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ON-LINE MONITORING OF CELL SIZE DISTRIBUTION IN MAMMALIAN CELL CULTURE PROCESSES

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Abstract: In this work, mammalian cell cultures were monitored on-line by measuring the culture capacitance over a range of excitation frequencies. The data collected during a fed-batch culture were used to build partial least squares (PLS) models for predicting the cell concentration, viability and size distribution. The models were validated on cultures performed in batch and perfusion modes and the predictions agreed well with off-line measurements performed with a Coulter Counter. The capacitance signals can also be used to follow the evolution of the mean cell diameter during a culture. Cell size distribution provides valuable information about the physiological state of cells in culture. Capacitance measurements at multiple frequencies can thus be a useful tool for the development, optimization, monitoring and control of cell culture processes.

Keywords: mammalian cell culture, on-line monitoring, capacitance, PLS modelling, cell size distribution.

1. INTRODUCTION

The reliable measurement of viable biomass is one of the most important parameters to monitor in every biotechnology process and especially in mammalian cell culture. This is due to the fact that the amount of viable and thus metabolically active cells is closely related with productivity. Many processes employ dynamic nutrient feeding strategies to reach high cell and product concentrations. Ultimately, the performance of these strategies rely on a proper quantification of viable cells since this measurement is required to accurately evaluate specific yields and kinetic rates. This is especially important in long-term perfusion cultures during which process behaviour may vary considerably over time. A successful culture typically requires tight control of the feed rate, which must be constantly adjusted in response to changes in cell density.

This has led to the development of numerous techniques for measuring the biomass content of a bioreactor. The most direct measurement methods such as light microscope counting/dye exclusion, flow cytometry and electrical cell counter are generally not applicable for on-line monitoring purposes. Moreover, these off-line methods are based upon frequent sampling and are resulting in additional risks of contamination. Consequently, many monitoring methods using indirect measurements have been proposed. Most approaches are based upon the correlation of metabolic rates with biomass or cell density e.g. glucose (Ducommun et al. 2001; Pelletier et al. 1994) or oxygen (Zhou and Hu 1994) uptake rates.

In recent years, many in-situ probes have been developed for the direct on-line determination of cell concentration based on several principles: infrared measurements (Merten et al. 1987), in situ microscopy and laser-based image analysis (Joeris et al. 2002), fluorescence measurements (Sonnleitner et al. 1992) and capacitance measurements (Degouys et al. 1993). The latter is becoming an established tool for the estimation of viable biomass in cultures of

many different cell types including yeast, bacteria, plant cells, insect cells and mammalian cells (Konstantinov et al. 1994; Markx et al. 1991; Zeiser et al. 1999). This method is based on the fact that biological cells act like small capacitors during the application of an electrical field to a cell suspension. The amount of biomass in a bioreactor can thus be correlated with the measured capacitance of the cell suspension.

Cell size distribution is also an important physiological parameter that can provide valuable information for process development, optimization and control. The size distribution is intimately link with the cell cycle and was also related with the specific productivity (Lloyd et al. 2000). Changes in cell size are already established as a tool to monitor the success of viral infections in culture (Henry et al. 2004). Normally, particle size distribution is determined by off-line methods involving a series of manual steps such as sample dilution and aggregates disruption. These steps may greatly affect the quality of the measurement. On-line size distribution analysis directly in the bioreactor would be more advantageous to get real-time information and avoid any interference with external sample manipulations.

In the present study, we have assessed the possibility of correlating online capacitance performed at several frequencies with changes in the cell size distribution. Using data from a fed-batch experiment as a calibration set, PLS models were built and used to predict the cell concentration, cell viability, cell diameter and size distribution. These models were validated by independent data from other experiments performed in batch and perfusion modes.

1.2 Principle of culture capacitance measurement

Applying an electrical field to a suspension of biological cells causes a polarization of the cells due to their insulating plasma membrane. Ions in the suspending medium as well as in the cytoplasm move toward the oppositely charged electrodes and are stopped by intact plasma membranes and cause the formation of a dielectric bilayer at the cell surface. Hence, biological cells act like tiny capacitors in an electrical field. Measuring the capacitance at alternating currents with different frequencies causes a spectrum which is called the "β-dispersion" (Figure 1). The capacitance is decreasing in the range of the β-dispersion starting on a low frequency plateau that is produced by the complete polarization of the cell membrane. At low excitation frequencies, there is sufficient time for the cells to become completely polarized, and the capacitance of the solution is high. As the excitation frequency is increased, the capacitance will decrease because of incomplete polarization. The high frequency plateau is due to the polarization of suspending medium or liquid when the frequency is too high to cause polarization of the cell membrane. As shown in Figure 1, the ΔC increases in direct proportion to the biomass and the critical frequency ($f_{C,}$, at which the capacitance is half-way between the two plateau values) depends mainly on the cell diameter and the conductivity. An increasing cell size causes an increase in the lower frequencies of the β-dispersion and finally leads to a smaller critical frequency. On the other hand, a decreasing cell size leads to lower capacitance values for the low frequencies and results in an increased critical frequency.

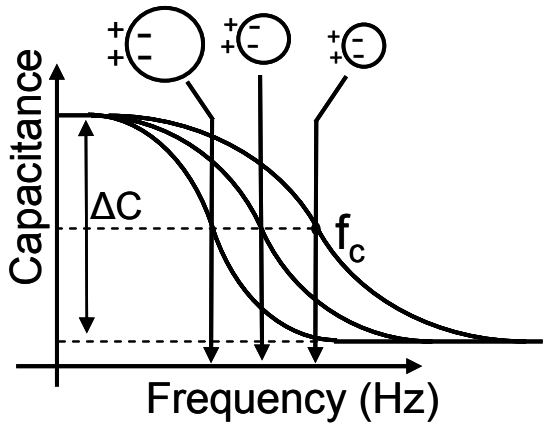

Fig. 1. Principle of culture capacitance measurements at several frequencies. ΔC is mostly a function of the biomass concentration. The characteristic (or critical) frequency f_c is the frequency at which the rate of polarization is one-half complete. f_C depends mainly on the cell diameter and the conductivity. (figure adapted from Cannizzaro *et al.* (2003)).

2. MATERIALS AND METHODS

2.1 Cell line and culture medium

All experiments were conducted using HEK-293 SF cells that are derived from the human kidney fibroblasts. Cells were grown in HSFM medium (Gibco) and were inoculated in the bioreactor at around $0.3x10^6$ cells/ml.

2.2 Bioreactor setup

The batch and perfusion cultures were performed in a controlled 3.5 L Chemap CF-3000 bioreactor (Mannedorf, Switzerland) equipped with 3 surface baffles. Agitation by two marine impellers was set at 100 RPM. The pH (Ingold pH probe, Andover, MA) was maintained constant at 7.2 by the addition upon demand of a 1N NAOH solution or adjusting the fraction of CO_2 in the gas inlet. Oxygen was supplied via surface aeration or sparging to keep the dissolved oxygen at 40 % air saturation. A water jacket maintained the temperature constant at 37°C. Off-gas measurements were performed using a Servomex 1400 infrared analyser (Norwood, MA). The bioreactor was also equipped with a dissolved CO_2 probe (YSI 8500 CO_2 monitor, Yellow Springs, OH). Process data were logged to a computer using the FIX software (Intellution, MA). For perfusion

culture, cells were retained in the bioreactor using an acoustic separator (BioSep 10L, Applikon Inc., Foster City, CA). Three pumps (Watson-Marlow Inc, Wilmington, MA) were used for medium feed, culture harvest and recirculation.

2.3 Capacitance and cell size distribution measurements

Cell size distribution was evaluated off-line using a Coulter Counter model Z2 (Beckman). Prior to measurement, a dilution of the cell suspension of 1:250 was performed. The upper and lower analysis limits were 7 and 30 μm, respectively. Culture capacitance was measured on-line using a Biomass Monitor 220 (Aber Instruments Ltd., Aberystwyth, UK) that allows the scanning over a frequency range of 0.1 MHz to 20 MHz. The time between the scans was set to 4 minutes.

3. RESULTS AND DISCUSSION

Figure 2A presents the total and viable cell concentration profiles during a fed-batch experiment. A simple discontinuous addition of glutamine was implemented as the feeding strategy. The bioreactor was inoculated at around $0.2x10^6$ cells/mL and a maximum cell density of $10x10^6$ cells/mL was achieved at 180 h. The culture was run until the viability decreased to around 50 %.

The corresponding raw capacitance measurements are shown in Figure 2B. Capacitance was measured at 25 frequencies ranging from 0.1 to 20 MHz. The onset of sparging at 65 h and the various glutamine additions had no evident effect on the capacitance profiles. Signals corresponding to the lower frequencies had the greatest amplitudes, but were also more corrupted by noise. To remove any potential effect due to background noise, the capacitance signal corresponding to the highest frequency (20 MHz) was subtracted from the signals collected at all lower frequencies. The filtered and variance-scaled signals are shown in Figure 2C.

During the exponential growth phase, the changes in cell size and other cellular properties are usually small. This was reflected in the capacitance signals which remain fairly similar at the beginning and throughout the exponential phase. However, notable differences were observed during the stationary and decline phases of the culture. Capacitance profiles measured at low frequencies exhibited a decrease concomitant to the reduction in viable cell concentrations. In contrast, high frequency signals showed almost no reduction and had a similar trend to that of total cell concentrations. This provided an early indication that meaningful information can be derived by considering the whole capacitance spectrum.

On-line and off-line measurements performed during this experiment were combined to build models describing the cell concentration, viability and size distribution as a function of the different capacitance signals. Partial least-squares was used to circumvent the collinearity problem due to highly correlated input data.

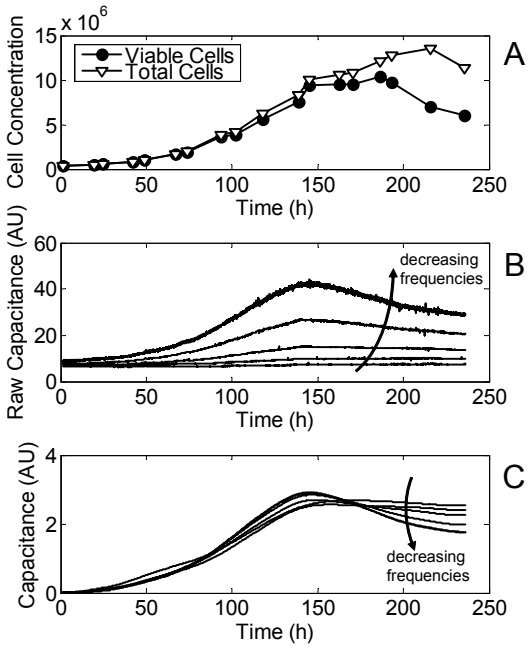

Fig. 2. On-line monitoring of capacitance at various frequencies during a fed-batch culture of mammalian cells. A) Viable and total cell concentration profiles determined by off-line microscope measurements B) Raw measurements from the multi-frequency capacitance system C) Filtered and scaled capacitance signals.

Results from the viable cell concentration PLS model with two latent variables are shown in Figure 3. The root mean square error of calibration was $9x10^5$ cells/mL. Adding another factor (latent variable) did not improve significantly the root mean-squared error. The obtained model was validated by comparing the predicted viable cell concentrations with independent experimental data collected during batch and perfusion cultures. The root mean square errors of prediction were $7.5x10^5$ cells/mL for the batch and $3.5x10^5$ cells/mL for the perfusion. Significant deviations were only observed in the late stage of the perfusion culture. These deviations were likely the result of changes in the conductivity of the culture medium due to various concentrate additions performed during the culture (data not shown). Significant aggregation and cell adhesion to the bioreactor wall were also observed and may have contributed to the measurement error.

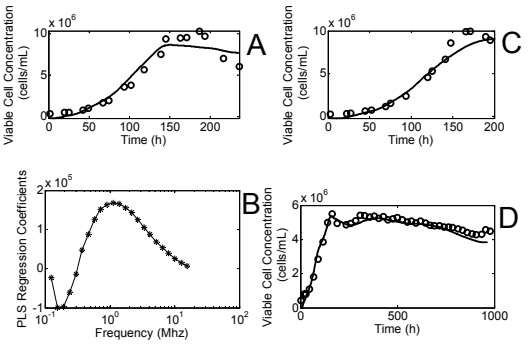

Fig. 3. On-line viable cell concentration estimation. A) PLS model calibration for viable cell number using data from a fed-batch culture. B) PLS model coefficients C) Viable cell estimation during a batch culture D) Viable cell estimation during a long-term perfusion culture

A similar approach was followed to model the cell viability and the results are depicted in Figure 4. The root mean square error was 5.8 % for the calibration, 5.6 % for the batch and 6.5 % for the perfusion. A slight offset is noted in the prediction for the perfusion culture (Figure 4D), again likely due to differences in the culture medium composition. From the regression coefficients, we note that the cell viability is negatively correlated with the measurements performed at low frequencies. Overall, these results demonstrate that the calibrated model is consistent with the experimental data and can be suited for close monitoring of a long-term perfusion run.

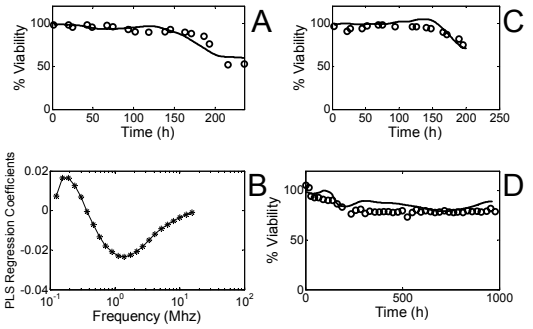

Fig. 4. On-line cell viability estimation. A) PLS model calibration for cell viability using data from a fed-batch culture. B) PLS Model coefficients
C) Cell viability estimation during a batch culture
D) Cell viability estimation during a long-term perfusion culture

In order to further interpret and understand the changes in the capacitance data, an analysis of the changes in the cell size distribution was undertaken. To this end, cells were arbitrarily divided into three groups: cells with a diameter smaller than 12 µm, cells between 12 and 16 µm and cells ranging from 16 to 25 µm. The last two groups were chosen around the mean cell diameter during the exponential phase (~16 µm), whereas the first group was chosen to encompass mostly non-viable cells.

As shown in Figure 5, during exponential growth, the culture is essentially composed of average and large cells and the proportions remain fairly constant. As expected from the drop in viability, when the cells enter the stationary phase, the proportion of small cells is increasing and there is a concomitant decrease in large cells. The regression coefficients obtained from the PLS model clearly shows that the concentration of large cells is correlated with low frequencies and negatively correlated with high frequencies. As expected, the opposite is true for the small cells. The root mean square errors of calibration were ranging from 2 to 4 % for the three groups.

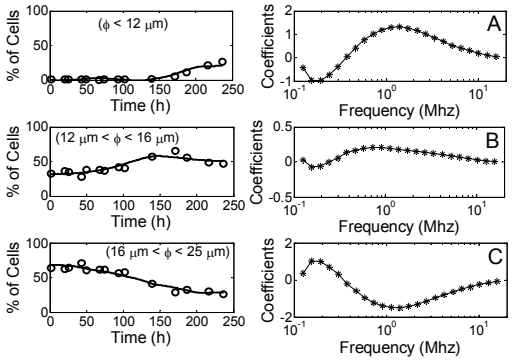

Fig. 5. PLS model calibration for the concentration of different cell sizes in a fed-batch culture.
A) Φ<12 µm B) 12<Φ<16 µm C) 16<Φ<25 µm

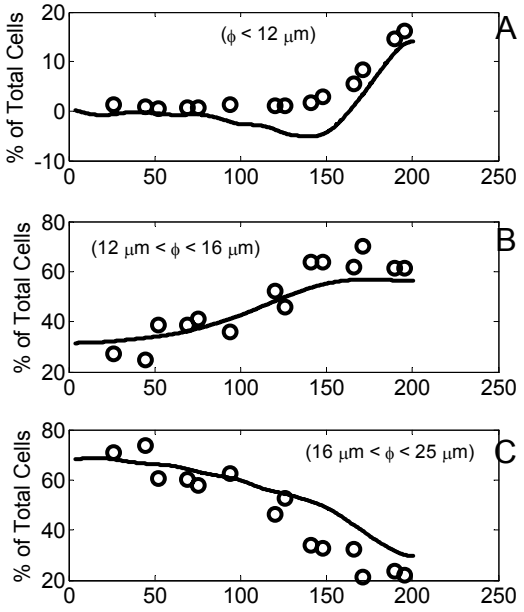

Fig. 6. On-line cell size distribution estimation. PLS models are compared with data derived from off-line coulter counter measurements.
A) Φ<12 µm B) 12<Φ<16 µm C) 16<Φ<25 µm

Compared with the experimental data from the batch experiment, predictions from the models agreed reasonably well for the three groups of cells defined

in the calibration dataset (Figure 6). The predicted measurement errors were 4.2, 6.8 and 9.8 % for the three groups. It should be emphasis that, if needed, the cell population can be split into more groups to obtain a more detailed cell size distribution.

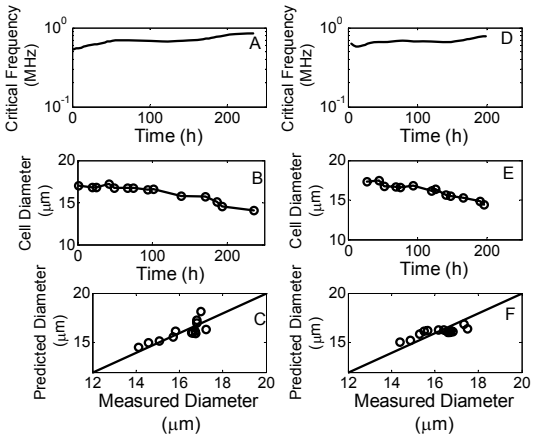

Fig. 7. Evolution of the critical frequency and mean cell diameter estimation. A) & D) Critical frequency profiles during fed-batch and batch cultures B) & E) Mean cell diameter profiles during fed-batch and batch cultures C) & F) Estimation of the average cell diameter from the critical frequency

From the capacitance spectrum, it is also possible to estimate the critical frequency (Figure 1), the point at which the fall in capacitance if half-completed. In turn, this characteristic frequency is expected to provide a good indication on the evolution of the average cell diameter. It should be mentioned that the conductivity of the suspending medium can also affect the critical frequency. The higher the conductivity, the higher will be the characteristic frequency because more ions in solution cause a higher rate of polarization. This effect is however much less significant for high-conductivity media, like in our case. The conductivity was also monitored during all the cultures and remained fairly constant (data not shown).

Values for the critical frequency obtained from the capacitance signals of the fed-batch culture are shown in Figure 7A. Throughout the culture, there is a trend towards an increasing critical frequency. This increase was caused by a decreasing capacitance at the low frequencies and consequently a lower relative volume represented by larger cells. Qualitatively, this observation corresponds with the results obtained from the Coulter Counter for this run and the decreasing volumetric mean cell diameter over time, also shown in Figure 7B. Similar trends for the critical frequency and the mean cell diameter were observed in the batch experiment (Figure 7D and E).

A simple regression model between the mean cell diameter and the inverse of the critical frequency was shown to give satisfactory results (Figure 7C) with an R^2 of 0.79 and a predicted measurement error of 0.61 μm. More importantly, this model derived from the fed-batch experiment was also able to adequately describe the data collected during the batch experiment (Figure 7F). The R^2 was 0.76 and the predicted measurement error was 0.59 μm.

4. CONCLUSIONS

Capacitance measurement is now an established tool for the online estimation of biomass. In this study, we have demonstrated that monitoring the capacitance at multiple frequencies allows not only to estimate viable cell concentration, but can also provide real-time quantitative information about the cell size distribution. As this is an important physiological parameter that can be related to cell growth, metabolism and productivity, such multi-frequency capacitance system can provide a useful tool for process development, optimization and control.

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