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RESEARCH ARTICLE

Effect of different parameters used for *in vitro* gene electrotransfer on gene expression efficiency, cell viability and visualization of plasmid DNA at the membrane level

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Abstract

Background Gene electrotransfer is a nonviral method used for DNA delivery into cells. Several steps are involved. One of them is the interaction of DNA with the cell membrane, which is crucial before DNA can enter the cell. We analysed the level of DNA–membrane interaction in relation to electrotransfer efficiency and the importance of the electrophoretic accumulation of DNA at the cell membrane. Systematic comparison of long-duration, short-duration and combinations of electropermeabilizing short (high-voltage; HV) and electrophoretic long (low-voltage; LV) pulses were performed. The effect of Mg^{2+} ion concentrations on electrotransfer and their effect on DNase activity were explored.

Methods To visualize the DNA–membrane interaction, TOTO-1 labeled DNA was used. Transfection efficiency was assessed with plasmid DNA coding for green fluorescent protein.

Results Higher relative electrotransfer efficiency was obtained by using longer pulses, whereas shorter pulses preserved cell viability. Short-duration pulses enabled higher (24%) overall transfection yield compared to long-duration pulses (12%), although a higher DNA–membrane interaction was observed. No significant difference in transfection was obtained between different HV-LV pulsing protocols, although the highest DNA–membrane interaction was observed with HV + LV pulses. The formation of the DNA–membrane complex depended on the Mg^{2+} concentration, whereas DNase inhibitor did not affect gene expression.

Conclusions Gene electrotransfer is a complex phenomenon, where many factors mutually affect the process and the DNA–membrane interaction only comprises the first step. We showed that longer electric pulses are optimal for higher transfection efficiency but reduce viability, whereas shorter pulses enable moderate transfection efficiency and preserve viability. Thus, each application needs a careful choice of pulsing protocol. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords electroporation; gene-transfer; microscopy; plasmid-DNA; plasmid-transfection

Introduction

Electroporation is a process by which transient permeability of the cell membrane can be induced by applying sufficiently high electric field pulses [1–3]. Consequently, small and large molecules, which otherwise cannot pass the cell membrane, can be introduced into the cytoplasm. Because of its efficiency, safety and easy application, electroporation has shown great potential for use in biomedical or industrial applications, such as electrofusion [4,5], electrochemotherapy [6,7], irreversible tissue ablation [8,9], gene therapy [10–12], DNA vaccination [13,14] and microbial nonthermal inactivation [15,16].

Gene electrotransfer was first described in early 1980s [17,18]. It was shown that electric pulses enable permeabilization of the cell membrane and DNA transfer into the cells. Subsequently, the method has been used to introduce DNA into prokaryotic, eukaryotic cells and *in vivo* into different tissues. Although, currently, viral vectors are the most effective for transfecting genes into living cells, viral transfection has serious limitations in terms of safety [19]. Therefore, the need for a nonviral transfection method has emerged, such as gene electrotransfer.

There are number of steps involved in successful gene electrotransfer: (i) formation of a complex between DNA and cell membrane; (ii) translocation of DNA across the permeabilized membrane; (iii) transfer of DNA from cytoplasm into the nucleus; and (iv) gene expression [20,21]. Several parameters, such as electric pulses [18,22–27] or medium properties [17,18,28–30], can influence each of these steps. By reversing the polarity of electric pulses, it was shown that the DNA–membrane complex becomes stable after a 1-s transition time [31]. Nevertheless, the molecular events involved in the formation of a complex between cell membrane and DNA, as well as its translocation across the membrane and through the cell cytoplasm, are still poorly understood and require further investigation [32–34].

Most electric pulse protocols used for gene electrotransfer consist of the application of long-duration millisecond pulses of moderate pulse amplitude [21,24] above the threshold for electropermeabilization of the cell membrane. For *in vivo* applications, electric pulse parameters have to be optimized for given electrodes positions and geometry, either experimentally [12,21] or numerically [35]. Electric pulses have two different effects that are important for efficient gene electrotransfer: (i) permeabilization of the cell membrane and (ii) electrophoretic migration of highly-charged DNA in the electric field [36,37]. Longer-millisecond pulsing protocols (e.g. 8×5 ms, 10×1 ms) combine both effects, whereas shorter-microsecond pulses (e.g. 8×100 μs, 4×200 μs) affect mostly cell membrane permeabilization because their short duration electrophoretic effect is much lower [37]. Comparison of short- and long-duration pulsing protocols on the transfection efficiency revealed that both long-duration millisecond pulses of moderate pulse amplitude and short-duration microsecond pulses of higher pulse amplitude can be used; nevertheless, a higher level of gene transfer and a longer expression is obtained by millisecond pulses *in vivo* [12,21,38]. The electrophoretic role of electric pulses was first proposed in 1992 by Sukharev *et al.* [39]. They demonstrated that high-voltage (HV) short-duration electric pulses can be used for cell membrane permeabilization, whereas low-voltage (LV) long-duration pulses below the electropermeabilization threshold provide the electrophoretic force needed for DNA–membrane interaction [36]. It was shown that higher gene electrotransfer efficiency can be achieved when applying the combination of HV and LV electric pulses compared to only HV pulses. The HV + LV protocol gave good results *in vitro* [26,37,39–41] and *in vivo* when applied to different tissues, such as muscle, liver, skin and tumour [36,42–45]. It was also demonstrated, that the role of LV pulse was crucial when a low concentration of plasmid DNA was used [26,41]. Under such conditions, an electrophoretic LV pulse applied after HV pulses improved DNA insertion into the permeabilized cell membrane [41].

Gene electrotransfer efficiency is also affected by the electroporation media [28–30,46–48]. Among other factors, divalent cations such as Ca^{2+} and Mg^{2+} in the media are necessary for the formation of DNA–membrane complex during the pulses. They act as a bridge between negatively-charged DNA and the negatively-charged cell plasma membrane and thus improve DNA–membrane binding [48]. During previous studies of the effect of Mg^{2+} ions on gene electrotransfer [30], we obtained a stronger interaction of DNA with the membrane, as well as a higher viability but lower electrotransfer efficiency for increased concentrations of Mg^{2+} ions. Altogether, the mechanisms of gene electrotransfer are still not completely understood. Because gene electrotransfer is a multistep process, it is necessary to study the influence of different parameters on each of these steps separately. In the present study, we focused on an analysis of the DNA–membrane interaction and its relationship with final gene transfer, and on the role of the electrophoretic accumulation of DNA and cell membrane permeabilization. We compared systematically typical pulsing protocols used for *in vitro* and *in vivo* gene electrotransfer: (i) long-duration millisecond pulses of moderate pulse amplitude; (ii) short-duration microsecond pulses of higher pulse amplitude; and (iii) different combinations of HV and LV pulses (HV, HV + LV, LV + HV, LV). These were selected to separate electropermeabilizing and electrophoretic effects of the pulses, where HV acts as a 'permeabilizing' pulse and LV acts as an 'electrophoretic' pulse below the threshold for electroporation.

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Furthermore, we analysed the effect of different Mg^{2+} concentrations on the DNA–membrane interaction and electrotransfer efficiency. Also, the effect of Mg^{2+} ions on DNA stability in the cytoplasm was explored using the DNase inhibitor $ZnSO_4$.

We compared all of the results obtained in relation to the DNA–membrane interaction, transfection efficiency, viability and total transfection yield. A theoretical evaluation of DNA accumulation at the cells membrane is also presented.

Materials and methods

Cell culture and electroporation media

Chinese hamster ovary cells (CHO-K1) were grown in Ham's F-12 tissue culture medium for mammalian cells with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Cells were plated as a monolayer culture in multiwells at cell density of $\rho = 5 \times 10^4$ cells/ml for 24 h at 37 °C in a humidified atmosphere in a 5% CO_2 incubator (Kambič, Semič, Slovenia).

For the present study, we used standard isoosmolar electroporation media with 1 mM $MgCl_2$ (10 mM KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.2). The influence of Mg^{2+} ions was also studied, using modified electroporation media with 50 mM $MgCl_2$ (10 mM KH_2PO_4/K_2HPO_4, 50 mM $MgCl_2$, 100 mM sucrose, pH 7.2). We used a concentration of 50 mM Mg^{2+} because our previous study showed that, with an increasing Mg^{2+} in the electroporation media, gene electrotransfer also decreases (minimum transfection being at 50 mM Mg^{2+}) [30]. In the present study, we further attempted to explain the cause of such an effect.

Plasmid DNA

Plasmid pEGFP-N_1 (Clontech Laboratories Inc., Mountain View, CA, USA) encoding green fluorescent protein (GFP) was amplified in Top10 strain of *Escherichia coli* and isolated with a HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). The plasmid DNA concentration was spectrophotometrically determined at 260 nm and confirmed by gel electrophoresis.

Electroporation

Different pulse generators were used to generate electric pulses: Cliniporator™ (IGEA s.r.l., Carpi, Modena, Italy), Jouan GHT 1287 (Jouan, St Herblain, France) or a prototype pulse generator, which enabled us to apply only HV pulses, only LV pulses or different combinations of HV and LV pulses [41].

Three types of pulsing protocols were used: (i) long-duration pulses of moderate pulse amplitude (ii) short-duration pulses with higher pulse amplitude and (iii) HV-LV pulsing protocols where different combination of HV and LV pulses were applied [26,41].

The present study was divided into three sets of experiments. In the first part, long-duration pulses of moderate amplitude (8×5 ms, $E = 0.7$ kV/cm, 1 Hz) or short-duration pulses with higher amplitude (4×200 μs, $E = 1.0$ kV/cm, 1 Hz) were applied. In the second part, different combinations of HV and LV pulses (HV-LV) were applied: only HV pulses (4×200 μs, $E = 1.4$ kV/cm, 1 Hz), a combination of HV + LV (first 4×200 μs, $E = 1.4$ kV/cm, 1 Hz, followed by 1×100 ms, $E = 0.137$ kV/cm), a combination of LV + HV (first 1×100 ms, $E = 0.137$ kV/cm, followed by 4×200 μs, $E = 1.4$ kV/cm, 1 Hz) or only LV pulse (1×100 ms, $E = 0.137$ kV/cm). It should be emphasized that HV pulses were the same as 'short-duration pulses' (4×200 μs, $E = 1$ kV/cm), except in the second part of the study where the TOTO–DNA interaction was analysed for HV-LV pulsing protocols, and where the amplitude of HV was increased to 1.4 kV/cm to obtain a higher interaction on the cell membrane, which was easier to observe.

In the third part, the influence of the Mg^{2+} ion concentration in electroporation media was investigated with 1 mM $MgCl_2$ or 50 mM $MgCl_2$. To maximize transfection efficiency, we used a long-duration pulse protocol. Therefore, 8×5 ms pulses, with a repetition frequency of 1 Hz and an electric field strength of 0.7 kV/cm were applied. A pair of parallel wire electrodes was used with the distance d between the electrodes being 2 mm for all above experiments. Electric field strength (E) can be obtained using the formula: $E = U/d$, where U denotes the applied voltage on the electrodes and d is the electrode distance.

When studying the role of Mg^{2+} ions on DNase activity, cells were exposed to a train of four square wave pulses with a duration of 200 μs. The electric field strength was 1.0 kV/cm, with a repetition frequency of 1 Hz. After the pulses, $ZnSO_4$ DNase inhibitor was added. To avoid possible interaction of Zn^{2+} ions with electropermabilization and the DNA–membrane interaction, we added $ZnSO_4$ after the electric pulses were delivered.

Because $ZnSO_4$ could also affect cell viability, we used a short-duration pulse protocol (and not a long-duration pulse protocol) to maximize cell viability.

DNA staining and visualization of the DNA–cell membrane interaction

TOTO-1 nucleic acid stain (Molecular Probes-Invitrogen, Carlsbad, CA, USA) was used to visualize the DNA

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interaction with cell membrane. Details of the protocol are provided in Golzio *et al*. [49]. Briefly, the plasmid pEGFP-N_1 was labeled with 2.3×10^{-4} M TOTO-1 DNA intercalating dye for 1 h on ice with an average base pair to dye ratio of 5. Cells were plated as a monolayer culture at a cell density of $\rho = 1 \times 10^5$ cells/ml in a Lab-Tek chamber (Nunc, Rochester, NY, USA) and incubated for 1 h in cell culture medium at 37 °C in a humidified 5% CO_2 atmosphere. Labeled plasmid was added to cells in concentration of 10 μg/ml and electric pulses were applied (see above).

Fluorescent microscopy (Zeiss 200; Axiovert, Oberkochen, Germany) with a × 100 oil immersion objective was used to monitor the interaction of DNA with the cell membrane. The images were recorded and TOTO fluorescence intensity profiles were measured and analysed using MetaMorph imaging software (Visitron, Puchheim, Germany) [49,50].

In the third part, where the effect of Mg^{2+} ions on binding strength between DNA and cell membrane was studied, first train of pulses was applied (8 × 5 ms, 0.7 kV/cm, 1 Hz) and the interaction of DNA with the cell membrane was observed. Approximately 2 min after the application of the first train of pulses, a second train of the opposite polarity (8 × 5 ms, 0.7 kV/cm, 1 Hz) was applied.

Gene electrotransfer

Gene electrotransfer was performed in accordance with previously described protocols [26,41]. Briefly, electroporation was performed on 24-h old cell culture. Culture medium was removed and electroporation media with plasmid DNA was added to cells at a final concentration of 10 μg/ml. Cells were exposed to square wave electric pulses to deliver plasmid DNA into the cells (see above), and incubated for 5 min at 37 °C to allow cell membrane resealing. Afterwards, cell culture medium was added and cells were incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. Gene electrotransfer efficiency was determined by fluorescent microscopy (Zeiss 200; Axiovert). The images were recorded using MetaMorph imaging software (Visitron). At least five fluorescence images were acquired in the area between the electrodes for each parameter. The cells were counted manually and gene electrotransfer efficiency was determined as the ratio between the number of green fluorescent cells and the total number of cells. Cell viability was obtained from phase contrast images as the ratio between the number of viable cells in the treated sample and the number of viable cells in the control sample.

In cases where the effect of DNase inhibitor on gene electrotransfer efficiency was studied, cell suspension was prepared by 0.25% trypsin/ethylenediaminetetraacetic acid solution (Sigma-Aldrich Chemie GmbH), centrifuged at 180 ***g*** for 5 min at 4 °C (Sigma, Germany). Cells were resuspended in different electroporation media (1 mM $MgCl_2$ or 50 mM $MgCl_2$) to a cell density of $\rho = 2.5 \times 10^6$ cells/ml. Plasmid DNA was added to cell suspension at a concentration of 40 μg/ml. For electroporation, cuvettes with built in aluminium electrodes were used (Eppendorf, Hamburg, Germany). Cells were exposed to square wave electric pulses to deliver plasmid DNA into the cells (see above). Immediately after pulses, 10 μl of 80 μM $ZnSO_4$ (Sigma-Aldrich Chemie GmbH) was added to inhibit intracellular DNases, as descibed previously [51,52]. After 24 h, cells were trypsinized, centrifuged at 180 ***g*** for 5 min at 4 °C and resuspended in phosphate-buffered saline to a cell density of $\rho = 1 \times 10^6$ cells/ml. The percentage of GFP-expressing cells was determined using a flow cytometer (Coulter EPICS Altra Flow Cytometer, Beckman Coulter Electronics, Brea, CA, USA) equipped with a laser emitting at 509 nm and 9000 events were recorded.

Statistical analysis

At least three independent experiments were performed and the results are presented as the mean ± SD. The results were analysed using an unpaired *t*-test analysis (SigmaPlot, version 11.0; Systat Software, Richmond, CA, USA). $p < 0.05$ was considered statistically significant.

Results

The aim of the present study was to analyse the relationship between the DNA–cell membrane interaction and gene electrotransfer efficiency. In the first part of the study, we compared two electric pulse protocols used for gene electrotransfer: long-duration pulses with a moderate amplitude (8 × 5 ms, $E = 0.7$ kV/cm) and short-duration pulses with a higher amplitude (4 × 200 μs, $E = 1.0$ kV/cm); in the second part, we analysed different combinations of HV-LV electric pulse protocols; and, in the third part, we analysed the effect of Mg^{2+} ions in electroporation media. The DNA–membrane interaction, electrotransfer efficiency and cell viability were determined for all pulsing protocols.

Effect of long- and short-duration pulses on DNA–membrane interaction and gene electrotransfer efficiency

Direct visualization of the DNA interaction with the cell membrane was performed for long-duration pulses with

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a moderate amplitude (8×5 ms, 0.7 kV/cm, 1 Hz) and short-duration pulses with a higher amplitude (4×200 μs, 1.0 kV/cm, 1 Hz). For both electric pulse protocols, a fluorescence intensity increase appeared at the membrane level and was restricted to the side of the cell facing the cathode (Figures 1C and 1D). This was the consequence of an electrophoretic drag in the opposite direction of the electric field, resulting in the accumulation of the labeled DNA at the permeabilized cell membrane surface. The fluorescence level was not homogeneous along the membrane; spots with various fluorescence intensities were observed. From Figure 1, it can be seen that more DNA interacted with the cell membrane for 8×5 ms pulses compared to shorter 4×200 μs pulses. Long-duration pulses gave approximately three-fold higher mean fluorescence intensity values (474 ± 158) than short-duration pulses (161 ± 3.8), meaning that more DNA interacted with the cell membrane when longer pulses with a moderate pulse amplitude were applied (8×5 ms, 0.7 kV/cm, 1 Hz).

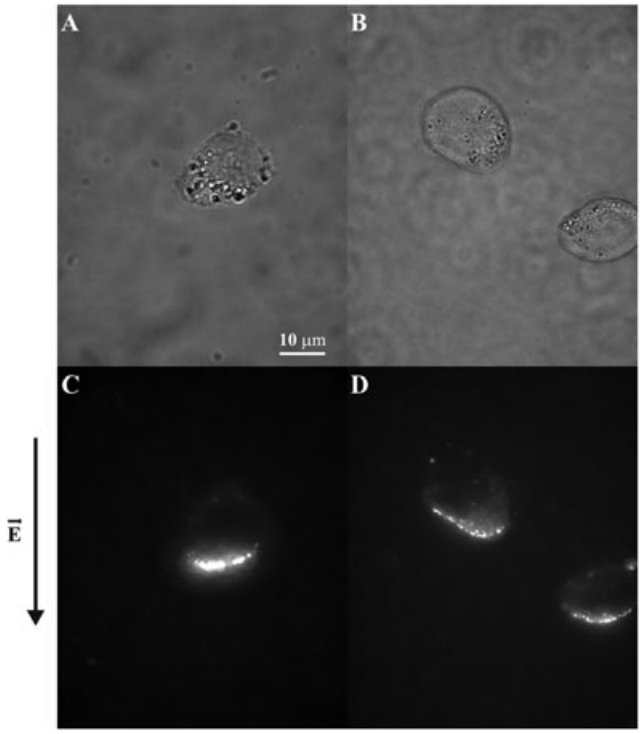

Figure 1. Fluorescence microscopy observation of the DNA–membrane interaction for long- and short-duration pulses. Plated cells were incubated in the presence of TOTO-1 labeled DNA (pEGFP-N_1). The concentration of labeled DNA in electroporation media was 10 μg/ml. Phase contrast images of treated cells for (A) long- and (B) short-duration pulses and fluorescence images of treated cells for (C) long- and (D) short-duration pulses are presented. Shortly after labeled plasmid was added to cells, a train of (A) and (C) long- (8×5 ms, 0.7 kV/cm, 1 Hz) or (B) and (D) short- (4×200 μs, 1.0 kV/cm, 1 Hz) duration pulses was applied. Images were acquired ($\times 100$ objective magnification) approximately 1 min after exposure of cells to electric pulses. The black arrow indicates the electric field direction.

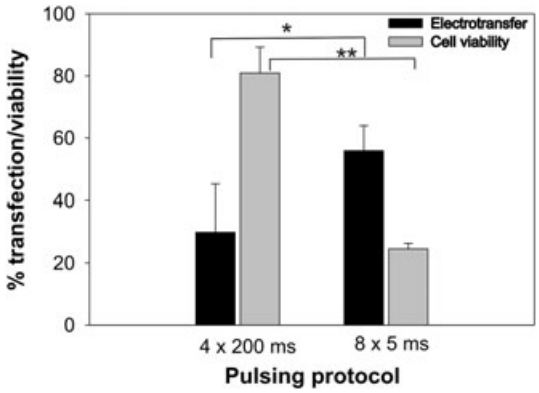

Figure 2. Effect of long- and short-duration pulses on gene electrotransfer efficiency and cell viability. The percentage of transfected cells, cells expressing GFP (black histogram), and the percentage of viable cells (grey histogram) as a function of long- (8×5 ms, 0.7 kV/cm, 1 Hz) or short- (4×200 μs, 1.0 kV/cm, 1 Hz) duration pulses is shown. Pulses were applied at room temperature ($T = 22\,^{\circ}C$). Cell density was $\rho = 5 \times 10^4$ cells/ml. The plasmid DNA concentration was 10 μg/ml. Values represent the mean ± SD. A statistically significant difference was obtained by the *t*-test: for percentage transfection, $^{*}p < 0.05$; for viability, $^{**}p < 0.001$.

Gene electrotransfer efficiency in terms of the percentage of effectively transfected cells is presented in Figure 2. It can be seen that gene electrotransfer efficiency was significantly higher ($p = 0.038$) for long-duration (55%) compared to short-duration pulses (30%). However, viability was drastically reduced ($p < 0.001$) for long-duration pulses, where it dropped to only 22%, whereas, for short-duration pulses, viability was maintained at 80%. Furthermore, it would be interesting to obtain the same results in terms of the overall transfection yield, where both transfection efficiency and viability are taken into account. If we define the percentage of overall transfection as the ratio between the number of successfully transfected cells and the number of all treated cells, we obtain: % overall transfection = % transfected cells × % viability/100. From this, we found that the short-duration electric pulse protocol gave a higher overall transfection yield (24%) compared to the long-duration pulse protocol (12%).

Effect of a combination of HV and LV pulses on the DNA–membrane interaction and gene electrotransfer efficiency

Different combinations of HV and LV pulses were used to separate permeabilizing and electrophoretic effects of electric pulses. This specific pulse combination allowed us to determine how the electrophoretic LV pulse

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contributes to DNA accumulation at cell membrane level. In addition, different sequences of HV and LV pulse application (HV + LV versus LV + HV) indicated the importance of cell membrane permeabilization on the DNA–membrane interaction. Direct visualization of DNA interaction with cell membrane for HV-LV pulsing protocols is presented in Figure 3. For all pulse combinations where the amplitude of the pulse was above the threshold for cell membrane electropermeabilization, DNA interaction with cell membrane facing the cathode was observed when HV pulses (Figures 3A and 3E), HV + LV pulses (Figures 3B and 3 F) and LV + HV pulses (Figures 3C and 3G) were applied. No fluorescence could be detected when only an LV pulse below the threshold for electropermeabiliation was applied, indicating that DNA–membrane complex formation did not take place (Figures 3D and 3H).

The fluorescence intensity was proportional to the amount of TOTO labeled DNA interacting with the cell membrane. Consistently, the highest mean fluorescence intensity was obtained for HV + LV electric pulse protocol, whereas, for HV and LV + HV electric pulse protocols, the fluorescence intensity was lower. At least seven images were acquired for each parameter and the mean fluorescence intensity was determined. After exposure to HV + LV pulses, the average mean fluorescence intensity (189 ± 6) on the cell membrane was higher than for HV pulses (161 ± 5) or LV + HV pulses (164 ± 2.5). For only LV pulses, the mean fluorescence intensity was 135 ± 2.

In Figure 4, we compare electrotransfer efficiency in terms of percentage of transfected cells and viability for different combinations of HV and LV pulses (HV, HV + LV, LV + HV and LV). No statistically significant differences between HV, HV + LV and LV + HV pulsing protocols were observed, whereas, for LV pulses alone, no gene transfection was observed.

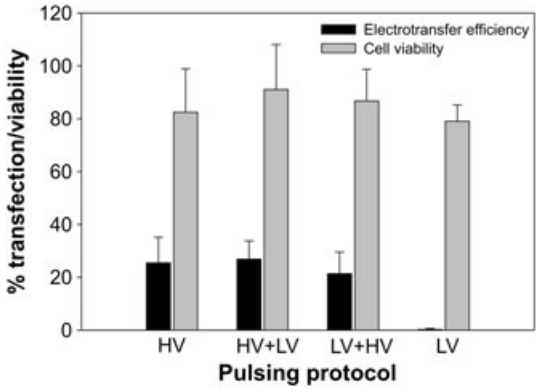

Figure 4. Effect of different combinations of HV-LV electric pulse protocols on gene electrotransfer efficiency and cell viability. The percentage of transfected cells, cells expressing GFP (black histogram), and the percentage of viable cells (grey histogram) as a function of different pulses are shown: HV (4×200 μs, 1.0 kV/cm, 1 Hz); HV + LV (4×200 μs, 1.0 kV/cm, 1 Hz and 1×100 ms, 0.075 kV/cm); LV + HV (1×100 ms, 0.075 kV/cm and 4×200 μs, 1.0 kV/cm, 1 Hz) and only LV (1×100 ms, 0.075 kV/cm). Pulses were applied at room temperature (T = 22 °C). Cell density was $\rho = 5 \times 10^4$ cells/ml. The plasmid DNA concentration was 10 μg/ml. Values represent the mean ± SD.

Effect of Mg^{2+} ions on the DNA–membrane interaction and gene electrotransfer efficiency

To understand the role of the DNA–cell membrane interaction in gene electrotransfer better, direct visualization

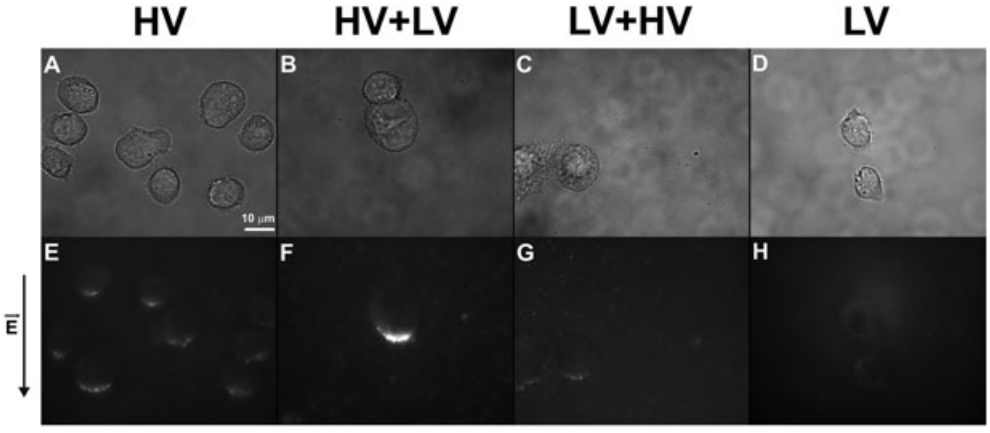

Figure 3. Fluorescence microscopy observation of the DNA–membrane interaction for different combinations of HV ($E_{HV} = 1.4$ kV/cm) and LV ($E_{LV} = 0.137$ kV/cm) pulses. Plated cells were incubated in the presence of TOTO-1 labeled DNA (pEGFP-N_1) in standard electroporation media. The concentration of labeled DNA in electroporation media was 10 μg/ml. Shortly after labeled plasmid was added to cells, different protocols of HV (4×200 μs, 1.4 kV/cm, 1 Hz) and LV pulses (1×100 ms, 0.137 kV/cm) were applied. Phase contrast (A, B, C, D) and fluorescence images (E, F, G, H) of treated cells are presented, when HV (A, E), HV + LV (B, F), LV + HV (C, G) and only LV (D, H) pulses were applied. Images were acquired (× 100 objective magnification) approximately 1 min after exposure of cells to electric pulses. The black arrow indicates the field direction.

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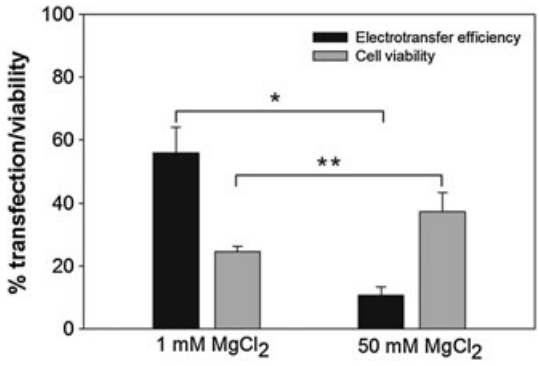

Figure 5. Effect of Mg^{2+} ions on gene electrotransfer efficiency and cell viability. The percentage of transfected cells, cells expressing GFP (black histogram), and the percentage of viable cells (grey histogram) as a function of different Mg^{2+} concentration (1 mM or 50 mM) in electroporation media is shown. 8×5 ms pulses with an electric field strength of 0.7 kV/cm and a repetition frequency of 1 Hz were used at room temperature (T = 22 °C). Cell density was $\rho = 5 \times 10^4$ cells/ml. The plasmid DNA concentration was 10 μg/ml. Values represent the mean SD. A statistically significant difference was obtained with the *t*-test: for percentage transfection, $*p < 0.001$; for viability, $**p = 0.025$.

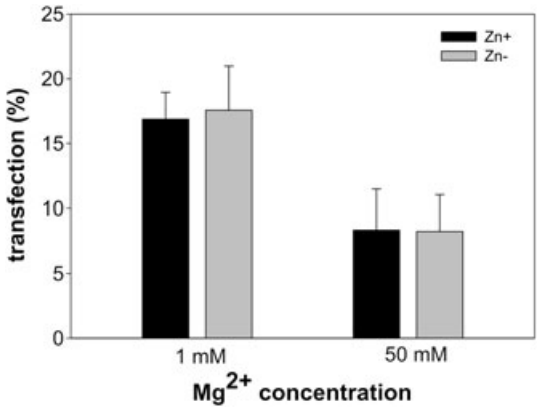

Figure 6. Effect of DNase inhibitor ($ZnSO_4$) on gene electrotransfer efficiency as a function of different Mg^{2+} concentration in electroporation media (1 mM and 50 mM). The percentage of transfected cells, cells expressing GFP when $ZnSO_4$ was added (black histogram), or when no $ZnSO_4$ was added (grey histogram) is presented. 4×200 μs pulses with an electric field strength of 1.0 kV/cm and a repetition frequency of 1 Hz were used at room temperature (T = 22 °C). Cell density was $\rho = 2.5 \times 10^6$ cells/ml. Values represents the mean ± SD.

was performed using TOTO-1 labeled plasmid DNA in standard electroporation media that contained 1 mM Mg^{2+} cations or enriched electroporation media with 50 mM Mg^{2+} ions. First, the effect of Mg^{2+} in electroporation media on gene electrotransfer efficiency and cell viability was determined. In Figure 5, the percentage of transfection and cell viability for 1 mM or 50 mM $MgCl_2$ media is shown for 8×5 ms, 0.7 kV/cm, 1 Hz pulses.

An increase in the Mg^{2+} concentration in the electroporation media (from 1 mM to 50 mM) resulted in a decrease of gene expression ($p \leq 0.001$) and in an increase in cell viability ($p = 0.025$), similarly to our previous study [30]. The percentage of transfection in electroporation media with 1 mM $MgCl_2$ media was approximately 55% with 24% of cell viability. When 50 mM $MgCl_2$ media was used, only approximately 7% of transfected cells were obtained with 37% of cell viability.

We further tested the possible effect of Mg^{2+} ions on nuclease inhibitors and, consequently, on gene electrotransfer efficiency. Because the DNase inhibitor $ZnSO_4$ has a profound effect on gene electrotransfer efficiency [52], it was added immediately after pulse application. We did not observe any improvement of gene electrotransfer efficiency by adding DNase inhibitor $ZnSO_4$ in 1 mM and 50 mM media (Figure 6). Adding a concentration of 80 μM of $ZnSO_4$ did not affect cell viability (data not shown), although treatment of cells with higher concentrations of $ZnSO_4$ (up to 2 M) resulted in lower or no cell viability.

Finally, we investigated the strength of the DNA–membrane interaction using TOTO labeled DNA by applying electric pulses in two opposite direction. Namely, two trains of pulses were applied and fluorescence intensity at cell membrane level was determined and the results are presented in Figure 7. When exposing cells to the first train of pulses [E_1 (8×5 ms, 0.7 kV/cm, 1 Hz)], DNA interacted with the cell membrane facing the cathode in both media (1 mM or 50 mM $MgCl_2$). After exposing cells to the second train of pulses of the opposite polarity [E_2 (8×5 ms, 0.7 kV/cm, 1 Hz)], the fluorescence decreased at the membrane in media with 1 mM $MgCl_2$, whereas almost no decrease was observed in 50 mM $MgCl_2$ media. TOTO fluorescence intensity profiles were measured along the membrane [53] of the same cell after the first and the second trains of pulses were applied. In both media (1 mM and 50 mM $MgCl_2$), the increase in fluorescence caused by the first pulses varied at the cathode side from 4.5- to eleven-fold compared to the anode side. After second train of pulses was applied, an approximate 1.5-fold decrease in fluorescence intensity in 1 mM $MgCl_2$ media was observed at the cathode side. However, no (or only a very small) decrease in fluorescence intensity in 50 mM $MgCl_2$ media was detected.

Discussion

Gene electrotransfer is a method that enables the delivery of plasmid DNA both into cells *in vitro* and *in vivo* by means of electric pulses. It is safer than viral transfection and has already been used in clinical trials [11]. Several

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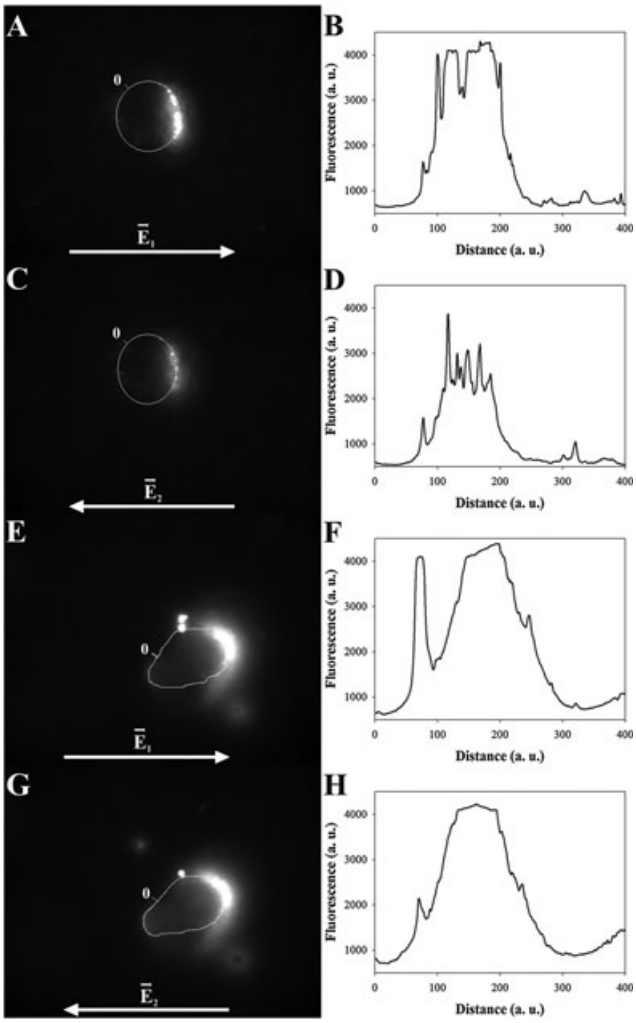

Figure 7. Fluorescence intensity profiles at the membrane level when using different Mg^{2+} ion concentrations (1 mM and 50 mM $MgCl_2$). Plated cells were incubated in the presence of TOTO-1 labeled DNA (pEGFP-N_1) in 1 mM or 50 mM $MgCl_2$ electroporation media. Immediately after labeled plasmid was added to cells, the first train of pulses (8 × 5 ms, 0.7 kV/cm, 1 Hz) was applied (A, E). The second train of pulses, pulses of the opposite polarity (8 × 5 ms, 0.7 kV/cm, 1 Hz), was applied 2 min after the first pulsation (C, G). Fluorescence images of treated cells in (A, C) 1 mM and (E, G) 50 mM Mg electroporation media. Images were acquired approximately 1 min after pulsation. The membrane was drawn in white on these images. Fluorescence intensity profiles of the permeabilized areas (B, D, F, H) were determined by quantifying the intensity values of the fluorescence at the membrane level along the white line drawn in (A), (C), (E) and (G). The white arrows indicate the field direction of the first (E_1) and the second (E_2) train of pulses applied. The same cells (A, C and E, G, respectively) were always observed after the first and the second train of pulses was applied.

steps are important for gene electrotransfer and it is necessary to understand each step to improve its efficiency. To better understand the process involved in gene electrotransfer and to expand our previous studies of the mechanisms [26,30,41], we designed several experiments aiming to study the relationship between the DNA–membrane interaction and gene electrotransfer efficiency using different electric pulse protocols and Mg^{2+} concentrations.

To clarify the relationship between DNA membrane interaction and its role in final percentage of transfected cells, we addressed the following questions: (i) is the quantity of DNA interacting with the cell membrane proportional to gene transfer; (ii) can electrophoretic pulse accumulate DNA at cell the membrane even if applied before permeabilizing HV pulse; and (iii) which protocol is more optimal for a given application?

The first question was addressed from two different points of view: first, by applying different electric pulse protocols resulting in different amounts of DNA detectable at cell membrane level and, second, by adding Mg^{2+} ions into electroporation media, resulting in a higher amount of the DNA accumulated at the cell membrane. In the second approach, additional experiments were needed because Mg^{2+} ions are known to activate DNase in the cell. This effect was separated from the bridging effect of Mg^{2+} ions by adding the $ZnSO_4$ DNase inhibitor. The relationship with the final percentage of transfected cells was clarified by determining the complex stability by applying electric pulses in the opposite direction. Namely, such pulses would release any unbound DNA from the cell membrane, as already reported by Faurie *et al*. [31].

Table 1 summarizes all of the results obtained for the DNA–membrane interaction (mean fluorescence intensity of TOTO-1 labeled DNA), percentage of transfection, percentage of viable cells and overall transfection for three types of pulsing protocols. Overall transfection represents the percentage of transfected cells relative to the initial population, which takes into account both the efficiency of transfection and viability. In addition, we present a calculation of DNA accumulation (A_{DNA}) at the cell membrane as a result of the electrophoretic force of the applied electric field (E). Accumulation linearly depends on electric force $F_E = eE$, where e is the electric charge of DNA molecules, and also on the total duration of electric pulses for a given pulsing protocol – t_E:

$$A_{DNA} = F_E \times t_E = eE \times t_E \quad (1)$$

Until now, a variety of pulsing protocols have been used for electrotransfer but, in general, they can be divided into protocols using long-duration pulses with a moderate amplitude (8 × 5 ms, 0.7 kV/cm, 1 Hz), short-duration pulses with a higher amplitude (4 × 200 μs, 1.0 kV/cm, 1 Hz) and HV-LV pulsing combinations consisting of electropermeabilizing HV pulses and electrophoretic (nonpermeabilizing) LV pulses. Nevertheless, only a few studies have directly

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Table 1. DNA–membrane interaction [mean fluorescence intensity (FL) of TOTO-1 labeled DNA], accumulation of DNA at the cell membrane (A_{DNA}) calculated from Eqn 1, percentage of transfection, percentage of viable cells, and overall transfection yield for three types of pulsing protocols

	Interaction FL (AU)	A_{DNA}/e – DNA accumulation (ms × kV/cm)	Transfection (%)	Viability (%)	Overall transfection yield (%)
Long-duration pulses					
8 × 5 ms, 0.7 kV/cm, 1 Hz	474 ± 158	28	56 ± 8%	25 ± 2%	12%
Short-duration pulses					
4 × 200 μs, 1.0 kV/cm, 1 Hz	161 ± 3.8	1.12	30 ± 15%	81 ± 8%	24%
HV-LV					
HV: 4 × 200 μs, 1.0 kV/cm, 1 Hz*					
LV: 1 × 100 ms, 0.075 kV/cm*					
HV	161 ± 5	1.12	25 ± 10%	82 ± 16%	20%
HV + LV	189 ± 6	14.82	27 ± 7%	91 ± 17%	25%
LV + HV	164 ± 2.5	14.82	21 ± 8%	87 ± 12%	18%
LV	135 ± 2	13.7	0.7 ± 0.3%	78 ± 7%	0.5%

*For observation of the DNA–membrane interaction, we used 1.4 kV/cm short-duration pulses instead of 1.0 kV/cm (see Materials and metkods) and, for LV, 0.137 kV/cm instead of 0.075 kV/cm. AU, arbitrary units

compared different types of pulsing protocols in terms of relative and total electrotransfer efficiency [37,54].

Electric pulse parameters for HV-LV were selected to separate the electrophoretic and electropermeabilizing effects of such pulses. Specific combinations of HV and LV pulses enable the separation of electropermeabilization (HV pulses) from electrophoresis (LV pulses), which are both crucial for efficient DNA–membrane interaction. For selected parameters, HV pulses permeabilized the membrane, whereas LV pulse provided only an electrophoretic drag of negatively-charged DNA towards the cell membrane [26,41].

On the one hand, millisecond pulses present both permeabilizing and electrophoretic compounds; therefore, it is difficult to establish which of those is affecting cell membrane DNA complex formation. On the other hand, microsecond pulses above the threshold for electroporation present mainly an electropermeabilizing effect of the electric field. In the present study, these two pulsing protocols were compared and, in addition, different combinations of microsecond permeabilizing HV pulses and low voltage electrophoretic LV pulses below the threshold were studied to determine the role each component in the DNA–cell membrane interaction. Special attention was paid to only the nonpermeabilizing LV electrophoretic pulse.

Relationship between the DNA–membrane interaction and electrotransfer efficiency for different pulsing protocols

Table 1 shows that the highest interaction and DNA accumulation is obtained with long-duration millisecond pulses, also resulting in the highest percentage of transfection. For short-duration and HV-LV pulsing protocols, the interaction is smaller for all pulse combinations (Table 1 and Figure 3) compared to a long-duration pulsing protocol. For only HV pulses, we obtained the smallest number of DNA interactions with the membrane because electrophoretic drag and DNA accumulation is much smaller compared to HV + LV or long-duration pulses. For only an LV pulse that is below the permeabilization threshold, no interaction (compared to control) and no transfection was obtained, despite sufficient electrophoretic force for the accumulation of DNA (Table 1). This is in agreement with our previous study [55] indicating that, for electrotransfection and interaction with the cell membrane, electropermeabilization is a crucial condition, and DNA can form a complex only with the permeabilized cell membrane. It can be seen (Table 1) that the calculated accumulation of DNA relates to some extent with the interaction and electrotransfer efficiency (i.e. for long-duration pulses, the highest accumulation, interaction and percentage transfection is obtained, whereas, for short-duration pulses, accumulation, interaction and percentage transfection are much lower). However, there is no direct correlation between interaction and final transfection efficiency because the HV + LV interaction is higher compared to only HV pulses, whereas, for the percentage of transfection, there is no significant difference.

If we compare HV + LV and LV + HV pulsing protocols, we determine that the electrophoretic LV pulse increased interaction only if it was applied after permeabilizing HV pulses, even though the calculated accumulation is the same. This further supports the observation that the first necessary step is cell membrane permeabilization, as already discussed. Therefore, HV + LV pulses enabled

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more DNA to be inserted into permeabilized cell membrane compared to LV + HV pulses. Interestingly, this did not result in a higher transfection efficiency (Figure 4) for HV + LV protocols compared to HV or LV + HV pulses. This is also in agreement with previous studies [26,41] indictating that the HV + LV protocol is most efficient only for low plasmid concentrations (1–2 μg/ml). These results again demonstrate that the increased accumulation of DNA at the cell membrane and a higher level of interaction does not necessarily result in a higher transfection efficiency. However, if there is limited number of DNA molecules (in case of sub-optimal plasmid concentrations), electrophoresis is crucial and HV + LV pulsing combination enables a higher transfection efficiency [26,41], which also explains the results of *in vivo* studies where the HV + LV combination was more efficient compared to only HV pulses.

Relationship between the DNA–membrane interaction and electrotransfer efficiency for different concentrations of Mg^{2+} ions

A further step aiming to clarify the relationship between the DNA–membrane interaction and its role in the final percentage of transfected cells was made in experiments where Mg^{2+} ions and DNase inhibitor were used. It was shown that divalent cations act as a bridge between negatively-charged DNA and the negatively-charged cell plasma membrane and, as a result, improve DNA–membrane binding [48]. We chose Mg^{2+} ions because it has previously been shown that they improve viability [30] and electrolyte cell homeostasis [56]. While larger concentrations of Ca^{2+} ions (above mM) decrease cell viability [29,57,58] and are therefore less appropriate for studying mechanisms of gene electrotransfer. Mg^{2+} ions can have several effects on transfection efficiency: (i) they act as a bridge between negatively-charged DNA and negatively-charged cell plasma membrane [48]; (ii) they affect cell viability [30,56]; and (iii) they could also contribute to the faster degradation of introduced plasmid DNA in the cell cytoplasm [59] because they are necessary for the enzymatic activity of intracellular nucleases (DNases) [60].

In the present study, we used a millisecond pulse protocol (8×5 ms, 0.7 kV/cm), whereas, in our previous study [30], a microsecond pulsing protocol was used. Namely, with long-duration millisecond pulses, we obtained a higher fluorescence intensity of the DNA–membrane complex and it was easier to analyse the effect of Mg^{2+} concentration during this step of gene electrotransfer. As previously reported [30], a higher Mg^{2+} concentration increases the strength of binding between DNA and cell membrane and, consequently, reduces electrotransfer efficiency. Furthermore, Mg^{2+} ions are also potent activating divalent cations for DNase enzymes [51], which could additionally explain the lower gene electrotransfer efficiency for 50 mM Mg^{2+} [60]. Therefore, to test this hypothesis, DNase inhibitor was added immediately after electroporation. To inhibit intracellular DNases, several inhibitors have already been described, such as actin [61,62], sodium dodecyl sulfate [63], $ZnSO_4$ [51,52], etc. We chose $ZnSO_4$ because it was already shown that Zn^{2+} ions at a a sufficiently high concentration (approximately 100 μM) have a strong inhibition effect on DNase enzyme, regardless of the concentration of Mg^{2+} ions (for the present study, we used a concentration of 80 μM $ZnSO_4$). It was also shown that Zn^{2+} can dislodge Mg^{2+} from its binding site on DNase enzyme [51]. Although some studies have reported an increase in gene electrotransfer efficiency when $ZnSO_4$ was added [52], we observed no such effect (Figure 6). We also added a higher concentration of $ZnSO_4$ (up to 2 M) to exclude the possibility that the concentration of Zn^{2+} (80 μM) was not sufficiently high to obtain any effect; however, cell viability was severely affected at such high concentrations (data not shown).

Under our conditions, we demonstrated that the inhibition of DNase activity did not improve gene transfection efficiency at a high Mg^{2+} concentration. Our results thus suggest that the effect of Mg^{2+} ions can be mostly attributed to Mg^{2+} ions at higher concentrations binding DNA at the cell membrane with such intensity that the second step needed for successful gene expression (DNA transfer across the cell membrane) is hindered. This finding was further supported by changing the orientation of pulse application, as already described by Faurie *et al.* [31]. In our additional experiments, cells were exposed to another set of the same electric pulses of the opposite polarity to analyse the effect of Mg^{2+} ions on the stability of the DNA–membrane complex. We observed the fluorescence decrease on the cell membrane when standard electroporation media containing 1 mM $MgCl_2$ was used (Figures 7A and 7C). Therefore, part of the DNA–membrane complexes was disrupted. On the other hand, no (or very small) fluorescence decrease on cell membrane was observed for 50 mM $MgCl_2$ (Figures 7E and 7G) indicating a strong interaction as a result of the Mg^{2+} bridging effect.

Comparison of different pulsing protocols in terms of electrotransfer efficiency and cell viability

Altogether, long-duration pulsing protocols of 8×5 ms were most effective in terms of the percentage of transfected cells (56%) compared to a short-duration

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pulsing protocol of $4 \times 200\,\mu s$ (30%) and HV-LV pulsing protocols (approximately 23%) (Table 1). However, the viability was drastically reduced to 20% for a long-duration pulsing protocol versus the very good viability (81%) obtained for the short-duration protocol. Furthermore, when we analysed transfection efficiency in terms of the overall transfection yield, we obtained significantly higher efficiency for short-duration pulsing protocols (24%) compared to only 12% for a long-duration pulse protocol (Table 1).

Clearly, both the physiological condition of the cell and cell viability are very important for efficient gene electrotransfer. Taking this into account, it becomes more understandable why a short-duration protocol, where a relatively small interaction is observed, can lead to a high percentage of absolute transfection compared to longer-duration electric pulse protocols. On the other hand, longer pulses are preferable if only high 'relative' percentage transfection is needed as is the case, for example, for certain biotechnological applications [15], or *in vivo* [64] and under similar conditions [65,66] where it was shown that longer pulses are more efficient. Namely, *in vivo*, lextracellular matrix hinders the transport of DNA in the proximity of cells, consequently leading to relatively low transfection; therefore, the electrophoretic force of longer pulses enables the efficient contact of the DNA molecule with the cell membrane [66–68].

To summarize, the results of the present study indicate that gene expression is not directly related to the number of DNA interactions with the cell membrane. Even though an interaction is a necessary step, the accumulation of DNA and the observed interaction do not correlate directly with final gene transfection. This is in agreement with other studies showing that electrotransfer is a complex phenomenon, where many factors mutually affect the process [26,31,41]. The DNA–membrane interaction is only the first step, whereas transfer across the membrane, towards and into the nucleus is also crucial and required for final gene expression. We have shown the role of the DNA–membrane interaction by applying various pulsing protocols and using different Mg^{2+} ions concentration.

Finally, our results show that, for translation of a given gene electrotransfer protocol to clinical practice, it is advisable to use long-duration millisecond pulsing protocols or a combination of HV + LV pulses [64]. On the other hand, for certain biotechnological applications where the total yield of transfected cells and/or preserved viability is crucial, short-duration protocols [26,41] might prove more optimal.

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