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Dielectrophoretic forces can be safely used to retain viable cells in perfusion cultures of animal cells

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Abstract

Dielectrophoresis is a well established and effective means for the manipulation of viable cells. However, its effectiveness greatly depends upon the utilization of very low electrical conductivity media. High conductivity media, as in the case of cell culture media, result only in the induction of weaker repulsive forces (negative dielectrophoresis) and excessive medium heating. A dielectrophoresis-based cell separation device (DEP-filter) has been recently developed for perfusion cultures that successfully overcomes these obstacles and provides a very high degree of viable cell separation while most of the nonviable cells are removed from the bioreactor by the effluent stream. The latter results in high viabilities throughout the culture period and minimization of lysed cell proteases in the bioreactor. However, an important question that remains to be answered is whether we have any adverse effects by exposing the cultured cells to high frequency electric fields for extended periods of time. A special chamber was constructed to quantitate the effect under several operational conditions. Cell growth, glucose uptake, lactate and monoclonal antibody production data suggest that there is no appreciable effect and hence, operation over long periods of time of the DEP-filter should not have any adverse effect on the cultured cells.

Introduction

Perfusion cultures represent the most cost effective mode of operation for the large scale production of therapeutic proteins, such as HBAg, tPA, erythropoietin, monoclonal antibodies, etc. The main characteristic of a perfusion culture is the retention of the cells in the bioreactor, i.e. no cells are removed by the effluent stream which results in high cell densities, increased volumetric productivity and reduced downstream purification requirements although the total volume to be processed is expected to be larger. A suitable cell retention device, located in the effluent stream of the bioreactor, is an important part of a cell perfusion bioreactor system. Existing cell retention systems are based on sedimentation (Berthold and Kempken, 1994; Hansen et al., 1993; Hülscher et al., 1991; Searles et al., 1994), centrifugation (Deo et al., 1996; Lee, 1989; Mahar, 1993) or conventional filtration (membrane-based) techniques (Avgerinos et al., 1990; Caron et al., 1994; Escalade et al., 1991; Hawrylik et al., 1994; Oh et al., 1994) and all of them have several significant limitations (Docoslis et al., 1997). A recent device (Doblhoff-Dier et al., 1994; Trampler et al., 1994) that employs ultrasonic resonance fields to transiently aggregate animal cells and are intermittently settled back in the bioreactor has also several limitations. The main disadvantage being its inability to selectively separate viable from nonviable cells and second the high power requirements that can cause excessive medium heating with possible detrimental effects to cellular viability. A commercial device is

currently available from Applikon which can be operated without the problem of medium heating, however it is situated outside the bioreactor and this could be a potential problem in regulatory compliance. Gaida et al. (1996) reported a modified acoustic filter that allows a small proportion of the cell debris to exit the bioreactor under certain operating conditions as the smaller cell debris have different acoustic properties than larger living cells.

As an alternative to the existing devices, we have developed a cell retention device based on the exploitation of dielectrophoretic forces (Docoslis et al., 1997). Dielectrophoresis (DEP) is the motion of neutral particles (e.g., spheres, bubbles, biopolymers, biological cells, etc.) under the influence of a divergent electric field. Numerous techniques have been developed using dielectrophoresis that include electroporation (Neumann et al., 1989), electrofusion (Abidor and Sowers, 1992; Neumann et al., 1989; Zimmermann, 1986), electroinjection (Neil and Zimmermann, 1993), measurements of the dielectric properties of the cell (Gascoyne et al., 1995; Gimsa et al., 1991; Krishna et al., 1989), as well as cell separation in low conductivity media (Archer et al., 1993; Huang et al., 1993; Krishna et al., 1989; Markx et al., 1994; Pohl, 1977). We have recently shown that there is a frequency range where selective separation of the viable from non-viable cells is achievable in high conductivity media typically found in cell culture systems (Docoslis et al., 1997). As Fuhr et al. (1994) has also shown using anchorage dependent mouse fibroblasts, cell separation in highly conductive culture media can only be achieved under negative DEP, using high frequency AC fields. Their results were also encouraging in that no adverse effects due to continuous exposure of the cells to high frequency electric fields were detected. They found that high field frequencies (above 10 MHz) had no significant effect on cell growth.

In this present work, we examine whether there are any adverse effects by exposing the cultured cells to high frequency electrical fields for extended periods of time. A special chamber was constructed and two versions (no-pass through) of the DEP-filter were microfabricated to quantitate the effect under several operational conditions. Cell growth, glucose uptake, lactate and monoclonal antibody production data are presented here that have been obtained from cultured cells exposed to high frequency dielectrophoretic fields over long periods of time. Finally, the inherent capability of the DEP-filter for preferential removal of apoptotic bodies is also discussed.

Dielectrophoretic forces

The net time-averaged induced dielectrophoretic force for AC electric fields is directly proportional to the cell volume, the real part of the medium permittivity, e_M, and the gradient of the electric field intensity squared, $\nabla \mathbf{E}^2$, i.e.,

$$< \mathbf{F}_{DEP} > \; = \; 2\,\pi\,r^3\,\epsilon_M\,\mathrm{Re}[\underline{K}_e(\omega)]\nabla \mathbf{E}^2 \qquad (1)$$

where r is the cell radius and **E** is the intensity of the electric field. The frequency dependence of the induced DEP force is given by the dimensionless Clausius-Mossotti function, $\underline{K}_e(\omega)$

$$\underline{K}_e(\omega) = \frac{\underline{\varepsilon}_{eff} - \underline{\varepsilon}_M}{\underline{\varepsilon}_{eff} + 2\underline{\varepsilon}_M} \qquad (2)$$

where the underlined parameters denote complex quantities. The complex permittivity of the surrounding medium is given by

$$\underline{\epsilon} = \epsilon_M - j\sigma_M/\omega \qquad (3)$$

where $j = \sqrt{-1}$ and ω ($= 2\mu f$) is the angular frequency of the applied field. According to the single-shell model (Kaler and Jones, 1990; Kaler et al., 1992), the cell can be represented as an ohmic, spherical particle, enclosed by a thin insulating shell (cellular membrane). The transmembrane conductance and surface conductivity are assumed to be negligible since mammalian cells do not have a cell wall and their membrane thickness is at least three orders of magnitude smaller than their cell radius (Pohl, 1977; Sukhorukov et al., 1993). If, furthermore, the cell cytoplasm is modeled as a linear ohmic dielectric fluid (i.e., there are no dielectric losses) of permittivity ϵ_c and conductivity σ_c, the resulting effective cell permittivity is given by (Kaler and Jones, 1990)

$$\underline{\epsilon}_{eff} = C_m r\left[\frac{1 + j\omega\tau_c}{1 + j\omega(\tau_m + \tau_c)}\right] \qquad (4)$$

where C_m is the area-specific membrane capacitance and $\tau_c = \epsilon_c/\sigma_c$, $\tau_m = C_m r/\sigma_c$ are time constants. As shown in Equation (1), the real part of $K_e(\omega)$ is directly related to $\mathbf{F}_{DEP}$. The sensitivity of $\mathrm{Re}[\underline{K}_e(\omega)]$ to cell size and various other electric parameters over a wide frequency range have been discussed elsewhere (Docoslis et al., 1997). Having estimated the unknown parameters in ϵ_{eff} through a series of levitation experiments, one can plot $\mathrm{Re}[\underline{K}_e(\omega)]$ versus ω and identify the frequency range where the induced DEP forces

are large for viable cells and essentially negligible for nonviable cells. Under these conditions, we can have preferential retention of viable cells in the bioreactor through the action of negative dielectrophoretic forces and simultaneous withdrawal of the nonviable cells by the effluent stream. This frequency range was found to be 5 to 50 MHz (Docoslis et al., 1997).

Description of the DEP-filtration system

Based on the above findings several 'DEP-filter' prototypes were microfabricated. The DEP-filter can be briefly described as a grid of interdigitated microelectrodes deposited on a silicon substrate, with the silicon etched through in the areas between adjacent electrodes (Figure 1). The filter was made using photolithography and silicon microfabrication techniques. The electrodes are made of gold deposited on a chromium layer. Structural details can be found elsewhere (Docoslis et al., 1997). The DEP-filter is mounted at the bottom of a housing device and the whole unit is submerged in the cell suspension. A schematic diagram with details of the whole system is given in Figure 1. To further illustrate the action of the DEP forces, in Figure 2 the forces that act on viable and nonviable cells are shown as well as their expected trajectories in the vicinity of the DEP-filter are also highlighted. An important characteristic of the DEP-filter is its 'inherent anti-clogging capability'. If a viable cell overcomes the repulsive DEP-forces and crosses the electrode surface, the DEP-force changes direction and pushes the viable cell away from the electrodes in the direction of the effluent stream. Actually the net force acting on the viable cell is now stronger as the drag force acts in the same direction. The presence of this 'anti-clogging force' is very important in maintaining the DEP-filter clean and functional over extended periods of time. It should be noted however that nonviable cells and cell debris do not induce such a DEP force and hence they can only be swept away by the effluent stream.

Our first results have shown that, at low flow rates, the retention of the viable cells can be very high (up to 98%) whereas it is extremely low, often below 15%, for nonviable cells regardless of the operating conditions. The latter is an indication that cell debris does not experience any dielectrophoresis. It was also shown that the retention of viable cells is subject to many factors, such as frequency of the applied electric field, voltage across the filter electrodes and flow rate of the effluent stream (Docoslis et al., 1997).

It is worth noting that the magnitude of the induced DEP-force (given by Equation (1)) on the cells is proportional to the cube of the cell radius. As a result, among the viable cells, or better among cells that have their membrane intact, the smaller ones should induce a weaker repulsive DEP-force. The direct ramification of this effect is the preferential retention of the larger viable cells in the bioreactor. Therefore, one may expect a preferential withdrawal of apoptotic bodies from the bioreactor before they undergo secondary necrosis (Cotter and Al-Rubeai, 1995), since apoptotic bodies are significantly smaller than viable cells. This is highly desirable as it maintains the culture medium cleaner. This preferential retention is not unique to our system but it has also been observed in other cell retention systems where cell size is an important factor, e.g., the inclined sedimentator proposed by Searles et al. (1994).

Effects on cell physiology and metabolism

The vast majority of the investigations on the biological changes induced during the exposure of cells to AC electrical fields has focused in the low frequency range (50 to 300 Hz). Such electric and/or electromagnetic fields are increasingly present in the human environment and they have been associated with a wide range of biological effects. Epidemiological studies (Sagan, 1992) point out a direct relationship between low frequency electromagnetic fields and increased rates of leukemia and brain cancer in humans. Loscher and Mevissen (1994) reported co-promotional effects of electromagnetic fields on tumor growth.

Despite the intense efforts that have resulted in numerous publications, the mechanism of electromagnetic field interactions with cells has not been fully revealed as of yet. Holian et al. (1996) concluded that electric fields act on cellular signal transduction and pointed out an important link between electric field-triggered, cell surface-mediated events and genomic activation by electromagnetic fields. Observed effects include the redistribution of specific membrane receptors (transferrin and low-density lipoprotein), increased intracellular calcium concentration and significant protein kinase C activity variations at different electric field strengths. Knedlitschek et al. (1994) showed that electric fields are capable of simultaneous activation of stimulatory and inhibitory pathways

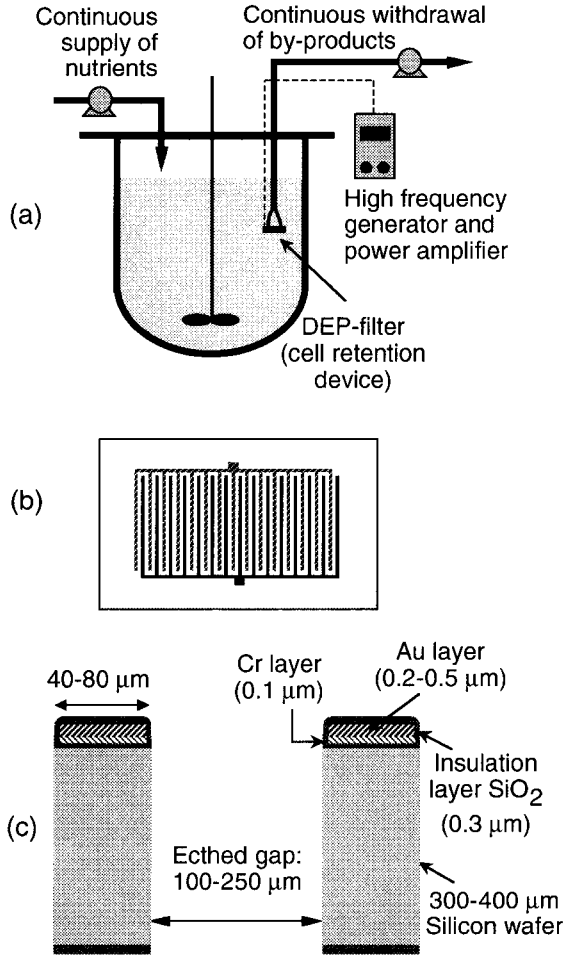

Figure 1. (a) Schematic diagram of a typical perfusion system with the DEP-filter in place, (b) Layout of interdigitated electrodes deposited on a silicon wafer (not to scale), (c) cross sectional view of two adjacent electrodes with microfabrication details.

of cAMP signal transduction. The net effect depends strongly on the frequency (increase at 50 Hz and decrease at 4000 Hz) as well as on the strength of the applied field. Cantoni et al. (1995, 1996) observed that the rate of repair of DNA single and double strand breaks in mammalian cells are not affected by 50 Hz electric and/or electromagnetic fields when caused by certain carcinogens, hydrogen peroxide or hydrogen peroxide/L-histidine. These results are in complete agreement with previous studies (Whitson et al., 1986) on human skin fibroblasts even at high values of field strength (10^5 V m^{-1}). In addition, Trombi et al. (1993) could not find any evidence that low frequency (50 Hz) electric fields are associated with fusogenic effects.

The only reported study on high frequency electric fields is that by Fuhr et al. (1994) on anchorage-dependent fibroblasts (3T3, L929). The cells were subjected to a permanent, high frequency (10 and 40 MHz) electric field with an intensity of 30 to 60 kV m^{-1} and their growth was observed over a three-day period. Their results showed no change in viability, fibroblast motility, anchorage and cell-cycle time compared to the control culture. An additional physiological parameter that is strongly affected by AC electric fields is the transmembrane potential, i.e., the potential across the two sides of the cell plasma membrane. It facilitates ion transport in and out of the cell. For mammalian cells the transmembrane potential is normally between 20 and 100 mV (Fuhr et al., 1994; Schwan, 1983).

Grosse and Schwan (1992) have developed a simple relationship (based on the analytical solution of the Laplace equation) between the transmembrane potential and the intensity of the applied AC field in

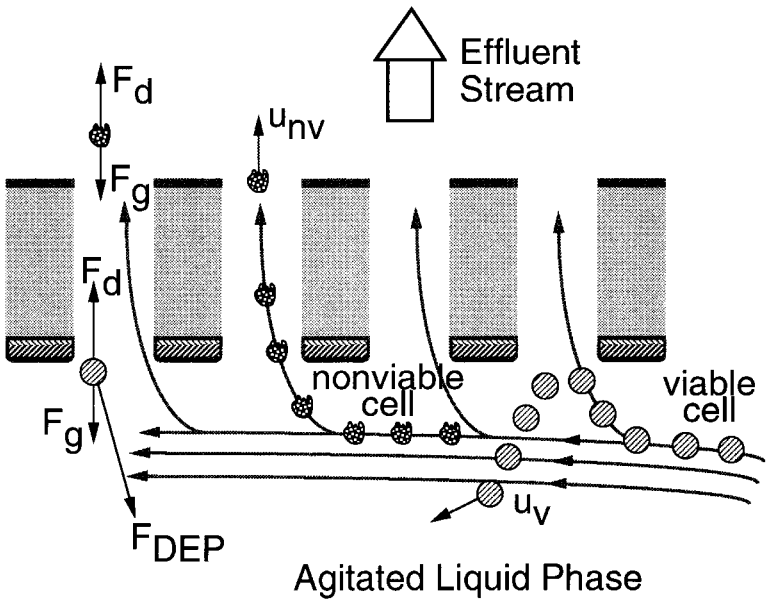

Figure 2. Schematic representation of a typical flow path of a viable and a nonviable cell in the agitated liquid phase in the bioreactor as the cells approach in the vicinity of the DEP-filter (u_v is velocity of viable cells and u_{nv} is the velocity of nonviable cells). The forces (gravitational F_g, drag F_d and dielectrophoretic F_{DEP}) acting on viable and nonviable cells are also shown.

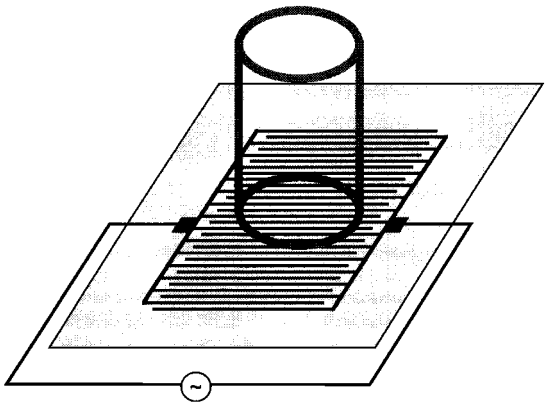

Figure 3. Schematic representation of the DEP-chamber (culture vessel). The chamber is comprised of a bottomless polycarbonate vial (5 ml) fixed permanently on top of an interdigitated microelectrode array that was deposited on an oxidized (insulated) silicon wafer. The vial was filled with 1 ml of cell suspension and a potential difference was applied by connecting the poles of the electrode array to a high frequency power generator.

aqueous electrolyte solutions under physiological conditions. The transmembrane potential is given as a function of the angular frequency of the applied external electrical field, the cell radius, the membrane capacitance and absolute permittivity, the membrane thickness, the cytoplasmic resistivity and the resistivity of the electrolyte solution surrounding the cell. Using this relationship and the dielectric parameters for the C174 mouse myeloma cell line (Docoslis et al., 1997) the transmembrane potential was computed as a function of the electric field frequency. Although at low frequencies (less than 10 kHz) the potential is nearly 3000 mV, at higher frequencies (more than 10 MHz) the induced potential drops significantly and becomes comparable with the physiological one (20 to 100 mV).

DEP-chamber construction

In order to further investigate the effect of exposure to high frequency electric fields, we constructed a culture

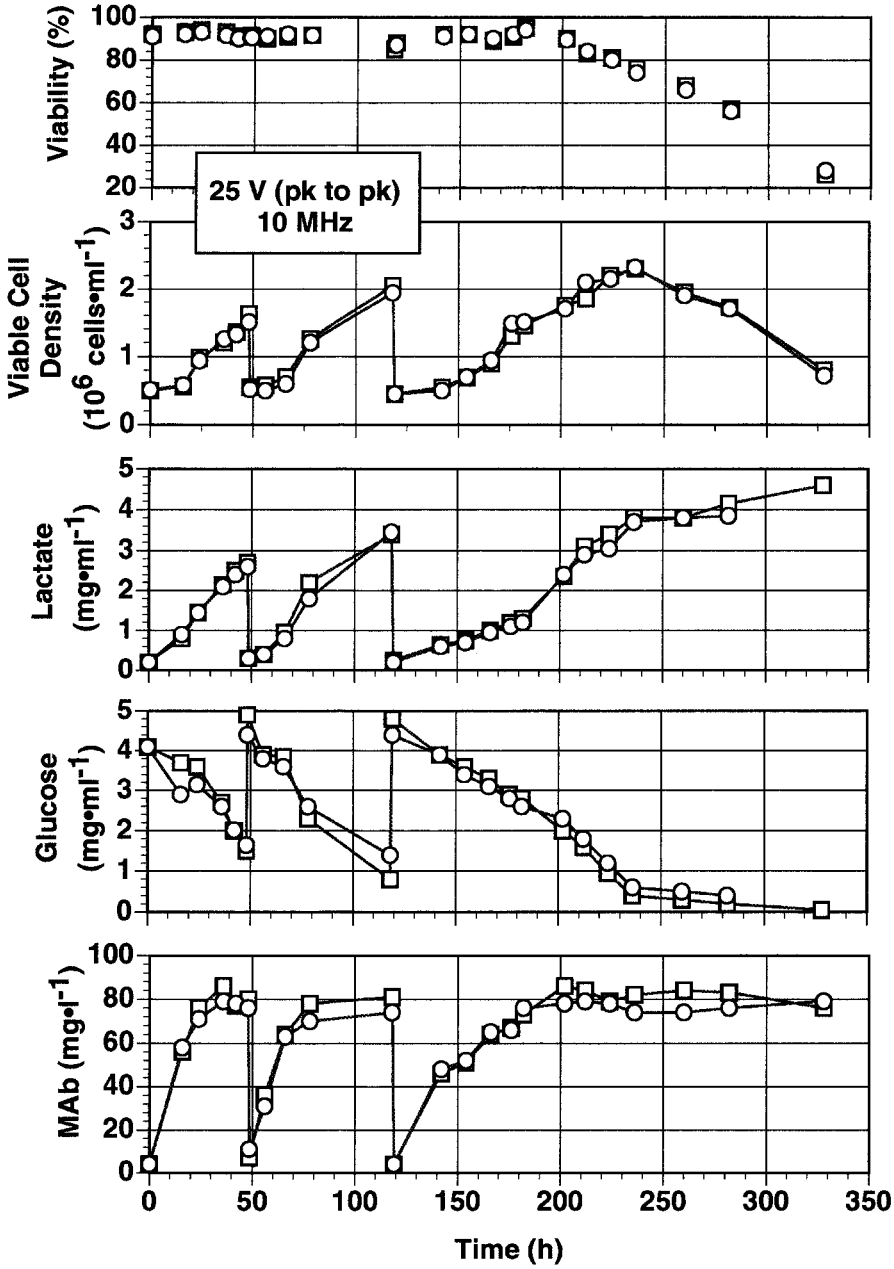

Figure 4. Viability, viable cell density, lactate, glucose and monoclonal antibody concentration profiles for HFN 7.1 cells cultured under continuous exposure to high frequency electric fields.

system that could mimic the conditions in the vicinity of the DEP-filter inside a bioreactor. The culture vessel was a suitably modified (open bottom) polycarbonate vial (5 ml capacity). The bottomless vial was glued onto an oxidized (insulated) silicon wafer where a grid of micro-electrodes were deposited (Figure 3). In this case the silicon wafer was not etched through in the areas between adjacent electrodes as it is normally done for the construction of the DEP-filter. The dimensions of the electrode grid was approximately 1.1 cm $\times$ 1.1 cm. The silicon wafer was about 2.5 cm $\times$ 2.5 cm. In earlier constructions where the size of the silicon wafer was sligthly larger than the electrode area, we observed heating of the culture

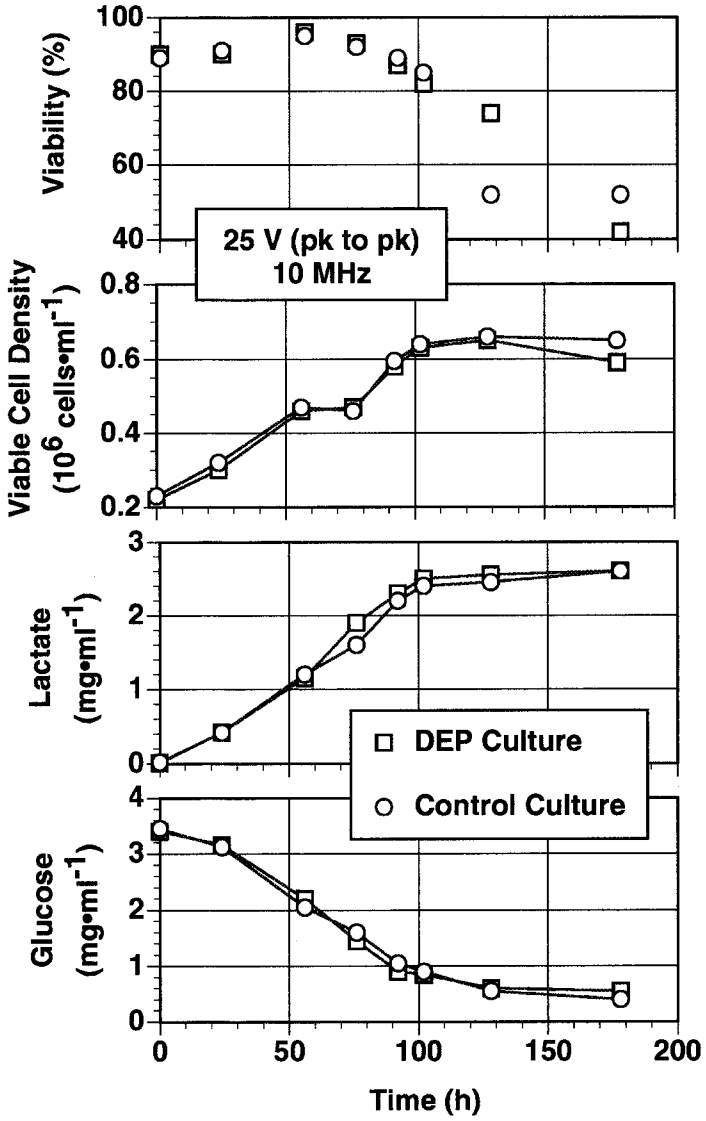

Figure 5. Viability, viable cell density, lactate and glucose concentration profiles for CHO cells cultured under continuous exposure to high frequency electric fields.

medium up to 2 °C. The larger silicon wafer was sufficient to dissipate the generated heat in the forced air CO_2-controlled humidifier. This simple growth chamber can accommodate the culture of the cells in the vicinity of the microelectrodes and the quantification of any potential adverse effects from the exposure to the high frequency electrical fields in a T-flask-like environment. In other words, no agitation was used. Actually the control culture, was carried out in a similar vial but without applying any high frequency voltage. The culture vessel in this case was essentially a 1 cm^2 round-bottom T-flask. An RF generator (BK PRECISION, 2005A) and a 7-watt power amplifier (Mini-Circuits, ZHL-1A) were utilized to apply a high frequency voltage to the microelectrodes. The frequency and magnitude of the applied voltage were monitored with the aid of an oscilloscope (Tektronix, 2213–60 MHz).

Materials and methods

The murine lymphocyte hybridoma HFN 7.1 producing an IgG antibody reactive with human fibronectin

(ATTC: CRL-1606) was used in these experiments. The cell culture medium was DMEM (Dulbecco's Modified Eagle's Medium, Sigma) supplemented with 10% Fetal Bovine Serum (Gibco/BRL) (heat deactivated at 58 °C for 30 min). In addition CHO/dhfr- cells (ATCC: CRL-9096) were grown in IMDM (Iscove's modified Dulbecco's medium) supplemented with 4 mM L-glutamine, 0.1 mM hypoxanthine and 0.01 mM thymidine and adjusted to contain 1.5 g l^{-1} sodium bicarbonate and 10% FBS after adaptation to grow in suspension. This medium was used intially for adaptation to grow in suspension and subsequently for cell maintainace. During the runs in the DEP-chamber reported here, a serum free medium was used for the CHO/dhfr- cells (CHO-S-SFM II by Gibco/BRL). The cells were grown in suspension at 37 °C in a 5% CO_2 and humidity controlled incubator (Forma Stericult). Temperature was monitored continuously in the DEP-chamber during preliminary runs with the use of a thermocouple immersed in the culture medium. The analysis of D-glucose and L-lactate was done by an YSI-2700 glucose analyzer (Yellow Spring Instruments). Monoclonal antibody titers were determined by ELISA. The ELISA was performed in 96-well plates (Corning). The wells were first coated with a goat anti-mouse IgG, Fc fragment specific (Sigma) antibody and were left overnight to equilibrate at 4 °C. Subsequently, the standard wells were coated with mouse IgG, whole molecule (Sigma), unconjugated. The rest of the wells were coated with the actual samples. The blocking buffer was PBS-3% BSA (Sigma) and the washing solution PBS (Life Technologies, Grand Island, NY). Finally, the horseradish peroxidase-conjugated Ab was goat anti-mouse IgG, F(ab')2 fragment specific (Sigma). The substrate was a solution of o-phenylenediamine in citric acid buffer. The reaction was quenched with 1 N HCl and the absorbance was read at 492 nm.

Aminoacid analysis was performed by measuring the absorbance at 340 nm of orthophthaldialdehyde (OPA) derivatives after separation on a reverse phase HPLC column (Supelco) using an HP-1090 liquid chromatograph equipped with an HP-1046A Fluorescence detector (Forestell, 1992). The cell count was performed using a haemocytometer and the viability was determined by the trypan blue (0.4%) exclusion test for nonviable cells.

Experimental results and discussion

A series of experiments were designed to examine whether cells staying in the vicinity of the DEP-filter electrodes suffer from any adverse effects. In a typical perfusion culture due to bulk agitation, the cells are expected to arrive and stay near the DEP electrodes only for a short period of time. To emulate this, we applied a high frequency (10 MHz) electric field (25 V pk to pk) intermittently. However, we were unable to see any adverse effect. Hence, it was decided to expose the cells continuously and examine if any changes in their growth characteristics and metabolism could be observed. In Figure 4, the results from three subcultures of HFN 7.1 cells are shown. The subcultures were performed at 48 and 120 h. In particular, the last subculture was monitored throughout the stationary phase in case the exposure effects are more pronounced during the latest stages of a culture. Comparison of viable cell density, viability, glucose uptake, lactate production as well as monoclonal antibody production data between the DEP-culture and the control suggests that there are no adverse effects. Even during the latest stages of the culture when the viabilitry had dropped below 60%, the glucose uptake, lactate and MAb production were essentially identical between the control and the DEP-culture. The same was observed with 15 aminoacids measured by HPLC analysis during the first subculture (data not shown).

In another series of experiments using CHO/dhfr- cells adapted to grow in suspension, no noticeable effect was observed from the exposure to high frequency electric fields during cultivation in serum-free media as shown in Figure 5.

Although the results may vary slightly with each cell line, we can conclude that cell exposure to high frequency electric fields does not cause any detrimental effects to cell growth and metabolism. However, the effect of the exposure on other cell functions at the sub-cellular level may warrant further investigation (e.g., induction of mutations, loss of plasmid DNA, etc.). It should be noted however, that prolonged and continuous cell exposure (more than 100 h) to high frequency electric fields represents a significantly exaggerated event as far as the operation of the DEP-filter is concerned. Due to the continuous agitation of the cell suspension in the bioreactor and the short range effect of the field, during a two-month long perfusion culture the average cumulative time period that an elementary liquid volume will interact with the electric field will be significantly less than that studied

here. In conclusion, no adverse effects on the physiology and metabolism of the cells should be expected during a perfusion culture where the cells are retained in the bioreactor with the aid of our DEP-filter.

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