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Feasibility study for cell electroporation detection and separation by means of dielectrophoresis

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Abstract

Electroporation is a phenomenon during which exposure of a cell to high voltage electric pulses results in a significant increase in its membrane permeability. Aside from the fact that after the electroporation the cell membrane becomes more permeable, the cells' geometrical and electrical properties change considerably. These changes enable use of the force on dielectric particles exposed to non-uniform electric field (dielectrophoresis) for separation of non-electroporated and electroporated cells. This paper reports the results of an attempt to separate non-electroporated and electroporated cells by means of dielectrophoresis. In several experiments we managed to separate the non-electroporated and electroporated cells suspended in a medium with conductivity 0.174 S/m by exposing them to a non-uniform electric field at a frequency of 2 MHz. The behaviour of electroporated cells exposed to dielectrophoresis raises the presumption that in addition to conductivity, considerable changes in membrane permittivity occur after the electroporation.

Keywords: Electroporation; Dielectrophoresis; Cell separation; Membrane permittivity

1. Introduction

In biotechnology, biology and medicine, it is sometimes important to be able to introduce specific molecules which are otherwise cell membrane impermeant. Electroporation is a widely-used technique for delivering a large variety of impermeable molecules, such as drugs [1–3] and genes [4–6] into cells, both in vitro and in vivo. This is a phenomenon during which exposure of a cell to an electric field results in a significant increase in its membrane permeability [7–9]. It is believed that pores are formed in the membrane due to induced electric potential difference across the cell membrane above some critical value (0.3 and 1 V). For this reason, the phenomenon was named electroporation [10,11]. Nevertheless, in order to emphasize on increased membrane permeability, also a more descriptive term electropermeabilization is in use for the observed phenomenon [12]. If electric pulse parameters like duration, number, amplitude and repetition frequency are chosen properly, so that resulting external electric field is not too intense, the cell is capable to reseal its membrane and electroporation is reversible [13,14]. On the other side, if the external electric field is too large, membrane rupture takes place and the electroporation becomes irreversible, resulting in cell death [15,16]. Beside the parameters of the electroporation pulse, the properties of the medium and the cell affect induced electric potential on the cell membrane and thus the electroporation itself [17,18].

Normally, a test whether a cell is electroporated or not is performed by exposing cells to dye (propidium iodide, trypan blue, lucifer yellow…) [19–21]. In these procedures the cell is destroyed and, as such, can not be further used. In many cases, researchers would prefer a more convenient method which would separate the non-electroporated and electroporated cells from the cell suspension. It would be especially convenient if the method would monitor the efficacy of cell electroporation in real time [22]. A very useful method for manipulation and

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separation of microscopic particles in practice is dielectrophoresis [23,10]. Dielectrophoresis (DEP) is a phenomenon in which a force is exerted on a polarisable particle, e.g., a biological cell, when it is exposed to a non-uniform electric field [24–26]. Besides cell manipulation and separation, the dielectrophoresis spectra of the cell may be also used to derive electric properties of the cell [27–29].

In this paper we report the dielectrophoresis behaviour of non-electroporated and electroporated cells. In the first part we described the preparation procedures of the cells and the medium and determination of the parameters necessary for successful cell electroporation. In the second part the influence of the electroporation on cell dielectrophoresis was investigated theoretically and experimentally. Finally, the possibility of the non-electroporated and electroporated cells separation is described.

2. Materials and methods

The dielectrophoretic force is generated through the interaction of the non-uniform electric field and the induced electric dipole of the cell. The direction and magnitude of the dielectrophoretic force depends on the dielectric properties of the cell and the suspending medium. If we assume that a biological cell is a spherical particle the dielectrophoretic force is defined as:

$$F_{\mathrm{DEP}} = 2\pi R^3 \varepsilon_{\mathrm{med}}\, Re[f_{\mathrm{CM}}]\, \nabla |E^2|, \tag{1}$$

where R is the radius of the cell, $\varepsilon_{\mathrm{med}}$ is the absolute permittivity of the suspending medium, E is the electric field acting on a cell and $Re[f_{\mathrm{CM}}]$ is the real part of the Clausius–Mossotti factor [25]. The $Re[f_{\mathrm{CM}}]$ describes Maxwell–Wagner relaxation and is given by:

$$f_{\mathrm{CM}} = \frac{\varepsilon'_{\mathrm{cel}} - \varepsilon'_{\mathrm{med}}}{\varepsilon_{\mathrm{cel}} + 2\varepsilon'_{\mathrm{med}}}, \tag{2}$$

where $\varepsilon'_{\mathrm{cel}}$ and $\varepsilon'_{\mathrm{med}}$ are the complex permittivities of the cell and medium, respectively and Re denotes the real part of a complex expression. Complex permittivity is defined as: $\varepsilon' = \varepsilon - j(\sigma/\omega)$, where ε is permittivity, σ is conductivity, ω is the angular frequency of the electric field and $j = \sqrt{-1}$. According to the single-shell model [30], the complex permittivity of the cell $\varepsilon'_{\mathrm{cel}}$ can be obtained from the Pauly–Schwan equation:

$$\varepsilon'_{\mathrm{cel}} = \varepsilon'_{\mathrm{mem}} \frac{2(1-a)\varepsilon'_{\mathrm{mem}} + (1+2a)\varepsilon'_{\mathrm{cyt}}}{(2+a)\varepsilon'_{\mathrm{mem}} + (1+a)\varepsilon'_{\mathrm{cyt}}}; \tag{3}$$

$$a = \left(1 - \frac{d_{\mathrm{mem}}}{R}\right)^3. \tag{4}$$

d_{mem} is the thickness of the membrane, $\varepsilon'_{\mathrm{mem}} = \varepsilon - j(\sigma_{\mathrm{mem}}/\omega)$ is the complex permittivity of the cell membrane and $\varepsilon'_{\mathrm{cyt}} = \varepsilon_{\mathrm{cyt}} - j(\sigma_{\mathrm{cyt}}/\omega)$ is the complex permittivity of the cytoplasm, as shown in Fig. 1.

Aside from the fact that after the electroporation the cell membrane becomes more permeable (which may also be the intended effect of the electroporation), the cells' geometrical [22] and electrical properties [31,32] change. Because the rate of electroporation is highly dependent on the medium conductivity [33], studies of geometrical and electrical properties of the cell were based on two media with different conductivity: a high-conductive medium Spiner's modification of Eagle's minimum essential medium (SMEM) (Life Technologies, Paisey, UK) having conductivity $\sigma_{\mathrm{SMEM}} = 1.58$ S/m that does not contain calcium and a low-conductive medium that contained phosphate buffer with 250 mM sucrose (PB) with conductivity $\sigma_{\mathrm{PB}} = 0.127$ S/m [22,31,32]. In our study we used medium M1 with conductivity $\sigma_{\mathrm{M1}} = 0.174$ S/m. Taking into account conductivity of the media and the data from studies [22,31,32] we calculated the dimensions and electrical properties of the cell, before and after electroporation in medium M1, with interpolation (see Table 1).

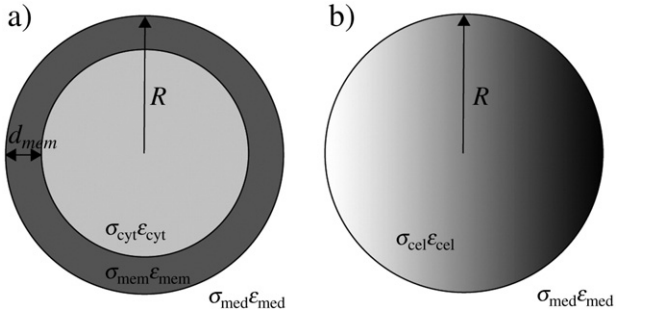

Fig. 1. Single-shell model of a spherical cell, where R is the radius of the cell, d_{mem} is the thickness of the membrane, σ is absolute conductivity and ε is absolute permittivity. The subscripts, med, mem and cyt denote respectively, medium, membrane and cytoplasm. Subscript cel denote cell as a homogeneous particle.

From Table 1 we can conclude that electrical conductivity of the cell membrane changes considerably after the electroporation, which has a large impact on the magnitude and the sign of $Re[f_{\mathrm{CM}}]$. Taking into account that the electroporated cell (because of different dimensions and electrical properties) has a different $Re[f_{\mathrm{CM}}]$ than the non-electroporated one, it can be suspected that it would be possible to separate the non-electroporated and electroporated cells at a specific frequency of the applied signal.

Fig. 2 shows two different $Re[f_{\mathrm{CM}}]$ spectra for the cells A and B. At a frequency of approximately $f_{\mathrm{sep}} = 5$ MHz (the separation frequency) the cell A has a positive $Re[f_{\mathrm{CM}}]$ whereas the cell B has a negative $Re[f_{\mathrm{CM}}]$. According to Eq. (1), the dielectrophoretic force applied to cell A is of the opposite direction as the dielectrophoretic force applied to cell B, which means that these cells can be separated. The most important frequency of $Re[f_{\mathrm{CM}}]$ is the crossover frequency f_0, at which $Re[f_{\mathrm{CM}}]$ changes its sign, either from the negative to the positive or vice versa (dielectrophoresis crossover).

2.1. Preparation of extracellular media

The conductivity of the medium has a significant role on the dielectrophoretic force [25] and electroporation [33]. Therefore, two media with different conductivity were prepared and

Table 1
Dimensions and electrical properties of a typical cell before and after electroporation in medium M1

Cell property	Symbol	Before electroporation in medium M1:	After electroporation in medium M1:
Radius	R	10^{-5} [m]	1.3×10^{-5} [m]
Membrane thickness	d_{mem}	5×10^{-9} [m]	3.0×10^{-9} [m]
Membrane conductivity	σ_{mem}	10^{-7} [S/m]	1.8×10^{-5} [S/m]
Cytoplasm conductivity	σ_{cyt}	0.5 [S/m]	0.5 [S/m]
Membrane permittivity	ε_{mem}	4.4×10^{-11} [As/Vm]	4.4×10^{-11} [As/Vm]
Cytoplasm permittivity	ε_{cyt}	7.1×10^{-10} [As/Vm]	7.1×10^{-10} [As/Vm]

The data were calculated with interpolation according to the conductivity of the medium M1 ($\sigma_{M1}=0.174$ S/m) and the data from studies [22,31,32].

used in the experiments: medium M1 (with conductivity $\sigma_{M1}=0.174$ S/m) and medium M2 (with conductivity $\sigma_{M2}=0.0012$ S/m). For preparation of medium M1 the following ingredients were dissolved in 200 ml distilled water: 272.2 mg KH_2PO_4, 348.4 mg K_2HPO_4, 19.02 mg $MgCl_2$ and 17 115 mg sucrose. Medium M2 was prepared by diluting 10 ml of medium M1 with 222 ml of distilled water and 19 016 mg of sucrose. The conductivities of both prepared media were measured by a Conductometer MA 5950 (Metrel, Slovenia). All experiments were carried out at room temperature (23 °C).

2.2. Preparation of cells

Mouse melanoma cell line, B16F1, was grown for four days in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) at 37 °C in humidified 5% CO_2 atmosphere in the incubator (WTB Binder, Labortechnik GmbH, Seekbach, Germany). For all experiments the cell suspension was prepared out of confluent cultures with 0.05% trypsin solution containing 0.02% ETA (Sigma-Aldrich Chemie GmbH). From the obtained cell suspension trypsin and growth medium were removed by centrifugation at 1000 rpm at 4 °C (Sigma-Aldrich Chemie GmbH) and the resulting pellet was resuspended in a medium with a specific conductivity and centrifuged again.

To meet the required concentration for electroporation, t.i. 2×10^7 cells/ml [29], the centrifuged cells were diluted with an appropriate medium once again. A 50 µl drop of diluted cell suspension (containing 10^6 cells) was placed between two parallel plate stainless steel electrodes spaced 2 mm apart and exposed to electric pulses. A train of eight rectangular pulses amplitude: 230 V, duration: 100 µs, repetition frequency: 1 Hz, was generated and monitored with the Cliniporator (IGEA, Carpi, Italy).

The fraction of the electroporated cells was determined by exposing cells to electric pulses in the presence of dye propidium iodide (PI). Similarly, the fraction of cell survival was established by adding PI in cell suspension 30 min after the electroporation, when the cell membranes of the survived cells were already resealed. Both procedures are in more detail described in Refs. [34,35].

2.3. Dielectrophoresis (DEP)

The dielectrophoretic manipulation of cells was performed by castellated microelectrode structures, which were fabricated on a 500 µm thick wafer of Pyrex glass using microtechnology processing steps. The structures were fabricated in the Laboratory of Microsensor Structures, Faculty of Electrical Engineering, University in Ljubljana, Slovenia. After processing, the

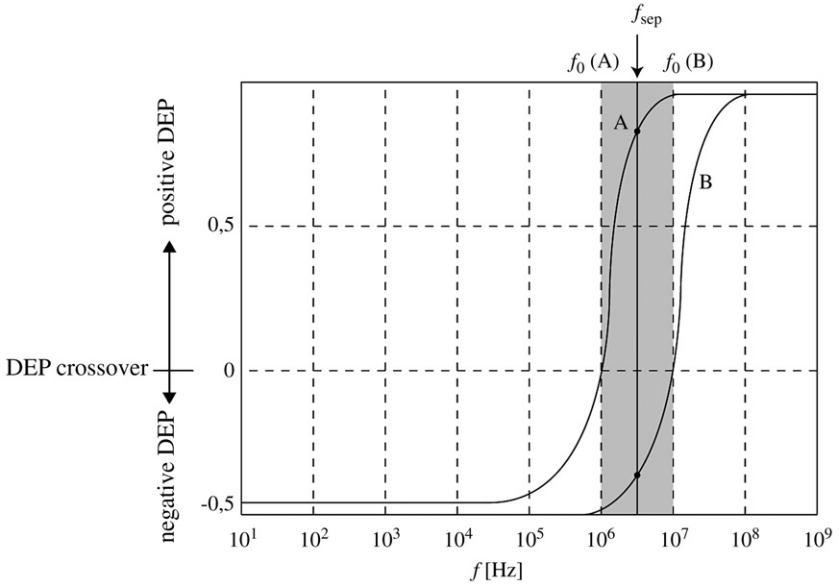

Fig. 2. Plot of the $Re[f_{CM}]$ spectra for two different particles. In the shaded area particle A experience positive dielectrophoresis and particle B experience negative dielectrophoresis, enabling separation at this window [25].

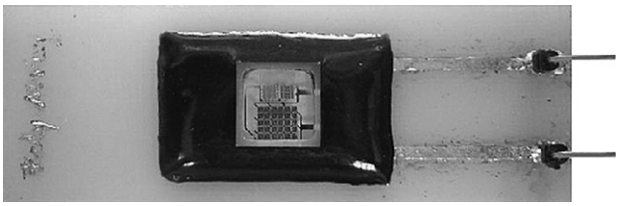

Fig. 3. Module with a microelectrode structure.

microelectrode structures were cut from the wafer and prepared as modules as presented in Fig. 3. The fabrication of the microelectrode structures was not a subject of this study and is in more detail described in Ref. [36].

Sinusoidal signals of magnitude 7 V_{pp} were applied to the pair of electrodes over the frequency range 5 kHz– 50 MHz using a function generator 33250A (Agilent, USA).

The cells were diluted with a medium with a selected electric conductivity, since the most appropriate concentration to observe cell motion under the microscope is 2×10^6 cells/ml. After placing the diluted cell suspension in the module, the cells were left for 5 min to swell and reseal before being examined by dielectrophoresis. To investigate dielectrophoresis, the cells were exposed to a non-uniform electric field and observed under the transmitted-light microscope (Zeiss 200, Axiovert, Jena, Germany). The frequency of the electric field was varied and the images were recorded with a camera (Visicam, Visitron Systems, Germany). Each experiment was repeated at least three times.

2.4. Numerical calculations

According to Eq. (1), it is obvious that the frequency depended $Re[f_{CM}]$ has the most significant impact on cell motion caused by dielectrophoresis. If $Re[f_{CM}]$ of a cell is negative, the cell is subjected to negative dielectrophoresis and moves away from the high-field region. However, if $Re[f_{CM}]$ of a cell is positive, the cell is subjected to positive dielectrophoresis and moves toward the high-field region. $Re[f_{CM}]$ was calculated from Eq. (2) using electrical properties of the cells and the suspending medium from Table 1. The spectra of $Re[f_{CM}]$ was determined for non-electroporated and electroporated cells in medium M1.

Another crucial parameter in Eq. (1) is the gradient of the square of the electric field. Electric field distribution is obtained by suitable design of the microelectrode structures. Several structures are suitable for development of large non-homogeneities of electric field [25]. In this work we used castellated microelectrode structures. Areas of high-and low-field intensity

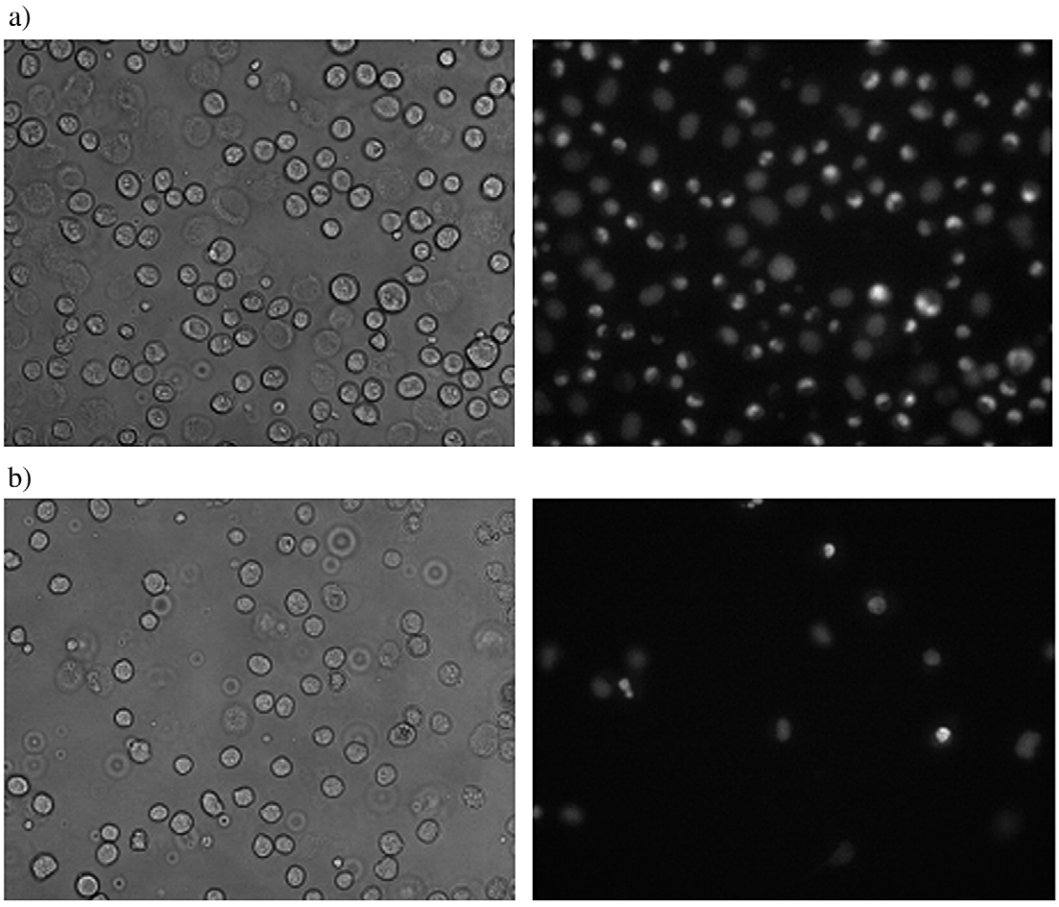

Fig. 4. Cells exposed to eight rectangular pulses amplitude: 230 V, duration: 100 μs and repetition frequency 1 Hz. Fraction of permeable cells (a) after the electroporation in the presence of propidium iodide is large and the fraction of dead cells (b) 30 min after the electroporation is small.

were determined by the numerical simulation using finite element modelling software FEMLAB [www.comsol.com].

3. Results

3.1. Electroporation parameters

The cells were electroporated with a train of eight rectangular pulses, amplitude: 230 V, duration: 100 μs, repetition frequency: 1 Hz. The electroporation procedure was repeated three times. The fraction of electroporated cells was high (see Fig. 4(a)) and the fraction of dead cells was low (see Fig. 4(b)). Consequently we concluded that under these conditions cells were reversibly electroporated.

3.2. Numerical results

In Fig. 5, $Re[f_{CM}]$ and f_0 of the non-electroporated and electroporated cells in medium M1 are illustrated. As we can see, both the non-electroporated and electroporated cells are exposed to a negative dielectrophoresis at low frequencies and to a positive dielectrophoresis at higher frequencies of the applied AC voltage. The calculated f_0 of the non-electroporated cells is approximately 0.4 MHz. The calculated crossover frequency f_0 of the electroporated cells, based on data from Table 1, is approximately 0.2 MHz.

The distribution of the non-uniform electric field generated with the castellated microelectrode structures at applied voltage between the electrodes is shown in Fig. 6. Dark shades represent areas of high-field intensity, whereas bright shades are those of low-field intensity. Therefore, if we expose the cells to a positive dielectrophoresis, they will move toward the edges of the electrodes where the high-field intensity is generated. Exposing cells to a negative dielectrophoresis would direct the cell motion towards the areas of the low-field intensity in the middle of the electrodes.

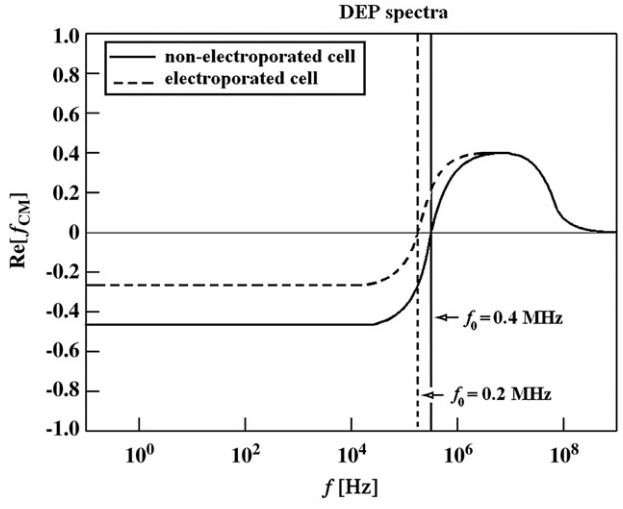

Fig. 5. $Re[f_{CM}]$ and f_0 of the electroporated and non-electroporated cells in medium M1, calculated according to the electric properties of cells and suspending medium (Table 1).

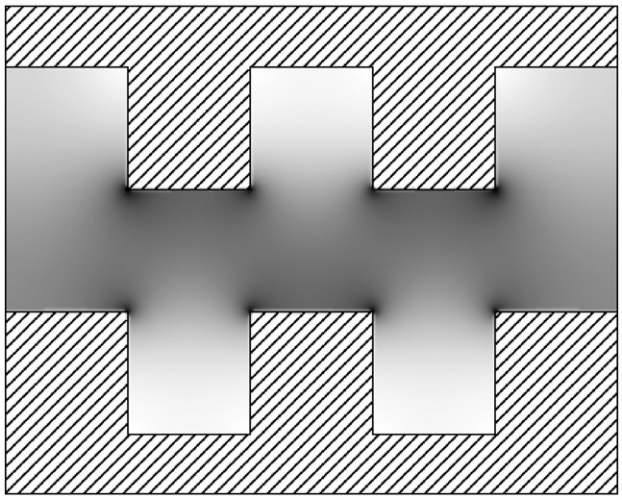

Fig. 6. Plot of the numerically calculated non-uniform electric field generated with the castellated microelectrode structures. Dark shades represent areas of high-field intensity, whereas bright shades those of low-field intensity. Hatched pattern represents castellated microelectrode structure.

3.3. Experimental results

Experiments revealed intense cell motion by exposing cells to dielectrophoresis. Cell motion in medium M2 was very intense for all frequencies of the applied voltage signal and it was not possible to determine the crossover frequency for cells suspended in this medium. For this reason, the investigation of this study was focused on cells suspended in medium M1, where a difference between f_0 of the non-electroporated and electroporated cells was considerable.

Fig. 7 (above) shows the results of motion of the non-electroporated cells in medium M1 at several different frequencies. At low frequencies the cells are exposed to negative dielectrophoresis. At a frequency of about 0.4 MHz, the cells started to move toward the edges of the electrodes where the intensity of the field is high (positive dielectrophoresis).

The movement of the electroporated cells in medium M1, see Fig. 7 (bellow), is very different. Cells are exposed to negative dielectrophoresis up to the frequency 10 MHz.

Some of the cells have moved on the top of the electrode structure. Because we have observed cell motion under the transmitted-light microscope, cells on the top of the electrodes can not be seen in Fig. 7. We repeated each experiment at least three times by increasing and decreasing the frequency of the applied signal and the results of experiments were repeatable for both the non-electroporated and electroporated cells in the medium M1.

4. Discussion

The aim of our study was to examine the possibility of separation of the electroporated cells from non-electroporated by means of dielectrophoresis. On the basis of theoretical findings we predicted that electroporated cells exposed to dielectrophoresis would behave differently. The theoretical predictions were than verified with experiments.

The parameters of the electric pulses for cell electroporation were determined, which assured successful electroporation. This procedure is important because later we assumed the cells which were electroporated with determined electric parameters as successfully electroporated cells. During investigation of dielectrophoresis it was impossible to dye cells and observe electroporated (dyed) and non-electroporated (non-dyed) cells at the same time, since the dye would adversely affect the microelectrode structures and change the electric properties of the medium and possibly of cells as well. Determined optimal parameters of electric pulses for electroporation in specific medium are in agreement to those obtained previously [33].

Based on our experience and published data we determined that the electric conductivity of the cell membrane changes considerably after electroporation (see Table 1) which has a strong influence on $Re[f_{CM}]$ mainly at low frequencies. We calculated $Re[f_{CM}]$ by applying a single-shell model on the electric properties of the cell. From the $Re[f_{CM}]$ spectra we can predict that dielectrophoresis crossover of the electroporated cells (f_0=0.2 MHZ, see Fig. 5) should appear at lower frequencies than of the non-electroporated cells (f_0=0.4 MHZ). If these theoretical results are compared with the experimental ones presented in Fig. 7, we can conclude that the calculated f_0 of the non-electroporated cells matches the experimentally obtained crossover frequency. Fig. 7 (above) shows the results of motion of non-electroporated cells by increasing frequency of the applied signal from 10 kHz up to 10 MHz. At low frequencies the non-electroporated cells move toward areas of the low-field intensity. At a frequency of about 0.5 MHz the cells start to move toward the edges of the electrodes were high-field intensity is generated. The cell motion was also observed by decreasing the frequency of the applied signal from 10 MHz to 10 kHz. In this case the cells begin to move toward the areas of the low-field intensity at a frequency of about 0.3 MHz. Experimentally determined f_0 of the non-electroporated cells suspended in medium M1 is therefore between 0.3 MHz and 0.5 MHz, which corresponds well to the calculated f_0=0.4 MHz.

The calculated f_0=0.2 MHz of the electroporated cells suspended in medium M1, however, did not match the experimentally determined crossover frequency. At low frequencies the electroporated cells move toward the areas of the low-field intensity (negative dielectrophoresis). The electroporated cells are exposed to negative dielectrophoresis up to a frequency of about 10 MHz, see Fig. 7 (below). According to theoretical predictions we expected that the crossover frequency f_0 of the electroporated cells is lower than that of the non-electroporated ones. However, the experiments revealed just the opposite. The theoretical predictions for the electroporated cells were based on Table 1 and on the single-shell model of a spherical cell. Therefore, one possibility is that we did not

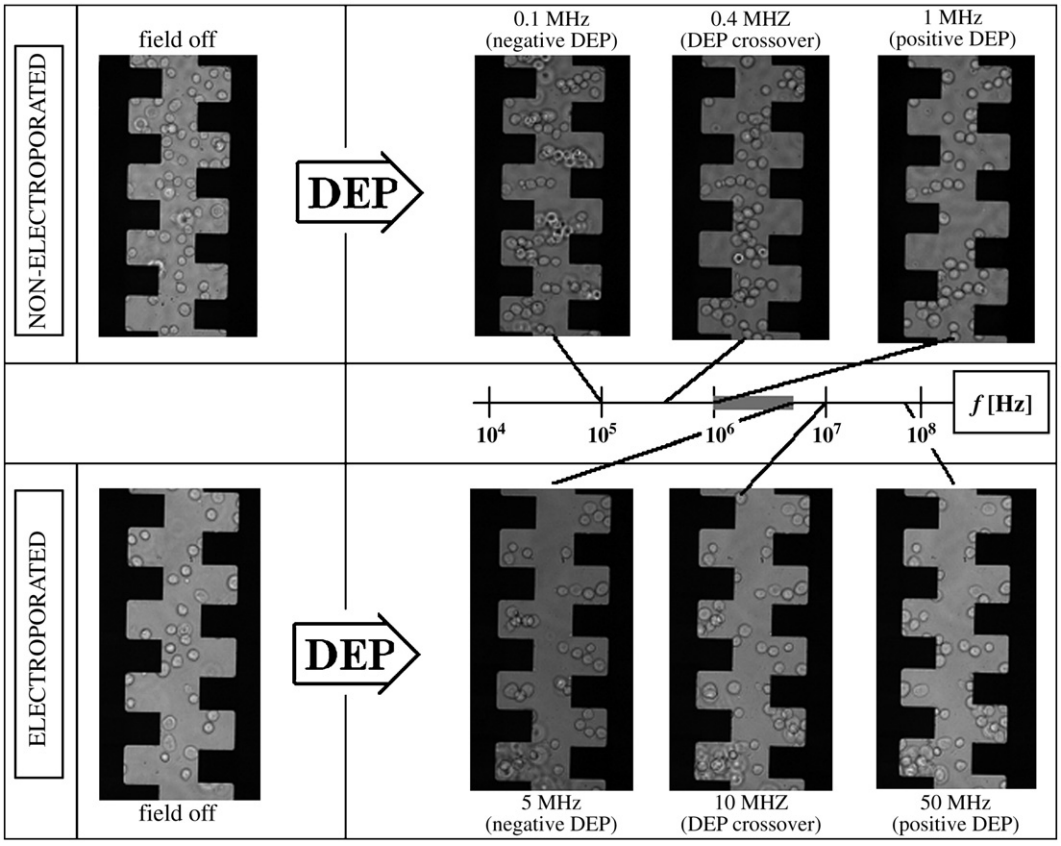

Fig. 7. Dielectrophoresis behaviour of the non-electroporated and electroporated cells in medium M1. For cell separation by means of dielectrophoresis it is important that cells have different crossover frequency. The crossover frequency of the non-electroporated cells is 0.4 MHz and the crossover frequency of the electroporated cells is approximately 10 MHz. Therefore, cells can be separated at frequencies between 0.4 MHz and 10 MHz, because non-electroporated cells are exposed to positive and electroporated cells to negative dielectrophoresis.

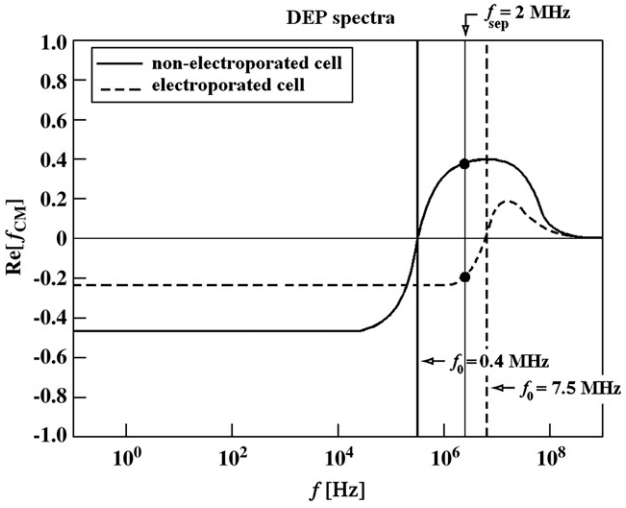

Fig. 8. *Re*[f_{CM}] and f_0 of the electroporated and non-electroporated cells in medium M1, considering 10 to 20 times lower permeability of cell membrane after electroporation.

succeed to describe a biological cell with the single-shell model, because the model was too simple. A more likely option is that at least one property of the electroporated cell, given in Table 1, is not correct. Taking into consideration that the dimensions of the electroporated cells are well investigated and documented [22,31,32], we can conclude that the electric properties of the cell membrane or cytoplasm given in Table 1 are incorrect. At electroporation, significant changes appear particularly on the cell membrane. There were many studies investigating the electric conductivity of the membrane, however, there are not many studies on cell membrane permittivity after electroporation. It is interesting that cell membrane permittivity in particular has a significant role on f_0. If we require the calculated f_0 of the electroporated cells suspended in medium M1 (see Fig. 8) to correspond to the experimental results, the cell membrane permittivity after electroporation should have been reduced for at least ten times of its value before the electroporation (from 4.4×10^{-11} As/Vm to around 4×10^{-12} As/Vm).

Considering that the non-electroporated and electroporated cells have different f_0 (0.4 MHz and 10 MHz), the cells could be separated with dielectrophoresis at a frequency of approximately 2 MHz as shown in Figs. 1 and 8. At this frequency, the electroporated cells move toward the middle of two adjacent electrodes, where the low-field intensity is generated, while the non-electroporated cells move toward the edges of the electrodes, where the areas of the high-field intensity are. In several separate experiments the non-electroporated and electroporated cells suspended in medium M1 were exposed to dielectrophoresis at frequency of 2 MHz. On the basis of cell arrangement around the microelectrode structures it was easy to determine if the cells were electroporated or not, see Fig. 9.

Cell separation using a castellated microelectrode structures is, of course, not the only possibility of cell separation using dielectrophoresis. There are many widely-used techniques that use dielectrophoresis force for cell separation [25]. Nevertheless, they are all based on the same principle.

5. Conclusion

The aim of our study was to research the influence of cell electroporation on the dielectrophoresis and to investigate the possibilities of separation of the non-electroporated and electroporated cells by means of dielectrophoresis. It was obvious from the experimental results that the behaviour of the non-electroporated and electroporated cells exposed to dielectrophoresis is different. The influence of the dielectrophoresis on the movement of the cells was investigated in the media with different conductivities: M1 ($\sigma_{M1}=0.174$ S/m) and M2 ($\sigma_{M2}=0.0012$ S/m). The motion of the cells suspended in medium M2 was more intense then in medium M1, but it was practically impossible to determine the conditions for cell separation. On the other hand, we were successful in separating the non-electroporated and electroporated cells suspended in medium M1 by exposing the cells to the electric field at a frequency of about 2 MHz. Taking into account the theoretical predictions and the behaviour of electroporated cells exposed to

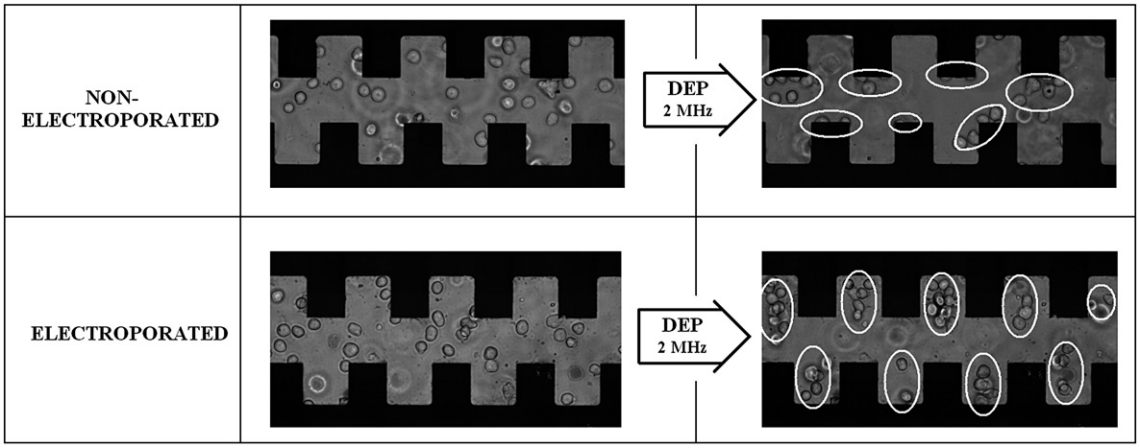

Fig. 9. Separation of electroporated and non-electroporated cells at a frequency of 2 MHz. Non-electroporated cells are exposed to positive dielectrophoresis, therefore they move toward the edges of the microelectrode. On the other side, electroporated cells move toward the middle of two adjacent electrodes because they are exposed to negative dielectrophoresis.

dielectrophoresis we presume that cell membrane permittivity decreases after electroporation for at least ten times.

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