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Publisher's version / Version de l'éditeur:

<https://doi.org/10.1002/btpr.347>

Biotechnology Progress, 26, 1, pp. 272-283, 2009-11-06

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Multifrequency Permittivity Measurements Enable On-Line Monitoring of Changes in Intracellular Conductivity Due to Nutrient Limitations During Batch Cultivations of CHO Cells

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DOI 10.1002/btpr.347

Published online November 6, 2009 in Wiley InterScience (www.interscience.wiley.com).

Lab and pilot scale batch cultivations of a CHO K1/dhfr⁻ host cell line were conducted to evaluate on-line multifrequency permittivity measurements as a process monitoring tool. The β -dispersion parameters such as the characteristic frequency (f_C) and the permittivity increment ($\Delta\epsilon_{max}$) were calculated on-line from the permittivity spectra. The dual-frequency permittivity signal correlated well with the off-line measured biovolume and the viable cell density. A significant drop in permittivity was monitored at the transition from exponential growth to a phase with reduced growth rate. Although not reflected in off-line biovolume measurements, this decrease coincided with a drop in OUR and was probably caused by the depletion of glutamine and a metabolic shift occurring at the same time. Sudden changes in cell density, cell size, viability, capacitance per membrane area (C_M), and effects caused by medium conductivity (σ_m) could be excluded as reasons for the decrease in permittivity. After analysis of the process data, a drop in f_C as a result of a fall in intracellular conductivity (σ_i) was identified as responsible for the observed changes in the dual-frequency permittivity signal. It is hypothesized that the β -dispersion parameter f_C is indicative of changes in nutrient availability that have an impact on intracellular conductivity σ_i . On-line permittivity measurements consequently not only reflect the biovolume but also the physiological state of mammalian cell cultures. These findings should pave the way for a better understanding of the intracellular state of cells and render permittivity measurements an important tool in process development and control. © 2009 American Institute of Chemical Engineers Biotechnol. Prog., 26: 272–283, 2010

Keywords: permittivity, on-line monitoring, CHO cell cultures, physiological state, metabolic shift

Introduction

On-line monitoring of bioprocesses is an important tool in process development and optimization and is fundamental for process control. Its foremost advantage is the monitoring of sudden changes in critical process parameters by a virtually unlimited sample number. Contamination risks are also reduced which are generally increased by frequent sampling. The demand for and interest in reliable on-line monitoring techniques has increased in recent years. This is at large due to FDA's guidance on process analytical technology (PAT)¹

that proposed a paradigm shift in biomanufacturing. This shift can be characterized by the move of quality control key aspects from laboratory-based to process-based locations, that is, off-line to at-line/on-line.^{2,3} On-line monitoring is furthermore critical to build better process models and develop reliable, well-controlled processes in a shorter period of time.⁴

One of the most important parameters to monitor in any cell culture process is the viable, that is, metabolically active biomass.^{5,6} Its estimation is almost a philosophic problem.⁶ In fact, its most commonly used definition is rather operational and might in some case also include necromass.⁷ Consequently, many different methods are available for biomass determination. On the one hand, classical on-line biomass measurement techniques like the calculation of the oxygen uptake rate (OUR) or the CO₂ evolution rate (CER) give indirect information on the biomass

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

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content. They are mainly related to the metabolic activity of the cell culture.^{8,9} On the other hand, common direct biomass measurement methods like turbidity or in situ microscopy rather follow the total cell concentration and are insensitive to changes in the physiological state of the culture.^{10–12}

Permittivity-based biomass probes are a valuable alternative and their application in cell culture processes is rather novel. They allow in situ application and perform on-line measurements with a quasi-continuous signal (i.e., a very short sample interval of a few seconds to minutes). Permittivity can be seen as a direct measure of the membrane enclosed volume fraction or biovolume of the cell suspension. The successful application of this technology in a wide variety of cell culture processes is described in the literature.^{5,6,9,13–16}

Several studies report measurements of the permittivity at one single or two frequencies (to reduce the effect of a changing media composition on the signal). Permittivity measurements are described as a tool to directly measure the viable biomass. However, this relationship does not always hold because stained cells are known to contribute to the permittivity.^{5,13,17–19} This observation is probably caused by the fact that the “remaining” permittivity of a nonviable cell is dependent on the mechanism by which the cell was “inactivated.”²⁰ The classical method of counting viable cells by dye exclusion can therefore not always be expected to give data that correlate linearly with the permittivity, in particular in the late stage of cultivations during which viability is low. The relationship is rather governed by a mechanism in which dying cells lose their polarizability (and hence their permittivity) progressively.²¹

Permittivity measurements do also reflect changes in cell physiology which has been demonstrated for a wide variety of cell types.^{5,9,13,22–24} Cell physiology is most commonly described by the term “physiological state.” This term is defined by several variables that impart significant information about the cellular state.²⁵ These variables include growth and metabolic rates (including their ratios), degrees of nutrient limitations, cellular morphology (cell size, membrane state), dielectric properties (intracellular conductivity, membrane conductivity), and the distribution of these variables. Although qualitatively intuitively clear, the quantitative description of the physiological state is, due to the complexity of the variables, rather elusive. It is therefore difficult to monitor, causing a complex situation when it should be used to control a bioprocess.^{25,26} Advances in bioprocess engineering will consequently ultimately depend on the level of understanding and control of the physiological state of a cell population.²⁵

Concerning the measurement of the physiological state, current sensor technology suffers from critical limitations. It is consequently impossible to uncover all aspects of the cellular state at an intracellular level.²⁶ The permittivity and the physiological state of a cell culture are intrinsically related. Permittivity is a function of cell size, membrane integrity, and properties and also of changes in the cell’s interior.²¹ One can monitor cell size and follow the cell cycle progression and division of budding yeast by measuring the permittivity at very low frequencies.^{27,28} Changes in cellular morphology, the capacitance per membrane area after viral infection^{29,30} and viability¹⁸ can also be monitored by permittivity measurements.

Permittivity measurements consequently yield rather complex information on the biovolume of a cell culture. This is an advantage of this technology when compared with, for example, OUR measurements that measure mainly the metabolic activity of cells. Single- or dual-frequency permittivity measurements result, however, in a parameter which might be difficult to interpret. One way to overcome this limitation is the measurement of permittivity at several frequencies in the physiologically important β -dispersion range (0.1–20 MHz). The obtained datasets are nevertheless highly correlated, that is, appropriate data treatment needs to be performed. Partial least square (PLS) models have been used to decorrelate datasets and retrieve information about the cell size distribution from on-line multifrequency permittivity measurements. This was shown to be a useful tool for the on-line monitoring and prediction of successful adenoviral production.³¹ Another study using the same method reports the monitoring of physiological changes and major transition points in CHO cell perfusion culture from multifrequency permittivity data.⁵

An alternative to PLS modeling are recently available software packages that allow the automated calculation of the most important β -dispersion parameters. The interpretation of these parameters, like the characteristic frequency (f_C), the permittivity increment ($\Delta\epsilon_{\max}$), and others result in additional information on the state of the culture. From changes in one of the β -dispersion parameters (f_C), the harvest time in CHO cell production processes was optimized.³² The onset in baculovirus production was also identified by the same parameter.²⁹ The use of only a few parameters that result out of the β -dispersion should be user-friendlier than the analysis of complex frequency scanning datasets, in particular for the application of this technology in industrial production settings.

In this study, a commercially available device, the Fogale BIOMASS SYSTEM[®] (BMS), was used for measurement of the permittivity and the BIOMASS+ software for the on-line calculation of the β -dispersion parameters. The technology was evaluated in batch cultivations of a CHO K1/dhfr[−] host cell line. It is demonstrated that multifrequency permittivity measurements are useful tools to monitor metabolic shifts and consequently the physiological state of mammalian cell cultures. This is possible because changes in the intracellular conductivity (σ_i) are reflected in one of the β -dispersion parameters, the characteristic frequency (f_C).

Theoretical Background

Dielectric permittivity and theory on dielectric properties of biological cells

The underlying theory on the dielectric properties of biological cells has been extensively described elsewhere.^{33–36} The permittivity of a cell suspension can be easily measured because a characteristic fall in permittivity, the β -dispersion (Figure 1), occurs with increasing frequency. The β -dispersion is caused by the polarization of cell membranes. The resulting permittivity increment ($\Delta\epsilon_{\max}$) is directly correlated to the membrane enclosed volume fraction (biovolume) of the cell suspension. For spherical cells at high viability (and therefore a low membrane conductivity) and for moderate values of P ($P < 0.2$), one can mathematically define this fall by three parameters ($\Delta\epsilon_{\max}$, f_C , α) and the equation of Schwan (Eq. 1)^{7,37}:

Figure 1. The β -dispersion for spherical cells within the frequency range of 0.1–100 MHz.

The β -dispersion is mathematically defined by three parameters ($\Delta\epsilon_{\max}$, f_C , α) that are dependent on the properties of the cell population. f_1 and f_2 represent the measurement frequencies of the Fogale BMS.

$$\Delta\epsilon_{\max} = \frac{9 \times r \times P \times C_M}{4}$$

where $\Delta\epsilon_{\max}$ is the permittivity increment (difference in permittivity at very low and very high frequency relative to f_C) ($F\ m^{-1}$), r is the cell radius (m), N is the cell density (m^{-3}), C_M is the capacitance per membrane area ($F\ m^{-2}$), and P is the volume fraction of cells (biovolume); $P = \frac{4}{3} \times \pi \times r^3 \times N$.

The first parameter $\Delta\epsilon_{\max}$ can be measured as the difference in permittivity at the low-frequency and the high-frequency plateau (permittivity increment). The second parameter, the characteristic frequency (f_C), is defined by a simplified equation (Eq. 2)⁷:

$$f_C = \frac{1}{2 \times \pi \times r \times C_M \times \left(\frac{1}{\sigma_i} + \frac{1}{2\sigma_m} \right)}$$

where σ_i is the conductivity of the cytoplasm/intracellular conductivity ($mS\ cm^{-1}$), and σ_m is the conductivity of the medium ($mS\ cm^{-1}$).

f_C is the frequency at which the permittivity has fallen half way (Figure 1). The equation for f_C changes for situations of low cellular viability or high membrane conductivity.^{37,38} In this study, this relationship could be neglected for the most important part of our results because viability remained high when important changes in f_C occurred.

A third parameter describing the β -dispersion is α (also: Cole-Cole α). It is an empirical parameter describing the fall in permittivity with increasing frequency in the Cole-Cole equation.³⁹ α is believed to increase when the distribution in cell electrical properties widens in the cell population.¹⁸

Practical considerations for the measurement of the β -dispersion

Measurements of the “lumped parameter”²² permittivity in single or dual-frequency mode result in a value which is dependent on many variables. To overcome this limitation, the β -dispersion parameters f_C , $\Delta\epsilon_{\max}$, and α were calculated in our experiments from the multifrequency permittivity data (measured from 0.3–10 MHz) by modeling the β -dispersion, that is, fitting the dataset to the Cole-Cole equation that

includes the dielectric parameters $\Delta\epsilon_{\max}$ and f_C (from Eqs. 1 and 2) and α .³⁹ Although this approach significantly reduced the amount of data, changes in these parameters are still dependent on many variables.

Equation 2 was therefore further simplified by neglecting the term $\frac{1}{2\sigma_m}$. This assumption is valid because the extracellular conductivity (σ_m) is much higher than the intracellular conductivity (σ_i) of mammalian cells. For yeasts, intact red blood cells and cultivated BHK cells, σ_i is in the range of 3–5 mS/cm.^{18,30,40,41} Only earlier work reported a wider range of 2–9 mS/cm.⁷ σ_i is therefore significantly lower than σ_m measured in this study (~ 18 –21 mS/cm).

After modeling the β -dispersion, a multiplication of the parameter $\Delta\epsilon_{\max}$ (Eq. 1) with f_C (Eq. 2) led to a further simplification. The resulting parameter $\Delta\epsilon_{\max} \times f_C$ is then only dependent on P (biovolume) and σ_i (Eq. 3):

$$\Delta\epsilon_{\max} \times f_C = \frac{\frac{9}{4} \times P \times \sigma_i}{2\pi} = P \times \sigma_i \times C_1$$

where $\Delta\epsilon_{\max} \times f_C$ is the mathematical product of the permittivity increment $\Delta\epsilon_{\max}$ and the characteristic frequency (f_C), and C_1 is a mathematical constant.

It is shown later in this study how this relationship can be useful for the interpretation of multifrequency permittivity measurements.

Materials and Methods

Bioreactor setup

A benchtop Biostat MCD (B. Braun Biotech, Melsungen, Germany) (3 L total volume) as previously described was used for cultivations in lab scale.²⁹

For the cultivations performed in pilot scale, a 25 L airlift bioreactor CF 3000 (Alfa Laval Chemap, Volketswil, Switzerland) was used. This system was additionally equipped with a turbidity probe (Aquasant Messtechnik AG, Bubendorf, Switzerland). All process parameters were recorded on a pen recorder, manually transferred into txt format, and filtered using Matlab software (Mathworks, Natick, MA).

Cell lines and media

A CHO K1/dhfr⁻ (dehydrofolate reductase negative) cell line was cultivated in batch mode in lab and pilot scale. A previously described formulation was used as culture medium.⁴² This formulation was, theoretically, in all experiments identical but included different preparations of critical components such as hydrolysates (Primateone RL) and surface acting agents (Pluronic[®] F68), resulting in different cell growth rates and maximum cell densities in the experiments.

Metabolite and product analyses

Amino acid analysis was performed on a HP 1100 Series HPLC (Palo Alto, CA) as suggested by the manufacturer.⁴³ Glucose concentration was determined with a Beckman Coulter Glucose Analyzer 2 system.⁴⁴

Biovolume, cell number, and cell size determination

A 0.05% (w/v) solution of Trypan Blue (Sigma-Aldrich, MO) was routinely used for staining and cell counting in a Neubauer hemacytometer. The Vi-CELL[™] XR system (VC) (Beckman Coulter, Fullerton, CA) which is also based on

Figure 2. Batch cultivation of CHO cells in pilot scale.

After inoculation at $\sim 2.5 \times 10^6$ c/mL, exponential growth took place at a specific rate of $\mu = 1.21 \pm 0.02$ d⁻¹. A maximum viable cell density of $\sim 3 \times 10^6$ c/mL was reached after ~ 80 h; viable cell count, hema (triangles, continuous line), total cell count, hema (circles, continuous line), turbidity (dashed gray line), $\Delta\epsilon_{\text{Fogale}}$ (thick continuous line), glutamine (solid squares, continuous line), glucose (solid triangles, continuous line), viability (crosses, dashed line).

Trypan Blue staining was used as an alternative method for cell counting and viability, and for measurement of cell size. A CASY®1 device (Innovatis AG, Bielefeld, Germany) was used to determine mean cell diameter and “total biovolume (CASY).”

Fogale BIOMASS SYSTEM® (BMS). The commercial in situ autoclavable DN 12 and DN 25 probes were used for the lab and pilot scale bioreactor system, respectively. The system setup was performed according to manufacturer’s instructions.

The BMS makes use of the β -dispersion to measure the biovolume. The system uses a dual-frequency mode with a high frequency f_2 (10 MHz) and a working frequency f_1 in the region of f_C . The dual frequency permittivity signal given by the BMS, $\Delta\epsilon_{\text{Fogale}}$, is the result of the difference in permittivity at f_1 and f_2 . f_1 is adjustable and it is hence possible to measure $\Delta\epsilon_{\text{Fogale}}$ for any given cell type in the region of f_C (for mammalian cells: ~ 1 MHz) and not in the low-frequency range of Schwan’s postulation (Eq. 1).³⁷ A constant $\Delta\epsilon_{\text{Fogale}}$ response can consequently be observed when the cell radius is changing at a constant biovolume. $\Delta\epsilon_{\text{Fogale}}$ is henceforth linear to the biovolume even in the case of cell size changes.⁴⁵ It can furthermore be mathematically proven that the relative profiles of $\Delta\epsilon_{\text{Fogale}}$ and $\Delta\epsilon_{\text{max}} \cdot f_C$ are identical over time (Fogale nanotech, personal communication, available to reviewers). This relationship can be used as a control of the β -dispersion modeling and is useful for a further interpretation of the results.

The BIOMASS+ software automatically analyzes the permittivity over a total of 20 frequencies from 0.3–10 MHz. The software then determines the β -dispersion parameters f_C , $\Delta\epsilon_{\text{max}}$, and α . To minimize noise in the calculated parameters, it is optional to either set the Cole Cole α to a constant (fixed) value or to calculate it depending on the results of the other β -dispersion parameters (value of α is variable). During our analyses, a calculated (variable) α did not affect the qualitative patterns of the other β -dispersion parameters. All analyses were therefore conducted with a calculated (variable) α as the setting of a fixed value did not result in a significant noise reduction.

Matlab software (Mathworks, Natick, MA) was finally used to filter the generated datasets and further minimize noise in all calculated parameters.

Results and Discussion

Monitoring nutrient limitations using on-line permittivity measurements

In preliminary experiments, batch cultivations (pilot scale) were performed using the Fogale BMS for on-line monitoring of the permittivity ($\Delta\epsilon_{\text{Fogale}}$) (Figure 2). The permittivity profile was characteristic for batch cultivations of mammalian and animal cells. In these processes, the permittivity typically increases during the growth phase and then decreases in an almost symmetric manner with the beginning of death phase, showing a single maximum. The permittivity profiles are cell-line and medium-dependent but sudden changes in the signal are most often not observed.^{16,31,32,46} In this experiment, the end of exponential growth phase coincided with the beginning of death phase when glutamine and glucose were simultaneously depleted (Figure 2). The turbidity signal correlated well with the viable cell count during the growth phase but also measured dead cells and debris and kept increasing during the first part of death phase (80–100 h). This behavior of turbidity measurements in comparison with permittivity measurements is in agreement with findings by others.³²

Subsequently, batch cultivations were conducted in lab and pilot scale (Figures 3–7). These experiments included the same medium composition with different lots of critical components (see Cell Lines and Media Section), resulting in improved cell growth. In the lab scale cultivation, a drop in $\Delta\epsilon_{\text{Fogale}}$ at ~ 60 h was observed (Figure 3). Although growth slowed down significantly at that time, no decrease in cell density was observed that could have explained this change in permittivity. The culture still showed a high viability and growth continued during which $\Delta\epsilon_{\text{Fogale}}$ increased until viability dropped at ~ 130 h and death phase begun. Medium conductivity increased rather constantly and could therefore

Figure 3. Batch cultivation of CHO cells in lab scale with improved medium composition.

After exponential growth until around 60 h at a growth rate of $\mu = 1.72 \pm 0.14 d^{-1}$, cell growth slowed down to $\mu = 0.14 d^{-1}$. A maximum viable cell density of $\sim 8 \times 10^5$ c/mL (hema) was then reached after ~ 100 h; viable cell count, hema (triangles, continuous line), total cell count, hema (circles, continuous line), $\Delta\epsilon_{Fogale}$ (thick continuous line), viable cell count, VC (triangles, gray continuous line), total cell count, VC (circles, gray continuous line), conductivity (dashed line), viability (crosses, dashed line).

Figure 4. Permittivity and off-line biovolume measurements for lab scale cultivation which was divided in three distinct phases (B I, B II, B III); PCV (circles, gray continuous line), total biovolume (CASY) (squares, dash-dotted line), mean diameter (CASY) (diamonds, continuous line), $\Delta\epsilon_{Fogale}$ (thick, continuous line).

be excluded as a reason for this fast drop in permittivity. The drop in $\Delta\epsilon_{Fogale}$ at ~ 60 h was not reflected in the off-line biovolume measurements (Figure 4). Total biovolume (CASY) and packed cell volume (PCV) showed a linear relationship with the permittivity during exponential growth phase ($R^2 > 0.99$) and slightly lower values when data from all growth phases was included (0.85 and 0.9, respectively, data not shown). The cell diameter increased at the beginning of the cultivation and decreased significantly during the exponential growth phase. Significant changes in cell diameter were, however, not found from ~ 60 – 120 h. The biovolume (CASY and PCV) kept increasing after 60 h. Both measurements reached a maximum value at around 100 h, corresponding with the maximum in viable cell density. The biovolume then decreased during death phase.

An explanation for the drop in $\Delta\epsilon_{Fogale}$ at ~ 60 h could be found by taking the nutrient concentrations into account (Figure 5). It is proposed to divide this batch cultivation in three distinct phases (Figures 4 and 5). During phase I (B I) (0–60 h), glutamine and glucose were consumed, and alanine

and lactate were produced. Phase B II started with the drop in $\Delta\epsilon_{Fogale}$ at ~ 60 h. From that time onwards, glutamine was depleted and glucose was consumed at a lower rate. Furthermore, alanine was now consumed resulting in a metabolic shift from the production of alanine to its consumption after glutamine was depleted. A similar metabolic shift has also been reported for hybridoma and other cell lines.^{47,48} Lactate remained at a constant concentration of ~ 30 mM in phase B II. $\Delta\epsilon_{Fogale}$ increased slightly and growth continued at a reduced rate. Phase B III started after glucose and alanine were depleted at ~ 130 h. This phase marked the beginning of death phase with strongly decreasing viability and decreasing permittivity. During death phase lactate was consumed. The different phase transition points could be also observed in the pattern of the OUR (Figure 5). Although the patterns of OUR and permittivity were generally similar, the OUR dropped more significantly at the time of glutamine limitation.

The results for the cultivation in lab scale were underlined by a parallel cultivation in pilot scale (Figures 6 and 7). In

Figure 5. Metabolite concentrations for lab scale cultivation; $\Delta\epsilon_{\text{Fogale}}$ (thick continuous line), volumetric oxygen uptake rate (circles, continuous line), alanine (gray stars, gray continuous line), glutamine (solid squares, continuous line), glucose (solid triangles, continuous line), lactate (crosses, continuous line).

Figure 6. Metabolite concentrations for pilot scale batch cultivation of CHO cells; $\Delta\epsilon_{\text{Fogale}}$ (thick continuous line), volumetric oxygen uptake rate (circles, continuous line), alanine (gray stars, gray continuous line), glutamine (solid squares, continuous line), glucose (solid triangles, continuous line), conductivity (dashed line), viability (crosses, dashed line).

Figure 7. Off-line biovolume, mean diameter and turbidity measurements; $\Delta\epsilon_{\text{Fogale}}$ (thick continuous line), volumetric oxygen uptake rate (circles, continuous line), PCV (circles, dashed line), total biovolume (CASY) (squares, dotted line), mean diameter (CASY) (diamonds, continuous line), turbidity (dashed line).

this run, conductivity increased by ~ 1 mS/cm after base addition for pH control at ~ 65 h. Despite this sudden increase in conductivity, a significant drop in $\Delta\epsilon_{\text{Fogale}}$ occurred at almost the same time. This observation substantiated our hypothesis that changes in $\Delta\epsilon_{\text{Fogale}}$ were not related

to changes in conductivity. Consequently, the changes in $\Delta\epsilon_{\text{Fogale}}$ were most likely related to the described metabolic shift caused by nutrient limitation that occurred in both cultivations. The cultivation was again divided in three phases according to the similar metabolic profile (phase B I-III).

Table 1. Comparison of Parallel Batch Cultivation in Two Different Scales

Measurement	2 L Lab Scale	25 L Pilot Scale
$\mu_{\max}$ (phase I) (d^{-1})	1.72 (± 0.14)	1.41 (± 0.10)
$T_{D \max}$ (phase I) (h)	9.71 (± 0.77)	11.88 (± 0.82)
$vcc_{\max}$ (10^6 c/mL)	8	5.7
Time($vcc_{\max}$) (h)	95	95
Perm _{max} (phase B I) (pF/cm)	5.7	5.25
Perm _{max} (phase B II)	6.2	5.2
OUR _{max} (phase B I) (mmol/L $\times$ h)	0.6	0.55
OUR _{max} (phase B II) (mmol/L $\times$ h)	0.56	0.53
PCV _{max} (μ L/mL)	10	7.3
Mean cell diameter (phase B II) (μ m)	12.64 (± 0.13)	13.15 (± 0.04)
Total biovolume (CASY) _{max} (μ L/mL)	6.7	5.9

$\mu_{\max}$ (phase I), maximum specific growth rate in phase I; $T_{D \max}$, doubling time at maximum specific growth rate; $vcc_{\max}$, maximum viable cell count; time ($vcc_{\max}$), time point of maximum viable cell count; perm_{max}, maximum permittivity ($\Delta\epsilon_{\text{Fogale}}$); OUR_{max}, maximum volumetric oxygen uptake rate; PCV_{max}, maximum packed cell volume; mean cell diameter (phase B II), average mean cell diameter in phase B II; total biovolume (CASY)_{max}, maximum total biovolume(CASY).

Phase B II was significantly shorter in pilot scale (25 when compared with 70 h). Similar to the cultivation in lab scale, a leveling of the biovolume (PCV and CASY) was observed at the time and after the drop in $\Delta\epsilon_{\text{Fogale}}$ (Figure 7). The cell diameter decreased in phase B I and remained almost constant from ~ 60 –100 h with a similar pattern as in lab scale. The turbidity signal substantiated our previous findings (Figure 2) according to which turbidity-based probes cease to correlate with the viable cell density after exponential growth phase and during death phase (Figure 7).

Table 1 compares the most important on-line and off-line measurements for the two batch cultivations (lab and pilot scale). The growth rate in pilot scale was significantly lower than in lab scale. This finding corresponded with a lower maximum viable cell density in pilot scale. The differences in the profiles were most likely caused by a prolonged lag phase in the airlift system, possibly due to a different aeration system (sparging in airlift system versus headspace aeration in benchtop bioreactor). Values in pilot scale (biovolume, cell density, growth rate, OUR) were generally found to be lower than in lab scale. However, the culture was showing a larger cell size in phase B II of the pilot scale which led to a higher biovolume than expected from the lower viable cell density. Despite differences in quantitative measurements, the qualitative patterns of the permittivity and other parameters (OUR, biovolume, cell size) were similar in the two cultivations.

Multifrequency permittivity measurements and analysis of calculated β -dispersion parameters

Changes in β -Dispersion Parameters. The calculated β -dispersion parameters (f_C , $\Delta\epsilon_{\max}$, $\Delta\epsilon_{\max}^*f_C$) were used to further interpret the observed changes in $\Delta\epsilon_{\text{Fogale}}$ (Figures 8 and 9). A significant drop in f_C of $\sim 40\%$ occurred at the time point of the drop in $\Delta\epsilon_{\text{Fogale}}$ during both cultivations (lab and pilot scale). In contrast, $\Delta\epsilon_{\text{Fogale}}$ only decreased by $\sim 10\%$ during that time. f_C decreased earlier and found its minimum at around the same time as $\Delta\epsilon_{\text{Fogale}}$. f_C was almost constant in

pilot scale during phase B II and increased only slightly during phase B II in lab scale. Starting with the beginning of phase B III, f_C then increased until the end of both cultivations. This prolonged dramatic increase in f_C from 0.8–2 MHz (pilot scale) and ~ 1 –1.8 MHz (lab scale) could be an indicator of the onset of death phase (phase B III). It was probably a result of a combination of a decreasing cell diameter (Figures 4 and 7) and an increase in membrane conductance during death phase.³⁸

The drop in $\Delta\epsilon_{\text{Fogale}}$ could not be observed in $\Delta\epsilon_{\max}$. This parameter kept increasing at the time of the drop in $\Delta\epsilon_{\text{Fogale}}$, reaching a maximum during phase B II. $\Delta\epsilon_{\max}$ then showed an almost constant value during phase B II and finally decreased with the beginning of phase B III.

$\Delta\epsilon_{\max}^*f_C$ showed a very similar pattern when compared with $\Delta\epsilon_{\text{Fogale}}$. This observation confirmed the mathematical relation between $\Delta\epsilon_{\max}^*f_C$ and $\Delta\epsilon_{\text{Fogale}}$ (see Fogale BIO-MASS SYSTEM[®] (BMS) Section). The value for the Cole Cole α changed only minimally during the two experiments with average values of 0.13 ± 0.05 and 0.12 ± 0.03 in lab and pilot scale, respectively (data not shown).

The overall qualitative pattern of all β -dispersion parameters was similar for both cultivations. In phase B II of the pilot scale cultivation, $\Delta\epsilon_{\max}$ gave higher values relative to $\Delta\epsilon_{\text{Fogale}}$ when compared with the lab scale cultivation. This was probably related to the larger cell diameters in the pilot scale cultivation (Figures 4 and 7, Table 1). Another difference was observed in pilot scale in which f_C was almost constant during the complete phase B II. This might be related to the significantly shorter duration of phase B II in pilot scale. Finally, a decrease in f_C at around 130 h (pilot scale) was observed which was caused by an unrelated technical problem.

Changes in Intracellular Conductivity (σ_i) Occur at the Time of Nutrient Limitations. f_C decreased in both cultivations at the transition from phase B I to B II (time of glutamine depletion). It is most likely that this led to the drop in $\Delta\epsilon_{\text{Fogale}}$ at the end of phase B I. This can be explained by the fact that the measurement frequencies f_1 and f_2 are adjustable but were not changed after the start of the cultivation. In other words, a decrease in f_C would “push” the β -dispersion towards lower values and a lower permittivity value will be read at f_1 (compare Figure 1). In the lab scale cultivation, f_C furthermore appeared to monitor the metabolic shift during phase B II. f_C recovered during that phase and showed a similar pattern to $\Delta\epsilon_{\text{Fogale}}$ and the OUR (Figure 5).

With the help of several assumptions, it was possible to identify the parameters responsible for the change in f_C at the transition from phase B I to B II. These are based on the underlying Eqs. 1 and 2 and our on-line and off-line measurements. Sudden changes in cell density, viability, and effects caused by medium conductivity (σ_m) were immediately excluded due to frequent sampling around the time of interest (compare results part) and on-line monitoring data (σ_m). The remaining parameters (r , C_M , and σ_i) are discussed in the following.

As changes in cell radius (r) affect the volume fraction P (Eq. 1), multifrequency measurements can be used to monitor r and also cellular division on-line.^{5,27,31,32} Because of off-line and on-line measurements, cell size changes could be nevertheless excluded as reason for the observed changes in f_C and $\Delta\epsilon_{\text{Fogale}}$ at the time of the metabolic shift (end of

Figure 8. β -dispersion parameters calculated by the BIOMASS+ software for CHO cell batch lab scale cultivation.

The values of these parameters are given starting ~ 24 h after the start of the cultivation. During the first hours of the culture, the biomass content was too low to allow for reliable measurements of the β -dispersion. $\Delta\epsilon_{\text{Fogale}}$ (thick continuous line), characteristic frequency (f_c) (dashed gray line), $\Delta\epsilon_{\text{max}} * f_c$ (gray continuous line), $\Delta\epsilon_{\text{max}}$ (dash-dotted line).

Figure 9. β -dispersion parameters for pilot-scale cultivation; $\Delta\epsilon_{\text{Fogale}}$ (thick, continuous line), characteristic frequency (f_c) (dashed gray line), $\Delta\epsilon_{\text{max}} * f_c$ (gray continuous line), $\Delta\epsilon_{\text{max}}$ (dash-dotted line).

phase B I). First, the drop in f_c is not in-line with a decrease in cell size but would only occur if r had increased (Eq. 2). Second, the CASY and VC off-line biovolume and cell size measurements gave almost constant values around the time of the drop in $\Delta\epsilon_{\text{Fogale}}$ for both cultivations (Figures 4 and 7). Finally, a decrease in cell size would have caused a drop in $\Delta\epsilon_{\text{Fogale}}$ at the end of phase B I because of a drop in permittivity measured at low frequencies (Eq. 1). In contrast, it was observed here that the permittivity at high frequencies (>1.5 MHz) decreased at the transition from phase B I to B II. The permittivity at low frequencies (<1 MHz) only leveled off (not shown). This observation substantiated our off-line biovolume measurements (Figure 4) because the measurement of biomass (i.e., the membrane enclosed volume fraction) takes place at low frequencies that give all cells sufficient time to completely polarize (Eq. 1). This is in-line with the values of $\Delta\epsilon_{\text{max}}$ which remained almost constant or even further increased during phase B II (Figures 8 and 9).

A second parameter that might have changed at the time of glutamine depletion is the capacitance per membrane area (C_M) (Eq. 1). C_M is generally interpreted as a measure of the “wrinkliness,” that is, the level of folding of the cellular

membrane.³⁸ Whereas originally reported to be a biological constant with a value of $1 \pm 0.5 \mu\text{F}/\text{cm}$,^{2,7,37} C_M was in more recent studies found to be depending on viability and physiological state.^{22,24} It also changes after viral infection and increases significantly after overexpression of cation channels,^{30,49} being particularly affected by processes directly concerning cellular membrane properties, for example, exocytosis (loss of microvilli), viral release, and apoptosis.^{20,29,38} One study reports that changes in C_M might, in combination with a rather small drop in cell size, be responsible for changes in permittivity. These authors attributed difference in permittivity to unknown changes in membrane structure (and therefore in C_M) and found an excellent correlation between the permittivity signal and physiological state during perfusion cultures of hybridoma cells.²² Yet, the conclusions were drawn based on a “limited” dataset in which the permittivity was measured at only one single frequency of 0.6 MHz which is not in the low frequency plateau (for which Eq. 1 is postulated). Permittivity measured at this frequency is therefore also affected by changes in f_c . In our present study, multifrequency measurements allowed a further analysis and interpretation of the results. In the case of a decrease in C_M , we should have also observed a drop in

$\Delta\epsilon_{\max}$ (Eq. 1). In contrast, $\Delta\epsilon_{\max}$ was found to continue increasing at the transition from phase B I to B II. Second, C_M would need to rise to cause a decrease in f_C (Eq. 2). This would then result in an additional increase of $\Delta\epsilon_{\max}$ (and possibly also $\Delta\epsilon_{\text{Fogale}}$). Its continuous increase with no additional change in slope at the transition from phase B I to B II did not support this assumption. Finally, it can be proven with the help of the underlying equations that C_M is not responsible for the observed change in f_C and $\Delta\epsilon_{\text{Fogale}}$. For both batch cultivations, the pattern of the mathematical product $\Delta\epsilon_{\max} \cdot f_C$ was identical to the pattern of $\Delta\epsilon_{\text{Fogale}}$. We can consequently extend Eq. 3 to Eq. 4 and get:

$$\Delta\epsilon_{\text{Fogale}} \times C_2 = \Delta\epsilon_{\max} \times f_C = \frac{\frac{9}{4} \times P \times \sigma_i}{2\pi} = P \times \sigma_i \times C_1$$

where C_2 is a mathematical constant.

According to Eq. 4, changes in $\Delta\epsilon_{\text{Fogale}}$ can be entirely explained by the parameters P and σ_i . As changes in the biovolume have been excluded as a reason for the decrease in f_C and $\Delta\epsilon_{\text{Fogale}}$, it is hypothesized that changes in the intracellular conductivity (σ_i) caused the drop in f_C .

The intracellular conductivity (σ_i) is a measure of the ability of the cell's interior to conduct electrical current. Only found a handful of studies were found in which σ_i and its changes were determined using different methods. In early and even recent work on dielectric properties of biological cells, σ_i is often presumed to be rather independent of the physiological state.^{7,50,51} σ_i is nevertheless known to increase during early apoptosis, possibly due to cell shrinkage, and decreases after induced morphological changes or in late-apoptotic and necrotic cells.^{52–54} Ion efflux leads to a decrease of σ_i , which can also be observed after an increase in membrane conductance, for example, because of antibiotic treatment.^{55,56} Differences in σ_i are most likely attributable to different ion concentrations in the cell's interior.⁴⁰ Based on the presented analysis, it is therefore assumed that significant changes in the intracellular environment (and hence, in σ_i) occurred at the time of glutamine depletion. At that time, f_C and $\Delta\epsilon_{\text{Fogale}}$ decreased in both batch cultivations. According to Eq. 2, a decrease in σ_i would lead to a drop in f_C and following the previously explained relationship also to a decrease in $\Delta\epsilon_{\text{Fogale}}$. Permittivity measurements are consequently sensitive for changes in physiological state caused by metabolic shifts. Multifrequency measurements therefore appear to solve at least in part the previous problem that the "lumped parameter" permittivity is depending on many variables.²² Intracellular monitoring techniques beyond simple viability measurements are needed to understand the physiological state of cells and to develop improved process models, combining extracellular and intracellular data.^{2,26}

The underlying mechanisms that caused a decrease of the intracellular conductivity still need to be identified, ideally using methods like dielectrophoresis or electrorotation. For now, it can only be speculated how nutrient depletions might cause changes in the cell's interior. Although changes in the specific parameter σ_i are rarely determined, the intracellular environment of cells is very well studied. The intracellular content of mammalian cells is variable, changes with starting and feed nutrient concentrations and might even be affected when extracellular concentrations of major nutrients remain constant.^{57–60} More commonly investigated

than σ_i is the intracellular pH (pH_i). Within the physiological range, it appears to be a general rule that with increasing pH_i the metabolic activities of cells increase.⁶¹ pH_i is therefore strictly regulated and can be used for process control and is an excellent marker of growth phase, growth rate, and physiological state.^{61–63} Changes in pH_i might serve as examples for possible alterations in the cell's interior that could possibly also be reflected in permittivity measurements.

The analysis of multifrequency permittivity measurements remains a complex task. During the presented batch cultivations, the cell diameter remained constant in critical phases. Yet, one cannot always benefit from such a situation. Significant cell size changes occurred in all cultivations and are particularly important in fed-batch processes and after viral infections (Ansorge et al., Submitted).²⁹ Cell size is a valuable indicator of the physiological state.^{5,64–68} In late stages of cultures, changes in cell diameter and viability typically occur, leading to changes in several parameters (C_M , σ_i , and others).^{20,29,38} An easily comprehensible example for the complexity of the analysis is that according to our results, an increase in f_C might occur either as a result of a decrease in cell size (Eq. 2), viability (beginning of death phase), or as an indicator of a metabolic shift of the culture (phase B II in lab scale). It is therefore inevitable to apply a combined logical analysis of process parameters to obtain meaningful results. This decorrelation is only possible when a sufficient amount of off-line measurements is available.

Conclusion

The on-line permittivity signal $\Delta\epsilon_{\text{Fogale}}$ was found to reliably follow the biovolume in batch cultivations of a CHO K1/dhfr[−] host cell line. During batch cultivations in lab and pilot scale, $\Delta\epsilon_{\text{Fogale}}$ showed a decrease in the same cultivation phase. Although not reflected in the off-line biovolume measurements, the decrease in $\Delta\epsilon_{\text{Fogale}}$ at the transition from exponential growth (phase B I) to a phase with reduced growth rate (B II) could be explained by the depletion of glutamine and a metabolic shift occurring at that time. The different phase transition points could be also observed in the pattern of the OUR. The on-line permittivity signal ($\Delta\epsilon_{\text{Fogale}}$) consequently not only indicated the biovolume of the cell suspension but also its metabolic or physiological state. Our observations made concerning the on-line monitoring of the metabolic shift (end of phase B I, start of B II) have not yet been described in the literature.

Second, the calculated β -dispersion parameters were analyzed. The analyses were based on observations from on-line and off-line measurements and theoretical assumptions. After excluding all other parameters as responsible for the drop in f_C , it was possible to identify a decrease in f_C as a result of a fall in σ_i (intracellular conductivity) at the transition from phase B I to B II. σ_i was hypothesized to be the responsible parameter that caused changes in $\Delta\epsilon_{\text{Fogale}}$ when nutrient limitations occurred in the presented cultivations. Alterations of the nutrient environment appeared to have an effect on the intracellular content (σ_i) rather than the membrane structure (C_M). σ_i hence seems to indicate changes in the physiological state, that is, more specifically changes in the nutrient availability of mammalian cell cultures.

The measurement of the permittivity at multiple frequencies to obtain the β -dispersion parameters is therefore advantageous and allows fully exploiting the information that can be obtained from this technology. Our findings might pave the way for a better understanding on the intracellular state of cells. Multifrequency permittivity measurements could be helpful tools to develop improved biomass sensors.

Our results should be useful for media or feed optimization and the optimization of the timing of feed additions. An additional study from our group evaluated the usefulness of our findings in fed-batch cultivations of CHO cells (Ansorge et al., Submitted). Using an on-line signal, harvest time points could also be determined reproducibly, not in a time-based but rather process- or event-based manner. Further applications should be processes in which changes in the physiological state are of primary importance for productivity, for example, viral vector production schemes and/or perfusion cultures.^{26,69}

To our knowledge, this article is the first report that interprets multifrequency permittivity measurements and the resulting β -dispersion parameters and relates them to the physiological state of mammalian cells. These measurements appeared to us as being a powerful on-line monitoring tool. The application of this tool in a wider variety of processes should lead to an increased understanding of the intracellular state of mammalian cells. Permittivity measurements should consequently be useful in process development and control.

Acknowledgments

The authors would like to acknowledge C. Ghommidh from Université Montpellier II and M. Biselli from the Aachen University of Applied Sciences (Department Juelich) for the stimulating and fruitful discussions and remarks, D. Zacher for conducting preliminary experiments, M. Foggetta, M. Siegrist, J.-M. Vonach, and J.-C. von Bueren for support with small scale experiments and setup of the bioreactor system and H. Remy for use of and help with the CASY[®]1 system. S. Ansorge was supported during his internship by a Hoffmann-La Roche AG fellowship. The presented results form a part of his diploma thesis at the Aachen University of Applied Sciences.

Notation

α = Cole-Cole α
 BMS/Fogale BMS = Fogale BIOMASS SYSTEM[®]
 CASY = CASY[®]1 system
 CER = CO₂ evolution rate
 C_M = capacitance per membrane area (F m⁻²)
 $\Delta\epsilon_{\text{Fogale}}$ = difference in permittivity measured at f_1 and f_2
 $\Delta\epsilon_{\text{max}}$ = permittivity increment (difference in permittivity at very low and very high frequency relative to f_C)
 $\Delta\epsilon_{\text{max}}^*f_C$ = mathematical product of $\Delta\epsilon_{\text{max}}$ and f_C
 f_C = characteristic frequency (MHz)
 f_1 = working frequency of BMS in the region of f_C
 f_2 = second measurement frequency of BMS (10 MHz)
 hema = hemacytometer
 N = cell density
 OUR = oxygen uptake rate
 P = volume fraction of cells (biovolume)
 PAT = process analytical technology

pH_i = intracellular pH

r = cell radius

σ_i = intracellular conductivity (mS cm⁻¹)

σ_m = conductivity of the medium (mS cm⁻¹)

VC = Beckman Coulter Vi-CELL XRTM system

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Manuscript received Apr. 8, 2009, and revision received Aug. 25, 2009.