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TECHNISCHE UNIVERSITÄT WIEN
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DISSERTATION

A Quality by Design Approach for Enhanced Process Understanding in Biopharmaceutical Process Development

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines Doktors der technischen Wissenschaften unter der Leitung von

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Biopharmaceutical products will be important drivers of improving medical treatments and the standard of living in the 21th century. The development and commercialization of biosimilar monoclonal antibodies is a major milestone to maximize patient benefit, as these products deliver equivalent clinical effect at lower costs than the originator product. One of the major challenges in biosimilar development is to adjust the quality profile of the drug substance with technological control strategies in a tight range which is ultimately determined by the characteristics of the originator product. This goal is in accordance with the quality by design (QbD) paradigm, which demands the thorough understanding of interactions between process parameters and product quality attributes to assure consistent process output.

This thesis focuses on the implementation of QbD tools into biosimilar process development aiming to understand links between process parameters and product quality attributes in a fed-batch recombinant CHO process producing a monoclonal antibody. In order to achieve this targeted knowledge, four essential steps have been identified and accomplished. First, standard risk assessment tools were tailored to address the above discussed unique characteristics of biosimilar development. These novel tools allowed a risk-based investigation of the complex interactions between critical process parameters (CPPs) and critical quality attributes (CQAs). Thereby, physiological features of the production cells were identified to have a vast impact on these interactions. Accordingly, the second step was to develop workflows for the quantification of physiological variables on the level of cell metabolism and to investigate the effect of multiple CPPs on these variables. Thereafter, novel control strategies were developed to steer physiological features such as the metabolic switch to lactate uptake as well as specific productivity in the fed-batch process. The control of physiological variables enabled the combination of standard QbD tools with the physiological approach. Consequently, the final step was to involve the controlled physiological features as input and output variables in design of experiment (DoE), multivariate data analysis (MVDA) and process analytical technology (PAT) tools.

The essential novelty of the presented work is the combination of QbD tools with the quantification of physiological variables in cell culture process development. This approach enables the generation of enhanced process understanding and the development of a novel control strategy to adjust a CQA of the product. The anticipated benefit of the presented workflow over conventional QbD approaches is the identification of novel CQA control strategies based on sound process understanding, an aspect especially relevant for biosimilar development.

Biopharmazeutische Produkte sind wichtige Treiber für die Verbesserung von medizinischen Behandlungen und Lebensqualität im 21. Jahrhundert. Dabei ist die Vermarktung von Biosimilars, die einen gleichwertigen therapeutischen Effekt wie das Originalprodukt zu einem niedrigeren Preis anbieten, ein wesentlicher Schritt, um den Patientennutzen von biopharmazeutischen Therapien zu maximieren. Eine der größten Herausforderungen in der Entwicklung von Biosimilars ist es das Qualitätsprofil des biosimilaren Arzneistoffes mit Hilfe von technologischen Kontrollstrategien in dem von dem Originalprodukt vorgegebenen engen Bereich einzustellen. Der risiko- und wissenschaftsbasierte Ansatz Quality by Design (QbD), der darauf abzielt, durch das vertiefte Verständnis der Zusammenhänge zwischen technologischen Parametern und Produktqualität gleichmäßig gutes Output im Herstellungsprozess sicherzustellen, bietet eine Lösung für diese Herausforderung.

Diese Doktorarbeit untersucht die Implementierung von QbD-Methoden in den Herstellungprozess von Biosimilars anhand des Beispiels der Produktion eines biosimilaren monoklonalen Antikörpers (mAb) durch rekombinante CHO-Zellen. Vier wesentliche Schritte wurden identifiziert und durchgeführt, um den Effekt von technologischen Parametern auf die Qualitätsattribute des produzierten Wirkstoffs zu verstehen. Als erster Schritt wurde eine an die Entwicklung von Biosimilars angepasste Methode der Risikobewertung entwickelt, um die komplexen Interaktionen zwischen kritischen Technologieparametern (critical process parameter, CPP) und kritischen Qualitätsattributen (critical quality attribute, CQA) zu untersuchen. Die Anwendung dieses strukturierten Ansatzes hat ergeben, dass diese Interaktionen letztendlich von den physiologischen Eigenschaften der Produktionszellen geprägt sind. Der zweite Schritt des Projektes war dementsprechend die Entwicklung von Methoden für die Quantifizierung von physiologischen Variablen auf der Ebene des Zellmetabolismus. Anschließend wurden neue Kontrollstrategien entwickelt, um physiologischen Variablen, wie zum Beispiel Laktat-Metabolismus und spezifische Produktivität, während des Herstellungsprozesses zu steuern. Diese Kontrollstrategien ermöglichten den letzten Schritt der Arbeit, nämlich die Verbindung des physiologischen Ansatzes mit Standardmethoden des QbD. In diesem Rahmen wurden die kontrollierten physiologischen Eigenschaften als Input- und Outputvariablen in die Methoden Design of Experiment (DoE), multivariate Datenanalyse (MVDA) und Process Analytical Technology (PAT) eingebunden.

Das Novum der Doktorarbeit ist die Kombination von QbD-Methoden mit der Untersuchung von Zellphysiologie in der Entwicklung eines CHO-Prozesses. Dieser Ansatz ermöglicht das

gründliche Verstehen von Interaktionen zwischen technologischen Parametern und den Qualitätsattributen der Wirkstoffe. Durch die Entwicklung einer neuen Kontrollstrategie für die Einstellung eines CQAs wurde die Anwendbarkeit und der antizipierte Nutzen des QbD-Ansatzes für die Entwicklung von Biosimilars demonstriert.

First, I would like to express my appreciation to Prof. Christoph Herwig for supporting and supervising this work. I am grateful for the stimulating discussions and his inspiring ideas which have shaped this thesis as well as my technical knowledge to a great extent.

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I am very grateful to Daniela Ehgartner, Bettina Lorantfy and Sebastien Bernacchi for their friendship, for the inspiring discussions at TopKino and for giving me a roof over my head in Vienna. My appreciation goes to Christian Dietzsch for the valuable advice and kind support right at the beginning of this project. Many thanks to Patrick Wechselberger for his expert advice in statistical tests and metabolic models. A big thank also to Tobias Klein, Aydin Golabgir and Paul Kroll for their help and contributions to the manuscripts.

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A huge thanks to my wonderful family for all their support and encouragement over the years. I am especially grateful to my parents - this thesis is the fruit of the strength and wisdom they have always given to me. I am also grateful to my brother for his clever advice and for asking smart questions about my research.

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Introduction

The pharmaceutical industry experienced an outstanding development throughout the 20th century. The first blockbuster drug, aspirin, was already introduced at the turn of the previous century. The swift scientific progress in pharmaceutical development around the time of the two world wars enabled the development of the first two biotechnology-derived medicines: insulin and penicillin. In the 1950s and 1960s health authorities introduced the foundations of a highly regulated market and meanwhile the pharmaceutical industry went global. The revolution in the development and manufacture of small molecule substances enabled the production of several blockbuster drugs, which led to great improvements in the standard of living in the second half of the century. In 1982, the first recombinant protein product was approved in the United States and since then the importance of biopharmaceuticals has been increasing in the treatment of several diseases. Around the turn of the millennium, the first monoclonal antibodies were introduced, allowing specific and effective treatment for many chronic diseases such as cancer and autoimmune disorders. The production of these drugs at low prices and high quantities could impact on human health in the 21st century to a similar extent as the blockbuster medicines of the 20th century (Ecker et al. 2015).

Biopharmaceuticals and biosimilars

The clinical performance of biopharmaceuticals is influenced by the molecular structure of the active drug substance. Recombinant glycoprotein products, the largest group of biopharmaceuticals in terms of sales volume and growth (Walsh 2014), possess a highly complex three dimensional structure. These proteins have several sites which are prone to variability, including disulfide bridges, C-terminal lysine residues as well as the glycosylation pattern at dedicated positions of the amino acid sequence. As changes in these molecular features affect the clinical performance (safety and efficacy) of therapeutic proteins, they are also referred to as product quality attributes (Eon-Duval et al. 2012). Even approved biopharmaceuticals show a considerable variability in quality attributes such as glycosylation as a consequence of changes in the manufacturing process (Schiestl et al. 2011) or lot-to-lot variability in raw materials (Gilbert et al. 2014).

Biosimilars are follow-on therapeutic protein products which aim to deliver equivalent clinical effect as the respective originator product; however, due to their lower costs they offer economic benefits for healthcare systems and patients. The term “biosimilar” has been introduced by the European Medicines Agency (EMA) based on the recognition that

biological products can be only similar to the reference product, but not the same, due to the high complexity of the protein structure (EMA 2015). However, market approval of biosimilars is under strict regulatory oversight. For example, the EMA has issued numerous general as well as product specific guidelines in order to define the requirements towards biosimilar applicants for the demonstration of similarity with the originator product. The cornerstone of similarity is a range of comparability studies at the level of structural biochemical properties, in other words the comparison of product quality attributes (Declerck 2013). Accordingly, the target quality attribute ranges of biosimilar products are ultimately determined by the characteristics of the originator product (Figure 1).

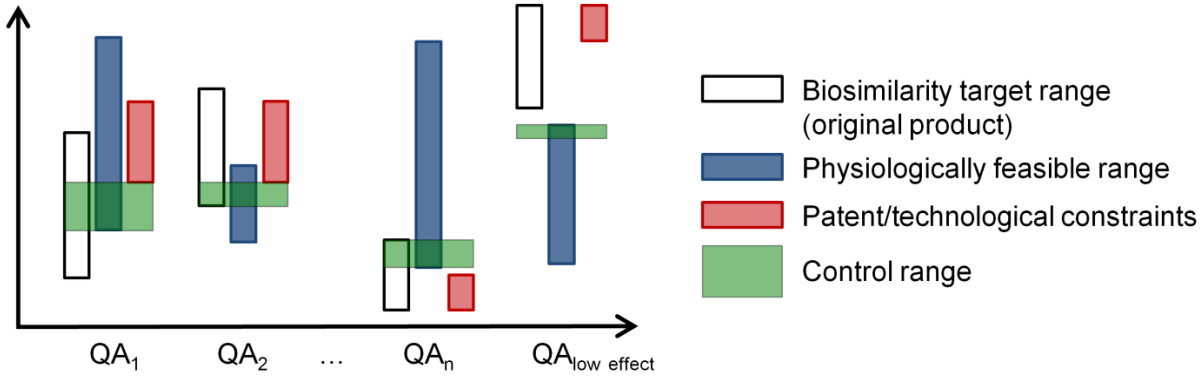

Figure 1. The challenge of targeting quality attribute ranges in biosimilar process development. Quality attributes (QA_{1-n}) with a relevant impact on clinical performance have to be strictly controlled within the biosimilarity target range. In contrast, for quality attributes (QA) with low effect on clinical performance and comparability ($QA_{low\ effect}$), a control range outside the original product's range can be also defined.

Meeting predefined quality attribute ranges with technological control strategies is one of the biggest challenges of biosimilar development. On the one hand, the physiological characteristics of the production clone determine which range is feasible at all. On the other hand, technological constraints such as scalability or more often upcoming patent issues can also narrow down the operational flexibility to target the range of biosimilarity with quality attributes. This is especially true for biosimilar monoclonal antibody (mAb) process development, where the rapidly increasing number of technological patents constrain the opportunities to adjust quality attributes to a high extent. Moreover, mAbs have numerous quality attributes due to their complex structure and post-translational modifications (Declerck 2013). The development of control strategies which steer product quality attributes into the predefined tight ranges is therefore essential for successful biosimiar mAb development. This aim is in accordance with the Quality by Design paradigm which calls for the development of control strategies based on sound science to ensure product consistency in terms of quality.

Quality by Design

QbD is defined in the International Conference on Harmonisation (ICH) Q8 guideline as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" (ICH 2009). In the context of QbD, process understanding can be interpreted as the structured scientific knowledge on how critical process parameters (CPPs) affect critical quality attributes (CQAs) in the manufacturing technology. For the pharmaceutical industry, the generation of process understanding has been one of the most important anticipated benefits of QbD (Cook et al. 2014). As discussed above, this knowledge is essential for biosimilar production, in order to keep product quality attributes in the predefined tight CQA ranges (Figure 2). Thus, the ultimate goal of QbD, which is to ensure product quality by the manufacturing process itself and not only by final product testing (Rathore and Winkle 2009), is an enabler of successful biosimilar process development. Control strategies which deliver consistent process output, and thus reduce the number of failed batches, are necessary to keep production costs low and ensure profitability in this highly competitive market segment. Accordingly, the implementation of QbD into biosimilar development is an obvious business interest for the pharmaceutical industry (Kenett and Kenett 2008; Luciani et al. 2015).

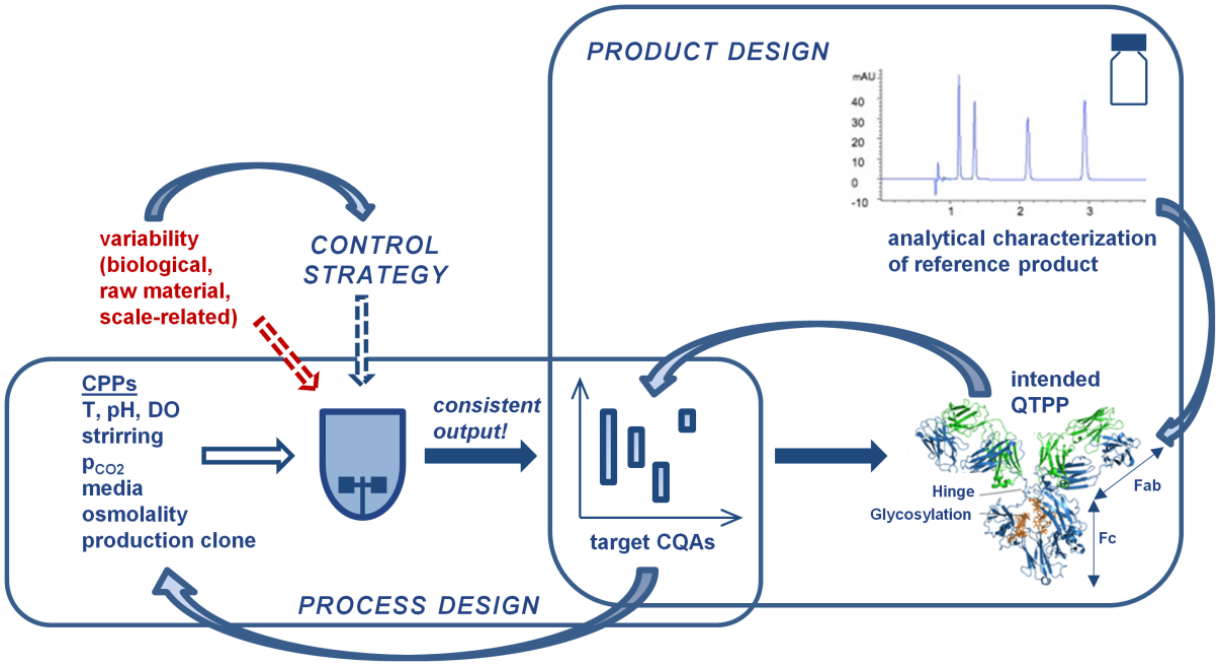

Figure 2. Quality by Design for biosimilars. Biosimilar process development starts with product design, which is the definition of the intended Quality Target Product Profile (QTPP) based on the extensive characterization of the reference product. This goal has to be kept considered throughout the selection of CQAs as well as the identification of CPPs (process design). For more details see text.

Process design to target CQAs

As discussed previously, process understanding starts with the characterization of CPP-CQA interactions. However, this task represents a scientific challenge in biopharmaceutical technology development due to the complexity of the process, the production host and the product itself. The high number of controlled and non-controlled process parameters necessitates laborious experimentation to identify the relationships between CPPs and CQAs. Hence, the anticipated extent of process understanding for QbD is usually constrained to the necessary minimum. First, the most critical process parameters are selected with risk management approaches. Thereafter, statistical tools, such as design of experiments (DoE) and multivariate data analysis (MVDA), are applied to define a multidimensional combination of CPPs (also referred to as the design space) within which the process delivers the product in the targeted CQA ranges (Abu-Absi et al. 2010; Rouiller et al. 2012). Although these approaches facilitate the characterization of CPP-CQA interactions, the high number of indirect or unidentified influencing factors renders the sound understanding of these interactions solely based on statistical considerations impossible.

Due to their biological nature, recombinant expression systems (i.e. the production cells) add an inherent variability to the production process, thereby influencing the characteristics of CPP-CQA interactions. An unforeseen biological variability in the process can lead to significant changes in product quality attributes and - in the worst case - result in out-of-specification batches. In order to develop control strategies, which are able to manage such variances, the behavior of the production cells in the manufacturing process has to be extensively characterized. For this, process understanding has to go beyond verifying CPP-CQA links with statistical approaches, and investigate these interactions on the level of cell physiology. Understanding cell physiology enables the identification of mechanistic links between process parameters, cell physiology and product quality. This knowledge is referred to as enhanced process understanding in this thesis (Figure 3).

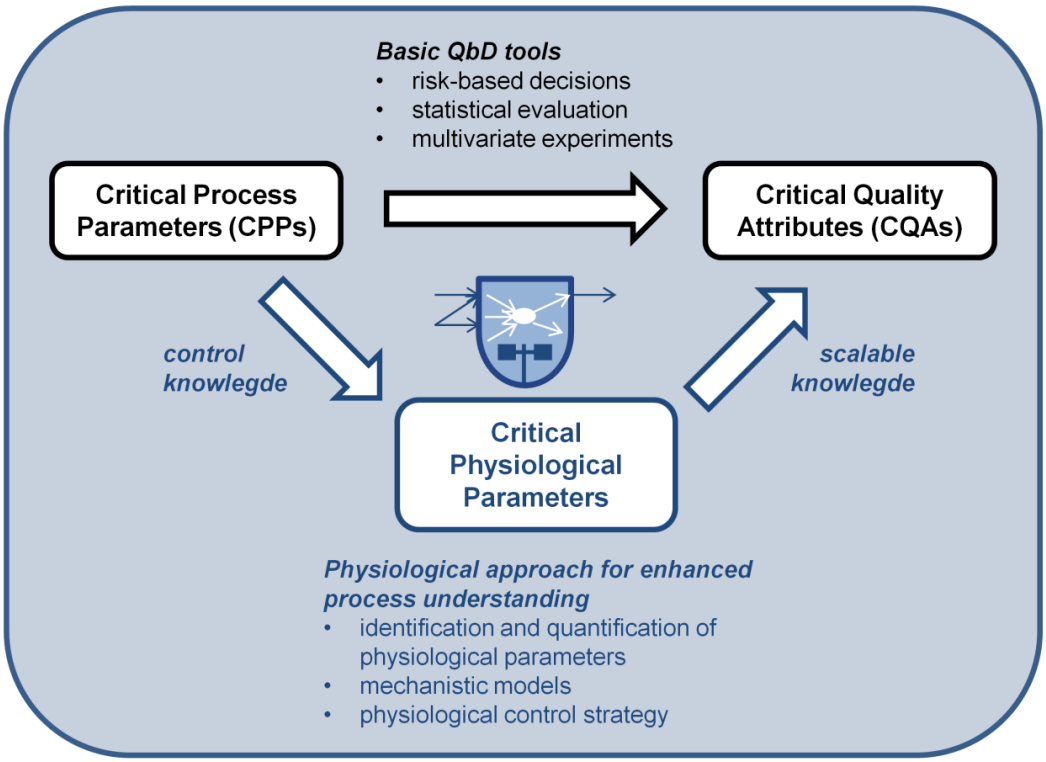

Figure 3. Basic QbD tools for process design and the physiological way through the investigation and control of physiological parameters [2].

Recent advancements in systems biology enable the investigation of different features of cell physiology (e.g. metabolome, transcriptome and proteome), which together define the physiological phenotype of the production cell (Dickson 2014; Heffner et al. 2014; Vishwanathan et al. 2014). It is already a well-accepted paradigm that the relationship between process input (CPPs) and process output (CQAs) parameters is ultimately determined by intracellular mechanisms occurring at multiple layers of cell physiology (Carinhas et al. 2012). In order to generate and to benefit from physiological understanding, two types of knowledge have to be gathered (Figure 3). First, the effect of process parameters on cell physiology has to be investigated. The goal is to gather a sound understanding of how technological control parameters and process variables affect physiological features such as protein expression, metabolic fluxes or the energetic status of the cells. A time-resolved investigation of these interactions is essential to understand the background of shifts in cell physiology during the production process. This knowledge can be subsequently used to steer cell physiology in the favored direction, for example by process parameter shifting or the addition of dedicated substances. The second type of knowledge to be gathered is the understanding how the physiological status of the cells affects the quality attributes of the product. All intracellular mechanisms which play a role in mRNA transcription, maturation and translation as well as in the post-translational modification and secretion of proteins might affect the quality of the recombinant product. Moreover, these mechanisms are

dependent on other, even more complex physiological features (e.g. cell metabolism or redox homeostasis). As interactions between cell physiology and product quality are determined on the cellular level, they are independent of the production scale and thus the knowledge on these interactions is scalable.

A considerable amount of studies is available in the scientific literature on how process parameters affect cell physiology. However, the link of product quality attributes to the cell's physiological status is still rare. In order to demonstrate the benefit of the physiological approach proposed in Figure 3, two steps are necessary. First, physiological variables should be identified which affect Critical Quality Attributes. Thereafter, the identified physiological variables ("Critical Physiological Variables") should be controlled at different levels in order to investigate their effect on CQAs.

Thesis scope: A quality by design approach for enhanced process understanding

The studies summarized in this thesis aim to demonstrate the implementation of QbD tools in process design for biosimilar production with the ultimate goal to adjust CQAs. Thereby, the focus is on the generation of enhanced process understanding via the investigation of cell physiology, and the development of science-based CQA control strategies as promoted by the QbD paradigm. The manuscripts included in this thesis are related to the different steps towards the implementation of the proposed physiological approach (Figure 4).

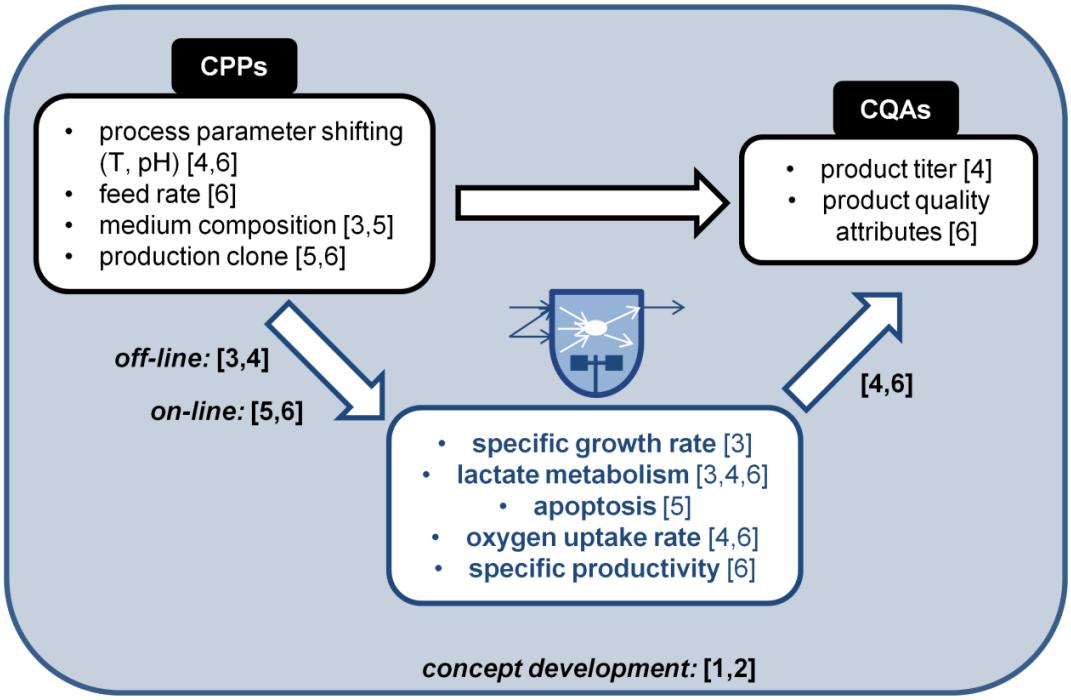

Figure 4. Critical Process Parameters, Critical Physiological Parameters and Critical Quality Attributes investigated in this thesis. Citations in square brackets refer to the manuscripts included in the thesis.

The first two manuscripts provide the conceptual basis for the proposed approach focusing on cell physiology as a key to enhanced process understanding. The very first manuscript is a methodological contribution which demonstrates how tailored risk management can accelerate process development for biosimilars [1]. Then, a review contribution summarizes the state of the art for the quantification and control of physiological variables in cell culture processes [2]. The subsequent manuscripts discuss experimental results generated in a total number of 46 cultivations in 10 different experimental setups, which demonstrate the implementation and benefits of the proposed physiological approach. CPPs identified in the initial risk assessment [1] were adjusted to different levels in order to investigate their effect on physiological features such as specific growth rate, cell respiration, the switch to lactate uptake and specific productivity. The links between process parameters and cell physiology were investigated using physiological variables calculated on the basis of off-line measurement results [3,4]. Moreover, the implementation of on-line monitoring tools for the assessment of physiological changes in manuscripts five and six enabled to investigate the effect of process parameters on cell physiology on-line [5,6]. The implementation of a feeding strategy based on the on-line monitored oxygen uptake rate (OUR) enabled to control specific productivity at multiple levels and to investigate the link between this physiological variable and product glycosylation [6]. Explaining the link between cell physiology and a product CQA, the last manuscript demonstrates the benefits of the proposed physiological approach for the development of novel CQA control strategies.

Quality by Design tools used within this thesis

As discussed in the previous sections, the application of QbD tools facilitate the generation of process understanding. Moreover, in order to achieve enhanced process understanding in biopharmaceutical processes, CPP-CQA interactions have to be investigated on a physiological level and not only on an empirical level.

This thesis can be considered as a case study which aims to combine standard QbD tools with the investigation of physiological variables in order to achieve enhanced process understanding (Table I).

Table I. QbD tools used in the different manuscripts of this thesis. The rows indicate the six manuscripts involved in the thesis with short titles and citation numbers in square brackets. Columns indicate QbD tools used in the different manuscripts. Blue fields indicate the tools which were applied in the respective manuscript.

	Risk management	**Process understanding via cell physiology**	**Process Analytical Technology**	**Multivariate experimental design**	**Control strategy**
Risk-based process development of biosimilars [1]	■				
Advanced development strategies review [2]		■	■	■	■
Process development along QbD principles [3]	■	■			
Combining mechanistic and data-driven approaches [4]		■		■	■
Impact of apoptosis on the on-line measured dielectric properties [5]		■	■		
Control of specific productivity to modify high mannose levels [6]		■	■	■	■

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Author Contributions

Contributions of Dénes Zalai in experimentation, data analysis and manuscript drafting are summarized in Table II.

Table II. Author contributions of Dénes Zalai (DZ) in the manuscripts cited in this thesis. The citation numbers in square brackets are used in the *Introduction* as well as the *Summary and Outlook* sections to cite the manuscripts.

Short title	Title	Contribution of Dénes Zalai (DZ)
Risk-based process development of biosimilars [1]	Risk-based Process Development of Biosimilars as Part of the Quality by Design Paradigm	DZ drafted the risk assessment methodology, gathered prior knowledge and drafted the manuscript
Advanced development strategies review [2]	Advanced Development Strategies for Biopharmaceutical Cell Culture Processes	DZ drafted the introduction and conclusion sections, conducted the literature research for the section "Media selection and optimization" and drafted the section
Process development along QbD principles [3]	Process Development Along QbD Principles: Characterizing the Effect of Process Parameters on Cell Physiology	DZ conducted the experiments, performed data analysis and drafted the manuscript
Combining mechanistic and data-driven approaches [4]	Combining Mechanistic and Data-Driven Approaches to Gain Process Knowledge on the Control of the Metabolic Shift to Lactate Uptake in a Fed-Batch CHO Process	DZ designed the experiments, conducted parts of the experiments and data analysis, and drafted the manuscript
Impact of apoptosis on the on-line measured dielectric properties [5]	Impact of Apoptosis on the On-Line Measured Dielectric Properties of CHO Cells	DZ performed parts of data analysis and drafted parts of the manuscript
Control of specific productivity to modify high mannose levels [6]	Control of Specific Productivity of CHO Cells to Modify the Abundance of High Mannose mAb Glycoforms	DZ constructed the control strategy, performed parts of the experiments, performed data analysis and drafted the manuscript

In the *Introduction* as well as the *Summary and Outlook* sections, the six manuscripts are indicated with the same numbers as in Table II. All other publications cited are indicated using a different citation format (Author name, year of publication) in these sections.

Risk-based Process Development of Biosimilars as Part of the Quality by Design Paradigm

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COMMENTARY

Risk-based Process Development of Biosimilars as Part of the Quality by Design Paradigm

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ABSTRACT: In the last few years, several quality by design (QbD) studies demonstrated the benefit of systematic approaches for biopharmaceutical development. However, only very few studies identified biosimilars as a special case of product development. The targeted quality profile of biosimilars is strictly defined by the originator's product characteristic. Moreover, the major source of prior knowledge is the experience with the originator product itself. Processing this information in biosimilar development has a major effect on risk management and process development strategies.
The main objective of this contribution is to demonstrate how risk management can facilitate the implementation of QbD in early-stage product development with special emphasis on fitting the reported approaches to biosimilars. Risk assessments were highlighted as important tools to integrate prior knowledge in biosimilar development. The risk assessment process as suggested by the International Conference on Harmonization (ICH Q9) was reviewed and three elements were identified to play a key role in targeted risk assessment approaches: proper understanding of target linkage, risk assessment tool compliance, and criticality threshold value. Adjusting these steps to biosimilar applications helped to address some unique challenges of these products such as a strictly defined quality profile or a lack of process knowledge. This contribution demonstrates the need for tailored risk management approaches for the risk-based development of biosimilars and provides novel tools for the integration of additional knowledge available for these products.

KEYWORDS: Biosimilars, Process development, Risk assessment, Quality by design

LAY ABSTRACT: The pharmaceutical industry is facing challenges such as profit loss and price competition. Companies are forced to rationalize business models and to cut costs in development as well as manufacturing. These trends recently hinder the implementation of any concepts that do not offer certain financial benefit or promise a long return of investment. *Quality by design* (QbD) is a concept that is currently struggling for more acceptance from the side of the pharmaceutical industry. To achieve this, the major goals of QbD have to be revisited and QbD tools have to be subsequently developed. This contribution offers an example as to how implement risk management in early-stage biosimilar development as part of the QbD concept. The main goal was to go beyond the conventional QbD workflow and to adjust risk management to the challenges of biosimilar products. Accordingly, instead of using methods like failure mode and effects analysis, recommendations of the ICH Q9 guideline were reviewed and put into practice by creating tailored risk assessment tools. The novelty of this contribution is to report those tailored tools ready-to-use for early bioprocess development of biosimilars along QbD principles.

Introduction

Biosimilars are a promising segment of the pharmaceutical market forecasted to reach $4–6 billion in sales by 2016 (1). It is generally recognized that biosimilar products offer equivalent medical treatment, both in efficacy and safety to the reference product, but at lower costs for patients and healthcare systems (2). Therefore, significant efforts have been

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made in the last years to create a supportive regulatory environment for the application process of these products (3–5). The requirement for biosimilar approval is an equivalent clinical performance and a high similar quality profile to the originator product (6, 7). The success of biosimilars depends on the ability to reach this target. Thus, risk-based approaches and enhanced product knowledge are key elements of their development (2). This progress is in accordance with the quality by design (QbD) paradigm, which promotes pharmaceutical development based on risk management and sound science (8).

The QbD workflow starts with the definition of the targeted clinical performance in the Quality Target Product Profile (QTPP) document (9), the basis of the manufacturing process to deliver a product that meets these quality specifications. Accordingly, QbD defines a systematic business process for pharmaceutical development where the major objective is product quality. Linking process understanding to product understanding and ultimately to the desired QTPP is therefore a cornerstone of QbD approaches (10, 11). These linkages are based on scientific knowledge and quantified through the assessment of criticality. *Criticality* measures the significance of certain variables in the investigated interaction. The assessment of criticality as it relates to product quality is determined as a function of risk (12). Consequently, risk management plays a key role in QbD by creating a platform for linking subsequent stages in pharmaceutical development.

QbD applications have been reported many times for biopharmaceuticals (9, 13, 14); however, these publications focused more on scientific understanding and did not detail the risk management process. Although the A-Mab case study demonstrated the risk management process more in detail, the study originated a significant amount of information from platform knowledge of xyz monoclonal antibodies (mAbs) (15). A major difference between biosimilars and original biopharmaceuticals is that biosimilars are designed to provide alternatives to originator products after patent expiration. Biosimilar development is therefore based on the characteristics of the originator product and relies on existing prior experience with the same drug substance and product. Risk management has to integrate this specific piece of information in decisions throughout the development process. However, to our knowledge, risk management approaches for biosimilars have not been reported until now in the scientific literature.

The present contribution reports the implementation of risk management as part of the QbD workflow in early-stage biosimilar development. Risk assessments were identified as logical linkages between product development stages and as tools to integrate prior existing product knowledge. The risk assessment process as defined by the International Conference on Harmonization for Quality Risk Management (ICH Q9) was reviewed and three elements of the risk assessment process were identified to have a major impact on its outcome. These elements were investigated in detail with special emphasis on their application in biosimilar development. This study proposes novel risk assessment tools adapted to early-stage biosimilar process development to address the unique challenges of these products.

Positioning Risk Assessments in the QbD Workflow

Biopharmaceutical product development is a multistage process that involves various activities from molecule design to process engineering. QbD connects these activities by creating a structured workflow based on risk management. According to the ICH Q9 guidance, a basic principle of quality risk management is that the assessment of risk to quality should always target the protection of the patient (16). In this respect, risk assessments connect consecutive stages of product development to each other and ultimately to the desired clinical performance recorded in the QTPP document.

Figure 1 traces a QbD workflow for early-stage product development. The first step is to select targeted indications, intended clinical performance, and dosage forms. These decisions can be performed with the help of a business risk assessment, but beyond the scope of this article. All details are summarized in the QTPP, which assures a platform to synchronize the development process with the proposed product characteristics. This document should contain all relevant information on the intended quality profile and has to be updated regularly throughout the lifecycle of the product.

The connection of product development and process development to the QTPP is an important part of the QbD paradigm. In this logical flow chart, linkages (diamonds on Figure 1) are the risk assessments, where the decisions on criticality with respect to prod-

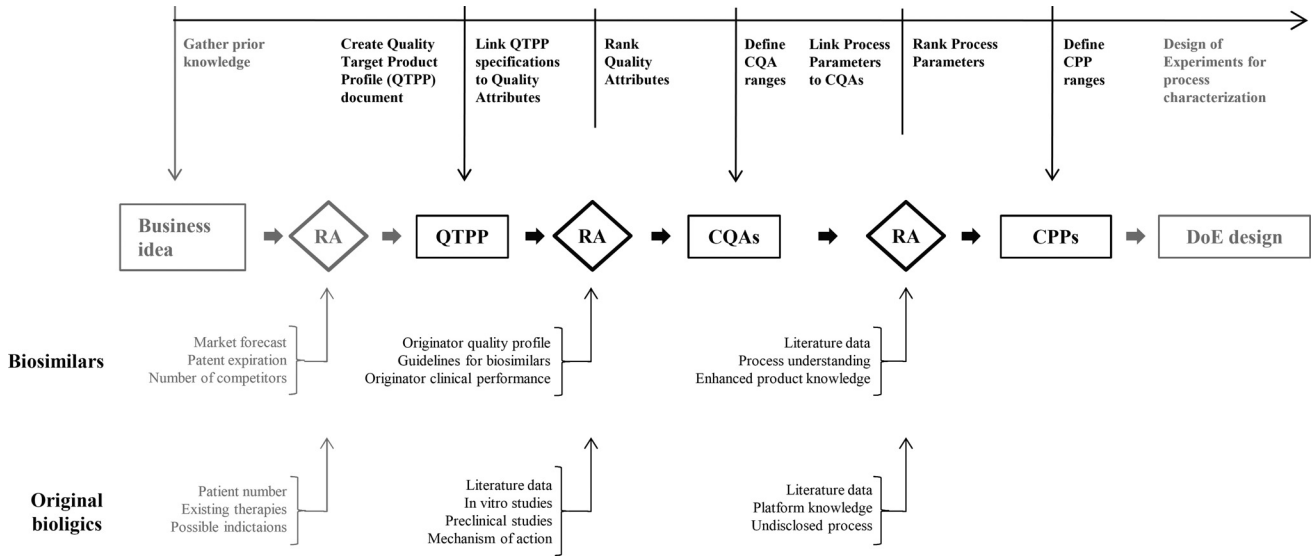

Figure 1

Differences in the source of information throughout product development of biosimilars and original biologics. Diamonds represent decision points where risk assessments are performed to select critical variables for the next step of product development. Those steps of product development that are not detailed in this contribution are colored grey.

uct quality are met. Finally, these decisions have a major impact on the experimental design of process development.

Although the above discussed workflow for product development is valid for both biosimilars and original biologics, a major difference is the source of information processed in risk assessments (Figure 1). Regarding product quality, strict specifications based on the originator's quality profile are defined for biosimilars, which have to be met accordingly. As the production process of the originator product is undisclosed, no process development and manufacturing knowledge is available for follow-on biologics producers. These differences should be considered in order to fit risk management to the purposes of biosimilar process development. The risk assessment process as described in ICH Q9 should be reviewed and developed accordingly.

The Risk Assessment Process

The most common operative manifestation of risk management in pharmaceutical development is risk assessment. Although ICH Q9 supports the selection of risk assessment methods on a case-by-case basis, it recommends a general model (Figure 2) as part of the quality risk management concept (16).

The three-step model initiates risk assessment with the identification of risk by understanding the linkage between the analyzed steps of product development, for example, between critical quality attribute (CQA) and critical process parameter (CPP) selection. In order to visualize the logical linkage and to sort all attributes by categories for the risk analysis, decision tools such as an Ishikawa diagram can be used. Afterwards, the *risk question*—a clearly formulated sentence referring to the goal of the risk assessment—is defined. Risk question formulation supports the process to obtain an agreement on the purpose of the risk assessment. The progress of risk question formulation might be a challenging task due to the complex nature of biologics and the diverse concerns of interdisciplinary risk assessment teams.

The second step focuses on the assessment of criticality with the help of risk analysis tools. According to the ICH Q9 guidance, risk is always defined as a function of the severity of potential harm. Consequently, a risk assessment tool always contains a factor that represents this severity. Additional factors can be chosen to optimize the tool for the exact purpose (13). After defining the score ranges for each factor, the maximal risk score can be calculated and subsequently the criticality threshold can be set. Although the threshold value has a great influence on the out-

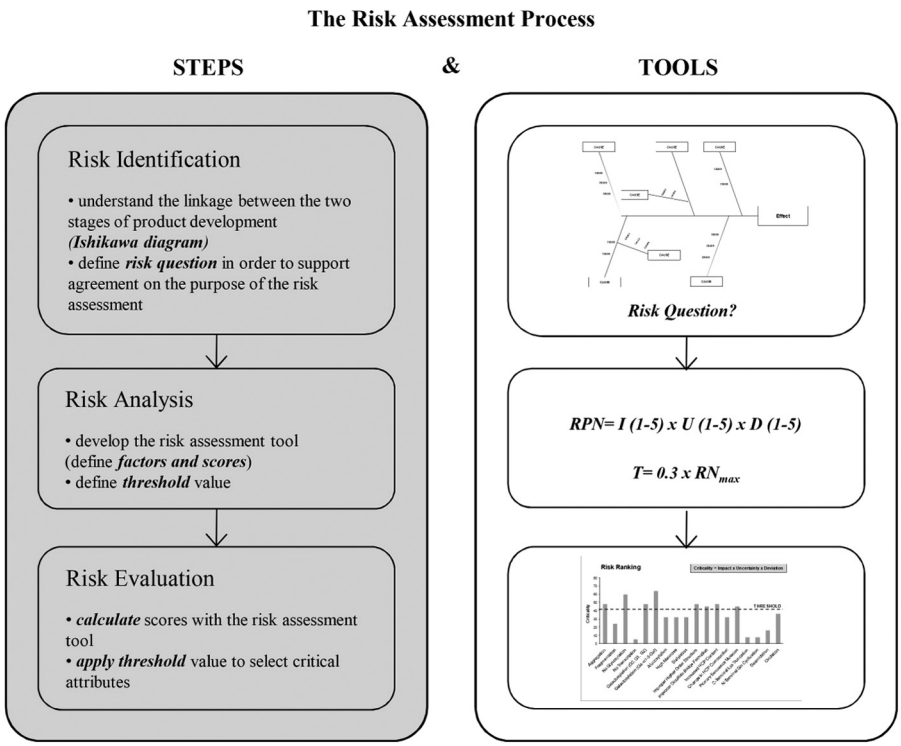

Figure 2

The general risk assessment process. The box on the left traces the three steps of risk assessment as described by ICH Q9, whereas the box on the right represents essential tools supporting the risk assessment process.

come of the risk assessment, there is no best practice reported for defining its value.

The last step of risk assessments is the calculation of risk scores and the subsequent ranking of the attributes. Finally, attributes receiving higher risk scores as the criticality threshold are designated as critical.

Eventually, the outcome of the risk assessment is dependent on the following elements of the process:

1. Risk question definition
2. Tailored risk assessment tool
3. Criticality threshold definition

However, even very sophisticated risk assessment tools can not extract valuable information from bad-quality input data (17). In other words, risk assessment is a decision tool to identify criticality and does not replace the need for scientific knowledge. Accordingly, lack of information in early-stage process development was reported to be a major obstacle of risk assessment approaches (13). However, the growing experience of the pharmaceutical industry for the production of biologics will serve as a robust input for such purposes in the future. In order to integrate this knowledge in biopharmaceutical development, tailored risk assessment approaches are needed that are able to process prior knowledge in risk decisions. Therefore, the three critical elements of risk assessment will be analyzed in the following sections as part of the early-stage process development of a biosimilar mAb.

1. Risk Question Definition and Target Linkages

In the QbD paradigm, risk assessments create junctions between successive stages of product development. Thereby, understanding of linkages between these stages is initiated by a suitable risk assessment approach. First, visual and interactive tools can be used to organize data in accordance with the recommendations of the ICH Q9 guideline (16). The type of the visual tool can be selected based on the nature of the applied risk assessment technique. In early-stage process development, risk assessment is a deductive

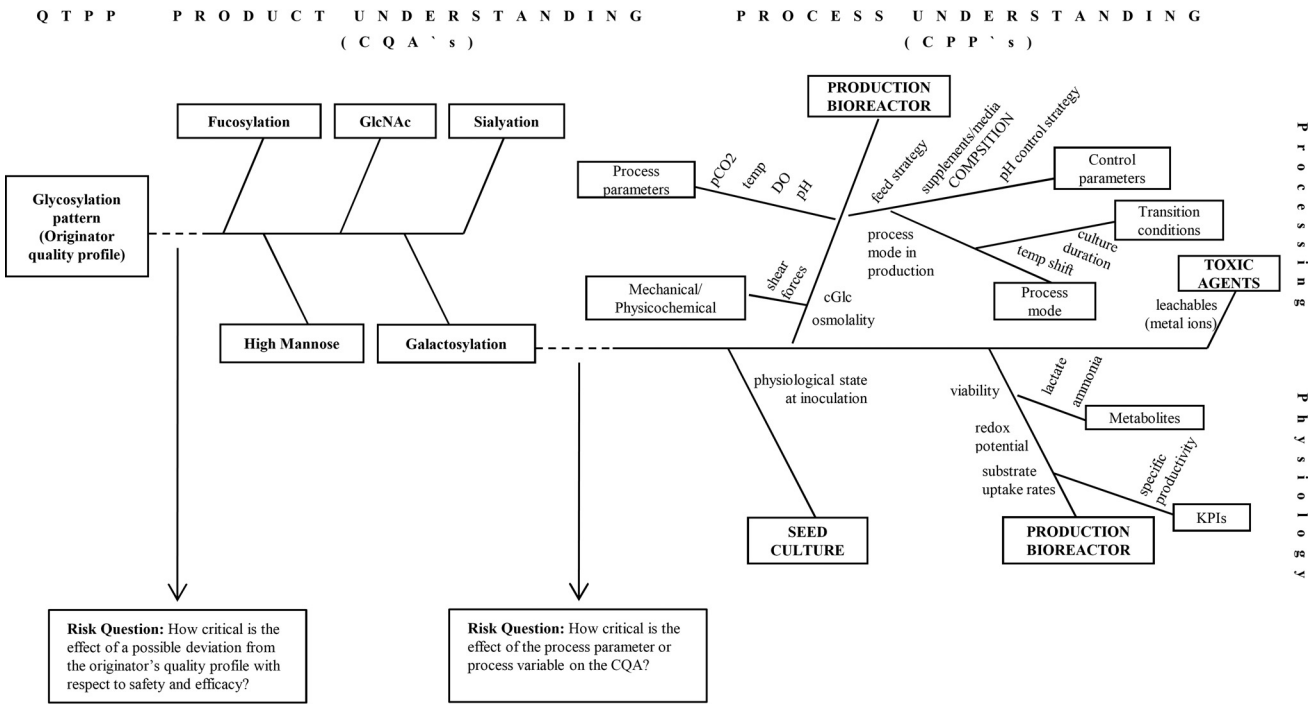

Figure 3

Multi-level Ishikawa diagram depicting the connection between process parameters, critical quality attributes, and the targeted quality profile. Dataset of a selected QTPP specification (glycosylation pattern) and a selected CQA (galactosylation) is shown as an example. Dashed lines represent the logical linkage between consecutive stages of process development, condensed in Risk Questions.

problem, answering the question: "What can cause a failure in the clinical performance of the product?". In order to trace back this problem to possible causes, visual tools such as Ishikawa diagrams are appropriate. In contrast, when the risk assessment addresses inductive problems answering the question "What can go wrong in the production process?", other types of visual tools such as flow charts or process mapping might be more suitable.

Ishikawa diagrams help to identify logical connections between different types of product- or process-related attributes and thus support a better understanding of linkages between clinical performance, product, and process. This enhanced understanding can be then condensed into risk questions in order to support the identification of critical attributes via targeted risk assessments. Although the formulation of risk questions has not yet been a routine task in risk assessments, their use can facilitate the development of tailored risk assessment tools. In this contribution, risk questions are used to tighten the focus of risk assessments by enhancing the logical background of the linkages (Figure 3). A well-defined risk question already denominates some factors of the risk assessment tool. For example, the risk question for CQA selection contains the word "deviation", which is also the third factor in the CQA risk assessment tool.

After the definition of CQAs, process parameters with a possible effect on these quality attributes are collected. Another benefit of Ishikawa diagrams is the possibility to structure the high number of parameters that influence cell culture performance. In Figure 3, process variables are divided into two groups and several subgroups in order to classify potential CPPs. This structure supported the understanding of the mechanism of action as to how different variables might affect product quality and served as a road map for the assessment of criticality. Accordingly, process variables were divided into two main groups: Processing and Physiology. The latter group was defined after the identification of cell physiology as a complex variable with major effect on product quality in cell culture processes. Although the complex physiology of mammalian cells (e.g., Chinese hamster ovary, CHO) has been investigated in many studies (18–20), there is little known about how

this information can be coupled to product quality (10). Therefore, physiological parameters such as specific rates have to be defined that support the extraction of scalable and science-based information on physiology–product quality interactions in bioprocess development (21). The identification of such physiological parameters in early-stage development has to be facilitated by structured risk assessment approaches.

Ishikawa diagrams and risk questions support the proper understanding of linkages targeted by risk assessments. With the help of these tools, interdisciplinary team members can come to an agreement on the scope of risk assessments. Due to high complexity of targeted bioprocesses, the approach depicts a promising tool for more efficient process development, especially in its early stages.

2. Development of Tailored Risk Assessment Tools

Risk assessment tools convert subject knowledge into quantitative information in order to assess criticality. The outcome is the *risk number* (RN), which is calculated by multiplying two or more factors. Although the ICH Q9 guideline lists a variety of risk assessment tools, it does not provide a clear definition as how to select the most appropriate one for the specific purpose. Some studies have reported the use of these tools during biopharmaceutical product development (13, 22), but they did not provide extensive information on the reasons for selection. As discussed already above, the factor "Severity" is always included in the risk assessment tool to express the potential harm on pharmaceutical quality as the basis for the determination of criticality. Additional factors are used to improve the risk assessment tool by breaking up the risk into multiple components. For example, "Uncertainty" is often used as a second factor beside "Severity" to include the quality of input data as a possible source of risk (15). This is especially relevant in early-stage process development, where scientific knowledge is often lacking to fully understand the linkage between product- and process-related parameters. The two factors "Severity" and "Uncertainty" were included in both CQA and CPP risk assessment tools within this study.

If additional information is available that can increase the selectivity of the risk assessment, the tool has to be appended in order to process all the information at hand. An example is the original product's quality profile for biosimilars. Biosimilar guidelines in the European Union and United States put emphasis on analytical comparability with the original product (23). Consequently, the quality profile of biosimilars is highly determined by the originator product. In order to involve this additional information in biosimilar development, a third factor called "Deviation" was added to the here-described risk assessment tool for CQA selection (Table I). This factor incorporates the extent of acceptable deviations from the originator product's quality profile. Quality attributes with minor importance would receive a low "Deviation" score, indicating a higher acceptable deviation. Thus, this factor helps to prioritize the quality attributes for product development based on their effect on biosimilarity. However, as communicated by regulatory bodies, the effect of a deviation from the originator in attributes with low relevance has to be justified as well in biological assays. The factor "Deviation" can also contain information about the purification capacity of downstream process steps if the risk assessment is conducted for the determination of CQAs in upstream process development (see Table I).

Another example to incorporate additional information into the risk assessment in this study was, considering the complexity of mechanisms, how process parameters can affect the investigated quality attributes. Accordingly, beside the factors "Severity" and "Uncertainty", a third factor called "Complexity" was added to the risk assessment tool of CPP selection. This factor quantifies as to which extent the mechanism of the CPP-CQA interaction can be described by a scientifically developed formula (Table I). The higher the score, the more complex the mechanism and the less information is available on its quantification. As the lack of reliable information on CPP-CQA interactions raise the "Uncertainty" score of almost each process parameters and variables in early-stage process development, including "Complexity" as a third factor helped to differentiate CPP candidates based on scientific considerations. Introducing this factor also emphasizes the scope of CPP risk assessment at this stage of process development, which is not to select critical parameters for a finalized manufacturing process but rather to rank parameters in order to prioritize experiments for process development. These considerations justify the development of novel tools as described above instead of failure mode and effects analysis (FMEA), which is commonly used to assess the criticality of process parameters in manufacturing processes (22).

Table I
Overview of the Risk Assessment Tools for CQA and CPP Selection

	CQA Risk Assessment	CPP Risk Assessment
Linkage	CQAs—QTPP specification	CPPs—CQA ranges
Risk Question	How critical is the effect of a possible deviation from the innovator's quality profile with respect to safety and efficacy?	How critical is the effect of the process parameter or process variable on CQAs?
RA tool	RN = Severity × Uncertainty × Deviation	RN= Severity × Uncertainty × Complexity
Scores for the third factor	**Deviation** (from the quality profile of the reference material) 1 no deviation in the quality profile 2 low deviation in the quality profile or robust purification method 3 deviation, limited purification efficiency 4 severe deviation in the quality profile limited purification efficiency 5 severe deviation in the quality profile, variant cannot be purified	**Complexity** (of the mechanism responsible for the CPP-CQA effect) 1 mechanism described by a physical law 2 simple mechanism with well-known characteristics 3 complex mechanism with previously reported quantitative interactions 4 complex mechanism without quantified characteristics 5 very complex mechanism

In order to demonstrate the effect of factor selection on the result of risk assessment, the ratio of critical attributes was calculated for the case of two or three factors. As shown in Table II, the addition of a third factor decreased the ratio both in CQA and in CPP risk assessment. This observation suggests that the integration of additional knowledge with an appropriate factor in the risk assessment tool leads to the reduction of critical attributes and hence simplifies experimental design for early process development. The lower number of critical attributes does not mean higher risk acceptance, but rather risk reduction by enhanced integration of prior knowledge into product development via efficient risk assessment tools.

Table II
Decreased Number of Critical Attributes with Risk Assessment Tools Using Three Factors. The values represent the percentage of critical attributes with respect to all attributes involved in the risk assessment. Threshold values for the selection of criticality were defined based on the rule of $T = 1/n \cdot RN_{max}$, where n is the number of factors.

	CQA Risk Assessment	CPP Risk Assessment
2 factors	67%	53%
3 factors	50%	39%

3. Threshold Definition for Risk Assessment

The criticality threshold expresses the level of risk that is accepted for a product or a process. As already stated above, its value has a great influence on the outcome of the risk assessment. However, only a few publications discuss the rationale behind threshold selection. The A-Mab case study considers risk rather as a continuum and consequently does not define a single threshold value but rather ranges for different categories of criticality (15). At the end, this approach also results in a single threshold value, which is the lower end of the category still defined as critical. Another study selects criticality threshold by consid-

Table III
Critical Attribute Selection Using Different Threshold Calculation Formula. Values represent the percentage of critical attributes with respect to all attributes. Criticality was assessed with tools using 2 or 3 factors as described above. RN_{max} represents the maximal value of the risk score (all factors at maximum score). Values in bold represent criticality corresponding to the $T = 1/n \cdot RN_{max}$ rule.

Threshold calculation formula	CQA Risk Assessment		CPP Risk Assessment	
	2 factors	3 factors	2 factors	3 factors
$1/5 \cdot RN_{max}$	89 %	78 %	99 %	72 %
$1/3 \cdot RN_{max}$	89 %	**50 %**	81 %	**39 %**
$1/2 \cdot RN_{max}$	**67 %**	11 %	**53 %**	6 %

ering the cardinality of factors which have scores above the minimum value (22). Finally, the threshold value is always determined based on the acceptable level of risk (24). In the literature, threshold values around $0.2 \cdot RN_{max}$ are commonly reported, resulting in an average of 70% of critical attributes (13, 15, 22). However, applying a static formula for criticality threshold calculation leads to variable outcomes when the number of factors is changed in the risk assessment tool. Thus, general formulas should be developed in order to deliver comparable results when adjustments in the risk assessment tool are made. Such a formula is $1/n \cdot RN_{max}$, which calculates the criticality threshold based on the number of factors (n) involved in the risk assessment tool.

In order to investigate the effect of threshold selection on the outcome of risk assessment (i.e., the number of attributes considered as critical), we applied different threshold values based on mathematical rationale (Table III).

The final goal of criticality threshold selection in early-stage process development is to identify factors for the design of experiments along what is executable in the very strict development timeline of biosimilars. In this respect, the number of critical parameters is an important indicator of factor selection because it has a major effect on the experimental design. Table III confirms the effect of threshold selection on the number of critical attributes. Moreover, it also demonstrates the need for threshold selection strategies that take the number of factors applied in the risk assessment tool into account. Accordingly, the results suggest that $1/n \cdot RN_{max}$ is an appropriate formula for threshold calculation.

Iteration of Risk Assessment

Risk assessments deliver a rank of attributes based on the calculated risk number. Moreover, attributes with a higher risk number than a predefined threshold are designated as critical. Thus, the final outcome of the risk assessment is the rank and the number of critical attributes. Based on these results, the most critical tasks for process development are prioritized and the first process characterization studies are designed. The knowledge gained in these experiments can result in increased process understanding and a subsequent variation in the criticality of some attributes. Therefore the risk assessment can be revised to integrate the obtained knowledge in process development decisions (see Figure 4).

Iteration of risk assessments as part of the QbD workflow has been already reported in the A-Mab study (15). The basis for the revision was the increased scientific knowledge gained in process characterization studies, which resulted in the subsequent reduction of some attributes' criticality. However, other reasons can also enforce the re-evaluation of risk assessment results, for instance, an unexpected high number of critical parameters assessed. By increasing the threshold value, the number of critical parameters can be reduced, resulting in a subsequent simplification of the experimental design for process development (25). Another reason for threshold modification is, for example, that important attributes are designated as noncritical by the risk assessment process. This indicates an initially too high defined threshold value, and a subsequent reduction is necessary.

The use of inappropriate factors in the risk assessment tool can lead to an unexpected rank of the attributes. In such cases, the risk assessment tool itself has to be revised and factors modified. Decision points and reasons for risk assessment iteration are visualized in Figure 4.

Although the outcome of risk assessments can be modified by iterative processes shown on Figure 4, the predefined goal has to be considered in order to avoid missing the aim of the risk assessment.In other words, revisions and subsequent adjustments in the threshold value or the risk assess-

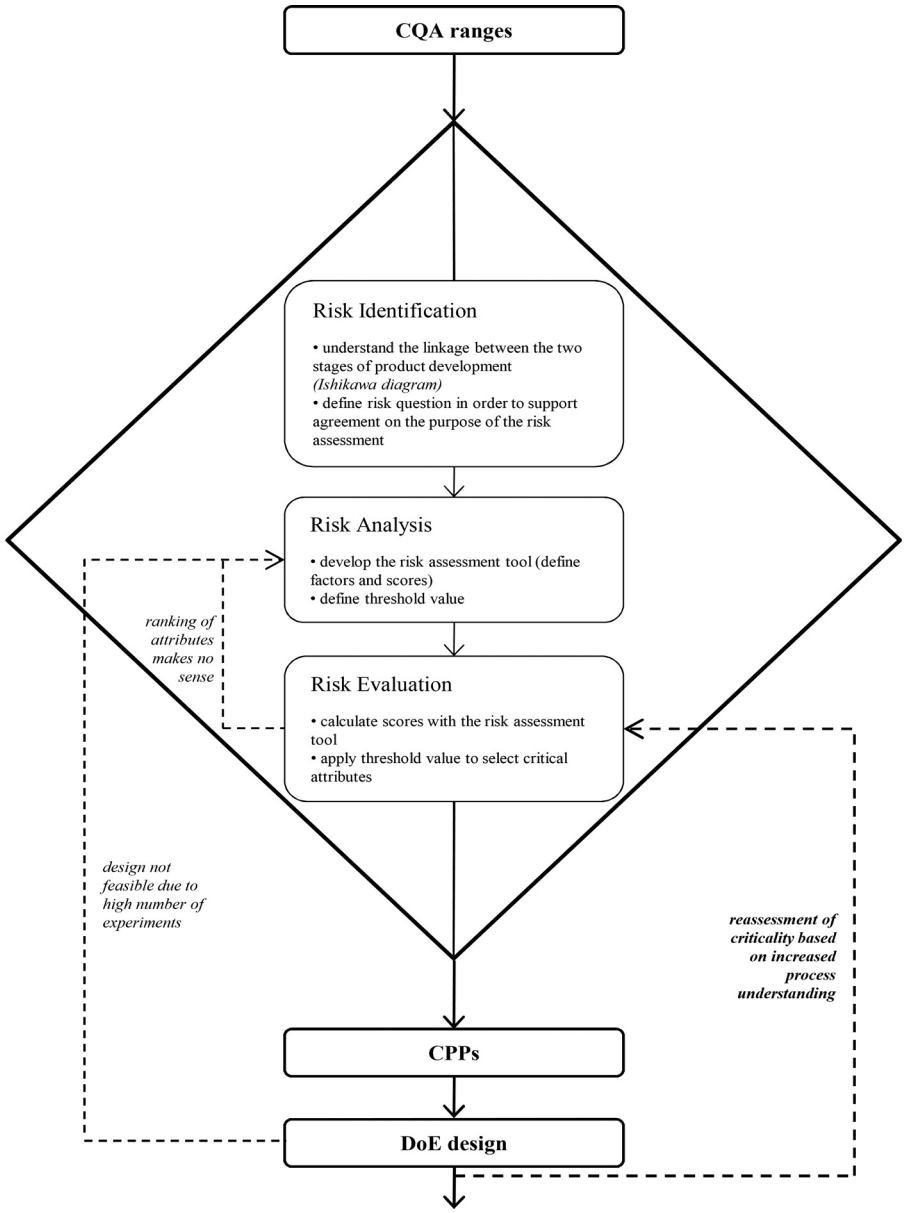

Figure 4

Iteration processes in CPP risk assessment. Dashed lines represent possible iteration reasons and paths.

ment tool should not compromise patient safety for the simplification of product development.

Another interesting issue in risk management is the handling of noncritical data. Variables with a lower score than the risk threshold are designated as noncritical and do not have to be included further in description of design space (12). However, the assessment of criticality is not static and might change with the increase of product and process understanding. Consequently, noncritical variables can become critical throughout product development. Therefore, handling of noncritical data is a major question of QbD approaches. Well-developed risk assessment tools enable a more accurate determination of criticality and reduce the possibility of underestimated risk. Moreover, scheduled iterations in the QbD workflow support the revision of previously taken incorrect decisions. All these conclusions highlight the key role of risk management in early-stage biosimilar process development.

Conclusions

QbD has been gaining an increasing acceptance from the pharmaceutical industry as well as from regulatory

authorities. The number of QbD drug submissions has been steadily increasing for small molecules, and the first QbD submissions have already been reported for biologics as well. One of the key elements on which the authorities focus on during the review process of QbD submissions is how the connection between defining QTPP, identifying CQAs, and selecting CPPs is established. The use of risk-based methods is anticipated to create these connections; however, the approaches are influenced by the characteristics of the product and the amount of existing prior knowledge.

The recent commentary discussed the application of risk management as part of the QbD paradigm for biosimilars. A QbD workflow for early-stage product development was traced to define a path from QTPP to the first set of CPPs based on scientific considerations and risk management principles. Thereby, major differences between the process development of biosimilars and original biologic approaches were identified:

- A key difference in the development of biosimilars is the source of prior knowledge. Both the targeted quality profile and the available clinical experience are determined by the original product. Risk assessments were proposed as a tool to integrate this additional information into biosimilar development. Consequently, risk assessments not only assure the logical integrity of risk-based QbD approaches but also help to process prior knowledge for biosimilars.

- Tailored risk assessment approaches are needed to process additional information in the risk-based development of biosimilars. Therefore, the risk assessment process as suggested by ICH Q9 was reviewed and three elements were identified to play a key role in targeted risk assessment approaches: proper understanding of target linkage, risk assessment tool compliance, and criticality threshold value.

- Increased process understanding is an important cornerstone of the QbD paradigm. The novel approaches reported in this study for CPP risk assessment (clustering of process parameters in processing and physiology, inclusion of the factor "Complexity" in the risk assessment tool) are an important step towards this paradigm change.

The integration of these findings into biosimilar applications via tailored risk management approaches triggers the ultimate goal of pharmaceutical development, which is product quality. As essential novelty of this contribution, those tailored tools and the proposed workflow, can now be used generically for early bioprocess development of biosimilars along QbD principles. The integration of similar risk-based approaches into QbD submissions is expected to be encouraged by the authorities, as indicated by the ICH Q10 guideline and QbD pilot programs. The advantage for the industry is not supposed to be any reduction of regulatory requirements but rather opportunities for more flexible approaches to meet these requirements. Over the long term, this flexibility can lead to the promised benefits of QbD such as reduction of development costs and time. These conclusions confirm risk management as an important element of the implementation of QbD principles for biosimilar development in the future.

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Conflict of Interest Declaration

The authors declare that they have no competing interests.

List of Abbreviations

CHO Chinese Hamster Ovary

CPP Critical Process Parameter

CQA Critical Quality Attribute

FMEA Failure Mode and Effects Analysis

ICH International Conference on Harmonization

mAb Monoclonal Antibody

QbD Quality by Design

QTPP Quality Target Product Profile

RN Risk Number

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Advanced Development Strategies for Biopharmaceutical Cell Culture Processes

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Advanced Development Strategies for Biopharmaceutical Cell Culture Processes

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Abstract: The shift from empirical to science-based process development is considered to be a key factor to increase bioprocess performance and to reduce time to market for biopharmaceutical products in the near future. In the last decade, expanding knowledge in systems biology and bioprocess technology has delivered the foundation of the scientific understanding of relationships between process input parameters and process output features. Based on this knowledge, advanced process development approaches can be applied to maximize process performance and to generate process understanding. This review focuses on tools which enable the integration of physiological knowledge into cell culture process development. As a structured approach, the availability and the proposed benefit of the application of these tools are discussed for the subsequent stages of process development. The ultimate aim is to deliver a comprehensive overview of the current role of physiological understanding during cell culture process development from clone selection to the scale-up of advanced control strategies for ensuring process robustness.

Keywords: Biopharmaceuticals, cell physiology, mammalian cell culture, Process Analytical Technology, process development, Quality by Design.

INTRODUCTION

Biopharmaceuticals have been a major contributor of growth in the pharmaceutical industry during the last decade. Thereby, recombinant protein therapeutics produced in cell culture processes have gained particular importance due to the growing portfolio and sales volume [1]. The economic success has triggered a scientific revolution to increase systems biology and process technology knowledge of mammalian production systems. The swift scientific progress has been resumed in excellent reviews covering different aspects such as cell line development [2-5], high throughput processing systems [6-8], omics approaches [9-13], process operations and monitoring [14-17], as well as process development [18]. Today's challenge is to integrate this expanding body of knowledge into advanced development strategies and facilitate science-based process development in order to

- increase product yields,
- be more efficient in terms of time to market,
- avoid fail batches and
- allow effective platform learning, which consequently will enable to transfer the knowledge to another process, product and site.

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In the biopharmaceutical industry, the available scientific knowledge has not yet been routinely implemented in process development; instead, heuristic approaches are still often in use which have been created to develop platforms for cell culture processes. However, such approaches mostly rely on empirical experimentation and rarely deliver a sound scientific understanding of the process. Thus, heuristic approaches often fail to run the process (or the production host) at its maximum performance potential. In contrast, this review focuses on the tools available to enable advanced process development. The cornerstone of advanced development strategies is the thorough understanding of the interactions between the production cell line and the process, which together define the physiological phenotype and ultimately the product [13]. The key of this understanding is the perception that the relationship between process inputs (process parameters and the production host) and process outputs (physiological phenotype and product quality) is ultimately determined by intracellular mechanisms occurring at multiple layers of cell physiology. Accordingly, advanced development strategies implement tools that:

1) Generate physiological knowledge through the identification and quantification of physiological markers.
2) Link the gained physiological knowledge to process output and subsequently use this information to facilitate process development.

Here, the goal is to identify which kind of tools are required for the two subsequent steps of the physiological ap-

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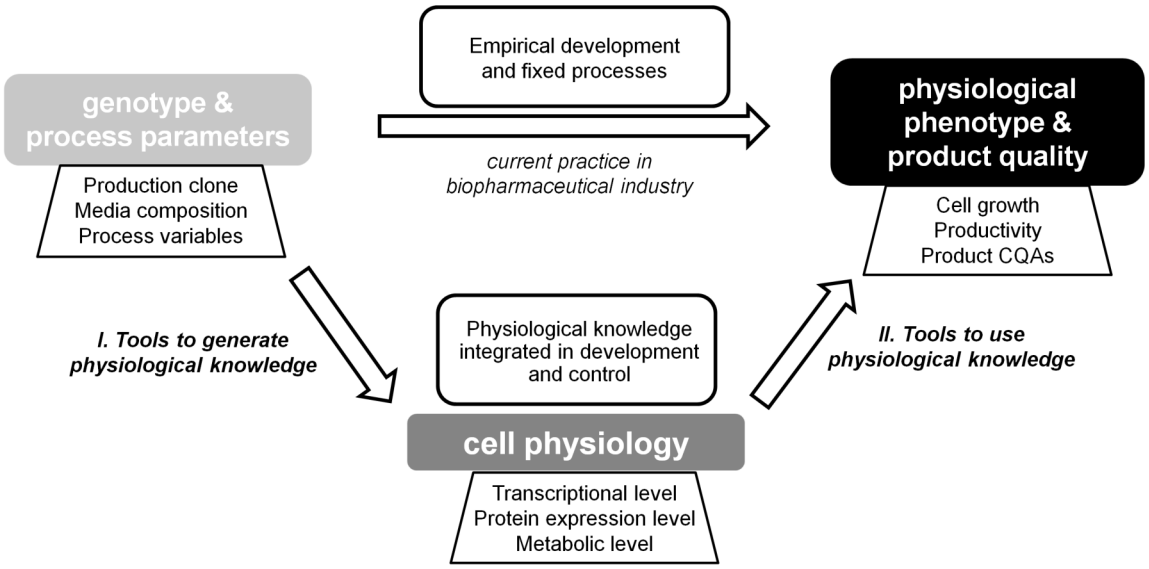

Fig. (1). The empirical and the physiological ways of process development and control. Empirical approaches describe direct relationships between process input and process output variables. Approaches based on physiological knowledge integrate scientific understanding and provide an opportunity for more efficient process development.

proach (Fig. **1**) and to demonstrate the perceived benefits of their application. As a structured approach in this review, we discuss the following tasks of process development (Fig. **2**).

1) Existing tools for the integration of physiological information in clone screening and selection.
2) Recent advancements in media selection and optimization using physiological approaches.
3) Tools for the model-based estimation of physiological variables in cell culture processes with special emphasis on possible real-time applications.
4) Implementation of physiological knowledge in process control strategies.

The implementation of physiological knowledge in process development and control is in accordance with the Quality by Design (QbD) and Process Analytical Technology (PAT) initiatives which were introduced by regulatory bodies to stimulate and enforce the generation of process understanding to ensure a quality product. A cornerstone of these initiatives is to understand the effect of Critical Process Parameters (CPPs) and Critical Quality Attributes (CQAs). Moreover, pharmaceutical companies are also interested to extend this understanding to key performance indicators (KPI), such as product titer, from an economical point of view. In biotechnological processes, the key to understand the relationship between CPPs, CQAs and KPIs is the generation of physiological knowledge [19]. Accordingly, the focus of this review is in alignment with the directives of QbD and PAT.

1. CELL LINE SELECTION

Generation of the producer cell clone is the first challenge to be faced during biopharmaceutical process development. It requires considerable amount of time, costs and labor representing a critical period in the timeline of the biopharmaceutical projects.

The generation of the producer cell line typically starts with the expression of the targeted product in various cell lines screening for the most appropriate host. Chinese hamster ovary cells (CHO), mouse myeloma cells, human embryonic kidney-293 (HEK-293), baby hamster kidney (BHK), and human embryonic retinoblastoma (PerC6) cell lines are the most frequently applied mammalian cell lines in the biopharmaceutical industry [20]. It is notable, that the majority of biopharmaceuticals are produced in the various cell lines of CHO [4, 5].

Although the generated clones (cell lines) originate from the same host, their physiological phenotypes (i.e productivity, product quality, cell growth) display high divergence [21, 22]. This high divergence explains the need of the insertion of a highly effective clone selection step into the development process. After DNA transfer of the sequence encoding the product into the selected host cell line(s), a few numbers of polyclonal pools are generated containing a large number of potential candidates of the production clone. The selection of the production clone from the cell pools occurs usually in two major rounds of selection. First, hundreds or thousands of individual clones are screened and examined generating a set of clones of tens, referred, as leader clones. Afterwards, these potential candidates are further screened to select the final production clone. The number of the host cell lines, pools, and individual clones are mainly dependent on screening capacities, timelines and usually varies in each project.

Here, we aimed to discuss the possible strategies which have been established to develop the efficiency of clone selection. Investigating the available tools of clone selection (Fig. **1**), empirical approaches seem to mainly dominate over physiology-based strategies. Most probably due to the high

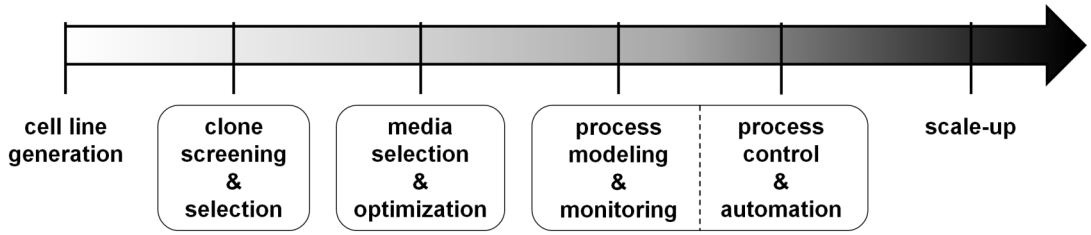

Fig. (2). A chronological overview of cell culture process development. This review focuses on the tasks marked with black boxes.

number of clones to be screened, the empirical clone selection primarily focuses on the analysis of the physiological phenotypes (i.e. high productivity, product quality), using them as selection markers, without the deeper understanding their cell physiological background, which we referred here as to physiology-based development. In the following few paragraphs, we aimed to give a short overview of both development strategies, illustrated with the examples of some recent studies, and also to discuss the possibility of the integration of physiological knowledge into clone selection.

1.1. Empirical Clone Selection

Focusing on the targeted physiological phenotype of the clone to be selected, the major drives are high productivity and the achievement of the desired quality profile of the product, i.e. glycosylation pattern, charge variant profile, aggregation, and correct folding. In order to improve the selection on these criteria, more strategies has been applied such as (1) the increase of the efficiency of selecting high-producers, (2) the increase of the number of selection markers, and (3) improve cultivation technology of clone selection (Table **1**).

1.1.1. Increase Selection Efficiency

Traditionally, limiting dilution has been used for clone selection. By high dilution, the survival of the single cell clones is very low increasing the risk to lose the rare occurring high-producing clones [23]. In order to avoid this, several advanced technical improvements have been developed by using high-throughput and robotics techniques for the elevation of the number of the examined clones and/or the enhancement of the probability to isolate high-level producers. Furthermore, these techniques also succeeded in coupling the measurement of productivity with single cell isolation. Fluorescence activated cell sorting have been employed for the rapid screening of millions of cells followed by the isolation of high-producing clones [24-26]. The efficiency of selecting high productivity clones were also achieved with various semi-solid media based techniques such as matrix-based selection assay (MBSA) [27], gel microdrop technology (GMT) [28], laser enabled analysis and processing (LEAP) [29], and also the automated combination of these techniques [30, 31].

1.1.2. Increase the Number of Selection Markers

Extension of the numbers of phenotypic parameters examined during clone selection can also contribute to the enhancement of process development strategies. Besides productivity, the early integration of the analysis of the product quality attributes into clone selection is also advantageous, because numerous quality attributes, i.e. protein glycosylation, charge variant profile, aggregation (folding), biological activity, are mainly defined by the physiological characteristics of the cell and these attributes can be modified only moderately by cultivation parameters [32-34]. The early analysis of quality attributes can enhance the probability to reach the targeted quality profile, an aspect especially important in the development of biosimilar products. The development of rapid high-throughput methods could be an alternative solution. Recently, a high-throughput method has been developed and used for the analysis of intraclonal variability of glycoprotein sialylation [35], while another array-based glycoprofiling technique has been developed for clonal selection [36]. For charge variant analysis, a newly developed screening assay on a chip has been developed and its applicability has been tested in case studies of biological process development [37]. Affinity screens, aiming the estimation of biological activity, can also be optional; techniques are available using surface plasma resonance or label-free biosensors [38, 39].

1.1.3. Improve Cultivation Technology of Clone Selection

Finally, the efficiency of clone selection can also be increased by its combination with "traditionally" subsequent process development elements such as media, feed and control strategy optimization; this can speed up then the entire process development and also cut timelines in the project. This idea has been supported, for example, by the comparative analysis of selected clones propagated under batch or fed-batch conditions [40]. Fed-batch cultivation is currently the major form for mammalian cell processes. The change from batch to fed-batch resulted in divergence in quality attributes (glycosylation and charge variants) of the product highlighting the advantage of inserting the production process parameters into the process development stream, as early, as possible, even into clone selection. The minimization of the cultivation parameter differences between clone selection and production process – naturally under possible circumstances – also reduce the risk to lose the phenotypes obtained during the clone selection.

1.2. Physiology-based Clone Selection

The physiological phenotype of a clone can be modified by various cultivation parameters, but it can be performed only within certain limitations, which are strongly determined by the genotype of the selected clone. The deeper understanding of these limitations on the cell physiological

level during clone selection could provide further valuable information for the subsequent process development steps to predict not only the physiological phenotype of the clones but also their potential for optimization. Potential tools for the generation of cell physiological knowledge (1.2.1) and that of subsequent application are discussed below (1.2.2) and summarized in Table **1**.

1.2.1. Generate Physiological Knowledge for Clone Selection

As discussed above, the traditional principal criteria for clone selection is obviously the generation of clones with high productivity. Since the physiological background of high productivity has not been completely clarified yet, deeper understanding of its complex trait has been aimed in several studies. Omics approaches, a tool for this, provide a global view of cellular biological activities through the quantitative analysis of gene expression at the mRNA level (transcriptomics), at the level of the expressed proteins (proteomics) or they give a complex overview of the metabolite species of the cell at a certain time point (metabolomics) [12, 41]. The most recent approach, fluxomics comprises a number of various methods with a common focus, metabolic flux analysis which aimes the measurement and estimation of biochemical reaction rates within an organism. The generated results share a critical link amongst genes, proteins and phenotype [42]. The recent publication of the first genomic next-generation sequencing of CHO-K1 cells was certainly a milestone in this field [43]. The type of the identified potential markers of high productivity varies from a single or small numbers to larger set(s) of key element(s). A recent transcriptome analysis of CHO cells resulted in the selection of one potential key factor regulating productivity in the mTOR signaling pathway [44]. Another analysis targeting a much wider (genome scale) range of genes in the combination of proteomics identified further potential genes and proteins [45]. Further combinations of multiple omics approaches (transcriptomics, metabolomics, and fluxomics) has been recently used to analyze recombinant protein production in HEK293 cells [46]. Instead of single key regulator(s) for high productivity, the meta-analysis using the data of previous transcriptome studies, has recently introduced a set of genes in different functional classes, such as cell growth and death control, energy metabolism, mRNA and protein processing [11]. Using metabolomics, a recent study has aimed to elucidate the key characteristics for high protein expression in CHO cells [47] while another one analyzed the clonal variations [48]. The physiological background of key physiological phenotypes such as culture growth rate and productivity could be investigated by large-scale microarray profiling [49]. The variation of clonal growth rate of CHO cells has been also characterized on transcriptome level providing more insight into the physiological background of the targeted phenotypes [50].

1.2.2. Use of Physiological Knowledge for Clone Selection

The deeper understanding of physiological phenotypes, i.e. high productivity, on genetic, RNA, protein or metabolite level are often discussed to provide potential targets for the generation of improved cell lines or for clone selection markers of high-expressors [45, 47, 51, 52]. Numerous successful cell engineering strategies have been developed to improve protein expression targeting a wide variety of mechanisms involving transcription, translation, folding, protein processing or secretion, as well, as mechanisms of cell physiology, such as cell proliferation and survival [53, 54] or to generate host cells for protein quality improvement for example with modified glycosylation potencies [5]. Thus the linkage between cell physiology and the targeted physiological phenotypes has already been successfully applied in the development of improved cell lines.

In contrast, the integration of the physiological knowledge into advanced high-throughput clone selection techniques are currently not yet in the routine use according to our knowledge; however, it may have potentials for advanced process understanding and optimization. For example, high productivity has been successfully predicted based on gene transcription data [55]. Such tools using physiological knowledge can be applied in the future to validate the physiological phenotype obtained in the empirical clone selection strategy. Furthermore, it has been shown that the majority of cells in transfected cell populations are functionally limited in terms of specific productivity; high productivity clones exceed this functional threshold in the cellular synthetic processes [56]. The monitoring of these limitations and competencies early in the clone selection might also deliver additional information for subsequent optimization of the clones. Based on transcriptional profiling in early-stage development, the prediction of other key physiological phenotypes such as syalilation capacity or stress resistance has been envisioned [52].

Table 1. Tools for advanced clone selection.

Clone selection		
Empirical development	**Physiological knowledge integrated in development**	
	I. Tools to generate physiological knowledge	II. Tools to use physiological knowledge
• Increasing the efficiency of selecting high-producers • Increasing the number of selection markers (i.e. quality) • Improve cultivation technology of clone selection (i.e. fed-batch)	• Gene expression (sequencing, transcriptomics) • Protein expression (proteomics) • Metabolites (metabolomics)	• Mostly used for the development of new cell line generation strategies • Predict physiological phenotypes

2. MEDIA SELECTION AND OPTIMIZATION

As basal and feed media vastly determine cell culture performance and product quality attributes, media selection and optimization have become one of the key steps in bioprocess development. Numerous chemically defined cell culture media products are commercially available [57] and several biopharmaceutical companies develop proprietary media platforms [58]. The commonly used heuristic approach for media screening is to compare several media formulations and select the best candidates based on product titer or quality profile. The following step is media optimization based on spent broth analysis information to overcome possible nutrient limitations [59] or the addition of supplements to adjust product quality profile [60]. However, a thorough understanding of interactions between medium composition, cell physiology and process performance is not delivered by such approaches. Moreover, heuristic approaches rely on trial-and-error experimentation characterized by a high number of experiments and a high probability to fail in finding the optimum. Advanced knowledge-based methods are needed to increase the efficiency of media development and to maximize process performance. Thereby, the first step is to understand the effect of media composition on cell physiology and to link this physiological information to process performance. This knowledge can be subsequently used to modify media composition in order to achieve the targeted physiological phenotype in the manufacturing process.

2.1. Approaches to Understand the Effect of Media Composition on Cell Physiology and the Link to Process Output

The most commonly considered level of cell physiology is metabolism in cell culture development. As demonstrated in numerous publications, investigating the interactions between media composition, metabolic data, and process performance can deliver useful information for media development. Recent studies reported differences in cell growth in different basal media formulations and detected correlations between metabolite concentrations and cell growth characteristics with MVDA methods [61, 62]. Interestingly, Dietmair *et al.* reported that the observed differences could not be explained on the basis of extracellular concentration patterns; profiling intracellular metabolites is also nessesary [62]. Accordingly, due to the complexity of mammalian cell metabolism and cell culture media, advanced analytical methods (e.g. determination of intracellular metabolite concentrations) and evaluation approaches (e.g. multivariate data analysis) are required in order to understand the impact of different media formulations on a metabolic level.

Advanced evaluation approaches should also include proper data processing to gain useful metabolic information. Instead of looking at concentration data, De Alwis *et al.* calculated specific rates and conducted partial least squares regression to uncover correlations between amino acid fluxes and the resulting physiological phenotype (growth rate, productivity and byproduct formation) [63]. Based on the results, the authors could derive valuable knowledge for media optimization from a few batch cultivations. However, specific rates are subjected to multiple type of errors (error propagated from prime variable measurements or the error of the calculation method), the sum of which can render the statistical analysis of specific rate datasets to be difficult [64]. Selvasaru *et al.* reported a workflow integrating specific rate calculation, data reconciliation, and elemental balancing to generate datasets for multivariate statistical analysis in order to detect correlations between metabolite fluxes and the physiological phenotype [65]. The authors identified possible sources of errors in specific rate calculations and offered methods to identify these types of errors and reduce their impact on the statistical evaluation.

The high connectivity of metabolic pathways in mammalian cells renders conclusions based on data-driven statistical approaches to be of limited use. As demonstrated by Selvarasu *et al.* in a later study, understanding the intracellular effects behind statistical correlations can be used to gain an enhanced knowledge of cell metabolism [66]. By conducting a systematic analysis of metabolite concentrations and metabolic flux analysis (MFA), the authors could identify intracellular oxidative stress as a cause of cell growth limitation and a potential target of media development. Kyriakopoulos and Kontoravdi used flux balance analysis (FBA) to understand the effect of feed medium composition on metabolism and on physiological features such as cell growth and productivity [87]. Thereby, FBA revealed a connection between high asparagine consumption and ammonia accumulation, which subsequently led to a limitation in cell growth. These results demonstrate that approaches delivering a comprehensive overview on intracellular metabolic fluxes such as FBA or MFA are key methods to interpret the effect of medium composition on cell metabolism. The drawback of the reported metabolic models is that they only consider main catabolic and anabolic pathways. However, other pathways such as nucleotide sugar metabolism play a key role in cell growth [62] and protein glycosylation [67]. Models describing nucleotide sugar fluxes in protein glycosylation have been recently reported [68]. The integration of these pathways in metabolic models would accelerate a mechanistic understanding of interactions between media composition, culture metabolism and process performance [69].

Metabolism is only one layer of cell physiology. The metabolic fingerprint of the cell is influenced by complex control mechanisms occurring on the transcriptomic and proteomic level. Accordingly, in order to understand the effect of media composition on cell physiology, the consideration of gene- and protein expression data might be necessary. A good example is basal medium copper content [70], which has been reported to affect lactate metabolism and culture performance [71]. Both a detailed metabolomic investigation [72] as well as a gene expression study [73] failed to uncover the exact physiological background of the phenomenon. However, a recent proteomic study reported that copper might exert its effect by influencing protein assembly in the mitochondrial electron transport chain [74]. The complex effect of copper on cell physiology demonstrates the need for complete omics approaches to facilitate the understanding of the effect of medium composition on a cellular level. The ultimate goal should be the development of mathematical models incorporating multiple layers of cell physiology. Meshram *et al.* reported the coupling of a population-balance model of apoptosis and a metabolic flux model to describe different stages of cell death in batch cultivations [75]. By

estimating the activity of the intrinsic apoptotic pathway using an ammonia accumulation-dependent term, the model established a connection between a metabolic byproduct and an undesirable physiological phenotype. Such a model can be used to assess the effect of modifications in media composition on metabolism and on the resulting physiological phenotype.

Beside the challenges of physiological characterization, media development is also characterized by complex experimental designs due to the high number of media components. In order to describe correlations between media composition and process performance, multivariate experimental designs investigating more concentration levels for the individual components should be performed [76]. Thereby, another challenge is the complex media formulation procedure which has to be repeated for each individual point of the experimental space. Accordingly, advanced approaches are needed for experimental design, media formulation, execution and data exploitation. First, the number of experiments can be kept low by choosing proper experiment designs. It has been reported that a screening design involving 31 runs was sufficient to investigate the effect of 15 media components as well as higher order interaction effects of these components on culture performance [77]. An advanced tool to overcome media formulation problems is media blending. In this approach, the levels of the individual components are adjusted by mixing different media formulations. By a systematic design of the blended formulations and sound statistical analysis, it is possible to detect the effect of individual components on culture performance [78]. Using automated media blending and a high-throughput cultivation system, Roullier *et al.* executed 376 cultivations in a single experiment campaign to test the effect of 43 components at three concentration levels [79]. Moreover, in order to support data exploitation from the large dataset, the authors applied advanced data analysis tools such as MVDA and score ranking.

For a detailed review of advanced microscale bioreactor systems the reader is referred to the works of Bareither and Pollard as well as Long *et al.* [6, 7]. Such systems are also an important tool for the implementation of integrated experimental approaches which investigate the multivariate effect of cell line, basal medium and feed medium on culture performance. A recent contribution of Xiao *et al*. reported a considerably shortened development time by using an integrated media optimization approach [80]. The authors conducted a multivariate experiment design where both basal and feed media were included as factors. This approach enabled to find the optimal media combination in three months compared to the one year long timeline of the classical sequential optimization. As the response on media development can be cell line-dependent [67, 81], defining cell line as a factor in integrated experimental designs is essential to understand the interactions between clone selection and media development [60].

2.2. Use of Physiological Knowledge in Advanced Media Development Approaches

The most important physiological features of a fed-batch cell culture process are high viable cell density, high specific productivity, and product quality attributes that meet the targeted CQA range. Thus, an advanced media development strategy has to use the available knowledge on the correlations between media composition and cell physiology in order to adjust the three key features of cell culture performance. There are already several examples in the scientific literature which show a successful application of physiological understanding for media development (Table **2**).

2.2.1. Cell Growth

Mechanistic knowledge on cell metabolism can be used to tailor media composition for improved metabolic and cell growth profiles. For example, interconnections between metabolic pathways can be used to reduce the production of toxic metabolites. Based on metabolic flux analysis results, Xing *et al.* successfully decreased ammonia accumulation and increased peak VCD by the targeted modification of feed media composition (e.g. decreasing glycine content and changing the ratio of glutamine and asparagine) [82]. The investigation of amino acid transport mechanisms can be also important for medium design. A recent paper combining metabolomics and transcriptomics reported an upregulation of gluthatione-associated amino acid transporters in the stationary phase [83]. This phenomenon indicates the onset of intracellular oxidative stress which can be overcome by adding antioxidants to reach higher viable cell densities [66]. Membrane synthesis is a key feature of cell proliferation; however, lipid metabolism is not routinely monitored during process development. Based on the analysis of lipid synthesis pathways using transcriptomic data, Schaub *et al.* successfully increased viable cell density by supplementing the basal medium with chemically defined lipids [84].

2.2.2. Productivity

As amino acids are the structural building units of proteins, media optimization strategies targeting the increase of cell specific productivity are focusing on balancing the amino acid content of basal and feed media. The detrimental effect of amino acid limitations on productivity has been demonstrated by several studies [85, 86]. In order to avoid such limitations, the amino acid content of the feed medium has to be adjusted to the metabolic characteristics of the producer cell line. An advanced method for this task is to determine specific amino acid uptake rates in batch cultures and accordingly modify feed medium composition [87, 88]. Moreover - although amino acid transport is not the rate-limiting step of protein synthesis [83] - knowledge on the synergistic effects at transporter proteins might be necessary to optimize the concentration of competing amino acids [88]. Based on the knowledge of metabolic pathways, amino acid supplementation can be used to control metabolic fluxes in order to achieve the targeted physiological phenotype [89]. For example, as high specific productivity has been reported to correlate with high TCA activity [90], strategies to enhance TCA fluxes can be used to enhance product titers. Accordingly, Sellick *et al.* supplemented the feed medium based on intracellular metabolite profiling data to reach higher TCA activity and reported the increase in culture productivity [91].

Table 2. Examples of using physiological knowledge in media development.

Toolset used	**I.**		**II.**		**Ref.**
Physiological phenotype/product quality	**Physiological level**	**Physiological understanding**	**Link to physiological phenotype / product quality**	**Action taken based on physiological knowledge**	
Cell growth	Metabolic	Interconnection between glycine and ammonia production	Toxic NH_4^+ production inhibits cell growth	Reduction of medium glycine content to decrease ammonia production	[82]
	Transcriptional	Bottleneck in lipid synthesis pathways on the level of protein transcription	Decreased cell growth due to low lipid availability	Optimization of medium lipid composition	[84]
	Metabolic	Intracellular glutathione concentration correlated with the transition to stationary phase	Oxidative stress plays an important role in growth inhibition	Addition of antioxidants	[66]
Productivity	Metabolic	Amino acid uptake rates are cell-line dependent	Balanced amino acid metabolism is required for maximal specific productivity	Determine amino acid uptake rates and define medium composition accordingly	[83]
	Metabolic	TCA cycle activity is dependent on amino acid metabolism	High TCA activity is required for high specific productivity	Increase the concentration of amino acids which are fueling the TCA cycle	[91]
Product quality	Metabolic	High NH_4^+ concentration leads to an increase in the intracellular pH	The intracellular pH has an effect on product glycosylation	Adjust medium composition to optimize metabolism and decrease NH_4^+ production	[92, 93]
	Metabolic	Bottlenecks in nucleotide sugar metabolism affect the availability of precursors for protein glycosylation	Nucleotide sugar precursors are required for appropriate glycosylation	Adjust medium composition to overcome glycosylation bottlenecks	[96]

2.2.3. Product Quality

The most frequently investigated quality attribute of monoclonal antibodies is N-linked glycosylation, as it shows high variability, has a great impact on the biological activity of the product and cannot be modified by standard downstream techniques. Fan *et al.* recently showed that combinations of different basal and feed media deliver very different glycosylation patterns [67]. Thus, the investigation of the effect of media development on a physiological level is essential to understand the responses in product glycosylation. The authors detailed three major mechanisms which can affect the glycan structure of the product: product-associated mechanisms, resident protein-associated mechanisms, and metabolic mechanisms. The detailed metabolic and gene expression analysis revealed that insufficient glycosylation can be caused by bottlenecks in nucleotide sugar metabolism. However, metabolic features which can be controlled by media development can also act on product quality through resident protein-associated mechanisms. Such a mechanism is the accumulation of ammonia leading to an increased intracellular pH, which subsequently affects the expression or the activity of glycosylation enzymes. Accordingly, medium composition was successfully adjusted to influence product glycosylation through the control of ammonia accumulation [92, 93]. Nucleosides, sugar precursors and trace elements have been used as media supplements in advanced experimental designs to precisely adjust product galactosylation [94]. Wong *et al.* demonstrated that these supplements can influence both the expression of glycosylation-associated Golgi proteins (resident protein-associated mechanisms) as well as the availability of nucleotide sugars within the Golgi lumen (metabolic mechanisms) [95]. A comprehensive study of Amand *et al.* investigating the effect of media supplementation on product glycosylation combined the benefit of advanced experiment designs and in-depth physiological analysis [96]. Based on the results, the authors could develop medium supplementation strategies to adjust the relative abundance of individual glycoforms. Moreover, the same authors also developed a mathematical model of product glycosylation to simulate the effect of different sugar nucleotide concentrations and glycosylation enzyme levels on the quality profile of the product [97]. Similar mathematical models might be a powerful tool to assess the effect of the supplementation of glycosylation precursors in the future. Furthermore, the detailed knowledge of the glycosylation pathways can be also used for the targeted activation or repression of selected steps in the glycosylation machinery by the addition of enzyme cofactors or inhibitors in cell culture medium [98].

2.3. Raw Material Variability in Cell Culture Media

Raw material variability, particularly lot-to-lot variability in cell culture media, is a major source of deviations in biopharmaceutical processes [99, 100]. Accordingly, the control

of medium variability is a key task already in process development. The first step is to identify the source of variability and assess its effect on cell culture performance. Due to the high complexity of cell culture media, advanced analytical tools are needed to detect differences in their composition. Spectroscopic methods have been demonstrated to be powerful tools to detect lot-to-lot variability in basal media formulations [101, 102]. The prediction of cell culture performance with multivariate tools based on the spectroscopic data of different lots of basal medium [103] and of soy hydrolysates [104, 105] has been also reported. Moreover, the use of advanced analytical tools such as UHPLC-MS-MS and GC-MS enabled the identification of soy hydrolysate compounds which triggered cell growth and IgG production [106]. A similar approach was successfully used by Richardson *et al.* to identify components in soy hydrolysates which were responsible for the observed variability in product yield in two different CHO processes [107]. Based on this information, the authors achieved an improvement in product titer by selectively adding one of the identified components to the process. Accordingly, a thorough understanding of raw material variability is not only important to ensure risk mitigation and increase process robustness, but such knowledge can be also used in advanced process development approaches to improve process performance.

3. REAL-TIME MONITORING OF MAMMALIAN BIOPROCESSES

As mentioned previously, enhanced process understanding can be achieved by implementation of tools which focus on (I) generation of physiological knowledge and (II) linking the gained physiological knowledge to process output and subsequent use of this information for process improvement (Fig. **1**). In this context, process monitoring strategies play an important role for generating physiological understanding by delivering physiological information. Moreover, physiological information can be also the basis of advanced control strategies, which necessitate timely measurements of the desired attributes. Bioprocess monitoring strategies can be differentiated as either off-line or real-time methods according to the availability of the information for subsequent control strategies.

Off-line methods, in which a sample is manually taken from the bulk of the bioreactor or downstream unit operation and analyzed in the laboratory with a time delay, provide often a variety of information with high precision. However, the obvious drawback of off-line monitoring is the inconsistent delay between sampling and analysis times, which complicates the use of results for improvement of running processes. In contrast, real-time monitoring techniques have the advantage of providing information during the process. The term "real-time" suggests that a pre-defined deadline should be upheld to produce the results of the analysis. Despite increasing interest towards implementation of real-time monitoring strategies in recent years, as reviewed by several authors [15, 17, 108, 109], most available analytical devices are not capable of directly measuring the physiological state of organisms; therefore, they do not contribute to the understanding of the process on a physiological level.

However, software sensors (soft-sensors), which combine mathematical models and real-time measurements, provide a powerful tool for the indirect monitoring of inaccessible process parameters, physiological characteristics, and quality attributes of bioprocesses [110]. Hence, soft-sensors can not only be used for linking process parameters and cell physiology (Fig. **3-I**), but also to link the physiological phenotype to product quality attributes (Fig. **3-II**). The implementation of real-time soft-sensing strategies requires advanced modeling as well as real-time monitoring techniques. The first part of this section reviews recent advances with respect to mathematical modeling of physiological aspects of mammalian cell cultures (empirical as well first-principle models), focusing on approaches which are particularly promising for implementation of real-time soft-sensing and control strategies. Subsequently, frequently employed real-time monitoring strategies, required for implementation of soft-sensors, will be reviewed. While this section considers soft-sensing applications for process monitoring, in section 4, utilization of soft-sensors for control applications will be reviewed.

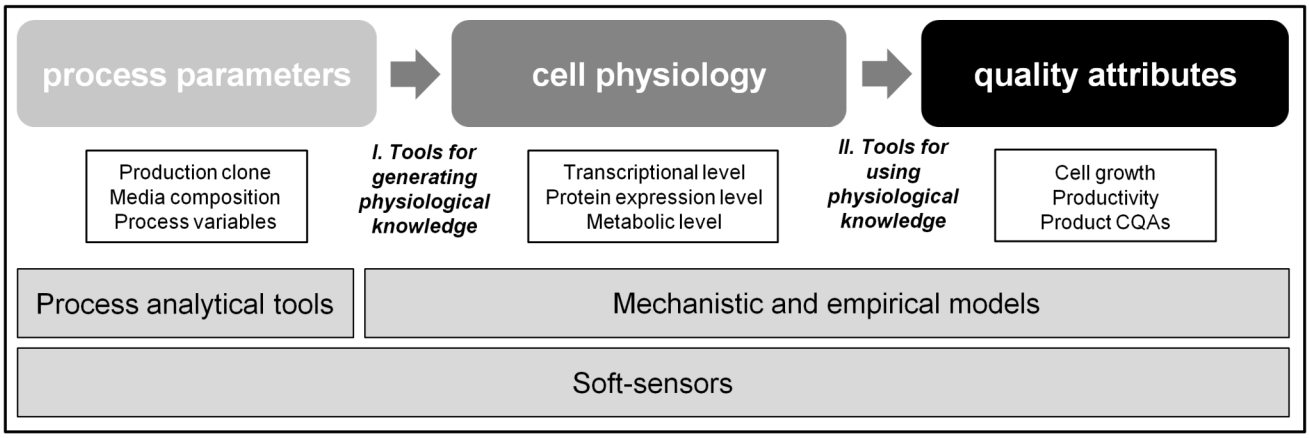

Fig. (3). Soft-sensors can be used as tools for generating physiological knowledge as well as using physiological knowledge for real-time monitoring of quality attributes. Soft-sensors combine measurements provided by process analytical technologies with mechanistic and/or empirical models.

3.1. Model-based Monitoring of Mammalian Cell Cultures

Physiological knowledge can be represented by mathematical models, which are either empirical (data-driven) or mechanistic (based on first-principles). Such models can be subsequently used for soft-sensing or control applications.

3.1.1. Empirical Models

Statistical methods can be employed for generation of physiological understanding as well as developing empirical models linking process parameters with cell physiology and quality attributes. In recent years, several authors have reported the application of statistical methods to cell culture bioprocess datasets for generation of physiological knowledge and monitoring techniques. For example, Le *et al.* applied kernel-based support vector regression (SVR) and partial least square regression (PLSR) to time-series data of 134 process parameters acquired through the inoculum train and production bioreactors of 243 runs to predict the final antibody concentration and final lactate concentration [111]. The authors found that the history of the culture (inoculum train) has a significant influence on the final process outcome. It was also possible to identify that the parameters related to lactate metabolism and cell viability have the highest influence on the process outcome. In addition, devising a real-time process monitoring strategy for predicting the final product concentration of a run could be envisioned. Similarly, Mercier *et al.* applied multivariate data analysis (MVDA) methods, namely principal component analysis (PCA) and PLS methods, on early development datasets of a human cell cultivation system in order to identify the effects of scale changes on the quality attributes of the process, concluding that none of the critical quality attributes were affected by scale-related variables [112].

Despite the usefulness of empirical modeling approaches for linking process parameters and quality attributes, the modeling often necessitates large amounts of training data, typically not available during bioprocess development stages. In addition, arriving at physiological interpretations based on the structure of empirical models is often challenging. For instance, whereas empirical models can be used to assess the influencing factors on quality attributes (Fig. **3-I**), the underlying mechanisms influencing process outcome (the physiological phenotype) cannot be readily extracted. Despite this limitation, empirical modeling provides an invaluable tool for identification of significant influencing factors, and additionally a ground for generating hypotheses about the underlying cellular physiology. Subsequently, mechanistic models, which rely on the fundamental principles of the system, have been gaining momentum in their application towards cell culture bioprocesses.

3.1.2. Mechanistic Models

Mechanistic models are mathematical formulations of the internal operation of systems in terms of their constituent parts and mechanisms [113]. By relying on fundamental principles, such as mass and charge balances, mechanistic models do not require exhaustive training datasets; however, since these models often contain an empirical part (e.g. enzyme kinetics parameters), some degree of model calibration using process datasets is necessary. An example of a recent effort is published by Jedrzejewski *et al.*, where a mechanistic modeling framework for linking the extracellular environment and intracellular metabolites of CHO cells with the glycosylation pattern of the final product has been developed [69]. Since this approach links process parameters (extracellular environment) with cell physiology (intracellular concentrations) and quality attributes (glycosylation), it can be considered to include both sets of toolsets reviewed here (Fig **3- I** and **II**). The underlying kinetic model is based on enzyme kinetic expressions, mainly Michaelis-Menten kinetics, for describing the concentration of intracellular nucleotide sugars as a response to extracellular concentrations. The intracellular nucleotide concentrations are subsequently used to predict the cumulative N-linked glycosylation of the antibody Fc region using another dynamic model describing monoclonal antibody glycosylation and nucleotide sugar donor transport within the Golgi apparatus [114]. The controllability of such a system, that is the feasibility of achieving specific glycan distributions by variation of process parameters, has been recently addressed [115]. Combined with a robust real-time-capable simulation system, such approaches could find utility towards solving a highly relevant industrial challenge, namely steering bioprocesses towards production of pharmaceuticals with desired glycan distributions.

The recently sequenced CHO-K1 genome has led to an increased interest in 'omics' approaches that are relevant for bioprocessing applications and constitute a deeper form of mechanistic understanding of the biological system [12]. Computational models of cell metabolism, which are used to simulate metabolic fluxes, can be coupled with extracellular process conditions (e.g. substrate concentrations) via kinetic expressions. When integrated with omics datasets (transcriptomic, metabolomic, or fluxomic) these approaches can lead to novel insights into the physiological characteristics of production systems. Works on the topic of dynamic metabolic flux analysis of CHO cell cultures have been reviewed previously [116]. While metabolic flux methods have often been employed for comparative analysis of fluxes between different process phases or conditions [117, 118], real-time-capable applications have been rare. Here, we present an overview of some research efforts which are promising with respect to applicability towards real-time bioprocess monitoring and control (Table **3**).

Combination of metabolic flux modeling with models of the bioreactor environment (e.g. dilution effects and uptake rates of extracellular metabolites) provides a promising tool for real-time monitoring and control applications. An interesting contribution by Meshram *et al.* presents an integrated model of extracellular metabolites and intracellular fluxes in addition to intracellular caspases, which are found to be an indication for apoptosis in CHO cell culture [75]. The model has been further enhanced by considering different subpopulations, so that monoclonal antibody production could be described after the exponential phase. Combined with real-time measurements of extracellular metabolites, such a model could potentially provide real-time information regarding the apoptotic state of different sub-populations.

Table 3. Summary of recent mechanistic modeling approaches suitable for real-time monitoring on physiological level.

Toolset used	I.		II.		Ref.
Physiological phenotype / product quality	**Physiological level**	**Physiological understanding**	**Link to physiological phenotype / product quality**	**Action taken based on physiological knowledge**	
Cell characteristics	Metabolic	Interconnection between extracellular metabolites and intracellular fluxes	Concentration of intracellular caspases influences apoptosis	Utilization of the model for monitoring monoclonal antibody production	[75]
	Metabolic	Physiological variables described as function of extracellular concentrations using Monod-type equations	Viable and dead cell density and mAb concentration linked to intracellular fluxes	Integrated model used for prediction of viable and dead cell densities, mAb concentration, and extracellular metabolites	[122]
	Metabolic	Metabolic flux analysis used for estimation of intracellular fluxes as a function of extracellular concentrations	NA	NA	[120]
Product characteristics	Metabolic	Extracellular environment has an effect on intracellular nucleotide concentrations	Intracellular nucleotide concentrations affect the N-linked glycosylation patterns of antibodies	Integrated process model was developed for prediction of glycosylation patterns	[69, 114, 115]

Computational platforms for realizing real-time physiological monitoring have been previously reported. For a comparison of software applications for quantitative metabolic flux analysis, the reader is referred to a recent publication by Dandekar *et al.* [119]. Goudar *et al.* reported the use of a quasi real-time combination of on-line and off-line data to estimate metabolic fluxes [120]. The utility of the presented approach was demonstrated using CHO cells cultivated in perfusion reactors where exposure to lower nutrient concentrations shifted cellular metabolism toward a more efficient state, namely increased flux into the TCA cycle. In addition, the authors performed sensitivity analysis in order to identify the necessary on-line and off-line measurement methods required for the estimations. Similarly, Henry *et al.* developed a system for on-line estimation of intracellular fluxes using a metabolic model for HEK-293 cells [121]. The authors envisioned control applications in which the physiological state of the cell is held constant. More recently, Ohadi *et al.* reported a soft-sensor based on an Extended Kalman Filter (EKF), combining fluorescence spectroscopy with a dynamic mechanistic model for prediction of viable and dead cells, recombinant protein, glucose, and ammonia concentrations [122]. The performance of this soft-sensor was compared with the performance of a purely data-driven soft-sensor based on fluorescence spectroscopy, with the EKF-based soft-sensor providing consistently better estimations for 10 CHO batch cultures.

Whereas these strategies provide suitable computational platforms for real-time applications, further integration with omics datasets (e.g. fluxomic) is necessary in order to arrive at verified predictions. In addition, most authors envision the implementation of the devised monitoring strategies for potential control applications; however, reports of such efforts are rare. In the future, as more knowledge about the intracellular mechanisms of mammalian cells becomes available, metabolic models incorporating a variety of omics data are expected to play a more pronounced role for real-time bioprocess monitoring and control applications, effectively aiming at improving process efficiency and product quality.

3.2. Process Analytical Technologies for Real-time Monitoring of Cell Culture Variables

Implementation of the aforementioned model-based monitoring approaches often necessitates the availability of real-time/on-line analyzers for measurement of various process variables, which provide input to the computational algorithms for either empirical or mechanistic models. This section of the review covers recent advances regarding monitoring of relevant cell culture process parameters and quality attributes, focusing on methods with a potential for real-time implementation. Despite the availability of several technologies for real-time measurement of extracellular components and biomass characteristics (e. g. in-line spectroscopic and capacitance methods) wide-spread industrial implementation of these methods is hindered by complicated calibration procedures (e.g. multivariate statistical modeling). In addition, the transferability of models across processing scales and equipment is an active area of research.

3.2.1. Extracellular Substrates and Metabolites

Direct real-time measurement of extracellular metabolites and substrates is one of the cornerstones of model-based soft-sensor implementations. For mammalian cell culture processes, typical components to be measured include glucose, lactate, glutamine, glutamate, and ammonium. In recent years, the applicability of vibrational spectroscopic methods has been demonstrated, and a detailed review of these methods and the accompanying modeling/chemometric methodologies are available [123]. While most reported works demonstrate the applicability of selected measurement techniques, demonstrations of real-time implementations are few. Here we highlight contributions with demonstrated real-

time implementation capabilities. For instance, real-time measurement of glucose concentration has been demonstrated in CHO bioprocesses via *in-situ* Raman spectroscopy [124]. With a consistent measurement interval of 6 minutes the authors were able to demonstrate successful control of glucose concentration using a closed-loop non-linear model-predictive controller. Similarly in another contribution, Abu-Absi *et al.* verified the feasibility of real-time measurements of glutamine, glutamate, glucose, lactate, ammonium using *in-situ* Raman spectroscopy with an excitation wavelength of 785 nm [125]. In a recent study, the performance of multivariate calibration models for measurement of several metabolites using Raman spectroscopy was compared across different processing scales, from 3 L up to 2000 L. Models generated in small scale fermentations were applied to larger scales, and for some metabolites, model predictions were found to be acceptable. The authors also suggested a workflow for generation and adaptation of models, providing a general approach for future applications [126].

3.2.2. Product Quality Attributes (Post Translational Modifications)

Recombinant protein biopharmaceuticals are mainly produced in mammalian cell culture processes due to the complex and stringent requirements with respect to post-translational modifications (PTMs). Glycosylation, a particular example of PTM and considered one of the most crucial quality attributes, is the addition of glycan structures to polypeptide chains. It is known to influence the physico-chemical properties of proteins (e.g. folding, solubility, electrical charge, stability) in addition to clinical function (e.g. efficacy, *in vivo* half-life, immunogenicity) [15]. Therefore, real-time monitoring of glycosylation patterns in cell culture bioprocesses is considered an important step towards implementation of the QbD paradigm, potentially enhancing regulatory acceptance and shortening the time to market. Furthermore, in the context of production of biosimilar products, regulatory authorities demand comparative studies showing the similarity of products with the original biologic products with respect to PTMs, especially glycosylation.

Traditionally, quantitative methods for characterization of glycosylation patterns are time and labor consuming, involving enzymatic digestion, labeling, derivatization and separation using chromatographic techniques (gel electrophoresis) or mass spectroscopy (MS). In recent years, several developments have emerged which focus on shortening the complexity and duration of the analysis method. As highlighted by Pais *et al.* by combining fluorescent labeling and ultra-performance liquid chromatography (UPLC), results can be attained within 90 minutes [15]. In another approach, an at-line assay to isolate MAb charge variants in near real-time was proposed as a quality control technique. The method could be performed by automatic sampling from the bioreactor and results could be attained within two hours [127]. The at-line application of analytic methods with high measurement accuracy is attractive; however, it often poses additional risk of contamination at the location of sampling and filtration. In this respect, *in-situ* methods involving spectroscopic techniques can provide advantages. In recent years, several authors have reported attempts utilizing vibrational spectroscopic methods, especially Raman, for monitoring protein quality [128-130]; however, such techniques are not used routinely for the monitoring of product quality attributes. As mentioned before, the complexity of calibration methods and lack of calibration datasets can often be a limiting factor for widespread adaptation of spectroscopic methods.

3.2.3. Cell Characteristics (e.g. vcd, Viability, Apoptosis)

In mammalian cell culture processes, cell-related variables, such as viable cell density (VCD), viability, and apoptosis, are known to have a large influence on the quality attributes of the process and recombinant protein titer [75]. In addition, viable cell density is often a key variable that needs to be controlled in perfusion systems to ensure the consistency of the process. Therefore, demand for real-time monitoring techniques of these variables is strong.

Radio-frequency impedance methods (dielectric spectroscopy), in which the permittivity of the culture fluid is measured at multiple frequencies, have been long used to measure parameters related to cells and are considered reliable because of their perceived simplicity and accuracy [131, 132]. A recent example of the estimation of VCD in CHO cell cultures using dielectric spectroscopy and multivariate regression models is provided by Lee *et al.* [132]. The authors compared the performance of different multivariate techniques with the classical Cole-Cole equation-based approaches and found that multivariate methods, particularly a locally weighted partial least squares model, offered the best estimation performance. Similarly, Párta *et al.* employed dielectric spectroscopy for online monitoring of VCD in industrial CHO cultivations, comparing the performance of various preprocessing and modeling techniques [133]. The authors were able to demonstrate the feasibility of on-line VCD measurement during both the growth as well as decline phases using multivariate calibration approaches.

Alternatively, many authors have reported the use vibrational spectroscopic methods for measurement of cell-related variables of cell culture processes. For instance, Sandor *et al.* compared the performance of MIR and NIR for monitoring total cell count (TCC) and viability in cell culture processes and reported that NIR provided slightly more accurate predictions when applied to an external validation dataset [134]. Abu-Absi *et al.* also reported the utility of in-line Raman spectroscopy for measuring TCC and TCD [125]. Comparing the cross-scale performance of calibration models for Raman spectroscopy, Berry *et al.* found that models for prediction of cell characteristics exhibited scale-dependent effects and were not suitable for transfer from development scale to manufacturing [126].

In-situ microscopy is another potentially real-time capable method and has a relatively long history of being applied for monitoring of mammalian cell cultures. More than a decade ago, Joeris *et al.* reported the measurement of cell density, cell size distribution, and degree of aggregation using *in-situ* microscopy and real-time digital image processing software [135]. More recently, Widemann *et al.* demonstrated real-time monitoring of viability using an *in-situ* microscopic cytometric method [136]. By coupling in-flow imaging capabilities, flow-cytometry, and high-throughput image analysis software, imaging flow cytometric ap-

proaches have been attracting more interest for applications involving morphological characteristics of organisms [137].

4. ADVANCED CONTROL TOOLS FOR MAMMALIAN CELL CULTURES

Mammalian processes are highly complex with diverse requirements for nutrient availability and susceptibility towards changes in process parameters. Accordingly, sophisticated control tools are required to tightly control process parameters and nutrient availability within the targeted ranges. This section of the review deals with technological process control strategies, such as feed profile, pH, temperature, and pO_2 control. One key aspect of mammalian processes is the control of overflow metabolism (e.g. lactate production). Many authors have shown that lactate overflow may be detrimental to product quantity and quality [138-140]. A recent review states that lactate metabolism is a major challenge in cell culture bioprocess development [14]. Balanced metabolism can be achieved by the proper adjustment of the process technological parameters. The possibilities for technological process control strategies to maintain a balanced substrate flux depend on the choice of process mode.

Batch

Batch is the most simple process mode. The nutrients are available for the cells in excess until the onset of limitations, thus nutrient availability cannot be controlled in a batch process. However, other process parameters (pH, temperature and pO_2 settings) can be adjusted to steer cell metabolism in the desired way.

Fed-batch

The feed rate of substrates (e.g. C-sources or amino acids) can be adjusted beside other process parameters (pH, temperature and pO2 settings) in order to adjust cell metabolism during the cultivation. Fed-batch is the most widely used process mode nowadays.

Continuous / Perfusion Process

This process mode offers the possibility to remove overflow and toxic metabolites such as lactate or ammonia, which have a detrimental effect on the cells. While the feeding rate in a continuous process is governed by the maximum specific growth rate, the perfusion rate in a perfusion process can be selected independently of the specific growth rate since the cells are retained within the process. Despite its advantages, this process mode requires the most complex technical facilities and sophisticated control strategies.

Control strategies can be differentiated with respect to the source of information for process manipulations. Open loop control strategies do not require measurement of real-time inputs. While open loop control has the advantage of being simpler to apply, it cannot be adapted to the highly dynamic and variable physiology of mammalian cell culture processes. Hence, open loop strategies are often not flexible enough or are only useful for very well characterized processes with limited variability. In contrast, closed loop strategies can be flexibly adapted to variations in the physiology; however, these strategies require real-time measurements of physiological variables to adapt. As reviewed in the previous section, a great amount of PAT tools have been reported offering the possibility to monitor a large number of different process variables in real-time, which are then available as an input for control strategies. However, open loop control strategies, for example bolus feed additions in pre-defined amounts, are still frequently used rather than timely adaptive control strategies. The potential of the application of PAT tools in closed loop control strategies to improve process performance is still not fully exploited. In order to facilitate the use of advanced control strategies, the ability of these tools has to be demonstrated in order to satisfy the most important criteria towards pharmaceutical processes (e.g. simplicity, transferability, robustness).

4.1. Control of Process Parameters: pH, Temperature and pO_2

These basic process parameters can be modulated in all process modes to steer cell metabolism towards a balanced substrate flux among production, biomass formation and catabolism. This is very simple to apply since these process parameters are routinely measured *in situ* in most bioreactor systems for mammalian cell culture.

4.1.1. Modulation of Process Parameters such as pH, Temperature, pO_2 etc. in Mammalian Cell Culture

Shifting of pH or temperature is often used for process optimization to maximize biomass productivity in the first cultivation phase while to promote high product titers and to limit by-product formation in the later phase [141]. The reduction of the temperature from 37°C to 32.5°C was shown to result in a decrease in growth, metabolite and lactate production [142]. Variation of the pH value within the physiologically viable range from 6.85 to 7.8 greatly effects lactate production. At a lower pH, a decreased specific lactate production rate was reported, which may have beneficial effect on viable cell density and productivity [143, 144]. Dissolved oxygen concentration at very low levels (< 10%) was shown to have an effect on lactate metabolism resulting in an increased overflow metabolism (lactate production) and a decreased viable cell density [145, 146].

4.1.2. Feed Control Strategies Mixed with Control of Other Process Parameters

pH-auxostat is a processing strategy which applies *in-situ* pH measurement to control the feed rate in fed-batch and continuous mode. This strategy was introduced in CHO perfusion processes; however, the lack of reliable real-time information on closed loop feedback control has prevented the application of advanced process control strategies [147]. More recently a pH-auxostat control strategy has been successfully implemented to control the feeding rate in a fed-batch culture [148].

As discussed at the media optimization section, numerous media supplements are known which can influence CQAs such as product glycosylation. Advances in the real-time determination of CQAs [149] will enable the implementation of control strategies which adjust the feeding rate of media supplements with respect to the real-time measured CQA profile.

4.2. Direct Closed Loop Feed Control Algorithms

There are two different approaches for feeding control: i) adaptation of the feeding rate based on the real-time measurement of a limiting substrate (e.g. glucose or glutamine); ii) measurement of viable cell density. In both approaches, the real-time measurement is directly linked to the control of the feed rate.

The implementation of a feedback control algorithm maintaining glutamine concentration at a level of 0.1 mM by on-line direct measurement of glutamine was reported to improve both cell densities and viral production [150]. An on-line monitoring and automated fed-batch system was used to control glucose and glutamine concentration which had a significant effect on cellular metabolism leading to an increased efficiency of nutrient utilization, altered byproduct synthesis, while it had no effect on cell growth rate [151]. Furthermore, a review from 2009 showed that near infrared spectroscopic techniques are extensively used for monitoring and control of cell culture processes [152].

Capacitance was measured to dynamically adapt the feeding rate in a fed-batch culture based on the measurement of viable cell density [131]. Other authors have compared the two different approaches (measurement of the limiting substrate or viable cell density) for automated dynamic feeding [153]. The first was based on the real-time estimation of viable cell density based on capacitance measurement, while the other was based on automated at-line glucose measurements. The authors found that measurement of glucose was superior to feed control based on measurement of VCD, although they also stated that not all cell lines profited equally from this approach and that the optimization of feed composition was also critical for optimization of the process.

4.3. Soft-sensor Closed Loop Feed Control Algorithms

As mentioned in section 3 of this review, a soft-sensor is an algorithm which is able to calculate the desired output(s) from multiple inputs using a mathematical algorithm (e.g. a mechanistic model which represents the growth kinetics of the culture). The output of the soft-sensor can be used to control the feeding rate in fed-batch and perfusion processes.

A soft-sensor based approach was used as early as 2003 to control a mammalian process reported by Frahm *et al.* [154]. The control strategy was based on an adaptive open loop control algorithm (without real-time measurement) with an interface for automated adaption to new information derived from off-line samples. In a later contribution, the authors tested different soft-sensor approaches to control the feeding rate in fed-batch cultivations. For well-characterized processes, the authors suggested the application of a fixed feed rate trajectory, while they recommended adaptive feeding control for new processes, because adaptive feeding strategies do not require extensive process knowledge [155]. The incorporation of on-line nutrient measurement information into adaptive feed control will also enable the adjustment of feeding strategies to the different nutrient demand of different cell lines in early phase process development [88]. Real-time Raman spectroscopy was used to determine residual substrate concentrations and the integration of these data in a model-based feed rate controller was demonstrated in fed-batch process [124]. More recently a model-based strategy has been used by Aehle *et al.* in a fed-batch process by using oxygen uptake rate as an input variable for the estimation of viable cell density and the subsequent adaptive control of the feeding rate [156]. The same authors have also found that their adaptive feed control approach delivers increased batch-to-batch reproducibility [157]. A similar feed control method was used by other authors to control the feed rate in a perfusion process. The feeding of amino acids was controlled on the basis of the determination of the oxygen uptake rate during perfusion; furthermore, the application of the adaptive control approach resulted in increased viable cell density and volumetric productivity [158].

4.4. Applicability for Commercial Production

4.4.1. Summary of Available Control Actions

Conventional (heuristic) process control is open loop and less flexible with respect to variability in cell physiology possibly appearing in manufacturing processes. Therefore, open loop control strategies should be used for well-characterized processes after excluding process variability during process development.

The potential of advanced process control strategies have been often shown to improve product yield and product quality (Table **4**). Advanced process control integrates the benefit of adaptive control and closed loop control strategies. Advanced control strategies use process understanding to control cell physiology based on real-time measured signals to balance substrate flux between production, biomass formation and catabolism. The proposed benefit of these advanced processing strategies is controlled and consistent process output and a reduction in the number of out-of-specification batches.

Table **5** summarizes the possible control actions of advanced processing strategies with respect to the applicable process mode (batch, fed-batch or perfusion) and the complexity of the control algorithm (direct or soft-sensor control).

4.4.2. Evaluate Complexity and Ease of Application

While advanced process control strategies and new process technological concepts are abundantly available in the literature, they are rarely applied in the industry for commercial production.

Typical acceptance criteria for advanced control strategies within the industry are simplicity of operation, transferability to similar processes or other scales and robustness with regard to probe failure (Table **6**). The modulation of basic process parameters within the knowledge space determined during process development is simple to realize and already used within the industry to a larger extent. Direct control action on the perfusion- or feed rate is also relatively simple and robust, if it is based on non-invasive/in-line probes. However, the transferability and flexibility to adapt the control strategy to similar processes and other scales is lower compared to soft-sensor control algorithms. If at-line/invasive sensor inputs are used, the transferability and robustness are reduced for both direct control actions and soft-sensor closed loop control algorithms.

Table 4. Overview of advanced processing tools published in the scientific literature.

Toolset used	I.		II.		Ref.
Physiological phenotype / product quality	**Physiological level**	**Physiological understanding**	**Link to physiological phenotype / product quality**	**Action taken based on physiological knowledge**	
Metabolic balance between substrates and products	Metabolic	Limited control of byproduct formation and onset of nutrient limitation	Increase productivity and / or improve product quality	Modulation of basic process parameters	[141-146]
		Full control of nutrient limitation on a desired level and limited control of byproduct formation		Act on feed-/perfusion rates	[88, 124, 150-155, 158]
		Full control of cell growth on a desired level and limited control of byproduct formation			[131, 153, 156, 157]

Table 5. Overview of process modes and control tools published in the scientific literature.

Batch	Fed-batch, Continuous	Perfusion	Direct control	Soft-sensor control
Basic controls (pH, temperature and pO_2)			[141-146]	[147, 148]
	Feed rate		[131, 150-153]	[88, 124, 155-157]
		Perfusion rate		[18, 154]

Table 6. Acceptance criteria for advanced control strategies within the industry.

Control strategy	Simplicity	Transferability	Robustness
Modulation of basic process parameters (e.g. pH shift)	+++	+	++
Direct control of perfusion- / feed rate based on non-invasive in-line sensor inputs (e.g. off-gas)	++	+	++
Direct control of perfusion- / feed rate based on invasive on- at-line sensors (e.g. filtration probes)	-	+	-
Soft-sensor closed loop control algorithms based on non-invasive in-line sensor inputs (e.g. off-gas)	-	+++	+
Soft-sensor closed loop control algorithms based on invasive on- at-line sensors (e.g. filtration probes)	--	++	--

CONCLUSIONS

An increasing number of scientific publications indicate that physiological knowledge is growing for mammalian cell culture technologies. This knowledge facilitates the move from empirical process development towards the routine use of physiology-based approaches. In order to support the implementation of such approaches, two major toolsets are required. The first challenge is to generate physiological knowledge by the quantification and system-level evaluation of physiological markers such as metabolic pathway activities, transcript levels or morphological information. The second toolset is required to link the physiological knowledge to process output, for example by predicting the physiological phenotype or product quality based on a physiological marker or a system-level model. The ultimate goal is then to implement the physiological knowledge in advanced process development and control strategies. A qualitative gap analysis is provided below in order to demonstrate the availability of the toolsets for the reviewed tasks of process development (Fig. **4**).

Numerous tools have been reported in the scientific literature for the identification and quantification of physiological markers. Although system-level models have been recently reported which integrate different types of physiological information, the application of such models in the reviewed process development tasks is still rare. Similarly, the implementation of physiological knowledge in process development is rather a future perspective as a routine exercise. For example, clone selection strategies which use physiological markers have not been reported in routine

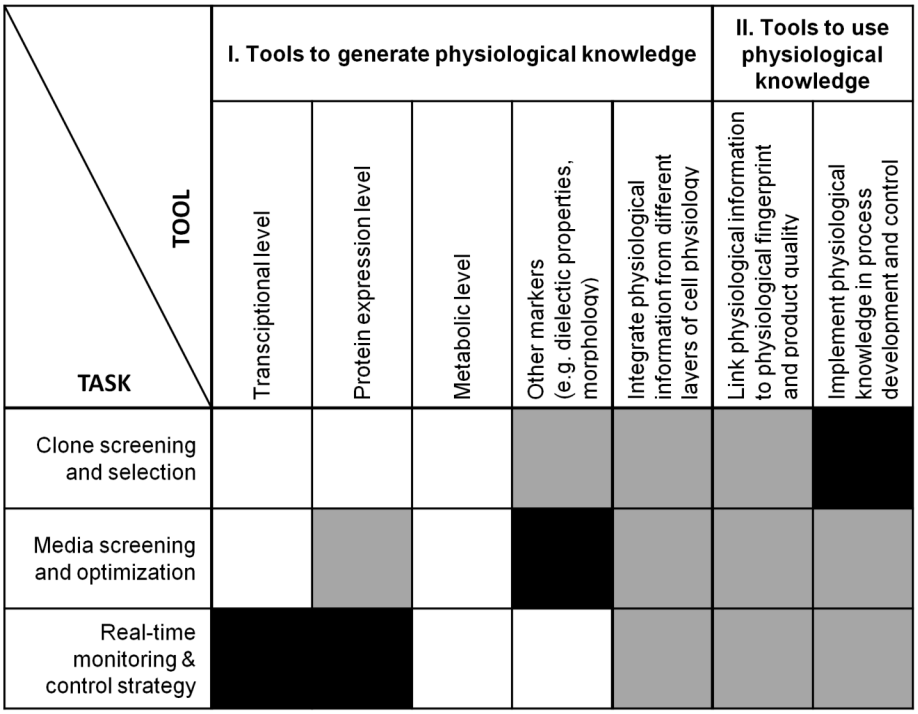

Fig. (4). Gap analysis of the availability of tools required for physiological approaches in process development. White coloring indicates routinely used tools, grey coloring indicates available but rarely reported tools and black coloring marks gaps where tools have been not reported in the scientific literature.

process development strategies. Also in the other reviewed process development tasks, the application of physiological approaches is limited to individual cases. However, these studies already perceive the benefits of integrating physiological information into process development, namely

- increased efficiency in process development by targeted optimization,
- increased process performance delivered by adaptive process control strategies which respond to physiological changes during the cultivation,
- model-based prediction of process optimum and in general, enhanced process understanding which can support QbD filings.

FUTURE PERSPECTIVES

Due to the high complexity of mammalian cell physiology and cell culture processes, empirical approaches were used for a long time in process development. The advent of Quality by Design provoked the identification of causality links between process parameters and product quality attributes. The next challenge is to implement systems biology knowledge in cell culture development in order to understand the mechanistic links between process input, cell physiology and the resulting phenotype. As demonstrated in this review, many of the necessary tools are already available; however the completion of the toolset with the missing approaches is a future task. For example, the integration of physiological data in system-level models is necessary to deliver a sound scientific understanding of the interactions between the host cell and the production process. Based on this knowledge, genetic engineering tools for the targeted modification of physiological features will be applied. Moreover, physiological markers will be used in early process development in order to increase the efficiency of clone and media selection. The integration of physiological information in process monitoring tools will also enable to develop adaptive control tools to drive robust processes at their potential performance maximum.

The routine application of available and novel physiological tools for cell culture process development will be facilitated by several factors in the near future. First, expanding biopharmaceutical product portfolios will necessitate subtle solutions in bioprocess development, for example to meet the strict quality specifications of biosimilar drug substances or to rapidly develop manufacturing technologies for personalized medicine products. Meanwhile, economical drivers will force pharmaceutical companies to reduce the number of out-of-specification batches and to increase the efficacy of process development, which goals can be ultimately achieved through enhanced process knowledge. Finally, as already forecasted in the Quality by Design framework, regulatory bodies will demand increased process understanding and the demonstration of control strategies which assure constant product quality in future process validation documents [159]. In accordance with this, the concept paper of the ICH Q12 guideline encourages the application of advanced development and control strategy approaches to provide scientific foundations for knowledge management throughout the lifecycle of the product.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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Process Development Along QbD Principles:

Characterizing the Effect of Process Parameters on Cell Physiology

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Abstract

Pharmaceutical companies make great efforts to improve bioprocess technologies in order to meet increasing demands for monoclonal antibodies. As cost and time are becoming factors with increasing importance in bioprocess development, enhanced approaches are needed to extract valuable information from a limited number of experiments. Thereby, the principles of Quality by Design can be used as a guide to improve data exploitation or to rationalize experimental effort. This contribution presents a workflow for the exploitation of the information in physiological variables from simple uni- or bivariate Chinese Hamster Ovary experiments. Specific rates with temporal resolution were calculated to extract quantitative information on cell physiology in different phases of fed-batch cultivations. Different aspects of bioprocess development such as stirring speed, medium composition or inoculation strategy were investigated and statistical tests were conducted in order to verify the effects of the experimental factors on cell physiology. The presented workflow shows existing tools of process development such as univariate experiments and statistical tests in order to demonstrate their interpretation in the Quality by Design context. Thereby, novel characteristics of the physiological responses to the investigated process parameters such as changes in specific growth rate or overflow metabolism could be identified.

Introduction

The amount of different monoclonal antibody products (MAbs) produced in the pharmaceutical industry has increased drastically in the last decades. Thereby a great amount of experience has been gathered about the expression of MAbs with mammalian hosts such as Chinese Hamster Ovary (CHO) cell lines. The implementation of Quality by Design (QbD) for biopharmaceuticals has accelerated the generation of process knowledge even further. However, the guidelines of the International Conference on Harmonization (ICH) have left open questions which kind of process understanding is necessary for QbD [1].

Numerous publications demonstrated how QbD tools such as risk assessments and multivariate experiment designs (DoEs) can be used to gain a better understanding of the manufacturing process [2-4]. Risk-based approaches support process characterization by integrating prior knowledge from other processes on the same platform or by decreasing the number of investigated process parameters based on scientific considerations [5]. Multivariate designs are subsequently used to gain enhanced process understanding in a reduced number of experiments. Despite these benefits of multivariate designs, univariate experiments are still

frequently used, since their experimental plans are easier to perform, especially if unexpected issues such as variations in inoculums material or cell culture medium components show up. It is important to apply the same quality requirements of statistical validity and reproducibility to simple experimental designs as for a full blown DoE approach [6]. We propose that - similarly to the workflows reported for the analysis of multivariate datasets [7,8] - univariate experiments should be carefully evaluated within a structured workflow to avoid redundant follow up experiments and to extract all the information the dataset contains. Thereby, basic principles of QbD such as the call for science- and risk-based methods should be used as a guide to develop enhanced workflows.

The first step is to gain scientific understanding by consequent data exploitation in order to extract the necessary information from a lower number of experiments. This starts with proper choice of process variables with high information content for the design and evaluation of the experiments [9]. Physiological variables such as specific rates deliver scale- and technology-independent information about the growth kinetics and metabolic behavior of cells in the cultivation. As these variables contain condensed process information, their use often leads to enhanced process understanding more directly compared to prime variables such as viable cell density or metabolite concentrations [10,11]. Specific rates quantify material fluxes related to a single cell in a finite time period, however, they are often calculated from prime variables measured at discrete time points. The standard error of prime variable measurements, as well as the slow dynamics of cell metabolism result in low signal to noise ratio (SNR) of the calculated specific rates in mammalian cell cultivations [12]. In order to decrease the relative error propagated from prime variables in the specific rates, many studies calculated these variables for the whole cultivation simply by looking at a long temporal history to compare different experimental setups [13-16]. However, choosing a too large time period for the time history decreases the temporal resolution of specific rates and compromises the detection of different phases and distinct events during the process. This may render the detection of physiological changes and process events typically occurring in mammalian fed-batch cultivations impossible. Accordingly, the time period of specific rate calculation has to be chosen short enough in order to achieve adequate temporal resolution of the physiological changes and to support scientific understanding throughout process development.

Quality by Design promotes the use of risk-based approaches for the design [17] as well as for the evaluation of experiments [18]. Statistical tests are valuable tools to challenge hypotheses, which have been previously imposed based on the investigated data, in a scientifically sound way. By the careful definition of acceptable risks for false positives or false negatives, which

one considers acceptable during process development, these tools can be used to decrease the number of experiments required for a decision. Tools for the planning and statistical evaluation of multivariate experiments, such as Design of Experiments (DoE), are available and routinely used in scientific and industrial research groups [2,4,19]. However, despite their ease of applicability, proper statistical evaluation of univariate experiments is often missing in scientific publications. A number of very simple univariate tests (e.g.: t-test, f-test) which are well documented in standard text books for statistical data analysis can be used for such purposes in analysis of variance (ANOVA). While these tests are easy to apply, a careful application of appropriate statistical tools and the definition of the risk threshold have a major impact on the outcome of the analysis.

In this study, a structured workflow was applied to analyze univariate experiments and simple bivariate designs from the development of a CHO cell culture process producing a monoclonal antibody. First, specific rates were calculated for distinct time periods in order to gain temporal resolution of physiological changes. These variables were used to understand the effect of selected process parameters on cell physiology. Analysis of variance was subsequently conducted in order to support hypothesis testing. The reported workflow enabled to assess the criticality of the investigated process parameters with respect to their effect on cell growth and metabolite formation. To our knowledge, a detailed investigation of the effect of these process parameters on cell physiology has either not been reported in the literature before (basal medium insulin content or inoculation strategy), or it was not analyzed in a temporally resolved fashion (stirring speed).

Materials and methods

Cell line and preculture

A Chinese hamster ovary (CHO-K1) cell line producing an IgG1 monoclonal antibody was cultivated in disposable shake flasks before inoculating the bioreactors. Stocks were revived in commercially available basal medium (ActiCHO P, GE Healthcare, Little Chalfont, UK), supplemented with 8 mM L-Gln (Life Technologies, Carlsbad, CA) and 5 mg/l Insulin (GE Healthcare, Little Chalfont, UK). The cells were sub-cultured every 3–4 days with a seeding density of $0.3 \cdot 10^6$ cells/ml and were grown in shake flasks of different scales. The shake flasks were incubated at 37°C with humidified air containing 5% CO2 at 110 rpm orbital stirring speed.

Bioreactor cultivations

Fed-batch cultivations were performed in bioreactors with 1 l maximal working volume (Sartorius AG, Göttingen, Germany). The bioreactors were equipped with on-line pH, temperature and pO2 probes and the process parameters were controlled by a Biostat BPlus Twin DCU (Sartorius AG, Göttingen, Germany). The targeted seeding cell density was $0.5 \cdot 10^6$ cells/ml. If not specified differently in the *Results and Discussion* section, the following parameters were used for the cultivations. Stirring speed was set to 125 rpm, cultivation temperature was 37 °C and the dissolved oxygen rate was maintained at 40 % of air saturation by air–oxygen mixture sparging. pH was maintained in the range of 7.15 ± 0.05 by automatic addition of 10 % H3PO4 solution or 0.5 M Na2CO3 solution. In order to reduce foaming, FoamAway (Life Technologies, Carlsbad, CA) was added. The basal medium was the same as the one used for the preculture (*vide supra*). Feeding was initiated on day 3 by adding feed medium to the cultivation every second day. The actual feed volume was determined as 5% of the current broth volume. The feed medium was a commercially available medium (Excell, Sigma-Aldrich Corp., St. Louis, MO) supplemented with amino acids.

Inoculation procedures

In the experiment investigating the effect of inoculation strategy two different inoculation procedures were applied. For the direct transfer in the bioreactor (marked with "D"), the calculated amount of inoculum broth was first loaded from the shake flask into a sterile vessel. The volume of the broth was refilled up to 100 ml with fresh basal medium. The vessel containing the cell broth was subsequently connected aseptically to the bioreactor and an overpressure was added on the vessel to initiate the transfer into the bioreactor. For the other inoculation procedure (marked with "C"), the calculated amount of inoculum broth was first centrifuged in sterile centrifuge tubes at 300 g for 10 minutes. The supernatant was discarded, the cell pellet was subsequently re suspended in 100 ml fresh basal medium and the cell broth was transferred into the bioreactor with a sterile vessel as described above.

Measurements

Real-time measurements

The bioreactors were placed on balances (BB30, Mettler Toledo, Zurich, Switzerland) to determine broth volume gravimetrically. At the end of the cultivations, the broth volume was measured volumetrically and the error of the gravimetric determination was verified to be less than 5% in all experiments. Feed medium and glucose stock solution volumes were also determined gravimetrically with balances (MS8001SE, Mettler Toledo, Zurich, Switzerland). The balance signals as well as on-line measured process parameters (pH, pO2, and

temperature) were collected by the Biostat BPlus DCUs and processed in the Citect SCADA system (Schneider Electric, Rueil-Malmaison, France) via local area network connection.

At-line and off-line measurements

At-line samples were taken every 24 hours or more frequently in order to measure several process variables. pH measurement for in situ pH meter re calibration was performed with a S47 SevenMulti pH meter (Mettler Toledo, Zurich, Switzerland). Viable cell density as well as viability was determined in triplicates by Countess automated cell counter (Life Technologies, Carlsbad, CA). Samples were centrifuged at 3000 rpm for 10 minutes (Rotanta 460 R, Hettich Zentrifugen, Kirchlengern, Germany) and cell-free supernatants were stored at -20 °C until further analysis. Glucose, lactate and ammonium concentrations were determined in duplicates by enzymatic assays (GM8, Analox Instruments, London, UK). Amino acid concentrations were determined by NMR (Bruker 500 MHz Avance III, Bruker Biospin, Rheinstetten, GE). Glutamine and alanine concentrations were determined with this method with a standard error lower than 5% as determined by measuring duplicates of three selected samples (data not shown).

Calculation of specific rates and statistical analysis

Specific rates were calculated in MS Excel (Microsoft Corporation, Redmond, WA) from the measured prime variables by using Equations 1-4.

$$IVCD = \int_{t_0}^{t} VCD\,dt \tag{1}$$

$$\mu = \frac{\frac{dX}{dt}}{X} \tag{2}$$

$$q_s = \frac{\frac{dS}{dt}}{X} \tag{3}$$

$$X = VCD \cdot V_{broth} \tag{4}$$

The time window (dt in equations 2 and 3) of specific rate calculation was determined to be 24 hours. This value was low enough to gain a sufficient temporal resolution of cell growth and metabolism, and high enough to minimize the effect of error propagation from prime measurement errors.

An in-house developed Matlab script (MathWorks Inc., Natick, MA) was used to perform analysis of variance (anova1 and anova2, http://www.mathworks.de/de/help/stats, retrieved February 21, 2014) on the specific rate datasets. The significance level α has to be carefully defined for statistical tests such as ANOVA. The significance level corresponds to the risk

that the effect in question occurred merely by chance. Since effects identified in early process development are usually analyzed repeatedly in the further stages of development, it may be acceptable to take a higher risk to decrease the number of required experiments, in order to rationalize cell culture development costs. Another script was used to calculate the statistical power and the probability for false negatives (type II errors) of a performed ANOVA test Model I (powerAOVI, Trujillo-Ortiz, A. and R. Hernandez-Walls. 2002). False negatives (type II errors) can be a much greater issue than false positives (type I errors), since it is generally more safe to have a closer look at any given problem, than neglecting the potential problem from start, only based on a limited amount of experiments. If one is forced to make a choice, it is probably better to "get lost in the woods", than "failing to find the woods in the first place" (Figure 1). Type II errors can potentially lead to very high cost and can potentially result in unexpected disaster later in the product life cycle, if it is decided not to look at a certain problem any further due to a false negative [20].

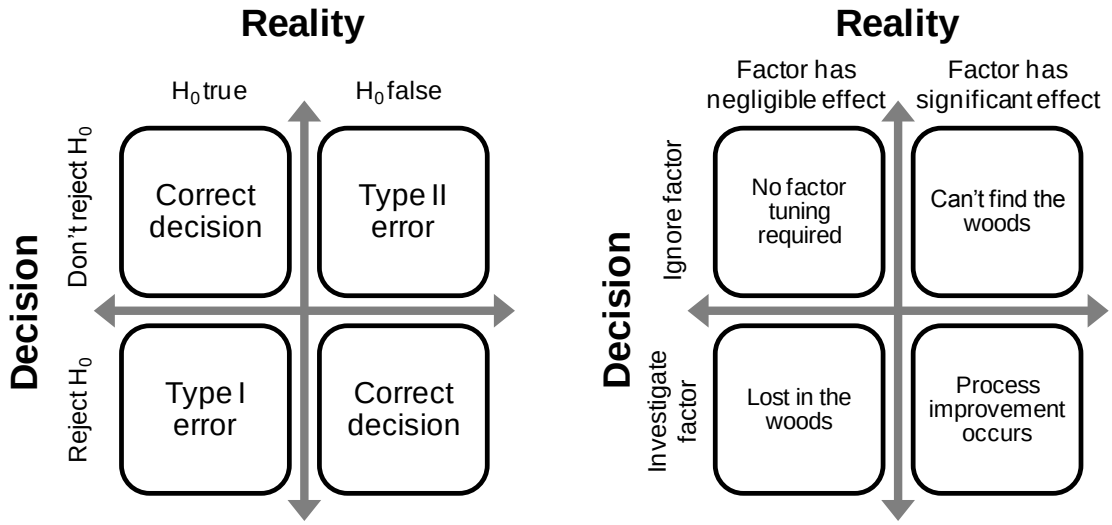

Figure 1. The alpha-beta war according to Propst [20]. A) Type I and Type II errors of decisions made based on the outcome of statistical tests. **B)** Consequences of decision correctness on process development.

The probability for false negatives can be especially high for low sample sizes (< 5) and a low threshold definition for the significance level α (Figure 2). Based on these considerations, the threshold of significance α was chosen to be 0.1. This means that the risk that the effect in question occurred merely by chance (= Type I error) is less than 10%. For α is equal to 0.1, the probability for a false negative (type II errors) and low sample sizes (<5) is only lower than 10% if the effects size is larger than 4-5 times the standard deviation. In contrast, for low α (= 0.01), much higher effects sizes are required to reach a probability lower than 10% (Figure 2).

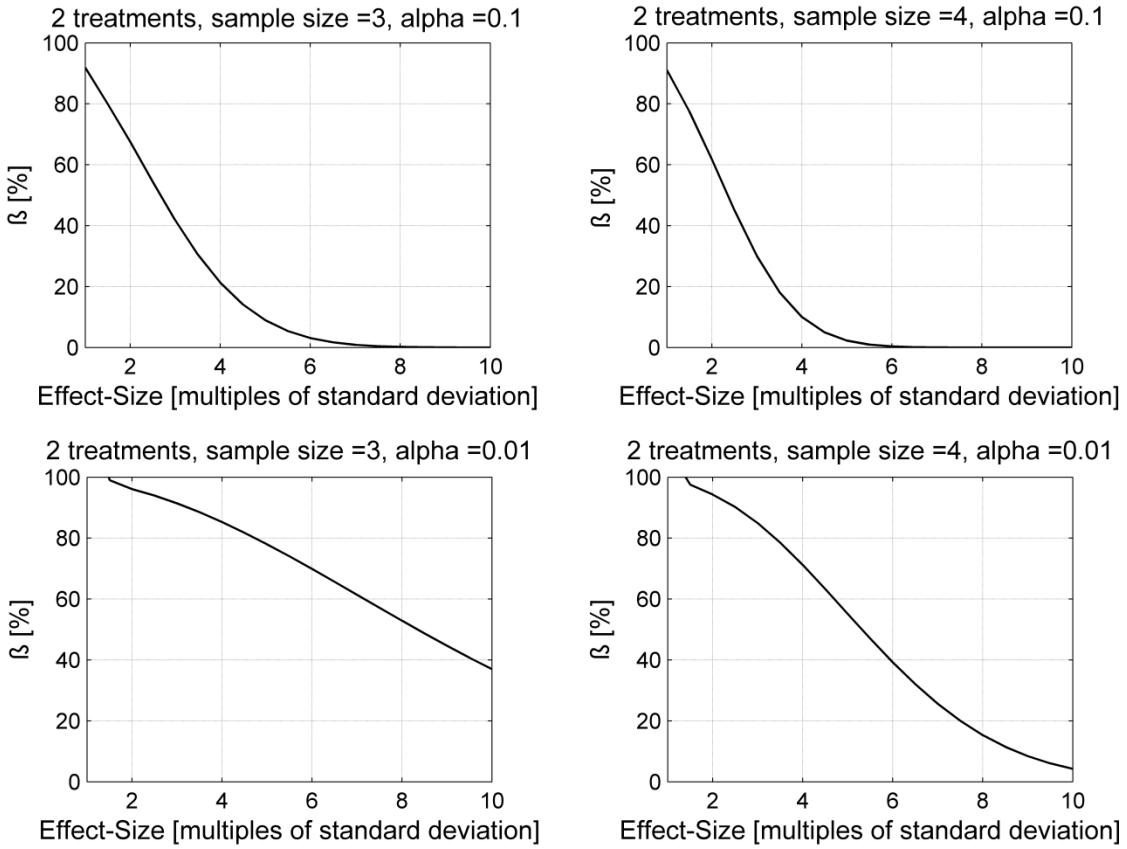

Figure 2. ß-error (the chance for false negatives) in dependence of the effect-size for two treatments (e.g. one factor with two levels). A) α=0.1 and sample size = 3 (e.g.: two levels of one factor with one replicate) (**B)** α=0.1 and sample size = 4 (e.g.: two levels of one factor with two replicates) (**C)** α=0.01 and sample size = 3 (**D)** α=0.01 and sample size = 4

Results and discussion

The goal of this study was to characterize the effect of different process parameters on physiological variables in simple uni- and bivariate mammalian cell culture experiments. The process parameters were selected based on a structured risk assessment we reported previously [5]. Although the risk assessment was conducted to assess the effect of process parameters on the critical quality attributes (CQAs) of the product, we investigated the links between process parameters and physiological variables in the recent contribution in order to generate physiological knowledge first [21].

One experimental design included the factor basal medium insulin content, which is related to medium formulation and the control of overflow metabolism, while the other two experimental designs (stirring speed and inoculation strategy) investigated factors related to scale-up activities. Table 1 reviews publications investigating the effect of similar parameters on CHO processes. The novelty of the recent contribution is the deeper understanding of these effects by using the reported workflow for the characterization of cell physiology.

Table 1. Factors chosen for this study and their reported effects.

Factor	Reported effect	Reference
stirring speed and stirrer type	decreased μ above tip speed 1 $m \cdot s^{-1}$ (μ calculated for the whole cultivation period)	Platas Barradas et al [22]
insulin	insulin increases viable cell density	Birch and Rachel [24]
inoculation strategy	culture duration of N-1 inoculum not critical for production bioreactor performance	A-Mab case study [3]

Stirring speed

In order to investigate the effect of shear forces on cell growth, cultivations with different stirring conditions were conducted (Table 2). First, stirring rates were varied in a range based on values reported in the literature for bioreactors with similar geometry [22]. Afterwards, the effect of stirrer type was assessed to represent both axial (Pitch blade) and radial (Rushton) type impellers.

Table 2. Parameter set for investigating the effect of stirring rate on cell growth.

	Parameters		
Impeller type	**Stirring speed [rpm]**	**Tip speed [m/s]**	**ID**
Pitch blade	50	0.14	P50
	125	0.34	P125
	200	0.54	P200
Rushton	125	0.33	R125
	200	0.52	R200

Although final VCD values were similar at the different stirring speed setpoints in all three cultivations conducted with Pitch blade impeller (P50, P125 and P200), a difference was observed between the time progressions of the different VCD curves (Figure 3A). Integral of Viable Cell Density (IVCD) values were calculated and plotted over time to allow a better comparison of cell growth (Figure 3B). A lower IVCD profile in the P200 cultivation indicated that cell growth might be inhibited at this stirring speed. In order to verify the effect

with a radial type impeller, Rushton impellers were used and two cultivations at 125 rpm and 200 rpm stirring speed were performed (R125 and R200, subsequently). Whereas the IVCD profile in the R125 experiment was very similar compared to the P125 run (Figure 3C), a decrease in IVCD was observed at the higher stirring speed (R200) also this time. Although the comparison of IVCD values revealed that high stirring speed inhibits cell growth in the investigated CHO system, it is not possible to acquire information on the temporal progress of this effect directly from IVCD profiles.

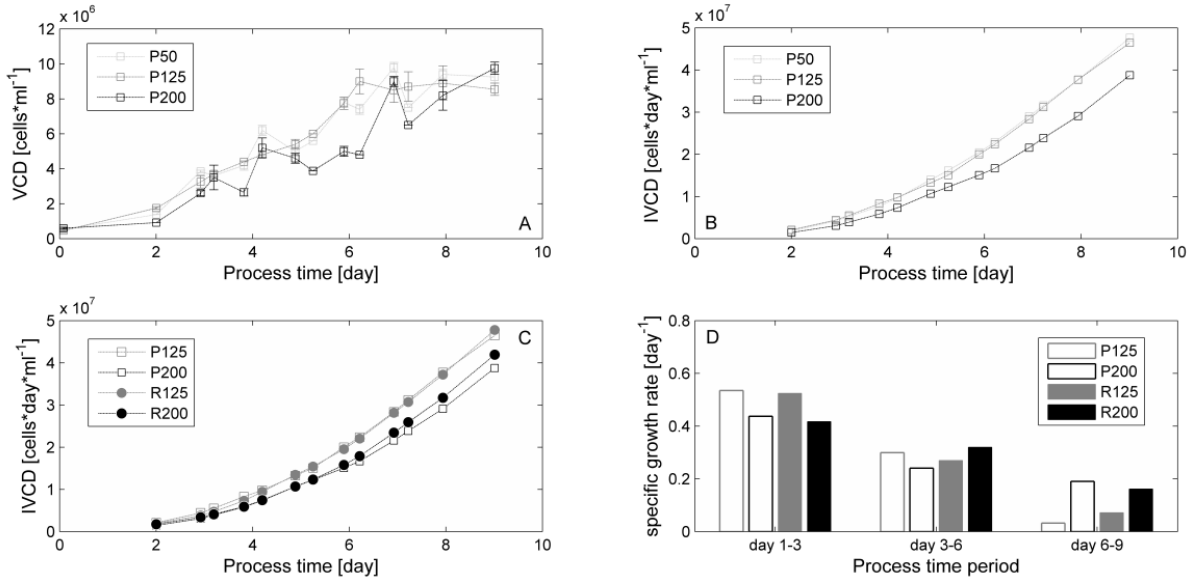

Figure 3. Cell growth in cultivations conducted with different stirring strategies. (A) VCD values in the three cultivations performed with Pitch blade impeller. Error bars indicate the standard variation calculated from parallel VCD measurements. **(B)** IVCD values in the three cultivations performed with Pitch blade impeller. **(C)** IVCD values in cultivations performed at 125 rpm and 200 rpm stirring speed with both impeller types. **(D)** Specific growth rates calculated for 72 hour time periods.

Such information on the temporal progress can be identified from specific growth rates, revealing differences in growth kinetics throughout the cultivations. However, when calculating μ values for time periods (dt in equations 2 and 3) of 24 hours, the low signal-to-noise ratio and reproducibility rendered the evaluation of the dataset difficult. Specific growth rate is very sensitive to measurement errors. Although Barradas et al successfully used the maximal specific growth rate (μmax) as a single value to compare mixing parameters of different lab scale bioreactors in temporally non-resolved fashion [22], another study demonstrated the challenges of using μ with temporal resolution to investigate the effect of stirrer type on culture performance [23]. By increasing the time period for the calculation of μ to 72 hours in our study, the signal-to-noise ratio for the specific growth rate was increased and the cell growth kinetic was resolved into three subsequent phases (Figure 3D). The size of

the time period was selected in accordance with the chronology of processing events such as the addition of bolus feeds. Another possibility is to select time window size by trial and error in order to achieve a pre-defined signal-to-noise ratio value.

The temporal resolution of growth kinetics revealed that high stirring speed has a negative effect only on μmax at the beginning of the growth phase. In the second phase of the cultivations, similar μ values were observed in all bioreactors. Interestingly, specific growth rates even turned out to be lower in the "125 rpm" bioreactors in the last phase of the experiment, probably due to faster growth initially and the onset of nutrient limitations in the late phase of these cultivations.

In order to investigate the statistical significance of the observed effect of high stirring speed on μmax, a two-way ANOVA was performed. The independent variables were impeller type (Pitch blade or Rushton) and stirring speed (125 rpm or 200 rpm), whereas the dependent variable was the specific growth rate calculated for 72 hour periods. As no replicate datasets were available for the four combinations of the independent variables, the statistical test was only able to assess the individual effect of the two factors, but not their interactions, due to lack of residual degrees of freedom for the ANOVA approach. A multivariate design which would have enabled to investigate factor interactions on the output variable, would have required conducting more experiments.

Table 3. 2-way ANOVA assessing the effect of impeller type and stirring speed on specific growth rate in the first three days of the cultivation. Significance level $\alpha = 0.1$.

	Impeller type	**Stirring speed**
F-value	11.86	518.83
p-value	0.179	0.028

The two-way ANOVA confirmed the inhibitory effect of high stirring speed on μmax in the first three days of the cultivation (Table 3). Additionally, the statistical test confirmed that impeller type has no effect on cell growth in the investigated range of stirring speed. The effect of stirring speed was not significant in the later phase of the cultivation (ANOVA not shown) indicating that this parameter is not critical in the stationary phase.

Although concentration profiles or cumulative values of VCD already revealed the inhibitory effect of high stirring speed on cell growth, the characteristics of cell growth could be investigated more in detail by investigating specific growth rates. By optimizing the time

window for specific rate calculations, the temporal resolution of growth kinetics was possible. The results suggest that high stirring speed has a significant effect on μmax in the early growth phase but it does not impair cell growth in the later phases of the cultivation, probably since other limitations such as nutrient depletion are more dominant. From a QbD point of view, stirrer speed is a key process parameter in the early growth phase of the investigated process with a significant effect on cell growth.

Insulin content of basal medium

Insulin is known as a growth factor for mammalian cells and is included in most chemically defined basal media [24,25]. Insulin was also reported to affect glucose uptake rates in CHO cells [26]. In order to test the effect of insulin addition in the basal medium on cell growth kinetics and metabolism, three cultivations differing in basal medium insulin content were conducted (Table 4). The variability in the initial VCDs was caused by the variations in the volume of the transferred inoculum.

Table 4. Parameter set for investigating the effect of insulin addition in basal medium. The last dataset in the table was derived from a culture initiated with the same cultivation conditions as the "w/o Ins" experiment (same parameters in the first three days).

Insulin	**initial VCD [cells*ml^{-1}]**	**ID**
-	$4.9 * 10^5$	w/oIns, $4.9 \cdot 10^5$
+	$5.7 * 10^5$	+Ins, $5.7 \cdot 10^5$
+	$4.3 * 10^5$	+Ins, $4.3 \cdot 10^5$
-	$4.3 * 10^5$	w/oIns, $4.3 \cdot 10^5$

Different VCD profiles were observed in the two "+Ins" cultivations (Figure 4A). The reason for these differences was the variability in the initial VCD. Consequently, when using concentrations from the experimental datasets, the statistical evaluation of the effect of basal medium insulin content was not feasible. However, the calculation of specific rates enabled the comparison of culture physiology in the three experiments (Figure 4B).

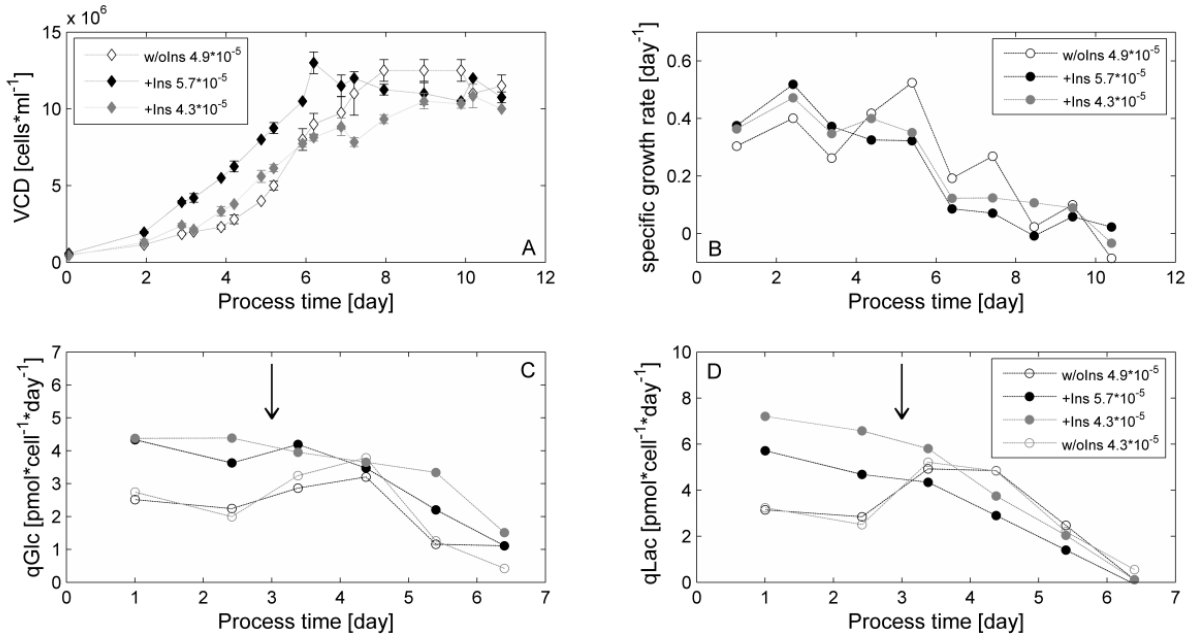

Figure 4. Effect of insulin addition in basal medium on cell growth and metabolism. (**A)** VCD values. Error bars indicate the calculated standard error of the triplicate VCD measurements. (**B)** Specific growth rates. (**C)** Specific glucose uptake rates. (**D)** Specific lactate production rates. Arrows indicate the time point when the feeding regime was initiated.

Higher specific growth rates were observed in the first days of the "+Ins "cultivations. On day 3, a bolus feed containing insulin was added to all cultivations. After this event, cell growth recovered in the "w/oIns" cultivation as indicated by an increase in specific growth rate and followed a similar pattern as in the "+Ins " runs. Based on these findings, culture physiology was resolved into two phases (batch and fed-batch phase) and the effect of basal medium insulin content on culture physiology was investigated in the batch phase.

An average of the specific growth rate was calculated for the batch period (day 1-3) in order to assess the statistical significance of the observed difference. An ANOVA was conducted ($F=10.1$; $p=0.19$) and as p-values were higher as the chosen threshold level of 0.1, the difference was not accepted as significant. In order to increase the degree of freedom of the analysis, data from the first three days of another cultivation without insulin in the basal medium ("w/oIns, $4.3 \cdot 10^5$"") was added to the statistical analysis. ANOVA was conducted again for the extended dataset, and the difference in μ ($p=0.04$) was found to be significant (Figure 5A). Accordingly, the dataset of a fourth experiment had to be involved in the ANOVA in order to decrease the probability of false negative decisions. However, even more additional experiments would have been necessary to show this effect based on concentration profiles as the effect of insulin was masked by the variability in initial VCD. The use of

variables with condensed information content (specific growth rate) reduced the problem into an univariate question by neutralizing the effect of the variation in the initial conditions.

Basal medium insulin content also affected glucose uptake in the batch phase of the cultivations, as indicated by the higher qGlc values in the "+Ins" experiments (Figure 4C). In accordance with this, higher qLac values indicated a faster overflow metabolism in these cultivations (Figure 4D). Both effects were confirmed to be significant by the statistical analysis of average specific rates (Figure 5B and 5C). As the effect of basal medium insulin content was proven to be statistically significant on μ, qGlc and qLac, the parameter was designated to be critical for the control of cell growth and overflow metabolism.

Inoculation strategy

This experiment aimed to investigate the effect of the physiological status of the inoculum and the inoculation procedure on culture performance in the production bioreactor. Therefore, bioreactors were inoculated with two different strategies on two different days from the same N-1 shake flask culture (Table 5).

Table 5. Parameter set for investigating the effect of inoculation strategy on culture performance.

Inoculation procedure	N-1 culture duration [day]	initial VCD [cells*ml^{-1}]	ID
N-1 broth centrifuged and resuspended	4	$4.8 \cdot 10^5$	C4
	6	$4.6 \cdot 10^5$	C6
Direct transfer from shake flask	4	$5.0 \cdot 10^5$	D4
	6	$4.9 \cdot 10^5$	D6

Process parameters and metabolite concentrations were monitored in the N-1 shake flask in order to gain information on the physiological status of the inoculum. Glutamine was depleted on day 4 and a metabolic shift was observed after this time point. The cells started to take up lactate and ammonium as indicated by decreasing concentrations of these metabolites. The pH of the cultivation broth started to increase and the pCO2 value decreased after day 4. However, the viable cell density increased further and reached a value of $8 \cdot 10^6$ cells/ml on day 6. The measured parameters suggested a different physiological status on day 4 and day 6 in the N-1 culture.

Furthermore, two different inoculation procedures were compared, which represent standard techniques in small scale (cells centrifuged and re-suspended in fresh medium, marked

with "C") and lab- as well as production scale (direct transfer in bioreactor, marked with "D"). A difference was expected to occur due to the medium change and consequently the removal of toxic metabolites such as lactate or ammonium.

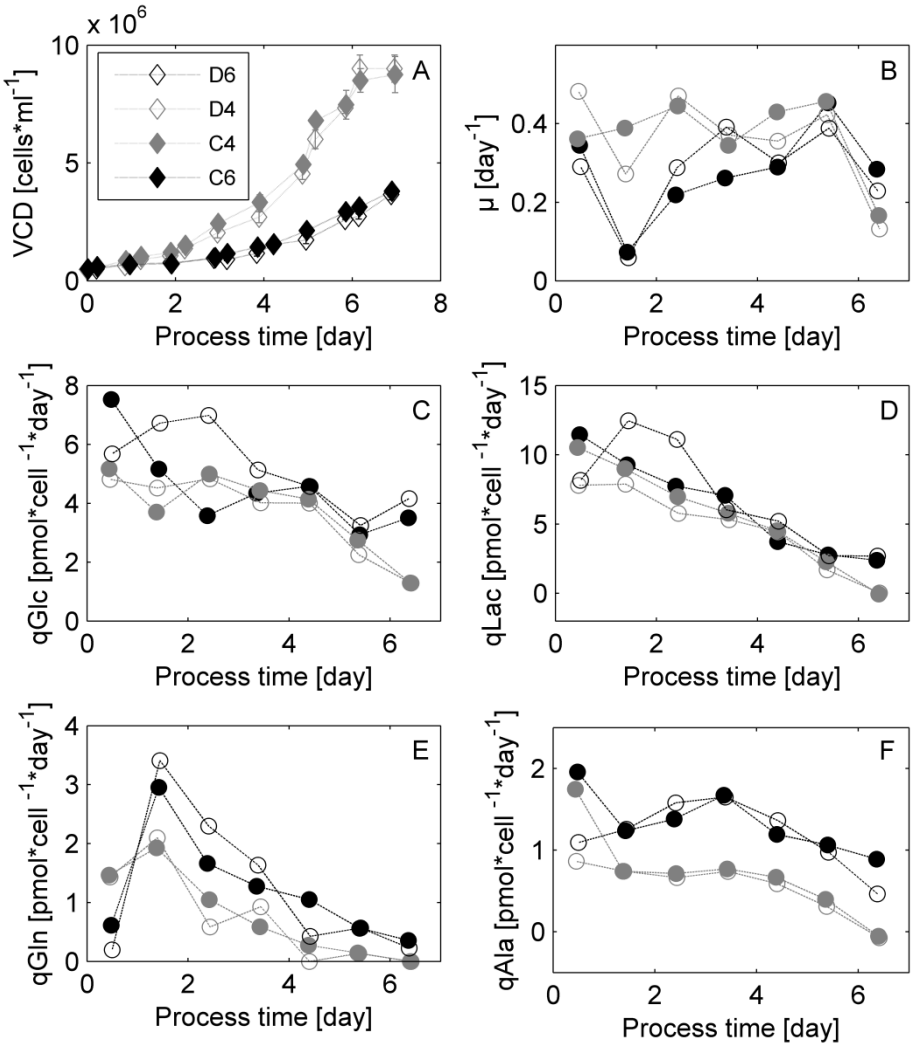

Figure 6. Effect of N-1 culture duration (4 or 6) and inoculation procedure (C or D) on cell culture performance. (**A)** VCD values. (**B)** Specific growth rates. (**C)** Specific glucose uptake rates. (**D)** Specific lactate production rates. **(E)** Specific glutamine uptake rates. (**F)** Specific alanine production rates.

Slower cell growth was observed in the bioreactors inoculated from the six day old N-1 culture (Figure 6A). The difference was reflected in the calculated specific growth rates in the first three days of the cultivation as well; however, the further temporal progression of μ showed that cell growth recovered in the C6 and D6 cultures after day 3, as indicated by similar μ values as in the C4 and D4 bioreactors (Figure 6B).

Very interesting differences were observed in the metabolic behavior of the cultures when specific metabolic rates with temporal resolution were plotted against process time (Figure 6C-F). Based on physiological events detected on the plots, the cultivations were divided in four phases: day 1, day 2-3, day 4-6, day 7. Two-way ANOVA was conducted to

investigate the effect of both N-1 culture duration and inoculation procedure on culture physiology in each phase (Table 6).

Table 6. 2-way ANOVA assessing the effect of N-1 culture duration and inoculation procedure on specific rates in different phases of the production culture. p-values in bold are smaller than the 0.1 threshold and indicate statistically significant effects.

	day 1		day 2-3		day 4-6		day 7	
Factor / Response	Day (4 or 6)	Procedure (C or D)	Day (4 or 6)	Procedure (C or D)	Day (4 or 6)	Procedure (C or D)	Day (4 or 6)	Procedure (C or D)
qGlc	0.28	0.38	0.49	0.41	0.37	0.99	**0.08**	0.50
qLac	0.26	**0.06**	0.43	0.72	0.26	0.70	**0.03**	0.34
qGln	0.12	0.45	0.18	0.67	**0.06**	0.63	0.10	0.50
qAla	**0.03**	**0.01**	**0.07**	0.64	**0.04**	0.69	0.17	0.47

N-1 culture duration had a significant effect only on alanine production rates, as higher qAla values were observed in the C6 and D6 reactors. This difference prolonged over the entire experiment (Figure 6F) and was confirmed to be significant until day 6 by the statistical analysis (Table 6). Interestingly, specific glutamine uptake rates were lower on the first day in the C6 and D6 reactors compared to those bioreactors inoculated from the 4 day old N-1 culture (Figure 6E). However, this difference was not shown to be significant by the statistical analysis ($p=0.12$). This may be a type II error (false negative), probably due to the higher standard deviation of the glutamine concentration measurement and the subsequent propagation of the error in the calculated specific rates. Another issue may be the low sample number (no replicate experiments conducted). Specific glutamine uptake rates recovered in the C6 and D6 cultivations after day 1 and remained higher as the values observed in C4 and D4 reactors throughout the experiment (effect shown to be significant in the day 4-6 phase). The correlation between high glutamine uptake rates and high alanine production is in accordance with published models of CHO metabolism [27,28].

Another significant difference was the production of the overflow metabolites lactate and alanine according to the inoculation procedure. In those cultivations inoculated after a medium change from the shake flask N-1 culture (C4 and C6) to the bioreactor, significantly higher qLac and qAla values were observed in the first day of the experiment. The values recovered until day 2 (Figure 6D and 6F) decreasing to levels observed in the directly inoculated cultivations. A fast production of overflow metabolites in the first day can be a

result of the lack of inhibition effects these metabolites exert on the enzymatic reactions which produce them [29]. However, concentrations of lactate and alanine were low in the directly inoculated cultivations as well (3 mM and 1.3 mM in average, respectively). Another possible reason of the accelerated overflow metabolism in the C4 and C6 cultures might be the total absence of extracellular signaling molecules after medium change leading to an environmentally induced increase in metabolic activity [30].

Specific lactate production rate increased to very high values in the D6 culture between day 2 and 3, but the reason for this phenomenon is unknown. However, the observed qLac profile is in accordance with the higher glucose uptake rate in days 2 and 3 (Figure 6C and 6D), indicating that lactate overproduction was the result of a high glycolytic flux. As no repetition of the experiments was performed, the ANOVA model could not show factor interactions and thus did not indicate the observed lactate overflow in the D6 experiment as significant.

Furthermore, the statistical test indicated significant effect of N-1 culture duration on qGlc and qLac values in the very last phase of the cultivation. This phenomenon is a consequence of the higher viable cell densities reached in the C4 and D4 cultivations leading to nutrient limitations and a subsequent decrease in glycolytic flux and overflow metabolism in these bioreactors (Figure 6C and 6D).

The results highlight the importance of selecting time periods for the detection of physiological changes based on specific rates. First, specific rates with the highest possible temporal resolution have to be analyzed to determine physiological events. Based on these observations, physiological phases can be defined and subsequently, specific rates can be calculated for these phases. Thereafter, statistical analysis can be performed to assess the significance of the observed effects. This workflow is applicable as a general tool for information extraction from univariate cell culture experiments (Figure 7). A careful consideration of the acceptable risk for false positives or false negatives, allows for the reduction of the number of experiments to investigate a previously imposed hypothesis. Furthermore, the use of physiological variables with condensed information content facilitates the generation of process understanding in the context of Quality by Design.

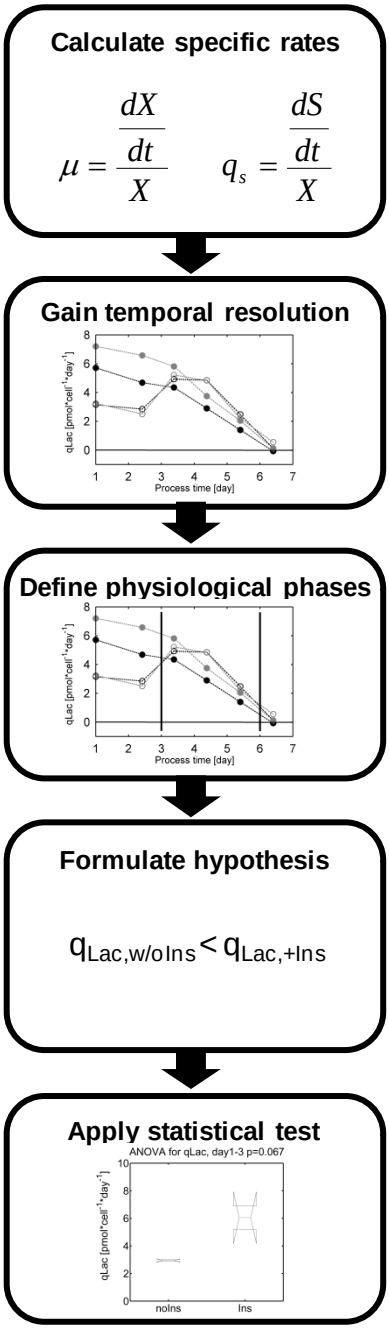

Figure 7. The proposed workflow to quantify physiological variables and to perform statistical tests for enhanced process understanding.

Conclusions

The recent study discussed different aspects of cell culture process development by investigating the effect of selected process parameters on cell growth and metabolite formation. The datasets of uni- and bivariate experiments were evaluated along Quality by Design principles in order to facilitate data exploitation. Accordingly, the cornerstones of the reported approach are:

- Use of physiological variables to extract information from a small number of experiments
- Temporal resolution of specific rates to understand the effect of the investigated process parameters on cell physiology
- Application of statistical tools to verify hypothesis

Based on these principles, the following workflow was proposed for the evaluation of cell culture experiments:

First, prime variable datasets have to be transformed into specific rates which contain condensed physiological information. These variables are not influenced by the effect of variations in initial experiment conditions such as cell density or broth volume. Thus, an important benefit of using specific rates is the scalable information content independent of initial conditions which enabled a reduction in the number redundant experiments in this study. The next step of the evaluation is to calculate physiological variables for finite time periods. The temporal resolution of specific rates enabled to identify novel characteristics of the effect of the investigated process parameters on cell physiology. The last step is to verify the formulated hypotheses with statistical tests. A statistical analysis estimates the probability of a false decision based on the existing dataset. Based on this probability one can decide if the level of risk is acceptable or if further experiments are needed to decrease the risk of a possible false decision in process development. Accordingly, statistical tests supported decision making in this study and helped to keep the number of experiments needed to prove a research hypothesis down.

The above discussed workflow does not contain entirely novel tools, but interprets existing tools of process development in the context of the QbD paradigm. By implementing this workflow, novel insights into the effect of the selected process parameters on cell physiology have been gained. Based on this enhanced knowledge, sophisticated physiological control strategies can be developed in the future to control cell growth or metabolic overflow. These strategies can be used subsequently to drive robust processes with optimal growth and metabolic profiles, leading to a reduction in out-of-specification production batches. A major challenge in the implementation of physiological control strategies is the acceptance of specific rates as Critical Process Parameters (CPPs) from the side of the regulatory bodies. In order to facilitate this paradigm change and to involve physiological variables in regulatory filings, similar workflows as the one demonstrated in this contribution are needed.

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Symbols

IVCD = integral of viable cell density (cells · day · ml^{-1})

q_s = specific substrate uptake rate (mmol · $cell^{-1}$ · day^{-1})

S = total amount of substrate in the cultivation broth (mmol)

t = time (day)

VCD = viable cell density (cells · ml^{-1})

X = total amount of cells in the cultivation broth (cells)

μ = specific growth rate (day^{-1})

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Combining Mechanistic and Data-Driven Approaches to Gain Process Knowledge on the Control of the Metabolic Shift to Lactate Uptake in a Fed-Batch CHO Process

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A growing body of knowledge is available on the cellular regulation of overflow metabolism in mammalian hosts of recombinant protein production. However, to develop strategies to control the regulation of overflow metabolism in cell culture processes, the effect of process parameters on metabolism has to be well understood. In this study, we investigated the effect of pH and temperature shift timing on lactate metabolism in a fed-batch Chinese hamster ovary (CHO) process by using a Design of Experiments (DoE) approach. The metabolic switch to lactate consumption was controlled in a broad range by the proper timing of pH and temperature shifts. To extract process knowledge from the large experimental dataset, we proposed a novel methodological concept and demonstrated its usefulness with the analysis of lactate metabolism. Time-resolved metabolic flux analysis and PLS-R VIP were combined to assess the correlation of lactate metabolism and the activity of the major intracellular pathways. Whereas the switch to lactate uptake was mainly triggered by the decrease in the glycolytic flux, lactate uptake was correlated to TCA activity in the last days of the cultivation. These metabolic interactions were visualized on simple mechanistic plots to facilitate the interpretation of the results. Taken together, the combination of knowledge-based mechanistic modeling and data-driven multivariate analysis delivered valuable insights into the metabolic control of lactate production and has proven to be a powerful tool for the analysis of large metabolic datasets. © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2015
Keywords: mammalian cell culture, lactate metabolism, metabolic flux analysis, PLS-R, multivariate experimental design

Introduction

In the last decades, significant efforts have been made to increase the productivity and robustness of cell culture processes, enabling the accumulation of an extensive knowledge on interactions between process control features, cell physiology and product formation.[1] Thereby, the investigation of cell metabolism has become a key method in bioprocess characterization and optimization.[2] Metabolomic analysis is essential to identify shifts in cell physiology typically occurring during industrial fed-batch cultivations and to adapt process control to the metabolic phenotype. Moreover, a thorough understanding of interactions between process control strategies and cell metabolism can be used to steer cell physiology towards a high producing phenotype.

Mammalian cell metabolism experiences different phases during fed-batch cultivation. The extensive cell growth in the first phase of fed-batch Chinese hamster ovary (CHO) processes is characterized by a high glycolytic flux and high lactate production rate. In this phase, NADH generated by the elevated glycolytic flux is re-oxidized to NAD via conversion of pyruvate to lactate.[3] At the end of the growth phase, the glycolytic activity is suppressed, followed by a subsequent decrease in the lactate production rate. At low glycolytic fluxes, CHO cells are able to switch from lactate production to uptake by converting the substance back to pyruvate.

Additional Supporting Information may be found in the online version of this article.

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The onset of lactate consumption was shown to be dependent on various initial parameters such as cell line or culture medium composition.[4–6] Moreover, process parameters such as cultivation pH and temperature have been demonstrated to influence lactate metabolism.[7] A linear correlation of specific lactate production rate and the setpoint of pH and temperature has been observed in batch cultivations,[8] and the metabolic switch to lactate uptake has been shown to be dependent on pH setpoint.[9,10] However, as pH and temperature do not only influence overflow metabolism, but also cell growth and productivity,[11,12] a biphasic strategy is often applied where the parameters are first controlled at a setpoint which favors extensive cell growth and then shifted to a lower setpoint where overflow metabolism is down regulated. Whereas most publications investigate the effect of the setpoint of pH and temperature shifts, the timing of these process events can be also critical to optimize their effect on lactate metabolism and cell growth. Although Nolan et al. considered timing as an important factor, they only investigated the effect of temperature shift timing.[13] Yoon et al conducted a simultaneous shift in temperature and pH at various time points of a fed-batch cultivation, however the authors focused on the effect of the parameter shifts on cell growth but not on metabolism.[14] As to our knowledge, a multivariate experimental design investigating the effect of pH and temperature shift timing on overflow metabolism has not been published in the scientific literature.

The metabolic control of lactate production on a cellular level has been frequently investigated, mostly focusing on the effect of glycolytic activity.[3] However, lactate metabolism has been recently reported to be influenced by numerous metabolic pathways beside glycolysis, such as tricarboxylic acid (TCA) cycle activity,[15] or the uptake of several amino acids.[16] Accordingly, a systematic investigation of cell metabolism is necessary to understand the effect of the experimental factors on the regulation of lactate metabolism. Recent studies demonstrated that stoichiometric metabolic models such as flux balance analysis[17,18] or metabolic flux analysis[19,20] are powerful tools to gain insight into mechanistic links between intracellular fluxes and lactate metabolism. However, metabolic models result in large datasets, the interpretation of which necessitates advanced evaluation tools. Wahrheit et al. recently demonstrated how the combination of MFA, linear regression, and heat maps can be used to identify the effect of experimental factors such as glutamine concentration on intracellular fluxes.[21] Although PLS-R and heat maps have been successfully used for knowledge extraction from large bioprocess development datasets,[22] the combination of multivariate data analysis methods with metabolic flux analysis has not been reported in the literature to our knowledge.

In this study, a Design of Experiments approach was used to gain process knowledge on the control of overflow metabolism. Temperature and pH shift timing were defined as experimental factors and their multivariate effects on cell growth, metabolism, and product formation were investigated. To support knowledge extraction from the experimental dataset, metabolic flux analysis was applied and the links between lactate metabolism and major intracellular fluxes were assessed by PLS-R. The applied methodology triggered process understanding by combining the benefits of statistical experimental design, multivariate data analysis, and the investigation of metabolic fluxes on a cellular level.

Materials and Methods

Cell line and preculture

A CHO-K1 cell line producing an IgG1 monoclonal antibody was cultivated in disposable shake flasks before inoculating the bioreactors. Stocks were revived in commercially available basal medium (ActiCHO P, GE Healthcare, Little Chalfont, UK), supplemented with 8 mM L-Gln (Life Technologies, Carlsbad, CA) and 5 mg/l Insulin (GE Healthcare, Little Chalfont, UK). The cells were sub-cultured every 3–4 days with a seeding density of $0.3 \cdot 10^6$ cells/mL and were grown in shake flasks of different scales. The shake flasks were incubated at 37°C with humidified air containing 5% CO_2 at 100 rpm orbital shaking.

Bioreactor cultivations

Fed-batch cultivations were performed in bioreactors with 1 liter maximal working volume (Sartorius AG, Göttingen, Germany). The targeted seeding cell density was $0.5 \cdot 10^6$ cells/mL. The bioreactors were equipped with on-line pH, temperature and pO_2 probes and the process parameters were controlled by a Biostat BPlus Twin DCU (Sartorius AG, Göttingen, Germany). Stirring speed was set to 125 rpm, initial cultivation temperature was 37°C, initial pH setpoint was 7.2 and the dissolved oxygen rate was maintained at 40% of air saturation by air–oxygen mixture sparging. The pH value was controlled in the range of ± 0.02 of the current setpoint by automatic addition of 10% H_3PO_4 solution or 0.5 M Na_2CO_3 solution. To reduce foaming, FoamAway (Life Technologies, Carlsbad, CA) was added. The shifting of temperature and pH was performed at timepoints according to the experimental design. The setpoints after the shift were 33°C and 6.90 for temperature and pH respectively and the shifts were performed by the change of the controller setpoint directly to the shifted parameter value. Both pH and temperature were adjusted to the new setpoint within a few minutes by the controller. The basal medium was the same as the one used for the preculture (vide supra). Feeding was initiated on the third cultivation day by adding feed medium every second day. The actual feed volume was determined as 15% of the current broth volume. The feed medium was a proprietary medium.

Measurements

Real-Time Measurements. The bioreactors were placed on balances (Mettler Toledo, Zurich, Switzerland) to determine broth volume gravimetrically. At the end of the cultivations, the broth volume was measured volumetrically and the error of the gravimetric determination was verified to be less than 5% in all experiments. Feed medium and glucose stock solution volumes were also determined gravimetrically with balances (Mettler Toledo, Zurich, Switzerland). The balance signals as well as on-line measured process parameters (pH, pO2 and temperature) were collected by the Biostat BPlus DCUs and processed in a Citect SCADA system (Schneider Electric, Rueil-Malmaison, France) via local area network connection.

At-Line and Off-Line Measurements. At-line samples were taken every 24 hours or more frequently to measure several process variables. pH measurement for in situ pH meter re-calibration was performed with a S47 SevenMulti pH meter (Mettler Toledo, Zurich, Switzerland). Viable cell

density as well as viability was determined in triplicates by Countess automated cell counter (Life Technologies, Carlsbad, CA). Samples were centrifuged at 3000 rpm for 10 minutes (Rotanta 460 R, Hettich Zentrifugen, Kirchlengern, Germany) and cell-free supernatants were stored at −20°C until further analysis. Glucose, lactate, glutamine, glutamate and ammonium concentrations were determined in duplicates by enzymatic assays (Cedex BioHT, Roche Diagnostics, Mannheim, Germany). Amino acid concentrations were determined by NMR (Bruker 500 MHz Avance III, Bruker Biospin, Rheinstetten, GE). The concentrations were determined with this method with a standard error lower than 5% as determined by measuring duplicates of three selected samples (data not shown).

Calculation of physiological variables

Specific rates were calculated in MS Excel (Microsoft Corporation, Redmond, WA) from the measured prime variables by using the equations shown as Supporting Information.

The time window of specific rate calculation was determined to be 12 hours or 24 hours. The goal was to define a time window value which is low enough to gain a sufficient temporal resolution of cell growth and metabolism, and high enough to minimize the effect of error propagation from prime measurement errors.

To assess the correlation between metabolic fluxes, yields were calculated using two different calculation methods. A cumulative yield ($Y_{CS1/CS2}$) was used for the statistical analysis to assess the effect of the experimental factors on the ratio of metabolic fluxes. The other yield variable ($Y_{qS1/qS2}$) was calculated by using specific rates to gain a temporal resolution of the changes in the mechanistic relation of metabolic fluxes.

Oxygen uptake rate (OUR) was determined with the stationary liquid phase method as described in the literature.[23] The effect of temperature on the Henry coefficient was considered, and kLa was determined as a function of broth volume and aeration rate for the accurate calculation of OUR.

Statistical analysis and evaluation

The experimental design was developed and evaluated using the design of experiments software MODDE (Umetrics, Sweden). The design resulted in 11 experiments, which were conducted in three successive cultivation campaigns. The three center point runs were performed in different campaigns. For the evaluation, the factors were orthogonally scaled and centered before fitting the model with multivariate linear regression (MLR). To improve model precision, non-significant model terms were excluded (backward elimination) until the maximum Q^2 was achieved.

To investigate the relationship between specific lactate production rate (q_{Lac}) and other metabolic fluxes, a PLS-R model was developed where q_{Lac} was defined as the predicted variable (Y) and other 67 intra- and extracellular rates were defined as explanatory variables (X). 8 PLS-R models were developed for the eight time points between nine sampling events where the specific rates were calculated (Supporting Information Figure 4). The data of all conducted experiments (11, according to the DoE design) was involved in the respective PLS-R model. The lack of fit estimates for the eight PLS-R models were: 0,74; 0,74; 0,78; 0,80; 0,87; 0,87; 0,85; 0,93. This value expresses the explained variance for X, where 1 corresponds to 100% explained variance. An objective criterion does not exist for the lack of fit estimate, however, the values for the eight PLS-R models were found to be appropriate for the purposes of the models, namely the identification of correlations between q_{Lac} and other intra- and extracellular fluxes. For improved model interpretation, the variable importance in projection (VIP) method was applied for the PLS-R analysis.[24] Finally, k-means cluster analysis was performed to detect groups of fluxes showing a similar relationship to q_{Lac}.

Time resolved stoichiometric metabolic flux analysis

Stoichiometric metabolic flux analysis was applied to estimate intracellular metabolic flux distributions of the CHO cell line over the course of the cultivation. A metabolic network of the central carbon metabolism of CHO cells was applied based on published works.[21,25] The network comprised glycolysis, pentose phosphate pathway (PPP), the TCA cycle and amino acid metabolism. Compartmentation was neglected and anaplerotic fluxes were lumped into one reaction allowing carbon exchange between phosphoenolpyruvate (PEP) and oxaloacetate. PPP activity was directly constrained by the amount of precursors and NADPH necessary for biomass formation.[17,20] Biomass composition of CHO cells was taken from literature[21,26] and an additional flux was introduced into the network to take the anabolic demand for antibody formation into account. Unbalanced pools of MTHF and NH_4^+ were introduced to allow transfer of C1 units and account for the loss of volatile NH_3 over the course of the cultivation as described before.[21] Balancing of redox cofactors was introduced for NADH and regeneration of excess NADH was taking place via respiration. Specific rates of uptake and production of metabolites at sampling time points were used as model inputs as well as well as specific OUR, which was calculated based on the determined OUR (*vide supra*) and could be subsequently used as a constraint for the activity of the respiratory chain.

The resulting stoichiometric matrix S encompassed 41 metabolites with 40 unknown and 29 known fluxes and could therefore be solved by the least squares method:

$$v_c = -\mathrm{inv}(S_c) \cdot S_m \cdot v_m \quad (1)$$

where v_c and v_m are the arrays of intracellular (calculated) and extracellular (measured) fluxes and S_c and S_m the respective corresponding stoichiometric matrices (Supporting Information Table 1 and SII). As additional constraint, irreversibility of certain reactions were introduced (Supporting Information Table 1). Briefly, reactions of PEP-kinase and pyruvate dehydrogenase, the decarboxylating reaction of the PPP and lumped amino acid degradation fluxes were considered irreversible. The matrix of unknown fluxes v_c was overdetermined with 41 linear independent equations and 40 unknown variables (fluxes), which allowed detection of gross measurement errors via data reconciliation as described before.[27,28] For a matrix of this size, an h-value above 42.6 was considered critical.

Redox variable R

The redox variable R was introduced by Nolan et al.[13] and describes the ratio between cytosolic NADH production to mitochondrial NAD+ regeneration via oxidative phosphorylation:

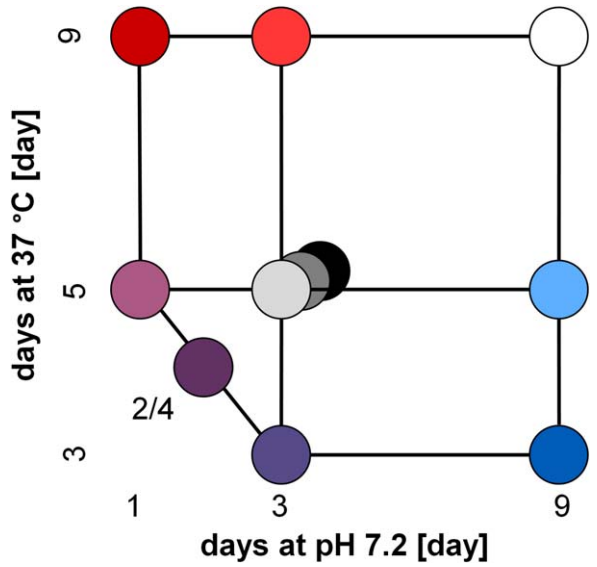

Figure 1. The experimental design.

The center point was performed three times in the three different cultivation campaigns. The experiments are marked with the same colors as on the figures below.

$$R = \frac{\sum q_{\mathrm{NADH,cyt}}}{q_{\mathrm{NAD^+,mit}}} \quad (2)$$

With $\sum q_{\mathrm{NADH,cyt}}$ as the sum of all NADH producing fluxes in the cytosol and $q_{\mathrm{NAD+,mit}}$ as the flux of oxidative phosphorylation taking place in the mitochondria.

An R value above 1 indicates an insufficient capacity of the respiratory chain, causing NAD^+ regeneration via fermentative pathways (lactate production). A value below 1 indicates complete regeneration of redox equivalents via the respiratory chain.

Results and Discussion

The goal of the study was to investigate the effect of pH and temperature shift timing on lactate metabolism in a fed-batch CHO process. To support the sound statistical analysis of the results as well as to identify possible interaction effects of the two experimental factors, a DoE experimental design was developed and executed.

The experimental design

The factor levels were defined based on typical timepoints for the shift of pH and temperature in fed-batch CHO processes.[14] The experimental design was planned with two quantitative factors at three discrete levels (Figure 1). The factor values were defined by calculating the number of days the culture run in the non-shifted condition.

An irregular design was chosen due to the expected low cell densities in the low-low corner of the experimental design. An important diagnostic parameter for irregular designs is the condition number, which is a measure for the quality of design and can be used to detect design problems, such as lack of orthogonality of the runs in the design. If the condition number is too high, the design matrix is called ill-conditioned and small errors in the estimation of the coefficients of the linear algebra problem will result in a large error in the prediction of the response. The calculated condition number was lower than 12 for each model (the threshold value suggested by the guidelines issued by Umetrics for their Design of Experiments software MODDE), a highest value of 9.7 for those models where all interaction and quadratic terms were involved. Accordingly, the design was found to be appropriate for the experiment scope.

To investigate the quality of the experimental dataset, we visualized center point variability of the measured (Figures 2A,B) and the calculated physiological variables (Figures 2C,D). Moreover, the relation of the center point error to the variability observed across the entire experimental design was assessed by investigating whether the "reproducibility" value calculated by the statistical software is above the recommended threshold of 0.5 (constrain true for each of the models, data not shown).

DoE evaluation

First, IVCD was defined as a response variable to identify the effect of the experimental factors on cell growth (for IVCD curves see Supporting Information Figure 1A). IVCD was affected by the timing of temperature shift, as indicated by the significant linear and quadratic terms of the model (Table 1). However, pH shift did not affect cell growth, which is also indicated on Figure 2A, where the two experiments with only pH shift („pH3T9" and „pH1T9", red colors) show very similar VCD profiles to the control run ("pH9T9", empty squares). Literature data on the effect of pH on cell growth in cell culture processes suggest that a shift to pH 6.9 affects specific growth rate only to a small extent, and the effect is much more pronounced at pH 6.8.[12,29,30] Considering this information and the cell line-dependent physiological response on process parameters, the absent effect of the shift to pH 6.9 on cell growth in our experimental system is not contradictory to literature data.

To investigate how the experimental parameters affected lactate metabolism, various physiological variables were defined as responses in the statistical evaluation (Table 1). Cumulative lactate production was divided with the integral of viable cell count (C_{Lac}/IVC) to investigate lactate metabolism with a cell count independent variable. pH shift timing had a significant positive effect on C_{Lac}/IVC, indicating that a shift in pH at an early cultivation timepoint decreased the specific lactate production rate of the cells and triggered the switch to lactate uptake. Interestingly, the timing of temperature shift had a significant negative effect on lactate production, probably as a consequence of low cell densities and high nutrient availability in cultivations with an early temperature shift. Accordingly, the cultivation where the temperature shift was performed already on day three ("pH9T3") showed higher lactate production rates as the control cultivation without temperature shift ("pH9T9") in the last four days of the process (Figure 2C). The process time where the specific lactate uptake rate (calculated for 12 hour periods) fell below 0 was also defined as a response variable (day@ $q_{\mathrm{Lac}} < 0$). For this response variable, the fitted model showed only a significant effect of pH, both for the linear and quadratic terms (Figure 3A). Interestingly, the interaction term of temperature and pH was also significant indicating that temperature shift induces the metabolic shift to lactate uptake when pH shift is also conducted during the cultivation. The curvature of the contour level lines in the low-low corner of the response contour plot indicates this interaction effect (Figure 3B).

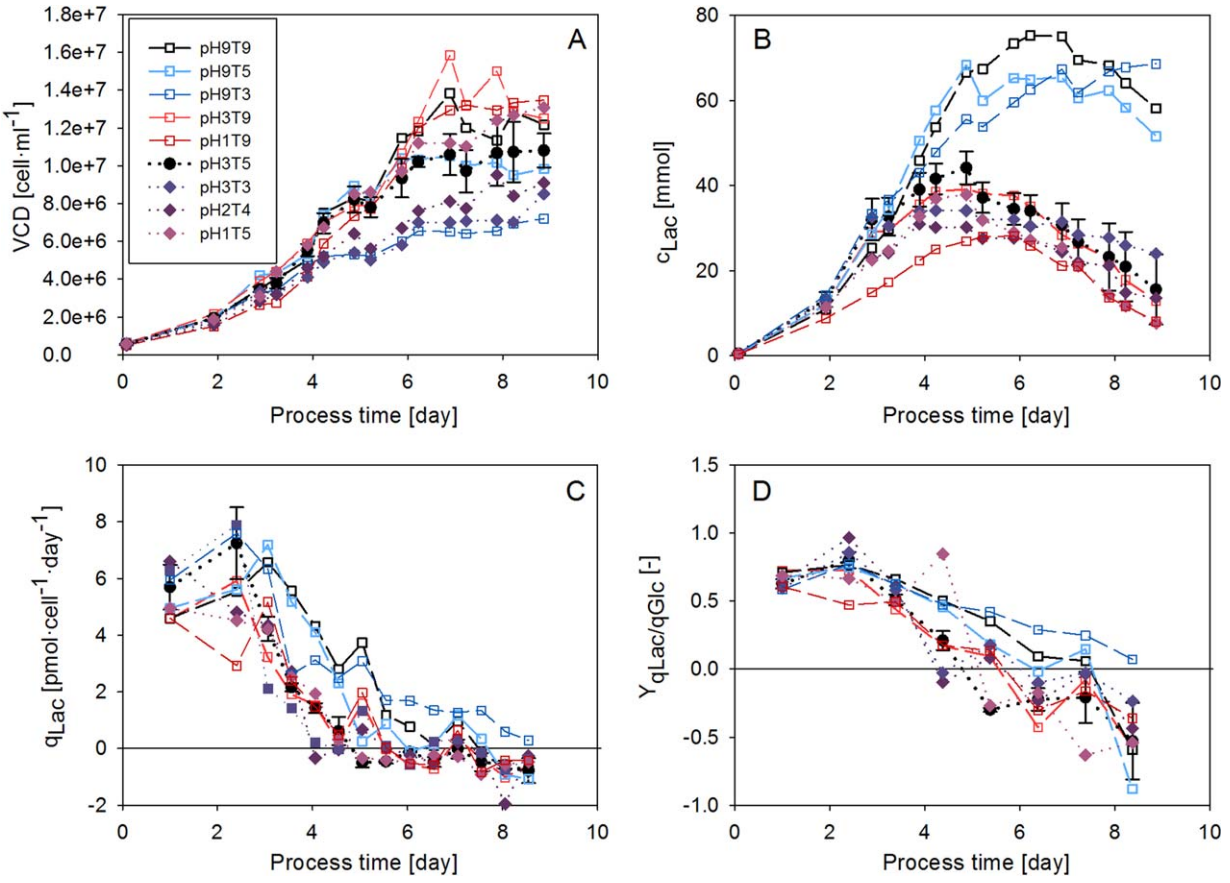

Figure 2. VCD and lactate concentration profiles, specific lactate uptake rates and the ratio of lactate and glucose uptake rates in the 11 experimental points.

Black circles represent the average value of the three center point runs („pH3T5"); error bars indicate the calculated standard deviations.

Table 1. Normalized Model Coefficients for the Analysis of the Effect of the Experimental Factors on Cell Growth and Lactate Metabolism

	IVCD	C_{Lac}/IVC	C_{Glc}/IVC	$Y_{CLac/CGlc}$	day@ $q_{Lac} < 0$
R^2	0.86	0.96	0.86	0.96	0.98
Q^2	0.69	0.85	0.73	0.91	0.94
Days at pH 7.2	0.28	**0.98**	**1.16**	**0.79**	**0.95**
Days at 37°C	**1.25**	**−0.47**	–	**−0.77**	0.14
pH*pH	–	0.30	–	0.38	**0.66**
temp*temp	**−1.48**	**0.60**	–	**0.78**	–
pH*temp	–	–	–	**0.47**	**−0.47**

Bold characters indicate significant terms. Terms marked with dash were not included in the model. day@$q_{Lac} < 0$ indicates the cultivation time where the cells shift to lactate uptake

The prediction plots confirm this interaction effect when the two factors are set to different values (Figure 4). If the pH shift factor is set to 9 (which means no pH shift is performed during the cultivation), an early temperature shift delays the metabolic shift to lactate uptake. This is in accordance with the observation that in the "pH9T3" cultivation the cells produced lactate until the end of the run and did not shift to lactate uptake (Figure 2C). However, if the pH shift is conducted on the third day of the cultivation (center point experiment, (days at pH 7.2] = 3), the model indicates that an early temperature shift triggers the metabolic shift to lactate uptake. This result is confirmed by Figure 2D where the center point runs ("pH3T5", black color) show the metabolic shift immediately after the temperature shift on the fifth cultivation day, whereas those runs where only pH shift was conducted ("pH3T9" and "pH1T9", red colors) start to take up lactate one day later. The earliest metabolic shift to lactate uptake was observed at day four in the experiments "pH2T4" and pH3T3". Taken together, the shift from lactate production to lactate uptake can be controlled in a broad time window (between day four up to the end of the cultivation) with the help of the two experimental factors. Moreover, an interaction effect of pH and temperature shifts on the metabolic switch to lactate uptake has been identified.

Combining metabolic flux analysis and PLS-R

To gain a deeper understanding of the physiological responses on the experimental factors, mechanistic and data-driven approaches were combined in the evaluation of the experimental dataset. First, time-resolved metabolic flux analysis was performed to estimate intracellular fluxes based on stoichiometric assumptions for each sampling point. Thereafter, a PLS-R model was developed where q_{Lac} was defined as the predicted variable to identify correlations between metabolic fluxes and lactate metabolism.

The time-resolved metabolic flux analysis enabled to analyze the distribution of intracellular fluxes as a function of process time. Accordingly, the effect of the process

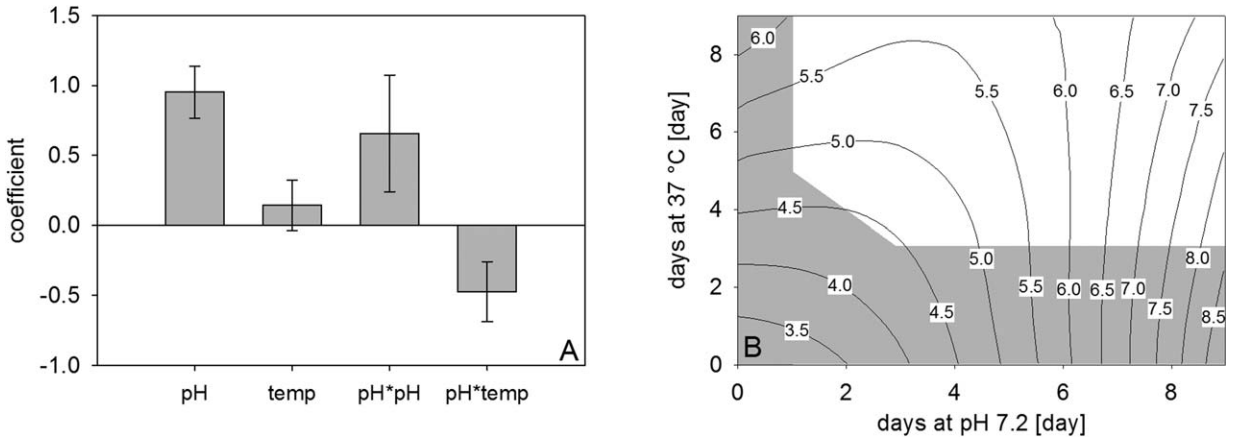

Figure 3. The effect of temperature and pH shift timing on the timepoint of the metabolic shift to lactate uptake.

(A) Coefficient plot with linear, quadratic and interaction terms. (B) Response contour plot. The value of the response indicates the process time [day] when the model predicts the onset of lactate uptake. The area marked with grey is the region where no experiments have been conducted.

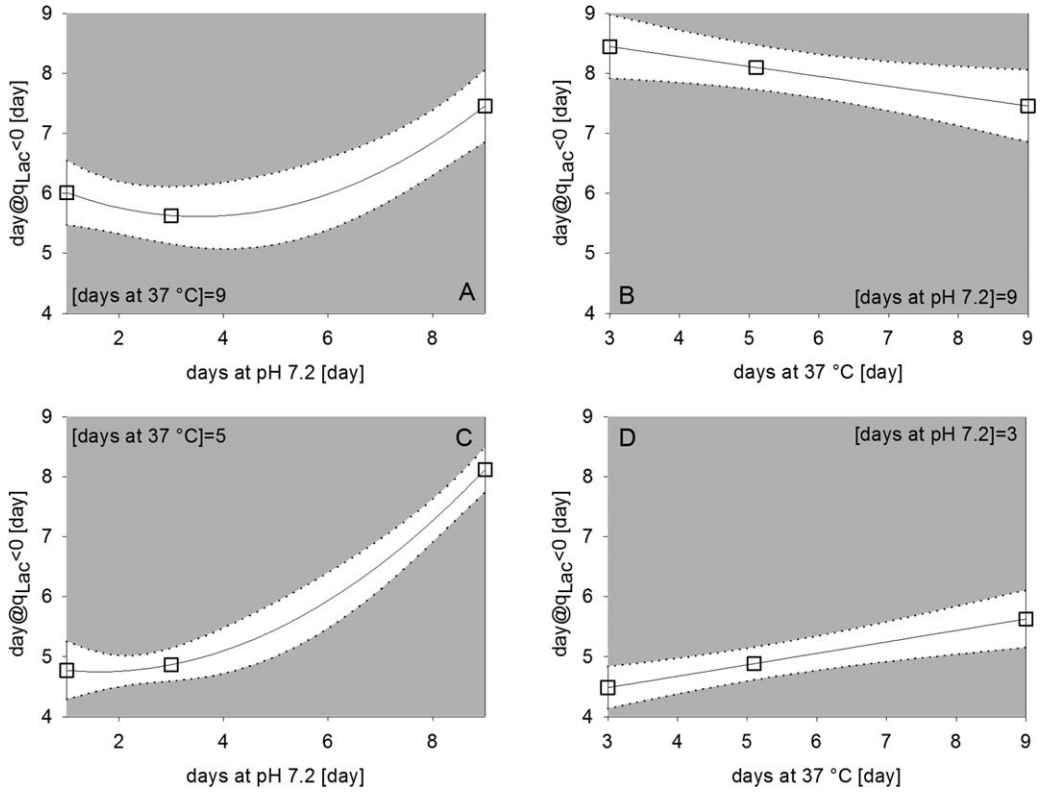

Figure 4. Prediction plots for the effect of the two factors on the onset of lactate uptake.

Confidence level = 95%. (A) [days at 37°C] = 9 (B) [days at pH 7.2] = 9 (C) [days at 37°C] = 5, center point level (D) [days at pH 7.2] = 3, center point level.

parameter shifts on the activity of metabolic pathways could be analyzed with time resolution. Signal noise in the model input variables (known specific rates) or false stoichiometric assumptions can lead to high residual error in the model. The statistical test value h can be used as a quantitative measure for the goodness of the model. The h-value was calculated for each time point in the cultivations and was checked to be below the threshold value 42.6, which was defined based on number of known fluxes (Supporting Information Figure 1B). The estimated fluxes of the main intracellular pathways are visualized on flux maps for four selected experiments (Supporting Information Figures 2 and 3). In accordance with the significant positive effect of pH shift timing on glucose uptake (C_{Glc}/IVC, Table 1), the MFA revealed a sudden decrease in glycolytic activity after the pH shift. In contrast, the experimental factors did not have a direct effect on TCA cycle activity, as indicated by the similar pattern of the TCA cycle fluxes up to day six in all cultivations. However, in the last three cultivation days, the experiments with pH shift showed lower TCA activities as the control cultivation.

The known extracellular fluxes and the estimated intracellular fluxes were used as explanatory variables in PLS-R models with q_{Lac} as predicted variable. At eight timepoints

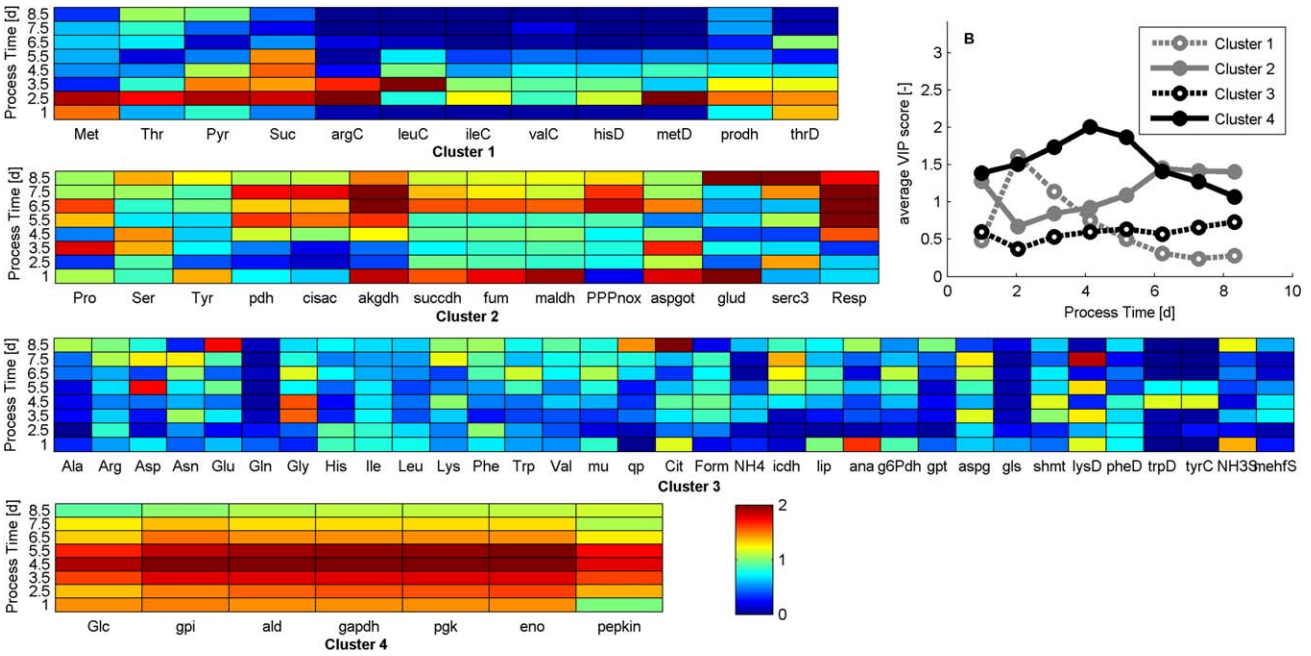

Figure 5. PLS-R model of q_{Lac} coupled with cluster analysis.

(A) Color plots representing the VIP values of the metabolite fluxes (columns) determined by PLS-R analysis in the eight process time periods (rows). The four plots represent the four clusters determined based on the analysis of the VIP values. The colors represent the values of the VIP scores. VIP > 1 indicates significant correlation with q_{Lac}. (B) Average VIP values of the four clusters.

(the time periods between the nine sampling events) eight PLS-R models were executed. Four principal components were used to explain the variability in the dataset. To facilitate the interpretation of the data-driven PLS-R analysis, the variable importance values (VIP) of the explanatory variables were analyzed. A VIP below one hints to variables which are not relevant for the prediction of the response, whereas a VIP above one indicates significant correlation with q_{Lac}.[24] Finally, *k*-means cluster analysis was performed and the calculated VIPs were clustered in four clusters on the variable dimension. The results of this data-driven analysis approach are visualized with the help of heat maps on Figure 5.

The first cluster showed high VIP scores in the first four days of the cultivation, indicating a correlation between the fluxes of the cluster and q_{Lac} in this process phase. Thereby, the two metabolites with the highest VIP scores were pyruvate and succinate, both of which can be used by the cells to fuel the TCA cycle when the glycolytic flux decreases, for example as a consequence of a pH shift. Accordingly, the uptake rate of pyruvate and succinate was found to be higher in the cultivations where pH shift was performed compared to the control run (data not shown). The average VIP score of the second cluster increased continuously during the cultivation, and showed a value above one in the last three days. This cluster included mainly the fluxes of the TCA cycle reactions, both upstream (pdh, cisac) and downstream (succdh, fum, maldh) from α-ketoglutarate. These fluxes, together with the respiratory activity of the cells, showed a high correlation to q_{Lac} in the last four days of the cultivation. As the cells switched from lactate production to lactate uptake in this cultivation phase in almost all cultivations, the correlation of TCA cycle activity and lactate metabolism suggested a connectivity between lactate uptake rate and the fueling of the TCA cycle reactions through the pyruvate dehydrogenase (pdh) flux. The majority of the variables were represented in the third cluster, which showed an average VIP value lower than one, indicating a low correlation to lactate metabolism. Most of the amino acids were clustered in this group of variables, indicating that the metabolic switch to lactate uptake is not coupled to changes in the uptake rate of amino acids in the investigated system. This is in accordance with the observation that the majority of amino acids are only used for anabolic reactions and do not contribute to catabolic processes.[15] The fourth cluster included the glycolytic fluxes, which showed high correlation with lactate metabolism almost over the entire cultivation. The highest VIP scores were observed between day four and six, where q_{Lac} decreased to very low values or the cells switched to lactate uptake as a result of the shifts in the process parameters (Figure 2C). This observation indicated that the decrease in lactate production and the shift to lactate uptake is connected to the decrease in the glycolytic flux, in accordance with literature reports.[3,15,31] Taken together, the PLS-R VIP analysis revealed two main cultivation phases with respect to lactate metabolism (Figure 5B). In the first phase, lactate is produced and the specific lactate production rate correlates with the glycolytic flux. In the second phase, after the metabolic shift to lactate uptake, q_{Lac} correlates with TCA cycle activity.

The connection of lactate metabolism, glycolysis, and TCA activity

The PLS-R analysis revealed a high connectivity between lactate metabolism and glycolysis in the first phase of the cultivation. In accordance with this, the DoE analysis revealed a significant effect of pH shift timing both on specific glucose uptake rate and specific lactate production (Table 1). These findings are also in accordance with the mechanism of glycolytic regulation in CHO cells proposed

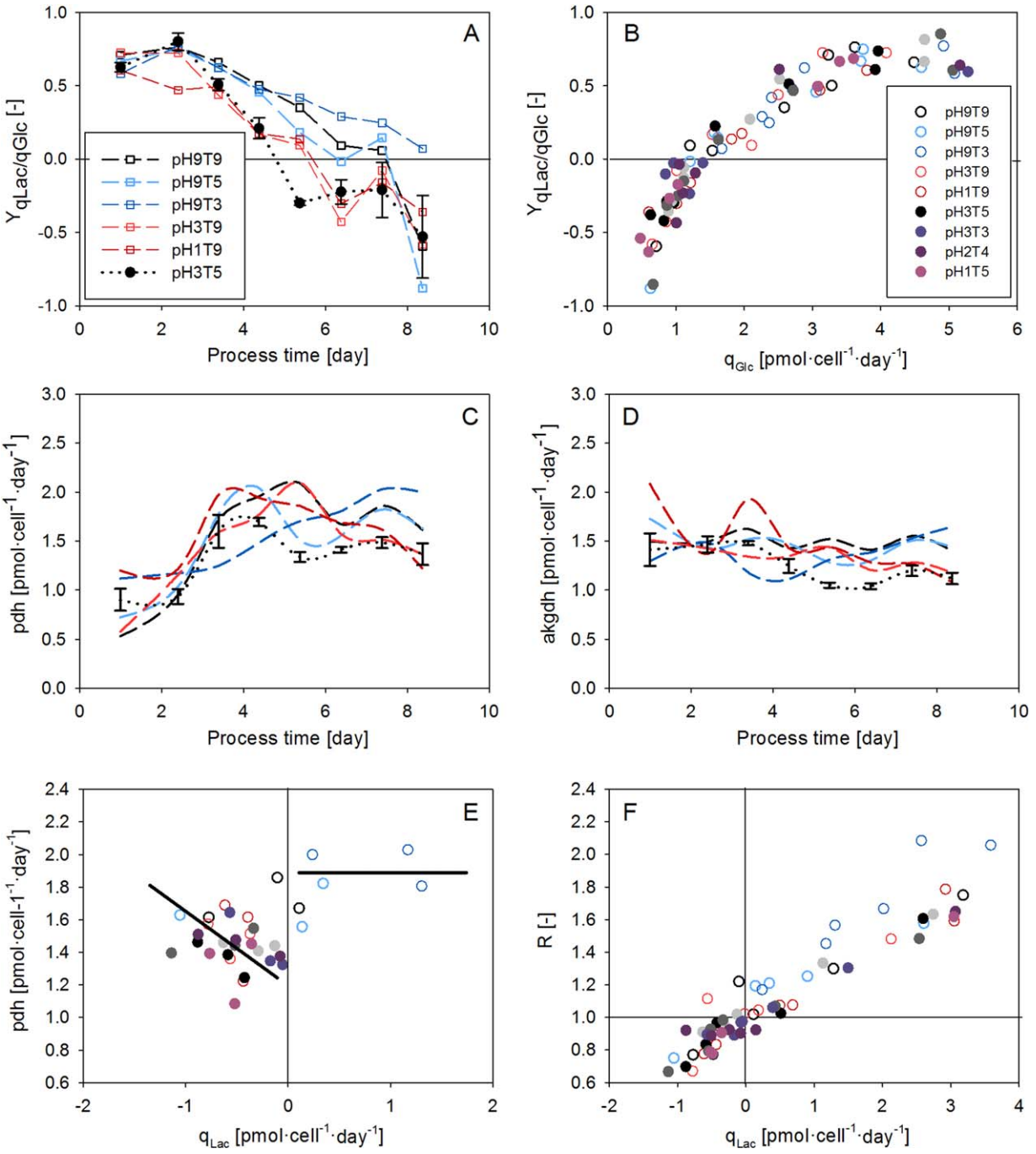

Figure 6. Analysis of lactate metabolism.

(A) Temporally resolved $Y_{qLac/qGlc}$ variable plotted against process time. Black circles represent the average value of the three center point runs; error bars indicate the calculated standard deviations. (B) The $Y_{qLac/qGlc}$ variable as a function of the specific glucose uptake rate. (C) Pyruvate dehydrogenase flux. (D) α-ketoglutarate dehydrogenase flux. (E) The pyruvate dehydrogenase flux plotted against specific lactate uptake rate in the last three days of the cultivations. (F) Relationship between the redox ratio and the specific lactate uptake rate.

by Wahrheit et al: the acidification of the cytosol results in a decreased glycolytic rate and a decrease of the lactate production rate.[15] However, the impact of the factor "days of shifted pH" was also significant on the calculated $Y_{CLac/CGlc}$ yield (Table 1), indicating that pH shift affected the two fluxes to a different extent. This was also confirmed by the analysis of the temporally resolved $Y_{qLac/qGlc}$ yield, which revealed that pH shift had a distinct negative effect on the ratio of lactate production and glucose uptake (Figure 6A). In contrast, the shift of temperature did not have an obvious effect on $Y_{qLac/qGlc}$. An early temperature shift did not affect the value of $Y_{qLac/qGlc}$ (compare experiments "pH9T9" and "pH9T3" after day3 on Figure 6A). Although a later temperature shift decreased the $Yq_{Lac\ /qGlc}$ yield in runs with and without pH shift as well (see cultivations "pH9T5" and "pH3T5" on Figure 6A after day five), the effect was more enhanced in those runs with pH shift. Accordingly, in the center point experiments the cells showed a shift to lactate uptake immediately after the temperature shift.

However, the $Y_{qLac/qGlc}$ value plotted against q_{Glc} showed the same pattern in all cultivations (Figure 6B). Thus, the mechanistic correlation between specific glucose uptake rate

and the $Y_{qLac/qGlc}$ variable was not affected by the shifts in pH and temperature. This observation supported the hypothesis generated based on the results of the PLS-R VIP analysis, namely that the experimental factors exerted their effect on lactate metabolism by influencing the glycolytic flux. This finding is in accordance with recent contributions on the regulation of glycolytic enzymes[3] and the connection between the regulation of glycolytic flux and lactate metabolism.[15]

The PLS-R analysis did not indicate a correlation of q_{Lac} and the TCA cycle fluxes in the first cultivation phase. The lack of correlation between the metabolic switch to lactate uptake and TCA activity indicates that the experimental factors affected lactate metabolism through mechanisms acting upstream from pyruvate on the glycolytic flux. A similar observation was reported for CHO cells by Martinez et al in a batch system; however the onset of lactate uptake was triggered by the exhaustion of glucose in a batch cultivation in their study.[18] Accordingly, independent of which mechanism triggers the decrease in the glycolytic flux, the metabolic switch to lactate consumption is rather provoked by the changes in the speed of glycolysis as in TCA cycle activity.

Interestingly, the PLS-R analysis indicated a high correlation between lactate metabolism and the flux of the TCA cycle reactions in the second phase of the cultivation (Figure 5, cluster 3). In this phase (the last three days of the process), only the cultivations without pH shift showed lactate production. These cultures also showed a higher TCA activity in the second process phase, indicating that lactate production is coupled to a higher TCA flux in this phase (Figures 6C,D). More interestingly, in the physiological state of lactate uptake, the lactate uptake rate seemed to correlate with the pdh flux, suggesting that the cells convert lactate back to pyruvate to fuel the TCA cycle subsequently through this flux (Figure 6E). In accordance with this, Mulukutla et al reported a slight increase in TCA activity after the metabolic shift to lactate consumption in the fed-batch cultivation of NSO cells.[3] Figure 6E summarizes the findings on the correlation of lactate metabolism and TCA cycle activity in the second cultivation phase. In the case of lactate production, a high TCA activity can be observed, which is independent of the lactate production rate (right side, positive q_{Lac} values). In contrast, the pdh flux and subsequently TCA cycle activity is correlated to q_{Lac} in the cultures which show lactate uptake (left side, negative q_{Lac} values). From these findings, we can hypothesize that the cells try to maintain a constant level of TCA cycle activity. The excess of pyruvate from the glycolysis is converted to lactate in the first phase of the cultivation. In contrast, in the second phase, lactate is used as additional carbon source to fuel the TCA cycle to maintain a certain activity of this pathway.[15]

According to studies investigating the cellular control of lactate metabolism, the most important trigger of the switch to lactate uptake is the intracellular redox environment, which is vastly influenced by the ratio of glycolytic flux and TCA cycle activity.[13,32] Nolan et al.[13] introduced the redox variable R to describe the ratio between cytosolic and mitochondrial NADH production/consumption. If the amount of NADH produced in the cytosol is lower than the amount consumed by the mitochondrial electron transport chain, the cells start to convert the accumulated lactate back to pyruvate. To prove this hypothesis, the redox ratio (R, see Equation (2)) was plotted against specific lactate uptake rate (Figure 6F). The observed correlation suggested that lactate metabolism is directly linked to the redox status of the cells in our system and that this relationship is independent of the shifts in pH and temperature. In case of a high glycolytic flux, the redox balance is high and the excess of NADH produced in the glycolytic reactions must be oxidized to NAD via lactate production. When glycolysis is down regulated as a consequence of pH shift,[33] the redox balance decreases. Finally, as R gets lower than one the cells switch to lactate uptake to fuel the TCA cycle. This is in accordance with a recent work of Ivarsson et al, where the authors also suggested that the effect of pH on lactate metabolism is guided by redox balancing and the regulation of glycolysis in hybridoma cells.[17]

Taken together, the correlation between the glycolytic flux, TCA cycle activity and lactate metabolism indicated by the PLS-R analysis could be verified by investigating the mechanistic relationship of the metabolic pathways in the different cultivation phases.

Product formation

To investigate the effect of the experimental factors on product formation, relative final mAb titer was defined as a response variable in the DoE analysis. Whereas the timing of pH shift did not have a significant term in the model, the factor "days at 37°C" had a significant positive effect on product concentration. However, this effect was probably a consequence of the lower cell densities in the cultivations with an early temperature shift. To test this hypothesis, specific product formation (P/IVC) was also defined as a response variable. The statistical analysis revealed a negative linear effect of the factor "days at 37°C" on specific productivity, indicating that an early temperature shift increases the specific productivity calculated on the whole cultivation period. The prediction plot (Figure 7A,B) visualizes the discussed effects, indicating that the optimum of the experiment space in terms of final product titer is close to the chosen center point setpoints ([days at 37°C]=5). Although one can increase the specific productivity with an earlier temperature shift even further, the negative effect of temperature shift on IVC would lead to a decrease in final titer. This effect is confirmed by Figure 7C, where the highest titer is observed in the center point runs.

The analysis of specific productivity with a temporal resolution showed similar q_p values in the first cultivation phase (Figure 7D), indicating that the parameter shifts (performed between the first and the fifth day of the cultivations) did not have a sudden effect on specific productivity. However, on the last day, significant differences in specific productivity were observed. Whereas the runs with an early temperature shift (see experiment "pH9T3" and "pH3T3") maintained a high q_p until the end of the cultivation, those cultivations without temperature shift showed a steep decline in q_p on the last day. As revealed by the metabolic analysis, this phenomenon is a consequence of the depletion of essential amino acids occurring at the high cell densities observed in these runs. Taken together, in contrast to other scientific papers which hypothesized a direct effect of temperature shift on specific productivity,[34,35] our findings suggest that the positive correlation between an early temperature shift and specific productivity is an indirect consequence of overcoming nutrient limitations which have a detrimental effect on the rate of product formation. This hypothesis is in accordance with a recent study of Vergara et al who demonstrated that an increase of specific productivity at low

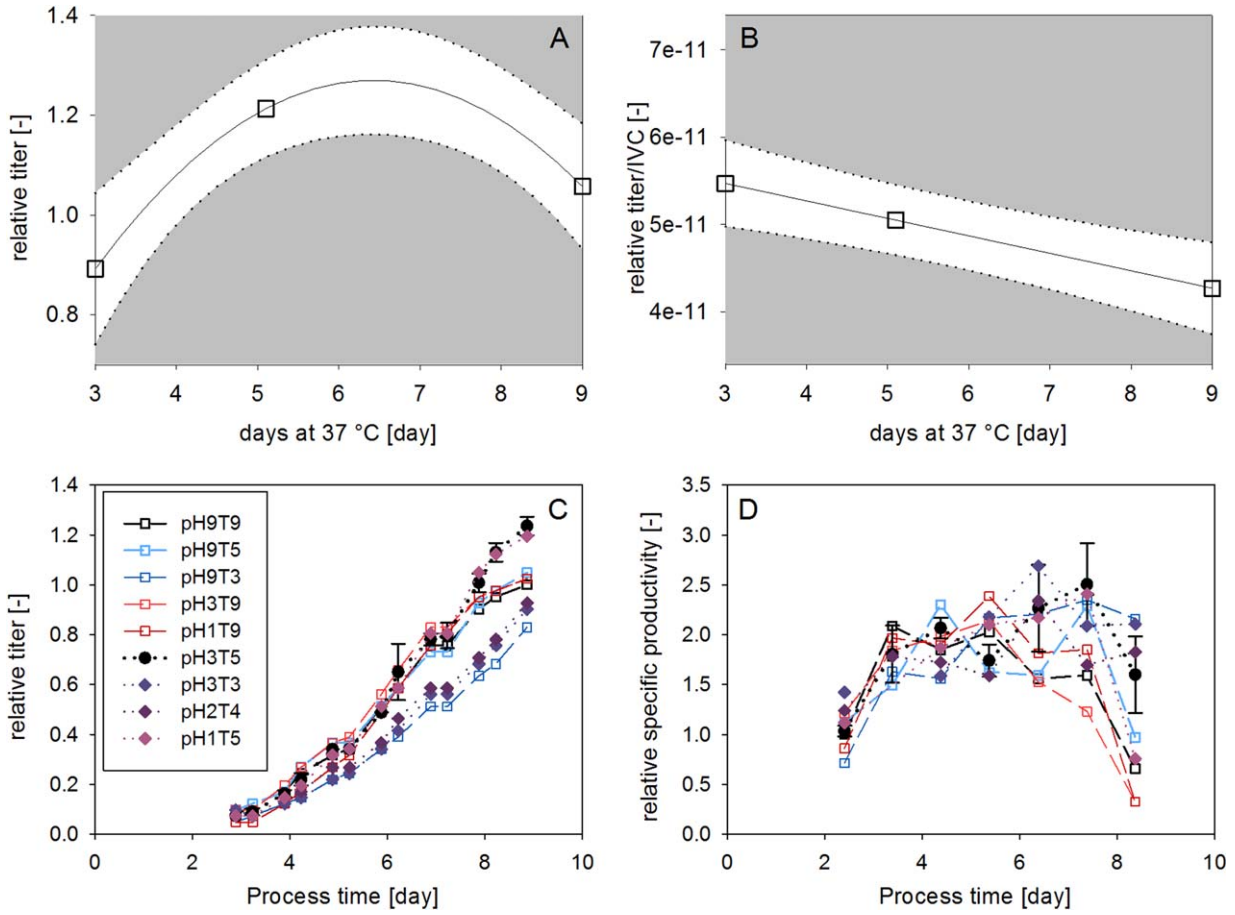

Figure 7. Effect of the experimental factors on relative final product titer and specific productivity.

(A, B) Prediction plot of the effect of temperature shift timing on relative final product titer and P/IVC. The setpoint of [days at pH 7.2] was set to 9 in both cases. Confidence level = 95%. (C) Relative titer plotted against process time. Black circles represent the average value of the three center point runs; error bars indicate the calculated standard deviations. (D) Relative specific productivity plotted against process time.

temperatures might be a result of indirect effects coupled to other physiological features such as specific growth rate.[36]

The PLS-R analysis revealed a significant correlation between lactate metabolism and specific productivity only on the last day of the process (Figure 5, cluster 2). This correlation suggests that the nutrient limitation which affected specific productivity on the last process day might have had an effect on lactate metabolism as well. However, the lack of correlation between lactate metabolism and specific productivity in the first eight days of the cultivation suggests that the shift to lactate uptake does not trigger an increase in product formation.

Conclusions

The goal of the recent study was to investigate the controllability of the metabolic shift to lactate uptake by process parameter shifting. The time points of pH and temperature shift were defined as experimental factors and a DoE was conducted to assess their effect on lactate metabolism. Whereas the timing of pH shift had a significant impact on the onset of lactate consumption, the effect of temperature shift was only significant when pH shift was also conducted. Identifying this interaction effect was one of the benefits of the DoE approach. Based on these results, a control strategy which targets an early metabolic shift to lactate consumption has to execute a shift in both temperature and pH setpoints.

With this contribution we present a novel methodology coupling time-resolved metabolic flux analysis, PLS-R and mechanistic analysis. First, MFA and PLS-R were used to detect correlations between lactate metabolism and the activity of the major intracellular pathways. Thereafter, specific rates and yields were plotted against each other to visualize these correlations on simple mechanistic plots. The PLS-R combined with VIP revealed two cultivation phases. In the first phase, lactate metabolism showed a high connectivity to the glycolytic pathway suggesting that the physiological switch to lactate uptake is triggered by the decrease in the glycolytic flux. The mechanistic relationship between q_{Glc} and the $Y_{Lac/Glc}$ yield showed the same profile in all experiments, confirming the strong link between glycolytic activity and the metabolic switch to lactate consumption. The second cultivation phase was characterized by lactate uptake in almost all points of the experimental design. The PLS-R VIP analysis revealed that lactate metabolism was more connected to TCA activity as to the glycolytic flux in this phase. Accordingly, the mechanistic analysis showed a correlation between lactate uptake rate and the pyruvate dehydrogenase flux fueling the TCA cycle.

The combination of metabolic flux analysis and PLS-R proved to be a successful tool to link a knowledge-based

mechanistic approach to data-driven analysis. Moreover, the verification of the detected metabolic links on simple mechanistic plots helped to generate process knowledge on the effect of the experimental factors on lactate metabolism. The presented toolset can be used in the future to facilitate knowledge extraction from large metabolic datasets and to detect mechanistic correlations between metabolic fluxes. Moreover, it can be also applied to define which metabolites should be monitored in different process phases to detect and characterize physiological events. For example, in our cultivation system, the measurement of glucose uptake and specific oxygen consumption would be sufficient to characterize lactate metabolism throughout the whole cultivation period. This knowledge can be then used to understand the effect of process parameters on cell physiology and to implement sophisticated control strategies targeting physiological effects such as the switch to lactate uptake or the maintenance of high TCA cycle activity.

Notation

CHO = Chinese hamster ovary
DoE = Design of experiments
MLR = Multivariate linear regression
OUR = Oxygen uptake rate
PEP = Phosphoenolpyruvate
PPP = Pentose phosphate pathway
TCA = Tricarboxylic acid
VIP = Variable importance in projection

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Impact of Apoptosis on the On-Line Measured Dielectric Properties of CHO Cells

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ORIGINAL PAPER

Impact of apoptosis on the on-line measured dielectric properties of CHO cells

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Abstract Apoptosis is a common type of cell death in biopharmaceutical cell culture processes which causes decrease in viable cell density and product yield. The progression of apoptosis has been reported to influence the dielectric properties of mammalian cells; however, the on-line detection of these effects has been rarely described. This study provides a comprehensive analysis of the on-line detectability of dielectric changes upon apoptosis induction in an industrial fed-batch process of CHO cells expressing a recombinant monoclonal antibody. Using capacitance signals, measured at 25 frequencies, the impact of apoptosis on the dielectric spectra was investigated in eight bioreactor cultivations in which various process conditions were combined with two different apoptosis induction strategies (camptothecin treatment and glucose starvation). To differentiate the apoptosis-related information from the cell concentration-associated variance in the multivariate capacitance datasets, principal component analysis (PCA) was used. A second principal component, explaining an explicit proportion (>20 %) of the variance, was identified to be related to dielectric changes induced by apoptosis. Furthermore, the analysis of caspase-3 and -7 activation and DNA fragmentation showed that the detected dielectric change occurred in the early phase of apoptosis. The presented results verify that apoptosis has a considerable impact on the dielectric features of CHO cells and it can be monitored on-line with the introduced tool-set combining capacitance measurement with multivariate data analysis.

Keywords Dielectric spectroscopy · Apoptosis · Mammalian cell culture · CHO · Multivariate data analysis

Introduction

In biopharmaceutical cell culture processes, both the yield as well as the quality of the product is strongly dependent on the viability of the cells. Mammalian cells are sensitive to changes in their environment which can lead to undesired responses such as cell death during the production process. Basically, there are two major forms of cell death: necrosis and apoptosis. Necrosis is generally induced by external stress factors causing cell swelling, ATP depletion, DNA hydrolysis and karyolysis followed by the rupture of the cellular membrane which leads to the release of the complete cell content into the extracellular space [1, 2]. In contrast, apoptosis is a strictly regulated cellular process in which cells "commit suicide". The proapoptotic signals activate a cascade of caspases, the central regulators of apoptosis, the initiator and subsequently the effector caspases which in turn proteolytically cleave a wide range of cellular substrates leading to cell death [3]. Typical morphological changes are cell shrinkage, increase of the cytoplasma density, nuclear condensation, depolarization of the mitochondric membrane, increase of cytosolic Ca^{2+} content, and blebbing of the plasma membrane, the place of the formation of apoptotic bodies which are membrane surrounded particles containing degraded cell organelles and cytoplasma [4–6]. In apoptotic cells, the nuclear DNA is also degraded; it is cleaved in the internucleosomal

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region resulting in $n \times 180$–200 bp fragments (DNA ladder) [7, 8]. The apoptotic events are consecutive in time; one of the earliest events is the exposition of phosphatidylserine on the cell surface, followed by blebbing, and finally by DNA fragmentation in the late phase of the process. Although apoptosis is associated with intense membrane re-arrangement; the membrane loses its integrity only in the late phase [9, 10].

Apoptosis was reported to be the major cause of cell death in CHO cells cultured in bioreactors [11, 12]. It can be induced by numerous factors such as depletion of substrates, pH alteration, hypo-, and hyperoxia, mechanical, shear and hydrodynamic stress, toxic ammonium concentration or viral infections [10, 13–15]. Since the product yield of a bioprocess strongly depends on its duration and the cell concentration, a decrease in the viable cell density by apoptosis during the production phase can easily reduce the final yield. Furthermore, apoptosis can also influence the quality attributes of the product (e.g. glycosylation pattern) [16]. Accordingly, postponing the onset of apoptosis in cell culture processes can be a key tool to maintain high volumetric productivity, or the targeted product quality. To achieve this, the detection of apoptosis should occur as early as possible. A prompt monitoring of the early events of apoptosis may contribute to recognize certain cultivation variables or their shifts which can induce apoptosis. In process development, these parameters or variables should be adjusted to avoid the induction of apoptotic events in the culture.

The apoptosis detection techniques greatly vary in the capability of which apoptotic phase is monitored. The fact that apoptosis is a cascade of molecular events in the cells raises the question which stage of apoptosis can be considered as death. Apoptosis is thought to be a reversible process; however, its detailed cellular switch control is still under investigation [17, 18]. In cell culture processes, a very critical point would be to determine the phase when apoptosis turns irreversible. There are two factors which hinder the detection of apoptosis before this critical time point. First, most of the methods used in daily laboratory routine (e.g. trypan blue exclusion) indicate apoptosis only in its late phase after the irreversible switch. Second, these techniques are performed at-line or off-line demanding extra sample collection to be taken from the bioreactors. The low sampling frequency (once or twice per day) and the resulting low temporal resolution gained with at-line or off-line methods in cell culture experiments is the second factor which hampers a precise apoptosis detection in time. This suggests that the development of a method for the on-line detection of apoptosis is a potential key task in cell culture development. Such a method could be subsequently applied to facilitate the development of novel processing strategies which postpone the onset of apoptosis in cell culture processes. Moreover, in accordance with the process analytical technology (PAT) initiative, the availability of on-line information about an apoptotic progress during cell cultivation could further enhance the sensitivity of multivariate process models towards physiological changes and thus enable the implementation of adaptive control strategies in the future. In order to achieve this goal, a method needs to be developed, which (1) applies a measurement technique which is compatible with the strict regulatory requirements of pharmaceutical production (e.g. GMP) and (2) transforms the measured signal into physiological information with tools which can be easily integrated into on-line process models (e.g. multivariate analysis methods).

On-line monitoring of cell cultures with dielectric spectroscopy (DEP) is a widespread method for measuring viable cell concentration [19]. This technique is based on capacity measurement using alternating current. In electrical terms, cells behave as small spherical capacitors surrounded by a poorly conducting lipid membrane [20]. In contrast, the inside of the cells (cytoplasma) is conducting and so is the environment of the cells (medium). In electrical field, cell membranes become polarized through charge separation and the capacitance of this electrical double layer can be measured between the electrodes placed in the cell culture. Increasing the frequency of the applied alternating current, the measured capacitance of the cell suspension drops, since the charge carrier particles of the cytoplasma has less time to reach, and thus to polarize, the membrane. This frequency-dependent phenomenon is called beta dispersion which occurs in a range of 0.5–3 MHz in most cell types [21]. At high frequencies, the measured capacitance (C_∞), deriving mostly from the dipole moment of water molecules, is negligible. The difference between the capacity plateaus belonging to the high- and low-frequency regions is called ΔC, while the frequency where the capacitance curve has its inflexion point is the critical frequency (f_C). For prediction of the biomass, the measurement is usually reduced on the determination of ΔC, because neither C_∞ nor f_C is considered to be influenced by the change of cell concentration as long as cells remain in a physiological steady state [22].

The dielectric features of the cells are determined by three major components: extracellular space (medium), intracellular matrix (cytoplasm), and the cell membrane. During apoptosis these major components, both the membrane, as well as the intracellular matrix, undergo remarkable structural and chemical changes suggesting alterations also in the measured dielectric features of the cells. Changes in the membrane and cytoplasm are the exposition of phosphatidylserine on the cell surface, formation of apoptosomes, blebbing, shrinkage of the cells, nuclear condensation, mitochondrial membrane permeability changes and

Ca^{2+} release. Accordingly, DEP measurements of human K562 cells after the induction of apoptosis with staurosporin showed an increase of cytoplasm conductivity, which might be explained by the reduction of cell radius (shrinkage) followed by the concentration of ionic material (ions, proteins, DNA, etc.) into the cytoplasm [23]. Similar dielectric changes were detected in apoptotic CHO cells by DEP measurement, suggesting that apoptosis has a strong impact on the dielectric properties of CHO cells [24].

Here, in our experiments, we intended to investigate the detectability of apoptosis-associated dielectric changes by on-line dielectric spectroscopy. Therefore, we induced apoptosis in an industrial cell culture process expressing a monoclonal antibody and investigated the changes in the dielectric properties of the culture with on-line capacitance spectroscopy. In order to provide a comprehensive analysis of the detected phenomenon, different apoptosis induction methods were applied at different cultivation phases. Moreover, the observed dielectric responses were verified using different process conditions such as altered temperature or two production cell lines. Parallel to the on-line determined dielectric changes, the progress of apoptosis was monitored in high time resolution collecting at-line samples which were then analyzed with conventional methods such as trypan blue exclusion and caspase activity measurement. Finally, as an ultimate novelty of this study, principal component analysis was performed for the detection of apoptosis-related changes in the capacitance spectrum.

Materials and methods

Cell line and preculture

Suspension cultures of two CHO-K1 derived cell lines expressing the same IgG1 monoclonal antibody were maintained in chemically defined and serum-free growth medium (ActiCHO P, GE Healthcare, Little Chalfont, UK) supplemented with 8 mM L-glutamine (Life Technologies, Carlsbad, CA) and 5 mg/l insulin (GE Healthcare, Little Chalfont, UK). Cells were cultured in disposable shake flasks using 110 rpm orbital agitation at 37 °C in humidified 8 % CO_2 incubator. Passages were carried out in every 3–4 days with an initial cell density of 0.3×10^6 cells/ml.

Bioreactor operation

Cells were cultivated in autoclavable 1 l glass culture vessels of BIOSTAT® B plus Laboratory benchtop system (Sartorius AG, Göttingen, Germany). The initial cell concentration was of 0.5×10^6 cell/ml in the bioreactors. Addition of a proprietary feed medium (5 % of the current broth volume) was started 72 h after the inoculation and it was repeated on every second day thereafter. The set point of temperature, pH, and DO were monitored on-line with in situ sensors. Data were managed and recorded in supervisory control and data acquisition (SCADA) system (CitectSCADA Version v7.10, Schneider Electric SA, France). The pH was controlled by the addition of 10 % H_3PO_4 or 0.5 M Na_2CO_3 solution, while the DO was controlled by sparging of air/oxygen mixture. Stirring speed was set to 125 rpm. Foaming was reduced by the addition of antifoam (FoamAway, Life Technologies, Carlsbad, CA).

At-line and off-line measurements

Depending on the measurement, at-line samples were collected daily or more frequently as indicated later. The following measurements were carried out: determination of glucose concentration (Cedex BioHT, Roche Diagnostics, Mannheim, Germany), pH measurement for the in situ recalibration of the on-line pH probe (S47 SevenMulti pH meter, Mettler Toledo, Zurich, Switzerland). Cell density and viability was determined with Countess automated cell counter (Invitrogen, Carlsbad, CA) using trypan blue stain (Life Technologies, Eugene, OR).

Camptothecin (CPT) treatment

Cells were treated with 70 μM CPT (Sigma Aldrich, St. Louis, MO) by using a stock solution prepared in DMSO. In the (mock) control cultivations, only DMSO was added to the culture parallel to the CPT induction.

Detection of apoptosis

The activity of effector caspases (caspase-3 and -7) was measured with a glow-type luciferase-based assay (Caspase-Glo 3/7 Assay, Promega GMBH, Germany) following the manufacturer's instructions. Briefly, the reactions were carried out directly in cell culture medium and immediately after sample collection using a 96-well plate format. In each reaction, 50 μl cell suspension containing 1.5×10^4 cells (total cell number) was mixed with 50 μl reagent and incubated for 1 h at constant room temperature. The luminescent signal was measured with Synergy 2 multi plate reader (BioTek, Winooski, VT).

Apoptosis-induced DNA fragmentation was detected in a modified version of the method described by Hermann et al. [25]. Briefly, 5×10^6 cells were washed with phosphate based saline buffer (PBS) and lysed in 100 μl lysis buffer (0.2 % Triton X 100, 20 mM EDTA, 50 mM Tris–HCl pH 7.5) at room temperature for 2 min. After centrifugation (Hettich Micro 22, Hettich, Tuttlingen,

Germany), the lysate was incubated with RNaseA for 30 min at 37 °C followed by Proteinase K digestion for 2 h at 56 °C in the presence of 1 % sodium dodecyl sulfate. After the DNA precipitation with ethanol (2.5 × volumes) and 3 M sodium acetate (0.1 × volume), the DNA was washed with 70 % ethanol, air-dried and finally resuspended in water. DNA was analyzed with agarose (1 %) gel electrophoresis using GelRed (Biotium, Hayward, CA) labeling.

Dielectric spectroscopy and Cole–Cole model fitting

Capacitance of the cultures was measured with Biomass Monitor 220 (Aber Instruments, Aberystwyth, UK) using 12 mm annular sensors at 25 frequencies (100, 120, 160, 190, 240, 300, 370, 470, 580, 720, 900, 1120, 1400, 1740, 2170, 2700, 3360, 4190, 5220, 6500, 8100, 10090, 12560, 15650, 19490 Hz). Scans were carried out every 8 min and the collected data were processed with AberScan Beta 4.2 software (Aber Instruments, Aberystwyth, UK).

The AberScan software was used to fit a Cole–Cole model on the collected dielectric spectra. The fitting algorithm was described in details by Dabros et al. [26]. The parameters (ΔC and f_C) of the fitted model were calculated by the software.

Statistical analysis

The capacitance datasets were structured in MS Excel (Microsoft Corporation, Redmond, WA). The datasets were treated by unit variance scaling and mean centering, and principal component analysis (PCA) was performed subsequently in SIMCA software (Umetrics, Umeå, Sweden). The number of principle components in the multivariate model was adjusted to achieve maximum Q^2 values.

Results

For the investigation of apoptosis-induced changes in the dielectric properties of CHO cells, CHO-K1 derived cells constitutively expressing the heavy and the light chains of an IgG type monoclonal antibody were cultivated in 1 l bioreactors. Apoptosis was induced with two different methods. Moreover, to gain process- and cell line-independent knowledge, apoptosis was induced in different cultures. In the first set of experiments (CPT1 and CPT2), cell line A was cultivated at different temperatures and apoptosis was induced by camptothecin addition in the early growth phase. In contrast, in the second set of experiments (GLC1 and GLC2), apoptosis was induced by glucose deprivation in the stationary phase of the cultivations using two different cell lines. The settings used in the four experiments are summarized in Table 1.

Induction of apoptosis by camptothecin addition

In order to avoid the formation of apoptotic cell populations—which is caused by nutrient depletion, aging etc., typically happening in the late phase of batch or fed-batch cultivations—apoptosis was induced early, 3 days (at a cell density of 2.5–3 × 10^6 cell/ml) after inoculation. At this time, the viability was still close to 100 % and intense cell proliferation occurred in all the cultivations. The induction was carried out by using camptothecin, an irreversible DNA topoisomerase I inhibitor [27], which is a well-established apoptosis inducing agent [28–30]. Two independent experiments (CPT1 and CPT2) were performed by running two parallel cultures with and without induction (CPT+ and CPT−, respectively). The cultivation parameters differed only in the cultivation temperature. The non-induced cultures served as control runs.

Cells showed extensive proliferation in all four cultivations until the time point of induction (Fig. 1a). The difference in growth rate between the CPT1 and CPT2 cultivations was the consequence of the different cultivation temperature. Accordingly, at the time point of induction, a higher cell density was observed in the CPT1 cultivations compared to the CPT2 runs. The addition of CPT had a rapid inhibitory effect on cell proliferation, as indicated by stagnating VCD values and a steep decline in viability in the post-induction phase of both CPT-induced cultivations. However, continuous cell growth was observed in the non-induced control cultures, while the

Table 1 Summary of the experimental settings in the eight cultivations

Experiment ID	Apoptosis induction	Runs in the experiment		Cell line	Physiological phase at induction	Cultivation temperature[a]
		Apoptosis induced	Control			
CPT1	Camptothecin	CPT1+	CPT1−	A	Growth phase	Standard
CPT2	Camptothecin	CPT2+	CPT2−	A	Growth phase	Altered
GLC1	Glucose deprivation	$GLC1^{dep}$	$GLC1^{ctrl}$	A	Stationary phase	Standard
GLC2	Glucose deprivation	$GLC2^{dep}$	$GLC2^{ctrl}$	B	Stationary phase	Standard

[a] Standard cultivation temperature: 37 °C

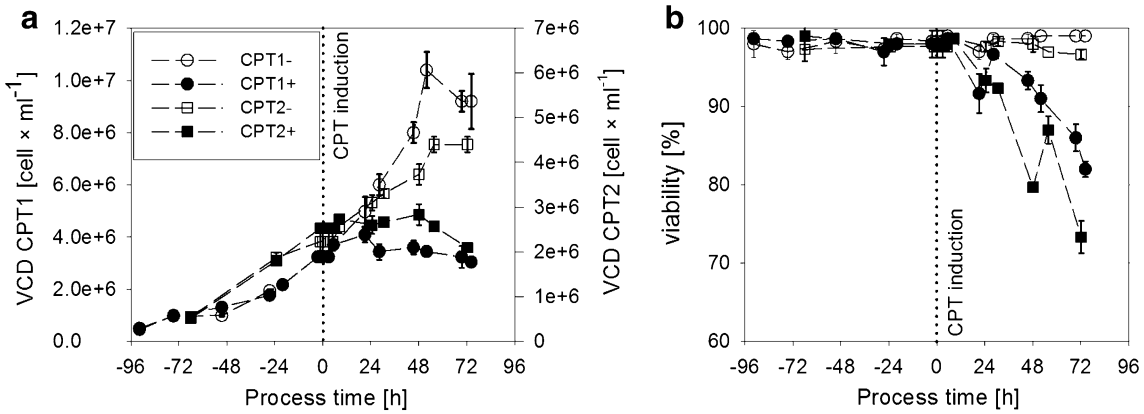

Fig. 1 Cell growth and viability in the CPT1 and CPT2 experiments. **a** Viable cell density, **b** viability determined with trypan blue exclusion. CPT-induced (+) and non-induced (−) cultures are shown

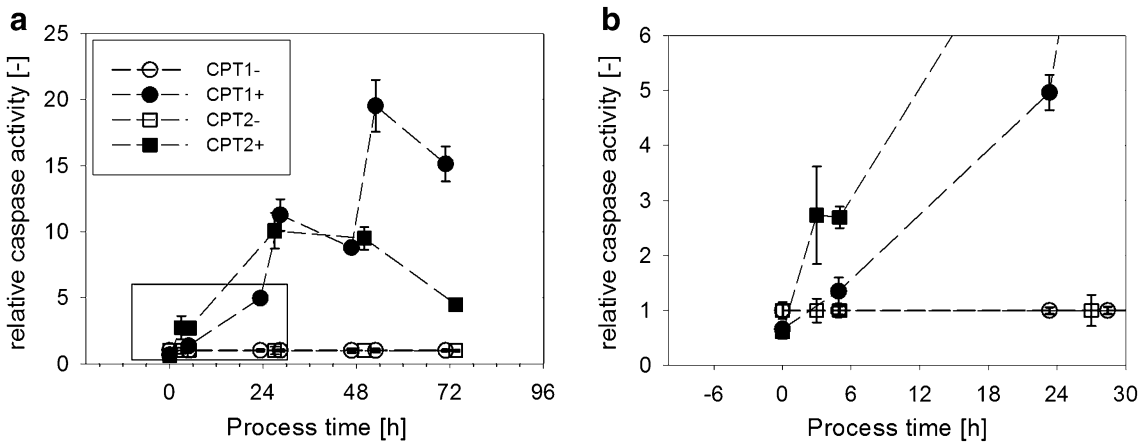

Fig. 2 Caspase activity measurements in the CPT1 and CPT2 experiments. The relative caspase activity was calculated by dividing the activity value measured in the CPT-induced cultivation with the activity value measured in the respective control run. The time window marked with *black box* in (**a**) is shown with higher resolution in (**b**). CPT-induced (+) and non-induced (−) cultures are shown

viability remained above 95 % (Fig. 1b). In the CPT-induced cultures, cell death was detected with trypan blue exclusion method 30–45 h after induction. This was indicated by a clear drop in viability (ca. 10 %), which further decreased until the end of the cultivation. This result was in a good correspondence with the fact that the cell membrane of apoptotic cells remains intact until the late phase of apoptosis. Using light microscopy, membrane blubbing was also observed nearly parallel with the appearance of trypan blue positivity (data not shown).

In order to verify the CPT-induced apoptosis in the cultivations, the enzymatic activities of the effector caspase-3 and -7 were measured using a luciferase-based assay. In apoptotic cells, caspase-3 and -7 are specifically activated early during apoptosis [3]. As shown in Fig. 2, clear evidence for caspase activation was found in the CPT-treated cultures. This occurred much earlier than the viability drop detected with Trypan blue exclusion. In apoptotic cells, the nuclear DNA is fragmented as a late event showing a well-described pattern of DNA ladder in the analysis of the DNA isolated from apoptotic cells. DNA was isolated from the cells at different time points after the CPT induction and analyzed with agarose gel electrophoresis. In the isolates from the induced culture, but not from the control runs, DNA fragmentation was detected revealing the progression of apoptosis (Fig. 1 in the electronic supplementary material).

Dielectric spectra were collected during the cultivations by simultaneous measurement of capacitance at 25 different frequency values in every 8 min. Principal component analysis was performed in order to reduce the dimensionality of the collected datasets. The observations were the measurement time points, and the measurement frequencies were defined as variables. Two separate PCA models were fitted on the CPT1 and CPT2 datasets. Both models required only two principal components (PC1 and PC2) to

explain more than 99 % ($R^2 > 99$ %) of the variability in the capacitance datasets.

The $t[1]$ in the induced and non-induced cultures displayed a very similar development until the induction of apoptosis in all four cultivations (Fig. 3a). The $t[1]$ value seemed to be continuously increasing in time; however, after the addition of camptothecin in the apoptosis-induced cultivations the $t[1]$ trajectory showed a plateau followed by a decline phase. The trajectory of $t[1]$ was very similar to the cell growth profiles suggesting that the increase in $t[1]$, which also explained the major part of variations in the dielectric spectra, is associated with cell (biomass) growth. The linear correlation between $t[1]$ values and the off-line measured VCD values confirmed this hypothesis (Fig. 3b).

The second principal component explained comparable ratio of the variance (24 % in CPT1 and 16 % in CPT2) and showed similar trajectories in the two experiments (Fig. 3c). The $t[2]$ values displayed similar constant values until CPT treatment in all the cultivations. After apoptosis induction, the trajectories were shown to be segregating; $t[2]$ started increasing in the cultures with induced apoptotic progression, while it remained nearly constant in the non-induced control cultures. Clear difference between the trajectories could be seen ca. 20 h after apoptosis induction. Accordingly, the increase in $t[2]$ seemed to be related to the onset of apoptosis. It is also notable that $t[2]$ changed only after apoptosis induction, while $t[1]$ was already continuously increasing before induction. This also supported our implication that $t[2]$ represented a type of variation in the dielectric spectra which can be in association with apoptotic events induced in the cultures. However, the change in $t[2]$ could not be attributed to changes in a distinct region of the beta dispersion curve, as indicated by the significant loading values of almost all 25 measurement frequencies (Fig. 2 in the electronic supplementary material).

Induction of apoptosis by glucose deprivation

In the second part of the study, apoptosis was induced by glucose deprivation in the stationary phase of fed-batch cultivations. Beside the cell line used in the previously discussed CPT runs, another CHO–K1 derived cell line

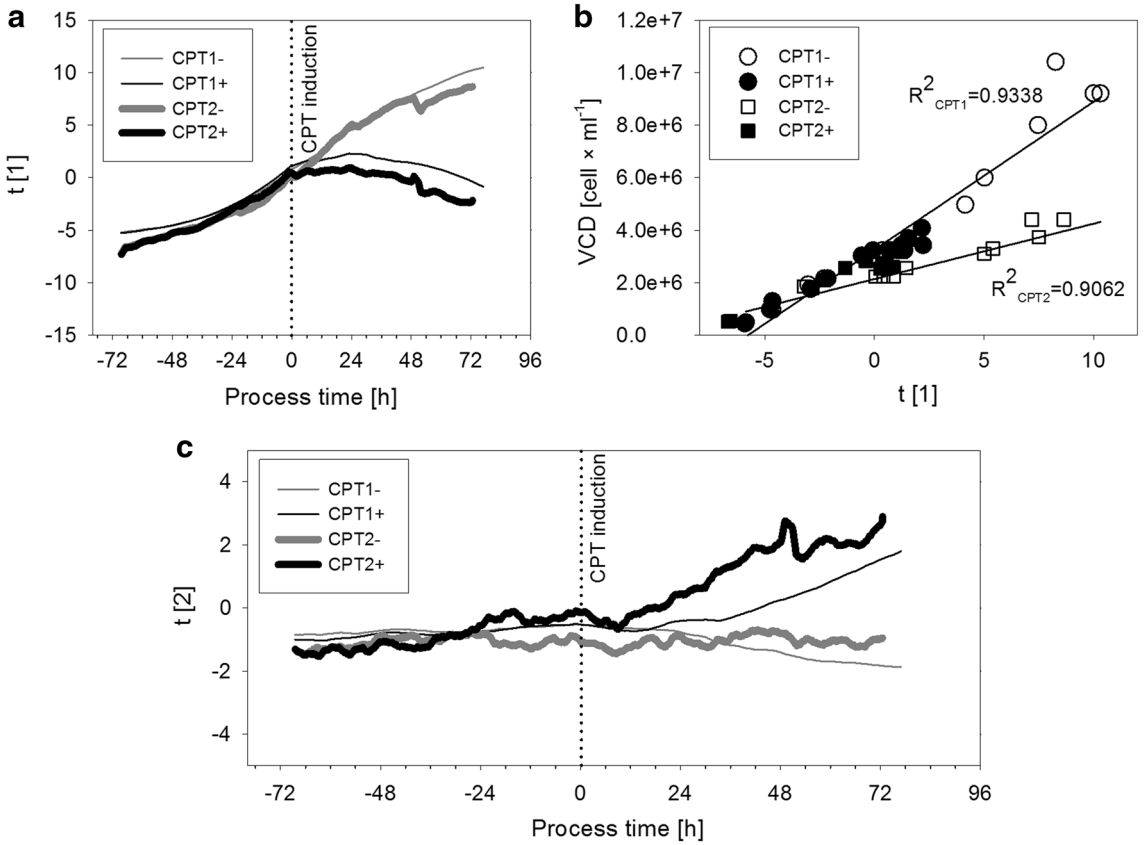

Fig. 3 Principal component analysis of the dielectric spectra in the CPT1 and CPT2 experiments. **a** The score values of the first principal components; $R^2_{PC1,CPT1} = 0.76$; $R^2_{PC1,CPT2} = 0.84$, **b** VCD values were plotted against the value of the first principal component at the time point of sampling events. Linear regression was performed on the CPT1 and CPT2 datasets separately. **c** The score values of the second principal components; $R^2_{PC2,CPT1} = 0.24$; $R^2_{PC2,CPT2} = 0.16$. CPT-induced (+) and non-induced (−) cultures are shown

was used. In order to demonstrate reproducibility, two independent experiments (GLC1 and GLC2) were carried out with the two different cell lines. In both experiments two parallel bioreactors were run (Table 1). First, after 13 days of cultivation, glucose feeding was terminated in one bioreactor ($GLC1^{dep}$ and $GLC2^{dep}$, respectively), while the glucose feeding was maintained in the parallel cultivations ($GLC1^{ctrl}$ and $GLC2^{ctrl}$, respectively) serving as controls. The deprivation of glucose lasted less than 2 days and the time point of glucose depletion was defined as zero process time (Figs. 4, 5, 6, 7). In both experiments, the glucose-depleted cultures ($GLC1^{dep}$ and $GLC2^{dep}$) were run until the viability drastically dropped; after reaching a viability of 30–40 %, the reactors were terminated. At the same time the viability was still close to 100 % in the control runs ($GLC1^{ctrl}$ and $GLC2^{ctrl}$).

Afterwards, to reproduce the phenomena observed during the previously described glucose termination, the glucose feeding was terminated in the remaining $GLC1^{ctrl}$ and $GLC2^{ctrl}$ control runs (Fig. 4b, c). This enabled to gain further verifications of our observations. Therefore, in all, we generated four replicas of glucose deprivation.

In the cultivations, the drop in VCD (Fig. 4a) and in cell viability (Fig. 4b) was detected at approximately 50 h after glucose deprivation. However, a rapid increase in the DO signal (Fig. 4d) indicated a decrease in the respiratory activity of the cells shortly after the deprivation of glucose. Interestingly, the increase in the DO curves showed a distinct profile with a local minimum approximately 20–30 h after the depletion of glucose in all four cultivations. After this short phase of increased oxygen uptake, the DO values converged to 100 % indicating the standstill of cell respiration. These observations suggested a very similar and reproducible physiological response on glucose deprivation in all the cultures showing no differences in the two investigated cell lines.

The induction of apoptosis was verified by the measurement of caspase activity after glucose depletion. Similar to the CPT induction, in all four GLC-depleted cultivations, the glucose deprivation resulted in a five- to

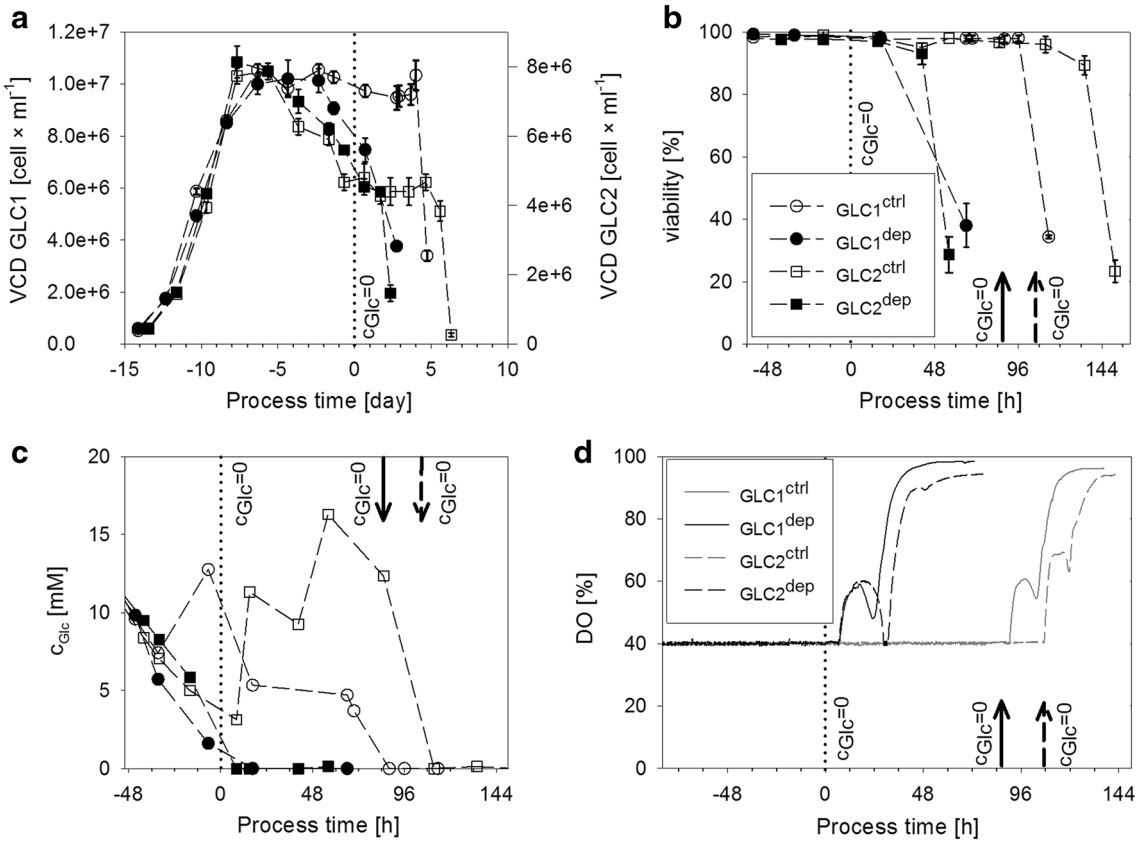

Fig. 4 Cell growth, viability, glucose concentration, and dissolved oxygen in the GLC1 and GLC2 experiments. In both experiments two parallel reactors were run. Glucose deprivation was first initiated in the runs $GLC1^{dep}$ and $GLC2^{dep}$ while the control runs ($GLC1^{ctrl}$ and $GLC2^{ctrl}$) were continued with glucose feeding. After termination of the glucose-depleted cultures, glucose starvation was also initiated in the control runs. The time point of glucose depletion in the $GLC1^{dep}$ and $GLC2^{dep}$ cultivations was defined as zero process time (*dotted line*, $C_{Glc} = 0$). Arrows indicate the time point of glucose deprivation in the $GLC1^{ctrl}$ (*solid arrow*) and $GLC2^{ctrl}$ (*dashed arrow*) cultivations. **a** Viable cell density, **b** viability determined with trypan blue exclusion, **c** off-line determined glucose concentration, **d** on-line measured dissolved oxygen signals

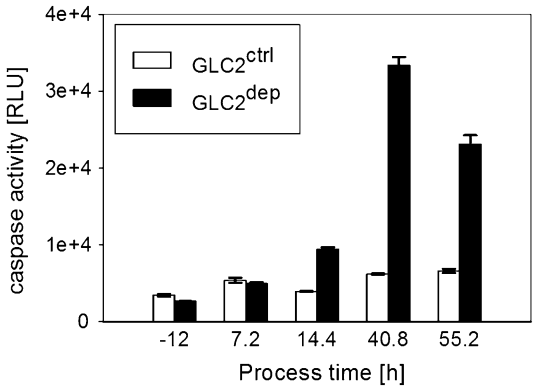

Fig. 5 Increase in caspase-3 and -7 activities after glucose depletion in the GLC2 experiment. Two parallel cultures were run. Apoptosis was induced by glucose deprivation in the $GLC2^{dep}$ culture while glucose feeding was continued in $GLC2^{crtl}$ run

tenfold increase in caspase-3 and -7 activities. (The relative increase was determined 48 h after glucose deprivation as discussed previously). In order to investigate the swiftness of apoptosis induction after glucose deprivation, the kinetics of caspase activity increase was estimated by measuring the activities at several time points in the GLC2 experiment. Compared to the control ($GLC2^{ctrl}$), a significantly higher caspase activity was observed 14 h after glucose depletion in the $GLC2^{dep}$ cultivation (Fig. 5). This indicated that the induction of this early intracellular apoptotic process happened between 7 and 14 h after the onset of glucose starvation in our experimental system.

In order to investigate whether the apoptosis-induced changes in the dielectric properties of the cells were similar to the behavior observed in the camptothecin-induced cultivations, PCA was performed using the on-line collected dielectric spectra. The second principal component captured a very similar ratio of variance in the two models (21 and 22 % in the GLC1 and GLC2 model, respectively). These values were also comparable to the R^2_{CP2} values observed in the CPT experiments (24 and 16 %). Moreover, the loading plot of the second principal component showed also very comparable profiles in the CPT and GLC experiments (Fig. 2 in the electronic supplementary material) indicating similar changes in the dielectric spectra after apoptosis induction in the two different experimental strategies.

The $t[2]$ values showed only small changes until the time point of glucose depletion; however, an increase in $t[2]$ occurred already short (<10 h) after the exhaustion of glucose in both GLC1 and GLC2 experiments (Fig. 6). Accordingly, the changes in the dielectric properties followed similar kinetics in both cell lines after glucose deprivation. Interestingly, this response was faster than the $t[2]$ increase in the CPT experiments after camptothecin induction.

Analysis of apoptosis-induced changes in the parameters of the Cole–Cole model

Beside multivariate data analysis, Cole–Cole modeling is also frequently applied to exploit physiological information from capacitance spectra. A Cole–Cole model was fitted on the capacitance datasets in order to investigate the response of the model parameters on the induction of apoptosis. Two major parameters of the model, ΔC and critical frequency (f_C) were calculated and plotted against time (Fig. 7).

The ΔC values showed very similar profiles to the $t[1]$ curves in the CPT experiments (Fig. 7a), confirming that the major variability in the dielectric spectra is related to the changes in the height of the beta dispersion curve as a result of cell growth or cell death. Similarly, the ΔC profiles in the pre-induction phase of the GLC experiments correlated with the kinetics of cell growth (Fig. 7c). However, in contrast to the moderate response on apoptosis induction in the CPT experiments, ΔC showed a rapid decline short after the depletion of glucose in the GLC

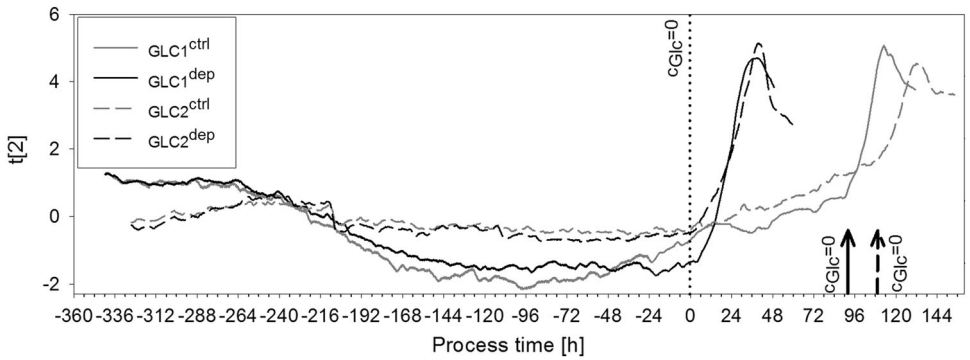

Fig. 6 Multivariate analysis of the capacitance spectra collected during the GLC1 and GLC2 experiments. The score values of the second principal components were plotted against process time; $R^2_{PC2,GLC1} = 0.21$; $R^2_{PC2,GLC2} = 0.22$. The time point of glucose depletion was defined as zero process time (*dotted line*, $C_{Glc} = 0$). *Arrows* indicate the time point of glucose deprivation in the $GLC1^{ctrl}$ (*solid arrow*) and $GLC2^{ctrl}$ (*dashed arrow*) cultivations

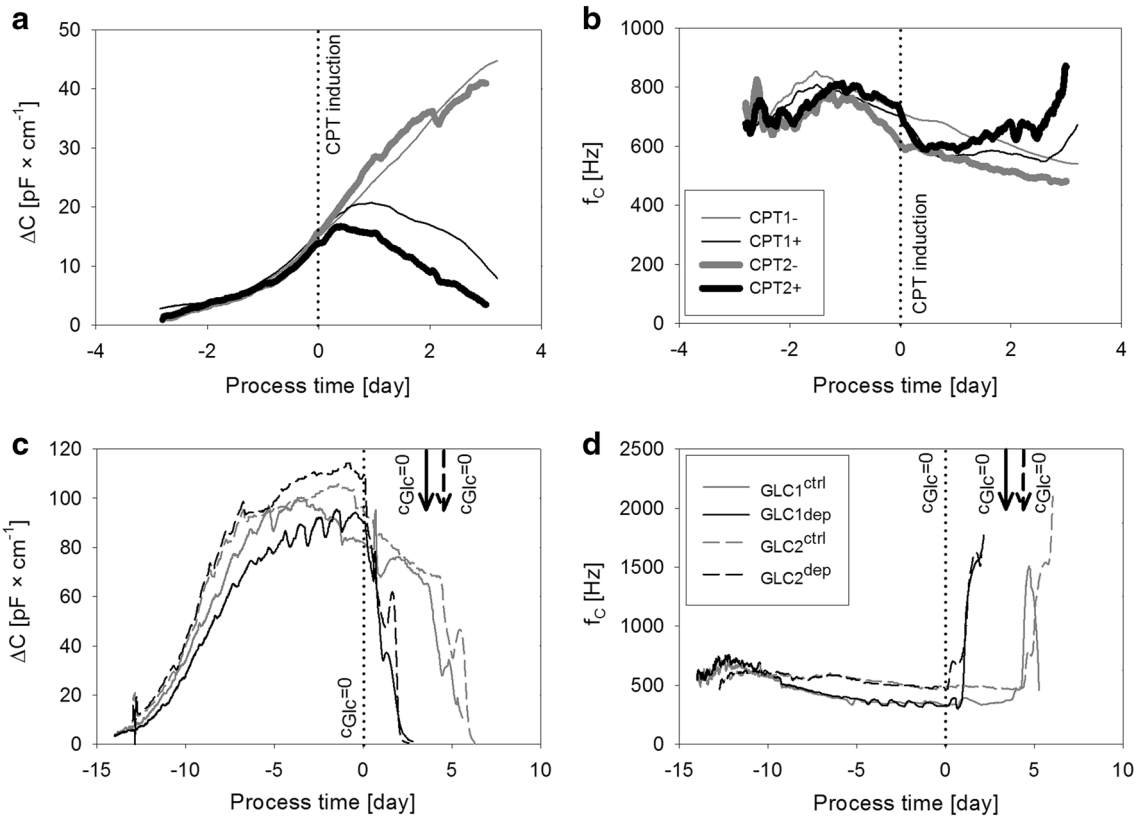

Fig. 7 Cole-Cole model parameters of the capacitance spectra collected during the CPT and GLC experiments. **a** ΔC values in the four CPT cultivations, **b** critical frequency values in the four CPT cultivations, **c** ΔC values in the four GLC cultivations, **d** critical frequency values in the four GLC cultivations. The time point of CPT induction and glucose depletion was defined as zero process time (*dotted lines*, CPT induction, and $C_{Glc} = 0$). *Arrows* indicate the time point of glucose deprivation in the GLC1^{ctrl} (*solid arrow*) and GLC2^{ctrl} (*dashed arrow*) cultivations

experiments. This difference in the ΔC response might have been a consequence of the different physiological status of the cells at the time point of apoptosis induction or the consequence of the different induction strategy.

The f_C values showed similar profiles in the pre-induction period of the CPT experiments (Fig. 7b). Compared to the CPT2− control run, the f_C value showed an increase around 20 h after camptothecin induction in both CPT1+ and CPT2+ runs. However, the f_C curve in the CPT1− cultivation showed an offset compared to the curve of the CPT1+ very shortly after CPT induction. Consequently, the f_C increase in the CPT-induced cultures as compared to the respective control f_C trajectories could not deliver an accurate estimation of the time point when the apoptosis-induced dielectric changes appeared. Accordingly, in contrast to the $t[2]$ curves (Fig. 3c), the f_C trajectories could not be used as a palpable marker of apoptosis-induced changes in the dielectric properties of the cells after CPT induction. In the GLC experiments, the value of f_C remained nearly constant in the 14 day pre-induction period (Fig. 7d), showing a similar trajectory to the $t[2]$ values of the PCA model. After glucose deprivation, f_C increased rapidly to very high levels, suggesting major alterations in the shape of the β-dispersion curve as a result of glucose starvation.

Discussion

In industrial processes, CHO cells undergo physiological changes, the detection of which is essential to gain process knowledge and maximize viable cell density and product yield. The changing dielectric properties of CHO cells in different physiological phases has been reported by Cannizaro et al. [31], who could assign major changes in the shape of the on-line measured dielectric spectrum to process events in a perfusion process. On-line capacitance screening has been successfully used to monitor dielectric changes in the decline phase in batch [32] and fed-batch [33] CHO processes; however, apoptosis as a cause of the detected changes was not investigated in these studies. Apoptosis-induced changes in the dielectric properties of CHO cells have been previously described with the help of at-line DEP measurements [23, 24]. In this study, on-line

measured capacitance spectra were used to investigate how apoptosis affects the dielectric properties of CHO cells in an industrial fed-batch process. Capacitance was measured on-line at 25 frequencies. An experimental design was executed comprising eight cultivations, where:

- control cultivations were performed,
- apoptosis was induced in different process phases with two different induction strategies,
- two different cell lines were used.

This experimental strategy allowed us to identify the dielectric changes which are related to apoptotic events independent of other, cell concentration or process-associated changes. Principal component analysis was performed to extract the relevant physiological information from the multivariate capacitance spectral datasets. More than 99 % of the variability in the capacitance spectrum could be captured by two principal components in all experiments. The first principal component captured the variability related to cell density as indicated by the linear relationship between $t[1]$ values and the at-line measured VCD. We identified a second principal component explaining an explicit proportion ($\sim$20 %) of the variance which appeared after the induction of apoptosis. The similar R^2_{PC2} values in all PCA models suggested that the effect of apoptosis on the capacitance spectrum was similar in both induction strategies. Therefore, we suggest that $t[2]$ is associated with the apoptosis-induced dielectric changes in our experimental system.

The changes in the $t[2]$ could not be linked to a distinct region of the capacitance spectra, suggesting that apoptotic events influence the shape of the whole spectrum. Downey et al. reported changes in the shape of the beta dispersion curve in the decline phase of a fed-batch CHO cultivation [34] which were probably caused by similar cell death-related processes as in our study. In our study, the shift in the f_C of the Cole–Cole model after the induction of apoptosis further confirmed the hypothesis that the shape of the capacitance spectrum is vastly affected by apoptosis-induced dielectric changes. In accordance with this, the increase of the f_C has been recently shown to associate with the increase of the number of apoptotic cells at the end of an adherent Vero cell culture process [35]. Our comparative analysis revealed similarities between the progression of f_C and $t[2]$ curves. However, $t[2]$ showed a more distinct response after the induction of apoptosis. Moreover, $t[2]$ can be calculated by standard PCA algorithms which are generally integrated in commercially available data acquisition and process control softwares. The application of multivariate data analysis for the detection of apoptosis-related changes in cell culture processes has been demonstrated recently; intact cell mass spectrometry datasets were successfully differentiated by PCA [36]. Similarly, principal component analysis in our study has been proven to be an easy-to-use and powerful tool to detect the apoptosis-related changes in the capacitance spectral datasets.

The response of CHO cells on glucose starvation could be analyzed in a timely resolved manner. The cellular pathways of the regulation of apoptosis and glycolytic activity share many regulator molecules [37, 38] suggesting that the fast response in the dielectric properties on glucose depletion observed in our study is linked to apoptotic processes. Although the induction of apoptosis in glucose starvation has been previously reported [15, 24, 39], a timely resolved analysis of the changes in the respiratory activity and dielectric properties of the cells is novel. Two molecular markers of apoptosis were investigated to timely allocate the onset of the dielectric changes with respect to the cellular progression of apoptosis. We showed that both camptothecin treatment and glucose deprivation were followed by caspase-3 and -7 activation considered as early events in the progression of apoptosis. Also apoptotic DNA fragmentation, a late apoptotic event, was analyzed in the CPT experiments. Although we cannot gain deep insight into the molecular mechanism causing the observed changes, our results let us suggest that the dielectric change characterized by the increase in $t[2]$ of the PCA model occurred in the early phase of apoptosis prior to the loss of cell membrane integrity detected with trypan blue exclusion.

As a future perspective, the combined analysis of biological apoptotic markers and on-line capacitance monitoring can be used to investigate the activation of apoptotic processes with time resolution after shifts in process parameters or the onset of nutrient limitations.

Based on the presented results, we propose that the combination of dielectric spectroscopy and PCA can be a powerful tool for the monitoring of apoptotic events in industrial processes. Using similar PCA models, capacitance spectra could be integrated into hierarchical process models, where the capacitance dataset is represented by a single variable (the score value of the apoptosis-related principal component) beside many other on-line measured signals. The proposed methodology could be used in the future to augment the sensitivity of multivariate process models towards physiological changes in cell culture processes.

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Control of Specific Productivity of CHO Cells to Modify the Abundance of High Mannose mAb Glycoforms

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Abstract

The integration of physiological knowledge into process control strategies is a cornerstone for the improvement of biopharmaceutical cell culture technologies. The present contribution investigates the applicability of specific productivity as a physiological control parameter in a cell culture process producing a monoclonal antibody (mAb) in CHO cells.

In order to characterize cell physiology, the on-line oxygen uptake rate (OUR) was monitored and the time-resolved specific productivity was calculated as physiological parameters. This characterization enabled to identify the tight link between the deprivation of tyrosine and the decrease in cell respiration and in specific productivity. Subsequently this link was used to control specific productivity by applying different feeding profiles. The maintenance of specific productivity at various levels enabled to identify a correlation between the rate of product formation and the relative abundance of high mannose glycoforms. An increase in high mannose content was shown as a result of high specific productivity. Furthermore, the high mannose content as a function of cultivation pH and specific productivity was investigated in a Design of Experiment approach.

This study demonstrated how physiological parameters could be used to understand interactions between process parameters, cell physiology and product quality attributes.

Introduction

The amount of biopharmaceutical products expressed in mammalian cell lines has been constantly increasing in the last decade (Walsh 2014). Parallel to the success of mammalian expression systems, the scientific understanding of complex interactions between process parameters and product quality attributes in cell culture processes has been also expanding. This knowledge has become especially critical in biosimilar development, where critical quality attributes (CQAs) have to be steered in a tight range defined by the original product (McCamish and Woollett 2011). The swift scientific progress enabled to identify the production cells' physiological attributes which ultimately determine the interactions between process input parameters and product quality (Carinhas et al. 2012; Dickson 2014). The successful integration of physiological knowledge into process control tools and their applicability to adjust product quality attributes have been recently reviewed (Zalai et al. 2015a).

A frequently investigated physiological parameter of recombinant cell culture processes is specific productivity (q_P), which quantifies the rate of protein expression per cell and time

unit. Product titer, a variable frequently defined as a key performance indicator, is affected by specific productivity to a great extent. Accordingly, the maximization of q_P is an important target of bioprocess development (Kou et al. 2011; Schaub et al. 2012; Templeton et al. 2013). Moreover, as specific productivity delivers time-resolved information on the kinetics of recombinant protein synthesis, it can be used to investigate the interactions between processing events, product formation and changes in post-translational modifications (Sou et al. 2015). Accordingly, q_P is a key parameter to understand links between cell physiology and product quality. An important basis of this knowledge should be the mechanistic understanding of interactions between the rate of product formation and the progress of post-translational modifications.

To investigate mechanistic interactions between specific productivity and product quality, strategies to control q_P at multiple constant levels are required. As to our knowledge, approaches reported in the scientific literature exclusively targeted maximal q_P and did not aim to control this physiological parameter at different levels. The reported approaches either used genetic engineering to enhance protein expression (Kober et al. 2013; Seth et al. 2007; Xiao et al. 2014) or applied process control strategies such as cell cycle arrest (Du et al. 2015) or medium development (Kang et al. 2012; Sellick et al. 2011) to increase product titer. The latter strategy is based on the recognition that a limitation of key nutrients (e.g. amino acids) leads to a decrease in specific productivity, which can be restored by the supplementation of these substances (Lu et al. 2013; Read et al. 2013; Sellick et al. 2011). However, these results also suggest that a targeted limitation and the subsequent continuous feeding of the limiting amino acids can be used to control specific productivity in fed-batch processes. The addition of nutrients in limiting amounts has been already applied in cell culture processes to adjust another physiological parameter, specific growth rate. Aehle et al added glutamine by using a simple open-loop control and successfully controlled the specific growth rate of the cells at four different levels (Aehle et al. 2011a). The same authors also developed a closed-loop control based on oxygen uptake rate (OUR) to control specific growth rate at a constant level for a long time period (Aehle et al. 2012). These studies already demonstrated the controllability of physiological parameters in cell culture processes. However, the control of specific productivity at multiple levels has not been reported previously in the literature to our knowledge.

In this case study, we demonstrate the use of specific productivity as a control parameter in a fed-batch CHO process. The oxygen uptake rate was used to monitor the metabolic activity of the culture and to detect the onset of nutrient limitations. Based on this on-line signal, a

feeding strategy was developed to obtain different q_P profiles. The control of q_P y at different levels allowed investigating interactions between the rate of product formation and post-translational modifications such as product glycosylation.

Materials and Methods

Cell line and preculture

Suspension cultures of two CHO-K1 derived cell lines (referred to as cell line A and B) expressing the same IgG1 monoclonal antibody were maintained in shake flasks before inoculating the bioreactors. Stocks were revived in commercially available basal medium (ActiCHO P, GE Healthcare, UK), supplemented with 8 mM L-Gln and 5 mg/l Insulin. The cells were sub-cultured every 3–4 days with a seeding density of $0.3 \cdot 10^6$ cells/ml and were grown in shake flasks of different scales. The shake flasks were incubated at 37 °C with humidified air containing 5% CO_2 and agitated at 100 rpm orbital shaking.

Bioreactor cultivations

Fed-batch cultivations were performed in bioreactors with 1 l maximal working volume (Sartorius AG, Germany). The targeted seeding cell density was 0.5·106 cells/ml. Temperature, pH and pO2 were controlled by a Biostat BPlus Twin DCU (Sartorius AG, Germany). Stirring speed was set to 125 rpm, initial cultivation temperature was 37 °C, initial pH setpoint was 7.2 and the dissolved oxygen rate was maintained at 40% of air saturation by air–oxygen mixture sparging. The pH value was controlled at the current setpoint ±0.02 by automatic addition of 10% H3PO4 solution or 0.5 M Na2CO3 solution. The shifting of pH and temperature was performed on cultivation day 3 and 5, respectively. Temperature was shifted to 33 °C and pH was shifted to pH 6.9. In the DoE experiments, pH was shifted to the pre-defined setpoint according to the experimental design (see Figure 6a). The basal medium was the same as the one used for the shake flask preculture cultivations (vide supra).

Feeding strategy

The feed medium was a proprietary medium. Bolus feeding was initiated on the 3rd cultivation day by adding a pre-defined amount of feed medium to the culture broth at a high feeding rate. Continuous feeding was carried out by applying Watson Marlow 120U and 101U/R pumps (Watson Marlow, UK) and PharmMed BPT pump tubings (Saint-Gobain Performance Plastics, France). The supplementary feed was a proprietary feed medium containing high concentration of essential amino acids dissolved at alkaline pH. Each pump and tubing combination was tested at several pump speed setpoints to obtain pump speed – feeding rate calibrations. These calibrations were subsequently used during the experiments to adjust

feeding rates to the pre-defined setpoints. Feeding rates were also determined gravimetrically during the experiments and pump speed was adjusted when required.

Measurements

Real-time measurements

The bioreactors and feed mediums were placed on balances (Mettler Toledo, Switzerland) to determine broth and liquid volumes gravimetrically. The balance signals as well as on-line measured process parameters (pH, pO_2 and temperature) were collected by the Biostat BPlus DCUs and processed in a Citect SCADA system (Schneider Electric, Rueil-Malmaison, France) via local area network connection. Capacitance of the cultures was measured with Biomass Monitor 220 (Aber Instruments, Aberystwyth, UK) using 12 mm annular sensors. Oxygen uptake rate was determined with the stationary liquid phase method as described in the literature (Ruffieux et al. 1998). The temperature-dependence of the Henry coefficient was considered (0.974 and 0.905, at 37 °C and 33 °C respectively). The value of kLa was determined as a function of broth volume and aeration rate ($kLa_{(V,\ aer)}$) in a preliminary DoE experiment using the same cultivation medium as for cell cultivation. An equation was determined based on the results of the preliminary experiment and was used to estimate $kLa_{(V,\ aer)}$ on-line.

At-line and off-line measurements

At-line samples were taken every 24 hours or more frequently in order to measure several process variables. pH measurement for in situ pH meter re-calibration was performed with a S47 SevenMulti pH meter (Mettler Toledo, Switzerland). Viable cell density as well as viability was determined in triplicates by Countess automated cell counter (Life Technologies, CA). Samples were centrifuged at 1000 g for 10 minutes and cell-free supernatants were stored at -20 °C until further analysis. Metabolite concentrations were determined in duplicates by enzymatic assays (Cedex BioHT, Roche Diagnostics, Germany). Spent broth analysis to determine amino acid concentrations was performed by HPLC using OPA and FMOC in-needle derivatization and an Agilent ZORBAX Eclipse AAA HPLC column. Product titer was measured by affinity chromatography using a POROS Protein A column (Thermo Fisher Scientific, MA) and applying gradient elution.

Determination of product glycosylation

Cultivation samples were centrifuged at 1000 g for 10 minutes (Rotanta 460 R, Hettich Zentrifugen, Germany) and the supernatant was purified using Protein A affinity chromatography. Enzymatic digestions were performed using trypsin, according to the protocol described before (Ozohanics et al. 2012; Turiák et al. 2011). UPLC-MS analysis of

the antibody digest was performed on a Nexera UPLC (Shimadzu Corporation) coupled to a high resolution micrOTOF-Q II mass spectrometer (Bruker Corporation). Chromatographic conditions were the following: reversed-phase column (Aeris Peptide 1.7 µm XB-C18 particles, Phenomenex Inc., USA), gradient elution (Solvent A: 0.1 v/v% formic acid in water; solvent B: 0.1 v/v% formic acid in 10% water and 90% acetonitrile mixture; flow rate: 225 µL/min flow rate, column temperature: 30 °C). Mass spectrometric conditions: positive electrospray ionization mode (capillary voltage: 4.5 kV; dry gas flow rate: 12.0 L/min; dry temperature: 200 °C; end plate offset: 500 V), scans acquired in the 140-2000 m/z range.

Results

The first goal of the study was to gain real-time information on cell physiology by monitoring the oxygen uptake of the culture on-line and to link this physiological information to the rate of product formation. The ultimate goal was then to develop a control strategy including specific productivity as a parameter and to investigate interactions between specific productivity and the glycosylation pattern of the recombinant product.

On-line detection of the dynamics of cell physiology during switches between nutrient limitation and excess

A typical fed-batch cell culture process was performed with cell line A, using bolus feed additions every second day. OUR was monitored on-line in order to gain information on the respiratory activity of the cells (Fig. 1). On the 5^{th} cultivation day, a temperature shift was performed leading to a decrease in the oxygen uptake of the culture. Another steep decrease in OUR was observed after the 7^{th} cultivation day, which could not be related to any processing events. Spent broth analysis revealed that the decrease in the respiratory activity was linked to the exhaustion of tyrosine in the cultivation broth. The concentration of all other amino acids remained above the limit of detection of the measurement method during the whole cultivation (concentrations of two other aromatic amino acids which also frequently show deprivation in cell culture processes due to their low solubility in cultivation media are shown in Figure 1). After the addition of the bolus feed (which contained tyrosine) on the 9^{th} cultivation day, the respiratory activity of the cells recovered and OUR showed an increase for several hours. However, as tyrosine was depleted again, similar to the previously described events, OUR showed repeatedly a decline and remained on a low level until the next feeding event.

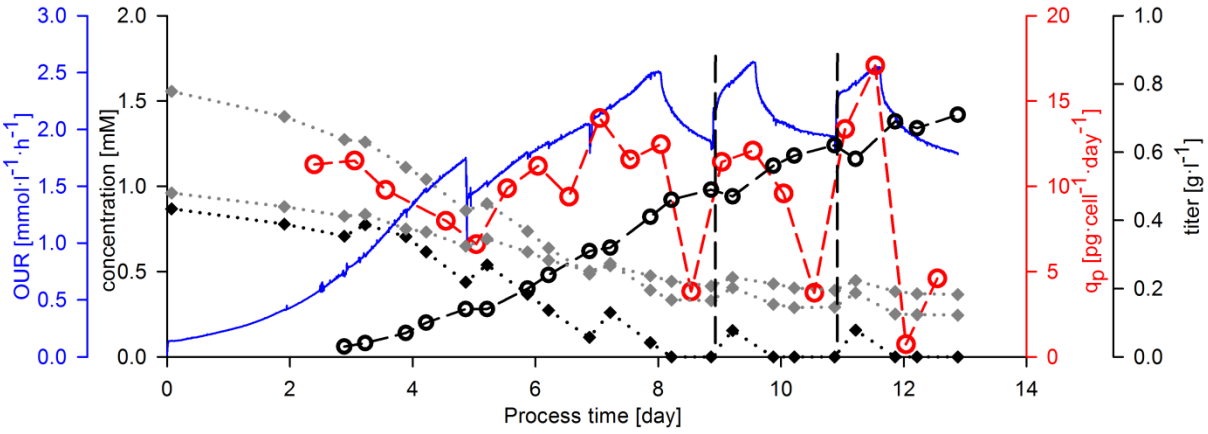

Fig. 1 Physiological response on the switch between nutrient limitation and nutrient excess. The concentration of tyrosine (black diamonds) and the concentration of two aromatic amino acids, phenylalanine and tryptophan (grey diamonds) are represented. The on-line determined oxygen uptake rate (OUR, blue), the product titer (empty black circles) and the specific productivity (empty red circles) is also demonstrated. Black dashed lines indicate the timepoint of the addition of the last two bolus feeds.

Whereas the investigation of product titer did not indicate variations in product formation during the switch between nutrient limitation and excess, the analysis of q_P revealed a steep decrease in the rate of product formation in the nutrient limited phases (Fig. 1). However, similar to OUR, q_P recovered after the feeding events. Taken together, the exhaustion of tyrosine led to a decrease in the oxygen uptake (OUR) as well as in the productivity (q_P) of the culture. The main benefit of OUR monitoring was the real-time detection of the changes in cell physiology.

Implementation of a feeding strategy to increase q_P based on real-time physiological information

Based on the above discussed observations, an experiment was designed where a supplementary feed containing tyrosine (and other essential amino acids) was added to the cultivation in order to overcome nutrient limitations. The supplementary feed was initiated after observing the first decrease of the OUR signal and it was terminated at the next bolus feeding event (Figure 2). Control cultivations were performed for both cell lines to obtain a similar physiological profile as shown in the previous experiment.

Interestingly, the first decrease in OUR was observed one day earlier in the cultivations with cell line B (day 7) compared to the cell line A cultures (day 8). Spent broth analysis (data not shown) revealed that this phenomenon was a consequence of the earlier exhaustion of tyrosine, probably due to the higher substrate uptake rates of cell line B. However, the on-line monitoring of OUR enabled to detect the earlier onset of nutrient limitations and to maintain a

high specific productivity by starting the supplementary feed one day earlier as in the cell line A cultivation.

After the bolus feeding events, the OUR of the control cultivation ("ctrl") with cell line A was monitored and when the decline in OUR was detected, the supplementary feed of the supplemented cultivations ("suppl") was started again for both cell lines. This strategy allowed to avoid nutrient limitation in the supplemented cultivation of cell line A from the first start of the supplementary feed until the end of the cultivation (data not shown). However, a decrease in OUR was observed in the supplemented cultivation of cell line B after the 11th cultivation day, suggesting the exhaustion of a further substance which was not added with the supplementary feed. The spent broth analysis revealed the exhaustion of leucine in this cultivation, which was indeed not added to the supplementary feed. The next step of process development would be to subsequently adjust the composition of the supplementary feed to the metabolic requirements of cell line B.

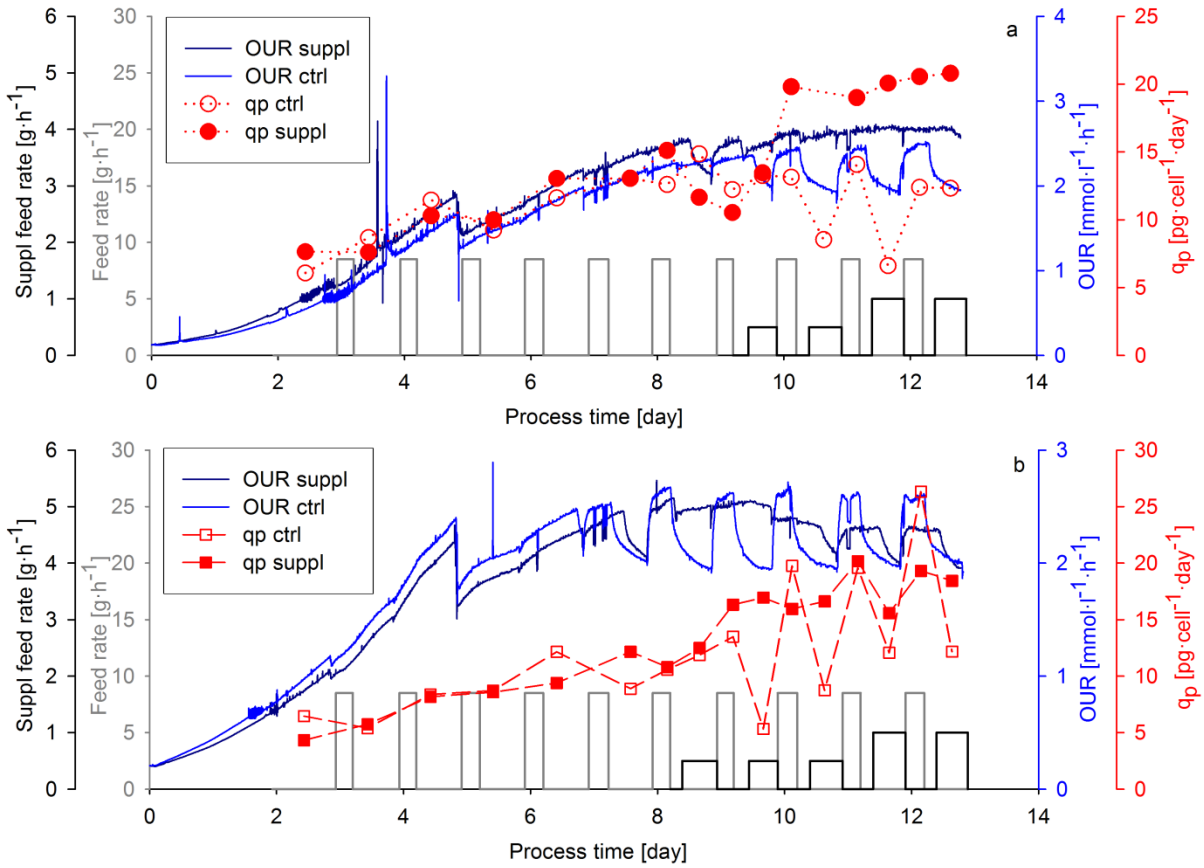

Fig. 2 Physiological response on nutrient limitation and on the addition of a supplementary feed. The feeding rate of the daily bolus addition of the standard feed medium is shown in grey. Black lines indicate the feeding rate of the supplementary feed, started after the detection of the decrease in OUR of the control cultivation. **a** Cell line A **b** Cell line B

The time-resolved analysis of q_P revealed that product formation rate followed the pattern of the OUR signals (Fig. 2). In the control cultivations, both cell lines showed high variations in q_P, in accordance with the changes in OUR. In contrast, the supplemented cultures of both cell line A and B showed a high and nearly constant q_P after the initiation of the supplementary feeding. Thus, the real-time adjusted feeding strategy enabled us to generate different q_P patterns with two different cell lines in a single experiment. The detected differences in cell respiration as well as in q_P suggested that the cells experienced a very different physiological status in the "ctrl" and in the "suppl" cultivations. Whereas the "ctrl" cultivations showed repeatedly physiological changes in nutrient limitation and excess, the addition of a supplementary feed maintained amino acid concentrations and a stable physiological status in the "suppl" cultivations.

Beside OUR monitoring, capacitance measurement, another on-line tool, was also performed to investigate changes in the dielectric properties of the cells during the cultivations. The capacitance signals measured at 580 kHz (C_{580}) frequency showed a linear correlation to the at-line measured VCD values for both cell lines in the growth phase of the cultivations (Fig. 3 a and b). The linear correlation between the capacitance signal measured at one frequency and cell density indicated a constant physiological status in this process phase. Interestingly, the capacitance signal showed a similar pattern to OUR in the control cultivations, indicating major changes in the dielectric properties of the cells as a response on the repeated switches between nutrient limitation and excess.

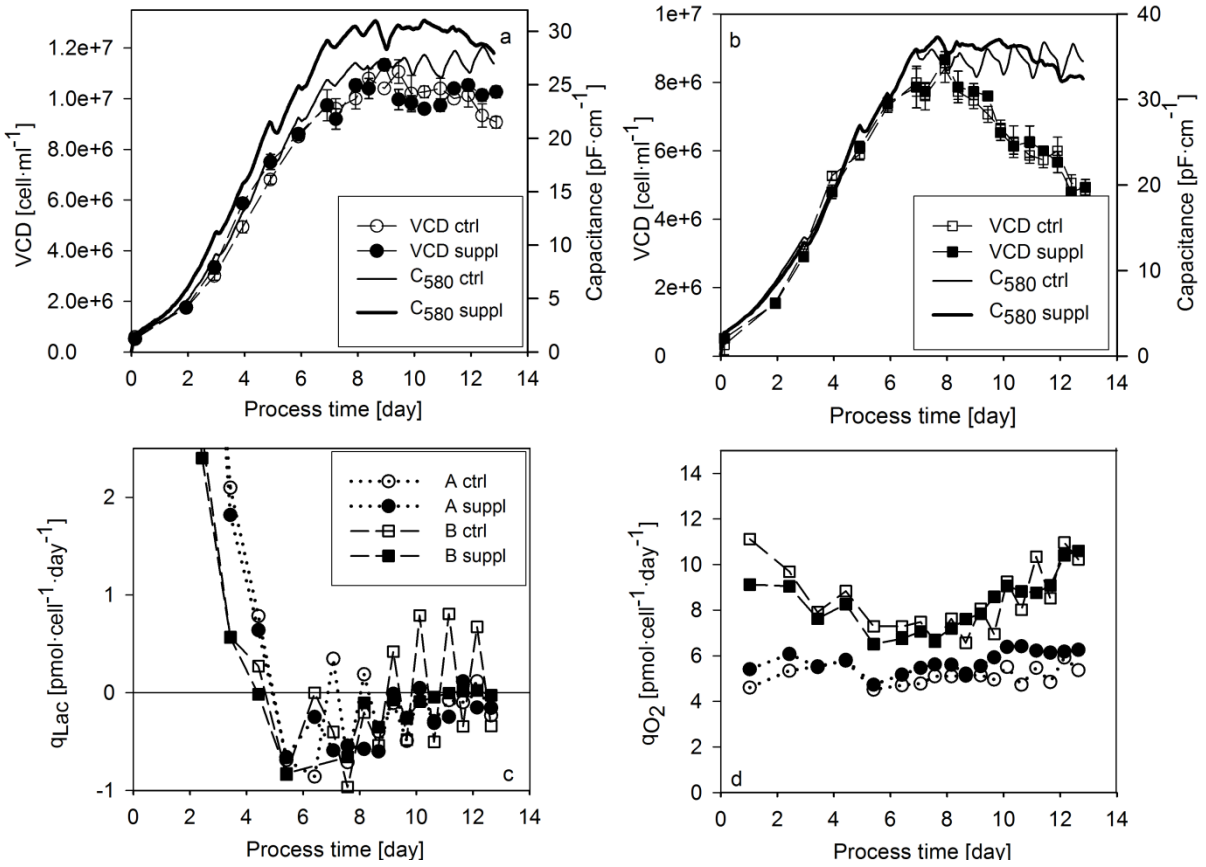

Fig. 3 Cell growth, lactate metabolism and respiration in the supplementary feeding experiments. a VCD and on-line measured capacitance for cell line A **b** VCD and on-line measured capacitance for cell line B **c** Specific lactate uptake rate **d** Specific oxygen consumption rate. Symbols are used the same way as in Fig. 3c

In order to gain insight into cell metabolism, specific lactate production rate and specific oxygen uptake rate (q_{O2}) were calculated. Both cell lines switched to lactate uptake after the temperature shift performed on the 5th cultivation day (Fig. 3c). Interestingly, cell line B showed an oscillation in lactate metabolism in accordance with the phases of nutrient excess and limitation. Whereas the cells produced lactate after bolus feeding events, they switched to lactate consumption after nutrient exhaustion. Cell line A showed a different pattern indicating that the effect of nutrient limitation on lactate metabolism may be cell line dependent. The calculated q_{O2} values showed an oscillation in the respiratory activity of the cells (Fig. 3d), in accordance with the OUR pattern. Interestingly, the q_{O2} values of the two cell lines differed in a great extent, indicating differences in the metabolic activity of the two cell lines (*vide supra*).

Specific productivity was plotted against q_{O2} in order to investigate the link between respiratory activity and product formation (Fig. 4a). A linear relationship was observed,

verifying the tight physiological link between cell respiration and the rate of product formation in our system of interest.

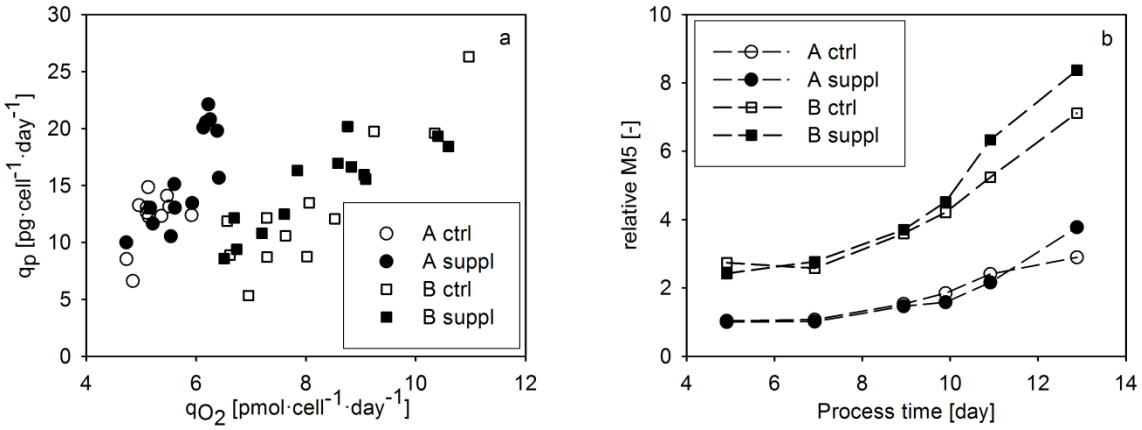

Fig. 4 The link between respiratory activity, product formation and product quality in the supplementary feeding experiments. a Specific productivity plotted against specific oxygen uptake rate **b** Relative abundance of M5 high mannose glycoform. The values were normalized by dividing with the value determined in first measurement point for cell line A

Product glycosylation – which is an N-linked biantennary oligosaccharide structure in the Fc region of the antibody – was analyzed at several time points of the cultivations. The seven most abundant glycoforms were investiagted: an afucosylated high mannose glycoform containing five mannose residues (M5), and further six glycoforms labelled according to the number of galactose in the core structure (G0, G1 and G2); each of them occurred in both fucosylated and non-fucosylated forms. The relative abundance of the glycoforms was calculated in order to investigate their distribution as a function of process time. Beside the cell line-dependent difference, a process-dependent difference was also observed in the glycosylation patterns. Generally, the relative abundance of high mannose (Fig. 4b) and other afucosylated glycoforms (G0, G1 and G2 on Fig. S1) was higher in the supplemented experiments. Both cell lines showed an increasing M5 pattern with very similar values until the 10th cultivation day in the respective control and supplemented cultivations (Fig. 4b). However, the relative abundance of M5 high mannose glycoform was higher in both supplemented experiments, suggesting that the differences in q_P-patterns led to differences in product quality. The successful adjustment of q_P enabled to identify a link between specific productivity and high mannose content. In order to investigate this link further, another experiment was conducted with cell line A, where q_P was controlled at two different levels (*vide infra*).

Application of dynamic feed profiles to adjust q_P to different levels

In this experiment, feed media were added continuously to two independent cultivations performed with cell line A. The dynamic feed ramps were initiated on the 8th cultivation day based on previous observations (*vide supra*) to avoid any nutrient limitations. In order to achieve a high q_P, both standard and supplementary feed media were added to the cultivation broth in experiment "HI". While, in experiment "LO", only the standard feed medium was used at a lower feeding rate to obtain a lower q_P value. Moreover, the feeding rate was reduced on the 11th cultivation day in the "LO" cultivation in order to trigger a decrease in q_P in the last two cultivation days (Fig. 5a). We found that both the on-line determined OUR and the calculated q_P were higher in experiment "HI", indicating that the proposed strategy to control q_P by adjusting the feeding rate was successful. Moreover, both OUR and q_P immediately followed the dynamic change in the feeding rate in experiment "LO" on the 11th cultivation day, suggesting a strong link between feeding rate, the respiratory activity and product formation in the nutrient-limited environment; the correlation of the latter two physiological features is also proven by the linear correlation of q_{O2} and q_P on Fig. 5b.

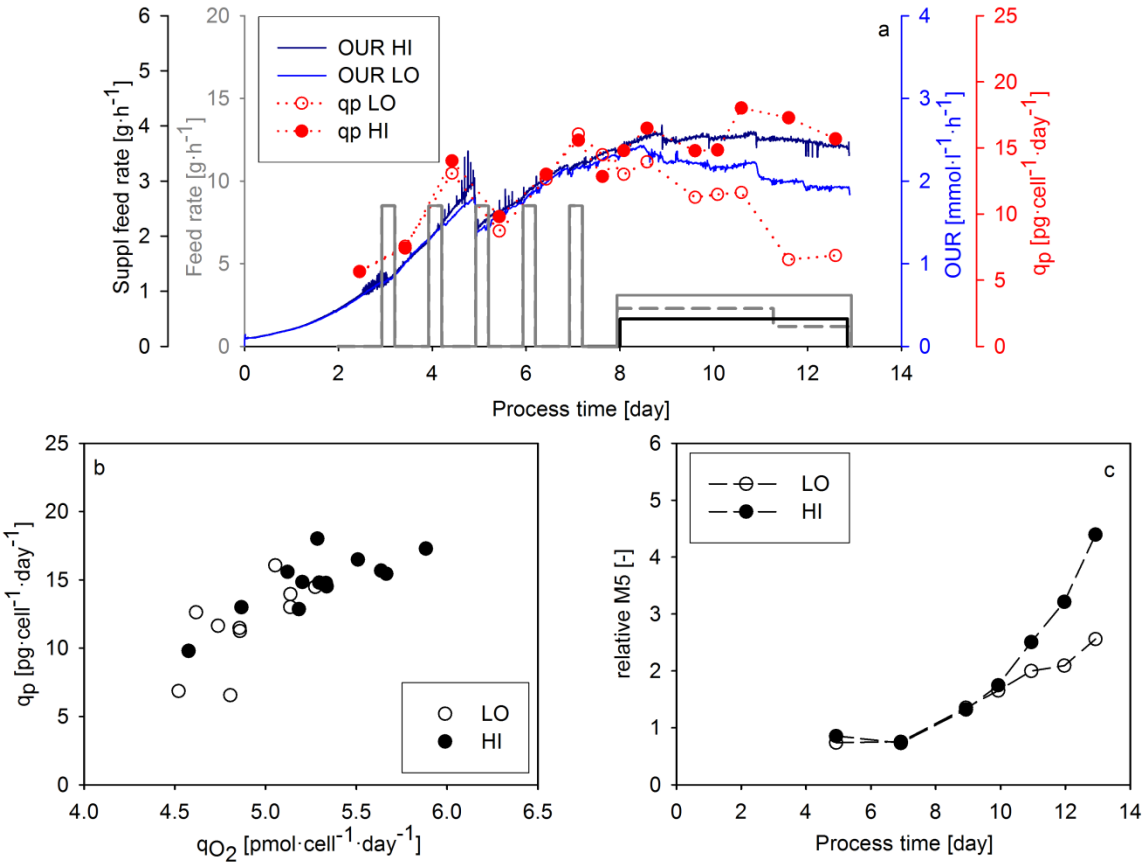

Fig. 5 Cell physiology, product formation and product quality in the dynamic feeding experiments conducted with cell line A. a Feeding rates, oxygen uptake rate and specific productivity (q_P) in the "LO" and "HI" experiments. Grey dashed line indicates the feed profile of the "LO" experiment. Grey and black

continuous lines indicate the feed profile of the "HI" experiment. **b** Specific productivity plotted against specific oxygen uptake rate **c** Relative abundance of M5 high mannose glycoform. The values were normalized by dividing the measured glycoform abundance with the value determined in the first measurement point of the bolus-fed experiment (Fig. 4b)

The relative M5 value plotted as a function of process time showed a distinct response on the level of specific productivity (Fig. 5c). When the productivity of the two cultivations diverged to a great extent from each other, the relative M5 values increased in experiment "HI" reaching much higher values than in experiment "LO". The relative abundance of G0F glycoform showed the opposite trend and the distribution of all other glycoforms remained very similar in both cultivations (Fig. S2). These results suggest that the abundance of high mannose glycoforms is higher when q_P is increased. The major achievement of this experiment was the adjustment of q_P on two constant levels, which enabled to identify its effect on high mannose levels independent of any other process parameters.

Application of q_P as a physiological factor in a multivariate experimental design

The benefits of using physiological parameters as experimental factors in DoE designs have already been demonstrated in microbial process development (Wechselberger et al. 2012). As a proof of concept for cell culture processes, a DoE approach involving specific productivity as an experimental factor was conducted in our study (Fig. 6a). Specific productivity was controlled at three levels ("high", "center point" and "low") in the last three cultivation days by applying different pre-defined continuous feeding profiles (Fig. S3a). The second experimental factor was chosen to be the pH shift setpoint, as pH was shown to effect mAb glycosylation (Jedrzejewski et al. 2013). The center point value of the pH-shift setpoints was 7.05.

We found that the experimental factors did not affect VCD (Fig. 6b), thus the implementation of a cell density-dependent feeding rate was not necessary. The cultivations showed a similar q_P-profile until the 8th cultivation day (Fig. 6c). After the initiation of the continuous feed profiles, the specific productivity curves diverged from each other. The two "q_P high" cultivations showed a high specific productivity, as expected, until the end of the experiment. In the center point experiments, q_P started to decrease after the 8th cultivation day and then remained on a constant level in the last three days of the experiments. The highly similar q_P profile of the three center point experiments proved the reproducibility of the control strategy (Fig. S3b). Interestingly, the specific productivity in the "q_P low" experiments showed a pH-dependent behavior. Whereas q_P started to decrease immediately after the initiation of the

continuous feeding in the "w/o pH-shift, q_P low" cultivation, specific productivity remained high until the 10th cultivation day in the "pH-shift, q_P low" run and decreased to a low level only in the last three days of the experiment. The reason behind the later decrease of product formation rate is the lower metabolic activity in pH-shifted conditions and a subsequently later onset of nutrient limitation in the "pH-shift, q_P low" cultivation. Indeed, the spent broth analysis verified that tyrosine exhausted on the 9th cultivation day in the cultivation without pH-shift and only on the 11th cultivation day in the "pH-shift, q_P low" cultivation. However, q_P decreased to similar values in both "q_P low" runs, enabling the use of specific productivity as a DoE factor independent of pH in the last three days of the cultivations.

The relationship of respiratory activity and specific productivity also showed a pH-dependent pattern (Fig. 6d). Although the values of q_{O2} and q_P followed a linear correlation in all runs, the cultivations without pH-shift formed a different cluster on the q_{O2}- q_P plot from those where pH-shift was performed. This suggested that the respiratory activity of the cells is dependent on pH. This was also confirmed by the OUR and q_{O2} profiles (Fig. 6e and f), where the values of the pH-shifted cultivations run lower between the timepoint of the pH shift (3rd cultivation day) and the initiation of the continuous feeds (8th cultivation day). Nutrient availability also affected the respiratory activity of the cells. Whereas q_{O2} increased in the "q_P high" cultivations after the 8th cultivation day, the "q_P low" cultivations, in which the feeding rate was low, showed a decline in q_{O2} after the initiation of the continuous feed profile. However, the relationship between q_{O2} and q_P was only affected by pH shift, and retained its linear nature at the different q_P levels of the DoE experiment.

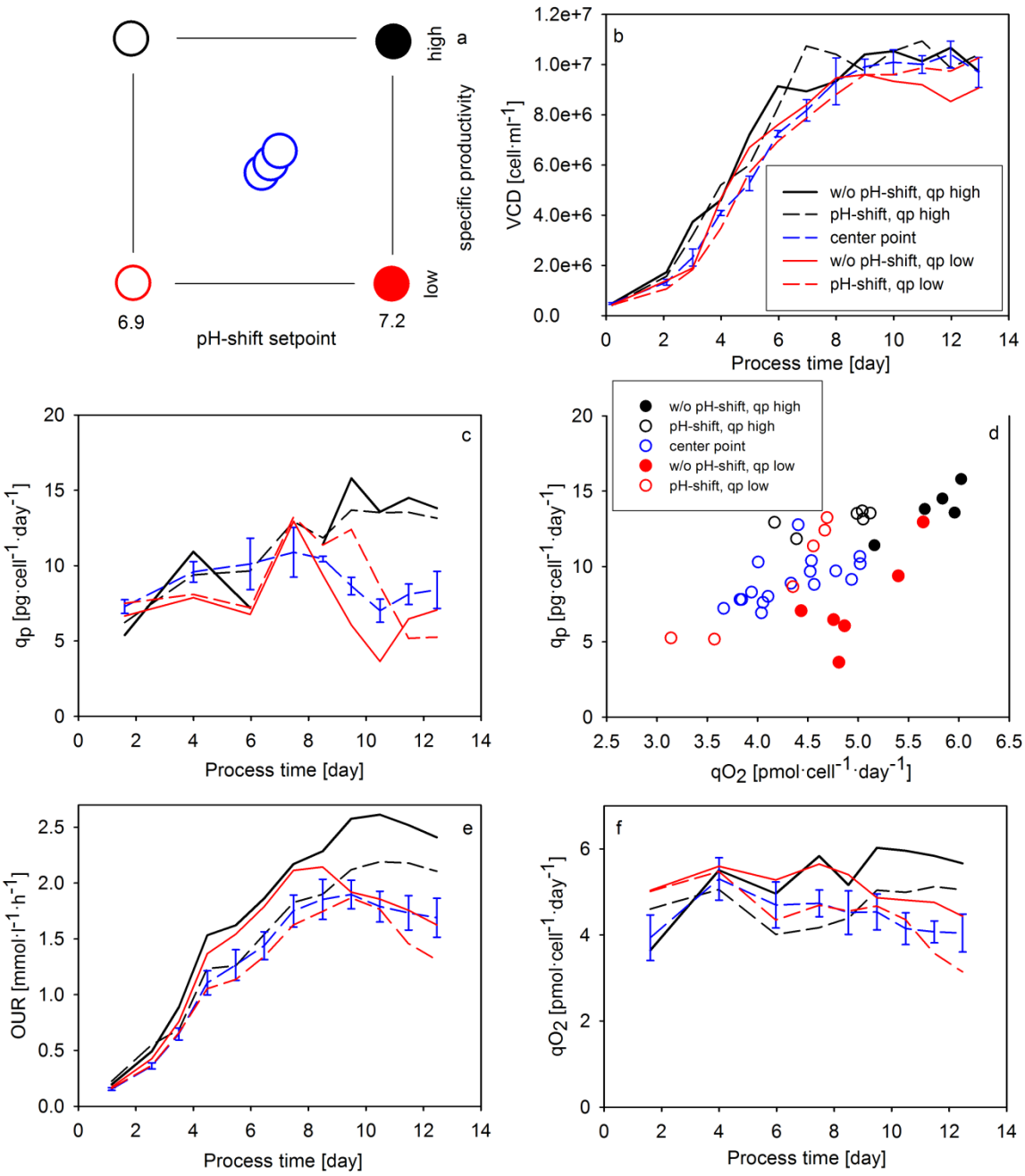

Fig. 6 The Design of Experiment approach conducted with cell line A. a The DoE design with specific productivity as an experimental factor **b** Specific productivity as a function of process time **c** VCD curves **d** Specific productivity plotted against specific oxygen uptake rate after day 7 (The values of the three center point runs are shown with empty blue circles) **e** Oxygen uptake rate **f** Specific oxygen uptake rate

In order to investigate the effect of the experimental factors on cell metabolism, the $Y_{Lac/Glc}$ variable was investigated (Fig. 7a). Similar to previous observations with the same cell line (Zalai et al. 2015b), the shift in pH to 6.9 on the 3rd cultivation day immediately affected the ratio of lactate and glucose uptake rates. Interestingly, the metabolic shift to lactate consumption was also dependent on the feeding rate in the cultivations without pH shift.

Whereas the cells did not switch to lactate uptake in the cultivation with a high feeding rate, lactate consumption was observed after the 8th cultivation day in the "w/o pH-shift, q_P low". Osmolality has been shown to affect high mannose content in cell culture processes (Shi and Goudar 2014). In this study, osmolality profiles clustered according to the setpoint of the pH-shift, however did not show a response on the q_P setpoint (Fig. 7b). Thus, the effect of q_P on high mannose content was not a consequence of interactions with osmolality effects.

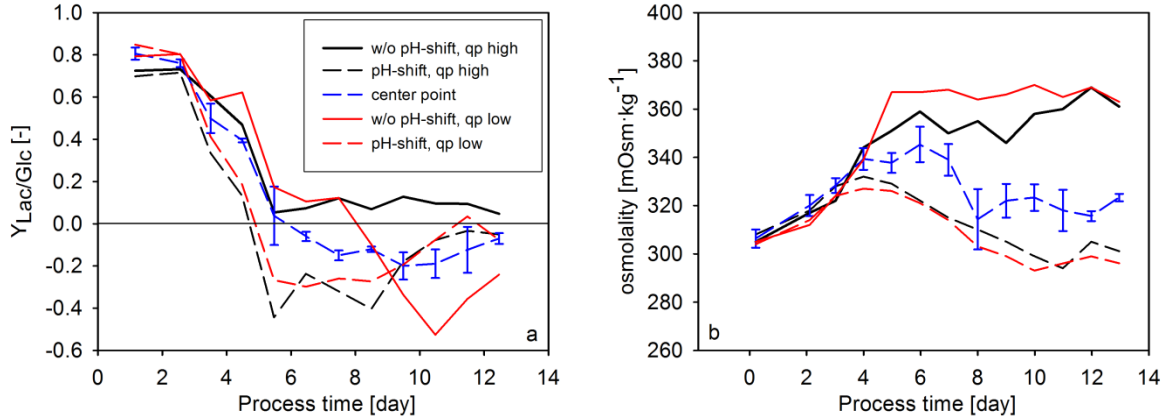

Fig. 7 Lactate metabolism and osmolality in the DoE experiments. a The ratio of lactate production and glucose uptake rates **b** Off-line determined osmolality values

High mannose content was determined in the DoE cultivations in order to investigate the effect of the experimental factors on the accumulation of this pre-mature glycoform. The center point runs showed very similar high mannose profiles during the whole cultivation period (Fig. 8a). Whereas the two cultivations without pH shift showed a same profile as the center point runs, the cultivations with a pH shift to 6.9 showed elevated relative M5 values already on the 10th day of the cultivation. However, the high mannose of these cultivations diverged in the last day of the experiment, according to the q_P setpoint. The relative M5 values in the two cultivations with pH shift were comparable to the values observed in the "HI" and "LO" experiments (Fig. 8b), verifying our previous observations that the increase of q_P leads to the accumulation of pre-mature glycoforms. However, the high mannose content remained low in the "w/o pH-shift, q_P high" cultivation, indicating that the phenomenon is pH-dependent. Taken together, the DoE experimental design enabled the identification of an interaction effect between pH and q_P, which lead to elevated high mannose levels in the "pH-shift, q_P high" experimental point.

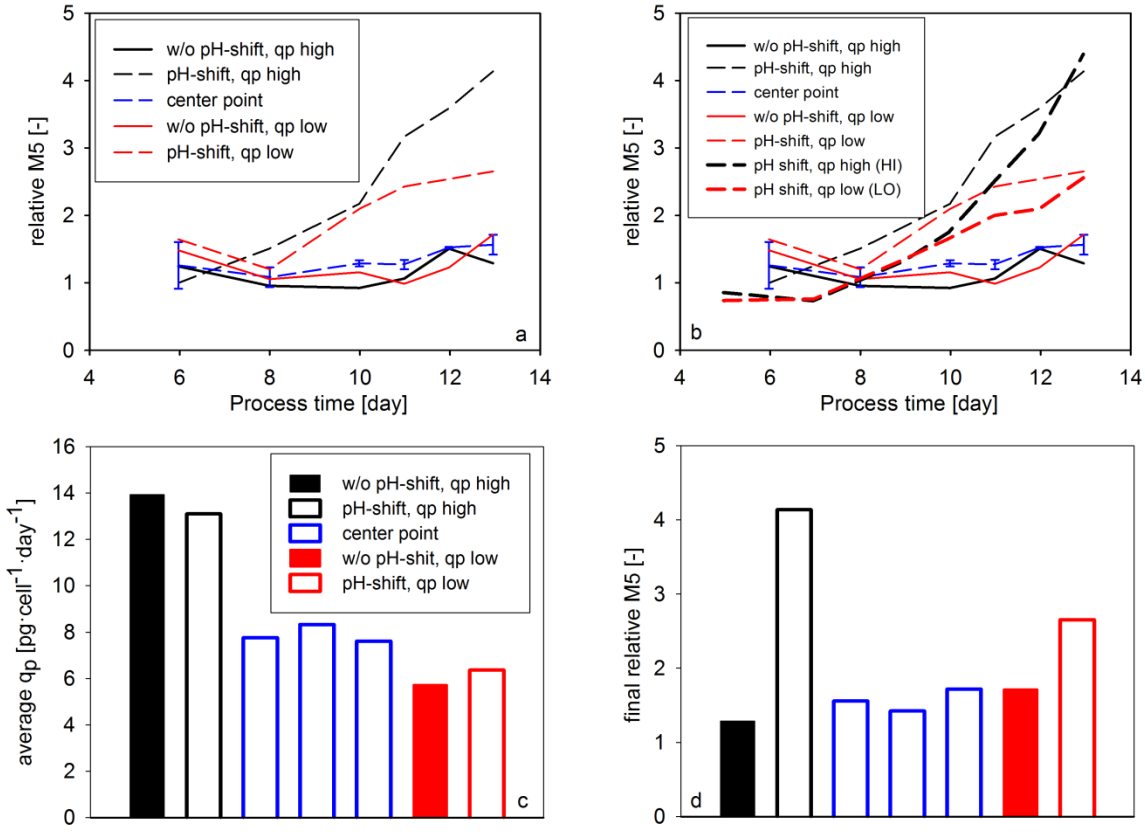

Fig. 8 Product formation and product quality in the DoE experiments. a The relative abundance of M5 high mannose glycoform as a function of process time **b** The relative abundance of M5 high mannose glycoform as a function of process time in the DoE as well as in the dynamic feeding experiments **c** Average q_P values calculated from the specific productivity values in the last three days of the cultivations **d** Final high mannose content. The values were normalized by dividing with the value determined in first measurement point of the bolus-fed experiment (Fig. 4b).

In order to involve q_P as an experimental factor in the statistical evaluation of the DoE data, average specific productivity in the last three cultivation days was calculated (Fig. 8c). The values in the "q_P low" and the center point runs showed only a small difference, leading to an asymmetric design. Consequently, the fitted mathematical model showed a low Q^2 value (0.52) as an indicator of the poor prediction capability. However, the statistical analysis identified the interaction term of pH and q_P as a significant model coefficient to explain relationship between the experimental factors and final high mannose content (Fig. 8d). This result verified the above discussed pH-dependence of the effect of q_P on high mannose content.

Discussion

Changes in cell physiology during switches between nutrient limitation and excess

In this study, the investigation of OUR and q_P enabled the identification of major changes in cell physiology during a fed-batch CHO cell culture process. Spent broth analysis revealed that the cause of the decrease in the metabolic activity of the cells (indicated by OUR) and productivity (q_P) was the depletion of tyrosine. After the addition of tyrosine with a bolus feed both physiological parameters recovered. These results suggest that cell metabolism shows a very prompt response on the depletion and subsequently on the addition of the essential amino acid tyrosine, as indicated by the steep increase in OUR after the feeding events. Although the exact time point of the onset of tyrosine limitation could not be determined, it can be hypothesized that the steep decrease in OUR happened shortly after the limitation. Ansorge et al. reported a similar OUR pattern in a fed-batch CHO cultivation, however, the authors could not detect the depletion of amino acids with the available analytical device and thus only hypothesized that the observed decrease in OUR is a consequence of nutrient limitation (Ansorge et al. 2010).

Interestingly, the capacitance of the culture, which was also monitored on-line, showed a similar response to OUR on the switches between nutrient limitation and excess. This observation is again in accordance with the results of Ansorge et al, who suggested that the observed phenomenon can be a consequence of the change in multiple physiological attributes influencing dielectric properties, such as cell size or intracellular conductivity (Ansorge et al. 2010). The changes in dielectric properties upon apoptosis induction have been recently demonstrated for the same cell line (Zalai et al. 2015c), showing that capacitance spectroscopy can be used to detect major changes in cell physiology. The exact reason for the observed variations in C_{580} in the recent study remains to be elucidated, however, it demonstrates the applicability of capacitance measurement to detect physiological changes in cell culture processes.

The change in the metabolic activity of the cells upon nutrient limitation was also verified by the q_{Lac} profile of cell line B in the experiments with bolus feeds (Fig. 3c). The cells produced lactate after each bolus feeding event and switched to lactate consumption when tyrosine was depleted. This observation is in accordance with literature data suggesting that the decrease of lactate production rate is a consequence of amino acid limitation (Read et al. 2013). Interestingly, cell line A did not show a similar fast response on bolus feeding events and remained in a metabolic status characterized by lactate uptake from the 5^{th} cultivation day

until the end of the cultivation (Fig. 3c). This constant metabolic status was probably a consequence of cultivation pH which was shifted to 6.9, and might have restricted metabolic fluxes to a higher extent as in cell line B. The observation that cell line A produced lactate throughout the cultivation where pH was controlled at 7.2 and a high feeding rate was maintained (Fig. 7a) supported this hypothesis further.

The kinetics of product formation was assessed by calculating q_P with a high time resolution. In accordance with several papers reporting an increase in product titer after the supplementation of limiting amino acids (Feeney et al. 2013; Read et al. 2013; Yu et al. 2011), we observed an increase in q_P after the supplementation of tyrosine (Fig. 2). However, these publications calculated q_P for either the whole cultivation period or for time windows of several days, and did not deliver an understanding how exactly specific productivity is affected by nutrient limitation. A q_P calculated with a low resolution (> 24 hours) would not indicate the dynamic changes in productivity upon nutrient depletion in our study. By calculating q_P for sufficiently short time periods (12 hours) and by monitoring other physiological variables on-line (OUR and capacitance), we were able to show a distinct response of cell physiology on nutrient limitation and feeding events.

Control strategy to adjust specific productivity

The swift response of OUR on tyrosine depletion suggested that OUR can be used to detect nutrient limitations and to implement control strategies which respond to the limitation by the addition of the limiting substrate. Accordingly, we implemented a feeding strategy based on the real-time monitoring of OUR to supplement tyrosine. As the investigation of physiological variables with high time resolution revealed a similar response of OUR and q_p on nutrient limitation and excess, we expected that maintaining the oxygen uptake of the culture at a high level will result in high productivity. Indeed, the dynamic OUR-based feeding strategy enabled us to keep specific productivity at a constant high level throughout the cultivation (Fig. 2). OUR has been already used as an input signal for feeding strategies targeting constant cell growth or metabolism (Aehle et al. 2011b; Zhou et al. 1997). Moreover, OUR has been used as an input signal to control the addition of amino acids in mammalian perfusion processes (Aehle et al. 2012; Feng et al. 2006). However, to our knowledge, approaches to adjust q_p based on OUR have not been reported previously in the literature. The applicability of the presented control strategy was verified with two different cell lines. In the cell line B cultivation, nutrient limitation occurred one day earlier as in the cell line A cultivation, as indicated by the on-line OUR signal. However, the real-time

adjusted feeding strategy enabled to respond on this difference and maintain a high q_p for cell line B, as well. This result demonstrated that a control strategy, which considers real-time physiological information, can be beneficial to respond on cell line-dependent differences during cell culture process development. Furthermore, our results support that the monitoring of OUR in cell culture process is a key PAT method, which can be used to implement sophisticated control strategies (Kroll et al. 2014).

The basis of the q_p-control strategy was the tight link between q_{O2} and q_P in our experimental system. The linear correlation of the two variables was verified in the experiments with bolus as well as with continuous feeding (Fig. 4a and Fig. 5b). As the respiratory activity correlates to TCA flux in mammalian cells (Nargund et al. 2015; Zagari et al. 2013), a similar linear correlation of q_P and TCA cycle activity can be assumed, which has been previously observed in a fed-batch CHO cultivation (Templeton et al. 2013). However, the q_{O2}-q_P relationship showed a different pattern in the two different cell lines, showing a steeper linear correlation in cell line A and further confirming the metabolic differences between the two cell lines (Fig. 4a). Although clone-to-clone differences in q_{O2} have been reported to correlate with clonal variations in productivity (Ghorbaniaghdam et al. 2014), in our study the higher q_{O2} of cell line B was not coupled to a higher q_P. The results of the DoE cultivations revealed changes in q_{O2} at different pH setpoints. Accordingly, the pH-dependence of q_{O2} and substrate uptake rates has to be considered when OUR-based feeding strategies are set up.

The effect of specific productivity on the abundance of high mannose glycoforms

The q_p-control strategy enabled to investigate the effect of product formation rate on glycosylation, one of the most critical quality attributes of monoclonal antibodies. Glycosylation, a form of protein post-translational modification, is a result of a complex cellular process which occurs intracellularly, enclosed in the compartments of the endoplasmic reticulum and the Golgi apparatus. The formation of protein-linked glycan structures is catalyzed by enzymes of the glycosylation pathway, however, this consecutive enzymatic process is not always fully accomplished resulting in a heterogenic mixture of various glycoforms. High-mannose glycans are generated early in the glycosylation pathway; the reason why these glycoforms are considered to be pre-mature structures. Increasing $q_{P,}$ which was achieved by supplemented feeding in this study led presumably to an elevated protein flux towards the glycosylation machinery. The increasing accumulation of high mannose forms, which was shown to be associated with the elevated q_P (Fig. 5c), may be the cause of putative bottlenecks in the later phase of the glycosylation pathway. Taking the high

complexity of protein glycosylation into account, the nature of these bottlenecks can be very diverse including the activation and the transport of substrates through cellular and intracellular membrane barriers or the expression level and the activity of glycosylation enzymes. For example, the identification of a bottleneck in protein translocation could help to overcome intracellular protein aggregation and enable to increase the productivity of the cells by overexpressing the enzyme responsible for protein translocation (Le Fourn et al. 2014). In a good accordance with our results, increased q_P has been reported to result in the accumulation of pre-matured oligosaccharides in mild hypothermic culture conditions (Sou et al. 2015); furthermore, high specific productivity was also discussed in the association with the increase of non-fully processed high-mannosylated glycans (Hossler 2012; Umaña and Bailey 1997). Our results together with the above cited considerations, allow to suggest that the rate of protein production may affect the output of post-translational modification.

Interestingly, the increase of high-mannosylated glycoforms was observed only in the cultivations with pH-shift. The DoE experiments revealed that the phenomenon did not occur at high pH setpoint, as indicated by the high mannose profiles were similar in the experiments conducted at pH setpoint 7.2. As extracellular pH influences the intracellular pH (L'Allemain et al. 1984), the activity of the enzymes responsible for protein glycosylation might change as a result of the pH-shift (Hossler et al. 2009; Rivinoja et al. 2009). Accordingly, the high pH setpoint in the DoE experiments could lead to sufficient enzyme activities in the Golgi resolving the bottleneck of the glycosylation machinery. However, in order to elucidate the exact mechanism behind the observed phenomenon, a more comprehensive physiological characterization (e.g. proteomic and transcriptomic measurements) would be required.The q_P-control strategy presented in this study was based on the addition of the essential amino acid tyrosine at limiting levels to influence the rate of product formation. Tyrosine has been reported to be replaced by the structurally similar phenylalanine during protein translation, leading to tyrosine misincorporation and an increase in the abundance of sequence variants in tyrosine limitation (Feeney et al. 2013). These results together with ours suggest that the effect of control strategies based on nutrient limitation has to be thoroughly investigated targeting all Critical Quality Attributes, for example post-translational modifications as well as the primary sequence of the produced monoclonal antibody.

The recent case study demonstrated the control of q_P in a fed-batch CHO process expressing a monoclonal antibody. The novel control strategy enabled the investigation of links between the rate of product formation and the glycosylation pattern. The increased abundance of high mannose glycoforms at high q_P suggest that the output of post-translational modifications is

dependent on the rate of product formation. Moreover, by involving specific productivity as an experimental factor in a DoE design, we could show that this phenomenon is dependent on cultivation pH. Our results demonstrate that the application of PAT tools, physiological characterization and multivariate experimental designs can facilitate the understanding of complex interactions between process input and output parameters. Such knowledge will contribute to the development of sophisticated control strategies to control product quality attributes in a tight pre-defined range, which is especially relevant in biosimilar process development.

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Supplementary material

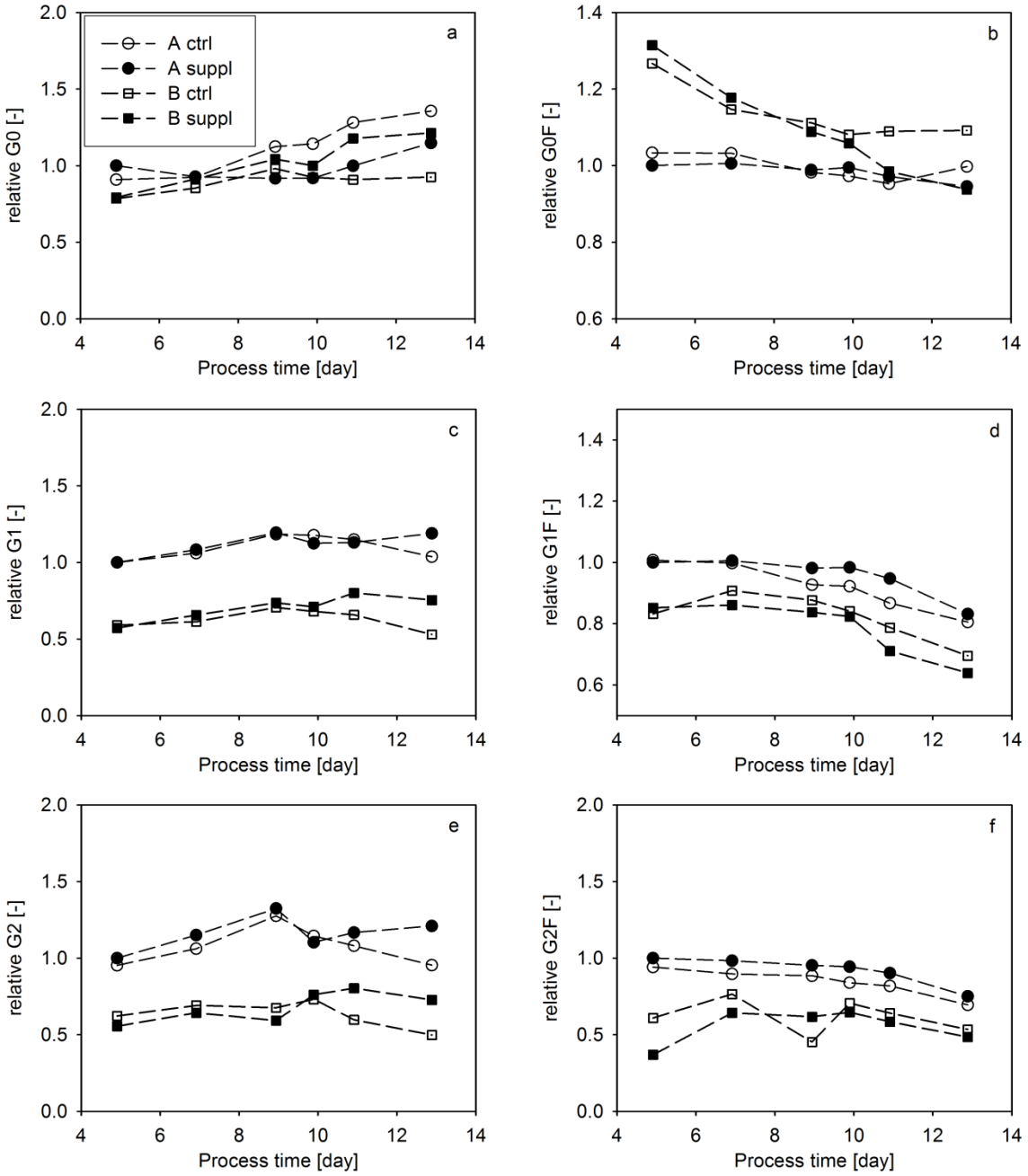

Fig. S1 The relative abundance of the major glycoforms in the bolus-fed experiments with supplementary feed (Fig. 4b). The values were normalized by dividing with the value determined in first measurement point for the respective glycoform for cell line A

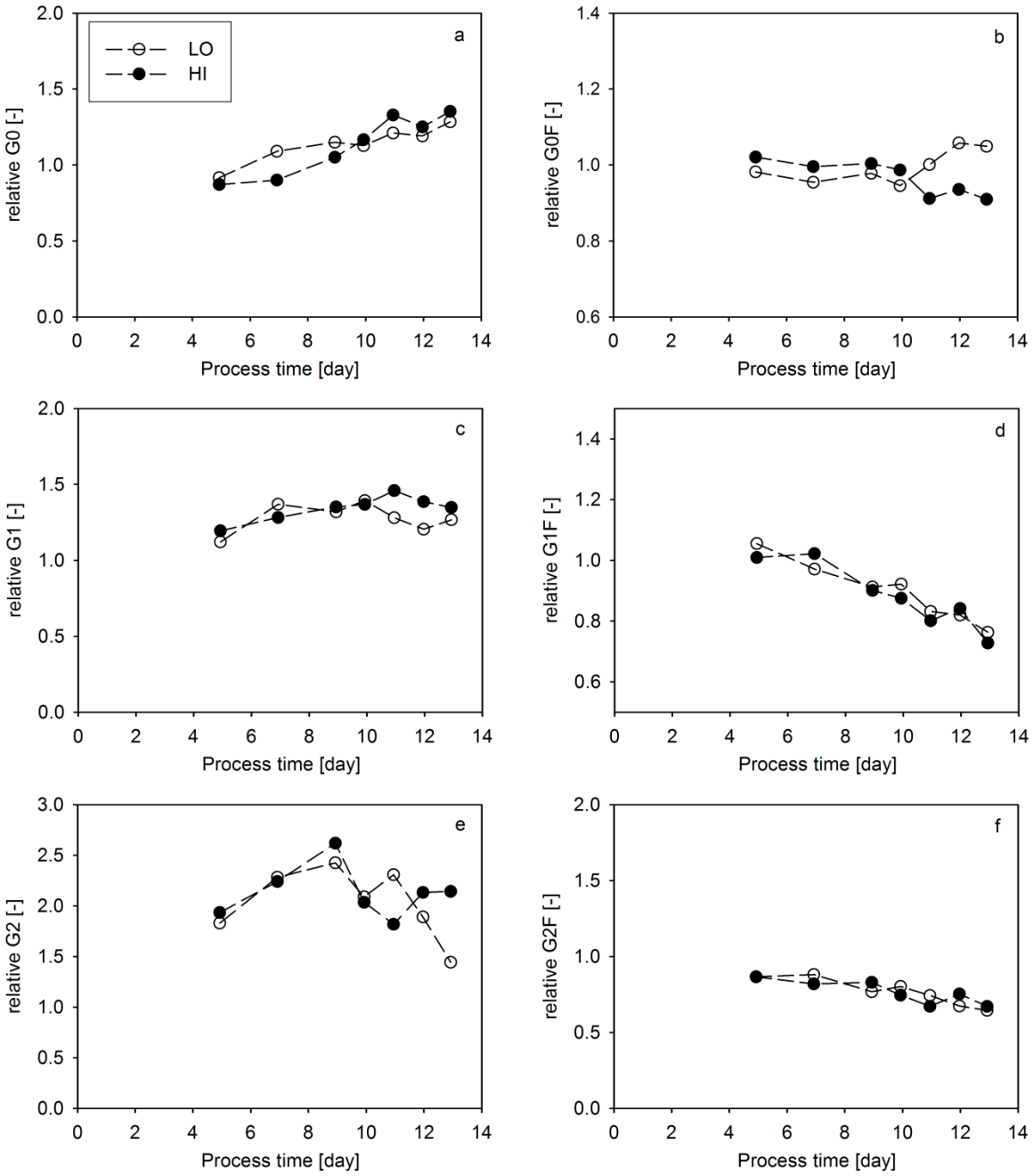

Fig. S2 The relative abundance of the major glycoforms in the dynamic feeding experiments conducted with cell line A (see Fig. 5). The values were normalized by dividing with the value determined in first measurement point of the bolus-fed experiment for the respective glycoform in the bolus-fed experiments (Fig. S1)

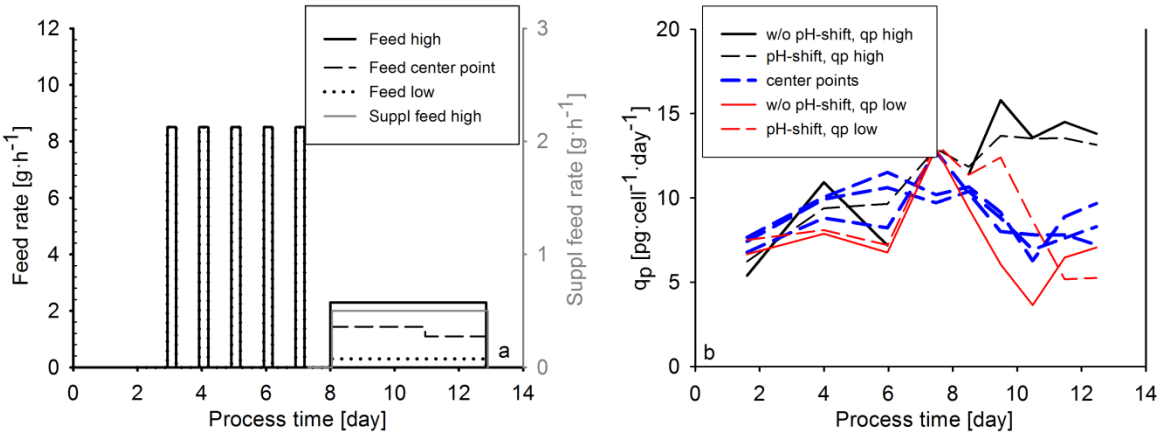

Fig. S3 a Feeding profiles in the DoE experiments. The supplementary feed was added exclusively in the "q_P high" cultivations. In the center point cultivations, the feeding rate was decreased on the 11th cultivation day based on previous observations showing an increase in q_P at a similar constant feeding rate. **b** Specific productivity profiles in the DoE experiments. Center point cultivations are represented with dashed blue lines

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Summary and Outlook

Within this thesis, the implementation of Quality by Design tools in biosimilar development is demonstrated. The main objective was to investigate the benefits of focusing on cell physiology to target enhanced process understanding. Accordingly, the manuscripts included in this thesis can be summed up in a two-dimensional logical structure (Figure 5).

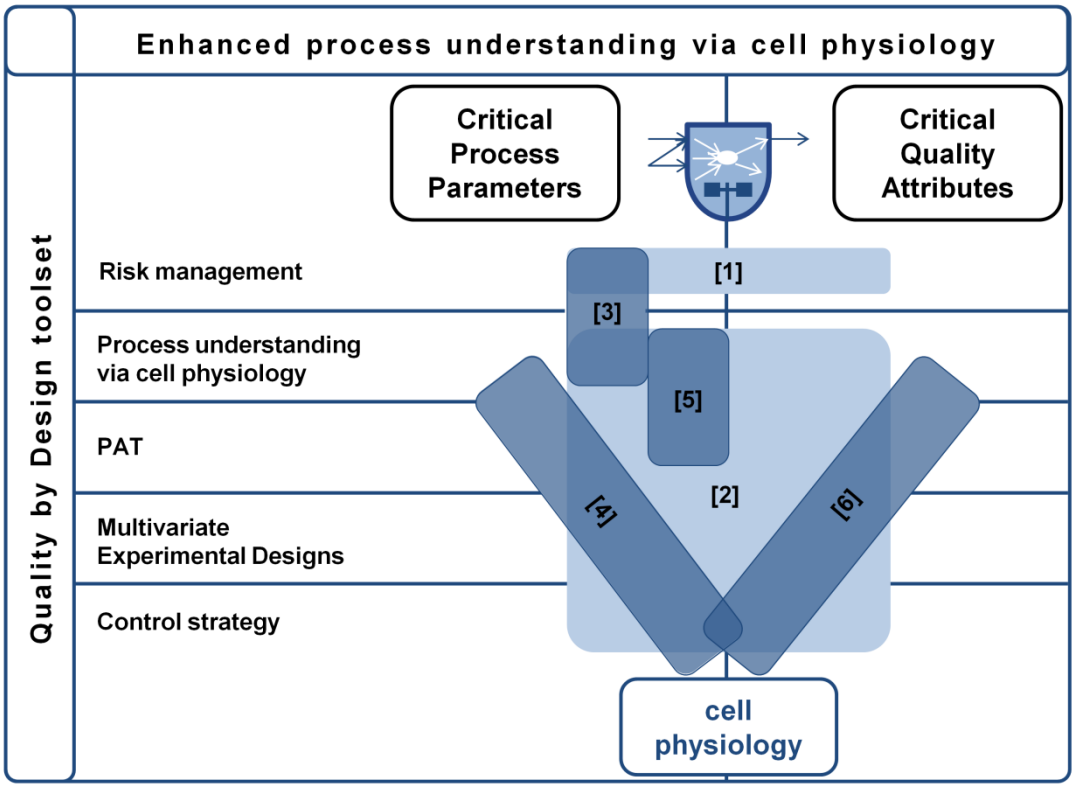

Figure 5. The localization of manuscripts in the two dimensional logical matrix of the thesis. Theoretical manuscripts for concept development are marked with light blue boxes. Manuscripts reporting experimental results are marked with dark blue boxes. Citations in square brackets refer to the manuscripts included in the thesis.

The first (horizontal) dimension indicates the variety of Quality by Design tools which were used in the studies in order to gain enhanced process understanding. The second (vertical) dimension indicates the two knowledge groups (see *Introduction – Process design to target CQAs*), which were used to achieve a sound scientific understanding of CPP-CQA interactions. According to this second dimensionality, the thesis can be considered as a methodological case study which investigates the applicability of the physiological approach in the development of cell culture processes.

Achievements

In this thesis, a methodological workflow is presented which triggered the generation of enhanced process understanding in a fed-batch CHO process producing a biosimilar monoclonal antibody. Based on this knowledge, a novel control strategy was applied to adjust the glycosylation pattern of the product [6]. Accordingly, the presented workflow enables to address a major challenge of biosimilar process development, namely the control of CQAs in the production technology.

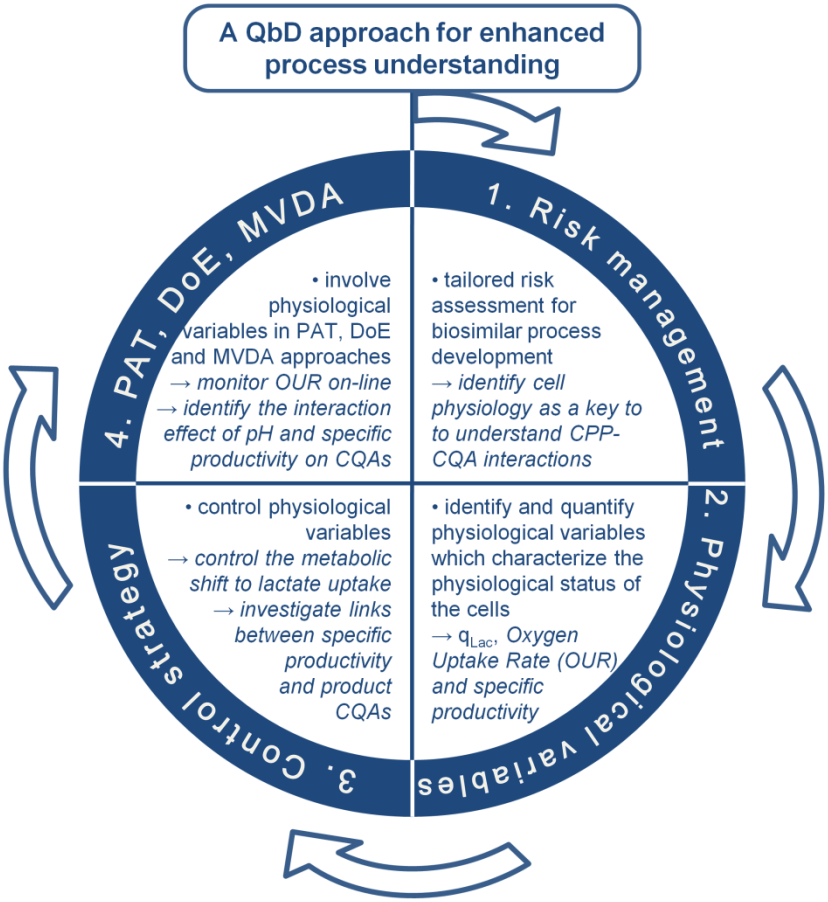

Figure 6. Summary of the four steps towards enhanced process understanding and product quality control. The four steps are shown on the arc of the circle. The achievements are summarized in the center.

In order to achieve the targeted process knowledge, the four essential steps presented in Figure 6 were necessary. Here, these four steps and the achievements in the manuscripts included in the thesis are summarized.

1. First, a **structured risk-assessment approach** enabled to focus on product quality from the very beginning of process development [1]. A novel risk assessment tool tailored for early-stage biosimilar development was developed in order to define critical process parameters with a risk-based approach. Thereby, cell physiology was identified to have a major effect on the relationship of CPPs and CQAs. This finding suggested that enhanced

process understanding can be achieved by investigating the effect of cell physiology on CPP-CQA links.

2. The second essential step was the **identification** and subsequently the **quantification of physiological variables** to gain enhanced process understanding. The thesis focuses mainly on the investigation of cell physiology on the level of metabolism. Although a sound understanding of cell physiology requires the assessment of additional physiological levels such as transcriptome or proteome [2], cell metabolism has been proven to be a good indicator of physiological changes throughout the investigated fed-batch cell culture process [3,4,6]. In order to identify the most important metabolites, advanced data evaluation tools such as mechanistic models and multivariate data analysis tools were applied [4]. The ability to reduce the number of metabolites to be measured for the monitoring of cell physiology offers a clear economic benefit for the industry as it reduces measurement costs as well as data evaluation efforts in routine bioprocess development. As demonstrated in this thesis, the quantification of lactate metabolism [4] or oxygen uptake [6] can be used to assess important physiological features associated with targeted phenotypes (e.g. reduced overflow metabolism or high productivity).
3. The third essential step was the development of **strategies to control physiological variables** in order to investigate their effect on CQAs. The basis of the physiological control strategies was the understanding of interactions between CPPs and cell physiology (referred to as *control knowledge* in the introduction). In [4], an important physiological feature of CHO cells, namely the metabolic switch to lactate uptake is controlled by shifting process parameters such as cultivation pH and temperature. Moreover, the control of specific productivity with dynamic feeding profiles in a nutrient-limited environment is presented in [6]. This novel control strategy enabled to identify a link between the rate of product formation and product glycosylation, and subsequently to adjust glycan heterogeneity. Accordingly, a physiological control strategy is successfully applied to understand cell physiology-CQA links (referred to as *scalable knowledge* in the introduction) in this thesis.
4. The novel physiological control strategy is based on a PAT approach monitoring OUR on-line. Moreover, as an ultimate novelty, specific productivity was involved as an experimental factor in a DoE design and an interaction effect of specific productivity and pH-shift on product glycosylation could be identified. Accordingly, the fourth essential step was the **involvement of physiological variables in standard QbD tools** such as PAT, multivariate experiment designs and multivariate data analysis, which enables to

exploit the full benefit of the physiological approach to gain enhanced process understanding and to control product quality.

Evaluation

The proposed steps of the physiological approach presented in this thesis enable to achieve enhanced process understanding. As discussed above, this knowledge is essential for the development of a novel physiological control strategy. However, some of the tools used in this project might not be essential or could be simplified in order to reduce development time and cost in the future.

For example, DoE designs have to be carefully selected or can be even replaced by univariate experiments in order to keep the number of experiments low [3]. Therefore, risk-based approaches could be used to select the most critical parameters for multivariate designs and identify those which have to be investigated with univariate experimentation.

Moreover, the targeted extent of process understanding should be considered by the selection of DoE designs. Whereas a simple screening (e.g. factorial) design is sufficient to identify interaction effects of different process parameters, an optimization (e.g. central composite) design is appropriate to develop a control strategy. Accordingly, conducting a screening design in [4] and an optimization design in [6] would have been more appropriate with respect to the overall goal of the case study.

Another strategy to speed up process development is the use of dynamic ramps in process parameters to obtain multiple levels of the experimental factors within a single experiment (Zalai et al. 2012). However, the applicability of dynamic strategies can be constrained by time- or memory effects, which are especially present in fed-batch cell culture processes. In [6], a dynamic feeding strategy was successfully applied to adjust specific productivity to multiple levels within a single cultivation; however, the effect of specific productivity on the investigated CQA was clearly process time-dependent.

The methodological novelty presented in this thesis is the integration of physiological knowledge into cell culture process development to achieve enhanced process understanding. The anticipated benefit of the presented workflow over conventional QbD approaches is twofold. On the one hand, physiological variables provide scale- and technology independent information about the production technology. Thus, the presented workflow can be considered as a platform development tool since it could be applied for diverse cell culture processes. For example, the on-line monitoring and control of physiological variables could be applied in continuous processes in order to keep the cells at an optimal physiological status

for a very long time period. On the other hand, the use of scale-independent control variables such as specific productivity is expected to accelerate process transfer and scale-up and ultimately leads to predictable performance in production scale.

Impact and applicability

The growing importance of biotechnological processes in pharmaceutical production triggers an increasing demand to better understand and control these complex technologies. The considerable effort of the biopharmaceutical industry to achieve enhanced process understanding is reflected by the steep increase in the number of scientific contributions in this field. As discussed in the introduction of this thesis, process understanding is especially relevant in biosimilar development, where tight CQA specifications necessitate the development of advanced control strategies. On the one hand, it has been already realized that the key to process understanding is to understand how the recombinant cells, which express the product, behave in the production technology. On the other hand, the swift scientific revolution in systems biology has enabled the high-throughput measurement of numerous physiological variables in a fast and easy way. Taken together, both the market need as well as the necessary measurement technology is available to gain insight into the physiological status of the cells. However, despite of the availability of a vast amount of physiological data, the integration of this valuable data into process development remains a challenging task. The transformation of physiological data to process understanding and control knowledge requires structured approaches.

The anticipated benefit of implementing the methodology presented in this thesis is exactly to fill this gap, namely to provide a conceptual platform for the generation and application of physiological knowledge in cell culture process development. Two necessary knowledge groups are identified: the understanding of CPP-cell physiology (control knowledge) and cell physiology-CQA (scalable knowledge) interactions (Figure 3). Targeting any of these two knowledge groups will enable scientists and development engineers to facilitate information extraction from bioprocess datasets.

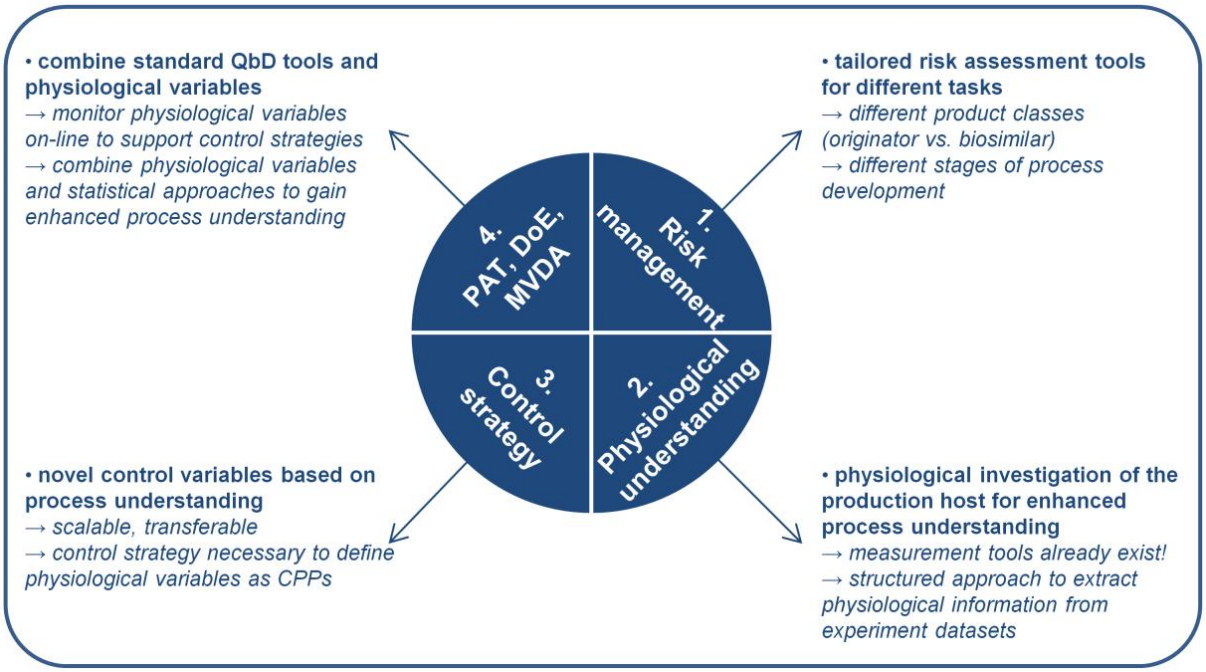

Figure 7. The applicability of the four essential steps of the thesis in bioprocess development activities.

It is expected that the four essential steps presented within this thesis to use physiological knowledge can be transferred and applied to facilitate bioprocess development activities (Figure 7). Here, possible applications of these four steps are discussed.

1. Risk management is routinely used in the pharmaceutical industry to focus on the ultimate goal of product development, which is product safety and efficacy. However, the risk management tool has to be carefully selected to effectively support process development. As demonstrated in [1], **tailored risk assessment tools** can be used to address the unique characteristics of different product classes or different stages of process development.
2. As discussed above, measurement techniques already exist to create physiological data, for example to determine intra- and extracellular metabolite concentrations. However, due to the high number of metabolites involved in mammalian cell metabolism, approaches are needed to **extract physiological knowledge** from these large datasets. Therefore, mechanistic and data-driven approaches can be combined, as presented in [4] (metabolic flux analysis combined with PLS-R VIP), to convert data into knowledge. Moreover, based on the derived physiological knowledge, a few benchmark components or fluxes can be selected which can be used to characterize and monitor cell physiology during the process. Similar advanced data evaluation tools can be applied in many different areas of bioprocess development to extract knowledge from large experimental datasets.

3. It is expected that enhanced process understanding will facilitate the transfer and regulatory filing of biopharmaceutical processes. **Novel control strategies** will enable to increase process robustness and facilitate site-to-site process transfer. Moreover, the involvement of scale-independent physiological parameters as control variables into regulatory filings will enable to reduce activities related to scalability issues. However, process development engineers will have to deliver robust physiological control strategies to exploit the above mentioned benefits. Workflows similar to the one presented in this thesis can be used to achieve the required knowledge.
4. The need to understand and monitor biopharmaceutical processes has triggered the development of several PAT concepts. However, conventional PAT approaches mainly use spectroscopic data and multivariate data analysis to predict process performance indicators or metabolite concentrations; hence, do not contribute to the understanding of CPP-CQA interactions on a physiological level. In this thesis, PAT tools are demonstrated which detect physiological changes such as the onset of apoptotic events [5] or the decrease in oxygen uptake rate [6]. Similar PAT tools will facilitate the detection of shifts in cell physiology and enable the development of adaptive control strategies in the future which respond real-time to physiological events.

   Moreover, the use of physiological variables as response variables or as experimental factors in DoE exercises and statistical evaluation (as demonstrated in this thesis in [4] and [6], respectively) will support the generation of enhanced process understanding.

Taken together, the implementation of the four steps demonstrated in this thesis is expected to facilitate the sound understanding of CPP-CQA interactions. The anticipated benefit of using the here proposed workflow is the development of control strategies which enable to run robust processes and to precisely target CQA ranges. This is especially relevant for biosimilar processes, in order to meet strict product quality specifications. Moreover, novel CQA control strategies are also essential for biobetters, a type of biopharmaceutical products, the clinical effect of which is enhanced by the targeted modification of CQAs.

The workflow presented in this thesis is also expected to generate platform knowledge for biopharmaceutical processes, which can be transferred to similar processes (e.g. similar expression systems or the same cultivation media). Although many physiological features show high variability in different host cells, understanding fundamental links between process parameters, cell physiology and product quality will ultimately facilitate biopharmaceutical process development.

Beside the above discussed aspects of the transferability of the presented approaches and knowledge, the applicability has been continuously assessed and verified in the pharmaceutical company where the work was conducted.

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