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Process Control in Cell Culture Technology

Using Dielectric Spectroscopy

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1 Abstract

In the biopharmaceutical industry, mammalian and insect cells as well as plant cell cultures are gaining worldwide importance to produce biopharmaceuticals and as products themselves, for example in stem cell therapy. These highly sophisticated cell-based production processes need to be monitored and controlled to guarantee product quality and to satisfy GMP requirements. With the process analytical technology (PAT) initiative, requirements regarding process monitoring and control have changed and real-time in-line monitoring tools are now recommended. Dielectric spectroscopy (DS) can serve as a tool to satisfy some PAT requirements. DS has been used in the medical field for quite some time and it may allow real-time process monitoring of biological cell culture parameters. DS has the potential to enable process optimization, automation, cost reduction, and a more consistent product quality. Dielectric spectroscopy is reviewed here as a tool to monitor biochemical processes. Commercially available dielectric sensing systems are discussed. The potential of this technology is demonstrated through examples of current and potential future applications in research and industry for mammalian and insect cell culture.

Keywords: dielectric spectroscopy, cell culture technology, PAT, biomass monitoring, process control

2 Introduction

Animal-, plant-, and insect cell cultures have become significant tools in the production of proteins and biopharmaceuticals for applications involving humans [Baldi et al., 2007, Daniell et al., 2001, Walsh, 2006, Wurm, 2004]. Animal cell lines, such as Chinese hamster ovary (CHO), hybridoma, human embryo kidney (HEK) or baby hamster kidney (BHK) cells are commonly used as production cells [Baldi et al., 2007, Butler, 2005, Walsh, 2006, Wurm, 2004]. Whole cells may also be the target product for example in stem cell therapy [Weber et al., 2007b]. Insect cells, which are for example used for the production of recombinant proteins, viruses or viral components [Nehring et al., 2006] are currently gaining an increased importance in the field of regenerative medicine [Negrete and Kotin, 2008, Stanbridge et al., 2003], especially in gene therapy [Alexander et al., 2007, Edelstein et al., 2007, Stanbridge, et al., 2003]. Cells may be grown in suspension, on surfaces, or even in three-dimensional solid matrices. Standard production units include stirred tank reactors, fixed and fluidized bed reactors, airlift reactors, wave reactors, spinner flasks, roller bottles, and Erlenmeyer type shake flasks.

The production processes for cells or cell related products are complex, and must comply with GMP requirements [Baldi, et al., 2007, Butler, 2005, Weber et al., 2008, Weber et al., 2007a, Weber, et al., 2007b, Wurm, 2004]. The intricate relationships of process parameters and process outcome may not be clear in all cases, especially on the industrial scale. However, for clinical applications when implementing research results on a larger scale, not only quality but also sufficient product quantity - recombinant protein, virus or cell - has to be delivered by monitored and controlled processes satisfying GMP.

Any tool that can contribute to supply direct on-line information on the biological state of a cell culture is extremely valuable. Dielectric spectroscopy (DS) can serve as such a tool with the advantages of rapid non-contact measurements that can be automated through a variety of sterilizable commercial sensors and systems.

2.1 Process Monitoring and Control

Process monitoring is an important tool for process characterization to demonstrate the robustness of manufacturing processes by establishing the relationships between key operating parameters and the desired product. To receive regulatory approval for a specific biopharmaceutical production process, process monitoring is generally required for documentation purposes. Cell growth, productivity (yield), product quality, medium conditions (temperature, pH, pO_2, pCO_2) and level of metabolites (glucose, glutamine, lactate, ammonia) are examples of important parameters for biopharmaceutical process characterization. These parameters are also important for subsequent legally required process validation [Li et al., 2006]. Many cell culture based processes are operated in perfusion or fed-batch mode, where control of the perfusion or feed addition rate is required to maximize recombinant protein production [Carvell and Dowd, 2006]. Biological systems are very sensitive to many process changes. The fundamental understanding of the intricate relationships between the very significant number of process parameters and the ultimate desired process outcome is often not complete. Kinetics and mass balances are sometimes difficult to obtain and describe. Despite heroic efforts to achieve reproducibility growth rates in cell cultures are often not easily reproduced and controlled [Butler, 2005].

These issues may be tackled through process monitoring. A simple example would be to monitor the temperature in a fermentation vessel, while a complex example may be the virus titer as a function of time. Even the apparently simple monitoring of temperature which is fairly easily achieved in the laboratory causes issues for scale-up since in larger physical systems the temperature will more likely show spatial inhomogeneities. Improved process monitoring can help to accelerate process development, and aids in achieving efficient production of consistently high quality products [Baldi, et al., 2007, Hoeber, 1910].

2.1.1 Methods of Monitoring

The key parameters to be monitored in cell culture are substrate and metabolite concentrations, temperature, pH, oxygen and carbon dioxide partial pressures, protein concentration and especially the concentration and state of entities such as cells or virus [Czermak et al., 2009]. This list is certainly not complete as far as the impact on cell culture is concerned. Conductivity or electrolyte content, trace minerals, and viscosity could be other examples and other significant parameters will surely emerge in the future.

Methods to monitor biochemical process parameters described in the literature can be roughly categorized as in-line, on-line, at-line and off-line analysis. In the case of in-line analysis the detector is directly interfaced to the process, while in on-line analysis process liquid is directed continuously through some kind of a recirculation loop past the detector and back to the process. At-line analysis is performed on a sample removed from the process and using an instrument in close physical proximity to the process (quasi-on-line) while for off-line analysis the sample is removed from the process and is transported to a remote location for analysis [Vojinovic et al., 2006]. Common methods for substrate and metabolite determination as well as product concentration analysis are briefly described below with a focus on methods for monitoring cell concentration and biomass.

2.1.1.1 Substrate and Metabolite Concentration

Standard substrates, e.g. glucose, glutamine and oxygen, and metabolites, e.g. lactate, ammonia, glutamate, oxygen, and carbon dioxide, are mostly determined by on-line and off-line methods using common probes, standard assay kits as well as chromatographic methods (HPLC, Ion Chromatography). Different glucose sensors (off-line and on-line) are available [Folly and B., 1996, Male et al., 1997, Pickup et al., 2005], but stability, calibration and validation of these probes remains an issue [Renneberg and Lisdat, 2008]. An example for a PAT-tool for glucose measurement is a continuous flow injection analysis based on chemiluminescence [Huang et al., 1991]. In-line methods to determine the concentration of glucose, glutamate, prolin, lactic acid, ammonia, and dissolved carbon dioxide are photometric infrared (IR) and near-infrared (NIR) spectroscopy which both conform to PAT requirements [Rathore et al., 2008]. Infrared spectroscopy suffers significantly from possible interference of background compounds [Boudreau and Benton, 2008, Vojinovic, et al., 2006]. Information gained from substrate consumption and base addition for pH stabilization was used as an indirect method to determine cell growth and cell concentration in a perfused system [Kussow et al., 1995].

2.1.1.2 Protein Concentration

Product (protein) concentration is commonly measured by HPLC, which conforms to PAT requirements [Rathore, et al., 2008] or ELISA [Li, et al., 2006]. Raman spectroscopy can be used in-line to monitor the homogenization process of an active pharmaceutical ingredient (API) according to Beer et al., 2006. Here the method also helped to understand the production process better [De Beer et al., 2006]. Several applications of Raman spectroscopy are reported as a PAT tool for process optimization, understanding, monitoring, and control [Rathore, et al., 2008].

2.1.1.3 Cell and Biomass Concentration

The biomass concentration is one of the centrally important parameters of cultivation processes. Real-time measurements are important for this key parameter [Gnoth et al., 2007]. Today, several in-line measurement methods are available to determine cell number and cell size distributions in biological processes. Most of these methods are based on image analysis. Examples are focused beam reflectance measurement, particle vision, and different types of in-situ microscopy. In all cases real-time analysis is possible, but these approaches are only suitable for well mixed systems, where homogeneity can be assumed throughout the process since only a very small volume fraction is generally monitored. On the other hand, these approaches may allow the monitoring of potential morphological changes of cells during the process in addition to cell concentration and volume. Optical sensors are unfortunately often prone to fouling especially over long culture times and are sensitive to changes in turbidity, light dispersion, presence of variable amounts of cell debris, gas bubbles, and similar interferences along the light path [Carvell and Dowd, 2006, Vojinovic et al., 2006]. Other

measurement principles include optical density measurements, acoustics, laser light- Raman-, and fluorescence spectroscopy [Vojinovic et al., 2006]. Infra-red sensors have also been described, but their range of cell concentrations is rather limited. Laser turbidity measurements are suitable for high cell density cultures [Carvell and Dowd, 2006]. Until 2002, most methods for the monitoring of cell concentrations were not applicable to immobilized cell cultures using microcarriers or other fixed bed carriers. Common methods now allowing the monitoring of these cultures include some optical techniques (see above) based on light absorbance and/or scattering, real-time imaging, particle size analysis and measurements of culture fluid density [Ducommun et al., 2002a].

Established off-line measurement methods for cell concentration determination are for example Cedex® by Roche Applied Sciences, and various systems by Beckmann Coulter [Rudolph et al., 2007]. The obvious drawback of these methods is the delay between sampling and the availability of results as well as the risk of contamination [Vojinovic et al., 2006]. Indirect methods, such as DNA concentration determination or fluorescent based measurements have been described [Myers, 1998, Rengarajan et al., 2002, Zipper et al., 2004]. Furthermore indirect off-line methods, where the determination of the oxygen partial pressure, lactate or glucose concentration in the medium is used to monitor the process and to indirectly calculate the cell concentration have been described [Vojinovic et al., 2006].

Not only is the concentration of the cells of importance for the process, but also their viability. Different off-line methods for viability measurement can be categorized into permeability assays, functional assays, reproductive assays, and morphological assays [Cook and Mitchell, 1989].

Biopharmaceutical production processes are generally overseen by agencies such as the Federal Drug Administration in the U.S. (FDA) and the European Medicines Agency (EMEA) and must satisfy GMP requirements. The methods summarized above must satisfy the requirements of the respective agencies. In recent years, these requirements have changed and new initiatives arose, such as the Process Analytical Technology (PAT) described in detail below.

2.1.2 Process Analytical Technology

The Process Analytical Technology (PAT) initiative was introduced by the FDA in 2004 and has been supported by the EMA, the Japanese Ministry of Health, Labor and Welfare (MHLW), and by the International Conference on Harmonization (ICH) guidelines [Rathore et al., 2008]. With this initiative, increased requirements for bioprocess monitoring were implemented to improve the understanding and control of biology-based manufacturing processes [Clementschitsch and Bayer, 2006]. The PAT initiative was started within the “GMP-Initiative for the 21st Century”. PAT can be described as a tool for designing, analyzing, and controlling manufacturing through timely measurements of critical quality and performance parameters with the goal of ensuring final product quality.

Present GMP standards are usually empirical and not scientifically confirmed [FDA, 2004]. Details on biopharmaceutical production processes are often not entirely known and are difficult and/or costly to establish. Small variations in a culture's progress due to subtle changes in process parameters are often not tracked since on-line monitoring is generally limited to relatively simple parameters such as temperature, pH, and dissolved oxygen. This limitation of on-line information is especially critical for biological systems, as they are subject to significant variations in their growth- and/or production rates due to small variations in process parameters that are below detection limits or are not monitored at all [FDA, 2004].

The FDA describes the goal of PAT as follows: “The goal of PAT is to understand and control the manufacturing process, which is consistent with the current drug quality system: quality cannot be tested into products, it should be built-on or should be by design” [Douennebier 2007, FDA, 2004]. The tools described by the FDA to reach PAT goals are multivariate tools for design, data acquisition and analysis, process analyzers, process control tools, continuous improvement, and knowledge management tools. In combination, PAT allows the control of the production process and its quality assurance [Kourti, 2006]. A better process understanding, improved yields, and minimization of waste,

rejects and reprocessing will result in overall cost reduction and on-time release of batches [Rathore et al., 2008]. The FDA suggests key PAT technologies: near infra-red spectroscopy (NIR), UV-VIS, NMR, MS, HPLC, fluorescence measurements, and imaging technology [Boudreau and Benton, 2008, FDA, 2004, Kourti, 2006, Rathore et al., 2008] promoting process understanding and process control for improved consistency of process performance and product consistency [Rathore et al., 2008]. Recent reviews on trends in PAT and PAT in biopharmaceutical processes are available [Chew and Sharratt, 2010, Rathore et al., 2008].

The application of DS for bioprocess monitoring is emerging as a promising tool for PAT [Clementschitsch and Bayer, 2006, Teixeira et al., 2009].

3 Impedance Spectroscopy as a Tool for PAT

Perhaps one of the more critical areas of biochemical engineering is the "upstream" section where the product (ranging from a molecule to a whole organism) is produced. This part of the process is the origin of impurities etc. that lead to significant separation and purification issues "downstream" [Rathore et al., 2008]. A promising tool for the on-line monitoring of cell cultures and biomass properties is DS, allowing non-invasive and non-destructive continuous process monitoring in bioreactors for suspension cultures and even cells attached to solid carriers [Carvell and Dowd, 2006].

DS has been used industrially for some time to monitor the viable cell density for example of yeast in the brewing process [Heggart et al., 2000, Austin et al., 1994]. The application of DS has already been discussed in the context of PAT [Boudreau and Benton, 2008, Gnoth et al., 2007] and has been suggested as a possibly accurate and reliable method for online monitoring of viable cell density in cell manufacturing processes [Carvell and Dowd, 2006]. Examples include cell cultivation on solid surfaces, on microcarriers in suspension, on porous glass carriers in fluidized bed reactors, and on porous discs in a fixed bed reactor. Reviewed cell types include bacteria, yeast, mammalian, and insect cells [Carvell, 2008, Carvell and Dowd, 2006, Ducommun et al., 2001, Fehrenbach et al., 1992, Noll and Biselli, 1998, Sarra et al., 1996, Zeiser et al., 1999].

The potential of DS, however, may well reach beyond measuring viable cell density to more intricate issues such as the physiological state of the cell or organism.

3.1 Basic Theory of Impedance Spectroscopy

3.1.1 Physical Principle and Applications

DS is based on the measurement of the passive dielectric properties of substances or biological units in a conducting medium. The term basically describes the measurement and analysis of the electrical capacitance and conductivity over a certain range of frequencies. The sample, called the dielectric, is placed in the electrical field between two electrodes. The change in the electric current-voltage relation in the presence of an alternating electrical field is then used to derive information on the sample. The focus of DS can either be the material that is placed between the electrodes, or the electrode interface itself. The electrode interface is investigated by DS techniques in corrosion research [Mansfeld, 2006]. This is a significant application of DS. The dielectric material placed between the electrodes is the focus of attention for applications ranging from materials such as cement paste to the yeast suspensions in brewing, and to cell cultures as discussed here.

The basic idea of DS is to apply a periodically alternating electrical field, perhaps at various frequencies, to a system (an aqueous cell suspension for example in our case). This is somewhat similar to application of an alternating mechanical stress at different frequencies to a solid material as in dynamic mechanical analysis of polymers, for example. The system for DS can be an entire multicellular organisms such as a human in medical applications of DS, or in the case of interest here a solution/suspension containing suspended or supported cells or unicellular organisms at least some of which are alive, along with low molecular solutes (salts, nutrients) and perhaps cell debris, virus,

and virus particles. If the frequency is in the correct range, then some components in the medium can respond for example by storing some energy as temporarily separated charges (polarization). When the electrical field is periodically reversed then some lag in the system response is detected (amplitude and/or frequency changes). This response is the basis of dielectric spectroscopy.

The AC electrical field applied to the samples may cause, depending on the frequency and field strength, a polarization, orientation or displacement of electrically charged entities that may range from single inorganic ions to whole cells or even multi-cell organisms. In the range between 0.1 – 10 MHz, the method is termed radio frequency impedance spectroscopy (RFI) and the polarization of non conducting entities with surfaces, such as cell membranes, occurs [Arnold, 2001]. This range represents a small fraction of the wide range of frequencies possible with DS. Intermediate wavelengths cause the change of orientation of dipoles while near infra-red and infra-red frequencies cause atomic relaxation. Electronic relaxation is observed in the range of visible light (see fig. 1) [Doerner, 2008].

It is qualitatively simple to recognize that at very high frequencies the system has no time to respond and no signals of interest are observed, while at very low frequencies the physical changes in the system are in phase with the change of the external field polarity and no useful signal is observed either.

In the radio frequency range, cells with intact plasma membranes basically act as capacitors, since the non-conducting nature of the generally lipid-based cell plasma membrane allows the buildup of charge [Noll and Biselli, 1998]. Living organisms actively maintain electrochemical potential differences across their membranes. The cell's dielectric properties give important information about the cell's physiology, in particular the properties of the membrane and the cell interior. Values that can be determined are the membrane capacitance C_{mem}, the effective membrane conductance G_{mem} and the intracellular conductivity σ_i. DS can also be applied to non-biological membranes for example to investigate subtle surface fouling [Watkins and Pfromm, 1999]. The membrane capacitance of living cells generally describes the amount of charge that can be stored across the cell membrane in an applied alternating electric field and is highly dependent on among other parameters on the folding of the membrane. The effective membrane conductance gives a measure of the permeability of the cell membrane, where movements of ions along the membrane also play a role. Capacitance values of viable cells with intact membrane are very high compared to non-viable cells, so that nonviable cells, leaking cells, cell debris, evolved gas bubbles and other media components are essentially invisible to RFI [Carvell, 2008, Ducommun et al., 2001, Ducommun et al., 2002a]. Finally, the mobility of ions in the cytoplasm determines the intracellular conductivity. Exchanges between the cytoplasm and the exterior may be monitored as these values change [Hoeber, 1910, Patel and Markx, 2008].

Fig. 2 describes the dependency of the capacitance signal of a biological sample with spherical cells as a function of the radio frequency. The increase in capacitance from high to low frequencies displays a sigmoid shape and is known as the ß-dispersion [Cannizzaro et al., 2003, Davey et al., 1993a, Esteban et al., 2008]. At low frequencies, the polarization process is slow, so that the resulting capacity is high. The working point for RFI-based sensors is near the critical frequency f_c. At high frequencies, there is hardly any polarization and only background capacitance due to the dipoles of water or other small molecules in the suspending medium is detected [Carvell and Dowd, 2006]. If an alternating electric field is applied, then the resulting capacitance is measured and is dependent upon the cell type, the cell size and, in a certain range, it is proportional to the concentration of the viable cells (see fig. 2) [Carvell, 2008]. The dependency of the signal on the cell size is shown in fig. 3. When measured at a fixed frequency close to the average f_c, the capacitance is independent of cell size.

In the case of adherent growing cells, signals differ from single suspended spherical cells. Ron et al. describe models for aggregation effects of spherical cells. Two models are described, were one simulates changes in the cell membrane being similar to anchoring junctions by adjacent cells. The strength of permittivity decreases significantly in comparison to spherical cells suspended in a homogenous solution, and a shift in the critical frequency is found [Ronet al., 2009]. Cell adhesion

experiments show similar results, describing smaller capacitance values for adherent cells than for suspended essentially spherical cells [Ehret et al., 1998].

3.1.2 Mathematical Description of Dielectric Spectroscopy

The detailed mathematical description of DS has been discussed in the literature and in textbooks [Arnold, 2001, Coster et al., 1996, Davey et al., 1993a, Davey et al., 1993b, Marszalek et al., 1990, Pethig and Kell, 1987, Barsoukov and Macdonald, 2005]. An overview is given below.

DS is based on the measurement of passive electrical properties of a material, the capacitance C and the conductance G (generally for 0.1-10 MHz). Both variables depend on the properties of the system that is investigated but also on the geometry and the size of the measuring electrodes, and on interactions at the electrode/system (here medium or broth) interface. The relative permittivity ε_T and the conductivity κ describe the relation independent from the electrodes given by the following equations:

$$C = \varepsilon_T \varepsilon_0 \left({}^{A}/_{d}\right) \tag{1}$$

$$G = \kappa \left({}^{A}/_{d}\right) \tag{2}$$

for plane-parallel electrodes where the area A is separated by distance d and the constant ε_0 is the permittivity of a vacuum (ε_0 = 8.854 pF m^{-1}). In 1957, Schwan correlated the capacitance of a suspension of ideal spherical cells with radius r and the capacitance per membrane unit area C_m as:

$$C = {}^{9}/_{4}\, rPC_m\, {}^{d}/_{A} \tag{3}$$

where P is the volume fraction of the cells in the suspension volume between the parallel plates of area A and distance d and is defined by the cell volume ($4/3\pi r^3$) and the cell density per unit volume N, so that the capacitance is a function of the cell radius and correlates linearly with the cell density and capacitance per membrane unit area:

$$C = 3\pi r^4 N C_m\, {}^{d}/_{A} \tag{4}$$

[Noll and Biselli, 1998]. A detailed description including the real and imaginary part of the complex impedance can be found elsewhere [Hoeber, 1910].

To establish a mathematical correlation of the capacitance signal to the desired biological and/or physical variables, a complex mathematical correlation is necessary, which is based on reference sample analysis for each of the desired parameters via established analytical methods [Ansorge et al., 2007, Cannizzaro et al., 2003, Ducommun et al., 2002a, Esteban et al., Noll and Biselli, 1998].

Clearly, both the medium and the cells or organisms (on carrier particles or suspended) impact the overall electrical properties of a suspension placed between the electrodes of a DS setup. In a dilute system (with respect to cell density) the direct-current resistance will for example be governed by electrolytes and especially salt concentrations in the liquid. The capacitance or ability to at least temporarily store electrical energy by charge separation, however, will exist mainly across cell membranes of living organisms.

3.2 Probes and Instrumentation

The DS technology allows a wide range of applications, but for sterile biopharmaceutical production processes, GMP requirements must be fulfilled. GMP compliant DS probes for commercial industrial bioprocesses are to the author's knowledge currently available from two manufacturers: Aber Instruments, Aberystwyth, UK, and FOGALE nanotech, Nimes, France. Both offer a range of instruments for the brewery and biotech industry but the focus is here on the instruments specifically developed for biotechnology.

Aber Instruments currently offers the Biomass Monitor 200, 210, 220 and 230 for in-line monitoring of biomass in bioprocesses. 12 and 25 mm diameter in situ steam sterilizable probes with an annular ring electrode configuration in various lengths are available. The instruments can be used with pre-set working frequencies for different types of cultures (bacteria, yeast or animal cell culture) but the more advanced models are also capable of performing a frequency scan. Depending on the model, up to four bioreactors can be monitored simultaneously with one instrument. Recently the company has introduced the new Futura line with improved accuracy and life cycle tracking for the probe.

FOGALE nanotech's Biomass System and Biomass System Pro have the option to connect up to four probes and allow frequency scanning. A range of stainless steel probes in various lengths for a variety of standard bioreactor ports is available. FOGALE nanotech has announced the introduction of the new BIOMASS 100, 465 and the BIOMASS PRO (see fig.4) with free choice of working frequency, and monitoring of sterilization cycles and hours of operation [Ansorge et al., 2007].

In addition, in 2008, a modular concept was announced for process instrumentation with broad bandwidth DS, designed for the integration in industrial plants. It utilizes multi-sinus technique (10 Hz – 10 MHz) and maximum length correlation for the high frequency range up to 5 GHz. The instrument performance is comparable to that of commercial network analyzers and being connected to a PC, they are also a versatile and affordable laboratory instrument [Nacke et al., 2008].

3.2.1 Disposables

The instrument manufacturers for DS equipment follow the market trend towards disposable probes. This eliminates the need of cleaning in place (CIP) sterilization in place (SIP), and may result in operating cost reduction. As trends in the pharmaceutical industry show, the use of disposable units for production processes is increasing and cGMP and GMP guidelines support their application. In 2008, Aber Instruments introduced a viable biomass probe for the increasingly popular disposable bioreactors. This probe is compatible with the Hyclone Single Use Bioreactor (HyClone Laboratories, Inc., Logan, Utah, U.S.A.). A standard reusable probe is available that can be inserted into the bag through a Pall Kleenpak connector (see fig. 5). A flow-through silicone tubing bypass loop can also be used, and a weldable probe for bags and other single-use bioreactors is available [Aber Instruments, 2008] (fig. 5). A similar probe for single use reactors is available from Fogale (fig. 6).

3.3 Applications

3.3.1 Applications in Research

Various applications of DS in cell culture research are described, going back to the early 20th century, when Hoeber [Hoeber, 1910] compared the low and the high frequency conductivity of erythrocytes and demonstrated the existence of the cellular membrane based on electrical properties. Nowadays, not only process monitoring in production processes, but also monitoring on the cellular level is described, such as differentiation processes of mesenchymal stem cells [Hildebrandt et al., 2010], dielectric membrane phenomena [Zhuang et al., 2010] and electrical properties of cells in general [Heubach et al., 2004, Wang and Jang, 2009]. Often, systems are described, where cells are grown on microplates or other small units with integrated electrodes, where the dielectric properties of single cells can be monitored. To assess cell adhesion and morphological changes of mouse cells, immortalized fibroblasts [Ceriotti et al., 2007a], cardiomyocytes [Qui et al., 2008], and mammalian cancer cells [Rahman et al., 2008] were monitored in a 96-well plate and in small volume detection chambers, where probes are integrated [Ceriotti et al., 2007a, Ehret et al., 1997, Ehret et al., 1998, Guo et al., 2006, Yu et al., 2006]. Furthermore, the monitoring of apoptosis and necrosis [Lee et al., 2009, Qiu et al., 2009] as well as monitoring of cytotoxic effects of various substances by DS have been described [Ceriotti et al., 2007b, Liu et al., 2009, Malleo et al., 2009]. Examples for the on-line monitoring and study of morphological changes of cells induced by receptors, such as the G –Protein coupled receptors on HeLa and CHO cells [Yu et al., 2006], as well as the analysis of a protein kinase inhibitory effect on 3T3 fibroblast cells [Huang et al., 2003] in microchambers equipped with DS electrodes have been reported [Huang et al., 2003, Yu et al., 2006]. The successful assessment of tissue growth within microporous polymer scaffolds has been reported [Dziong et al., 2005]. Sensor

design is an important aspect for the mentioned measurements, such as for differentiation of stem cells and differentiated cells [Dalmay et al., 2009]. A recent summary of single cell dielectric measurements is given by Sun and Morgan [Sun and Morgan, 2010]. DS has also been proposed to detect living organisms in extreme environments such as Martian soil [Miller and Warmflash, 2011].

Virus detection is a serious issue in the biopharmaceutical industry. DS can be useful for cultivation of virus producing cell lines [Bragos et al., 2006]. The detection of small virus concentrations (down to a MOI of 0.0006) of herpes simplex infecting Vero cells (green monkey kidney) is possible using a specially designed electrode based chip and DS. The state of infection was clearly reflected by the impedance signal and spectra correlated to cell/cell or cell/substrate junctions, determined by off-line methods. Furthermore, a correlation to different MOI was detected [Bragos et al., 2006].

In summary, the field of DS of cell cultures is rapidly expanding. Increasingly sophisticated information related to cells in a whole culture down to individual cells is being extracted from the DS signals.

3.3.2 DS Applications in Production Processes

Several examples of the application of DS in biopharmaceutical production processes have been reported in the literature. While many and sometimes unknown parameters may impact a cell culture some of the main issues are the cell concentration, cell death, due to shear, toxic chemicals, virus infection, changes in cell size and metabolism, osmotic stress, and immobilization processes of adherent growing cells. It will be shown below that all of these main issues can be successfully monitored by DS.

There are certainly limitations of DS in cell culture. Insertion of DS electrodes into a hollow fiber module for example did not lead to reliable results [Blute et al., 1988]. Cultures where cells grow either in suspension or on solid carriers in relatively conventional vessels such as stainless steel reactors or disposable systems are the focus here.

3.3.2.1 Examples of Mammalian Cell Cultures

Noll et al. [Noll and Biselli, 1998] describe the use of DS to monitor suspended and immobilized hybridoma cells in a fluidized bed reactor. DS together with Schwan's equations [Pethig and Kell, 1987] allowed the development of correlations of the capacitance to derive the membrane area, changes in cell physiology, cell size, apoptosis, monitoring of nucleotide triphosphates and ATP, cellular protein content, and the glutamine consumption rate. For the immobilized cells, the progress of immobilization could be monitored, as the measured capacitance increased proportional to the progress of cell immobilization. Immobilized cells change their size according to reduced available carrier surface as the cell density increases, but on the other hand, the increased immobilized cell density compared to suspended cells improves the signal to noise ratio. For the immobilized cell culture, the comparison of the cell density and the capacitance during the fermentation showed deviations, which result from a drastic decrease in the cell specific capacitance, C_s. Further results indicated that changes in the cell physiology during a batch culture are reflected in the measured capacitance. An excellent correlation between the specific capacitance and the specific amount of nucleotide triphosphates in the cells could be shown. It was also shown that during a batch culture, the cell specific capacitance signal changes by about 45%, having a maximum value at the maximum growth rate [Noll and Biselli, 1998].

Similar correlations were found for CHO cells in a perfusion culture [Cannizzaro et al., 2003]. Monitoring of batch and fed-batch processes of CHO cells with DS has been recently reported. [Opel and Amanullah, 2010]. Linear modeling, Cole-Cole modeling, and partial least squares regression were used to correlate the DS data with routine biomass measurements of viable packed cell volume, viable cell concentration, cell size, and the oxygen uptake rate. Excellent correlations were found during the growth phase, but the stationary and declining phases showed weakened correlations which could not be improved when taking the changing cell radius in account. Examination of the correlation between permittivity, oxygen uptake rate, and viable cell concentration showed how critical the definition of viability is when analyzing biomass online. Furthermore, values of critical frequency, α

(cole-cole parameter) and dielectric parameters of static internal conductivity and membrane capacitance per area were calculated. As these values did not remain constant throughout the process, important information for correlations was gained. Conclusions included that the common assumption of constant size and dielectric properties used are not always valid during later phases of cell cultures processes. Dielectric spectroscopy was found not to be a surrogate for viable cell concentration, but rather a measurement of viable biomass, providing information on the physiological state of a culture [Opel and Amanullah, 2008].

DS applied to fed-batch CHO expansion processes with two different cell lines has been reported [Ansorge et al., 2007]. Nutrient availability was monitored by the critical frequency and the online dual frequency permittivity signal. Feeding resulted in a sudden increase in permittivity and critical frequency and a corresponding decline was reported when nutrient depletion occurred. When the cell radius remained constant, a good correlation between the oxygen uptake rate and the critical frequency was found, indicating that metabolic activity is reflected in the critical frequency. When changes in cell size were monitored, data were difficult to interpret. The authors hypothesize that multi-frequency measurements can give information on the intracellular or physiological state of the cells, allowing to optimize and better understand feed strategies. This was confirmed for other cell lines and systems [Ansorge et al., 2007]. In 2010, similar results were published for a batch process of CHO cells on the laboratory and pilot scale [Ansorge et al., 2010].

DS measurements of adherent growing Vero cells producing rabies virus in a stirred tank reactor on Cytodex 1 carrier were recently described [Rourou et al., 2010]. Batch cultures, perfused cultures, and cultures in animal component free media showed a linear relationship between the permittivity and the viable cell density measurements during the growth phase as well as for the virus production phase. Calculations of the critical frequency allowed monitoring of cell lysis. Changes in the membrane capacitance and intracellular conductivity where monitored through changes in the critical frequency.

Applications of RFI measurements for mammalian cell cultivation have been reviewed [Carvell and Dowd, 2006]. Table 1 shows a summary of available literature based on the literature cited here and in Carvell and Dowd's paper.

Monitoring of cell death by DS has been reviewed [Patel and Markx, 2008]. Cell death is often associated with a loss of the integrity of the cell membrane or a change in membrane permeability. This can be monitored by DS, as capacitance values decrease and membrane conductance increases [Hoeber, 1910]. Scientists ascribe an increase in the capacitance to cell shrinking and membrane smoothing with discussion still ongoing. Several types of cell death are described. In the case of apoptosis, smoothing of the membrane occurs, cells shrink, and are becoming spherical. The cytoplasm condenses and finally the cells lyse and break down into vesicles. This phenomenon was monitored by large changes in the cell's dielectric properties in HL60 and Jurkat cells. The effects of osmotic stress, shear and other mechanical stress, infection of cells by viruses, and the toxic effects of solvents intercalating the membranes have all been related to changes in the dielectric spectra.

3.3.2.2 Examples of Insect Cell Cultures

Insect cell cultures are widely used in the production of recombinant proteins for a variety of different purposes utilizing the Baculovirus Expression Vector System (BEVS). Reports of DS as an on-line monitoring tool for insect cell cultures are relatively scarce.

Zeiser et al. [Zeiser et al., 1999] appear to be the first to report capacitance measurements for cell cultures of uninfected and infected *Spodoptera frugiperda* (Sf-9) insect cells. The relative permittivity correlated well with the viable cell density for uninfected cells and a culture infected with a baculovirus encoding for β-galactosidase (MOI 10). As signals showed a further increase of permittivity after infection, it was shown to be possible to monitor a synchronous infection (with high MOI and arrested cell division). This was mainly thought to be due to a significant increase in cell diameter after infection. Interestingly, a plateau in the permittivity signal was observed 18-24h post-infection, which could be correlated to a temporary cell size leveling during this time. Beyond post infection, the

following cell lysis could be detected, as the signal decreased due to a decrease in cell concentration and size. Parallel to a further signal decrease due to cell size reduction, the highest β-galactosidase titer could be detected.

The same group as above reported in 2000 on monitoring of high cell density fed batch cultures, where the infection strategy was of special interest. Uninfected and infected Sf-9 cells [Elias et al., 2000] were cultivated and correlations of impedance signals with physiological parameters during growth and infection of two different insect cell cultures (Sf-9, High-5) [Zeiser et al., 2000] were made. During the cultivation of the Sf-9 cells, a signal plateau was detected, which corresponded to a peak in the CO_2 evolution rate and the release of virus particles into the medium. Infection at a lower MOI of 0.001 did not result in a cell division arrest at the time of infection and CO_2 evolution rates were delayed compared to the results obtained for infections at higher MOI values. In addition, infection of another insect cell line (High-5), (MOI 10) generally matched the results in Sf-9 cell cultures. The authors concluded that both CO_2 evolution rate and relative permittivity can be used to directly measure the physiological state of the cells during the entire process and suggest that the relative permittivity measurement could be used to optimize or detect the time of harvest reliably. This is especially important for products requiring post-translational modifications.

DS applied to Sf-9 insect cell cultures has been reported more recently [Ansorge et al., 2007]. Off-line biovolume measurements correlated well with the permittivity signal of DS measurements for a limited time after infection. Only off-line analysis of the viable biovolume (by Vi-CELL) and the total biovolume (by CASY 1) correlated well with the permittivity measurement throughout the entire time the culture was tracked. Multiple frequencies over the range of 300 kHz to 10 MHz were observed by DS to obtain the β-dispersion and its characteristic values. It was found that the characteristic frequency changes during the culture correlated with changing cell diameter but changes in cell membrane properties are suspected to also have an influence on this parameter.

A large-scale production process for recombinant adeno-associated vectors (rAAV) using the BEVS system in Sf-9 cells was reported to have been monitored and optimized using DS [Negrete et al., 2007]. They compared the profiles obtained at different frequencies and used the relative permittivity measurement to determine the optimum time of harvest. In this case a second local maximum in the permittivity profile could be observed at about 48 h post-infection, which correlated to the maximum product yield.

4 Summary and Outlook

DS appears to be a promising non-invasive on-line tool for the monitoring of cell cultures, in research as well as for production processes. The potential to follow cell concentration, cell size, metabolic state, cell morphology, apoptosis, immobilization, viral infection, and virus release as well as a number of other parameters in real-time gives important information and enables improved process understanding, process control, and optimization while satisfying PAT requirements. Not only suspension cultures but also adherent growing cell lines have been successfully monitored, opening new possibilities for process monitoring in this area. In comparison to other common methods, the DS allows in-line process monitoring and control, probes can be sterilized in-situ, and are available as disposable units. The technique may be less susceptible to particles or fouling effects than optical turbidity measurements and is an ideal candidate as a tool for process automation in biopharmaceutical production processes.

Some areas of DS in cell culture monitoring and optimization can likely be addressed through further development. Possible approaches are outlined here according to the literature and the author's own experience. The changes in cell size during cultivation and/or infection impact the ideal critical frequency f_c. This might be addressed through continuous frequency scans.

Both components in the solution besides the cells to be monitored, and changes in simple non-biological parameters impact DS. The optimum measurement frequency has to be calibrated by frequency scans of cell-free and cell-containing solutions of different conductivity [Noll and Biselli, 1998]. Since the temperature greatly influences the dielectric behavior of cells and cell suspensions, temperature should be kept constant or temperature correction is required, which leads to a significant reduction of the thermal contribution to experimental noise [Asami et al., 1999]. Solid particles can cause a significant displacement of cell-containing solution, which may reduce the measured capacitance. This may require separate calibration measurements.

A fundamental issue is the assumption of spherical cells which simplifies the mathematical treatment. Non-spherical cells, cell protrusions, or cell deformation could probably be included in the mathematical description if independent information is available on these subtle geometric issues to essentially calibrate the DS measurement [Zeiser et al., 1999, Zeiser, et al., 2000]. However, the measurements made to provide a reference for DS measurements are often not without ambiguities either.

Organisms or cells that contain organelles such as mitochondria or other membrane structures can impact the DS signal through internal events rather than the external membranes [Noll and Biselli, 1998].

The capabilities and the potential of DS seem to outweigh the disadvantages especially for industrial applications. The relative simplicity and ruggedness of the probe is a significant advantage. DS can improve process stability, product quality and cost reduction for biopharmaceuticals. The growing area of regenerative medicine including cell therapy and gene therapy with examples such as production of encapsulated mesenchymal stem cells could be considered a good application for DS. , Controlled cell expansion is often required for mesenchymal stem cell production [Weber et al., 2008, Weber, et al., 2007a, Weber, et al., 2007b]. In-line monitoring by DS for cell density and the time for culture harvest might be especially useful when stem cells are grown adherent or on carriers in fixed bed reactors since it is difficult to take samples.

DS may also be useful in more fundamental aspects such as research on the differentiation of stem cells. Structural information can be obtained using integrated electrodes allowing the monitoring of growth and differentiation [Bragos et al., 2006].

In medicine, viruses can serve to prepare vaccines, but are also an element of gene therapy [Alexander et al., 2007, Edelstein, et al., 2007, Stanbridge, et al., 2003]. These viruses are mainly produced in mammalian packaging cell lines [Merten, 2004] or in insect cell lines using baculovirus [Negrete et al., 2007, Stanbridge et al., 2003]. The same cell culture system can be used to produce recombinant proteins for vaccines. The application of DS may be suitable for monitoring of these production processes [Nehring et al., 2006], allowing process control under PAT and GMP [Negrete et al., 2007]. The infection process of cells can be monitored directly by DS.

DS has been shown to be a useful and in some respects advantageous asset for cell culture. Applications range from the fundamental to process optimization and control. The ability to derive rather sophisticated information on a cell culture without the danger of breeching sterility or the time delays of more conventional measurements is a significant advantage. The ruggedness of the needed probes, along with availability of systems and probes ranging from sterilizable probes for standard ports to probes for disposable bags from reputable suppliers allows integration in processes even under strict regulatory requirements. It seems likely that DS technology will further grow in importance for cell culture applications.

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7 Figures

Fig. 1 Relaxation processes tracked by ε_r' (permittivity of the sample in the dielectric) and ε_r'' (permittivity of the surrounding dielectric) across a spectrum of electromagnetic wave frequencies (radio frequency range RW, intermediate frequency range MW, infra red IR, visible light (V) and ultraviolet light (UV). Entities responding to these frequencies are schematically shown, from left to right: a living cell, a polar molecule, atomic level vibration, and electronic response. [generously provided by S. Doerner, Faculty of Electrical Engineering and Information Technology., Otto-von-Guericke-Universität Magdeburg); by permission, Mensch und Buch Verlag, Germany [Doerner, 2008].

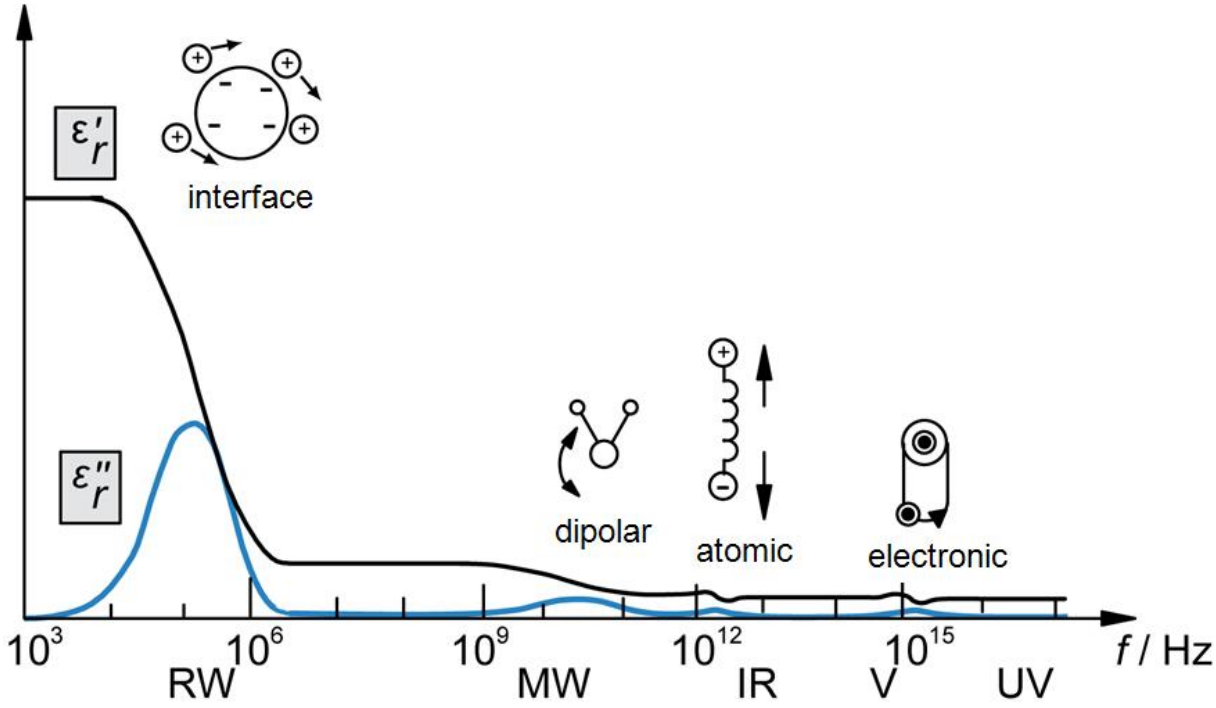

Fig. 2 Capacitance Δc, critical frequency f_c, and membrane capacitance c_m at increasing cell densities in suspension [Cannizzaro et al., 2003]; reprinted with permission of John Wiley Sons, Inc.)

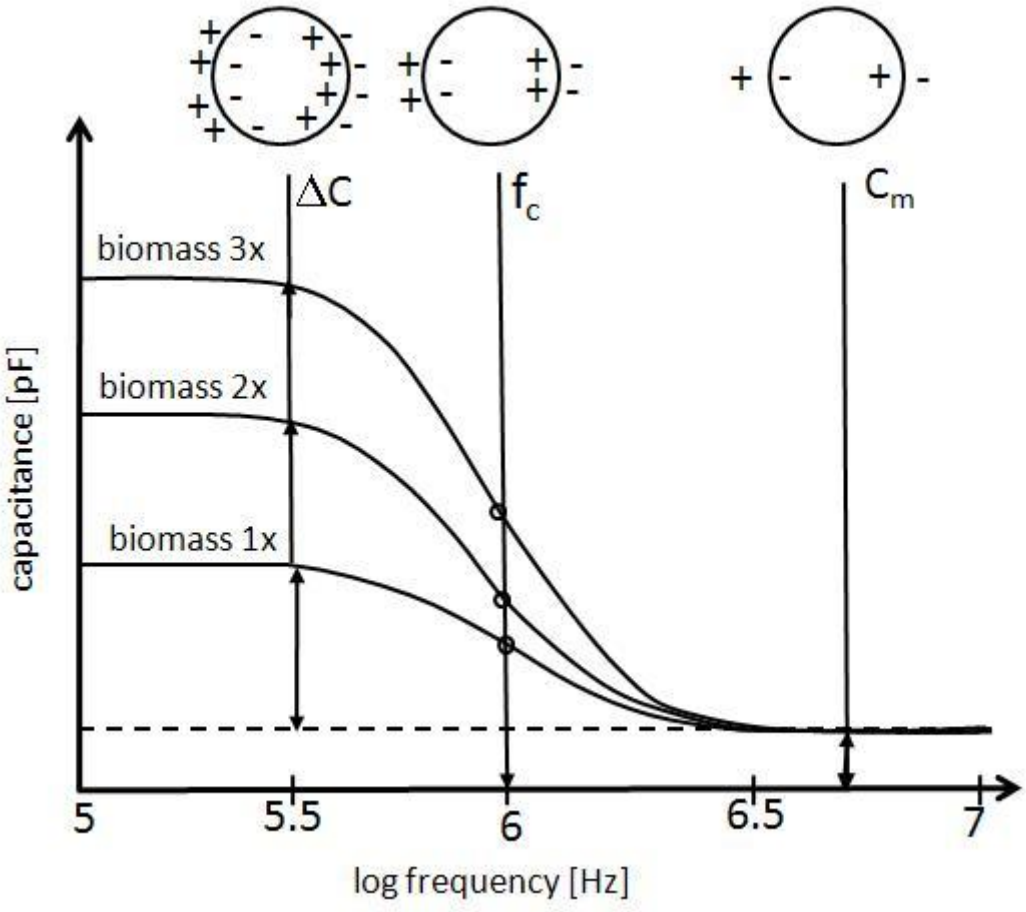

Fig. 3 Increase of the critical frequency f_c with cell size. This graph is valid only for a remaining biovolume, even though the cell size changes. [Carvell and Dowd, 2006]; reprinted with permission of John Wiley Sons, Inc.)

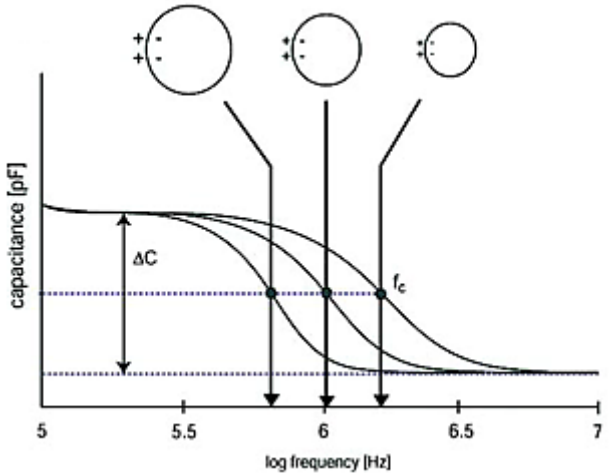

Fig. 4 FOGALE nanotech Biomass 465 with pre-amplifier and 25 and 12 mm reusable probes; (generously provided and with permission by R. Luemen, Cellogics GbR, Muenster, Germany).

Fig. 5 Top: Aber reusable biomass probes inserted into disposable 50 L Hyclone bioreactor, Pall (Dreieich, Germany), Kleenpak connector; bottom left: Single use biomass probe connected to lightweight Futura (Aber product line) pre-amplifier for Biomass Monitor; bottom right: Single use biomass probe welded into 50L Hyclone SUB (Photographs courtesy of Hyclone, Logan (Utah), USA, generously provided and with permission from Carvell, J., et al.., On-line monitoring of the live cell concentration in disposable bioreactors. Aber Instruments Ltd. Science Park, Aberystwyth, Wales, UK SY23 3AH).

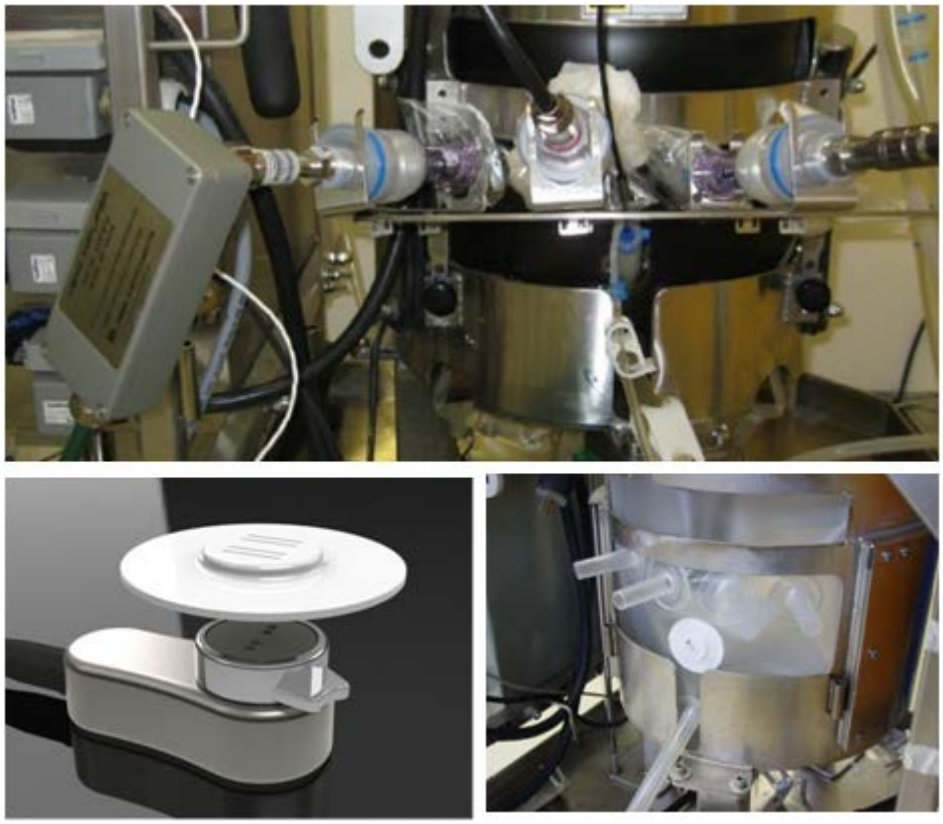

Fig. 6 FOGALE nanotech biodis series biomass sensor for single use bags; (generously provided and with permission by R. Luemen, Cellogics GbR, Muenster, Germany).

Legends

Abbreviation	
A	surface
BEVS	Baculovirus expression vector system
BHK	Baby hamster kidney
C	Capacitance
CHO	Chinese hamster ovary
CIP	Cleaning in place
C_M	Membrane capacitance
C_S	Cell specific capacitance
d	Distance
DNA	Deoxyribonucleic acid
DS	Dielectric Spectroscopy
ELISA	Enzyme linked immuno sorbent assay
EMEA	European Medicines Agency
f_c	Critical frequency
FDA	Food and Drug Administration
G	Conductance
GMP	Good Manufacturing Practice
HEK	Human embryo kidney
HeLa	Henrietta Lacks
HPLC	High pressure liquid chromatography
MOI	Multiplicity of infection
MS	Mass spectrometer
N	Cell density
NMR	Nuclear magnetic resonance
P	Volume fraction
PAT	Process Analytical Technology
PC	Portable computer
pCO2	Carbon dioxide partial pressure
pO2	Oxygen partial pressure
r	Radius
rAAV	Recombinant adeno associated vector
RFI	Radio frequency impedance spectroscopy
Sf-9	*Spodoptera frugiperda*
SIP	Sterilization in place
UV-VIS	Ultra violet-visible
κ	Conductivity
ε_0	Constant permittivity in vacuum
ε_T	Relative permittivity
σ_i	Intracellular conductance
ϵ_r'	Permittivity of the sample immersed in the dielectric (medium)
ϵ_r''	Permittivity of the medium

8 Tables

Table 1 – Summarized applications of DS in cell culture

Summary of applications of DS (adapted from Carvell et al., 2006 [Carvell and Dowd, 2006] and further cited literature)

Cell line	Culture conditions	Summary	Ref.
CHO	Immobilized in a packed bed of FibraCell discs	DS to characterize the packed bed reactor; monitoring of maximum cell concentration and protein production rate; evaluation of the effect of temperature on specific metabolic rates during 3 months of continuous culture in a 40 L industrial process; characterization of growth kinetics up to $4x10^7$ cells/mL of packed bed.	[Ducommun et al., 2002a, Ducommun et al., 2002b]
CHO	Airlift reactor	Monitoring of exponential growth and death phases, and changes in the physiological state of the cells.	[Schmid and Zacher, 2004]
CHO	Batch and fed-batch in a stirred tank reactor	Modeling by linear, cole-cole and partial least squares models with good correlations for the growth phase. The assumption of constant cell size throughout the process was discarded for later phases of the process.	[Opel and Amanullah, 2008]
CHO K1/dhfr$^-$ and CHO recombinant for antibody production	Fed batch process in a 3L stirred tank reactor	Permittivity and critical frequency allow the monitoring of nutrient depletion and feed points giving information on the intracellular or physiological condition of the cells. Optimization and better understanding of fed-batch processes. Results may also be applicable for other cell lines and systems.	[Ansorge et al., 2007]
CHO K1/dhfr$^-$	Batch process		
CHO 320	Suspension culture	Linear correlation between cell number and capacitance; 0.5 MHz optimum measurement frequency.	[Cerckel et al., 1993]
CHO 320	Anchorage cells on Cytopore1 microcarrier	More accurate information than with data obtained by protein content determination.	[Guan and Kemp, 1997]
CHO 320	Suspension of immobilized cells	Relationship between capacitance and viable cell concentration.	[Davey et al., 1997]
CHO 320	Stirred aerobic batch culture	Combination of DS with microcalorimetric measurements.	[Guan et al., 1998]
CHO	Perfusion culture	Optimization and control of the system, automated perfusion feeding based on live cell concentration via DS data. Optimized process operation through cell specific perfusion rate (based on DS) keeps the cell concentration constant and optimizes recombinant protein production rates.	[Dowd et al., 2003]
HeLA	Suspension culture	Linear correlation between cell number and capacitance; 0.5 MHz optimum measurement frequency.	[Cerckel et al., 1993]
HTC	Anchorage dependent cells on Cytodex 5g/L in	DS at 0.8 MHz enables accurate estimates of cellular biomass from the capacitance signal.	[Degouys et al., 1993]

	spinner flask		
Hybridoma	Immobilized in suspension of porous microcarrier in batch cultivations in spinner flasks as well as in fluidized bed reactor in continuous mode	DS data reflects changes in cell physiology and could be correlated to cell metabolic activity. For the continuous culture, this enabled a closed loop control of the glutamine feed rate according to the metabolic needs of the cells.	[Noll and Biselli, 1998]
SP2/0	Perfusion process	Excellent correlation of capacitance and cell concentrations over a culture period of 40 days ($2x10^5$-$17x10^6$ cells/mL); mean size of a population changes in response to media changes, possible detachment of adherent growing cells from the surface, substrate limitations and osmotic stress. The correlation between capacitance and live cell number is lost, when the dielectric properties of the cells change (for example due to substrate limitation or osmotic stress).	[Carvell and Dowd, 2006]
Vero producing rabies virus	Batch, perfused, with animal component free medium on Cytodex1 in a 2L stirred tank reactor	Linear correlation between the permittivity and viable cell concentration for the growth phase and virus production phase. Cell lysis monitoring by critical frequency calculation using the cole-cole model.	[Rourou, et al., 2010]