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Optimization of Insect Cell Based Protein Production Processes - Online Monitoring, Expression Systems, Scale-Up

**Damir Druzinec, Denise Salzig, Alexander Brix, Matthias Kraume, Andreas
Vilcinskas, Christian Kollewe and Peter Czermak**

Abstract Due to the increasing use of insect cell based expression systems in research and industrial recombinant protein production, the development of efficient and reproducible production processes remains a challenging task. In this context, the application of online monitoring techniques is intended to ensure high and reproducible product qualities already during the early phases of process development. In the following chapter, the most common transient and stable insect cell based expression systems are briefly introduced. Novel applications of insect cell based expression systems for the production of insect derived antimicrobial peptides/proteins (AMPs) are discussed using the example of *G. mellonella* derived gloverin. Suitable in situ sensor techniques for insect cell culture monitoring in disposable and common bioreactor systems are outlined with respect to optical and capacitive sensor concepts. Since scale-up of production processes is one of the most critical steps in process development, a conclusive overview is given about scale up aspects for industrial insect cell culture processes.

Keywords BEVS, *Drosophila* S2, In situ monitoring, Gloverin, FBRM, Scale up

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1 Introduction

Over the last decades, insect cells were more and more established for recombinant protein production. Insect expression systems were thereby used for both, basic research and large-scale commercial applications. One key factor for their popularity is their ability to produce large quantities of posttranslational modified eukaryotic proteins in a relatively short time period. A relatively new field of application is the production of insect derived antimicrobial peptides/proteins (AMPs), which are intended as novel therapeutics in medical applications. Further, the handling of insect cells is quite easy compared to mammalian cells which also produce functional posttranslational modified recombinant proteins. This means that insect cells grow in suspension at 28°C, are tolerant to osmolality and no carbon dioxide is needed for their cultivation [1]. The simplicity of insect cell cultivation makes them very attractive for recombinant protein production.

To exploit the maximum potential of insect cell cultivation, the production process has to be well controlled and monitored. With regard to PAT for pharmaceutical processes it became more and more important to ensure product quality by design and during the entire process. In this context, many online tools have been investigated. Some of them are still routinely used in industrial processes. Beyond the standard parameters temperature, pH and oxygen, the key parameters which have to be monitored are the viable insect cell biomass, the metabolic activity and product quantity.

Scale up of well-established processes to industrial scales is the final challenge for the production of recombinant proteins in insect cells. Therefore the specific cultivation parameters should be kept constant. Geometric similarity of the cultivation vessels in small and large scale eases the scale up procedure. Nowadays simulation tools as CFD help a lot in the understanding and scale up of processes even at different cultivation setups in small and large scales. Scale up of insect cell production is relatively rewarding since insect cells usually grow in suspension. The main point in scale up is to guarantee homogeneity to ensure sufficient access to nutrients. In the following chapter the main insect cell expression systems are introduced. Issues related to the application of insect cell expression systems for the production of *G. mellonella* derived gloverin, as an example for insect derived AMPs, are discussed. Modern online monitoring tools

are presented and valued for their application in insect cell processes. Finally, scale up considerations for BEVS based production systems are reflected.

2 Recombinant Protein Production in Insect Cells

The possibilities to produce recombinant proteins in insect cells are manifold. A multiplicity of expression systems, cell lines and vectors are available. Nevertheless the majority in industrial production uses the baculovirus expression system with great success. Recently stable expression systems became prominent for insect cells as well. Compared to mammalian cells or bacteria, the insect cells combine the advantages of relatively short production times and the ability to produce complex post-translational modified proteins.

2.1 Common Insect Cell Lines for Recombinant Protein Production

Insect cell culture began with the establishment of an insect cell line from the pupal ovarian tissue of the moth *Antheraea eucalypti* in 1962 [2]. In the meanwhile over 500 insect cell lines from several species and tissue sources have been established. These cells are widely used in research. However, the number of cell lines used and engineered for recombinant protein production in routine industrial processes is relatively low [3]. In the following, only the most common insect cell lines utilized for recombinant protein production are introduced.

2.1.1 Spodoptera frugiperda Derived Cells

Sf9 and Sf21 cell lines are traditional cell lines originally isolated from the pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda* [4,5]. The Sf9 cell line is thereby a clonal isolate of IPLBSF21-AE (Sf21 cells) [4]. Both cell lines are spherical with some granular appearance, whereas Sf9 cells are more regular in size. Reported doubling times for Sf21 and Sf9 cells vary between 26 to 30h and 24 to 31h, respectively [6]. Both cells grow well in monolayer and suspension and are adaptable to serum- and protein-free medium. They are both suitable for viral transfection, resulting in high virus stocks and expression of recombinant proteins. In some cases Sf21 cells may express more protein than Sf9 cells [7].

Functionality of proteins is often dependent on the right glycosylation pattern. Glycosylation in insect cells is different and less complex compared to mammalian cells. In general N-glycans of insect cell proteins mainly contain high

mannose or trimannose structures which are truncated and unsialylated (Fig. 1). This pauci-mannose-type is very ordinary for invertebrates. In contrast O-linked glycosylation of insects is similar to mammalian cells [8].

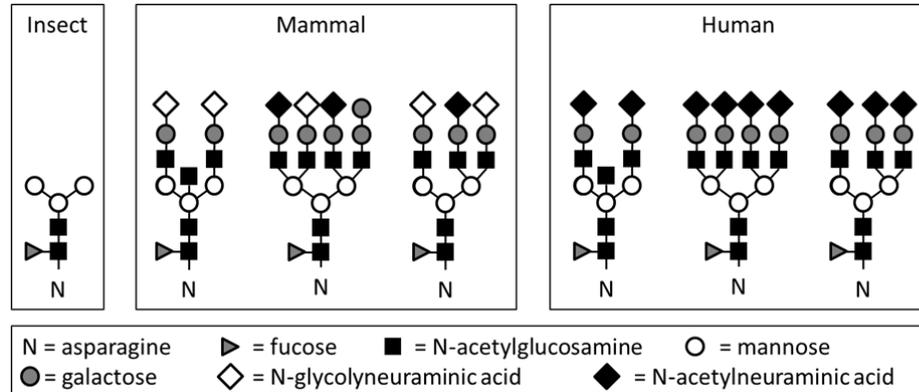


Fig. 1 Major differences between human, mammalian and insect glycosylation pattern.

To guarantee full functionality of recombinant insect-produced proteins, a special cell line was developed (SfSWT-1). These cells are transgenic Sf9 cells that have been engineered to produce recombinant proteins with terminally sialylated N-glycans like those found in mammalian systems [9]. Their doubling time and growth characteristics are identical to Sf9 cells except for the fact that serum is required for cell cultivation.

2.1.2 *Drosophila melanogaster* Derived Cells

The most common used *Drosophila melanogaster* cell line is the Schneider 2 cell line, usually abbreviated as S2 cells. These cells have been derived from a primary culture of late stage (20-24h) *Drosophila melanogaster* embryos [10]. The cell phenotype has many characteristics of cells from a macrophage-like lineage. The cells are spherical in shape and possess a granular cytoplasm. Similar to the *Spodoptera frugiperda* derived cell lines, S2 cells can grow as loose, semi-adherent monolayer in tissue flasks or suspension culture in dynamic systems, with doubling times around 24h. Optimum temperature is 28°C and CO₂ is not required for pH regulation during cultivation. Cultivation in serum-free and even protein-free medium is possible for these cells. Beside S2 cells, other *Drosophila melanogaster* cell lines such as S3 cells and a few derivatives of the Kc cell line were used for recombinant protein production [11].

2.1.3 Other Cells

The High Five or Tn5B1-4 cell line has been established from the ovarian cells of the cabbage looper, *Trichoplusia ni* [12]. A characteristic of this cell line is a population doubling time of less than 24h. The cells grow in adherent culture, but form irregular monolayers. Suspension culture and serum-free cultivation is possible. Compared to Sf9 cells, High five cells provide 5-10 fold higher secreted recombinant protein expression [13]. However, this high productivity may be more evident in low passage cells [12].

2.2 Expression Systems for Insect Cells

Insect cells can express recombinant proteins via transient and stable expression. In the case of insect cells, transient expression mostly means lytic expression where cells were infected with a lytic recombinant virus carrying the gene of interest. Stable expression in insect cells is similar to the stable expression in mammalian cells, with an insertion of the expression vector followed by a selection and screening of high producer clones. The following section focuses on the introduction of the most prominent insect cell expression systems.

2.2.1 Baculovirus Expression Vector System (BEVS)

Many types of viruses can infect insect cells. The most common ones belong to the family of *Baculoviridae*. The BEVS used today are based on the *Autographa californica* multiple nucleopolyhedrovirus (*AcMNPV*) which mainly infects *Lepidoptera* derived insect cells. Therefore, this type of virus is mainly used in combination with Sf- or Hi-5 cells. The expression of recombinant proteins in the BEVS is under control of a very late viral promoter, often the strong polyhedrin promoter [14] or the p10 promoter [15]. The p10 promoter has been shown to be activated earlier in the infection time course than the polyhedrin promoter, which results in an earlier initiation of the expression of some recombinant proteins. Several systems use the BEVS in order to express the foreign gene under control of an immediate-early promoter (IE), which is active in uninfected cells as well as infected cells during the early infection phase. These systems have shown to be superior for the expression of eukaryotic secretory glycoproteins with high activity [16].

An elegant way to create recombinant Baculoviruses (BV's) is the bacmid technology. A bacmid is a shuttle vector between *Escherichia coli* and BV's. It replicates in *E. coli* and generates the recombinant viral DNA via site-specific transposition. After that, insect cells are transfected by the bacmid in order to produce the recombinant BV's [17]. The advantage of this method is the short development time necessary to generate the recombinant viruses. In addition, helper viruses are not required. BV's are not harmful to humans and due to the late expression also cytotoxic proteins can be produced. Disadvantages of the BEVS are the lysis of cells following infection as well as the proteolysis of recombinant proteins. Furthermore, the infection is an extra process step which often results in difficulties in reproducibility and process stability dependent on the quality of the virus stock. The heterogeneity of the infection affects the glycosylation pattern as well. Perfusion mode can be hardly performed with this system [18].

2.2.2 Stable Expression System

Virus infected cells have a finite life span. Therefore, untreated cells need to be infected in order to produce a fresh batch of recombinant protein. To overcome this limitation, non-lytic expression systems have been developed for insect cells as well. The stable expression system of insect cells is similar to that of mammalian cells. An expression vector carrying the gene of interest is stable integrated in the insect genome. The expression and secretion of the recombinant proteins requires no lysis of the cells. Traditionally, S2 cells were used with this type of expression system, but also for Sf- and Hi-5 cells stable expression systems are available. A wide variety of vectors allows the expression of recombinant proteins in S2 cells. For induced expression, a vector with the metallothionein promoter is used. Thereby the expression of the recombinant protein is induced with copper sulfate [19]. Constitutive expression of recombinant proteins in S2 cells is often from the actin 5 promoter[20]. More global stable expression systems for insect cells use the baculovirus-derived immediate-early OpIE promoters. These promoters originally derived from the Baculovirus *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (*OpMNPV*) allow constitutive recombinant protein expression in a variety of cells types. These include Sf9, Sf21, Hi-5, S2, Kc and *Lymantria dispar* as well as mosquito

cell lines. Both *OpIE* promoters (*OpIE1* and *OpIE2*) provide relatively high levels of recombinant protein expression. Nevertheless, the *OpIE2* promoter has been shown to be up to 10-fold stronger than the *OpIE1* promoter[21].

The stable expression system has several advantages. It allows a homogenous glycolysation profile of the recombinant protein and guarantees a higher reproducibility and process stability. Almost every cultivation mode can be used for protein production with this expression system. Due to the missing cell lysis during recombinant protein production, the contamination with host cell proteins is very low. This is superior for protein purification and avoids proteolysis of the target protein. The main disadvantage of this system is its time intensity compared to the BEVS, since the establishment of stable cell lines is a time consuming venture [18].

2.3 Comparison of the BEVS with Stable Insect Expression Systems

Several studies compared the BEVS with stable insect expression systems. Expression of two different proteins (extracellular vascular cell adhesion protein [VCAM], trans-membrane dopamine D4 receptor) was compared with the Baculovirus/Sf9 system and stable transfected *Drosophila* S2 cells. VCAM was produced in both systems in similar amounts and appeared identical within the framework of the performed analytics. D4 was also expressed similarly in both systems [22]. The expression of several antibody fragments was evaluated in the BEVS and in stable transfected S2 cells. Reported maximum concentrations of the recombinant antibody fragments were 9 mg/L in the BEVS and 0.4 mg/L in S2 cells [23]. Production of recombinant human IL-7 in the BEVS and stable transfected Sf cells revealed in 10 times higher expression levels for the BEVS [24]. Up to now the investigations on recombinant protein production do not show BEVS being superior to the stable insect expression systems. It strongly depends on the protein which is intended to be produced. For the choice of the best expression system not only the quantity of the recombinant protein is crucial, but also its activity which results from the right post-translational modifications.

2.4 Application of Insect Cell Based Expression Systems for the Production of Insect Derived Antimicrobial Peptides/Proteins (AMPs)

The use of insect cell based expression systems for the production of recombinant insect derived peptides/proteins is a relatively new field and emerged more and more as an attractive alternative to other commonly utilized expression systems. Especially for the production of insect derived proteins with a potential antimicrobial or antifungal activity, the insect cell based production systems can be a promising tool in order to provide satisfactory product yields and the specific glycosylation patterns. The increasing demand for new AMPs is even more emphasized due to the increasing number of human pathogens showing resistance against available antibiotics. AMPs as novel anti-infective therapeutics could be an alternative to fight multiresistant bacteria strains. The lepidopteran model host *G. mellonella* has recently been reported as an attractive source for various AMPs such as gloverin, cecropin or the insect metalloproteinase inhibitor (IMPI) [25]. Gloverins are glycine-rich and heat stable basic proteins which primarily exhibit activity against gram-negative bacteria such as *E. coli*. [26-29]. However, also activity against gram-positive bacteria or fungi has been reported in the literature [30,31]. The BEVS based expression of a *G. mellonella* derived gloverin, which has been fused to a green fluorescent protein for better protein detection (*GmGlv*-GFP), represents a good example on how the protein itself might influence the production process. Figure 2 depicts an exemplary production of *GmGlv*-GFP in a 3 L stirred tank bioreactor. Once the uninfected Sf21 insect cell culture has been diluted to approximately 3×10^6 cells mL⁻¹ at 48 h cultivation time and simultaneously infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 0.1, the cell density and permittivity based biomass (see chapter 3.8: dielectric spectroscopy) revealed the typical time course of a secondary infection dependent kinetic. The strong increase in biomass signal within the first 24 h post infection includes the typical swelling of Sf cells following baculovirus infection as well as cell growth.

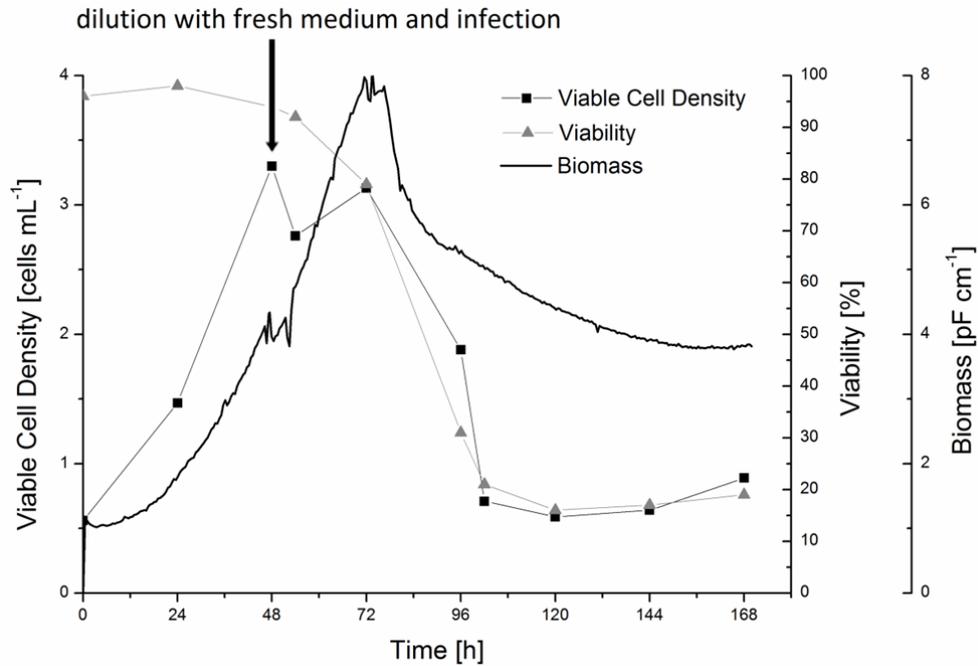


Fig. 2 Exemplary production process for *GmGlv-GFP* in a 3 L bioreactor system using the BEVS with Sf21 insect cells, TOI $\sim 3 \times 10^6$ cells mL⁻¹, MOI = 0.1, cultivation temperature: 28 °C, dissolved oxygen concentration (dO₂): 40 % of air saturation, utilized medium: Sf-900 II SFM, the biomass signal is based on permittivity measurements via dielectric spectroscopy (see chapter 3.8), viable cell densities were determined via trypan blue exclusion method

After approximately 72 h cultivation time the biomass signal and cell density decreased as a result of cell lysis. A comparison of the cell death kinetic during *GmGlv-GFP* production with the time course of cell death during expression of only GFP reveals a much faster dying of the cells for the *GmGlv-GFP* production process (Fig 3). For both baculovirus based production processes the same cell system (Sf21), the same virus (AcMNPV) as well as the same 3 L bioreactor system have been utilized.

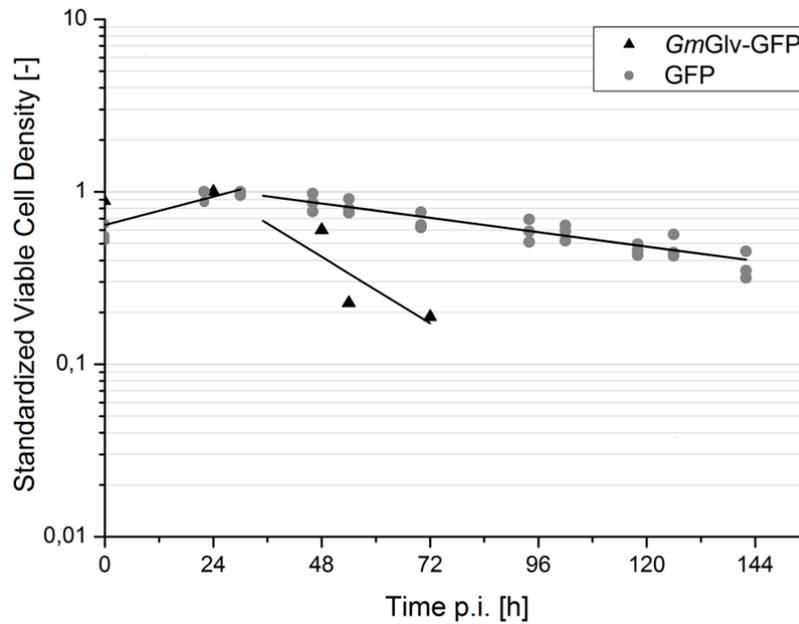


Fig. 3 Time courses for the standardized viable cell densities of BEVS based production processes for GmGlv-GFP and GFP in a 3 L bioreactor system, insect cell system: Sf21, baculovirus vector: AcMNPV, MOI = 0.1, TOI = $1 - 3 \times 10^6$ cells mL⁻¹, dissolved oxygen concentration (dO₂): 40 % of air saturation, utilized medium: Sf-900 II SFM, viable cell densities were determined via trypan blue exclusion method

Obviously the baculovirus induced *GmGlv*-GFP production in Sf21 insect cells leads to distinctive cell stress, which finally results in a faster dying of the culture. Moreover, Moreno-Habel et al. [32] recently found that *Menduca sexta* derived gloverin (*MsGlv*) leads to inactivation of budded baculoviruses (AcMNPV) by disrupting the viral envelop. An inactivation of budded baculoviruses affects infection kinetics and could lead to falsified infection doses as well as increased process variability. This finally can affect product titers. Typical product concentrations observed for *GmGlv*-GFP produced in the BEVS range from 5 to 20 mg/L, which is quite low when compared to the theoretical productivity of the system. Gloverin as example shows that the production of insect derived molecules in insect cells can be very challenging. On the one side the production of these molecules in insect cells is a logical consequence as it is very likely to produce an active molecule with 100% correct folding and modifications. On the other side it is possible that the recombinant molecule influences the production system either in a positive or, as observed for gloverin, in a negative manner. In this special case it has to be evaluated if gloverin only influences the baculovirus

and sufficient product yields can be reached with the *Drosophila* S2 System which works independent from viruses.

3 Online Monitoring

Modern bioprocesses demand for high efficiency, productivity and reproducibility in order to reduce costs and to ensure a satisfactory final product quality already during the ongoing production process. For that purpose, the Food and Drug Administration (FDA) launched the process analytical technology (PAT) initiative which is intended to achieve these goals by implementing modern process monitoring tools for critical process parameters during all stages of the production processes. Particularly in cell culture processes, small changes of critical parameters such as temperature, hydrodynamic conditions, pH, nutrient supply or dissolved oxygen concentration can lead to alternations in cell metabolism and finally influence product yield and quality [33]. Moreover, from the industrial point of view low process efficiencies and productivities can even result in unprofitability of processes [34]. Biopharmaceutical manufacturing processes still suffer from performances far below their theoretical potential since improvement is a time and cost consuming venture once the existing process has been approved by the regulatory authorities. Thus, real-time process monitoring of critical process parameters can allow for product definition already during the manufacturing process in order to avoid losses of production batches due to quality issues [35,36]. Additionally, there is a growing need for bioprocess monitoring in disposable bioreactors which has been driven by the increasing demand for single use technologies in the biopharmaceutical industry [37]. Up to now, the connectivity of PAT-devices to disposable bioreactors is one of the major drawbacks [38].

In modern bioprocesses, monitoring of various parameters such as temperature, pH or dissolved oxygen concentration via common electrodes is already well established. However, there is still a strong demand for monitoring various other parameters of interest e.g. medium components, product concentration as well as cell density and viability, which allow a deeper understanding and insight into manufacturing processes [39]. Despite the increasing demand for a high and consistent product quality in the biopharmaceutical industry and the high potential for process improvement, available in situ monitoring techniques have been rarely

established for insect cell culture processes. Due to the similarity of insect cell systems to other eukaryotic production systems, this contribution also includes application examples for various other animal as well as mammalian cell culture processes. Related in situ sensor concepts for the most common optical and capacitive monitoring techniques are introduced as well as their potential in monitoring cell culture processes and, if applicable, insect cell processes.

3.1 In Situ Analysis and its Requirements

Bioprocesses are usually sampled in order to gain information about parameters that may have a significant impact on culture performance. This finally facilitates a fundamental understanding and allows for process control in order to reach effective processing [38]. For instance, the concentrations of key substrates and, with respect to BV infected insect cell cultures, the time of infection (TOI) and time of harvest (TOH) are well known to influence product yield [40]. Gaining information about these parameters usually requires sampling from the bioreactor for offline analysis. However, major drawbacks of this approach are the time delay between sampling and analysis as well as the risk of contaminations [39]. In order to avoid these concerns, an in situ monitoring system can be applied to facilitate real time measurement directly in the bioreactor or in a bypass [41]. Bypass configurations are particularly applied in systems where, for instance, gas bubbles in the bioreactor would interfere the measurement signal [38]. However, for bypass-measurements it needs to be considered that within the bypass changes in state of the medium e.g. temperature, dissolved oxygen concentration, can cause falsified results when compared to measurements performed directly in the bioreactor [34]. Another possibility is to utilize the sensors externally (ex situ). In this case, a sterile sample removal system forwards the medium directly to the sensor (on line) [38,42]. Since in situ sensors are placed directly in the sterile environment of the bioreactor, they have to fulfill special requirements. In situ sensors need to be autoclavable and should provide a stable and reliable signal over an extended period of time. This is especially the case when in situ sensors are applied for cell culture monitoring purposes since these processes are usually conducted over several days. A calibration previously performed has to be stable even after a sterilization cycle. Alternatively, there should be an option to calibrate the sensor after sterilization without sacrificing sterility of the system

[38]. Moreover, disposable in situ sensors for single-use applications have to fulfill additional requirements. These sensors should be cheap enough to ensure economic efficiency, however, a long life time is not necessary. Technologies that could be mentioned in this context are semiconductor devices such as pH-ion-sensitive field-effect transistors (IS-FETs). These devices, for instance, can be installed directly into the cultivation medium to facilitate pH, temperature and dissolved oxygen analysis. Alternatively, it is possible to use non-disposable optical sensors in combination with transparent observation windows. So far, continuous sterile sampling devices for single-use applications which facilitate on line analysis via ex situ measurements are not commercially available [42]. Generally, the choice of which process analytical technology is necessary to monitor and control the bioprocess is also dependent on the employed biological system. While prokaryotic systems provide a fast increase in cell density, which comes along with a fast metabolic activity, eukaryotic systems only exhibit slower growth rates and, consequently, a slower metabolic activity. Therefore, eukaryotic cultures require a lower recording frequency and analysis time for the sensor signal compared to prokaryotic systems [38,34].

3.2 Infrared Spectroscopy

Spectroscopic methods such as the near infrared (NIR) spectroscopy and mid infrared (MIR) spectroscopy offer the advantage of monitoring various critical process parameters simultaneously. The function principle is based on the absorbance of energy and the resulting molecular vibrations and rotations once the related molecules have been excited at specific wavelengths. This energy absorption is finally identified and provides information about the molecules of interest [43]. The spectral signatures of organic molecules are thereby classified into three categories based on specific spectroscopic wave number ranges: far infrared ($10\text{-}200\text{ cm}^{-1}$), MIR ($200\text{-}4000\text{ cm}^{-1}$), and NIR ($4000\text{-}13000\text{ cm}^{-1}$). Compared to the NIR signal, the MIR signal provides more defined peaks in the absorption spectra which facilitates a better assignment of molecules [39]. Especially the “fingerprint region” ($500\text{-}1500\text{ cm}^{-1}$) in the MIR spectra allows for very specific identification of organic molecules since this region exhibits the most specific absorption patterns [38]. However, the applicability of this technique is limited due to the strong infrared absorption of water below wave

numbers of 4000 cm^{-1} . NIR spectroscopy has been reported to be a suitable tool for monitoring of substrates and products in biotechnology [38,44]. Hydrogen bonds which exhibit distinctive stretch vibrations between 3600 and 2400 cm^{-1} allow for the detection of molecules that contain hydrogen bound to a heteroatom. Further potential analytes are proteins with their N-H bonds as well as O-H bonds of alcohols and C-H bonds of aliphates and aromates [39]. The possibility of utilizing IR spectroscopy for in situ applications in bioprocess monitoring was facilitated by the development of attenuated total reflectance (ATR) probes. A description of the measuring principle of ATR-IR spectroscopy is given by Lindner et al. [42]. Despite the fact that ATR-IR probes are commercially available, an application of the ATR-IR technology to disposable reactor systems remains a cost intensive venture since the expensive ATR crystals would have to be replaced after each cultivation process [42]. Furthermore, signal interpretation of multidimensional IR spectroscopic data is not straightforward and requires chemometric techniques. These techniques usually include data reduction via principal component analysis (PCA) as well as the development of calibration models via multivariate regression methods such as principal component regression (PCR) or partial least square regression (PLS). Also artificial neural networks (ANN) can be applied, especially when the correlation between the spectral data and target variables exhibit distinctive non linearity [36]. Various authors utilized IR spectroscopic techniques in cell culture applications. The most applications of NIR and MIR spectroscopy have been performed to monitor glucose, lactate as well as glutamine and ammonia in Chinese hamster ovary (CHO) cell culture processes [45-47]. Beside glucose, lactate and ammonia, Henriques et al. [48] also employed NIR spectroscopy to determine cell density in mammalian cell culture processes for monoclonal antibody production. Sellick et al. [49] simultaneously predicted glucose, lactate as well as monoclonal antibody concentrations in supernatants of CHO and murine myeloma (NS0) cell cultures via fourier-transform (FT)-MIR spectroscopy. An application of NIR spectroscopy for in situ monitoring of glucose and lactate during bioreactor cultures of adherent Vero cells attached to microcarriers is reported by Petiot et al. [50]. Only Riley et al. [51] applied off-line NIR spectroscopy to monitor the glucose and glutamine concentrations during growth of Sf-9 insect cells cultivated in Sf-900 II serum-free media. Despite the complexity of the culture media,

glutamine and glucose concentrations could be predicted with standard errors of 0.51 and 1.46 mM, respectively, indicating the feasibility of IR-spectroscopic techniques in monitoring insect cell culture processes.

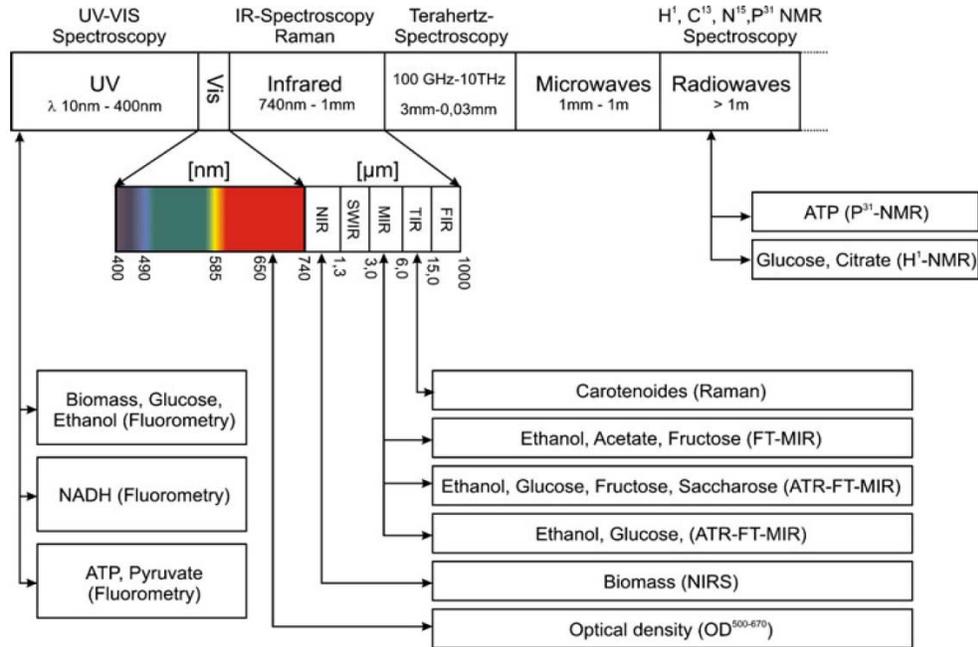


Fig. 4 Electromagnetic wavelength spectrum and the corresponding wave length ranges for various optical and spectroscopic sensor techniques (Beutel et al. [38]; with kind permission from Springer Science and Business Media)

3.3 Fluorometry

Direct measurement of fluorescence in an in situ application represents a promising tool for bioprocess optimization since various biologically active substances such as amino acids, enzymes, cofactors and vitamins exhibit fluorescent activity when excited at specific wave lengths. First applications of fluorometry for monitoring purposes was limited to only one pair of excitation and emission wavelength, and thus, to a single fluorophore [36]. In these applications, the nicotinamide adenine dinucleotide NADH and its phosphorylated form NADPH has been the fluorophore of choice since the intracellular NAD(P)H concentrations could be utilized to estimate biomass concentrations [52]. However, major drawbacks of this technology are the dependency of the fluorescence signal to environmental conditions such as pH or temperature as well as the possibility for overlapping signals caused by the presence of other medium

components fluorescing at the same wavelengths as the component of interest [53,34,36]. Further concerns that have been described to decrease fluorescence yield are inner filter effects, cascade effects and quenching phenomena [54,55,36]. Therefore, multivariate chemometric techniques are necessary to enable reliable data interpretation. In contrast to the single wavelength fluorometers, 2D fluorometers allow the detection of multiple fluorophores at once. The principle is based on the application of various excitation and emission wavelengths resulting in a complete fluorescence spectrum [38,36,34]. However, fluorometry has only scarcely been applied in monitoring eukaryotic cell culture processes. A possible reason might be the complexity of the culture media. In mammalian culture media, for instance, various fluorescent amino acids need to be provided since mammalian cells are not capable of synthesizing these amino acids on their own [56]. Once these amino acids are incorporated into recombinant or cellular proteins, their fluorescent properties might change. For instance, electrostatic interactions of tryptophan with neighbored charged amino acid residues of e.g. glutamate, lysine or aspartate, can lead to a significant shift in the emission wavelength of tryptophan [57]. This and other phenomena, such as quenching effects, make direct correlations between the growth of mammalian cells and fluorescence signals a difficult task [36]. Anders et al. [58] utilized in situ fluorometry to measure the NAD(P)H dependent culture fluorescence in uninfected and infected Sf-9 insect cell cultures. The fluorescence signal correlated well with the total cell density. A decrease in the fluorescence signal could be detected as a result of the decrease in viable cell density due to nutrient depletion. Comparable experimental results were obtained in serum containing Grace and TC – 100 insect cell medium as well as serum free Ex – Cell 401. In mammalian myeloma NSO cell culture, Hisinger et al. [59] demonstrated the use of 2D fluorescence spectroscopy for monitoring the GFP concentration during a GFP production process. Teixeira et al. [56,60] applied traditional 2D fluorescence spectroscopy as well as synchronous fluorescence spectroscopy for monitoring the production of recombinant glycoprotein IgG1-IL2 and monoclonal antibody IgG4 in Baby Hamster Kidney (BHK) and CHO cell culture processes, respectively. Besides product formation, they also demonstrated the suitability of these techniques for monitoring viable cell densities. Especially the synchronous fluorescence spectroscopy provides the potential for bioprocess monitoring in a

real-time context since this technique exhibits improved peak resolution and recording speed [60].

3.4 Raman Spectroscopy

The principle of Raman spectroscopy is based on shifted wavelength scattering of molecules due to inelastic collisions of photons with the molecules once excited with monochromatic light. Bioprocess applications of Raman spectroscopy are feasible since adjustable lasers are available. The technique can be applied for multi-analyte measurements as well as differentiated measurements of certain compounds [61]. Resulting molecular fingerprints are well defined with high chemical specificity even in aqueous systems [62]. Despite these advantages, Raman spectroscopy has not been extensively utilized for in situ applications in cell culture processes, which might be related to the difficulties arising with the use of this technique. One of the main problems is the considerable fluorescence activity of several biological molecules that might overlay the Raman scattering bands [60,61,63]. This is even more a problem when the fluorescent compounds cannot be eliminated, as is the case for culture broths. A common approach to overcome this issue is the use of wavelengths outside the excitation range of the fluorescing molecules. Another problem arising from the use of charge-coupled device (CCD) detectors, which might exhibit pixel-to-pixel sensitivity, is the appearance of stable variations. Magnitudes of these variations can even exceed magnitudes of the measured signals [38]. Especially in bioprocess applications, bubbles from aeration and biomass can cause light scattering which results in signal attenuation. In this case, internal referencing can be utilized for correction purposes [61]. The high potential of using Raman spectroscopy in monitoring of cell culture processes was successfully demonstrated by Abu-Absi et al. [62]. In this work, Raman spectroscopy was employed to monitor glucose, glutamine, glutamate, lactate, ammonium, as well as the viable and total cell density in 500 L CHO cell culture processes. Chemometric techniques have been applied to the training datasets in order to obtain the required calibration models. Especially the possibility of monitoring the cell viability, which can be calculated from the predicted viable and total cell densities, represents an additional advantage.

3.5 Optical Chemosensors for O₂, pH and CO₂ Determination

The application of optical chemosensors (optodes) facilitates measurements of parameters not accessible via direct measurements. Measuring principles of these sensors are based on indicators with optical properties such as photoluminescence, reflection and absorption. Available concepts are suitable for common bioreactors as well as disposable systems. The externally placed optical detector unit can be interfaced to the transducer via optical fibers [42]. O₂, CO₂ and pH can be measured in a non-invasive manner from outside the bioreactor via transparent observation window. Expendable sensor patches, containing the immobilized indicator, are thereby placed inside the bioreactors. Alternatively, invasive measurements are possible via probes immersed in the culture broth and equipped with an optical window and indicator spot at the tip. This set up allows the application of optodes even in stainless steel bioreactors without optical windows. Compared to the classical electrodes, optical chemosensors represent a promising alternative for conventional bioreactors as well as disposable systems and can easily be implemented in cell culture processes. For instance, optical oxygen sensors have been successfully applied for oxygen monitoring in human mesenchymal stem cell cultures in fixed bed bioreactor systems [64-69].

3.5.1 Optical O₂ Sensors

Optical oxygen sensors are based on fluorescence quenching by molecular oxygen [70,71]. After excitation with light e.g. by a light emitting diode (LED), the lifetime and intensity of a fluorescence signal is proportional to the oxygen concentration present around the dye. Emitted fluorescence light is transmitted via the optical fiber and segregated from the reflected excitation light by a dichroic mirror. Subsequent fluorescence detection is achieved by a photodiode or photomultiplier. Optical oxygen sensors are autoclavable without loss of sensitivity [42]. Compared to conventional Clark electrodes, commercially available O₂ optodes (e.g. available at PreSens, Regensburg, Germany / Ocean Optics Inc., Dunedin, FL, USA) are calibration-free and do not require polarization time or replacement of the electrolyte solution prior to use. A major drawback that needs to be mentioned is the limitation of long-term stability caused by photobleaching [42]. Therefore, the sensor patches need to be replaced on a regular basis.

3.5.2 Optical CO₂ Sensors

CO₂ sensors have been based on potentiometric function principles such as the Severinghouse electrodes [72]. These sensors are equipped with a pH and a reference electrode surrounded by a hydrophobic CO₂ permeable membrane. The electrodes are thereby placed within a carbonate buffer. When CO₂ concentration outside the permeable membrane increases, CO₂ molecules diffuse into the carbonate buffer and change the pH value, which finally can be described by the Henderson-Hasselbalch equation. Fiber-optic CO₂ optodes are based on a similar pH-sensing system, where the electrodes are replaced by a pH-sensitive dye [73]. Compared to these classical CO₂ optodes, the development of solid type optical CO₂ sensors, with ion-pairs directly immobilized in the membrane, resulted in faster response times as well as a reduced sensitivity to ionic strength [74]. Determination of CO₂ via optical sensors can either be based on absorbance or fluorescence. Intensity based quantification methods have been the preferred ones since problems associated with the short life time of the fluorescence signal increased the need for sophisticated instrumentation. A method to overcome this drawback is based on the conversion of the intensity signal or the luminescence decay time of a pH-sensitive dye into a long-lifetime signal via resonance energy transfer (RET) [73].

3.5.3 Optical pH Sensors

Measurements of the pH via optical sensors can either be based on absorbance or fluorescence indicator dyes [75]. Today, pH can be measured in a range between pH 1 – 11 [76]. Also the cross-sensitivity to ionic strength for fluorescence based pH sensors, which has been reported as one of the drawbacks [77], could be reduced by the use of esterified fluorescein derivatives [78]. Covalently bound fluorescent dyes are suitable to decrease the loss of sensitivity due to elution of the dye or temperature treatment during autoclaving [79].

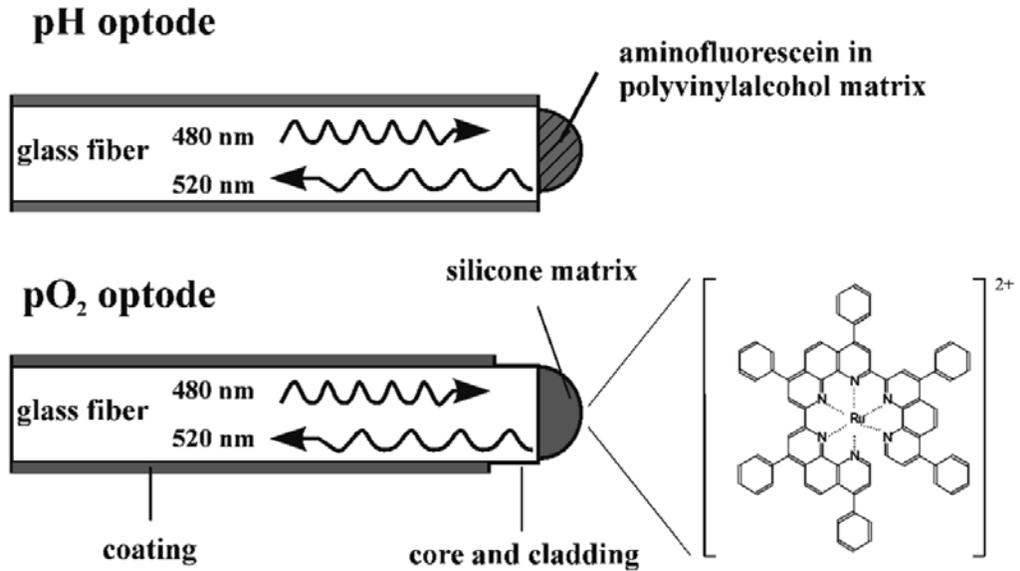


Fig. 5 Working principle of optical pH and pO₂ chemosensors (Glindkamp et al. [80]; with kind permission from Springer Science and Business Media)

3.6 In Situ Microscopy

In situ microscopy (ISM) is a promising tool for bioprocess monitoring in cell culture applications since the technology offers real time information about various parameters such as cell concentration, cell size distribution or cell morphology. The probe is thereby directly immersed in the culture broth. Images taken by the CCD-camera are analyzed by sophisticated image analysis algorithms. Generally, in situ microscopes can be subdivided into two different groups, the incident light microscopes with an optically defined analysis zone and the transmitted light microscopes containing an optically or mechanically defined analysis zone [81,82]. However, in the studies published so far only the transmitted light in situ microscopes have been applied for cell culture monitoring purposes. A transmitted light in situ microscope that meets the demands for industrial applications was described by Frerichs and Jöris in 2002 [83,84].

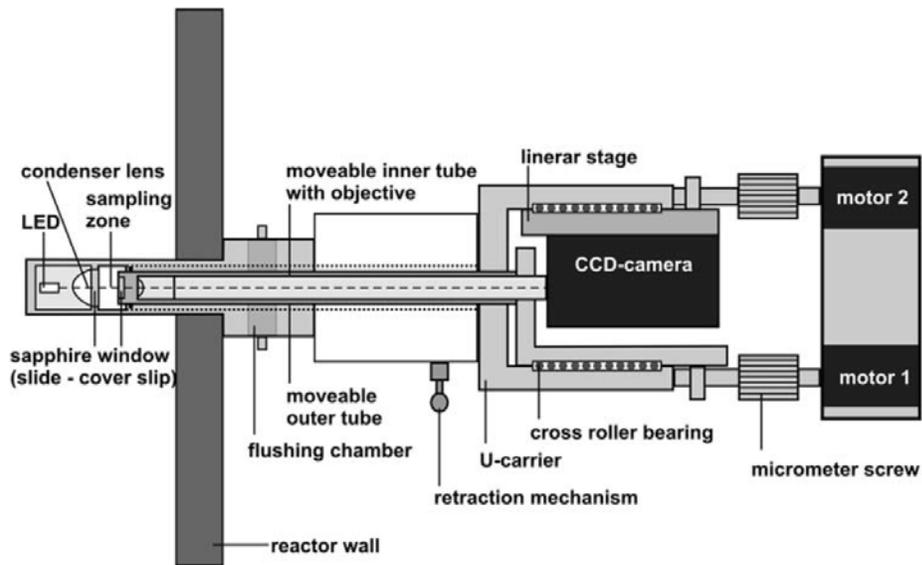


Fig. 6 Construction scheme of an *in situ* microscope developed by Frerichs and Joeris (Höpfner et al. [81]; with kind permission from Springer Science and Business Media)

In order to allow the adaption to changing process conditions, this microscope contains a mechanical defined variable-volume flow-through sampling zone. The sampling volume can be adjusted by a sampling zone tube which is connected to a movable slide. Beside the sampling zone tube, the microscope also contains two additional tubes, an outer probe tube as well as an inner objective tube. Proper focusing of the image is facilitated by the inner objective tube mounted to a second movable slide. Both slides are connected to a U-shaped profile and can be controlled by two separate stepper motors or micrometer screws. Illumination of the sampling zone via LED provides sufficient light for the visualization of cells by the CCD-camera. Since the reactor segment of the microscope can be separated from the optical segment, the sensor can be sterilized in the autoclave. The microscope has been employed for the monitoring of CHO and BHK cell cultures as well as adherent mouse fibroblast cells (NIH-3T3) on microcarriers [83-85,81,86]. In order to recognize cells attached to the surface of microcarriers, the optical density of the microcarriers turned out to be an important parameter. Cytodex 1 microcarriers were found to be suitable for microscopic cell observations and image analysis due to the low optical density of the polydextrin matrix. Especially the reported change in the greyscale distribution between microcarriers without cells and overgrown with cells was assumed to be applicable for an estimation of cell densities [85]. In this context, Rudolph et al.

[86] demonstrated the suitability of different greyscale distributions in order to determine the planting efficiency and level of colonization during cultivation of NIH-3T3 cells on Cytodex 1 microcarriers.

The application of a transmitted light in situ microscope with optically defined sampling zone utilized to monitor BALB/c hybridoma cells was presented by Guez et al. [87]. The microscope described in this study is based on a pulsed illumination of the sampling zone via LED, which is synchronized with the image generation of the CCD-camera. This setup facilitates image generation of moving cells without motion blur. The LED is thereby mounted to an outer tube angularly to an optical quartz window. In order to facilitate proper focusing, the microscope contains a movable inner tube equipped with a 40 x magnification objective. Since the microscope does not enclose a defined sample volume mechanically, information about the cell concentration is derived from a virtually defined volume (*depth from focus* – procedure [88]).

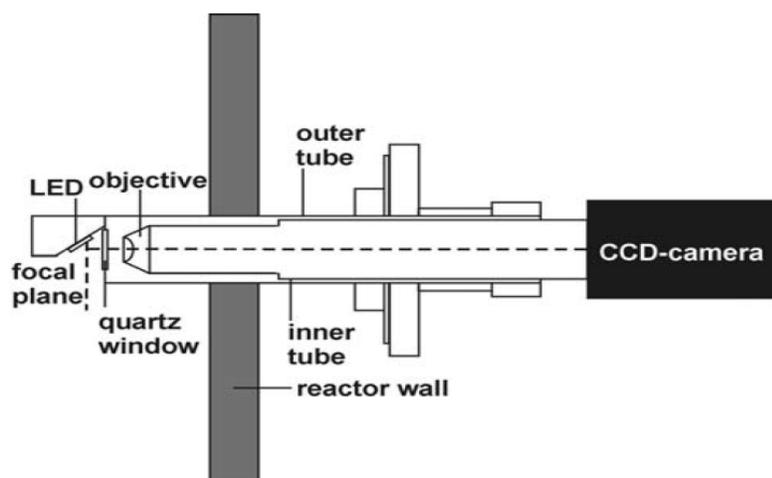


Fig. 7 Construction scheme of a transmitted light in situ microscope equipped with an optically defined sampling zone (Höpfner et al. [81]; with kind permission from Springer Science and Business Media)

Beside information regarding cell density, cell size distribution and cell morphology, the development of an in situ dark field microscope also facilitates measurement of the cell viability. The dark field microscope developed by Wei et al. [89,90] is based on the transmitted light in situ microscope described by Frerichs and Jöris in 2002 [83,84]. For the purpose of dark field microscopy, the illumination of the original microscope has been modified. The original condenser

was replaced by a dark field condenser. The application of Support Vector Machine (SVM) classifiers, which have been trained by a dataset of images with either living or dead cells, facilitates an automated determination of cell densities and viabilities. So far, the technology was only applied for fermentations of *Saccharomyces cerevisiae*, but also represents a promising method in cell culture applications. Another important point is the applicability of in situ microscopy in disposable systems. Up to know the use of ISM in disposable systems is not examined, but different approaches are already envisioned in the literature [42].

3.7 Focused Beam Reflectance Measurement (FBRM)

The FBRM technology as an in situ monitoring tool for cell culture processes offers the potential to gain real-time information about various important process variables such as cell density, cell size distribution as well as cell morphology. Inside the FBRM system, a laser light generated by a laser diode is passed to the probe assembly via fiber optics. The probe, which can be immersed in the culture broth, contains optical components that transfer the incoming laser light to an eccentrically rotating lens. The rotating lens finally facilitates a constant circular movement of the laser beam (2 m/s) as well as a focusing of the laser light to a small spot. As the laser beam intersects particles passing through the measurement zone, light is backscattered towards the probe. The backscattered light is then collected by the optics and forwarded to a photo diode, where the optical signal gets detected [91].

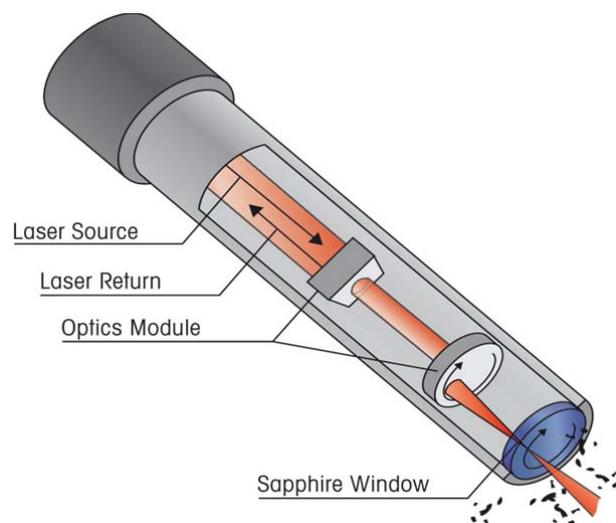


Fig. 8 Layout of a FBRM probe (with kind permission from Mettler - Toledo)

The duration of reflection multiplied by the rotation velocity of the laser beam finally results in a chord length, which considerably depends on the particle size as well as the particle shape. Compared to the rotation velocity of the laser, the velocity of the particles passing through the measurement zone can be neglected. The measurement range is 1 – 1000 μm and the measured particles are sorted into different channels according to measured chord lengths, resulting in a characteristic chord length distribution. Thus, the technique does not deliver the particle size distribution directly. Empirical or theoretical methods are applicable in order to determine the particle sizes from the chord length data [92-94]. Although the probe has already been utilized for offline characterization of microbial [95-98] and plant cell systems [99-101], applications in cell culture processes are not yet reported. The latest version of the G400 FBRM system distributed by Mettler-Toledo is fully autoclavable and therefore allows the integration of this technology even in cell culture processes. As indicated in figure 9, the system provides reliable information regarding the exponential growth of *Drosophila* S2 insect cells cultivated in a sparged and agitated 1L bioreactor system. Air bubbles generated by the O-shaped sparger did not interfere with the FBRM cell counts. The measured amount of cell counts per second in the chord length range of 10-50 μm exhibited a linear correlation with the offline total cell densities and reflected the exponential growth of the cells. Further research needs to be done in order to examine whether this technology can be utilized to follow the changing mean cell diameter after baculoviral infection of insect cells as observed for the *Spodoptera frugiperda* derived cell lines Sf9 and Sf21. In this case, the change in mean cell diameter would result in a corresponding variation of the mean chord length. As a result, the mean chord length signal could be utilized to evaluate a successful infection of the cells.

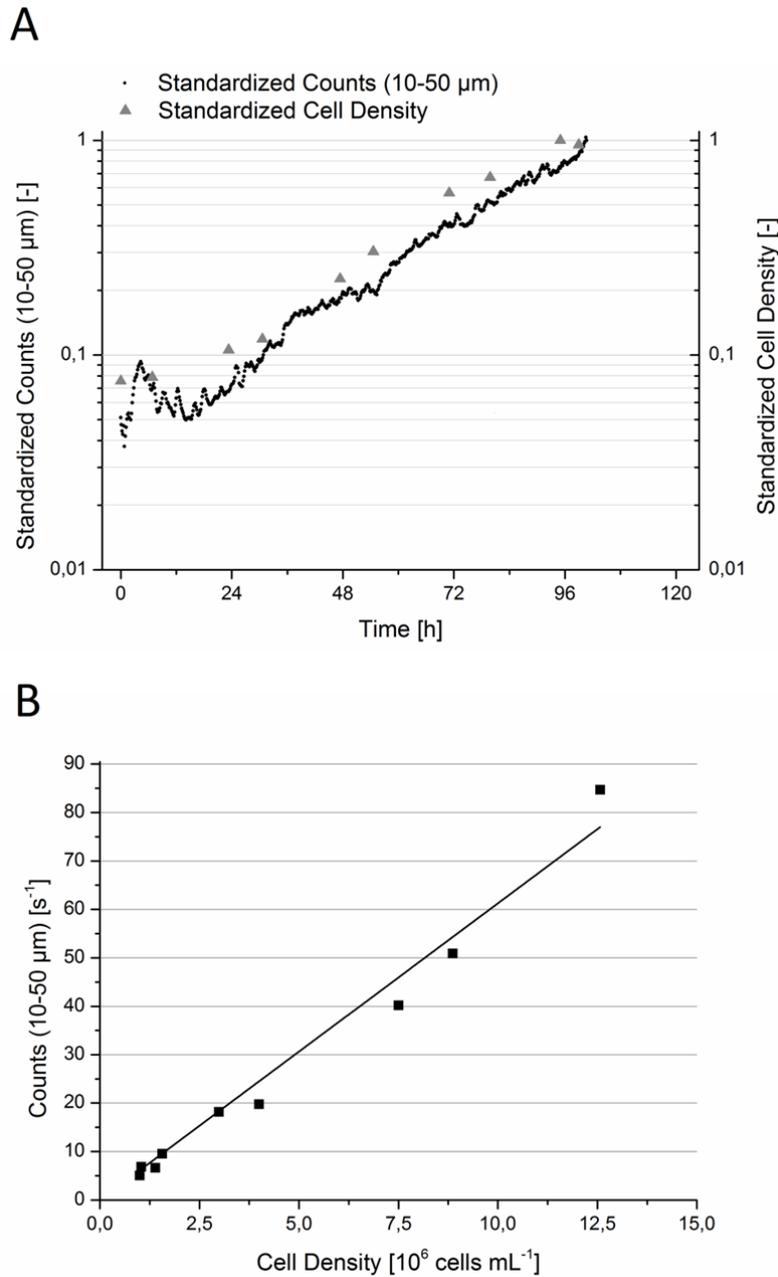


Fig. 9 A) Standardized FBRM counts in the range of 10-50 μm and the corresponding standardized offline cell density for a *Drosophila* S2 insect cell culture process; B) Linear correlation between the FBRM counts (10-50 μm) and the offline total cell density for a *Drosophila* S2 insect cell culture process

3.8 Dielectric Spectroscopy

The so called dielectric or impedance spectroscopy sensors belong to the group of capacitance and conductivity sensors. The technology is based on the passive

dielectric properties of e.g. cells in a conducting medium. Beside the surrounding culture medium, the cytoplasm of the cells also contains conductive solutes such as salts and nutrients. Due to the dielectric properties of the lipid – based cell membrane, electrically charged ions accumulate at these cell membranes when an alternating electric field is applied to the cell suspension. In this case, the cell membranes act as small capacitors leading to a buildup of electrical charge (polarization). The measured overall capacitance is thereby dependent on the applied frequency of the alternating electric field, which is usually in the range between 0.1 and 10 MHz, as well as the cell size and cell concentration. At low electric field frequencies the ions have sufficient time to reach and polarize the cell membranes leading to a high overall capacitance of the cell suspension. With increasing excitation frequencies the polarization and thus the capacitance of the cell membranes decreases. In this case, the ions do not have enough time to move and accumulate at the cell membranes before the electric field changes direction. The observed drop in the measured capacitance from low to high frequencies exhibits a sigmoid shape which is also known as the β -dispersion spectrum [102]. As indicated in figure 10, the β -dispersion contains the critical frequency f_c which represents the working point for impedance sensors in the frequency range mentioned above. Since the polarization is dependent on the ability of the cell membranes to stop ion movement, only cells with intact cell membranes are involved in the buildup of the capacitance signal. Thus, dead or leaking cells are not captured by this technology [103]. Additionally, the capacitance signal is reported to be insensitive to gas bubbles and microcarriers facilitating the monitoring of cell culture processes in common sparged as well as fixed bed bioreactors [102,104,105]. However, very high volume fractions of non-biomass materials close to the sensor may influence the capacitance signal since the polarizable cells are replaced by non-polarizable materials [102].

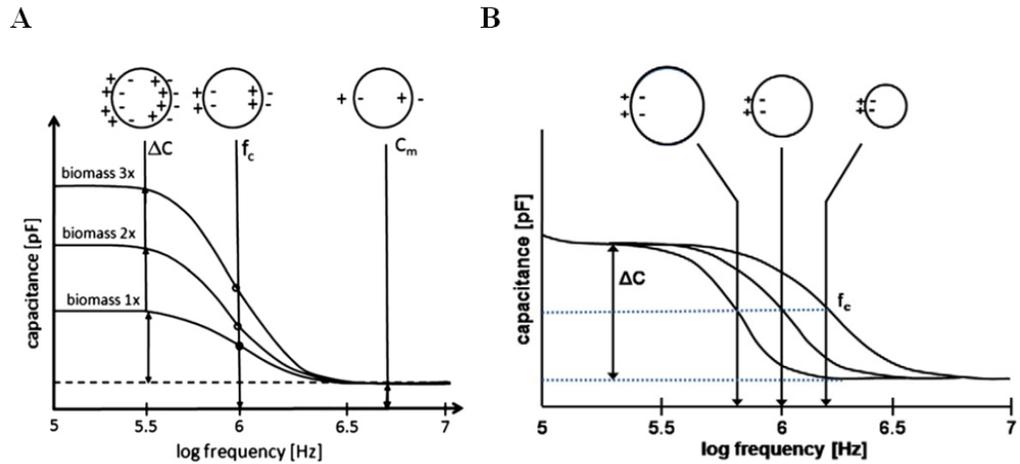


Fig. 10 A) β -dispersion spectra for increasing cell densities with the capacitance ΔC , critical frequency f_c , and medium capacitance C_m ; B) β -dispersion spectra for changing cell sizes, the indicated variation in the critical frequency f_c is valid for a constant biovolume (Cannizzaro et al. [106]; with kind permission from John Wiley and Sons)

The increasing demand for disposable cultivation systems emphasized the development of compatible sensors. Today, several solutions for the application of dielectric spectroscopy in single use bioreactors are available from the manufacturers (Aber Instruments, Aberystwyth, UK and Fogale nanotech, Nimes, France) [107,42].

Dielectric spectroscopy offers a great potential in monitoring insect cell culture processes, especially the baculovirus related production processes, since the arrest of cell growth after infection as well as the swelling and lysis of infected cells can be monitored in real-time. The first online monitoring of infected and uninfected Sf9 insect cell cultures using dielectric spectroscopy was reported by Zeiser et al. [108]. The obtained results for uninfected insect cells indicated a linear correlation between the relative permittivity and the viable cell density during growth. After synchronous infection with a recombinant baculovirus encoding for β -galactosidase (MOI = 10), which resulted in an arrest of cell growth, the permittivity signal further increased as a result of the increasing cell size. Finally, the signal coincided well with the decrease in cell viability and size during cell lysis in the late infection phase. In order to enhance the β -galactosidase yield, the same work group focused on infection and feeding strategies and demonstrated the use of dielectric spectroscopy for monitoring high cell density cultivations of High-5 [109] as well as Sf9 insect cells [109,110]. In either case, physiological

parameters correlated well with the impedance signal. An observed peak in the CO₂ evolution rate during Sf9 cultivation could be related to a temporary plateau in the relative permittivity signal, reflecting the onset of the release of virus particles into the culture broth. However, this signal plateau has not been detected in the case of lower MOI infections of 0.001. In this case, the missing simultaneous cessation of cell growth resulted in a delay of the CO₂ evolution rates [109]. Furthermore, the results indicated that further optimization and automation of the fed batch processes potentially could be based on the permittivity signal [110]. Negrete et al. [111] employed dielectric spectroscopy in order to analyze and characterize the production of recombinant adeno-associated vectors (rAAV) in a 40 L tank using the baculovirus expression vector system with Sf9 insect cells. The permittivity signal allowed a determination of the infection time since the viable cell density and growth rate of non-infected cells could be monitored in real-time. Moreover, the increase in cell diameter could be correlated to the yield of rAAV as well as the optimum harvest time (TOH). Ansoerge et al. [112] monitored infected Sf9 cell cultures and found a good correlation between the permittivity signal and the viable biovolume (determined by Vi – CELL[®]) as well as the total biovolume (determined by CASY[®] 1). After infection, the characteristic (critical) frequency f_c was observed to change according to the changing cell diameter. However, impact of the cell membrane properties on f_c could not be excluded. Besides the monitoring of insect cell cultures, dielectric spectroscopy has been extensively utilized to monitor mammalian cell cultures such as hybridoma, CHO, Vero or HeLA cultures. These applications have already been reviewed by other authors [113,107].

Tab. 1 Summarized applications of monitoring techniques in insect cell cultures

Cell line	Technology	Application	Reference
Sf9	NIR Spectroscopy	Monitoring of glucose and glutamine in insect cell culture samples (offline)	[51]
Sf9	Fluorometry	In situ monitoring of NAD(P)H dependent culture fluorescence in infected and uninfected cultures; correlation with cell density	[58]
Sf9	Dielectric Spectroscopy	In situ monitoring of infected and uninfected cultures; correlation with viable cell density during growth; signal increase due to increasing cell size after infection	[108]
Sf9; High-5	Dielectric Spectroscopy	In situ monitoring of high cell density cultivations up to 50×10^6 cells/mL; detection of virus release after simultaneous infection	[109]
Sf9	Dielectric Spectroscopy	In situ monitoring of fed-batch processes for β – galactosidase production; increase in permittivity after infection at 14×10^6 cells/mL could be attributed to cell size increase	[110]
Sf9	Dielectric Spectroscopy	Characterization of a rAAV production process in a 40L large-scale vessel; permittivity signal allowed a determination of the infection and harvest time	[111]
Sf9	Dielectric Spectroscopy	In situ monitoring of cell cultures during growth; change in critical frequency f_c following infection was assumed due to changes in cell size and membrane properties	[112]

4 Scale Up of Industrial Insect Cell Culture Processes

4.1 The Baculovirus Expression Vector System for Commercial Vaccine Production

The Baculovirus Expression Vector System (BEVS) is nowadays considered an established and matured manufacturing technology for the commercial production of a wide array of recombinant proteins in an industrial scale, e.g. of virus like particles (VLP) for vaccines [114]. As of mid-2012, a total of five human or veterinary vaccine products based on the BEVS system have been approved. Examples include GSK's CERVARIX® (Human Papillomavirus) and Boehringer Ingelheim's CircoFLEX® (Porcine Circovirus Type 2). Several other products are already in development and BEVS-based vaccines are considered for more and more diseases, including for the field of emerging diseases. Recent literature provides a good overview over the current state of commercial production utilizing the BEVS [114,115].

4.2 Process Scale-Up and Implications for Processes Utilizing the Baculovirus Expression Vector System

The BEVS production system requires the growth of an insect cell line, preferably in suspension culture, and its infection with a specific recombinant BV carrying the genetic information for the protein of interest. The infection can either be carried out subsequently to cell growth or simultaneously with cell planting into a vessel. For the commercial vaccine production it is crucial to establish an efficient, cost-effective and robust large-scale process to manufacture the required quantities of the protein of interest [115]. Taking the process developed initially in the milliliter or liter scale and scaling it up to industrial production volumes of hundreds or thousands of liters is a challenging procedure. Maintaining the productivity of the small scale process as well as critical process and product quality attributes are the essential goals of the scale up procedure [116-118].

A well developed, optimized and characterized small scale process is essential for a successful scale up [119]. Implementation of strategies like Quality by Design (QbD) or Process Analytical Technology (PAT) and the utilization of high throughput systems help to achieve these goals by increasing the process

understanding, monitoring and control. The use of suspension insect cell lines readily facilitates the possibility of large scale processes as required for the commercial production of vaccine proteins and it is probably safe to assume that most commercial BEVS processes are performed in stirred tank reactors (STR). Biological process parameters of importance in the BEVS system are generally considered to include cell density at infection, multiplicity of infection, time of infection and physiological state and age of the cells at the time of infection [115]. But engineering parameters like agitation rate (mixing and shear), dissolved oxygen concentration or pH are also considered to be of significant importance for the process.

Cell culture condition, and therefore eventually the cells physiological state, affects and determines product quantity as well as quality. Scale up of suspension culture systems like the BEVS and its related equipment is performed based on well understood principles of scaling relevant parameters. Often it is based on geometric similarity of the small and large scale vessels [120]. In such a case, one or more specific parameters are then kept constant from the small to the large scale. Literature provides a good overview about the potential criteria for scale up [116]. They include, but are not limited to, power input, impeller tip speed or shear rate, Reynolds Number Re , oxygen transfer coefficient, gas flow rate per unit volume, mixing time and similar. It should be noted that because some of these criteria are partially associated with each other, keeping one of them constant can result in a change of the other parameters during scale up, in turn causing a change in the physical environment the cells experience [121]. Nevertheless, geometric similarity is often not given, for example when transferring or scaling up an established process into production facilities where equipment is already installed or if equipment is designed as multi-purpose equipment. Also, single use systems may not always be of the same geometry as the small scale or the stainless steel STR currently used. In such a case the process and its control need to be modified accordingly. In any case, the equipment used obviously needs to meet the current regulatory as well as process specific requirements. More recently, the application of Computational Fluid Dynamics (CFD) has also become more popular in the field of bioprocess scale up [116].

Volumetric scalability is considered as one of the key benefits of the BEVS systems, indicating the relative ease with such a system can be scaled up to commercial scales [122,123]. Several key aspects need to be considered when scaling up a BEVS based process from the laboratory to large scale. Generally speaking, system heterogeneity increases with increasing scale, which has some important implications for the scale up procedure. Sufficient mixing via impellers has to be ensured at the large scale to allow sufficient mass transfer and to avoid major nutrient, oxygen or pH concentration gradients in the culture. Impeller agitation related shear stress has long been thought to be a major issue for (not only) insect cell cultures, but nowadays is not considered a major issue anymore. The use of multiple and supposedly shear sensitive impellers as well as medium additives like Pluronic F68 further helps to reduce the impact of this type of physical damage [118,124]. Similarly nowadays it should not be a problem anymore to achieve the required oxygen transfer rates to supply the insect culture with sufficient oxygen. But even while the oxygen demand of insect cell cultures is relatively low compared to aerobic bacterial processes, surface aeration is generally not sufficient at the large scale and sparging of air or oxygen is therefore usually required [124,125]. This leads to the problems of bubble induced cell damage as well as potential foaming issues which require the selection of the correct sparger type as well as gas composition and flow rates. Bubble size is also an important parameter to consider. Smaller bubbles, for example generated by sintered spargers, can cause more cell damage in insect cell cultures than large bubbles [125,124]. Bubble size also affects CO₂ removal from the culture which can be considered another significant parameter to evaluate during scale up of a BEVS process [126]. Overall, an acceptable equilibrium between the parameters in question has to be found for the specific process and equipment used (see Figure 11).

LS Cell Culture Process Constraints

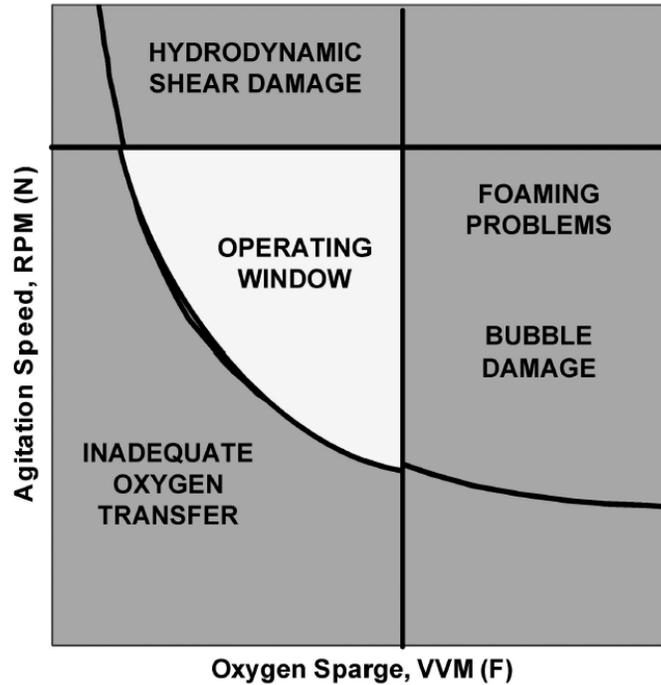


Fig. 11 Large scale cell culture process constraints (Marks [125]; with kind permission from Springer Science and Business Media)

4.3 Single-Use Systems in Baculovirus Expression Vector System Processes

In recent years single use systems like the Single-Use Bioreactor (HyClone) or WAVE® (GE Healthcare) have been become more and more utilized in the biotech industry in general but also in BEVS processes [127,128]. Main advantages of single use systems are their reduction in cross contamination potential as well as cleaning costs, increase of flexibility and decrease of the turnaround time. Main disadvantages on the other hand are the risk of leachables or extractables, sometimes insufficient material strength and difficulty of handling, pressure as well as temperature limitations, increased costs of waste disposal and scale limitations [115,129]. Regardless of the specific system employed, the basic principles for scale up and transfer of a BEVS process into such a system are the same as for the standard stainless steel stirred tank equipment.

5 Conclusions

For the production of biologically active recombinant proteins the insect cell expression system is considered to be a suitable alternative to bacterial or mammalian cells. Compared to bacterial fermentations recombinant protein production in insect cells produce fully post-translational modified proteins. This is also possible with mammalian cells but is much faster and easier to implement with insect cells. BV expression vectors are commonly used in combination with lepidopteran species such as *Spodoptera frugiperda* (Sf9 and its parental line Sf21). However, infection of Sf9 cells by the baculovirus leads to cell lysis and a consequent dying of the culture. Here, the use of stably transfected *Drosophila melanogaster* S2 cells can be a promising alternative. In contrast to the BV induced protein production in Sf9 cells, S2 cells are not lysed during cultivation facilitating even continuous process modes and protein production at high cell densities. Comparisons of the BEVS with the stable S2 expression system regarding protein yields clearly indicate that neither of the systems can be assumed superior. The amount of protein produced in both systems as well as process performance strongly depends on the properties of the protein itself. This has been further demonstrated using the example of a production process for GmGlv-GFP. In order to choose the right expression system, not only protein yields should be considered but also the quality of the proteins resulting from post-translational modifications.

In situ technologies suitable to provide real-time information regarding nutrient consumption, product and metabolite formation, cell morphology, cell size, cell density and even cell viability, result in a deeper understanding of cell culture processes and allow the early detection of potentially unfavorable changes during production processes that might have a negative impact on product formation and quality. Hence, in situ monitoring contributes to meet the “quality by design” (QbD) requirements imposed by the FDA, resulting in a high and consistent product quality in the biopharmaceutical industry. Moreover, in situ monitoring reduces the risk for contaminations caused by common sampling procedures and represents an ideal tool for process control and automation. Despite the increasing demand for monitoring techniques in disposable systems, commercially available solutions which facilitate the connection of optical in situ sensors to disposable reactors are still rare. However, various methods are already described in the

literature. Monitoring techniques such as the dielectric spectroscopy, in situ microscopy or FBRM are suitable to provide information on cell responses after viral infection making these technologies interesting for the optimization of baculovirus related production systems. In this context, interrelations of critical process parameters such as the MOI, TOI and TOH can be evaluated. The high potential of optical monitoring techniques to determine nutrient and metabolite concentrations for optimization purposes in cell culture processes have not been fully exhausted yet. However, due to the fast developments in the area of in situ online monitoring systems driven by the PAT initiative and the increasing demands for high quality biopharmaceuticals, further applications of these technologies can be expected in the near future.

Also in industrial large scale processes, online monitoring techniques are intended to implement QbD strategies by increasing the process understanding and control. The use of suspension insect cell lines enables production processes in common stirred tank reactors. This is beneficial from the industrial point of view since stirred tank reactors can be used as multi-purpose equipment. However, in order to transfer small scale insect cell production processes into production scale, various aspects need to be considered. These aspects include biological parameters as well as engineering parameters. Geometric similarity is often not given especially when equipment is already installed and used as multi-purpose equipment. Moreover, disposable systems may differ significantly from small scale production vessels. In order to maintain culture performance, the process and the belonging process control needs to be modified in a way that regulatory as well as process specific requirements are considered. Finally, these goals can only be achieved by finding a compromise that ensures satisfactory yields of high quality products as well as the implementation of equipment specific requirements.

Up to now process optimization and process understanding for insect cell based processes is still immature. Some process engineering principles can be adopted from bacterial and mammalian fermentations which particularly are intensively investigated since decades. Nevertheless, the biological reaction of the insect cells to process changes cannot be simulated but can only be experimentally determined. Same is true for online monitoring of insect cells where mammalian cell processes can only give suggestions. The transferability of the online

monitoring technology to insect cell processes still needs intensive research. Summarizing, insect cell processes still provide room for improvement to further increase the maximum yields of active recombinant proteins.

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