Pharmaceutical BIOPROCESSING

Ex situ online monitoring: application, challenges and opportunities for biopharmaceuticals processes

Monitoring of bioprocesses, especially biopharmaceutical production processes, is of utmost importance to ensure constant product quality and availability. Economical bioprocess development depends on time-efficient process development, aiming for in-depth understanding of the bioprocess and its critical parameters. Online monitoring of bioprocesses allows the assessment and processing of critical process data in real time. This is a prerequisite to permit real-time process management. Various devices for automated sampling and analysis, as well as data analysis and processing, have emerged over the last decade. In this review, we will cover the most important developments and novelties in the field of bioprocess monitoring from a methodological point of view. We focus on consolidation and processing of big amounts of data generated during a bioprocess and discuss how the proper interaction of hardware and software will improve bioprocess monitoring and control in the future.

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Background

Biopharmaceutical processes must satisfy numerous demands. Next to high productivities for commercial success, they are under tight supervision of regulatory authorities. Recent regulatory initiatives such as the Process Analytical Technology (PAT) and Quality by Design (QbD) initiative demand a high degree of process understanding to ensure constant product quality [1–3].

Process understanding can be achieved through sound, science-based process development [4]. This includes prior knowledge, sound experimentation and systematic observation of the bioprocesses, referred to as bioprocesses monitoring [5]. In order to reduce the number of necessary experiments, suitable statistical design of experiments (DoE) can be applied. In addition, this approach can serve not only to quantify bioprocesses, but also to determine optimal settings of process parameters [6,7]. Furthermore, DoE acts as a tool to establish the design space as necessary in the context of QbD [8]. Especially in recent years, the reduction of time on market has also come more and more into focus. One promising strategy for rapid bioprocess development is parallel processing in small scale [9–12]. These systems pose great challenges to process monitoring. For the monitoring of such systems, multiplexing-enabled measurement devices are an absolute necessity to cope with the high number of samples [11].

Bioprocess monitoring includes a system for acquiring process data and a system for data interpretation. In the simplest case, this includes optical observation by a human operator. Measurements can take place in-line, which means inside the reactor by specific probes (pO2, pH and near IR). Spectroscopic measurement techniques are especially used for this [13–15]. Using in-line probes has the big benefit that no physical interaction across system boundaries takes place during the process, reducing the risk of contamination greatly. Consequently, the use of in-line probes is strongly recommended by regulatory authorities such as the US FDA, whenever possible. However, many important parameters cannot be assessed by in-line measurements. The sample has to be removed from the bioreactor and usually

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Key Terms

Multiplexing: To distribute and analyze samples from multiple bioreactors in an automated and traceable way.

Bioprocess monitoring: The monitoring of all important key parameters of a bioprocess using in-line, at-line and online measurements.

Online monitoring: The completely automated removal, processing and analysis of a representative sample from a bioreactor.

Representative sample: A sample that represents the actual condition and/or concentration of the analyte (metabolites, protein product or biomass).

further processed before the analysis. The simplest way of dealing with these samples is at-line measurements. Here, the sample is manually processed for the following analysis. While the technical requirements are relatively low, the personal demand is highest here. Online measurements on the other hand provide fully automated sampling and measurement outside of the bioreactor. While this reduces the involvement of an operator to a minimum, the technical expense for setting up such systems can be enormous. Advantages of online measurements are that relevant outcomes such as metabolite concentrations, product concentrations and cell numbers can be determined directly or using a simple linear calibration model. In principle, all standard analytical methods such as HPLC can be connected to any bioprocess [16,17]. For this, even spectroscopic methods such as near IR can be applied [16,18–19]. Moreover, by multiplexing, the monitoring of parallel processes is possible. This is especially important in the context of multibioreactor processing. A further advantage of these systems is that storage of reference samples for later analysis and documentation is possible. The methods developed for bioprocess monitoring must finally be transferable and validated in production scale. The requirement in manufacturing differs strongly from those in development. The main task of monitoring here is to ensure that the process runs in the predefined parameter ranges (or within the design space in a QbD context), and hence the product quality is guaranteed. Thus, monitoring during production processes mainly includes real-time event detection, which requires high-frequency measurements. To avoid manual interaction, automation is of interest in a manufacturing environment. Furthermore, requirements for data management must be considered [20].

Various conflicting definitions of online monitoring can be found in the literature [21–23]. They reach from strictly using *in situ* measurements to *ex situ* and at-line measurements. We support the definition of online monitoring as *ex situ* measurement combined with real-time processing of the measurement data for the determination of critical process parameters. This review is intended to give an overview of the available methods, challenges and solutions for online monitoring. We elucidate the workflow in detail starting from the bioreactor interface, automatic sampling, measurement methods as well as data and information processing (Figure 1). We highlight current applications [24,25] as well as future trends and needs for biopharmaceutical bioprocesses.

Sampling & sample treatment

The task of any sampling system is to provide a representative sample for further analysis. This is considered a major challenge in establishing an online monitoring system [26]. Especially in recent years, the application of multibioreactor systems has increased [10]. Thus, modern sampling devices must additionally be able to remove and distribute samples from multiple bioreactors in an automated and traceable way (multiplexing). The sterility of the bioprocess must not be jeopardized by the sampling. Therefore, a sterile barrier is needed. This ultimately ensures that there are no interactions between the system and the environment. Sterile barriers can be membranes or sterilizable sluice systems. All types of sampling have in common that reference samples can be taken. These are important for a proper documentation. The sampling system is dependent on the type of sample that shall be analyzed. The different types of samples can be roughly divided into three main categories:

- Gas samples
- Liquid samples without biomass
- Liquid samples with biomass

The type of sample determines the type of sampling systems necessary for the online system. In the following section, the characteristics of the respective samples are briefly described.

Gas sample

Gas samples have the lowest demands on the bioreactor interface. It is not invasive and can be connected to any existing system. For this reason, application of online monitoring via the gas phase is widely used [27–30]. In connection with multibioreactor systems, the step to multiplexing is necessary. The miniaturization leads to novel challenges in areas of low gas flow rates in order to get a representative sampling [31]. Due to the high back-mixing occurring in the gas phase, gas lines must be sufficiently rinsed with process off-gas. Thus, the sample frequency is a function of the gas flow rate considering the back-mixing of the gas phases.

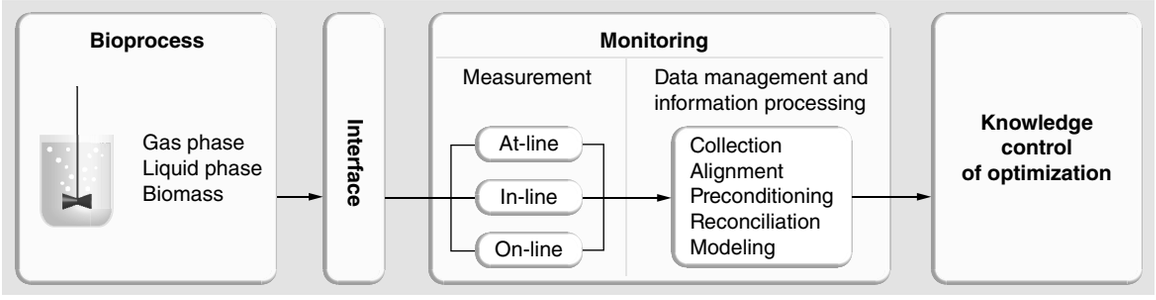

Figure 1. The different stations of online monitoring from the running bioprocess include sampling and sample treatment; analysis of key metabolites and biomass; and alignment and reconciliation of processing data in real-time. The acquired information can be used for knowledge generation, bioprocess optimization and bioprocess control.

To increase the measurement frequency, especially in highly dynamic systems such as fed-batch cultivations, a mix of submerse and headspace aeration was proposed [32]. If multibioreactor systems are applied, additionally the number of parallel running reactors must be considered here.

Liquid sample without biomass

Liquid samples of culture supernatants provide important information about production and consumption of metabolites, which are prerequisites for the establishment of feeding strategies and process event detection. Selective sampling of cell-free supernatant via membrane-based probes offer a solution for continuous or discontinuous sampling. The membrane serves both as a sterile barrier and as a filter. For this purpose, different porous membranes are applied in literature (e.g., IBA, Heiligenstadt, Germany), which proved applicable for online bioprocess monitoring. A comparison of membranes can be found in Spadiut *et al.* [33]. They were assessed to be applicable for small metabolites. However, representative sampling of proteins was not simply possible but was correctly estimated using a correction factor. Such membrane probes were also used recently by Sagmeister *et al.* to separate biomass and liquid supernatant as fast as possible in order to avoid corruption of the sample at high cell densities, which would occur during manual processing [34]. Moreover, automated liquid sampling using membrane probes allows high sampling rates and thus a high resolution of process dynamics in the range of seconds to minutes [35]. The mode of sampling has to be chosen carefully, it can be performed continuously or batch wise [25]. In both cases, changes of permeability can lead to leaky or ultimately to blocked membranes. Increasingly clogged pores were especially found to alter the required volume exchange for representative sampling [33]. In addition, continuous sampling consumes more sampling volume, which will decrease the reactor volume over time and, since the biomass is retained, increase the biomass concentration in the system. This effect is almost negligible in large reaction volumes, but can be quite significant in small fermenters used in bioprocess development. Alternative methods comprise the use of automated centrifugation devices [36] or flow injection analysis [37]. However, the latter has the disadvantage that retaining a reference sample is usually not considered.

Liquid sample with biomass

Biomass sampling can yield important information about the amount of biomass in the bioreactor as well as the state of the biomass or used for segregation of the biomass into subpopulations. Liquid samples containing biomass comprise great challenges in terms of sterility. These samples are taken without a sterile barrier to protect the reactor from contamination. Steam sterilizable sluice systems can be used but are technically complex and expensive. However, these systems are the only ones that replace the manual sample taking. Recently, commercial available systems such as the BaychroMAT® (BAYER, Leverkusen, Germany) or the Groton Biosystems Automated Reactor Sampling System® (Groton Biosystems, MA, USA)were introduced into the market, allowing flexible and sterile handling of liquid samples with biomass. A recent study described the application of the BaychroMAT system for sampling and sample processing of long-term mammalian cultures [24]. The modular setup of these systems allows the connection of further devices and thus, a wide range of analytics for biomass can be covered simultaneously. Direct measurement methods may be used to quantify biomass, for example, cell counting in mammalian cell culture bioprocesses [24] or even retrieve information about biomass state and allow biomass segregation via online flow cytometry [38]. Subsequently, the biomass can be also deposited from the sample, followed by any analyses that are possible for liquid samples without biomass.

Table 1. Available analysis methods for online monitoring of the different sample types: gas, supernatant and biomass.

Risk management	Method	Analyte/ measurement parameter	Advantages	Disadvantages	Ref.
Gas					
No additional risks compared with the standard process	MS	CO_2, O_2, H_2O, H_2 and volatile compounds (ethanol, CH_4)	High accuracy, high sensitivity, flexible and mass balancing possible	Expensive, complex systems and prior knowledge necessary for proper application	[40]
	Specific sensors	CO_2, O_2, H_2O, H_2 and CH_4	Low priced, accuracy sufficient for most applications and easy implementation	Only applicable to certain metabolites and most sensors do not include H_2O measurements	[28]
Supernatant					
Additional risks of contamination and danger of membrane blocking and fouling	HPLC	Organic acids, alcohols, sugar, lipids and so on	High accuracy, high sensitivity and low priced	Complex samples can result in difficult to analyze chromatograms and method development can become time and resource consuming	[41,42]
	Enzymatic photometric analyzer	Glutamate, glutamine, glucose, glycerol, acetate, ethanol, NH_4^+ and PO_4^-	High specificity, high sensitivity and high accuracy	Assays are not available for every analyte, assays are sensitive to temperature and pH and running costs are high for assays	[25,43]
	Enzymatic membrane analyzer	Glucose, lactate, glutamate and glutamine	High specificity, high sensitivity and membranes provide (some) protection of enzymes against environmental influences	Assays are not available for every analyte, analyte may interact with the membrane material and accuracy is lower than for photometric assays	[44,45]
Biomass					
Additional risks of contamination and more complex sterilization	Image-based cell analysis	Cell number, aggregation, viability and morphology	Data resolution on the single cell level, information on functional homogeneity/ inhomogeneity in culture population is provided and multiparametric analysis for highly correlated data	Versatile applicability is linked to the use of fluorophores and complex staining protocols and comparatively long overall time for sample workup and analysis	[38,46–49]
	Flow cytometry	Cell number, aggregation, morphology, viability, apoptosis, cell cycle synchronization, metabolic activity and intracellular product			

Measurement methods

Measurement methods must fulfill a number of requirements to be applicable for online bioprocess monitoring. The prerequisite is of course that sample processing and subsequent analysis can be performed completely automated. Furthermore, the method must be robust and able to deal with strong changes of the sample composition over the process. This comprises interfering substances such as salts but also drastic changes in analyte concentrations. Regarding the latter, a high accuracy and precision of the measurements must be ensured over the whole range of occurring concentrations of the different analytes. Finally, a reduction of costs in biopharmaceutical process development means

reduction of the time to market [39]. Thus, a measurement device must allow high-throughput analysis of a multitude of samples, especially with regard to the increasing number of multibioreactor systems. In the following section, different measurement methods and devices applicable to the task of bioprocess online monitoring will be discussed with regard to the above discussed aspects. The link between the sampling and measurement methods is shown in Table 1.

Measurement of off-gas composition

In the pharmaceutical production, off-gas analyzers find more and more application in recent years. The main advantages of these measurement methods are the easy installation in the nonsterile area and the high information content of the measured data. The most important measured variables are the concentrations of oxygen and carbon dioxide in the off-gas.

Due to the continuous gas flow, the off-gas measurement can be performed at high frequency. The most common methods of analysis are specific sensors and the mass spectrometer. Mass spectrometers are versatile applicable and have a very high sensitivity [40], but are very expensive. For example, with proton transfer reaction–MS (PTR–MS), other components with high resolution can be measured [27,50].

Especially for production processes, the application of the cheaper specific sensors becomes more common. Aehle *et al.* showed on the example of BlueInOne® (BlueSens gas sensor GmbH, Herten, Germany)that quantitative measurements using a specific sensor are equal in sensitivity and accuracy compared with mass spectrometers [28]. The amount of water in the inlet gas stream must be additionally quantified to fully close the volume balance around the system. Alternatively, water content can also be estimated wet as demonstrated. With the data from gas analyzers, the process can be physically and reactions kinetically quantified online [51–53].

Liquid sample analysis without biomass

Enzymatic analyzers

Enzymatic analyzers, as the name implies, make use of specific enzymatic reactions for the detection of metabolites or specific proteins. There are two main setups for enzymatic analyzers: either the respective enzymes are in solution (photometric analyzers [54]) or immobilized on a membrane (amperometric analyzers [55]). Various devices using both types of analyzers can be found on the market: the YSI Biochemistry Analyzer series (YSI Life Sciences, OH, USA) or the BioProfile Analyzers (Nova Biomedical, MA, USA) as examples for membrane analyzers, and CuBiAn® XC (Optocell Technology, Bielefeld, Germany) and Cedex Bio HT (Roche Diagnostics, Mannheim, Germany) as prominent examples for photometric analyzers.

Membrane analyzers have the advantage that the enzymes are immobilized on a membrane, which provides a certain amount of protection. The coated membranes can be stored at room temperature for days to weeks and exposed to strong pH shifts or used in the presence of solvents. Moreover, the analyte can be washed from the membrane, allowing a repeated usage of the membrane [44]. By contrast, enzymatic assays used in photometric analyzers use enzymes in solution, which is discarded after the measurement. They are much more sensitive to environmental influences. Usually, reagents must be stored in a cooled environment and the stability of an assay can vary between weeks or only days [44,45].

However, analytes can also stick or interact with the membrane material of enzymatic membrane analyzers. Thus, the superiority of photometric analyzers in terms of accuracy and linearity compared with membrane analyzers has been demonstrated recently for Cedex Bio HT [43]. In summary, the choice of the analytical device is a tradeoff between robustness and accuracy and thus, strongly depends on the specific demands imposed by the sample.

HPLC

HPLC is being used extensively as an off-line method to quantify numerous compounds by UV/refractive index detection. The most common mode of detection is by UV absorption, where most organic compounds, especially with conjugated pi systems, are detectable. The RI detector covers substances with polarizable electrons comprising [56], yet not exclusively, lipids [57], proteins [58], sugars and polyalcohols [59], and also organic acids [41]. Online HPLC systems can be automated to a great degree, which decreases operator effort for high frequency sampling extensively. Monolithic columns will presumably help to facilitate a further increase in sample throughput by decreasing method duration drastically [60]. Classical [61] and experimental design-based [62] optimization of methods can improve accuracy and decrease the noise on the measurement, which in return decreases the amount of required sampling frequency required for resolution of dynamic changes in concentrations [63].

Liquid sample analysis with biomass

Picture analyzer

Over the last years, systems for whole-cell analysis have gained increasing importance and rapidly became an integral part of state-of-the-art monitoring and biopharmaceutical production regimes. Among the most prominent examples in this class are automated and

Key Terms

Data processing: Extraction and statistical validation of physiological meaningful information from raw process data.

Data alignment: The restructuring of data from different sources to one common format suitable for further processing.

semi-automated systems for determining cell number [64], morphology [65] and viability [66]. They use either image-based analysis with dye-exclusion assays (Cedex HiRes, Roche Diagnostics, and ViCELL™ series, Beckman Coulter, CA, USA) and fluorescence labeling (NucleoCounter® series, ChemoMetec, Allerod, Denmark) or label-free, electrical current exclusion methods (CASY, Roche Diagnostics). The main advantage of these systems is a standardized, reproducible and fast measurement routine, compliant with GMP regulations such as 21 CFR part 11 or ISO 13319. Online implementation of such devices in combination with suitable sampling systems (e.g., SEG-FLOW®, Flownamics, WI, USA) allows for accurate determination of phase transitions in the culture, for example, from the exponential to stationary phase [67], which may be of relevance for deciding on the optimal time point for induction or harvesting.

Flow cytometry

Flow cytometry (FC) has for a long time represented a key technology in cell biological and medical research, and with the ability for automatization is a highly interesting tool for improved bioprocess control strategies. FC may, according to its measurement principle, be regarded a more advanced version of optical and fluorescence-based cell counting and viability analyzers; it is more complex to operate but offers a considerably higher versatility with regard to the possible measurement parameters. Using a spectrum of dyes, FC allows the discrimination of living cells; metabolically active; viable but dormant cells; and dead cells with compromised membrane integrity [68–70]. This enables the stratification of total biomass into subsections, and gives valuable information on culture homogeneity/inhomogeneity. The variable use of fluorescent reporter proteins offers a convenient method for real-time monitoring of product formation, also in expression systems without active secretion [71,72]. Moreover, DNA-selective staining protocols allow for detailed assessment of cell cycle kinetics and synchronization [73].

The main obstacle for successful implementation of advanced, cell function-related FC analysis protocols in real time is given in the need for establishing a fully automated cascade of sample processing. Thereby, the required manipulation steps range from the initial withdrawal out of the bioreactor to temporary sample retention for dilution, staining and washing, until the final transfer to the actual bioprocess analysis in the FC device. Despite the inherent complexity associated with this approach, the value of the information obtained has driven forward the research on such systems and multiple successful examples have been reported up to date [38].

Data & information processing

Information derived from online measurements is highly useful to make manufacturing and process development decisions and can directly be used for feedback process control of processes. Hence, next to the bioreactor interface and the measurement device, attention should be drawn to sound data processing from (multiple) online analyzers.

For the real-time use of online data, it is a prerequisite to use a suitable online data collection, alignment and preconditioning system, processes here summarized under the term 'data processing'. Subsequently, using prior knowledge (e.g., data-driven process models and mechanistic process models or fuzzy knowledge) in combination with the online information, the process state can be identified (process identification) and predictions can be made (here summarized under the term 'information processing'. On this basis, control actions can take place. The basic elements of an online data and information processing system are described in Figure 2 and further explained in detail within this section.

Data collection & alignment

Data collection involves the storing of data of (multiple) online analyzers in one common environment where it is accessible for further processing. Data alignment is the restructuring of data from different sources to one common format suitable for further processing, for example, aligning of data points on one uniform time axes using interpolation. This step is vital since data preconditioning procedures typically require data on one single time axis or in matrix form. Powerful tools for data collection and alignment are SIPAT (Siemens, Berlin, Germany), Discoverant (Accelrys, CA, USA), S-Matrix (S-Matrix, CA, USA) and SmartDataCockpit (AGU, Leverkusen, Germany).

Data preconditioning & rate calculation

Online measurements are corrupted by random errors (noise) as well as gross errors. Poor data quality can lead to wrong design, analysis and control decisions. Hence, it is necessary to evaluate and denoise online data prior to its use. This task can be achieved via data preconditioning. As outlined in Figure 2, the task of

data preconditioning succeeds data alignment. Filtering (e.g., low pass filters) can be useful for the easier identification of process trends. However, it should be considered that the use of filters (e.g., low-pass filtering or Savitzky-Golay filtering [74]) can reduce the information content of the respective signal.

Process data in the form of concentrations can be converted into more significant and interpretable forms by the calculation of (specific) rates and yield coefficients. This is also referred to as data to information conversion, since specific rates and yield coefficients provide simplified insight into metabolic fluxes and flux distributions [75]. This requires rate calculation on the basis of online signals. Formulas for the calculation of reaction rates are derived from the general mass balance for the respective components and are given in several recent contributions [76,77], whereby methods of finite differentiation are prevailing or the use of Savitky-Golay filtering [74]. For handling of reaction rates, a matrix approach introduced by van der Heijden [78–80] is frequently applied [76–77,81–84]. Mathematically, rates are often calculated by taking the numerical derivative of data at discrete time points. Here, analogies to the use of derivatives of spectra aiming at feature detection can be drawn. Similar to the use of derivatives in spectroscopy [85], the calculation of rates and yield coefficients from online concentration data reduces the signal-to-noise ratio of the respective signal. Therefore, filtering methods, also referred to as convolution methods, can be applied in conjunction with online rate calculation [85]. For this purpose, moving average methods were shown to be applicable for the real-time extraction of specific rates and yield coefficients using online devices [37]. However, the use of the computationally fast Savitzky-Golay filter [74] is a promising alternative for the calculation of smoothed turnover rates.

Data reconciliation

Complementary to filtering techniques, reconciliation is useful for enhancing the signal-to-noise ratio of online bioprocess data and to detect gross errors [35,76–77,84,86]. As outlined in Figure 2, data reconciliation is typically applied on the basis of turnover rates. In principle, reconciliation makes use of linear constrain relationships within a set of data, identifying the most likely set of rate or concentration estimates in least squares sense fulfilling the constraints imposed on them. The underlying optimization algorithm is computationally fast and is described extensively in the literature [76,86]. Reconciliation is a key procedure for the correction and assessment of gross, systematic and random errors in the chemical process industry, which, for instance, makes use of the total mass balance as a constraint [87]. For complex biological systems, the use of the total mass balance is typically not applicable since not all species involved in a reaction can be quantified. Nevertheless, the powerful tool of reconciliation can be applied using linear constraints such as elemental, charge or energy balances. Typically, reconciliation is applied on the basis of conversion rates, hence following rate calculation and smoothing (see Figure 2). Mathematical formulation of basic linear constraint relationships as well as their use for gross and systematic error detection are discussed by a series of articles by Van der Heijden *et al.* [78–80]. In practice, the carbon balance as well as the degree of reduction balance are widely employed as linear constraints in reconciliation procedures for microbial processes [35,83,88–89]. Reconciliation results in a higher signal-to-noise ration of the processed data [77], in case the imposed constraints are valid. Validity of constraints can be checked by employing a statistical test checking whether the adaptation of measurements can be attributed to white noise or whether gross measurement or systematic errors are present [79,80].

In practice, data preconditioning methods and data reconciliation can be performed in a technical computing environment capable of performing matrix operations, such as MatLab (Mathworks, MA, USA) This requires an interface to the data management environment, as provided, for example, by SIPAT. Alternatively, high level programming interfaces directly implemented in process management systems can be used, as provided, for example, by Lucullus PIMS (Secure Cell, Schlieren, Switzerland).

Information processing

Information delivered by the data processing system can be used to extract real-time knowledge on the process state (process identification). This can be achieved using prior knowledge and/or the use of mathematical models, as illustrated in Figure 2. In the simplest case, this involves an operator interpreting the information on a screen, for example, interpreting a carbon dioxide evolution rate and drawing a conclusion concerning the process state. However, for complex systems utilizing (multiple) online analyzers, it is more advisable to use an automatic information processing system, which typically employs a mathematical process model.

Based on the model used, three different types of information processing methods can be distinguished: data driven-, mechanistic- and hybrid model-based information processing methods. Data-driven information processing uses large data sets for the training of a statistical model. This does not require a mechanistic description of the system and hence can be faster considering the model-building process [90]. Draw-

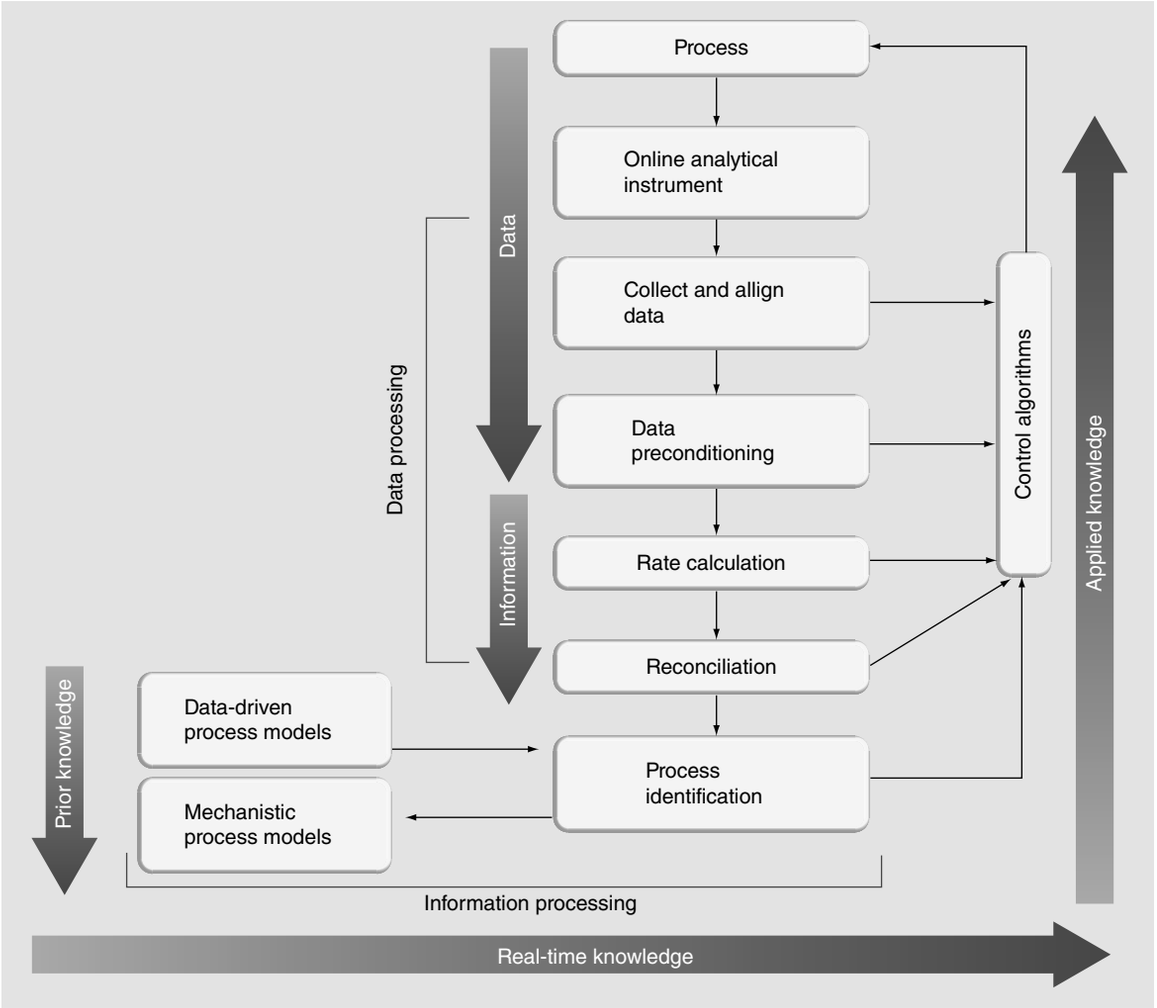

Figure 2. Workflow for online data and information processing from processes over measurement to data alignment tools for process identification and process control.

backs of data-driven models are that the extrapolation from the training data sets is dangerous since the space of validity is unknown. Furthermore, little mechanistic insight in the underlying system is granted. A review of data-driven bioprocess information processing methods with relevance to bioprocesses can be found elsewhere [91]. A highly promising, but for bioprocesses underrepresented, class of data-driven information processing strategies are multiway methods. Process datasets can be expressed in three dimensions: process variables (J) are recorded for a series of time points (K) for multiple batches (I), forming a 3D matrix with the dimensions IxJxK [92]. Multiway methods are capable of processing three or more dimensional data matrices efficiently for the benefit of a more stable model, as reviewed recently [93].

In contrast to data-driven methods, mechanistic process models use a physical or biochemical mathematical description of the process, for example, using first principle relationships (e.g., mass and elemental balances) in combination with a description of the system typically in the form of ordinary differential equations. These approaches are typically more time intensive with respect to model building [90] and a high degree of system expertise is needed. However, efficient automated model-building strategies using a library of mechanistic process models are also reported [94]. In case a process model in state-space formulation is available and the errors on online measurements are known, a Kalman filter can be used for precise process identification in the presence of noisy measurements. Since bioprocesses typically show nonlinear process dynamics, the nonlinear versions of the Kalman filter (extended Kalman filter and unscented Kalman filter), belonging to the class of nonlinear observers, are typically used [95].

The combination of both data driven and mechanistic models is referred to as hybrid modeling [96]. This follows the idea that well understood elements of the system are described using mechanistic models,

whereby data-driven methods such as artificial neural networks are used for the modeling of parts of the system, where there is no deterministic relation established [96]. Hybrid modeling combines drawbacks and benefits of data-driven and mechanistic methods.

Rate-based information processing

The signal-to-noise ratio of rate-based information obtained through finite differentiation depends upon the biologic variability, the error of the measurements involved and the time window of calculation [77]. Rate-based information can be compared or used for the establishment of process models, for example, correlating specific growth rates with specific product formation rates, [97,98]. In case regression models are established, it is a prerequisite that all observations (rates) show homoscedasticity. For rate-based information obtained through finite differentiation of (online) concentration measurements, this can be achieved by adjusting the time window of calculation, as discussed recently [77]. The described method can be used for the sound determination of sampling intervals from online analyzers [77].

Soft sensors

Software sensors, also referred to as soft sensors, are information processing methods that use a model to compute nonmeasured process variables from a combination of real-time process information obtained from multiple hardware sensors. Hence, they perform estimation of nonmeasured process states or process information. They can be used to substitute an existing hardware sensor or to compute nonmeasureable variables (e.g., the specific growth rate) or process events based on online data. The type of model used can be either data driven, mechanistic or of hybrid type. Data driven soft sensors are reviewed in detail elsewhere [90,99].

We have recently reported benefits and applications of first principle, rate-based soft sensors requiring little prior knowledge for the estimation of the biomass concentration, specific rates and yield coefficients [35,83,89,100]. First principle, rate-based soft sensors use reconciled metabolic rates obtained from data processing of online measurements to estimate nonmeasured rates (e.g., the biomass formation rate). Via numerical integration of the estimated rate, concentrations of process states are obtained, for example, the biomass concentration. Using a statistical test value obtained from the reconciliation procedure, the quality of the estimation can be checked in real time. We propose that mechanistic soft sensors are especially powerful for process development purposes where the data basis necessary for data driven models is not sufficient.

Applications

Off-gas analysis

Due to the high measurement frequency, off-gas analyses are very well suited for real-time characterization of fermentation processes. Typical outputs of the analysis are the oxygen uptake rate (OUR) and carbon evolution rate (CER). The OUR and CER include information about the metabolic activity (metabolism) and physiology (respiratory quotient) of the respective culture [51,101–102]. For this reason, the data obtained by off-gas analysis are well suited as inputs to software sensors for biomass estimation. In particular, the research group led by Lübbert has made an enormous contribution in this area. Jenzsch *et al.* and Aehle *et al.* showed that the OUR and CER are good inputs for soft sensors for biomass estimation, which can be applied to both microbial and mammalian cultures [28,103]. On the basis of these soft sensors, process control could be improved, resulting in an increased reproducibility, especially regarding mammalian cell cultures [104,105]. Thus, the application of the off-gas analysis is an important step towards fulfilling the requirements of the PAT initiative.

To close the gas balances different boundary conditions must be considered. The focus of the papers is not in the description of detailed assumptions for real-time determination of gas-related rates. Bicarbonate-buffered systems, such as those used in cell culture, would have the stripping effects taken into account when estimating the CER [101,106–107]. Moreover, in the scale-up of these systems, the transport mechanisms must be considered [108]. Another benefit of the off-gas analysis is the estimation of dissolved carbon dioxide (pCO_2). This is not trivial because pCO_2 is a function of pH, temperature, osmolality and more [101,106–107]. Thus, detailed prior knowledge is necessary to perform this estimation. This has a direct impact on the physiology and thus performance of the bioprocess [109].

Liquid sample analysis without biomass

Online enzymatic analyzers

While enzymatic analyzers are one of the major tools in modern diagnostics, their application for bioprocess monitoring is less established, especially considering online applications. Dietzsch *et al.* described the online application of the Cubian XT analyzer for online measurement of substrates and products during *Pichia pastoris* cultivations [25]. The reliability of the online measurements was verified by carbon balances. The online application of this device for online cell culture monitoring and process control is a current topic of research. In addition, the application of Cedex Bio HT as a tool for microbial and mammalian bioprocess monitoring has been discussed recently [43] and

its application for cell culture process monitoring has been demonstrated [110]. However, online application of the Cedex Bio HT device has not been published yet but is currently a topic of research in our institute.

Online HPLC

A typical application of online HPLC is the quantification and subsequent control of substrate concentration within a fed-batch experiment [111]. Especially in dynamic experiments, monitoring of residual substrate concentration is of great concern [112]. The utilization of an online HPLC does not only reduce sampling effort, it enables a simultaneous quantification of possibly accumulating metabolites (e.g., acetate). This feature is of special interest during early strain characterization. In addition, product analytics can be performed online with HPLC, the online signal of the product titer facilitates the decision about the optimal time point of harvest during an fermentation process [113]. Furthermore, during an digital signal processing unit operation of a bioprocess, an online HPLC was shown as a PAT tool to facilitate real-time decisions about fraction pooling [42]. Whereas HPLC methods for quantification of various molecules have become a standard tool for process characterization at-line, the online HPLC application including feedback for process control although established [42,111,113–114] have not become everyday routine yet. This circumstance may be owned by the highly complex sample handling in order to secure representative sampling and the required extensive effort for system and method validation.

Comparison between chromatographic & enzymatic analysis

Enzymatic analyzers make use of enzyme-catalyzed reactions for the quantification of the metabolite of choice. Enzymatic assays have the advantage that the applied enzymes are highly specific for the desired metabolite and also very sensitive. This allows for the detection of metabolites also in complex samples such as crude cell extracts or in cell culture media [54]. Here, chromatographic methods often show complex chromatograms with overlapping peaks. As a consequence, quality control of the measurements can quickly become labor intensive to avoid matrix effects and enable proper separation of substances [115]. At the same time, enzymatic analyzers are limited to the number of available enzymatic assays in the choice of analytes and running costs (enzymatic kits) are typically higher. Here, chromatographic methods have the advantage, since theoretically any substance can be quantified if proper conditions for separation and detection have been found. Thus, often a combination of both enzymatic analyzers and chromatographic methods is the best way for reliable and robust online analysis.

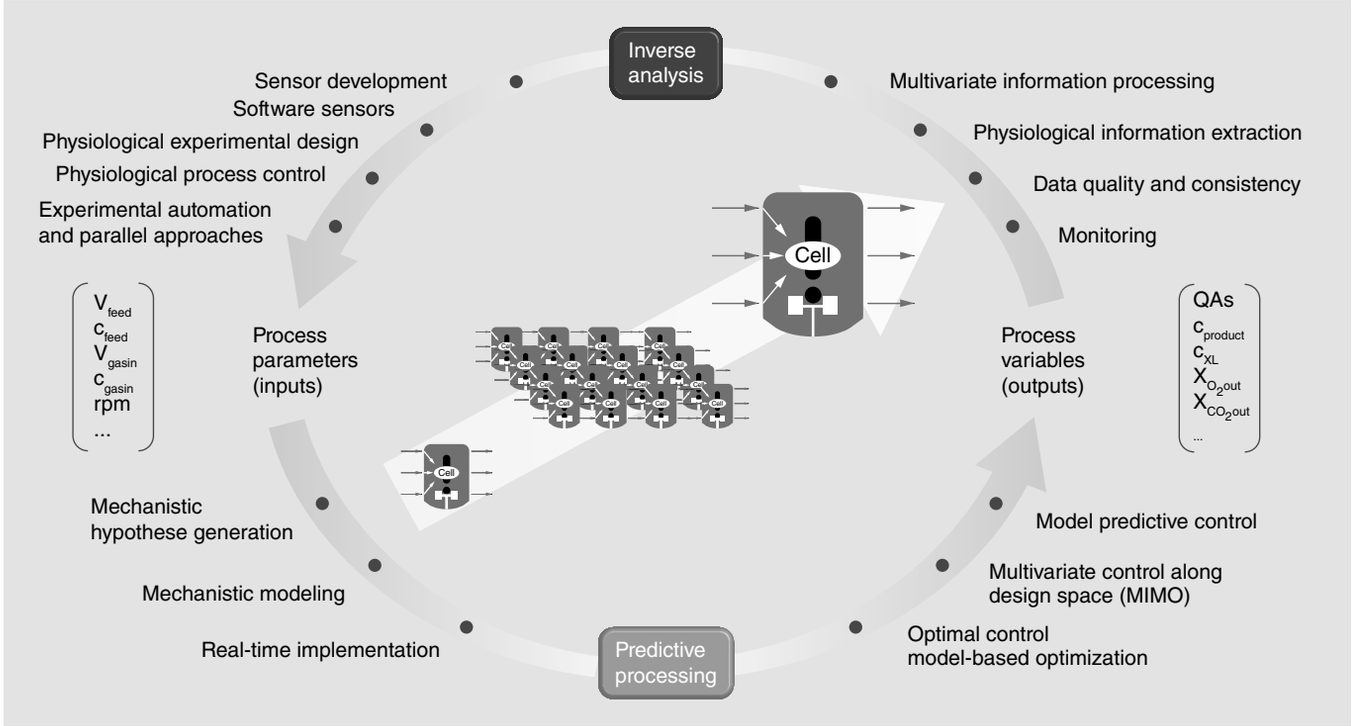

Figure 3. Task cycle for future process development and control: starting from measurement and data processing to process modelling for mechanistic process understanding and predictive process control.

Liquid sample analysis with biomass

Online estimation of biomass is still one of the bigger challenges in bioprocess monitoring. However, several approaches have been proposed to solve this problem. Automated biomass sampling and subsequent cell counting using the BaychroMAT system have been described recently [24]. Kacmar *et al.* implemented a 'cytostat' with FC-based online measurement of cell concentration, size and culture inhomogeneities in both CHO and yeast fermentations, with a temporal resolution down to 7 min [46,47]. The sampling interface consisted of a microchamber for cell collection, dilution and staining reactions, and a pressure-driven injection valve for transferring the mobile phase to the FC device. In combination with automated feed regulation, the system not only allowed for tight control of the cell number in suspension, but also facilitated a precise assessment of growth dynamics at biomass levels far below that usually required for good reproducibility. In the same group, an online FC protocol was used to study cell cycle kinetics of CHO cultures in bioreactors during nutrient deprivation, with a resolution of 25 min over 4.5 days of culture [48]. Automated staining protocols allowed for identifying previously hard to detect transitions and oscillations in culture state, which may have a profound impact on product titer and quality. Moreover, the use of online FC results for establishing a feedback-regulated control strategy for fed-batch culture expansions was impressively demonstrated [49]. Similar systems were described recently for online monitoring of *P. pastoris*, *Saccharomyces cerevisiae* and *Escherichia coli* bioprocesses, where the formation of an intracellular, green fluorescent protein-labeled product was followed over time [38]. The setup comprised a defoaming section, an injector and a dilution unit, with pumps and valves being actuated by a programmable automation controller. This way, online FC measurements could be conducted in 5-min intervals over the entire process duration. The FC results were in good agreement with *in situ* and off-line data on the mean population fluorescence intensity, but additionally revealed that only a certain fraction of cells in the bioreactor actually expressed the desired product. Furthermore, microscopy-based methods for combined cell counting and morphology analysis, as recently described by Posch *et al.* could be further developed for online application with reasonable effort [116].

Future perspective

The greatest challenge in pharmaceutical bioprocesses is and will remain to be to guarantee product quality. Furthermore, the long time needed from development to market introduction is still limiting the fast supply of novel, effective biopharmaceuticals. To master these tasks in the future, a multitude of various analytical and data processing methods are available. Those must be combined in a reasonable way, to ensure mechanistic process understanding, which is a prerequisite for predictive process control (Figure 3).

The required bioprocess monitoring formulated in the PAT initiative lead to the development of numerous novel systems for sampling and sample analysis. Owing to the modular design of most online monitoring systems, they can be easily expanded by additional measurement devices. The big challenge will be the adaptation of these systems from single reactor application to the task of handling multiple bioreactors at the same time. Multiplexing sample devices such as BaychroMAT or Groton Biosystems Automated Reactor Sampling System must be combined with upcoming multibioreactor systems for automated sampling. Due to the small culture volumes between 1 and 10 ml of these bioreactor systems, the next big step will be the development of smart sampling strategies. These will be absolutely necessary to gain the maximum of information per milliliter of culture broth. Furthermore, more effort must be put in the development of analytical devices for automated and traceable high-throughput analysis of the arriving flood of samples from multibioreactor systems. This increasing automation will allow higher measurement frequencies and manual errors can be minimized.

The mass of process data can only be handled using robust and automatable data processing tools. Some software platforms already exist for automated data handling. However, these platforms must be adapted and further expanded specifically for the analysis of fermentation bioprocesses. Future research must therefore focus on the development of generic and easy-to-handle software tools, which do not only consolidate data but perform automated processing using data-driven as well as generic models to provide the user with process information rather than raw process data. This will enable a tight and reproducible process control within the mandatory design space [1–3]. Automated data processing in addition to bioprocess models such as soft sensors will enormously contribute to process understanding and control. With increasing understanding of the bioprocess and the complex relationships between host strain, product and process parameters, the product quality could also be predicted by customized bioprocess management. Further research and development will save resources and reduce bioprocess development time and process costs in the long run significantly.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial inter-

est in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Online monitoring is a prerequisite for fast and sound science-based bioprocess development and quality control.
- Different measurement methods are available for different kinds of samples (gas, supernatant and biomass).
- Without proper data management and processing, the huge amount of data cannot be handled.
- Only the combination of reliable and robust interface, sampling, analytics, data management and processing results in sophisticated online monitoring.
- Future research must be focused on improved hardware devices for sampling and analysis as well as easy-to-handle, generic software tools to cope with the increasing flood of data.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1 US FDA. Guidance for industry PAT-A framework for innovative pharmaceutical development, manufacturing, and quality assurance. www.fda.gov/downloads/Drugs/Guidances/ucm070305.pdf

2 ICH. Q9, Quality Risk Management. www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf

3 ICH. Q10, Pharmaceutical Quality System. www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q10/Step4/Q10_Guideline.pdf

4 Wechselberger P, Seifert A, Herwig C. PAT method to gather bioprocess parameters in real-time using simple input variables and first principle relationships. *Chem. Eng. Sci.* 65(21), 5734–5746 (2010).

• **Gives a good example for application of first principle-based soft sensors for real-time bioprocess monitoring.**

5 von Stockar U, Valentinotti S, Marison I, Cannizzaro C, Herwig C. Know-how and know-why in biochemical engineering. *Biotechnol. Adv.* 21(5), 417–430 (2003).

6 Weuster-Botz D. Experimental design for fermentation media development: statistical design or global random search? *J. Biosci. Bioeng.* 90(5), 473–483 (2000).

7 Mandenius C-F, Brundin A. Bioprocess optimization using design-of-experiments methodology. *Biotechnol. Prog.* 24(6), 1191–1203 (2008).

8 Rathore AS. Roadmap for implementation of quality by design (QbD) for biotechnology products. *Trends Biotechnol.* 27(9), 546–553 (2009).

9 Bareither R, Bargh N, Oakeshott R, Watts K, Pollard D. Automated disposable small scale reactor for high throughput bioprocess development: a proof of concept study. *Biotechnol. Bioeng.* 110(12), 3126–3138 (2013).

10 Weuster-Botz D. Parallel reactor systems for bioprocess development. *Adv. Biochem. Eng. Biotechnol.* 92, 125–143 (2005).

11 Weuster-Botz D, Puskeiler R, Kusterer A, Kaufmann K, John GT, Arnold M. Methods and milliliter scale devices for high-throughput bioprocess design. *Bioprocess. Biosyst. Eng.* 28(2), 121 (2005).

•• **Development and application of a sophisticated multibioreactor system for parallelization of bioprocess development with subsequent real-time monitoring.**

12 Neubauer P, Cruz N, Glauche F, Junne S, Knepper A, Raven M. Consistent development of bioprocesses from microliter cultures to the industrial scale. *Eng. Life Sci.* 13(3), 224–238 (2013).

13 Clavaud M, Roggo Y, Von Daeniken R, Liebler A, Schwabe J-O. Chemometrics and in-line near infrared spectroscopic monitoring of a biopharmaceutical Chinese hamster ovary cell culture: prediction of multiple cultivation variables. *Talanta* 111, 28–38 (2013).

14 Kessler RW. Perspectives in process analysis. *J. Chemometr.* 27(11), 369–378 (2013).

15 Voura C, Posch A, Hörl G *et al.* Robust monitoring of pharmaceutical manufacturing operations based in combined NIR and Raman spectroscopy. Presented at: *AIChE 2009 Annual Meeting.* Nashville, TN, USA, 8–13 November 2009.

16 Foley DA, Wang J, Maranzano B *et al.* Online NMR and HPLC as a reaction monitoring platform for pharmaceutical process development. *Anal. Chem.* 85(19), 8928–8932 (2013).

17 Jung B, Lee S, Yang IH, Good T, Coté GL. Automated on-line noninvasive optical glucose monitoring in a cell culture system. *Appl. Spect.* 56(1), 51–57 (2002).

18 Havlik I, Lindner P, Scheper T, Reardon KF. On-line monitoring of large cultivations of microalgae and cyanobacteria. *Trends Biotechnol.* 31(7), 406–414 (2013).

19 Klimkiewicz A, Mortensen PP, Zachariassen CB, van den Berg FWJ. Monitoring an enzyme purification process using on-line and in-line NIR measurements. *Chemometr. Intell. Lab. Syst.* 132(0), 30–38 (2014).

20 US FDA. Part 11: electronic records; electronic signatures – scope and application. US FDA, MD, USA (2003).

21 Rathore AS, Bhambure R, Ghare V. Process analytical technology (PAT) for biopharmaceutical products. *Anal. Bioanal. Chem.* 398(1), 137–154 (2010).

22 Bork C, Holdridge S, Walter M, Fallon E, Pohlscheidt M. Online integrity monitoring in the protein A step of mAb production processes – increasing reliability and process robustness. *Biotechnol. Prog.* 30(2), 383–390 (2014).

23 Olsson L, Schulze U, Nielsen J. On-line bioprocess monitoring – an academic discipline or an industrial tool? *Trends Anal. Chem.* 17(2), 88–95 (1998).

24 Chong L, Saghafi M, Knappe C, Steigmiller S, Matanguihan C, Goudar CT. Robust on-line sampling and analysis during long-term perfusion cultivation of mammalian cells. *J. Biotechnol.* 165(2), 133–137 (2013).

25 Dietzsch C, Spadiut O, Herwig C. On-line multiple component analysis for efficient quantitative bioprocess development. *J. Biotechnol.* 163(4), 362–370 (2013).

26 Mattiasson B, Håkanson H. Sampling and sample handling – crucial steps in process monitoring and control. *Trends Biotechnol.* 11(4), 136–142 (1993).

27 Schmidberger T, Gutmann R, Bayer K, Kronthaler J, Huber R. Advanced online monitoring of cell culture off-gas using proton transfer reaction mass spectrometry. *Biotechnol. Prog.* 30(2), 496–504 (2014).

28 Aehle M, Kuprijanov A, Schaepe S, Simutis R, Lübbert A. Simplified off-gas analyses in animal cell cultures for process monitoring and control purposes. *Biotechnol. Lett.* 33(11), 2103–2110 (2011).

29 Jenzsch M, Simutis R, Lübbert A. Optimization and control of industrial microbial cultivation processes. *Eng. Life Sci.* 6(2), 117–124 (2006).

30 Teixeira AP, Oliveira R, Alves PM, Carrondo MJT. Advances in on-line monitoring and control of mammalian cell cultures: supporting the PAT initiative. *Biotechnol. Adv.* 27(6), 726–732 (2009).

31 Klein T, Schneider K, Heinzle E. A system of miniaturized stirred bioreactors for parallel continuous cultivation of yeast with online measurement of dissolved oxygen and off-gas. *Biotechnol. Bioeng.* 110(2), 535–542 (2013).

32 Eyer K, Oeggerli A, Heinzle E. On-line gas analysis in animal cell cultivation: II. Methods for oxygen uptake rate estimation and its application to controlled feeding of glutamine. *Biotechnol. Bioeng.* 45(1), 54–62 (1995).

33 Spadiut O, Dietzsch C, Posch A, Herwig C. Evaluating online sampling probes for substrate concentration and protein production by a design of experiments screening approach. *Eng. Life Sci.* 12(5), 507–513 (2012).

34 Sagmeister P, Langemann T, Wechselberger P, Meitz A, Herwig C. A dynamic method for the investigation of induced state metabolic capacities as a function of temperature. *Microb. Cell Fact.* 12, 94 (2013).

35 Sagmeister P, Kment M, Wechselberger P, Meitz A, Langemann T, Herwig C. Soft-sensor assisted dynamic investigation of mixed feed bioprocesses. *Process. Biochem.* 48(12), 1839–1847 (2013).

36 Allen SF, Gross M, Pang WS, Pedrazzini GA, Rauskolb RF, Tanumihardja H: US6060022A (2000).

37 Herwig C, Marison I, Von Stockar U. On-line stoichiometry and identification of metabolic state under dynamic process conditions. *Biotechnol. Bioeng.* 75(3), 345–354 (2001).

•• **The power of first principles for real-time on-line monitoring of transient states in chemostat cultures of *Saccharomyces cerevisiae.***

38 Broger T, Odermatt RP, Huber P, Sonnleitner B. Real-time on-line flow cytometry for bioprocess monitoring. *J. Biotechnol.* 154(4), 240–247 (2011).

39 Herwig C, Posch, A. Herausforderungen und trends für zukünftige bioprozesse. *Pharmind* 75(10), 1688–1694 (2013).

•• **In this publication, challenges and future trends for bioprocess development considering Quality by Design guidelines.**

40 Heinzle E, Oeggerli A, Dettwiler B. On-line fermentation gas analysis: error analysis and application of mass spectrometry. *Anal. Chim. Acta* 238(0), 101–115 (1990).

41 Lopez EF, Gomez EF. Simultaneous determination of the major organic acids, sugars, glycerol, and ethanol by HPLC in grape musts and white wines. *J. Chromatogr. Sci.* 34(5), 254–257 (1996).

42 Rathore AS, Wood R, Sharma A, Dermawan S. Case study and application of process analytical technology (PAT) towards bioprocessing: II. Use of ultra-performance liquid chromatography (UPLC) for making real-time pooling decisions for process chromatography. *Biotechnol. Bioeng.* 101(6), 1366–1374 (2008).

43 Druhmann D, Reinhard S, Schwarz F, Schaaf C, Greisl K, Notzel T. Utilizing Roche Cedex Bio analyzer for in process monitoring in biotech production. *BMC Proc.* 5(Suppl. 8), P106 (2011).

44 Sheldon RA. Enzyme immobilization: the quest for optimum performance. *Adv. Synth. Catal.* 349, 1289–1307 (2007).

45 Yang c, Zhang Z, Shi Z, Xue P, Chang P, Yan R. Development of a novel enzyme reactor and application as a chemiluminescence flow-through biosensor. *Anal. Bioanal. Chem.* 397(10), 2997–3003 (2010).

46 Kacmar J, Gilbert A, Cockrell J, Srienc F. The cytostat: a new way to study cell physiology in a precisely defined environment. *J. Biotechnol.* 126(2), 163–172 (2006).

47 Kacmar J, Srienc F. Dynamics of single cell property distributions in Chinese hamster ovary cell cultures monitored and controlled with automated flow cytometry. *J. Biotechnol.* 120(4), 410–420 (2005).

48 Sitton G, Srienc F. Growth dynamics of mammalian cells monitored with automated cell cycle staining and flow cytometry. *Cytometry A* 73(6), 538–545 (2008).

49 Sitton G, Srienc F. Mammalian cell culture scale-up and fed-batch control using automated flow cytometry. *J. Biotechnol.* 135(2), 174–180 (2008).

50 Luchner M, Gutmann R, Bayer K *et al.* Implementation of proton transfer reaction-mass spectrometry (PTR-MS) for advanced bioprocess monitoring. *Biotechnol. Bioeng.* 109(12), 3059–3069 (2012).

51 Eyer K, Oeggerli A, Heinzle E. On-line gas analysis in animal cell cultivation: II. Methods for oxygen uptake rate estimation and its application to controlled feeding of glutamine. *Biotechnol. Bioeng.* 45(1), 54–62 (1995).

52 Oeggerli A, Eyer K, Heinzle E. On-line gas analysis in animal cell cultivation: I. Control of dissolved oxygen and pH. *Biotechnol. Bioeng.* 45(1), 42–53 (1995).

53 Heinzle E, Oeggerli A, Dettweiler B. On-line fermentation gas analysis: error analysis and application of mass spectrometry. *Anal. Chim. Acta* 238, 101–115 (1990).

54 Passonneau JV, Lowry OH. *Enzymatic Analysis: A Practical Guide.* Humana Press, NJ, USA (1993).

55 Helm I, Jalukse L, Leito I. Measurement uncertainty estimation in amperometric sensors: a tutorial review. *Sensors (Basel)* 10(5), 4430–4455 (2010).

56 Pagni R. Modern physical organic chemistry (Eric V. Anslyn and Dennis A. Dougherty). *J. Chem. Ed.* 83(3), 387 (2006).

57 Hopia AI, Ollilainen VM. Comparison of the evaporative light-scattering detector (Elsd) and refractive-index detector (Rid) in lipid analysis. *J. Liq. Chromatogr.* 16(12), 2469–2482 (1993).

58 Wen J, Arakawa T, Philo JS. Size-exclusion chromatography with on-line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. *Anal. Biochem.* 240(2), 155–166 (1996).

59 Chavez-Servin JL, Castellote AI, Lopez-Sabater MC. Analysis of mono- and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection. *J. Chromatogr. A* 1043(2), 211–215 (2004).

60 Xu RN, Fan L, Rieser MJ, El-Shourbagy TA. Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. *J. Pharm. Biomed. Anal.* 44(2), 342–355 (2007).

61 Kromidas S. *HPLC Made to Measure: A Practical Handbook for Optimization.* Wiley, NY, USA (2006).

62 Vera Candioti L, De Zan MM, Cámara MS, Goicoechea HC. Experimental design and multiple response optimization. Using the desirability function in analytical methods development. *Talanta* 124(0), 123–138 (2014).

63 Wechselberger P, Sagmeister P, Herwig C. Model-based analysis on the extractability of information from data in dynamic fed-batch experiments. *Biotechnol. Prog.* 29(1), 285–296 (2013).

64 Sonnleitner B. Automated measurement and monitoring of bioprocesses: key elements of the m3c strategy. In: *Measurement, Monitoring, Modelling and Control of Bioprocesses.* Mandenius C-F, Titchener-Hooker NJ (Eds). Springer Berlin Heidelberg, Berlin, Germany, 1–33 (2013).

65 Posch AE, Spadiut O, Herwig C. A novel method for fast and statistically verified morphological characterization of filamentous fungi. *Fungal Genet. Biol.* 49(7), 499–510 (2012).

66 Brognaux A, Han S, Sorensen S, Lebeau F, Thonart P, Delvigne F. A low-cost, multiplexable, automated flow cytometry procedure for the characterization of microbial stress dynamics in bioreactors. *Microb. Cell Fact.* 12(1), 100 (2013).

67 Maiti SK, Srivastava RK, Bhushan M, Wangikar PP. Real time phase detection based online monitoring of batch fermentation processes. *Proc. Biochem.* 44(8), 799–811 (2009).

68 Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat. Prot.* 1(3), 1458–1461 (2006).

69 Bradner JR, Nevalainen KM. Metabolic activity in filamentous fungi can be analysed by flow cytometry. *J. Microbiol. Methods* 54(2), 193–201 (2003).

70 Wang L, Liu X, Yu J. Rapid assessment of cell viability of *Lactobacillus casei* after freeze-drying by measurement cell membrane using flow cytometry and fluorescence probes. In: *Proceedings of the 2012 International Conference on Applied Biotechnology (ICAB 2012).* Zhang, T-C, Ouyang, P, Kaplan, S, Skarnes, B (Eds). Springer Berlin Heidelberg, Berlin, Germany, 1799–1806 (2014).

71 Helman D, Toister-Achituv M, Bar-Shimon M *et al.* Novel membrane-bound reporter molecule for sorting high producer cells by flow cytometry. *Cytometry A* 85(2), 162–168 (2014).

72 Jarmander J, Janoschek L, Lundh S, Larsson G, Gustavsson M. Process optimization for increased yield of surface-expressed protein in *Escherichia coli*. *Bioprocess Biosyst. Eng.* doi:10.1007/s00449-014-1141-5 (2014) (Epub ahead of print).

73 Jayat C, Ratinaud MH. Cell cycle analysis by flow cytometry: principles and applications. *Biol. Cell* 78(1–2), 15–25 (1993).

74 Savitzky A, Golay MJE. Smoothing and differentiation of data by simplified least squares procedures. *Anal. Chem.* 36(8), 1627–1639 (1964).

75 Sagmeister P, Wechselberger P, Herwig C. Information processing: rate-based investigation of cell physiological changes along design space development. *PDA J. Pharm. Sci. Technol.* 66(6), 526–541 (2012).

76 Wechselberger PS, Sagmeister P, Herwig C. Real-time estimation of biomass and specific growth rate in physiologically variable recombinant fed-batch processes. *Bioprocess Biosyst. Eng.* 36(9), 1205–1218 (2012).

77 Wechselberger P, Sagmeister P, Herwig C. Model-based analysis on the extractability of information from data in dynamic fed-batch experiments. *Biotechnol. Prog.* 29(1), 285–296 (2013).

78 van der Heijden RTJM, Romein B, Heijnen JJ, Hellinga C, Luyben KCAM. Linear constraint relations in biochemical reaction systems: I. Classification of the calculability and the balanceability of conversion rates. *Biotechnol. Bioeng.* 43, 3–20 (1994).

79 van der Heijden RTJM, Romein B, Heijnen JJ, Hellinga C, Luyben KCAM. Linear constraint relations in biochemical reaction systems: II. Diagnosis and estimation of gross errors. *Biotechnol. Bioeng.* 43, 11–20 (1994).

80 van der Heijden RTJM, Romein B, Heijnen JJ, Hellinga C, Luyben KCAM. Linear constraint relations in biochemical reaction systems: III. Sequential application of data reconciliation for sensitive detection of systematic errors. *Biotechnol. Bioeng.* 44, 781–791 (1994).

81 Wechselberger P. Real-time quantification of specific rates in bioprocess. Presented at: *European Symposium on Biochemical Engineering Science.* Bologna, Italy, 6–8 September 2010.

82 Wechselberger P, Sagmeister P, Engelking H, Schmidt T, Wenger J, Herwig C. Efficient feeding profile optimization for recombinant protein production using physiological

information. *Bioprocess Biosyst. Eng.* 35(9), 1637–1649 (2012).

83 Sagmeister P, Wechselberger P, Jazini M, Meitz A, Langemann T, Herwig C. Soft sensor assisted dynamic bioprocess control: Efficient tools for bioprocess development. *Chem. Eng. Sci.* 96, 190–198 (2013).

84 Jobe AM, Herwig C, Surzyn M, Walker B, Marison I, von Stockar U. Generally applicable fed-batch culture concept based on the detection of metabolic state by on-line balancing. *Biotechnol. Bioeng.* 82(6), 627–639 (2003).

85 O'Haver TC, Begley T. Signal-to-noise ratio in higher order derivative spectrometry. *Anal. Chem.* 53(12), 1876–1878 (1981).

86 Dabros M. Data reconciliation of concentration estimates from mid-infrared and dielectric spectral measurements for improved on-line monitoring of bioprocesses. *Biotechnol. Prog.* 25(2), 578–588 (2009).

87 Crowe CM. Data reconciliation – progress and challenges. *J. Process Control* 6(2–3), 89–98 (1996).

88 Wechselberger P, Sagmeister P, Herwig C. Real-time estimation of biomass and specific growth rate in physiologically variable recombinant fed-batch processes. *Bioprocess Biosyst. Eng.* 36(9), 1205–1218 (2012).

89 Sagmeister P, Langemann T, Wechselberger P, Meitz A, Herwig C. A dynamic method for the investigation of induced state metabolic capacities as a function of temperature. *Microb. Cell. Fact.* 12(1), 94 (2013).

90 Kadlec P, Gabrys B, Strandt S. Data-driven Soft Sensors in the process industry. *Comput. Chem. Eng.* 33(4), 795–814 (2009).

91 Mandenius C-F. Recent developments in the monitoring, modeling and control of biological production systems. *Bioprocess Biosyst. Eng.* 26(6), 347–351 (2004).

92 Gurden SP, Westerhuis JA, Bro R, Smilde AK. A comparison of multiway regression and scaling methods. *Chemometr. Intell. Lab. Syst.* 59(1–2), 121–136 (2001).

93 Acar E, Yener B. Unsupervised multiway data analysis: a literature survey. *IEEE Trans. Knowl. Data Eng.* 21(1), 6–20 (2009).

94 Schaich D, Becker R, King R. Qualitative modelling for automatic identification of mathematical models of chemical reaction systems. *Control Eng. Pract.* 9(12), 1373–1381 (2001).

95 Zorzetto LFM, Wilson JA. Monitoring bioprocesses using hybrid models and an extended Kalman filter. *Comp. Chem. Eng.* 20, S689–S694 (1996).

96 Schubert J, Simutis R, Dors M, Havlik I, Lübbert A. Bioprocess optimization and control – application of hybrid modeling. *J. Biotechnol.* 35(1), 51–68 (1994).

97 Sagmeister P, Schimek C, Meitz A, Herwig C, Spadiut O. Tunable recombinant protein expression with *E. coli* in a mixed-feed environment. *Appl. Microbiol. Biotechnol.* 14, 14 (2013).

98 Levisauskas D, Galvanauskas V, Henrich S, Wilhelm K, Volk N, Lübbert A. Model-based optimization of viral capsid protein production in fed-batch culture of recombinant *Escherichia coli*. *Bioprocess Biosyst. Eng.* 25(4), 255–262 (2003).

99 Luttmann R, Bracewell DG, Cornelissen G *et al.* Soft sensors in bioprocessing: a status report and recommendations. *Biotechnology J.* 7(8), 1040–1048 (2012).

100 Wechselberger P, Sagmeister P, Herwig C. Real-time estimation of biomass and specific growth rate in physiologically variable recombinant fed-batch processes. *Bioprocess Biosyst. Eng.* 36(9), 1205–1218 (2013).

101 Bonarius HPJ, de Gooijer CD, Tramper J, Schmid G. Determination of the respiration quotient in mammalian cell culture in bicarbonate buffered media. *Biotechnol. Bioeng.* 45(6), 524–535 (1995).

102 Garcia-Ochoa F, Gomez E. Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnol. Adv.* 27(2), 153–176 (2009).

• **A very good overview over oxygen transport processes in bioreactors with on-line monitoring of oxygen tranport rates.**

103 Jenzsch M, Simutis R, Eisbrenner G, Stueckrath I, Lübbert A. Estimation of biomass concentrations in fermentation processes for recombinant protein production. *Bioprocess Biosyst. Eng.* 29(1), 19–27 (2006).

104 Aehle M, Kuprijanov A, Schaepe S, Simutis R, Lübbert A. Increasing batch-to-batch reproducibility of CHO cultures by robust open-loop control. *Cytotechnology* 63(1), 41–47 (2011).

• **Soft-sensor based on off-gas measurement for on-line monitoring and control of Chinese hamster ovary cell culture processes.**

105 Jenzsch M, Gnoth S, Kleinschmidt M, Simutis R, Lübbert A. Improving the batch-to-batch reproducibility of microbial cultures during recombinant protein production by regulation of the total carbon dioxide production. *J. Biotechnol.* 128(4), 858–867 (2007).

106 Frahm B, Blank H-C, Cornand P *et al.* Determination of dissolved CO_2 concentration and CO_2 production rate of mammalian cell suspension culture based on off-gas measurement. *J. Biotechnol.* 99(2), 133–148 (2002).

107 Minkevich IG, Neubert M. Influence of carbon dioxide solubility on the accuracy of measurements of carbon dioxide production rate by gas balance technique. *Acta Biotechnol.* 5(2), 137–143 (1985).

108 Sieblist C, Hageholz O, Aehle M, Jenzsch M, Pohlscheidt M, Lübbert A. Insights into large-scale cell-culture reactors: II. Gas-phase mixing and CO(2) stripping. *Biotechnol. J.* 6(12), 1547–1556 (2011).

109 Gray D, Chen S, Howarth W, Inlow D, Maiorella B. CO_2 in large-scale and high-density CHO cell perfusion culture. *Cytotechnology* 22(1–3), 65–78 (1996).

110 Boniface R, Schageman J, Sanderson B *et al.* Profiling of glycosylation gene expression in CHO fed-batch cultures in response to glycosylation-enhancing medium components. *BMC Proc.* 7(Suppl. 6), P99 (2013).

111 Kurokawa H, Park YS, Iijima S, Kobayashi T. Growth characteristics in fed-batch culture of hybridoma cells with control of glucose and glutamine concentrations. *Biotechnol. Bioeng.* 44(1), 95–103 (1994).

112 Dietzsch C, Spadiut O, Herwig C. A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for Pichia pastoris. *Microb. Cell Fact.* 10, 14 (2011).

113 Zhang J, Zhou H, Ji Z, Regnier F. Monoclonal antibody production with on-line harvesting and process monitoring. *J. Chromatogr. B Biomed. Sci. Appl.* 707(1–2), 257–265 (1998).

114 Foley DA, Wang J, Maranzano B *et al.* Online NMR and HPLC as a reaction monitoring platform for pharmaceutical process development. *Anal. Chem.* 85(19), 8928–8932 (2013).

115 Masson P. Quality control techniques for routine analysis with liquid chromatography in laboratories. *J. Chromatogr. A* 1158(1–2), 168–173 (2007).

116 Posch AE, Koch C, Helmel M *et al.* Combining light microscopy, dielectric spectroscopy, MALDI intact cell mass spectrometry, FTIR spectromicroscopy and multivariate data mining for morphological and physiological bioprocess characterization of filamentous organisms. *Fungal Genet. Biol.* 51, 1–11 (2013).