Scalability of Single-Use Biopharmaceutical Manufacturing Processes using Process Analytical Technology (PAT) Tools

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“Had I really succeeded at anything else,

I might never have found the determination to succeed

in the one arena I believed I truly belonged.”

J.K. Rowling

Abstract

Monoclonal antibodies (mAbs) are key molecules in biopharmaceutical manufacturing with important therapeutic use such as anticancer drugs. Mammalian cells serve as production hosts for mAbs. However, mammalian cell culture processes are complex and development timelines for new processes are long. To overcome these challenges the industry is moving towards high-throughput, single-use bioreactors and intensified processes. The scalability in both directions (scale-down and scale-up) is a key step towards fast and economic process development. Moreover, novel Process Analytical Technology (PAT) tools aim at improving process understanding and establishing process control and automation resulting in high and consistent product quality.

In the first part of this PhD thesis a method to transfer an existing Chinese Hamster Ovary (CHO) cell culture fed-batch process platform into a semi-perfusion process with threefold higher cumulative product titers was developed. Design of Experiment (DoE) as a powerful PAT tool to screen medium and feed compositions speeded up significantly the semi-perfusion process development. The process transfer to a small scale, single-use bioreactor enabled process control for important parameters such as pH and dissolved oxygen (DO) while keeping the experimental costs low. However, important process attributes (e.g. Viable Cell Concentrations (VCC)) were measured offline limiting the automation possibilities.

The second part of this thesis demonstrated how the implementation of an advanced inline capacitance sensor can support online monitoring of important biomass related changes in cell culture. The sensor implementation was proven to be scale-independent in single-use bioreactors (50 L up to 2000 L). Additionally, the transferability of the method to different CHO fed-batch processes was demonstrated. The Wet Cell Weight (WCW) and Viable Cell Volume (VCV) were predicted for the complete cultivation duration within an acceptance criterion based on the offline reference method. The VCC, however, correlated with the permittivity signal only until the end of the exponential growth phase due to the single-frequency measurement dependency on cell diameter changes.

The third part of this PhD thesis successfully tested the inline capacitance sensor in frequency scanning mode to predict VCCs over the complete culture time by establishing a robust Multivariate Data Analysis (MVDA) model. A small scale bioreactor system served as method development tool. Therefore, a fast and economic development of a robust MVDA model was demonstrated, highlighting the benefits of scale-down models in biopharmaceutical manufacturing.

Key words: mammalian cell culture, single-use bioreactors, process analytical technologies (PAT), scalability

Kurzfassung

Monoklonale Antikörper sind Schlüsselmoleküle in biopharmazeutischen Herstellungsprozessen aufgrund ihres wichtigen therapeutischen Nutzens, wie beispielsweise als Antikrebs-Medikament. Tierzellkultivierungen dienen als Produktionswirt für monoklonale Antikörper. Tierzellkultivierungsprozesse sind jedoch komplex und Entwicklungszeiträume dauern lange an. Um diese Herausforderungen zu überwinden strebt die Industrie eine Entwicklung in Richtung Hochdurchsatz-, Einwegbioreaktoren und intensivierte Prozesse an. Die Skalierbarkeit in beide Richtungen (Runterskalierung und Hochskalierung) ist ein Schlüssel für schnelle und wirtschaftliche Prozessentwicklung. Des Weiteren ermöglichen neue Technologien zur Prozessanalytik (engl. *Process Analytical Technology*, PAT) ein verbessertes Prozessverständnis, Prozesskontrolle und Automation, sodass eine konstante und hohe Produktqualität gewährleistet wird.

Im ersten Teil dieser Doktorarbeit wurde eine Methode entwickelt, um einen *Chinese Hamster Ovary* (CHO) Fed-Batch Zellkulturprozess in einen semi-Perfusionsprozess mit dreifach erhöhter kumulativer Produktausbeute zu übertragen. Statistische Versuchsplanung (engl. *Design of Experiment*, DoE) wurde als PAT Tool genutzt, um verschiedene Medium- und Feed-Kompositionen zu untersuchen und führte zu einer signifikant schnelleren Entwicklung des semi-Perfusionsprozesses. Der Prozesstransfer zu einem kleinvolumigen Einwegbioreaktor ermöglichte die Prozesskontrolle wichtiger Parameter, wie pH und gelöstem Sauerstoff, mit geringen experimentellen Kosten. Wichtige Prozessattribute (z.B. Lebendzellzahl) wurden jedoch offline gemessen und führte zu einer Limitierung der Prozessautomation.

Der zweite Teil der Arbeit zeigt, wie die Implementierung eines inline Kapazitätssensors das online-Monitoring wichtiger Biomasseänderungen in der Zellkultivierung unterstützt. Die Skalierungsunabhängigkeit des Sensors wurde in Einwegbioreaktoren mit Volumina von 50 L bis 2000 L erwiesen. Zusätzlich wurde die Übertragbarkeit der Methode auf andere CHO Fed-Batch Prozesse dargelegt. Die Feuchtbiomasse und das lebensfähige Zellvolumen konnten über die gesamte Kultivierungsdauer innerhalb eines Akzeptanzkriteriums auf Basis der offline Referenz vorhergesagt werden. Die Lebendzellzahl korrelierte mit der Dielektrizitätskonstante aufgrund der Durchmesserabhängigkeit bis zum Ende der exponentiellen Wachstumsphase.

Im dritten Teil der Doktorarbeit wurde der inline Kapazitätssensor im Scanning Modus evaluiert, um mit Hilfe eines multivariaten Datenmodells die Lebendzellzahl über den gesamten Kultivierungszeitraum vorherzusagen. Ein kleinvolumiger Bioreaktor diente zur Entwicklung der Methode. Aus diesem Grund konnte das robuste multivariate Modell schnell und wirtschaftlich entwickelt werden, was die Vorteile von kleinvolumigen Bioreaktoren in biopharmazeutischen Herstellungsprozessen hervorhebt.

Schlagwörter: Tierzellkultivierung, Einwegbioreaktor, Technologien zur Prozessanalytik, Skalierbarkeit

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List of Abbreviations

BHK21	Baby Hamster Kidney21
CHO	Chinese Hamster Ovary
CPP	Critical Process Parameter
CQA	Critical Quality Attribute
D_i	Impeller Diameter
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
DoE	Design of Experiment
FB	Fed-Batch
FDA	Food and Drug Administration
ICH	International Conference on Harmonization
k_La	Mass Transfer Coefficient
mAb	monoclonal Antibody
mRNA	messenger Ribonucleic acid
MVDA	Multivariate Data Analysis
N	Impeller Speed
N_P	Power Number
NIR	Near-Infrared
OPLS	Orthogonal Partial Least Square
OTR	Oxygen Transfer Rate
p	Fluid Specific Gravity
P_0	Impeller Power
PAT	Process Analytical Technology
PCA	Principle Component Analysis
PC	Principle Component
pCO_2	Partial Pressure of CO_2
PFR	Plug Flow Reactor
PLS	Partial Least Square
P/V	Power Input per Unit Volume of Liquid
QbD	Quality by Design
Re_i	Impeller Reynolds number
STR	Stirred Tank Reactor
VCC	Viable Cell Concentration
VCV	Viable Cell Volume
WCW	Wet Cell Weight

1 Introduction and Goals

1.1 Introduction

In 1953, James D. Watson and Francis Crick revealed the structure of “the molecule of life”. The discovery of the Deoxyribonucleic acid (DNA) structure was a breakthrough in molecular biology and opened up the way for what we know as biopharmaceutical manufacturing nowadays. After the DNA structure was revealed one significant discovery was followed by the next one: In 1956, Arthur Kronberg discovered the DNA polymerase as being the enzyme that synthesizes the DNA. In 1958, John C. Kendrew described the complex structure of the protein myoglobin. Francois Jacob and Jacques Monod discovered in 1960 how DNA can be transcribed to messenger ribonucleic acid (mRNA) that is translated to protein sequences.

A second fundamental discovery in biotechnology, that led to modern biopharmaceutical processes, started with the development of a “DNA sequencing method” by Allan Maxam and Walter Gilbert. Finally, Stanley Cohen and Herbert Boyer used the novel knowledge to create *in vitro* a new recombinant gene. In 1975, Goerges J.F. Köhler and César Milstein developed a method to produce monoclonal antibodies (mAbs) first with an *in vivo* production method and later on with *in vitro* production processes. mAbs are highly target-specific protein molecules used in diagnostics and as therapeutic drugs. Ultimately, this resulted in the first biotech company called Genentech going public in 1980.

Even today mAbs are still the raising star within biopharmaceuticals. First-time approvals of mAbs raised from 27% (2010 – 2014) to 53% nowadays (2015 – July 2018). Because of their crucial role in diagnostics and cancer research, mAbs are one of the most important biopharmaceutical class for the future. Today mammalian cell lines, especially Chinese Hamster Ovary (CHO) cells, mainly serve as host for mAb production. Even though, mAbs became the most significant biopharmaceutical drug, the cell as production system is still considered as a black box.

To overcome the challenges in modern biopharmaceutical manufacturing and to better understand cell metabolism and process parameter correlations, the industry is moving towards high-throughput, small scale and single-use systems offering significant cost advantages and flexible experimental designs. Scalability in both directions (scale-down and scale-up) plays a key role in efficient bioprocessing to provide process understanding on the one hand and economic production processes on the other hand. Combined with the trends of process intensification and automation a better process understanding should lead to autonomous production laboratories of the future enabling fast access of new and safe biopharmaceuticals for patients.

To optimize current bioprocess development the Food and Drug Administration (FDA) launched the Quality by Design (QbD) guideline to implement high and consistent product quality already during the development of new drugs. A toolbox allowing for QbD are Process Analytical Technologies (PAT). PAT tools support process understanding, online monitoring and automated control strategies in many different ways, e.g. the integration of spectroscopic inline sensors, the application of Multivariate Data Analysis (MVDA) or advanced risk analysis.

1.2 Goals

The goal of this work is to combine the novel industry approaches such as process intensification, single-use bioreactors and PAT tools to support the scalability of biopharmaceutical processes and to gain deep process understanding by innovative solutions.

As a first step an established fed-batch process is transferred to a semi-perfusion process keeping the bioreactor equipment and available medium and feeds the same. This way, an established process platform can be utilized for further process intensification. The establishment of the new semi-perfusion process includes PAT tools like Design of Experiment (DoE). Proof of principle in an automated small scale, single-use bioreactor is investigated that can be used for early process development in future. Moreover, online PAT sensors for important process attributes or parameters (e.g. viable cell concentrations, VCC) are implemented into mammalian cell culture processes to enable real-time monitoring for a better

understanding of the process and the cell culture. This PhD work contributes to implement control strategies in future, such as automated feeding rates in fed-batch processes or perfusion rate control in intensified processes.

Overall, the benefits of robust scale-down models, especially in process and method development, are highlighted and scale-independency of the selected PAT tools are investigated. Through the implementation of the developed methods in this work, a biopharmaceutical manufacturer will be supported to intensify and automate his single-use facility from laboratory up to production scale.

2 Theoretical Background

2.1 Biopharmaceutical Manufacturing

mAbs are key molecules in modern research and therapeutic areas. Because of their universal binding capability with a high precision to the target molecule, mAbs are frequently used in diagnostics or clinical research such as an anticancer drug or against autoimmune diseases [1–3]. However, the production of mAbs is a challenging and demanding task. The correct antibody quality is essential for their therapeutic activity and the risk reduction of causing any biological harm. As bioassays or even animal assays cannot be performed for every pharmaceutical product or batch, physical features or chemical structures serve as criteria to judge the quality of mAbs (e.g. post-translational modification). One important post-translational modification of a mAb is the glycosylation pattern. Glycans affect solubility, pharmacokinetics and they are critical for biological activities. The need for a specific glycosylation pattern leads to mammalian cells as being the workhorse for mAb production [4–6].

Mammalian cells are able to produce complex molecules with post-translational modification resulting in similar protein structure compared to equivalent produced molecules in human bodies. Another benefit of mammalian cells as production host of mAbs is that the protein is often secreted into the medium resulting in easy processing and purification of the product. Commonly used expression systems in research and industry are CHO cells, Baby Hamster Kidney (BHK21) cells, or murine myeloma cells [7, 8].

For the expression of mAbs in mammalian cells a suitable production system is necessary. Bioreactors offer the possibility to grow mammalian cells under controlled conditions. Several bioreactor types exist that underlie different basic principles of how the bioreactor is controlled and performed. Plug flow reactors (PFR), stirred tank reactors (STR) and rocking motion bioreactors are commonly used systems [9]. There are different types of processes for a mammalian cell cultivation, depending on the cultivation mode. Batch processes are short processes where the cell culture and all medium components are inside the bioreactor from

the beginning. Thus, the bioreactor volume is kept constant over the complete cultivation time. Nutrient levels in a batch process decrease constantly and inhibiting metabolites, such as NH_4 or lactate accumulate over the process time. The most common used cell culture processes are operated in fed-batch (FB) mode. A FB process consist of a short batch phase and is followed by feeds of nutrients. The feeding rates are optimized based on the cells' needs and the current growth phase. Thus, the nutrient level is adjusted based on the cell metabolism. Inhibiting metabolites, however, accumulate in the bioreactor. The bioreactor volume is increasing over time and the cell growth is expected to be higher compared to a batch process. A third way of cultivating cells inside a bioreactor is in perfusion mode. A perfusion process consists of a continuous exchange of medium and feeds during a process including a cell retention device. Therefore, the cells are kept in the bioreactor with a constant supply of fresh nutrients and inhibiting metabolites are constantly removed. The volume is kept constant but a high cell growth and productivity can be expected. In contrast to batch and FB processes, the product can be harvested continuously if the product is secreted into the medium, offering advantages for unstable molecules (e.g. recombinant Factor VIII) [9]. In the last years, the bioreactor equipment drastically improved enabling perfusion processes to be back in the focus of the pharmaceutical industry and academia [10–12]. The suitable combination of the bioreactor and operation mode depends on the cell type, the specific product and the available equipment.

Besides the trend of intensifying the operation mode with perfusion processes, other trends and technical developments have significantly changed the research in biopharmaceutical manufacturing in the recent past. The industry moved from the classical stainless steel bioreactors towards single-use facilities offering high flexibility, lower cross contamination risks, fast time to market and a cost reduction [13–15]. However, a transfer from established processes, methods and bioreactor setups is not easily done when moving from stainless steel to single-use facilities.

Moreover, the development of high-throughput small scale bioreactors offers the flexibility for a fast process development and to gain intensive process understanding keeping experimental costs low and parallelizing and automatizing the work [16–18]. However, the scalability of the established processes is still a key step towards successful manufacturing. Only a process that reaches production stage meets the patients´ needs and is economical convenient. The following chapter gives a detailed overview of the scalability of biopharmaceutical processes.

2.2 Scalability of Upstream Manufacturing Processes

The main goal of a biopharmaceutical process is to achieve a robust and economic process with a constant, reproducible and high product quality in production scale. To achieve the goal, the development process can be divided into three stages. In a first step, the process development takes place in a laboratory scale. Main targets at this scale are a fast screening of cell lines, process parameters and operation modes. After the establishment of a robust process, first tests in pilot plants do follow. The pilot plant enables to define optimal operating spaces and process conditions. However, clinical trials in phase I/II/III of the novel drug need several kg of product. Thus, a scale-up to larger bioreactors is necessary to achieve an approved biopharmaceutical. The final biopharmaceutical process is transferred to production scale. The product can be produced economically for commercial applications only in production scale [19]. However, scalability is not one directional. The scale-up from small to large scale is as important as the establishment of a suitable scale-down model [20]. Therefore, each bioreactor from laboratory scale until production scale plays an important role in the scalability. Figure 1 gives an overview of a single-use bioreactor family from small scale (0.15 L) to large scale (2000 L) systems.

For all these steps, scalability between the different bioreactor sizes is essential. The product quality as well as cell productivity and the cellular physiological states need to be kept constant in all bioreactors. A scalable process symbolizes a good prediction of the process performance and a specification of operating conditions switching from one scale to the other [6, 21].

Figure 1 Overview of a single-use bioreactor family (0.15 L - 2000 L) of Sartorius Stedim Biotech GmbH. [22]

When scaling up bioprocesses the geometry of the bioreactors can either be similar or non-similar. Similarity of bioreactors describes the ratios between the main parts being responsible for the chemical and physical environment of the bioreactor, such as the ratio height over diameter or the impeller configurations. In bioreactors of different sizes that are geometrically scaled, all length dimensions are scaled proportionally to the bioreactors scale. However, the change of bioreactor size can influence and change the chemical and physiological environment for the cells inside the bioreactor leading to unfavorable cell reactions or product qualities. Table 1 gives an overview of important biological, chemical or physical factors that influence the scaling approaches of cell culture processes. Major effects of the bioreactor scale are detected on the oxygen gas transfer, the shear forces for the cells, the compression force, the heat transfer in the bioreactor and the CO_2 removal [6].

Table 1 Summary of different factors that influence the scalability of mammalian cell culture processes

Biological factors	Chemical factors	Physical factors
Contamination risk	Carbohydrate concentration	Aeration
Mutation probability	Foam formation	Agitation
Number of generations	Medium quality	Back pressure
Pellet formation	Nitrogen concentration	Heat transfer
Selection pressure	pH control agents	Hydrostatic pressure
	Product concentration	Medium sterilization
	Redox potential	Mixing
	Water quality	Tank configuration
		Temperature control

Several classical scaling factors are suggested to be kept constant during a scale-up of mammalian cell cultivations, such as mixing time, volumetric oxygen transfer coefficient (k_La), power input per unit volume of liquid (P/V), Impeller Reynolds number (Re_i) and superficial gas velocity [9, 19, 23–25].

A constant power input per unit volume of liquid is a frequently used scaling approach. The power input is dependent on the impeller power (P_0) as described in the following equation (Eq. 1) [26].

$$P_0 = N_P N^3 D_i^5 p \quad (1)$$

N_P is the Power number, N is the impeller speed, D_i is the impeller diameter and p describes the fluid specific gravity. Keeping the power input per unit volume constant has several effects on other important process and bioreactor parameters. The agitation rate decreases with increasing scale. The impeller tip speed increases with an increase of the bioreactor scale. Mixing times increases with increased scales and the pumping capacity per volume decreases with increased scales [26].

The oxygen transfer rate (OTR) is another key aspect when scaling from one bioreactor to larger bioreactors. The OTR depends on the mass transfer coefficient (k_La) and the driving force $(C^*_{O_2} - C_{O_2})$, described in the following equation (Eq. 2). $C^*_{O_2}$ is the broth DO concentration at saturation and C_{O_2} is the measured broth DO concentration [27, 28].

$$OTR = k_L a \, (C^*_{O_2} - C_{O_2}) \quad (2)$$

Equation 2 indicates that high OTRs can be achieved with an increase in the mass transfer coefficient (k_L), the interfacial area (a) or the driving force. Additionally, pure oxygen can be used to improve OTR. However, the sparging in the bioreactor influences the CO_2 removal and therefore OTR changes need to be balanced based on the interaction of parameters [28].

To conclude, a bioreactor is a complex system and each parameter is connected and influenced by each other. Therefore, keeping all parameters constant during scale-up is difficult and many times compromises are needed.

To understand the complexity of all parameters in the process, robust small scale models can offer big advantages. Therefore, in the last time the tendency in academia and industry led to miniaturized stirred tank reactors. Recently, bioreactors of sizes below 1 L working volume gained intensive interest for process development. The scalability between such small scale bioreactors and production bioreactors is a challenging task that needs to be investigated. In the following, recent scale-down and scale-up approaches will be further discussed.

2.2.1 Scale-Down Approaches

Early process development and characterization in biopharmaceutical manufacturing requires many experiments, leading to high development costs, resources and long development timelines. Recently, the interest in high-throughput small scale bioreactors increased significantly, because these bioreactors offer a solution to both development drawbacks: development costs and timelines. The highly parallel, single-use bioreactors with working volumes below 1 L enable fast process development and reduce the overall costs of experiments (e.g. applying DoE studies).

Scale-down is far more than a simple reversion of the scale-up in biopharmaceutical manufacturing [21]. Scale-down models are not only advantageous for process development and characterization. Once a process reached pilot plants or production plants a robust small-scale model can improve the process understanding, such as being a troubleshooting tool for root cause analysis of failures that occurred in large scale bioreactors.

One system offering the possibility of developing robust small scale models of mammalian cell culture is the ambr® 250 bioreactor system. These highly-parallel, small scale bioreactors were selected in many novel studies to compare scale-up from laboratory scale to 1000 L bioreactors. The performance of the scale-down model was comparable in regard to process

performance and product quality in fed-batch mammalian cell culture processes [29–33]. The ambr® 250 system offers advantages when used as scale-down model. The volume of the bioreactors is low (0.25 L), but important scalability factors can be considered already in the scale-down model. The bioreactor is geometrically similar to large systems and process parameters, such as pH, DO, stir speed and temperature can be individually controlled for each bioreactor [17].

However, the availability of data sets for the scalability between the high-throughput, small scale bioreactors compared to commercial bioreactor scales of 2000 L and higher is limited. Manahan *et al.* successfully demonstrated for the first time the ambr® 250 as a scale down model for two commercial CHO cell lines producing mAbs in bioreactors larger than 10,000 L [17].

As already mentioned, the industry moves towards continuous manufacturing and perfusion process. The development costs for such processes without a scale-down model are high. A small-scale system can significantly improve perfusion development and increase the possibilities. Especially in process development and cell line screening, small scale bioreactors reduce costs and resources. Sewell *et al.* demonstrated that the automated ambr® 15 system with bioreactor volumes below 0.015 L represents a profitable process development tool being used for pseudo-perfusion processes [18].

To conclude, a robust small-scale process enables fast development at low costs and resources. Therefore, the biopharma industry can provide new treatments and medicine faster to the patients and positively impact their life. The potential of high-throughput, single-use, small scale bioreactors will impact the future of biopharmaceutical manufacturing, even though still many challenges do exist when scaling from smaller volumes in process development to production scale. A lot more research on miniaturized systems is necessary and the limitations need to be carefully considered [21].

2.2.2 Scale-Up Approaches

Due to the limitations in the configuration and design of large scale bioreactors, three main scale-up strategies for mammalian cell culture are established: (1) Constant k_La and constant specific impeller pump rate; Geometric similarity (2) Constant k_La and constant maximum shear; Geometric similarity (3) Constant k_La, constant impeller tip speed and constant impeller pump rate [19, 34–36].

The three scale-up strategies match with the most important needs of the cell culture. The main critical factors for cell cultures are oxygen supply, mixing times and CO_2 removal.

The oxygen supply to a mammalian cell culture is a critical factor as the process is aerobic. However, a simple increase in power input is not possible as many mammalian cell lines are shear sensitive. The development and use of surfactants such as poloxamers reduced shear stress and bubble damage supporting scalability for mammalian cell cultures [36, 37]. Nevertheless, the agitation rate constrains the scale-up of cell culture processes.

The relatively low agitation rates and power inputs impact another critical factor, the mixing time. Process parameters such as pH or nutrient levels can form gradients with insufficient mixing in large scale bioreactors [36, 38].

Xhu *et al.* experienced variations in cell growth, viability and productivity during their scale-up study with an increasing partial pressure of CO_2 (pCO_2) in large scale bioreactors [39]. In an extended scale-up study of the same group a 5000 L bioreactor was characterized resulting in longer mixing times, lower oxygen transfer and lower pCO_2 removal rates for the large scale bioreactor [36]. The study revealed that an increase in bottom air sparging was more efficient than an increase in the power input in regard to oxygen transfer and pCO_2 removal rates.

Brunner *et al.* demonstrated in their scale-up studies that pH, DO and pCO_2 variations have a strong impact on cell growth and productivity [40]. Moreover, this study revealed that pH and pCO_2 interactions impact the product quality.

To conclude, for scalability of CHO cell cultures the deep understanding of how cell metabolism and productivity respond to process parameters variations is essential. Process scale-up can favor unwanted changes in the chemical environment of the cells such as pH variations that leads to changes in the cell productivity or product quality [36, 40–42]. To reduce the risk of failures during process scale-up advanced online monitoring and control tools need to be available and comparable in all bioreactor scales.

Therefore, besides the classical scaling factors, PAT enables a deep understanding on how scale translations affect the process. Thus, PAT tools can improve the scalability between different bioreactor sizes. The following chapter provides a detailed description of PAT tools.

2.3 Process Analytical Technology

The International Conference on Harmonization (ICH) launched the ICH Q8 guideline in 2004 including the QbD initiative to improve and sustain high quality in the development of pharmaceutical products [43]. The QbD guideline supports systematic approaches including continuous risk assessments, the identification of product attributes that are of significant importance to the product's safety and establishment of robust control strategies to ensure consistent process and product performance [44]. Main goal of the QbD approach is to monitor and control Critical Process Parameters (CPPs) that influence Critical Quality Attributes (CQAs) of the pharmaceutical product to prevent variations in the CQAs. QbD implementation leads to a well-controlled manufacturing process within a pre-defined design space avoiding the detection of critical deviations after completion of the production processes.

PAT is a toolbox that facilitates the process control aspect of QbD. The FDA launched the PAT initiative in the same year as the QbD guideline. PAT tools enable the establishment of consistent process performance, process control strategies and high product quality by monitoring and controlling CPPs in the complete life cycle of a manufacturing process [45–47].

The PAT toolbox consists of many different parts that can be applied to a pharmaceutical process including software tools (e.g. Design of Experiments or Multivariate Data Analysis) or advanced process analyzers for online monitoring (e.g. Capacitance measurement).

PAT tools cover different steps from designing experiments, analyzing the process, monitoring CPPs and finally online control to achieve a robust, reliable and consistent process performance from cell line development until fill and finish of the product. The advantages of PAT tools are obvious: enhanced process understanding, improved product yields, prevention of process failures, cost savings and the possibility for real-time release of products [48–51]. For chemical processes PAT is commonly applied and already integrated into FDA approved processes including real time releases [52]. However, the integration of PAT tools into biopharmaceutical processes is a challenging topic. Besides the sterility and integration of inline, online and at-line sensors into complex bioreactors, the transition to single-use systems and the scalability through all bioreactor systems represent demanding tasks for the industry. A biopharmaceutical upstream process with mammalian cells consists of many different reactions, production and consumption of several nutrients at the same time and the cell and its metabolism is still partially seen as a black box [44, 52]. Because of these challenges and the complexity of protein production, PAT tools and their integration are crucial to move from a black box towards a well-controlled process.

In the last years, many attempts have been done to integrate different PAT tools into biopharmaceutical processes. Rowland-Jones *et al*. demonstrated in a powerful way how DoE can lead to a fast and efficient approach to create a design space in cell line screening and compare different PAT tools for online monitoring of nutrients such as ammonia, glucose and lactate in small scale systems [53]. Within the three selected PAT tools Near-Infrared (NIR), 2D-Fluorescence and Raman spectroscopy the Raman spectroscopy offered best results in the investigated CHO process.

Raman spectroscopy was successfully tested in many other applications including large scale bioreactors or downstream processing [54–59].

Furthermore, NIR, 2D-Fluorescence and other PAT tools such as online High Performance Liquid Chromatography, at-line Fluorescent Activated Cell Sorting or soft sensors were investigated for online monitoring of CPPs in a variety of production processes [52, 60–64].

Recently, Moore *et al.* reported for the first time the integration of a capacitance sensor into a commercial mammalian cell culture process being implemented as online tool to control seed train inoculations or feeding rates [65]. Capacitance sensors are preferred tools and frequently used to monitor online biomass related changes in the cell broth [66–72]. Key benefits are their easy integration into the sterile setup of a bioreactor and relatively low implementation costs [65, 73, 74].

Even though the PAT initiative was published 2004 and many work has been done already, the integration of PAT tools into industrial processes remains a big challenge and more research to achieve standardized and reliable solutions is needed.

2.3.1 Design of Experiments

In the beginning of every new process development the question arises on what the optimum conditions look like and how they can be found. The number of process settings can easily exceed three process factor combinations when looking at complex processes, such as mammalian cell cultivation for mAb production: the cell line, medium and feed medium or process conditions, such as temperature, DO or pH are some examples that need to be considered at defined process development steps. Many times the best combination of process factors, that achieves a high product yield, is not sufficient. Other requirements might include establishing a process with the lowest possible costs and high product quality with short production times.

The QbD approach aims at defining an optimal normal operating space within a defined design space of a manufacturing process. Figure 2A illustrates the understanding of a design space. A design space is a well-understood process range within the investigated knowledge space.

After the definition of the design space, the process can be performed in a controlled space within the design space.

In mammalian cell culture processes univariate analysis of each parameter is complex and demanding with the requirement of a large number of experiments. Development times and costs do not allow for such approaches to identify the optimal design space. DoE however, aims at keeping the experimental work as low as possible and increases the understanding of the process response and data at the same time. The selection of characteristic parameter combinations, application of mathematical modelling and statistical methodologies optimize the process to move from the knowledge space towards the design space in a systematic way [75]. Therefore, DoE applications can be used as powerful tools to save costs, resources and time, while gaining deep information about the process.

There are three different basic problems where DoE is commonly applied: Screening, optimizing and robustness testing [76]. The purpose of screening DoEs is to identify most influential parameters within the investigated process. Moreover, screening DoEs analyze the knowledge space and give indications about the parameter ranges that are applicable to the process. In early process development of pharmaceutical manufacturing a screening DoE can be used to screen for different media components, the ratio between each components and the right feeding times of the nutrients to maximize product yield and quality [77–80]. After screening an optimization DoE is commonly applied to find the optimum within the design space based on previously determined responses and CQAs. In many complex cases the optimum is defined by a compromise that maximizes the outcome while considering all parameters and response factors. In a mammalian cell culture process an optimization DoE can be applied to optimize bioreactor settings such as pH, temperature or DO set points [40, 81, 82]. The last step of a DoE investigation consists of robustness trials that demonstrate the ruggedness of the process towards expected inevitable parameter variations at the chosen set points inside the design space under which the process results in CQAs within acceptable

ranges. Questions in regards to product specification and reproducibility of the results are covered within robustness trials.

The DoE design depends on the scope of the experiments and the amount of investigated parameters. However, the amount of experiments needed and information received by the DoE strongly depends on the selected DoE design. Commonly used designs (full factorial, fractional factorial and composite designs) are summarized in Figure 2B [75, 76, 83, 84].

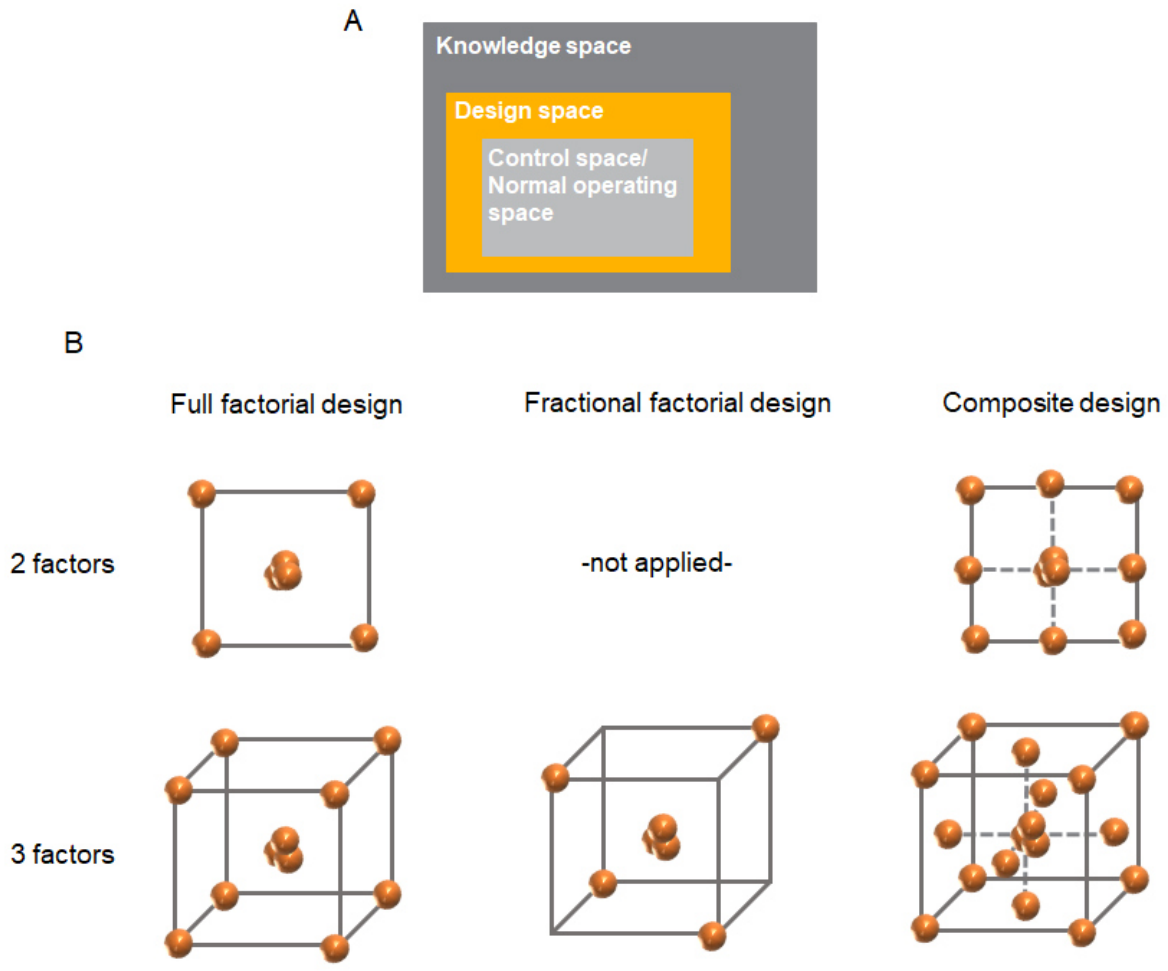

Figure 2 A Schematic description of the definition for knowledge space, design space and control space/normal operating space. B Selected DoE designs for 2 or 3 factors.

In the last years, DoE became the method of choice to improve process understanding and speed up development processes in academia as well as in industry. Therefore, many examples of DoE applications, especially in biopharma, can be found in the literature [85–90].

2.3.2 Multivariate Data Analysis

The complexity of data generated during a biopharmaceutical process is continuously increasing. High-throughput, automated small scale bioreactors, the implementation of DoEs and integration of PAT tools into each process intensify the need for systematic and multivariate analysis of the available data sets to separate and understand overlapping effects and parameter relations. Improved process knowledge cannot be acquired by univariate data analysis. MVDA is a proposed key method to accomplish the PAT and QbD guidelines in pharma processes by reducing and classifying the data and separating important information from noise [91]. MVDA allows for a detailed process understanding and delivers relevant and crucial process information by analyzing large data sets and enabling easy interpretation of complex parameter relationships [45, 46].

Traditional MVDA methods were used for a diverse range of problem statements in varying industries. The methods include multiple linear regression, linear discriminant analysis, canonical correlation or factor analysis [92, 93]. These methods were applied to independent and conditioned variables. Pharmaceutical processes are complex and variables might be driven by a few inherent and latent variables compared to the overall number of observed variables. One simple approach to overcome these challenges within pharmaceutical processes is the implementation of MVDA projection methods such as Principle Component Analysis (PCA) and Partial Least Squares projections to latent structures (PLS). Both approaches aim at reducing the number of variables in a k-dimensional space down to a lower-dimensional space or hyper-plane [93].

One basic method behind MVDA is PCA [93–96]. As described in Figure 3A, for PCA the experimental data is summarized in a matrix consisting of K variables and N observations.

The variables represent the data from a pharmaceutical manufacturing process, such as data from pH and temperature sensors, chromatographic methods or spectroscopic PAT tools (e.g. Raman, NIR or capacitance frequency scanning) [59, 91, 97].

PCA reduces the dimensionality of the data set by integrating lines, planes and hyperplanes in the K-dimensional space and applying the least square approximation while keeping as

much information as possible about the variation of the data. Therefore, the original data set is transferred into a set of uncorrelated variables, the Principle Components (PCs). Figure 3 B demonstrates the principle of establishing PCs with the least square approach. Commonly, PC1 describes the largest variation of the data set. Each following PC is orthogonal to the existing PCs until the amount of PCs is sufficient to describe the variation in the complete data set. The coordinate value along the PC-line generated by the projection of an observation onto the PC-line is defined as score. The PCA approach can reveal groups of observations, trends, and outliers and defines relationships between observations and variables [92, 93].

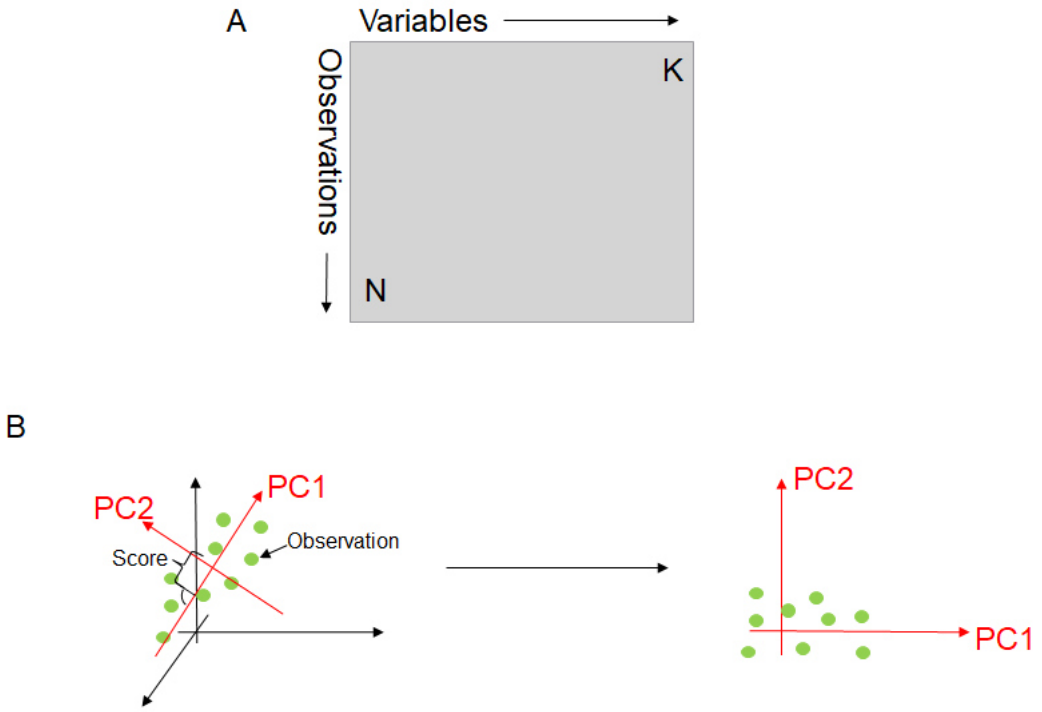

Figure 3 A Overview of data matrix for MVDA analysis. B Determination of the first and second principle component (PC) in a coordinate system using the least square approach. Each data point is defined as observation. The coordinate value along the PC-line generated by the projection of an observation onto the PC-line is defined as score. Based on the PCs a new reduced coordinate system is defined.

One drawback of PCA is that the method reduces unsupervised the dimensions in the variables. In contrast to PCA, PLS offers the possibility to link the information of two data matrices by a linear multivariate model, where one matrix is assigned to the variables (also called predictors) and the other matrix belongs to the responses. Thus, PLS enables analysis of collinear or even incomplete variables in both matrices and can be seen as a regression

method to model the association between the predictors' matrix and the response matrix [93, 98].

MVDA is frequently used in biopharmaceutical manufacturing to increase process understanding and knowledge [44, 46, 99–101]. Moreover, MVDA enables online monitoring and control of process responses (e.g. CPPs) based on selected variables, such as spectroscopic sensor data [63, 64, 74, 97].

3 Results and Discussion

This doctoral thesis can be separated into three different parts that are closely linked to each other. Each part was published separately in a peer-reviewed journal.

The first publication named "*A protocol to transfer a fed-batch platform process into semi-perfusion mode: The benefit of automated small scale bioreactors compared to shake flasks as scale-down model*" focuses on process intensification. Process intensification and continuous manufacturing are upcoming trends in biopharmaceutical manufacturing. Due to the increasing number of new biopharmaceuticals and a high pressure for low cost manufacturing, the industry is moving towards development of high yielding processes with consistent product quality. To achieve the ambitious goals PAT tools and small scale bioreactors are crucial.

The publication demonstrates how DoE as a PAT tool enables a powerful and fast development of a semi-perfusion process based on an established fed-batch process platform. Therefore, the method can be used to transfer existing process platforms to intensified processes with significant increase in product yield while keeping the same process timelines. A simple increase in nutrient supply was beneficial for high cell counts as long as the medium composition did not exceed the physiological limits of the cells (e.g. high osmolality). Finally, the new established semi-perfusion process was transferred to an automated, high-throughput bioreactor system. The proof of concept in the miniaturized bioreactor empowers future development in a controlled bioreactor environment speeding up timelines, reducing costs and enabling deep process understanding for easy scale-up.

The development of intensified mammalian cell culture processes reveals once again the importance of VCC as a process attribute. VCCs can be used for automation and control of perfusion processes, such as automated bleed or perfusion rates based on the online information about the cell growth. However, VCCs are still measured offline.

Therefore, the second part of the doctoral thesis focuses on the scale-independent development of a PAT tool for online monitoring of biomass related changes in cell culture.

The publication *"Monitoring online biomass with a capacitance sensor during scale-up of industrially relevant CHO cell culture fed-batch processes in single-use bioreactors"* uses the capacitance measurement principle to correlate the permittivity signal with important biomass parameters: The Wet Cell Weight (WCW), the Viable Cell Volume (VCV) and the VCC. For method development an established and well-defined, industrial fed-batch process is used.

The principle of capacitance sensors can be found in various literature under different names, such as radio frequency impedance, bio-capacitance, dielectric spectroscopy or multi-frequency permittivity [73, 81, 97, 102–104]. A compact overview on the methodology used in this thesis is given in Carvell *et al*. [69].

The principle behind capacitance sensors is the application of an electrical alternating current in the ionic cell solution. The ions in the highly conducting cellular cytoplasm move in the direction of the electrical field towards the non-conducting cellular membrane where their movement is limited. Thus, a charge separation within the cells take place and the cells polarize [69]. As a result, the measured capacitance is dependent on the polarizability of the cells that is linked to the cell properties.

A capacitance sensor supplies information about the physical properties capacitance and conductivity of the sensor electrodes in interaction with the cell suspension. The capacitance and conductivity are frequency dependent and need to be combined with the cell constant of the sensor, taking the electrode geometry into account, to give information about the dielectric properties of the cells. Thus, the capacitance component of the sensor provides the permittivity signal of the cell suspension [105, 106].

As a result of the capacitance measurement principle, dead cells and other impurities in the cell broth are not polarizable as they have no intact cell membrane [69, 107]. Therefore, they

do not influence the measurement and the difference in permittivity can be correlated to viable biomass related changes.

The second publication demonstrates scale-independency of the capacitance measurement principle in single-use bioreactors ranging from 50 L scale up to 2000 L bioreactor volume. The method is investigated for two different industrial fed-batch processes. As expected from the literature, the method is successful for WCW and VCV in both processes. However, the VCC correlation is limited to the exponential growth phase. As soon as a significant cell diameter change occurred, the correlation to VCC is not given with single-frequency measurements anymore.

The polarizability of the cells is frequency dependent. Depending on the selected frequencies, the cells can either according to a given excitation completely polarize (leading to high permittivity) or the cell polarization is incomplete with increasing frequency (resulting in a decrease of permittivity). This phenomenon is described as ß-dispersion curve in literature [69]. Thus, measuring permittivity at one frequency leads to a cell diameter dependency. Changes in permittivity can result from changes in cell counts as well as constant cell counts with changes in cell diameters.

To overcome the challenge of single-frequency measurements first attempts to measure permittivity at multiple frequencies in combination with mathematic modelling were applied in the recent past to online monitor VCCs [66, 74].

The third publication "*Multivariate data analysis of capacitance frequency scanning for online monitoring of viable cell concentrations in small scale bioreactors"* uses a method to combine MVDA analysis of frequency scanning data to correlate and predict online VCCs. The method is applied to one of the previous used industrial fed-batch processes. The MVDA model, including all frequencies, leads to a successful correlation and prediction of the VCC throughout the complete cultivation time. Moreover, the method development is done in a small scale bioreactor enabling fast and economic development timelines. The publication demonstrates once again the importance and benefits of suitable scale-down models in

combination with PAT tools. To conclude, the presented method on frequency scanning in combination with MVDA enables online monitoring of the important process attribute VCC. The method is proposed to be used as process control tool leading to improved process understanding and consistent process performance.

3.1 A protocol to transfer a fed-batch platform process into semi-perfusion mode: The benefit of automated small scale bioreactors compared to shake flasks as scale-down model (Biotechnology Progress, 2018)

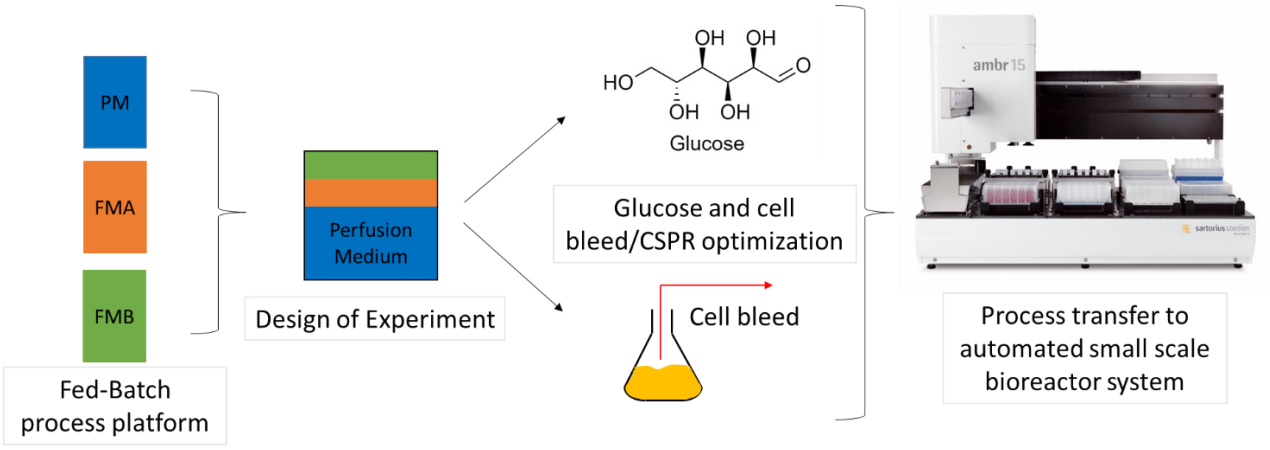

Figure 4 Graphical abstract of "A protocol to transfer a fed-batch platform process into semi-perfusion mode: The benefit of automated small scale bioreactors compared to shake flasks as scale-down model". [22]

Nowadays, the need for fast pharmaceutical development with high product yield and quality is increasing constantly. The following section focuses on process intensification to achieve a product yield increase within the same process time and by keeping the original equipment. Goal of the work was to develop in a short timeframe a semi-perfusion CHO process based on an existing conventional fed-batch process, increasing the product titer significantly, while keeping a healthy cell culture with end viabilities above 70%.

The first step towards a semi-perfusion process was to identify the optimal medium composition based on the process medium and feeds of the conventional fed-batch. A DoE approach in shake-flasks enabled a fast medium screening with a low number of experiments. The optimal basic medium formulation consisted of 91.2% process medium, 8% feed medium A and 0.8% feed medium B. After the DoE identified the optimal medium composition for the semi-perfusion process, the glucose concentration was investigated and optimized. To deliver best nutrient supply to the high VCCs in the culture a glucose spike to keep the concentration above 2 g/L was implemented. Additionally, the cell bleed was investigated as it relates to the cell specific perfusion rate that influences the viability of the cell culture. The final semi-perfusion process design was fixed to a cell bleed to 20 million cells/mL, a glucose spike to

keep the concentration above 2 g/L and the medium formulation revealed from the DoE studies. The cumulative mAb titer was successfully increased to 10 g/L being threefold higher compared to the conventional fed-batch process.

The optimized semi-perfusion process was transferred from shake-flask to an automated, small scale bioreactor system offering a controlled bioreactor environment for important process parameters, such as pH or DO. In the small scale bioreactor the best overall result for the cell specific productivity (38.9 pg/(cell*day)) in contrast to semi-perfusion shake flasks (36.0 pg/(cell*day)) and the fed-batch process (24.2 pg/(cell*day)) was achieved. The microscale bioreactor represents a powerful scale-down system enabling early process development in controlled bioreactor environments. Thus, the media screening and process development takes place in an environment similar to the final production bioreactors. The presented method can be used for process intensification, saving costs and reducing the footprint of biopharmaceutical production plants.

AIChE

A Protocol to Transfer a Fed-Batch Platform Process into Semi-Perfusion Mode: The Benefit of Automated Small-Scale Bioreactors Compared to Shake Flasks as Scale-Down Model

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*Continuous processes such as perfusion processes can offer advantages compared to fed-batch or batch processes in bio-processing: improved product quality (e.g. for labile products), increased product yield, and cost savings. In this work, a semi-perfusion process was established in shake flasks and transferred to an automated small-scale bioreactor by daily media exchange via centrifugation based on an existing fed-batch process platform. At first the development of a suitable medium and feed composition, the glucose concentration required by the cells and the cell-specific perfusion rate were investigated in shake flasks as the conventional scale-down system. This lead to an optimized process with a threefold higher titer of 10 g/L monoclonal antibody compared to the standard fed-batch. To proof the suitability and benefit as a small-scale model, the established semi-perfusion process was transferred to an automated small-scale bioreactor with improved pH and dissolved oxygen control. The average specific productivity improved from 24.16 pg/(c*d) in the fed-batch process and 36.04 pg/c*d in the semi-perfusion shake flask to 38.88 pg/(c*d) in the semi-perfusion process performed in the controlled small-scale bioreactor, thus illustrating the benefits resulting from the applied semi-perfusion approach, especially in combination with controlled DO and pH settings.* © 2018 American Institute of Chemical Engineers *Biotechnol. Prog.*, 2018
Keywords: continuous mammalian cell cultivation, small-scale bioreactor, DoE, mAb, process optimization

Introduction

Production of monoclonal antibodies (mAbs) with mammalian cell cultivation processes is an important and challenging step in modern medicine to achieve the final pharmaceutical product. Pharmaceutical companies must balance strong market requirements with the pressure to decrease costs of production. Therefore, improved process efficiency due to implementation of intensified processes such as perfusion has raised attention with the recently improved perfusion equipment. Establishing perfusion processes can offer advantages for specific cell lines in product yield, manufacturing costs, and flexibility compared to fed-batch or batch processes.[1–3] The characteristics of intensified processes are based on a continuous removal of inhibiting metabolites as well as continuous addition of fresh medium. Therefore, high cell concentrations and high productivity can be achieved in relatively small volumes of bioreactors.[1,4] Thus, the adaptation of an industrially relevant fed-batch process to perfusion mode leads to higher product yields and an increase in efficiency.

Viable cell concentrations (VCCs) for CHO cell cultures in batch cultures were reported from 0.13 million cells/mL up to 6 million cells/mL.[5–7] VCCs of CHO fed-batches range between 1 and 26 million cells/mL,[8–10] whereas in perfusion processes constant VCCs from 27 million cells/mL to high concentrations of more than 200 million cells/mL were reported.[6,11,12] Product titers of batch processes were reported for mAbs with titers up to 0.9 g/L.[5,12] Fed-batch processes showed advantageous antibody concentrations in the range of 1–13 g/L.[10,13] Recently, perfusion

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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processes were established with 10-fold higher mAb production up to 1.9 g/L per day of cultivation, that can lead to cumulative titers up to 25 g/L of mAb and more in one cultivation process.[14,15]

Nevertheless, intensified mammalian cell culture processes have to overcome challenges. To keep the cell culture in a pseudo-steady-state, feed rates and uptake rates need to be well-known and controlled during the complete cultivation time.[16] The cell-specific perfusion rate (CSPR) is one indicator giving the relation of the chosen perfusion rate and existing cell concentration in a process (see Eq. 1). The CSPR optimum depends on each cell line, medium composition and process.[17] For a stable perfusion culture, this value should be monitored and controlled leading to an optimal cell growth and productivity.[18] There are many other influencing factors, like the medium and glucose concentration and the implementation of a cell bleed.[19–21]

$$\mathrm{CSPR} = \frac{\text{perfusion rate}}{\text{viable cell concentration}} \quad (1)$$

One important and critical step in perfusion processes is the cell retention device that keeps the cells inside the bioreactor while the medium with the inhibiting metabolites leaves the reactor. Depending on the cell line and the process characteristics, the product is either kept in the bioreactor or it is part of the medium that leaves the reactor.[22] A cell retention device allows the removal of inhibiting metabolites, like lactate and ammonia, and enables fresh nutrient supply through the medium exchange during cultivation.[23] For this step, different approaches and devices are available that are based on either filtration principles, sedimentation, or centrifugation.[24] One commonly used method in industry is to implement a filtration step during the process. The two common used applications within filtration are crossflow membrane filtration called alternating tangential flow (ATF) filtration and tangential flow filtration (TFF).[11,25,26] Moreover, cell retention can be implemented through devices like spin filters, inclined settlers, continuous centrifugation, or ultrasonic separators.[4,6,22,25]

Besides process development, the transfer of pharmaceutical processes to automated bioreactors and the subsequent scale-up are essential and delicate steps in biopharmaceutical manufacturing. Critical process parameters (e.g. temperature, pH, or the volumetric oxygen transfer coefficient) need to be studied and considered for process transfers. Especially, the cell retention step in a perfusion process needs to be adjusted during process scale-up and automation.

The goal of the reported work was to develop a semi-perfusion protocol and to show an easy transfer of an optimized semi-perfusion process from classical shake flasks to an automated small-scale bioreactor. Thus, fast and efficient process development of a semi-perfusion process based on an established fed-batch process platform was investigated and optimized. Further on, the process transfer and the possibility to equally use the automated small-scale bioreactor as scale-down model with increased process control compared to shake flasks was tested. Due to existing limitations of continuous medium exchange in shake flasks and disposable small-scale bioreactors, the medium exchange was applied on a daily basis by centrifugation resulting in a semi-perfusion process. During the centrifugation step, there is no aeration and process control that can lead to a short period of starving of the cells. Despite this technical restriction of missing process control during the centrifugation step, the developed protocol still provides a good approximation of a genuine perfusion process. Semi-perfusion operation modes in small-scale systems have been successfully investigated to simulate perfusion processes for screening and process development.[17,27,28] Based on these results, a strategy was developed to meet the requirements for a perfusion process.

The standard medium formulation with its proprietary feeds used in fed-batch processes was adapted to the nutritional requirements in perfusion cultivations. Afterward, critical process variables, like CSPR, glucose concentrations, and cell bleed were investigated for the semi-perfusion operation. Finally, the newly established semi-perfusion process was transferred from shake flasks to an automated small-scale, single-use, stirred tank bioreactor system. The small-scale bioreactor offers advantages for future testing of the critical process parameters in early process development of continuous processes and enables conditions close to production scale due to pH and DO control.

Micro-scale bioreactors are commonly used as a scale-down model for fed-batch processes.[29–31] Recently, the additional application of small-scale bioreactors for semi-perfusion processes with a sedimentation approach for media exchange was investigated.[17,32,33] The sedimentation approach showed limitations and is in conflict with the advantages of such controlled micro bioreactors as sedimentation times with no bioreactor control of 40 min are reported (i.e. DO and pH control). [33] Therefore, this study focuses on transferring the centrifugation approach from the shake flasks to the small-scale bioreactors with a maximum of 15 min without bioreactor control. The centrifugation method drastically reduces the time of non-controlled process parameters in the bioreactors. Accordingly, it efficiently supports the advantages of the small-scale bioreactors in contrast to shake flasks or other small-scale models for semi-perfusion processes. The goal of this work was to develop an easy perfusion-based protocol using existing devices to reduce costs and maximize the benefits from process intensification in terms of product increase.

Materials and Methods

Cell lines and medium

A DG44 CHO cell line expressing an IgG1 mAb was used in this study (Sartorius Stedim Cellca GmbH). All medium and feeds used were part of the Sartorius Stedim Cellca medium platform and were chemically defined. In total, stock medium for the seed culture (SM) and production medium as basal medium for production (PM) were used. Moreover, two different feeds, Feed Medium A (FMA) for macro nutrients (e.g., glucose) and Feed Medium B for micro nutrients (e.g., amino acids) (FMB) were used (Sartorius Stedim Cellca GmbH).

Seed culture

A cryo vial containing 1 mL CHO suspension (passage 8) at a concentration of 30 million cells/mL was thawed and transferred in a 15 mL Falcon® tube (Sarstedt) with 10 mL pre-warmed (36.8 °C) seed medium. This suspension was centrifuged (Centrifuge 3–30 K, Sigma) at 190*g* at room temperature for 3 min to remove all components of the freezing medium. After decanting the supernatant, the pellet was resuspended with 10 mL pre-warmed seed medium and transferred

into a 500 mL Erlenmeyer flask (Corning) filled with 150 mL pre-warmed seed medium. The shake flask was incubated in an incubation shaker (Certomat CTplus, SSB) at 36.8 °C and 7.5% pCO_2 with a shaking rate of 120 rpm and 85% humidity. Cells were passaged for five times every 3–4 days until inoculation of the production culture was done.

Semi-perfusion shake flask studies

Semi-perfusion shake flask cultures were inoculated from the seed train with a starting cell concentration of 2.5 million cells/mL. All cultivations were performed in 125 mL baffled shake flasks (Corning) with a vent cap and with a working volume of 25 mL. The experiments were performed in an incubation shaker (Certomat CTplus, SSB) with the same conditions as described for the seed culture. Each medium exchange took place in a 24 h rhythm. For sample analysis, shake flasks were moved to the biological safety cabinet, and 1 mL was removed after sufficient shaking. The remaining cell suspension was transferred into a 50 mL Falcon tube by pipetting, and centrifuged at 500*g* for 5 min at room temperature (3-30K, Sigma). Following centrifugation, 2 mL of the supernatant were taken for IgG quantification, and the remainder was discarded by decanting. The cell pellet was resuspended with 25 mL of pre-warmed, fresh medium by gentle pipetting, transferred into the shake flask, and placed back into the incubator. The total duration of each centrifugation step was not exceeding a total time of 15 min to keep the time as less as possible. The medium composition of each experiment is described in detail in the corresponding paragraphs.

Design of experiment study

The experimental set-up and the analysis of the results were carried out with the design of experiment (DoE) software MODDE 12 (Umetrics, Sartorius Stedim Data Analytics). A full factorial design was used to evaluate the influence of different medium compositions on the VCC as a single and quantitative response using a quadratic regression model, with FMA (three levels) and FMB (three levels) as the controllable and qualitative mixture factors, and PM (nine levels) as the fill factor (to 100%).

Cell bleed in semi-perfusion experiments

The cell bleed in shake flasks was applied based on the defined conditions for each experiment, and was calculated according to Eq. 2 based on the daily VCC and the total volume of the culture (V_c). The calculated volume of cell suspension was removed by manual pipetting and replaced with fresh, pre-warmed medium. Therefore, the cell bleed was applied in a semi-continuous mode.

$$V = \left(1 - \frac{VCC\text{target}}{VCC\text{measured}}\right) \cdot V_c \qquad (2)$$

Parallel, automated small-scale bioreactor

The automated small-scale bioreactor system ambr® 15 with up to 48 disposable cell culture bioreactor vessels (Sartorius Stedim Biotech GmbH) was used in the experiments. The working volume of each bioreactor can be in the range of 10 mL and 15 mL. Placed in a biological hood, the ambr® 15 can be used sterile and fully automated due to pH and DO control and independent gassing for each bioreactor of O_2, CO_2, and N_2. Each culture station containing up to 24 bioreactor slots can be temperature controlled and run at fixed impeller speeds. Sampling and feeding is applied by the liquid handler inside the hood.

Each bioreactor was inoculated from the seed train with a seeding density of 2.5 million cells/mL. To achieve this higher seeding density, cells from the seed train were centrifuged at 500*g* at room temperature for 5 min (3-30K, Sigma) and resuspended in pre-warmed medium for the semi-perfusion process. The total duration of each centrifugation step was not exceeding a total time of 15 min to keep the time as less as possible. All cultivations were performed in a 15 mL ambr® 15 system with sparged cell culture vessels (Sartorius Stedim Biotech GmbH). The working volume of each bioreactor was 10 mL. The temperature set point was chosen at 36.8 °C and the pH was controlled at 7.1 through CO_2 sparging. Agitation of each bioreactor was set to 1300 rpm and dissolved oxygen was controlled at 60%. A constant N_2 flow was set to 150 µL/min to allow gas transfer. To prevent foaming 20 µL of a 2% solution of Antifoam C Emulsion (30%) was added every 24 h (Sigma). The process lasted 12 days with one vessel volume exchange (VVD) of medium per day. This was carried out by spinning down the cells in the vessel every 24 h (centrifuge 6K15, Sigma), and resuspending the cell pellet afterward in 10 mL fresh medium using the liquid handler of the ambr® 15 system. 2 mL of supernatant before the daily medium exchange were stored at −20 °C for further IgG quantification. In addition, supernatant was taken after the daily media exchange. The volume of the second supernatant sample was variable to keep the working volume at 10 mL in each bioreactor.

Offline analytics

Process specific values for pH, glucose and lactate concentrations were measured in a blood gas analyzer (ABL800 Basic, Radiometer). The osmolality was determined (Osmomat 030, Gonotec) for every sample. The VCC indicating the amount of viable cells in the cultivation and the viability of cells according to the total cell concentration, as well as the cell diameter, were analyzed with the Trypan Blue Assay based Cedex HiRes Cell Counter and Analyzer system (Roche). The CSPR, used to monitor the specific medium supply per cell per day was calculated according to Eq. 1. The integral viable cell concentration (IVCC), also called cumulative cell hours, indicates the total amount of time the cells have produced mAb and is calculated by trapezoidal integration of the VCC over the time (t) (Eq. 3).[34]

$$IVCC_i = \int_{t=0}^{t} VCC(t)dt \approx \sum_{i=1}^{n} \frac{VCC_i + VCC_{i-1}}{2} \cdot (t_i - t_{i-1}) \qquad (3)$$

Analysis for IgG quantification

The cell-free supernatants of all samples were stored in the freezer at −20 °C before IgG titer quantification with high-performance liquid chromatography (HPLC) was performed using a Dionex UltiMate 3000 HPLC System (Thermo Scientific) and a Yarra 3 µm SEC 3000 (Phenomenex). As buffer

solution a mixture of 1 M Na_2SO_4, 0.5 M NaH_2PO_4 + 0.5 M Na_2HPO_4 (Sigma) and water (1:1:8) with a pH at 6.6 was used. Before analysis, samples were thawed and diluted with water, and filtered through Minsart RC4 0.2 µm syringe filters (Sartorius) into autosampler cups (Carl Roth) using 1 mL syringes (Optifine-F). The calibration curve was measured based on an IgG stock solution (Cytoglobin 5%, human Immunoglobulin G, Bayer Vital GmbH).

To calculate the indicated cumulative product, first the measured product concentrations c_i were normalized to a volume of 1 L resulting in the product amounts m_i (Eq. 4). The product amount of the supernatant after centrifugation $m_{1,i}$ and the product amount of the resuspended cells after the exchange of medium $m_{2,i-1}$ were considered for the calculation of the cumulative product m_{total} (Eq. 5) considering 1 L working volume. By plotting the cumulative product against the integral viable cells (IVC), which are calculated with Eq. 6, the productivity of each process is indicated.

$$m_i = c_i \cdot 1\,L \tag{4}$$

$$m_{\text{total}} = \sum_{i=1}^{n} (m_{1,i} - m_{2,i-1}) \tag{5}$$

$$IVC = IVCC \cdot 1\,L \tag{6}$$

Calculation of cell-specific productivity and the lactate yield coefficient from glucose

The cell-specific productivity (Q_p) was calculated to compare the key experiments from each bioreactor system, the shake flask and the automated small-scale bioreactor, and the new established semi-perfusion process. The Q_p was calculated according to Eq. 7.[35]

$$Q_P = \frac{V_c(m_i - m_{i-1})}{t_i - t_{i-1}} \left(\frac{V_c(VCC_i + VCC_{i-1})}{2} \right)^{-1} \tag{7}$$

The lactate yield coefficient from glucose ($Y_{Lac/Glc}$) (Eq. 10) is calculated based on the specific glucose consumption (q_{Glc}) (Eq. 8) and the specific lactate production (q_{Lac}) (Eq. 9), with the glucose concentration (c_{Glc}), the lactate concentration (c_{Lac}) and the geometric mean of the VCC ($\bar{x}$).[35]

$$q_{Glc} = \frac{V_c\,(c_{Glc,i-1} - c_{Glc,i})}{(t_i - t_{i-1})} \frac{1}{\bar{x}} \tag{8}$$

$$q_{Lac} = \frac{V_c\,(c_{Lac,i} - c_{Lac,i-1})}{(t_i - t_{i-1})} \frac{1}{\bar{x}} \tag{9}$$

$$Y_{Lac/Glc} = \frac{q_{Lac}}{q_{Glc}} \tag{10}$$

Results and Discussion

An overview of the experiments performed in this study is presented in Figure 1. First of all, the medium composition for the semi-perfusion process was optimized. For this purpose, different blends from a CHO-fed-batch platform-medium and its proprietary feeds were investigated applying DoE. The basal process medium (PM) was used as a basis for the new medium formulation. FMA was added to the DoE as it is known to deliver macro nutrients (e.g., glucose) to the cells. FMB was investigated in the DoE as it is necessary for micro nutrients (e.g., amino acids) in the cell culture. The optimized medium was used afterwards for further process optimization. In particular, the glucose addition to the cell culture and the cell bleed were investigated to improve the final viability. The medium and process optimization was conducted in shake flasks as a scale-down model using centrifugation for media exchange. In a last step, the optimized semi-perfusion process was transferred to an automated small-scale bioreactor system in which a continuous DO and pH control can be achieved. By transferring the process to the automated small-scale bioreactor, the great potential of the bioreactors to replace the classical shake flasks as parallel scale-down system was investigated.

DoE studies for medium optimization

In the first part of the studies, medium optimization based on an existing fed-batch process was performed. Therefore, FMA, FMB, and the PM were combined in different blends to receive one medium that meets the requirements of higher cell concentrations in the semi-perfusion process. Therefore, the PM was enriched with FMA and FMB to receive one media formulation for the semi-perfusion. For optimization and fast progress with as few experiments as necessary a DoE study was implemented in shake flasks as shake flasks are commonly used as scale-down model for process development.[17]

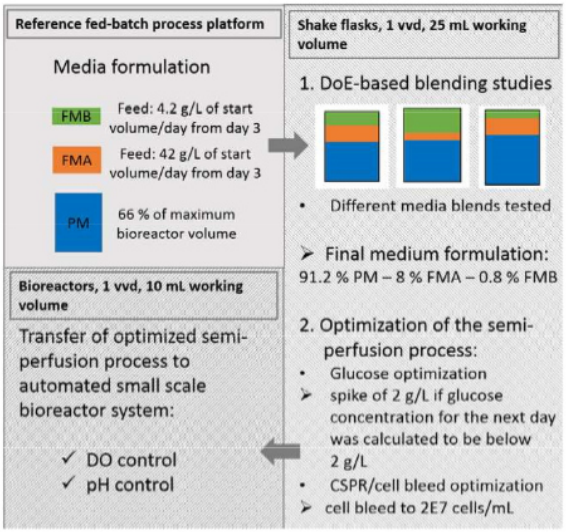

Figure 1. Schematic overview of the experimental structure in this work. The fed-batch process platform was used as a base for further process improvements meeting the requirements for process intensification. The PM and the respective feeds, FMA and FMB, were investigated with DoE to find an optimal medium mixture for semi-perfusion processes. The process strategy was further optimized by adjusting the glucose concentration and the CSPR by implementation of a cell bleed. The optimal conditions of the semi-perfusion process were transferred to a small-scale bioreactor system with DO and pH control. Each VVD of medium per day is displaced for the semi-perfusion processes.

Table 1. DoE Set-up for Medium Development in Shake Flasks Suitable for the Semi-Perfusion Process

Composition ID	PM (%)	FMA (%)	FMB (%)
PF1_DoE1	100.0	0	0
PF1_DoE2	99.2	0	0.8
PF1_DoE3	92.0	8	0
PF1_DoE4	91.2	8	0.8
PF1_DoE5	96.0	4	0
PF1_DoE6	95.2	4	0.8
PF1_DoE7	99.6	0	0.4
PF1_DoE8	91.6	8	0.4
PF1_DoE9	95.6	4	0.4

The variations of each fraction within the DoE were highly beneficial to understand the nutrient requirements of the high cell concentrations. The medium exchange was applied on a daily basis by centrifugation. Therefore, the VVD for each experiment was 1. Conditions of each shake flask run and the center point PF1_DoE9 that was performed in a triplicate is shown in Table 1. The DoE design was based on a full factorial central composite face-centered design (CCF) containing the three factors PM, FMA, and FMB. The VCC was integrated as response factor. The experimental design was based on an optimization response surface methodology with a quadratic process model.

The criteria for optimization were the VCC and viability of the cell population (Figure 2A). VCCs up to 45 million cells/mL were obtained, which is twice the amount of cells compared to the previous fed-batch process (Supporting Information Figure S1). As it can be seen from the VCC and viability data, exclusively in the medium blends PF1_DoE4 and PF1_DoE8, the viability and VCC were high over a period of 250 h. In all other shake flasks, the viability decreased dramatically after 170 h. Therefore, one indication from the DoE is the addition of both feeds, and 8% FMA is necessary for a stable process. This demonstrates that only in the highlighted medium compositions the nutrient supply is sufficient for long productivity.

However, the increase of the FMA concentration is also limited due to higher osmolality with rising nutrient concentration. The glucose profile (Figure 2B) showed a lack of glucose for nearly every cultivation after 150 h. Comparing the cultivation time, the lack of glucose corresponded with a strong decrease in viability and VCC. The correlation of the glucose concentration and the decreased viability and VCC indicated a possible glucose limitation during the cell cycle and other metabolic processes (e.g., nucleotide synthesis). Optimization is necessary in further steps.

The CSPR was analyzed for each semi-perfusion process based on Eq. 1 taking into consideration the VCC after each 24 h interval. The CSPR values for the DoE study (Figure 2B) indicate a lower limit for the CSPR at 50 pL/(c*day). However, the critical CSPR needs to be further investigated in the following experiments. The cumulative product summarizes the product concentrations before and immediately after centrifugation based on the integral of viable cells in the cell culture according to Eqs. 5 and 6. The cumulative product in the semi-perfusion processes showed a clear improvement up to 12 g compared to the normal fed-batch process with an average of 3 g in the corresponding working volume of 1 L (Supporting Information Figure S1).

Besides the results of the beneficial medium compositions PF1_DoE4 and PF1_DoE8 seen from the offline measurements in viability, VCC and CSPR (Figure 2), the DoE response surface plot showed strong tendency that with 8% FMA a local optimum was reached in the presented DoE setup

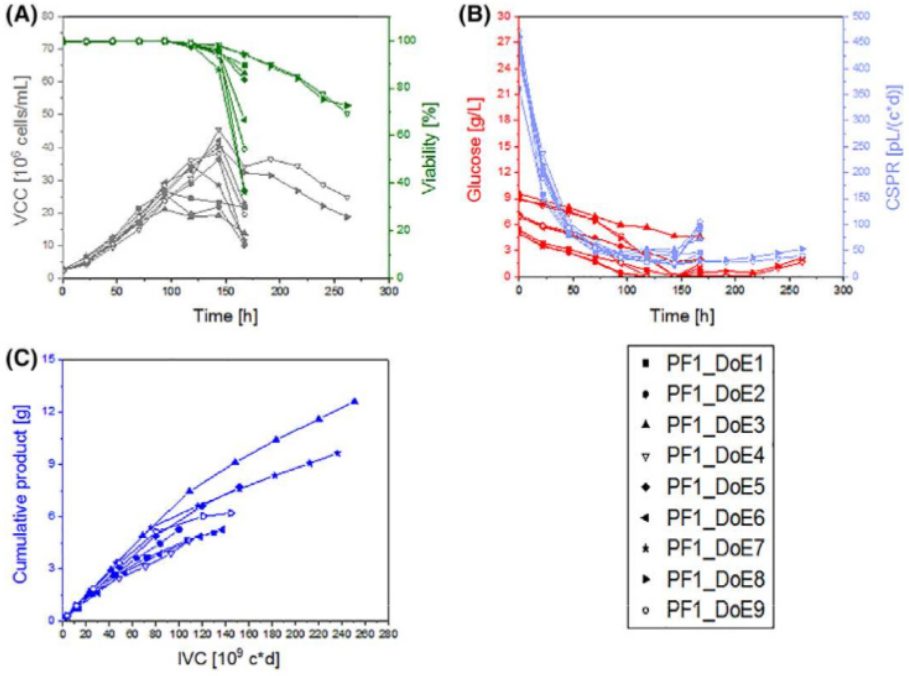

Figure 2. Results of DoE experiments for semi-perfusion medium optimization with a mAb producing DG44 CHO cell line. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) of each shake flask run are shown. Center point PF1_DoE9 based on $n = 3$.

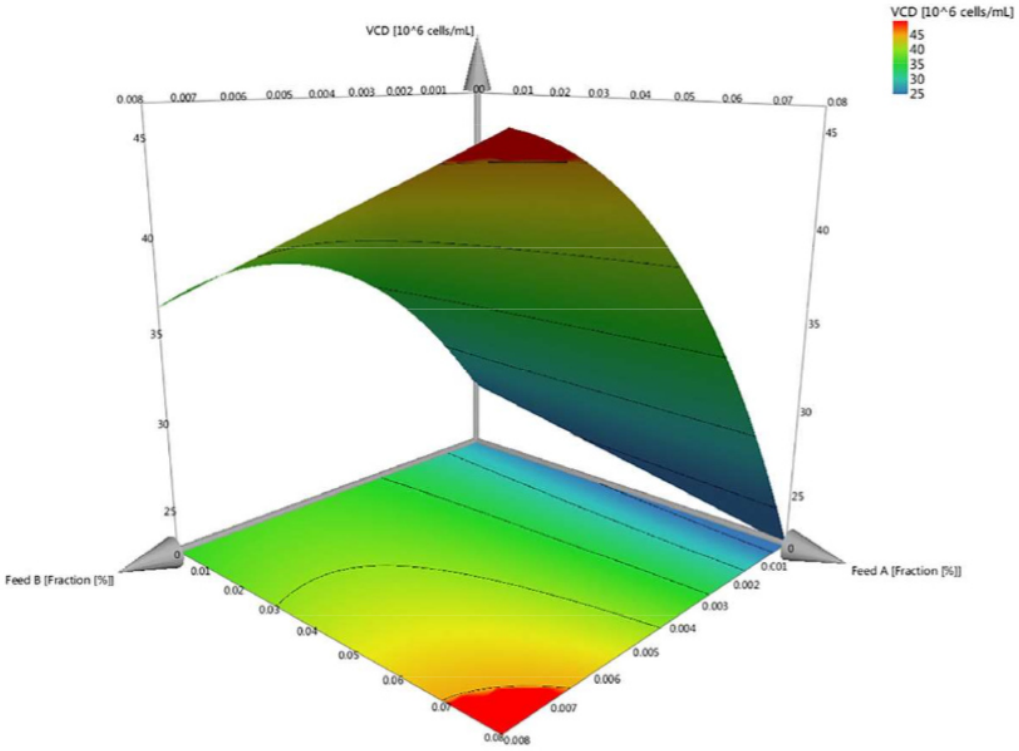

Figure 3. Response surface plot of DoE experiment. The evaluation is based on VCC and different feed fractions (%) in the medium blends. The response of the target variable is color-coded, ranging from blue (low) to red (high).

(Figure 3). However, further increase of the fractions FMA and FMB to optimize the first screening DoE is likely to result in an osmolality exceeding 400 mOsmol/kg. Compared to literature, an elevated osmolality from 320 to 435 mOsmol/kg reduced production rates by 50% for CHO cells.[36] As further refinement of the media formulation requires detailed information on the original recipe, no additional optimization runs were conducted. The resulting local optimum was used as a satisfactory compromise between performance and osmolality.

Optimization of glucose concentrations for stability in the semi-perfusion process

One critical factor during process transfer from fed-batch mode to perfusion processes is the glucose concentration. As it can be seen from the DoE study (see Section "DoE studies for medium optimization"), it is necessary to optimize the amount of glucose in accordance to the higher cell concentrations obtained in the semi-perfusion process. Therefore, two different approaches of glucose bolus were analyzed. In the first part, different compositions of PM, FMA, FMB together with glucose (added directly to the compositions) were analyzed. These compositions were tested to gain a better understanding of the correlation between each component (Supporting Information Figure S2 and Table S1). In a second step, glucose spikes were added directly to the culture after the daily medium exchange (Figure 4).

Both experiments showed similar culture behavior achieving a longer stability and the favored 12 days of cultivation with high cumulative products. The control composition PF2_Glc1 without any additional glucose showed the same glucose depletion after five cultivation days as in previous experiments (Supporting Information Figure S2B). Best results in viability were achieved in PF2_Glc4, PF2_Glc5, and PF3_GlcSpike1. All of those approaches contained 91.2% PM, 8% FMA, and 0.8% FMB. Being aligned with the previous DoE results, this medium composition was used in every following experiment and is now called semi-perfusion medium.

The glucose addition was highly beneficial for the stability of the new established semi-perfusion process with VCCs achieved up to 40 million cells/mL. The results are in good agreement compared to perfusion cultures in the literature.[12] Analyzing the CSPR values of the different approaches, it is indicative, that below a CSPR of 50 pL/(c*day) a decrease in viability can be seen in the following days. Glucose additions starting from 2 g/L up to 8 g/L were beneficial for higher CSPR values. The best glucose concentration in the range between 2 g/L and 8 g/L needs to be further specified in the following experiments.

As no difference between the two tested approaches of how to add glucose to the cell culture was observed, the glucose spike was further tested as it offers advantages in easier handling and better adaption to batch to batch variations in cell growth.

However, the viability in both experimental setups decreased to 60% on the last two cultivation days (Supporting Information Figure S2 and Figure 4). As viabilities below 70% are common harvest criteria,[10,37,38] further improvement of the process stability and higher CSPR values are desirable.

Influence of the cell bleed on cell culture stability

After improving the medium in the DoE (see Section "DoE studies for medium optimization") and the process strategy by

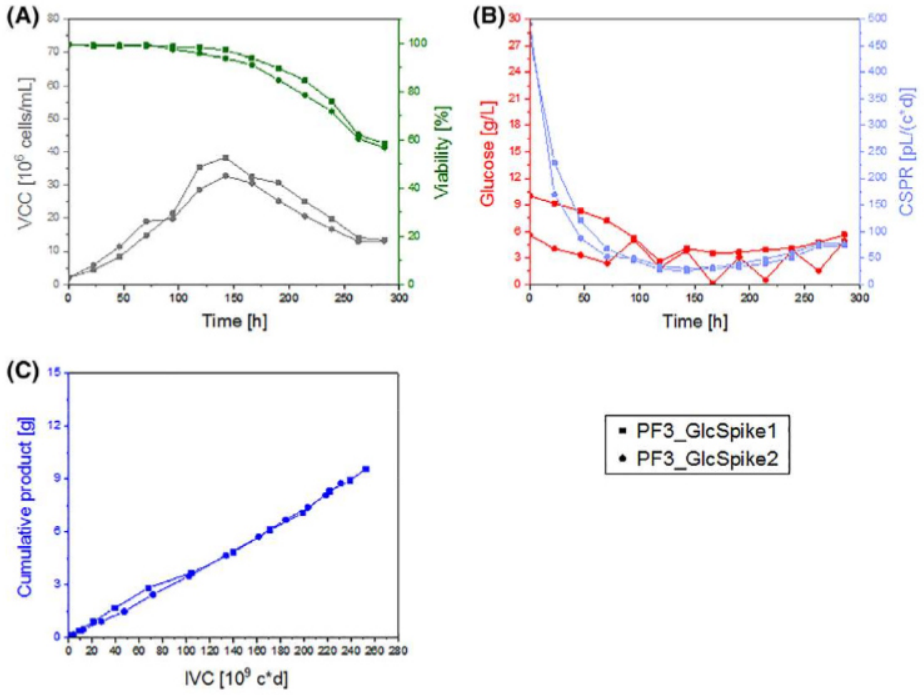

Figure 4. Performance of shake flasks with implementation of additional glucose spikes in the semi-perfusion processes of a mAb producing DG44 CHO cell line. For PF3_GlcSpike1 glucose was spiked with 4 g/L daily after Day 5 of cultivation. The medium composition for PF3_GlcSpike1 was 91.2% PM, 8% FMA, and 0.8% FMB. The glucose spike for PF3_GlcSpike2 was applied on a daily basis starting from Day 1 if the subtraction of the glucose consumption of the last 24 h from the actual glucose concentration was below 2 g/L. In that case, a spike of 4 g/L was carried out. The medium composition for PF3_GlcSpike2 was 100% PM in the first 3 days and 91.2% PM, 8% FMA, and 0.8% FMB in the following days. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the produced antibody in relation to the IVC (C) of each shake flask run are shown. Depicted are the mean values of duplicate measurements (n = 2).

adapting glucose additions (see Section "Optimization of glucose concentrations for stability in the semi-perfusion process") another key factor in the new established semi-perfusion process, the cell bleed, was investigated. During a cell bleed, the VCC is reduced to a fixed target bleed VCC after medium exchange. The cell bleed protects the culture from being limited by nutrients provided with a fixed, daily medium exchange. To move from a fed-batch culture to perfusion cultures, the cell bleed is essential to maintain a stable VCC over prolonged process time provided that the VVD of 1 is kept constant. As the established process is based on semi-perfusion, a requirement of a fixed VVD of 1 was set for the experiments. Therefore, different cell bleed strategies were tested (Table 2) instead of increasing the VVD. In addition, to further improve the glucose concentrations and the correlation of cell bleed and glucose spikes, different combinations of bleed and glucose additions were tested in the cell bleed studies. The cell bleed was combined with the daily media exchange. Therefore, the cell bleed is considered as being applied in semi-continuous mode within the semi-perfusion process.

In all approaches applying the cell bleed to the semi-perfusion shake flask cultures, peak VCCs of 25 million cells/mL or higher were achieved (Figure 5). However, the first bleed condition PF4_Bleed1 with a bleed VCC of 40 million cells/mL and a high glucose addition to 4 g/L from Day 5 on, showed a decrease in viability to 65% on the last cultivation day. This viability is lower than the demanded viability of 70% at the end of the semi-perfusion culture. Therefore, the high bleed VCC is not beneficial for a stable semi-perfusion process with the given VVD of 1. Moreover, the VCC of PF4_Bleed1 was similar to the VCC achieved in PF4_Bleed4 with a bleed threshold of 25 million cells/mL. In both approaches, the CSPR fell below 50 pL/(c*day) after 110 h of cultivation, thus resulting in a decrease of viability and inhibition of further cell growth to higher VCCs. The result indicates that the bleed should be applied at an earlier stage with lower target bleed VCCs. Higher VVDs might support stable cell populations with higher VCCs without the need to bleed, but increase the medium consumption and costs significantly.

Indeed, every bleed condition with a target bleed concentration of 20 million cells/mL (PF4_Bleed2 and PF4_Bleed3) or 25 million cells/mL (PF4_Bleed4) showed viabilities above 70% on the last day of cultivation with similar peak VCCs around 30 million cells/mL. In addition, the glucose concentration was not limited in the semi-perfusion medium over the complete process time (Figure 5B). Interestingly, PF4_Bleed2 with no additional glucose spiking showed no glucose limitation and was characterized by the highest end viability of above 80%. This indicates that the bleed in a semi-perfusion process enables constant CSPR values above the critical CSPR and therefore is fundamental for culture stability. The detected importance of the CSPR for the semi-perfusion culture is in good agreement to results from literature that report controlling, modeling, and optimizations of semi-perfusion processes based on the CSPR.[16] Even more, the highlighted condition

Table 2. Cell Bleed Strategies Tested During the Semi-Perfusion Processes in Shake Flasks. All Batches with 91.2% PM, 8% FMA, and 0.8% FMB

Composition ID	Glucose Addition	Bleed VCC
PF4_Bleed1	From Day 5 adjusted to 4 g/L	Cell bleed to 4E7 cells/mL
PF4_Bleed2	No addition	Cell bleed to 2E7 cells/mL
PF4_Bleed3	if: glc^{d}-[$glct^{d-1}$-glc^{d}] ≤ 2	Increase bleed level by 0.3E7 cells/mL every third day, starting with 2E7 cells/mL
PF4_Bleed4	if: glc^{d}-[$glct^{d-1}$-glc^{d}] ≤ 2	Cell bleed to 2.5E7 cells/mL

was constantly bled to the smallest investigated bleed level of 20 million cells/mL. According to previous observations, the decrease of the CSPR below 50 pL/(c*day) in each shake flask run, was aligned with a lower viability in the following culture days. Concluding with the cumulative product (Figure 5C), the improvement of the cumulative product was significantly up to 10 g in comparison to the fed-batch process (Supporting Information Figure S1).

As shown, medium optimization and corresponding cell bleed adaptation led to successful semi-perfusion cultures with final viabilities of more than 70% after 12 days of cultivation. Based on these findings, the process was fixed to 91.2% PM, 8% FMA, and 0.8% FMB and a target bleed concentration of 20 million cells/mL. This semi-perfusion process can be transferred into an automated small-scale bioreactor system in the following experiments. Ultimately, this will enable more process control and easier future investigations of the new process. Due to the direct transfer of the optimized process into the small-scale bioreactor, the advantage of the automated bioreactor as scale-down model for process optimization over the classical shake flask approach was investigated.

Use of automated small-scale bioreactor system to control pH and DO

After successful conversion of an existing industrially relevant CHO fed-batch process to a semi-perfusion process in shake flasks, the new process was transferred to an automated small-scale bioreactor system. The transfer into the bioreactors enables higher process control of relevant process parameters (e.g., DO, pH). In addition, a better process understanding can be achieved due to monitoring of the key performance indicators and the future possibility to establish automated medium exchanges. By applying the centrifugation approach in a small-scale bioreactor, the system's advantage in process control was fully used compared to previous literature that were using sedimentation-based approaches to exchange the media.[17,32,33] As mentioned before, the medium composition of 91.2% PM, 8% FMA, and 0.8% FMB and a cell bleed target of 20 million cells/mL were applied. Due to improved process control of the parameter set points and especially the pH control at 7.1 in the automated bioreactor, higher cell growth rates were expected. Therefore, glucose was additionally spiked

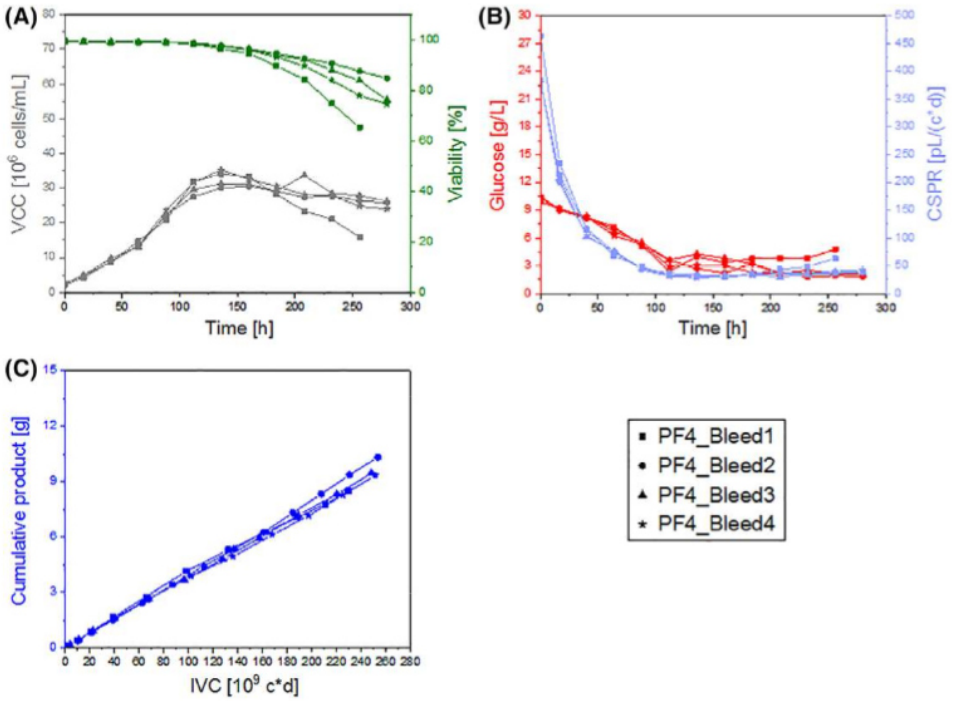

Figure 5. Implementation of cell bleed in the semi-perfusion processes of a mAb producing DG44 CHO cell line. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) of each shake flask run are shown. Depicted are the mean values of duplicate measurements (n = 2).

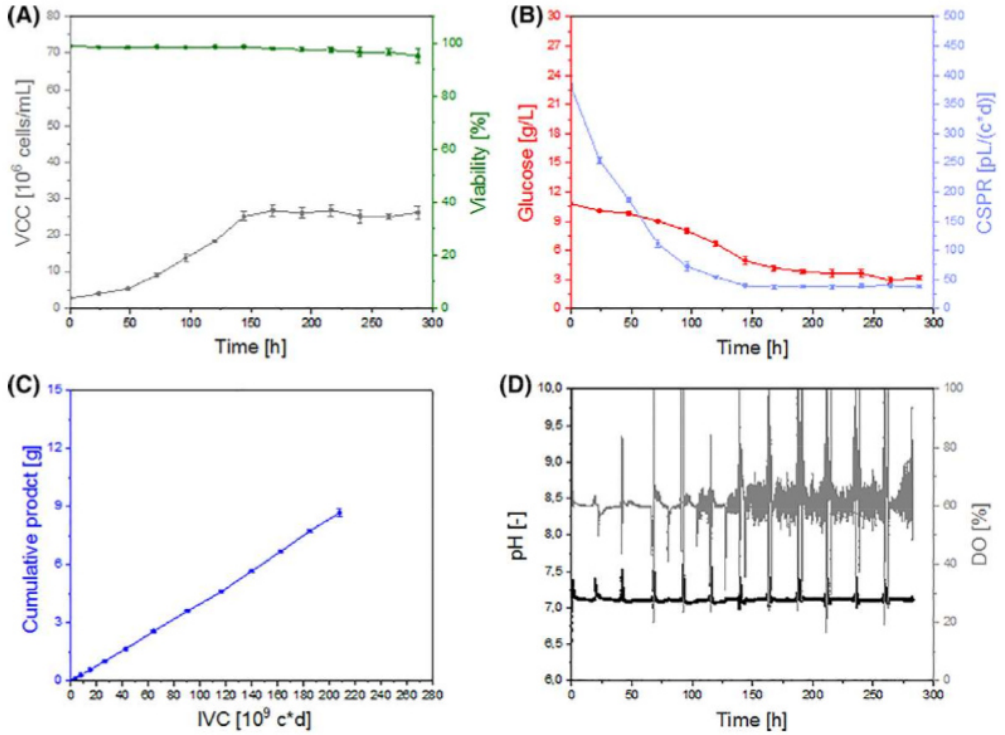

Figure 6. Transfer of the semi-perfusion processes of a mAb producing DG44 CHO cell line to an automated small-scale bioreactor system. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) are shown. Error bars are reported for each value. The controlled parameters, here the pH value and the DO of the process are shown (D). Depicted are the mean values of triplicate measurements (n = 3).

with 2 g/L to each bioreactor whenever the resulting glucose concentration for the following day was calculated to be below 2 g/L based on the specific glucose consumption per cell. The glucose spike was implemented to maintain a stable culture over the total cultivation time.

Figure 6 displays the results of the process transfer to the automated system. For all critical parameters shown in the figure, the favored criteria were achieved. The culture was stable over the complete culture time of 12 days. Even more, 20–30 million cells/mL were accomplished and kept as steady-state concentration with the help of precise cell bleeds (Figure 6A). In addition, the viability was above 98% for half of the cultivation time and decreased to 95% once the previous detected critical CSPR of 50 pL/(c*day) (Figure 6B) was reached. Therefore, it was shown that keeping the CSPR above 50 pL/(c*day) results in a stable cell culture with high viabilities. With the help of the bioreactor system, the viability was significantly higher than in any other shake flask experiment and the process was more stable than in uncontrolled shake flasks. The process was controlled to the pH set point of 7.1 and the DO set point of 60% over the complete culture time (Figure 6D). Compared to the pH values in the shake flask experiments (Supporting Information Figures S4–S6), a huge improvement of the pH control was observed. In addition to that an oscillation of DO could be detected in the automated bioreactors over the cultivation time. At later stages, high cell densities led to an increased oxygen demand of the culture that caused a more active control loop of the system and subsequent oscillations in the DO signal. Based on the generated data for DO and pH control experienced users will be able to have a sound understanding of the process's behavior at larger scales. Therefore, the use of small-scale bioreactors

Table 3. Lactate Yield Coefficient from Glucose in the Fed-Batch Process Platform, the semi-perfusion shake flask, and the ambr® 15 Semi-Perfusion Process, n = 3

Day of Culture	$Y_{Lac/Glc}$ (Fed-Batch Process Platform)	$Y_{Lac/Glc}$ (Semi-Perfusion Shake Flask)	$Y_{Lac/Glc}$ (ambr® 15 Semi-Perfusion)
1	0.91	0.45	0.5
2	0.58	0.19	0.08
3	0.31	−0.16	0.06
4	0.30	−0.07	−0.06
5	1.58	−0.06	−0.01
6	−0.07	0.02	−0.02
7	−0.03	0.10	−0.00
8	0.03	−0.02	0
9	−0.37	0.03	0.00
10	0.14	0.02	0.00
11	−0.15	−0.02	0.00
12	−0.01	−0.09	0.00

Table 4. Comparison of the Cell Diameter Increase Over Process Time, the Average Specific Productivity, the Medium Consumption Based on the Reactor Volumes (RV) and Other Key Process Parameters of the Fed-Batch and the Semi-Perfusion in Shake Flask and ambr® 15

Parameter	Fed-Batch Process Platform	Semi-Perfusion Shake Flask	ambr® 15 Semi-Perfusion
Cell diameter increase over culture time	3.14 µm	2.33 µm	2.69 µm
Average specific productivity	24.16 pg/(c*d)	36.04 pg/(c*d)	38.88 pg/(c*d)
Cumulative product	3.62 g	10.32 g	8.67 g
Integral of viable cells	180.71 10E9 c*d	253.62 10E9 c*d	208.15 10E9 c*d
Overall process time	12 days	12 days	12 days
Medium consumption	1.4 RV	12 RV	12 RV
End viability of cells	86.0%	83.55%	95.23%

is highly favored to gain deep knowledge for intensified bioprocesses. However, in a large-scale bioreactor the process will be implemented as a fully continuous process with a suitable cell retention device. The change from centrifugation in small-scale bioreactors to cell retention in large-scale bioreactors will enable culture control and process monitoring over the complete process time.

Applying glucose spikes whenever the glucose concentration was below the threshold of 2 g/L led to a sufficient nutrient supply for the semi-perfusion cell culture. The cumulative product of 8.7 g in the semi-perfusion process was three-fold higher than in the corresponding fed-batch process (Supporting Information Figure S1). The resulting standard deviations showed the three bioreactors to perform consistently throughout the process. The product amount over the total culture time can even be improved by cultivating the semi-perfusion culture in the controlled system for longer periods in the future. The possibility of longer cultivations is given by the higher stability and high viability in the controlled bioreactors. Due to the consistent and controlled semi-perfusion process in the small-scale bioreactors, the conclusions can be compared and used to improve process understanding for continuous processes and the implementation of perfusion processes in suitable bioreactors with cell retention devices.[17] Furthermore, the results can be used to predict and optimize the scale-up to production scales.

To conclude the presented study, the fed-batch process, the successful implementation of semi-perfusion processes in shake flasks and the process transfer in the automated small-scale bioreactor can be compared. This comparison can be based on the $Y_{Lac/Glc}$, the specific productivity, the increase in cell diameter, the cumulative product as well as the IVC and the end viability of each process (Tables 2 and 3). $Y_{Lac/Glc}$ allows deeper information about the cell state and the metabolism in the cells.[35] Glucose is used in different pathways based on the metabolic state and the nutrients in the environment. Lactate is a waste product in unfavorable pathways that can inhibit the cell growth. Therefore, a low $Y_{Lac/Glc}$ indicates efficient cell metabolism with low lactate production or lactate consumption by the cells.[35,39] Especially in the beginning of each process, $Y_{Lac/Glc}$ is significantly smaller in the semi-perfusion process indicating a more efficient metabolism than in the corresponding fed-batch cultivation (Table 3). The biggest positive effect was achieved in the automated small-scale bioreactor to values around 0, so that the ratio of glucose consumption and lactate production indicates a highly productive and healthy cell culture. These findings are supported by the lactate production over time in each of the three cultures (Supporting Information Figure S3). Especially in the beginning of the historic fed-batch a significantly higher lactate production was detected before the cells switched into lactate consumption.

Even more, the increase in the cell diameter is much less for the established semi-perfusion process compared to the historic fed-batch indicating a more viable cell culture (Table 4).[40] Another benefit of the semi-perfusion process is the low osmolality around 310 mOsmol/kg at the end of the process in comparison to an osmolality around 360 mOsmol/kg in the fed-batch. Besides the effect that apoptotic cells show larger diameter, the higher osmolality can additionally support the increase in diameter in the fed-batch.

Finally, the average specific productivity was increased to a final productivity of 38.88 pg/(c*day) in the automated small-scale bioreactor. The cumulative product and the IVC increased significantly in the semi-perfusion processes during the same culture time compared to the fed-batch. The automated small-scale bioreactor performed better with regards to the end viability of 95.23% compared to the semi-perfusion in shake flask or the fed-batch process. Therefore, the ambr® 15 performed best in the demonstrated studies regarding product yield and viability of the cell culture. The benefit of the automated small-scale bioreactor as scale-down model was clearly shown compared to the shake flasks.

Conclusion

With this work the conversion of an existing, industrially relevant fed-batch to a semi-perfusion process was successfully demonstrated by using the same platform medium and its proprietary feeds. The product yield normalized to 1 L of the corresponding fed-batch process increased from 3 g up to a cumulated product amount of 10 g in the semi-perfusion process for the same cultivation duration. However, in further experiments the product quality attributes of the newly established semi-perfusion process need to be compared to the product quality from the historic fed-batch. With the presented methodology, a powerful but low resource demanding approach to transfer an existing CHO fed-batch process into a semi-perfusion process implementing DoE studies and perfusion specific handling was established. The developed strategy to establish a semi-perfusion process can be adopted and used for different cell lines. In addition, the suitability of the process for a multiparallel controlled micro-bioreactor system as scale-down model has been proven so that early stage development of a semi-perfusion process can be done in a controlled system in future. Moreover, in a next step, process scalability and transfer will be tested in different bioreactor systems and scales. Changing to intensified cultivations enables high product yields with time and cost savings as well as keeping the same size of biopharmaceutical plant capacities or even reducing them.[3]

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3.2 Monitoring online biomass with a capacitance sensor during scale-up of industrially relevant CHO cell culture fed-batch processes in single-use bioreactors (Bioprocess and Biosystems Engineering, 2019)

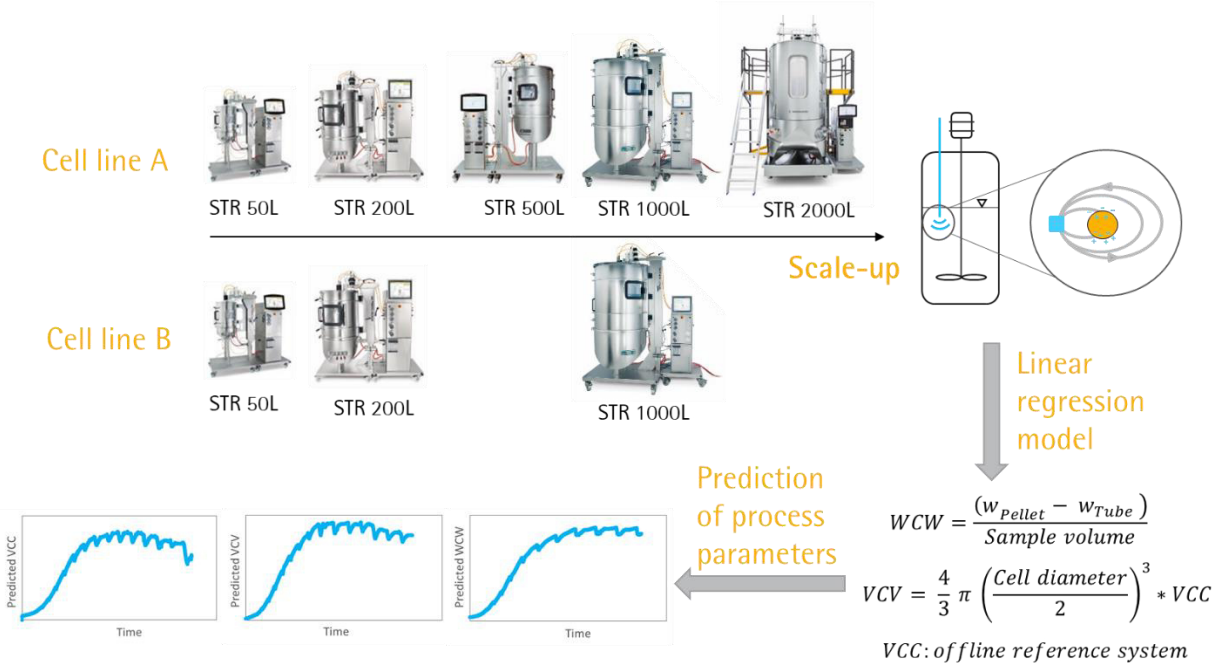

Figure 5 Graphical abstract of "Monitoring online biomass with a capacitance sensor during scale-up of industrially relevant CHO cell culture fed-batch processes in single-use bioreactors". [22]

VCC is an important process attribute in all mammalian cell culture processes. The previous section demonstrated the great potential of process intensification leading to high product yields. Perfusion processes require a high degree of process understanding and automation. Online monitoring of VCCs can be used for cell bleed adaption or for perfusion rate automation. However, VCC is still measured offline limiting the opportunities for the further development and automation of the semi-perfusion process. The offline sample for VCC reveals additional drawbacks, such as low temporal resolution or high contamination risks due to interaction of the operator with the bioreactor. Therefore, this section focuses on the establishment of a PAT tool to monitor online VCCs. The method development required a scale-independent method for single-use bioreactors that can be applied to all different process types. Thus, a capacitance sensor operated in single-frequency mode was investigated to provide online information about

biomass related changes. For the proof of concept a well-established fed-batch platform was used and performed in all available single-use bioreactors from 50 L up to 2000 L. The permittivity signal was correlated to selected parameters (WCW, VCV and VCC) and a linear regression model served the online prediction of the parameters. The proof of concept was repeated with a second industrial relevant fed-batch process to give an indication about the method transferability.

The linear regression model based on the inline capacitance sensor successfully monitored the WCW with a coefficient of determinations of 0.79 for Process A and 0.99 for Process B. The VCV correlated best to the permittivity signal with high coefficients of determination of 0.96 (Process A) and 0.98 (Process B), respectively. However, the linear correlation between permittivity and VCC was only valid for the exponential growth phase. In agreement with literature results the regression model excluded values after the peak cell concentration. The linear regression for the exponential growth phase was successful for all cultivations with high coefficients of determination of 0.99 (Process A) and 0.96 (Process B), respectively.

For all investigated parameters and in both fed-batch processes, no influence of the bioreactor size was observed. Therefore, scale-independency of the method was demonstrated offering a deep process understanding during future scale-up or scale-down. The presented method enables process control and online monitoring of important process parameters.

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RESEARCH PAPER

Monitoring online biomass with a capacitance sensor during scale-up of industrially relevant CHO cell culture fed-batch processes in single-use bioreactors

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Abstract
In 2004, the FDA published a guideline to implement process analytical technologies (PAT) in biopharmaceutical processes for process monitoring to gain process understanding and for the control of important process parameters. Viable cell concentration (VCC) is one of the most important key performance indicator (KPI) during mammalian cell cultivation processes. Commonly, this is measured offline. In this work, we demonstrated the comparability and scalability of linear regression models derived from online capacitance measurements. The linear regressions were used to predict the VCC and other familiar offline biomass indicators, like the viable cell volume (VCV) and the wet cell weight (WCW), in two different industrially relevant CHO cell culture processes (Process A and Process B). Therefore, different single-use bioreactor scales (50–2000 L) were used to prove feasibility and scalability of the in-line sensor integration. Coefficient of determinations of 0.79 for Process A and 0.99 for Process B for the WCW were achieved. The VCV was described with high coefficients of determination of 0.96 (Process A) and 0.98 (Process B), respectively. In agreement with other work from the literature, the VCC was only described within the exponential growth phase, but resulting in excellent coefficients of determination of 0.99 (Process A) and 0.96 (Process B), respectively. Monitoring these KPIs online using linear regression models appeared to be scale-independent, enabled deeper process understanding (e.g. here demonstrated in monitoring, the feeding profile) and showed the potential of this method for process control.

Keywords Mammalian CHO cell culture · Process monitoring and control · PAT · Capacitance · Impedance · Scale-up

Introduction

Scalability is a key aspect for biopharmaceutical companies to transfer a process that is producing an important protein or product from development stage to production scale. A bioprocess must reach production scale to be further considered for clinical trials in a pharmaceutical company and finally reach commercialization [1, 2]. A fast and reliable scale-up method enables faster development timelines and earlier entrance to the market with saving money and plant capacities. Therefore, it is desirable to have the ability to monitor all relevant parameters with the same measurement type in each process scale to keep the product quality and product quantity high and within GMP compliance [3–6]. Moreover, besides monitoring the process parameters, the demand for process control is strongly increasing [3, 7, 8]. The implementation of PAT supports online monitoring and real-time process control strategies. The application of PAT tools in biopharma processes is strongly suggested by the US Food and Drug Administration (FDA), underlined by the PAT initiative published in 2004. PAT enables real-time monitoring and control of critical process parameters (CPPs) that lead to consistent process performance and product quality [9–11].

The viable cell concentration (VCC) is one of the most important key performance indicator (KPI) during upstream technologies in mammalian cell culture [3]. However, often the VCC is measured by an offline method that stains dead

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cells with Trypan Blue and a cell count is done based on microscopic image analysis [12]. Online monitoring of biomass remains challenging as new technologies are more complex to calibrate or integrate into processes [13–16]. The limited samples per cultivation day as well as the time delay to respond to process changes are significant limitations of offline measurements that prevent efficient process monitoring and control of important CPPs. Several online methods to monitor the cell concentration of mammalian cell cultures have been investigated and developed in the last years (e.g. radio frequency impedance, Raman spectroscopy or near-infrared spectroscopy) [16–22].

One highly promising method to monitor online, the cell concentration is the radio frequency impedance measurement in the cell broth. The sensor principle is based on the polarization of the cells by applying a periodic alternating electric field to the system. Cells with an intact membrane can be seen as closed compartments in an aqueous system containing different ions, like salts or nutrient. Positive-charged ions will move towards the field and negative-charged ions will move in the contrary direction [13]. Both movements are limited by the plasma membrane being a barrier for both, the ions inside the membrane and the ions in the aqueous suspension. This effect generates a polarization at the poles of the cells that means that a charge separation takes place. These polarized cells change the relative permittivity of the liquid, and therefore the capacitance or impedance measurement changes. As only viable cells are polarizable, because of their non-disrupted membrane, capacitance measurements can be used to correlate viable cell correlations [23]. The capacitance measured in Farads describes the magnitude of the polarization that is induced by the field in the cell suspension [13]. By converting capacitance into absolute permittivity, the resulted values are normalized to the cell constant of the measurement arrangement, which corresponds mainly to the electrode geometry [24]. The absolute permittivity (ε) in pF/cm is calculated by the following:

$$\varepsilon = C \times K \tag{1}$$

The measured capacitance can be used to calculate the relative permittivity (ε_r) using:

$$\varepsilon_r = C \times \left(\frac{K}{\varepsilon_0}\right) \tag{2}$$

The relative permittivity is dimensionless as it is relative to the electric constant (ε_0) called the permittivity of free space that is equal to 8.854×10^{-12} F/m. C is measured in Farads and K is the cell constant measured in 1/m [24].

Dead cells or impurities in the cell culture broth are not detected by this method, because the outer cell membrane needs to be intact to enable polarization [13, 23]. One major difference of the measurement method compared to traditional VCC offline methods is that with increasing cell diameter, the cell polarization is different and the signal contribution of each cell increases. Therefore, in the death phase/apoptosis of a mammalian CHO cell culture, where the cell diameter is increasing, the permittivity signal does not correlate with the viable cell concentration anymore [25, 26]. There is, however, the discussion among the scientific community for strong evidence that permittivity is more robust than VCC for certain applications. Measuring cell mass or online viable cell volume (VCV) can be better for automated feeding strategies, because larger cells usually demand more nutrients and that is not accounted for by the traditionally used cell count [27].

Capacitance sensors were frequently used in the past to monitor different cell lines in bioprocesses (e.g. mammalian cells, insect cells or microcarrier cultures) [28–30]. Many times the signal was treated and correlated to different process parameter such as the VCV, the VCC, or the total cell count [13, 27, 31]. Besides linear regression to correlate the permittivity with selected parameters, linear mixed effects (LME) models or multiple frequency measurement analysis via more complex mathematical modeling such as Cole–Cole modeling or Partial Least Square Regression (PLS) can be done to achieve online monitoring of important parameters [13, 27, 32–35]. However, measuring the capacitance at one frequency offers several advantages: The method is easy to implement, and offers a fast measurement principle which is important, especially in rocking motion bioreactors. Additionally, no sophisticated data processing is needed. Linear regressions were reported to deliver satisfying results for many applications and parameters [26].

Even though, research was done on the field of capacitance measurements, this technique is not yet a standard measurement principle for process monitoring or control in industry or research departments dealing with mammalian cell cultivation.

In this work, the scalability and transferability of the capacitance measurement principle from small scale to large scale and single-use bioreactors up to 2000 L were investigated. Besides the scalability, the comparability and prediction ability of KPIs were studied using single-use bioreactors. Single-use bioreactors raised high attention in the last years enabling fast turnover times, reducing costs, and providing dynamic plant capacities [36–39]. The work investigates the use of a capacitance sensors from process development to production scales. A fully scalable process in single-use bioreactors with monitoring KPIs and CPP control by PAT tools can significantly optimize new product development and fast market entrance for biopharmaceutical companies. The presented studies analyze the possibilities to provide a scale-independent linear regression model for the VCC, the VCV, and the wet cell weight (WCW) based on online capacitance measurements. Traditionally VCC is

the main parameter used to monitor the cell growth of a mammalian cell culture. WCW is important for downstream processing and the correct selection of devices to purify the product. VCV as a KPI is increasing in interest and a correlation between permittivity and VCV is expected to show best results based on the measurement principle [27]. Moreover, the work investigates applications for two different cell lines to prove, whether the approach can be applied on a large variety of mammalian CHO cell cultures.

Materials and methods

Cell lines and media

Two DG44 CHO cell lines expressing different monoclonal antibodies were used in this study (Cell line A and cell line B). Seed medium (SM), basal medium for production (PM), and two different feeds: feed medium A (FMA) and feed medium B (FMB) were used for all studies (Sartorius Stedim Cellca GmbH). All media and feeds were chemically defined.

Seed culture for Process A and Process B

The seed culture was performed similar for both processes. A cryovial containing 1 mL CHO suspension (passage 8) at a concentration of 30 million cells/mL was thawed and transferred into a 15 mL Falcon® tube (Sarstedt) with 10 mL thermalized (36.8 °C) seed medium. This suspension was centrifuged (Centrifuge 3-30 K, Sigma) at 190*g* at room temperature for 3 min to remove all components of the freezing medium. After decanting the supernatant, the pellet was resuspended with 10 mL pre-warmed seed medium and transferred into a 500 mL Erlenmeyer flask (Corning) filled with 150 mL pre-warmed seed medium. The shake flask was incubated in an incubation shaker (Certomat CTplus, Sartorius Stedim Biotech) at 36.8 °C and 7.5% pCO_2 with a shaking rate of 120 rpm and 85% humidity. Cells were passaged five times every 3–4 days until inoculation of the production culture was done.

To increase the volume of the pre-culture for the large bioreactor scales (50–500 L) in the main stage, the last pre-culture steps were moved from shake flasks to rocking motion bioreactors (BIOSTAT® RM 20/50, Sartorius Stedim Biotech). The temperature set point was chosen at 36.8 °C and the pH was controlled at 7.1 (for Process A) and 7.15 (for Process B) through CO_2 sparging. The rocking rate of each bioreactor was set to 30 rpm with an angle of 10° and dissolved oxygen (DO) was controlled at 60%.

The largest main culture bioreactors (1000–2000 L) were inoculated with a pre-culture in a stirred-tank bioreactor (BIOSTAT® STR 200/500, Sartorius Stedim Biotech). The temperature set point was chosen at 36.8 °C and the pH was controlled at 7.1 (for Process A) and 7.15 (for Process B) through CO_2 sparging. The stir speed of each bioreactor was set to 120 rpm (200 L) or 96 rpm (500 L), respectively. The DO was controlled at 60%.

Main culture for Process A

The main culture was inoculated with 0.3 million cells/mL. The main process was conducted at 36.8 °C ± 0.05 °C. The pH was set to 7.0 and controlled by the addition of CO_2. Once in a day the pH was measured offline (see 2.6 Offline Analytics) and compared to the online measurement. If the result deviated by more than 0.05, the online sensor was recalibrated.

The set point for DO was set to 60%. For inoculation, N_2 gas was sparged to adjust the DO to 60%. The initial gassing rates were adjusted using N_2, air, and oxygen to keep a k_La of 7.9 1/h (based on previous process engineering characterizations). The bioreactors were stirred according to their scale with 162 rpm (50 L), 121 rpm (200 L), 96 rpm (500 L), 86 rpm (1000 L), and 70 rpm (2000 L), respectively. The stir speed was adjusted according to the scale-up strategy in keeping the k_La of 7.9 1/h constant in all scales. On the day of inoculation, antifoam (2% Antifoam C Emulsion, Sigma-Aldrich®) was added (0.001% of the cell suspension volume). During the cultivation, antifoam was added manually by the operator depending on the foam level.

Starting from inoculation day, which was day 0, the cultivation lasted for 12 days. FMA and FMB were supplied from day 3 in a ratio of 10:1 (FMA: FMB) with a feed amount of FMA of 42 g/L start volume/day and FMB, accordingly. The volume changes inside the bioreactor based on the feeding strategy are demonstrated in Fig. S1.

Starting from day 5, depending on when the glucose level dropped below 5 g/L, glucose was added to the cell broth as a bolus to hold the glucose concentration at 5 g/L.

Main culture for Process B

Similar to Process A, the main culture was inoculated with 0.3 million cells/mL and the main process was conducted at 36.8 °C ± 0.05 °C. The pH was set to 7.15 and controlled by the addition of CO_2. If the offline pH deviated by more than 0.05 the online sensor was recalibrated on a daily basis.

The set point for DO was set to 60%. For inoculation, N_2 gas was sparged to adjust the DO to 60%. The initial gassing rates were adjusted using N_2, air, and oxygen to keep a k_La of 7.9 1/h. The bioreactors were stirred according to their scale with 170 rpm (50 L), 124 rpm (200 L), and 86 rpm (1000 L), respectively. The stir speed was adjusted according to the scale-up strategy in keeping the k_La of 7.9 1/h constant in all scales. On the day of inoculation, antifoam was added (0.001%

of the cell suspension volume). During the cultivation, antifoam was added manually by the operator depending on the foam level.

Starting from inoculation day, which was day 0, the cultivation lasted for 17 days. FMA and FMB were supplied from day 3 in a ratio of 10:1 (FMA: FMB) with a feed amount of FMA of 43.2 g/L start volume/day and FMB accordingly. Equally to Process A, the volume changes inside the bioreactor based on the feeding can be seen in Fig. S1.

Starting from day 5, depending on when the glucose level dropped below 5 g/L, glucose was added to the cell broth as a bolus to hold the glucose concentration at 5 g/L.

Online capacitance measurements

Capacitance measurements were conducted with an impedance probe (BioPAT® ViaMass, Sartorius Stedim Biotech for single-use applications and a Futura 12 mm Probe, Aber Instruments Ltd for multi-use applications). The sensor was set to cell culture mode measuring at one single frequency at 580 kHz and a filter over 30 values was used. The probe was either directly connected to a BioPAT® DCU (Sartorius Stedim Biotech), BioPAT® MFCS/win was used for data acquisition and the data were finally stored in an internal database or the probe was connected with a connection hub (Futura Connect, Aber Instruments Ltd) to a PC. On the PC it was processed by the Futura Tool® software (Aber Instruments Ltd.). The data were stored as .csv format and imported to an Excel® file (Microsoft Cooperation) for further treatment.

Offline analytics

The viable cell concentration indicating the amount of viable cells in the cultivation and the viability of cells according to the total cell concentration, as well as the average cell diameter, were analyzed with the Trypan Blue Assay based Cedex HiRes Cell Counter and Analyzer system (Roche). The pH and the glucose concentration were measured offline in a blood gas analyzer (ABL800 Basic, Radiometer).

For the WCW measurement, 5 mL of the cell broth was transferred into a 15 mL Falcon® tube (Sarstedt). The cell suspension was centrifuged (Centrifuge 3–30 K, Sigma) at 5000*g* at room temperature for 5 min. After centrifugation the media was removed and the pellet was weighed (Genius, Sartorius AG). The WCW was calculated according to Eq. 3, with w_{Pellet} indicating the weight of the tube together with the pellet and w_{Tube}, the empty weight of the falcon before adding the cell suspension. The sample volume described the amount of cell broth that was added to each tube before centrifugation.

$$\mathrm{WCW} = \frac{(w_{\mathrm{Pellet}} - w_{\mathrm{Tube}})}{\mathrm{Sample\ volume}} \tag{3}$$

The VCV was calculated based on the offline VCC, the diameter measured in the Cedex and the equation of a sphere indicating the cell shape according to:

$$\mathrm{VCV} = \frac{4}{3}\pi\left(\frac{\mathrm{Cell\ diameter}}{2}\right)^3 \times \mathrm{VCC} \tag{4}$$

Results and discussion

Comparison of biomass related online and offline measurements in the smallest (50 L) and the largest (2000 L) bioreactor scale

In the following experiments, the suitability of online capacitance measurements for biomass monitoring during scale-up was investigated.

As a first step, the online signal was compared and correlated to three different offline methods for biomass estimations in the smallest investigated bioreactor size of 50 L (Fig. 1) and the largest size of 2000 L (Fig. 2). The VCC was measured by Trypan Blue Assay in a semi-automated system. The same system detected the diameter so that by calculation (Eq. 3), the VCV was determined. Finally, the WCW was achieved in a fully manual assay according to Eq. 3. To better understand the deviations that are in an acceptable range for the correlations, the errors of each method are listed in Table 1.

For both scales the offline data points that are describing the biomass in the process are showing the same tendencies and results as the online permittivity signal depending on the culture time (Figs. 1a, 2a). As long as the cells were in the exponential growth phase, the VCC, the VCV, the WCW, and the permittivity are overlapping and correlating with each other. An advantage of the capacitance measurement compared to the offline references can be seen immediately in both figures (Figs. 1a, 2a). The dips starting from 72 h on reflect the feeding in the fed-batch processes. Thus, in the permittivity signal, it is possible to monitor the feeding online. Information about the feeding profile and the effect on the cells can be detected online with the sensor integration.

In Fig. 1b–d and Fig. 2b–d, a linear correlation used to describe the relationship of the online permittivity signal with the corresponding offline parameters and the coefficient of determination was investigated.

The WCW is a manual assay with a high expected error of up to 25% in the measurement principle itself (see Table 1). In the smallest scale (Fig. 1b), this resulted in a coefficient of determination of 84.5%, which is the lowest detected coefficient of determination for the investigated parameters. For the 2000 L scale, the correlation between WCW and permittivity improved to 94.1%. This result

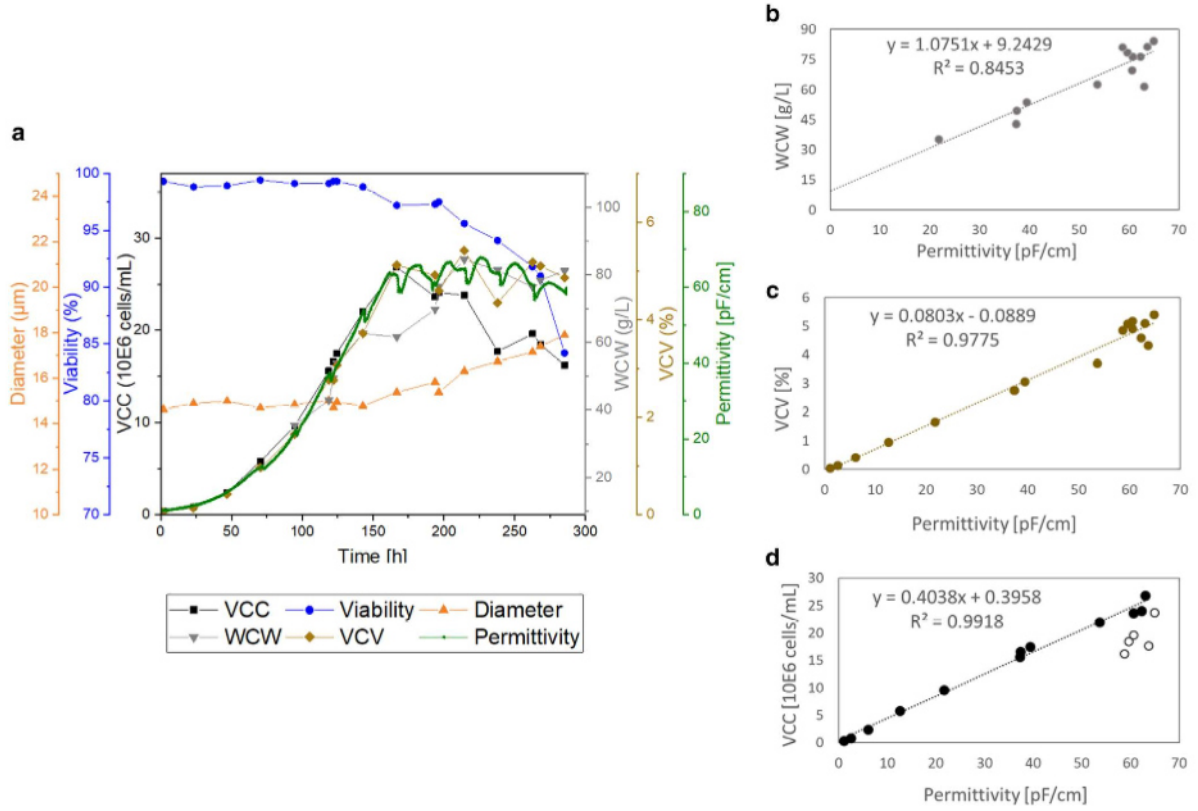

Fig. 1 Overall cultivation results of the STR50 cultivation of process A. Top **a**: time-dependent cultivation results for cell diameter, cell viability, viable cell concentration (VCC), wet cell weight (WCW), viable cell volume (VCV), and online permittivity signal. Bottom: correlation of the permittivity signal with WCW (**b**), VCV (**c**), and VCC (**d**). Full circles in **d** represent the data points incorporated into the linear regression model (up to peak VCC) and empty circles represent the values excluded from the linear regression (stationary and apoptotic cell growth phase)

from the 2000 L run shows that the online permittivity can describe the WCW within an acceptable range. The deviation, especially in the 50 L scale, was most likely caused by manual operations, changes in the operator or wrong execution of the reference method. Furthermore, there were few data points taken at low WCW in the beginning of each cultivation and the data points at the end of the cultivations showed a large scatter. A straight line through the origin was expected with WCW measurements. However, due to the non-symmetric point's distribution, the linear regression model resulted in high y axis intercepts in both scales. Therefore, the correlations of the WCW shows relatively high errors at small WCWs. Using the online permittivity signal to estimate the WCW instead of offline samples could even improve the WCW determination throughout the complete cultivation.

Referring to the correlation of online permittivity and VCV in both scales, the coefficient of determination was 97.8% (Figs. 1c, 2c). Therefore, it can be concluded that the online sensor is capable to describe the VCV in a highly accurate manner.

As the permittivity signal has deficits in describing the VCC in the death phase, differences after the peak cell concentration were expected. As described in the introduction, the permittivity is increasing with higher cell concentration as well as higher cell diameters. Indeed, deviations in the data points of the 50 L and the 2000 L scale were detected with increasing diameter and decreasing viability (Figs. 1a, 2a).

Therefore, the correlation of the VCC and the online signal was based on a selected set of data points until the end of the exponential growth phase (Figs. 1d, 2d, full circles). The values in the death phase were not considered (Figs. 1d, 2d, empty circles). For the WCW and the VCV, the complete process duration was included in the correlations.

The VCC in the exponential cell growth could be monitored by the online sensor with a coefficient of determination of 99.2% for the 50 L scale and 99.0% for the 2000 L

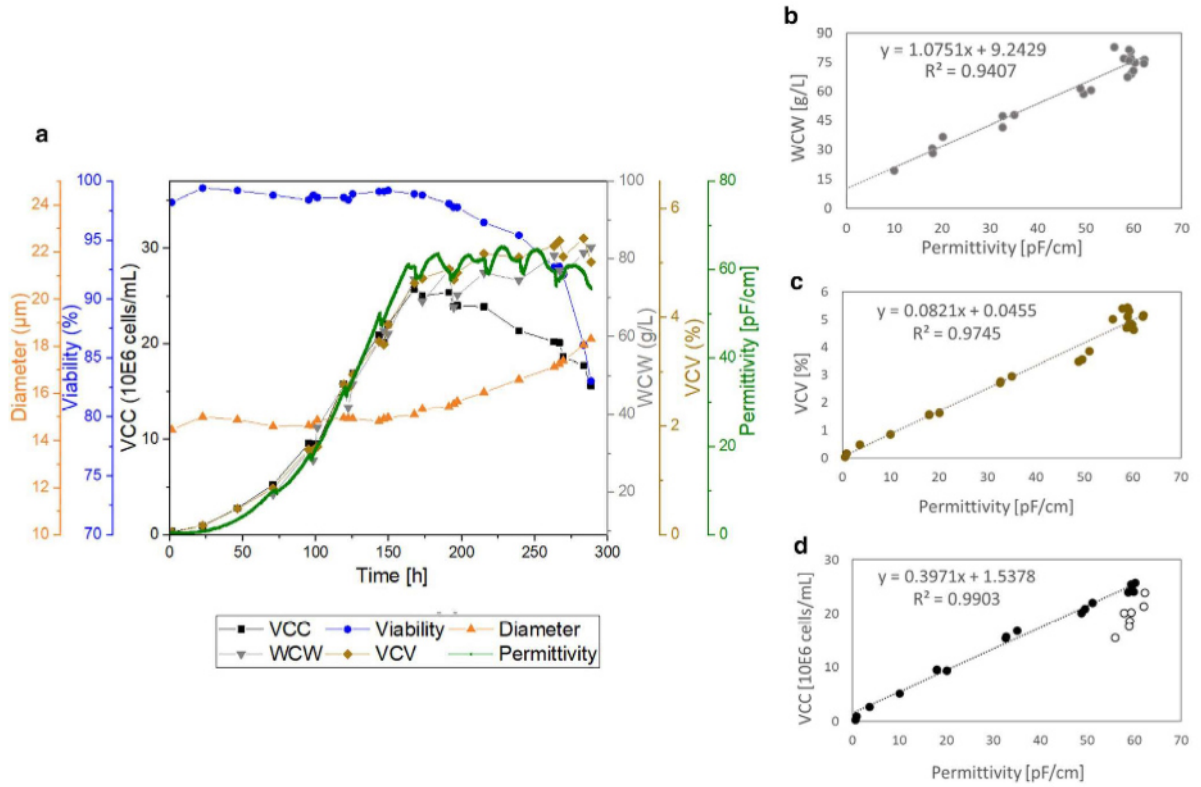

Fig. 2 Overall cultivation results of the STR2000 cultivation of process A. Top **a**: time-dependent cultivation results for cell diameter, cell viability, viable cell concentration (VCC), wet cell weight (WCW), viable cell volume (VCV), and online permittivity signal. Bottom: correlation of the permittivity signal with WCW (**b**), VCV (**c**), and VCC (**d**). Full circles in **d** represent the data points incorporated into the linear regression model (up to peak VCC) and empty circles represent the values excluded from the linear regression (stationary and apoptotic cell growth phase)

Table 1 List of errors for measurement methods that are presented in the studies

Method	Error expectations (%)	Reference for error expectations
Wet cell wet	5–25	Error range detected during performed measurements
Viable cell concentration	10	Typical detected error in all performed measurements is not better than the stated error and in agreement with reports in the literature [22]
Viable cell volume	16	Error propagation based on expected Viable Cell Concentration error and 2% estimated error in diameter detection that was detected during the presented and previous measurements

scale. Compared to the 10% measurement errors that exist in the offline reference (Table 1), the permittivity signal is robust and reliable. Therefore, the sensor is suitable for both bioreactor scales, the small bioreactor of 50 L and the large bioreactor of 2000 L. The permittivity signal can successfully monitor the VCC during the exponential growth phase of the cells.

These results give a first indication for the suitability of an online capacitance sensor to monitor cell growth in the given cell culture process during scale-up experiments. In the following, the transfer to other scales (200 L, 500 L, and 1000 L) as well as other cell clones was tested to achieve a broader correlation that predicts VCC, VCV, and WCW based on the online permittivity signal.

Investigation of scalability of single-use bioreactors based on online permittivity trajectories

After successful demonstration of the correlation of the online permittivity signal to different biomass related process parameters in the smallest and largest bioreactor size, the trajectory of the permittivity signal should be analyzed through all involved bioreactor scales (50 L, 200 L, 500 L, 1000 L, and 2000 L).

To investigate the scalability of the permittivity signal, the different single-use bioreactor cultivation data sets were combined and included in each linear correlation for WCW, VCV, and VCC (Figs. 3, 4, 5). Within the correlation to each offline value of the parameters, the corresponding online value was selected and included in the linear model. The linear regression model was used to predict the respective values based on the complete online permittivity data sets. In the linear regression, each batch was colored individually

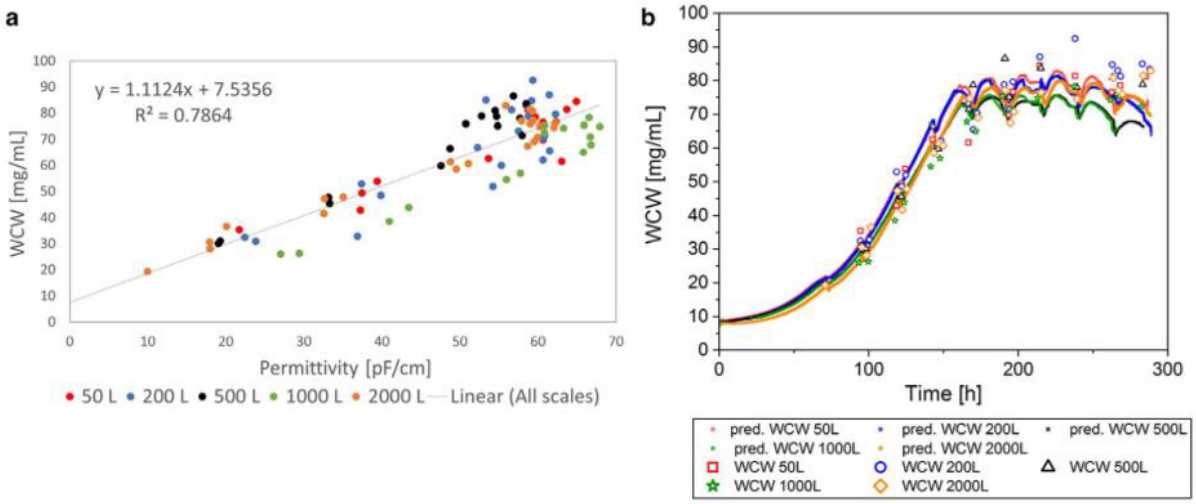

Fig. 3 Linear regression model of permittivity and wet cell weight (WCW) for Process A including all cultivations from 50 L up to 2000 L bioreactor volume (**a**). Predictions (pred.) based on the online permittivity signal for all bioreactor scales using the equation from the linear regression (**b**)

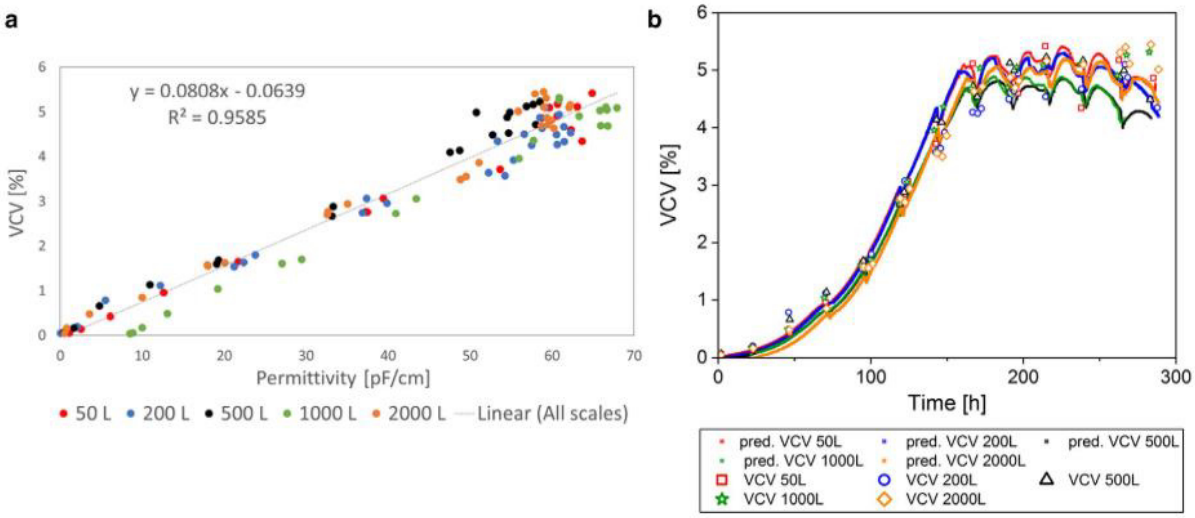

Fig. 4 Linear regression model of permittivity and viable cell volume (VCV) for Process A, including all cultivations from 50 L up to 2000 L bioreactor volume (**a**). Predictions (pred.) based on the online permittivity signal for all bioreactor scales using the equation from the linear regression (**b**)

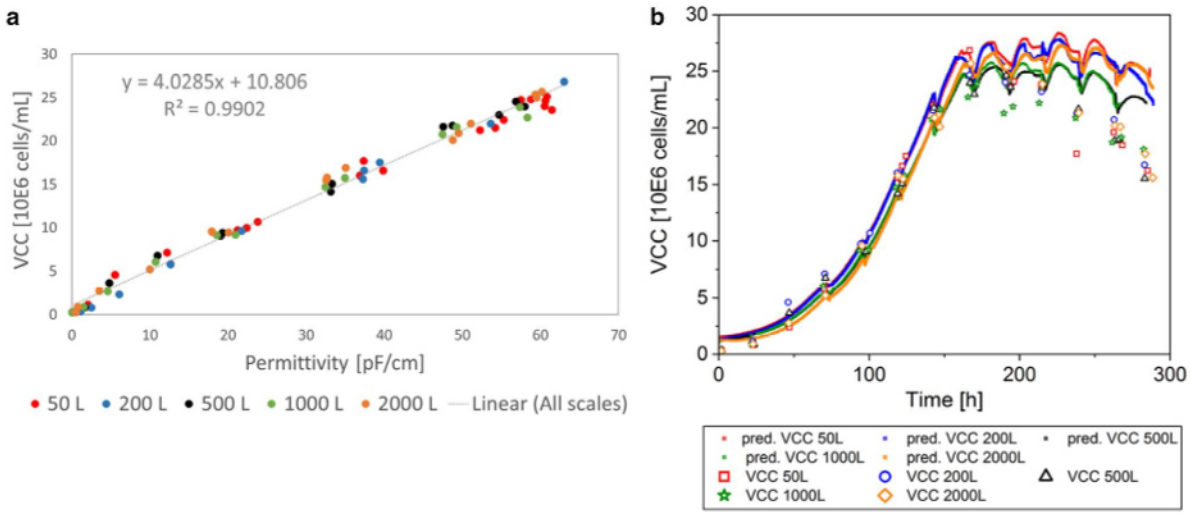

Fig. 5 Linear regression model of permittivity and viable cell concentration (VCC) for Process A, including all cultivations from 50 L up to 2000 L bioreactor volume (**a**). VCC only considered up to peak VCC for linear regression. Predictions (pred.) based on the online permittivity signal for all bioreactor scales using the equation from the linear regression (**b**)

to investigate the impact of a specific bioreactor scale or cultivation on the regression model (Figs. 3a, 4, 5a). However, there was no systematic difference in each regression model between scales and cultivations detected. This result indicates a robust and scalable process that enables a scale-independent linear model to predict the WCW, VCV, and the VCC (VCC only until end of exponential growth phase).

As described above and in Table 1, the WCW is a manual assay, which can have a large error up to 25% based on the method itself and influence of the operator. Based on the theory, a good correlation between permittivity that represents the volume of viable cells and WCW should be given. Surprisingly, combining all scales in one linear model, led to a relative low coefficient of determination of 78.6% (Fig. 3a). The measurement values were highly scattered and only few data points were available for small WCWs for all processes. Therefore, the available data set was insufficient for a representative regression. The highest deviation could be seen in the apoptotic cell status (Fig. 3b). This result might indicate strong manual deviations depending on the operator and frequently changed operators over the process time. As a result of the poor regression, there was no straight line through the origin detected in contrast to previous expectations. In summary, the low coefficient of determination can be seen as a result of an inconsistent offline method with errors up to 25% between each measurement and a very inhomogeneous sample distribution over the measurement range (Table 1). Even though much better results were expected, considering the error of the offline method for each measurement point, the online signal might be used to predict the WCW during scale-up in the future.

The correlation of the VCV and the online permittivity signal had a coefficient of determination of 95.9% (Fig. 4a) in a combined linear model for all scales. Especially, in the exponential growth phase, the VCV was described highly accurate (Fig. 4b), whereas in the death phase, the predicted values deviated as seen in the WCW. Remembering that the offline method results in errors of up to 16% (Table 1), the resulted coefficient of determination implies a stable prediction and can be used for online monitoring of the VCV in different bioreactor scales.

The VCC was correlated only for the exponential growth phase, as described previously. In the exponential growth phase, the linear model for all scales described 99.0% of the data points (Fig. 5a). The prediction of the VCC in the exponential growth phase based on the online signal, described the offline data reference precisely (Fig. 5b). The online signal was not capable to predict the VCC in the death phase of the cells. Nevertheless, the scalability of the linear model and the transfer from scale to scale was working in the exponential growth phase. Therefore, the online signal can be a useful tool to support process scale-up. Even though limitations of the permittivity signal for the VCC prediction in the death phase could be detected, real time process monitoring of the VCC during the exponential growth phase was possible. By including the cell diameter into the calculations as it is shown in the VCV, an accurate linear model to describe all bioreactors was given. The real-time information based on the permittivity signal enables to monitor and control the bioprocess, as process deviations can be immediately recognized.

Proof of concept with different CHO cell line and cross verification

To prove the results during scale-up of the single-use bioreactors that are shown in Process A, selected single-use bioreactor scales (50 L, 200 L, and 1000 L) were cultivated and the same parameters were investigated with a different CHO cell culture fed-batch process, here named Process B. The same linear regressions were made for WCW, VCV, and VCC, including all bioreactor scales done for Process B (Figs. 6, 7, 8). As previously shown for Process A, each cultivation for Process B was colored individually within the linear regression model to prove scale independency (Figs. 6a, 7, 8a). The offline data points were well mixed for each correlation and there was no trend for a specific bioreactor scale or cultivation detected. Therefore, it can be concluded that the bioreactor scale itself had no influence on the measurement method and linear regression model.

For Process B, the coefficient of determination for the WCW was drastically higher with 99% (Fig. 4a) compared

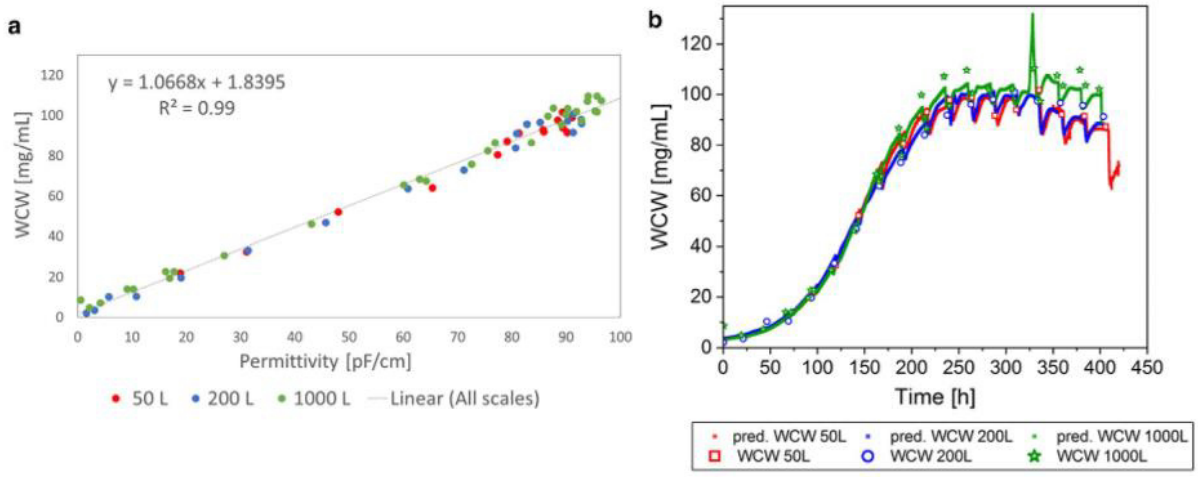

Fig. 6 Linear regression model of permittivity and wet cell weight (WCW) for Process B, including all cultivations from 50 L up to 1000 L bioreactor volume (**a**). Predictions (pred.) based on the online permittivity signal for all bioreactor scales using the equation from the linear regression (**b**)

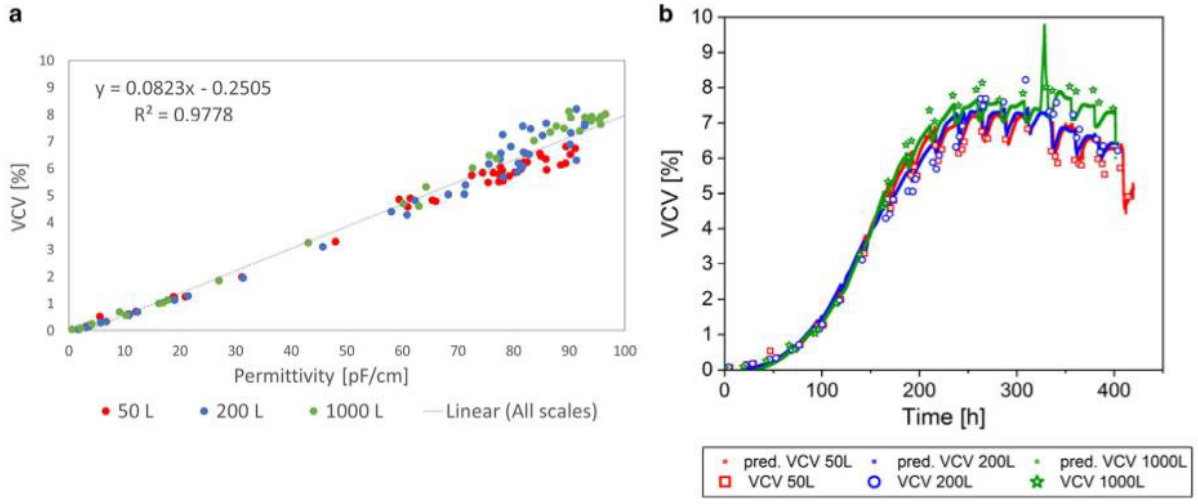

Fig. 7 Linear regression model of permittivity and viable cell volume (VCV) for Process B, including all cultivations from 50 L up to 1000 L bioreactor volume (**a**). Predictions (pred.) based on the online permittivity signal for all bioreactor scales using the equation from the linear regression (**b**)

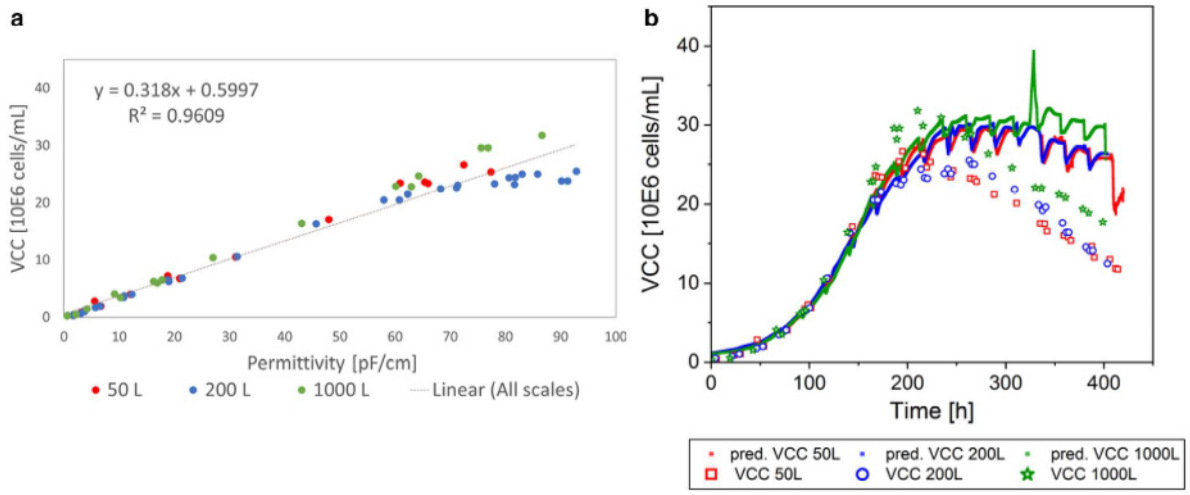

Fig. 8 Linear regression model of permittivity and viable cell concentration (VCC) for Process B, including all cultivations from 50 L up to 1000 L bioreactor volume (**a**). VCC only considered up to peak VCC for linear regression. Predictions (pred.) based on the online permittivity signal for all bioreactor scales using the equation from the linear regression (**b**)

to 78.6% in Process A (Fig. 6). In contrast to Process A, more data points were incorporated in the correlation, especially at low WCW in the early process phase. Moreover, the scattering of the measurement values was reduced resulting in a regression with a straight line through the origin. The performance of the offline reference method was strongly improved compared to previous results that might be aligned with fewer operators and a more detailed standard operating procedure. The high coefficient of determination shows the potential of correlating permittivity and WCW measurements as it was expected based on the theory of each measurement method. To conclude, the prediction of the WCW based on the online signal is in agreement with the offline data and can be used to describe the WCW accurately during scale-up, as long as the linear regression is based on a representative data set with a high amount of measurement values. Even more process deviations can be detected thanks to the online signal. A peak after 330 h of cultivation was detected in the 1000 L bioreactor for all predicted offline parameters (Figs. 6b, 7, 8b). After verification with the DO measurements of the cultivation, a DO blackout was recognized. The peak in the permittivity signal might be related to a stress response of the cells that increases in diameter rapidly. In addition to the classical DO probe, the online permittivity signal detected the problem immediately and gave information about the cell response and not only the bioreactor environment due to the blackout. The combination of both probes saved the batch, due to fast reaction times and increased process understanding. This example shows that the online capacitance probe is a powerful tool to give insights into cell metabolic states, to control bioprocesses, and to keep the process within an approved trajectory.

The coefficient of determination of the linear regression of the permittivity and the VCV was 97.8% (Fig. 7a). The error of the linear regression was within the range of 16% determined for the offline VCV error (Table1). Therefore, the prediction of the VCV is consistent in all scales and is suitable as online monitoring tool for all bioreactor scales (Fig. 7b).

The VCC in the exponential phase was described with a coefficient of determination of 96.1% (Fig. 8a) and the prediction was suitable for all scales (Fig. 8b). Similar to the case of process A, a clear deviation of the prediction and the offline measurement was detected after the VCC peak was reached. This result was expected based on the described limitations in the measurement method.

The presented results show the scalability of the online bio-capacitance measurement to describe the WCW, the VCV, and the VCC (in the exponential phase) given in all bioreactor scales and for different CHO fed-batch processes. In addition, the online monitoring enabled fast operative steps to keep the process within the pre-defined trajectory. This shows the method´s potential for automated process control to keep a process within the accepted design space during a cultivation.

In a final step, the prediction ability of the online measurement was challenged regarding creating a process-independent model. Therefore, a linear regression was applied using the data from Process A and Process B. To see the process dependency of the model, each model was colored

Table 2 Summary of results and comparison to the literature

Key performance indicators	R^2 in Process A	R^2 in Process B	Expected error of reference method (%)	Literature comparison for linear correlations with capacitance
Wet cell weight	0.79	0.99	1–25	–
Viable cell volume	0.96	0.98	10	0.99—in exponential growth phase of a mammalian cell line [27] 0.98—in exponential growth phase and 0.75—in stationary phase for viable packed cell volume of a mammalian cell line [26]
Viable cell concentration	0.99	0.96	10	0.96—for a mammalian cell line [26] 0.98—in a perfusion process of a mammalian cell line [16]

individually (Fig. S2). Thus, each process was clearly distinguished for the WCW and the VCC, especially with prolonged culture time. Process B was cultivated for a longer period than Process A. For VCC correlations, it can be discussed whether in a combined approach, the criteria of incorporating VCC values until peak cell concentration is suitable. The peak cell concentration for Process A took place at an earlier process time (~150 h) compared to Process B (~200 h). Moreover, the high error in the offline reference hamper the exact determination of the peak cell concentration. Comparing the diameter at both process times, a clear difference appeared with the prolonged process time between each peak cell concentration (Fig. S3). As capacitance is influenced by diameter changes, this can have an impact on the VCC result for the common correlation that is supported by the better result for VCV correlations for a common approach (Fig. S3b). Thus, the criteria of which values to include VCC correlations might be changed for a combined approach of both processes. Other criteria can be the inflection point of the VCC, curve, or using a fixed change in diameter as criteria. However, in these studies, a fixed diameter change would increase the coefficient of determination, but each process would remain distinguishable (Fig. S2c). Therefore, with the presented method of single-frequency measurements in this work, it is not recommended to use one model for the two differently presented CHO processes or a process-specific calibration is needed in the beginning of each cultivation.

Table 2 summarizes the results in the presented study. In comparison to the literature values and the measurement errors, the coefficients of determination for the selected KPIs were within comparable and acceptable error ranges. Thus, the linear regression models based on the capacitance sensor integration were suitable for each process and process parameters (WCW, VCV, and VCV) resulting in scale-independent online predictions for each process. Detailed process information was gained with online monitoring in regards to the feed profile. Furthermore, the sensor integration enabled process monitoring with fast reaction times to process failures, indicating strong potential for process control (e.g. feed control or determination of harvest point).

Conclusion

In the presented work, it was shown that the online permittivity signal is capable to describe different KPIs for biomass dynamics during process scale-up of different CHO cell culture processes. The sensor integration was successfully demonstrated for single-use bioreactor scales ranging from 50 L up to 2000 L reactor volume and scale-independence of the method was shown. However, limitations of the measurement method for the presented processes in the stationary growth phase and the death phase based on cell diameter changes of apoptotic cells were detected, confirming previous results from the literature. Nevertheless, the correlations of permittivity with VCC in the exponential growth phase and WCW, respectively, VCV throughout the whole process reached coefficients of determination up to 99% that were comparable to the literature reports and the error of the offline reference methods. The sensor implementation to all bioreactor scales enabled a robust and reliable online monitoring of cell growth in all fed-batch cultures. This can lead to faster process development and mitigation of process risks, and therefore a more robust production process. The presented approach applied in process control can lead to save resources and prevent cultivations from failure by keeping the batch within an approved trajectory.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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3.3 Multivariate data analysis of capacitance frequency scanning for online monitoring of viable cell concentrations in small scale bioreactors (Analytical and Bioanalytical Chemistry, 2019)

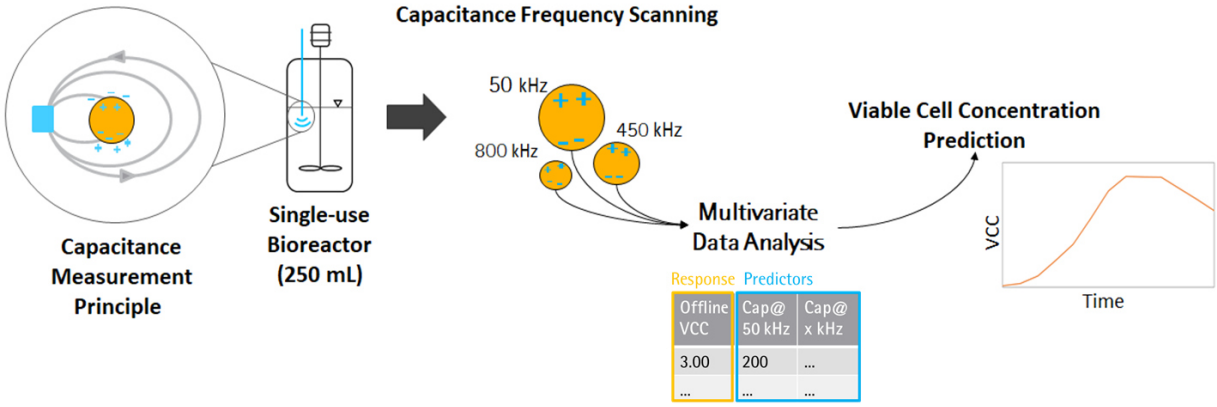

Figure 6 Graphical abstract of "Multivariate data analysis of capacitance frequency scanning for online monitoring of viable cell concentrations in small scale bioreactors".

The previous two sections successfully described how the combination of PAT tools and small and large scale models lead to process intensification and improved process understanding. After identification of VCC as an important process attribute for mammalian cell culture process (especially in perfusion processes), the capacitance sensor was evaluated and successfully implemented as a scale-independent tool for online monitoring of biomass related changes. However, a drawback of the single-frequency measurement method did not allow for a VCC prediction over the complete process time. Therefore, this section presents a novel methodology to optimize capacitance sensors applying frequency scanning and mathematic-statistic modelling for online VCC monitoring.

In this section, several standard fed-batch cultivations served as training set to develop a MVDA model that predicts the VCC from the frequency scan of the capacitance sensor with 25 different frequencies. The MVDA model successfully enabled a VCC prediction throughout the complete cultivation time with relative errors ranging from 5.5% to 11%, which fulfils the acceptance criterion based on the offline reference method accuracy (approximately 10%

relative error). The prediction capabilities for VCC strongly improved with the MVDA model compared to single-frequency results (16% to 23% relative error).

After the VCC MVDA model was established with standard fed-batch cultivations, robustness trials were conducted to investigate the model´s predictive ability under challenging conditions. The selected process deviations consisted of dilution steps in a first experiment and feed variations in a second robustness trial. All process deviations were detected immediately in the online prediction of the VCC with relative errors between 6.7% and 13.2%. The online VCC monitoring led to superior process information compared to the conventional offline reference method. A batch evolution model detected the feed variation immediately, whereas the corresponding offline reference did not reveal clear changes compared to the standard fed-batches.

The method development in a suitable scale-down model (single-use bioreactor with a volume of 0.25 L) was fast and economic including a sufficient amount of cultivations for a robust MVDA VCC model.

To summarize, the combination of an inline PAT sensor, frequency scanning and MVDA in the mammalian CHO fed-batch culture enables online VCC prediction over a complete cultivation time being a major process attribute for future monitoring and control strategies.

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RESEARCH PAPER

Multivariate data analysis of capacitance frequency scanning for online monitoring of viable cell concentrations in small-scale bioreactors

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Abstract
Viable cell concentration (VCC) is one of the most important process attributes during mammalian cell cultivations. Current state-of-the-art measurements of VCC comprise offline methods which do not allow for continuous process data. According to the FDA's process analytical technology initiative, process monitoring and control should be applied to gain process understanding and to ensure high product quality. In this work, the use of an inline capacitance probe to monitor online VCCs of a mammalian CHO cell culture process in small-scale bioreactors (250 mL) was investigated. Capacitance sensors using single frequency are increasingly common for biomass monitoring. However, the single-frequency signal corresponds to the cell polarization that represents the viable cell volume. Therefore single-frequency measurements are dependent on cell diameter changes. Measuring the capacitance across various frequencies (frequency scanning) can provide information about the VCC and cope with changing cell diameter. Applying multivariate data analysis on the frequency scanning data successfully enabled direct online monitoring of VCCs in this study. The multivariate model was trained with data from 5 standard cultivations. The model provided a prediction of VCCs with relative errors from 5.5 to 11%, which is a good agreement with the acceptance criterion based on the offline reference method accuracy (approximately 10% relative error) and strongly improved compared with single-frequency results (16 to 23% relative error). Furthermore, robustness trials were conducted to demonstrate the model's predictive ability under challenging conditions. The process deviations in regard to dilution steps and feed variations were detected immediately in the online prediction of the VCC with relative errors between 6.7 and 13.2%. Thus in summary, the presented method on capacitance frequency scanning demonstrates its suitability for process monitoring and control that can save batches, time, and cost.

Keywords Mammalian cell culture · PAT · Capacitance frequency scanning · MVDA (multifrequency permittivity, impedance spectroscopy, small-scale bioreactor, process monitoring and control)

Introduction

Mammalian cell culture processes for the production of monoclonal antibodies are challenging and complex processes that are crucial in modern medicine to generate pharmaceutical products such as anticancer drugs. Pharmaceutical companies face a strong market demand and a high pressure to provide safe products according to quality guidelines and legislations.

To improve and sustain high quality in pharmaceutical products, the International Conference on Harmonization (ICH) launched the "Quality by Design" (QbD) initiative within the framework of the ICH Q8 guideline in 2004 [1]. The QbD approach involves, inter alia, the identification of product attributes that are of significant importance to the

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product's safety and establishment of robust control strategies to ensure consistent process performance [2]. The initiative focuses on the detection of critical process parameters (CPPs) that have influence on critical quality attributes (CQAs) of the product (e.g., glycosylation profile of a monoclonal antibody). Moreover, the CPPs should be monitored and controlled online to achieve a constant and high product quality.

The QbD concept of CPPs that influence CQAs in the regulatory framework can be transferred and used also in the perspective of a pharmaceutical manufacturer. Besides the CQAs, a manufacturer focuses on selected process attributes that are important for the process performance and the economy of the production. Thus, process attributes need to be monitored and key process parameters (KPPs) that influence the process attributes require to be well-controlled as well [3].

Process analytical technology (PAT) is a toolbox allowing for the implementation of QbD. When the Food and Drug Administration (FDA) launched the PAT initiative in 2004, they aimed at establishing a consistent process performance, process control, and a high product quality by monitoring and controlling KPPs or CPPs that affect process attributes or CQAs, respectively [4–6]. PAT tools are significant to fulfill regulatory needs resulting in high product quality as well as optimizing the process performance based on selected process attributes.

From a pharmaceutical manufacturer perspective, the antibody titer and the process yield are some of the most important process attributes to achieve an economic process. The viable cell concentration (VCC) reflects the amount of viable cells in a cell suspension that is responsible for the antibody production. Thus, VCC is strongly linked to product titers and is considered process attribute, too [3]. Monitoring the VCC enables process optimization and control that leads to higher titers and efficient processes. One example can be the adjustment of the feeding rate based on online VCC values leading to an optimal feed consumption in every process that reduces medium costs in the production facility. Current state-of-the-art VCC measurements comprise offline methods like trypan blue assays. Major drawbacks are the low temporal resolution of the VCC, the temporal delay between sampling and measurement. Further, operator-dependent measurement errors can alter the results and increase risks of contaminations [7, 8]. Offline measurements for important process attributes conflict with the online control and QbD requirements biopharmaceutical processes have to meet. In recent years, many online PAT tools have been investigated in pharmaceutical processes to monitor cell concentrations of mammalian cultures (e.g., radio-frequency impedance, Raman spectroscopy, or near-infrared spectroscopy) [9–14].

Radio-frequency impedance was selected as a preferred tool for mammalian cell culture monitoring because of relatively low implementation costs, a sensitivity to cell numbers, and easy implementation into the sterile surrounding of a bioreactor [15–17]. Online radio-frequency impedance measurements as a PAT tool have been used for many years in order to measure the permittivity of mammalian cell cultivations and derive information about cell growth and biomass [15, 18–21]. Therefore, the theory and principles of radio-frequency impedance have been presented in various literature, so here only a short summary of the basic terminology and theory will be given [19, 22, 23].

In the context of radio-frequency impedance, the terms used to describe the same measurement approach differ from author to author. The same approach is described as (bio-)capacitance [15, 24, 25], dielectric spectroscopy [7, 17, 26], multifrequency permittivity [27], or impedance measurements [7].

Impedance measurements provide capacitance and conductivity as physical properties where capacitance and conductivity are generally frequency-dependent. Combined with the cell constant (which corresponds mainly to the electrode geometry), capacitance leads to dielectric properties of the analyte where the capacitance component delivers the permittivity signal measured in picofarads per centimeter of the analyte [28]. Therefore, in the following, the measurement signal of a capacitance sensor is referred to as permittivity.

When an electric field is applied to cells within an ionic solution, a charge separation occurs within the cells and the poles of the cells polarize. The polarization occurs because the electric field forces ions to move within the highly conducting cellular cytoplasm until they reach the non-conducting cellular membrane which impedes their further movement. The polarizability of the cell suspension corresponds to the permittivity of the cell suspension. This means with higher cell concentrations, more cells contribute to the polarization, leading to a higher permittivity. Furthermore, the polarizability is frequency-dependent. In the case of mammalian cells with diameters in the range of several micrometers, excitation frequencies below 100 kHz leave enough time for the cells to completely polarize, resulting in a high permittivity of the solution. An increase in the excitation frequency leads to a decrease in permittivity because the cells cannot polarize completely. This loss in cellular polarization is referred to as β-dispersion. Figure 1 illustrates the schematic overview of a ß-dispersion curve for spherical cells that describes the principle of permittivity measurements. The frequency at which the rate of polarization is half complete is the characteristic frequency (f_C). Depending on the polarizability and the size of the cells, the characteristic frequency (f_C) can change. Dead cells and impurities of the cell broth are not polarizable and therefore do not impact the capacitance measurements [19, 29]. Moreover, before inoculation of the cells, the signal of the medium is zeroed. Therefore, only changes during cultivation due to the cell culture will be detected in the permittivity signal. A cultivation broth does not consist of many polarizable species

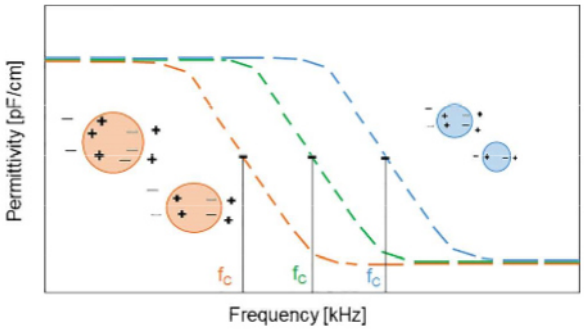

Fig. 1 Schematic overview of a ß-dispersion curve for spherical cells. With increasing frequency, the permittivity of cells in suspension moves from a low-frequency plateau that corresponds to a maximal cell polarization down to a high-frequency plateau that corresponds to a minimal cell polarization, respectively. This typical occurrence is known as ß-dispersion. The extent of the polarization is measured by the permittivity of the electrical double layer. Depending on the polarizability of the cells, the characteristic frequency (f_C) can change

or polarizable species appear in concentrations below the limit of detection of a capacitance probe. Thus, the capacitance probe is a promising tool to monitor cell growth online.

As a result of the measurement principle, the permittivity signal measured at one frequency increases with higher VCCs in a culture as well as with a larger cell diameter that can occur during cell aging or as stress responses. The influences of VCC and cell diameter on the permittivity signal are not distinguishable with one frequency measurement. Thus, single-frequency measurements correlate with the viable cell volume (VCV) instead of the VCC [17, 30]. With radio-frequency impedance spectroscopy (here called capacitance frequency scanning), the dielectric properties of a cell suspension are measured with multiple frequencies resulting in a higher degree of cell information. Recently, it was reported that applying capacitance frequency scanning and mathematic modeling (e.g., Cole-Cole modeling of the ß-dispersion curve or partial least squares regression) on the frequency scanning data revealed online information of the VCC, cell apoptosis, or nutrient limitations [24–27, 31–33].

Frequency scanning captures the permittivity at various frequencies over the course of cultivation, delivering a large amount of complex data. The interaction between different effects that influence the measurement requires advanced statistics/analysis such as multivariate data analysis (MVDA).

The handling of multivariate data and advantages of orthogonal partial least squares (OPLS) models have been well-described in literature [34–36]. Unlike PLS models, OPLS models separate the variation within the predictors into two parts: one being correlated, predictive to the model response; and one being uncorrelated, orthogonal to the model response. However, OPLS and PLS result in identical models. The main difference between OPLS and PLS is the easier interpretability when using OPLS models [35]. By definition of the OPLS algorithm, orthogonal components are orthogonal to the target information. Besides noise, they mainly cover changes due to matrix effects. Applying OPLS (or PLS) to frequency scanning data will support the predictability of VCCs as other potential polarizable species will not necessarily follow the trend over time for VCC.

This work aims to demonstrate the superiority of frequency scanning compared with single-frequency measurements in terms of VCC determination. Within this study, a new quantitative multivariate model based on OPLS regressions is developed correlating offline VCC measurements of an industrially relevant CHO fed-batch process to frequency scanning data.

As a result, the model is able to display the correct VCC throughout a cultivation reducing the need of manual sampling. Moreover, in contrast to single frequency, the multivariate model predicts the VCC in all cell growth phases including the cell death phase. Lee et al. reported a direct data-driven PLS modeling approach to achieve online VCC models for batch cultivations with low cell counts, claiming the need to verify their results in cultivations with higher cell counts such as fed-batch or perfusion processes [26]. To the best of our knowledge, this study presents for the first time a direct data-driven model based on OPLS for VCCs greater than 10 million cells/mL in a fed-batch process. Moreover, robustness of the MVDA model is investigated with fed-batches including deliberate process changes.

Material and methods

Cell lines and media

A DG44 CHO cell line expressing a monoclonal antibody was used in this study (Sartorius Stedim Cellca GmbH). Seed medium (SM), basal medium for production (PM), and two different feeds, i.e., feed medium A (FMA) and feed medium B (FMB), were used in all experiments (Sartorius Stedim Cellca GmbH). All media and feeds were chemically defined.

Seed culture

The seed culture process comprised the thawing and passaging of the cells before inoculation of the bioreactor. A cryo vial of 1 mL CHO cell suspension with a concentration of 30 million cells/mL was thawed and transferred into 10 mL pre-warmed seed medium (36.8 °C) that was stored in a 15-mL falcon tube (Sarstedt). In order to remove the preservation medium, the falcon tube was centrifuged at 190*g* for 3 min (3–30K Centrifuge, Sigma). The supernatant was decanted and the pellet re-suspended in 1 mL of seed medium. The cell suspension was transferred into a single-use 0.5-L Erlenmeyer flask (Corning) containing 150 mL pre-warmed seed medium.

All seed cultures were kept in an orbital shaking incubator (CERTOMAT® CT plus, Sartorius Lab Instruments GmbH) at a temperature of 36.8 °C, a pCO_2 of 7.5%, 85% humidity, and a shaking rate of 120 rpm (rpm) with an orbital diameter of 50 mm. The cells were passaged 5 times in a rhythm of 3–4 days before inoculation of the main culture.

Main culture

The main culture was inoculated with 0.3 million cells/mL (day 0) and lasted 12 cultivation days. All experiments were carried out in a small-scale multiparallel bioreactor system (ambr® 250 modular, Sartorius Stedim Biotech GmbH) with a maximum working volume of 250 mL per vessel. The process temperature set point for all experiments was set to 36.8 °C ± 0.05 °C. The pH was controlled using CO_2 additions to maintain a pH set point of 7.1. Once a day, the pH was measured offline and re-calibrated if the result deviated by more than 0.05 units between the online and offline signals.

The set point for DO was set to 60% and the control loop adjusted the DO by modulating air and oxygen additions. The small-scale bioreactor was stirred at 855 rpm. On the inoculation day, antifoam was added depending on the actual bioreactor volume (0.001% of the total culture volume). During subsequent cultivation, antifoam was added manually depending on the foam level in the bioreactor.

FMA and FMB were added to the culture from day 3 in a ratio of 10:1 (FMA:FMB) according to Table 1.

From day 5 on, glucose was supplied as a bolus feed to the bioreactor once the glucose level dropped below 5 g/L to achieve a daily maximal glucose concentration of 5 g/L.

Dilution experiment

The seed and main culture of the process with integrated dilution steps were conducted as described in the corresponding section. The dilution with PM amounted to 30 vol.% of the cell broth. Two dilution steps were applied at a process time of 123 h and 194 h. For the dilution, the calculated amount of cell suspension was removed by sampling of the bioreactor and the same amount of pre-warmed PM was pumped to the culture. The PM was pre-warmed in an orbital shaking incubator (CERTOMAT® CT plus, Sartorius Lab Instruments GmbH) at a temperature of 36.8 °C, a pCO_2 of 7.5%, and 85% humidity until the addition of the PM into the main bioreactor was completed. Therefore, the temperature and pH were close to process conditions. The pre-conditioning of DO was not necessary, because the main bioreactor was fully controlled and the DO set point was achieved in short process times.

Altered feed strategy

The seed and main culture of the process with applied altered feed strategy were conducted as described in the corresponding section with exception of the feed and glucose strategy. Glucose was added on day 5 as a bolus once the glucose concentration depleted below 4 g/L to keep the set point at 4 g/L glucose. The feed control recipe of FMA and FMB was programmed according to Table 2.

Offline analytics

The viable cell concentration and the viability of the cell suspension, as well as the cell diameter, were analyzed with the trypan blue assay–based Cedex HiRes Cell Counter and Analyzer system (Roche). The pH and the glucose concentration were measured offline in a blood gas analyzer (ABL800 Basic, Radiometer).

Online capacitance measurements

Frequency scanning measurements were conducted with an impedance probe (FUTURA pico, Aber Instruments). The sensor scanned the permittivity at 25 discrete frequencies between 50 and 20,000 kHz resulting in a new measurement value for each frequency every 30 s. The probe was connected via a connection hub to a PC, and the data was processed by the FUTURA SCADA software (Aber Instruments). The data was stored as CSV format and imported into an Excel® file (Microsoft Corporation) for further treatment. The single-frequency excited permittivity was measured in parallel at the frequency signal of 607 kHz via the same impedance probe. The single-frequency data was processed by the FUTURA SCADA software (Aber Instruments), and it was stored as CSV format for further data treatment in an Excel® file (Microsoft Corporation).

Table 1 Feeding strategy of feed medium A (FMA) and feed medium B (FMB). The medium was added as volume percent of the cell broth. Day 0 was the day of inoculation

Day	3	4	5	6	7	8	9	10	11
Volume FMA (%)	3.9	3.8	3.6	3.5	3.3	3.2	3.0	2.9	2.8
Volume FMB (%)	0.39	0.38	0.36	0.35	0.33	0.32	0.30	0.29	0.28

Table 2 Feeding strategy of feed medium A (FMA) and feed medium B (FMB) for the altered feed strategy. The medium was added as volume percent of the cell broth. Day 0 was the day of inoculation

Day	3	4	5	6	7	8	9	10	11
Volume FMA (%)	5.1	5.0	4.8	4.7	4.6	4.4	4.3	4.2	4.1
Volume FMB (%)	0.71	0.68	0.66	0.64	0.62	0.61	0.58	0.57	0.56

Data analysis and data treatment

The MVDA model was composed of frequency scanning data from cultivations and the respective VCC offline measurements. The time points when offline and online data were available were identified and summarized. The data summary was generated in Excel® (Microsoft Corporation) and imported into the model building software (SIMCA®, Version 15, Sartorius Stedim Data Analytics). The scanning frequencies were marked as the model predictors and mean-centered. The VCC was scaled to unit variance and marked as the model response. OPLS was used to create all MVDA models. For predictions, the full dataset of the frequency scan of the selected fed-batch was imported into the OPLS model and marked as the prediction set.

Single-frequency results were analyzed in Excel® (Microsoft Corporation). A linear regression was applied including the offline and corresponding online values up to a diameter change greater than 0.5 µm compared with the averaged previous diameter values was detected. Therefore, the linear regression model did not contain any values from the end of the exponential growth phase, the stationary growth phase, or the death phase. The resulted equation from the linear regression was used to predict the VCC values based on the single frequency at 607 kHz.

The predicted VCC trajectories for both approaches (single-frequency and frequency scanning) were plotted in a graphing and analysis software (Origin® 2018, OriginLab Corporation). Within this software, the data were smoothed applying the Savitzky-Golay filter (second polynomial order) over a window of 30 data points.

The resulting trajectories were 1-point calibrated. As the inoculation cell concentration is usually a known parameter, the offsets of trajectories were adjusted to the corresponding inoculation cell concentration. For this purpose, the first 30 predicted VCC points were averaged after being smoothed. The difference between that averaged predicted value and the inoculation cell concentration was added or subtracted from all predicted data points of the corresponding batch.

For all predicted fed-batches, the root mean square error of prediction (RMSEP) was calculated to investigate the quality of the predicted values in comparison to the values observed with the offline reference (Eq. 1). y_{pred} describes the predicted VCC value based on the applied model and y_{obs} describes the observed VCC value based on the offline reference method. The number of observed and predicted value pairs is described by n.

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n}\left(y_{\mathrm{pred}} - y_{\mathrm{obs}}\right)2}{n}} \quad (1)$$

In addition, a Batch Evolution Model (BEM) was created by importing the mean-centered permittivity values of all frequencies into the model building software (SIMCA®, Version 15, Sartorius Stedim Data Analytics). The BEM displays the averaged trajectory of the selected dataset, and the standard deviations serve as limits to monitor the trajectory of independent cultivations online. The trajectories displayed in a BEM are also known as golden batch trajectories. In this work, the OPLS method was used to create the BEM. The selected robustness fed-batches that were monitored with the BEM were imported as prediction sets. Thus, the setup enables an online comparison of the current fed-batch compared with the golden batch trajectory and can be used as alarm system once a deviation occurs.

Validation of MVDA model

The OPLS model was validated applying a leave-one-batch-out (LOB) approach. Five independent models (models A–E) were created always leaving the complete dataset from one batch out that was then used as the prediction set. Table 3 summarizes the different models that were created. Each standard fed-batch served once as a prediction set and was predicted with a model comprising all other corresponding standard fed-batches. The RMSEP was calculated (models A–E in Table 3) from these predictions compared with the actual data values. The robustness trials (FB#6–8) served as prediction sets for the final model including all standard cultivations (model F in Table 3).

Within each OPLS model (e.g., model A), the root mean square error of calibration (RMSEC) was calculated. For this purpose, an identical calibration and validation dataset was used. Each data point of the dataset was predicted by the model resulting in the RMSEC.

Table 3 Overview of generated MVDA VCC models

Model	Included fed-batches (FB)	RMSEC (10E6 cells/mL)	Predicted fed-batch (FB)	RMSEP (10E6 cells/mL)	Relative error (%)	R^2 (%)	Q^2 (%)	Principle components (predictive + orthogonal)
A	FB#2–FB#5	1.37	FB#1	1.17	6.6	95.3	95.0	1 + 2
B	FB#1, FB#3–5	1.41	FB#2	1.27	8.3	95.6	95.0	1 + 2
C	FB#1, FB#2, FB#4, FB#5	1.52	FB#3	0.99	5.5	94.3	93.8	1 + 2
D	FB#1–3, FB#5	1.27	FB#4	1.04	5.7	96.2	95.7	1 + 2
E	FB#1–4	1.00	FB#5	2.22	11.0	97.3	97.1	1 + 2
F	FB#1–5	1.36	FB#6	1.33	6.7	95.4	95.0	1 + 2
F	FB#1–5	1.36	FB#7	1.78	8.8	95.4	95.0	1 + 2
F	FB#1–5	1.36	FB#8	2.71	13.2	95.4	95.0	1 + 2

Results and discussion

Establishment of a MVDA model using a leave-one-batch-out approach

In this work, capacitance frequency scanning was applied to an industrially relevant fed-batch process. After integration of the inline sensor into the single-use bioreactor, standard cultivations were performed to create and validate the MVDA model. The online data from the different frequencies served as predictors in the model, and the offline reference represented the response. Therefore, the presented method to correlate the permittivity information with VCC was a data-driven approach and did not need any further calculations like in previous approaches from literature (e.g., Cole-Cole modeling) [24, 25, 27].

In the following, the 5 standard fed-batch processes are analyzed (FB#1–FB#5). The standard fed-batch processes were conducted under the same culture conditions without changes to the process parameters and cultivation strategy. Typical sources of process variations as seed and medium lots differed for each cultivation. Each step followed the internal standard operating procedure (SOP) as described in the "Material and methods" section. To further understand the capabilities of the established frequency scanning MVDA model, the LOB method was applied. Table 3 summarizes the prediction results and the RMSEP for each cultivation being discussed in the following chapters.

Figure 2 a represents the VCC trajectory and other process parameters of one selected fed-batch (FB#4). The cell growth represented in VCC started with a short lag phase (0–48 h), followed by an exponentially growth phase with a peak cell concentration of 18 million cells/mL (48–168 h) and finally reached the stationary and death phases at the end of the process. At the end of the exponential growth phase (for FB#4 after 150 h), a strong increase of the cell diameter was detected in all standard fed-batches as exemplarily demonstrated in Fig. 2 a. With inoculation of the main bioreactor, the viability kept constantly high until day 8 of cultivation. After that day, the viability decreased to 92% for FB#4. The end viabilities for all standard fed-batches were in the range of 87% and 93% (see Electronic Supplementary Material (ESM) Table S1). The peak cell concentrations, end cell concentrations on day 12, the end viability, and the average cell diameter increase from day 0 to day 12 are summarized in Table S1 (see ESM) for all standard fed-batches.

Figure 2 b represents the predicted VCC trajectories of FB#4 based on the permittivity of one frequency and on the MVDA VCC model based on frequency scanning using the LOB method. The VCC prediction based on the MVDA model represents the VCC offline values over the complete cultivation time within the accepted error range of 10% of the offline reference method. The authors assume the measurement error of the widely used and accepted offline method to be at least 10%. This conservative estimation is based on experimental experience and is in agreement with previous literature reports [14]. However, for further discussion, 10% error is taken as acceptance criterion for the MVDA VCC model. Table 3 indicates the RMSEP and the relative errors for the prediction of all standard fed-batches within the LOB method. In this study, the RMSEP varied between roughly 1 and 2 million cells/mL corresponding to relative errors between 5.5 and 11% for the fed-batches FB#1 to FB#5 (Table 3). The coefficient of determination for all presented models was above 94%. Therefore, the prediction error of the frequency scanning model matches the uncertainty of the offline reference method. Thus, frequency scanning is a suitable technique for online monitoring of VCC and facilitates the implementation of advanced control strategies.

The online single-frequency permittivity trajectory and the trajectory based on the VCC prediction model reveal the great potential of the measurement approach itself (Fig. 2b): Each feed addition can be monitored online resulting in dips, starting with the first feed after 72 h of cultivation time. Moreover, the impact of the feed in the permittivity signal increased over time resulting in stronger dips in the stationary

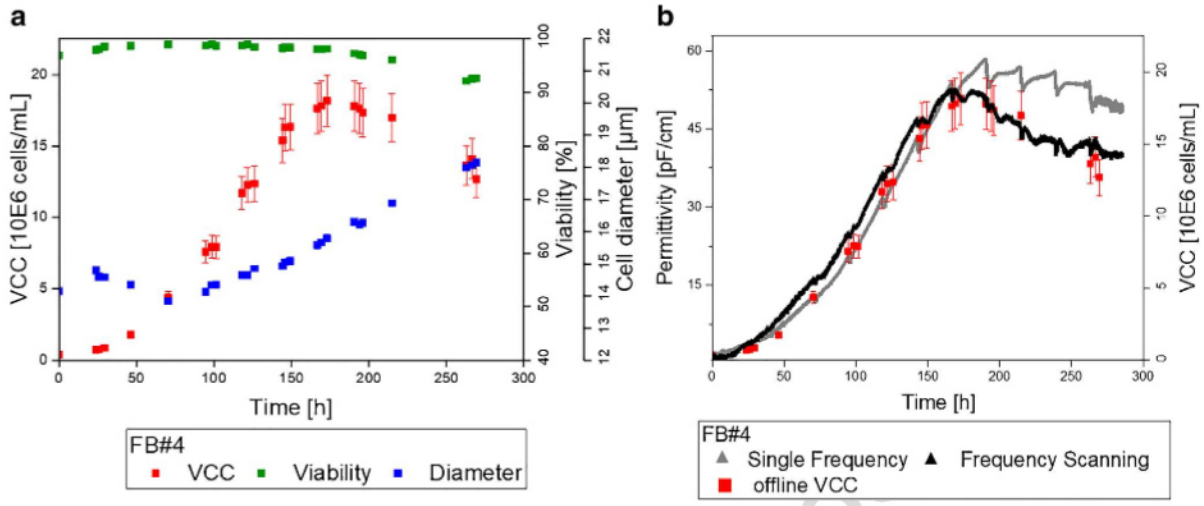

Fig. 2 Results of the standard fed-batch cultivation FB#4. **a** Viable cell concentration (VCC), viability and cell diameter trajectories for the investigated fed-batch process over a cultivation time of 12 days (288 h). **b** Corresponding online permittivity signal (single frequency), the prediction based on the multivariate model (frequency scanning) and the offline reference VCC values for FB#4. The indicated error bars for the offline reference VCC values describe the prediction error acceptance criterion of 10%

and apoptotic phases. The feed dips in the permittivity signal of single-frequency measurements were showing a decrease in the VCC higher than the pure dilution effect of the cells. It can be concluded that the permittivity signal might include information about metabolic cell changes. This effect was described by Ansorge et al. who were able to correlate feed-related changes in the permittivity to the cell metabolism [18]. However, this result needs to be confirmed in future experiments. The MVDA trajectory did not show such strong feed dips at the end of the cultivation. The OPLS algorithm excludes orthogonal information (noise) that does not correlate to the VCC. Therefore, the cross-sensitivity to other cell metabolic effects is reduced.

The permittivity trajectory of the single-frequency measurement correlated with the offline VCC within the exponential growth phase up to a significant cell diameter change (Fig. 2b). In the apoptotic cell culture phase at the end of the cultivation, the permittivity and the offline values were no longer correlated. The strong increase in the cell diameter resulted in a higher VCV and therefore a higher single-frequency permittivity signal compared with the VCC reference. This result is in good agreement with previous publications and confirms the expectations based on the literature [17]. The next section provides a more detailed comparison of the single-frequency measurements versus the MVDA analysis of the frequency scan.

Comparison of the MVDA model and single-frequency measurements

In analogy to the MVDA analysis for the frequency scanning, the LOB method was applied to the single-frequency measurements. Therefore, five separate linear regressions consisting of 4 fed-batches each were created. The individual left out fed-batch served as prediction set. The single-frequency measurement at 607 kHz and the offline VCC reference up to a cell diameter change greater than 0.5 µm were used to create a linear regression model. The criterion of the cell diameter change was selected based on the previously discussed literature indicating an impact of the cell diameter on single-frequency measurements [17]. The selected criterion was in agreement with observations concerning the cell diameter behavior in all standard fed-batches that can be seen exemplarily in Fig. 2 a for FB#4. The total diameter change of the cells over the complete cultivation time ranged from 1.9 up to 4.6 µm (see ESM Table S1). The equation from the linear regression model was used to predict the VCC of each fed-batch. Table S2 (see ESM) gives an overview of the different linear regressions presented in this work.

In Fig. 3, the predicted VCCs for each fed-batch using the LOB method are plotted against the VCC reference data points for the frequency scanning VCC model (Fig. 3a) and for the single-frequency correlations (Fig. 3b). The dotted straight line through the origin with the same distance to the *x*-axis and *y*-axis at every point corresponds to a perfect correlation between the observed and predicted values. The gray lines in the figure indicate the 10% acceptance criterion for the VCC prediction. A prediction within this error range is comparable to the current state-of-the-art measurement method and indicates a successful implementation of the online sensor approach for VCC predictions. In the MVDA model, there was a strong consistency between predicted and observed VCC values with few exceptions for all tested standard cultivations (Fig. 3a). As already mentioned previously, the

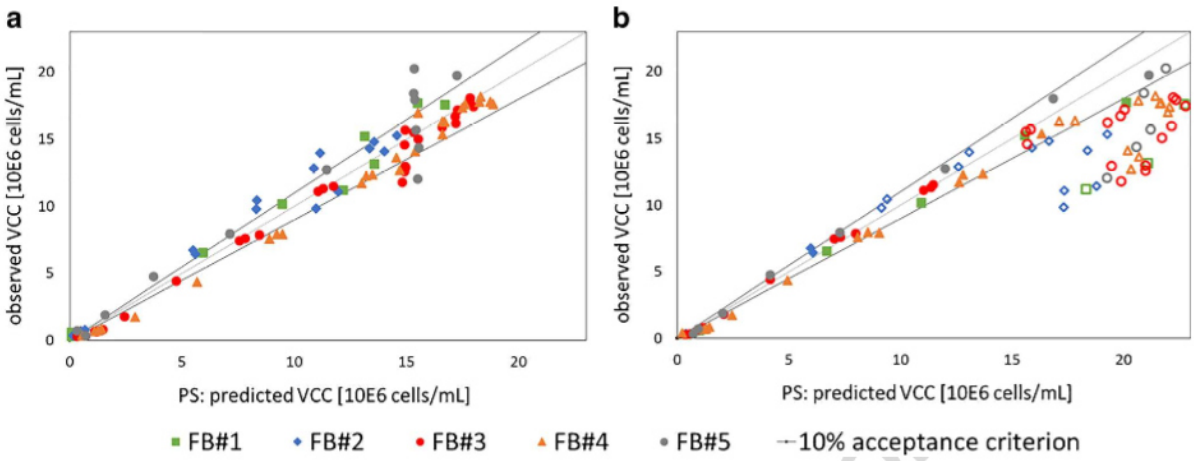

Fig. 3 Observed versus predicted plot. **a** Comparison of the observed viable cell concentration (VCC) and the predicted VCC from the prediction set (PS) based on the leave-one-batch-out (LOB) method for the multivariate data analysis. **b** Comparison of the observed VCC and the predicted VCC from the PS based on the LOB method for the single-frequency correlations. The empty symbols reflect the measurement values that were excluded from the correlations based on linear regression

RMSEP for the multivariate analysis resulted to be between 1 and 2 million cells/mL with relative errors between 5.5 and 11% for the fed-batches FB#1 to FB#5 (Table 3).

Figure 3 b displays the results of the LOB method applied to the single-frequency measurements. However, the single frequency did not provide a correlation within the 10% acceptance criterion over the complete cultivation time. In the exponential growth phase below VCCs of 15 million cells/mL, a good correlation was given. With higher VCCs, the observed and predicted values were not comparable to each other and out of the 10% acceptance range. Empty symbols indicate the VCC values that were not included into the linear regression because of the diameter change greater than 0.5 μm.

The RMSEP for the single-frequency analysis (between 3 and 4 million cells/mL) was much higher compared with the RMSEP values achieved with the MVDA model (Table 3 and ESM Table S2). The relative error for the prediction based on single frequency ranged from 15.8 to 22.7%. The accuracy of the single-frequency measurement was not comparable to the offline reference method.

When dealing with such numbers, one should keep in mind that single-frequency measurements do not correlate to VCC if cell diameter changes occur [17, 30]. Therefore, the error of this method is strongly dependent on the data point selection and distribution. Many data points within the death phase of the cells (with higher cell diameter) will increase the error values for VCC predictions automatically as they were not considered in the linear regression (empty symbols). Using linear regression models to predict the values in the death phase necessarily results in high deviations. To better compare the single-frequency VCC prediction and the MVDA VCC model, the RMSEP of the values after a significant cell diameter change of 0.5 μm that served as criterion for the linear regression was calculated. Table S3 (see ESM) summarizes the RMSEP and relative errors for the VCC values after the cell diameter change. The superiority of the frequency scanning MVDA model compared with the single-frequency prediction is clearly demonstrated for the stationary and death phases of the cells. The RMSEP for the MVDA VCC model ranged from 1 to 3 million cells/mL (rel. errors between 5.4 and 15.2%) whereas the RMSEP for the single-frequency prediction was calculated to be between 4 and 6 million cells/mL (rel. errors between 25 and 34%). The relative error of the VCC prediction in the cultivation period with an increased diameter was only for the MVDA model inside the acceptable range with one exception of FB#5 with 15.2% that was only close to the acceptance criterion. The exception is probably caused by VCC variations within the standard process and a small amount of cultivations in the calibration set. The results can likely be improved by a larger set of calibration runs in future.

To conclude, the results of the LOB method demonstrate that the MVDA model provides significant benefits compared with single-frequency measurements in predicting VCCs over the complete culture time for the presented fed-batch process. The frequency scanning itself is a powerful tool for monitoring the process attribute VCC with a high accuracy and with reduced cross-sensitivity to cell diameter changes. For future applications, use of frequency scanning in combination with MVDA for VCC online monitoring is therefore recommended.

MVDA model properties including all standard cultivations

The previous section demonstrates that the MVDA model based on standard cultivations predicts the VCC of an independent fed-batch with a high accuracy. For future

applications, the MVDA model will be trained and extended with every successful cultivation. In accordance with future applications, the most robust model containing all 5 cultivation training sets is described and used for further analysis and robustness trials.

Figure 4 a shows the regression plot and therefore the influence of each frequency on the MVDA VCC model. The frequencies can be divided into 3 different regions that are indicated by different hills or valleys. Within each region, the resolution of the permittivity measurement is different. The information within one valley or hill might represent the same effect in different resolutions. Therefore, the information might correlate to the same effect within one region. In the investigated frequency range, three affected sub-ranges are revealed. The three different sub-ranges support the amount of selected principle and orthogonal components of the MVDA model (Table 3). For all MVDA VCC models, 1 predictive and 2 orthogonal components were used. In addition to the regression plot (Fig. 4a), the R^2/Q^2-summary of fit plot supports the selection of components (see ESM Fig. S2). It is common to stop adding principle component in case an additional component does not result in an improvement of more than 10%. Moreover, the comparison of R^2/Q^2 and RMSEC/RMSEP (Table 3) should be roughly similar. Based on the presented results, 1 predictive and 2 orthogonal components were used for all MVDA VCC models (Table 3).

In comparison to single-frequency measurements at 607 kHz, the result of three different sub-ranges indicates that frequency scanning delivers additional information in lower frequencies. This additional information is included in the MVDA model and improves the predictability of VCC values. The analysis of the score contribution plots in a point-to-point comparison of two data points within one fed-batch underlined the increase of information in lower frequencies compared with the single frequency at 607 kHz. Figure S1 (see ESM) demonstrates the score contribution plot for each frequency in a point-to-point comparison for the cultivation days 5 and 12 in FB#3. The VCC values on the selected days were in a comparable range (11.51 million cells/mL for day 5 and 11.79 million cells/mL for day 12), but a strong change in the cell diameter was detected (14.7 µm for day 5 and 18.4 µm for day 12). A strong effect at low frequencies, especially at 50 kHz, was identified. Therefore, the information from low frequencies might include information about the cell diameter changes in the late exponential growth phase, the stationary phase, and the death phase.

The use of multiple frequencies and a MVDA VCC model resulted in a higher accuracy of VCC predictions especially in

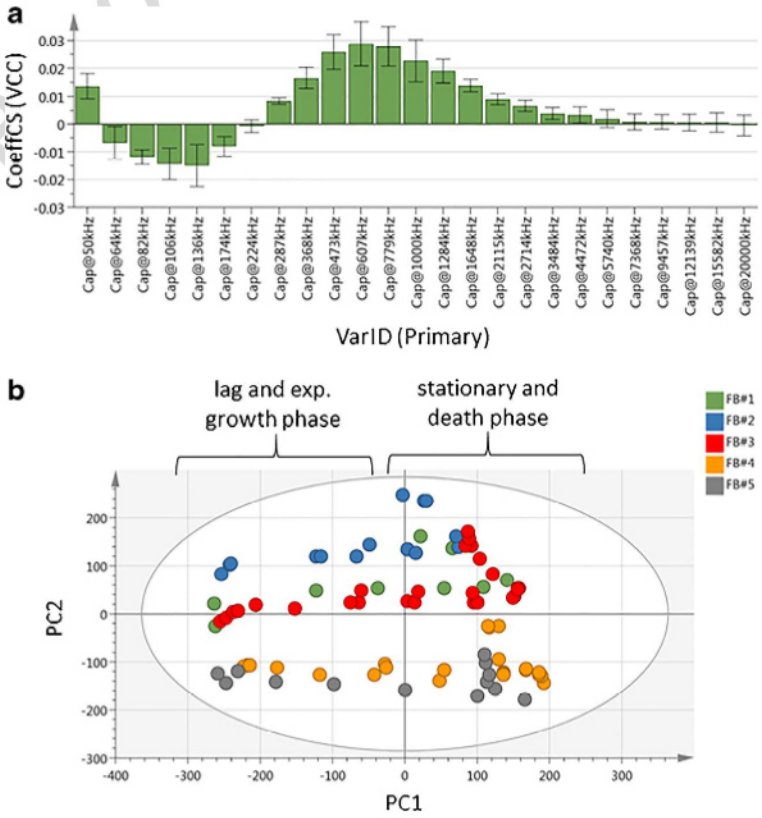

Fig. 4 Overview of the multivariate model containing all standard fed-batches FB#1 to FB#5. **a** Contribution of each of the 25 measured frequencies to the multivariate model. **b** Score plot indicating the distribution of the fed-batch cultivations (FB#1–FB#5) dependent on the predictive principle component (*x*-axis) and the first orthogonal principle component (*y*-axis). The ellipse around the plotted data points indicates the 95% confidence bound based on Hotelling's T2 statistics

the death phase of each cultivation (Fig. 3). Furthermore, in future applications, it might be possible to follow the approach to reduce the amount of detection frequencies from 25 frequencies down to one frequency per identified valley or hill. A reduction of frequencies enables an improved process control by a higher measurement frequency and results in smaller databases for the online calculations.

Figure 4 b shows the score plot including all standard cultivations regarding the first two principle components (PC1 and PC2). PC1 is the predictive component located on the *x*-axis, and PC2 is the first orthogonal component on the *y*-axis. Each fed-batch is colored individually. The scores of each cultivation can be separated based on the cultivation time of each score. The scores in the lag phase and the exponential growth phase did not distribute in PC2 within one fed-batch. However, the scores of each fed-batch in the declining phase distributed in PC2. In agreement with the statements above, this result indicates that the MVDA model improves the VCC predictability especially during the death phase because of additional information described by further PCs based on different frequencies. Moreover, the score plot displays the distribution of the standard cultivations that were carried out. Each standard cultivation started from a separate seed train and was carried out at a different time leading to different medium charges and other variances. All these variances represented the accepted variation according to SOPs in the presented fed-batch process. The process parameters were not changed between runs. However, a variation within PC2 from process to process was detected. FB#2 and FB#5 were divided and distinguishable with an offset in the PC2. This difference was also reflected by comparing the RMSEC for the model containing FB#1–FB#4 (1 million cells/mL) and the resulting RMSEP (2 million cells/mL) of FB#5 (Table 3). Thus, for FB#5, the RMSEP was more than doubled compared with the RMSEC of the corresponding multivariate model. Therefore, FB#5 seems to be an important fed-batch that needs to be included into the MVDA model even though FB#5 was performed according to the same protocol as all other fed-batches. This result is in agreement with the higher relative error for FB#5 that was previously detected when predicting the VCCs only after the cell diameter change of 0.5 µm (see ESM Table S3). To conclude, there are variations from process to process that should be accounted for in the model building process. A sufficient number of standard cultivations are necessary to create a robust and optimized MVDA model that is able to describe variations between standard fed-batches according to SOPs. This is in good agreement with other publications which stated that a robust model should contain at least 5 batches [16, 26]. In this work, a single-use, small-scale bioreactor was used for all standard cultivations. The benefit of using small-scale, single-use systems is fast turnover times with low experimental costs. Therefore, this approach provides a rapid and an economic method to achieve a sufficient number of cultivations for a robust MVDA model that can be used for future online monitoring and control of the process. The automated small-scale bioreactor enables the possibility to develop a robust model in one single run using several bioreactors at the same time and setting up a design of experiment (DoE). Thus, the presented approach can be applied in early process development where DoEs and multiparallel small-scale bioreactors are commonly used. However, the scalability of the established MVDA model to other bioreactors should be investigated in the future. The application of the MVDA VCC model in large-scale bioreactors is critical to move from early process development towards production. Therefore, it might be possible that the MVDA VCC model needs to be further developed during scale-up by including larger bioreactor scales into the model. This approach is commonly used for advanced models and leads to strong and robust predictions of the target value.

Robustness test—dilution series

The robustness trials were applied to ensure that the prediction of the MVDA VCC model is not based on any correlation to standard process behavior or process time. The first robustness trial consisted of two dilution steps of the cell broth during one cultivation (FB#6). The cell broth was diluted by 30 vol.% with pre-warmed PM. The advantage of using a dilution with PM as first robustness trial was to implement simple changes to the permittivity signal without significantly influencing the process parameters and cell biology. Therefore, the change in the permittivity was expected to be related predominantly to the dilution steps itself and therefore the VCC changes.

The dilutions were applied after 123 h and 194 h of cultivation time (Fig. 5). As described previously, cell diameter changes can have an impact on the permittivity signal. Therefore, the timing of each dilution was selected to cover process changes in the exponential growth phase (constant cell diameter) and in the stationary phase (incl. cell diameter changes), respectively.

Besides a low-frequency disturbance (probably caused by grounding issues of the used setup with temporarily electromagnetic interference) and CO_2 supply perturbations, the dilution steps were detectable in the online signal (Fig. 5). The CO_2 supply perturbations had no apparent effect on the complete cultivation and can be seen as unintended robustness tests. The influence of the CO_2 supply perturbations on the cells was visible in the permittivity signal, leading to an increased real-time process understanding on the cellular level. Each offline reference sample that was measured after the dilution steps was matching to the predicted online VCC value. Moreover, the RMSEP of the MVDA VCC model for the complete cultivation was calculated to be 1 million cells/mL (Table 3). The prediction resulted in a relative error of 6.7%

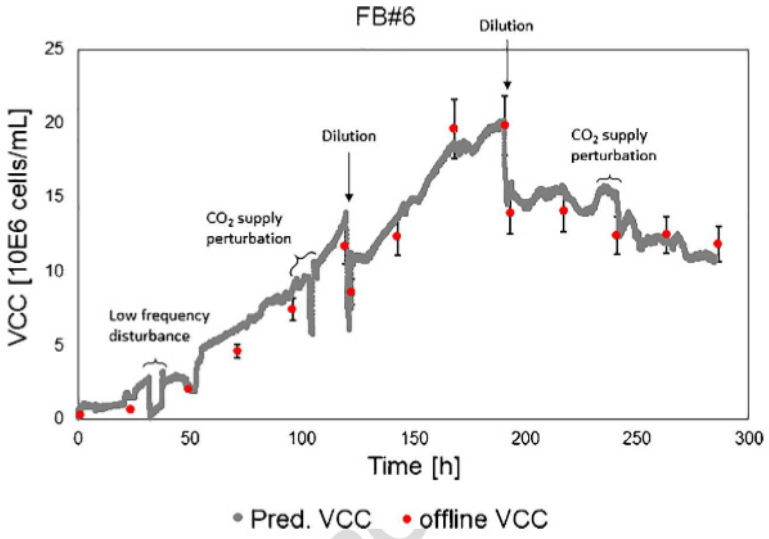

Fig. 5 Prediction results based on the multivariate model including all standard cultivations for the dilution robustness trial (FB#6). Dilutions were applied after 123 h and 195 h of cultivation time. The online prediction of the viable cell concentration (VCC) was compared with the offline reference method. The indicated error bars for the offline reference VCC values describe the prediction error acceptance criterion of 10%

for FB#6. The predicted VCCs for the diluted fed-batch process were within the 10% acceptance criterion and comparable to the offline reference. Moreover, process disturbances (e.g., CO_2 supply perturbations) were detected in the online signal immediately. Therefore, the online signal can be used for early fault detection. Such an alarm system will enable fast corrective actions to minimize process deviations that might lead to process failures and lost batches.

This result indicates that the MVDA model based on the 5 standard cultivations is a robust tool for VCC online monitoring. The process changes were detected immediately even though no induced process variation was previously included in the MVDA VCC model.

Robustness test—varying feeding strategy

The dilution in the previous robustness trial was selected as being a significant process deviation with direct impact on the VCC and immediate detection in the offline reference method. To further investigate the MVDA model regarding its behavior towards less obvious process deviations, different feeding strategies were applied to the standard cultivation. The feed was slightly decreased compared with the standard feeding strategy (Tables 1 and 2). Even small process disturbances (e.g., different nutrient levels in the cell culture) in the exponential growth phase might influence the investigated process attribute (VCC) in a later stage of the cultivation or the quality attributes of the product itself [37–40]. Thus, the feed robustness trial aims at investigating the model robustness with changes having an indirect impact on the cell growth. For this purpose, a multivariate BEM based on the frequency scanning data and deviations in the univariate offline parameter were compared.

A process control chart of the offline VCC for FB#1 to FB#5 was generated applying two respectively three standard deviations as control limits (Fig. 6). This control chart was used to compare the offline VCC of the feed variation trials FB#7 and FB#8. The offline VCC values were mainly inside the range of two standard deviations for the complete culture time. FB#7 showed two outliers outside the two standard deviations after 120 h and 144 h of cultivation time. However, remembering the minimum of 10% acceptance criterion, these outliers are not considered critical for the processes. Moreover, in the literature, deviations up to 3 standard deviations are a common acceptance criterion [41]. Applying three standard deviations to the presented offline reference results in no critical outliers for any of the two batches. For FB#8, the offline VCC was within the two standard deviations for the complete cultivation time except of the measurement value at 240 h that was still within the three-standard-deviation range. For both cultivations, no consecutive outliers were detected. Consecutiveness of outliers can serve as trigger for alarm functions and reduces the likelihood of false alarms due to single-event faulty measurements. In summary, offline VCC measurements did not detect induced process variations based on the varying feeding profiles.

The online monitoring capabilities were tested by applying the BEM to the fed-batches with the altered feeding profile (Fig. 7). The multivariate BEM displays an average trajectory for the frequency scanning data dependent on the process time with two standard deviations. Compared with the offline reference in Fig. 6, a strong deviation in both altered feed fed-batches (FB#7 and FB#8) was detected. After 140 h of cultivation, both fed-batches were outside the alarm limits of two standard deviations. Compared with the univariate control chart based on offline VCC reference, the BEM allowed for a significantly earlier detection of the induced process

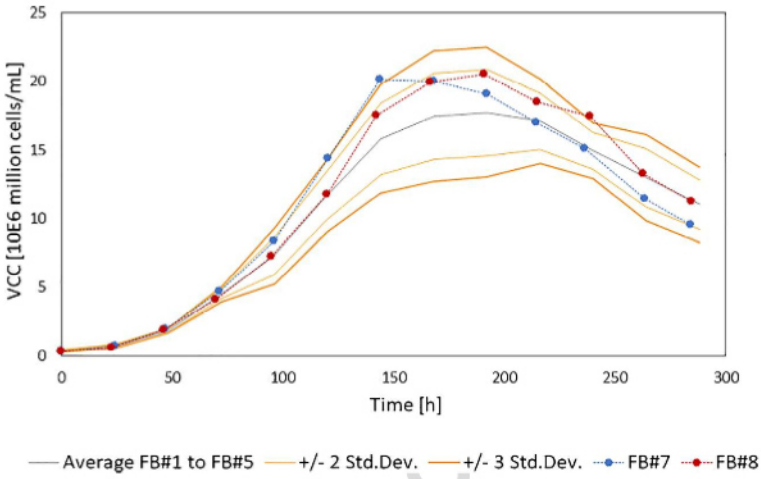

Fig. 6 Comparison of viable cell concentration (VCC) offline reference values of the feed variated processes (FB#7 and FB#8) and the standard cultivations (FB#1–FB#5). The VCC values for the standard cultivations were averaged and the 2 and 3 standard deviations were plotted

deviation. Therefore, applying the online monitoring enables a fast identification of process deviations in regard to the investigated process attribute (VCC).

Applying MVDA and using the offline reference VCC values to build the model are an indirect measurement method. The absolute error of the reference method rises with increasing VCC. Therefore, the VCC prediction in the death phase of the cell culture results inevitably in higher prediction errors compared with the beginning of a process. Considering a common reference VCC measurement device (in industry and academia), the error of the MVDA VCC model is comparable to the reference uncertainty over the complete culture time and can be equally used for decision-making processes in the death phase of a cell culture.

Table 3 summarizes the predictions of FB#7 and FB#8 based on the MVDA VCC model including all standard cultivations (FB#1–FB#5). The relative errors of the prediction were calculated to be 8.8% for FB#7 and 13.2% for FB#8. Both fed-batches were predicted with low errors, and FB#7 was below to the 10% acceptance criterion for VCC predictions even though the MVDA model used for the predictions was simply based on standard cultivations only. The relative error of FB#8 was slightly higher but still comparable to the offline reference system error. The prediction accuracy and model robustness can likely further be improved by including the process robustness trials into the MVDA model in future.

In summary, combining MVDA and frequency scanning can lead to be a powerful tool for future control strategies with implemented alarms saving batches, costs, time, and resources. Furthermore, the use of the single-use, small-scale bioreactor enables investigations of purposely induced process deviations by reducing medium and maintenance costs for each cultivation (including the dilution trial and the changed feed strategies). This result leads to the conclusion that the MVDA VCC model provides high accuracy even with changing process conditions.

Conclusion

Within this work, the superiority of frequency scanning over single-frequency measurements was demonstrated,

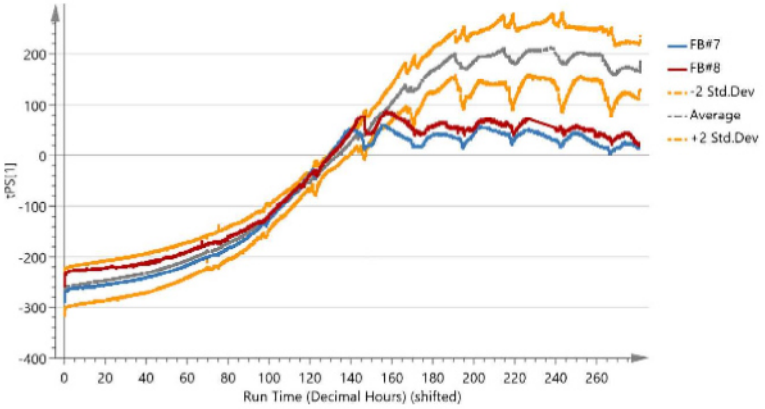

Fig. 7 Batch Evolution Model (BEM) created from all standard cultivation (FB#1–FB#5). The BEM displays the golden batch trajectory based on the zeroed permittivity data for all frequencies. The zeroed permittivity frequency scans of the fed-batches with a varied feed strategy (FB#7 and FB#8) were included as prediction sets and monitored online in the BEM over the complete cultivation time

particularly during the death phase of the investigated fed-batch process. The relative errors for the data points ranged between 5.4 and 15.2% for the MVDA VCC model after the cell diameter changed (more than 0.5 μm compared with the averaged previous cultivation days). In contrast to this, the single-frequency VCC prediction resulted in relative errors between 25 and 34%. Including the complete cultivation time, the MVDA model predicted the VCC with relative errors between 5.5 and 11% in standard cultivations. The robustness of the MVDA model was successfully proven resulting in relative errors between 6.7 and 13.2%. A BEM model provided immediate information about process deviations that were difficult to detect in the corresponding offline reference on its own.

The use of a small-scale, single-use bioreactor provided a fast and economic development of a robust MVDA model with a sufficient amount of standard cultivations. Moreover, the use of the small-scale bioreactor enabled the proof of model robustness with deliberately induced process deviations. In the small scale, medium and maintenance costs for each cultivation were reasonably low.

To conclude, the combination of a capacitance probe as an inline monitor tool with MVDA enabled predictions of the VCC as a major process attribute for cell cultivation processes. Thus, the method presented here is recommended for future monitoring and control strategies such as feed control or the endpoint determination of a cultivation process based on online VCC predictions. The various monitoring and control possibilities of the presented method lead to economic, safe, and robust mammalian cell culture processes according to FDA's PAT and QbD approaches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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4 Summary and Outlook

Modern biopharmaceutical manufacturing aims at intensifying and automating mammalian cell culture processes in single-use facilities to reduce costs, increase product safety and deliver high flexibility in the facility.

Scalability and online monitoring or control by PAT tools are crucial factors for fast and economic process development and deep process understanding. The implementation of PAT tools in all available bioreactor scales, starting from scale-down models up to production scales, enable advanced process monitoring, control and automation. Thus, PAT tools lead to consistent and high product quality following the QbD requirements of the FDA.

The presented work focused on demonstrating the benefits of PAT tools in different single-use bioreactor scales to fulfill the industries' needs for process intensification and automation. The application of DoE studies in high-throughput, small scale bioreactors enabled rapid process development of a semi-perfusion process with increased product yield. The presented method is a powerful tool that can be used to intensify existing fed-batch process platforms in future as well as to develop new perfusion processes.

To further automate the presented cell culture processes a capacitance sensor was successfully investigated as online monitoring tool for biomass related changes (WCW, VCV, VCC) in single-use bioreactor volumes from 50 L up to 2000 L. Especially, the process attribute VCC requires a continuous measurement principle that can be used for automation, e.g. feeding rates in perfusion or fed-batch processes. However, VCC prediction was limited to the exponential growth phase with the single-frequency measurement mode of the capacitance sensor.

Thus, the capacitance sensor was used in frequency scanning mode and the application of MVDA to the frequency scan optimized the online prediction of VCCs with great success. The MVDA model development was performed in single-use, small scale bioreactors resulting in a fast and low cost method development. The novel method can provide extensive process

understanding and lead to powerful control strategies. As a future step, the transferability of the developed VCC MVDA model to larger bioreactor scales should be investigated.

This work demonstrates that the combination of PAT tools and single-use bioreactors can positively impact the process development and understanding. The application range of the tools developed in this work is broad. First results of applying the tools in the process automation were recently presented at an international conference (ESACT 2019, Poster Matuszczyk *et al.*). A capacitance sensor that monitored online the VCC in the pre-culture, performed automated the inoculation of an n-stage bioreactor. The automation led to reduced operator handling and less contamination risks. However, further work is required in future to automate mammalian cell culture processes with the help of PAT tools. The establishment of autonomous production facilities with complete online monitoring and control remains an ongoing task. Future work of high interest is to develop a connected and automated cell culture process from cell thaw until downstream processing using the developed online PAT tools.

Multi-parallel bioreactors with implemented PAT tools as small scale models can support fast troubleshooting and enable advanced understanding of process parameter interactions and their impact on product quality. The developed tools can be adopted and transferred to FDA approved biopharmaceutical manufacturing. Small scale systems and easy scale-up significantly reduce development timelines of new drugs resulting in faster clinical trials and finally faster access for patients to new pharmaceuticals.

Even though the final implementation of these tools into FDA approved biopharmaceutical processes remains a challenge and regulatory work is needed, the high potential of these methods is driving the industry forward. This PhD thesis contributes to implement online control tools into mammalian cell culture processes that lead to autonomous laboratories of the future. The reduction of operator interaction in biopharmaceutical processes can significantly decrease the risk of failures in productions and increase a consistent product quality.

5 References

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Publications and Conference Contributions

Publications

(1) **Sabrina Janoschek*** **(75%)**, Markus Schulze (20%), Gerben Zijlstra, Gerhard Greller, Jens Matuszczyk *A protocol to transfer a fed-batch platform process into semi-perfusion mode: The benefit of automated small scale bioreactors compared to shake flasks as scale-down model.* Biotechnology Progress 2018, doi: 10.1002/btpr.2757.

SJ (75%): Planning and execution of experiments; Data analysis; Preparation of manuscript
MS (20%): Support of shake-flask experiments
GZ, GG, JM (in total 5%): Discussions and support of results interpretation; Paper review

(2) **Sabrina Metze*** **(85%)**, Sebastian Ruhl (10%), Gerhard Greller, Christian Grimm, Jochen Scholz *Monitoring online biomass with a capacitance sensor during scale-up of industrially relevant CHO cell culture fed-batch processes in single-use bioreactors.* Bioprocess and Biosystems Engineering 2019, doi: 10.1007/s00449-019-02216-4.

SM (85%): Planning and execution of experiments; Data analysis; Preparation of manuscript
SR (10%): Support of large-scale experiments
GG, CG, JS (in total 5%): Discussions and support of results interpretation; Paper review

(3) **Sabrina Metze*** **(85%)**, Stefanie Blioch (10%), Jens Matuszczyk, Gerhard Greller, Christian Grimm, Jochen Scholz, Marek Hoehse *Multivariate data analysis of bio-capacitance frequency scanning for online monitoring of viable cell concentrations in small scale bioreactors.* Analytical and Bioanalytical Chemistry 2019, doi: 10.1007/s00216-019-02096-3.

SM (85%): Planning and execution of experiments; Data analysis; Preparation of manuscript
SB (10%): Support of robustness experiments
JM, GG, CG, JS, MH (in total 5%): Discussions and support of results interpretation; Paper review

Conference Contributions

(1) Sabrina Janoschek*, Sebastian Ruhl, Jens Matuszczyk, Adrian Stacey, Jochen Scholz, Gerhard Greller, Christian Grimm *Using Process Analytical Technologies (PAT) to compare*

the process trajectory of an industrial fed-batch process in different single-use bioreactor scales. The Bioprocessing Summit 2017, Boston (Poster)

(2) Jens Matuszczyk, Markus Schulze, Sabrina Janoschek*, Gerben Zijlstra, Gerhard Greller *High cell density cell cultures (>100E6 c.mL-1) in 2D bags with integrated filter for seed train process intensification.* The Bioprocessing Summit Europe 2018, Lissabon (Poster)

(3) Jens Matuszczyk, Markus Schulze, Sabrina Janoschek*, Gerben Zijlstra, Gerhard Greller *Cost Effective 2D Rocking Motion Bioreactors with Internal Filters are Compatible with High Cell Density Perfusion Cultures (up to 170 Million Cells per mL), and Offer Significant Advantages for Seed Train Handling.* BioProcess International 2018, Boston (Poster)

(4) Sabrina Janoschek*, Markus Schulze, Gerben Zijlstra, Gerhard Greller, Jens Matuszczyk *Moving from a fed-batch process to perfusion mode using a semi-continuous approach in shake flasks and an automated small scale bioreactor.* Laupheimer Biotech Days 2018, Laupheim (Poster)

(5) Alexander Graf, Sabrina Janoschek*, Johannes Lemke, Sebastian Wolff, Bernd Hitzmann, Karsten Rebner, Marek Höhse *Raman Spectroscopy for Bioprocess Monitoring: From Re-Usable Large-Scale to Single-Use Miniaturized Bioreactors.* Herbstkolloqium AK-PAT 2018, Hannover (Poster)

(6) Alexander Graf, Sabrina Janoschek*, Johannes Lemke, Sebastian Wolff, Bernd Hitzmann, Karsten Rebner, Marek Höhse *Raman Spectroscopy for Bioprocess Monitoring: From Re-Usable Large-Scale to Single-Use Miniaturized Bioreactors.* Doktorandenseminar AK-PAT 2019, Berlin (Oral Presentation)

(7) Sabrina Janoschek*, Jens Matuszczyk, Marek Hoehse, Jochen Scholz, Gerhard Greller, Christian Grimm *Multivariate data analysis for online monitoring of viable cell concentrations in small scale bioreactors.* Doktorandenseminar AK-PAT 2019, Berlin (Oral Presentation)

(8) Sabrina Janoschek*, Jens Matuszczyk, Marek Hoehse, Jochen Scholz, Gerhard Greller, Christian Grimm *Online monitoring of viable cell concentrations in small bioreactors.* ESACT 2019, Copenhagen (Poster)

(9) Jens Matuszczyk, Johannes Lemke, Markus Schulze, Sabrina Janoschek*, Gerben Zijlstra, Gerhard Greller *Scalability of Cost Effective 2D Rocking Motion Bioreactors with Internal Filter Membrane for Perfusion Processes. High Cell Densities of up to 170 mln Cells per mL and Spectrometric PAT Sensors Allow for an Intensified Seed Train Handling and Automated Inoculation by Maintaining CQAs and Titers.* ESACT 2019, Copenhagen (Poster)

**Name change from "Janoschek" to "Metze" in 04/2019*

Curriculum vitae

Personal Information

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2002 - 2011	German school leaving certificate at Aloisiuskolleg, Bonn

Professional Experience

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