9 Bioreactor Sensing and Monitoring for Cell Therapy Manufacturing

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9.1 MONITORING AND CONTROL OF ATMPs QUALITY ATTRIBUTES THROUGH PROCESS ANALYTICAL TECHNOLOGY

As the field of cell therapy is maturing, the necessity for well-controlled large-scale bioprocesses is imperative. These bioprocesses should allow for the robust expansion of stem/progenitor cell populations, meeting the target number and quality, while at the same time guaranteeing product safety and efficacy (Abraham et al. 2017). In light of the growing need for cell technologies (e.g., for cell therapy and cell-based drug screening), where cost of goods (CoGs), process efficiency, and logistics become critically important for the clinical and commercial translation (Dodson and Levine 2015), a plethora of bioreactor systems has been employed during the last 15 years with increasing scales of operation.

While the introduction of automated bioreactor systems is already a significant step towards the industrialization of cell production processes (Hourd et al. 2014), in a "black box" bioreactor it is still unlikely to efficiently produce cells since it is impossible to precisely define (and later effectively control) the process that is going on inside. Therefore, the incorporation of monitoring technologies in or around these bioreactors systems is a logical continuation of the industrialization of the cell culture process since it is a necessary precondition of providing confidence in the manufacturing process. Additionally, the monitoring results provide the basis for comparability or equivalency of the process (e.g., after the introduction of a process change or the switch to another production location) that is required by the regulators (Williams et al. 2016).

Regulatory bodies such as the Food and Drug Administration (FDA) and European Medicine Agency (EMA) have suggested Process Analytical Technology (PAT) guidelines, which outline a set of tools and methods for making (bio)pharmaceutical manufacturing processes more reliable and efficient (Simaria et al. 2014). Following this direction, regulatory bodies and the academic research institutes have been active in pursuing these tools in the cell therapy field, suggesting that a variety of aspects such as Quality-by-Design, on-line sensors, and statistical experimental design should be incorporated in process development initiatives. Based on the guidelines of the FDA for PAT guidance, there are six aims that should be attained through the implementation of PAT tools: (1) reducing production time; (2) preventing rejection of batches (detrimental for autologous, patient specific, advanced therapy medicinal product (ATMP) manufacturing); (3) enabling real-time release of ATMPs; (4) increasing automation; (5) improving efficiency of material use; and (6) allowing the implementation of continuous processing (important for allogeneic (donor-derived) ATMP manufacturing) (Mandenius and Gustavsson 2015). These goals are expected to be applicable to all biomanufacturing sectors, bio-therapeutic protein-based drugs and gene therapy vectors, as well as cell therapy products with appropriate customization and adaptation in each case.

Cell therapies are either autologous (derived from a single patient, for that patient) or allogeneic (coming from a banked donor source, for many patients). The former does not face the risks associated with cell rejection. However, they are expected to be much more expensive in terms of manufacturing and logistics costs than the latter (Simaria et al. 2014). The differences encountered between the two biomanufacturing paradigms and the high risks associated in their production call for data

driven strategies that will allow for the quantification of bioprocess performance metrics and their association with product quality attributes. Specifically, regarding the monitoring of the culture process, the autologous case imposes generally more challenges. For example, while the allogeneic case profits from a clear economies of scale advantage regarding the cost for quality control (QC), the patient-specific autologous production requires an individual QC approach. In most cases, this QC depends at least partially on end-points analysis, thereby increasing the release time of the product significantly (or worse, receiving the final QC data after administration of the cells). While also beneficial for the efficient manufacturing of allogeneic cells, implementing on-line monitoring systems that provide pre-validation data on the release criteria are therefore more critical for autologous processes.

9.2 BASIC CONCEPTS AND DEFINITIONS REGARDING BIOREACTOR MONITORING

The primary goal of bioreactor monitoring is to provide a quantitative description of cell state and fate inside the vessel (e.g., the critical process parameters), which for ATMP manufacturing is closely linked to the resulting cell quality (i.e., the critical quality attributes). There are many modes of operation for sensing (Wendt et al. 2009) that will be discussed in the following paragraphs. Depending on the specific goal of the read-out and considering other factors such as costs, user-friendliness, and required specificity of the results, any of the following strategies can be appropriate to implement in a bioreactor process.

It is important to note that while sensor systems are used to validate the manufacturing process, the sensor systems themselves, and especially (the interpretation of) their results, need validation too. Therefore, it is important to understand the limitations and optimal implementation of the specific sensor under consideration. For example, sensor accuracy (i.e., the magnitude of the error on the reading), sensitivity (i.e., the lowest and higher detectable limit), specificity, response time, and signal drift all must be considered when choosing a specific sensor for an application.

9.2.1 On-Line or In-Line *versus* Off-Line or At-Line (Biechele et al. 2015)

1. On-line or in-line sensing strategies generally refer to methods that allow for receiving continuous up-to-date information on the state of the system in real-time or near real-time, respectively. To be able to capture dynamic responses of the stem/progenitor cells in culture, the sample frequency and signal resolution of the on-line or in-line sensor should be at least twice the frequency at which the variable under consideration changes (Nyquist sampling frequency). While on-line or in-line sensing strategies are technically more challenging to implement for certain types of analysis (e.g., secreted protein concentrations), this high-frequency data is ideal for continuous bioreactor process control. pH and dissolved oxygen tension are typical parameters for on-line monitoring in bioreactors.
2. Off-line or at-line sensing strategies do not provide the operator with instant read-outs. On the contrary, most off-line or at-line sensing strategies require

considerable manual preparation and analysis by the operator. While the informational load of the analysis can be extremely high due to the large range of possible analytical systems to be used, these sensing strategies are less optimal for automated control of bioreactors due to the discontinuity between measurement and result (i.e., the result is a retrospect). A frequently used example of off-line monitoring is the analysis of cell surface markers by flow cytometry or high-performance liquid chromatography (HPLC) for medium analysis (e.g., metabolites).

9.2.2 Invasive *versus* Non-Invasive *versus* Indirect Sensing

1. Invasive monitoring is based on *in situ* probes (or labels such as fluorescent tags) that have a direct interface with the cells or culture medium inside the bioreactor. Generally, they can provide direct information on the state of the culture in the vessel in which they are embedded. A typical example of an invasive sensor is an electrochemical sensor where the culture medium must come in close contact with the functionalized membrane of the sensor. Unless these sensor probes can be sterilized in place or installed as a disposable and pre-sterilized component, invasive sensors are generally not preferred for clinical production due to the possible contamination risk, possible interference from the functionalized membrane with the medium and the risk of degradation of the readout quality by sensor fouling.
2. Non-invasive methods can infer information from the culture environment without physical contact to the medium or cells. A typical example is an optical pH or O_2 sensor with a sensor patch with a pH or O_2 sensitive complex that is glued to the (transparent) inside wall of the culture vessel. Plus, it has an optical fiber at the outside of the vessel that allows a contactless interrogation of the bio-environment. While the transparency of the vessel and often relatively lower sensitivity of the sensor must be considered, their ease of use and simpler handlings to maintain sterility make such sensors ideal for bioreactor monitoring.
3. Indirect sensing *(ex-situ)* makes use of sampling methods, either automated via (recirculating) sampling lines, or manual via sampling ports that allow a sterile connection to the culture vessel with a syringe, for example. A very common use of this strategy is found in bioprocesses where samples are drawn from a sample port and analyzed for lactate and glucose concentrations.

9.2.3 Destructive *versus* Non-Destructive

Destructive testing is an end-point analysis that renders the cells unsuitable for further use. This type of analysis potentially results in readouts with a very high sensitivity and specificity. In certain cases, however, at the start of an autologous culture process where cell material is scarce, the resulting cell loss is undesirable. Non-destructive methods do not require using/manipulating the cells at all (e.g., microscopy, analysis of spent medium samples), nor are they able to return the sampled cells to the vessel via a sterile recirculation sampling-loop.

Keeping in mind the end goal of large-scale automated production of clinically relevant cells, this chapter will focus on non-invasive and non-destructive technologies. Special attention will be given to on-line or in-line methods that are essential for the continuous process control, leading to reproducible high-quality products. Additionally, the cost-effectiveness will be discussed since certain monitoring strategies contribute significantly to the CoGs.

9.3 MONITORING OF BIOREACTORS BASED ON THEIR MODE OF OPERATION AND DESIGN

Large-scale expansion and differentiation of different stem cell populations in a monitored and controlled environment are of immense importance in the field of cell therapy and regenerative medicine. The use of bioreactors is considered to be the most efficient method for this and currently we have a plethora of different systems available, differing in design and operation based on user requirements. The following section aims at a concise review of the different bioreactor systems used for stem cell culture and the implications of the vessel design on the monitoring strategy. The reactors are classified according to their mode of operation and according to the method of cell growth within the systems. Most importantly the implication of bioreactor mode of operation with sensor compatibility and readout/data quality will be also discussed.

9.3.1 Modes of Operation

Batch: A batch mode of bioreactor is a closed system, wherein the bioreactor is seeded with a defined volume of medium and cells at the initial time point, with no further addition or withdrawal throughout the culture period. At the end of the culture period, the content of the whole reactor is harvested in one go. Cells in a batch operated reactor follow the well-established cell growth pattern while consuming nutrients and producing waste metabolites until the nutrients are completely depleted and/or maximum cell density for the system is reached. Monitoring of processes in reactors under batch mode allows for continuous and regular on-line monitoring of various parameters like pH, metabolite concentrations, dissolved oxygen, and so on, wherein their absolute values can be obtained at different time points. However, due to the closed nature of the system, off-line analysis of parameters-like secretory products, phenotype markers can only be carried out at the end of the total culture period. This may result in loss of information for the user. The closed nature of the system also does not allow dynamic process changes based on gathered process data. As a result, bioreactors with this mode of operation have been mostly used for preliminary analysis of culture parameters for mammalian cells (Dalili et al. 1990, Merten et al. 1990).

Fed-Batch: In a fed-batch system, an intermittent or continuous feed of nutrient is supplied to the reactor without any withdrawal of cells or supernatant. The nutrient level in such a system is controlled by optimizing the feed rate in a way to reduce the production of waste metabolites without completely

depleting the medium of nutrients. Mammalian cells can be cultured for weeks in a fed-batch reactor and results in higher product concentration in comparison to batch reactors. Monitoring tools and methods in a fed-batch system are essentially similar to batch reactors but the change in system volume makes it a relative reading and requires the users to take in to account the feed rate and medium volume of the system for analysis purposes. In the field of mammalian cell culture, fed-batch reactors have been used for different cell types, such as baby hamster kidney (BHK) cells, Chinese hamster ovary (CHO) cells, and so on (Lenas et al. 1997, Andersen et al. 2000).

Perfusion reactor system: A perfusion reactor is a continuous system with constant inflow of fresh medium along with an outflow of cell-free supernatant, keeping the culture volume constant throughout the culture period. This system has a relatively homogenous mixture of nutrients and dissolved oxygen resulting in a high final cell density. Most current day dynamic stem cell culture systems use the perfusion mode of operation for different bioreactor designs. The monitoring system and resulting analysis are more complex in a perfusion system in comparison to batch and fed-batch reactors since the dilutive effect of the influx of fresh medium has to be taken into account during the analytics, and the measurement values of different parameters could have different values at different positions in the system (e.g., at the inlet *versus* the outlet of the system). Experimental data and computational modeling has shown that cell growth and matrix formation are affected by the scaffold position within a perfusion system due to different flow patterns, shear stress, and so on (Papantoniou et al. 2014, Guyot et al. 2016). As a result, it is imperative that measurements and monitoring tools be used at different locations within the system to extract every possible data for the system. As an example, it has been reported that oxygen concentration in a scaffold-based perfusion system is different at the inlet and outlet. While at the inlet, the concentration remains almost constant throughout the experiment, a decrease in oxygen concentration is measured at the outlet. This information can be used to provide an estimation of oxygen consumption by the cells (Simmons et al. 2017). Similar measurements of oxygen concentration at different positions have been used in mechanistic models to link such monitored parameters to cell growth and health (Lambrechts et al. 2014).

9.3.2 Bioreactor Design

Stirred-tank reactor: Stirred-tank reactors are cylindrical vessels with an impeller for stirring motion and are one of the oldest and widely used dynamic cell culture systems. Stirred-tank reactors are easy to operate and scaling-up is a relatively simple task using vessels of different sizes, thus reducing the chances of vessel-to-vessel variability. Other advantages include the possibility of expanding suspension cells, as well as anchorage-dependent cells, feasibility of on-line monitoring, and automation of the system and parameters involved like nutrients, dissolved oxygen, pH,

and so on. Although in most cases it is used under fed-batch, continuous feeding is also possible as a perfusion system. One of the earliest known stirred-tank reactor-based expansion of stem cells was published by Zandstra and co-workers in 1994, when they cultured hematopoietic stem cells in a spinner flask. Since then, experiments have been carried out on various other stem cell types under variable growth conditions. Pluripotent stem cell (both embryonic stem cells [ESCs] and induced pluripotent stem cells [iPSCs]) culture has also been reported in bench top spinner flasks either as cell aggregates or on attachment surfaces like microcarriers, scaffolds, and hydrogels (Fok and Zandstra 2005, Cormier et al. 2006, Chen et al. 2010, Azarin and Palecek 2010, Zweigerdt et al. 2011, Gupta et al. 2016, Ashok et al. 2016, Badenes et al. 2016, Abecasis et al. 2017). Unlike pluripotent stem cells (PSCs), mesenchymal stem/stromal cells (MSCs) from different sources have been extensively expanded using stirred-tank reactors of different scales, which range from 15 mL bench top systems to liter scale reactors (Baksh et al. 2003, Sart et al. 2010, Jung et al. 2012, Rafiq et al. 2013, dos Santos et al. 2014, Chen et al. 2015, de Soure et al. 2016, Heathman et al. 2016). Spinner flask systems have also been used for the differentiation of stem cells towards more committed lineages like cardiomyocyte, osteogenic, chondrogenic, and neurogenic. Although shear stress within a stirred-tank reactor is heterogeneous in nature, proper spin rate within stirred-tank reactors is essential for having a homogenous mixing of metabolites, dissolved oxygen, and proper cell growth within the system. The more or less homogenous culture environment allows the users to have sample-based monitoring of the system along with continuous probe-based ones. The suspension method of culture in case of stirred-tank reactors also allows users to use spectroscopic methods for monitoring the process. Near Infrared (NIR) spectroscopic method, Raman spectroscopy, and impedance spectroscopy have been used for measuring and monitoring cell process status, concentration of medium nutrients, and cell concentration in a suspension bioreactor (Zhao et al. 2015)

Parallel plate reactors: Parallel plate bioreactors consist of a vessel wherein medium is perfused parallel to the 2D cell layer resulting in uniform shear stress over the cells. These reactors are straightforward as the required operations resemble the standard flask-based culture, they are easy to use, have automation options, and are mainly used as micro or bench top systems. However, periodic harvest from this type of system is virtually impossible. Hematopoietic, mesenchymal, and pluripotent stem cells have been cultured using parallel plate type systems (Palsson et al. 1993, Dennis et al. 2007, Wolfe and Ahsan 2013, Belair et al. 2015). While most 2D cell cultures are monitored by microscopy, the multi-plate configuration brings additional challenges for visual inspection. Commercial multi-plate bioreactors, such as the Pall Xpansion® Multiplate Bioreactor system, therefore developed a specialized microscope (Ovizio Imaging Systems) for monitoring cell growth in combination with on-line pH and

O_2 sensors (Leferink et al. 2015, Lambrechts et al. 2016). Since mixing in parallel plate bioreactors is limited and nutrient gradients might exist, for example, perpendicular to the plates, care should be taken for sample-based monitoring techniques.

Hollow-fiber reactor: Hollow-fiber bioreactors are double compartment systems, usually consisting of fiber bundles encased in a vessel with ports for intra-capillary and/or extra-capillary medium flow. This type of a system allows for surface areas larger than parallel plate systems but results in more challenging harvesting and scale-up. Despite their disadvantages, embryonic, mesenchymal, and hematopoietic stem cells have all been expanded using hollow-fiber bioreactors by several groups (Housler et al. 2012, Lambrechts et al. 2016). The 3D configurations of the opaque fibers do not allow visual inspection of the cells, and at the same time, due to the relatively large length of the fibers, nutrient gradients are potentially introduced over the length of the fibers. Therefore, precise monitoring of the culture environment is required so the homogeneity of the nutrient availability can be controlled via the interplay between perfusion and recirculation in the extra-capillary environment of the hollow-fiber bioreactor.

Rotating wall vessel: The rotating wall vessel (RWV) bioreactor was designed by NASA's Biotechnology group as a means to have a dynamic culture with relatively low shear and turbulence. The bioreactor simulates a microgravity environment, offers attractive options for stem cell culture, and has been used for culturing different stem cells including embryonic, mesenchymal, hematopoietic and neural stem cells in suspension (Gerecht-Nir et al. 2004, Lin et al. 2004, Liu et al. 2006, Chen et al. 2006). Differentiation of MSCs have been also carried out using the RWV system (Song et al. 2006). The rotating wall makes it challenging to implement probe or patch-based sensors and for visual inspections.

Fixed-bed reactor: Fixed-bed reactors consist of scaffolds or particles fixed within a column. Medium is perfused through the "packed bed" and supplies nutrients, oxygen, and shear stress to the cells. A fixed-bed reactor can provide 3D environment for stem cells, allows for greater cell-cell and cell-extracellular matrix interaction resulting in better mimicking of *in vivo* cell niche. However, due to the high cells density per cubic meter these systems may also give rise to spatial concentration gradient of nutrients oxygen etc. Fixed-bed type reactors with scaffolds have been used extensively for the expansion and/or differentiation of different types of stem cells (Alves da Silva et al. 2011, Sonnaert et al. 2017). They have also been used for the formation of neo-tissues making them extremely important in the field of tissue engineering. These systems are usually used as a perfusion system with in-line and off-line measurements and monitoring carried out in a similar way as a perfusion system (de Peppo et al. 2013, Sonnaert et al. 2015, Sabatino et al. 2015).

9.4 MONITORING BASIC PHYSICOCHEMICAL PARAMETERS

Bioreactor process variables are of a chemical, physical, or biological nature and can be measured in the gas, liquid, and solid phases of a bioprocess. Concentration changes are informative of cell growth, metabolism and productivity. A typical biosensor comprises three parts, a biological detection component that is immobilized on a signal transducer unit, followed by an amplification, and signal conversion unit. The biocomponent recognizes the analyte either through a catalytic mechanism (e.g., cells) or through binding (e.g., antibodies, membrane receptors) (Thevenot et al. 2001). There are also novel components such as thermostable enzymes or aptamers (Song et al. 2008). Monitoring can be performed with in-line sensors or via analytical systems that can be coupled with sampling devices as at-line sensor systems. Biosensors for many different analytes have been developed to date (Mulchandani and Bassi 1995, Bracewell et al. 2002, Rhee et al. 2004, Borisov and Wolfbeis 2008). Typical implementation of these types of sensors are the Clark-type electrode for O_2 or optical readings from O_2 or pH sensitive sensor patches (Sections 9.4.2 and 9.4.3).

9.4.1 Glucose

Most widely implemented biosensor application is that of determining glucose concentration since this consists of the most important energy source for cells during culture. Glucose and lactate levels have been used as in-process control parameters to indicate the active metabolism for differentiation of stem cell populations. Glucose concentration can have an impact on the cell differentiation process and, in high concentration, has been seen to suppresses embryonic stem cell differentiation into cardiomyocytes (Yang et al. 2016) and inhibit the proliferation and migration of bone marrow mesenchymal stem cell (Zhang et al. 2016). In addition, for both PSCs and MSCs, it has been seen that culture under constant low glucose concentration in the bioreactor feed, glucose consumption, and lactate production were limited (Schop et al. 2008, Chen et al. 2010).

In the case of glucose, electrochemical and optical sensing methods have been extensively investigated. However, continuous glucose monitoring is still limited and potentially could be incorporated in the process development stage by the adoption of automated parallelized platforms offering on-line glucose monitoring such as the ambr® system (Rafiq et al. 2017, Xu et al. 2017). Many more electrochemical detection methods (Borisov and Wolfbeis 2008) are in use for glucose monitoring than optical ones. Glucose sensors, according to a comprehensive review of Steiner and co-workers, can be classified in four categories: (a) monitoring of optical properties of enzymes, their cofactors and co-substrates, (b) measurement of enzymatic oxidation products of glucose oxidase, (c) the use of boronic acids, and (d) the use of glucose binding proteins. In the literature, there are also examples of using spectroscopic methods such as NIR and mid infrared (MIR) spectroscopy for measuring glucose (Rhiel et al. 2002, Henriques et al. 2009).

9.4.2 Oxygen

Oxygen tension is one of the most important parameters in cell culture monitoring. It has been seen that long-term culture of human MSCs under hypoxic conditions (2% O_2) results in a decrease in cell proliferation but not in an increased apoptosis for up to 24 days of culture (Grayson et al. 2007). In addition, it has been reported that hypoxia induced down-regulation of osteoblastic genes in human MSCs *in vitro* (Potier et al. 2007) and low dissolved oxygen values (1% vs 21%) affected osteogenic differentiation with a metabolic switch to anaerobic glycolysis (Hsu et al. 2013). In general, low dissolved oxygen tensions have been seen to maintain progenitor cell populations maintaining stemness of human MSC populations (Mohyeldin et al. 2010).

Semiconducting, electrochemical and paramagnetic sensors are commonly used for oxygen analysis in the gas phase. The dissolved oxygen concentration in the medium (liquid phase) can be measured by electrochemical and optical chemical sensors (chemosensors). Membrane-covered amperometric electrodes, known as Clark-type electrodes, are the most common electrochemical sensor used. Optical measurement of the partial pressure of oxygen is based on the fluorescence quenching of an indicator by molecular oxygen (Harms et al. 2002). In addition, optical sensors can be easily miniaturized (Beutel and Henkel 2011).

9.4.3 pH

pH has been seen to influence osteogenic differentiation and ECM mineralization. In a relevant study by Monfoulet and colleagues, the presence of mineralized nodules in the extracellular matrix of human bone marrow MSCs was seen to be fully inhibited at alkaline (>7.54) pH values. In addition, for a pH range between 7.9 and 8.27, proliferation was not affected while osteogenic differentiation was seen to be inhibited (Monfoulet et al. 2014). In addition, reduction of extracellular pH from 7.8 to 7.0 was seen to negatively affect MSC osteogenic differentiation *in vitro* (Tao et al. 2016). Moving on to glucose, mineral deposition of MSCs during osteogenesis in high-glucose medium is greater than that of MSCs in low-glucose medium (Li et al. 2007).

pH must be controlled in nearly all bioprocesses to keep the cells at optimal cultivation conditions. Electrochemical techniques for pH are based on potentiometric and amperometric measurements, with potentiometric sensors being more commonly used. A working (measurement) electrode (working potential) is correlated to a reference electrode (Henkel and Beutel 2013). Other systems are the ion-sensitive field effect transistors (ISFETs). Apart from the classical electrochemical sensors, also optical chemosensor systems are used. Fiber optic pH sensors are based on absorbing or fluorescing pH indicators. Frequently used pH-sensitive indicators for absorbance are cresol red, phenol red, bromopherol blue, and chlorophenol red (Gupta and Sharma 1998). These indicators are immobilized on solid substrates or in polymers that are mounted directly on a transparent carrier (glass or optical fibers). The pH optodes can easily be miniaturized and have response times in the range of milliseconds. However, they suffer from cross sensitivity to ionic strength, a limited

dynamic range, and the loss of sensitivity during sterilization or cleaning procedures (Beutel and Henkel 2011). Nowadays only γ-sterilized optical biosensors are commercially available and are implemented in pre-sterilized disposable bioreactor systems. Finally, pH levels can also be measured by spectroscopic methods, such as MIR and NIR spectroscopy (Schenk et al. 2008).

9.4.4 Carbon Dioxide

Monitoring of carbon dioxide in bioprocesses is essential as it is a product of cell respiration and can pass through cell membranes to influence pH inside the cells. Thus, variations of dissolved CO_2 concentration can affect cell morphology, their metabolic products, and consumption/productions rates. Most optical CO_2 sensors operate in the same way as the classical Severinghaus electrodes. They are typically based on the fluorometric or colorimetric changes of pH-sensitive indicators that are added to the buffer system and separated from the analyte solution by a CO_2—permeable membrane.

For monitoring dissolved carbon dioxide, the use of traditional electrochemical sensors can be problematic because they are bulky and invasive (Chatterjee et al. 2015). Disposable optical sensors have a lot of advantages, like high sensitivity and easy miniaturization, and they are only partially invasive. However, there are the disadvantages regarding the toxicity of the patch and the photo-toxicity of illuminating light. Gupta and colleagues (Gupta et al. 2014) used a completely non-invasive approach to monitor dissolved carbon dioxide in disposable small-scale cell culture vessels based on the diffusion through permeable vessel walls, which can be applicable in sensitive cells. A strong limitation of this approach was that it cannot continuously measure the process. A completely non-invasive rate-based technique, measuring the initial diffusion rate, allowed for the measurement of the partial pressure of CO_2. This system has been tested in spinner flasks and the results were comparable to those measured with fluorescence-based patch sensors. The main difficulty regarding the integration and use of carbon dioxide sensors is their limited stability and dependency on the ionic strength of the buffer (Glindcamp et al. 2009). A "credit-card" microsystem for the determination of carbon dioxide for fermentation monitoring was also recently reported (Calvo-Lopez et al. 2016).

9.4.5 Spectroscopic Sensors

Spectroscopic analyses are based on the interaction of electromagnetic waves and molecule bonds. Common spectroscopic methods for bioprocess monitoring are focused on the spectral range from UV to MIR, including fluorescence and Raman Spectroscopy. Various process variables can be measured in different spectral ranges (16–21). The advantages of spectroscopic sensors include no sampling is needed (except for calibration), there is no interaction between the sensor and the analytes, and several different process variables can be determined simultaneously. One major drawback is that chemometric data analysis is needed to extract relevant process information from the generated data.

9.5 IMAGE-BASED SINGLE CELL MONITORING DURING BIOPROCESSING

Despite new advances and technological breakthrough in monitoring, visual analysis of cells is still one of the most versatile and powerful tools (Molder et al. 2008). Image-based monitoring of bioprocess parameters is increasingly recognized as a valuable tool in the field, and the use and development of in-situ image acquisition sensors is rising (Bluma et al. 2010). However, today image-based results often depend on manual analysis, which is time consuming and subject to very high variability. Thus, there is a need for non-invasive automated visual cellular analysis that allows objective quantification of critical quality attributes (CQA). In this section, we review the different optical modalities that are currently available and under development in the bioreactor field, as well as the future perspective for imaging of bioreactor-based bioprocesses.

The developments and trends in the field will be reviewed from the most basic optical techniques point of view (i.e., bright field microscope), to the latest high-tech developments (i.e., lens-free imaging), naturally following a quasi-chronological order. In the last decade, different types of imaging modalities have been adapted to bioreactors in *in-situ* setups. Most of these are gathered in Table 9.1.

From this table, one can see that the first innovations in the field consisted in adapting bright field microscopes to bioreactors, using *in-situ* probes in contact with cell suspension and commercially available products (i.e., *in-situ* microscope type III XTF, Sartorius). The probe allows monitoring of a sample volume (assumed to be representative of the batch). By using image processing algorithms, it can provide estimates of the cell concentration, cell size or degree of aggregation (Joeris et al. 2002). Using neural network algorithms trained on manual analyses, Rudolph and co-workers were able to build a model-based monitoring of colonization of microcarriers by cells (Rudolph et al. 2008).

However, bright field systems show inherent limitations for live cell imaging (and processing) because of the low contrast of transparent specimen. Furthermore, the sample volumes imaged with these systems can be quite small compared to the bioreactor volume, making the extrapolated data less representative of the cell suspension. To overcome the contrast limitations, Wei and collaborators adapted a dark field condenser into an existing *in situ* probe (Wei et al. 2007). However, direct phase quantification is not possible with this technique, as it involves a non-linear phase to amplitude conversion, which can result in artefacts in the images (Mann et al. 2005). This applies also to other imaging modalities, like phase contrast or differential interference contrast imaging, which provide good contrast for transparent specimens.

Quantitative phase imaging allows the computation of the optical thickness of a transparent specimen, which gives information on the physical thickness of the samples and their optical index variation (Mann et al. 2005). Lens-free imaging techniques are rising in the field for imaging of 2D planar cell culture as they provide direct phase quantification, great contrast of transparent samples, decoupling of the field-of-view to the resolution, and larger field of views (Greenbaum et al. 2012). Depending on the spatial and temporal coherence of the light source on the image

TABLE 9.1
An Overview of Available Imaging Techniques for Visualization of Cell Features. Advantages and Shortcomings of Each Technology Are Also Listed

Imaging Modality/Setup	Measurements	Pros	Cons	Representative References
Bright field imaging/probe inside stirred system under batch operation.	Cell concentration, cell size, cell volume. As population averages. Cell concentration, cell volume. As population averages Cell concentration, cell size distribution, degree of aggregation.	Real-time imaging.	Probe is invasive. Relatively small field of view.	Joeris et al. (2002)
	With neural network validated on manual analysis → level of microcarrier colonization modelled	Real-time imaging.	Probe is invasive. Computationally heavy methods required. Relatively small field of view.	Rudolph et al. (2008)
Dark field imaging/probe inside stirred system under batch operation.	Cell density. Cell viability reading using a machine-learning algorithm validated against live dead manual analysis.	Real-time imaging.	Probe is invasive. Computationally heavy methods required. Relatively small field of view.	Wei et al. (2007)
Differential interference contrast imaging/probe inside stirred system under batch operation.	Confluence of cells on micro-carriers, cell density, cell distribution.	Real-time imaging.	Probe is invasive. Computationally heavy methods required. Relatively small field of view.	Odeleye et al. (2017)
Phase contrast imaging used at-line	Cell concentration, cell size distribution, 2D attachment morphology,	Non-invasive imaging. Good contrast of transparent cells.	Suited for planar cultures, not for 3D setups. At-line operation.	Jaccard et al. (2015)
Digital holographic imaging	Cell number, cell area, thickness and volume.	Real-time, non-invasive imaging. Excellent contrast. Large field of view, decoupled from resolution. Compact devices used on-line.	Not suited for non-planar cultures.	El-Schish et al. (2010)

reconstruction algorithms, several lens-free imaging (LFI) techniques have emerged (Kim et al. 2012). In general, the interest for digital holography imaging (DHI) has increased in the field of cell monitoring (Carl et al. 2004, Rappaz et al. 2005, Molder et al. 2008, Biechele et al. 2015) to become a powerful quantitative, non-invasive imaging technique for 2D planar cell cultures nowadays. Due to the design simplicity of the technique, DHI allows the development of compact, light, and cost-effective devices. Several commercially available DHI systems (such as iLine S, Ovizio® with Pall®; iLine M, Ovizio®; LUX2, CytoSMART™; and Cytonote, iPRASENSE) exist for the monitoring and controlling of cell bioprocesses with compact designs fitting inside incubators, a wireless connection to computer, wide field of views, and high resolutions. Recently, an integrated optics-based methodology was reported that consistently and accurately assesses viable cell number, confluence, and cell distribution of embryonic stem cells cultured on micro-carriers sampled from suspension bioreactors (Odeleye et al. 2017).

9.6 VISUAL MONITORING OF 3D GROWTH IN SCAFFOLD-BASED PERFUSION BIOREACTORS

Concerning tissue engineered ATMPs, bioreactors are used to enhance tissue proliferation and cell differentiation. The field of tissue engineering is evolving towards the *in vitro* creation of complex 3D tissues and, currently, the gold standard for assessing engineered tissue construct quality is by using destructive end-point assays such as histological sectioning (Doroski et al. 2007, Leferink et al. 2016). The field is developing a growing interest in novel methods for non-destructive 3D construct visualization over time with a decent spatiotemporal resolution (Mather et al. 2007). Most optical microscopy techniques show major constraints for these applications as they have limited penetration depth (Vielreicher et al. 2013). The applicability of a given imaging modality highly depends on the type of carrier structure used for the tissue and the biological components of interest. In this section, we review the currently used or promising imaging technologies for *in vitro* 3D tissue formation in bioreactors.

9.6.1 Micro-Computed Tomography

X-ray computed tomography (CT) exploits the variations in the X-ray absorption properties of a sample to form a 3D reconstructed image. The spatial resolution achieved with this technique depends on the X-ray source spot size, which, more generally, depends on the coherence of the X-ray beam (Appel et al. 2013). To date, by using this technique nanometer scale resolution has been achieved. As the contrast in the reconstructed pictures is dependent on the X-ray absorption properties of the materials, a good contrast can be achieved between denser tissues like bone and softer tissues (i.e., cartilage, fibrous tissue). However, achieving a decent contrast to distinguish between two soft tissues and the background is challenging. Two arising approaches to overcome this limitation in soft tissue engineering consist in using chemical contrast agents that stains the structures of interest (Papantoniou et al. 2014)

combining the use of micro-CT with X-ray phase contrast imaging (Zhu et al. 2011, Appel et al. 2015, Bradley et al. 2017). Using synchrotron approaches, even single cell resolution could be achieved without the use of contrast (Voronov et al. 2013). These approaches go one step further in the development of X-ray micro-CT based on-line monitoring tool for tissue engineering.

9.6.2 Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) has shown to be a promising modality for the imaging of soft tissues (Lalande et al. 2011, Kotecha et al. 2013). Few advances have been achieved in the coupling of MRI devices with bioreactors for longitudinal monitoring of tissue growth (Crowe et al. 2011, Gottwald et al. 2013). The use of contrast agents with MRIs has also allowed cell tracking (Feng et al. 2011, Di Corato et al. 2013). Although this modality inherently provides better contrast than CT for soft tissue 3D visualization, the acquisition of MRI scans with decent resolutions (micro-MRI) require high-field MRI scanners and long scanning times. Such devices capable of applying strong enough magnetic fields for micro-MRI are expensive, and their implementation in tissue engineering laboratories is not yet feasible (Leferink et al. 2016).

9.6.3 Impedance Monitoring

Radio frequency impedance (RFI) monitoring has long been recognized as an accurate and reliable method for on-line and off-line measurement of live cell biomass as the impedance (measured capacitance) is proportional to the volume of living cells in the bioprocess (Carvell and Dowd 2006). ABER® Instruments' biomass sensor, HP® Colloid Dielectric Probe and CellSine are a few RFI systems currently commercially available. Furthermore, the use of this sensor to scaled-down systems (down to 1 mL) and single-use bioreactors has been reported (Carvell et al. 2014).

9.7 HIGH-THROUGHPUT BIOREACTOR MONITORING THROUGH THE ADOPTION OF OMICS TOOLS

There is a clear need for the development and application of novel methods that enable the comprehensive characterization of cell state, improving the current conventional set of measurements for monitoring cell and tissue quality (Lipsitz et al. 2016). In this context, the development of "omics" technologies could further contribute in capturing intracellular characteristics and, hence, enhance the ability to monitor bioreactor processes.

The term "omics" refers to the quantification and characterization of various biological molecules present at a specific time or condition. These approaches include genomics, transcriptomics, proteomics, and metabolomics, which, respectively, examine genes, messenger RNA (mRNA), proteins, and metabolites present in a particular cellular environment. Each set of biological molecules and their levels can be analyzed as a molecular signature for the cell and condition of interest. This

information is a key for understanding cellular physiology and using a systembiology approach to rationally improve cellular performance. Omics technologies, although relatively new, have been rapidly implemented in biotechnology, leading to "revolutions" in the field of cancer biology (Vucic et al. 2012), biofuel production (de Jong et al. 2012), and in the process optimization of industrial mammalian systems (Vernardis et al. 2013). Additionally, major biotechnological-biopharmaceutical companies like Bristol-Myers Squibb, Bayer, Pfizer, and Life Technologies (part of ThermoFisher Scientific) had already conducted studies with the goal of integrating omics technologies in the bioprocessing field (Xu et al. 2011, Slade et al. 2012).

Recently, the adoption of omics technologies in the field of stem cell bioprocessing has been initiated. More specifically, Silva and colleagues (Silva et al. 2015) used genome-transcriptome profiling as a critical evaluation tool for the robustness of the expansion process of human ESCs in stirred-tank bioreactor cultures. Furthermore, they explored the use of omics tools and, more specifically, metabolomics to investigate the physiological and metabolic changes during human ESC expansion in the aforementioned bioprocess. They claimed that this new information can guide bioprocess design and medium optimization. In the same context, Alves and co-workers (Gomes-Alves et al. 2016) explored the use of omics tools for the characterization of the *in vitro* expansion of human cardiac progenitor cells (CPC). They used gene expression microarrays and mass spectrometry-based approaches to compare transcriptome, proteome, surface markers, and secretion profiles of human CPC cultured in static monolayers and in stirred microcarrier-based systems. In this work, the authors used omics tools as a quality control for the developed bioprocess, and they underlined the importance of this methodology as a future potency assay of their stem cell population. In another study, Abecasis and colleagues (Abecasis et al. 2017) used stirred-tank bioreactors for the expansion of human iPSCs as aggregates. Extensive cell characterization was conducted by using whole proteomic analysis as a quality control tool for the maintenance of cells' pluripotent character during the culture.

Although bioreactor-based ATMP manufacturing still relies on empirical techniques, it is clear that this is a rapidly evolving landscape. The use of omics represents a unique opportunity to develop improved methods to characterize and understand cell culture state, and rationally optimize bioreactor cultivation processes as illustrated in the comprehensive review of Lewis and co-workers (Lewis et al. 2016). The information gained from omics approaches can be used for the critical evaluation of the impact of cell culture conditions but also to define potency criteria. All the above can help even to redesign operating conditions, coupled with medium formulations and bioreactor design approaches (Clarke et al. 2011). In addition, the identification of sets of efficacy-specific biomarkers, which could be easily monitored on-line with existing sensors, is another important aspect of the use of omics strategies.

9.7.1 Limitations of Omics Technologies in Stem Cell Bioprocessing

Although they possess a great potential for the rational improvement of stem cell bioprocessing, omics technologies face some critical limitations that should be addressed before this technology could be adopted in industrial practice. These

limitations should be mostly identified in the data analysis cost required for such large and complex data sets. Despite great advancements in the bioinformatics field, a strong limitation of omics methodologies is the limited ability to meaningfully interpret the large amount of data that are generated by these methods. For that purpose, there is continuing need to develop more efficient computational tools that would facilitate the extraction of conclusions from such large data sets at a higher frequency. Furthermore, the identification of meaningful links between omics datasets and desired phenotypes is a major challenge. Large data sets can be used to identify specific biomarkers of cell physiology. However, validation of these biomarkers is a critical and often neglected step for monitoring purposes. Once validated, these biomarkers can be routinely monitored. However, biomarkers can be cell line dependent, and, as a result, omics strategies need to be developed to incorporate this methodology into the rapid timelines of the industrial bioprocesses. As biomarkers can be cell line specific, there is also the need to develop omics strategies for integrating this methodology in the rapid timelines of the industrial bioprocesses.

9.8 MODEL-ENHANCED MONITORING AND PROCESS CONTROL

In automated bioreactors, it is standard practice that measurements of physicochemical or environmental variables (i.e., critical process parameters (CPPs) such as pH and O_2) are used as input for a controller that, in turn, takes care of providing suitable conditions for cell culture inside the bioreactor. It is then presumed, based also on the mapping of a design space, that these suitable environmental conditions will deliver cells with the proper quality attributes for clinical application. While currently these CQAs are verified by end-point and destructive analysis, it would be more efficient to have continuous feedback on the CQAs during the culture process and implement controllers that directly optimize the CQAs (instead of indirectly via the CPPs), ultimately leading to higher process certainty and more potent cell-based products.

As it can be seen from the previous overview of monitoring techniques, it is not necessarily a problem of measuring the CQAs, but the limiting factor, to date, is the lack of non-invasive sensor systems with a measurement rate that allows on-line monitoring of the CQAs. Furthermore, sensor hardware development is required to have cost-effective on-line and non-invasive CQA measurements from inside the culture vessel (e.g., replace classic off-line methods for immunophenotyping or quantitative polymerase chain reaction [qPCR]). Alternatively, it is a promising approach to estimate the unmeasurable CQAs in real-time, based on a combination of CPP measurements that can be linked to the CQA of interest by means of a model. This model-enhanced monitoring strategy is also sometimes referred to as a "soft(ware) sensor" or "inferential measurement" methods (de Assis and Maciel 2000, Kadlec et al. 2009). In practice, the models are used to translate sensor data into more informative process read-outs that can be used to make informed process decisions.

This "model-based monitoring" strategy, combined with a desired output trajectory can be used as the basis for "model-based control" in which the measured process data and a model are used to determine or predict the most appropriate controller setting to reach the desired state of the process. Although more often used in bioreactors for mammalian cell culture (Kovarova-Kovar et al. 2000, Ramaswamy

et al. 2005, Aehle et al. 2012), only limited examples of model-based control can be found in literature for stem cell bioreactors (Csaszar et al. 2012, Lambrechts et al. 2014). While the clinical use of these adaptive or predictive manufacturing strategies might require adjustments to the current regulatory guidelines, they provide a possible robust and objective strategy to deal with heterogenic input material (e.g., donor-related variability in autologous processes).

9.9 CONCLUDING REMARKS

The steady increase in the production scale of cell-based therapeutics highlights the continuous maturation of the field. However, there is still considerable room for the adoption of additional sensors and monitoring strategies that will further aid the understanding of bioprocesses. This is necessary to face the challenges encountered during the transition from the early preclinical stage to the late commercial stage of manufacturing. In addition to the adoption of novel biosensors, it is crucial to develop model-based strategies for making most of the existing sensor-derived data sets. There is a need for determining additional cost-effective metrics allowing for the definition of process efficiency and how model-based analysis of bioreactor processes could significantly contribute to this. Moreover, the identification of the risks associated with batch failure through model-based monitoring could also serve as an excellent tool for addressing important bottlenecks in the current field of stem cell bioprocessing, which is growing into a personalized biomanufacturing sector.

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