



CELLULAR & MOLECULAR BIOLOGY LETTERS
<http://www.cmbi.org.pl>



Received: 21 June 2012
Final form accepted: 17 December 2012
Published online: 27 December 2012

Volume 18 (2013) pp 102-119
DOI: 10.2478/s11658-012-0042-3
© 2012 by the University of Wrocław, Poland

Research article

REVERSIBLE AND IRREVERSIBLE ELECTROPORATION OF CELL SUSPENSIONS FLOWING THROUGH A LOCALIZED DC ELECTRIC FIELD

WŁODZIMIERZ KOROHODA*, MACIEJ GRYS and ZBIGNIEW MADEJA*

Department of Cell Biology, Faculty of Biochemistry, Biophysics and
Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387, Cracow, Poland

Abstract: Experiments on reversible and irreversible cell electroporation were carried out with an experimental setup based on a standard apparatus for horizontal electrophoresis, a syringe pump with regulated cell suspension flow velocity and a dcEF power supply. Cells in suspension flowing through an orifice in a barrier inserted into the electrophoresis apparatus were exposed to defined localized dcEFs in the range of 0-1000 V/cm for a selected duration in the range 10-1000 ms. This method permitted the determination of the viability of irreversibly electroperforated cells. It also showed that the uptake by reversibly electroperforated cells of fluorescent dyes (calcein, carboxyfluorescein, Alexa Fluor 488 Phalloidin), which otherwise do not penetrate cell membranes, was dependent upon the dcEF strength and duration in any given single electrical field exposure. The method yields reproducible results, makes it easy to load large volumes of cell suspensions with membrane non-penetrating substances, and permits the elimination of irreversibly electroperforated cells of diameter greater than desired. The results concur with and elaborate on those in earlier reports on cell electroporation in commercially available electroporators. They proved once more that the observed cell perforation does not depend upon the thermal effects of the electric current upon

* Authors for correspondence: e-mail: w.korohoda@uj.edu.pl, tel.: + 48 12 664 61 25, fax: +48 12 664 69 02; e-mail: z.madeja@uj.edu.pl, tel.: + 48 12 664 61 42

Abbreviations used: DC – direct current; dcEF – direct current electric field; Eth BR₂ – ethidium bromide; FBS – fetal bovine serum; FDA – fluorescein diacetate; HSF – human skin fibroblasts; IRE – irreversible electroporation; PBS – buffered saline without or without calcium and magnesium ions; RBC – red blood cells; RE – reversible electroporation

cells. In addition, the method eliminates many of the limitations of commercial electroporators and disposable electroporation chambers. It permits the optimization of conditions in which reversible and irreversible electroporation are separated. Over 90% of reversibly electroporated cells remain viable after one short (less than 400 ms) exposure to the localized dcEF. Experiments were conducted with the AT-2 cancer prostate cell line, human skin fibroblasts and human red blood cells, but they could be run with suspensions of any cell type. It is postulated that the described method could be useful for many purposes in biotechnology and biomedicine and could help optimize conditions for *in vivo* use of both reversible and irreversible electroporation.

Key words: Irreversible electroporation, Reversible electroporation, Flow through electric field, Fluorescent dye loading, Cell viability, Direct current electric field, Focused electric field, Electrophoresis apparatus, Cell suspension electroporation system

INTRODUCTION

Reversible electroporation (RE) is often used to introduce substances into cells, such as dyes, drugs, proteins and nucleic acids, which otherwise do not penetrate through cell membranes [1-3]. In recent years, increasing attention has been paid to irreversible electroporation (IRE), which was found to be an effective technique for neoplastic tissue ablation without scar formation or local bleeding [4-8].

There is commercially available equipment for electroporation, based on the application of electric pulses to cell suspensions or tissues. Its design limits the influence of the investigator on the experimental procedure. The amplitude (strength), duration and number of electric pulses applied to cells can be modulated only in a narrow range, and the disposable chambers for electroporation of cell suspensions are usually of low volume (less than 0.5-1 ml) [9-11].

The experiments presented in this report were carried out in order to develop a research-appropriate system that would be free of some of the limitations associated with the use of commercially available electroporators and electric pulse generators.

The second goal was to quantitatively examine the efficiency of the developed method of cell electroporation, described herein as flow through a localized electric field. These measurements focused on confirming and elaborating on earlier studies performed using commercial electroporators. It is suggested that the described alternative method of cell electroporation facilitates further research in this field and could be useful in the search for improved conditions of electroporation *in vivo*, which requires the use of conventional commercial electroporators. This method is expected to open new possibilities for broader and easier application of cell electroporation in research related to biotechnology and medicine.

MATERIAL AND METHODS

Chemicals

Ethidium bromide, diacetate fluorescein, Alexa Fluor 488 Phalloidin, gentamicin, calcein and Trypsin-EDTA were obtained from Sigma. Fetal bovine serum (FBS) was purchased from Gibco (Invitrogen). Carboxyfluorescein was from Fluka-biochemist (Buchs, Switzerland) and culture medium RPMI 1640 with L-glutamine from Lonza. PBS-buffered saline without calcium and magnesium ions and PBS-buffered saline with calcium and magnesium ions were obtained from Biomed, and sucrose from Merck.

Cells

Experiments were carried out on the well-characterized AT-2 rat prostate cancer cell line grown in 25-cm² flasks (Sarstead) as described previously [12]. For some experiments, normal human skin fibroblasts (HSF) were grown *in vitro* as described previously [13]. Human red blood cells isolated from blood drawn from healthy laboratory volunteers were also used. Before the electroporation experiments, the cells were washed in Ca,Mg-free PBS by centrifugation and suspended in an electroporation solution. The electroporation solution, if not modified as described in the Results section, was 9.5% sucrose and PBS with Ca/Mg ions in the ratio 19:1.

Cell electroporation system

Cell electroporation was carried out with a setup consisting of a standard EP 1201-1EA apparatus for horizontal electrophoresis (Sigma-Aldrich, UK), a PS 304 Electrophoresis Power Supply (Gibco BRL, Life Technologies, USA; not shown on the photo), a Type 610 BS Syringe Infusion Pump (Medipan, Warsaw, Poland; not shown on the photo), power supply cords with plugs, a silicon rubber tube with an inserted 3-cm long segment made of a dialysis tube 1 mm in diameter (made from Sigma dialysis tubes), an exchangeable plexiglass barrier (1 to 5 mm thick) with an orifice 1 mm in diameter, and probe electrodes situated at two sides of the barrier and connected to a Multimeter G-1004.500 voltmeter (RFT, Germany) to measure the dcEF strength across the barrier. The tube was immersed in the same electroporation buffer as the cell suspensions. The linear velocity of the cell suspension flow was estimated by dividing the volume of the suspension pumped in one second by the diameter of the orifice in the barrier. Continuous velocity of the flow was assured by the syringe infusion pump – peristaltic pumps are not suitable for this purpose. The duration of cell suspension exposure to high dcEFs focused in the segment of the tube passing through the barrier orifice was calculated from the known thickness of the barrier and the linear velocity of the flow of the cell suspension.

Cell viability examination

Cell viability was determined using the fluorescein diacetate (FDA) test, which is based on the preservation of esterase activity, and the ethidium bromide (Eth Br₂)

test, which is based on an examination of the maintenance of cell membrane integrity [14-17]. The viability of the electroporated cells was examined 15-30 min after electroporation. In each sample, at least 250 cells were observed and counted under an epi-fluorescent microscope (Jenavert, Carl Zeiss Jena, Germany). Green fluorescent cells were counted as alive and red fluorescent cells as dead or irreversibly electroporated (IRE; Fig. 1). Sporadically, the viability of cells loaded with carboxyfluorescein or calcein through electroporation was tested by washing in PBS and seeding in culture medium to cell culture flasks. Cells attaching to the plastic and actively spreading on its surface 2-5 h after electroporation were counted as alive.

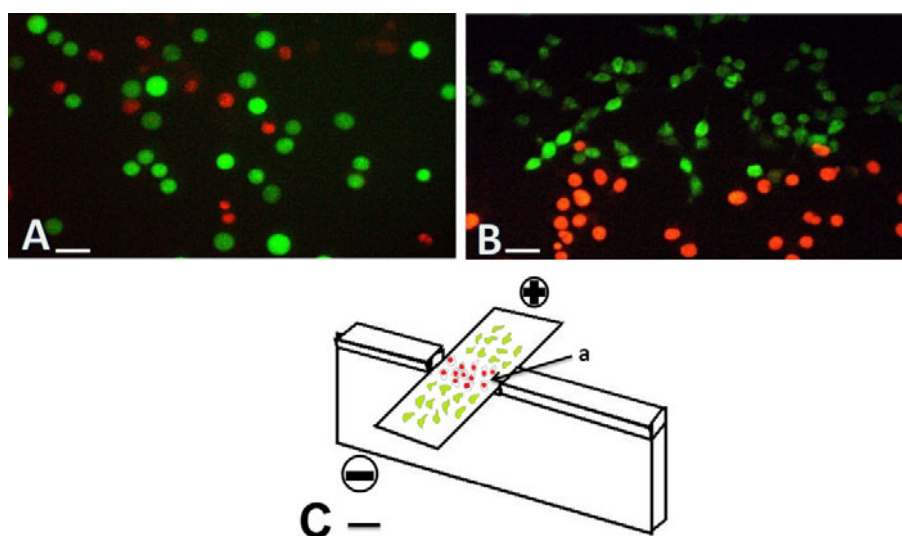


Fig. 1. Cell viability examination. A – Fluorescein diacetate/ethidium bromide test of the viability of the electroporated AT2 prostate cancer cell line was performed 10 min after electroporation. The fluorescent dyes were added on top of the spread cells. Viable cells fluoresce green, while dead (irreversibly electroporated) cells fluoresce red. Photos from a Zeiss epi-fluorescent microscope, magnification 100x, scale bar = 30 μm . B – Response of irreversibly electroporated AT2 cells to one pulse of 400 V/cm dcEF acting locally for 1000 ms. The cells at the end of the channel (the notch at point a in the plexiglass barrier) in which the electric field is focused are all dead, whereas cells outside the channel remain alive. Scale bar = 30 μm . C – The experiment to monitor the effect of a dcEF of 500 V/cm for 1000 ms focused on cells attached and spread on cover glass placed in a 10 x 3 x 1 mm notch (a) in a plexiglass barrier. Scale bar = 5 mm.

Cell size measurement

The sizes of spherical cells suspended in the electroporation medium were measured with a Coulter Z2 Cell and Particle Counter (Beckman Coulter, USA). The average diameter of the human red blood cells was 5.4 \pm 0.9 μm , that of the AT 2 rat prostate cancer cells was 14 \pm 1 μm , and that of the human skin fibroblasts was 18.67 \pm 2.3 μm .

RESULTS

The dcEF acting on cells can be focused and measured, as in the methods applied for cell electrophoresis or in the systems used for research on cell galvanotaxis [18-24]. With this method, strong electric fields can be localized and sharply separated in the designated region of the electric current circuit. The voltage gradient is calculated according to the Ohm law or directly measured with probe electrodes at the ends of the channel [20, 23, 24]. An experiment was

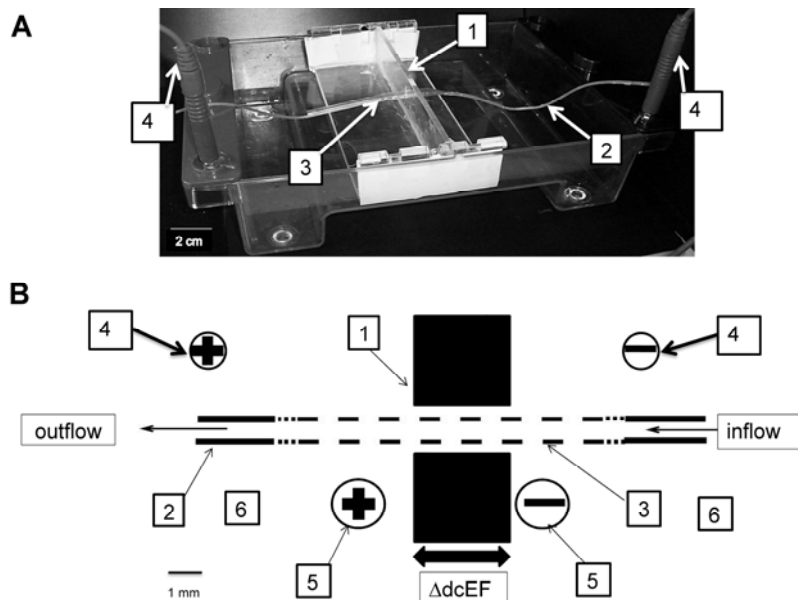


Fig. 2. Experimental setup applied for cell electroporation using a localized dcEF. A – Photo of setup. 1 – Transverse barrier made of plexiglass with a 1-mm diameter orifice, through which a tube passes in which the cell suspension flows. 2 – Silicon rubber tube with an inserted 3-cm long segment made of dialysis membrane (3) 1 mm in diameter (from Sigma dialysis tubes). 4 – Power supply cords with plugs. Scale bar = 2 cm. B – Diagram of the cell suspension flow circuit in which cells are exposed to electric fields. 1 – Exchangeable plexiglass barrier (1 to 5 mm thick) with an orifice 1 mm in diameter, through which a tube 1 mm in diameter passes. 2 – Silicon rubber tube with a 3-cm long insert made of an electric current-conductive dialysis membrane of low resistance to ensure that the electric field was focused within the tube in its region located within the barrier orifice. The inflow end of the tube is connected to a needle attached to the syringe infusion pump; the outflow end is attached to the sample collector (Petri dishes, test tubes or microscope slides). 3 – A 3-cm long segment of the tube made of cellophane dialysis membrane. 4 – Power supply cords with plugs joined with the electrophoresis apparatus. 5 – Probe electrodes situated either side of the barrier and joined to a Multimeter G-1004.500 voltmeter (RFT, Germany) to measure dcEF strength across the barrier. 6 – The external solution in which tube is immersed. Scale bar = 1 mm.

performed to monitor the effect of dcEF on cells on a cover glass. Cells were attached and spread on a cover glass, which was placed in a 10 x 3 x 1 mm notch in the plexiglass barrier. All the cells within the notch were killed when exposed to a dcEF of 500 V/cm for 1000 ms. Cells 100 μm away from this channel remained alive. The thickness of the borderline between the region of the notch in which a strong dcEF acted on cells and the remaining low electric field area is limited to a few dozen micrometers, i.e. the diameter of a single cell layer (Fig. 1). All the other experiments were carried out with a setup permitting the controlled bulk flow of cells through the limited region of the channel in which the strong dcEF is focused, as described in the Materials and Methods section (Fig. 2). The duration of the flowing cells' exposure to dcEF is determined by measuring the length of a sector of the channel exposed to the dcEF and the linear velocity of the flow of cell suspension. This corresponds to the single rectangular dcEF pulse applied to cells suspended in a stationary medium in disposable chambers attached to commercial electroporators.

The reproducibility of the results was tested by repeating the estimation of the percentage of AT2 cells surviving a single exposure for 870 ms to a given dcEF strength acting on flowing cells. The results shown in Fig. 3 confirm the reproducibility of the results of identical experiments on cell irreversible electroporation performed on two consecutive weeks.

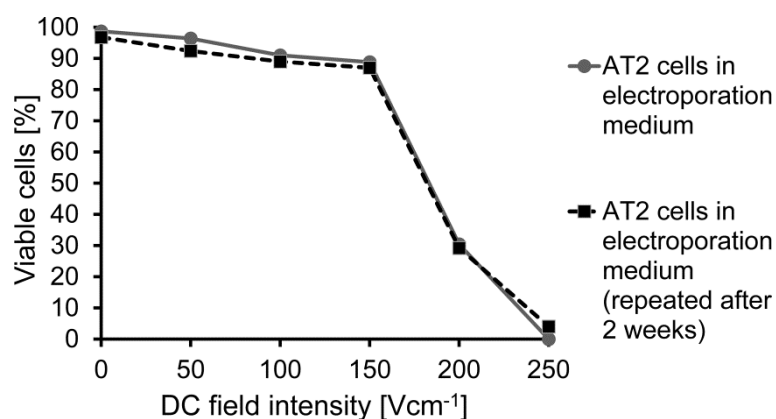


Fig. 3. Reproducibility of results of experiments carried out with the flow through a localized dcEF system. AT2 prostate cancer cells were exposed for 900 ms to a dcEF in an electroporation medium (Ca/Mg-free PBS and 9.5% (w/w) sucrose solution in a 1:19 ratio). The Y-axis shows the percentage of viable cells as determined with FDA-Eth BR₂ tests. The two curves represent the results of two separate experiments performed on two consecutive weeks.

Further experiments were carried out to confirm that both types of electroporation, RE and in particular cell IRE (for which stronger dcEFs must be applied), are not associated with the thermal effect and thermal coagulation of proteins [25]. AT2 rat prostate cells in three solutions significantly differing in

electric conductivity (0.98 mS/cm, 1.92 mS/cm, 3.6 mS/cm for solutions I, II and III, respectively) were electroporated. The results are shown in Fig. 4. In these solutions, heat production at the same dcEF differs several-fold when an electric current is passing through the suspension. Yet the shift of the thresholds for IRE expressed in dcEF strength is only a few percent, probably because of the effects of ionic strength on the electrical stability of cell membranes [26]. This shows the lack of correlation between biological effects of dcEFs causing irreversible electroporation with heat produced by the electric current under experimental conditions, when media with relatively low conductivity are used.

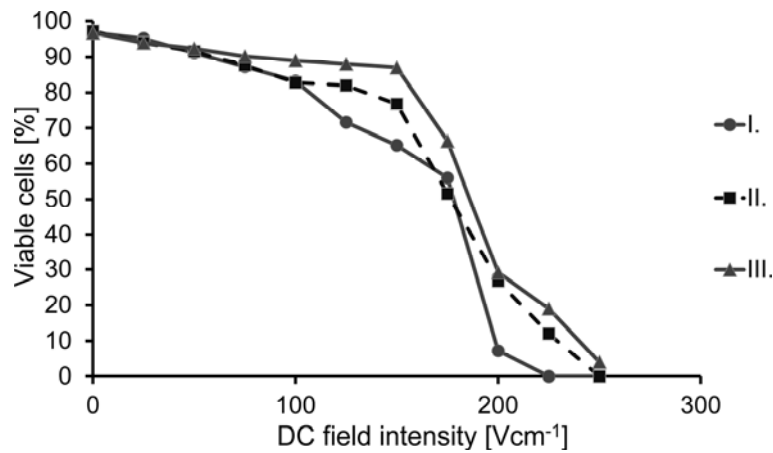


Fig. 4. Results of three experiments carried out in media of different electrical conductivity (I – 0.98 mS/cm, II – 1.92 mS/cm, III – 3.6 mS/cm) showing no correlation between Joule heat production and irreversible electroporation in low conductivity media.

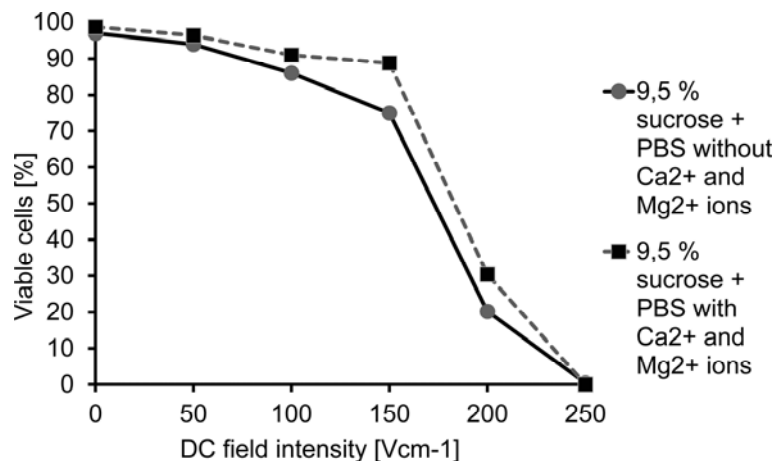


Fig. 5. Viability of AT2 cells electroporated in the presence and absence of calcium and magnesium ions. Cell exposure to dcEF for 900 ms.

Similar observations on the lack of medium conductance influencing the thresholds of IRE (RE) were reported in [27], but the authors did not discuss that this excluded significant effects of heat production on cell electroporation carried out in low conductivity media.

The presence or absence of calcium ions (when PBS with and without Ca/Mg was used) was found to have a very limited effect on the dependence of AT2 cell survival on dcEF strength at 900 ms exposure time, as shown in Fig. 5.

Fig. 6 presents a series of curves showing that the percentage of AT2 cells surviving a single exposure to dcEF was dependent on dcEF strength and exposure time. Each curve corresponds to a different duration of cell exposure to dcEF and each point shows the percentage of living cells when at least 300 cells in the sample were counted. These results show that the dcEF strength required to cause IRE at a longer single exposure (in the range of 500-1000 ms) can be a few times lower than for a single short exposure (i.e. 80-200 ms).

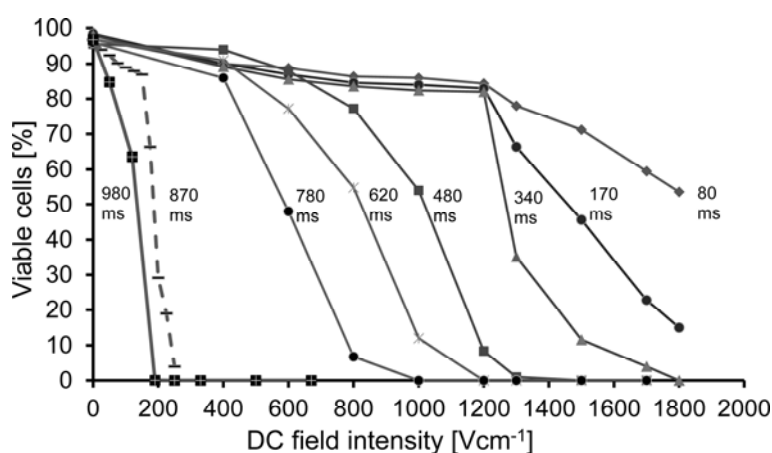


Fig. 6. Viability of AT2 cells exposed to dcEFs of different durations. Each curve corresponds to a different exposure time.

The next series of experiments concerned RE and the uptake of dyes that do not penetrate the membranes of living cells. Fig. 7A, B and C respectively show the curves of AT2 cell survival as a function of single exposure to dcEFs of a given strength applied to cells for 340, 620 or 980 ms. Survival was measured by the uptake of ethidium bromide 15-30 min after electroporation (cf. Materials and Methods), showing the effectiveness of IRE together with corresponding curves for calcein uptake showing the effectiveness of RE. The uptake and maintenance of calcein AM in cells is often used as a marker of cell viability [28, 29]. The decline in the uptake of calcein after exceeding the optimal values of dcEF at a given duration of the field action corresponds therefore to an increasing number of cells that are unable to retain calcein. The RE is caused by much weaker dcEFs and/or shorter exposure times to dcEFs than the IRE. The curves

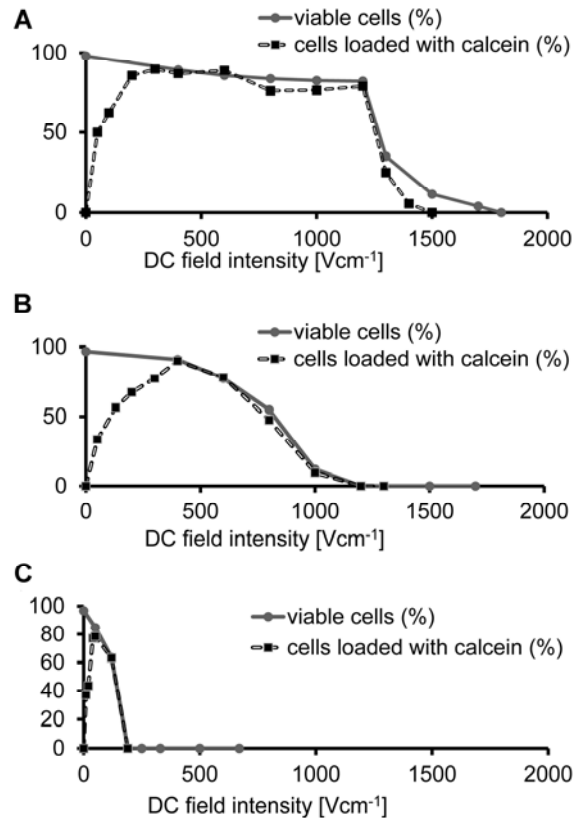


Fig. 7. Viability tested with the FDA/ethidium bromide method, and the uptake and maintenance of calcein in AT2 cells as a function of dcEF strength for three different times of cell exposure to the electric field. A – 340 ms, B – 620 ms, C – 980 ms.

show that with shorter exposure times to a single dcEF action, it is easier to establish conditions in which a high proportion of the cells is reversibly electroporated and remains alive. The viability of the cells that took up the dyes present in the cell suspension when electroporated was additionally tested by seeding the electroporated cells to cell culture conditions. Electroporated cells under these conditions attach to the bottom of the cell culture flasks and spread within 2-3 h (data not shown).

The conclusion that shorter exposure times are favorable for RE, whereas longer exposure times are more effective for cell killing through IRE was confirmed in further experiments (Fig. 8). The comparison of the effect of a single exposure to electric fields, causing either cell loading with calcein (RE) or cell death (IRE), shows that the shorter the time of exposure, the greater the difference between the dcEF strength required for RE and IRE.

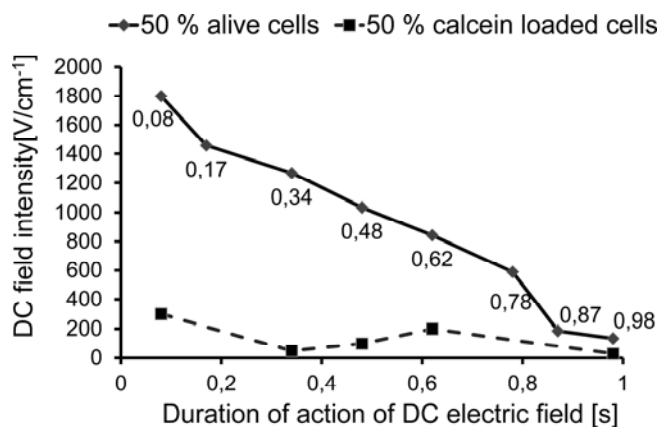


Fig. 8. ED_{50} of the electric field strength causing 50% cell loading with calcein (lower curve) and 50% cell death (upper curve) as dependent upon the duration of cell exposure to the electric field. Numbers below the upper curve show the duration of cell exposure to the electric field.

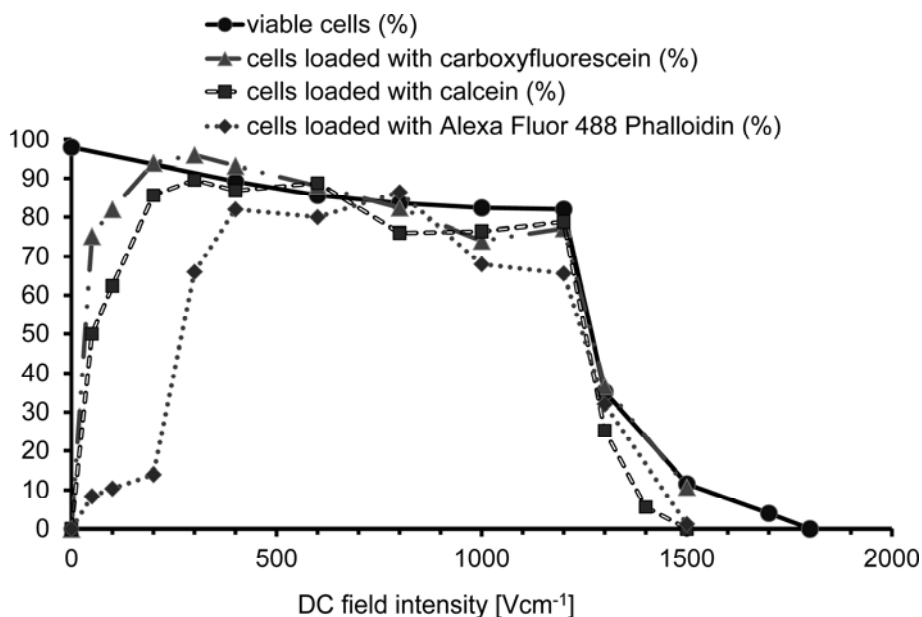


Fig. 9. Viability, uptake and retention of three fluorescent dyes (6-carboxyfluorescein, calcein and Alexa Fluor 488 Phalloidin) in AT2 cells exposed to a dcEF for 340 ms.

Fig. 9 presents a series of curves showing cell viability and the uptake and retention of three different fluorescent dyes as dependent upon dcEF strength when exposed to dcEFs for 340 ms. The larger the dye molecule (in order: carboxyfluorescein, calcein, Alexa Fluor 488 Phalloidin), the stronger the field must be to induce an effective RE of cells and uptake of the tested dye. None of

these dyes penetrate the cell membrane of AT2 cells under standard experimental conditions in the cell growth media or in the electroporation solution. The MW of the tested dyes for carboxyfluorescein is 332.306 Da, for calcein is 622.55 Da, and for Alexa Fluor 488 Phalloidin is 1320 Da. When the dcEFs increase and reach values sufficient to cause IRE and cell death, the uptake of dyes gradually decreases. For smaller molecules, it is easier to find conditions when almost 100% of cells are reversibly electroporated (take up the dye) and remain alive.

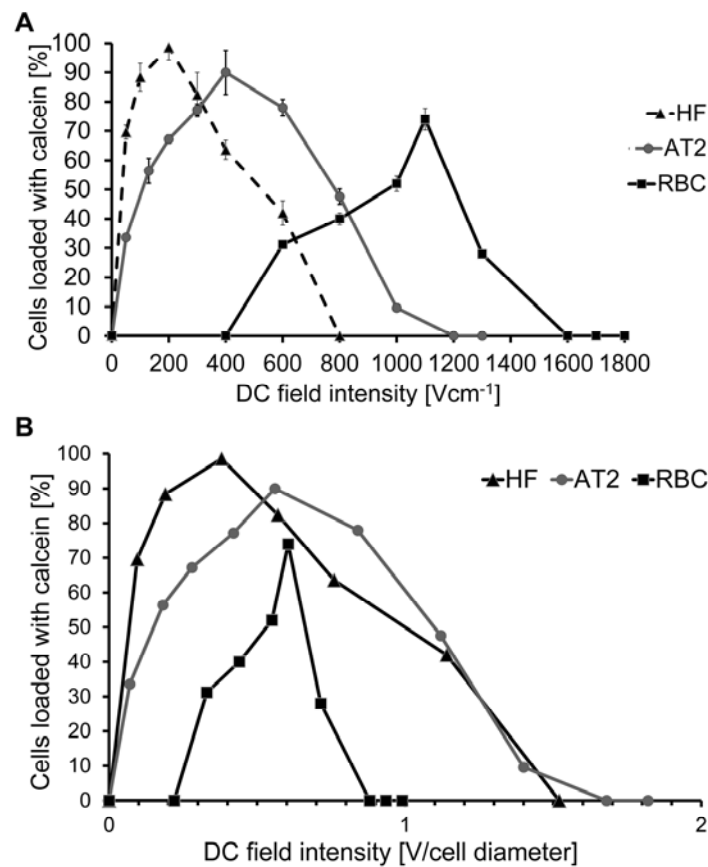


Fig. 10. Selective IRE of cells based on their diameter. A – Loading of cells and maintenance of calcein as a function of dcEF acting for 620 ms on three cell types differing in size: human RBC (5.4 μm in diameter), AT2 prostate cancer cells (14 μm in diameter) and human skin fibroblasts (HSF; 18.7 μm in diameter). B – The same curves but with dcEF strength expressed as a voltage gradient per cell diameter.

It was reported that IRE can be applied to eliminate and kill cells with diameters greater than a selected size threshold [30, 31]. Subsequent experiments were therefore performed to examine whether the method applied in our experiments can be useful for this purpose. The RE of viable cells and IRE when calcein is no

longer kept in the cells were estimated for human skin fibroblasts (HSF) of $18.67 \pm 2.3 \mu\text{m}$ in diameter, AT2 cells of $14 \pm 1 \mu\text{m}$ in diameter, and red blood cells (RBC) of $5.4 \pm 0.9 \mu\text{m}$ in diameter. The cells were given a single exposure to dcEF for 340 ms. The results are shown in Fig. 10A. Much stronger dcEFs were required for both RE and IRE of RBC than for AT2 cells. The ED_{50} for RBC was 966.7 V/cm, for AT2 cells was 194 V/cm and for HSF was 35.9 V/cm. LD_{50} for IRE was 1204 V/cm for RBC, 784 V/cm for AT2 cells and 525.9 V/cm for HSF. When these values were normalized to the cell diameter, they corresponded to dcEF values causing 50% RE, as shown in Fig. 10B. The values expressed as dcEF/cell diameter are: 0.53 V/cell for RBC, 0.27 V/cell for AT2 cells, and 0.07 V/cell diameter for HSF. LD_{50} was: 0.7 V/cell for RBC, 1.1 V/cell for AT2 cells, and 1 V/cm for HSF.

DISCUSSION

Our results show that the described method of electroporation of cell suspensions exposed to a short localized dcEF is very versatile. It permits the adjustment and control of the strength of the electric field and time of its action on flowing cells. In addition, the described system is based on the use of standard laboratory equipment.

The results concerning cell viability and cell loading with cell membrane non-permeable substances are reproducible and the method can be used for electroporation of small volumes of cell suspensions (0.1 ml) as well as for large suspension volumes (5-10 ml). The dcEF strength acting locally on flowing cells can be regulated in the range of 0-2000 V/cm and the duration of exposure of the flowing cells to the dcEFs can be adjusted in the range of one to a few thousand ms.

The experiments were limited to the effects of a single exposure of cells to dcEFs of known strength and duration. We studied the influence of the duration of AT2 cell exposure to dcEFs of variable strength upon cell survival (IRE) and/or upon reversible perforation (RE) and cell loading with membrane non-permeable substances (fluorescent dyes). We found that for IRE, it is favorable to use relatively long lasting (500-1500 ms) cell exposure times to dcEFs.

External dcEFs acting on suspended cells influence the cell membrane as a primary target [32, 33]. The cell interior, nucleus, mitochondria and other organelles within the cell remain screened from the externally acting dcEFs. However, when the dcEF acts on the cells for a longer time (i.e. milliseconds), pores appear within the first phase of the cell exposure to dcEF. As a consequence, the electric field can later act within the cells and its effects are no longer limited to the cell membranes. The process of pore formation is very fast, but the pores remain open for much longer, depending on temperature [34-37]. Thus, once the pores are formed, the ionic current begins to flow through the cytoplasm and the dc electric field begins to act within the cell interior on the nucleus and cytoplasmic structures. Cell death due to IRE, in particular with longer exposure times (in the range of dozens and hundreds of milliseconds) or when cells are

subjected to a series of electric pulses, for example of 100 ms duration, can therefore also depend on the electric field acting within the cells alongside its effects on the cell membrane. This might be similar to exposure of cells to ns pulses, which directly act on the nucleus and cytoplasm [38-40]. This question requires a separate set of experiments to be unequivocally answered.

The conditions under which flowing cell suspensions are reversibly electroporated are more favorable to cells than when the cells are electroporated within electroporation chambers. Aluminum or stainless steel electrodes are generally mounted in the conventionally used disposable electroporation chambers [10]. Such electrodes polarize, causing the dcEFs, applied as rectangular pulses in the solution, to change shape with time. Polarizable electrodes also cause gas bubble formation, which can disturb the distribution of the dcEF within the chamber and may injure the cells. Because ions are characterized by high electrokinetic mobility, the dcEF can induce local changes in pH and ion concentration near the surface of the electrodes. These inconveniences are absent when cells flow through a locally focused electric field. The exposure of cells located in a narrow canal in which dcEFs are focused was also recently tested [41]. The authors used needle steel electrodes and this led to issues associated with the appearance of gas bubbles. These bubbles disturbed the electric field and caused cell injury. The authors therefore concluded that dcEFs are not suitable for electroporation of cells located in the narrow channel between electrodes. To avoid these difficulties, they applied pulses of alternate polarity. Similar difficulties were found years ago when stainless steel or aluminum polarizable electrodes were tested in research into cell electrophoresis or cell galvanotaxis. Such difficulties in research on cell electrophoresis or cell galvanotaxis were avoided by the use of reversible Ag/AgCl electrodes, and often by additional separation of electrodes with agar bridges from the chambers or capillaries containing the cells. In the same cases, the effects of electrode products upon cells were eliminated by focusing the dcEF at a significant distance from the surface of the electrodes [12, 18-22].

The cell flow system for electroporation that we used is similar to that used in experiments on cell electrophoresis and cell galvanotaxis [12, 23]. The dcEF was focused about 12 cm from the electrodes of the electrophoresis apparatus, and flowing cells were separated from the electrode-containing solution by a dialysis membrane. In some experiments, reversible Ag/AgCl electrodes were also used, but this did not change the results. The protection of cells from the harmful effects of electrode products and good heat dispersion in our experiments could be the reason why high percentages of cells were reversibly electroporated, preserving over 90% cell viability, which is much higher than usually reported in experiments with conventional electroporators.

In summary, the results of our experiments with the flow of cell suspensions through localized dcEFs have shown that:

1. Experiments with such a system would be easier than those with commercial electroporators, and researchers could more precisely control experimental conditions.
2. The system can be used for RE and loading of large volumes of cell suspensions with cell membrane non-permeable substances. In the case of loading cells with vital fluorescent dyes it can replace the need for expensive derivatives of the dyes (AM calcein, BCECF, etc.).
3. The use of electrodes that are separated from the cells protects the cells against harmful electrode products and permits the preservation of good viability of electroporated cells, to over 90% when RE is performed.
4. The decrease in Joule (Ohmic) heating of the cell suspension thanks to low-electric conductivity media and good heat dispersion confirmed that RE and IRE are not related to cell heating [22, 27, 42, 43].
5. The estimation of strength-duration curves for RE and IRE shows that a strong dcEF acting for a short time is favorable for RE, whereas longer action times and weaker dcEFs are effective for cell killing by IRE. It is easier to ensure conditions for a high percentage of cell loading with membrane non-permeable molecules. More than 90% of cells survive this procedure using a single dcEF action on flowing cells for less than 100 ms. On the other hand, for IRE, a longer exposure time was more efficient than for short ones.
6. The efficiency of RE and IRE strongly depends upon the size of the cells exposed to dcEFs; the differences in dcEF strength and duration causing RE and IRE of different cells depend not only on differences in the physical and chemical properties of the cell membranes but correlates with cell diameters and cell surface curvature [30, 44-48]. With the described method, it is easy to selectively kill cells of greater size than required in great cell suspension volumes, i.e. to obtain results similar to subjecting flowing cells to a series of rectangular electric pulses [30].

The described method of cell electroporation by short exposure of flowing cell suspensions to localized dcEFs can be used to search for improved conditions for RE and IRE experiments *in vivo*. The method can be applied for many purposes, e.g. for the introduction of membrane impermeable compounds to large amounts of cells, for the elimination of cells of size greater than required from mixed cell suspensions, or for the exact determination of parameters of the dcEF acting on cells. We believe that the conclusions from the results of *in vitro* experiments can be useful for planning the optimal conditions for *in vivo* clinical use of both RE and IRE.

Acknowledgements. This study was supported by the European Regional Development Fund within the Operational Programme Innovative Economy (UDA-POIG.01.03.01-14-036/09-00 “Application of polyisoprenoid derivatives as drug carriers and metabolism regulators”).

REFERENCES

1. Chang, D.C., Chassy, B.M., Saunders, J.A. and Sowers, A.E. **Guide to Electroporation and Electrofusion**, Academic Press Inc., San Diego, 1992.
2. Li, S. (Editor). **Electroporation Protocols. Preclinical, and Clinical Gene Medicine**, Humana Press, Totowa, New Jersey, 2008.
3. Neumann, E., Schaeffer-Ridder, M., Wang, Y. and Hofschneider, P.H. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. **EMBO J.** 1 (1982) 841-845.
4. Gissel, H., Lee, R.C. and Gehl, J. Electroporation and cellular physiology. In: **Clinical Aspects of Electroporation** (Kee, S.T., Gehl, J. and Lee, E.W., Eds.), Springer, New York, 2011, 9-17.
5. Kee, S.T., Gehl, J. and Lee, E.W. (Eds.). **Clinical Aspects of Electroporation**. Springer, New York, Dordrecht Heidelberg, London, 2011.
6. Miller, L., Leor, J. and Rubinsky, B. Cancer cells ablation with irreversible electroporation. **Technol. Cancer Res. Treat.** 4 (2005) 699-705.
7. Rubinsky, J., Onik, G., Mikus, P. and Rubinsky, B. Optimal parameters for the destruction of prostate cancer using irreversible electroporation. **J. Urol.** 180 (2008) 2668-2674.
8. Rubinsky, B. (Ed.). **Irreversible Electroporation**, Springer-Verlag, Berlin, Heidelberg, 2010.
9. Chen, N., Garner, A.L., Chen, G., Jing, Y., Deng, Y., Swanson, R.J., Kolb, J.F., Beebe, S.J., Joshi, R.P. and Schoenbach, K.H. Nanosecond electric pulses penetrate the nucleus and enhance speckle formation. **Biochem. Biophys. Res. Commun.** 364 (2007) 220-225.
10. Raptis, L. and Firth, K.L. Electrode assemblies used for electroporation of cultured cells. **Methods Mol. Biol.** 423 (2008) 61-76.
11. Yumura, S., Matsuzaki, R. and Kitanishi-Yumura, T. Introduction of macromolecules into living *Dictyostelium* cells by electroporation. **Cell Struct. Funct.** 20 (1995) 185-190.
12. Djamgoz, M.B.A., Mycielska, M., Madeja, Z., Fraser, S.P. and Korohoda, W. Directional movement of rat prostate cancer cells in direct-current electric field: involvement of voltage-gated Na⁺ channel activity. **J. Cell Sci.** 114 (2001) 2697-2705.
13. Krzysiek-Mączka, G. and Korohoda, W. Surface anisotropy orients cell divisions in contact guided cells. **Folia Biol.** 56 (2008) 13-19.
14. Karmiol, S. Cell isolation and selection. In: **Methods of Tissue Engineering**. (Atala, A. and Lanza, R., Eds), Academic Press, San Diego, 2002, 19-35.
15. Kemp, R.B., Meredith, R.W.J., Gamble, S. and Frost, M. A rapid cell culture technique for assessing the toxicity of detergent-based products in vitro as a possible screen for eye irritancy in vivo. **Cytobios** 36 (1983) 153-159.

16. Szydłowska, H., Zaporowska, E., Kuszlik-Jochym, K., Korohoda, W. and Branny, J. Membranolytic activity of detergents as studied with cell viability tests. **Folia Histochem. Cytochem.** 16 (1978) 69-78.
17. Zaporowska-Siwiak, E., Michalik, M., Kajstura, J. and Korohoda, W. Density-dependent survival of Ehrlich ascites tumor cells in the presence of various substratum for energy metabolism. **J. Cell Sci.** 77 (1985) 75-85.
18. Abramson, H.A., Moyer, L.S. and Gorin, M.H. **Electrophoresis of Proteins and the Chemistry of Cell Surface**. Reinhold Publ. Corp., NY, 1942.
19. Cooper, M.S. and Schliwa, M. Electrical and ionic controls of tissue cell locomotion in DC electric fields. **J. Neurosci. Res.** 13 (1985) 223-244.
20. Ericson, C.A. and Nuccitelli, R. Embryonic fibroblasts motility and orientation can be influenced by physiological electric fields. **J. Cell Biol.** 98 (1984) 296-307.
21. Korohoda, W. Effect of electric field on cell movement. In: **Tenth School on Biophysics of Membrane Transport**. School proceedings, Poland, 1990, 178-191.
22. Korohoda, W. and Kurowska, A. Quantitative estimations of the thresholds of electrotactic responses in Amoeba proteus. **Acta Protozool.** 7 (1970) 375-382.
23. Korohoda, W., Mycielska, M., Janda, E. and Madeja Z. Immediate and long-term galvanotactic responses of Ameba proteus to dc electric fields. **Cell Motil. Cytoskeleton** 45 (2000) 10-26.
24. Nuccitelli, R. and Erickson, C.A. Embryonic cell motility can be guided by physiological electric fields. **Exp. Cell. Res.** 147 (1983) 195-201.
25. Shafiee, H., Garcia, P.A. and Davlos, R.V. A preliminary study to delineate irreversible electroporation from thermal damage using the Arrhenius equations. **J. Biomech. Eng.** 131 (2009) 074509-074514.
26. Seaman, G.V.F. Electrophoresis using a cylindrical chamber. In: **Cell Electrophoresis** (Ambrose, E.J., Ed.), J.& A Churchill LTD., London, 1965, 4-21.
27. Pucihar, G., Kotnik, T., Kandušer, M. and Miklavčič, D. The influence of medium conductivity on electroporation and survival of cells in vitro. **Bioelectrochemistry** 54 (2001) 107-115.
28. Ammar, D.A., Noecker, R.J. and Kahook, M.Y. Effects of benzalkonium chloride-preserved, polyquad-preserved, and sofzia-preserved topical glaucoma medications on human ocular epithelial cells. **Adv. Ther.** 27 (2010) 837-845.
29. Schlieve, C.R., Lieven, C.J. and Levin, L.A. Biochemical activity of reactive oxygen species scavengers do not predict retinal ganglion cell survival. **Invest. Ophthalmol. Vis. Sci.** 47 (2006) 3878-3886.
30. Craiu, A. and Scadden, D. Flow electroporation with pulsed electric fields for purging tumor cells. **Methods in Molecular Biology**, The Humana Press, Totowa, NJ, 2008, 301-310.

31. Eppich, H.M., Foxall, R., Gaynor, K., Dombkowski, D., Miura, N. and Cheng, T. Pulsed electric fields for selection of hematopoietic cells and depletion of tumor cell contaminants. **Nat. Biotechnol.** 18 (2000) 882-887.
32. Kotnik, T., Pucihar, G. and Miklavčič, D. The cell in the electric field. In: **Clinical Aspects of Electroporation** (Kee, S.T., Gehl, J. and Lee, E.W., Eds.), 2011, 19-43.
33. Phez, E., Faurie, C., Golzio, M., Teissié, J. and Rols, M.P. New insight in the visualization of membrane permeabilization and DNA/membrane interaction of cells submitted to electric pulses. **Biochim. Biophys. Acta – Gen. Subjects** 1724 (2005) 248-254.
34. Teissié, J. and Rols, M. Manipulation of cell cytoskeleton affects the lifetime of cell membrane electroporation. **Ann. N. Y. Acad. Sci.** 720 (1994) 98-110.
35. Teissié, J., Escoffre, J.M., Rols, M.P. and Golzio, M. Time dependence of electric field effects on cell membranes. A review for a critical selection of pulse duration for therapeutic application. **Radiol. Oncol.** 42 (2008) 196-206.
36. Tsong, T.Y. Time sequence of molecular events in electroporation. In: **Guide to Electroporation and Electrofusion** (Chang, D.C., Chassy, B.M., Saunders, J.A., Sowers, A.E., Eds.) Academic Press, Inc., San Diego, Calif., 1992, 47-61.
37. Pucihar, G., Kotnik, T., Teissié, J. and Miklavčič, D. Electroporation of dense cell suspensions. **Eur. Biophys. J.** 36 (2008) 173-185.
38. Joshi, R.P. and Schoenbach, K.H. Bioelectric effects of intense ultrashort pulses. **Crit. Rev. Biomed. Eng.** 38 (2010) 255-304.
39. Long, G., Shires, P.K., Plescia, D., Beebe, S.J., Kolb, J.F. and Schoenbach, K.H. Targeted tissue ablation with nanosecond pulses. **IEEE Trans. Biomech. Eng.** 58 (2011) 2161-2167.
40. Nuccitelli, R., Chen, X. and Pakhomov, G.A. A new pulsed electric field therapy for melanoma disrupts the tumor's blood supply and causes complete remission without recurrence. **Int. J. Cancer** 125 (2009) 438-445.
41. Ziv, R., Steinhardt, Y., Pelled, G., Gazit, D. and Rubinsky, B. Micro-electroporation of mesenchymal stem cells with alternating electrical current pulses. **Biomed. Microdevices** 11 (2009) 95-101.
42. Davalos, R.V. and Rubinsky, B. Temperature considerations during irreversible electroporation. **Int. J. Heat Mass Transf.** 51 (2008) 5617-5622.
43. Fox, M.B., Esveld, D.C., Valero, A., Luttge, R., Mastwijk, H.C., Bartels, P.V., van der Berg, A. and Boom, R.M. Electroporation of cells in microfluidic devices: a review. **Anal. Bioanal. Chem.** 385 (2006) 474-485.
44. Fabczak, S., Korohoda, W. and Walczak, T. Studies on the electrical stimulation of contraction in Spirostomum. I. Conditions of the quantitative measurements. **Cytobiologie, Eur. J. Cell Biol.** 7 (1973) 152-163.
45. Hui, S.W. Overview of drugs delivery and alternative methods to electroporation. In: **Electroporation Protocols. Preclinical, and Clinical**

- Gene Medicine.** (Li, S., Ed.), Humana Press, Totowa, New Jersey, 2008, 91-107.
46. Sixou, S. and Teissié, J. Specific electropermeabilization of leukocytes in a blood sample and application to large volumes of cells. **Biochim. Biophys. Acta** 1028 (1990) 154-160.
 47. Teissié, J. and Rols, M. An experimental evaluation of the critical potential difference inducing cell membrane electropermeabilization. **Biophys. J.** 65 (1993) 409-413.
 48. Towhidi, L., Kotnik, T., Pucihar, G., Firoozabadi, S.M.P., Mozdarani, H. and Miklavcic, D. Variability of the minimal transmembrane voltage resulting in detectable membrane electroporation. **Electromagn. Biol. Med.** 27 (2008) 372-385.