematic diagram of a batch processing with hold steps, enabling ease of critical product quality attributes (CQA) measurements offline.

Leading pharmaceutical companies such as Genzyme, Bayer, Lonza, Novartis, Janssen, and Merck-Serono have manufactured approximately 19 recombinant products using elements of continuous processing. In addition to advantages such as reduced cost, flexibility and smaller ecological footprint, continuous processing enables better process control without manual intervention[3].

In our opinion, the optimum outcome when shifting from batch to continuous processes arrives when:

**a.** All unit operations can run in continuous mode, AND
**b.** the unit operations can be linked in continuous mode.

While significant improvements have been done for continuous single unit operations, fulfilling criterion a, there is a significant lack in tools for linking unit operations in continuous mode.

We strongly believe that the main enablers for the latter are:

1. PAT-analyzers, providing timely controlled and comprehensive characterization of the performance and the outflow of each unit operation.
2. Data Science tools which allow quick elaboration of complex process understanding.

The task is to:

**a.** adjust the process parameters of the current unit operation A using process understanding, hence on the knowledge how critical process parameters (CPPs) and critical product quality attributes (CQAs) are linked[1].

**b.** but in addition allow compensation for the variability in unit operation A by modulating succeeding unit operations B and C to achieve desired CQAs.

**3.** Control algorithms to process a multitude of input variables, allowing consistent and robust output and holding multiple quality attributes in the acceptable range. Such control systems are already available in other market segments, but hardly deployed in biotechnological applications.

The current book chapter proposes a novel concept linking PAT analyzers and data science tools to enable continuous processing.

## 2. PAT for CQA Monitoring

The design, analysis and control of biopharmaceutical processes require the monitoring of critical quality attributes (CQAs) of the drug product. A CQA is defined as "a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution

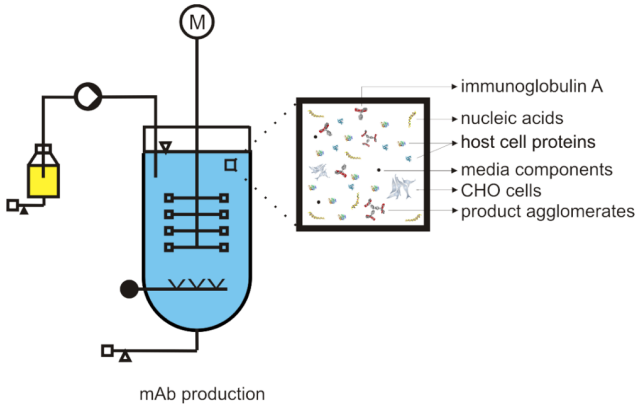


**Fig. 2** Typical impurities generated and present in a typical mAb production process.

to ensure the desired product quality"[1]. Typical CQAs are process related impurities such as nucleic acid or host cell protein concentrations in the cell culture, or characteristics of the drug product, such as glycosylation pattern or aggregate formation e.g. in a monoclonal antibody (mAb; e.g. IgA) production process (Figure 2).

In classical batch processing, CQAs can be measured subsequent to each unit operation in the hold step between two unit operations. This is required to assess the performance of the processing step and ensure that the process delivers the intended product quality. However, in continuous processes, there are no hold steps between unit operations because, by definition, the process is run in continuous mode. Therefore, as also illustrated in Figure 1B, for continuous processes, it is mandatory to measure CQAs directly in the process. This demands that the analytics of CQAs is performed close to the process using appropriate process analytical technology (PAT). Therefore, we consider process analytical technologies to measure CQAs as key enabler for the success of continuous biopharmaceutical manufacturing processes.

Direct measurement of CQAs is cumbersome as product specific measurements, such as specific activity or impurity concentration, are hard to analyze in a complex background matrix with different impurities. Most established analytical techniques for quantification of different impurities and determination of CQAs are offline, demand elaborate sample preparation and are therefore time consuming and typically performed in a separate analytical laboratory. However, in order to use this information for process monitoring and to set counteractions, information in real-time is necessary. Therefore, there is increasing need for fast analytical tools for enabling real-time determination of CQAs.

## 3. HPLC Fingerprinting: A promising tool to enable in-process CQA analytics

In order to accelerate the development of continuous processes, we present a novel technology that is highly promising for the measurement of process related impurity CQAs, hence assessing generic components independent of the specific product.

For decades, high performance liquid chromatography (HPLC) has been considered the "gold standard" for the accurate quantification of a variety of analytes. To date, the processing of spectral data from HPLC is mainly performed via simple peak integration and simple calibration models.

However, for process analytical applications, the complex matrix results in a high number of overlapping peaks, resulting in a characteristic fingerprint chromatogram (Figure 3). To the HPLC practitioner this seems unfavorable at first, since the quantification of analytes is not possible through simple peak integration. However, the chemical information, including information on protein structure, process- and product-related impurities, is contained within the chromatograms. This information can be extracted from the fingerprint chromatograms using chemometric methods.

## Characteristics, Bath and Continuous

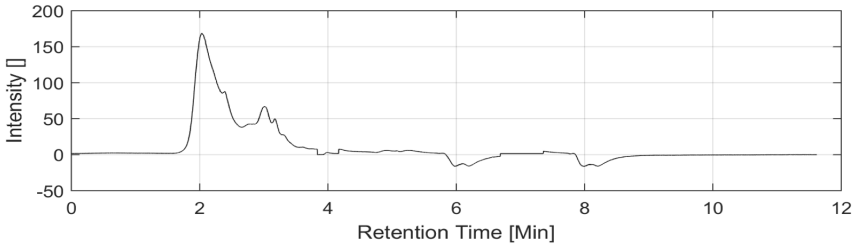


**Fig. 3** Chromatographic fingerprint of a bioprocess sample. The chemical information is contained inside the fingerprint and can be used for the determination of CQAs using chemometric methods.

Hence, we present a promising technology for timely resolved determination of CQAs, using the HPLC principle and impurity fingerprinting as a novel PAT tool. We use monolithic columns as they render high resolution separations, high reproducibility and can be operated at high flow rates and therefore allow high measurement frequency[4].

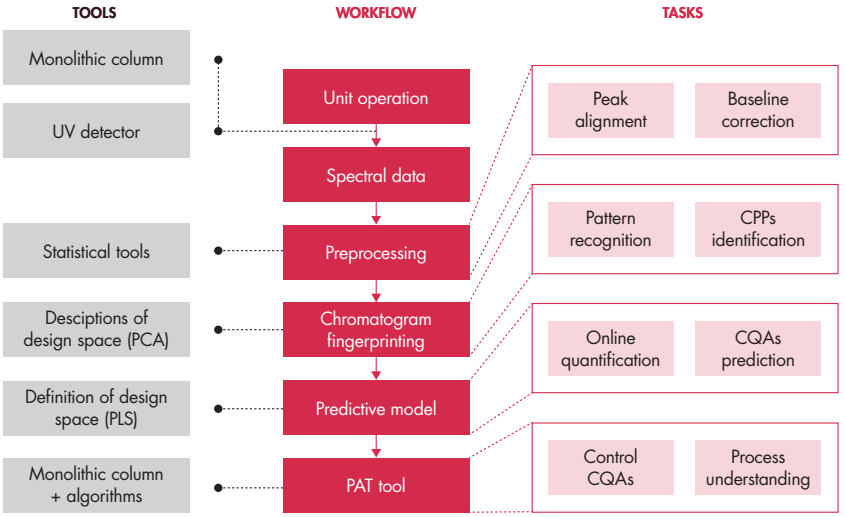


**Fig. 4** Workflow for the establishment of the PAT tool. The integrated PAT tool combines the HPLC hardware (such as monolithic columns and detectors) with data science tools for data acquisition, data pre-processing but also multivariate fingerprinting.

The proposed PAT tool is aimed to tackle process parameter variations in the upstream process on impurity release and enable modulation of downstream unit operations, with chromatogram fingerprinting and advanced chemometric techniques. The workflow establishment of this PAT-tool is shown in Figure 4. The workflow of the proposed PAT tool is as follows:

- The overall process requires samples from a biopharmaceutical process in a timely resolved way and from all unit operations. The samples procured from the cell culture along the process are analyzed using a HPLC system to obtain chromatographic fingerprints. Nucleic acids and proteins contribute to the major portion of the impurity content in a bioprocess. Hence, UV spectral data at two different wavelengths, namely 260 and 280nm, provide information pertaining to nucleic acid and protein content, respectively.

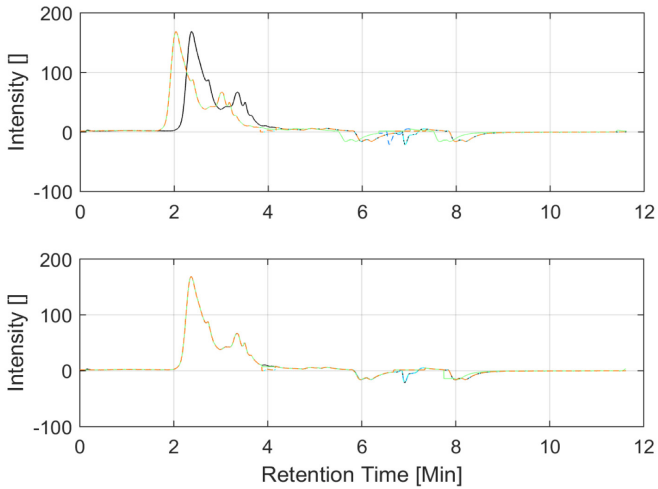


**Fig. 5** Alignment of chromatograms. A) Chromatograms show a shift in the x-axis (retention time). B) Using peak-alignment algorithms, chromatograms can be aligned and true differences between the chromatograms are emphasized.

- The main hurdles in using spectral data for extracting vital process information are noisy signals, shifts in retention profiles, shifts in baseline, peak tailing and artifacts. In order to overcome the aforementioned challenges, pre-processing of spectral data is mandatory. New advances in data science treatment have provided a plethora of statistical tools for tackling signal variations. The two main variations in spectral data to be considered are shifts in retention profile and baseline. Figure 5 shows an example of tackling the shifts in the retention profile.

- By definition, a chromatographic fingerprint is the chromatographic pattern for identification of some (bio-) chemical components in a complex sample[5]. In chromatogram fingerprint analysis pre-processing plays an important role as the acquired spectral data for timely resolved samples from a bioprocess must explain the same phenomena and highlight variations between samples.

- The pre-processed data are used for performing different chemometric techniques, such as principal component analysis (PCA) or principal component regression (PCR), for identifying variations in spectral data and recognizing patterns in spectral changes.

## 3.1 Application Example

With information from the techniques mentioned above, CPPs, which trigger or play a major role in impurity release in a cell culture in Upstream Processing, can be identified in a time resolved manner. As a case study, samples from a Pichia pastoris cultivation where the production phase was conducted at three different temperatures namely 20, 25 and 30°C, were taken. The raw chromatogram fingerprints of samples were recorded using a UV detector at 260nm as shown in Figure 6A. After preprocessing, PCA was done on the aforementioned spectral data. The score plot from PCs indicated trends in the variation with respect to temperature. The score plot depicting the clusters for samples having similar ribonucleic acid (RNA) release pattern at different temperatures is shown in Figure 6B. Similarly, the same methodology has

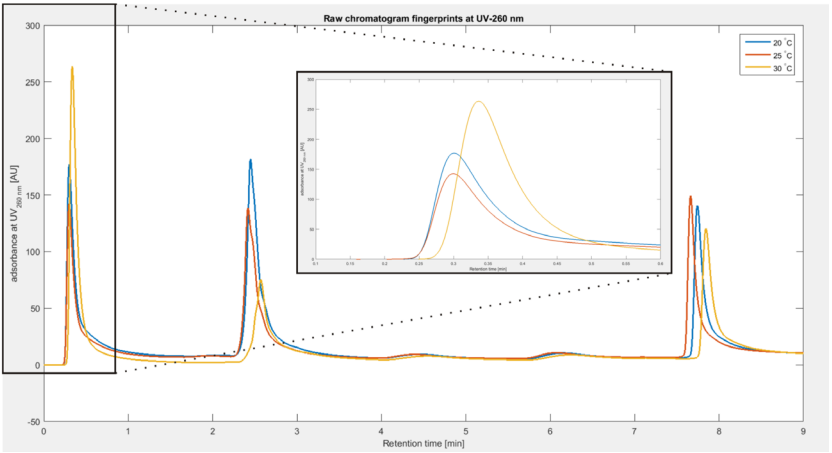


**Fig. 6A** An example highlighting identification of CPPs triggering impurity release using chromatogram fingerprints. Raw/not preprocessed chromatogram fingerprints of *Pichia pastoris* cultivation samples at different production temperatures.

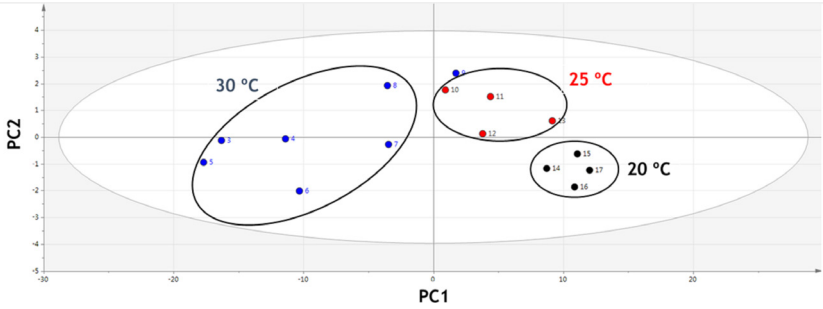


**Fig. 6B** Score plot of the first vs. second principal components depicting clear clusters at different temperatures.

been done for UV chromatogram fingerprints at 280nm to follow host protein release. Based thereon, mechanistic correlations between RNA and host cell protein release and product purity in the process can be established. The identified CPPs which influence the impurity release and in turn the CQAs, are aimed to be controlled in a continuous biopharmaceutical process with the aid of the proposed PAT tool.

Furthermore, the PAT tool can be implemented for downstream samples to follow the impurity pattern and CQAs and thereby modulating subsequent unit operations to achieve the desired product quality.

## 4. Conclusion

- Continuous biopharmaceutical processing is becoming an attractive segment in the industry due to various advantages. However, process control in continuous Bioprocessing is a tedious task, as analytical tools for real-time determination for CQAs and enhanced process control is not available to date. The presented PAT tool is envisaged to pave the way for continuous Bioprocessing without the need for manual intervention.

- As product CQAs depend on different factors, such as strain, media composition, mode of cultivation, the proposed PAT tool will also enable modulating downstream unit operations to compensate for upstream variations and achieve desired product quality.

- With the implementation of the proposed PAT tool, we intend to:
  - o identify and allow control of CPPs which influence impurity release
  - o determine CQAs in a timely resolved way, and
  - o enable horizontal process control to deliver the expected product quality along the entire process

- The advantages of the suggested impurity based PAT tool are:
  - o As impurities (such as host cell proteins and nucleic acids) are independent of the specific product, the method provides generic applicability.
  - o The HPLC chromatograms do contain actual information on specific compounds, which can be identified beyond pure fingerprinting. Therefore, the proposed tool is superior to conventional spectroscopic methods.
  - o Use of the HPLC principle as being accepted reference analytical device for easy incorporation in regulatory dossiers.
  - o Horizontal deployment across the full process, enabling easy operator training, direct comparability between unit operations and following the course of purification in real-time.

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# Insect Cells and ATF

## About the Author:

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Dr. de Jongh (South African) obtained a Bachelor degree followed by a M.Sc. in Chemical Engineering from the University of Stellenbosch, South Africa. Thereafter, he was awarded a doctorate in Biotechnology from the Technical University of Denmark in 2006. Dr. de Jongh has seven years' experience in the pharmaceutical industry in molecular biology; project management; process development; and process transfer to cGMP manufacturing.

## 1. Introduction

The choice of a manufacturing platform and production mode are some of the most important strategic decisions in recombinant subunit vaccine development. *Drosophila* S2 insect cell expression is less known than the extensively used Spodoptera (Sf9) or Trichoplusia ni (Hi-5) insect cell based Baculovirus expression system (BEVS). Nevertheless, S2 cells have been used in research for almost 40 years. The cell line was derived from late stage *Drosophila* melanogaster (Fruit fly) embryos by Dr. Schneider in the early 1970s, who named the cell line *Drosophila* Schneider line 2 (synonyms: S2, SL2, D.mel. 2). The S2 system has unique advantages for low-cost production compared to BEVS as it is a stable cell line based, non-viral and non-lytic system. This allows for a wide variety of upstream processing options compared to the obligatory batch process approach of the high-yielding, but lytic BEVS system.

The field of neglected diseases is specifically relevant for the application of process intensifying and cost reducing processing production modes. Particularly, the geographic distribution of malaria and the philanthropic funding sources involved require production to be as cost-effective as possible. Single-use bioreactors combined with perfusion production mode provide manufacturing flexibility and economic advantages, both highly desirable in this type of process. ExpreS$^2$ion Biotechnologies aims to develop cost-effective *Drosophila* S2 based production processes combining its ExpreS$^2$ constitutive insect cell expression system with single-use bioreactor and perfusion technology.

ExpreS$^2$ion has established collaborations with The Jenner institute, Oxford University and The Center for Medical Parasitology, Copenhagen University, to develop the protein production processes for the blood-stage malaria vaccine antigen protein (referred to as Protein2 in the text) and the placental malaria vaccine antigen VAR2CSA, respectively. In addition, a Virus-like particle (VLP) was also included in this study. VLPs are multimeric protein structures that mimic the organization and conformation of native viruses but lack the viral genome. Due to the complex multiprotein structure of VLPs, they can be extremely fragile and sensitive to shear forces. The production of these complex protein vaccine antigens provides an ideal opportunity to apply advanced processing technologies.

## 2. Methods

Batch, fed-batch and perfusion modes were compared for growth profiles and product yield. A truncation variant of the **VAR2CSA** placental malaria vaccine antigen, a full-length **Protein2** and a **VLP** were cloned into a pExpreS$^2$ vector and transfected into *Drosophila* S2 insect cells. Stable cell lines were established in three weeks using antibiotic selection in T-flask culture. The cells were expanded and inoculated at between 5E6 and 8E6 cells/ml for batch, fed-batch, or concentrated fed-batch in 1L DasGip, 2L B Braun or CellReady3L bioreactors. The batch production runs were harvested after 3 or 4 days, fed-batch after 7 days and perfusion cultures after 6 to 9 days. Alternating Tangential Flow (ATF) technology from Repligen was employed for concentrated fed-batch production. The bioreactor conditions were 25°C, pH6.5, and 110—350 rpm stirrer speed using a Marine impeller. The perfusion rates were set to 0.5 to 3 Reactor Volumes (RV) per day and was increased significantly faster for the CellReady 3L perfusion run compared to the B Braun runs, with 3 RV per day reached by day 6 vs. day 9 for the Braun runs.

## 3. Results

### Cell counts achieved using perfusion technology

S2 cells normally grow to cell densities of 40–50E6 cells per mL in batch mode. A fed-batch approach can increase the cell counts to 60-80E6 cells per mL. However, further increases of up to 104E6 cells per mL have been reported (Wang et al. 2012) when using a floating filter in a wave bioreactor. ExpreS$^2$ion has achieved 140E6 cells per mL using the Biosep perfusion technology in 2L and 5L B Braun bioreactors (Poulsen and de Jongh 2014). Recently, the application of ATF perfusion technology has improved the cell density to 300-350E6 cells per mL in both B Braun 2L and Cellready3L bioreactors (see figure 1).

### Effect of feed strategy

Significant effects on growth and production were seen depending on feed strategy. The increased growth rate observed for the CellReady3L perfusion run compared to the B Braun bioreactor was due to a feed profile designed to allow maximum growth rate in the Cellready3L (see figure 2A). The feed profile for the B Braun run was designed to obtain linear growth. Under

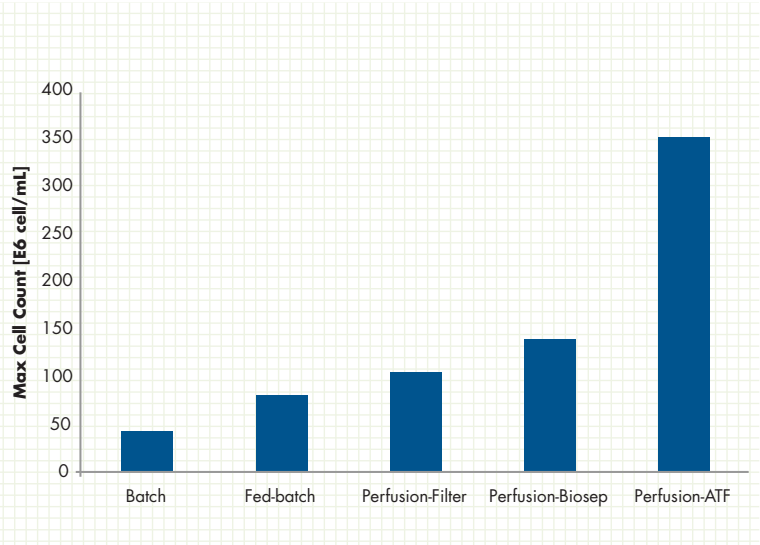
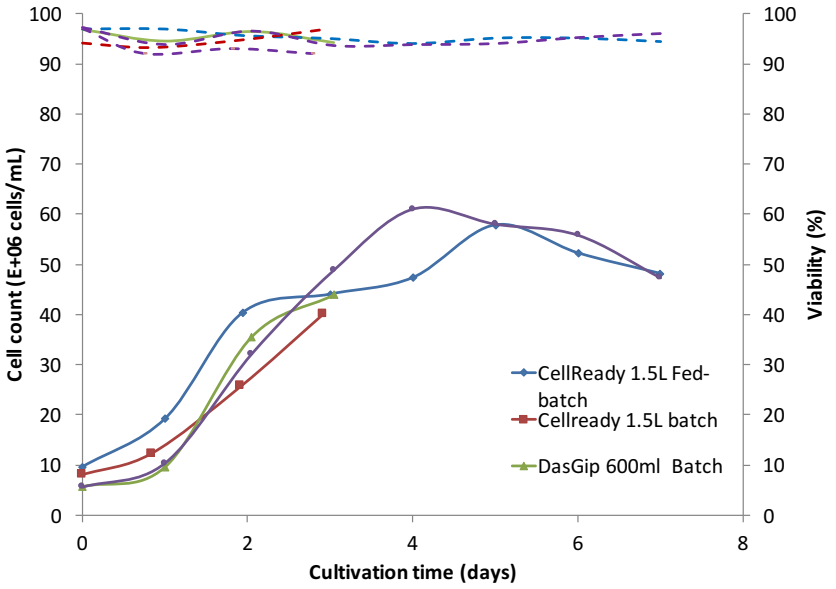



**Fig. 1** Maximum cell counts achieved in different production modes. The Perfusion-Biosep run was performed using the 10L BioSep (Applikon) in a 2L B Bruan bioreactor and the Perfusion-ATF run was performed using the ATF2 (Repligen) in a CellReady3L (Merck-Millipore). The Perfusion-Filter experiment (Wang et al. 2012) was performed using the Wave system (GE Healthcare) in a 2L CellBag using a floating filter with nominal pore size of 7 µm.

exponential growth a maximum VCD at 350E6 cells/mL was achieved, and the production was only stopped because the maximum flow rate of the ATF filter was reached. However, it is clear that the specific productivity of the S2 cells during exponential growth conditions was significantly reduced when compared to the linear growth conditions. Similar yields were achieved on days 1 through 6 in both bioreactors, even though the cell counts were up to three fold higher in the exponential growth experiment (figure 4A). Similarly, a linear growth profile was maintained for the VAR2CSA concentrated fed-batch run. However, on day 9 the perfusion rate was increased from 2 to 3 RV/day, which lead to a large increase in cell number. As this was the maximum possible perfusion rate with the ATF2, the increased cell count could not be maintained with an increased perfusion rate, which led to a drastic decrease

in cell viability. This demonstrates the need to maintain a minimum perfusion rate to achieve high viability. ExpreS$^2$ion estimates the needed perfusion rate using the standard approach of attempting to maintain a constant flow rate per cell per day throughout the run.

Applying the results from perfusion experiment 1, a linear perfusion profile appeared to be optimal for productively. A second medium was included since it has previously showed to stabilize VLPs as well as increase growth rate and viability of the cell line. Both experiments were ended at day 8 due to technical difficulties with the oxygen supply and the base addition pump but up to that point were progressing as expected. In this experiment, it was observed that the higher cell count, supported by medium 2, did not result in increased VLP titer. In fact, a 27% decrease was observed (figure 4B). Lower productivity caused by higher growth rate does follow what was seen in the first perfusion experiment. A possible solution could be a reduced perfusion rate when using medium 2.



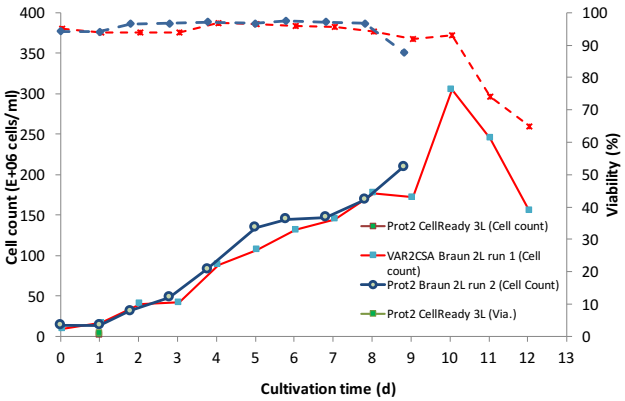



**Fig. 2** (A) Growth curves for Batch and Fed-batch S2 cultures cultivated in either CellReady3L or 1L DasGIP bioreactors, (B) Growth curves for perfusion runs using the ATF2 in 2L B Braun or CellReady3L bioreactors for either a VAR2CSA or Protein2 producing cell line.

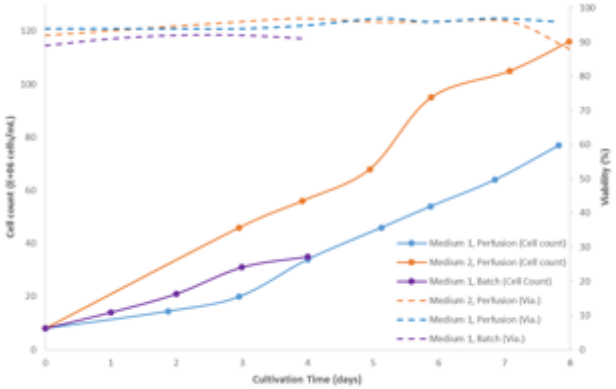



**Fig. 3** Growth curves for batch and ATF2 perfusion runs in 2L B Braun.

### Yield improvements achieved using Fed-batch and Concentrated fed-batch

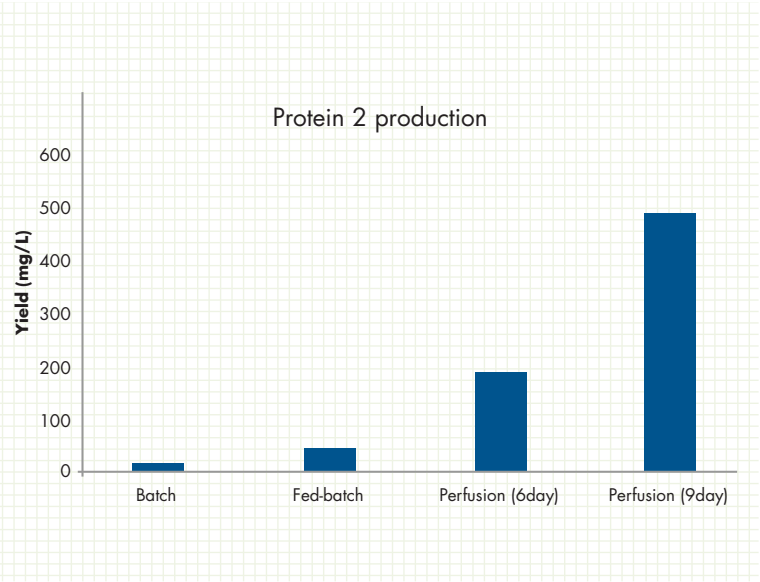
The VAR2CSA truncation variant was expressed in batch and fed-batch culture in 1L DasGip Bioreactors. A higher than 30% yield increase was achieved when using a fed-batch approach compared to batch production. Concentrated fed-batch was also performed on the cell line in a B Braun 2L bioreactor, and extremely high cell counts of 350E6 cells/mL were achieved. Unfortunately, no quantitative analysis technique was available to determine the yield increases achieved. An ELISA method is currently under development, but from western blot analysis it could be seen that significant yield increases were achieved.

The production of Protein2 was also compared in batch, fed-batch and concentrated fed-batch using both CellReady3L and glass bioreactors. Significant yield increases were obtained going from batch to fed-batch production, and again from fed-batch to concentrated fed-batch. Comparable yields were obtained in both CellReady3L and B Braun bioreactors (see figure 3). Furthermore, **350E6 cells/ml** were achieved in concentrated fed-batch mode using the ATF and CellReady3L. Concentrated fed-batch lead to final Protein2 yields of 210mg/L and **500mg/L** after 6 or 9-day production runs.

The VLP producing cell line was compared in batch and concentrated fed-batch mode in two different cell culture mediums. Again, yield increases were observed when using perfusion compared to batch. A **3.7 fold** increase in the overall titer for medium 1 and 2.7 fold for medium 2. Based on a linear product accumulation curve (data not shown), this increase could have been significantly higher if the experiment had been continued for longer.

### Protein stability

Strikingly, it could be observed that decreased cell viability on the last two days of the VAR2CSA perfusion run due to a too low perfusion rate led to extensive product degradation. Clearly, this degradation could be avoided by maintaining cell viability. Similarly, SDS-page analysis of the purified of Protein2 from a day 8 harvest from Fed-batch culture, or a day 10 harvest from the perfusion culture, showed increased intensity of bands corresponding to two degradation products of Protein2 when compared to the fed-batch culture



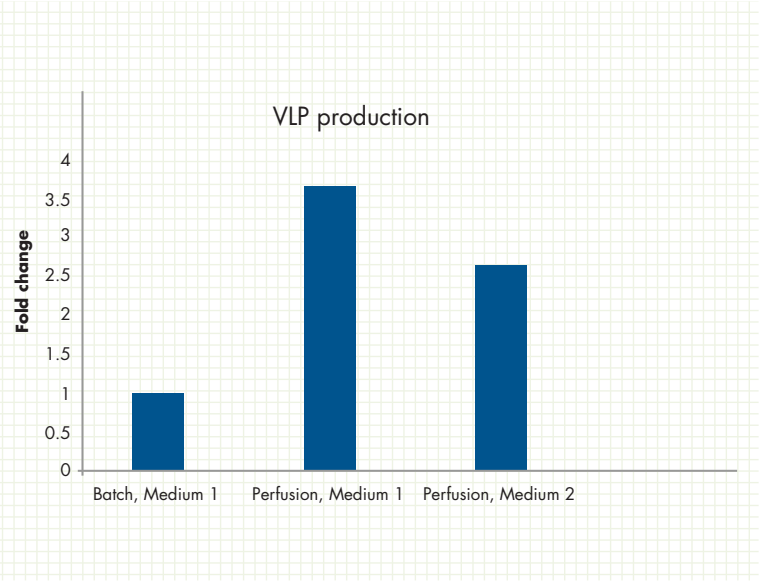




**Fig. 4** (A) Protein2 yield from Batch, Fed-batch and concentrated fed-batch cultures. (B) VLP yield comparison from Batch and concentrated fed-batch in two different culture media.

(figure 5). In this latter case the product cleavage was less severe, although it was also present even while the culture was maintained at high viability.

The VLP titer was determined using a conformational sandwich ELISA and for medium 1, an almost linear titer increase was observed. This indicates this VLP, had no / very little issues with shear from the ATF filter.

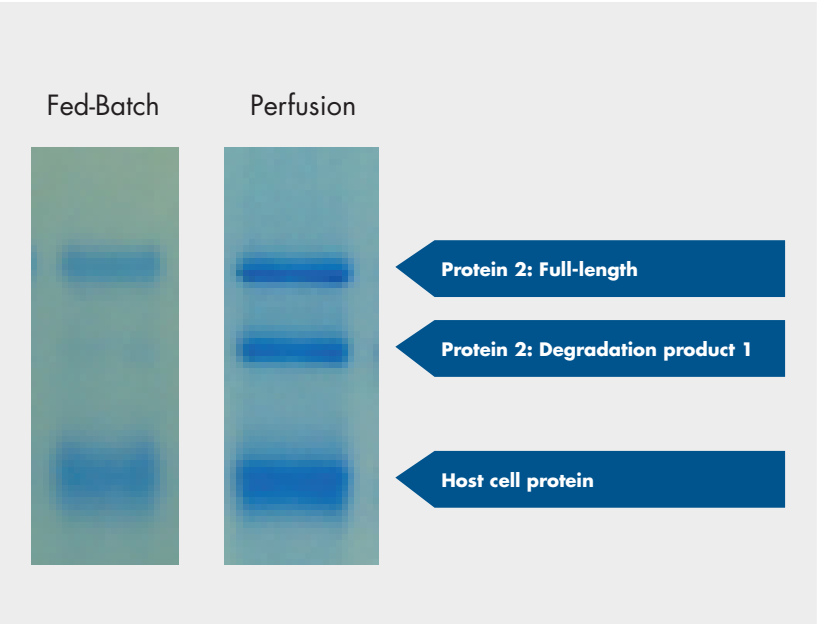



**Fig. 5** SDS-page analysis of purified Protein2 from a day 8 harvest from Fed-batch culture, or a day 10 harvest from the perfusion culture.

## 4. Conclusion

The protein stability issues observed for Protein2 (and to a lesser extent for VAR2CSA and the VLP) demonstrate one of the key weaknesses of concentrated fed-batch technology, namely the need for product stability to enable extended product residence times in the bioreactor. However, for degradation prone

proteins, the option of performing standard perfusion using the ATF offers a simple solution by reducing the product residence time to less than 24 hours with direct harvest at 4°C. It is therefore necessary to evaluate each protein based on stability before deciding on applying either concentrated fed-batch or standard perfusion. For instance, the VAR2CSA truncation variant and the VLP could be successfully produced using concentrated fed-batch on the condition of high cell viability. Significant yield increases through increased cell counts, and consequent production scale reductions are possible in both cases. Concentrated fed-batch or standard perfusion using the ATF technology therefore offers attractive process intensification approaches for cost sensitive protein production needs.

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## Acknowledgements:


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# Scale up and Technology Transfer in a Continuous Processing Environment

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Dr. Sadettin Ozturk, the head of Process and Analytical Development at Massbiologics has had a long career in cell culture process development, technology transfer, product licensing, and commercial manufacturing.

His early contributions to the field focused on applying chemical engineering principles and process control strategies to the optimization and scale-up of cell culture processes. He was responsible for the development of numerous cell culture based processes and novel technologies that helped not only the companies that he worked for (Verax, Bayer, GlaxoSmithKline, and Johnson & Johnson), but contributed to the rest of the field through his numerous presentations and publications. Sadettin led process development activities and played a key role in the licensing and commercialization of two monoclonal antibodies, Stelera®, and Simponi®. In addition, he transferred and supported the commercial manufacturing of Kogenate®, BeneFix®, and Remicade®.

Sadettin has published numerous research articles, given presentations, delivered keynote lectures, and edited books. He is a member of several societies including ESACT, American Association for the Advancement of Science, New York Academy of Sciences, American Chemical Society, and American Institute of Chemical Engineering. Sadettin is involved in these scientific organizations

## 1. Introduction

Even though it is more complicated by comparison to a batch or fed-batch process, many companies have successfully developed, scaled-up, validated, and commercialized biotherapeutics using processes that included continuous cell culture. Since 1990s, about fifteen biopharmaceutical products utilizing continuous upstream processing have been approved by regulatory agencies for commercial production[1]. Therapeutic enzymes, blood clotting factors, and monoclonal antibodies are manufactured using semi-continuous (draw-and-fill), continuous chemostat, and perfusion type of cell culture operation. Perfusion operation is preferred by the industry as it leads to higher cell densities, thus higher volumetric productivities. In addition, these systems run at steady state providing a consistent environment to the cells. Under these conditions, a more consistent product quality is expected[2,3]. Due to low residence times in the bioreactor, product degradation issues are also minimized in a continuous process. Despite all these advantages, implementation of a continuous process into manufacturing platform needs to be balanced against the challenges it brings to the scale up and technology transfer steps.

In this article, we will focus on continuous perfusion processes and discuss the challenges in their design, scale-up, and technology transfer for clinical and commercial applications.

## 2. Utilization of a Continuous Process in Biomanufacturing

Continuous cell culture processing uses the same seed and inoculation train steps of a batch culture[4]. It also may use the same medium, same equipment, and the same process parameters for these steps (Figure 1). Both modes of production can employ N-1 Perfusion to intensify the seed train to become more efficient. Two main differences can be noted here; (a) the bioreactor size used for production is typically smaller for continuous perfusion and (b) continuous processing requires less number of vessels for inoculation train. The mode of the operation for the production bioreactor necessitates other differences as well; (i) the use of a cell retention device for perfusion operation, (ii) one or more feed stream for fed-batch, and (iii) continuous feed and harvest streams for perfusion.

Cell clarification step for continuous culture (if necessary) is very similar to what is used for batch and it utilizes centrifugation and depth filtration. Compared to batch, cell clarification step can be much simpler for open cell retention systems (lower cell density in the harvest), or it can be completely eliminated for filtration-based cell retention systems. To enhance the productivity of the culture

batch cultures are typically fed by specially formulated feed solutions. Similarly, a continuous perfusion culture can utilize a specially formulated (enriched) medium to achieve higher yields with fewer liters per day required.

The use of continuous cell culture operation requires an intensification of downstream processing (1-3 days processing instead of every 14 days or so). Although there have been attempts to develop and implement continuous chromatography to biomanufacturing, downstream processing at scale is currently performed in batch mode using the same resins for both batch and continuous cell culture. Therefore scaling up and transferring the downstream operation for continuous cell culture is almost identical to batch cultures. In summary, the continuous cell culture process differs from batch culture mainly because of the production bioreactor and our discussion below will focus on how the configuration and the operation of production bioreactor impact the scale up and technology transfer.

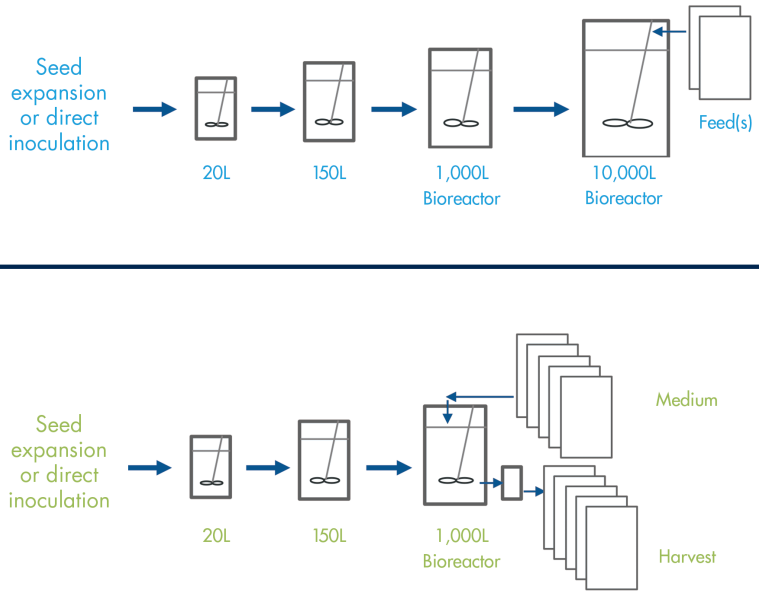


**Fig. 1** Upstream process steps commonly used for the manufacture of recombinant proteins. Fed-batch (top) and continuous perfusion (bottom) type of operations are illustrated.

## 3. Complexities of Designing, Operating, Scaling up, and Transferring Continuous Processes

The production bioreactor systems used for continuous perfusion operation differ from batch counterpart by the following features:

1) Cell retention device
2) High cell density
3) Longer run time
4) Process control

The complexities introduced by these features will be discussed below with their impact on bioreactor operation, bioreactor design, process scalability, process control and consistency, and process characterization and validation.

### Cell Retention Device

The cell retention system is an essential part of the perfusion bioreactor operation as it allows retention of the cells in the bioreactor while the harvest is being pulled out continuously. These systems have been described at length in the literature[5] and they can be classified as open perfusion (gravitational settlers, centrifuges, and acoustic separators) and filtration-based systems (tangential filtration, ATF). Cells are partially retained in open perfusion devices whereas they are completely retained in filtration-based systems.

Cell retention systems are often used as an external device, which requires cells spend time outside the bioreactor. Residence time in the cell retention device can impact the performance of the cells as they are exposed to an environment without environmental control. Drop in temperature, dissolved oxygen, nutrient levels, and pH can have a prolonged effect on the cells. Most of the external cell retention requires a circulation loop creating additional complexity and shear damage to the cells. These mechanical and physiological issues need to be addressed and the design and operation of cell retention device needs to be optimized for a successful perfusion run.

Use of a cell retention device adds to the complexity of scale up and technology transfer of cell culture processes. Bioreactor scalability in terms of vessel geometry, hydrodynamics, mixing, and aeration can be performed using

conventional scale-up methods rather easily. However, design and scale up of a cell retention device is not straightforward and is not widely understood. In addition, the potential impact of cell retention device on culture performance should be considered to capture the interactions between the cell retention device and the bioreactor.

Complexities in the design and mechanical construction inherently limit the capacity of cell retention device in terms of volumetric output, maximum cell density, and the run length it can support. It is very difficult to achieve perfusion rates greater than 500-1000 L/day for single cell suspensions and the actual limit can change from one cell retention device to another. Spin filters, for instance, can hardly achieve more than 10-15 million cells/ml because of issues related to clogging of the screen and continuous loss of cells through the openings on the screen. The XCell ATF system, on the other hand, has a maximum capacity of 500-2000L/day (process dependent) even with the largest commercially available hollow fiber cartridge and the diaphragm (ATF-10). Some cell retention systems can run for months while some require a changeover after a month.

Scale-up parameters for cell retention system are not fully understood although some guidelines are available. For gravimetric cell separation devices, the volumetric throughput (L/day) can be scaled based on settling area. Similarly a filtration based cell retention system can be scaled up based on filtration area. Unfortunately, scaling up with a single parameter like surface area does not secure the success. Other factors such as the hold-up volume, circulation rate, cycling frequency, cross flow, shear rate, and residence time need to be considered as well. The culture conditions in the bioreactor, cell density and viability, cell size and degree of aggregation, dissolved oxygen, and pH, can have significant impact on the performance and they need to be characterized and maintained for a successful scale up and technology transfer activity.

Cleaning, sterilization, automation, and validation (or qualification) of the cell retention device need to be addressed for a successful perfusion operation. Connecting the cell retention device to the bioreactor, integrating the system to the supporting utilities, and installation of ancillary equipment such as

pumps, pressure sensors, etc should be considered in the overall design of the bioreactor and the facility.

### High Cell Density

Continuous perfusion cultures typically reach 2-5x higher cell densities than the batch and operate at 30-80 Million cells/ml occupying about 10-30% of the volume in the bioreactor (Figure 2). Maintenance and control of high cell density cultures can be very difficult and they require attention to bioreactor design and appropriate process control strategies.

Issues related to mixing can cause irreversible damage to the cultures in continuous perfusion system. Local gradients in terms of pH and dissolved oxygen can establish more readily at high cell densities (see Ozturk, 1996). High levels of mixing is critical to homogenize the feed and base at the point of addition, to prevent the clumping and aggregation, and to obtain an uniform environment for the cells throughout the bioreactor. In addition a good mixing system will be needed for dispersion of the bubbles for aeration.

Design and scale up a mixing system for high cell density cultures are more difficult because of the process intensity and high metabolic demand of the cells. In addition, cells themselves can impact the hydrodynamics and mixing characteristics in the bioreactor and in the cell retention system. Axial flow impellers such as pitch blade impellers are preferred as they provide more homogenization and blending. Many companies have used scaling up the impeller system using power per volume instead of tip speed with success.

Aeration of high cell density systems needs to be well optimized not only for oxygen delivery but also for $CO_2$ removal. A cell density of 80 Million cells/mL will require an oxygen mass transfer rate of about 10-30 mM/hr, which can only be achieved at very high gas flow rates (and/or high agitation rates) for a macrosparger based aeration system. The use of a microsparger is preferred for high cell density cultures over a macrosparger because of bubble size. A microsparger is roughly 10x more efficient in supplying the oxygen to the culture for a given gas flow rate. $CO_2$ removal, on the other hand, is not effective for microsparger systems resulting an accumulation of $CO_2$ in

the culture, negatively impacting the cells. Use of two separate spargers, one microsparger for oxygen delivery, and one macrosparger for $CO_2$ removal, is recommended for a successful operation and scale up of a continuous perfusion culture.

High cell density can also impact the operation and performance of cell retention device. For acoustic cell separation and for inclined settlers, the volume fraction occupied by the cells can impact the retention efficiency negatively. For filtration-based systems, the run length before clogging is shortened at high cell densities.

High cell density cultures are extremely dynamic and they are more difficult to control and maintain. The nutrients and oxygen have to be delivered rapidly to the cells to keep up with their total metabolic demands. A small glitch in media feed, gas flow, and an interruption of pH controller can cause irreversible damage to the culture. The process control system has to be tightly tuned and all systems need to be in perfect working conditions for a continuous perfusion operation.

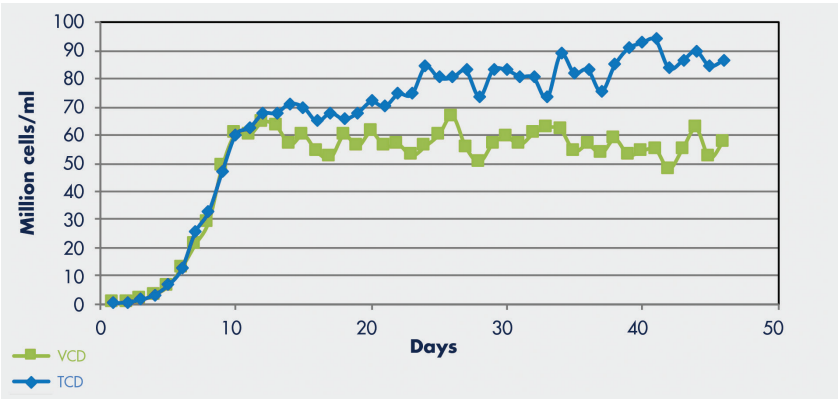


**Fig. 2** Viable and total cell densities for a continuous perfusion bioreactor using ATF as a cell retention device. The culture was operated at 2 volumes/day perfusion rate and the cell densities are maintained at 60 MM/ml by cell bleed at 0.2 volumes/day.

## Longer Run Time

Another complication for the scale up and technology transfer of continuous perfusion operation is the longer run time. Compared to a fed-batch culture, which runs about 2 weeks, a continuous cell culture is expected to continue for 2-6 months at steady state to fully utilize the benefits of a perfusion operation. The run time of a continuous operation is determined by consideration of several factors including cell line stability, culture productivity, product quality process economics, and operational reliability and consistency.

Cell line stability is an important issue for continuous cell culture and the genetic stability of the cells needs to be demonstrated for regulatory approvals. This applies to both batch and continuous cultures and same methods are used to establish the cell line stability. The duration of a stability study for continuous culture is longer so that it will cover the run time of the operation. Typically the stability studies are incorporated into the clone selection process before the cell bank, thus unstable clone candidates are eliminated from the process at the beginning. However, there is still a risk of demonstrating genetic stability by

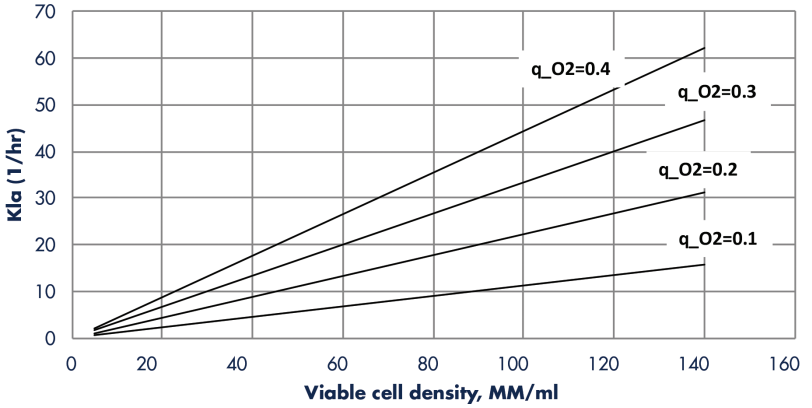


**Fig. 3** Oxygen mass transfer coefficients required to maintain a dissolved oxygen concentration at 50% air saturation for different cell specific oxygen consumption rates (q_O2 in umol/MM-hr). These calculations use pure oxygen in gas phase, if air is used instead of oxygen, almost 5x higher the mass transfer rates are needed.

comparing the end of production cells with the cell bank. Running the culture in perfusion mode for the desired run length can mitigate this risk.

Productivity and product quality may change during the continuous perfusion process. While a drop in productivity is not a serious issue as it mainly impacts the process economics, it can raise questions about the cell line stability. Changing product quality during a continuous culture can be a major issue for process validation and for regulatory filing. However, the continuous culture runs at almost steady state and the risk of getting different product profile during the run is expected to be very low.

Longer run time for continuous perfusion operation makes it more difficult for process validation and product commercialization. Process consistency needs to be demonstrated by at least three separate runs and as each run can take 2-4 months, the validation efforts are dramatically longer compared to a batch process. In addition, the product needs to be characterized at early, middle, and late phase of the run. This also adds more work to validation efforts.

### Process control

The bioreactors used for continuous processing use often the same ones used for batch cultures and the same type of process control strategy is used to control the cellular environment (temperature, pH, and dissolved oxygen, etc.). However, perfusion bioreactors require additional controls for feeding and harvesting, for maintaining cell density, and for the operation of the cell retention device.

Cell retention device should work reliably and consistently during the perfusion run that can take 2-6 months. Specially designed controllers are required to operate cell retention system systems at steady state. Continuous perfusion also requires a tight control of bioreactor volume (or weight), perfusion rate, and cell density. Use of on-line cell density probes, cell density estimations, special algorithms, and a control strategy using Cell Spefic Perfusion rate (CSPR) are typically used for this purpose (Ozturk, 1996, 2014).

## 4. Conclusions

Continuous processing for cell culture is a viable alternative to batch culture and it is used already for the production of several biotherepeutics. There are clearly several advantages of using a continuous process as it provides higher volumetric yields, uniform environment to the cells, and results in consistent product quality. There are, however, complexities and challenges to overcome for the design, scale up, and technology transfer of continuous processes as illustrated here. These issues can definitely be worked out by process understanding, by designing and implementing required control strategies, and by characterizing the process and the product. Cell retention devices are complicated to design and operate but there are several options to choose from. Operating a high cell density culture requires proper mixing, aeration, and control strategies in place. Continuous processes run longer compared to batch and they can have issues related to cell line stability, process validation, and process consistency. However, these issues can be managed by selecting stable cell lines, and by demonstrating the product quality during consistency run.

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# Continuous Multicolumn Chromatography Processes

## About the Author:

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Within Pall, Marc is responsible for development and implementation of continuous bioprocessing technologies.

Prior to joining Pall, Marc was CSO at Tarpon Biosystems, the company that developed the BioSMB technology. Marc held several scientific and management positions in the field of process development departments in Batavia Biosciences, Xendo. He has established a significant track record in completing projects related to continuous chromatography and continuous downstream processing. With over 250 projects completed, he is one of the leading scientists in this area.

Marc holds a PhD in biochemical engineering and an MSc in chemical engineering from Delft University in The Netherlands. He is listed as (primary) inventor on various patents related to continuous process technologies. He is a lecturer in in various post-graduate courses in Europe.

## 1. Introduction

Chromatography has been, and will probably remain for the foreseeable future, the most important work horse in the purification of biopharmaceutical products. A wide variety of chromatography products are commercially available, offering the possibility to separate the product of interest based on affinity interactions, electrostatic interactions (ion exchange), hydrophobic interactions, size and combinations of these. In the biopharmaceutical landscape, there is not a single product that is not purified using at least one chromatographic purification step and most biopharmaceutical products require at least two chromatographic purification steps.

Although powerful in terms of removing contaminants, chromatographic processes have a few disadvantages. In general, multi-step batch processes exhibit poor productivity and often this leads to scalability limitations. Even capture processes with new high capacity chromatographic media cannot always cope with the high titers that are becoming more prevalent in cell culture processes. For example, a fed batch cell culture bioreactor of 2000 liter with a 5 gm/L expression level produces more antibody than can be bound on a column with a one (1) meter diameter, even if that column is cycled twice per batch.

In other process industries, these limitations have been successfully dealt with by implementing continuous multicolumn chromatography processes. Notable examples of this approach is the use of simulated moving bed (SMB) technology for separating fructose from glucose and many chiral separations common in purifying API's made through organic synthesis. Although the traditional simulated moving bed technology is mainly applied for binary fractionations, continuous multicolumn chromatography systems have also found large scale applications in capture processes. Examples of these are the purification of L-lysine and antibiotics from fermentation broth and the production of ascorbic acid (vitamin C).

With increasing cell culture expression levels, the capacity bottleneck in biomanufacturing has shifted from the upstream process to the downstream process. This has generated a need to address the limitations of batch

chromatography in biopharmaceutical applications. This has resulted in various designs for multicolumn chromatography systems for the purification of biotherapeutics.

## 2. Key Features

The principle of multicolumn chromatography is to create a (simulated) movement of the chromatography columns in opposite direction of the process solutions. This results in a countercurrent contact between the liquid and the chromatography media, which allows overloading the columns beyond the dynamic binding capacity without suffering loss of material. When product breaks through from the first column, it will be captured on a second column in the load zone. With this, countercurrent chromatography processes can offer a significant gain in capacity utilization.

Another benefit of the countercurrent contact approach is that it eliminates idle zones in the process. In a batch chromatography column, the mass transfer zone only covers a small portion of the overall chromatography volume. The media above the mass transfer zone is in equilibrium with the feed solution and has no additional capacity to bind more product. The media below the mass transfer zone is in contact with depleted feed solution and hence is waiting for the first product to arrive. In a countercurrent process, these idle zones are eliminated and the load zone can be designed to only cover the length of the mass transfer zone. This is generally corresponds to a small part of the batch column volume. These two features are schematically demonstrated in Figure 1.

In batch processes, the column size is proportional to the total mass of protein that needs to be purified and hence there is a direct relationship with the feed concentration. In a continuous chromatography process, the load zone is mainly designed around the contact time associated with mass transfer zone. The total volume of chromatography media in the load zone thus hardly depends on the static binding capacity and the feed concentration. Instead, the process is designed around the volume that needs to be processed, or more precisely, the feed flow rate.

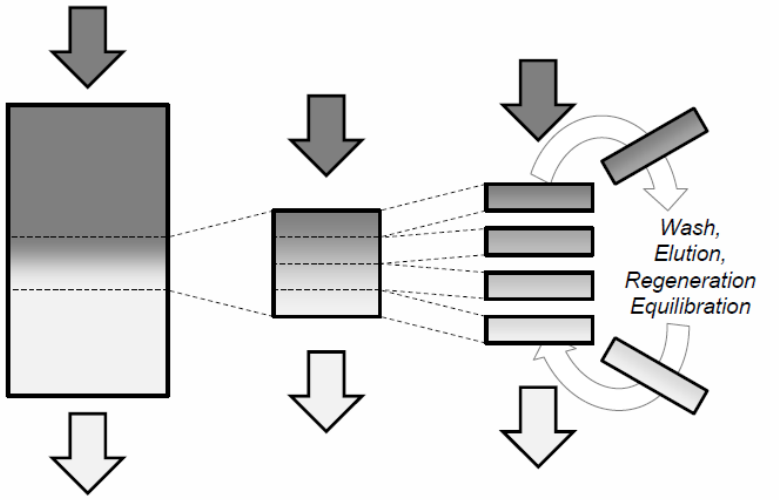


**Fig. 1** Schematic comparison between a batch process (left) and a multicolumn countercurrent chromatography process (right).

The number of columns that is required to run a continuous process does depend on titer. In order to transform the load step into a continuous countercurrent step, at least two columns are needed. This brings the minimum number of columns for a continuous process to three, provided that one column provides sufficient time to do all wash steps, elution, regeneration and re-equilibration steps. As soon as the load volume becomes relatively low, which is the case for medium and higher titers, the load time becomes proportionally smaller and one column is no longer sufficient. For these scenarios, the ability to connect extra columns to the system without adding complexity to the valve system is a valuable attribute. For polishing processes, where the chromatographic resolution is not as straightforward as in affinity separations, additional columns may also be needed in the elution zone and/or wash zones.

## 3. Continuous and disposable

There is a substantial gain in specific productivity over batch offered by continuous chromatography while the size of the overall chromatography system – including its columns – becomes significantly more compact. The

columns are cycled many times throughout each batch, usually by design up the chromatographic media lifetime. This process design enables a viable disposable chromatography process.

The Cadence™ BioSMB technology commercialized by Pall Life Sciences is designed around a completely disposable product contact/ fluid path. Most importantly, the Cadence BioSMB valve cassette, a single-use acrylic block containing all the valving and integrated fluid connections to run a multi-column process, can operate up to 16 columns or other single use devices such as membranes or monoliths. Each of the valves in the cassette can be individually addressed, thereby providing all the flexibility that is required to operate virtually any chromatography process in a multicolumn configuration. In addition to this, the pumps, tubing and sensors are also available in disposable format.

When the Cadence BioSMB system is combined with prepacked columns, membrane adsorbers or any other chromatographic devices designed for single-use applications, the entire chromatography process can be translated into a viable single-use option. With this, the Cadence BioSMB technology provides a promising answer for those companies who are developing completely disposable strategies for the entire biomanufacturing process.

## 4. Optimization Strategies

Continuous chromatography processes have more degrees of freedom than batch chromatography. This offers more flexibility in optimizing the process to meet the specific requirements of each manufacturing situation.

### 4.1 Clinical Manufacturing

In clinical manufacturing, the cost contribution of the consumables such as the chromatography media to the total COGs (Cost of Goods) is quite significant. This is mainly due to the fact that these consumables cannot be exploited to their full extent. Even expensive chromatography media such as Protein A affinity media are depreciated within a single clinical manufacturing campaign. The optimization strategy for this situation should target the total installed volume of chromatography media or the specific productivity (expressed as grams of protein purified per liter of chromatography media per hour).

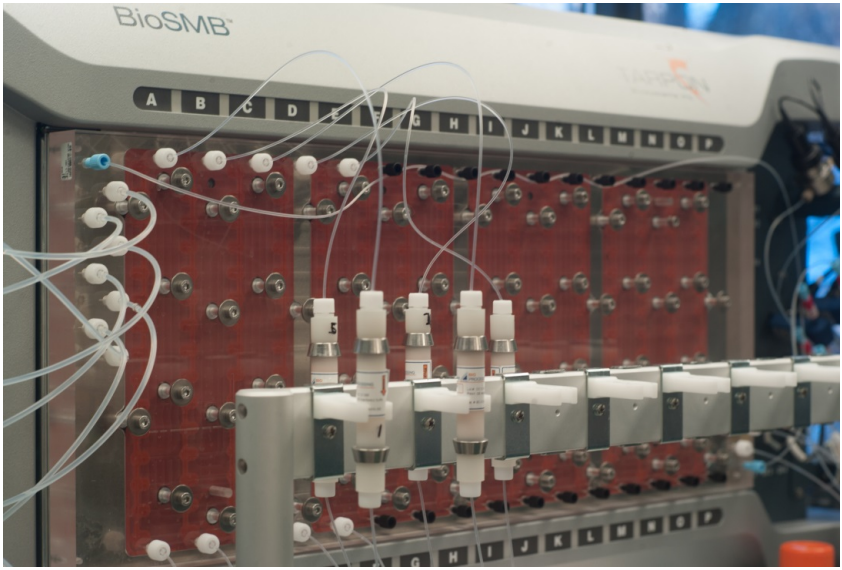


**Fig. 2** A BioSMB system for process development use with five prepacked chromatography columns connected to the system.

In a Cadence BioSMB process, this translates into process conditions that target a short contact time between the liquid and the chromatography media. This can be achieved by operating below the highest possible capacity utilization and in the process accepting sub-optimal savings in buffer consumption. In clinical manufacturing, the buffer consumption is generally not the limiting factor and the impact of the total costs of the campaign is negligible in most cases.

### 4.2 Commercial Manufacturing

Recently, data was presented demonstrating reliable and consistent scale-up from process development scale to manufacturing scale, using the Cadence BioSMB Process system. The data shows direct scalability over two orders of magnitude, maintaining critical quality attributes [6]. The Cadence BioSMB

Process system is fully cGMP compliant and the single-use assembly has been characterized for leachables and extractables and is USP VI compliant.

In commercial manufacturing, chromatographic media is depreciated over many more cycles than in clinical manufacturing. It is not uncommon to validate media life time up to 100 or even 200 cycles. In these situations, the cost contribution of the chromatography media is no longer related to the specific productivity of the process, but to the amount of product that is purified in each cycle per liter of chromatography media. This optimization strategy for continuous multi-column chromatography thus targets capacity utilization optimization. With this, the savings in buffer consumption will also be optimized.

In order to achieve the maximum capacity utilization, the load step will require a certain contact time, which has an impact on the specific productivity. In commercial manufacturing, however, the specific productivity does not affect the COG other than through capital costs.

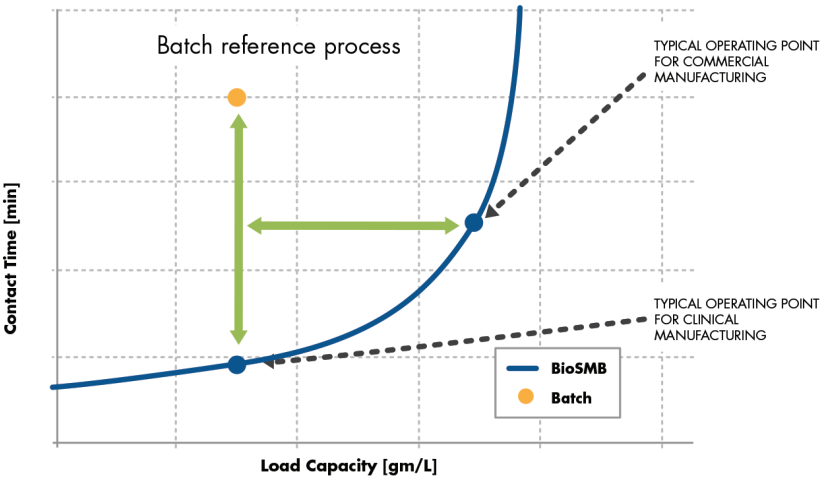


**Fig. 3** Schematic representation of the typical operating points for clinical manufacturing and commercial manufacturing in a Protein A based BioSMB process.

## 5. Application Areas

The flexibility offered by the Cadence BioSMB disposable valve system makes it a very versatile technology, allowing a wide range of applications. For various chromatographic processes, the impact of the technology has been investigated. A brief summary of some of the case studies is listed in Table 1.

| References | Chromatographic mode | Specific productivity |
|---|---|---|
| Capture of antibodies | Protein A chromatography | 2 – 6 x batch |
| Aggregate removal | Hydrophobic interaction (HIC) | 2 – 3 x batch |
| Aggregate removal | Ion Exchange | 4 – 8 x batch |
| Capture of recombinant proteins | Ion Exchange | 2 – 5 x batch |
| Capture of VLP vaccines | Ion Exchange | 3 – 7 x batch |
| Polishing of a VLP vaccines | Size Exclusion (SEC) | 6 – 14 x batch |

**Table 1** Some examples for which BioSMB technology has been successfully tested [2, 3, 4, 5]

The case studies listed above were performed with traditional chromatography media in prepacked columns. In addition to this, the combination of BioSMB technology with alternative chromatography formats has been successfully demonstrated. This includes the use of monolithic columns and membrane adsorbers to establish a continuous capture process. This has been done with membrane adsorbers ion exchange and with affinity ligands.

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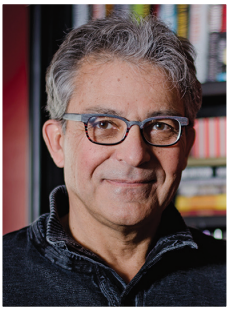
# Impact of Single-use Technology on Continuous Bioprocessing

## About the Author:



**William G. Whitford**

Bill Whitford is Strategic Solutions Leader, GE Healthcare in Logan, UT with over 20 years experience in biotechnology product and process development. He joined the company as an R&D Leader developing products supporting protein biological and vaccine production in mammalian and invertebrate cell lines. Products he has commercialized include defined hybridoma and perfusion cell culture media, fed-batch supplements and aqueous lipid dispersions. An invited lecturer at international conferences, Bill has published over 250 articles, book chapters and patents in the bioproduction arena. He now enjoys such activities as serving on the Editorial Advisory Board for Bioprocess International and the European Medical Journal.

## 1. Single-use in Bioprocessing

Single-use in Bioprocessing refers to materials or equipment that can be used in one processing batch or campaign, and have at least one product contact surface element that is disposable. Such equipment ranges from single material, very simple stand-alone items such as tubing – to complex and controlled systems of many components and materials, such as a bioreactor[1]. Relatedly, the application of this equipment ranges from an instrument with a single, simple function to assemblies, skids or pods housing entire or even combined unit operations. Most of the more complicated single-use (SU) systems contain re-usable non-product-contact elements, for such purposes as support. SU systems have been taken up in the biopharmaceutical industry in general because of the numerous enabling features they provide (Table 1A). Over the past 15 years or so both the number of individual process activities and operations addressed, as well as the number of systems commercially available to support each operation, have grown substantially (Table 1B). Examples of newer products seen in the past few years include SU heat exchangers, SU flowpath auto-sampling and large-scale SU fermenters[2].

| A: Features Provided by SU | B: Operations Supported by SU |
|---|---|
| Reduced contamination risks while supporting faster changeover | Cell culture for seed expansion and production |
| Reduce processing footprint and lower initial investment costs | Media, buffer and process liquid preparation |
| Lower facility and operating cost | Liquid pumping, filtration, collection, and shipping |
| Reduced classifications/utilities | Storage, transfer and delivery of dry powders and sterile microcarriers |
| Reduced operator requirement | On-line contents monitoring sensors/ samplers |
| Enhance process efficiency while enabling orphan/personalized drugs | Transport/storage of intermediate and product |
| Reduce time to market and promote remote facility replication | Cryopreservation of seeds and intermediates |

**Table 1** Upstream single-use technology features and systems

## 2. Continuous Processes in Upstream Bioproduction

By far the most common approach to continuous processing in upstream animal cell-based bioproduction is through perfusion culture[3,4]. Here, culture medium is removed using one of many available devices to retain cells in the bioreactor as fresh medium is proportionally added[5,6]. The rate of exchange and cell culture density to maintain are monitored and controlled by a variety of means and to a particular set of process parameters. Quite a number of such research- and production-scale perfusion bioreactor systems have been devised[7] (Table 2).

Although many perfusion processes for either suspended or adherent animal cells are known to be used in manufacturing-scale production, proprietary details on their design and operation are not always publicly available. Terminology in this dynamic field can get fuzzy as well, for example, continuous processing is also referred to as continuous bioproduction, continuous-flow Bioprocessing or continuous biomanufacturing (Table 3). And, minor categorical distinctions are sometimes made between approaches. Depending on the periodicity of either entire component production episodes or of the association and timing of more discrete individual component operations, some even apply such terms as semi-, quasi-, or pseudo-continuous operation[8]. Nevertheless, both interest in the field[9,10,11,12] and significant stakeholder investment continues to increase[13] and new commercial instrumentation to support its incorporation in SU or SU/cleanable hybrid applications continues to appear. The value and many benefits of, especially up-and downstream integrated, continuous bioproduction are reviewed elsewhere in this volume. It is sufficient here to remind that CB can provide such benefits as reduced plant footprint, technical transfer activities, operator attention, in process storage volumes and reactor residency time – while increasing product homogeneity (quality) and processing speed.

## 3. Single-use in Continuous Biomanufacturing

SU technologies supply a number of values to any mode of Bioprocessing, and their power in continuous biomanufacturing (CB) can be seen as two fold. First, some SU components provide specific and enabling features of particular value in CB implementations[14,15,16,17]. But more important is the fact that most SU materials simply provide acceptable functionality and robust support of

| System | Description |
|---|---|
| Dialysis | Primary/secondary metabolites exchanged across a membrane |
| Extraction | A two-phase system which lowers some secondary metabolites |
| Perfusion | Media continuously exchanged (by, e.g., gravity/ filter/centrifuge) |
| Enhanced perfusion | Media continuously exchanged with cells greatly concentrated |
| Perfusion-like | Any of the growing "not-quite" or "semi" perfusion approaches |
| Steady-state | Equilibrium-like, but establish with balanced inputs and outputs |
| Internal concentration | Integral cell concentration by, e.g., inclined ramp or hydrocyclone |
| Internal filtration | Media exchanged and cells retained through a (static/spin) filter |
| External filtration | Media exchanged through some external (virtual) filtration unit |
| Hollow Fiber Perfusion | Media changed and cells retained within a hollow fiber cartridge |
| Continuous | Prolonged control of input/output maintaining a controlled state |
| Chemostat | A steady-state type where culture expansion equals dilution rate |
| Repeated | A fraction of the biomass provides seed for the next culture cycle |
| Attached continuous | 2-D stacked array multiplate or stirred suspended microcarrier |
| | 3-D scaffold fixed or moving packed-bed perfused bioreactors |

**Table 2** Concepts in upstream continuous processing in techniques. Some with overlapping features, and local definition or connation in usage.

| System | Description |
|---|---|
| Single-Use Systems | Processing equipment or material used in one manufacturing cycle and then retired from use. Often composed of disposable materials in re-usable housings. |
| Mechanically Agitated Suspension Bioreactor | Bioreactors supporting the culture of free or particulate-bound cells (e.g., microcarriers) through continual mixing (e.g., by an impeller or paddle). |
| Continuous Biomanufacturing | Manufacturing of biological products employing continuous end-to-end processes and an integrated control strategy– beginning with starting materials and yielding final dosage units. Also commonly refers to continuous flow unit operations demonstrating the potential to be a component of the full process. |
| Perfusion Culture | The culture of cells through their isolation and the exchange/renewal of either culture medium metabolites/gasses or the whole culture medium itself. Can be classified by the type of cell (e.g., suspension/ adherent), exchange (e.g., metabolite/whole medium), cell isolation process (e.g., filtration/settling), or culture mode (e.g., stirred tank/packed bed). |
| SU Perfusion Bioreactor | Single-use bioreactors supporting some type of perfusion culture. In biomanufacturing it is implied that they are of manufacturing scale and commercially available as either an integral system or as complementary systems components of acceptable assembly. |

**Table 3** Manufacturing-scale SU CB terms

| System | Description |
|---|---|
| SU Filter Bioreactor | Supports mechanically agitated intensified batch or continuous perfusion culture through the retention of cells/microcarriers by an external or internal (e.g., fixed, floating or spinning) porous membrane, fiber or screen. Clogging of screen is greatest limitation and is ameliorated by various technologies. |
| SU Packed bed Bioreactor | Supports direct culture of suspension/adherent cells within an integral macro-porous matrix. They can be further classified into fixed (majority) and moving bed. |
| SU Hollow Fiber Perfusion Bioreactor | Supports direct culture of suspension/adherent cells employing integral micro-porous hollow fibers. Can be further classified into intra-fiber extra-fiber culture. |
| SU Centrifugation-based Perfusion | Supports mechanically agitated intensified batch or continuous culture, without such invasive components as membranes, through the retention of cells/microcarriers by continuous flow centrifugation of various rotor design. They can provide consistent performance with no mid-run degradations such as filter ageing or clogging. |
| SU Acoustic Wave-based Perfusion | Supports mechanically agitated intensified batch or continuous culture through the retention of cells/microcarriers by ultrasound-based isolation of without the need of invasive components such as membranes, or the moving components required by centrifuges or spin filters. These non-fouling cell retention devices are variously referred to as ultrasonic resonators, separators or filters or acoustic wave separation (AWS). |

**Table 3 (Cont'd)** Manufacturing-scale SU CB terms

CB operations while maintaining many of their original design advantages. So, we see SU either enabling or complementing CB in support of not only existing but especially a number of emerging manufacturing goals (Table 4). CB has introduced an interesting twist on the standard paradigm of the concept of iterations of equipment usage. There has always been a bit of wiggle in the distinction between the concept of "single-use" and "disposable" and "limited-use". Presented here is an introduction of how CB has determined a re-examination of a few related concepts in this regard (Table 5) and how SU and SU/cleanable hybrid equipment supports such upstream CB approaches as intensified perfusion culture.
Most operations in a CB process train are now supported by a commercially available single-use, or at least hybrid, solution. First of all, many of the SU equipment and solutions being developed for batch bioproduction have the same or related application in CB systems. Examples here range from simple equipment as tubings and connectors, to even more complex systems applications supporting the hydration and conditioning of powdered culture media and buffers. The list of CB-supporting SU technologies being developed is large and continually growing (Table 6).

A SU advantage in process development is its support of an open architecture approach and a number of hybrid designs. Such "hybrid" designs include combinations between SU materials of divergent composition, between reusable and SU systems, or between different suppliers of particular equipment. Especially in bioproduction, this flexibility contributes to a manufacturing platform's efficiency, adaptability, and operational ease[18]. Advances in SU transfer tubing styles and diameter, manifold design, connector engineering and container porting also supports creativity in process design. This is of particular value in designing a process with such demands as entirely new equipment, flow paths or lot designations - such for CB.

SU systems upstream provide a reduced footprint and eliminate the need for cleaning and sterilization service. This perfectly complements perfusion culture's inherently smaller working volume and independence from cleaning for extended periods of time. Neither demand extensive classified space or entire categories of QA procedures, documentation and support personnel.

Several newer approaches to formulating process fluids support the concept of CB. Single-use mixing systems are typically constructed of a rigid containment system with a motor and controls driving radiation-sterilized single-use bags equipped with disposable impeller assemblies. From a variety of manufacturers there are a number of distinct approaches to motor/disposable impeller assembly linkages, tubing lines and connections. Also appearing are a number of exciting SU sampling, sensing, and monitoring and control solutions specifically designed with SU support in mind. Single-use powder containers permit seamless transfer between powder and liquid formulation steps, and the ridged mixing containers are available in jacketed stainless steel for heating and cooling requirements. Newer container dimensions and impeller style/configurations are providing improved SU mixing performance to the 2000L scale. Surprisingly, and for especially continuous operations, the "topping-up" of large-scale single-use fluid containers with newly prepared

| Manufacturing Goals with Single-Use |
|---|
| Trend toward personalized and orphan entities |
| Anticipated lower mass "next-gen" products |
| Demand for reduced development times |
| Globalization of production competition |
| Trend toward contract manufacturing |
| Desire for local sourcing / production |
| Development of less stable products |
| Explosion in biosimilar development |
| Price-sensitive / controlled markets |
| Growth of multi-product facilities |
| Demand for process flexibility |
| Need for pandemic response |
| Increased volumetric yield |

**Table 4** Developing manufacturing goals calling for the features of CB and SU

| Concept | Definition | CB-specific Modification |
|---|---|---|
| Reusable | Equipment or material intended for use in a process for an indefinite number of times: especially in different production cycles or batches, and after salvaging or preparation by special treatment or processing. | None |
| Multi- or limited-use | Equipment or material intended for use in a process for a limited number of times: determined by validated procedure or subsequent testing. | As CB, by definition, can increase the time and throughput volumes involved in each "use", review of the number of iterations addressed is advised. |
| Single-use | Equipment or material intended for use in a process for one time and then retired from use. | As CB, by definition, can increase the time and throughput volumes involved in each "use", review of the validation requirement is advised. |
| Hybrid | Equipment or material composed of both reusable and single-use components or materials from disarate vendors or systems. | None |
| Disposable | Equipment or material intended for use either for one time or for use in a process in a limited number of times, and then retired as waste. | Same alteration as either "Single-use" or "Multi-use", depending upon the intent. |

**Table 5** Schematic representation of the typical operating points for clinical manufacturing and commercial manufacturing in a Protein A based BioSMB process.

buffer to provide a virtually unlimited and contiguous constant supply of each buffer/media type can be validated for GMP manufacturing procedures.

Continuous, automated in-line culture media and buffer dilution and conditioning have been attempted for decades, and interest in them remains high[19]. Of late, advancements in the mass flow technology and monitoring/feedback control required to establish and maintain process fluid specifications

| SU Technologies Supporting CB |
|---|
| Preparation and storage of media/buffers in SU mixers |
| SU liquid and gas filtration of many types, including TFF |
| SU valving and SU peristaltic or diaphragm(s) pumps |
| SU process fluid heat exchange/manifold distribution |
| Storage of media and buffers for CP feeding in SU BPC |
| SU storage and metered distribution of dry powders |
| SU or hybrid bioreactor cell culture in seed generation |
| Production in SU or hybrid-SU perfusion bioreactors |
| Continual appearance of new SU probes and sensors |
| SU real-time automated online multi-analysis interface |
| SU flow-path on-line real-time controlled feed porting |
| Clarification by SU centrifugation or filtration into BPC |
| Purification in SU traditional or PCC chromatography |
| Vaccine-specific activity support such as SU virus filtration |
| Bulk formulation, filtration and polishing in SU equipment |
| In eukaryotic and now even some prokaryotic SU platforms |
| Final fill in SU and/or automated and closed apparatus |

**Table 6** Continuous Bioproduction Related Single-use Technologies

are now allowing such approaches to become a reality[20]. The compact size and portability of the equipment involved allows it to produce fluids at the "point of use" and supports the incorporation of SU. Its support of significantly reduced buffer prep tank sizes also allows for the application of single-use BPCs containers and manifolds. So, in-line preparation and fluid conditioning would provide benefits to Bioprocessing in general, support CB in particular and provide specific features supporting single-use technology application in CB.

Process flexibility is a key feature in both SU and CB. CB contributes to overall process flexibility in that its smaller equipment tends to be easy to clean, inspect and maintain– and generally promotes simple and rapid product changeover. SU systems can provide similar flexibility and ease product changeover because they tend to be more modular and transportable than much of the older batch equipment. In fact the size, configuration and reduced service requirements of SU systems actually encourage diversity of physical location within a suite or plant - as well as re-location to other manufacturing sites[18].

Due to its inherent demand for immediate process data and control capabilities, CB supports such initiatives of coincident demand as continuous quality verification (CQV), continuous process verification (CPV), and real-time release (RTR)[21,22,23]. Although CB will not be feasible for all products and processes, many implementations well-support a "platform" approach, in which a single process supports more than one product. CB most always shortens the process stream, reduces downtime, and greatly reduces handling of intermediates. These features complement the operational efficiencies of SU systems, contributing to a greatly reduced cumulative processing time for the API. Furthermore, in that both greatly simplify production trains they inherently facilitate application of closed processing approaches to individual operations and even processes. Especially in bioproduction, the modularity and integral gamma irradiation sterility of SU, combined with the sustained operation of CB, promise the appearance of platforms of unparalleled operational simplicity, efficiency and convenience.

The heart of a CB approach is the bioreactor. Perfusion bioreactors have been

successfully employed in bioproduction, even biopharmaceutical production, for decades. And, rather remarkably, disposable bioreactors have been available for over 20 years. At the research scale there have even been single-use hollow fiber perfusion bioreactors available from a variety of vendors for over 40 years. And, we have many publications appearing on entities being launched following the successful application of available SU and hybrid production-scale perfusion-capable equipment[24,25,26] (Table 7).

The production-scale CB enabling SU bioreactor technologies appearing include single-use and hybrid perfusion-capable reactors (Figure 1, 2); a growing variety of SU and hybrid monitoring probes and sensors; SU pumps and fluid delivery automation of various design; SU fluid heat exchangers and automated SU online sampling, interface, valving and feeding technologies. Their coordinated implementation in actual production settings with appropriate control is now beginning.

Justified or not, concerns in the implementation of CB include performance reliability (incidence of failure), validation complexity, process control and economic justification[27,28,29]. But for many processes, such previous limitations — or their perception — are being alleviated by advances in CB processing technology and OpEx driven advances bioprocess understanding, reactor monitoring and feedback control[30,31]. In fact, the ongoing movement toward digital biomanufacturing supports this initiative. However, while some CB attributes inherently provide immediate advantages (such as reduced reactor residency time) others do present challenges (such as cell-line stability concerns).

Due to the limited contribution of API manufacturing to small-molecule pharmaceutical cost, the limited bottom-line financial savings of CB has been a concern here. However, biopharma is a different animal in general, and as such trends as globalization and biosimilars alter the picture even further, the financial benefits of CB are becoming even stronger (Table 4)[32].

Environmental sustainability objectives are supported by the fact that CB

operations can reduce:

1) amount of equipment to be cleaned and/or steam sterilized
2) the process steps, footprint, service and energy consumption demanded in the process
3) the number of operators (and their commute) required.

Environmental impact assessments (including life cycle analyses (LCA)) for both continuous manufacturing approaches[33] and SU material use have been well reviewed[34,35]. Due to both the surprising sustainability potential of SU materials, as well as the growing interest in these goals, such studies are now in the process of being greatly extended and will be available shortly.

The fact that many SU systems are constructed of standards-compliant and animal product-free materials supports CB applications in a wide variety of product types and classification. In fact, SU systems are available for almost any process format (e.g., microcarriers and suspension), platform (e.g., cell line, vectors, culture media), mode (e.g., batch or enhanced perfusion) or scale (e.g., through rapid, inexpensive scale-out) (Table 8). "Futureproofing", or supporting the sustainability of a new CB process in the face of product life cycle or an emerging technology imperative, is supported by many SU features[36].

**Fig. 1** Single-use intensified perfusion-based bioproduction process development in a Perfusion Cellbag™ bioreactor (GE Healthcare).

| SU Perfusion Bioreactors | SU / Hybrid PC Application |
|---|---|
| Fixed packed-bed | SU fully-controlled PC. Adherent and some suspension culture. |
| Hollow Fiber | SU fully-controlled PC. Suspension and adherent culture. |
| Moving packed-bed | SU fully-controlled PC. Suspension and some adherent culture. |
| Roller bottle | SU closed media exchange with some CPP control. Adherent culture. |
| Wave-action based | SU PC suspension and adherent (e.g., microcarrier) culture from some manufacturers. |
| **SU Perfusion Capable** | **SU / Hybrid PC Application** |
| Stacked array flask | SU closed media exchange with some CPP control. Adherent culture. |
| Stirred tank (mechanically mixed) | SU PC potential with perfusion-enabling technology. Suspension and adherent (e.g., microcarrier) culture. |
| Wave-action based | SU PC potential with perfusion-enabling technology. Suspension and adherent (e.g., microcarrier) culture. |
| **Perfusion Enabling Tech** | **SU / Hybrid PC Application** |
| Centrifugal media exchange | SU PC when combined with certain bioreactors. Suspension or adherent (e.g., microcarrier) culture. |
| Hydrocyclone media exchange | SU PC when combined with certain bioreactors. Suspension culture. |

**Table 7** Single-use perfusion-type culture at biomanufacturing scale. SU: Single-Use. PC: Perfusion Culture.

| Perfusion Enabling Tech | SU / Hybrid PC Application |
|---|---|
| Hollow fiber media exchange | SU PC when combined with certain bioreactors. Suspension or adherent (e.g., microcarrier) culture. |
| Sonic wave cell isolator | SU PC when combined with certain bioreactors. Suspension culture. |
| Spin Filter media exchange | SU PC when combined with certain bioreactors. Suspension or adherent (e.g., microcarrier) culture. |

**Table 7 (Cont'd)** Single-use perfusion-type culture at biomanufacturing scale. SU: Single-Use. PC: Perfusion Culture.

**Fig. 2** Hybrid continuous bioproduction accomplished in a GE Healthcare XCellerex™ XDR bioreactor (left) supported by a Repligen XCell™ ATF System (right).

Examples here include CB/SUs low initial facility, service and equipment cost and especially SU's undedicated manufacturing suites and ease of process train reconfiguration. Most recently two new initiatives have appeared. Supply-chain issues in both the materials and manufacturing security-of-supply has become a key issue in specifying a supplier. Customers are demanding a QBD approach in the design of comprehensive risk-mitigation initiatives, as well as redundant manufacturing capabilities.

Advanced single-use solutions are being successfully applied throughout the production train in bioproduction. This, and other developments in the field, are promising the design of continuous, disposable, integrated and even closed bioproduction systems supporting enterprise control in digital biomanufacturing facilities.

| SU Perfusion Equipment Summaries |
| --- |
| SU equipment and technologies provide perfusion complementing or enabling features in continuous biomanufacturing implementations. |
| Commercially available SU and hybrid production-scale perfusion-support has appeared for most any process format, platform or mode. |
| Many large-scale SU perfusion systems including mechanically agitated suspension, hollow fiber, floating filter and packed bed reactors. |
| SU accessories supporting perfusion culture range from hollow fiber exchange to continuous flow centrifugation to acoustic wave separation. |
| Environmental objectives are supported as CB reduces the equipment to be CIP/SIP and their required services and energy consumption. |

**Table 8** Single-use Perfusion Equipment Summary

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# Continuous Bioprocessing's Impact on Facility Design

## About the Author:

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Morten Munk's career is comprised of 30 years of experience within the global biopharmaceutical industry.

One common denominator for his work has been to ensure a holistic and broad perspective on biomanufacturing challenges from idea to established facilities. His key focus is to ensure compliant and cost-effective production through the optimal use of all relevant and available knowledge and technologies, such as single-use systems and continuous processing. Morten combines his technological expertise with a thorough business understanding coupled with a great personal interest in and practical understanding of stakeholder relations and change management.

Morten frequently gives technical presentations at international conferences and he is highly motivated by sharing knowledge and experiences in how to meet the key objectives of the pharmaceutical industry.

Furthermore, he prioritises and appreciates being a member of scientific committees for various international conferences, as well as a member of the CMC Biologics Technical Advisory Committee, PDA Biotechnology Advisory Board and the Advisory Board for Master studies at Copenhagen University.

Morten joined NNE as Global Technology Partner in 2015. In 2001 he co-founded CMC Biologics, after working 14 years at Novo Nordisk.

## 1. Introduction

Over the last decade, Biopharmaceutical manufacturing facilities and operations have changed, following the successful adoption of Single-Use Technology (SUT). The manufacturing methods for biological products have, however, not fundamentally changed. Manufacturing is still based mainly on a traditional batch based concept, with individual operated unit operations that are heavily dependent upon off-line analysis during the manufacturing process. The product quality is furthermore primarily relying on release analysis of the final product. Thus, it can be argued, that quality is tested into the product rather than being *built* into the product and the production method.

This production method might be compared to other large volume industries e.g., the automobile industry. In the automobile industry, a batch production method would mean that one or a number of identical cars are produced in one go from start to the end, and only the cars that pass a final quality test would be sold. This concept does work for a small number of high value cars, but not for large-scale low cost production. The solution in the car industry was to introduce the conveyor belt. The key driver was to reduce cost, which worked out very successfully. Moreover, this technology offered two additional benefits to the automobile industry, which could be relevant for the pharmaceutical industry. First and foremost, it enabled a higher quality level, as the conveyor belt requires good process understanding to be performed successfully. If a quality or manufacturing issue occurs during a production, the conveyor belt stops the entire process. This creates a "burning platform" for finding a reliable solution fast. The best way to avoid such stops to have built in quality (Quality by Design) and a continuous process improvement and monitoring system (Continued Process Verification). The second added advantage is increased flexibility, which has two aspects. The conveyor belt makes adjusting the production output to the marked demand relatively easy. The speed of the conveyor belt can be reduced when the demand decreases, and turned back up when the demand increases. The second type of flexibility is the possibility to make slightly different types of cars, (e.g., different colors), based on the same platform.

The pharmaceutical industry is obviously different in many ways compared to the automobile industry. One difference is the automobile industry's higher focus on manufacturing costs. This is probably one of the reasons why the pharmaceutical industry is generally viewed to have a quite conservative approach to manufacturing solutions and a relatively high reluctance to the introduction of continuous processing and other emerging production technologies.

In recent years, cost and quality of pharmaceutical manufacture has risen to the top of the agenda, and the regulatory bodies led by the FDA have actively promoted the use of continuous processing and other emerging technologies. The main reasons are most likely due to the higher quality levels anticipated when shifting to continuous processing. Moreover, the regulatory bodies also show awareness of their need to play a more active role in reducing manufacturing costs. This effort began in 2004 with the introduction of FDA's report on pharmaceutical quality for the twenty-first century. Moreover, several high-ranking FDA officers, led by Janet Woodcock, Director of FDA & CDER, have publicly voiced support for taking advantage of continuous processing. This initiative from the FDA includes an expectation that the industry establish and demonstrate higher levels of process understanding and a risk based

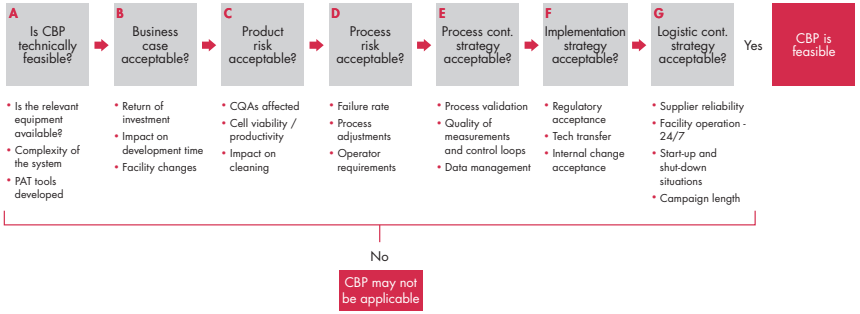


**Fig. 1** Illustration of the different elements to be considered when initiating a project for implementation of Continuous Bio-Processing.

approach to quality, which are key enablers and a requirement for successful implementation of continuous processing.

Changing to the more flexible and agile production method that continuous processing offers is not a trivial mission. It not only presents a challenge to develop manufacturing processes in new ways, but also requires that facilities be designed differently.

Before establishing a Continuous Bio-Processing (CBP) facility, a few key questions have to be considered. Figure 1 Illustrates a structured approach for some of those key questions.

## 2. Continuous Processing impact on facility design

When the fundamental requirements for continuous approach have been established and a construction of a new continuous facility is the next step, the basic elements of building a facility should be investigated.

Figure 2 represents an illustration of the complexity of facility construction projects. It is understandable why such projects require experienced third party support to ensure a successful result.  A good starting point would be with the

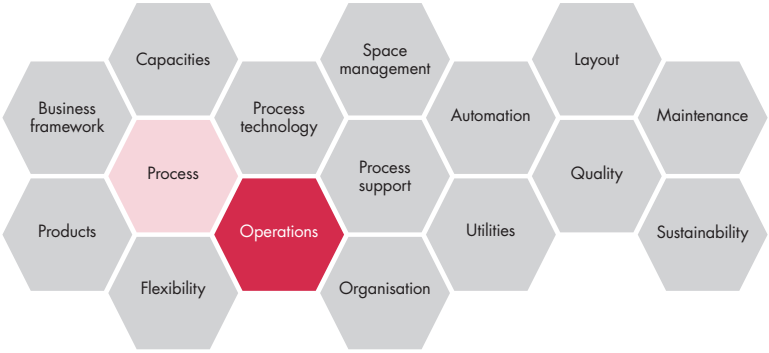


**Fig. 2** Selected main factors considered in facility design. The process is the central starting point in a "from inside-to-outside" facility design approach. Operations for continuous Bioprocessing introduce important shifts in focus areas for virtually all facility design aspects. The key impact is the requirement to keep the "conveyor belt running" without compromising product quality. The list is not exhaustive for reasons of clarity.

core process and perspective operations, process technology, etc., that relate to the business aspects of the process. Some of these aspects might not be affected by the fact that the processes will be continuous while others would be. The process technology is significantly changed and it is more complicated to define, order, test, qualify and validate continuous processes, at least initially. For example, the complexity of calibration of the complex sensor technology for NIR and other technologies used for continuous control is significantly more complex than traditional process instruments.

Thus, the involvement of all the aspects mentioned in the model require a systematic approach that brings them together to ensure early requirements, well-defined interfaces and a clear scope of each of the elements as well as the overall project.

The design should keep the daily operations in mind, as the operational modes are considerably different for equipment running as a 24/7 operation for several weeks or even months compared to a batch-based manufacturing

set-up, with inevitable pauses between the batches. It means that unplanned changes are a large challenge in a continuous facility and thus critical to be taken into consideration when planning and designing the facility.
In the design of the facility, the most significant differences are in the overall design of the manufacturing system. A holistic approach that outlines all the main modules for all the process steps is where to begin, just like designing a batch-based facility. However, the important differences are in the continuous processing modules and their process support systems. Therefore, a module-based overview is important.

For a company starting on this path, there is no reason to aim for a 100% continuous facility from the start. Reaching for the low hanging fruits and being able to demonstrate the advantage of continuous processing in selected parts of the process can help in convincing skeptics. A hybrid solution will often be the most cost effective way and give the highest chance for a successful outcome. In a hybrid solution, an important design decision is which process modules to be operated continuously and which ones in a batch mode, due to the large impact on the equipment design and the space needed for operation. The batch-based approach typically requires larger areas. This is not only for the core process equipment but also for the supporting vessels such as solvents, holding tanks, etc. This fact offers the possibility for a conversion of some of the batch processes to continuous operation at a later point in time, when the experience with continuous operation has increased and the positive impacts on cost and flexibility have been verified. It is naturally a large advantage to be proactive and, during the design and construction phases, include the possibility of an increased use of CBP at a later point in the facilities life cycle.

Some of the challenges relate to the technical complexity of the systems, which requires more knowledge of the processes and how to control manufacturing. Thus, Process Analytical Technology (PAT) and other types of advanced process models for multivariate process control, which are based on a high level of process and product understanding, are key enablers for successful continuous operation.

| Characteristic | Batch | Continuous |
|---|---|---|
| Process operation | • Process steps are independent<br>• Break down of one process step, has limited impact on the rest of operation<br>• Hold steps needed<br>• Batch definition is reactor volume | • Process steps are linked directly<br>• If one process step stops, the whole process stops<br>• No hold/storage steps<br>• Batch definition can be chosen based on volume, time or amount of product |
| Automation | • Low automation possible<br>• Medium amount of data to be handled | • High automation required<br>• Large amount of data to be processed and handled |
| Process Control:<br>• Feedback loops<br>• Feed forward loops<br>• Multivariate model based control | • Implemented to some extent<br>• Limited use of historic data for future batches<br>• Possible to be operated, even it might be suboptimal, with low level of process control and understanding | • Real time /online monitoring and control required – PAT<br>• Online data used for current campaign and future campaigns<br>• High level of process control and understanding needed |
| Quality Control | • Quality tested in after batch conclusion<br>• Can be off-site | • Quality build in – real time quality assurance<br>• Need to be on-site |
| Capacity flexibility | • Capacity is fixed<br>• Increased capacity based on scale-up – require larger equipment or new facility | • Capacity can be adjusted<br>• More options to increase capacity<br>• Can be based on scale-out – more systems in parallel<br>• Adjust processing time to meet demand |
| Facility Size | • Larger, due to larger and extra equipment | • Typical 4 to 10 times higher output per $m^2$ production |

**Table 1** Comparing batch and CBP operation, including impact on facility designs

| Characteristic | Batch | Continuous |
|---|---|---|
| Manning | • 1 or 2 shifts<br>• Increased staffing for manual operation<br>• Lower level of skills required<br>• Higher risk of contamination from operators<br>• Higher risk of people related GMP deviations<br>• Less interaction between teams need<br>• Manufacturing can be separated from Development, QC and QA | • 24/7 operation – 4 shifts<br>• Lower staffing, focus on surveillance – due to higher automation<br>• Higher level of skills required<br>• Lower risk of contamination from operators<br>• Low risk of people related GMP deviations<br>• All have to work as "One Team"<br>• Manufacturing needs to be integrated with Development, QC and QA |
| Equipment | • Simple construction<br>• Larger size<br>• Single Use for small scale<br>• Low utilization of each piece of equipment<br>• Option to use same equipment for different steps in the process | • Integrated construction, more automation<br>• Smaller size<br>• Single use as an enabler<br>• High utilization – all equipment runs at all time<br>• Same equipment cannot be reused for different steps |
| Infrastructure | • Less need for optimal infrastructure | • A well organized and designed infrastructure is key |
| Quality | • Average product quality and reproducibility | • Improved product quality and reproducibility |
| Scale Up / Tech Transfer | • Time and effort required to scale-up R&D to manufacturing scales<br>• Tech transfer to alternative sites can take time to adapt to different or legacy equipment | • R&D scale is commercial scale<br>• Tech transfer can mean copy/paste to a similar plant – since there is little legacy equipment |

**Table 1 (Cont'd)** Comparing batch and CBP operation, including impact on facility designs

Some of the differences to be considered when designing and operating biopharmaceutical based on a batch or a CPB mode are summarized in Table 1.

## 3. Continuous Processing and Single Use Technology

Single Use Technologies (SUT) have been a key enabler for flexible manufacturing, and as a result is used to some extent in nearly all the new products being tested for early clinical trials – trends that now are continuing to see increased use of SUT in commercial manufacturing.

This has fueled a fast development of a broad range of SUT components being used over a wide range of industrial applications. SUT components must meet the associated high quality requirements including robustness, reproducibility, reliable supply and most importantly, that the SUT components do not negatively affect the product or the process. Often referred to as "Extractables and Leachables", the leakage of unwanted materials in to the process has been a large concern and area of focus, where the developed solutions have improved dramatically over the last years.

The higher requirements have also increased the price for the SUT components, which could have a negative impact on the future growth in SUT applications in the industry. CBP might offer a solution for increasing the added value when applying SUT in Biopharmaceutical manufacturing.

The combination of SUT and CBP offers a number of synergetic advantages:

- CBP systems generally require a large number of valves, and low dead volumes in the piping, and should preferably offer a large flexibility to accommodate different CBP set-up's. SUT offers an option to construct an integrated and pre-assembled single use tubing and valve set which can be made to be optimal for a specific CBP set-up. In case another new CBP set-up is requested, then a new valve and tubing set can be constructed to fit that new set-up. Thus, a larger flexibility, which do not need to be build upfront and as a hard-pipes system.

- CBP systems are generally more complex and require a large number of components, which increase the challenge to ensure sufficient cleaning of

all vessels, piping and valves. Using SUT reduces the cleaning challenges, as all those components can be discharged upon completion of a CBP campaign.

- Combination of CBP and SUT offers a possibility to manufacture in closed systems, which reduces the requirements to room classification. Previously, chromatographic columns have been the proverbial Achilles heel when trying to obtain a closed system. The CBP approach requires smaller size columns, which makes it more attractive to use pre-packed disposable columns.

- CBP and pre-packed disposable columns are two technologies that complement each other. Large pre-packed disposable columns that only need to be used few times are generally not a cost effective solution. As noted above, CBP requires considerably smaller columns sizes, where each column is used for many runs in each CBP campaign. This will typically mean that the column resin is used closer to its full potential, which is rarely the case when chromatography steps are performed in batch mode. This results in less resin cost per gram of product and less capital bound in resin.

- The requirement for smaller column sizes as well as the smaller size of the CBP system, reduce the clean room foot print requirements. In general, a single use setup has a smaller size (bioreactors, columns, filters etc.,), which add to the positive impact on investments and running costs.

- CBP opens an opportunity to use single use components for a longer period. A SUT component can only be used for one batch, when producing in batch mode. In CBP mode, a SUT component might be used for a full campaign, which might be up to 50 days or more. This extended use of the of the SUT components might also have a quality advantages, as the leachables originating from the SUT components will be dilutes into large volumes.

Introduction of Single use and continuous technologies have a significant impact on the facility design due to the smaller footprint and substantial lower cleaning requirements, which reduces the utility requirements. SUT decouple

the equipment from the building, removing the need for the otherwise widely used complex distribution matrixes, which have an inherent risk for operational failures and cross-contamination. The decoupling of equipment from the building furthermore generates valuable flexibility, which include easier introduction of new technologies. An observed trend is that 50% of facility designs where single use bioreactors are included for fed batch manufacturing initially, also includes an option to convert from fed batch to perfusion mode using e.g. ATF modules and continuous capture, at a later stage.

Large scale fed batch manufacturing typically yields a high amount of product, which results in a requirement for large column diameters for chromatography as well as equipment to handle large intermediate product hold volumes. Downstream operation furthermore requires several connections and disconnections during manufacturing, especially when operating chromatographic columns. Due to this type of traditionally open operations, downstream operations typically have been performed either clean room Class D or Class C. In continuous Bioprocessing however, process equipment including chromatographic columns are significantly reduced in size and storage of process intermediates are either eliminated or reduced to low volumes in surge bags. Pre-packed columns (currently only available up to 60cm diameter), may also be employed to further simplify operations.

## 4. Concluding remarks

The combination of the possibility for supporting closed operations as well as the high frequency of introductions of new SUT solutions designed for CBP introduces a paradigm shift in Biopharmaceutical facility design. Those new technologies introductions include; single use XCell™ ATF, pre-packed (disposable), columns and single-path TFF. Assembling several unit operations as a closed system allows a reconsideration of clean room requirement and room segregation. Thus, continuous Bioprocessing is likely to fuel facilities designed with the so-called ballroom or dance floor concepts with less room segregation, (if any ultimately). Such a continuous production line consisting of, for example, one 500L single use bioreactor and a simulated moving bed setup consisting of 10L chromatographic columns may have an annual capacity of up to 500-1000kg drug substance within a footprint of a few hundred $m^2$.

This is a considerably smaller facility footprint than an equivalent fed batch facility would require and, given the shorter lead times, the decision to build the facility can consequently be made later in clinical development thereby reducing risk. The additional advantage of CBP and SUT supporting closed operations might in the future allow for manufacturing CNC area or maybe even in to grey space. Critical connections in the closed system could then potentially be in a closed environment (RABS isolator) to allow interference during the manufacturing if needed.

The industry is definitely transforming, and it will be interesting to follow how broadly CBP will be accepted by the conservative biopharmaceutical industry and fulfill the regulatory bodies' wishes for introduction of emerging technologies to the benefit of patients.

# CONTINUOUS BIOPROCESSING