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CELL BIOLOGY

Effect of Cell Passage Time on the Electrotransfection Efficiency

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Abstract—Gene electrotransfer is an effective and promising gene delivery technique in clinical applications, such as DNA vaccination and gene therapy. An improved gene therapy protocol depends on the the proper establishment of the gene transfer method. Electroporation has been widely employed in in vitro and in vivo protocols, and increaing its transfection efficiency has been the field of research. In order to achieve the the maximal introduction of plasmid DNA into cells with optimal cell viability, electro transfection conditions for every single cell type should be determined individually. In this work, the effect of cell passage time on the electrotransfection efficiency of CHO cells is determined. The selected cell passage times of 24 and 48 h prior to the electroporation are considered for the analysis. It is shown that electrotransfection efficiency with all plasmid concentrations significantly differs when comparing 24 and 48 h cell passage times. However, only slight change in the cell viability is observed at 24 and 48 h of cell passage times.

Keywords: plasmid DNA, electroporation, electrotransfection, optimization, cell passage time

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INTRODUCTION

Gene therapy is the emerging technology to treat or prevent disease by replacing a defective or missing gene (Wirth et al., 2013). A wide range of candidate genes for gene therapy have been divulged but very few have turned into target therapies because of the poor delivery of the nucleic acid (Rao and Zacks, 2014; Mostaghaci et al., 2016). Many viral and non-viral gene delivery methods have been used in cell transfection. Viral vectors have been thoroughly investigated and demonstrated to have high transfection efficiencies. However, they have potential risks such as immunogenicity, insertional mutagenesis, oncogenicity, etc. (Mulligan, 1993; Li and Huang, 2000; Kohn et al., 2003). Non-viral (chemical or physical) gene delivery approaches have been developed to overcome the inherent problems of viral gene vectors.

Among physical approaches, electroporation or electropermeabilization is one of the most promising methods for gene electrotransfer (Chopra and Satkauskas, 2018; Neumann et al., 1982; Rols et al., 1998; Satkauskas et al., 2002). Electroporation technique is based on the application of external electric field that permeabilizes the cell membrane by inducing transmembrane potential (Bonnafous et al., 1999; Neumann et al., 1999; Gehl, 2003; Gift and Weaver, 2000; Somiari et al., 2000; Phez et al., 2005). When the transmembrane potential exceeds the threshold, transient pores form in the membrane. These pores allow the entry of impermeable molecules to the cell (Rae and Levis, 2002). Based on the electroporation parameters such as electric field strength, pulse duration and the number of pulses, the cell membrane permeability may be either reversible and the cell viability is preserved or irreversible and leads to cell death (Kanduser et al., 2006). For gene electrotransfer, reversible electroporation is used (Mir, 2008).

Several studies have demonstrated different mechanisms of gene electrotransfer (Aihara and Miyazaki, 1998; Rols et al., 1998; de Gennes, 1999). Electromediated gene delivery is a complex, multistep process (Rosazza et al., 2016). Cell membrane must be permeabilized for plasmid/membrane interaction to happen as plasmid interacts only with permeabilized cell membrane (Teissié et al., 2008). The researchers have demonstrated that no plasmid/membrane interaction occurs if plasmid is added after electric field application(Tsong, 1991; Ganeva et al., 1995). After the formation of the complex, plasmid has to be translocated into the cytoplasm in order to reach the nucleus by crossing the nuclear membrane for its expression.

Membrane permeabilization is crucial but not enough for efficient gene transfer. The nuclear envelope has been thought to be a major impediment for

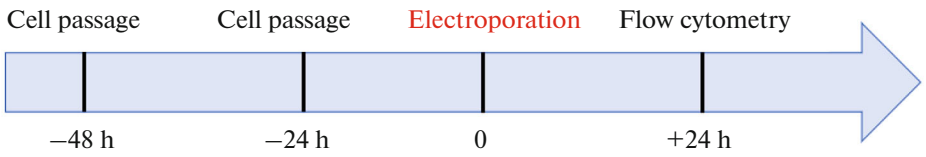

Fig. 1. Schematic representation of cell passage time during experimental investigation.

effective gene transfer (Bai et al., 2017). Capecchi has showed that the cytosolic injection of naked pDNA results in no gene expression whereas the nuclear injection of the same plasmid results in transgene expression in 50–100% of cells (Capecchi, 1980). Therefore, the fundamental limitation to gene expression is the inability of plasmid DNA to migrate from the cytoplasm into the nucleus. If the cell transfection happens during G2/M phase of the cell cycle when the nuclear envelope has disappeared, transfection efficiency has been shown improve (Escriou et al., 2001).

While the existing literature on electroporation research is vast, there is an apparent lack of conclusive studies detailing the relationship between cell passage time and transfection efficiency. The objective of this work is to investigate the dependence of electrotransfection on the elapsed time after cell passage at three levels: cell size, plasma membrane permeabilization and gene expression. The results presented here are important for the optimization of non-viral gene delivery. The timeline schematic of cell passage time is shown in Fig. 1.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary (CHO) cells (European Collection of Authenticated Cell Cultures) were grown at 37°C (5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM). DMEM supplemented with 1% penicillin-streptomycin (Sigma, P0781), 10% fetal bovine serum (FBS) (Sigma, F7524), and 1% of L-glutamine (Sigma, G7513). Cells were grown in a 10 cm diameter cell culture plate. Cells were passaged every 2–3 days and always a day before the experiment.

Plasmid

pMAX-GFP (3486 bp) (Amaxa, Cologne, Germany) plasmid driving the expression of green fluorescent protein (GFP) under the control of the CMV promoter was used for the electrotransfection. Plasmid was transformed in *Escherichia coli* DH5α and purified using endo-free plasmid Giga Prep kit (Qiagen, Valencia, CA, USA) according to the manufacturer guidelines. The purity, concentration and quality of plasmid were checked using NanoDrop spectrophotometer (Thermo Fisher, Washington, DC, USA) and gel electrophoresis.

Cell Electroporation

DNA electroporation was performed using BTX T820 electroporator (Harvard Apparatus, Holliston, MA, USA). For electroporation, the cells were trypsinized and resuspended in an electroporation medium that had 0.1 S/m conductivity, 270 mOsm osmolarity and 7.0–7.2 pH. The constituents of electroporation medium were sucrose (242 mM), Na_2HPO_4 (5.5 mM), NaH_2PO_4 (3 mM) and $MgCl_2$ (1.73 mM). The cell concentration was set to be 2×10^6 cells/mL. For each experimental point, 45 µL of cell suspension (9×10^4 cells) were supplemented with 5 µL plasmid DNA of different concentrations (10, 100, 200, 300, 400 and 600 µg/mL final concentration). Stainless steel electrodes with 2 mm gap were used to electroporate the cells. Cells were electroporated with 2 high voltage (HV) pulses (1400 V/cm, 100 µs, 1 Hz). Electroporation was carried out at different elapsed time since cell passage (24 or 48 h) to find out the optimal conditions for electrotransfection.

Evaluation of DNA Electrotransfer Efficiency

After the electric field application, cells were incubated for 10 min and then diluted with 950 µL of growth medium (DMEM). Then 900 µL of cell suspension was transferred to 24 well plate (Plastibrand; Wertheim, Germany) and incubated for 24 h in 37°C. The growth medium was removed from each well of the plate. Cells were harvested by trypsinization and resuspended in 200 µL of phosphate buffer saline (PBS). 10^4 cells per sample were measured in each sample. The percentage of GFP positive cells was detected using a flow cytometer (BD Accuri C6, BD Biosciences). The cells were excited by using 488 nm laser and the fluorescence was collected using 533/30 nm bandpass filter. BD Accuri C6 software was used to analyze the results, obtaining the percentageof GFP positive cells and the mean cell fluorescence (MCF) of the transfected cell population. The gating strategy used in this process is shown in Fig. 2.

The total fluorescence (TF) was calculated by the equation (1) and expressed as:

$$TF = \frac{TE \times 10000 \times MCF}{100}. \quad (1)$$

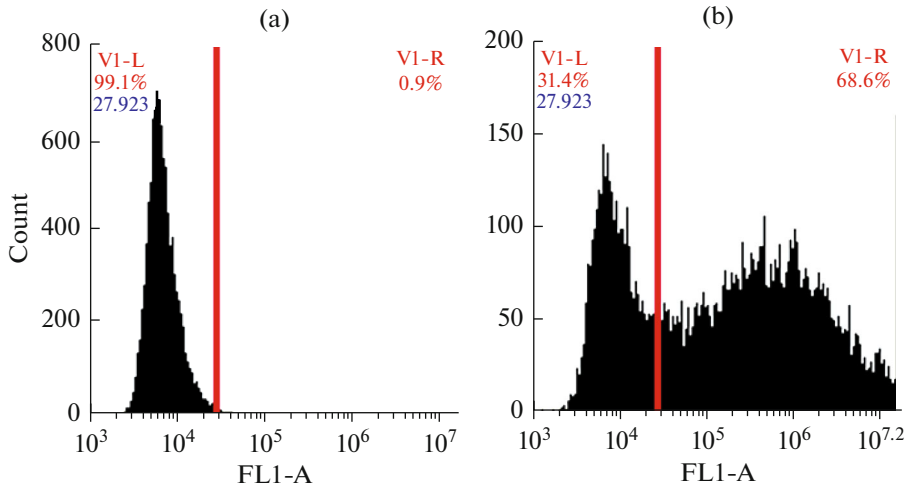

Fig. 2. Gating strategy f cell fluorescence measurements using flow cytometer (BD Accuri C6). Excitation was 488 nm, and the emission was collected with 533/30 nm bandpass filter. Part (a) represents autofluorescence in the control (no electric field treatment) group of the cells. All cells that have higher fluorescence than autofluorescence are considered to be transfected with GFP. Part (b) is the fluorescence of cells after successful electrotransfer of GFP coding plasmid. Mean fluorescence was obtained only form cells that have a higher emission that autofluorescence level.

Where TF is total cell fluorescence, TE is transfection efficiency (percentage of transfected cells) and MCF is mean cell fluorescence.

Cell Size Measurements

Chinese hamster ovary (CHO) cells were grown at 37°C in a 5% CO_2 humidified incubator for 24 and 48 hours prior to the experiment. Afterwards, cells were trypsinized, centrifuged (300 RCF) and suspended in PBS at concentration of 2×10^6 cells/mL and placed in Neubauer chamber. After 5 min incubation, cells were imaged using an inverted microscope (Nikon eclipse TS 100) with mounted camera (Moticam 2300/3.0M Pixel). Cell diameters were assessed with open-source imaging software ImageJ. At least 100 cells were counted to evaluate the diameter change at each cell passage time.

Viability Assay

Clonogenic assay (CA) was performed to determine cell viability. After electroporation, 100 μL of diluted cell suspension from each experimental point were added to 900 μL of growth medium. Then 44.4 μL of cell suspension (~400 cells) were seeded to a 40 mm diameter Petri dish containing 2 mL of growth mediumand grown for 6–7 days. The cells were fixed with 70% ethanol for 10 minutes and then stained with 10% crystal violet solution. For evaluation of results, the colonies were scanned with a scanner (CanoScan LiDE220, Canon, Newyork, USA) and counted using open-source imaging software ImageJ (National Institutes of Health, USA).

Statistical Analysis

Each experimental point was performed in triplicates with at least two separate experiments conducted on different days. Two-tailed Student's *t*-test was done using Microsoft Excel and SigmaPlot 12.5 software was used to produce the graphs. The statistical significance between the experimental points of different passage time was evaluated. The p values <0.05 were considered statistically significant.

RESULTS

The CHO cells were used in order to determine the effect of time elapsed since cell passage on the electrotransfection efficiency. The cells were passed at two different time points: 24 and 48 hours prior to the electrotransfection with different concentrations (10, 100, 200, 300, 400 and 600 μg/mL) of GFP coding plasmid. Since 1400 V/cm field strength was determined to be the most effective for efficient electrotransfection of CHO cell line in our previous work (Chopra et al., 2019), 2 HV pulses of 1400 V/cm were chosen for the electroporation to investigate the effect of cell passage time on electrotransfection efficiency. The percentage of GFP positive cells is shown in Fig. 3a, and the total fluorescence in these cells presented in Fig. 3b. It can be clearly observed that there is significant difference in both the number of transfected cells and the total fluorescence of cell population between the cells with different times elapsed since the passage. The highest difference in the number of transfected cells was observed at 400 μg/mL plasmid concentration. At these parameters, the transfection efficiency in the cells that were passaged 24 h prior experiment is $62.87 \pm 0.33\%$, but this number decreases to $34.55 \pm$

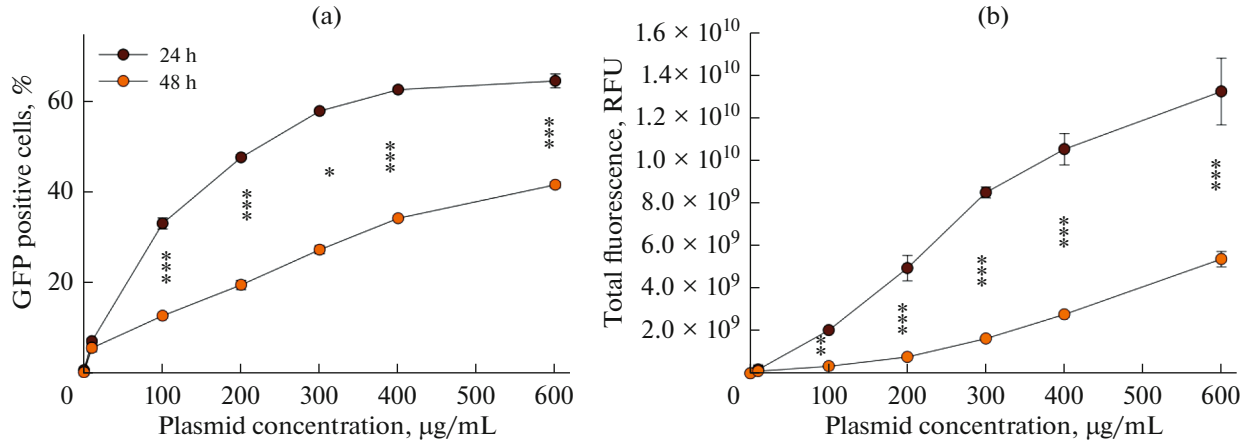

Fig. 3. The change in the percentage of transfected cells (electrotransfection efficiency) (a) and the total fluorescence of the transfected cells (b) in dependence on the time elapsed since cell passage. 2 HV pulses at 1400 V/cm pulse strength, 100 μs duration and 1 Hz frequency were used to transfect the cells with 10–600 μg/mL GFP coding plasmid. Error bars represent standard error of mean (SEM). The $p < 0.05$ of two tailed Student t-test is marked as *, $p < 0.01$ as **, and $p < 0.001$ as ***.

0.34% when the cell passage was done 48 h before the experiment. Similarly, a significant difference in the total fluorescence was observed between cells that were passaged 24 and 48 h prior the electroporation. However, no significant differences were observed after electroporation using 10 μg/mL plasmid concentration.

We also evaluated the cell viability after electrotransfection with the same conditions as those depicted in Fig. 3. The results of these experiments are shown in Fig. 4. It can be seen that there is no significant change in the cell viability when the cells passaged 24 and 48 h before the experiment in lower electric field strengths, but cell viability when experiment is done with the cells passaged 48 hours before the experiment is significantly lower when plasmid concentration is above 400 μg/mL.

The dependence of cell viability on the plasmid concentration prompted us to calculate the transfection efficiency of all transfected cells, rather than just the ones surviving the treatment. The transfection efficiency of all treated cells is depicted in Fig. 5a, and the total fluorescence of all treated cells—in Fig. 5b. It can be seen that these curves have different shape that the ones based on surviving cells (see Fig. 3 for comparison). Both the percentage of transfected cells and the total fluorescence of all cells show increasing, peak and decreasing parts. At all points, the percentage of transfected cells (Fig. 4a) and the total fluorescence in these cells (Fig. 4b) is higher when the cells are electroporated 24 hours after passage.

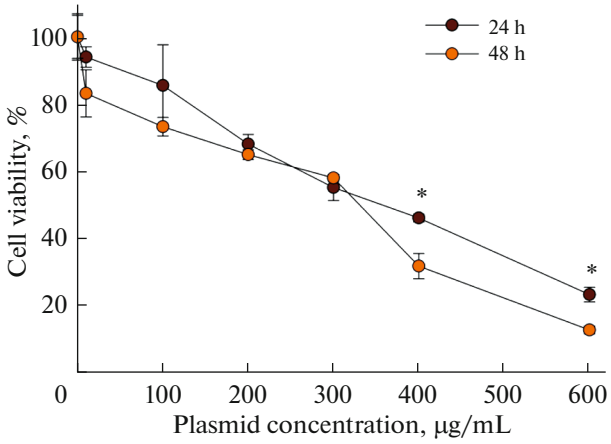

Fig. 4. The change of cell viability in dependence on cell passaging time. 2 HV pulses with 1400 V/cm pulse strength and 100 μs pulse duration at 1 Hz frequency were used to transfect the cells with 10–600 μg/mL GFP coding plasmid. Error bars represent standard error of mean (SEM). The $p < 0.05$ of two tailed Student t-test is marked as *.

DISCUSSION

The presented results show the significant differences in transfection efficiency when treatment is performed after 24 and 48 hours have elapsed since the last cell passage. One of the possible reasons for this is the difference in the cell size. According to our measurements, the average cell size 24 h after the passage is 9.47 μm. This decreases to 8.61 μm at 48 h after the passage as the Petri dish becomes more populated and the cells are pushed by their neighbours. It is well known that the cell permeabilization depends on the cell size (Sixou and Teissie, 1990). An increase in the cell size is associated with more efficient permeabilization at given electric field strength (Neumann et al., 1982). This can be numerically calculated using Schwan equation to calculate the magnitude of induced transmembrane potential $\Delta\psi i$ (Marszalek et al., 1990; Kotnik et al., 1997):

$$\Delta\psi i = frE\cos\theta, \quad (2)$$

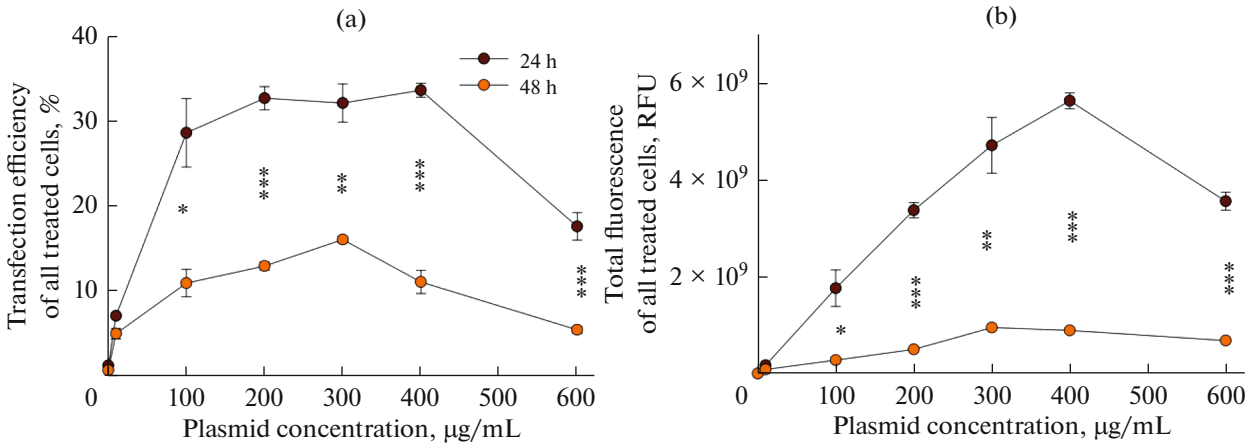

Fig. 5. The change of electrotransfection efficiency (a) and total fluorescence (b) of all treated cells in dependence on cell passaging time. 2 HV with 1400 V/cm pulse strength and 100 μs pulse duration at 1 Hz frequency were used to transfect the cells with 10–600 μg/mL GFP coding plasmid. Error bars represent standard error of mean (SEM). The $p < 0.05$ of two-tailed Student's *t*-test is marked as *, $p < 0.01$ as **, and $p < 0.001$ as ***.

where *f* is the cell shape factor (equal to 1.5 for a spherical cell), *r* is the radius of the cell, *E* is the strength of the applied electric field, and θ is the angle between the direction of the electric field and the selected point on the cell membrane. As the cell radii changed based on the time elapsed since last passage, so did the transmembrane potential induced by equal electric pulses. According to Eq (2), 1.99 V transmembrane potential was induced on the electrode facing pole of the cell that was passaged 24 hours prior the measurement, but only 1.81 V transmembrane potential was induced on the pole of the cell that was passaged 48 h prior. Therefore, the membrane area affected by the applied electric field was larger in the cells grown for 24 h, which in turn means that in these cells, a larger area was available for DNA/membrane interaction and, consequently, DNA delivery.

Other possible reason on the observed differences in transfection efficiency of the cells passaged at different times before the experiment are the differences of the cell cycle phases at which the electric field treatment was performed. Indeed, it was already shown that cell cycle synchronization at G2/M phase significantly increased the electrotransfection efficiency (Golzio et al., 2002). Once inside the cytoplasm, plasmid DNA must migrate to the nucleus through the nuclear envelope for the successful gene expression. However, at G2/M phase, nuclear envelope disappears(Cervia et al., 2018; Schwachtgen et al., 1994). Therefore, it is easier for the plasmid DNA to enter the nucleus in the absence of nuclear envelope and the efficiency of gene expression increases.

Hendrick and colleagues have demonstrated 1×10^5 CHO-K1 cells planted in commercially available Petri dishes grow linearly for 24 h and reach plateau after 48 h (Hendrick et al., 2001). As similar quantityof the same cell line have been grown for the same amount of time for our experiments, we can assume that the growth dynamics are similar. If the celldupliication speed decreases 48 h after passage in comparison 24 h after passage, it stands to reason that the distribution of cells in different cell cycle phases will also be different. As the lower cell duplication rate can be explained through the process of cell cycle arrest at G0 phase in plateau, it leads to a conclusion that a smaller number of cells will be in G2/M phase 48 h after cell passage. Because, as stated above, cell electrotransfection in G2/M phase leads to the highest transfection efficiency, the reduced cell duplication rate in 48 h sample can explain why significantly higher transfection efficiency and encoded protein expression are observed in the cells electroporated 24 hours after cell passage in comparison to the cells electroporated 48 hours after cell passage.

CONCLUSIONS

Our results reveal that cell passage time is a key factor to achieve the optimal gene electrotransfection. We show that the transfection efficiency is more efficient after 24 h of cell paasage time in comparison to the cells passage time of 48 h prior to the electroporation. Therefore, passaging the cells 24 h before the electroporation leads to the best transfection efficiency.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. Authors declare that they have no conflicts of interest.

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Statements on the welfare of animals. Animals were not a part of this work at any stage.

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