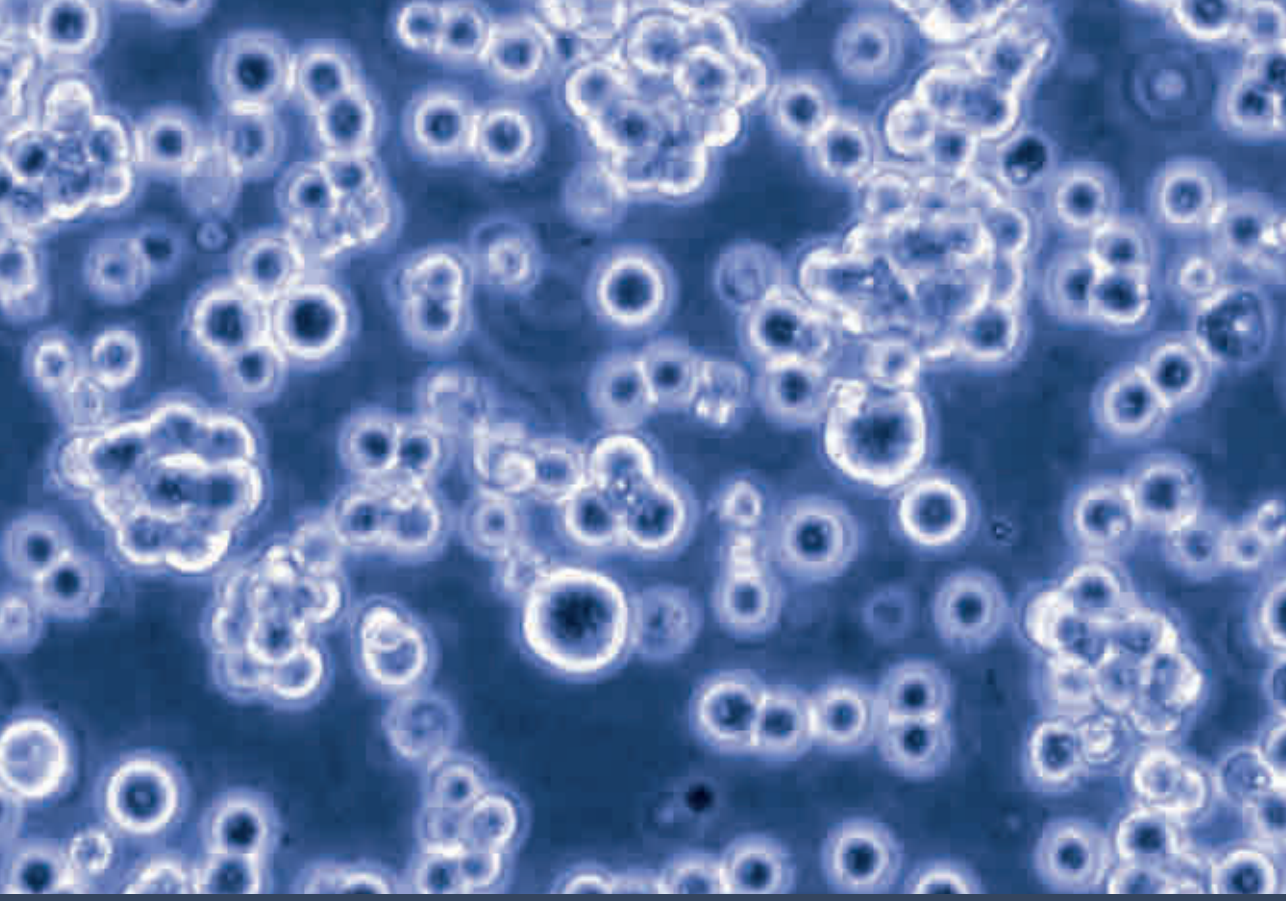
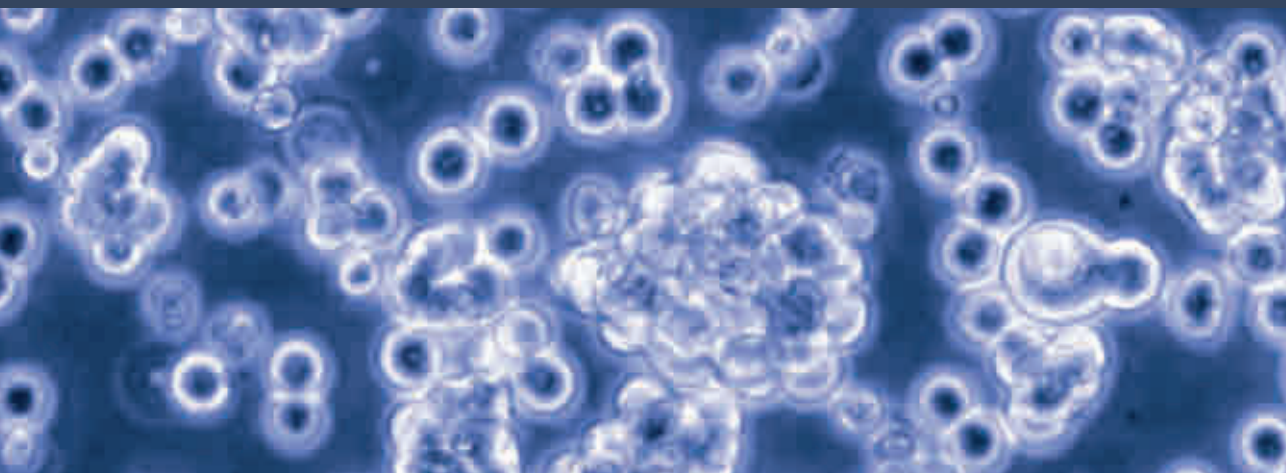


PAT for PER.C6® perfusion cultivation

Sarah M. Mercier



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PAT for PER.C6® perfusion cultivation

Sarah M. Mercier

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Il faut bien que je supporte deux ou trois chenilles si je veux connaître les papillons.

From Le Petit Prince, Antoine de Saint-Exupery

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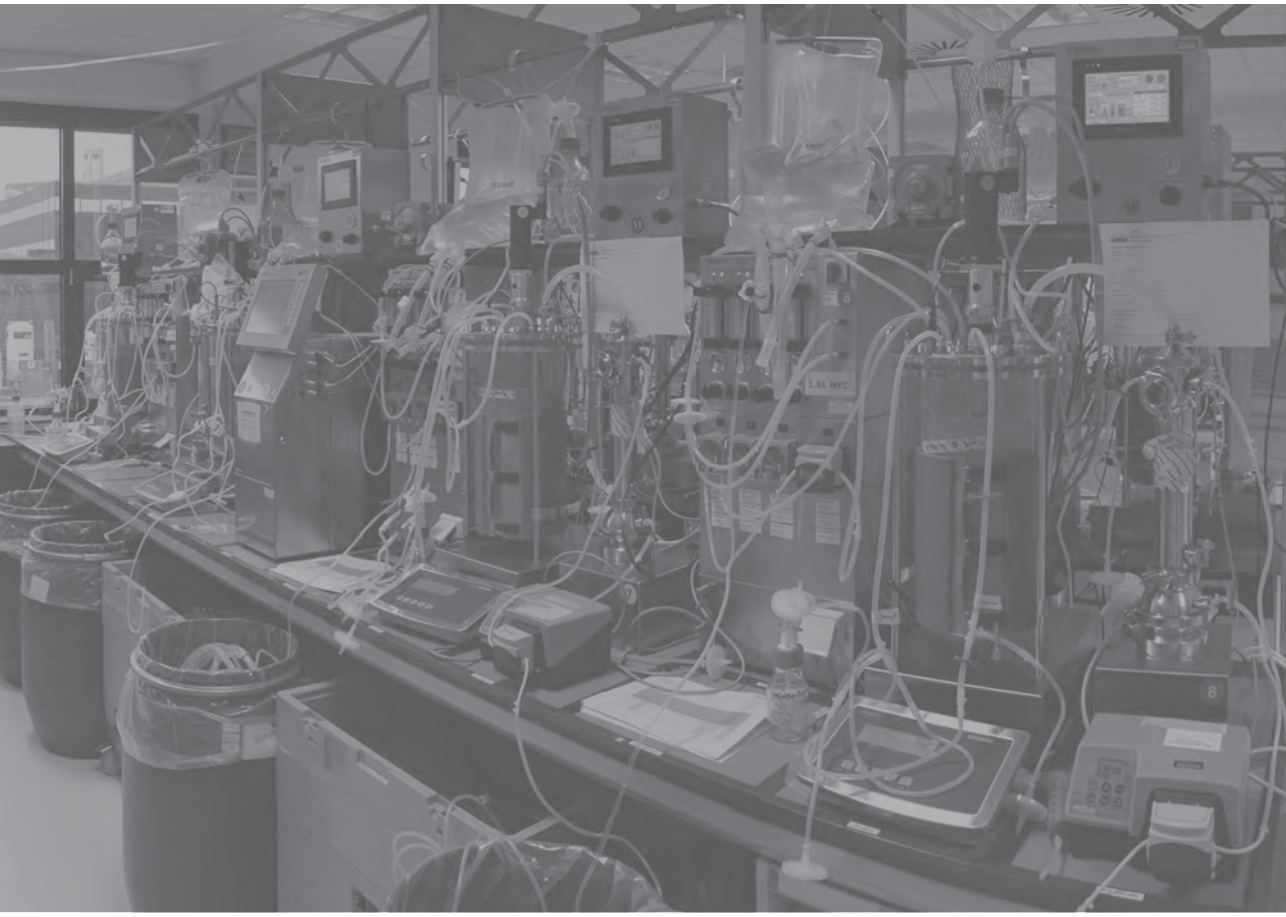
Chapter 1

Introduction and thesis outline



1 Background

The traditional approach to release of biopharmaceutical products relies on quality-by-testing, where a series of analytical assays typically measuring quality attributes related to product safety and efficacy (i.e. identity, purity, potency, stability) are tested after the manufacturing process is completed. The list of disadvantages of this approach is long, from limited understanding of the manufacturing processes and consequences of deviations on product quality, to extensive documentation and to long lead times for product release post-manufacturing (1). To improve this sub-optimal situation, the FDA launched several guidelines inviting the (bio)pharmaceutical industry to shift its quality paradigm towards the use of Process Analytical Technology (PAT) principles and towards implementation of Quality by Design (QbD) rather than quality by testing (2, 3). The aim is to increase the level of control of their manufacturing processes and ultimately to ensure consistent and high product quality.

Although PAT was launched over a decade ago, biotechnological companies have not all embraced this concept and PAT is not always applied to all process steps that form the complex train of operation units for the manufacturing of drugs or vaccines. In this context, the research described in this thesis has been initiated to apply PAT principles to a perfusion PER.C6® cell cultivation process, which is an operation unit of a viral vaccine production. This process aims at expanding PER.C6® cultures to high cell densities in perfusion mode, to seed the next process step of virus production. The quality of the cells used as a substrate for virus propagation was hypothesized to be critical for the performance of the virus production process. This thesis therefore focused on understanding the critical quality features of PER.C6® cells used for virus production, and on identifying an appropriate process monitoring strategy using the PAT principles. This research was conducted in collaboration between Crucell (a Janssen Pharmaceutical company) and Wageningen University. The structured and systematic application of PAT principles has led to the development of new analytical methods and data analysis tools used to increase the scientific understanding of the perfusion PER.C6® cultivation process. This work is the basis for the design of improved process control strategies.

2 PAT for biopharmaceutical processes

PAT was defined by the FDA as "*a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality*" (3). PAT is therefore a toolbox for process monitoring, control and continuous improvement. QbD is a broader concept stating that the quality of a product is built into its production process in order to ensure consistent high product quality during manufacturing (2, 4). The basis of QbD is that the product is designed to meet the patient's needs and the process is designed to yield consistent high level of product quality, by controlling all critical sources of process variation. In practice, QbD starts with the identification of the critical process parameters (CPPs) that affect the critical

quality attributes (CQAs) of the product during manufacturing. Ultimately, QbD yields the definition of a process design space, in which the variations of CPPs have demonstrated to provide high product quality. In this context, PAT is therefore a QbD enabler that aims at improving operational systems and risk management on manufacturing processes.

The PAT implementation path can be summarized in three major steps (Figure 1). Firstly, the process design phase focuses on identifying the product CQAs the CPPs. The way in which the CPPs and possibly their interactions affect the CQAs should also be characterized. Secondly, appropriate analytical tools need to be implemented to capture all relevant information on the CPPs or on the CQAs if the relations between the CQAs and CPPs are not clearly understood. In order to make the fullest use of the data collected by these analyzers, the analytical results must be available in a frame-frame that enables real-time decision making. Indeed, the third step is to use the combination of the process understanding gained during the process design phase and the data collected by the process analyzers, to achieve real-time process control. The ultimate process control strategy should ensure consistent process performance and more importantly product quality, while the process is running. This implementation exercise is a circle as new information and understanding gained on the process should be used to continuously improve the process design and its control strategy.

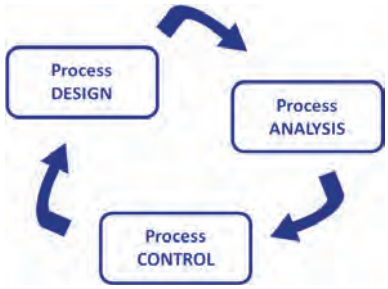


Figure 1. The three major steps for implementation of PAT during the development life cycle of a biopharmaceutical process. Modified from Rathore *et al*. (2010).

3 PER.C6® cell line and its applications

PER.C6® is a human immortalized cell line which was developed in the late '90s (5). These cells are derived from embryonic retinoblast cells obtained from an aborted fetus and were immortalized by transformation with the E1A and E1B-encoding sequences of Adenovirus 5 (5, 6). Expression of E1A stimulates the cells to enter the S phase of the cell cycle by disabling their ability to enter the quiescent G0 phase (7, 8). This way, cell proliferation is stimulated. To ensure cell integrity (e.g. number of chromosomes and accuracy of DNA replication), apoptotic cell death is initiated upon expression of E1A (9, 10). Expression of E1B results in two proteins E1B 55-kDa and E1B 19-kDa (11). E1B 55-kDa blocks of the function of the tumor-suppressor protein p53. E1B 19-kDa is a homolog of the anti-apoptotic protein Bcl2 (10, 12). These two proteins contribute to down

regulating apoptosis induction in the cells. The co-expression of E1B therefore prevents the defensive apoptosis induction caused by E1A (13, 14). Overall, immortalization of the PER.C6® cell line lead to proliferation at high rates and regulation of apoptosis.

PER.C6® is a safe and cost-effective production cell line for biopharmaceutical production (6, 15-18). These cells can grow in suspension in fully animal-derived component free and chemically defined medium. High cell densities of PER.C6® can be reached in continuous perfusion processes (19). Additionally, the immortalization technique used for PER.C6® makes these cells especially suited for complementing and propagating replication-incompetent Adenoviruses. PER.C6® is therefore an excellent platform to replicate Adenoviruses (20-23) and other types of viruses such as Influenza (16, 24, 25) and poliovirus (26). High productivities of immunoglobulin G (IgG) and IgM in PER.C6® cells have also been reported (27, 28), making it an all-round biopharmaceutical production platform. Several products produced using PER.C6® are currently in different stages of pre-clinical and clinical development (25, 29-31).

PER.C6® is used as a basis in a vaccine production platform based on intensified technology, which aims at maximizing process yield (Figure 2). A large volume and high density cell bank of PER.C6® is used to inoculate a wave-rocking bioreactor (Cultibag) for cell recovery post-thawing and seed train expansion. The following two steps are performed in perfusion bioreactors using an alternating tangential flow (ATF) filtration system for cell retention. The PER.C6® cultures are first further expanded and grown to high cell densities, to inoculate the subsequent intensified virus production process. PER.C6® cultures are infected with a virus seed and process conditions are adapted depending on the virus type to produce (e.g. Adenovirus, Influenza or Poliovirus).

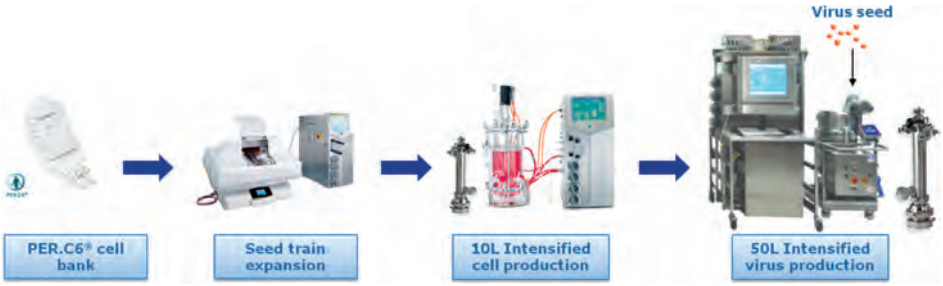


Figure 2. Schematic of a pilot-scale PER.C6®-based vaccine production platform from cell bank to virus production. Pictures on courtesy of Sartorius, Hyclone and Refine.

4 Research aim and thesis outline

The quality of cell substrate is critical for performance of a virus production process. Variation in performance of the virus production process is hypothesized to be caused by the quality of the cells. The characteristics, or CQAs, of mammalian cells used as a substrate for virus propagation are largely unknown. The relation between the

CQAs of these cells and virus attachment, infection, and propagation are also not fully understood. The objective of this thesis is to apply PAT principles to the perfusion PER.C6® cell expansion process. This thesis focuses therefore first on identifying the CQAs of the PER.C6® cells used for virus production and second on the implementation of the most appropriate analytical tools for optimal process monitoring.

The PER.C6® perfusion cell cultivation process studied in this thesis has been originally developed in 2008-2009 at Crucell. This process is the basis for the development of several vaccine development programs, which are mostly in early clinical development phases. The first step to implement PAT on this process is to extract the most relevant process information from the large and elaborate datasets typically generated from these processes. Multivariate data analysis (MVDA) is a statistical method of choice for this purpose. **Chapter 2** discusses the importance of MVDA for industrial bioprocess data and presents recent studies on MVDA as a PAT tool to analyze data from biopharmaceutical cultivation processes. In **Chapter 3**, MVDA was applied to historical data on the PER.C6® perfusion cultivation process. Although the data available from early development contain gaps and are not generated from statistically designed experimental sets, a thorough multivariate analysis expanded understanding on the process and revealed its sensitivity to scale and input materials.

Subsequently, the research focuses on the quality of the PER.C6® cells during the expansion process. To identify the CQAs of the PER.C6® cells used for virus propagation, biological and cellular parameters that are expected to reflect the cell's suitability for virus attachment, infection and replication, are studied. Firstly, in **Chapter 4**, the dynamics of PER.C6® cell growth and cell death are measured throughout the perfusion process and compared to those of batch cultures. Flow cytometry-based assays are used to measure apoptosis in the different cultures, targeting three steps of the apoptosis cascade. Secondly, in **Chapter 5**, a novel assay is developed to measure the cell's infectability for adenoviruses, or in other words their ability to be infected by viruses and to replicate viruses. Kinetics of infection and of virus production are compared for exponentially growing and high viability cultures and for cells sampled from the perfusion process within normal operating conditions and under suboptimal cultivation conditions, as well as on PER.C6® batch cultures exposed to various stresses.

The main aim of **Chapters 4 and 5** is to study the robustness of the PER.C6® cells in the perfusion process, and to assess whether variation in cell quality occurred over process time. On the one hand, apoptosis is found to be maintained at a constant low level during the entire perfusion process (**Chapter 4**), and one the other hand, infectability of PER.C6® cultures with adenoviruses shows to be very robust throughout the perfusion cultivation and under suboptimal process conditions (**Chapter 5**). Overall, these studies demonstrate the robustness of the PER.C6® cell line cultivated in perfusion and suggests that PER.C6® cell quality for virus propagation can be sufficiently assessed by the measurement of culture viability.

The next step focuses on the online PAT tools that are the most relevant to monitor and ultimately control the perfusion PER.C6® cultivation process. These PAT tools should capture information on the culture viability, which is the PER.C6® cells' CQA defined in the previous chapters, as well as the key performance attributes of the perfusion cultivation

process. The business capacity of this process is reflected in the cell specific growth rate, as the time to reach the cell density required to inoculate the virus production phase should ideally be reached as fast as possible. In **Chapter 6**, a combination of dielectric spectroscopy (DS) and near infrared spectroscopy (NIRS) is shown to be complementary to monitor the product CQA and the key performance attributes of the perfusion PER.C6® cultivation process. Using either simple linear regressions or more complex multivariate models, data from DS and NIRS are used for accurate online predictions of viable cell density as well as of glucose and lactate concentrations.

Finally, **Chapter 7** summarizes the results presented in this thesis and discusses the PAT application presented here in the context of the current and prospective efforts to implement PAT and QbD for continuous cell cultivation processes.

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Chapter 2

Multivariate PAT solutions for biopharmaceutical cultivation: current progress and limitations

Abstract

Increasingly elaborate and voluminous datasets are generated by the (bio) pharmaceutical industry and are a major challenge for application of PAT and QbD principles. Multivariate data analysis (MVDA) is required to delineate relevant process information from large multi-factorial and multi-collinear datasets. Here the key role of MVDA for industrial (bio)process data is discussed, with a focus on progress and limitations of MVDA as a PAT solution for biopharmaceutical cultivation processes. MVDA based models were proven useful and should be routinely implemented for bioprocesses. It is concluded that although the highest level of PAT with process control within its design space in real-time during manufacturing is not reached yet, MVDA will be central to reach this ultimate objective for cell cultivations.

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Glossary

Design of experiments (DoE) (1): DoE is a rigorous, structured and systematic approach to designing statistically relevant experimental set where the data generated ensures sound conclusions and decision making. DoE aims at generating the most informative results by eliminating experimental error while minimizing runs, time and resources.

Design space (2): Design space is "*the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality*". Operation of a process within its established design space is not considered as a change, as product quality is ensured within the entire design space.

Critical process parameter (CPP) (2): CPP is "*a process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality*". Any measurable input material attribute, operating parameter and process state variable of a process step affecting product quality or consistency of process performance is a potential CPP.

Critical quality attribute (CQA) (2): CQAs are "*physical, chemical, biological or microbiological properties or characteristics that should be within an appropriate limit, range, or distribution to ensure the desired product quality*". In QbD, CQAs are related to safety, efficacy and stability of the drug product or drug substance and must be directly or indirectly controlled throughout the manufacturing process.

Multivariate data analysis (MVDA) (3): MVDA makes use of statistical techniques to analyze datasets composed of more than one variable or more than one type of variable. With MVDA, variables are analyzed simultaneously to perform exploratory, regression or classification studies.

Partial least square (PLS) (4): PLS is a MVDA technique using projections and linear regressions to study relations and interactions among variables. Predictive models can be made using PLS when variables are highly collinear and when several input parameters correlated to one or more output parameters.

Principal component analysis (PCA) (5): PCA is a MVDA technique using orthogonal projections of the original dataset into new uncorrelated (latent) variables called principal components to study underlying structure of the dataset. Main trends or clusters among batches or data points can be identified using PCA.

1 Quality by Design and Process analytical technology for bioprocesses

Quality by Design (QbD) and Process Analytical Technology (PAT) are guiding principles stimulating the biopharmaceutical industry to improve understanding and control of their manufacturing processes, and to ultimately build product quality into the process

(Box1) (2,6-12). To date, classical chemical pharmaceutical production has achieved significant advances regarding QbD and PAT (13). For unit operations such as mixing, milling, crystallization or drying, process parameters affecting quality attributes are very well defined and PAT analyzers are widely used to control product quality and process performance (14-16). Implementation of QbD and PAT is however slower and more challenging for bioprocesses due to their distinct complexity. Biopharmaceuticals such as monoclonal antibodies or viruses are highly complex products because of their large size and biological nature and they are known for being sensitive to their manufacturing processes (17). Consortia of leading biotech companies and FDA officials gave an important impetus to QbD and PAT for biological processes in the A-Mab and AVAX case studies, paving the road with concrete examples for practical implementation (http://www.casss.org/associations/9165/files/A-Mab_Case_Study_Version_2-1.pdf and http://www.pda.org/HomePageContent/CMC-VWG-A-VAX.aspx).

Several PAT applications have been published on unit operations of downstream biprocessing such as protein refolding or chromatography (18,19). The main challenges facing PAT in this part of bioprocesses are the short execution time of these unit operations and their interdependency, which reduce the possibilities of real-time process control based on on-line monitoring (20). Similarly, efforts towards implementation of PAT to cell cultivations have been developed using a combination of data analysis systems, analytical tools and quality control principles (21-23). However, PAT applications on this unit operation are restrained by its unique complexity. Cells themselves have a sophisticated metabolism and product expression is a result of many elaborate and interacting cellular mechanisms often described as black boxes (24). Additionally, cultivation medium and feed typically have a complex composition with many nutrients, vitamins and co-factors each consumed at different rates (25). Finally, process control systems use cascade controllers for numerous process parameters such as aeration, agitation, pH and temperature. In most processes, cells therefore need to adapt to a continuously changing environment. All these dynamic parameters are monitored to varying extent and resolution, resulting in complex and elaborate datasets.

A multivariate approach is needed for the analysis of these multidimensional datasets from cell cultivations (26,27). Multivariate data analysis (MVDA) can translate the large amount of data recorded during bioprocesses into relevant, understandable information. MVDA is therefore largely recognized as essential for successful PAT and QbD implementations (Figure 1).

We discuss the increasing importance of MVDA for industrial bioprocess data in general and recent studies on MVDA as a PAT tool to analyze data from biopharmaceutical cultivation processes. We focus on current progress and challenges to reaching the highest level of PAT with MVDA-based models and on-line analyzers fully exploring the process design space.

2 MVDA for industrial bioprocess data

Linear statistical techniques are widely used to analyze most industrial datasets. However, when applied to multi-dimensional and multi-collinear data, univariate analyses or even

Box 1. PAT and QbD principles for biopharmaceuticals

Process analytical technology (PAT) is concept introduced by the American Food and Drug Administration (FDA) to encourage the pharmaceutical and biopharmaceutical industries to increase the efficiency and the control of the production of biopharmaceuticals (6). The goal of PAT is to monitor and control all critical process parameters (CPPs) that affect one or more of the critical quality attributes (CQAs) of the final product, preferably on-line, to ensure consistent and adequate product quality during manufacturing.

PAT is a key enabler of Quality by Design (QbD), the modern approach to quality which is also promoted by the FDA (7). Traditionally, the biopharmaceutical industry relies on the quality-by-testing paradigm, where product release is based on post-manufacturing testing. QbD oppositely promotes the use of thorough scientific understanding of the product and its manufacturing process, together with risk-based approaches, to build quality into the process, rather than quality being a variable output (2,8,9). Implementation of QbD and PAT for bioprocesses starts with the definition of the target product profile and results in a production process that routinely runs within a defined design space that has been proven to yield the specified product quality (10,12).

Similar quality principles based on improving analysis, monitoring and control of processes have been implemented for decades in many industries such as automotive, tobacco or petrochemical production (27). There, relations between end product and manufacturing processes are rather straight forward and can be defined very rigorously. Products from the biopharmaceutical industry are of particular complexity as their quality features (CQAs) must be linked to safety and specific therapeutic effects in humans, before they can be related to critical parameters of the manufacturing process (CPPs). In the early 2000's, it became apparent that the lack of understanding (both mechanistic and statistic) of the manufacturing processes of biopharmaceuticals contributed to a long list of industrial and business drawbacks including inability to predict, analyze and understand root causes for process failure, dependency on intense post-manufacturing quality testing for product release, limited flexibility for post-approval process changes, high number of batch rejects and recalls and heavy and long review times by the regulation authorities (11). The launch of the PAT and QbD initiatives by the regulation authorities was essentially driven by this situation of the biopharmaceutical industry regarded as sub-optimal by both the industry and the regulatory authorities.

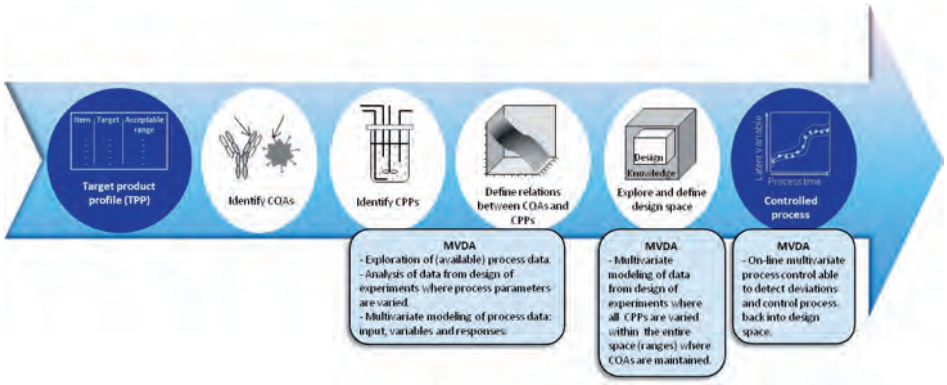

Figure 1. Typical approach for QbD and PAT implementation in bioprocess development and role of MVDA in this process.

bivariate regressions can lead to misinterpretation of data and to misleading conclusions (28). MVDA offers solutions to handle large datasets containing measurement errors and missing data (29). When a deviation is detected with a univariate approach, each process variable has to be analyzed and its contribution to the root cause has to be evaluated individually (Figure 2). This can be challenging especially when deviations result from an interaction of parameters. However, when the relations among process variables are studied upfront by using multivariate batch modeling and structured experimentation, the root cause of a process deviation can be readily identified (Figure 2).

Multivariate analysis methodologies for continuous and batch process monitoring were first proposed in the early 1990's (30). Among the panel of MVDA tools, multi-way principal component analysis (PCA) and multi-way partial least squares (PLS) regression have been successfully applied to visualize the non-linear dynamics of batch processes (4,5,31). Both are projection methods that reduce the original multidimensional dataset into a lower number of uncorrelated variables explaining most of the variance contained in the original data. PCA aims at identifying patterns and main trends within the dataset, whereas PLS analyses the covariance between the process variables and the process output.

MVDA has been widely accepted and is common practice in several industries, with focuses ranging from polymers to semi-conductors, food or the environment (32-34). The aim for MVDA in these industries is to maximize the exploitation of process data in order to deepen understanding of the processes and to improve the level of process control. The interest of the pharmaceutical industry in MVDA techniques largely increased after the launch of the PAT and QbD guidelines, where MVDA was explicitly recommended by the regulators as a key enabling tool for PAT and QbD. Applications were primarily on classical, small molecule pharmaceutical products, for which production steps are rather simple and very well defined. In this field, the use of spectroscopic tools for process monitoring increased the need for MVDA applications (35,36). An example of the importance of MVDA to support QbD for drug development was published by

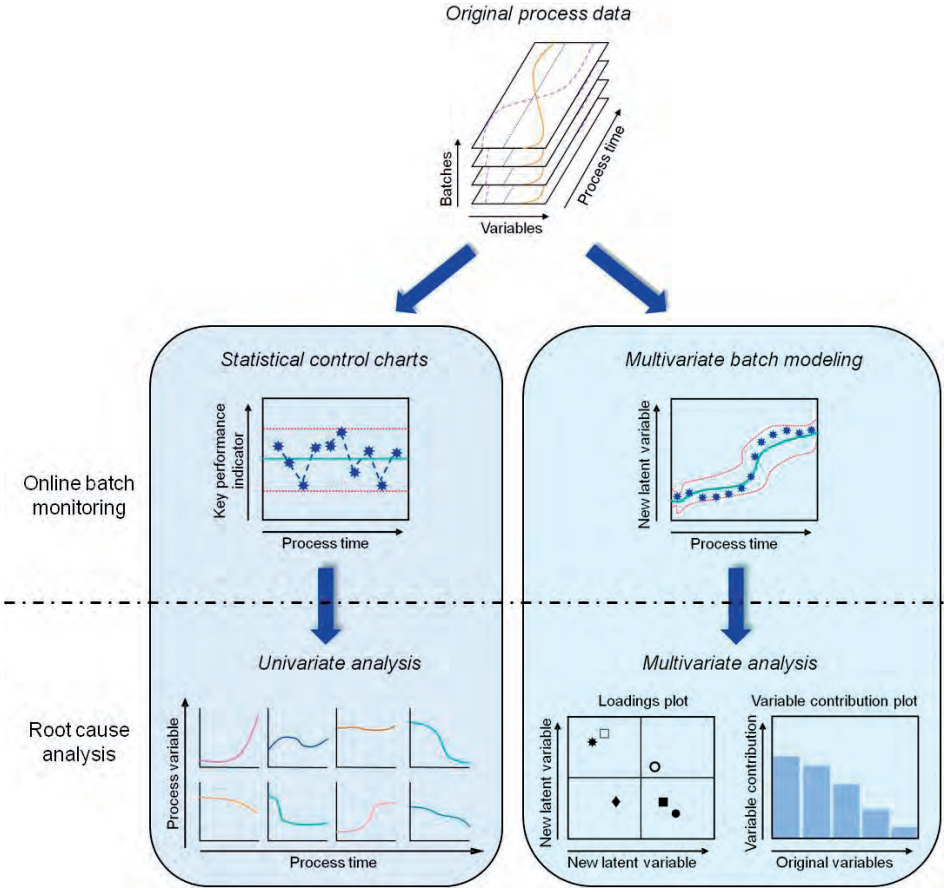

Figure 2. Comparison of classical univariate analysis (left) and advanced multivariate analysis (right) for bioprocess data. A multivariate approach helps to identify variables causing process deviations as the interactions and relations among process variables are known and used to build multivariate batch models. Conversely, with a univariate approach, process variables have to be analyzed individually in order to identify root causes for process deviations.

Huang et al. (37). An integrated multivariate approach was used in a QbD application for a pharmaceutical product, where statistically designed experiments and MVDA were applied during development to establish a process design.

Because the biopharmaceutical industry generates the most complex datasets, it has also greatly beneficiated from MVDA as shown in several reviews on this topic (3,26,27,38). Applications have been published in the past decade both on upstream and downstream unit operations. The use of MVDA on upstream cell cultivation data will be discussed in detail in the next section. In the field of downstream bioprocessing, examples relate mainly to chromatographic purification to characterize the effect of process conditions on process performance and robustness (39,40).

Furthermore, technological advances more frequently used for the innovation and production of biopharmaceuticals contribute greatly to increasing the amount of data to be modeled and interpreted. Systems biology and the omics technologies (genomics, transcriptomics, metabolomics, proteomics) support the development of a holistic methodology to investigate the complex interactions within biological systems, shifting from the current vision where cells are black boxes to systems in which cells define the product (41,42). Additionally, high-throughput, small-scale, automated systems were developed to facilitate parallel testing and screening to meet the demand for both low-cost development and extensive experiments required to understand processes and define design spaces (43). These innovative improvements are obvious QbD enablers as they make increased experiment throughput possible and they enhance understanding of biological processes. The future needs for MVDA in this field will therefore keep increasing together with the size of the datasets, especially for cultivation processes.

3 MVDA as a PAT tool for cell cultivation data

The use of MVDA as a PAT tool for biopharmaceutical cell cultivation steps has greatly increased over the last decade. The aim is often to exploit available datasets mostly originating from pilot or manufacturing plants, in order to find correlations pointing to causes for bioprocess variation and to improve bioprocess understanding. Since the launch of the PAT Initiative, there have been a variety of studies published on MVDA application to datasets originating from both microbial and mammalian cell cultivation processes. These studies range widely in complexity and scope, from exploration of early development runs to the complete mapping of a process design space. We propose to classify these studies into 3 different PAT levels (Table 1), depending on the level of statistical structure in the experimental design, the use of additional advanced multivariate process analyzers, and the resolution of the measurements (i.e. on-line or off-line). The PAT levels are defined as follows:

1. Datasets containing classical process variables for explorative studies;
2. Datasets containing process variables and basic off-line critical quality attributes (CQA) assessments for process development and scale up;

Table 1. Classification of PAT levels for cell cultivation processes, based on experimental designs, process analyzers and data resolution.

PAT level	Stage of process life-cycle	Data recorded
Level 1	Proof of concept, process capacity exploration	Basic process monitoring (pH, temperature, DO, etc.)
Level 2	Process optimization, scale-up, using small experimental sets	Same as level 1 + off-line CQA assessments
Level 3	Process characterization, using full statistical designs	Same as level 2 + on-line PAT tools and biological assessments of CQAs

3. Datasets containing the abovementioned and additional on-line PAT tools capturing relevant information related to the CQAs for predictive process modeling studies and basic design space exploration.

The structure of the datasets of these studies is described through the scale and origin of the batches, the process variables that are monitored, the use of PAT tools and the method of MVDA application (Table 2). It is important to note that to date no studies report the implementation of a design space model at manufacturing scale.

Level 1: MVDA on datasets containing classical bioprocess data

Two studies are limited to using basic readily available process variables in relatively simple applications and were therefore classified at Level 1 (Table 1). Gunther *et al.* (44) applied PCA to a pilot-scale industrial fed-batch cell culture process. A similar study was performed by Nucci and co-workers (45) on a *Bacillus* production of penicillin G acylase, where MVDA was used to generate on-line multivariate control charts. In these examples, each dataset contained information exclusively related to the process. MVDA models therefore only assessed quality of technical execution of the process, enabling detection of process faults or deviations. This is relevant for process monitoring in a manufacturing setting, for example. However, because no biological responses were captured to measure product quality or even process performance, the relations and interactions between process parameters and process performance or product quality remain unknown. Corrective actions in case of a deviation are difficult to implement when understanding of these relations is lacking. These studies can therefore be considered as the first, most basic level of MVDA and PAT.

Level 2: MVDA on datasets containing process variables and basic offline CQA assessments

In 7 studies simple off-line measurements of process performance or product quality (namely cell density, viability, end product concentration or purity) were used in addition to standard on-line data from the bioreactor control system, to create more elaborate multivariate models. With these analyses, the level of PAT increased because process performance was linked to actual product quality attributes (Table 1).

Within this second level of PAT, a first group of 4 studies focused on using raw unprocessed offline data to build explorative MVDA models. Kirdar *et al.* (46) used a set of off-line output variables to model a cell cultivation process at two different scales. A second study from the same group used similar off-line measurements and additional on-line monitoring of process variables to identify process parameters and their interactions that adversely impacted process performance (47). Similarly, Thomassen *et al.* (48) applied MVDA to exploit available data of an inactivated polio vaccine manufacturing process run at commercial scale. More recently, Mercier *et al.* (49) used a set of unstructured runs from early development and demonstrated that MVDA can even provide valuable handholds for further development.

Table 2. Applications of MVDA on cell cultivation processes with analysis of PAT level and of structure of the datasets.

PAT level	Dataset		
	Batches	Process variables	PAT tool
1	23 pilot scale batches	On-line process parameters (e.g. agitation, temperature, gas flows, etc., and controller outputs)	None
1	6 small scale batches	On-line process parameters (agitation, pH, temperature, dissolved oxygen, and gas flows)	None
2	14 small scale batches 11 pilot scale batches	Off-line metabolites and cell growth measurements	None
2	152 small scale batches 14 pilot scale batches 5 commercial scale batches	On-line process parameters (pH, dissolve oxygen and temperature) Off-line gas levels, metabolites and cell growth measurements	None
2	50 batches at two commercial scales	On-line process parameters (e.g. agitation, pH, temperature, gas flows, etc. and controller outputs) Off-line metabolites concentration and cell growth measurements	None
2	17 small scale batches	On-line process parameters (e.g. agitation, pH, temperature, dissolved oxygen, and gas flows) Off-line metabolites and gas concentrations, osmolality and cell growth measurements	None
2	20 sets of seed data at pilot scale 75 sets of seed and main fermentation data at commercial scale	On-line process parameters (e.g. agitation, pH, temperature, gas flows, etc.) Calculation of carbon dioxide evolution rate and oxygen uptake rate	None
2	108[1] and 243[2] batches including inoculation and production data at various scales	On-line process parameters (e.g. agitation, pH, temperature, dissolved oxygen, gas flows, etc. and controller outputs) Off-line metabolites concentration and cell growth measurements	None
3	16 pilot scale batches	On-line process parameters (carbon dioxide and oxygen concentration in the exhaust gases and dissolved oxygen in the broth) Calculation of carbon dioxide evolution rate and oxygen uptake rate	On-line DS measuring capacitance and conductance
3	small scale batches (number not available)	None	On-line multi-wavelength fluorescence

Dataset		
Process responses	MVDA application and outcome	Refs
None	Fault detection and diagnosis in industrial fed-batch cell culture. Identification of deviating batches and of the root cause for each deviation; findings matched observations from process engineers.	[44]
None	On-line detection of abnormal bacterial cultivation behavior	[45]
Off-line viable cell density, viability, osmolality and product purity	Application of MVDA to support key cell culture process activities. Gain of process information related to scale-up, process comparability, process characterization and fault diagnosis.	[46]
Off-line product yield	Identification of process parameters and their interactions adversely impacting process performance. Design of experimental work to confirm and correct the root cause.	[47]
Off-line cell concentration and product yield	Exploitation of historical data to gain understanding on the process. Identification of known outliers using MVDA.	[48]
Off-line culture viability and growth rate	Increased understanding of cell cultivation process. Identification of causes for batch deviations and of process differences among batches scale.	[49]
Off-line mycelial volume and pH for seed Off-line product yield for main fermentation	Assessment of seed quality and influence on productivity of an industrial antibiotic production process. Relation of final productivity at both pilot and production scales to seed fermentation quality.	[50]
Off-line product concentration	Prediction of process production performance days prior to harvest using cell culture stage-specific models.[1] Identification of hidden relations between process outcome and process parameters.[2]	[51][1] [52][2]
Off-line product concentration	Detection of dissimilarities among batches, prediction of final product concentration and identification of variables influencing process productivity. Improvement of knowledge on industrial fermentation process.	[56]
Off-line biomass and glucose concentration and carbon dioxide production rate	Prediction of process responses from fluorescence data in batch cultivations. Decrease in prediction accuracy when models calibrated with batch data were applied to fed-batch data, demonstrating that proper calibration of PLS models is critical for accurate predictions.	[57]

Table 2. *Continued*

PAT level	Dataset		
	Batches	Process variables	PAT tool
3	4 large scale batches	None	On-line Raman spectroscopy
3	5 small scale batches	Off-line metabolites and cell growth measurements	On-line NIRs
3		On-line process parameters (e.g. agitation, pH, temperature, gas flows, etc.) Off-line metabolites concentration (glucose and acetate)	On-line NIRs In-line electronic noise mapping
3	10 commercial scale batches	Off-line glucose concentration and osmolality	On-line NIRs
3	12 small scale batches, designed using DoE methodology	On-line process parameters (pH, dissolve oxygen and temperature) Off-line metabolites and bacteria density measurements	On-line NIRs Off-line microarray for determination of RNA expression profile

Additional relevant information can be extracted from standard on-line monitoring tools available for cell culture processes. Raw datasets can be complemented with new information that better reflect the biological features of the processes. Cunha *et al.* (50) added carbon dioxide evolution rate and oxygen uptake rate to their dataset initially consisting of main on-line process variables and a basic assessment of seed quality for a bacterial fermentation. These new computed variables encompassed the biological mechanisms taking place during cell growth better than the raw measurements in the multivariate models and this was achieved without additional analytical tools.

Classical measurements can also be used to yield predictive models for process performance. These applications go beyond process exploration and enable predictive modeling from raw material quality or process execution data. Examples are shown in two studies that used MVDA on manufacturing cell culture data from Genentech, Inc. (51,52). The authors did not use the typical PCA and PLS multivariate tools but rather support vector machines, which are supervised learning models for pattern classification and regression analysis (53). Charaniya *et al.* (52) first used several algorithms to integrate the large set of data and then showed that run performance can be reliably predicted several days before culture harvest. Le *et al.* (51) built models predicting the final concentrations of antibody and of lactate using data from the early phase of the production process.

In all the studies classified in the second PAT level, MVDA is useful for increasing understanding of cell cultivation unit operations *a posteriori*, meaning after the batches are terminated. However, when only off-line, classical and simple (univariate)

Dataset Process responses	MVDA application and outcome	Refs
Off-line viable and total cell densities, and main metabolites concentrations (glucose, glutamine, glutamate, lactate and ammonium)	Demonstration that prediction models for cell density and main metabolites concentrations based on Raman spectra can be used for on-line process monitoring.	[58]
Off-line viable cell density	Modeling of near-infrared spectroscopy data for monitoring of an antibody production process. Identification of outliers and detection of a contamination.	[59]
Off-line biomass and product concentration	Prediction of key process responses during bacterial cultivation process. Detection of abnormal cultivations and of contamination. Use of on-line biomass prediction to control cultivation feed.	[60]
Off-line cell density, culture viability, packed cell volume and product concentration	Detection of abnormal cell cultivation batches. Monitoring and accurate in-line prediction of seven cell culture parameters using control charts.	[63]
Off-line product quality score	Exploration and modeling of the design space of a bacterial vaccine cultivation process. Fingerprinting of process based on on-line measurements, modeling of the design space within the ranges studied.	[64]

measurements of process responses are available, the totality of the process parameters impacting process performance or product quality cannot always be fully captured and predictions on product quality cannot be reached.

Level 3: MVDA on datasets containing data from advanced and on-line process monitoring tools

Implementation of on-line or at-line measurements of CPPs or directly of CQAs enables data analysis and process modeling in real-time. This facilitates, for example, early fault detection at a time where corrective actions can be taken, maximizing both process success rate and probability of obtaining the desired end product quality (54). Five examples of MVDA applications illustrate this stage where complementary and advanced analytical data related to the CQAs were collected (Table 2), which makes them a distinctly more advanced PAT level.

Several on-line PAT tools capture information related to cellular features of cultivations, or cell state (23,55). Ferreira *et al.* (56) modeled on-line capacitance and conductance measured with dielectric spectroscopy (DS) on an industrial fermentation process, in combination with on-line dissolved oxygen and composition of the outlet gas stream data, to predict final product concentration and to identify faults influencing process productivity. Jain *et al.* (57) developed calibration models with on-line multi-wavelength fluorescence spectroscopy data from a batch *Escherichia coli* cultivation process to predict key process responses.

Efforts are also directed towards using broad spectroscopic techniques to fingerprint the complex matrix of cell cultures. These techniques capture all information related to the many interacting process parameters, at a high frequency. MVDA is ideal to translate the massive amount of data generated into relevant process information, which is the main challenge associated with these analyzers. Abu-Absi *et al.* (58) used Raman spectroscopy for simultaneous and accurate prediction of main cultivation parameters in large scale bioreactors. Henriques *et al.* (59) modeled on-line Near-infrared spectroscopy (NIRs) data for monitoring mammalian cell cultures producing a monoclonal antibody to identify batch deviation and to predict cell density and main cultivation metabolites. In another study, accurate calibration models linked data from on-line NIRs to very subtle process changes to generate robust process control (60). A major objective of PAT was achieved in this example, as process understanding was used to build accurate models from advanced on-line data, not only to monitor the process but most importantly to control it in real-time.

NIRs techniques outnumber other advanced PAT tools for cell cultivation, as four of the seven papers in this third PAT level used NIRs (Table 2). NIR and Raman are both vibrational spectroscopy techniques yielding very large datasets as each spectrum consists of more than 2000 points and measurement frequency can be as high as every few seconds (61,62). Raman enables more accurate identification of chemical compounds in small molecules applications and NIRs prevails for bioprocess fingerprinting. Of course these complex tools are relevant for PAT only when information related to product quality (or process performance) have been proven to link to or be contained in the signal from these analyzers. This needs to be established experimentally prior to implementing these tools.

All applications examined so far were conducted in laboratory or pilot plant settings. A manufacturing scale application of MVDA and PAT tools was published by a group at Hoffmann-La Roche (63), in which NIRs measurements were acquired in a Good Manufacturing Practices (GMP) compliant set up. Multidimensional control charts linked off-line information on cell growth, culture viability and protein concentration to the on-line NIRs signal. This study is a proof of concept of PAT and QbD application to existing processes. In such an approach, routine manufacturing data consisting of both the on-line control system data and results from the off-line test panel (e.g. release tests) can be used together with data from failed or rejected runs to map the process design space and to build on-line process control. However, when "retrofitting" a design space model to an existing process, the exact borders of the design space cannot be accurately defined, as the process is typically run at set-point.

The goal and highest level of PAT and QbD requires strong scientific and mechanistic understanding of the process gained during process development, before manufacturing (Figure 1). Such a comprehensive and structured approach was found only in one study. Streefland *et al.* (64) generated a hierarchical process fingerprint model from DNA microarray data, used as a biological measurement of a bacterial suspension's quality, and from a combination of on-line NIRs data and on-line measurements of classical process variables. A design of experiments (DoE) was used to explore ranges of process

parameters and a single model was developed, describing the process in the domain of the experimental design. Such models can be used to assess if the process is executed within the investigated design space, to push a deviating process back into the design space, and to make real-time release decisions when the investigated design space is validated to ensure desired product quality. The scope of this study was extensive but it lacked validation of the design space model and scalability testing scalability in a manufacturing setting. Nevertheless, the approach of first developing highly structured datasets and then hierarchical models to define a process design space as well as the strong emphasize given to the assessment of product quality and its link to process control clearly tends towards the highest level of PAT and QbD, where quality of the end product is built in the process design.

4 Concluding remarks and future perspectives

Addressing PAT and QbD for cell cultures is challenging, partly due to the many varying parameters and thus the complex analyzers necessary to capture all bioprocess variance and also to the resulting complex datasets. MVDA is therefore essential for PAT and QbD implementation on these bioprocesses.

We have highlighted the strong link between the nature and features of the bioprocess datasets, the necessity for MVDA approaches, and the level of the PAT implementation. The most powerful applications of MVDA are typically obtained when applied to highly structured datasets that comprise most of the process design space and include both data from on-line fingerprinting tools as well and data on product quality and process performance. MVDA models based on these datasets will allow highly accurate control of product quality in real-time, reducing failure and rework and thus reducing cost while increasing safety for the patient. However, this highest level of PAT and QbD has not been reached yet, probably because these studies typically take place in later process development and are therefore not disclosed because of company strategy. A QbD-based design space submitted by Genentech/Roche has been approved by the FDA at the end of 2013, but no PAT elements were included. To our knowledge, although a lot of applications are published during process development, there has never been a filing of a formal PAT/QbD based cell culture process to regulatory agencies.

Overall, given all the limitations underlined in this paper, we expect that full PAT and QbD implementations for cell cultivation will not be achieved in a near future. Nonetheless there is a clearly growing interest in exploring and modeling data to increase process understanding and control. Progress has been made in early stages of process development, where multivariate analyses are becoming common. Structured and statistical approaches (DoE for instance) are also being more frequently used during process characterization or late stage of process development.

Considering MVDA applications for cell culture only, this technique is not a "one-size fits all" solution, but rather an enabling tool that generates unique models to address defined questions or issues on a specific process. With a continuous rise in scientific understanding

of bioprocesses and more researchers learning the power of MVDA for bioprocess data, we expect that more advanced MVDA examples will be published in the future.

The next step to reach full PAT and QbD implementation for cell cultures will be to build complex hierarchical MVDA models for establishing process control strategies from multivariate PAT tools. Inputs and outputs of all process steps should be considered in these models, from seed expansion to production cultivation, possibly even including downstream processing. Efforts will need to focus on validation and confirmation of these models. In the end, release decisions of intermediate and final products should be based on these models, immediately after completion of a process step. The question remains how fast this rather high-tech approach to manufacturing will be implemented in an industry that traditionally sticks to technology that has been proven over decades.

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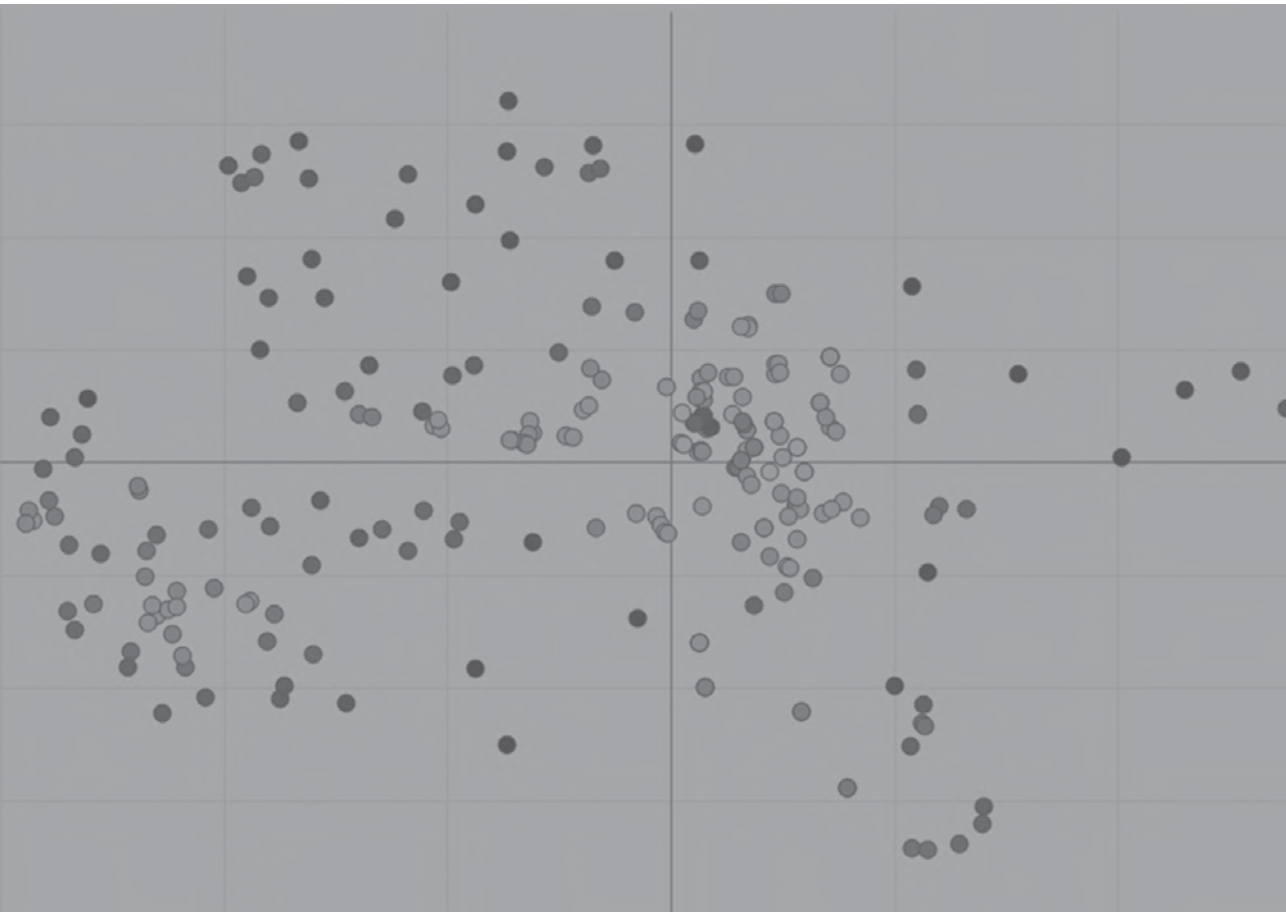
Chapter 3

Multivariate data analysis as a PAT tool for early bioprocess development data



Abstract

Early development datasets are typically unstructured, incomplete and truncated, yet they are readily available and contain relevant process information which is not extracted using classical data analysis techniques. In this paper, we illustrate the power of multivariate data analysis (MVDA) as a Process Analytical Technology tool to analyze early development data of a PER.C6® cell cultivation process.

MVDA increased our understanding of the process studied. Principal component analysis enabled a thorough exploration of the dataset, identifying causes for batch deviations and revealing sensitivity of the process to scale. These findings were previously undetected using traditional univariate analysis. The lack of structure and gaps in the early development datasets made it impossible to fit them to more advanced partial least square regression models. This paper clearly shows that MVDA should be routinely used to analyze early development data to reveal relevant information for later development and scale-up. The value of these early development runs can be greatly enhanced if the experiments are well-structured and accompanied with full process analytics. This up-front investment will result in shorter and more efficient process development paths, resulting in lower overall development costs for new biopharmaceutical products.

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List of abbreviations

ATF	Alternating tangential flow
CPP	Critical process parameter
CQA	Critical process attribute
DO	Dissolved oxygen
DoE	Design of experiment
FDA	Food and drug administration
MFCS	Multi-fermentation control system
MVDA	Multivariate data analysis
PAT	Process analytical technology
PC	Principal component
PCA	Principal component analysis
PDT	Population doubling time
PID	Proportional-integral-derivative
PLS	Partial least square
QbD	Quality by design

1 Introduction

Upstream processes for production of biopharmaceuticals, such as mammalian cell cultivation, are among the most complex industrial processes because of the very sensitive relation between the manufacturing process and the complex large biomolecules produced. The more complex the process, the more elaborate the dataset. Full analysis of these highly elaborate datasets is time consuming and difficult. However, analysis of bioprocess data is key for building the solid understanding of the production process required for commercialization of a biopharmaceutical product.

The Process Analytical Technology (PAT) initiative, launched by the United States Food and Drug Administration (FDA) in 2004, encourages biopharmaceutical companies to develop *"a system for designing, analyzing and controlling manufacturing through timely measurements of critical quality and performance attributes ... with the goal of ensuring final product quality"* (1). The aim of PAT is to monitor and control all critical process parameters (CPPs) that affect process performance or critical quality attributes (CQAs) of the final product, to ensure consistent and adequate product quality during manufacturing (2). Ultimately, PAT implementation leads to obtaining new information, which is used to make (real-time) process control decisions. To reach this aim, PAT relies on implementation of adequate data analysis systems, as well as on development of new analytical tools and on application of process quality control principles (3). Efforts to implement PAT on cell cultivation processes range from characterization and screening of complex raw materials (4,5), and use of analytical tools for online monitoring of bioreactor cultivations (6,7), to application of multivariate analysis of data for online batch performance modeling and quality prediction (8). Quality-by-design (QbD) is a quality concept that makes use of scientific understanding of the manufacturing process to build quality in, instead of testing products' quality post-manufacturing (2,9-11). Scientific understanding of a bioprocess typically includes two aspects. Firstly, statistical analyses should be applied in order to identify the relations and interactions among process parameters.

Secondly, mechanistic principles underlying the biological process should be studied, in order to explain the mechanisms and principles behind these interactions, and to support the findings of the statistical analyses. This combination can be used to build sound process control systems with the required knowledge of the process, to ultimately ensure product quality and patients' safety. In this context, PAT is therefore a tool enabling the implementation of QbD.

The initial steps for the implementation of PAT on an upstream bioprocess focus on gaining understanding of the process and on implementing appropriate analytical tools capturing relevant information on the end product and on the process dynamics, typically in the early phases of a process development (Figure 1). Statistical analyses on the complete dataset containing the additional information should lead to the identification of correlations between process parameters and product quality. The ultimate goal of QbD using PAT is to define the process design space, which is the *"combination and interaction of input variables and process parameters that have been demonstrated to*

provide assurance of quality" of the end product (11). The design space is typically explored with statistical experimental designs, during process characterization (late phase of a process development) (Figure 2).

Multivariate data analysis (MVDA) plays a key role in the implementation of PAT and QbD for biopharmaceuticals, as it enables the transformation of the enormous amount of data generated by process analyzers into relevant and crucial process information (12). Large amounts of data are generated during the (early) development phases of bioprocesses, in which parameters are intendedly or unintendedly varied in experiments for screening or optimizing process settings (Figure 2). In these early development datasets, statistical experimental design techniques like design of experiments are often not applied, and the data may be incomplete or not fully documented. MVDA on early development batches where unforeseen variations occur frequently would therefore likely reveal relevant information about the interactions between process input and output variables.

In this article, we present a detailed case study of MVDA applied to early development data of a biopharmaceutical cell cultivation process, as a preliminary step in the implementation of PAT on this process. The initial purpose of these runs was to develop a working process suitable to find failure limits and that can be a basis for further development. These runs are therefore feasibility studies and the preliminary steps in process development, where small experimental designs were performed testing side-

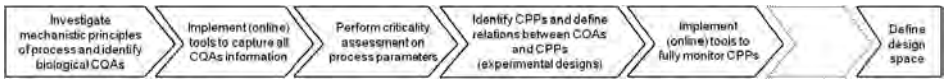

Figure 1. Proposed workflow to address initial steps of PAT and QbD: building process understanding and implementing analytical tools.

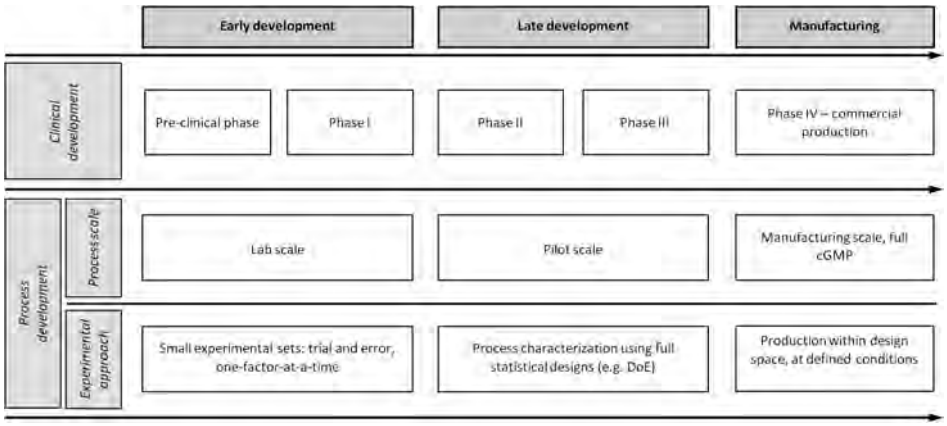

Figure 2. Biopharmaceutical development life cycle: phases of clinical and process development and associated process scale and experimental approaches.

by-side different conditions. The objective was to evaluate the strengths and limitations of MVDA for these unstructured, incomplete and truncated datasets. We successfully showed that MVDA of early development data enables the identification of gaps in process knowledge and of experimental shortcomings, which are crucial information to further develop a robust and well controlled bioprocess.

2 Background

2.1 Features of bioprocess datasets

The many variables routinely recorded for biopharmaceutical cell cultivation processes can be divided into three categories (Figure 3): input variables (e.g. setpoints, raw materials), process variables (e.g. pH or DO trends) and output variables (e.g. sample analyses, product quality).

These bioprocess datasets are difficult to analyze for several reasons. Firstly, variation in input variables, such as quality of raw materials or viability of the preculture cells, causes poorly understood effects on process responses like cell growth rate or metabolic profiles (4,13,14). Secondly, most of the process variables recorded are dynamic. This time-related-variation can be very different for each variable, making the complete analysis of these process variables highly complex. Thirdly, the frequency of measurement and of data recording (online versus offline) can be considerably different from a variable to another. For instance, variables such as temperature or pH are typically recorded every second, while process responses such as cell density, culture viability or end product concentration are only measured once or twice per day, which results in a dataset with a complex structure. Fourthly, interactions and correlations between process variables (e.g. cell and main metabolite concentrations, gas demand, etc.) are inherent in such datasets because of the biological nature of these processes. Fifthly, quantity and quality of the end product of a bioprocess are the cumulative result of complex biological reactions taking place for up to several weeks, and can therefore vary substantially from batch to batch.

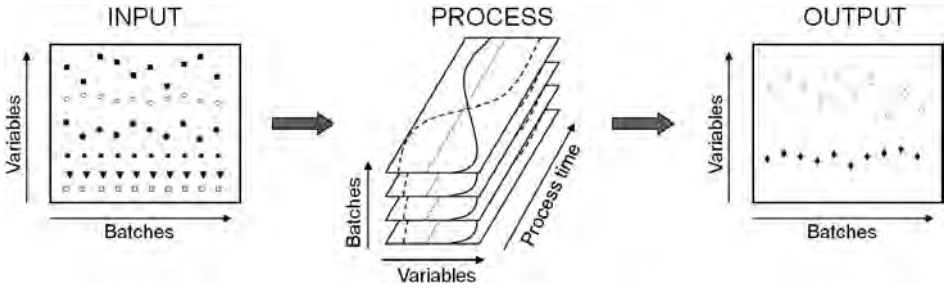

Figure 3. Schematic representation of bioprocess datasets. Process input (e.g. raw material quality) typically contains variation in quality whereas processes are run at pre-determined, fixed and controlled set-points, which can lead to substantial variation in process output (quantity or quality).

2.2 Multivariate analysis for bioprocess data

These large bioprocess datasets therefore require more advanced statistics to evaluate the quality of the process than the traditional linear principles based statistical process control charts (15). Classical linear techniques using univariate analyses or bivariate regressions applied to such complex datasets may result in misleading interpretations and conclusions (16). Indeed, with the use of univariate statistical control charts, several correlating parameters can be flagged as deviating in case of an abnormal batch behavior. In such a case, it is often difficult if not impossible to isolate or clearly identify the root cause of the deviation, since parameter dynamics and interactions are not considered in the analysis.

Bioprocess data is therefore best analyzed using multi-variate techniques, as they address the multidimensionality nature of bioprocess datasets (12). Multi-way principal component analysis (PCA) and multi-way partial least squares (PLS) regression are two common MVDA techniques that have been proven useful in the biopharmaceutical field as they account for complex behavior and correlations in dynamic batch processes (17-19). PCA is typically used in a first step to study the structure of a dataset. After projection along different orthogonal axes, underlying structures (trends or clusters) in the dataset can be identified. PCA provides handholds for further investigations to identify previously unknown causes for structures in the dataset. In a second step, PLS can be used to capture and describe in a regression model the relations and interactions among process variables. On one hand, PLS enables the identification and understanding of the relations between process outcome and process variables. On the other hand, after proper calibration and validation, PLS models can be used to predict the evolution of process variables and process outcome in newly generated datasets.

The use of MVDA as a PAT tool for biopharmaceutical processes has gained considerable interest over the last decade (12,20-22). The examples mostly use datasets originating from (large scale) pilot or manufacturing runs, which are typically complete yet poorly structured, meaning that the runs are intended to be identical and experiments are not designed to identify interactions among variables or explore process capabilities. Already on these datasets where batches are mostly similar, MVDA often reveals correlations that remained undiscovered using traditional linear statistics.

3 Materials and methods

3.1 Bioreactor cell cultivation process

The dataset comprised 17 2L and 10 10L PER.C6® cell cultivation runs operated in perfusion. Human immortalized retinal PER.C6® cells (Crucell, Leiden, The Netherlands) were thawed and cultivated in Crucell's proprietary and chemically defined PERMEXCIS® medium (Crucell, Leiden, The Netherlands), supplemented with 4mM L-Glutamine (Invitrogen, Paisley, UK). Cultures were expanded in roller bottles (Corning Costar Inc., Amsterdam, the Netherlands) or BIOSTAT® Cultibags RM (Sartorius Stedim Biotech, Aubagne, France) every 3-4 days before inoculation of either 2L or 10L autoclavable

glass bioreactors (B. Braun Biotech, Melsungen, Germany). pH, DO, temperature and stirrer speed were controlled at constant levels. Online process variables were recorded using a multi-fermentation control system (MFCS/win, Sartorius, Goettingen, Germany).

An alternating tangential flow (ATF) system (Refine, Pine Brook, NJ) with a 0.2 μm hollow fiber module was used for cell density driven perfusion with PERMEXCIS® based medium (Crucell, Leiden, The Netherlands). This perfusion process was designed to expand the PER.C6® culture to extremely high densities before viral infection. The cells were retained in the bioreactor vessel and only filtrate of waste medium was continuously extracted while fresh feed was supplied to keep a constant working volume. Samples from bioreactors were taken daily for the measurements described in table 1.

Table 1. Parameters measured on bioreactor samples and corresponding analyzers.

	Analyzer	
Parameter measured	**Type**	**Supplier**
Cell concentration Viability	CASY	Roche Innovatis, Almere, The Netherlands
pH	Bench pH meter	WTW, Weilhem, Germany
pCO_2 pO_2	ABL5	Radiometer, Zoetermeer, the Netherlands
Ammonium Glucose Glutamate Glutamine Lactate Potassium Sodium	Bioprofile 400	NOVA Biomedical, Rödermark, Germany
Phosphate	Vitros DT60 II	Johnson & Johnson, Rochester, NY
Osmolality	Fiske 210 Micro-sample	Advanced Instruments Inc., Norwood, MA

3.2 Multivariate data analysis

3.2.1 Background on the techniques used

The two MVDA techniques used to analyze the available dataset were multi-way PCA and multiway PLS. These techniques first unfold the three-way datasets (batches, process time interval and process variables, see Figure 4). PCA summarizes information of the original multidimensional dataset into a smaller set of uncorrelated variables called principal components (PC), by the mean of projections (23). The resulting models fit the data as closely as possible in the least squares sense, meaning that variance of the original data is maximized when projected onto the new coordinates, and the squared residual variance is minimized. PLS is a linear regression method based on the calculation of new variables as well. PLS models analyze the covariance between a matrix of process variables, and a matrix of process responses. The aim is to relate the variable matrix to the response matrix and to predict the latter (24).

3.2.2 Software

Raw process data were first assembled in Excel (Microsoft, Redmont, WA), and then SIMCA-P+ 12 version 12.0.1 (Umetrics AB, Kinnelon, NJ) was used for MVDA.

3.2.3 Data collection

Datasets from 2L and 10L runs were assembled similarly. For each set, offline variables (e.g cell count, nutrients concentrations, osmolality, etc.) were recorded in a digital format. Online process variables recorded by MFCS were extracted from this data base and averaged every 30 minutes to reduce the size of the dataset. This averaging timescale was chosen as the biological reactions occurring during the process are rather slow (the process ran for at least 7 days), and the timescale of most of the process perturbations appeared to be higher than this time interval. Relevant information on incoming material (e.g. performance of seed, medium and additives lot numbers, etc.) and starting conditions (e.g. process set-points, equipments, operators, etc.) were collected and selected. Online variables, offline variables and initial conditions for all runs were finally assembled in an Excel spreadsheet and imported into SimcaP+ 12.

3.2.4 Data pre-processing and variables selection

Gas flow variables displayed spiky profiles since they were supplied on demand throughout the process as a response to the control loops feedback for dissolved oxygen and pH. Such noisy profiles caused disturbance when generating models. Online air, O_2 and CO_2 flow data were therefore summed at each 30 minutes interval (i.e. smoothed) and cumulative gas production or consumption was considered. Additionally, every offline and gas related variable was normalized to bioreactor volume when necessary, thereby removing obvious dependency on bioreactor scale.

Based on *a priori* process knowledge and iterative exploration, variables best representing process behavior were included in the models. The original and selected data sets of process variables are showed in table 2, where the unit for each variable and the rational for exclusion from multivariate models are detailed. During the variable selection process, the aim was to exclude variables with low impact and low reliability, and to avoid unnecessary redundancy caused by strong correlations among variables or by the fact that some variables are generated by direct calculations from measurements or control outputs.

For each variable and each observation, up to 50% of missing values were tolerated and accounted for by SimcaP+, thanks to the projection methods used for the modeling. Below this default tolerance limit, variables or observations were excluded for the fit and prediction analyses.

The output variables, or process responses, selected to build the models were population doubling time (PDT) between inoculation and harvest and viability of the culture at the end of the runs. The ranges of the output variables contained in the dataset are given in table 3.

Batches included in this study were of different lengths. Two approaches were explored to overcome time alignment challenge (Figure 4). In one case, only batches

Table 2. Original and selected sets of process variables including units and rationale for exclusion from multivariate models.

Type of variables	Variable (underlined variables were included in multivariate models)	Unit	Rationale for exclusion from multivariate models
Offline	Total cell density	Cell/mL	Obtained from direct calculation using viable cell density and viability
	Viable cell density	Cell/mL	
	Viability	%	Process response
	Cell diameter	μm	
	Population doubling time	hrs	Process response
	Feed rate	mL/day	Obtained from direct calculation using specific perfusion rate set-point and viable cell density
	Total culture volume	L	Low impact, constant throughout process
	Glutamine concentration	mM	Data not available for 56% of the batches
	Glutamate concentration	mM	Data not available for 56% of the batches
	Glucose concentration	mM	
	Potassium concentration	mM	Data not available for 56% of the batches
	Lactate concentration	mM	
	Sodium concentration	mM	Data not available for 56% of the batches
	Ammonium concentration	mM	Data not available for 56% of the batches
	Phosphate concentration	mM	
	pH	-	
	Osmolality	mOsm/kg	
	pCO_2	mmHg	Data not available for 56% of the batches
	pO_2	mmHg	Data not available for 56% of the batches
Online	Dissolved oxygen	%	
	pH	-	
	Temperature	ºC	
	Actual air flow	L/min	Spiky profile causing disturbances in the models
	Actual CO_2 flow	L/min	Spiky profile causing disturbances in the models
	Actual O_2 flow	L/min	Spiky profile causing disturbances in the models
	Normalized cumulative air demand *	mL/L	Low impact, controlled at set-point and did not vary from batch to batch
	Normalized cumulative CO_2 supply *	mL/L	
	Normalized cumulative O_2 supply *	mL/L	
	Sodium bicarbonate pump totalizer	mL	Low reliability due to operating errors
	Permeate pump totalizer	mL	Obtained from direct calculation using specific perfusion rate set-point and viable cell density
	Feed pump totalizer	mL	Obtained from direct calculation using specific perfusion rate set-point and viable cell density
	Bioreactor weight	Kg	Low impact, constant throughout process

Table 3. Range of the output variables contained in the dataset.

Output variable	Population doubling time (h)	Viability (%)
Range	29.1 – 92.6	70.2 – 86.3

longer than 11 days were considered and the shorter batches were excluded. In the second case, all batches were truncated at 7 days of culture, which is the length of the shortest batch.

3.2.5 Multivariate analysis and modeling techniques

In order to keep as much information as possible for the explorative analysis of the large dataset available, online and offline data were analyzed separately. Indeed, data reduction needs to be applied when these data collected at different frequencies are combined, which may lead to a loss of information.

In the present study, the steps taken to analyze the data followed the complete and detailed descriptions of Kirdar et al. (25,26) (Figure 5), and are therefore only briefly explained here.

First, the evolution of batches' behavior over their duration was monitored using *observation level modeling*. At this stage, PLS was used to relate the process data to a response variable representing local batch time or run maturity, in order to explore run progression over time. Obvious outliers that deviated as a result from *a priori* known root-causes (e.g. technical or operating deviations) were identified and excluded from the analysis.

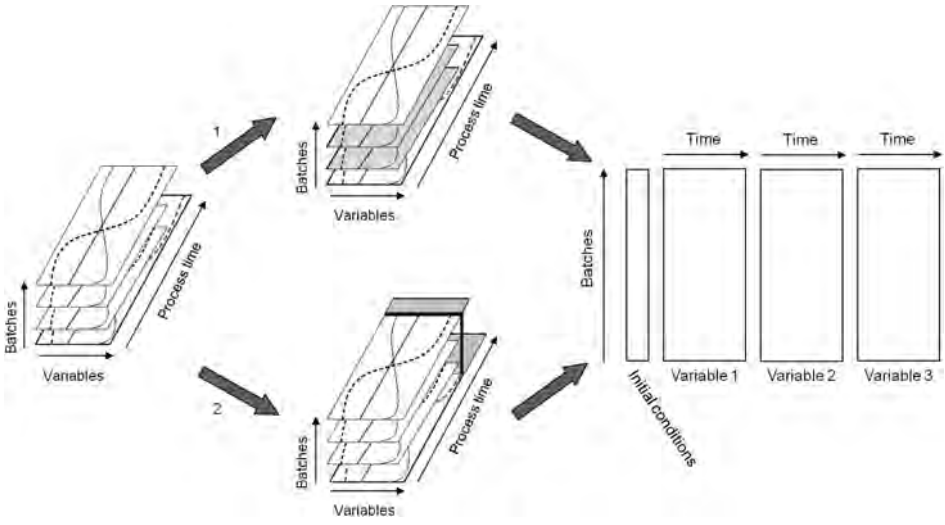

Figure 4. Schematic representation of data multiway unfolding. In option 1, only batches longer than 11 days were considered, the shortest batches are excluded. In option 2, all batches are truncated at 7 days of culture. For both options, data excluded are shaded.

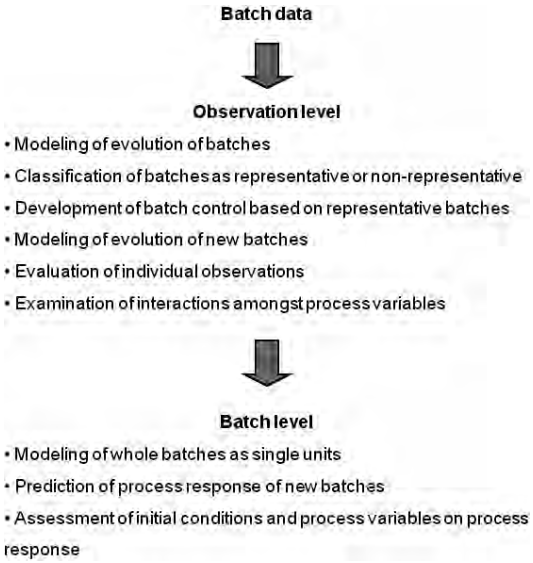

Figure 5. Overview of batch statistical process control via batch modeling. Figure adapted from Kirdar et al. 2008.

Next, *batch level modeling* was developed, where batches were modeled as single units. PCA models were first developed to analyze overall batch behavior. Secondly, PLS models were developed to understand how process responses were influenced by process data or initial conditions. For both PCA and PLS, score plots were used to study the structure of the dataset: in short, batches plotted close to each other behaved similarly whereas batches plotted far away from each other behaved differently. Diagnosis using cluster or pattern identification was done. Outliers still present in the dataset were identified on the score plots if they fell outside the 95% Hotelling's T2 ellipse (see Figure 6), or if they were identified as outliers in the corresponding residuals model. Loadings were used to identify the variables having the greatest contribution to the variance of the data, therefore the greatest contribution to the models.

4 Results and discussion

4.1 PCA and evaluation of the structure of the dataset

The first step in applying MVDA on bioprocess data is to investigate the structure of the dataset using PCA. In this way, clusters of batches, process deviations or disturbances, and process trends can be identified. This analysis is necessary to exclude strongly deviating batches or to compensate for trends that would hamper further modeling (e.g. time alignment, variables scaling, etc.).

Table 4 details the variance in the dataset captured by each principal component (PC), as well as the cumulative variance captured by the PCA models obtained from

Table 4. Variance captured in each principal component (PC) and cumulative variance captured by the PCA models for offline and online variables.

Type of variables used in model	PC	Variance captured (%)	
		in each PC	cumulative
Offline	1	21.5	21.5
	2	15.3	36.8
	3	11.7	48.6
	4	10.5	59.1
	5	7.7	66.8
	6	6.6	73.4
Online	1	51.1	51.1
	2	15.8	66.8
	3	14.4	81.2
	4	5.2	86.4
	5	3.1	89.5

offline and online variables. Note that when models contain several PCs, most of the information generally lies in the projections on the first two or three PCs, but additional information may still remain in the other PCs. The choice of PCs represented in figure 6 and figure 7 was made based on a systematic analysis of the score plots, to ensure that the selected plots display most of the relevant information.

Figure 6 and figure 7 show PC1 and PC2 for the offline variables model, and PC1, PC2 and PC3 for the online variables model respectively. In these models, all batches were truncated after 7 days of cultivation (option 2 of Figure 4). Models generated from the analysis of the sub-set of batches lasting at least 11 days (option 1 of Figure 4) are not shown since no additional relevant information was gained compared to the data of the first 7 days only. This shows that the structured variance that is captured in the PCA models is present in the data collected from the early phase of the run.

Clustering based on process scale was strong for the model of offline data (Figure 6) and was even more obvious for the models of online data (Figure 7), although batch 18, which is a 10L batch, is grouped with the 2L runs in the offline score plot (cause unknown). Process scale appears to cause a clear operational difference in process variables monitored both online and offline, despite the normalization to bioreactor working volume (Table 2). Analysis of raw online data suggested that O_2 and CO_2 demand throughout the process was greater for 2L cultures than for 10L cultures ($P \leq 0.01$). The cell density and associated population doubling time profiles were comparable at 2L and 10L, therefore this observation is not imputable to a difference in performance of the batches at these two scales. After closely reviewing the scaling of the process, the most probable explanation was found in the aeration strategy which was not linearly scaled between the two bioreactor volumes: the constant air flow supplied per culture volume varied between the two systems, which

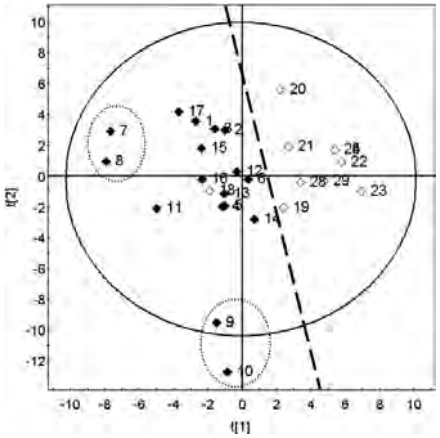

Figure 6. PCA score scatter plot and 95% Hotelling's T2 ellipse from offline variables of 2L (closed diamonds) and 10L (open diamonds) batches truncated after 7 days of culture. Circled batches are further analyzed in the text. 6 principal components explained 66% of the variation contained in the dataset. The dashed line separates the clusters based on process scale.

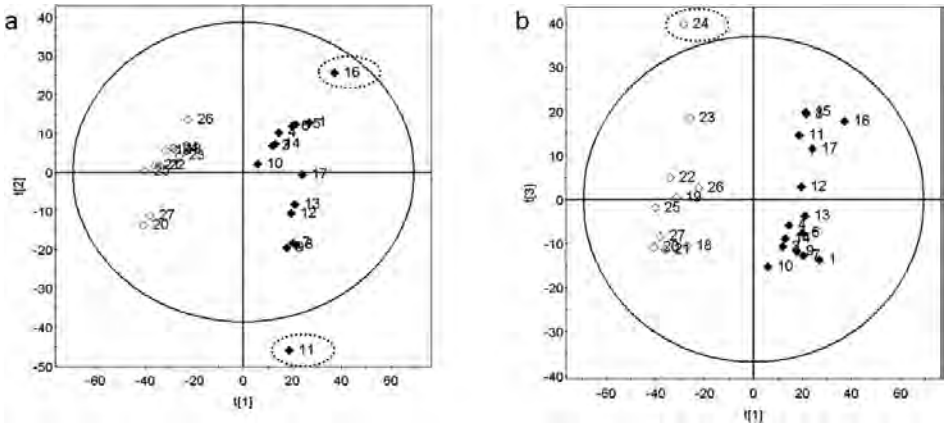

Figure 7. PCA score scatter plots and 95% Hotelling's T2 ellipse of PC1 vs PC2 (a) and PC1 vs PC3 (b) from online variables of 2L (closed diamonds) and 10L (open diamonds) batches truncated after 7 days of culture. Circled batches are further analyzed in the text. 5 principal components explained 89% of the variation contained in the dataset. Note that batch 26 is identified as an outlier due to high residuals.

was reflected in the cumulative gas demand throughout the process. In addition, detailed investigation of raw offline data revealed cell diameter on average 1.1 μm smaller for the 10L cultivations than the 2L ($P \leq 0.01$). Further analyses of the operating parameters of the ATF showed that the cross flows inside the fibers of the ATF module were different in the two bioreactor scales. The shear rates in the two systems were therefore different, which can have caused the observed difference in cell diameter.

Overall, PCA enabled to unveil technical process variations between 2L and 10L scales, which were previously not obvious, and for which the effect on process behavior were not known.

Furthermore, batch diagnosis was performed from the PCA modeling. The batches presenting a different score on the PCA model than the main cluster of batches were further analyzed for individual investigation of the causes for these deviations. The process variables included in the models and contributing to the scores of these batches were used to identify the corresponding process deviations from the raw data. Table 5 is an overview of process diagnosis achieved using PCA modeling. Batches of interest for diagnosis (batches 7, 8, 9 and 10 for offline model and batches 11, 16 and 24 for online model) are circled on the score plots in figure 6 and figure 7.

Sensitive changes in process behavior were caused by altered concentrations of additives in feed medium during the perfusion (this batch was part of an experiment where lower starting glucose and glutamine concentrations were tested), by an inoculation at twice the target cell density for the offline variables (operational error), and by a change in the procedure for medium preparation (powder lot of medium hydrated inhouse versus use of medium hydrated by the supplier) and by a deviation in DO probe calibration for the online variables (operational error). Note that for two batches deviating in the PCA models, no process deviation could be found. Overall, PCA enabled a fast and systematic analysis of the dataset and the identification of technical procedures which alter the process behavior.

Table 5. Overview of batch diagnosis from PCA models for offline and online variables: deviations observed in the models and corresponding process observations.

Variables used in model	Batch	Deviation in model	Process observation
Offline	7-8	Score on first component; contribution of cell diameter and osmolality during second half of process	Altered concentration of additives in feed medium (lower starting glucose and glutamine concentrations)
	9-10	Score on second component; contribution of viable cell density throughout process	Inoculation cell density twice the target
Online	11	Score on second component; contribution of pH throughout process	Powder medium hydrated in-house, whereas all other batches used liquid medium hydrated by the supplier
	16	Score on second component; contribution of pH and CO_2 demand throughout process	No deviation identified
	24	Score on third component; contribution of DO throughout process	Deviation in calibration of DO probe (calibration at 105% instead of 100% at the start of the run)
	26	Residuals larger than critical limit	No deviation identified

4.2 PLS modeling and analysis of relations between process parameters and process responses

The aim of PLS is to establish correlations between process parameters and process responses, and to identify parameters that influence the quality of process response. Process responses or product CQAs considered in this study were population doubling time (PDT) throughout the runs and viability at the end of the runs. These CQAs were chosen as the aim of the cell expansion process studied here is to produce a high viable cell density seed in a short time, to seed a larger scale virus production process. Based on previous knowledge, a batch was considered of high quality when viability at the end of the run was above 85% and when PDT was lower than 40h (note that high PDTs reflect low growth rates and vice versa).

A preliminary analysis of the dataset showed that there was no significant difference in process performance between the 2L and 10L batches, for both PDT and viability (data not shown). However, due to the apparent effect of bioreactor scale on process behavior (Figure 6 and Figure 7), 2L and 10L runs were analyzed separately in this section. The main source of variation in the dataset was excluded in this way, to give the modeling a better chance to unveil more subtle sources of variation that would otherwise be obscured by the large scale effect.

The PLS models showed a poor fit and did not bring additional information on the process, therefore they are not shown. Relevance of models is typically expressed as the percentage of variation contained in the process variables dataset and used in the model, and as the percentage of variation in process responses explained by the model. In all the PLS models generated here, a limited part of variation in process variables (maximum of 55%) was modeled and used to explain up to 75% of variation in process responses. This means that on one hand, a lot of variation contained in the process variables dataset was not structured (e.g. not large, not redundant or very noisy) and could not be captured in the models. On the other hand, even if the portion of variation in process responses captured was relatively high in some models, further investigations of raw data did not lead to major information on interactions between process parameters and process responses.

5 General discussion

Multi-variate analysis of available historical data of the cell expansion process described in this study highlighted new relevant information on the process. A thorough exploration of the dataset was done using PCA. The impact of changes of procedures for the batches preparation (medium preparation, probes calibration, bioreactor inoculation) was previously unknown and was clearly exposed using PCA.

Additionally, PCA proved to be a valuable tool to pinpoint the sensitivity of the process to scale, whereas scale effects were previously not considered to be relevant between 2L and 10L. No significant difference in performance (for both viability and PDT) was observed between the batches operated at 2L and at 10L. This shows that the

technical deviations causing the clustering of the batches based on process scale do not affect process performance. The technical differences between 2L and 10L processes are therefore not critical for process outcome.

Moreover, the process deviations other than scale effect could not be related to process performance using PLS. This indicates that the process variables considered, and potentially the way they were measured or monitored (e.g. method, frequency), do not correlate with the CQAs as currently defined. A conclusion can be that the process is robust and that process responses are not affected by variations in process parameters. However, as mentioned above, this process is at the early phase of its development, which means that full optimization, characterization and investigation of full process operating ranges have not been performed yet (Figure 2). Only at the late stage of development, when all CPPs are identified and their optimal ranges to ensure good level of CQAs are known, can a process be considered robust.

Two other possible causes can explain the lack of fit of the PLS models obtained from the studied dataset. On one hand, process parameters were not varied in broad enough ranges to reveal their effect on CQAs. In other words the dataset lacks structure. On the other hand, the process variables currently monitored or the chosen process responses do not capture all the relevant information, either on the cells' quality attributes side (intermediate product), on the process monitoring side, or on both sides. The implications of these issues for the use of MVDA to analyze early development data and for the use of PAT in early development are discussed below.

5.1 The importance of dataset structuring for MVDA analysis

Common knowledge built on decades of experience in the field of cell culture typically identifies pH, temperature, DO and culture agitation as critical process parameters. These parameters were controlled at pre-defined set-points in the batches studied here and exhibited limited or no variance. As a consequence their effect on the CQAs could not be captured. This means that almost all variation in the dataset must have originated from other input variables, or starting materials, such as the medium (inoculation medium, feed and additives) and the cells (seed source and performance).

When analyzing historical data or data from experiments done very early in the process development path, a structured approach is often not used or not in place yet. This case study showed that MVDA can still be used even on an unstructured experimental dataset, largely consisting of trial and error runs or one-factor-at-a-time experimental designs, to gain understanding on the process (e.g. sensitivity of the process to scale), and to identify relevant process steps which have been standardized (medium preparation, probes calibration). This information was previously not exposed using classical univariate analysis (i.e. trending), which shows that MVDA, which is a PAT tools commonly used in late process development or routine manufacturing stages, is a very useful tool for exploration of early development data.

Ideally, in order to identify the CPPs, and properly define the complete process design space and the corresponding safe operational ranges for the CPPs, the parameters

would have to be varied in a fully structured and designed way, for instance by using a full factorial Design of Experiments (DoE) approach. DoE is a methodology of choice to identify optimal process settings and interactions among process parameters (27,28). DoE leads to generating structured datasets, meaning datasets containing structured variation (29,30), which can be readily analyzed using multivariate techniques to build stable and robust models for the exploration of the process design space (31). Although this approach is usually an effort performed in later development stages, it is valuable to have structured data available in early development. Usually the most critical process parameters are known beforehand. By using fractional factorial designs or at least introduce structured variation for the most critical parameters, additional process understanding can be gained during early development. This understanding would already go beyond the typical yes-or-no information gained from simple one-factor-at-a-time type of designs.

5.2 Capturing all relevant process information

5.2.1 CQAs

At first sight, population doubling time and viability at the end of a run are obvious product quality attributes for this process, as the aim is to produce high densities of viable cells in a short time, to be able to seed a larger scale virus production process. However, since the cells produced in the process studied here are an intermediate product, quality requirements should be related to the subsequent process step. This means that the CQAs should be linked to the cells' ability not only to grow, but also to bind virus particles and to replicate viruses, in short to the cell's infectability. Biological features that characterize the best physiological conditions for cell infection are complex and not fully understood. Each stage of a viral infection process can be influenced by the physiological state of the cell (e.g. cell cycle, apoptosis, etc.). Therefore as a next step, additional measurements capturing biological features of the culture need to be investigated and related to the infectability of the host cells. For this, understanding of the mechanisms of cells' infection and subsequent virus replication are needed to develop biological measurements capable of reflecting the cell's infectability. For instance, analyses at the cellular or molecular level capturing the biochemical reactions taking place during cell's infection are required. Such assays are essential to relate variation in process parameters to variation in this newly defined process outcome. This step of identification of "real" product CQAs is a prerequisite for the identification of the CPPs and the implementation of further (online) PAT tools.

5.2.2 CPPs

In this study, only classical measurements typically performed on bioreactor cultures were available: cell count, metabolites concentrations, pH, DO, temperature, etc. As these process variables did not correlate to the CQAs defined in this study, critical measurements are likely missing on the process monitoring side as well. To complement the classical univariate measurements done on bioprocesses, broad spectroscopic

analyzers such as near infrared or Raman spectroscopy could be used to capture process fingerprints (32,33). A process fingerprint describes the main patterns and trends occurring over the process time course and it typically yields qualitative information on the complete trajectory of a process. All information collected is not necessarily fully characterized and understood, but the use of multivariate techniques to build fingerprint models enables batch to batch comparison and detection of global process deviations (34). This approach is also supported by the comprehensive overview of applications of online monitoring to mammalian cell cultures, including associated MVDA methods provided by Teixeira et al. (35).

In this particular case, spectroscopic tools may therefore capture the relevant information related to the cell's infectability in a single online measurement. Multivariate models can be used to assess whether this additional data captures information related to the CQAs directly, or related to surrogate process parameters which affect the CQAs, in other words related to the CPPs. The ultimate goal of PAT is to implement online analytical tools able to monitor the CPPs and which can eventually be used to control the CPPs in order to yield the desired level of quality of the CQAs.

6 Conclusions

Application of MVDA to early development of cell cultivation processes is rarely published, although all relevant process data is typically available. We demonstrated that systematic analysis of early development data using MVDA increased our understanding of the process studied. Deviating batches were identified in a more objective way using PCA than with classical approaches and PCA enabled targeted analysis of the causes of their deviation. Relevant steps in medium preparation, probe calibration and culture inoculation procedures have been optimized and standardized based on the outcome of this study. Moreover, PCA proved to be a valuable tool to reveal relevant features of the process, such as sensitivity of the process to scale. The scale effect between 2L and 10L processes were previously not considered to be relevant and this is now a topic for further research.

MVDA is typically used on late development or manufacturing datasets. Despite the inherent shortcomings of datasets from early development, this study clearly shows the added value of using MVDA on these datasets as it helps to identify knowledge gaps, experimental flaws and uncontrolled variance. Gaining this information during early development is key to further develop robust and well controlled bioprocesses.

In order to make full use of MVDA as a PAT tool throughout the entire process development path, MVDA should be routinely used and coupled with simple experimental designs providing the datasets with basic structure, with fundamental research on the process dynamics, and with the use of appropriate analytics. This strategy, which requires a larger investment up-front in the development path than classical approaches, will lead to shorter and more efficient process development and scale-up trajectories and will result in lower overall development costs for new biopharmaceutical products.

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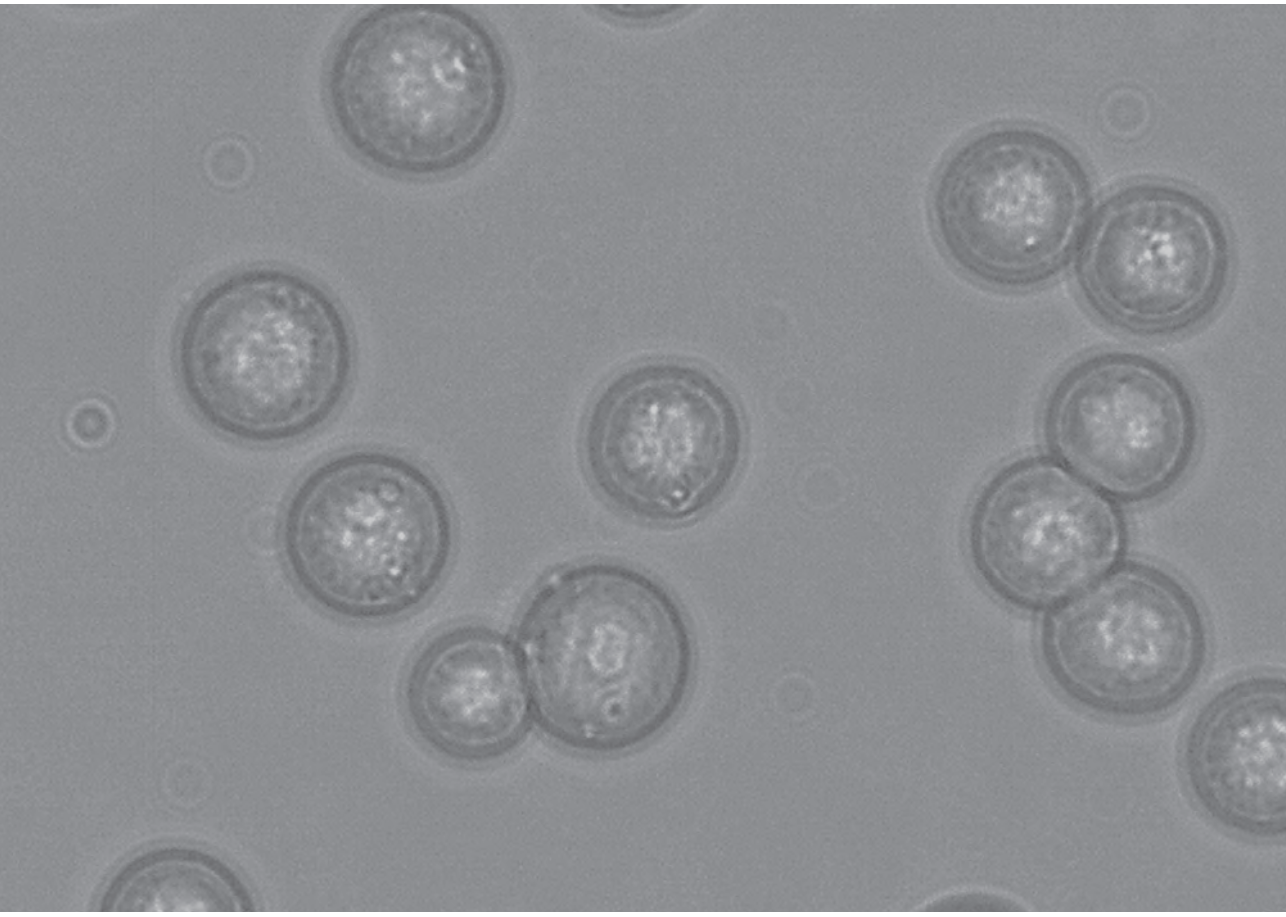
Chapter 4

Characterization of apoptosis in PER.C6® batch and perfusion cultures



Abstract

Preventing or delaying cell death is a challenge in mammalian cell cultures for the development and optimization of production processes for biopharmaceuticals. Cell cultures need to be maintained highly viable for extended times in order to reach maximum production yields. Moreover, programmed cell death through apoptosis is often believed to occur without being detected by classical viability measurements. In this study, we characterized cell death in PER.C6® batch and perfusion cultures using three flow cytometry techniques measuring different steps of the apoptosis cascade: DNA fragmentation, caspases activation and phosphatidylserine externalization. We showed that apoptosis is the main pathway of PER.C6® cell death in batch cultures after depletion of main carbon sources. In high cell density perfusion cultures fed at a constant specific perfusion rate, both high viability and very limited apoptosis were observed. When extending this perfusion process far beyond standard operations, cultures were exposed to suboptimal process conditions, which resulted in an increase of apoptotic cell death. Moreover, we showed that the reference viability measurement using trypan blue exclusion properly assesses the level of cell death in PER.C6® cultures. This study is a first step in understanding the mechanisms of PER.C6® cell death, which will be helpful to support applications of the cell line.

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1 Introduction

Understanding the mechanisms of cell growth and cell death in mammalian cell cultures is essential for the development and optimization of production processes for biopharmaceuticals. To reach maximum production yields (e.g. for monoclonal antibodies or more complex products such as viruses), cell cultures need to be maintained highly viable for extended times. Minimizing cell death is therefore an important challenge for industrial processes (1,2).

Cell death in mammalian cell cultures occurs in two ways: passive death called necrosis and programmed death called apoptosis. Necrosis occurs when cells are exposed to sudden and acute environmental stresses that cause irreversible damages to the cells. This results in cell swelling, loss of cell membrane integrity and finally uncontrolled release of cellular structures and organelles after cell disruption (3,4). In contrast, apoptosis is an actively regulated and genetically programmed way of cell death often referred to as cell suicide, which is triggered by either intrinsic or extrinsic stress or death signals (5). Specific morphological changes characterizing apoptosis include cell shrinkage, appearance of cytoplasmic blebs at the cell surface giving rise to apoptotic bodies, and condensation of chromatine followed by endonuclease fragmentation of nuclear DNA into 180-200 bp fragments (6). Cells that undergo apoptosis do not release their cellular content into the surrounding broth; they separate in small vesicles containing parts of the cell contents. Apoptosis and its mechanisms in mammalian cells are well understood and extensively described, as shown by the numerous elaborate reviews on this topic (6-8).

Several biochemical steps of the apoptotic cascade are of importance for the detection and measurement of apoptosis in cell cultures mostly using flow cytometry methods. Caspases (cysteine-aspartic proteases), which are expressed in an inactive form in healthy cells, are activated in cascade during apoptosis (6). Active caspases can be detected with labeled caspase substrates or inhibitors (9). During apoptosis, DNA is broken down into 180-200 base pairs fragments by calcium and magnesium-dependent endonucleases (10). The free 3'OH nick ends of these DNA fragments can be detected by specific incorporation followed by straining of deoxyuridine triphosphate (11). Expression of cell surface markers is also widely used to detect apoptosis. For example, the translocation of phosphatidylserine from the inner- to the outer-layer of the cell membrane during apoptosis can be detected using specific binding proteins such as Annexin V. (12). Additionally, DNA staining with readily detectable dyes such as propidium iodide (PI), ethidium bromide, 7-Aminoactinomycin D (7-AAD) or Hoechst 33342 can also be used to detect either morphological changes such as chromatin condensation, or plasma membrane permeability to the dyes (13,14). With a combination of these flow cyotmetric methods, a detailed analysis and quantification of apoptosis in cell cultures is achieved (15).

While necrosis is usually triggered by acute stressors like shear or sudden high osmotic pressure, apoptosis is triggered by more gradual events such as depletion of nutrients, accumulation of metabolic by-products or damage of the central genome. During cultivation in a controlled bioreactor system, apoptosis is therefore more likely

to happen. The first evidence of apoptotic cell death in *in vitro* cultures of commercially relevant cell lines has been exposed in the 1990's (16-18). Since then, dynamics, mechanisms and causes of cell death in cell cultivation processes have been widely studied for the cell lines commonly used by the biopharmaceutical industry. Several papers describe apoptosis in the well-known Chinese hamster ovary (CHO) cell line, which is commonly used for monoclonal antibody production (5,19-23). Studies on apoptosis in CHO cultures often aim to develop strategies to inhibit or delay cell death in cultivation processes by improving the extracellular environment either by supplementing cultivation media or optimizing process conditions (24-26).

Another way to delay apoptosis is to alter natural apoptotic pathways by engineering cell lines with anti-apoptotic genes or by silencing the expression of apoptotic genes. These processes have been widely described for example for CHO (27-29) and for human embryonic kidney HEK-293 (30,31). Another example of a genetically immortalized cell line that is developed by introducing anti-apoptotic genes is the human PER.C6® cell line. PER.C6® cells are derived from embryonic retinoblast cells and immortalized similarly to HEK-293 by transformation with the E1A and E1B-encoding sequences of Adenovirus 5 (32,33). Briefly, expression of the E1A gene results in deregulation of the cell cycle: cells lose the ability to exit the cell cycle and enter the G0 or quiescent phase and they are stimulated to enter into the S phase and to proliferate (34). The E1A product also activates defense mechanisms that initiate apoptosis (35,36). E1B counters this apoptosis induction. E1B encodes two proteins that on the one hand inhibit the function of p53, a tumor suppressor protein, and on the other hand are homologous to Bcl-2, an anti-apoptotic protein, thereby negatively regulating several apoptosis induction pathways (36,37). The combined mechanisms of these two genes therefore lead to cell lines able to regulate and delay apoptosis and to proliferate at high rates.

Several biopharmaceutical PER.C6®-based products are current in (pre-)clinical development, including Adenovirus-based vaccines (38-40), of Influenza vaccine (41-43) and of Polio vaccine (44). High level expression of IgGs and IgMs has also been reported in PER.C6® (45,46). To support these applications of the PER.C6® technology and to facilitate process optimization, fundamental studies of the cell line are required. The functions of apoptosis regulation of the E1 genes are well described in literature. However, the effect of this immortalization strategy on PER.C6® apoptotic cell death in industrial cell cultures has never been studied. Therefore in the present study, we describe and characterize the dynamics of cell growth and cell death in batch and high cell density perfusion cultures of suspension PER.C6® cells. Apoptosis was measured with three methods targeting different steps of the apoptosis cascade: phosphatidylserine externalization, caspases activation and DNA fragmentation. We show that although a low basal level of both apoptosis and necrosis is always measured, apoptosis is the main pathway for cell death at the end of batch cultures as well as at the end of perfusion cultures when suboptimal cultivation conditions are reached.

2 Materials and methods

2.1 Cell line and growth condition

PER.C6® cells (Crucell, Leiden, the Netherlands) were thawed and cultivated in Crucell's proprietary and chemically defined PERMEXCIS® medium (Lonza, Verviers, Belgium) supplemented with 4 mM L-Glutamine (Invitrogen, Paisley, UK). The parental PER.C6® cells used did not express proteins nor produced viruses in the processes studied, in order to be able to establish the basal apoptosis levels of the cell line as such.

2.2 Batch cultures

Batch cultures were done in 250 mL disposable shaker flasks (Corning Costar Inc., Amsterdam, the Netherlands) with a working volume of 25 mL. Cultures were inoculated at 0.5 x 10^6 viable cells mL^{-1} and incubated in a humidified incubator at 37 ºC in 10% CO_2 for up to 14 days. Agitation was set to 100 rpm on a IKA KS 260 shaker plateau (VWR International B.V., Amsterdam, the Netherlands).

2.3 Perfusion cultures

After expansion of PER.C6® seeds in roller bottles (Corning Costar Inc., Amsterdam, the Netherlands) or BIOSTAT® Cultibags RM (Sartorius Stedim Biotech, Aubagne, France), 13 L autoclavable bioreactor cultures (B. Braun Biotech, Melsungen, Germany) equipped with two marine impellers were inoculated at 0.8 x 10^6 viable cells mL^{-1} in a 10L working volume. Temperature was maintained at 37°C by a heating blanket. The pH was controlled at 7.3 by addition of sodium bicarbonate or by adding CO_2 to the gas inlet. Dissolved oxygen (DO) was controlled at 40% by sparging air and pure oxygen. Perfusion was run with an alternating tangential flow (ATF) system (Refine, Pine Brook, NJ, USA) and cells were continuously retained in the bioreactor due to the 0.2 μm hollow fiber module used (GE Healthcare, Breda, the Netherlands). Spent medium was extracted continuously while fresh medium was supplied to hold a constant working volume. The same medium was used for inoculation and feed. A constant specific perfusion rate (m^3 $cell^{-1}$ s^{-1}) was applied to maintain PER.C6® cultures in growth phase. Perfusion cultures were normally stopped on day 10, but in order to study the effect of process deviations encountered when extending the process, 3 perfusion cultures were prolonged until day 14. Between day 10 and 11, when cell density exceeded 100 x 10^6 viable cells mL^{-1}, the pumps supplying fresh medium and extracting spent medium reached their maximum output flow. From then and until the end of the runs, this maximum perfusion rate was maintained. Cultures were therefore no longer fed at the same specific perfusion rate and were thus exposed to suboptimal growth conditions. Samples were taken daily.

2.4 Cell counting and reference viability measurements

Cell number and viability based on trypan blue exclusion were measured using a Vi-cell XR (Beckman Coulter Inc., Woerden, the Netherldands), according to manufacturer's protocol. Cell specific growth rate between two sampling points was calculated as follows:

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

where μ represents cell specific growth rate, X_1 and X_2 representing total cell density of two samples (in x 10^6 cells mL^{-1}) and t_1 and t_2 representing the time at which the two samples were taken.

2.5 Biochemical assays

Main metabolite concentrations (glucose, lactate, glutamine, glutamate and ammonia) were measured using a NOVA bioprofile 400 (LA Biosystems BV, Waalwijk, the Netherlands). pH was measured on a blood gas analyzer (ABL5, Radiometer, Zoetemeer, the Netherlands). Osmolality was determined using a Gonotech Osmomat 3000 osmometer (Salm en Kipp BV, Breukelen, the Netherlands).

2.6 Flow cytometry

Three kits were used to measure the level of apoptosis in PER.C6® cultures using a Guava PCA Base flow cytometer (Merck Millipore, Amsterdam, the Netherlands). Externalization of phosphatidylserine (PS) was detected with Annexin V using the Nexin kit (Merck Chemicals BV, Amsterdam, the Netherlands). Activated caspases were labeled with a sulforhodamine-valyl-alanyl-aspartyl-fluoromethyl-ketone (SR-VAD-FMK), a fluorochrome-conjugated inhibitor of caspases, in the MultiCaspases SR kit (Merck Chemicals BV). In both Nexin and MultiCaspases kits, 7-Aminoactinomycin D (7-ADD) was present to stain DNA of dead cells with a permeable membrane. Finally, the Tunel kit (Merck Chemicals BV) was used, where bromo-deoxyuridine (BrdU) was incorporated into fragmented DNA and subsequently labeled with a tetramethylrhodamine (TRITC)-conjugated anti-BrdU antibody.

For the three assays, protocols recommended by the supplier were applied. Data were acquired and analyzed using the EasyCyte system of the CytoSoft software version 6.0.2 (Merck Millipore, Amsterdam, the Netherlands). The results in percentage of the total cell population were determined independently for each category detected by the assays.

2.7 Data analysis

For the batch cultures, as it was possible to record replicates of measurements at the exact same time, the mean and standard error of measurements was reported on the graphs. For the perfusion cultures, as practicalities made these simultaneous observations impossible, a linear model was adjusted on the data. The mean and 95% confidence interval (CI) of the mean from this linear regression were calculated as described elsewhere (47). CI is the interval of the mean responses conditional to the time. Briefly, CI was computed after logit

transformation of the data to constraint percentage value between 0 and 100%. Interval was then back transformed into original scale before plotting. The mean and 95% CI of the mean were reported on graphs to illustrate the kinetic of apoptosis.

3 Results and discussion

3.1 Cell growth and cell death in batch PER.C6® cultures

3.1.1 Performance of batch cultures

To describe cell growth and cell death dynamics of PER.C6® cultures in PERMEXCIS® medium, 14-day batches were conducted in triplicate. Cultures were in growth phase for 6 days, after which cells entered stationary phase followed by a decline phase from day 10 onwards (figure 1a). Culture viability measured based on trypan blue exclusion remained above 85% for 4 days. Viability then steadily decreased to a minimum of 25% on day 14, when the cultures were stopped. Lactate, a by-product formed from inefficient metabolism of glucose, accumulated to a maximum concentration of 27.5 mM at the moment of glucose depletion (day 4) and was afterwards consumed (figure 1b). Both glutamine and glutamate were depleted during growth phase although a low residual level of glutamate was detected again from day 6 onwards (figure 1c). Ammonia is known for being produced during metabolism of glutamine and glutamate and it has a negative effect on cell growth in mammalian cell lines (48). Ammonia was present at a maximum of 3.5 mM at the end of the growth phase. These data describe a typical PER.C6® batch culture.

Apoptosis was measured using the Tunel, MultiCaspases and Nexin assays on three independent samples on each sampling day for all three batch cultures.

3.1.2 Apoptosis in batch cultures

DNA fragmentation

The use of the Tunel assay enabled to discriminate two cell populations: TRITC positive cells, which presented fragmented DNA and therefore were apoptotic and TRITC negative cells without 3'OH DNA nick ends, which were viable. During batch PER.C6® cultures, accumulation of cells with fragmented DNA is very clear and reached 57% on day 14 (figure 2a).

Regulation of caspases

Monitoring of apoptosis in the same cultures with the MultiCaspases assay enabled discrimination of 4 populations. Cells positive for sulforhodamine-valyl-alanyl-aspartyl-fluoromethyl-ketone (SR-VAD-FMK), which binds to active caspases, were apoptotic. Within this population of apoptotic cells, cells positive only for SR-VAD-FMK maintained their membrane integrity while activating caspases (early apoptotic), whereas cells positive for both SR-VAD-FMK and 7-Aminoactinomycin D (7-AAD) had active caspases and leaking membranes (late apoptotic). Double negative cells were considered live

and non-apoptotic. Finally cells only positive for 7-AAD were necrotic as they present permeable membranes but no active caspases.

Results from the MultiCaspases assay showed that 93% of the total dead cells had active caspases at the end of the experiment (figure 2b). The portion of necrotic cells started at 12% of the total population and fluctuated around 25% during the first 8 days of culture. Early and late apoptotic cells however increased from 3 to 11% and from 3 to 27% respectively. After day 8, during the decline phase, the portion of early apoptotic cells remained constant around 12% for the rest of the culture whereas late apoptotic cells increased to 62% and necrotic cells decreased to 4%.

Phosphatidylserine externalization

With the Nexin assay, three cell populations can be distinguished: double positive cells with externalized phosphatidylserine (PS) and permeable membranes to 7AAD that were dead, cells positive for Annexin V only that had externalized PS and were therefore apoptotic, and double negative cells that were viable non apoptotic cells. The population of dead cells increased from 10 to 30% in the first 4 days and further increased from 30% to 70% in the last 6 days of the batches (figure 2c). This is consistent with the 7AAD staining observed in the MultiCaspases assay, when considering cells both positive and negative for active caspases. The percentage of Nexin apoptotic cells stayed constant at a low level between 5 and 9% throughout the batch runs.

Upon staining with the Nexin assay solely, it is impossible to differentiate necrotic from late apoptotic cells within the population of double positive dead cells. Indeed, it is reported in literature that Annexin V may be able to penetrate cells with compromised membranes integrity and stain PS on the inner layer of plasma membrane (49).

Overall, the data collected from these three assays suggested that during the growth and stationary phases of a batch PER.C6® culture (day 0 to day 8), both apoptosis and necrosis occurred at relatively low levels. However during the death phase, apoptosis cell death became dominant. Moreover, we showed that DNA fragmentation and active caspases are clear apoptosis markers in PER.C6® cultures.

3.2 Cell growth and cell death in perfusion PER.C6® cultures

3.2.1 Performance of perfusion cultures

In the process studied here, PER.C6® cells were cultivated to high cell densities in perfusion mode. Perfusion enables to prevent both depletion of nutrients and accumulation of toxic by-products which, as shown in figure 1b and c, both occur in batch cultures. Typically, this perfusion process runs for 10 to 11 days, when the desired cell density required for the next process step is reached.

Cell growth, viability and main metabolite concentration profiles for a representative perfusion run are showed in table I. The constant specific perfusion rate applied between day 0 and day 11 enabled to maintain the cultures in growth phase with a high cell growth rate between 0.017 and 0.022 h^{-1}. The viable cell density obtained after 11 days of perfusion was 33-fold higher than the maximum viable cell density obtained in batch

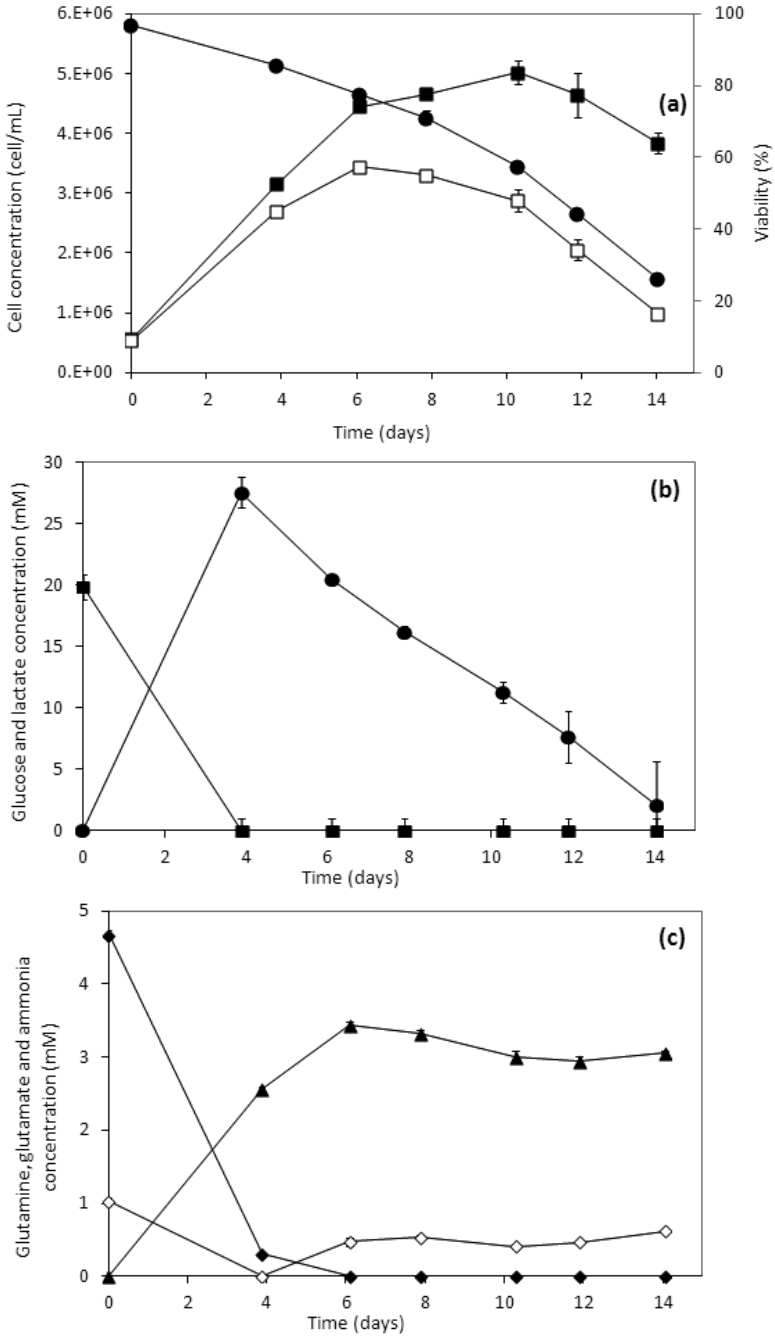

Figure 1. Evolution of cell growth and main nutrient concentrations in batch PER.C6® cultures (average of 3 runs). a) Total (■) and viable (□) cell densities, percentage viability (●). b) Glucose (■) and lactate (●) concentrations. c) Glutamine (♦), glutamate (◊) and ammonia (▲) concentrations. Error bars show the standard deviation of the three cultures.

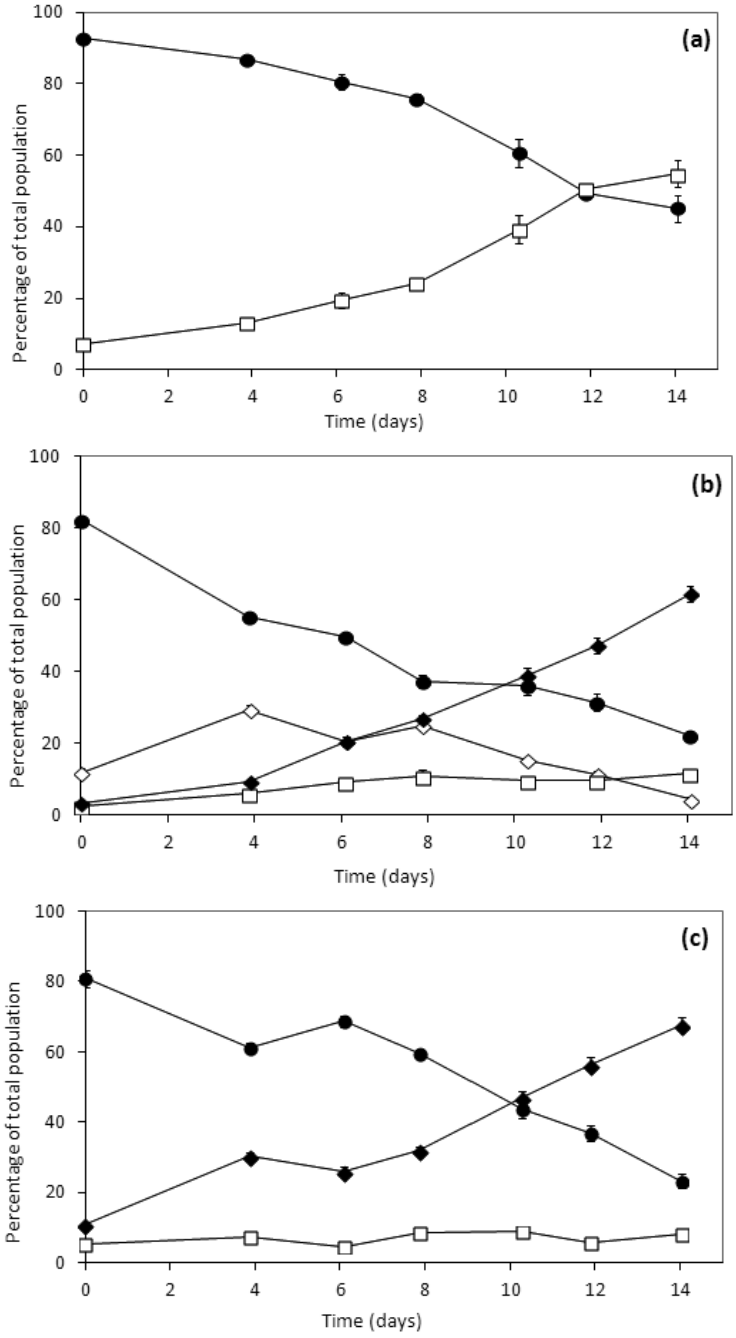

Figure 2. Progression of apoptosis (average of 3 runs) in 14-days PER.C6® batch cultures. a) Tunel assay: percentage of non-apoptotic (●) and apoptotic (□) cells. b) MultiCaspases assay: percentage of viable (●), early-apoptotic (◊), late-apoptotic (♦) and dead (□) cells. c) Nexin assay: percentage of viable (●), apoptotic (♦) and dead (□) cells. Error bars show the standard deviation of the three cultures.

on day 6 (figure 1a and table I). Additionally, classical process parameters such as pH, DO or agitation speed, were controlled at set-point during this first process phase.

Three perfusion runs were prolonged and ran for a total of 14 days, to characterize the progression of apoptosis outside cultivation conditions normally applied. Extremely high cell densities of 367 x 10^6 cell mL^{-1} with a viability of 83% were obtained on day 14. Bioreactor set-up was not adapted prior to performing these extended runs to cope with such high cell densities. Cultivation systems therefore reached maximum capabilities as indicated in table I. Briefly, when cell density exceeded 100 x 10^6 viable cell mL^{-1} (around day 11), the feed pumps reached their maximum capacity and perfusion rate was no longer increased with cell density (Table I). Additionally, DO could no longer be maintained at 40% from day 12 onwards, as the maximum capacity of the mass flow controllers supplying air and oxygen to the cultures was reached. Oxygen was therefore still supplied but dissolved oxygen could no longer be measured in the cultures (data not shown). Note that carbon dioxide did not accumulate after day 11, when flows of air and oxygen no longer increased with cell density, indicating that mass transfer was still sufficient to remove CO_2 from the culture. Finally, on day 13, pH control was manually turned off because the amount of sodium bicarbonate required to maintain pH at set-point caused an increase in osmolality towards known toxic levels. Stopping pH control caused a decrease by 0.5 units within 2 to 3 hours.

A consequence of extending the perfusion process is nutrient limitation, as the change in feed regime affected evolution of main metabolite concentrations. Glucose level decreased from 6.0 to 4.5 mM between days 11 and 13 and was completely depleted on day 14. Metabolic by-products accumulated in the cultures: ammonia concentration doubled between day 12 and 14 from 1.4 mM to 3.1 mM, and lactate increased by almost 8 mM over the last culture day (table I). Concurrently, cell specific growth rate decreased slightly from 0.022 h^{-1} on day 4 to 0.017 h^{-1} on days 10 and 11. Day-to-day growth rate calculated between days 11 and 14 showed large fluctuations, most likely due to imprecisions in sampling and cell counting. Calculations of growth rate between days 11 and 13 and days 11 and 14 showed a trend towards slower cell growth rates (0.018 and 0.015 h^{-1}, respectively). Growth rate remained high after day 11, when specific perfusion rate was no longer maintained at set-point. The maximum perfusion rate applied at the end of the cultures seem to have provided sufficient nutrients at sustain high cell density at high viability and to support cell replication at high rates. This observation is in accordance with the slow decrease in main nutrients observed after day 11.

3.2.2 Apoptosis in perfusion cultures

Apoptosis was measured for a total of 18 perfusion runs. The Nexin assay was used for all runs whereas MultiCaspases and Tunel assays were used for 9 and 13 runs, respectively.

The three apoptosis markers targeted in the flow cytometry assays consistently showed no increase in the portion of apoptotic cells and dead cells during the standard phase of the perfusion process (figure 3). Apoptosis and necrosis both occurred, at a low basal level of around 10% each. Overall, PER.C6® cell cultivation in perfusion mode enabled to increase maximum cell density, maintain high viability and limited apoptosis compared

Table I. Representative total cell density, viability and main metabolites concentrations for a 14-days perfusion culture and description of cultivation conditions.

Process time (d)	Total cell density (x10 ^ 6 cell/mL)	Cell growth rate (h^{-1})	Viability (%)	Normalized perfusion rate (%)	Metabolites concentration (mM)					Cultivation conditions
					Glucose	Lactate	Glutamine	Glutamate	Ammonia	
0	0.87	-	89	0.8	22.1	3.7	4.7	0.9	0.6	Perfusion rate increasing with cell density
4	6.64	0.022	91	6	7.6	22.0	1.2	0.7	1.7	
10	74.5	0.017	91	68	5.7	22.6	1.1	Bllq	1.3	
11	123	0.017	93	100	6.0	24.8	1.0	0.2	1.6	Perfusion at constant rate
12	159	0.013	91	100	4.1	21.7	0.6	Bllq	1.4	Same as day 11; dissolved oxygen no longer controlled
13	286	0.021	91	100	4.5	21.4	1.2	Bllq	2.1	Same as day 12; pH no longer controlled
14	367	0.011	83	100	Bllq	29.3	Bllq	0.3	3.1	Same as day 13

Bllq = below low limit of quantification with the NOVA bioprofile 400.

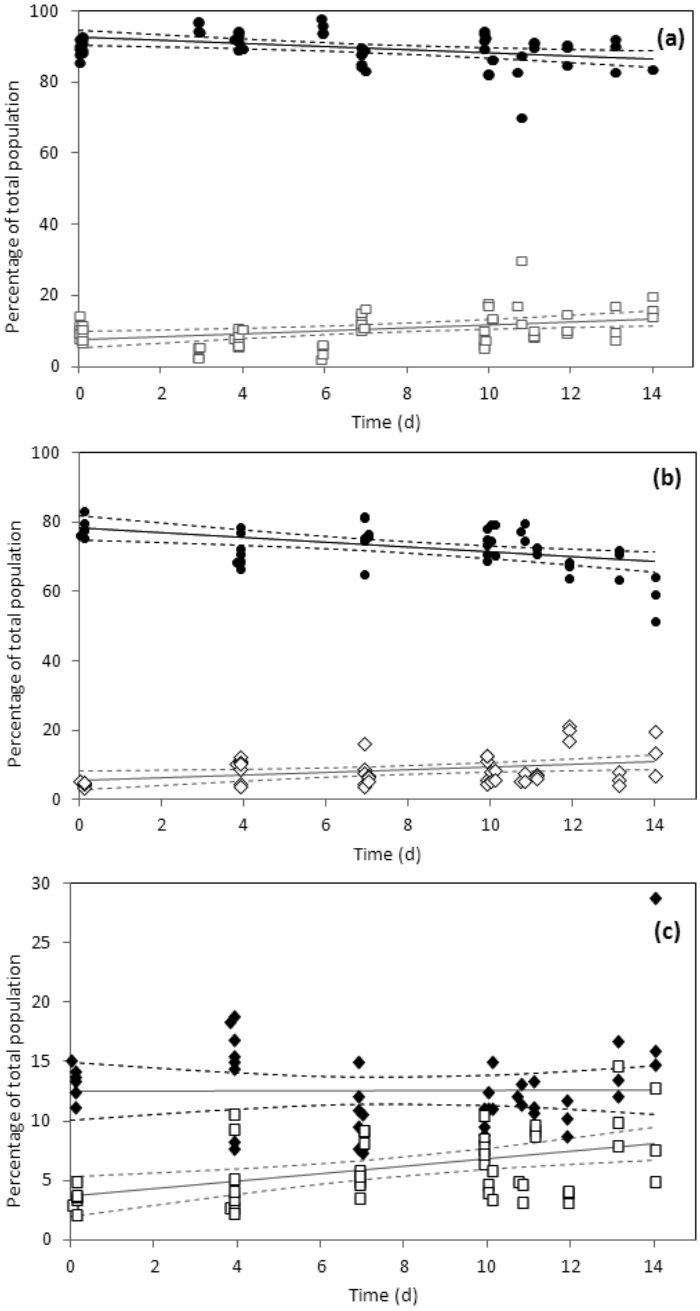

Figure 3. Progression of apoptosis (average of 3 runs) in 14-days PER.C6® perfusion cultures. a) Tunel assay: percentage of non-apoptotic (●) and apoptotic (□) cells. b and c) MultiCaspases assay: percentage of viable (●), early-apoptotic (◊), late-apoptotic (♦) and dead (□) cells. d) Nexin assay: percentage of viable (●), apoptotic (♦) and dead (□) cells. Dots are raw data; straight lines are mean from linear models and dashed lines are 95% confidence intervals of the mean.

to cultures conducted in batch mode. This can be due to the supply of nutrients, or the removal of inhibitory metabolism by-products, or the combination of both.

Culture viability and progression of apoptosis were not drastically affected by perfusion process extension and suboptimal cultivation conditions. Trypan blue viability was maintained above 90% until day 13, after which it decreased to 83% on day 14. Cells positive for TRITC (TUNEL staining) increased slightly from 12% to 14% between day 11 and day 14 (figure 3a), reflecting the small increase in apoptotic cell death. The level of late-apoptotic cells from the MultiCaspases assay remained constant around 13%, but the early-apoptotic cells increased from 6 to 12% throughout the run. Similarly to what was observed in batch, no increase in apoptosis was captured with the PS staining in the Nexin assay (figure 3d). Necrosis, determined from the population of cells positive for only 7AAD with the MultiCaspases assay, also marginally increased throughout the run from approximately 3% to a maximum of 8% on day 14 (figure 3c). Overall, data from the three apoptosis assays showed that under underfeeding and suboptimal cultivation conditions, perfusion PER.C6® cultures underwent only limited increase in cell death, primarily through apoptosis. The low basal level of necrosis remained constant.

Nevertheless, extended perfusion cultures beyond day 11 were clearly exposed to process deviation and suboptimal growth conditions. Firstly, from day 12 onwards, oxygen could no longer be measured in the cultures, which means that the oxygen uptake rate by the cells exceeded the oxygen transfer rate into the medium. Secondly, pH drifted by 0.5 units in a few hours after the control was turned off on day 13. Finally, culture density and viscosity increased with cell density, which may have resulted in increased shear forces. All these factors can potentially lead to necrotic cell death. For example, it has been showed that CHO and HEK293 cells can die of necrosis as a consequence of shear exposure above a certain threshold (25). However, it is difficult to translate the extremely unfavorable condition inducing necrosis to *in vitro* cultures (3). Based on the very small increase in cell death we observed, we conclude that the pH drift, oxygen deprivation and increased shear exposure underwent by the PER.C6® cells at the end of the perfusion did not induce necrosis.

3.3 Comparison of different methods to determine viability

Measurement of culture viability is critical for bioprocesses as viable cells represent the production capacity of the process. Methods commonly used to determine viability are based on membrane permeability, using either large molecular weight dyes such as trypan blue or stains that bind to DNA. Trypan blue is a negatively charged molecule that cannot penetrate the selective plasma membrane of viable, healthy cells. Viable cells with intact membranes "exclude" trpyan blue. However cells with damaged or leaking membranes are not able to exclude trypan blue and get stained with a characteristic blue color, detectable with a microscope (50,51). Trypan blue exclusion is the primary method used to determine viability of PER.C6® cultures in Crucell's processes. Trypan blue exclusion is compared to 7-AAD staining and Tunel labeling in figure 4 for PER.C6® batch and perfusion cultures. Tunel labeling and trypan blue exclusion yielded comparable viabilities throughout batch

cultures, except on the last culture day where they differed by 20% (figure 4a). Average 7-AAD viabilities calculated from Nexin and MultiCaspases assays were lower than those of trypan blue and Tunel until day 12, with a peak of 20% divergence on day 4, in the middle of the growth phase. Similar observations were made from perfusion data: trypan blue and Tunel viabilities were similar and 7-AAD viabilities were overall lower than with the other two methods, both when cultures were maintained at high viability (first 11 days) and under suboptimal cultivation conditions (days 11 to 14) (figure 4b).

Trypan blue and 7-AAD staining detect a late stage of cell death, when cell membrane integrity is lost. It is assumed that Tunel also capture a late phase of the apoptosis cascade, as DNA fragmentation depends on endonucleases activation by active caspases (6). The similarities between these two methods for viability measurement were therefore expected.

Additionally, 7-AAD viability, which was obtained from averaging Nexin and MultiCaspases results, showed a larger variance compared to the other methods. This variance in reflected

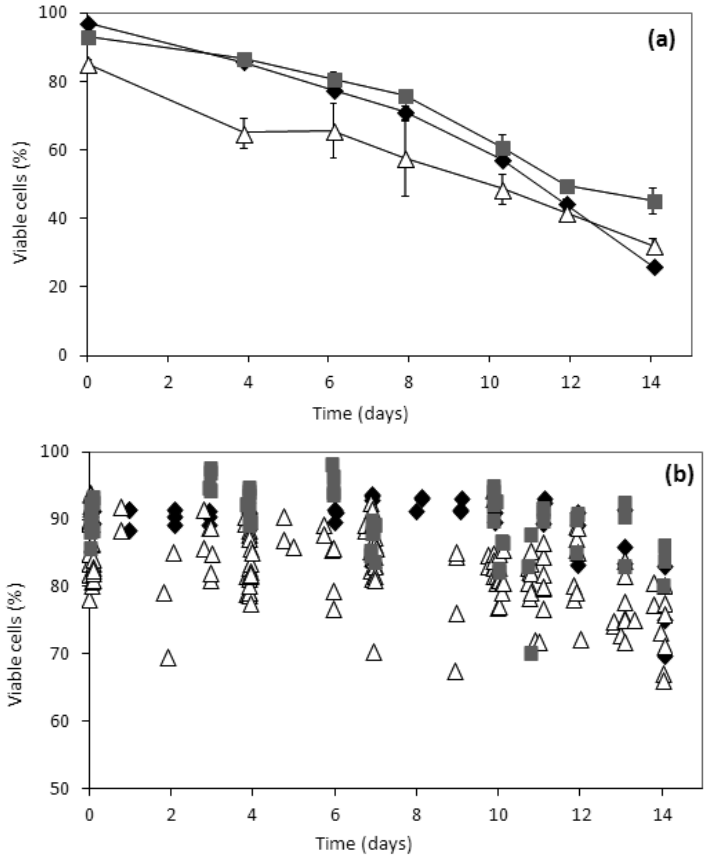

Figure 4. Comparison of viabilities from trypan blue (♦),Tunel (■) and 7-ADD (Δ) from Nexin and MultiCaspases assays for batch (a) and perfusion (b) cultures. Nexin and MultiCaspases viabilities are averaged for batch cultures (a). Error bars in (a) show the standard deviation of the three cultures.

in the error bars for batch cultures and in the spread of the data points for perfusion cultures (figure 4). Dyes used for flow cytometry are known for being able to penetrate live cells after prolonged exposure (52). This could explain on one hand the larger variation for 7-AAD, as the protocols for Nexin and MultiCaspases assay were different (number of cell washes and incubation times), and on the other hand the overall lower viability measured with this dye.

Interpretation of culture viability depends on the method chosen for the measurement. It is acknowledged in literature that the concept of viability is not clearly defined in the industry as it highly depends on the cell biological features used to determine it (53,54). In this study, data showed that trypan blue exclusion, the reference methods used at Crucell to measure viability of PER.C6® cultures, properly assessed the level of cell death both in batch and in perfusion. We therefore conclude that trypan blue exclusion is an appropriate method for routine monitoring of culture viability.

3.4 Characterization of apoptosis in PER.C6® cultures

Amongst the assays used in this study, the MultiCaspases kit offered the possibility to detect intermediate apoptotic state, as cells with an intact membrane and active caspases could be detected. However, we observed that this portion of cells positive for SR-VAD-FMK only slowly increased from 3% to 12% throughout the batch runs and from 8 to 11% for the perfusion runs (figure 2b and figure 3c). Accumulation of apoptotic PER.C6® cells therefore occurred without a visible transition via the state where caspases are active and membrane integrity is not lost yet. This observation in PER.C6® cultures is different from that of Wei et al. (5) observed in CHO cultures. There, a peak of early apoptotic cells with active caspases representing up to 25% of the total cell population occurred between days 5.5 and 8.5 of the cultures, and these cells afterwards transitioned into a late-apoptotic state with permeable membranes for 7-AAD.

Likewise, externalization of PS was not captured as a marker of early apoptosis. The portion of cells positive only for Annexin V remained constant at a low level between 5% and 9% throughout the batch cultures (figure 2c). In the perfusion process, this fraction did not increase at all even after day 11, when process deviation occurred and caspases activation started to be detected (figure 3b and c). This indicates that PS is a biological marker of apoptosis that is not useable for PER.C6® cultures. Contradictory conclusions can be found in literature on whether PS exposure at the cell membrane is a universal phenomenon during apoptosis or not. Engeland et al. (12) stated that this phenomenon is universal and has been reported not only in mammalian cells but also in insect and plant cells, whereas Frey (55) showed that Annexin V staining can be lacking in apoptotic populations of several human cell lines (namely lymphoma and hematopoietic cells HL60, U937 and Raji). It is therefore not possible to conclude on the occurrence of PS externalization in the PER.C6® apoptosis cascade. Additionally, the basal and low level of Annexin V positive cells throughout the batch and perfusion cultures is not due to non-specific binding of the dye, as Annexin V was proven to be very specific to PS (56).

The low level of detection of cells with only externalized PS and only active caspases, without compromised membrane integrity can be explained by a very short duration

of these phases during the apoptosis process. This would explain why cells did not accumulate in these phases. Literature on the duration of the different phases of apoptosis process is scarce (6,57).

4 Conclusions

With the use of three flow cytometry assays, we showed that apoptosis is the main pathway of PER.C6® cell death in batch cultures after depletion of main carbon sources. In high cell density perfusion cultures fed at a constant specific perfusion rate, both high viability and low apoptotic fractions were maintained. Extending this perfusion process led to suboptimal cultivation conditions and partial culture starvation, but with limited induction of cell death. In these conditions, PER.C6® cultures underwent primarily apoptotic cell death, while the low basal level of necrosis remained constant. Leads for potential process improvements have been identified based on this study. As decreasing the cell specific perfusion rate caused a slow decrease in nutrient levels and limited cell death, there may be opportunities for further optimizing the feeding regime. Similarly, interruption of process parameter controls such as pH and DO had slow and mild effects on the cultures, which suggests the possibility to optimize the current control strategy of these parameters.

Preventing or delaying cell death is a challenge in industrial bioprocesses, and programmed cell death through apoptosis is often believed to occur without being detected by classical viability measurements. Here, we show that apoptosis only occurs in PER.C6® cultures under starvation conditions such as the end of a batch, or suboptimal perfusion cultivation conditions which are far beyond how these processes are typically operated. Moreover, the reference viability measurement using trypan blue exclusion properly assesses the level of cell death in PER.C6® cultures.

To deepen the knowledge gained from this study on PER.C6® cell growth and cell death dynamics, experiments in chemostat cultivations could be conducted to study the effect of individual process parameters. Variations in inoculation cell density of batch and perfusion cultures, or in process design (limitation of oxygen transfer for instance) could also be useful to further study the limits of the standard processes and equipments. Moreover, similar studies on a monoclonal producing cell line or on a virus production process would enable to relate the kinetics of PER.C6® cell growth and cell death to productivity. Nevertheless, this study on characterization of apoptosis in different cultivations systems enabled further fundamental understanding on PER.C6®, which will be helpful to support applications of this industrially relevant cell line.

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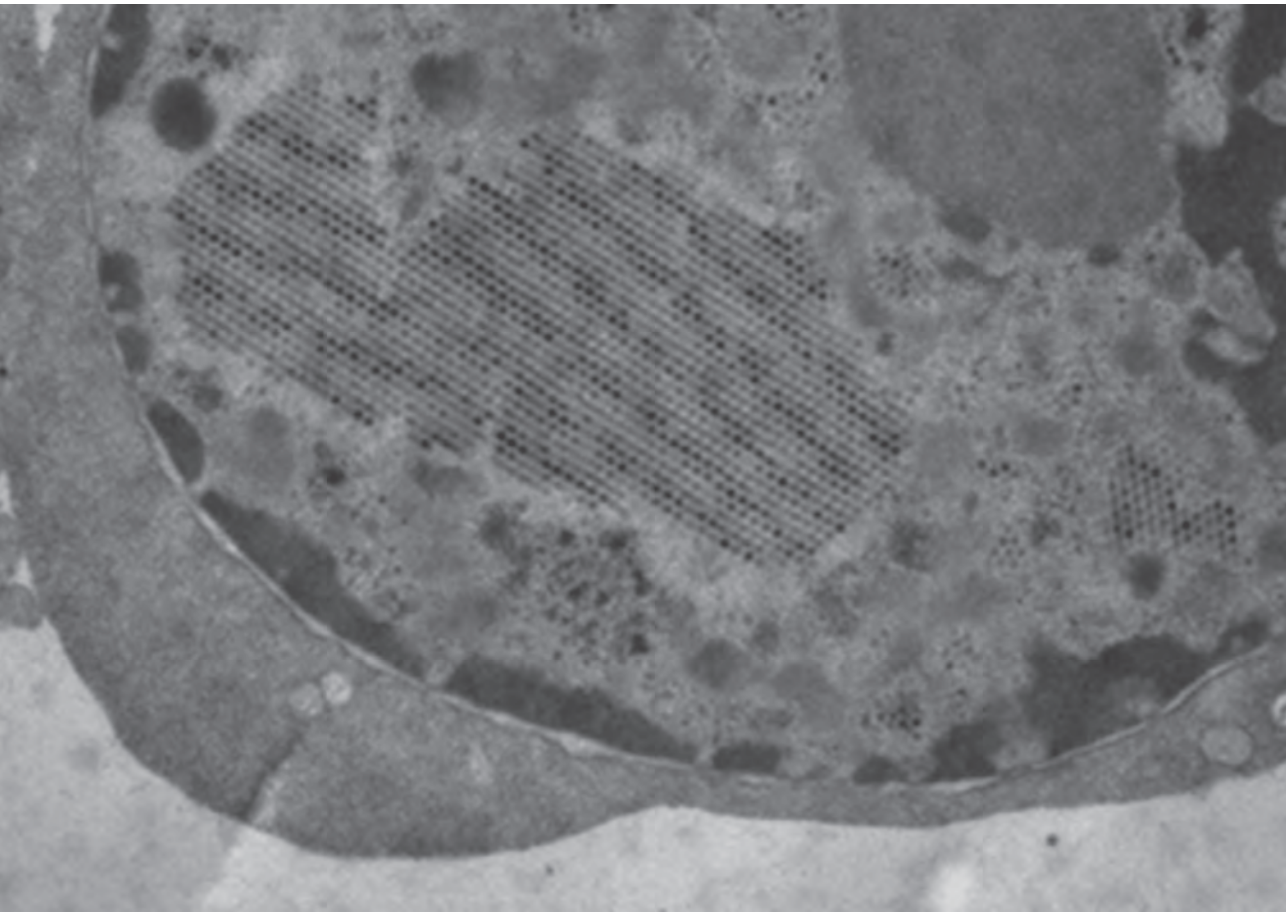
Chapter 5

Robustness of PER.C6® cell line for Adenovirus propagation



Abstract

Large-scale production processes are needed to cope with the increasing demand for production of adenoviruses (AdV) for gene therapy, cancer therapeutics or viral vaccines. To ensure good quality of the AdV produced, safety and quality features of the virus seeds and of the cells used as a substrate need to be controlled. However, it is difficult to characterize the quality of cells regarding virus propagation. Therefore we developed an assay to study the fitness of PER.C6® cells for AdV propagation, or the cells' infectability. PER.C6® is an efficient and safe complementing cell line for propagation of replication-deficient human AdV (hAdV). Infectability of PER.C6® cells was defined as their ability to both be infected and to replicate hAdV. On the one hand, kinetics of the infection process were measured by detection of intracellular hexon protein using flow cytometry. On the other hand, kinetics of virus production were measured through quantitative RT-PCR. First, kinetics of infection and of virus production were determined in exponentially growing and high viability cultures, to establish reference infectability data. Infectability was then measured on cultures exposed to various stresses as well as on cells sampled from a perfusion process within normal operating conditions and after a series of technical deviations causing suboptimal cultivation conditions. Overall, regardless of the effect of these stresses or perfusion process deviations on cell growth and cultures viability, infectability of PER.C6® cultures was not affected by any the stressors applied. These results show that PER.C6® is a robust cell platform for hAdV production.

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1 Introduction

Adenoviruses (AdV) are versatile delivery vectors for applications ranging from gene therapy to cancer therapeutics to viral vaccines (1-3). As the number of clinical trials for AdV-based products increases and the commercialization of these products is nearing, there is a need for efficient and robust large-scale production processes for these vectors (4-6). Platform processes developed for production of AdV aim at yielding consistent high virus titers. For these processes to be reliable and economically competitive, performance and yield must be not only high, but also repeatable and predictable. To achieve this, robustness of the substrate cell line for virus propagation therefore needs to be ensured.

Efficiency of infection processes are often related to process conditions during the virus production step. Parameters such as cell density at the moment of infection, cultivation conditions and medium composition and refreshment have been shown to be of importance (7-11). Definition and characterization of the optimal physiological state of substrate cells that relate to their ability to propagate viruses are however less widely published. In this line, increased osmotic pressure from 250 to 410 mOsm during the growth phase of HEK 293 cultures showed to improve AdV-5 productivity by 11-fold (12). Ferreira *et al.* showed that changing properties of HEK 293 cultivation medium influenced intracellular pH, which in turn affected specific AdV-5 productivity (13). Finally, decrease of culture temperature from 37°C to 33°C during the growth phase of E1-tranformed cell lines showed to increase AdV productivity 2 to 3-fold (14). Cellular responses to changes in growth conditions are complex and the effects in turn on virus production are not clearly elucidated in these studies.

Process changes can potentially be reflected at different levels of cell physiology and can in turn affect cell ability to propagate viruses at highest yields. Cell cycle distribution has showed to be of importance for AdV titers in HEK 293 (15,16), where increased portion of cells in the S phase improved virus production. A higher level of expression of receptors specifically binding AdV has also showed to increase HEK 293 cell-specific AdV productivity (17). Changes in HEK 293 cell metabolism are also known to occur upon infection with AdV (18-20), which suggests that the metabolic state of the cells at the moment of infection could be relevant for efficiency of virus replication.

The development of effective and productive AdV production processes could benefit from a better understanding of the effect of cultivation conditions to cell physiology and to productivity. Several methods have been developed to monitor effectiveness and efficiency of virus propagation. Kinetics of infection at the cell population level can be measured using flow cytometry and specific staining to discriminate between infected and non infected cells (21-23). An alternative is to quantify virus production over time upon infection (8,24), which can be done with quantitative real-time polymerase chain reaction (RT-PCR) (25-28). In the applications published, the aim was to either compare cell lines for replication of a virus, or to compare the replication behavior of different virus strains in one cell line, but never to focus on capturing the quality features of cells for virus production.

An interesting cell line for AdV production is the human PER.C6® cell line. PER.C6® was immortalized by transformation with adenoviral E1 genes (29,30), and is therefore a productive platform for propagation of replication-deficient AdV (11,31-33). In the

present work, the fitness and robustness of PER.C6® cells for AdV propagation were assessed under various normal and stressed culture conditions. The fitness of the PER.C6® cells was defined as their infectability, which includes cells' ability to both be infected by and replicate AdV. A multi-level assay was developed to measure the infectability of PER.C6® cells for AdV propagation. Kinetics of the infection process were measured by detection of intracellular hexon protein and kinetics of virus production were based on quantitative RT-PCR measurements. Infectability was firstly assessed for exponentially growing PER.C6® cultures exhibiting high viabilities. The resulting data were used to characterize reference infectability behavior for standard PER.C6® cultures. Infectability was secondly measured for cells cultivated in perfusion bioreactor both under normal operating conditions and after process extension, which yielded suboptimal process control conditions. The aim was to compare the fitness for AdV replication of PER.C6® cells cultivated in perfusion mode in bioreactor to that of reference cultures growing exponentially and with high viability, and to assess whether the perfusion cultivation could be extended while maintaining cells' fitness. Finally, infectability was measured on PER.C6® cultures exposed to severe osmolality, pH and starvation stresses, as these parameters were suspected to affect cells' ability to replicate AdV.

2 Materials and methods

2.1 Cell line and growth conditions

PER.C6® cells (Crucell, Leiden, the Netherlands) were thawed and cultivated in Crucell's proprietary and chemically defined PERMEXCIS® medium (Lonza, Verviers, Belgium) supplemented with 4 mM L-Glutamine (Invitrogen, Paisley, UK), referred to as "supplemented PERMEXCIS® medium" in the rest of this article. Seed trains were propagated twice per week with an inoculum density of 0.25 x 10^6 viable cells mL^{-1} in 850 cm^2 roller bottles (Corning Costar Inc., Amsterdam, the Netherlands) at 250 mL working volume. Cell counts and culture viability were measured using a Vi-CELL® XR counter (Beckman Coulter Inc., Woerden, the Netherldands). Main metabolites concentrations (glucose, lactate, glutamine and ammonium) were measured on a NOVA Bioprofile 400 (LA Biosystems BV, Waalwijk, the Netherlands). Cultivation bottles were rolled at 2 rpm in Cellroll systems (Micronic BV, Lelystad, the Netherlands) in humidified incubators at 37 °C in 10% CO2.

2.2 Bioreactor perfusion cultures

Perfusion PER.C6® cultures were inoculated in 13 L glass bioreactors (B. Braun Biotech, Melsungen, Germany) at 0.5 x 10^6 viable cells mL^{-1}. Temperature was controlled at 37°C by a heating blanket. Dissolved oxygen (DO) was controlled at 40% by sparging air and pure oxygen. The pH was controlled at 7.3 by addition of CO_2 to the gas inlet or sodium bicarbonate to the cultures. The same medium was used for inoculation and feed. An alternating tangential flow (ATF) filtration system (Refine, Pine Brook, NJ, USA) equipped with a 0.2 μm hollow fiber (GE Healthcare, Breda, the Netherlands) ensured cell retention during perfusion. Spent medium was extracted continuously while fresh

supplemented PERMEXCIS® was supplied to hold a constant working volume. Cultures were sampled daily except on day 5 for cell counting, viability determination and measurement of main metabolites concentration and osmolality as described above. A constant specific perfusion rate (m^3 $cell^{-1}$ s^{-1}) was applied throughout the process until cell density exceeded 100 x 10^6 viable cells mL^{-1}. At this point, a maximum feed rate was reached and maintained constant for the remainder of the culture duration.

2.3 Culture stresses

Two PER.C6® cultures were maintained in roller bottles for 7 and 10 days respectively without medium refreshment. For all other stresses, a PER.C6® seed train was split into four 850 cm^2 roller bottles (Corning Costar Inc., Amsterdam, the Netherlands). One culture was used as a control and grown for 4 days as described above. The other cultures were grown for 2 days as described above and thereafter independently exposed to several stresses as follows. Osmolality was increased from 290 to 430 mOsm by adding 5 M NaCl (Sigma-Aldrich, Zwijndrecht, the Netherlands). The pH was decreased by approximately 0.4 pH units by adding 4 M HCl (Sigma-Aldrich, Zwijndrecht, the Netherlands). One culture was exposed to 42 °C for 30 min in a water bath before being placed back in an incubator at 37 °C. Cultures were maintained under stressing conditions for another 2 days. Before and after exposure to the stresses, cell density and main metabolites concentration were measured as described above, osmolality was measured using a Gonotech Osmomat 030 (Salm en Kipp, Breukelen, the Netherlands) and pH was measured using an ABL5 (Radiometer, Zoetermeer, the Netherlands).

2.4 Measurement of infectability

2.4.1 Infection conditions

Cells from roller bottles or bioreactor perfusion cultures to test for infectability were medium-exchanged by centrifugation at 300 g for 5 min and resuspended in fresh supplemented PERMEXCIS® medium. 125 mL shaker flasks were inoculated at a working volume of 15 mL and at a target density of 1 x 10^6 viable cells mL^{-1}. Agitation was set to 100 rpm on an IKA KS 260 shaker plateau (VWR, Amsterdam, the Netherlands).

Cultures were infected with two human AdV (hAdV) serotypes, hAdV-A and hAdV-B. Both vectors are expressing different proteins. The same crude seeds of hAdV-A and hAdV-B were used for the entire study and their total viral particles concentration was determined using HPLC. For each cell source tested for infectability, shaker flask cultures were infected at two multiplicities of infection (MOI) for each virus, as described in figure 1. The MOI for each vector are given in table 1. Samples were taken from each shaker flask culture at regular time intervals post infection (p.i.).

2.4.2 Detection of infected cells by flow cytometric analysis

Samples from infected cultures containing 3-4 x 10^5 cells were fixed as follows. Cells were first washed twice in phosphate buffer saline (PBS, Life Technologies, Gent, Belgium) and

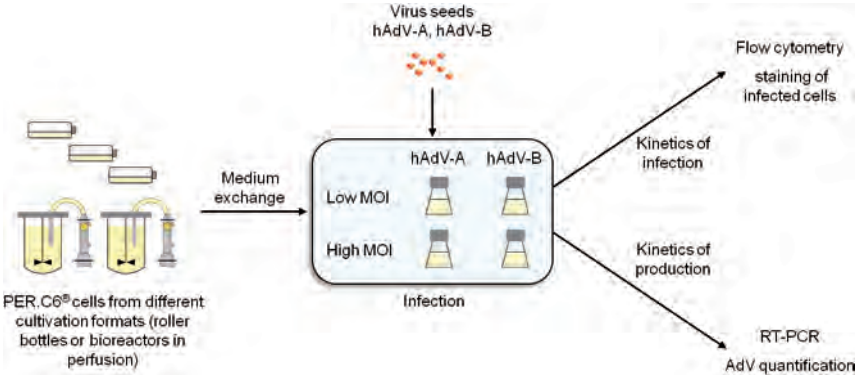

Figure 1. Schematic representation of the infectability assay.

incubated in 250 µL 1X CellFix (BD, Breda, the Netherlands) diluted in distilled water for 20 min at 4 °C. Cells were then washed again twice with PBS and stored at 4 °C until staining.

Intracellular viral capside protein hexon was specifically stained. Cells were first permeabilized in 700 µL of 1X Perm/Wash (BD, Breda, the Netherlands) diluted in distilled water (Life Technologies, Gent, Belgium). 1X Perm/Wash was used as a washing and dilution buffer for all subsequent steps. Cells were incubated for 30 min at 4 °C in 100 µL of a mouse anti-hexon monoclonal antibody (MAB8051, Merck Millipore, Amsterdam Zuidoost, the Netherlands) diluted to a concentration of 1 µg mL^{-1}. After two washes, cells were incubated for 30 min at 4 °C in 100 µL of a R-PE goat F(ab')2 anti-mouse IgG (I.T.K. Diagnostics, Uithoorn, the Netherlands) diluted to a concentration of 1 µg mL-1. After two final washes, cells were resuspended in 250 µL of 1X CellFix and stored at 4 °C in the dark until analysis.

Flow cytometry analyses were performed on a Guava PCA (Merck Millipore, Amsterdam Zuidoost, the Netherlands) using the Express module of the EasyCyte system with the CytoSoft software version 6.0.2 (Merck Millipore, Amsterdam Zuidoost, the Netherlands). Single cells were gated based on the forward light scatter. The fluorescence of 1500 single cells was analyzed. A negative control consisting of mock infected cells was used to gate non infected cells and cells outside of this gate were considered infected.

2.4.3 Virus particles titration by quantitative RT-PCR

Crude infected samples were lysed in 1% triton-X100 (Sigma-Aldrich, Zwijndrecht, the Netherlands) to release all virus particles from cells. DNA that was not encapsidated or DNA from incomplete virus particles was digested by DNAseI (Roche Diagnostics, Almere, the Netherlands) during a 15 min incubation with 5 µL of a solution at a final concentration of 10 mg mL^{-1}. Quantitative RT-PCR were carried out using the Applied Biosystems® TaqMan® Gene Expression master mix (Life Technologies, Gent, Belgium) with a total reaction volume of 15 µL. The primers and probe designed to amplify the hAdV promoter (Life Technologies, Gent, Belgium) were used at a final concentration of 667 mM and 6.67 µM, respectively. 5 µL of the sample DNA was added to 10 µL

Table 1. Summary of kinetics of population infection and of virus production for reference cell infected with hAdV-A and hAdV-B at high and low MOI.

		Kinetics of population infection			Kinetics of virus production			
Virus serotype	MOI (VP cell^{-1})	Onset (h p.i.)	Maximum infected population (%)	Time at which maximum is reached (h p.i.)	Onset (h p.i.)	Maximum titer (^{10}log VP mL^{-1})	Time at which maximum is reached (h p.i.)	Cell-specific productivity (VP cell^{-1})
hAdV-A	100	26	92	60	26	9.9	>97	8,218
	900	0	87	26	<26	10.3	47	19,053
hAdV-B	1	30	94	60	<26	11.5	>97	354,812
	70	13	90	24	<26	11.3	30	169,754

VP: virus particles; p.i.: post infection.
MOI: multiplicity of infection

of reaction mix. Each sample was tested in triplicates. Purified hAdVA and hAdV-B with known virus titers were used as a standard. A 10-fold dilution series was performed and 5 μL of each standard dilution was analyzed in triplicate to obtain a 7-points standard curve. A negative control reaction contained 5 μL of dilution buffer. Thermal cycling was performed using a ViiA7 RT-PCR system (Life Technologies, Gent, Belgium) with first an incubation at 95 °C for 8 min followed by 35 cycles of 95 °C for 10 s and 60 °C for 30 s. The number of DNA copies in the samples was calculated using the standard curve and the average of the triplicates was expressed in $\log_{10}$ virus particles (VP) mL^{-1}.

Cell-specific virus productivity was calculated by firstly subtracting the input virus from the mean estimate maximum virus titer and secondly dividing this number by the viable cell density at the moment of infection.

2.5 Data analysis

First, specifications were set using reference data on the two infectability responses, namely kinetics of population infection and kinetics of virus production. A four-parameter logistic regression (4PL) model was found suitable for the analysis of such kinetics. Two global 4PL models with covariates for hAdV serotype and MOI were thus estimated on the two responses and the 95% prediction intervals (PI) were computed using simulations. The interpretation of such an interval is that 95% of future individual measurements with similar kinetic behavior should be found within the interval. Then, new data coming from test experiments (stressed cultures and perfusion cultures) were added to the graphs of the reference models, and were compared to the 95 % PI obtained using the reference data. Equivalence was accepted if most of the new individual data (ideally, 95%) was found within the reference interval. All results were obtained using SAS 9.3 for Windows 7.

3 Results and discussion

3.1 Characteristics of infectability of PER.C6® cultures

3.1.1 Establishment of reference infectability data

In order to characterize the infectability behavior for standard PER.C6® cultures, infectability was measured on 6 independent PER.C6® cultures with high viability (above 90%) and in exponential growth phase. The level of cell population infection was determined by measuring intracellular hexon protein, and the dynamics of virus production were determined by quantification of full DNA copies from complete encapsulated virus particles. The mean estimates of these two infectability responses and the corresponding 95% prediction intervals (PI) are shown in figure 2. Note that the 95% PI could not be determined for hAdV-B virus titer at high MOI because the parameters from the 4PL model were highly correlated for this particular condition, given the number of replicates available.

The range of the 95% PI for the two infectability responses for these standard PER.C6® cultures represents the variation of the biological assay. To capture variation related to cell source, these 6 standard cultures originated from 4 development PER.C6® cell banks, which all had different passage numbers from the master cell bank. However the variation

measured in this dataset was not caused by the cell bank of origin and the number of doublings of the cultures at the moment of measuring infectability (data not shown).

The two aims here were first to capture the variation from the individual biological responses and to represent a prediction of where future measurements will fall, and second to assess whether the infectability measurements obtained after process change were in the same range as the reference measurements. Therefore PI are presented rather than confidence intervals (CI). Indeed, CI would only reflect the uncertainty around the estimation of the mean kinetic of each cultivation condition. On the other side, PI describe the individual data instead of their mean and additionally include the variation of the measurements. Obviously, the PI are larger than CI, standard deviation or standard error, that are generally presented in other publications studying kinetics of viral infection in different cell lines (23,24,35,36).

3.1.2 Effect of variation of MOI on kinetics infection and virus production

For infectability measurements, PER.C6® cultures were infected with two hAdV serotypes at two MOI each: 100 and 900 VP cell^{-1} for hAdV-A and 1 and 70 VP cell^{-1} for hAdV-B. The main characteristics of infectability in reference PER.C6® cultures for hAdV-A and hAdV-B at high and low MOI given are summarized in Table 1, which is directly derived from figure 2. MOI is known to influence kinetics of cell infection (6,21,23). Two MOI (high and low) per hAdV serotype in the infectability assay were included to increase the assay resolution by challenging the cells with different virus inputs. During the development of the infectability assay, high and low MOI for each hAdV serotype were screened to match the kinetics of cell population. Therefore the high and low MOI values for the two hAdV serotypes are very different (Table 1).

Cultures infected with low MOI of hAdV-A and hAdV-B were both fully infected after 60h p.i. and cultures infected with high MOI of hAdV-A and hAdV-B were fully infected after 24h and 26h p.i., respectively. For both hAdV serotypes, the onset of population infection was earlier at high MOI than at low MOI, respectively 0 vs 20 h p.i. for hAdV-A and 13 vs 25 h p.i. for hAdV-B. Similar to studies conducted with other cell lines (6,21,23), increased MOI of hAdV accelerated the progression of infection in PER.C6® cultures.

For both hAdV-A and hAdV-B, similar maximum titers were obtained at the two MOI. A plateau in virus titers was captured for the two high MOI conditions, whereas titers still increased at 100 h p.i. for the two low MOI. Full population infection was therefore only completed for the high MOI conditions. Cell-specific virus productivity was calculated for each infection condition (Table 1) and was on average 19-fold higher for hAdV-B than for hAdV-A. This difference can be related to serotype-specific replication features and to expressing of the transgenes present in the two hAdV constructs.

3.2 Infectability of perfusion PER.C6® cultures

After establishing reference infectability data for standard PER.C6® cultures in exponential growth phase and with high viabilities, infectability of cells cultivated in perfusion bioreactor both under normal operating conditions and under suboptimal

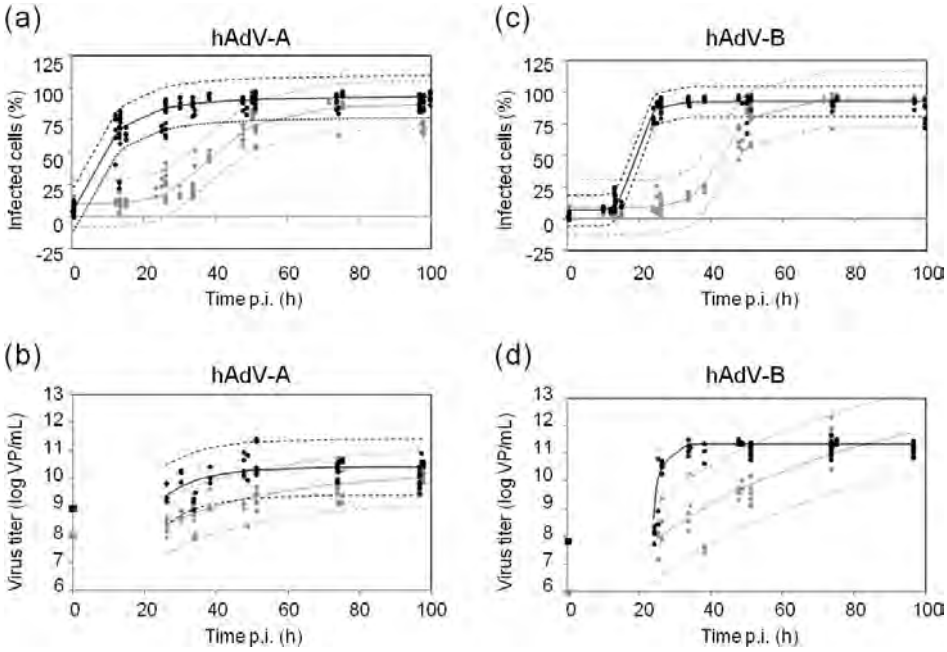

Figure 2. Infectability of reference PER.C6® cultures in exponential growth phase and with high viabilities above 90%. Time courses of infected cells and virus titer upon infection with hAdV-A (a and b) and hAdV-B (c and d) at low MOI (grey lines) and high MOI (black lines). Mean model estimates are shown by bold lines. Dashed lines represent 95% PI. Dots represent the raw data and squares the input virus concentration at t=0.

process control conditions was measured. The aim of this study was to asess whether the operating conditions applied to cultivate seeds of PER.C6® cells are optimal for virus production, and also to evaluate whether these conditions could even be extended to generate more cells, to potentially increase the yield of the perfusion process.

Under normal operating conditions of the perfusion process, a constant specific perfusion rate enables a fast exponential cell growth. The process generally ends after 10 or 11 days, when the cell density required to start the subsequent virus production process is reached. In a first experiment, infectability of cells from 3 perfusion cultures was measured on day 4, 7 and 10 of the process. In a second experiment, 3 perfusion runs were extended in order to study cells' fitness beyond normal operating conditions. A representative cell growth and viability profile for an extended perfusion run is shown in figure 3. A maximum total cell density of 307 x 10^6 viable cells mL^{-1} was obtained on day 14 at a viability of 83%. For the extended runs, the bioreactor set-up was the same as for standard cultures terminated on day 10 or 11, meaning that the systems were not specifically modified to support cell densities beyond the normal operating range. Upon process extension, nutrients and mass transfer requirements for the culture exceeded the capacities of the control system to maintain optimal conditions, which led

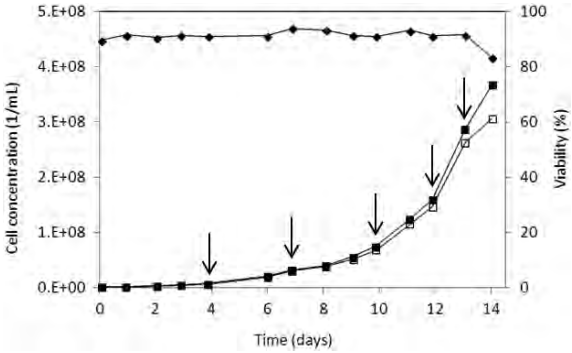

Figure 3. Cell growth profile for a perfusion PER.C6® cell expansion culture. Total (■) and viable (□) cell concentrations and viability (♦) are showed for a representative run for 14 days. Arrows show when samples were taken for infectability measurements.

to suboptimal process control and cultivation conditions. On day 11, perfusion rate no longer increased with cell density but was maintained constant. On day 12, disolved oxygen (DO) could no longer be controlled at setpoint as maximum capacity of the mass flow controller supplying air to the system was reached. Finally on day 13, pH could no longer be controlled at setpoint because the volumes of base required to maintain pH became critical for the osmolality of the culture, which ultimately resulted in the abortion of the experiment. During this second experiment, cell samples were taken for infectability measurements within normal operating conditions on day 7 and 10, as well as beyond normal operating conditions on day 12 and 13.

Infectability profiles of cells from the perfusion process are plotted against the estimate of the mean infectability and the 95% PI of the reference cultures previously described (Figure 4). The kinetics of infection and of virus production of cells from the perfusion process were very similar among each other. Only 11 data points out of the 648 shown in figure 4 fell outside of the 95% PI, which is well below the expected 1 data point out of 20 outside these PI. Data from figure 4 were further analyzed to evaluate if culture day in the perfusion process affected infectability (data not shown). As we did not observed any trend based on this parameter, data points are not represented by specific symbols in the figure. The extremely high cell density reached in prolonged perfusion runs, as well as the decreased specific feed rate and interruption of both DO and pH controls were expected to affect the fitness of PER.C6® cells for hAdV replication. Nevertheless, infectability of PER.C6® cells was maintained even under these suboptimal conditions.

3.3 Infectability of stressed batch PER.C6® cultures

In order to test the robustness of the PER.C6® cell line for hAdV production when cultivated under defined and severe stress conditions, infectability was measured after exposure of cultures to several isolated cultivation stresses. Instead of perfusion cultures we now used batch PER.C6® cultures exposed to high osmolality, low pH, high

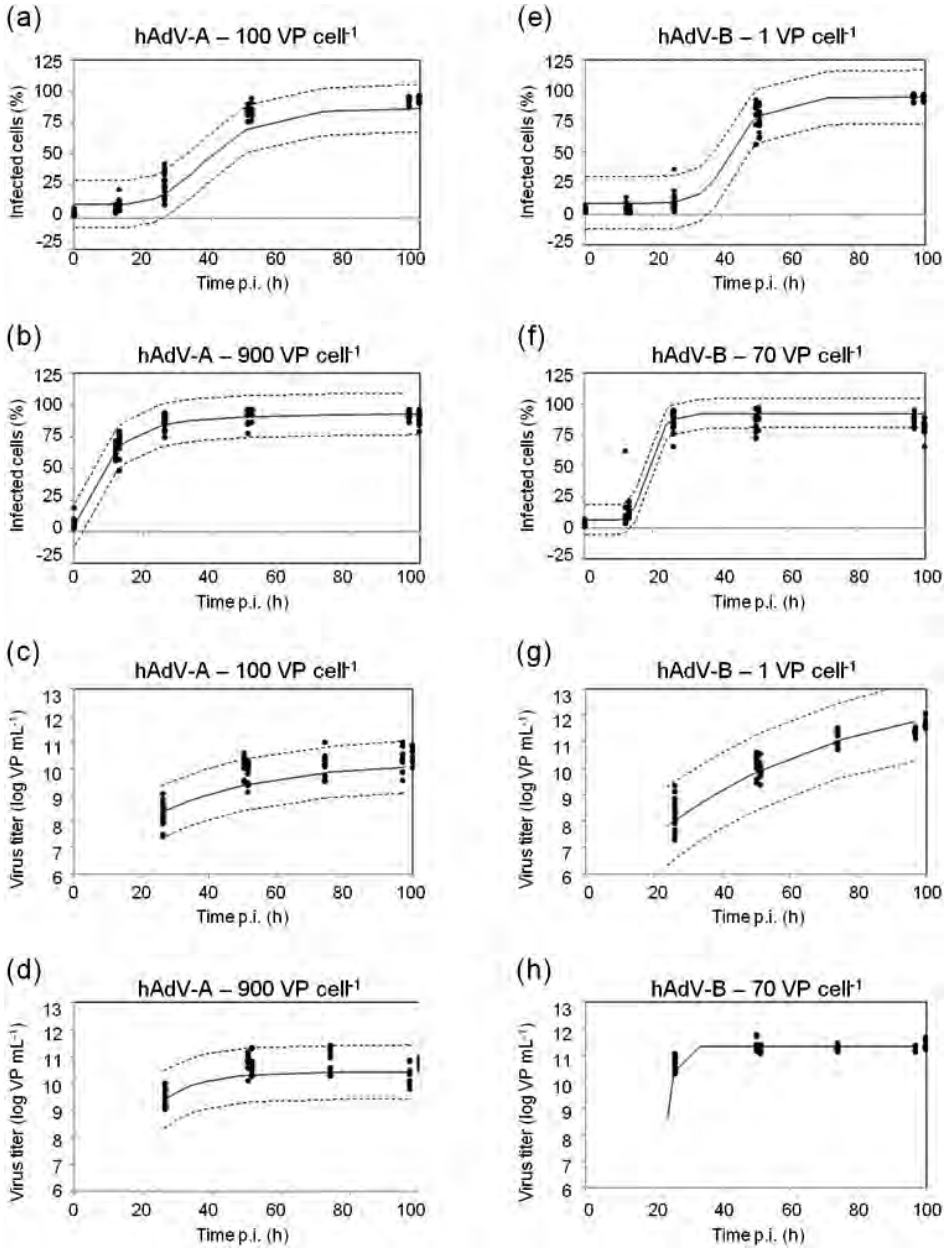

Figure 4. Comparison of infectability of reference PER.C6® cultures and cultures from perfusion expansion process on day 4, 7, 10, 12 and 13 (•).Time courses of infected cells and virus titer upon infection with hAdV-A (a, b, c and d) and hAdV-B (e, f, g and h) at low MOI (a, c, e and g) and high MOI (b, d, f and h). Mean model estimates are shown by bold lines. Dashed lines represent 95% PI.

temperature and starvation stresses, as these parameters have shown to affect hAdV productivity of HEK-293 (10,13,14,18-20). Cell growth and metabolism parameters of control and stressed cultures immediately before infectability measurements are presented in table 2. Osmolality increase was the most drastic stress as it caused a 10% loss in viability and a stop in cell growth. The 7-days and 10-days cultures were exposed to depletion of glucose and glutamine and to higher concentrations of lactate and ammonia. Cell density of the 10-days culture reached levels at which PER.C6® cells are at the end of the exponential growth phase. Oppositely, pH stress did not affect culture growth rate nor viability. Temperature stress caused a 30% decrease in growth rate compared to the control culture, while viability was maintained. Concentration of main metabolites in these two stress conditions mainly varied in relation to cell growth. Note that the higher glucose concentration in the culture with pH stress, reaching 138% of that of the control, is an exception which could not be fully explained. This could be either due to analytical variation or to an effect of pH stress, resulting in more efficient use of other nutrients as carbon sources than glucose (e.g. amino acids).

Infectability profiles of stressed cultures were compared to those of the reference cultures (Figure 5). Note that for the kinetics of cell population infection, the first data point at 12 h p.i. is missing because of a technical error for all stressed cultures with the exception of the 7-days culture (for the two virus serotypes at the two MOI) and for the culture with temperature stress with hAdV-B at MOI 70VP $cell^{-1}$. The kinetics of infection and of virus production of stressed cultures were overall very similar among each other. The majority of the data points (141 data points out of the 145 shown) fell within the 95% PI of the reference cultures or in a near neighborhood. This shows that infectability of stressed and starved PER.C6® cultures was maintained. The only data points falling outside the 95% PI are the last point of the infection kinetics (at 96.5 h p.i.) for all stressed cultures infected with hAdV-B at high MOI, except for the 7-days culture (Figure 5f). The reasons for this decrease in percentage of infected cells at the end of the infection process are unclear and this was not reflected in the virus productivity of these cultures (Figure 5h).

Several groups have investigated changes in cell physiology during growth phase prior to AdV virus production, with the aim of improving virus yields. Cultivation of HEK-293 cells under hyperosmotic stress yielded increased AdV titers. On the contrary, a decrease in intracellular pH of these same cell line reduced AdV productivity (12,14). As pH and temperature stresses did not drastically affect the physiological characteristics of PER.C6® cultures, it was not expected that infectability would be affected by these stressors. Oppositely, the drastic effects of osmotic stress on cell growth and culture viability were expected to affect cells' fitness for hAdV propagation. Yet in our experiments, the changes in PER.C6® cultivation conditions did not affect productivity in any way, positive or negative.

Table 2. Effect of stresses applied to PER.C6® cultures on cell growth and metabolism. Measurements were performed right before transfer of the cells to the infectability assay.

Culture condition	Viability (%)	Viable cell density (x10^{6}cell/mL)	Growth rate (h^{-1})	Osmolality (mOsm)	Metabolites concentration (mM)			
					Glucose	Lactate	Glutamine	Ammonia
Control	94	1.58	0.024	279	7.3	19.8	2.27	1.29
Osmoloality stress	84	0.62	0.03	429	9.2	18.3	2.42	1.71
pH stress	92	1.70	0.023	284	10.1	13.8	2.2	1.47
Temperature stress	95	1.66	0.017	277	6.9	18.8	2.03	1.23
7–days culture	92	3.74	0.019	271	1.6	21.5	0	2.16
10-days culture	87	5.19	0.013	261	0	11.7	0	2.13

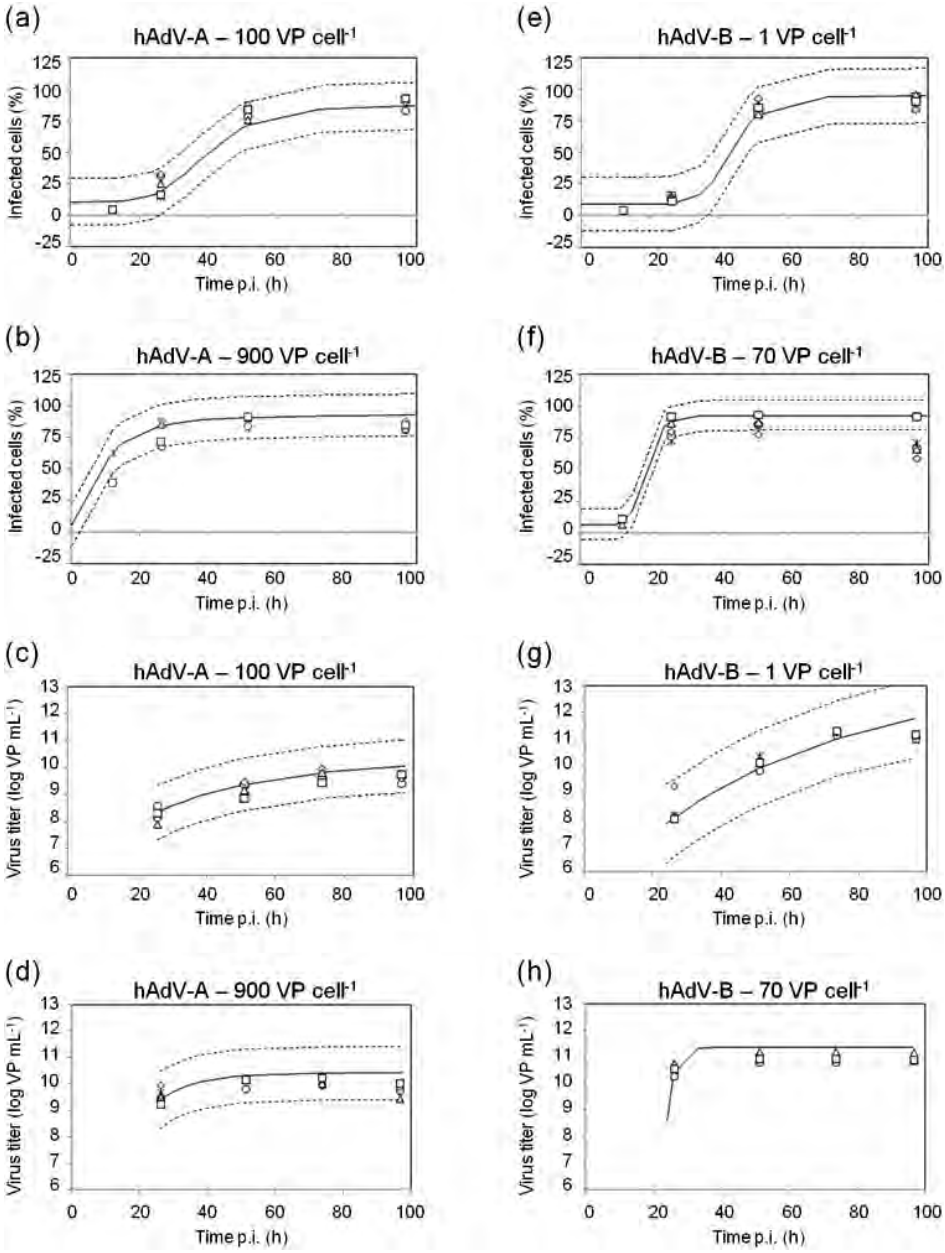

Figure 5. Comparison of infectability of reference and stressed PER.C6® cultures. Time courses of infected cells and virus titer upon infection with hAdV-A (a, b, c and d) and hAdV-B (e, f, g and h) at low MOI (a, c, e and g) and high MOI (b, d, f and h). Mean model estimates are shown by bold lines. Dashed lines represent 95% PI. Stressed cultures: (✱) 10-days culture, (□) 7-days culture, (Δ) temperature stress, (◊) pH stress, (**O**) osmolality stress.

4 Conclusions

In this study, a broad range of stressors was applied to PER.C6® cultures in order to mimic a variety of aberrant processing conditions. The stressors induced several complex effects at the cellular level, from physico-chemical environmental changes (osmolality increase and pH decrease), to metabolic activity changes (reflected in the different metabolites concentrations measured in the cultures). In all cases, regardless the effect of these stresses or of these process deviations on cell growth and cultures viability, the resulting infectability profiles were comparable to those of optimally growing reference cells.

We did this work to study whether the cells grown in perfusion prior to hAdV infection were prone to variation in quality attributes that could affect the subsequent virus infection process. This study clearly shows that cells at all stages of normal operation and even when subjected to severe stressors retain their ability to be infected by and propagate hAdV. Robustness of the cell line used as a substrate for virus production is key to develop high performing platform processes suitable for large scale processes for late stage clinical testing or product commercialization. Overall, this study shows that PER.C6® is a robust cell platform for hAdV production.

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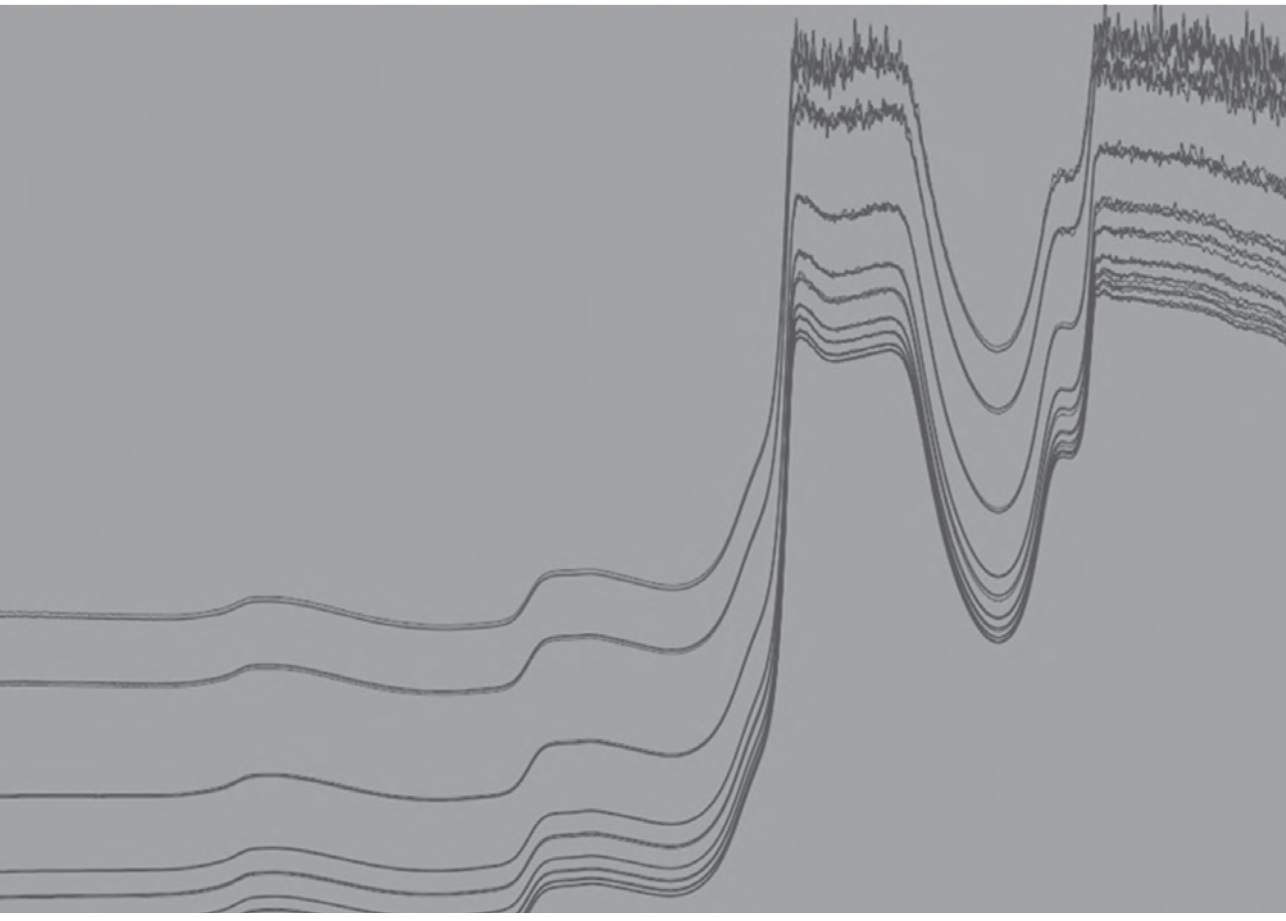
Chapter 6

PAT tools for a perfusion PER.C6® cultivation process: dielectric and near-infrared spectroscopy



Abstract

Cell cultivations are the most complex operation units of a bioprocess to produce biological therapeutics. In these processes, good process performance and good product quality can be ensured by online monitoring of critical process parameters. These can be used in real-time for process control, as suggested by the Process Analytical Technology (PAT) initiative. Today, solutions for real-time monitoring of critical parameters such as concentrations of cells, main nutrients and metabolism by-products are developing, but their applications in industrial settings are still limited. We evaluated in this study the use of dielectric spectroscopy (DS) and near-infrared spectroscopy (NIRS) as PAT tools for a PER.C6® cell expansion perfusion process, defining the optimal calibration strategy for these two tools. With DS and NIRS, accurate predictions of viable cell density and of glucose and lactate concentrations were achieved. We show that DS and NIRS are complementary PAT tools for monitoring of a perfusion PER.C6® cultivation process. We also show that the perfusion stream offers great opportunities for non invasive, yet frequent process monitoring. With the current trend towards more continuous bioprocesses, accurate online monitoring of critical process parameters with PAT tools is contributing to the continuous effort to increase control of process output.

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1 Introduction

Mammalian cell cultivations for the production of biological therapeutic products are complex. In order to ensure good process performance and consistent product quality, process parameters should be monitored online and used in real-time for process control. The US Food and Drug Administration advises (bio)pharmaceutical companies to use Process Analytical Technology (PAT) for better control of bioprocesses in order to consistently deliver high quality products (1). In applications of PAT to cell cultivation processes, parameters such as pH, temperature or dissolved oxygen are often controlled using data from well established classical sensors (2,3). Solutions for real-time monitoring of other relevant analytes such as concentrations of cells, main nutrients and metabolic by-products (glucose, glutamine, lactate, *etc.*) are also being developed, but their applications in industrial settings are still limited.

Among the established methods for online biomass monitoring, dielectric spectroscopy (DS) measuring permittivity provides the most direct information on biomass concentration (2,4). DS measures the capacity of the cells to store electrical charges. This measurement is therefore related to both the density of viable cells with intact cytoplasmic membranes, and the electrical properties of the membranes (5,6). Applications of DS for cell culture monitoring have been reviewed in several publications (5,7-9). DS has been used for mammalian and insect cell lines to establish correlations between permittivity and viable cell volume and to develop prediction models for cell density (4,10-13). Direct translation of permittivity into cell densities is only possible when variations in cell diameter are small throughout the process (4,14,15). However, in most fed-batch and perfusion processes cell diameter is not constant. Assessing whether the accuracy of viable cell density predictions could be improved by accounting for changes in cell diameter in such processes has not been reported yet.

Another interesting tool for online monitoring of cell cultivations is near-infrared spectroscopy (NIRS). NIRS is a non-invasive and non-destructive vibrational spectroscopy technique capturing real-time measurements of multiple variables in one single measurement (16,17). The use of NIRS for cell cultivation has been established for over a decade for predictions of total or viable cell density and concentrations of main metabolites (mainly glucose and lactate), which are parameters routinely monitored off-line (18-22). To obtain these predictions, multivariate models are calibrated to correlate variations in NIRS spectra with changes in concentration or level of the parameters to monitor. Partial least squares (PLS) regressions are the most widely used multivariate technique for NIRS calibration (23,24). The co-linearity among process variables due to metabolic stoichiometry during cell growth causes in turn challenges for accurate NIRS calibrations. This is often overcome by the use of semi-synthetic calibration samples to break the correlations (25,26). Accurate predictions of mammalian cell density and main metabolites concentrations have been shown from at-line NIRS measurements using samples extracted from the bioreactor cultures (27-29) and also from online NIRS measurements using *in situ* probes submerged in the cultures (19-22,26,30). In all these studies, batch or fed-batch processes were monitored. Here we report the application of NIRS on a high density perfusion culture of PER.C6® cells.

PER.C6® is an industrial human cell line used for the production of several vaccines (31-33), which can be grown to densities exceeding 150 x 10^6 viable cells mL^{-1} in perfusion (34,35). In perfusion systems optimal growth conditions are maintained because nutrients required for cell division are continuously supplied and by-products inhibiting cell growth are removed. Application of PAT on a perfusion process therefore aims at monitoring and controlling the process parameters to ensure optimal cell growth.

The objective of this study was to evaluate both DS and NIRS as PAT tools for a PER.C6® perfusion process. The focus was on defining the optimal calibration strategy for these two tools. Accurate predictions of viable cell density and of glucose and lactate concentrations were achieved using a combination of DS and NIRS. The possibilities this creates to use these PAT tools to further improve process control are discussed.

2 Materials and methods

2.1 Perfusion cultures and sampling methods

PER.C6® (Crucell, Leiden, The Netherlands) perfusion cultures were inoculated in 13 L glass bioreactors at 0.5 x 10^6 viable cells mL^{-1} in a 10 L working volume. PERMEXCIS® medium (Lonza, Verviers, Belgium) supplemented in house was used. Temperature, pH, dissolved oxygen (DO) and stirrer speed were controlled at set-point. Perfusion was operated with an alternating tangential flow (ATF) system (Refine, Pine Brook, NJ) equipped with a 0.2 μm hollow fiber (GE Healthcare, Breda, the Netherlands). Cells were retained in the bioreactor vessel while filtrate of spent medium was continuously extracted. Fresh medium was added at a constant specific feed rate to maintain a constant working volume. A total of 35 perfusion bioreactors were used as a support for this study.

In this perfusion process, total cell density typically increases exponentially, reaching approximately 75 x 10^6 cell mL^{-1} on day 10 (Figure 1a). Although perfusion starts immediately after inoculation, main metabolite concentrations evolve similarly to a batch culture during the first 4 days, with glucose consumption and lactate accumulation (Figure 1b). From day 4 onwards, metabolite concentrations are maintained constant.

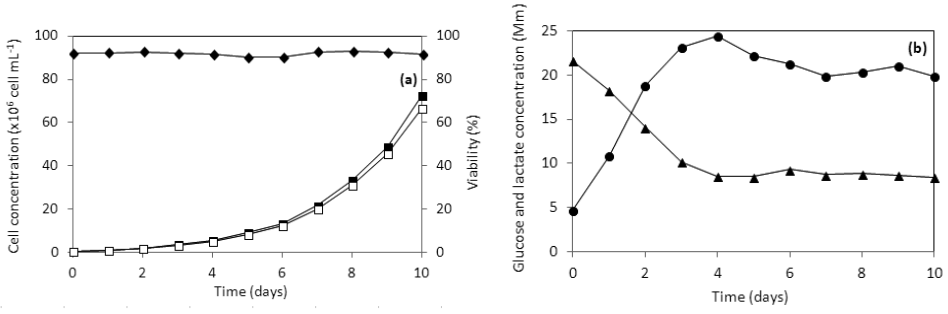

Figure 1. Representative cell growth (a) and main metabolite concentrations (b) during the perfusion PER.C6® cell expansion process. Total (■) and viable (□) cell densities, viability (♦), glucose (▲) and lactate (●) concentrations.

Bioreactor samples were obtained by direct sampling of bioreactors (Figure 2). These samples contained cells and all other components of the cultures. Permeate samples were collected from the permeate line of the ATF, after filtration through the hollow fiber.

2.2 Off-line reference analyses

Cell density, viability and mean cell population diameter were measured using a ViCell Counter XR (Beckman Coulter Inc., Woerden, The Netherlands). Glucose and lactate concentrations were measured on a NOVA BioProfile 400 (LA Biosystems BV, Waalwijk, the Netherlands).

2.3 Dielectric spectroscopy (DS)

2.3.1 Online DS measurement

Capacitance was measured online with a Fogale iBiomass 465 system and directly converted into permittivity (Applikon, Delft, The Netherlands). Measurements were taken every 12 minutes as described elsewhere (11). The frequency scans were obtained between 1 and 10 MHz.

2.3.2 DS data analysis

Two analysis strategies were applied. Firstly, a linear regression was used to directly correlate viable cell density (VCD) and permittivity ($\Delta\varepsilon$) (Equation 1). The intercept was constrained to zero as permittivity is assumed to be zero when no cells are present.

$$VCD = k_1 \times \Delta\varepsilon \quad (1)$$

Secondly, changes in mean cell population diameter were used to predict VCD from permittivity. For that, viable cell volume (VCV) was calculated from VCD and mean cell diameter measured off-line (with a ViCell Counter XR), using the formula of a sphere

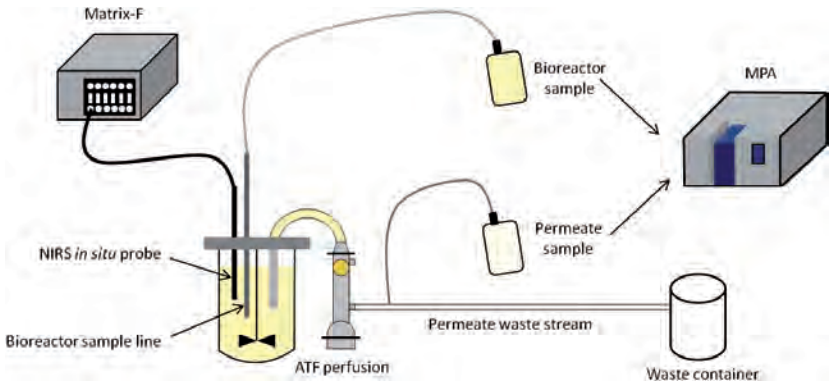

Figure 2: Procedure for sample collection for NIRS measurements on a perfusion cell culture process. Samples were collected both directly from the bioreactor (bioreactor samples) as well as from the permeate line of the ATF. These samples were analyzed at-line using an MPA FT-NIR analyzer. Online NIRS measurements were done by a Matrix F FT-NIR analyzer using an in situ probe.

volume (Equation 2), where V is the culture volume (in L). Then permittivity was correlated to VCV using a linear regression with the intercept also constrained to zero (Equation 3). Changes in cell population mean diameter ($\widehat{D}$) over time (t) during the cultivation process were described using a linear regression (Equation 4) (c represents the intercept). The resulting estimation of mean cell diameter over process time was used to calculate VCD from VCV, also using the formula of a sphere volume (Equation 2).

$$VCV = \frac{4}{3} \times \pi \times VCD \times V \times (\frac{D}{2})^3 \quad (2)$$

$$VCV = k_2 \times \Delta\varepsilon \quad (3)$$

$$\widehat{D} = k_3 \times t + c \quad (4)$$

2.4 Near-infrared spectroscopy (NIRS)

2.4.1 NIRS spectra acquisition

Bioreactor samples and samples from the permeate waste line were measured at-line (Figure 2). To enlarge the ranges of metabolite concentrations and to break correlations naturally occurring because of the PER.C6® metabolism, semi-synthetic samples were generated by spiking glucose (Lonza, Breda, The Netherlands) and lactate (Sigma-Aldrich, Zwijndrecht, The Netherlands) from stock solutions of known concentration.

At-line NIR spectra were measured in triplicate with a multipurpose analyzer (MPA) Fourier transform near-infrared (FT-NIR) analyzers from Bruker Optics (Delft, the Netherlands). Samples were homogenized and pre-heated at 30°C in glass disposable cuvettes of 8 mm optical path length.

Online NIRS analyses were performed in situ using a FT-NIR Matrix-F equipped with a transflectance probe with a path length of 2 mm (Bruker Optics, Delft, the Netherlands) that collected both transmitted and reflected light every 8s. The dataset was reduced prior to further analysis to one spectrum every 4 hours because this time period is sufficient to capture dynamics of a mammalian cell cultivation process.

Each spectrum, at-line or online, was an average of 16 scans with 8 cm^{-1} of spectral resolution recorded in the wavenumber range of 4 000 to 12 000 cm^{-1}, resulting more than 2 000 points for each spectrum. A single background spectrum was used with every sample spectrum to set up the baseline intensity.

2.4.2 NIRS data analysis and chemometric approach

Model calibration

The saturated regions due to absorption of water, between 4000 and 5340 cm^{-1} and between 6430 and 7200 cm^{-1}, were removed as they add noise add noise and unnecessary complexity to spectra (36). Several mathematical pretreatments of the spectra were used: standard normal variation (SNV), multiplicative scatter correction (MSC) and first derivative, as described by Næs *et al.* (37).

For at-line bioreactor samples, two types of partial least squares (PLS) calibration techniques were used: PLS type 1 (PLS1) and PLS type 2 (PLS2). With PLS1, parameters

are predicted individually and models can be fully optimized to obtain the most accurate predictions. Oppositely, with PLS2 all parameters of interest can be predicted simultaneously with one global model, but the modeling settings are applied for all parameters to predict. As optimal NIRS regions were better identified with PLS1 than PLS2, the PLS2 model was constrained to only use the NIRS regions identified with PLS1. For at-line permeate samples from the permeate line of the ATF and for online measurements, only PLS1 models were calibrated. The number of spectra and the origin of the samples used to calibrate all models are given in table 1.

Model validation

For at-line models, a cross-validation was first used to identify the optimal number of latent variables (LV), spectral pretreatment techniques and NIRS regions to be used. Best models displayed the lowest root mean square error of cross-validation (RMSECV), calculated as described elsewhere (38). Secondly, a test set validation was performed. The predictive ability of the models was evaluated using the correlation coefficient R^2 between reference and predicted values and the root mean square of prediction (RMSEP), calculated as described elsewhere (38). RMSEP was also expressed in percentage of the calibration range, by dividing the RMSEP values by the calibration range and multiplying by 100. Best prediction models presented the lowest RMSEP (in initial unit or in % of calibration range) and the closest R^2 to 1.

For online models, only a cross-validation was possible as reference values were not available for other spectra than those used in the calibration data set. Best models displayed the lowest RMSECV.

Software

PLS1 modeling was done using OPUS Quant2 (OPUS 6.5, Bruker, Germany). PLS2 modeling was done using SIMCA 13.0 (Umetrics AB, Kinnelon, NJ).

Table 1. Composition of calibration and prediction datasets for multivariate prediction models built from bioreactor and permeate NIRS spectra.

Measurement and modeling technique	Sample type	Dataset	Number of spectra		Number of bioreactor runs included
			from natural samples	from semi-synthetic samples	
At-line (PLS1 and PLS2)	Bioreactor	Calibration	231	30	10
		Prediction	84	15	5
	Permeate	Calibration	279	30	16
		Prediction	216	15	11
Online (PLS1)	Bioreactor	Calibration	12	0	1
		Prediction	80	0	1

PLS: partial least square.

3 Results and discussion

3.1 Dielectric spectroscopy to monitor viable cell density

Online permittivity using dielectric spectroscopy (DS) and off-line cell density, viability and metabolite concentrations were measured in 6 perfusion runs. Two modeling strategies were compared to predict viable cell density (VCD) from permittivity, either using a direct prediction or accounting for changes in cell diameter. For the first strategy, the model was built based on the correlation between permittivity and VCD (Figure 3a). For the second strategy, correlation between permittivity and viable cell volume (VCV) (Figure 3b) and changes in mean cell population diameter over process time (Figure 3c) were both used. Predictions of VCD were done using the two models on 5 independent perfusion runs (Figure 4). The two models accounting or not for changes in cell diameter closely fitted the data, with RMSEP of 6.8% and 7.2% of the calibration range, respectively and a R^2 between predicted and measured values of 0.96 in both cases.

These data showed that permittivity measured by DS can be modeled with simple linear regressions to monitor VCD in a PER.C6® perfusion process with very high cell concentrations above 90 x 10^6 viable cells mL^{-1}. When including changes of cell diameter to the prediction model, prediction accuracy did not change considerably. Mean diameter of the PER.C6® population increased during the perfusion process from approximately 15.5 to 17.5 μm (Figure 3c). These limited variations in cell diameter were expected as mammalian cell diameter is known to vary over cell cultivation process time due to changes in growth conditions, substrate concentrations or osmotic pressure (39). Including an additional variable in a model (in this case cell diameter) should be considered with caution, as it increases the complexity of the final model, increasing in turn the risk to over fit the original data. Here we showed that accounting for limited changes in cell diameter is not needed to obtain accurate VCD predictions.

The RMSEP achieved were low considering that the intermediate precision of the reference method (ViCell cell counter) is ± 6% according to the manufacturer. To our knowledge, the lowest standard error on prediction reported for the growth phase of mammalian cultures are of 0.135 x 10^6 viable cells mL^{-1} or 2.2% of the calibration range (0.35 to 4 x 10^6 viable cells mL^{-1}) with a linear regression (10). In the present study, the best prediction model yields a standard error of prediction of 6.8 % of the calibration range, which is close to the 6% reported for the off-line method used a reference to build the model. Different modeling techniques suggested in other studies could potentially increase further the accuracy of VCD prediction, for instance using multivariate modeling or the Cole-Cole empirical model, which relates permittivity to the dielectric properties of the cell suspension (10,40,41).

3.2 NIRS to monitor cell and metabolite concentrations

The process parameters predicted from NIRS spectra in this study were total cell density and glucose and lactate concentrations. It has been shown that glutamine and glutamate are poorly predicted from NIRS, especially when their concentration range was small (21,26,42). These two metabolites were thus not considered for predictions here.

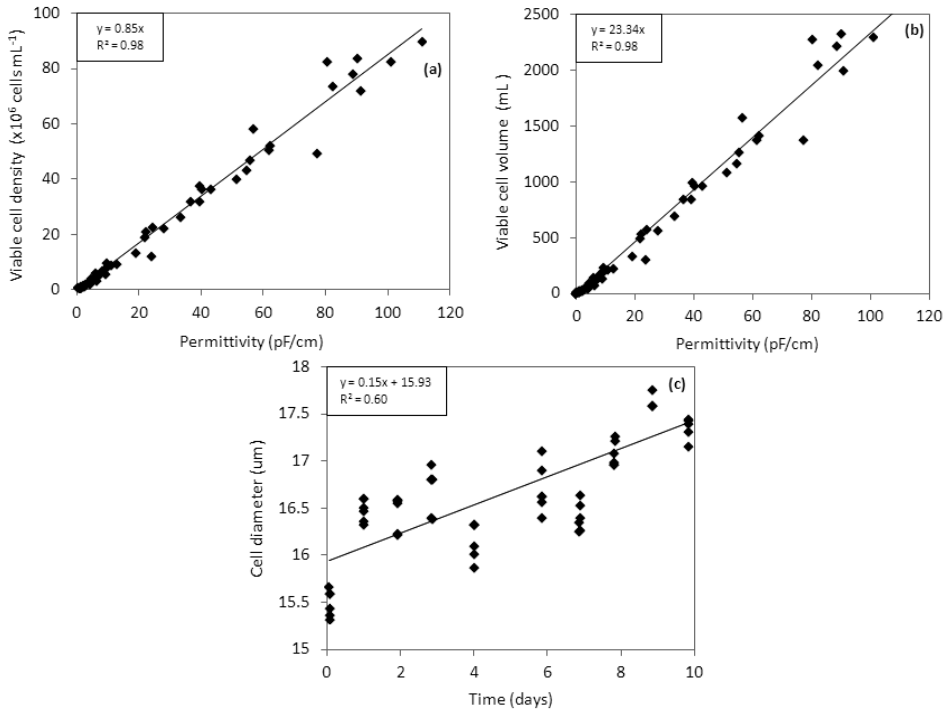

Figure 3. Calibration of DS models for direct prediction of viable cell density. Correlations between viable cell density and permittivity (a), between viable cell volume and permittivity (b) and between mean cell population diameter and process time (c) for 6 perfusion runs. The solid lines represent linear regression.

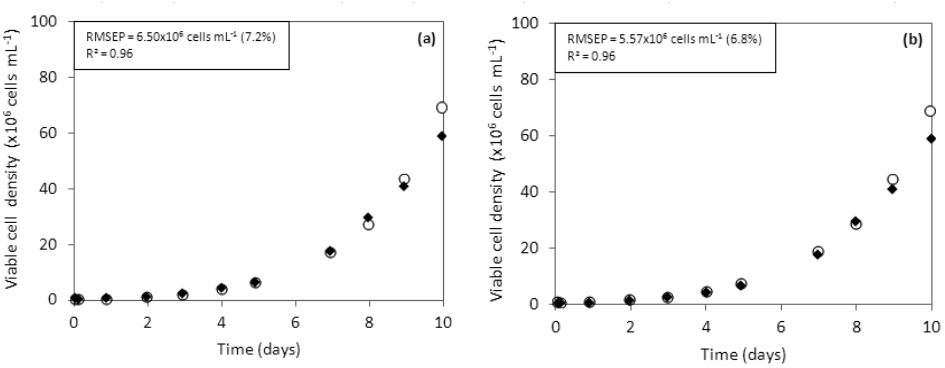

Figure 4. Prediction of viable cell density from DS. Comparison of viable cell density measured with reference method (♦) and predicted from online permittivity model including changes in cell diameter (O) for a model predicting viable cell density directly (a) or taking into account changes in mean cell population diameter (b). Data are shown for one representative run but RMSEP and R^2 were calculated from the 5 independent runs used to validate the models. Calibration range was 0.6 - 90.2 x 10^6 viable cells mL^{-1}.

Additionally, because of the strong correlation between viable and total cell density (R^2 > 0.999) in the perfusion process (Figure 1a), it was not possible to predict accurately both parameters. As DS enabled to monitor viable cell density, we decided to restrict the NIRS predictions to total cell density only.

3.2.1 Comparison between PLS1 and PLS2 modeling

Unlike DS, NIRS raw data cannot be directly correlated to the process parameters to monitor. Multivariate calibration using PLS is needed. To compare the performance of the two modeling techniques, PLS1 and PLS2 models were calibrated with at-line spectra measured from bioreactor samples (containing both cells and metabolites, figure 2) from 10 perfusion runs. The calibration dataset included 231 spectra from natural samples and 30 spectra from semi-synthetic samples spiked with known concentration of glucose and lactate, in order to break natural correlations between these metabolites (Table 1).

The calibration and prediction summary of these models is given in table 2. Regarding the calibration, the PLS2 model was rather simple as it had similar number of LV as PLS1 models and required no data pretreatment. For all three parameters to predict, the RMSEP of the PLS2 model was 1.6 to 1.7-fold higher than those of PLS1 models. Lactate predictions were particularly poor with PLS2, with a RMSEP of 23% of the calibration range. R^2 for the prediction of glucose and lactate concentrations also decreased in the PLS2, from 0.78 to 0.56 and from 0.69 to 0.25, respectively. An exception is seen for TCD, for which the same R^2 of 0.99 was achieved with both PLS1 and PLS2 models. This is most likely due to the fact that the spectral regions used for the calibration of the PLS2 model included all the specific regions required to predict TCD identified with PLS1 (5361 to 5489 cm^{-1} and 6144 to 6464 cm^{-1}). As a consequence, TCD was very well predicted with the PLS2 model. However, the PLS2 model also used additional spectral regions that were not necessary to predict glucose and lactate concentrations. Therefore noise was introduced for the prediction of these parameters and model accuracy decreased.

Model optimization is less flexible with PLS2, which leads to lower prediction performance for some process parameters of interest. Even though PLS2 presents the advantage that only one global model is needed, the data presented here showed that PLS1 modeling provides the most accurate predictions. Therefore to predict multiple process parameters accurately, several PLS1 models should be preferred over one overarching PLS2 model for these parameters.

3.2.2 Comparison of predictions from several types of samples from perfusion cultures

Effect of sampling and measurement procedure on raw NIRS spectra

NIRS spectra were collected in three different ways from the perfusion process: online with an *in situ* probe and at-line from the bioreactor vessel and from the permeate line of the ATF (Figure 1). The differences in sample types (bioreactor and permeate) and measurement methods (online versus at-line) were reflected in the corresponding raw NIRS spectra (Figure 5). Spectra from bioreactor samples presented a clear shift in baseline

Table 2. Calibration and prediction summary of PLS1 and PLS2 models from at-line bioreactor NIRS spectra.

		Calibration				Prediction	
Modeling technique	Parameter	Pretreatment	Spectral regions (cm^{-1})	Number of LV	RMSECV	RMSEP (% calibration range)	R^2
PLS1	TCD ($.10^6$ cells mL^{-1})	None	5361-5489 6144-6464	3	2.3	2.0 (2.2%)	0.99
	Glucose (mM)	First derivative	5732-6063 7301-8131	5	4.7	2.4 (10%)	0.78
	Lactate (mM)	SNV	5774-6102 8127-11834	8	4.6	3.4 (14%)	0.69
PLS2	TCD ($.10^6$ cells mL^{-1})	None	5361-5489 5732-6464 7301-11834	5	3.8	4.7 (5%)	0.99
	Glucose (mM)	None	5361-5489 5732-6464 7301-11834	5	4.5	4.0 (17%)	0.56
	Lactate (mM)	None	5361-5489 5732-6464 7301-11834	5	3.8	5.7 (23%)	0.25

PLS: partial least square; LV: latent variable; RMSECV: root mean square error on cross validation; RMSEP: root mean square error on prediction; R^2: correlation coefficient; TCD: total cell density; SNV: standard normal variation. Calibration ranges for TCD, glucose and lactate concentrations were 0.76 to 91.62 x 106 cells mL^{-1}, 7.4 to 31.5 mM and 1.9 to 27.1 mM, respectively.

absorbance with increased time and therefore increased cell density. This shift was caused by the light scattering from the cells and associated cell debris. Oppositely, spectra from the cell free permeate samples overlapped perfectly, which means that the extracellular environment during perfusion culture was constant. Online and at-line spectra from bioreactors also presented differences with absorbance values between 0.5 and 2.5 units lower for online spectra than at-line. These differences were caused by the measurement technique itself (measurement mode in transmission for at-line and transflectance for online, use of optical fibers to transmit the signal for online NIRS, or path length) (38).

Process monitoring from the perfusion permeate line

To study whether glucose and lactate concentrations in a perfusion process can be monitored using the external waste line of a cell retention device, PLS1 models were calibrated from NIRS spectra measured at-line from bioreactor samples and from samples taken on the permeate line of the ATF (Figure 2). Model performance was compared for the calibration and prediction of glucose and lactate concentrations (Table 3). The glucose and lactate models calibrated from at-line NIRS on permeate sample had similar complexity in terms of number of LVs and spectral regions used as models built from bioreactor samples. The glucose predictive ability from bioreactor and

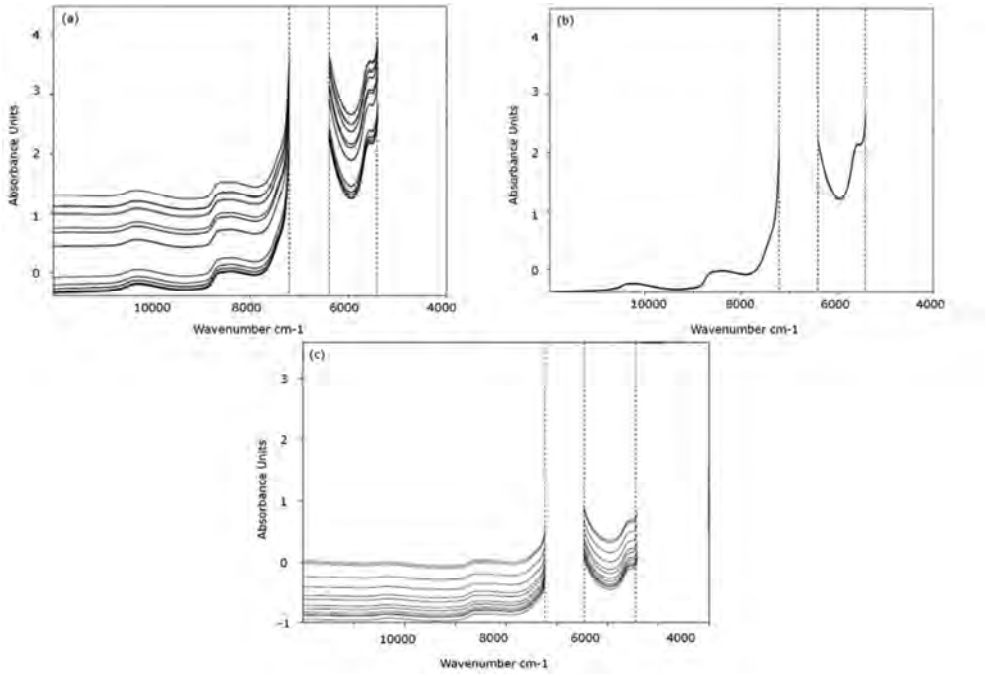

Figure 5. Overlay of 27 at-line NIRS spectra from bioreactor (a) and permeate (b) samples and of 12 online NIRS spectra (c), representative of an entire perfusion cell cultivation process. Spectral regions bordered by dashed lines correspond to the high absorption regions caused by water, which were excluded from the analysis.

permeate samples was comparable with RMSEP of 10 and 12% of the calibration range and R^2 of 0.73 and 0.78, respectively. Lactate predictions were more accurate with the model from permeate samples, as the RMSEP over the calibration range decreased from 14% to 10% and the R^2 increased from 0.69 to 0.89. Precision of reference methods were of 6% and 5% for TCD measured on ViCell and for glucose and lactate concentrations measured on NOVA Bioprofile 400, respectively. In the case of the metabolite concentrations, at-line NIRS RMSEP over the calibration range is higher (10 to 14%) than its reference counterpart (5%). Nevertheless, this is believed acceptable for an at-line measurement that can be replicated with no additional sample preparation and treatment, allowing for instance higher frequency of measurement.

Here, we showed that similar accuracy of prediction for glucose and lactate concentrations can be obtained from samples collected from the permeate line of a perfusion system. The continuous permeate stream obtained after a 0.2 µm filtration offers a great potential for process monitoring. Although the medium flow rate was low at the beginning of the process, when cell density was low, samples collected from the permeate line had a chemical composition close to that of bioreactor samples throughout the process, with R^2 of 0.82 and 0.98 for glucose and lactate concentrations, respectively (data not shown). Moreover, despite the variation in flow rate in the permeate

Table 3. Calibration and prediction summary of PLS1 models from online and at-line bioreactor and permeate NIRS spectra.

			Calibration					Prediction	
Measurement	Sample type	Parameter	Pretreatment	Spectral regions (cm^{-1})	Calibration range	Number of LV	RMSECV	RMSEP (% calibration range)	R^2
At-line	Bioreactor	TCD (.10^6 cells/mL)	None	5361-5489 6144-6464	0.76 – 91.62	3	2.3	2.0 (2.2%)	0.99
		Glucose (mM)	First derivative	5732-6063 7301-8131	7.4 – 31.5	5	4.7	2.4 (10%)	0.78
		Lactate (mM)	SNV	5774-6102 8127-11834	1.9 – 27.1	8	4.6	3.4 (14%)	0.69
	Permeate	Glucose (mM)	SNV	5446-6102 7498-9404	7.4 – 30.9	5	2.8	2.8 (12%)	0.73
		Lactate (mM)	SNV	5446-6102 7498-9453	3.2 – 26.4	6	2.2	2.2 (10%)	0.89
Online	Bioreactor	TCD (.10^6 cells/mL)	None	5774-6101 8451-9403	0.83 – 71.95	3	1.7	-	-
		Glucose (mM)	None	5450-6102	8.2 – 23.7	4	2.0	-	-
		Lactate (mM)	None	5774-6102	2.6 – 23.2	6	3.1	-	-

PLS: partial least square; LV: latent variable; RMSECV: root mean square error on cross validation; RMSEP: root mean square error on prediction; R^2: correlation coefficient; TCD: total cell density; SNV: standard normal variation.

line, prediction accuracy did not vary over process time. Therefore we present here a novel non invasive alternative for perfusion cell cultivation monitoring by measuring NIRS outside the sterile containment of the process.

Online in situ monitoring

NIRS predictions models for TCD, glucose and lactate concentrations from online and at-line bioreactor samples are also compared in table 3. The calibration ranges for TCD, glucose and lactate concentrations varied because two different sets of perfusion runs were used. Validation of the models from online NIRS could not be done using an independent prediction dataset, as reference data was not available for other spectra than those used to calibrate the models. The cross-validation step already indicated that the models from at-line and online samples performed similarly, with RMSECV of the same order of magnitude for all three parameters.

Evaluation of the predictive ability of the online models was done by plotting predictions from online spectra every 4h and process parameters measured daily with off-line reference methods (Figure 6). The exponential cell growth was accurately predicted by NIRS (Figure 6a). Negative values were predicted during the first day of the process, probably due to the small increase of TCD by 0.5 x 10^6 cells mL^{-1} on that first day of culture. Between days 2 and 6, measured and predicted TCD values were similar. When cell density exceeded 20 x 10^6 cells mL^{-1}, the discrepancy between measured and predicted values increased exponentially. This could be caused either by fouling of the online transflectance probe during the process, or by the use of the light scattering to calibrate TCD from NIRS, which is caused both by the cells and by other impurities also accumulating throughout the process. Glucose and lactate concentrations were predicted by online NIRS very closely to the values measured by the reference method (Figure 6b and c). As reference data was available only once per day, it was not possible to assess whether the small variations of +/- 2-3 mM predicted between day 5 and day 10 are real fluctuations of metabolites concentration or noise from online NIRS measurements.

It is important to note that online NIRS was measured on one perfusion run, for which 12 samples have been analyzed with reference methods. The experiment could not be repeated for practical reasons. The online model therefore does not account for run to run variability. For routine application, the calibration model should be trained with a much higher number of spectra from several experimental runs, as was done with at-line models. We expect that the predictive ability for TCD and metabolites concentration would consequently be lower than those we present here.

The main benefit of predictions from online NIRS is clearly depicted in figure 6: accurate monitoring of the cell cultivation process can be delivered much more frequently (on minute scale) than the traditional daily samples. Here, we chose to predict cell cultivation parameters every 4 h as we estimated that this time frame was sufficient to monitor the 10-days process and to take corrective actions in case of a deviation. Online spectra were actually collected as often as every 8 s, which offers the possibility for an even closer monitoring of the process.

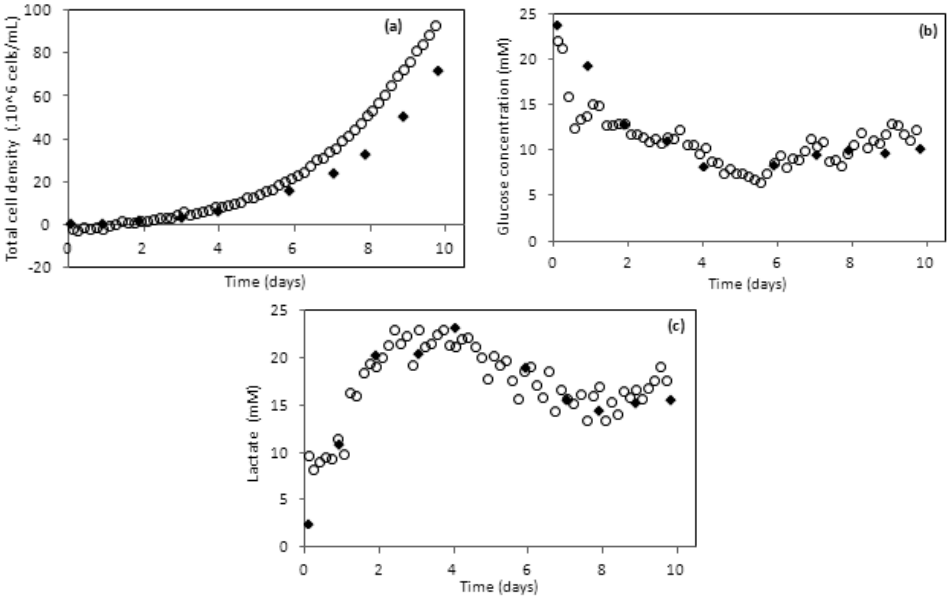

Figure 6. Comparison of total cell density (a), glucose (b) and lactate (c) concentrations measured with reference method (♦) and predicted with PLS1 models calibrated from online NIRS spectra on bioreactor samples (o).

Online NIRS measurements were performed *in situ* for the present study, which enabled to monitor information related to cell density. Based on the similar performance of the at-line NIRS prediction models from the permeate line of the perfusion system, we conclude that an ideal monitoring of this process should be done online and on the permeate line of the ATF retention device.

4 Conclusions

This paper shows that for perfusion processes, a combination of online *in situ* DS and online NIRS measured on the perfusion stream would be ideal to monitor cell density and main metabolites concentrations. The perfusion stream offers a great opportunity for safe process monitoring without the need for invasive probes or frequent sampling of the bioreactor contents. Moreover, daily samples, besides being laborious and causing risks of culture contamination, may not be sufficient to capture all relevant dynamics of a process where cells double every 20 to 30 h. Online monitoring is therefore a major advance compared to the current daily sampling regime. Accurate online monitoring of key process parameters with PAT tools such as DS and NIRS is clearly where the bioprocessing industry is moving towards in a continuous effort to increase control of process output.

The primary aim of DS or NIRS applications published for cell cultivations is process monitoring. Although efforts focus on controlling the processes based on these advanced

PAT tools, this is only seldom reached. An example is given by Dowd *et al.* (43), where the perfusion and feed rate of CHO cultures was automatically adjusted based on the cell concentration information extracted from online DS. Although several examples are published for microbial cultivations (44-46), to our knowledge, NIRS is not yet used to actively control mammalian cell cultivation processes.

DS and NIRS could also be used to not only monitor the perfusion process studied here, but also to improve the control strategy. Online predictions of viable cell density from DS could be connected to the control loop driving feed and perfusion rate, in order to ensure a tight control of the specific perfusion rate. Monitoring of glucose and lactate concentrations in real-time with NIRS could serve to confirm that cell metabolism and cell environment are constant, and could also be used in the control loop of the feed and perfusion flows. Given the recent trend in the industry to move towards more continuous manufacturing processes using perfusion, the need for continuous real time information on critical process parameters will become a crucial aspect of modern biopharmaceutical manufacturing.

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Chapter 7

General discussion

1 Introduction

Process analytical technology (PAT) is "*a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality*" (1). All critical quality attributes (CQAs) of a (bio) pharmaceutical product, which contribute to its safety and efficacy once administered into patients, need to be identified and controlled (shown in green in Figure 1). Shortly after the PAT initiative, additional guidelines were released inviting the (bio)pharmaceutical industry to adopt Quality by Design (QbD) (2,3). QbD promotes the definition of a process design space in which process parameters can vary without affecting product quality (shown in purple in Figure 1). QbD is therefore a modern approach to process design based on scientific understanding of the process and strong risk management strategies. As a result of PAT and QbD, product quality must be built in the process and ensured during its production rather than relying on post-manufacturing testing.

In this thesis, critical parameters for the quality of PER.C6® cells in a perfusion culture used for Adenovirus infection were studied and how these parameters are best monitored was addressed. In this chapter, the growing interest of the biopharmaceutical industry to develop continuous and perfusion manufacturing processes and the opportunities and challenges associated with implementation of QbD and PAT on such processes are discussed.

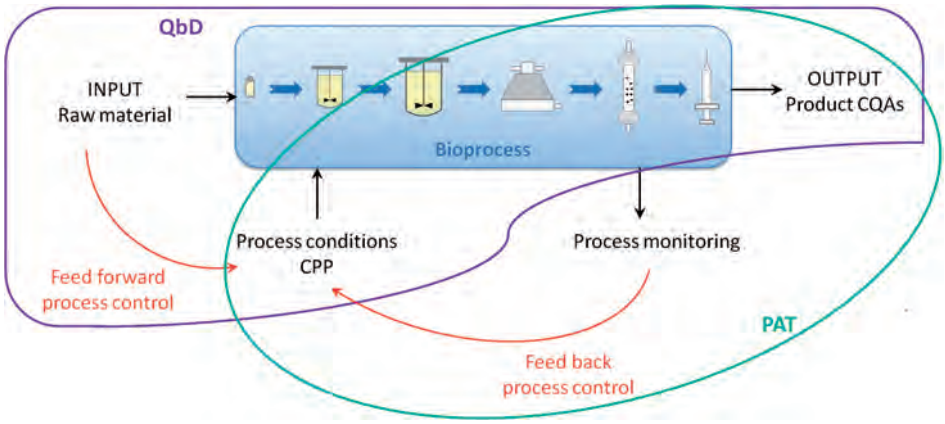

Figure 1. Roles of QbD and PAT-based process control for biopharmaceutical productions.

2 PAT for continuous cell cultivation

2.1 Continuous bioprocessing

Several industries from petrochemical, chemical, food and pharmaceutical, have developed intensified production processes by switching from classical batch-wise operation to continuous manufacturing (4-6). Across these different industries, continuous

manufacturing presents a series of similar advantages ranging from high volumetric productivity to small equipment and facility size and to reduced capital expenditure (7). The biopharmaceutical industry follows a similar path and is now rapidly developing more continuous processes.

Cell cultivation processes can be run in different modes to operate either batch-wise or continuously (Figure 2). Batch and fed-batch processes are both used for batch-wise productions. These processes are very dynamic and process parameters and control output vary continuously. Cell environment is never constant and it even deteriorates towards the end of the runs, as cells die and toxic metabolic by-products accumulate.

In continuous processes, the aim is to provide a constant favorable environment for the cells to grow at high rates and for the quality attributes of the product to be maintained, for as long as possible. Chemostat is the simplest continuous system, with incoming feed of unconcentrated medium that equals the outgoing bleed containing cells and spent medium. In chemostat, most process conditions are maintained constant: nutrient levels, cell density, gas flows, *etc.* However because cell densities are usually rather low (lower than in batch) and product is continuously washed out, this system is not very often used in manufacturing for cell cultivation, except for the most fragile products (8). Perfusion is a modified chemostat in which the cells are partially or fully retained in the system, usually by a filter system. Perfusion therefore combines the increased cell densities of the fed-batch with the continuous refreshment of the bioreactor contents of the chemostat. Because of that, this system is mostly considered for continuous biopharmaceutical manufacturing.

Several technologies have been developed to run perfusion cell cultivation processes and demonstrations of the resulting high productivity have been published (9-13). Several leading biotechnology companies such as Janssen, Genzyme, Merck-Serono, Boehringer Ingelheim and Bayer have implemented these processes in manufacturing settings for years (14). Because the aim of continuous processes is to have most process conditions maintained constant, control strategies for these processes are simpler

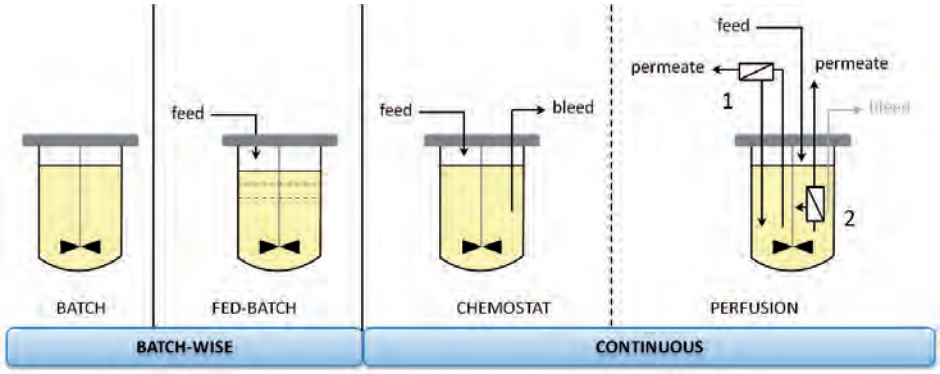

Figure 2. Schematic representation of the main cell cultivation modes. For perfusion processes, bleed is optional and cell separation and retention device can be external (1) or internal (2) to the bioreactor vessel.

than for batches or fed-batches, where all controller outputs vary continuously. These constant conditions are an advantage for PAT implementation. Moreover, with increased volumetric productivity and culture longevity, perfusion processes require smaller reactor volumes than batch or fed-batch processes to reach the same product yield. This decreases development costs and increases flexibility in manufacturing plants. However, with fewer runs per year compared to other (fed-batch) processes, the capital at stake for each batch is higher and failures are more expensive. For this reason, there is a special incentive for PAT and QbD for continuous processes. This combined with the fact that continuous processes are more efficient and easier to keep under control make them particularly interesting for investing in PAT tools.

2.2 PAT for perfusion cell cultivation: status and challenges

Efforts to implement PAT on cell cultivation steps have been widely published over the last decade (15-18). This process step is of particular importance in bioprocessing because the quality features of a product are often related to the process step where the product is formed, namely during cell cultivation (19). Therefore proper control of cell cultivations is required to ensure product quality.

In perfusion cultures, cell growth rate and nutrient concentrations are key process parameters that need to be controlled at constant levels and therefore should ideally be monitored online. Several techniques enable the monitoring of cell density online, such as capacitance measurements, soft-sensors for computation of oxygen uptake rate, automated flow cytometry or in situ microscopy probes (20-23). Metabolite concentrations can be measured at-line in cell cultures using for example high performance liquid chromatography, near-infrared spectroscopy or multi-functional analyzers coupled with automated sampling systems (24-26). Implementation of these tools on industrial continuous cultivations is not widely published, most likely because of the protection of companies' intellectual property and know-how. An example was presented recently at a conference (23rd European Society for Animal Cell Technology (ESACT) Meeting, 2013) by Nanjegowda *et al.*, where online capacitance was used to measure cell density in a perfusion culture and in the permeate stream, to monitor performance of the cell retention device (27). These measurements were also used to indicate time of switch of new cell retention filters and to control perfusion rates.

Overall, monitoring and control of cell density and metabolite levels is relevant in any cell cultivation process, regardless of the operation mode (batch or continuous). PAT tools for cell cultivations are therefore globally universal, and the applications that are most widely published on batch and fed-batch processes can largely be leveraged to continuous perfusion processes. Yet process control strategies to implement are largely dependent on operation mode. The major challenge for PAT implementation on continuous cell cultivations is therefore not essentially on the identification of the appropriate tools, but more importantly on defining the most appropriate utilization of these tools for optimal process control.

3 PAT for PER.C6® perfusion cultivation

The process studied in this thesis is a perfusion cell expansion process, which aims at generating high density PER.C6® cultures to seed virus production bioreactors. In this case, perfusion is run with an alternating tangential flow (ATF) filtration system, which continuously retains the cells in the cultivation vessel. The main reason for using ATF is that this system prevents fouling of the hollow fiber filter and enables very short residence times outside the bioreactor. Cell densities above 300 x 10^6 cells mL^{-1} can in this way be achieved (Chapters 4 and 5).

3.1 Exploitation of historical process data

PAT starts with understanding the underlying biological reactions taking place during the bioprocess that leads to the formation of the product and of the cellular biomass. Therefore the first logical step to increase understanding of an existing process is to explore the data already available. Cell cultivation processes yield particularly complex datasets, and because of the co-linearity among the dynamic process variables monitored, multivariate statistics are the most appropriate tool for exploring these data. Chapter 2 reviews the applications of multivariate data analysis (MVDA) for cell cultivation processes. This review shows that when applying MVDA, most information can be extracted from datasets that are complete and originate from series of well-designed experiments. Unfortunately this is not always the case, especially not for early development data. Chapter 3 shows the actual application of MVDA on a set of early development PER.C6® perfusion runs. Despite the fact that these datasets were incomplete and did not originate from well-designed experiments, MVDA proved to be very useful to expand understanding on the process. Causes for batch-to-batch variation and sensitivity of the process to scale were identified, whereas these findings were previously undetected using traditional data analysis techniques. In this chapter, the limitations of the dataset available were also shown, suggesting that some relevant information on the process (input, output or process variables) was most likely lacking.

3.2 Identification of product CQAs

In continuous cell cultivation processes, a number of process parameters are controlled at set point and these stable conditions should be maintained as long as possible. Perfusion mode enables to operate under constant metabolite concentrations, pH and culture viability for example. However parameters such as cell density, culture viscosity, shear stress, gas flows, *etc.* continuously increase during these processes. A challenge for application of PAT principles in such a process is to make sure that the product quality remains optimal throughout the process despite variation of certain parameters.

In Chapters 4 and 5 we studied the biological dynamics taking place during the perfusion process and the effect on the quality of the end product.

In chapter 4, several phases of programmed cell death through apoptosis were measured at different time points of the PER.C6® perfusion process. Unlike in batch

mode, where nutrients depletion and accumulation of metabolic by-products induce apoptotic cell death, this study shows that perfusion enabled to maintain apoptosis at a very low level. This is a clear benefit of this cultivation mode over for instance fed-batch, where cell death typically increases towards the end of the culture. Chapter 5 focuses on the features of PER.C6® cultures that could influence their suitability to be infected with viruses and propagate these viruses at high yields. An assay was developed to capture the infectability of PER.C6® cultures for Adenovirus replication. We showed that cells' fitness for virus propagation is maintained throughout the perfusion process. Overall, the conclusions from Chapters 4 and 5 are that the quality features of perfusion PER.C6® cultures were maintained throughout the culture time. We therefore conclude that the main CQA of the high density PER.C6® cells obtained from perfusion is the cells' viability.

3.3 PAT tools and online process monitoring

Besides attributes related to product quality, indicators of process performance are also relevant in PAT implementation, to assess the capability and efficiency of the process. Here, cells' viability was identified as the main CQA of the perfusion PER.C6® cultures. Moreover, cell growth rate is a key performance attribute of the process, as ideally high cell densities should be reached in the shortest process time possible. The PAT tools to monitor and control this process therefore need to capture accumulation of viable cells. Chapter 6 shows how dielectric spectroscopy (DS) and its permittivity measurements can accurately predict online viable cell density. The signal measured informs not only about the amount of viable cells generated at a certain time-point in the process, but it also enables to calculate cell specific growth rate, the key performance attribute of the process. Additionally, near-infrared spectroscopy (NIRS) was used for accurate online predictions of cell density and glucose and lactate concentrations. Online monitoring of metabolites level is of great importance during a perfusion process, since the aim is to maintain these parameters constant.

As shown in Chapter 6, the filtrate perfusion stream presents a great opportunity for monitoring and possibly controlling the process from outside the sterile containment of the vessel. This stream has similar physical-chemical contents to the main cultivation broth, but without the noise created by cells and debris. This is particularly true when high perfusion flow rates are used, as the residence time of spent medium in the waste line is then relatively short. Using online analytical tools on this stream therefore enables an accurate non-invasive and external monitoring of the process. This strategy would also be beneficial from a good manufacturing practices (GMP) perspective since the sensors are physically separated from the product and the implementation of these tools would not require any modification or revalidation of the production vessel itself.

In conclusion, for industrial perfusion cultivations there is a clear possibility to demonstrate that the steady state is maintained by using online PAT tools that monitor and control the CPPs or process outputs (28). DS and NIRS are suitable PAT tools in that respect.

4 Outlook of PAT and QbD for cell cultivations

4.1 Online process monitoring to replace culture sampling

Data generated by online PAT tools should ultimately substitute daily sampling that is usually done on mammalian cell cultivations. Relying on samples taken only once per day presents a series of disadvantages. If a process deviation is only at its initial stage at the moment of sampling, the sample taken the following day could come too late to take corrective actions on the deviation. Chapter 6 showed that accurate predictions of key process parameters such as viable cell density and concentrations of glucose and of lactate can be obtained from online PAT tools. In the examples provided in this chapter, glucose and lactate concentrations were predicted every 4 h from NIRS. This significantly increased the sampling resolution compared to the daily off-line measurements. In principle the technique even allows for continuous monitoring at time intervals as small as a few minutes. Similarly, in this chapter predictions of viable cell density are shown only every 24 h, but DS could yield measurements every 12 min. Overall, online process monitoring presents the advantage of collecting direct information on CQAs and CPPs and at a high frequency. These two points are beneficial to optimize process control.

4.2 Beyond process understanding: process control

The goal of PAT and QbD is real-time control of product quality and ultimately real-time product release based on online process control. QbD submissions to regulatory agencies are far from standard. To date, only one case of process control based on a design space has been submitted and approved by the FDA (http://qbdworks.com/qbd-biologics-gazyva-design-space/). This approval demonstrates that the pharmaceutical industry and authorities can reach the implementation of this new, science-based approach to quality on complex biopharmaceuticals. However to our knowledge, this approved design space relied only on classical process control, without including additional PAT tools.

Reaching PAT-based process control, without considering a design space from QbD, is also difficult as shown by the limited number of publications exposing this stage (Chapter 2). Applications of PAT tools used for process control found in literature are mostly on microbial cultures run in batch or fed-batch modes (29-32). In these examples, advanced analytical tools such as mid- or near-infrared spectroscopy, dielectric spectroscopy or radio frequency impedance measurement are used for online control of viable cell density or concentration of metabolites or product. What is achieved in these examples is still far from the ideal real-time product release based on PAT tools and on process control within a pre-defined design space.

A great number of steps need to be taken before real-time product release of a complex biopharmaceutical can be envisioned solely based on in-process control within a design space and using PAT tools. Gaining scientific understanding on the processes needs to become a main focus during development. Process monitoring and control strategies need to be defined with robust datasets and using reliable advanced analyzers and modeling tools. Finally, criteria for product release need to be defined based on the data available and the process knowledge. In this thesis and in most publications on PAT, the principles were

implemented on one process step. The major challenge in the future will be to demonstrate that QbD and PAT principles can be implemented on a complete bioprocess line, where high product quality will be ensured from thawing of a cell bank to storage of the finish product.

5 Investments and challenges for implementation of QbD and PAT

Despite the obvious benefits of QbD and PAT, which mainly lie in the increase in product quality, and the fact that the adoption of these initiatives were recommended by the FDA over 10 years ago, these concepts are not embraced by all biopharmaceutical companies yet. The reasons for this include underestimation of the increased flexibility brought by a QbD- and design space-based process control, resistance to change in quality mindset in some organizations, strong pressure on time-to-market and competition between business priorities and QbD and PAT implementation (33).

A major change to process development that is required to implement QbD and PAT is the amount of data generated to define the relations between process output, process input and process parameters and to explore the process design space. It is indeed acknowledged that process characterization for the submission of a design space requires a larger number of small scale development runs than classical approaches (34). The way the data are generated is also substantially different, with more investment in the early phases of product and process development, and with the use of statistical tools such as Design of Experiments (DoE) or MVDA, in order to gain strong scientific knowledge from experimental work (35). Although early process development is often driven by short timelines to deliver clinical trial material, stronger understanding of the process in an early stage increases the chances of success of the later process development. Finally, the way these data are used is also fundamentally different. With the traditional approach, submission files consist of detailed descriptions of process steps and their control strategies, whereas with QbD and PAT, the focus is to use the knowledge gained during the development of the process to explain why each process step is needed, how the objectives of these process steps are reached, and finally to demonstrate that the performance of these steps can be fully controlled and predicted (36).

The implementation of QbD and PAT and of the associated changes is slower than technical innovations in the field, but this process should be accelerated by the exposition of the benefits in terms of product quality, process efficiency and costs of goods. More examples from the companies that dare to pioneer in this field will continue to show the return on investment from QbD and PAT.

Conclusions

QbD and PAT principles for bioprocesses can be defined as systematic and structured approaches for applying common sense rules. The focus lies first on the quality requirements of the end product, and then shifts towards the design of the most apporpriate process

that ensures control of all critical sources of variation, to guaranty product quality. These principles are therefore strongly relying on the use of scientific knowledge to design manufacturing processes. In this line, this thesis gives a demonstration of the efforts that need to be deployed to identify the CQAs of a product and to understand how they are affected by process parameters. A structured approach to experimental design and to data analysis is required in the early phases of the clinical development of a drug candidate. Gaining this fundamental understanding on the process and on the interactions between process and product are absolute must-haves before further QbD and PAT steps can be taken and proper process control can be designed.

With the increasing number of successful demonstrations of QbD and PAT implementation, these concepts will become the common quality standard in industry in the coming years. The most important benefit from QbD and PAT will be that the biopharmaceutical industry delivers the safest, most efficacious and most affordable high-quality products to patients.

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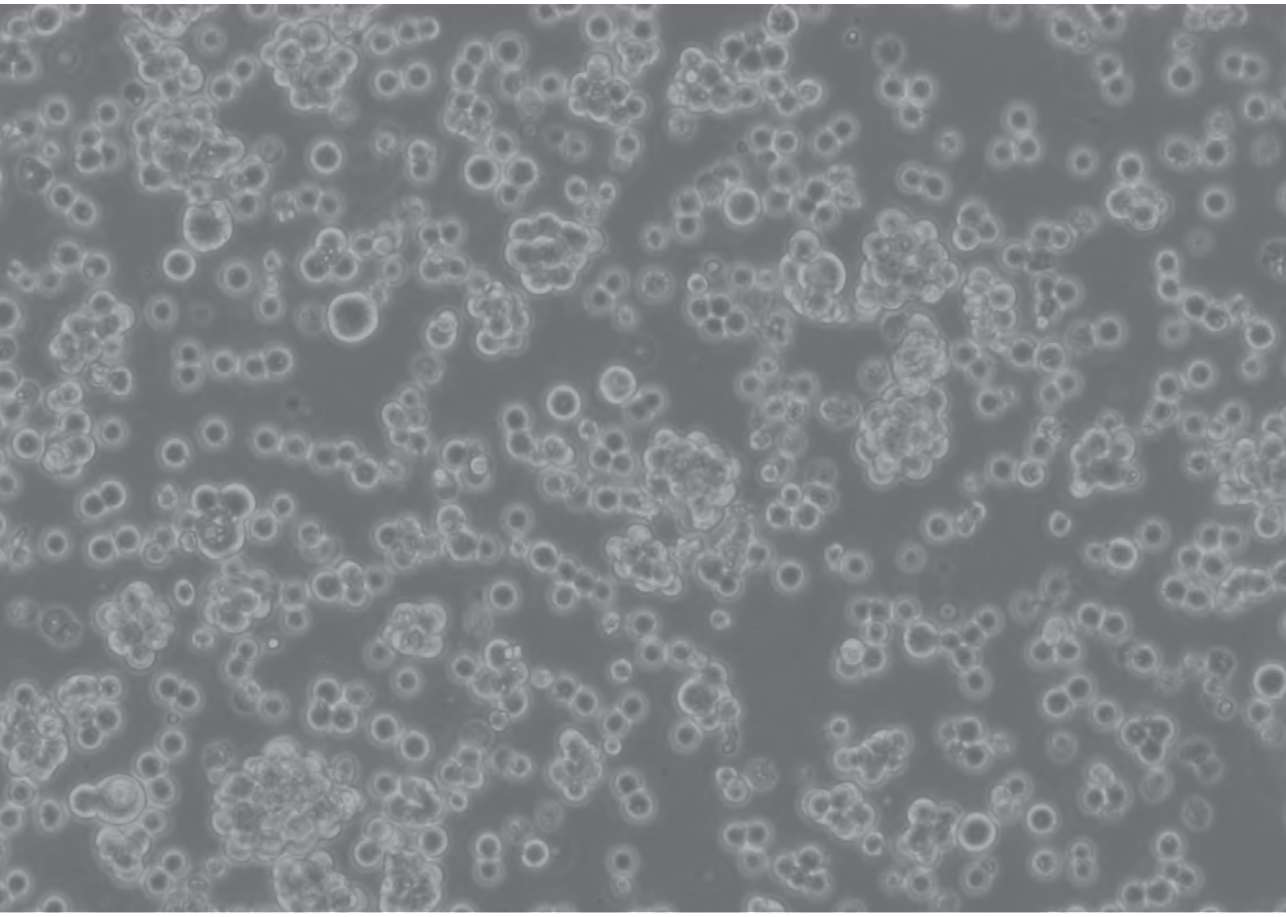
Summary
Samenvatting
Résumé
Curriculum Vitae
List of publications
Training activities



Summary

Process Analytical Technology (PAT) is an initiative introduced by the American Food and Drug Administration (FDA) in the early 2000s. (Bio)Pharmaceutical companies are encouraged by the PAT guideline documents to improve process monitoring and control of the quality of the product manufactured. PAT aims at monitoring and controlling all critical process parameters (CPPs) that affect the critical quality attributes (CQAs) of the end product or the process performance. The ultimate objective is to ensure consistent and high product quality during manufacturing, based on the use of scientific understanding of the production process to control all interacting CPPs.

Since its release, the PAT initiative has been largely embraced by the small molecule pharmaceutical world, where the manufacturing processes are overall simpler and the relations between product and process are generally well understood and characterized. Implementation of PAT for bioprocesses, and in particular the cell cultivation step of these process, is more challenging. First, biopharmaceutical molecules are larger and more complex in nature than small molecules (e.g. entire organisms or subunits), and therefore understanding the interactions between the manufacturing process and the quality of these complex molecules is more difficult. Secondly, cell cultivations are of particular importance in the manufacturing of biopharmaceuticals, as it is in this process step that the product formation actually takes place.

The research described in this thesis focuses on applying PAT principles to a perfusion PER.C6® cell cultivation process for viral vaccine production. The aim of this process is to expand PER.C6® cultures to high cell densities in perfusion mode, to seed the following process step of virus production. Variation in quality of these high density cultures was hypothesized to cause variation in performance of the subsequent virus production process. Therefore, a research project was started to identify which process parameters would affect the quality and suitability of these cells for virus production. However, the quality features, or CQAs, of substrate PER.C6® cells for virus propagations are not well defined, and the effect of variation in PER.C6® cell quality on virus attachment, infection, and propagation are also not fully understood. The objectives of this thesis were therefore to identify the CQAs of the PER.C6® cells used for virus production and to implement the most appropriate analytical tools for optimal monitoring of the perfusion cell cultivation process.

First, the most appropriate data analysis tool for transforming the large, multi-factorial and multi-collinear datasets generated during cultivations into relevant process information needed to be identified. Multivariate data analysis (MVDA) is a statistical tool able to explore these elaborate datasets to deepen process understanding and study the relations between process parameters and process outcome. The importance of MVDA for industrial (bio)pharmaceutical data was shown, particularly for cell cultivation processes where MVDA-based models are useful to characterize and build stronger understanding on the processes. MVDA therefore is a key element in for successful PAT and QbD implementations.

Multivariate analysis was used to explore the available data of the PER.C6® perfusion cell cultivation process. This process had been routinely run as part of early development of vaccine production processes; therefore data from these historical runs was available for further analysis. Principal component analysis (PCA) and partial least squares (PLS) are the two MVDA techniques used to exploit these early process development data, which were not generated from statistically designed experimental sets and contained data gaps. Nevertheless, the systematic and structured analysis of these data increased the understanding of the process studied. New process insights were revealed, such as root causes for batch deviations and process sensitivity to scale. MVDA helped to identify knowledge gaps and incentives for further monitoring of process parameters and of process responses.

Next, the research focused on understanding the biological dynamics taking place during the perfusion cell cultivation process and to assess the quality of the cells used for virus propagation. Biological and cellular parameters that were expected to reflect the cell's suitability for virus attachment, infection and replication were studied in order to identify the CQAs of PER.C6® cells. First, dynamics of PER.C6® cell growth and cell death were compared during perfusion and batch cultures. Flow cytometry assays were used to measure three phases of the apoptosis cascade. This showed that in batch mode, nutrients depletion and accumulation of metabolism waste products induced apoptotic cell death. However, in perfusion cultures apoptosis was kept at a constant low level. Having established this, the next step was to evaluate parameters that could influence the suitability of PER.C6® cells to be infected with viruses and propagate these viruses to high yields. A novel assay was developed to capture the fitness of PER.C6® cells for Adenovirus propagation, shortly referred to as "infectability". In this assay both kinetics of the infection process and kinetics of virus production were measured. Infectability measurements were compared from exponentially growing and high viability cultures and from cells sampled from a perfusion process within normal operating conditions and under suboptimal cultivation conditions. This study showed that infectability of PER.C6® cultures with adenoviruses is very robust throughout the perfusion cultivation and under suboptimal process conditions.

Overall, these studies on the CQAs of PER.C6® cells showed the robustness of the PER.C6® cells in the perfusion process. It was therefore postulated that the main CQA of the high density PER.C6® cells obtained from perfusion can be assessed by the measurement of culture viability. Consequently, the last step was to identify the most relevant online PAT tools to monitor this process. In addition to capturing information related to the cells' CQAs, PAT tools should also monitor key performance attributes of the process, related to business capacity and efficiency of the process. Specific growth rate was identified as a key performance attribute as sufficient cell densities to inoculate the subsequent virus production process should be reached in the shortest time possible. Dielectric spectroscopy (DS) and near infrared spectroscopy (NIRS) are two advanced PAT tools that can be used complementarily to monitor the product CQAs and the key performance attributes of the perfusion PER.C6® cultivation process. The models developed enabled accurate predictions of viable cell density and of glucose and lactate concentrations from

DS and NIRS data, respectively. In addition, this study showed that the perfusion stream closely reflects the actual cellular matrix and thus provides an excellent opportunity to do on line measurements without using invasive probes or excessive sampling.

This thesis gives a demonstration of the structured and systematic approach needed to apply PAT principle on a perfusion cell cultivation process. The identification of the CQAs of the PER.C6® cells and the evaluation of PAT tools for process monitoring enabled to gain fundamental understanding on the process and the cell line studied. These efforts are an absolute must before further QbD and PAT steps can be taken and proper process control can be designed.

Samenvatting

'Process Analytical Technology' (PAT) is begin jaren 2000 een door de Amerikaanse Food and Drug Administration (FDA) geïntroduceerd initiatief. (Bio)Farmaceutische bedrijven worden aangemoedigd om de kwaliteit van hun producten te verbeteren en te monitoren met behulp van PAT toepassingen. PAT richt zich op het monitoren en controleren van alle kritische proces parameters (CPPs), die een effect hebben op de kritische kwaliteitseigenschappen (CQAs) van het eindproduct of het proces. Het ultieme doel is om constante en hoge product kwaliteit al tijdens de productie te waarborgen, gebaseerd op wetenschappelijk inzicht in het productie proces.

Sinds de start is het PAT initiatief grotendeels omarmd voor laag-moleculaire geneesmiddelen waarvoor de productie processen veelal simpeler zijn en de relaties tussen product en proces vaak beter begrepen en gekarakteriseerd zijn. Het implementeren van PAT voor bio-processen, en in het bijzonder voor de celkweek stap van het process, geeft meer uitdagingen. Allereerst zijn biofarmaceutische moleculen van nature groter en complexer dan kleine moleculen (bijvoorbeeld hele organismen of sub-units van eiwitten). Dit maakt het begrijpen van de interacties tussen het productieproces en de kwaliteit van deze complexe moleculen moeilijker. Ten tweede is de celkweek stap van van bijzonder belang, aangezien dit de stap is waar het product daadwerkelijk wordt gemaakt. Celkweek is een bijzonder ingewikkeld proces, met vele parameters die interactie hebben met elkaar.

Het onderzoek beschreven in dit proefschrift richt zich op het gebruik van de PAT principes op een PER.C6® celkweek perfusie proces voor productie van virale vaccins. Het doel van dit proces is om PER.C6® culturen te laten groeien tot een hoge celdichtheid met behulp van perfusie. Omdat deze cellen vervolgens worden gebruikt om de volgende stap in het virus productie proces te starten, wordt onderzocht welke proces parameters de kwaliteit en geschiktheid van deze cellen voor virus productie beïnvloeden. Deze kwaliteitseigenschappen (of CQAs) waren vooralsnog niet gedefinieerd voor PER.C6® cellen, die dienen als substraat voor virus productie. Daarnaast is het effect van variatie van de kwaliteit van deze cellen op virus infectie en proliferatie niet volledig begrepen. De doelstellingen van dit proefschrift zijn dan ook om de CQAs van PER.C6® cellen gebruikt voor virus productie vast te stellen en om de meest geschikte analytische instrumenten (PAT) voor het optimaal controleren van perfusie cel cultuur processen te implementeren.

Allereerst moest de meest geschikte data analyse tool worden geselecteerd om de uitgebreide, multi-factoriale en mulit-collineaire datasets, gegenereerd tijdens celkweek, om te zetten in relevante proces informatie. Multivariate Data Analysis (MVDA) is een statistische methode, waarmee dit soort datasets kunnen worden geanalyseerd om het begrip van het proces te verbeteren en om relaties tussen proces parameters en product eigenschappen te bestuderen. MVDA is van groot belang voor industriële (bio) farmaceutische data, met name voor celkweek processen, waarin op MVDA gebaseerde modellen gebruikt kunnen worden om processen te karakteriseren en beter te begrijpen. MVDA is daarom een sleutelstuk voor het succesvol implementeren van PAT en QbD.

MVDA is gebruikt om de beschikbare data van het PER.C6 perfusie celkweek proces te onderzoeken. Dit proces werd routineus gedraaid als onderdeel van de beginfase van de ontwikkeling van vaccin productie processen; hierdoor was er data van historische runs beschikbaar voor verdere analyse. Twee MVDA technieken zijn gebruikt om deze data uit te diepen: Principal Component Analysis (PCA) en Partial Least Squares (PLS). Hoewel de data niet voortkwam uit statisch ontworpen experimenten en daardoor niet altijd compleet en gestructureerd was, vergrootte deze systematische analyse van de data het begrip van het bestudeerde proces. Nieuwe inzichten in het proces werden verkregen, zoals de oorzaak van batch deviaties en de gevoeligheid van het proces voor de schaal waarop het uitgevoerd wordt. MVDA hielp de kennisgaten en drijfveren voor het controleren van procesparameters en -gedrag te identificeren.

Vervolgens heeft het onderzoek zich gericht op het begrijpen van de biologische dynamiek, die plaats vindt gedurende het perfusie cel cultuur proces, en om de kwaliteit van de cellen gebruikt voor virus propagatie vast te stellen. Eerst zijn de dynamiek van de PER.C6® celgroei en afsterving in perfusie en batch culturen vergeleken. Flow cytometrie analyses zijn gebruikt om 3 fases van de apoptose cascade te meten. Deze analyses lieten zien dat nutrient depletie en de accumulatie van metabolieten apoptotische celdood induceerde in batch culturen. In perfusie culturen bleef apoptose echter op een constant laag niveau. Nadat dit vast gesteld was, was de volgende stap om de parameters, die de bruikbaarheid van PER.C6® om virus tot een hoge opbrengst te kunnen propageren, te evalueren. Een nieuwe test werd ontwikkeld om de geschiktheid van PER.C6® (de "infectability") voor adenovirus propagatie te meten. In deze test worden zowel de kinetiek van het infectie process als de kinetiek van de virus productie gemeten. Iinfectability metingen zijn vergeleken tussen exponentieel groeiende batch culteren met een hoge viabiliteit en perfusieculturen met standaard en substandaard celkweek condities. Deze studie liet zien dat de infectability van PER.C6® zeer robuust is gedurende het hele standaard cultivatie proces alsook onder substandaard condities. Deze studies over de CQAs van PER.C6® cellen toonden de robuustheid van de cellen in het perfusie proces aan. Daarom is aangenomen dat de belangrijkste CQA voor PER.C6® cellen bij een hoge celdichtheid verkregen uit een perfusie celkweek de viabiliteit van deze cultuur is.

Vervolgens werd gekeken wat de meest relevante online PAT tools om het proces te monitoren zijn. Naast het verzamelen van informatie over de CQAs van de cellen, moeten PAT tools ook de 'key performance attributes' kunnen monitoren, die gerelateerd zijn aan de economische performance en efficiency van het proces. Dielectric Spectroscopy (DS) en Near Infrared Spectorscopy (NIR) zijn twee geavanceerde PAT tools, die gebruikt kunnen worden om zowel de CQAs van het product alsook de 'key performance attributes' van het PER.C6® perfusie celkweek proces te kunnen monitoren. De modellen die ontwikkeld zijn hebben het mogelijk gemaakt om vanuit DS en NIRS data nauwkeurige voorspellingen te doen van respectievelijk de dichtheid van levende cellen, en de concentratie van glucose en lactaat. De studie liet verder zien dat de filtraat vloeistofstroom uit het perfusieproces een goede weerspiegeling is

van de daadwerkelijke cellulaire matrix. Hierdoor geeft deze stroom een uitstekende mogelijkheid om online metingen te doen zonder de kweek zelf te bemonsteren of gebruik te maken van invasieve probes.

Dit proefschrift beschrijft de gestructureerde en systematische aanpak, die nodig is om het PAT principe toe te passen op een perfusie celkweek proces. De identificatie van de CQAs van de PER.C6® cellen en de evaluatie van de PAT tools om processen te monitoren maakte een fundamenteel begrip van het proces en de bestudeerde cellijnen mogelijk. Deze pogingen zijn van cruciaal belang om verdere QbD/PAT stappen te kunnen maken en om goede proces controle te kunnen ontwerpen.

Résumé

Process Analytical Technology (PAT) est une initiative qui a été présentée par l'instance américaine de réglementation des produits alimentaires et de santé (Food and Drug Administration, FDA) au début des années 2000. Cette initiative encourage les industries pharmaceutique et biotechnologique à améliorer le suivi des procédés de production et le contrôle qualité des produits issus de ces industries. PAT a pour objectif de contrôler tous les paramètres opérationnels de production critiques (critical process parameters, CPPs) ayant un effet sur la qualité des produits (critical quality attributes, CQAs) ou sur le rendement des procédés de fabrication. Le but final est d'assurer de façon constante la bonne qualité des produits, et ce durant la totalité du procédé de fabrication. Pour ce faire, le concept PAT s'appuie sur les connaissances fondamentales et scientifiques des procédés de production ainsi que sur le contrôle de tous les CPPs.

Depuis la présentation de PAT par la FDA, cette initiative a été largement adoptée par l'industrie pharmaceutique classique (chimique), pour laquelle les procédés de fabrication sont globalement plus simples que ceux de l'industrie biotechnologique. De plus, l'impact de ces procédés sur la qualité des produits est généralement bien compris et caractérisé. La mise en œuvre de PAT pour les procédés de biotechnologie, et en particulier pour l'étape de culture cellulaire de ces procédés, est plus délicate. Les biomolécules produites sont beaucoup plus grosses et de nature plus complexe que les produits chimiques, puisqu'elles peuvent être par exemple des micro-organismes entiers ou des sous-unités de ces organismes. Comprendre et caractériser l'influence des procédés de production sur ces biomolécules complexes est donc plus difficile. De plus, l'étape de culture cellulaire est d'une importance particulière dans les procédés de production des biomolécules puisque c'est lors de cette étape que se forment les produits d'intérêt.

Le projet de recherche décrit dans cette thèse se concentre sur l'application des principes de PAT en biotechnologies, et plus particulièrement à un procédé de culture de cellules PER.C6® opéré en perfusion pour la production de vaccins viraux. Cette opération unitaire a pour objectif de cultiver les cellules PER.C6® en perfusion jusqu'à atteindre une très haute densité cellulaire. Ces cellules sont alors utilisées comme substrat pour la production de virus au cours d'une seconde opération unitaire. Une hypothèse a été formulée quant au lien entre la fluctuation de la qualité de ces cultures PER.C6® à haute densité et le rendement de l'étape suivante de production de virus. Par conséquent, un projet de recherche a été initié afin d'identifier les paramètres du procédé de culture cellulaire en perfusion affectant la qualité des cellules PER.C6® ainsi que l'aptitude de ces cellules à produire des virus. Cependant, les caractéristiques qui déterminent la qualité des cellules PER.C6® pour la production de virus (en d'autres termes les CQAs) ne sont pas clairement définies. De plus, l'influence de la qualité des cellules PER.C6® sur l'attachement des virus à ces cellules, sur le processus d'infection et sur la réplication des virus n'est pas entièrement comprise. Les objectifs de cette thèse sont donc d'identifier les CQAs des cellules PER.C6® utilisées pour la production de virus et de mettre en place les outils de PAT les plus appropriés pour un suivi optimal du procédé de culture cellulaire en perfusion.

Les procédés de culture cellulaire génèrent des ensembles de données qui sont particulièrement denses, complexes, multi-factoriels et multi-colinéaires. L'identification d'un outil d'analyse capable de transformer ces ensembles de données en information pertinente a été un prérequis à cette étude. L'analyse multivariée de données (multivariate data analysis, MVDA) est un outil statistique capable d'explorer ces ensembles de données complexes pour approfondir la compréhension des procédés et pour étudier les relations entre les paramètres opérationnels et les réponses du procédé (qualité du produit et rendement). L'importance de la MVDA pour analyser les données des industries pharmaceutique et biotechnologique est présentée dans cette thèse, en particulier pour les procédés de culture cellulaire pour lesquels des modèles issus de la MVDA sont très utiles pour acquérir une compréhension plus approfondie des procédés. La MVDA est donc un élément-clé pour la réussite de la mise en œuvre des principes de PAT.

Une analyse multivariée MVDA a été utilisée pour étudier les données disponibles sur le procédé de culture de cellules PER.C6® en perfusion. Ce procédé a été exécuté régulièrement pendant les phases précoces de développement pour la production de vaccins. Par conséquent, de plus en plus de données historiques étaient disponibles pour de plus amples analyses. Les analyses des composantes principales (principal component analysis, PCA) et des moindres carrés (partial least squares, PLS) sont les deux techniques de MVDA qui ont été utilisées pour modéliser les ensembles de données disponibles. Ces derniers n'avaient pas été générés à partir de plans d'expérience conçus avec des outils statistiques et contenaient des données manquantes. Néanmoins, l'utilisation de la MVDA pour une analyse systématique et structurée de ces données a permis d'améliorer la compréhension du procédé étudié. De nouvelles informations ont été révélées, comme les causes de déviation du procédé et la sensibilité du procédé à l'échelle à laquelle il est exécuté. La MVDA a permis d'identifier ces lacunes et a mis en évidence le besoin d'améliorer le suivi des paramètres opérationnels et des réponses du procédé.

Le projet de recherche a ensuite porté sur la compréhension des processus dynamiques qui ont lieu au cours du procédé de culture de cellules PER.C6® en perfusion, et sur l'évaluation de la qualité de ces cellules utilisées pour la production du virus. Les paramètres biologiques et cellulaires qui affectent l'aptitude des cellules PER.C6® à fixer les virus, à être infectées et finalement à répliquer les virus ont été étudiés afin d'identifier les CQAs de ces cellules. Tout d'abord, les cinétiques de croissance et de mort des cellules PER.C6® ont été comparées au cours de procédés de culture en perfusion et en batch. La cytométrie de flux a été utilisée pour détecter trois étapes différentes dans le processus d'apoptose des cellules. Cette étude a montré que lors des cultures en batch, l'épuisement des nutriments et l'accumulation des déchets métaboliques induit la mort cellulaire par la voie de l'apoptose. En revanche, dans des cultures en perfusion, l'apoptose est constante et à un faible niveau. L'étape suivante a ensuite consisté à évaluer les paramètres pouvant influencer la capacité des cellules PER.C6® à être infectées par des virus et à propager ces virus à des rendements élevés. Un test innovant a été mis au point pour capturer l'aptitude des cellules PER.C6® à répliquer l'Adénovirus, en d'autres termes leur "infectabilité". Lors de ce test, les cinétiques des processus à la

fois d'infection de la population cellulaire et de production de virus ont été mesurées. Les mesures d'infectabilité ont été comparées pour des cellules en phase de croissance exponentielle et avec une viabilité élevée, et pour des cellules cultivées en perfusion dans des conditions de fonctionnement soit normales, soit sous-optimales. Cette étude a montré que l'infectabilité des cellules PER.C6® par des Adénovirus est très robuste tout au long de la culture en perfusion et même sous des conditions de culture sous-optimales.

Dans l'ensemble, ces études sur les CQAs des cellules PER.C6® ont montré la robustesse de ces cellules lorsqu'elles sont cultivées en perfusion. Il a donc été postulé que le principal CQA des cultures de cellules PER.C6® à haute densité obtenues par un procédé en perfusion peut être évalué par la simple mesure de la viabilité de ces cultures. La dernière étape de ce projet de recherche a finalement consisté à identifier les outils PAT les plus pertinents pour suivre en ligne le procédé de culture en perfusion. En plus de collecter des informations relatives aux CQAs des cellules, ces outils PAT doivent également permettre de suivre les paramètres rendant compte de la performance du procédé (key performance attributes, KPAs), qui sont liés à la productivité et au rendement économique du procédé. Le taux de croissance cellulaire spécifique a été identifié comme étant un KPA du procédé de culture en perfusion. En effet, les hautes densités cellulaires nécessaires avant d'entamer l'opération unitaire de production virale doivent idéalement être atteintes en un minimum de temps. La spectroscopie diélectrique (dielectric spectroscopy, DS) et la spectroscopie proche infrarouge (near-infrared *spectroscopy*, NIRS) sont deux outils PAT qui peuvent être utilisés pour suivre les CQAs des cultures PER.C6® ainsi que les KPAs du procédé de culture en perfusion. Des modèles statistiques ont été développés pour prédire précisément la densité cellulaire et les concentrations en glucose et en lactate. De plus, cette étude a montré que le flux de perfusion reflète étroitement les conditions de culture présentes dans un bioréacteur et donc offre une excellente opportunité de suivre les cultures en ligne sans besoin de sondes additionnelles ou d'échantillonnage excessif.

Pour conclure, cette thèse apporte une démonstration de l'approche structurée et systématique à suivre pour appliquer le principe de PAT à un procédé de culture de cellules en perfusion. L'identification des CQAs des cellules PER.C6® et l'évaluation des outils PAT pour le suivi de leur procédé de culture a permis d'acquérir des connaissances et une compréhension fondamentales sur le procédé et sur la lignée cellulaire étudiée. Ces efforts sont absolument indispensables avant que la mise en œuvre plus approfondie du concept PAT puisse être envisagée et que la stratégie de contrôle du procédé puisse être optimisée.

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(*) Quote from Fernando Sabino (1923 - 2004)

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Curriculum vitae

Sarah Mercier was born on July 25, 1984in Cosne sur Loire, France. She attended elementary, middle and high school in the region of Puisaye where she grew up. She obtained her scientific baccalaureate in 2002. She moved to Dijon, France to start her university studies, joining the biology engineering program of the *Insitut Universitaire de Technology* (IUT) of Dijon. With a major in food processing, she did an internship at the Institute for Food Research (IFR) in Norwich, UK in 2004. There she used NMR to study the effect of high pressures on egg white preservation. Sarah obtained her DUT diploma in 2004.

Sarah then pursued her studies in Marseilles, France. She started in 2004 a Master's program at the engineering school of Luminy: *Ecole Superieure d'Ingenieurs de Luminy* (ESIL), in the biotechnology engineering curriculum with a major in pharmaceutical biotechnology. There she did an internship at the Center of Immunology of Marseilles Luminy (CIML) in 2006, where she conducted preliminary studies for the analysis of signalization pathways in T cells. She moved to San Francisco, USA, in 2007 to conduct an internship at Genentech. She studied the effect of hydrolysates on cell culture performance and on monoclonal antibody production. She graduated with her Master's in October 2007 and returned to Genentech, where she continued to work on the same project.

Since 2009, Sarah works at Crucell (part of Janssen pharmaceuticals since 2010), in Leiden, the Netherlands. She was hired as an assistant scientist and performed cell culture process development for various vaccines, in the up-stream processing group. In July 2010, she started her PhD project in collaboration with the Bioprocess Engineering group of Wageningen University and Crucell. Her research focused on applying the principles of Process Analytical Technology (PAT) on a PER.C6® perfusion cultivation process, the results of which are presented in this thesis.

Sarah is currently a junior scientist in the up-stream processing group of Crucell, where she pursues cell culture development work for vaccines.

List of publications

PAT tools for a perfusion PER.C6® cultivation process: dielectric and near-infrared spectroscopy.
Sarah M. Mercier, Perrine M. Rouel, Pierre Lebrun, Bas Diepenbroek, René H. Wijffels, Mathieu Streefland. 2014, submitted for publication.

Robustness of PER.C6® cell line for Adenovirus propagation.
Sarah M. Mercier, Pierre Lebrun, Bas Diepenbroek, Jort Vellinga, René H. Wijffels, Mathieu Streefland. 2014, submitted for publication.

Characterization of apoptosis in PER.C6® batch and perfusion cultures.
Sarah M. Mercier, Bas Diepenbroek, Dirk E. Martens, René H. Wijffels, Mathieu Streefland. 2014, accepted in a revised form at Biotechnology and Bioengineering.

Multivariate PAT solutions for biopharmaceutical cultivation: current progress and limitations.
Sarah M. Mercier, Bas Diepenbroek, René H. Wijffels, Mathieu Streefland. Trends in Biotechnology 2014, 32(6), 329-36.

Multivariate data analysis as a PAT tool for early bioprocess development data.
Sarah M. Mercier, Bas Diepenbroek, Marcella C.F. Dalm, René H. Wijffels, Mathieu Streefland. Journal of Biotechnology 2013, 167(3), 262– 270.

Training activities

Discipline specific activities

Course on MVDA for fermentation data (Umetrics, Malmo, Sweden, 2010)
Training on perfusion cell cultivation process (Percivia, Cambridge, USA, 2010)
Umetrics user meeting (Frankfurt, Germany, 2011)
8th World meeting on PBP (Istanbul, Turkey, 2012) [1]
QbD forum (Berlin, Germany, 2012) [2]
Course on Bio-statistics in JMP (Crucell - Arlenda, Leiden, the Netherlands, 2013)
IFPCA (Baltimore, USA, 2013) [2]
ESACT (Lille, France, 2013) [1]
3rd Workshop for ENVVP (Frankfurt, Germany, 2013) [2]
Nederland Biotech Congress, workshop (Wageningen, the Netherlands, 2014) [2]
Cell culture Engineering XIV (Quebec city, Canada, 2014) [1]

General courses

Basic bio-statistics (Crucell, Leiden, the Netherlands, 2010-2011)
Effective communication (Crucell, Leiden, the Netherlands, 2011)
PhD Competence Assessment (WGS, Wageningen, the Netherlands, 2011)
Project and time management (WGS, Wageningen, the Netherlands, 2011)
Techniques for writing and presenting scientific articles (WGS, Wageningen, the Netherlands, 2012)
Lean culture (Crucell, Leiden, the Netherlands, 2013-2014)
Career perspectives (WGS, Wageningen, the Netherlands, 2014)

Optional activities

Preparation of research proposal (2010)
PhD excursion to the USA (Wageningen University, Bioprocess Engineering group, 2010) [1]
PhD excursion to Spain (Wageningen University, Bioprocess Engineering group, 2012) [2]
PhD brainstorm days (2012, 2013 [2], 2014)

[1] Poster presentation
[2] Oral presentation

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