Online- and offline- monitoring of stem cell expansion on microcarrier

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Received: 9 February 2011 / Accepted: 22 April 2011 / Published online: 12 May 2011
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Abstract In the biopharmaceutical industry, adherent growing stem cell cultures gain worldwide importance as cell products. The cultivation process of these cells, such as in stirred tank reactors or in fixed bed reactors, is highly sophisticated. Cultivations need to be monitored and controlled to guarantee product quality and to satisfy GMP requirements. With the process analytical technology (PAT) initiative, requirements regarding process monitoring and control have changed and real-time on-line monitoring tools are recommended. A tool meeting the new requirements may be the dielectric spectroscopy for online viable cell mass determination by measurement of the permittivity. To establish these tools, proper offline methods for data correlation are required. The cell number determination of adherent

cells on microcarrier is difficult, as it requires cell detachment from the carrier, which highly increases the statistical error. As an offline method, a fluorescence assay based on SYBR[®]GreenI was developed allowing fast and easy total cell concentration determination without the need to detach the cells from the carrier. The assay is suitable for glass carriers used in stirred tank reactor systems or in fixed bed systems, may be suitable for different cell lines and can be applied to high sample numbers easily. The linear dependency of permittivity to cell concentration of suspended stem cells with the dielectric spectroscopy is shown for even very small cell concentrations. With this offline-method, a correlation of the cell concentration grown on carrier to the permittivity data measured by the dielectric spectroscopy was done successfully.

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Keywords Microcarrier culture · Process analytical technology · Fluorescence · Dielectric spectroscopy · Biomass monitoring

Introduction

Adherent growing cells, such as stem cells, have gained importance as biopharmaceuticals. Whole cells may also be the target product for example in stem cell therapy (Weber et al. 2007b, 2010a, b, c; Freimark et al. 2010). Standard cell cultivation systems for cell expansion and production of

biopharmaceuticals on microcarrier are described in a variety of culture systems (Eibl et al. 2008). Examples for cultivations of stem cells on microcarrier include the application of borosilicate glass carrier (BSKG) in a fixed bed reactor system (Weber et al. 2010a, b; c) and several types of carrier in different reaction systems, mostly spinner flasks (Zweigerdt 2009; Velden-de Groot 1995; Fernandes et al. 2009; Schop et al. 2008; Oh et al. 2009; Frauenschuh et al. 2007).

Cells or cell related products, which work as biopharmaceuticals must satisfy GMP guidelines given by the authorities (Wurm 2004; Baldi et al. 2007; Butler 2005; Weber et al. 2007a, b, 2009).

In 2004, the FDA introduced the Process Analytical Technology (PAT) initiative within the “GMP-Initiative for the 21st Century”. This initiative focused on bioprocess monitoring, which eventually aim at improving the understanding and control of manufacturing processes (Clementsich and Bayer 2006; FDA 2004).

Nowadays, the application of dielectric spectroscopy (DS) for process monitoring is handled as a promising tool for PAT satisfying production processes (Clementsich and Bayer 2006; Teixeira et al. 2009). For stem cell expansion processes, monitoring of the cell concentration is one of the key parameters. Therefore, a proper correlation between online (e.g. permittivity) and offline (e.g. cell number) data is required.

For the fast offline determination of the cell number, several methods are available. Cell counts and nuclei counts (Levine et al. 1979; Butler and Spearman 2007) are the most commonly used direct methods, but can be laborious for multiple samples. Beside cell counts using a hemocytometer, systems such as the Cedex[®] by Roche and various systems by Beckman Coulter are available (Rudolph et al. 2007). The obvious drawbacks of these methods are the delay between the sampling and the analysis as well as the risk of contamination (Vojinovic et al. 2006). In all cases, adherent growing cells have to be detached from their surface enzymatically and one must assure to have a representative sample. The two step process (cell detachment/lysis and cell count/nuclei count) causes an expenditure of time and a high error rate. Nuclei counts are prone to miscounts as cells may become binucleated, may not completely be lysed and are difficult to differentiate from cell

debris. Furthermore, the treatment for cell lysis is cell concentration dependent, which may cause a high error (Butler and Spearman 2007).

Indirect methods for cell concentration determination, which are often applied to cultures of adherent growing cells, involve the chemical analysis of metabolites (Vojinovic et al. 2006), protein or DNA content (Myers 1998; Rengarajan et al. 2002; Zipper et al. 2004). The DNA determination is probably one of the best indicators of cell concentration in solid tissue.

Myers (1998) published a sensitive, reproducible and simple assay for cell number determination of adherent cells using DNA intercalating SYBR[®]GreenI in micro-well plates. Cells were fixed, permeabilized and stained by the fluorescence dye. The dye itself had a very low fluorescence, when not bound to DNA, so that there was no need to remove the dye after staining the cells. The assay allowed linear correlation of fluorescence intensity to cell concentrations.

Today, several on-line measurement methods are available to determine cell number of adherent cells in biological processes. Common methods allowing monitoring these cultures include optical techniques based on light absorbance and/or scattering, real-time imaging, particle size analysis and measurements of culture fluid density (Ducommun et al. 2002a; Carvell and Dowd 2006; Vojinovic et al. 2006). Other measurement principles include optical density measurements, acoustics, laser light, Raman and fluorescence spectroscopy (Vojinovic et al. 2006; Butler and Spearman 2007). Additionally, infra-red sensors are described, but their operation is limited in the range of tested cell concentrations.

As mentioned above, DS may be a suitable tool for online monitoring of viable biomass. Successful applications for microcarrier cultivations as well as the theoretical background have been described in literature. A recent summary is given in Justice et al. (Justice et al. 2011). In case of adherent cell cultures monitored by DS described in literature, cell concentration determination was done by cell or nuclei counts after cell detachment or cell lysis (Davey et al. 1997; Ducommun et al. 2002a, b). Examples include the monitoring of HCT cells grown on Cytodex 3 in spinner flasks (Degouys et al. 1993), hybridoma cells on macroporous carrier in a fluidized bed (Noll and Biselli 1998), immobilized CHO in a packed bed of

FibraCell discs (Ducommun et al. 2002a, b) or CHO cultures on Cytopore1 microcarrier. Results showed that with data gained from DS, more accurate information can be obtained than with data obtained by protein content determination (Guan and Kemp 2002).

The current study describes the development of an assay for the cell density determination of cells on microcarrier. With this assay, correlation of cell concentration and DS in an expansion process for mesenchymal stem cell line (hMSC-TERT) was done (Justice et al. 2010). These strictly anchorage dependent cells are of increasing importance as medicinal products and underlie GMP guidelines and accordingly PAT. To our knowledge, no monitoring for stem cell expansion processes on microcarrier is described in literature yet. As an offline method for cell concentration determination a new fluorescence-based assay was developed which avoids cell detachment.

Material and methodology

Cell expansion in T-flasks and stirred tank reactor

Human mesenchymal stem cells with reverse telomerase transcriptase (hMSC-TERT) were obtained from CellMed, Alzenau, Germany.

Two-dimensional hMSC-TERT expansion in T-flasks (Sarstedt, Germany) was done by seeding the cells ($5 \cdot 10^3 - 1 \cdot 10^4$ cells/cm²) in Eagle Minimal Essential Medium (EMEM), supplemented with 10% fetal calf serum and 2 mM glutamine (all PAA, Germany). Culture conditions were 37 °C, 5% CO₂, 95% humidity. Cells were grown to densities of $5 \cdot 10^4 - 5.5 \cdot 10^4$ cells/cm² and then harvested enzymatic with 0.5 mg/mL concentrated trypsin—EDTA (1:250 PAA, Germany).

For three-dimensional cultivation, a 2 l stirred reactor system (d = 0.13 m) with stirrer, temperature and pH control (Applikon Biotechnology, Netherlands) at a height-diameter ratio of H/D = 1 was used. The reactor was equipped with a marine impeller. hMSC-TERT were cultured on RapidCell (RAC) (MP Biomedicals, USA) microcarrier at 37 °C in serum (10% fetal calf serum) and glutamine (2 mM) supplemented high glucose Dulbecco's Modified Eagles Essential Medium (DMEM-HG)

(all PAA, Germany) at 120–160 rpm. Inoculation density was $7 \cdot 10^3$ cells/cm² and the process was done in 4 cycles of each 2 min stirring and 45 min non-stirring. The process was online monitored by pO₂, temperature, pH and permittivity online measurement and by sampling twice a day for cell number determination by the developed fluorescence assay. Furthermore, glucose-, lactate concentration as well as microscopic analysis were performed.

Fluorescence assay

The fluorescence dye SYBR[®]GreenI (SG) ($1 \cdot 10^4 \times$; Sigma–Aldrich Chemie GmbH, Germany), which intercalates DNA, was used to determine the cell number. A stock solution of SG (400× in DMSO) was prepared from which the reaction solution (20× in TRIS (10 mM)—EDTA (1 mM) (Carl Roth, Germany; Sigma–Aldrich, Germany) at pH8) was made fresh daily. Dilutions for the analysis of different concentrations were made (2× and 0.2×).

Measurements were done in black 96 well plates (FluoroNunc, Nunc, Denmark) with a multimode reader for micro plates (Synergy HT, Biotek Instruments GmbH, Germany) with an excitation filter of 485 ± 20 nm and an emission filter of 528 ± 20 nm. Measurement temperature was 20 °C.

For BSKG (count as 50 µL) five carriers were set per well, for RAC, 50 µL of the desired carrier concentration for reactor cultivations were used per well. For estimating the calibration line, 50 µL of carrier, 50 µL of harvested single cell suspension of hMSC-TERT of different concentrations in PBS ($2 \cdot 10^3 - 1.5 \cdot 10^4$ cells/cm²) and 50 µL SG solution filled up to a total volume of 200 µL with PBS were measured. Measurement was done by removing the media, addition of 50 µL SG solution and PBS to fill up the volume to a total of 200 µL. For both carrier types and this cell line, no cell pretreatment was necessary, as SG penetrates viable and non-viable hMSC-TERT. For cell concentration determination, cells were detached enzymatically and counted with a hemocytometer.

As studies were done with single cell suspension added to the wells and not being adherent, convenience was required, that correlations do not differ when cells are grown adherent to the carrier. Therefore, cells were seeded with $4 \cdot 10^3$ and $6 \cdot 10^3$ cells/cm² on BSKG in the wells and grown for

5 days. Measurements were done daily by fluorescence measurement as well as via enzymatic cell detachment and hemocytometer count. Additionally, theoretical cell concentrations were calculated from known growth kinetics with an initial cell density of $5 \cdot 10^3$ cell/cm².

Permittivity measurements

In T-flasks cultured hMSC-TERT were harvested and cell concentrations were determined using a hemocytometer. After centrifugation (5 min, 230 g), single cells were resuspended to the desired cell concentration and measured in a stirred beaker using a 12 mm DS probe with the i-Biomass 465 (Fogale Nanotech, France). Permittivity measurements were done at room temperature. For different cell concentrations, serial dilutions were made by removing the volume of cell suspension (harvested single cells) and replacing it by medium.

hMSC-TERT grown on RapidCell microcarrier were monitored by DS throughout the whole cultivation time in the bioreactor.

Results and discussion

Offline fluorescence assay

Based on the publication by Myers (1998), a fluorescence assay was developed for cell concentration determination of hMSC-TERT growing on microcarriers.

The carriers of interest, RapidCell (RAC) and borosilicate glass carrier (BSKG) are both colorless and are not prone to influence fluorescence intensity. To avoid light scattering, black 96 wells were used for assay development. Experiments to evaluate possible effects on the background (Fig. 1) showed that medium must be replaced by buffer and that the carriers had hardly any influence on the fluorescence intensity. Data are shown exemplary for sensitivity 80, but ratios were similar for different sensitivities. Data for RAC were similar (data not shown).

Analysis of different SG concentrations (0.2–20×) showed that the most significant linear increase of the fluorescence intensity for the cell concentration range, was found for the 20× concentrated SG solution (Fig. 2).

Figure 3 shows the influence of the sensor sensitivity on the fluorescence measurement. A linear correlation of the fluorescence intensity as a function of the cell concentration was given for all sensitivities analyzed. Sensor sensitivity was decided to be 70 for further measurements. Higher sensitivities resulted in signal overflow and smaller values resulted in too small slopes in the correlation function.

Figure 3 includes studies of adherently grown cells on the carriers and single cell suspensions added to the carrier. Cell concentrations were calculated from known growth kinetics and correlated to the fluorescence intensity linearly. The correlation equals the one gained with experiments, where single cells were added to the carriers. Therefore proof was given that attached and loose single cells result in the same correlation (Fig. 3). Experiments in which cells were detached from the carriers and counted with a hemocytometer showed no correlation to the ones found when cells were added to the carriers loosely (data not shown). Microscopic analysis showed, that the cells could not all be detached from the carriers within the well. Furthermore, cell counting itself is prone to high error.

For fluorescence assay measurements with RAC, similar correlations were found, depending on the RAC concentrations and the chosen sensor sensitivities (data summarized in Table 1). Values were averages of 3×8 measurements each, resulting in standard deviations of the values smaller than 15%. For BSKG, no concentration changes occur so that a number of five carriers was chosen to be the sample size for one well. Repeats and average value calculations of one sample should be done by measuring at least 8 wells to reduce the statistical error.

The cell concentration information gained by this assay represents the total cell concentration. As primary viable cells remain adherent and stay on the carrier and non adherent cells are removed, the total cell concentration on the carrier should reflect the viable cell concentration. For other cell lines, a cell pretreatment of the cells may be required, as the fluorescence dye may be excluded by the cells.

Online-permittivity measurement

The DS allows monitoring of viable cell biomass. For our applications the cell concentration range, which has to be monitored, varied from 0 to $3.5 \cdot 10^5$ cells/

Fig. 1 Effects of different substances on fluorescence intensity for borosilicate glass carrier (BSKG). Measurements are averages of 8 measurements at sensor sensitivity 80, exemplary

Fig. 2 Effects of different SB concentrations (0.2–20×) on the fluorescence intensity for borosilicate glass carrier (BSKG) with addition of serial diluted cell suspension. Measurements are averages of three measurements at sensor sensitivity 80

mL for hMSC-TERT in suspension. The determination of the critical frequency for the cell line was essential for the concentration measurement. This was done by measuring the capacitance for a dilution series of hMSC-TERT cells in EMEM medium at different frequencies. As the hardware was limited to 300 kHz, no classical β -dispersion could be shown for hMSC-TERT (data not shown). The manufacturer then offered a hardware update allowing smaller frequencies to be tested ($1.8 \cdot 10^2 - 1.5 \cdot 10^5$ kHz

range), which resulted in similar results (data not shown).

Figure 4 shows the correlation of the permittivity to the cell concentration of single cell suspensions for different frequencies (300–1,117 kHz). For almost all tested frequencies, linearity was shown. The smallest possible frequency was 300 kHz, for which linearity was shown for even the small concentration range. Therefore, for further correlation analysis, the critical frequency of $f_c = 300$ kHz was chosen. This value

Fig. 3 Fluorescence assay for BSKG with hMSC-TERT at different sensor sensitivities. Measurements are averages of 2×8 measurements. Also shown are measurements of sample, where hMSC-TERT were grown on the microcarrier. Fluorescence assay for BSKG with hMSC-TERT was

Table 1 Linear functions of fluorescence intensity over the cell concentration for different carrier concentrations with each cell density of $2 \cdot 10^3 - 1.5 \cdot 10^4$ cells/cm²

Carrier concentration	Sensitivity	Slope	R ²
25 g/l	60	$0.038 \times$	0.9844
	70	$0.1 \times$	0.9897
	80	$0.19 \times$	0.9786
12.5 g/l	60	$0.014 \times$	0.9984
	70	$0.05 \times$	0.9976
	80	$0.14 \times$	0.9971

could also be confirmed when using the updated hardware with the extended frequency range.

Measurements at $f_c = 300$ kHz were repeated to assure data significance. As Fig. 5 shows, data were reproducible and showed that data analysis was possible for low cell densities.

Frequency determination was used to set the critical frequency to monitor expansion processes of hMSC-TERT growing on non-porous glass surface RapidCell carrier in a stirred system. This was done to allow homogenous conditions for permittivity measurements. RAC offer the same surface as BSKG but have a much lower density of 1.03 g/mL, so that homogenous suspensions can easily be generated.

performed at sensitivity 70 and $20 \times$ concentrated SG. Cell concentration values for seeded cells were calculated from the growth rate μ that resulted from growth kinetics for experiments with 5,000 cells/cm² seeding density. Measurements are averages of 2×8 measurements

Three repeated cultivations with similar boundary conditions were monitored showing repeatability in cell growth (Fig. 6).

Data from cell expansion offline monitoring with the developed fluorescence assay and online monitoring with the DS showed a similar progress and allowed data correlation (Fig. 7). Further information can be received from the online permittivity measurements. Obvious effects of aeration can be seen. Cell expansion increases after aeration, indicating, that the aeration itself has no harmful effect on the cells, but provides the required oxygen. Under forced oxygen deficiency, cells tend to get necrotic and detach from the surface and change their form to spheres (Tavernarakis 2006; Shimizu et al. 1996). This effect could be shown during the cultivation, when the oxygen partial pressure reached zero. The permittivity increased, which could be explained by the form change of the cells detaching from the carrier. Spherical forms result in an increase of capacitance in comparison to flat cells attached to a surface (Rahman et al. 2008).

The critical frequency was determined using harvested spherical cells by measuring cell suspensions at different frequencies. The single cells in suspension are spherical and might have tattered membranes due to enzymatic cell detachment. In

Fig. 4 Permittivity as a function of the cell concentration of hMSC-TERT in suspension at room temperature for different frequencies

Fig. 5 Permittivity as a function of the cell concentration of harvested hMSC-TERT in suspension at room temperature at a frequency of 300 kHz; two different measurements. Values are averages of 8 data points for one cell concentration

comparison, adherent cells are flat and have an intact membrane. Therefore, the critical frequency was to be approved using cultivation data with adherent cells on carriers. The critical frequency of 300 kHz could be confirmed by analyzing cultivation data at different frequencies, which is shown for all three cultivations done (Fig. 8).

As Fig. 8 shows, the critical frequency allowed monitoring the progress of cell adhesion, as the critical frequency decreased tremendously within the first few hours of culture. As cells start the exponential growth phase (>50 h), the change in the critical frequency decreased to very constant values varying between 300 and 400 kHz. The critical frequency of 300 kHz determined with suspended cells was the smallest

value determined during cultivations of the adherent cells as well. Greater values indicate a decreased cell volume. As the cell concentrations were low, the critical frequency of 300 kHz was chosen to achieve significant changes in permittivity, even though cell growth was slow and cell concentrations remained low in comparison to for example high cell density suspension cultures. As cell detachment related to oxygen limitation was assumed according to the permittivity values, the critical frequency of the culture (filled spheres) showed a decline in its values parallel to the gain in the critical frequency, supporting the theory of cell detachment from flat to spherical form.

With this confirmed information for the critical frequency, correlation of offline and online data was done. Linear correlation for all three cultivations was found (Fig. 9).

All three cultures showed a similar behavior, but for one cultivation, data only fit for permittivity values smaller 60 pF/cm. Offline microscopic data (not shown) for this cultivation showed, that carrier formed single clots, which may be responsible for the increased permittivity, even though the average cell concentration determined using the offline assay is similar to the other two data sets.

Conclusion

Within this work, the successful development of a fluorescence based offline assay for cell concentration

Fig. 6 Cell concentration as function of the culture time during three similar expansion processes. Data chosen were from offline data obtained using the developed fluorescence assay. Values are each averages of 8 measurements

Fig. 7 Cell concentration and permittivity as function of the culture time during one of the representative expansion processes (exemplary for three different cultivations). *Red*: offline-data from the fluorescence assay; *black*: online permittivity data

determination for anchorage dependent cells was shown. There is no need to detach the cells from the carrier. This assay indicated to be more reliable than cell counts when detaching the cells from the carrier. The assay is fast and easy, there is no need to remove unbound dye, it is easily done unsterile in micro-well plates and easily applicable to multiple samples.

The developed offline assay was of great use to monitor cell expansion processes of stem cells for their application for regenerative medicine. With this

assay, permittivity measurements could be correlated to cell concentrations. As PAT will soon be part of the GMP regulations, offline and online monitoring of cell expansion processes, especially for adherent growing cells, remains a difficulty. This work shows that the developed offline fluorescence assay and the DS are tools to fulfill these requirements.

Online measurements allow direct process information, process control and help to understand it. According to PAT, the major issues can be solved

Fig. 8 Frequency scans during cultivation of three repeated expansions of hMSC-TERT using DS

Fig. 9 Linear correlation of the cell concentration determined by the offline fluorescence assay as a function of the measured permittivity at 300 kHz. Cell concentrations are total cell concentrations and viability is set 100% as non-viable cells do not remain on the microcarrier. Values not considered for the linear correlation have signs with spotted lines

regarding the cell concentration of the process with the DS as an online tool. The linear correlation of permittivity data and cell concentration ease process monitoring and control, to allow for example the monitoring of the cell adhesion process and the start of the exponential growth phase by means of the critical frequency as well as the determination of the ideal point of harvest to guaranty highest product quality. Online monitoring of the process with the DS can even help to monitor process

irregularities, such as contaminations or necrotic behavior due to substrate limitation. The information may therefore lead to the abort of the process to save costs and can optimize processes by stopping it at the ideal point.

Acknowledgments We would like to thank the Hessen State Ministry of Higher Education, Research and the Arts in Germany for the financial support within the Hessen initiative for scientific and economic excellence (LOEWE-Program). Moreover, the authors would like to thank the Federal Ministry

of Economics and Technology of Germany (KF2268901UL9) for the financial support.

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