

On-line monitoring of responses to nutrient feed additions by multi-frequency permittivity measurements in fed-batch cultivations of CHO cells

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Abstract Changes in the nutrient availability of mammalian cell cultures are reflected in the β -dispersion parameter characteristic frequency (f_C) and the on-line dual frequency permittivity signal. Multi-frequency permittivity measurements were therefore evaluated in fed-batch cultivations of two different CHO cell lines. Similar responses to nutrient depletions and discontinuous feed additions were monitored in different cultivation phases and experimental setups. Sudden increases in permittivity and f_C occurred when feed additions were conducted. A constant or declining permittivity value in combination with a decrease in f_C indicated nutrient limitations. f_C correlated well with changes in oxygen uptake rate when cell diameter remained constant, indicating that metabolic activity is reflected in the value of f_C . When significant cell size changes occurred during the cultivations, the analysis of the β -dispersion parameters was rendered complex. For the application of our findings in other systems it will

be hence required to conduct additional off-line measurements. Based on these results, it is hypothesized that multi-frequency permittivity measurements can give information on the intracellular or physiological state in fed-batch mode. Similar observations were made when using different cell lines and feeding strategies, indicating that the findings are transferable to other cell lines and systems. The results should lead to an improved understanding of routine fed-batch processes. Additional studies are, however, required to explore how these observations can be used for fed-batch process development and optimization.

Keywords Permittivity · Physiological state · Fed-batch · CHO cell culture · Characteristic frequency

Abbreviations

BMS/Fogale BMS	Fogale Biomass System [®]
CASY	CASY [®] 1 system
C_M	Capacitance per membrane area ($F\ m^{-2}$)
$\Delta\epsilon_{\text{Fogale}}$	(online dual-frequency) permittivity signal; 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)

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$\Delta\varepsilon_{\max} * f_C$	Mathematical product of $\Delta\varepsilon_{\max}$ and f_C
eq.	Equation
f_C	Characteristic frequency
fig.	Figure
hema	Hemocytometer
N	Cell density
P	Volume fraction of cells (biovolume)
pH _i	Intracellular pH
r	Cell radius
VC	Beckman Coulter Vi-CELL XR TM system

Introduction

Fed-batch processes continue to be the most relevant culture mode for the vast majority of high-yielding commercial bioprocesses due to their ease of operation and reliability (Wurm 2004; Chee Fung Wong et al. 2005). Improvement in process monitoring and subsequent optimization of CHO cell-based processes is consequently of particular interest for the production of biotherapeutics (Chee Fung Wong et al. 2005). It can be hypothesized that advances in the optimization of bioprocesses can in particular be achieved by an improved understanding and control of the metabolic activity and physiological state of a cell population. However, with current sensor technology, on-line monitoring of the physiological state remains a challenging task, rendering its use as a bioprocess control parameter complex (Konstantinov 1996; Henry et al. 2007).

Permittivity-based in situ probes are now an established tool for the measurement of biomass in real-time in a wide variety of cell culture processes (Kamen et al. 1996; Zeiser et al. 1999; Elias et al. 2000; Zeiser et al. 2000; Ducommun et al. 2001, 2002; Cannizzaro et al. 2003). Permittivity is a direct measure of the membrane enclosed volume fraction or biovolume of the cell suspension. These measurements also reflect changes in cell physiology which has been demonstrated for several cell types (Fehrenbach et al. 1992; Noll and Biselli 1998; Zeiser et al. 2000; Ducommun et al. 2002; Cannizzaro et al. 2003; Sarrafzadeh et al. 2005).

Multi-frequency permittivity measurements can be used to monitor nutrient limitations during batch

cultivations of CHO cells (Ansorge et al. 2010). In this previous study, a decrease in the on-line dual frequency permittivity signal ($\Delta\varepsilon_{\text{Fogale}}$) was an indicator for a metabolic shift after glutamine depletion. This decrease was caused by a drop in the characteristic frequency (f_C) which is a function of the intracellular conductivity (σ_i). It was possible to identify σ_i as the parameter that was responsible for the decrease in $\Delta\varepsilon_{\text{Fogale}}$ and f_C . Changes in the nutrient availability are consequently reflected in the β -dispersion parameter characteristic frequency (f_C) and the permittivity signal ($\Delta\varepsilon_{\text{Fogale}}$).

We consequently evaluated the use of on-line permittivity measurements as monitoring tool in fed-batch cultivations of two different CHO cell lines (CHO I and II). It was hypothesized from previous results that the β -dispersion parameters are indicative of changes in metabolic activity or physiological state of cell cultures. We therefore investigated if nutrient additions, typically performed in discontinuous mode (bolus additions), would have an effect on f_C and $\Delta\varepsilon_{\text{Fogale}}$. The results give first insights into how permittivity measurements might provide additional information on, and understanding of, the physiological state of mammalian cell cultures.

Materials and methods

Bioreactor setup

A benchtop Biostat MCD (B. Braun Biotech, Melsungen, Germany) (3 L total volume) as previously described was employed for cultivations in lab scale (Ansorge et al. 2007b).

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 (Aqua-sant 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

Two different suspension-grown in-house CHO cell lines were employed in this study. First, a CHO K1/dhfr⁻ (dehydrofolate reductase negative) host cell line was cultivated in fed-batch mode (Ansorge et al. 2010).

Second, cultivations were conducted with a recombinant CHO cell line, expressing a human monoclonal antibody (CHO II). An in-house medium mixture (DHI) was used as basal medium for both CHO cell lines (Schlaeger 1996). Final nutrient concentrations, e.g. glutamine and glucose concentrations and other media supplements, differed for each medium composition and were cell line dependent. Discontinuous feeding (bolus addition) consisted of an addition of 4% (v/v) (CHO I) or 2% (v/v) (CHO II) of a concentrated nutrient mixture per day.

Metabolite and product analyses

Amino acid analysis was performed on a HP 1100 Series HPLC (Palo Alto, CA) as suggested by the manufacturer (Gratzfeld-Huesgen 1998). Glucose concentration was determined with a Beckman Coulter Glucose Analyzer 2 system (Beckman 1999).

The human monoclonal antibody produced by cell line CHO II was quantified by a standard sandwich ELISA assay and by ProSep A chromatography (Millipore, Billerica, MA) according to the manufacturer's instructions.

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 manual cell counting in a Neubauer hemacytometer. A CASY®1 device (Innovatis AG, Bielefeld, Germany) was employed to determine mean cell diameter and 'total biovolume (CASY)'. Furthermore, the Vi-CELL™ XR system (VC) (Beckman Coulter, Fullerton, CA) was used for automated cell counting and for measurement of viability and cell size. The VC allows distinguishing between the diameters of non-stained (viable) and stained (non-viable) cells. Total biovolume (PCV) was measured using disposable PCV measurement tubes (Techno Plastics Products AG, Trasadingen, Switzerland) (Stettler et al. 2006).

Permittivity measurements with the Fogale Biomass System® (BMS)

The underlying theory on the dielectric properties of biological cells has been extensively described

elsewhere (Kell and Harris 1985; Harris et al. 1987; Pethig and Kell 1987; Markx and Davey 1999).

In summary, the permittivity of a cell suspension can be easily measured because a characteristic fall in its value, the β -dispersion, occurs with increasing frequency. This decrease is caused by the polarization of cell membranes and characterized by several β -dispersion parameters. The first parameter, the dielectric increment ($\Delta\epsilon_{\max}$) can be measured as the difference in permittivity at the low-frequency and the high-frequency plateau and is defined by the equation of Schwan (1957) (Eq. 1):

$$\Delta\epsilon_{\max} = \frac{9 \times r \times P \times C_M}{4} \quad (1)$$

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 the cell radius (m), N the cell density (m^{-3}), C_M the capacitance per membrane area ($F m^{-2}$), and P the volume fraction of cells (biovolume) defined by:

$$P = \frac{4}{3} \times \pi \times r^3 \times N \quad (1.1)$$

The second parameter, the characteristic frequency (f_C) is the frequency at which a permittivity of $\frac{\Delta\epsilon_{\max}}{2}$ is reached. f_C is a function of cell size, capacitance per membrane area (C_M) and also intracellular (σ_i) and medium conductivity (σ_m) and defined by a simplified equation (Eq. 2) (Harris et al. 1987):

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

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

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 (Cole and Cole 1929). α is believed to increase when the distribution in cell electrical properties widens in the cell population (Davey 1993).

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 . Here, commercial in situ autoclavable DN 12 and DN 25 probes were employed for the lab and pilot scale

bioreactor system, respectively. The system setup was performed according to manufacturer's instructions. The 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 $\Delta\epsilon_{\text{max}}$ (Eq. 1) (Schwan 1957). 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 correlating with the biovolume even in the case of cell size changes (FogaleNanotech 2004). The BIO-MASS+ software automatically analyzes the permittivity over a total of 20 frequencies from 0.3 to 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

The two CHO cell lines (CHO I and CHO II) used in this work showed differences when correlating the permittivity signal ($\Delta\epsilon_{\text{Fogale}}$) from preliminary batch and fed-batch experiments to off-line cell counts and biovolume measurements (Table 1). Linear regression resulted in a linear relationship ($R^2 = 0.86$) of the viable cell count and $\Delta\epsilon_{\text{Fogale}}$ for cell line CHO I in batch experiments (during which only small cell size changes were observed). For the same cell line, a lower R^2 value ($R^2 = 0.76$), was observed for total cell count and $\Delta\epsilon_{\text{Fogale}}$, caused by values from later stages of the cultivation when viability and $\Delta\epsilon_{\text{Fogale}}$ decreased. In contrast, the permittivity signal correlated better with the total cell count for cell line CHO II ($R^2 = 0.89$ vs. 0.74 for the viable cell count). In later cultivation phases this effect seemed to be even

Table 1 Linear regression values for permittivity signal ($\Delta\epsilon_{\text{Fogale}}$) (Y) and off-line cell counting/biovolume determination (X)

A		Slope $\left(\frac{\text{pF}}{10^6 \text{cells} \times \text{cm}}\right)$	R^2
CHO I	vcc	0.82	0.86
	tcc	0.58	0.76
CHO II	vcc	1.45	0.74
	tcc	1.22	0.89
B		Slope $\left(\frac{\text{pF} \times \text{mL}}{\mu\text{L} \times \text{cm}}\right)$	R^2
CHO I	CASY	0.926	0.99
	PCV	0.675	0.99
CHO II	CASY	1.35	0.97
	PCV	0.54	0.98

Remarks: vcc: viable cell count (hema); tcc: total cell count (hema); data from a total of four cultivations were included in the table: (CHO I: 1 batch, 1 fed-batch cultivation (both in 25 L scale); CHO II: 1 batch, 1 fed-batch cultivation (both in 2 L scale))

(A): Values for regression of off-line cell counts (X) and $\Delta\epsilon_{\text{Fogale}}$ (Y); only data from batch cultivations are represented (see text for details); cell diameter variation for samples represented in the table: $13.78 \pm 0.72 \mu\text{m}$ (CHO I), $16.97 \pm 0.82 \mu\text{m}$ (CHO II)

(B): Values for regression of biovolume measurements (CASY and PCV) (X) and $\Delta\epsilon_{\text{Fogale}}$ (Y)

Only samples for which viability was $>70\%$ were considered for the regression

more pronounced, but cell diameter and viability changes made it difficult to interpret the results (data not shown). The differences in slope (i.e. the permittivity signal per cell) were most likely related to larger cell diameters of cell line CHO II and other cell-specific properties. It was also observed for cell line CHO I that, at the end of batch cultivations in different scales, $\Delta\epsilon_{\text{Fogale}}$ returned to its baseline value when viability tended to zero. Yet, for cell line CHO II, a remaining permittivity signal was observed when viability had reached zero. This value was clearly caused by non-viable/stained cells as disintegration of the cell suspension by sequential ultrasonication decreased the signal close to the baseline value before inoculation (results not shown). As viability and cell size changes occurred simultaneously in fed-batch cultures, these data were not considered for the regression values in Table 1A.

For both cell lines, a regression of $\Delta\epsilon_{\text{Fogale}}$ and off-line biovolume measurements (PCV and CASY) resulted in very high R^2 values for batch and fed-batch cultures, being independent of cell size changes in fed-batch and decreasing viability until very late stages of the cultures (Table 1B).

These results appeared to be independent of the chosen bioreactor scale, with cultivations in different bioreactor systems resulting in slightly different but overall similar correlations for both cell lines (Ansorge et al. 2010 and unpublished data). This indicates that the permittivity signal is not in all cases reflecting off-line viable cell counts by dye exclusion. The degree to which non-viable cells contribute to the permittivity signal seems to rather depend on the level of disintegration of cells after inactivation or the conservation of membrane properties (Patel et al. 2008a, b).

In a subsequent fed-batch cultivation with cell line CHO I, the goal was first to evaluate the impact of feed additions on $\Delta\epsilon_{\text{Fogale}}$ and f_C . We hence

performed feed additions at different physiological states (Fig. 1), expecting that both parameters would reflect the metabolic activity/nutrient availability of the cultures. The cultivation could be divided in four distinct phases (FB I–IV). During phase FB I (0–60 h), cells started growing exponentially with a specific growth rate of $\mu = 1.59 \pm 0.14 \text{ d}^{-1}$ (Fig. 1b). When the cell density reached $4 \times 10^6 \text{ cells/mL}$, the first feed addition ($\sim 60 \text{ h}$) was performed at a time when none of the analyzed nutrients was depleted (amino acid data not completely shown). A small decrease of $\Delta\epsilon_{\text{Fogale}}$ was observed at the time of the feed addition representing the dilution effect of the bolus addition. Otherwise there was no change in $\Delta\epsilon_{\text{Fogale}}$ or its slope. Our hypothesis is that the physiological state of the culture was not significantly changed after this first feed addition.

The second addition took place when glutamine was already depleted and alanine started to get consumed. Under these conditions, $\Delta\epsilon_{\text{Fogale}}$ leveled

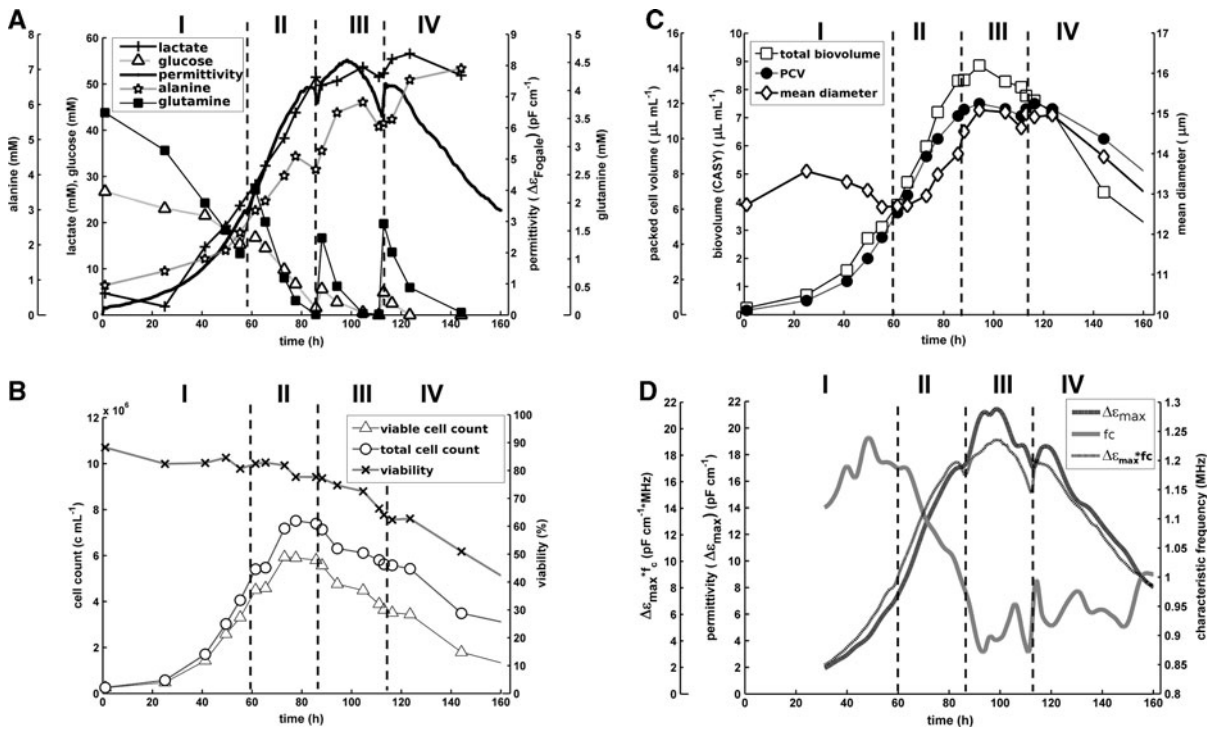


Fig. 1 Fed-batch cultivation of cell line CHO I in pilot scale. Feed additions are marked by vertical dashed lines and divide the cultivation in phases FB I–IV. **a** permittivity and metabolite

concentrations. **b** viability and cell counts (hema). **c** off-line biovolume (CASY, PCV)/cell size (CASY) measurements. **d** β -dispersion parameters

off (~ 85 h) and its slope tended to zero. This time the addition resulted in a significant change of $\Delta\varepsilon_{\text{Fogale}}$. The third and last feed addition (~ 112 h) was deliberately conducted at a time when the culture had already entered death phase and the viability had decreased substantially. $\Delta\varepsilon_{\text{Fogale}}$ had a negative slope and glucose as well as glutamine were depleted. At that time, both lactate and alanine were consumed. Adding extra nutrient resulted in a less prominent change of the on-line signal.

We observed significant changes in cell diameter during phase FB I, similar to those observed at the start of the cultivation for previously reported batch experiments (Ansorge et al. 2010) (Fig. 1c). A striking difference compared to the batch runs was a dramatic increase in cell diameter from ~ 12.5 to $15 \mu\text{m}$ starting at ~ 70 h (phase FB II) until late stages of the culture (phase FB IV). As in batch mode, we observed that changes in biovolume could not explain sudden changes in $\Delta\varepsilon_{\text{Fogale}}$ at the time of nutrient limitations and additions. The data for PCV and total biovolume (CASY) did not follow the sudden changes in $\Delta\varepsilon_{\text{Fogale}}$ after or before the feed additions in phases FB III–IV. The OUR, however, roughly matched the pattern of $\Delta\varepsilon_{\text{Fogale}}$, showed similar changes after, and more significant decreases before the three feed additions (not shown).

We analyzed the β -dispersion parameters ($\Delta\varepsilon_{\text{max}}$, f_C , $\Delta\varepsilon_{\text{max}} * f_C$) to further characterize the cultivation (Fig. 1d). f_C increased at the beginning of the cultivation (~ 25 – 45 h), probably because of a decreasing cell diameter (Fig. 1c). At the end of phase FB I and immediately after the first feed addition (in phase FB II), f_C remained constant at ~ 1.2 MHz. This supported our initial hypothesis after which there was no change in the physiological state of the culture (because of no change in $\Delta\varepsilon_{\text{Fogale}}$ or its slope). f_C decreased in phase FB II, most likely as a result of the increase in cell size after ~ 63 h (Eq. 1). This decrease continued until ~ 78 h when $\Delta\varepsilon_{\text{Fogale}}$ leveled off and glutamine was depleted. Although the second feed addition should have resulted in a change in the nutrient availability there was no direct change in f_C . Thus, f_C was affected by changes in nutrient state as well as cell size which is in-line with the underlying theory (Schwan 1957; Harris et al. 1987; Ansorge et al. 2010). This became even more evident in phase FB III when cell size remained almost constant for the

entire cultivation phase and the first part of phase FB IV. At that time (~ 90 h), f_C increased sharply when none of the analyzed nutrients were limiting and dropped shortly after (~ 110 h) as soon as glutamine and glucose were depleted and alanine was again consumed. At that point, changes in f_C seemed to indicate a metabolic shift as described for previous batch cultivations (Ansorge et al. 2010). The following third and last feed addition (~ 115 h) caused a sudden increase in f_C , indicating higher metabolic activity and improved physiological state.

$\Delta\varepsilon_{\text{max}}$ followed the pattern of $\Delta\varepsilon_{\text{Fogale}}$ very closely and only showed a slightly different profile in phase FB III, during which it gave a higher relative value compared to $\Delta\varepsilon_{\text{Fogale}}$. We could also observe that $\Delta\varepsilon_{\text{max}} * f_C$ gave an identical pattern compared to $\Delta\varepsilon_{\text{Fogale}}$. $\Delta\varepsilon_{\text{Fogale}}$ is only dependent on the biovolume and the intracellular conductivity σ_i when the pattern of $\Delta\varepsilon_{\text{max}} * f_C$ and $\Delta\varepsilon_{\text{Fogale}}$ are identical (Ansorge et al. 2010). Yet, it was impossible to draw conclusions based on the dataset of this cultivation because the biovolume (based on PCV and CASY) did not remain constant for an extended period (Fig. 1c).

Viability had already decreased significantly in phases FB III and IV. Yet, the underlying equations are only valid for cells at high viability. For this cell line, however, we observed that $\Delta\varepsilon_{\text{Fogale}}$ follows the viable cell density/biovolume in all cultivation phases (Table 1; Ansorge et al. 2010). This means that, for cell line CHO I, dead cells did not significantly contribute to the permittivity signal and indicates that $\Delta\varepsilon_{\text{Fogale}}$ only reflected changes in the dielectric properties of viable cells.

In summary, changes in the physiological state seemed to be reflected in $\Delta\varepsilon_{\text{Fogale}}$ and f_C in fed-batch mode with cell line CHO I (in particular after feed additions). However, cell size was significantly changing during the cultivation. As $\Delta\varepsilon_{\text{Fogale}}$ is a function of the biovolume of the cell suspension it is directly affected by changes in cell size (Eq. 1). f_C is also directly related to the cell diameter (Eq. 2) and it was therefore difficult to draw conclusions on the reasons for the changes in the different parameters.

After this first evaluation of the impact of feed additions on $\Delta\varepsilon_{\text{Fogale}}$ and f_C using the host cell line CHO I, we continued our efforts to evaluate permittivity measurements in fed-batch cultivations of a recombinant cell line (CHO II).

A lab scale fed-batch cultivation was conducted. Feed additions were performed independent of changes in the permittivity signal ($\Delta\epsilon_{\text{Fogale}}$), following a strictly time-based feeding strategy. At each feed addition, 2% (v/v) of a concentrated nutrient mix was added (Fig. 2a). The first feed addition was performed at ~ 50 h cultivation time at a cell density of $\sim 1.5 \times 10^6$ c/mL. One feed addition per day was then performed over a period of 9 days. The maximum in viable cell density of 3.5×10^6 c/ml was reached after ~ 100 h. The viable cell count then remained constant over a period of 3 days until viability decreased significantly after ~ 170 h. The total cell count, however, kept increasing and this was most likely the reason why $\Delta\epsilon_{\text{Fogale}}$ showed an increase until the beginning of death phase at ~ 170 h. Stained cells can, depending on cell line and bioreactor system, contribute significantly to the permittivity which seemed to have been the case for cell line CHO II (Table 1; Davey 1993; Guan et al. 1998; Ducommun et al. 2002; Cannizzaro et al. 2003; Ansoerge et al. 2007a).

Similar to previously described cultivations we divided this run in phases FB I–X (Fig. 2a). A sudden slope change and an increase in $\Delta\epsilon_{\text{Fogale}}$ was observed after each feed addition from phase FB II–VI (Figs. 2a, 3). Thereafter, $\Delta\epsilon_{\text{Fogale}}$ leveled off and only ~ 10 h after each feed addition its slope tended to zero again. When viability had significantly decreased (FB VII–X), $\Delta\epsilon_{\text{Fogale}}$ did not further increase but rather showed a continuous decrease with sudden short rises when feed was added.

The cell diameter did not change significantly in the first part of the cultivation (until ~ 140 h) with average values of $16.57 \pm 0.25 \mu\text{m}$ (CASY) and $16.61 \pm 0.25 \mu\text{m}$ (VC) (Fig. 2b). This was a significant difference compared to the fed-batch cultivation of CHO I (see Fig. 1). Dramatic increases in cell size occurred in both cultivations when nutrient concentrations had significantly decreased, i.e. starting at around ~ 70 h for CHO I but not before ~ 150 h for CHO II. At that time, viability dropped in both cases and the biovolume had almost reached its maximum value.

For cell line CHO II, the mean diameter increased until death phase started in phase FB X (~ 250 h). At high viability, the viable mean diameter (VC) showed an almost identical pattern but increased much more starting in phase FB V and VI from a mean of ~ 16.5 to $\sim 23 \mu\text{m}$ in phase FB X. In contrast, the mean

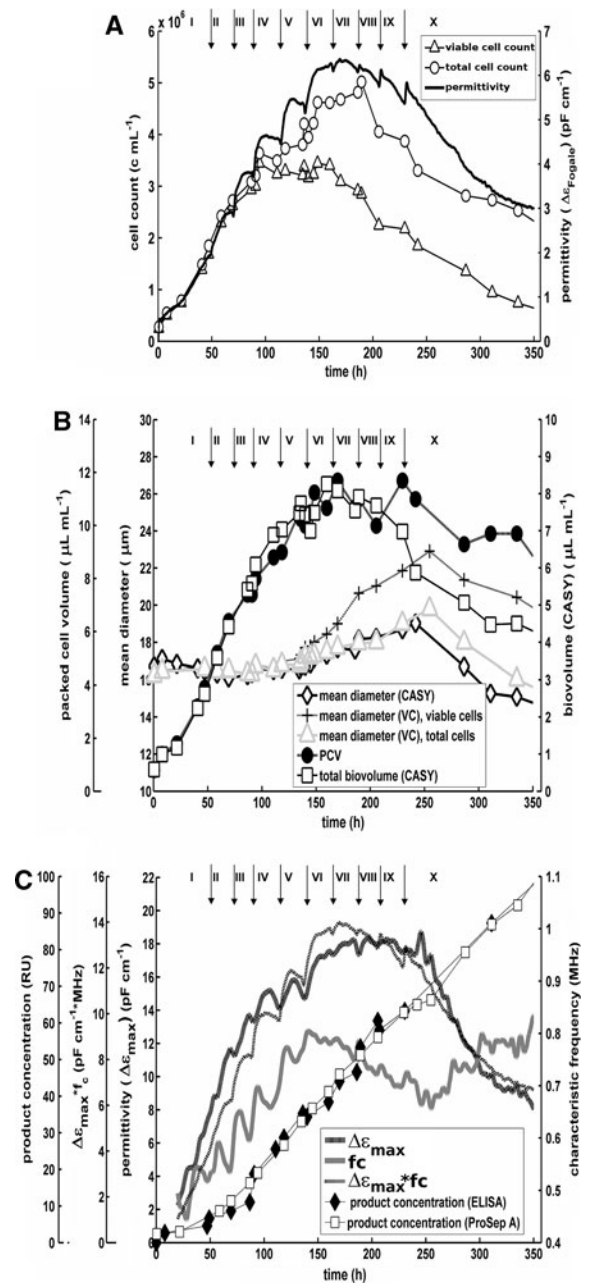
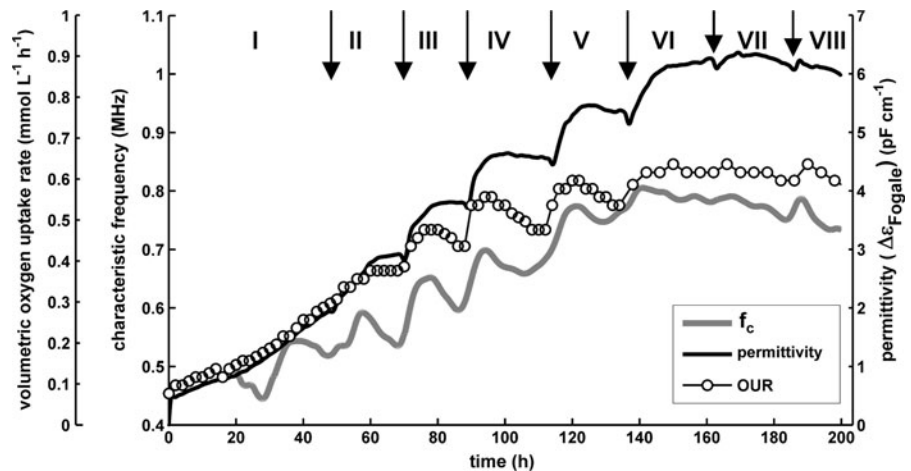


Fig. 2 Fed-batch lab scale cultivation of CHO II. Feed additions are marked by vertical arrows and divide cultivation in phases FB I–X. **a** permittivity and cell counts (hema). **b** biovolume measurements and cell diameter. **c** β -dispersion parameters and product concentration; product concentration, increased linearly over time from FB II–FB X: $\text{conc (mAb(ProSep A))} = 0.31 \times -8.7, R_2 = 0.997$ (regression not shown in figure)

diameter of stained cells (VC) was showing constant values ($\sim 14 \mu\text{m}$) over the whole time course of the cultivation (not shown).

Fig. 3 On-line measurements until 200 h of fed-batch cultivation with cell line CHO II (graph presents data for same cultivation also shown in Fig. 2)



The off-line biovolume measurements (PCV and CASY) generally followed the pattern of $\Delta\epsilon_{\text{Fogale}}$ (Fig. 2b), explaining the overall increase in permittivity signal until ~ 150 h. In late phases of the process (FB VII–X), the PCV gave higher values relative to $\Delta\epsilon_{\text{Fogale}}$.

The product concentration showed a linear increase over the whole time course of the cultivation (Fig. 2c). Hence, volumetric productivity ($q_{\text{prod}} = 0.31$ RU/h (from phase FB II–X; see legend for Fig. 2c)) was constant although the number of viable cells was decreasing after ~ 170 h. This might have possibly been an indication for the fact that the remaining viable cells (with a larger (viable) cell diameter (Fig. 2b)), showed a higher specific productivity. This observation was also observed in a batch cultivation of CHO II (not shown) and is supported by literature findings according to which productivity is also a function of cell size (Lloyd et al. 2000; Berdichevsky et al. 2008). Another possibility is that stained cells were partly metabolically active and contributed to antibody production in late stages of the culture. It could also not be excluded that cells growing on the inner surface of the bioreactor were still productive. A final conclusion explaining the product kinetics could therefore not be drawn because only a limited dataset was available for this study. These results also make clear that more studies are needed to further shed light on a relationship of on-line permittivity signals and their evolution, the physiological state, product formation and overall process performance.

For the analysis of the β -dispersion parameters (Fig. 2c) it is important to note that the cell diameter was almost constant from 20 to 140 h. Viability was also high ($>80\%$) during that time of the process. This was an advantage for the analysis of the calculated parameters compared to the previous CHO I fed-batch process.

We could observe a behavior of f_c which was much more corresponding to our expectations than the first fed-batch cultivation with cell line CHO I concerning the relationship of f_c and the physiological state/metabolic activity of the cell suspension. After each feed addition from phase FB II–IV, f_c increased significantly. It then decreased shortly after when $\Delta\epsilon_{\text{Fogale}}$ leveled (see also Fig. 3). This finding was in-line with our previous results according to which f_c indicated nutrient availability and limitations. A decrease in f_c and hence σ_i was indicating a depletion in nutrients in batch cultivations (Ansorge et al. 2010). During these batch cultivations, OUR and f_c correlated well at the time of glutamine depletion and the metabolic shift with regression coefficients of $R^2 > 0.82$ when cell size was constant (data not shown). During fed-batch cultivations, we observed here increases in f_c as responses to nutrient feed additions. The hypothesis that the changes in f_c were related to the metabolic activity was substantiated in fed-batch where a linear correlation of f_c and OUR was found from FB II–IX (Fig. 3, $R^2 = 0.92$; regression not shown), when cell diameter was subject to only small changes. Compared to the on-line permittivity signal, the OUR also correlated with $\Delta\epsilon_{\text{Fogale}}$

after the feed additions (phase FB II–VI; Fig. 3), although resulting in a lower R^2 value ($R^2 = 0.86$ for FB I–VIII). This means that the responses of f_C to the feed additions were also, in part, reflected in $\Delta\epsilon_{\text{Fogale}}$. The responses of the oxygen consumption to nutrient additions were more pronounced than the respective changes in the permittivity signal (Fig. 3). Whenever $\Delta\epsilon_{\text{Fogale}}$ leveled and its slope tended to zero the OUR decreased, reaching almost the lower value it showed before the feed addition. Changes in the oxygen uptake rate after the addition of nutrients have been described for hybridoma cultures (Ramirez and Mutharasan 1990). These authors found typical peaks in OUR after additions of limiting nutrients.

The results show that OUR and f_C better reflected changes in the metabolic activity of the culture whereas permittivity is rather an indicator of the biovolume (Fig 2b; Table 1). The changes in OUR found here were similar to those observed in f_C and $\Delta\epsilon_{\text{Fogale}}$. It was consequently hypothesized that decreases in OUR and f_C before the addition of feed were an indicator for a nutrient limitation of the culture. Yet, our standard metabolite analyses (amino acids, glucose) did not reveal any clear limitations or depletions of major nutrients during this cultivation (not shown). For cell line CHO II, the observed changes in $\Delta\epsilon_{\text{Fogale}}$ and f_C could hence not be attributed to one single nutrient component. They were possibly either a result of a complex interaction of changes in the concentration of several nutrients or caused by one or several unidentified medium components. In later stages of the culture (>150 h), an increase in cell size in combination with decreasing viability was probably responsible for the less pronounced responses to feed additions. During phases FB VI–IX, only small, temporary changes in f_C and $\Delta\epsilon_{\text{Fogale}}$ were observed when feed was added. Similar to previous findings, a significant continuous increase in f_C (from 0.7 to 0.8 MHz) marked the beginning of death phase in phase FB X.

Again, $\Delta\epsilon_{\text{max}}$ followed the pattern of $\Delta\epsilon_{\text{Fogale}}$ very closely during the first part of the cultivation. It showed a slightly different profile in phases FB VII–X (until ~ 250 h) with higher values relative to $\Delta\epsilon_{\text{Fogale}}$. This was probably related to the significantly increased cell diameter at that time and was in-line with the higher biovolume (Fig. 2b). As observed earlier, $\Delta\epsilon_{\text{max}} \cdot f_C$ gave an identical pattern compared to $\Delta\epsilon_{\text{Fogale}}$.

In summary, it was demonstrated how our previous findings from batch cultivations might be used when analyzing changes in β -dispersion parameters during fed-batch cultivations. When nutrient availability was high (CHO I, phase FB I), no responses to feed additions were observed in $\Delta\epsilon_{\text{Fogale}}$ and f_C . In contrast, sudden increases in $\Delta\epsilon_{\text{Fogale}}$ and f_C were observed in later culture stages at decreasing viability and viable cell count (CHO I, phases FB II/III and FB III/IV) but also at high viability and increasing viable cell count (CHO II, FB I–VI) in fed-batch cultivations of two different CHO cell lines. The OUR correlated well with changes in f_C when cell size remained constant during the fed-batch cultivation of CHO II. For cell line CHO I, a direct correlation of OUR and f_C in fed-batch was not possible because dramatic cell size changes occurred. However, batch cultivations resulted in comparable relationships at the time of glutamine depletion.

In addition to our previous findings (Ansoerge et al. 2010), an increase in f_C might therefore serve as an indicator of the nutrient availability/metabolic activity of mammalian cell cultures after feed additions. When the cell diameter remained constant in critical phases of the cultivation, the analysis was significantly simplified. In batch cultivations, this allowed identifying σ_i as underlying parameter indicating changes in the intracellular state of the culture after nutrient depletions (Ansoerge et al. 2010). For the fed-batch cultivations presented here, it was, however, not possible to unambiguously identify σ_i as responsible for the sudden increases in $\Delta\epsilon_{\text{Fogale}}$. This is due to the fact that σ_i is reflected in f_C but not directly determined by multi-frequency permittivity measurements. Despite the off-line measurement of cell size and biovolume it can consequently remain difficult to separate simultaneously occurring changes in biovolume, viability, cell diameter, C_M and σ_i which is consistent with other reports (Patel et al. 2008a, b). This complex analysis is certainly a challenge when interpreting datasets of β -dispersion parameters. However, the development of metabolic models for the determination of the physiological state with a full analysis of consumption rates for amino acids and other components is possibly an even greater challenge (Henry et al. 2007). Additionally, as we observed for cell line CHO II, analyses of concentrations of major nutrients do not necessarily lead to the identification of limitations in complex systems

(which is also in agreement with other non-published observations in our lab).

In general, permittivity measurements appear to be advantageous for processes in which a scale-up is intended and/or different scales need to be compared. This also holds for turbidity measurements whilst the calculation of the OUR is often hampered by the complexity of differences in mass transfer and aeration setups in different bioreactors. The OUR mainly indicates changes in the metabolism of cultures (Kussow et al. 1995; Zeiser et al. 2000). In contrast, turbidity and infrared sensors appear to measure the viable cell count during growth phases but also dead cells and debris in later phases of cultivations (Merten et al. 1987; Konstantinov et al. 1992; Ansoerge et al. 2007a). The majority of methods for biovolume measurements give identical patterns in situations where viability is high (growth phase) (Ansoerge et al. 2007b). It is generally more difficult in late phases to identify which part of any on-line signal is caused by debris or dead cells and which by viable cells.

Possible applications of the findings would include the on-line control of feed additions based on the permittivity signal although this approach would likely be sensitive to noise and algorithms including several process variables such as the β -dispersion parameter f_C are preferable. This could allow maintaining or driving mammalian cell cultures towards a more desirable productive state (Konstantinov 1996; Henry et al. 2007).

Conclusion

The on-line permittivity signal ($\Delta\varepsilon_{\text{Fogale}}$) and the characteristic frequency (f_C) were indicators of nutrient limitations and depending on nutrient availability. Responses to feed additions could be monitored in real-time in the values of $\Delta\varepsilon_{\text{Fogale}}$ and f_C . Correlations of OUR and f_C confirmed that the characteristic frequency reflects the metabolic activity. It is likely that changes in intracellular conductivity were the cause of the variations in the on-line signals, as was previously observed in batch cultivations. Permittivity measurements have thus the potential to give important information on the intracellular state of mammalian cell cultures.

Multi-frequency permittivity measurements provide more information on the culture than classical on-line monitoring methods but the resulting dataset seems to be more difficult to interpret. The most important information provided by the technology remains the real time in situ monitoring of the biomass content. Secondly, the metabolic activity and physiological state of mammalian cell cultures is reflected in the on-line signals. To obtain meaningful results and for the application of our findings in other systems it will be required to conduct additional off-line measurements for an 'in-depth' characterization of the respective production system.

We give here first insights into how permittivity measurements could be used for the development and optimization of fed-batch cultivations. This study is however only a first step towards an improved understanding of the metabolic/physiological and, in particular, productive state of cell cultures in fed-batch mode. Future work will have to investigate the relationship of nutrient limitations, f_C , σ_i and other parameters such as the OUR to reveal possible underlying mechanisms and directly provide usable process control parameters from the measurements. It is possible that improvements in productivity can be achieved when these measurements are employed to identify and minimize nutrient limitations or control feed additions.

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