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Research

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Analysis of DNA mobility and gene electrotransfer in 3D *in vitro* collagen gel model

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ABSTRACT

Background: Gene electrotransfer is an established method that enables transfer of DNA into cells with electric pulses. Several studies analyzed different parameters; however the question of the mechanisms involved in gene electrotransfer remains open. One of main obstacles toward efficient gene electrotransfer *in vivo* is relatively poor DNA mobility in tissues.

Objective and method: In order to analyze the effect of impaired mobility on gene electrotransfer efficiency, we applied electric pulses with different durations on plated cells, cells grown on collagen layer and cells embedded in collagen gel (3D model) and compared gene electrotransfer efficiency and viability of cells.

Results: We obtained the highest transfection of plated cells, while transfection efficiency of embedded cells in 3D model was lowest and similar as in *in vivo*. To further analyze poor DNA mobility in 3D model, we applied DNA in top of or injected it into 3D model and showed that former way increases gene electrotransfer efficiency as was shown in *in vivo* studies.

Conclusion: We reported empirically and theoretically evidence that DNA has impaired mobility and diffusion in collagen environment. In addition our method provides resembling *in vivo* situation, where gene electrotransfer mechanisms can be studied.

Keywords: 3D *in vitro* model, collagen gel, gene electrotransfer, GFP, high-voltage pulse, low-voltage pulse, different polarity pulses.

1. BACKGROUND

For DNA vaccination or gene therapy applications efficient delivery of plasmid DNA [1] or short RNA molecules [2] is crucial. Gene therapy is based on insertion of healthy genes or alteration or removal of defective genes responsible for disease development [3]. The most efficient method used for gene therapy is viral transfection [4]. Although viral vectors have been very efficient, the safety of their use has been questioned [5–7]. Thus, there is a great interest in developing non-viral methods for gene delivery [8]. For the past 20 years a huge variety of non-viral gene therapy methods, including chemical and physical ones, have been developed to introduce DNA into the cell *in vivo*, but many of them are either toxic or have poor gene expression [8–11]. Almost four decades ago a physical method for delivery of molecules by use of electric pulses (electroporation) was described [12]. It is based on the significant increase in the permeability of the cell plasma membrane caused by an externally applied electrical field. Electroporation is already successfully applied in different biomedical applications, including: electrofusion [13,14]; electrochemotherapy [15,16]; irreversible tissue ablation [17]; DNA vaccination [18,19] and gene electrotransfer [20–22]. Today gene electrotransfer is widely used to introduce DNA into different cells [23,24] and tissues [1,25,26] due to its efficiency, safety and easy application. It is also relevant in a variety of clinical settings including cancer therapy, modulation of pathogenic immune responses, delivery of therapeutic proteins and drugs [27,28]. Importantly, in the last decade DNA vaccination using electroporation became a very efficient approach in various settings, since it was demonstrated that electric pulses provide additional stimuli to the immune system [29]. DNA vaccination using electroporation has been successfully used for vaccination in different disease, among others AIDS [30], various infectious diseases and very recently also for vaccination against of COVID-19 [31].
Although the mechanisms of gene electrotransfer are not yet fully understood, it was shown that several steps are needed for successful transfection: (i) migration of DNA towards the cell; (ii) DNA insertion into the permeabilized cell membrane; (iii) DNA translocation across the membrane; (iv) migration of DNA towards the nucleus; (v) transfer of DNA across the nuclear envelope and finally (vi) gene expression [22,32–34].

Despite the fact that gene electrotransfer efficiency *in vitro* is quite high, efficient gene electrotransfer in *in vivo* conditions still presents a challenge. One of main causes of low *in vivo* electrotransfer efficiency was found to be relatively low mobility of DNA in tissue compared to mobility in *in vitro* conditions. In different tissues, extensive network of extracellular matrix hinders DNA mobility to migrate towards the cell by reducing its diffusion and especially its electrophoretic mobility during electric pulse application [35–39].

Since DNA has to be in close proximity of the cell, when electric pulses are applied in order to achieve electrotransfer of DNA [33,40–44], poor mobility of DNA in extracellular matrix reduces amount of DNA molecules in the vicinity of the cell, thus resulting in decreased transfection efficiency. Many parameters have been described, which may influence the efficiency of gene electrotransfer *in vitro* [45], [32], [46–58] and *in vivo* [59–68]. Several studies have also shown that more efficient transfection can be achieved by using the combination of high-voltage (HV) short duration pulse, followed by a different number of low-voltage (LV) long duration electric pulses [41,61,69–71] specifically for muscle or tissue. It was suggested that HV pulses are crucial

for permeabilization of cell membrane, while LV pulses electrophoretically drag DNA to the cell. Also changing the polarity of electric field during the electric pulse delivery was shown to increase gene electrotransfer as it allows interaction of DNA molecules on many sides of the cell membrane perpendicular to direction of electric field [72]. Moreover different combinations of pulses were used in order to induce alteration of core envelope and to enhance gene electrotransfer efficiency [73]. Nevertheless, there is still a need for additional *in vivo* studies in order to overcome the problem of poor DNA migration towards the cell (first step needed for successful gene electrotransfer).

There is a great potential for 3D models in various fields of research in order to complement more traditional testing methods [74], to improve treatment planning [75], to validate protocols in order to forestall invasive surgical procedures [76], to propose a reliable alternative to animal experiments [37] *etc*. Moreover, there are also diverse electroporation-based applications, exploiting 3D models either to study electropermeabilization [77], irreversible electroporation [78], electrochemotherapy [79] or gene electrotransfection [38,80]. In latter class of application, authors perceived successfully transfected cells solely located on the upper surface of the constructed 3D tissue, where close contact of DNA was established. In addition gene electrotransfer efficiency was strongly depended on DNA mobility within tissue-rich in collagen [38].

We have previously developed 3D model of CHO cells embedded in 3D collagen matrix [37] that enabled studies of DNA electromobility and optimization of gene electrotransfer protocol. Furthermore 3D model represents a valid biological tool that enables us analysis of various mechanism with very defined parameters [80,81].

The aim of this study was therefore to analyze different parameters of gene electrotransfer inside 3D collagen model and to theoretically analyze DNA diffusion and electrophoretic mobility and their effects on gene electrotransfer efficiency. Cells were grown: (i) in a monolayer, (ii) on top of collagen layer or (iii) were embedded into 3D collagen gel. We investigated different relevant pulsing parameters: pulse durations, HV + LV combinations and the polarity of electric pulses. Furthermore, two different protocols of DNA application were used: DNA applied on top, or injected DNA directly to the 3D model thus mimicking the *in vivo* administration of pDNA.

2. RESULTS

3.1. Experimental results

In our previous study we have already developed a 3D *in vitro* model made of collagen gel with embedded cells, where successful gene electrotransfer was obtained [37].

In this study, in order to assess the influence of DNA mobility on gene electrotransfer efficiency, cells were (see Fig. 1): (A) plated as a monolayer culture; (B) grown on top of collagen layer and (C) embedded in collagen gel (3D model).

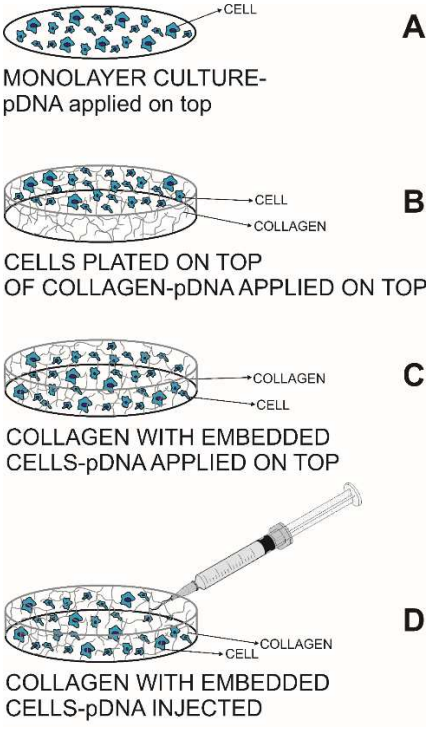

Figure 1: Cell culture and pDNA application

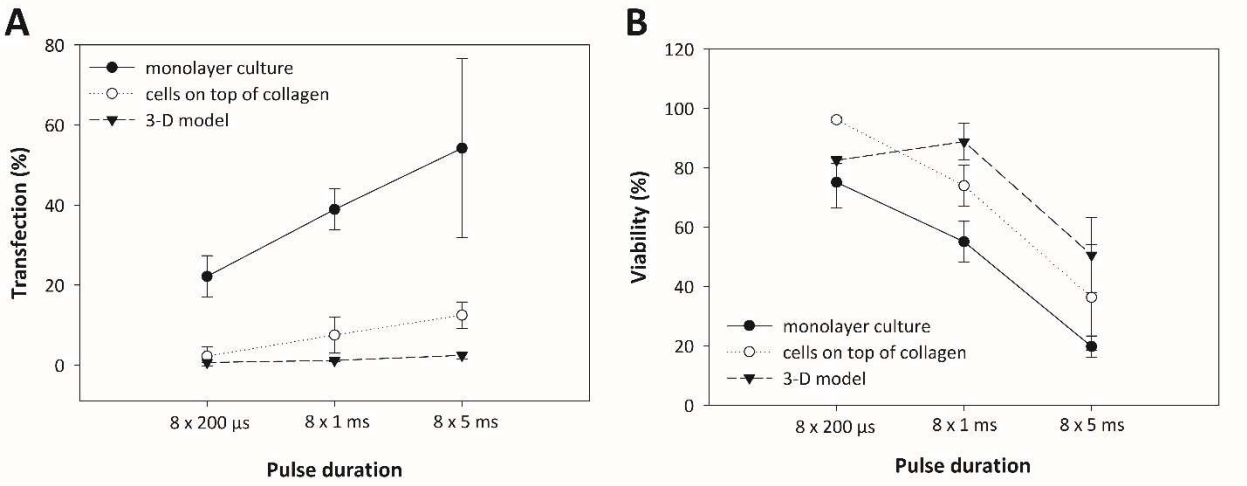

Figure 2: Effect of different pulse duration on gene electrotransfer efficiency (A) and on viability (B) for: (●) monolayer culture; (○) cells grown on top of collagen layer; (▼) cells embedded in collagen gel (3D model). Eight pulses of different durations, pulse repetition frequency of 1 Hz and E = 0.8 kV/cm were applied. pDNA concentration in electroporation media was 90 µg/ml.

In Fig. 2 the efficiency of gene electrotransfer and viability for different pulse durations of plated cells (monolayer culture), of cells grown on top of collagen layer and of cells embedded in

3D collagen gel are shown. We observed that gene electrotransfer efficiency was always significantly higher when cells were plated as a monolayer culture. Also, more cells were successfully transfected when they were grown on top of collagen layer compared to cells embedded in 3D model. Expectedly, the highest electrotransfer efficiency was obtained when we applied 8 × 5 ms pulses. Under this condition 54.2% of viable plated cells, 12.5% of viable cells grown on top of collagen layer and 2.5% of viable cells embedded in 3D model were transfected.

The viability of cells embedded in 3D model was for almost all conditions higher compared to plated cells or cells grown on top of collagen layer. The highest viability was observed when we applied shorter pulses (8 × 200 µs). Under this condition 75% of plated cells, 96% of cells grown on top of collagen layer and 82% of cells embedded in 3D model survived. At longer pulses viability of cells was significantly lower. At 8 × 5 ms 19.8% of plated cells, 36% of cells grown on top of collagen layer and 50% of cells embedded in 3D model survived.

Several studies have demonstrated that HV + LV pulsing protocols enable efficient electrotransfer in *in vivo* conditions [41,61,69–71]. In the second part of our experimental study, we analyzed the effect of high-voltage (HV) and low-voltage (LV) pulses on gene electrotransfer efficiency in a 3D collagen model. We used different combinations of HV and LV pulses, the parameters are presented in Table 2. Furthermore, pulses with alternating polarities were used (Table 2) to evaluate the effect of such pulsing protocols gene electrotransfer efficiency in a 3D model.

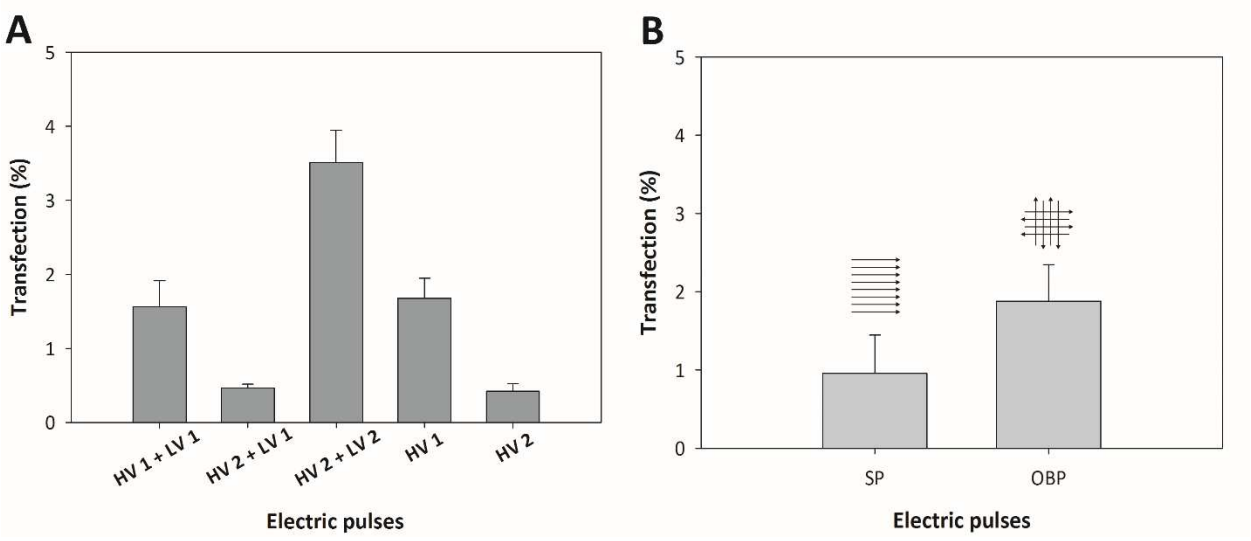

Figure 3: Effect of different pulsing protocols on gene electrotransfer efficiency in a 3D collagen model with embedded CHO cells, pDNA was administrated on top of the gel. (A) Different combinations of high-voltage (HV) and low-voltage (LV) pulses were applied. The number and duration of pulses, field strength and repetition frequency were as follows: HV 1 (5 x 1 ms; 0.8 kV/cm; 1 Hz), HV 2 (8 x 200 µs; 0.8 kV/cm; 1 Hz), LV 1 (1 x 100 ms; 75 V/cm) and LV 2 (1 x 100 ms; 150 V/cm). The time lag between HV and LV pulse was always 20 ms. (B) 8 x 1 ms pulses (repetition frequency 1 Hz) and *E* = 0.8 kV/cm with single polarity (SP) or orthogonal both polarities (OBP) were applied; Cells

were embedded in collagen gel (3D model). The percentage of transfected cells is plotted as a function of different electric pulses used. pDNA concentration in electroporation media was 90 μg/ml. Results are presented as a mean and vertical bars represent standard deviation.

In Fig. 3A gene electrotransfer efficiency is presented for different combinations of HV and LV pulse protocols for cells embedded in collagen gel (3D model). When 5 × 1 ms pulses (HV 1), with *E* = 0.8 kV/cm and 1 Hz repetition frequency were applied, one LV pulse (1 x 100 ms; 75 V/cm - LV 1) did not significantly contribute to gene transfection. Therefore, we used the same HV pulses (HV 1) and increased electric field strength of the LV pulse from 0.75 V/cm to 150 V/cm (LV 2). At those conditions (HV1 + LV2) cell viability was severely reduced and as a consequence gene electrotransfer efficiency was close to zero (data not shown). Therefore we reduced the duration of HV pulse in order to preserve cell viability and defined the following protocols: 8 × 800 μs pulses (HV 2) with *E* = 0.8 kV/cm and 1 Hz repetition frequency were followed by LV 1 or LV 2 pulse. It can be seen that when HV 2 pulse with increasing electric field strength of LV pulse was applied (HV2 + LV 2), gene electrotransfer efficiency significantly increased compared to HV 2 + LV 1 protocol. Maximum gene electrotransfer efficiency was obtained using HV 2 + LV 2 pulsing protocol (8 × 200 μs; 0.8 kV/cm; 1 Hz + 1 x 100 ms; 150 V/cm), where approximately 3.5% of cells in 3D model were successfully transfected. The cell viability was around 50% for those parameters (results not shown). Applying only LV pulse, no transfection was obtained (data not shown).

In Fig. 3B gene electrotransfer is presented for pulses with single or orthogonal both polarities for cells embedded in collagen gel (3D model). The higher gene transfer in 3D model was obtained, when pulses with different polarities were used (OBP) compared to single polarity pulses (SP) in agreement with *in vitro* study in classical monolayer conditions [72]. For OBP pulsing protocol (8 x 1 ms, *E* = 0.8 kV/cm) 1.88% of cells in 3D model were successfully transfected. The cell viability was around 75% for both pulse protocols-SP and OBP (results not shown).

To further analyze how reduced mobility of pDNA inside 3D collagen matrix affects gene electrotransfer efficiency, we compared gene electrotransfer efficiency for different pulsing protocols for two cases: in the first pDNA was administred on top of 3D model and in the second pDNA in electroporation media was injected into 3D model (Fig. 4). In general, for both methods of pDNA application the increase in gene electrotransfer efficiency was observed when longer pulses or pulses with higher *E* were used. For pDNA injected into the 3D model we consistently obtained higher gene electrotransfer efficiency compared to pDNA applied on the top of a 3D model. The highest efficiency for both methods of pDNA application was obtained when we applied 8 × 2 ms pulses with the applied electric field *E* = 1.0 kV/cm. When pDNA was injected into 3D model 6% of viable cells were transfected, but when pDNA was applied on top of 3D model the transfection efficiency was decreased to 4 %.

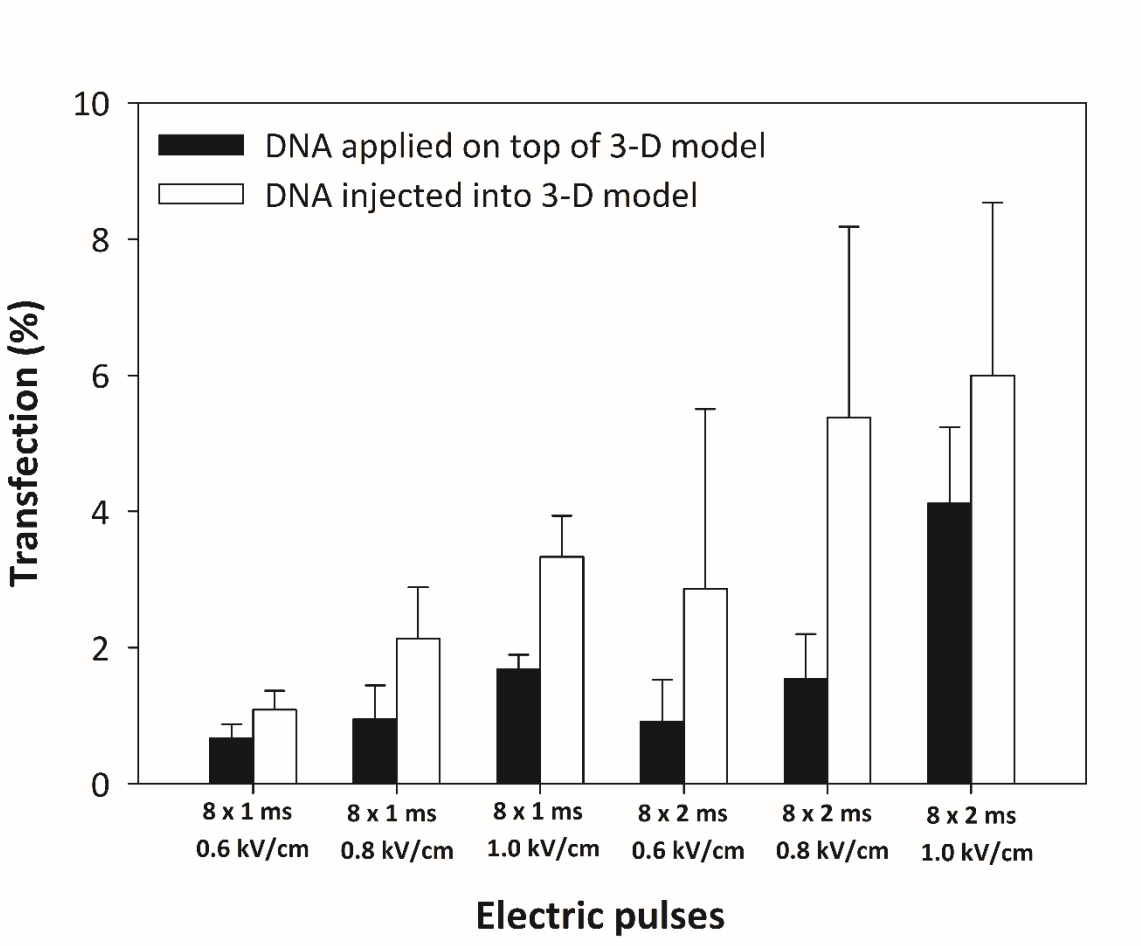

Figure 4: Effect of different pulse durations on gene electrotransfer efficiency of cells embedded in collagen gel two modes of DNA administration: (■) pDNA was administered on top of 3D model and (□) pDNA was injected into 3D model. The percentage of transfected cells is plotted for different electric pulses: 8 × 1 ms and 8 × 2 ms pulses of different electric field strength *E* (kV/cm) were used. Cells were embedded in collagen gel (3D model). In all experiments same amount of pDNA was administered (18.2 µg). Results are presented as a mean and vertical bars represent standard deviation.

3.2. Theoretical analysis

3.2.1. Quantifications of electrophoresis of plasmid DNA inside collagen matrix

Electrophoresis is another mechanism which was shown to be important for the delivery of DNA molecules into cells by electric pulses [32,34–36]. During pulse application, the electrophoretic driving force acts (*F*) on the negatively charged DNA molecule and drags it toward the cathodic side of the cell membrane. It depends on the local electric field (E_{loc}) and on the effective charge of a given molecule:

$$F = e_{eff} E_{loc} \,, \tag{1}$$

where the effective charge depends on the ionic strength of the solution and length of the pDNA (e_{eff} = 0.066 per base pair × 4.7 k bp for our 4.7 kbp pDNA). DNA molecule moving in an aqueous solution under external electric field *E* reaches the steady state velocities *v* practically immediately - in approximately 3 × 10^{-11} s [36], therefore during pulse application steady-state conditions can be assumed. Under steady-state condition frictional force equals electrophoretic force, therefore electrophoretic mobility μ is:

$$\mu = e_{eff} \,/\, f = \frac{e_{eff}}{6\pi\eta R_g}, v = \mu E \,, \tag{2}$$

and it depends on the friction drag *f* and effective charge. For standard *in vitro* experiments in suspension or on plated cells, one can use viscosity of water for η for the viscosity of electroporation buffer.

Clearly, in a 3D gel matrix such as extracellular matrix, agarose or collagen gel the friction *f* and viscosity depend on the composition of collagen matrix. Furthermore, the above equations are valid for supercoiled pDNA where we can assume globular geometry (Ogston model). However, as shown by Zaharoff and Yuan, 2002 [35], the electric field will also stretch DNA along the electric field and thus more complex models of DNA inside such 3D matrix are valid. Thus, instead of direct analytical calculation of electrophoretic mobility from Eq. 2, we have used measured electrophoretic mobilities from Zaharoff and Yuan, 2004 [36] for 0.5% - 3% agarose gel and extrapolated their values from Fig. 1 in reference [36] to our less dense (0.35%) collagen gel.

3.2.2. Diffusion of pDNA inside collagen matrix – calculation of the concentrations distributions a 3D collagen gel

Free diffusion (classical diffusion in zero field *E* = 0) of a given molecule within one dimension can be described by one dimensional diffusion equation:

$$c(z,t) = \frac{1}{\sqrt{4\pi Dt}} \int_{-\infty}^{\infty} c_0(z') \exp\left[-\frac{(z-z')^2}{4Dt}\right] dz' \,, \tag{3}$$

where *D* is the diffusion constant of a specific molecule in a given medium. If we now consider specifically our geometry of pDNA diffusion inside collagen matrix where pDNA was applied on the top of the collagen gel (*z* = 0) (see Fig. 1C), diffusion equation in half space can be applied:

$$c(z,t) = erfc(z / 2\sqrt{Dt}) \,, \tag{4}$$

where c (z, t) describes a time-dependent spatial concentration distribution of pDNA where z is the distance from the top of the gel to the given point inside the gel (see Fig. 5) and D is the diffusion constant. For estimation of D we have used measured diffusion coefficients from Zaharoff and Yuan 2004 [36] for 0.5% - 3% agarose gel. Since our 3D collagen gel with embedded cells is much less dense (0.35% collagen) we have extrapolated curve from Fig. 1 to lower gel percentages and obtained $D = 3 \times 10^{-8}\text{cm}^2/\text{s}$, which we have used in our theoretical model of pDNA diffusion inside a 3D collagen matrix.

From Eq. 4 one can obtain concentration distribution for the given initial concentration distribution $c_0(z, t = 0)$ – the initial conditions. For any given time of pDNA incubation ($t = t_{inc}$) one can thus obtain spatial concentration distribution c (z, t_{inc}):

$$c(z,t_{inc}) = erfc(z / 2\sqrt{Dt}_{inc}) . \tag{5}$$

Since electrotransfer efficiency depends on the local concentration $c\ (z,\ ti_{nc})$ of pDNA in the vicinity of a cell it is important to relate pDNA concentration with the probability of transfection. In gene electrotransfer experiments, usually percentage of transfected cells is evaluated (%TR) and this parameter is directly dependent to the probability of transfection of a single cell - P_1, where:

$$P_1(z) = k \times c(z, t_{inc}) . \tag{6}$$

Thus, percentage of transfected cell (%TR) is proportional to the integral of the concentration distribution for z = [0, d - thickness of a 3D gel] of all probabilities P_i:

$$\%TR = K \int_0^d c(z,t_{inc}) , \tag{7}$$

where constant K is proportional to number of cells and other parameters that determine the final probability of transfection (*e.g.* pulse parameters). We have determined K based on our experimental results for plated CHO cells [32] where %TR approximately linearly increased with pDNA concentration up to 10 µg/ml. The next assumption which we used is that very high pDNA concentrations are toxic and therefore also reduce transfection efficiency above c > 40 µg/ml.

From the above equations, one can calculate how pDNA diffuses inside and through the collagen matrix for the case where pDNA is administrated on the top of a 3D collagen with embedded cells. The diffusion equation (Eq. 5) enables us to calculate the concentration

distribution c (z, t_{inc}) depending on the distance from the top of 3D gel (z) and on the time of incubation t_{inc} , where at t = 0 we add suspension with pDNA on the top of the gel. In Fig. 5 we present calculated distribution of pDNA concentration – c (z) after diffusion for different times of pDNA incubation before the application of the electric pulses. It can be seen, that pDNA will in 1 h penetrate few tens of millimeter inside the collagen gel. In order to reach 1 mm, very long incubation time would have to be used.

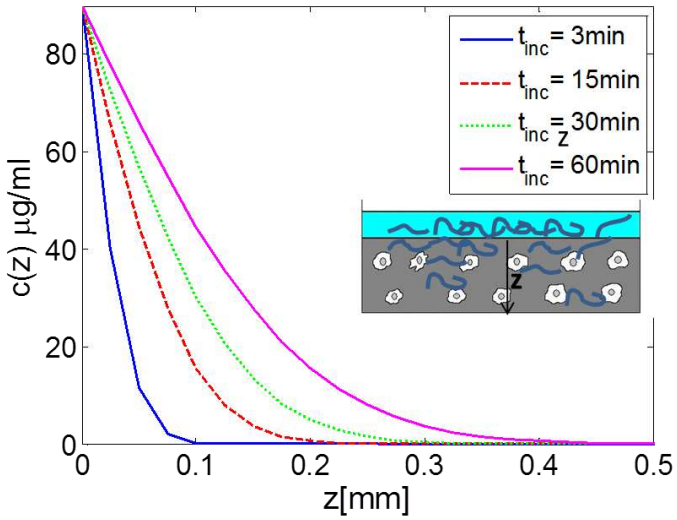

Figure 5: Diffusion of pDNA into 3D collagen gel, where pDNA was applied on the top of the gel. The distribution profile c (z, t_{inc}) is calculated from Eq. 5, where z is the distance from the top of 3D gel and t_{inc} is the time of pDNA application (incubation) on the top of the gel, the diffusion constant is: $D = 3 \times 10^{-8}/cm^2$.

From the calculated pDNA distribution c (z , t_{inc}) we can calculate the % electrotransfection (%TR) by integrating probability of transfection for all planes over z, taking into account that probability of transfection is approximately linear up to some maximum (10 µg/ml), where it reaches saturation. We have also assumed that for very high pDNA concentrations (c > 40µg/ml), when cell viability is affected, %TR drops [82].

In Fig. 6 and 7 we show comparison of the experimental values with the theoretically calculated %electrotransfection (%TR) based on the diffusion model (Eqs. 5-7) for different incubation times t_{inc} and different initial pDNA concentrations applied on the top of the gel (Fig. 7) - c_0 . It can be seen that it is crucial to allow enough incubation time with pDNA before pulse application, in order to achieve efficient transfection and that above some maximal initial plasmid concentration %TR is not increased. The theoretical model can approximately describe the experimental dependency on t_{inc} and c_0.

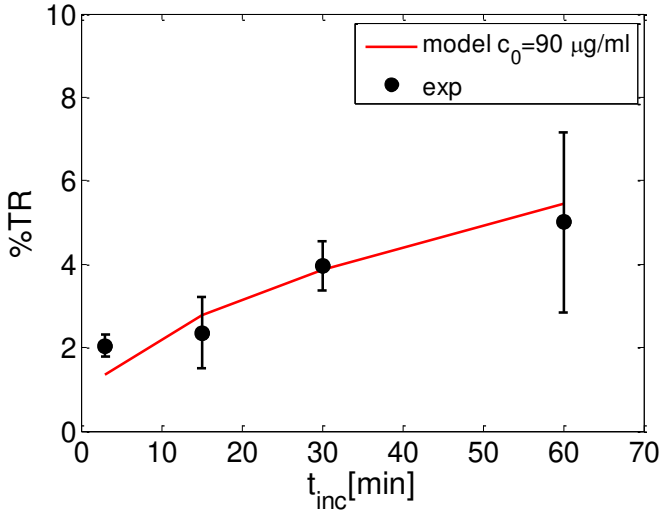

Figure 6: Comparison of theoretically calculated % electrotransfection (%TR) based on the diffusion model (see Eqs. 5-7) and experimental values for different times t_{inc} and for initial pDNA concentration on the top of the gel c_0= 90μg/ml, $D = 3 \times 10^{-8}/cm^2$ for 8 × 5 ms electric pulses, E = 0.8 kV/cm. Results are presented as a mean and vertical bars represent standard deviation.

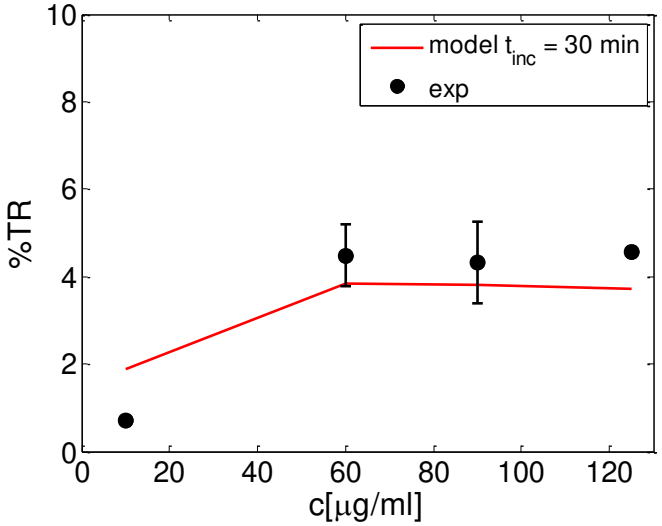

Figure 7: Comparison of theoretically calculated % electrotransfection (%TR) based on the diffusion model (Eqs. 5-7) and experimental values for initial pDNA concentrations c_0 and for following parameters: t_{inc} = 30 min, $D = 3 \times 10^{-8}/cm^2$ and 8 × 5 ms electric pulses, E = 0.8 kV/cm. Results are presented as a mean and vertical bars represent standard deviation.

3.2.2. Quantifications of pDNA electromobility inside 3D gel

As described in M&M section and in different studies [35,36] electromobility of pDNA inside 3D gels is a complex function of electric pulse parameters and density of the gels. Thus direct

analytical calculation form Eq. 9 is not possible and measured electrophoretic mobilities μ from Zaharoff and Yuan [36] for 0.5% - 3% agarose gel were used to extrapolate their values from Fig. 1 [36] to our less dense (0.35%) collagen gel. For the extrapolated mobilities μ for pulsing protocols 8 × 200 µs, 8 × 1 ms, 8 × 5 m, 8 × 10 ms are given in Table 1, where relation between the measured %TR, $U^2 \times t_E$ (proportional to electric energy of the pulses) and electromobilities are presented.

Table 1: The relation between measured %TR, electric pulse energy given in terms of $U^2 \times t_E$ and electromobilities (extrapolated from Zaharoff and Yuan, 2004) for different pulsing protocols: 8 × 200 µs, 8 × 1 ms, 8 × 5 ms, 8 × 10 ms, E = 0.8 kV/cm.

Pulse parameters	Electromobility μ [cm^2/Vs]	$U^2 \times t_E$ [V^2 s]	%TR
8 × 200 µs	0.2×10^{-8}	40.96	0.65
8 × 1 ms	1×10^{-8}	204.8	1.55
8 × 5 ms	2.4×10^{-8}	1024	3.58
8 × 10 ms	2.8×10^{-8}	2048	2.16

From Table 1 it is clear that longer pulses increase electromobility and also lead to higher %TR. For very long pulses electromobility reaches a plato and for 8 × 10 ms, %TR is even decreased since very long pulse reduce cell viability. Therefore, from the estimated mobility we can calculate the displacement L due to the electrophoretic drag during the pulses of total duration t_E:

$$L = v\, t_E = \mu E t_E \,, \tag{8}$$

from which we obtain that the maximal distances of pDNA movement for 8 × 5 ms are between 10 – 20 µm. Similarly, as we have shown previously [34], from Eq. 8 we can estimate number of pDNA molecules N_{DNA} in the volume V, which are available for the contact with the permeabilized part of the cell membrane. The strength and length of the electric pulses t_E determine the distance L from which pDNA can access the cell and electric field strength determines the area of the membrane which is electropermeabilized S:

$$N_{DNA} = c\,(z,\, t) \times V = c(z,\, t_E\,) \times \mu\, E\, t_E \times S\,. \tag{9}$$

From the above equation, it is clear, that the number of pDNA molecules available for contact with the membrane and consequently probability of electrotransfer linearly increases with the local pDNA concentration *c* (z, t).

3. DISCUSSION & CONCLUSION

Gene electrotransfer is an established method to deliver genes both *in vitro* and *in vivo*. The main problem in gene electrotransfer of mammalian cells *in vivo* is still relatively low efficiency [22,25,26]. *In vitro* the DNA can easily reach cells and is therefore directly in contact with the cell membrane, which is one of the crucial steps in gene electrotransfer, while *in vivo*, extracellular matrix hinders diffusion and electromobility of DNA in proximity of cells consequently leading to relatively low transfection. Studying different parameters of gene electrotransfer in 3D gel systems *in vitro* that more closely resemble the *in vivo* conditions, where especially mobility is drastically reduced, offers the possibility to study the mechanism and to enable optimization of the protocols for more efficient gene transfer *in vivo*.

In order to have more realistic *in vivo* model system, we used previously described 3D collagen model [37], which we have developed for analysis of the gene electrotransfer. In the first part of our study, we compared gene electrotransfer efficiency on: (i) plated CHO cells (standard monolayer culture), (ii) CHO cells grown on top of collagen layer (which represent the intermediate step between classical cell culture and *in vivo* model system) and (iii) CHO cells embedded in a 3D model. As we expected, gene electrotransfer efficiency was substantially higher when cells were plated as a monolayer culture, compared to cells grown on top of collagen layer or cells embedded in a 3D model for all pulsing protocols. The result of our experiments showed that maximum gene electrotransfer efficiency was obtained, when pulses of longer duration were used, where 54% of plated cells, 13% of cells grown on top of collagen layer and 2.5% of cells embedded 3D model were successfully transfected. The difference in gene electrotransfer efficiency can be mostly explained by the fact that pDNA transport through the collagen matrix is relatively slow, especially when cells are embedded in the 3D model. Our results of %TR in 3D collagen *in vitro* model are comparable to the results of *in vivo* experiments, where similar gene electrotransfer (around 2%) was obtained [83].

To understand if the decrease in gene electrotransfer efficiency is not a consequence of reduced cell viability, we tested survival of cells 24 h after electric pulse application for all three variants: plated cells, cells grown on top of collagen layer and cells embedded in 3D model. Expectedly, [45], when we increased pulse duration also cell viability was reduced in agreement with several other studies [22,33,45]. Indeed, pulse duration should be optimized to obtain sufficient gene electrotransfer efficiency, avoid irreversible cell damage and this optimization has to be performed for each specific cell line and electrotransfer protocol. Interestingly, when cells were embedded in the 3D model, cell viability was higher when longer pulses were used (8 x 5

ms) compared to the plated cells or cells grown on top of collagen layer for the same pulse parameters. We suggest that higher viability in the 3D collagen gel is partially a consequence of smaller pDNA concentration in a vicinity of cells, since it was shown by others, that high levels of pDNA can be toxic [82]. Also cell osmotic swelling [84] and other factors are altered when cells are embedded in a 3D matrix, which can be beneficial for cell survival.

Since it was shown by many *in vivo* [41,61,69–71,85] studies that short high-voltage (HV) microsecond pulses in combination with long low-voltage (LV) millisecond pulses contribute to higher gene electrotransfer efficiency, we analyzed in the third part of our study the influence of different combinations of HV and LV pulses on gene electrotransfer efficiency in 3D model. We obtained higher gene electrotransfer efficiency when using HV2 (8 × 200 μs; 0.8 kV/cm) pulse in combination with higher low-voltage pulse LV 2 (1 x 100 ms; 150 V/cm) pulse, compared to using only HV 2 pulse or lower LV 1 pulse. At those conditions also cell viability was preserved. Our results are in agreement with previous *in vivo* [85] and *in vitro* studies [32,40], where it was suggested that LV pulses are in *in vivo* conditions crucial for efficient transfection as they electrophoretically move DNA towards the cells [32].

The main limitation of our 3D model was very low cell viability for LV pulses with longer durations (more than 200 ms long). We therefore could not analyze the influence of even longer LV pulses on gene electrotransfer efficiency in 3D model.

In the second part of our study we analyzed the influence of changing the electric field orientation on gene electrotransfer efficiency in 3D model. It was already suggested, that by changing the polarity of the pulses the membrane area that is competent for DNA entry into the cell increases. Our results in 3D model were consistent with previous results [46,72] where gene transfection is increased when the electric field orientation between electrical pulses is changed (OBP protocol) compared to single polarity pulses, however the electrotransfer efficiency was low.

Furthermore, *in vivo* pDNA is usually delivered to the target cells by means of a local injection [69], [60], [41], [70] and consequently only cells in vicinity of injected site are in close contact with high concentrations of pDNA. Therefore, we next analyzed pDNA mobility in a 3D model by applying pDNA on top or injected it into the 3D model. We observed that the latter way of application showed higher transfection efficiency compared to the former one. The highest gene electrotransfer efficiency was obtained for both ways of pDNA application, when 8 × 2 ms pulses with E = 1.0 kV/cm was used. At those conditions the highest transfection obtained was around 6% when pDNA was injected into 3D model, compared to approximately 4%, when pDNA was applied on the top of the 3D model. We also observed that more cells were successfully transfected near the injection site (Fig. 8). Cell viability was for both ways of pDNA application similar (data not shown).

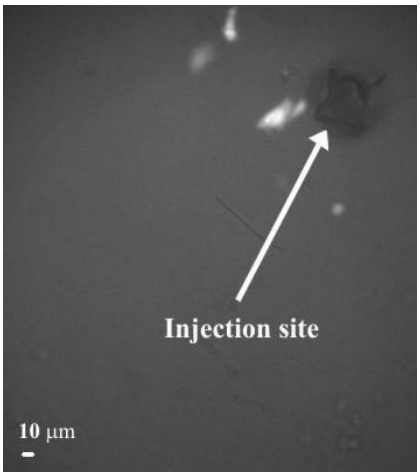

Figure 8: Representative picture, which shows that especially cells in the 3D model, which were near the injection site were successfully transfected. Eight pulses of 2 ms long, pulse repetition frequency of 1 Hz and *E* = 0.8 kV/cm were applied. pDNA concentration in electroporation media was 90 μg/ml.

Classical 2D cell cultures do not reproduce the morphology and biochemical features that cells possess in tissue. As alternative, cells grown in more *in vivo* like environment such as collagen gel, offer the possibility to study different parameters of gene electrotransfer. We therefore experimentally and theoretically analyzed mobility of pDNA during gene electrotransfer in a 3D model of CHO cells embedded in collagen gel. The results demonstrate that it is crucial that pDNA is in closer proximity of cells in order to achieve successful gene electrotransfer and that hindered diffusion of pDNA inside matrix lead to low transfection efficiency compared to *in vitro* conditions. Results obtained by different combinations of HV and LV pulses and changing the polarity of pulses in a 3D model resemble data obtained *in vivo*, namely that LV pulses and/or changing the polarity can increase transfection efficiency. We showed, that our 3D *in vitro* model can be used to study mechanisms of gene electrotransfer and to enable optimization of the protocols. Such approach enables faster optimization of parameters for electroporation based gene delivery *in vivo* thus reducing the number of animals used for *in vivo* experiments.

Furthermore, we present theoretical quantification of pDNA diffusion in collagen matrix that show good agreement with the experimental results (see Figs. 5 and 6). We demonstrate that in a 3D gel models or in tissues it is very important to allow long incubation time after application/injection of pDNA before pulse application allowing diffusion in larger area. We estimate, that for *in vitro* 3D models we can assume pDNA mobility due to diffusion in range few tens of mm, while in real tissue that has more dense collagen structure as shown by Zaharoff and Yuan [35] the diffusion is almost negligible (less than 1 μm).

Thus, our results show that in dense ECM matrix (*e.g.* in tumor tissue, skin, muscle) it is crucial to administrate – inject pDNA in several sites thus enabling coverage of larger area of

tissue with sufficient local pDNA concentration which will enable efficient gene electrotransfer [86].

Moreover, as stressed in previous papers [35,36] the advantage of electrophoretic drag of DNA over diffusion increases with both transport distance and gel concentration. A 3D gel can be considered as a distribution of larger voids connected by narrower passages or pores. When the radius of a molecule is comparable to the mean pore size (few tens of nm in collagen), transport is significantly hindered by frictional and steric interactions between the pDNA molecule and the pores [35]. Electrophoresis elongates pDNA in the direction of field and movements, and decreases its diameter in the perpendicular direction and therefore decreases entropy. The narrow passages function as entropic barriers to the transport of pDNA, thus application of longer pulses or HV-LV pulses could reduce the height of the entropic barrier.

However, in dense tissue even with very strong pulses electromobility during the pulses is very small (around 1 μm) – therefore we suggest that electrophoretic pulses are more crucial for formation of a contact between pDNA in vicinity with the membrane as we explain in our paper [34], but cannot be used to »drag« pDNA through ECM. This could also explain experimental data where in some studies HV-LV pulses could increase transfection efficiency in muscle tissue [41,69], while in tumor tissue this effect was less pronounced. Also Heller *et al.* showed very efficient electrotransfection for only HV pulses [87].

To conclude, we show that our 3D collagen model resembles the *in vivo* situation more closely than conventional 2D cell cultures and that the efficiency of gene electrotransfer in 3D model resembles the efficiency in *in vivo* environment. Thus, our 3D model provides an intermediate between *in vitro* and *in vivo* conditions to study mechanisms of gene electrotransfer especially for biotechnological/biomedical protocols where diffusion or electromobility are one of crucial mechanisms.

4. MATERIALS AND METHODS

2.1. Cell culture

For the experiment Chinese hamster ovary cells (CHO-K1) were used (European Collection of Cell Cultures, Salisbury, UK). Cells were: (A) grown as a monolayer culture in 24-multiwell plate, (B) grown on top of collagen gel layer and (C) embedded in collagen gel (3D model), where DNA was applied on top or injected into 3D model (see Fig. 1). For cells, culture medium F-12 HAM (Dulbecco's modification of EMEM) supplemented with 10% fetal bovine serum and 0.15 mg/ml L-glutamine (Sigma-Aldrich, St. Louis, MO) was used.

2.1.1. Preparation of cells grown as a monolayer culture

CHO-K1 cells were plated as a monolayer culture (Fig. 1A) in Ham's tissue culture medium in 24-multiwell plate in cell density of $\rho = 5 \times 10^4$ cells/ml. The plate was stored for 24 h at 37°C in a humidified 5% CO_2 atmosphere in the incubator (Kambič, Slovenia).

2.1.2. Preparation of cells grown on top of collagen gel layer

Type I collagen from rat tail was obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany) as a powder and mixed with diluted acetic acid (28.5 ml glacial acetic acid/litter) to achieve collagen solution concentration 4.0 mg/ml and stored at 4°C. After 24 h 1x PBS, pH=7.4 was added to collagen solution, in the ratio of 1:8. pH of mixture was adjusted to 7.2-7.6 with 0.1 M NaOH. To prevent gelation, temperature of mixture was maintained at 2–8°C. 200 µl of collagen was pipeted into each space of 24-multiwell plate and stored for 1 h at 37°C in a humidified 5% CO_2 atmosphere in the incubator. Collagen polymerized and formed a gel layer.

After 1 h incubation of collagen layer at 37°C, CHO-K1 cells were added on top of collagen layer as a monolayer culture (Fig. 1B) in Ham's tissue culture medium in cell density of $\rho = 5 \times 10^4$ cells/ml. The plate was placed back into the incubator (37°C, 5% CO_2) for 24 h.

2.1.3. Preparation of collagen gel with embedded cells (3D model)

Collagen solution was prepared as described above. After 24 h incubation of collagen solution at 4°C, collagen mixture was prepared as already described before [37]. Briefly, 2.3 parts of chilled collagen solution was mixed with 0.5 part of Ham tissue culture medium for mammalian cells and 0.5 part of 1x PBS, pH=7.4. CHO-K1 were prepared as a cell suspension and cell pellet was re-suspended with liquid collagen solution to a cell density of $\rho = 5.6 \times 10^5$ cells/ml. 180 µl of collagen with cells was pipetted into each space of multiwell dish and stored for 1 h at 37°C in a humidified 5% CO_2 atmosphere in the incubator. After raising the temperature to 37°C, collagen polymerized and formed a gel with embedded cells inside (3D model) (Fig. 1C, D and Fig. 9). Ham's tissue culture medium was then gently added and cells were stored for 24 h at 37°C in a humidified 5% CO_2 atmosphere.

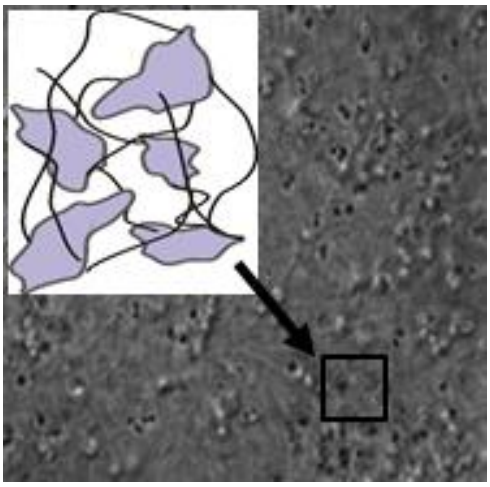

Figure 9: Cells embedded in collagen gel.

2.2. Plasmid DNA

Plasmid pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA) encoding green fluorescent protein (GFP) was amplified in Top10 strain of *Escherichia coli* and isolated with HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid DNA (pDNA) concentration was spectrophotometrically determined at 260 nm and confirmed by gel electrophoresis.

2.3. Gene electrotransfer

Our study was divided into three sets of experiments. In the first part, gene electrotransfer was performed on plated cells, on cells grown on top of collagen layer and on cells embedded in 3D model (to show how DNA mobility-which was lowest in 3D model-affects gene electrotransfer efficiency) (Fig. 1 A-C). In the second part gene electrotransfer was performed on cells embedded in 3D model, where DNA was administered on top or injected into the 3D model (to show, how injected DNA can come closer to the cells and by that gene electrotransfer efficiency could be increased) (Fig. 1C, D) and in the third part we analyzed gene electrotransfer efficiency in 3D model by using different pulsing protocols – combinations of high-voltage and low-voltage pulses, single polarity pulses and orthogonal both polarities pulses (to show, that also in our 3D model different pulsing protocols are affecting gene electrotransfer efficiency).

Electroporation was performed on 24 h old cell culture with standard electroporation media (pH 7.4, 10 mM NaH_2PO_4/Na_2HPO_4, 1 mM $MgCl_2$ and 250 mM sucrose). On the day of the experiment culture medium was removed and cells were incubated with 200 µl of electroporation media with pDNA that codes for GFP for 30 min at a room temperature (22°C). Plasmid DNA concentration in electroporation media was 90 µg/ml.

In first two parts of the experiment for pulsing Jouan GHT 1287 electroporator (Jouan, St. Herblain, France) and an oscilloscope Wave surfer^{TM} 422 (Le croy, Chestnut Ridge, New York, USA) to monitor pulse shape were used. The distance between a pair of two plate stainless steel parallel electrodes was *d* = 4 mm.

In third part of the experiment for pulsing pulse generator Cliniporator™ (IGEA s.r.l., Carpi, Modena, Italy) was used which enabled different combinations of high- (HV) and low-voltage (LV) pulse. The distance between a pair of two plate stainless steel parallel electrodes was *d* = 4 mm. For analyzing gene electrotransfer efficiency by changing pulse polarity a high-voltage prototype generator (EP-GMS 7.1) was used [72], which allowed application of relatively homogeneous electric field in different directions. An oscilloscope Wave surfer^{TM} 422 (Le croy, Chestnut Ridge, New York, USA) monitored pulse shape. Especially designed electrodes allowing delivery of electric field in different directions and at the same time providing relatively homogeneous electric field distribution were used [72]. No electric pulses were applied to cells in a control sample.

In the first part of the experiment, electroporation media with pDNA was applied on top of plated cells, cells grown on top of collagen layer and cells embedded in 3D model. A train of eight square wave pulses of different pulse durations: 200 µs, 1 ms and 5 ms were used to deliver pDNA into the cells. Electric field strength was 0.8 kV/cm, with repetition frequency 1 Hz for all pulsing protocols.

In the second part, two ways of pDNA application were studied in 3D model: (i) electroporation media with pDNA applied on top of 3D model or (ii) electroporation media with pDNA injected into the 3D model (see Fig. 1C and 1D). Electric pulses of two different pulse durations were used: 8 × 1 ms and 8 × 2 ms to deliver pDNA into the cells. Electric field strengths used were 0.6 kV/cm, 0.8 kV/cm and 1.0 kV/cm, with repetition frequency 1 Hz for all pulsing protocols.

In the third part, electroporation media with pDNA was applied on top of cells embedded in 3D model. Different types of pulsing protocols were used to deliver pDNA into the cells as shown in Table 2 and Figure 10. Also different incubation times and pDNA concentrations were tested.

Table 2: Pulsing protocols for gene electrotransfer in 3D model. The time lag between HV and LV pulse was always 20 ms.

Electric pulse parameters
HV 1 (5 × 1 ms; 0.8 kV/cm; 1 Hz)
HV 2 (8 × 200 µs; 0.8 kV/cm; 1 Hz)
LV 1 (1 × 100 ms; 75 V/cm)
LV 2 (1 × 100 ms; 150 V/cm)
Single polarity pulses-SP (8 × 1 ms; 0.8 kV/cm; 1 Hz)

Orthogonal both polarities pulses-OBP (8 × 1 ms; 0.8 kV/cm; 1 Hz)

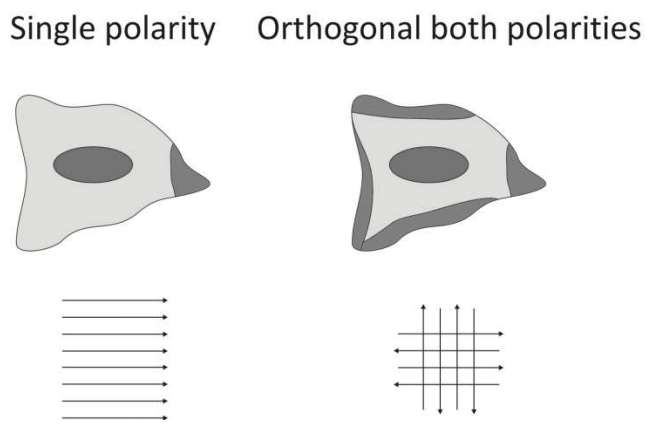

Figure 10: In single polarity (SP) electric pulses are applied between two opposite electrodes. While in orthogonal both polarities (OBP) electric pulses are applied between both opposite pairs of electrodes.

After exposing cells to electric pulses, 70 µl of fetal calf serum was added (35% of sample volume) to preserve cell viability. Cells were then incubated for 15 min at 37°C to allow cell membrane resealing and then grown for 24 h in cell culture medium at 37°C in a humidified 5% CO_2 atmosphere in the incubator.

Gene electrotransfer efficiency was determined by fluorescent microscopy (Zeiss 200, Axiovert, ZR Germany) with excitation light at 445 nm generated with a monochromator system (PolyChrome IV, Visitron, Germany) and emission was detected at 488 nm. The images were recorded using imaging system (MetaMorph imaging system, Visitron, Germany). At least ten fluorescence images were acquired in the area between the electrodes at 10× objective magnification per each parameter. The cells were counted manually and gene electrotransfer efficiency was determined by the ratio between the number of green fluorescent cells (successfully transfected) and the total number of cells. Three independent experiments were performed for each parameter and results are presented as a mean values ± standard deviation.

2.4. Cell viability

Cell viability was determined by measuring propidium iodide (PI) uptake 24 h after applying pulses as was already described [37]. Briefly, culture medium was removed and 200 µl of PBS with 6 µl of 0.15 mM PI was added to cells. After 5 min incubation cell viability was determined by fluorescent microscopy. The cells were counted manually under the fluorescent microscope and cell viability was determined by the ratio between the number of dead cells (cells with incorporated PI) and the total number of cells.

5. DECLARATIONS

5.1. Ethics approval and consent to participate

Not applicable.

5.2. Consent for publication

Not applicable.

5.3. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

5.4. Competing interests

The authors declare that they have no competing interests. The authors declare that there are no conflicts of interest.

5.5. Funding

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5.6. Authors' contributions

SHM and MP performed the experiments with cells in collagen, analyzed and interpreted the data. MP theoretically analyzed DNA diffusion and electrophoretic mobility and interpreted the theoretical data.

Both authors equally contributed in writing the manuscript. All authors read and approved the final manuscript.

5.7. Acknowledgement

Not applicable.

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Figures

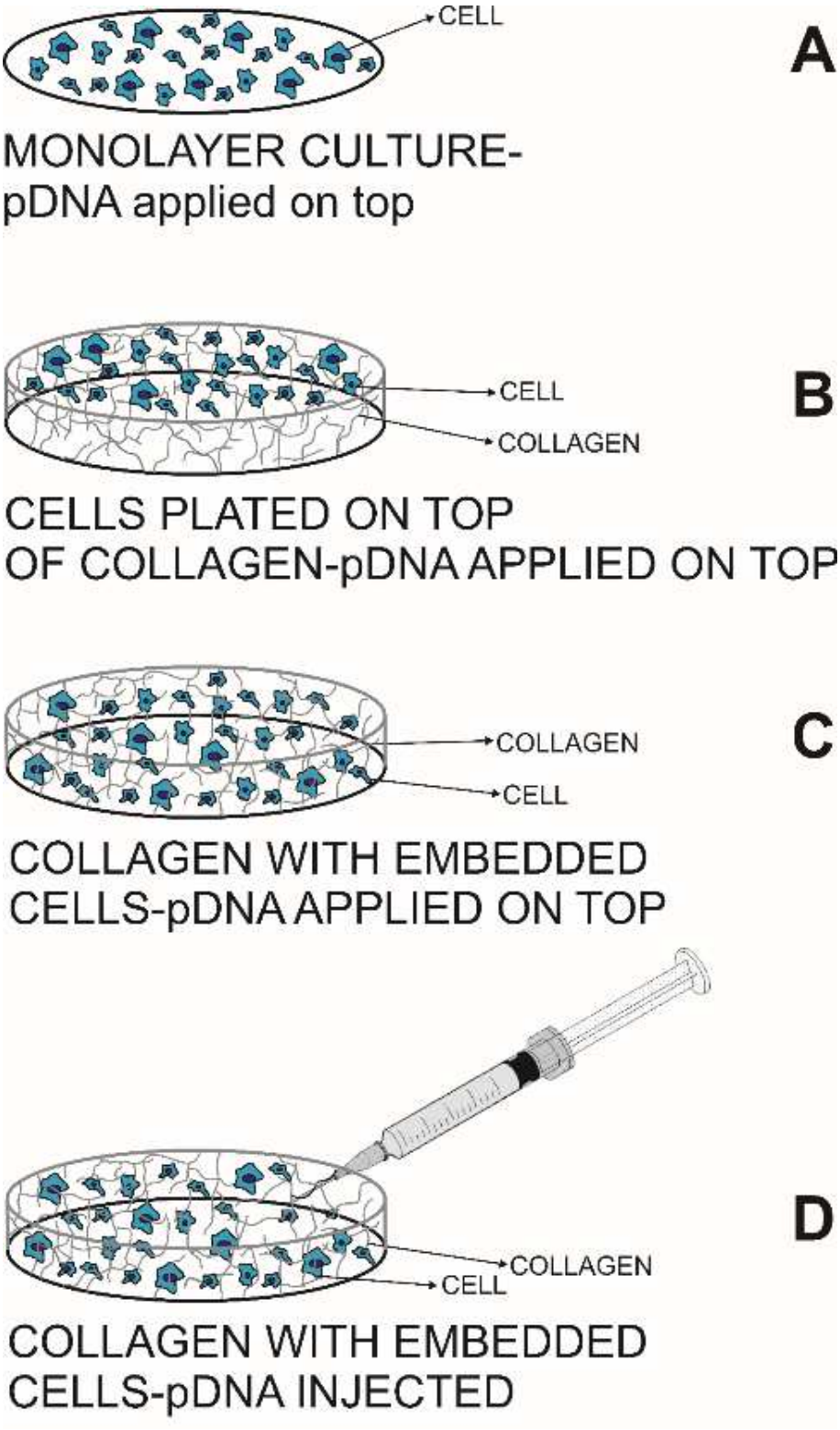

Figure 1

Cell culture and pDNA application

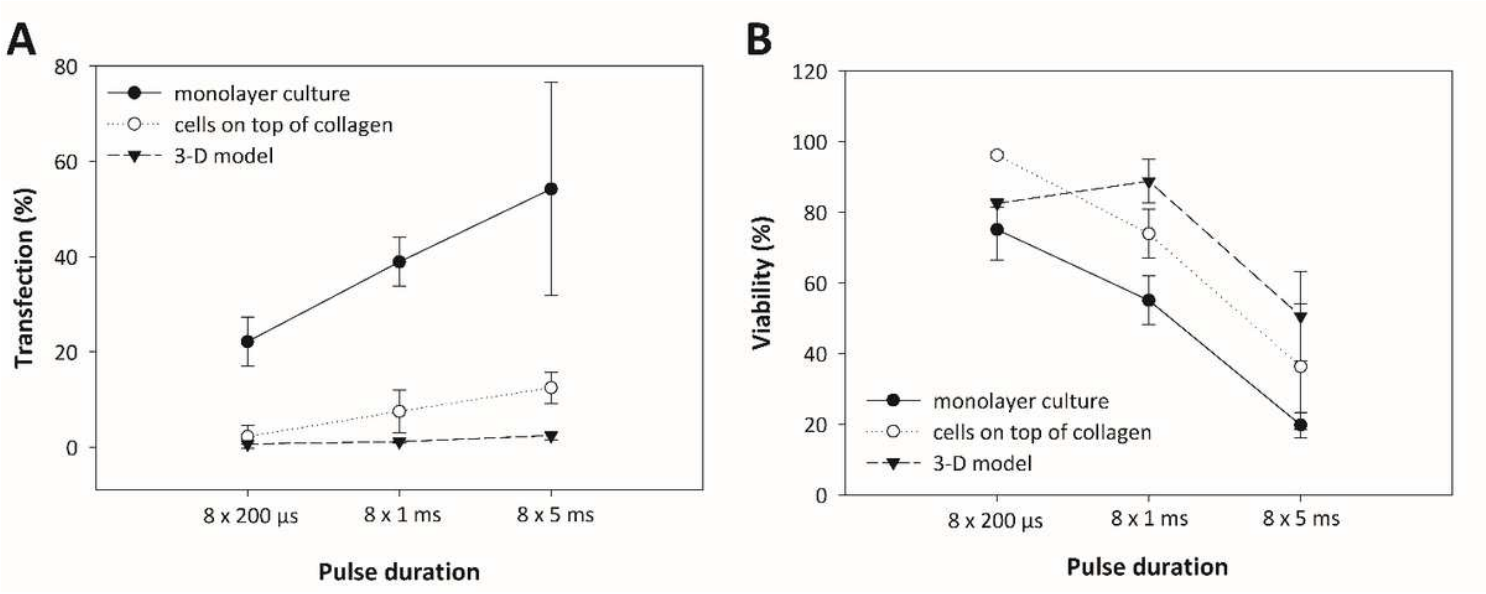

Figure 2

Effect of different pulse duration on gene electrotransfer efficiency (A) and on viability (B) for: (□) monolayer culture; (□) cells grown on top of collagen layer; (□) cells embedded in collagen gel (3D model). Eight pulses of different durations, pulse repetition frequency of 1 Hz and E = 0.8 kV/cm were applied. pDNA concentration in electroporation media was 90 µg/ml.

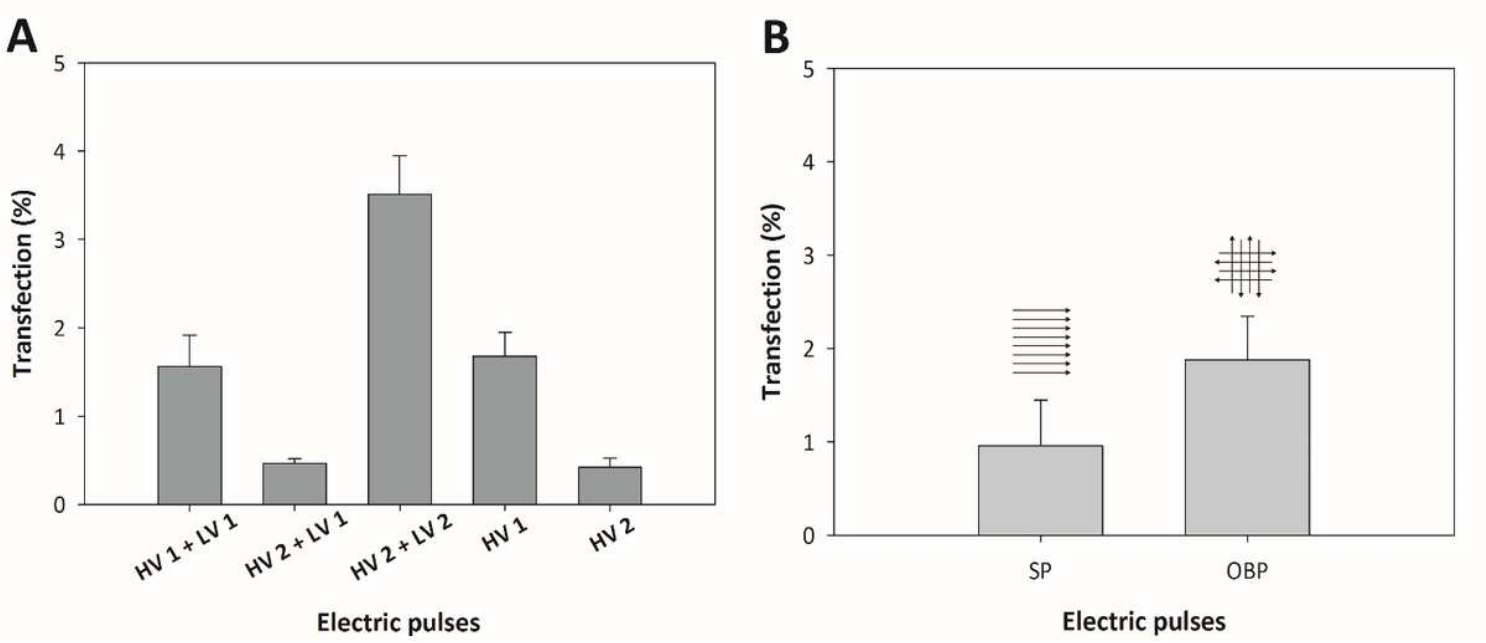

Figure 3

Effect of different pulsing protocols on gene electrotransfer efficiency in a 3D collagen model with embedded CHO cells, pDNA was administrated on top of the gel. (A) Different combinations of high-voltage (HV) and low-voltage (LV) pulses were applied. The number and duration of pulses, field strength and repetition frequency were as follows: HV 1 (5 x 1 ms; 0.8 kV/cm; 1 Hz), HV 2 (8 x 200 µs; 0.8 kV/cm; 1 Hz), LV 1 (1 x 100 ms; 75 V/cm) and LV 2 (1 x 100 ms; 150 V/cm). The time lag between HV and LV pulse

was always 20 ms. (B) 8 x 1 ms pulses (repetition frequency 1 Hz) and E = 0.8 kV/cm with single polarity (SP) or orthogonal both polarities (OBP) were applied; Cells were embedded in collagen gel (3D model). The percentage of transfected cells is plotted as a function of different electric pulses used. pDNA concentration in electroporation media was 90 μg/ml. Results are presented as a mean and vertical bars represent standard deviation.

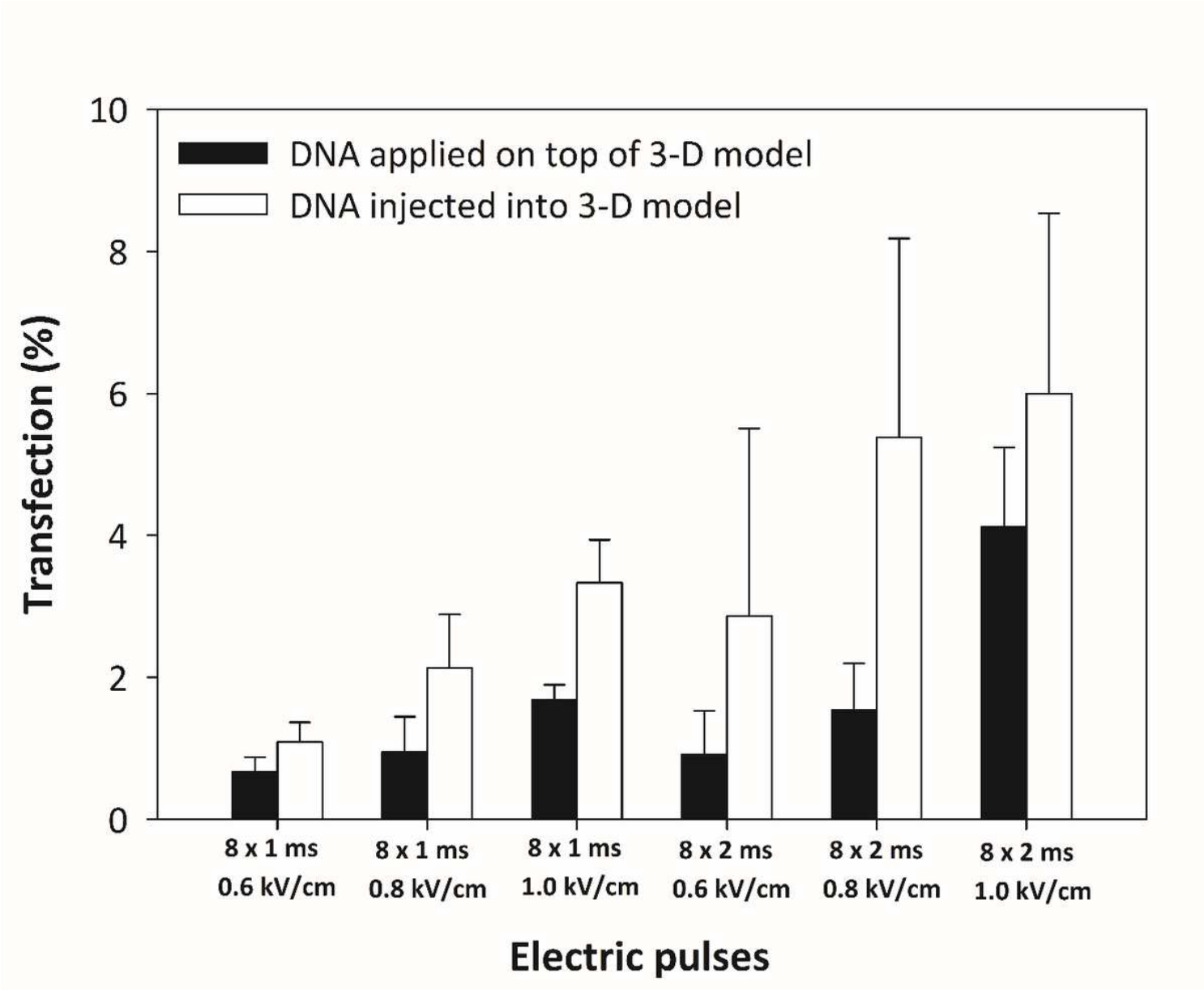

Figure 4

Effect of different pulse durations on gene electrotransfer efficiency of cells embedded in collagen gel two modes of DNA administration: (□) pDNA was administered on top of 3D model and (■) pDNA was injected into 3D model. The percentage of transfected cells is plotted for different electric pulses: 8 × 1 ms and 8 × 2 ms pulses of different electric field strength E (kV/cm) were used. Cells were embedded in collagen gel (3D model). In all experiments same amount of pDNA was administered (18.2 μg). Results are presented as a mean and vertical bars represent standard deviation.

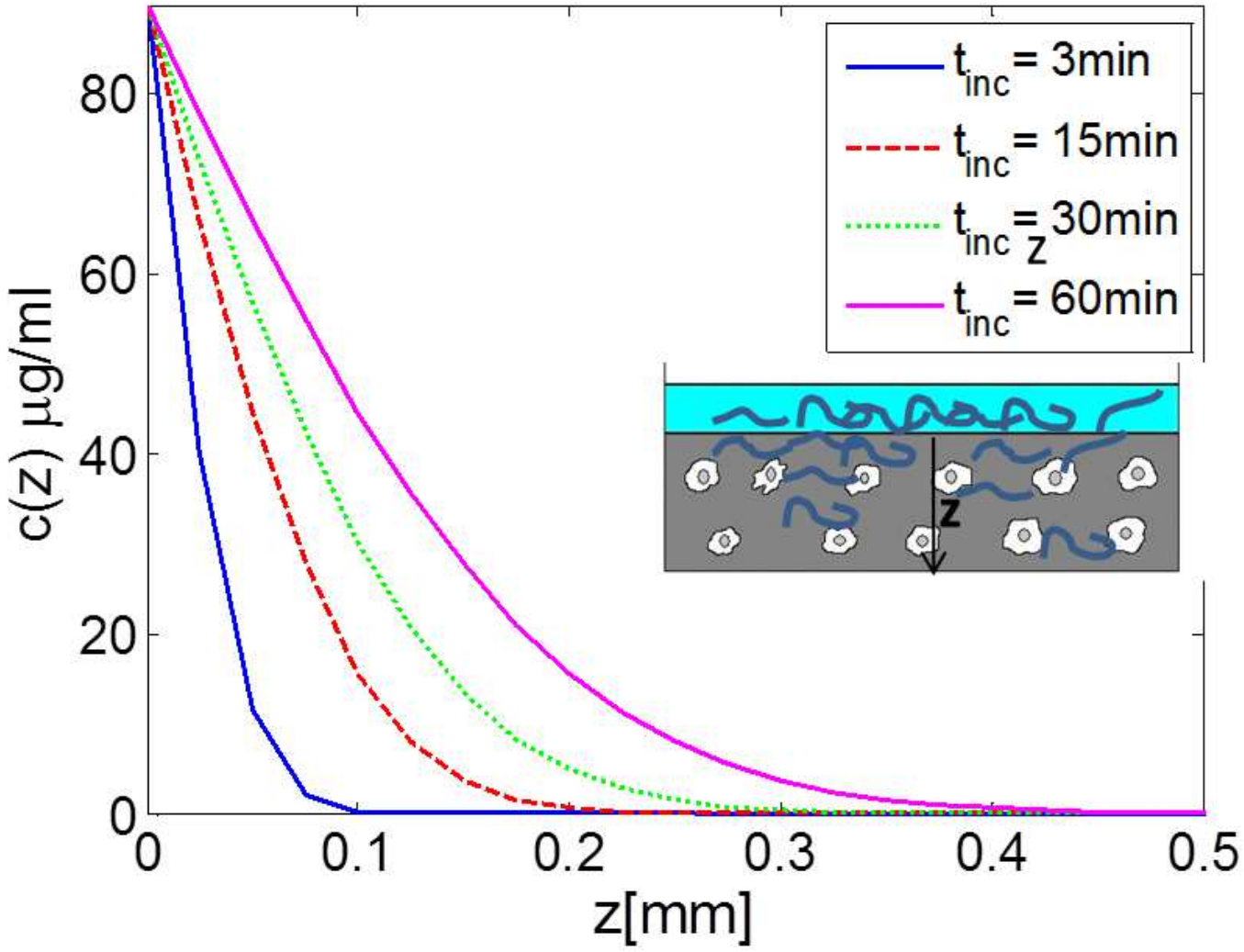

Figure 5

Diffusion of pDNA into 3D collagen gel, where pDNA was applied on the top of the gel. The distribution profile c (z, tinc) is calculated from Eq. 5, where z is the distance from the top of 3D gel and tinc is the time of pDNA application (incubation) on the top of the gel, the diffusion constant is: D = 3 × 10-8/cm2.

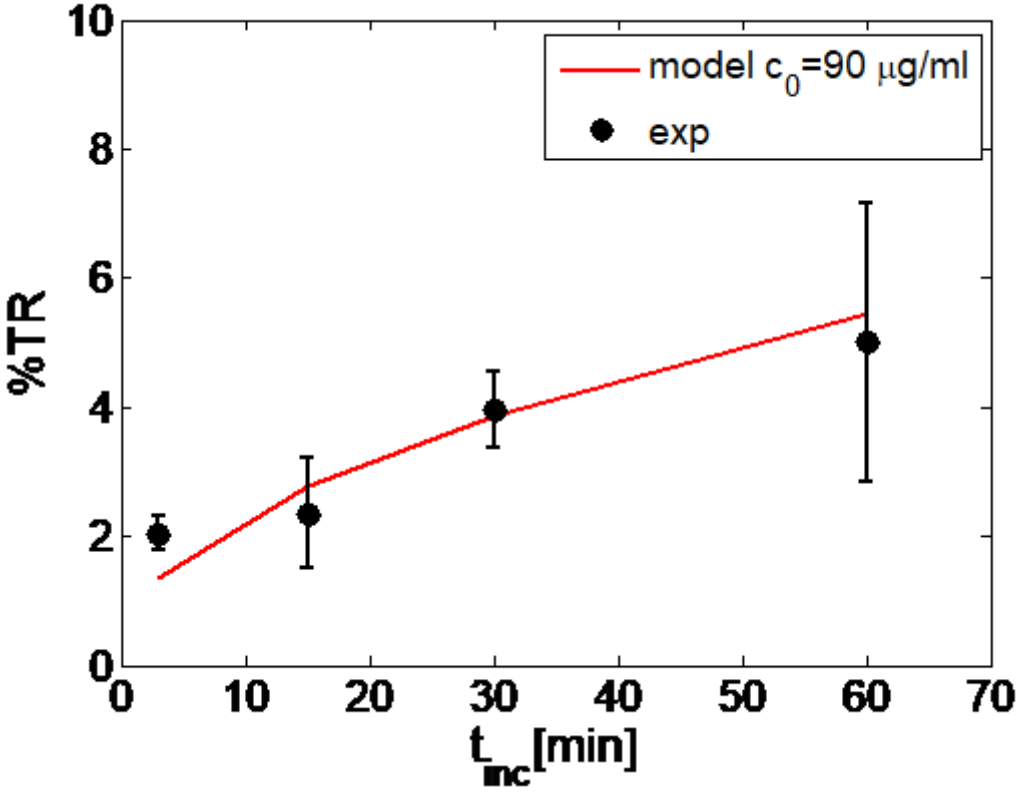

Figure 6

Comparison of theoretically calculated % electrotransfection (%TR) based on the diffusion model (see Eqs. 5-7) and experimental values for different times tinc and for initial pDNA concentration on the top of the gel c0= 90μg/ml, D = 3 × 10-8/cm2 for 8 × 5 ms electric pulses, E = 0.8 kV/cm. Results are presented as a mean and vertical bars represent standard deviation.

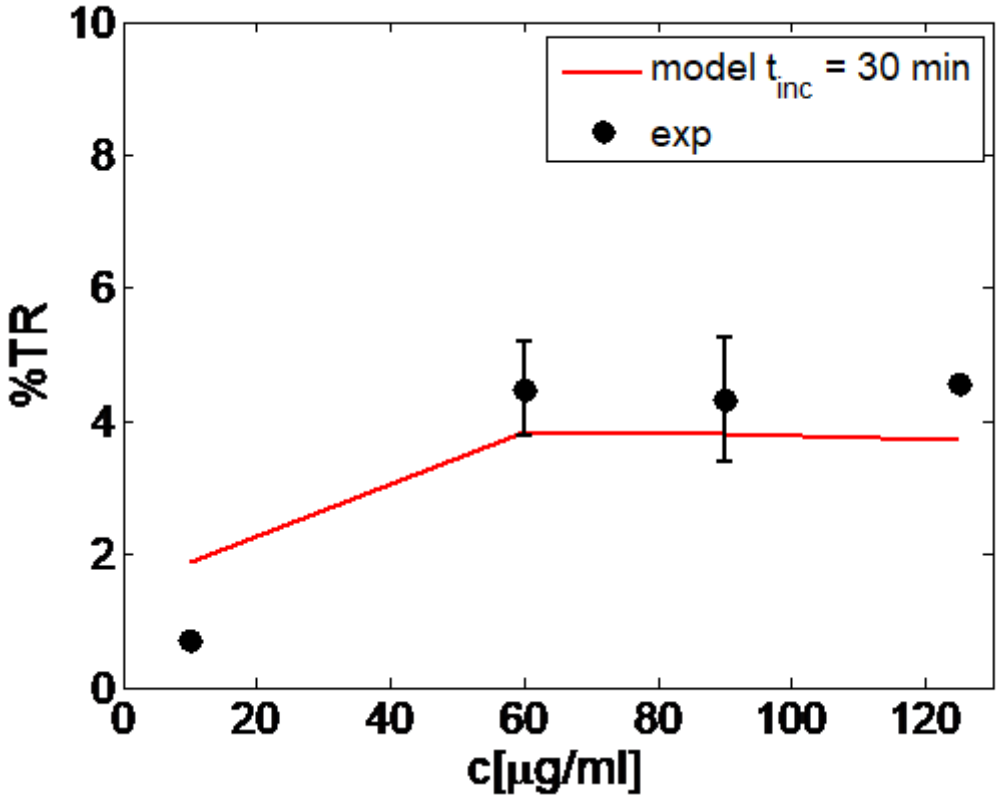

Figure 7

Comparison of theoretically calculated % electrotransfection (%TR) based on the diffusion model (Eqs. 5-7) and experimental values for initial pDNA concentrations c0 and for following parameters: tinc = 30 min, D = 3 × 10-8/cm2 and 8 × 5 ms electric pulses, E = 0.8 kV/cm. Results are presented as a mean and vertical bars represent standard deviation.

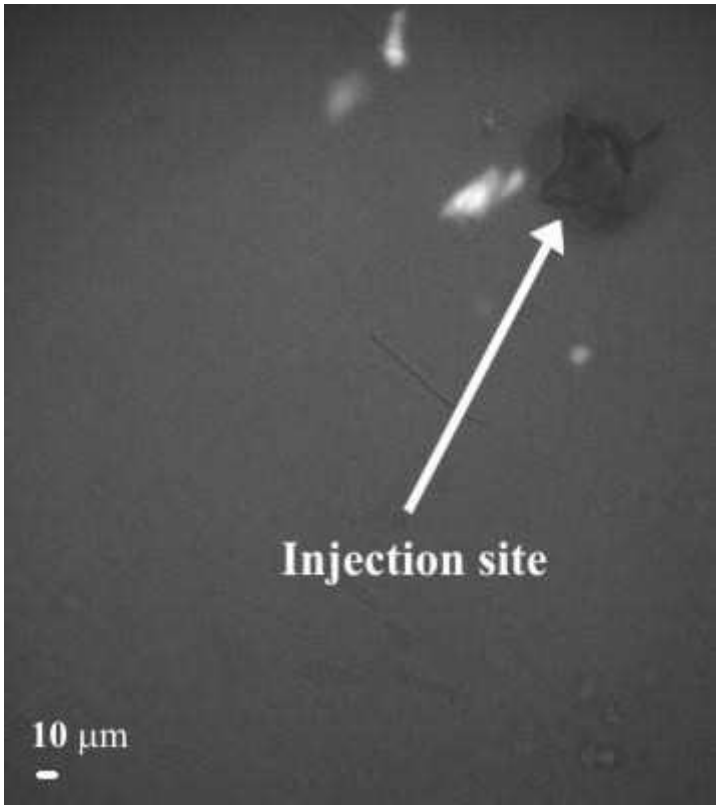

Figure 8

Representative picture, which shows that especially cells in the 3D model, which were near the injection site were successfully transfected. Eight pulses of 2 ms long, pulse repetition frequency of 1 Hz and E = 0.8 kV/cm were applied. pDNA concentration in electroporation media was 90 μg/ml.

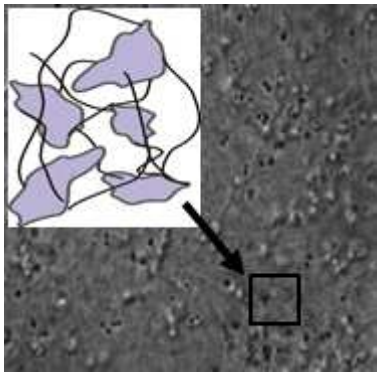

Figure 9

Cells embedded in collagen gel.

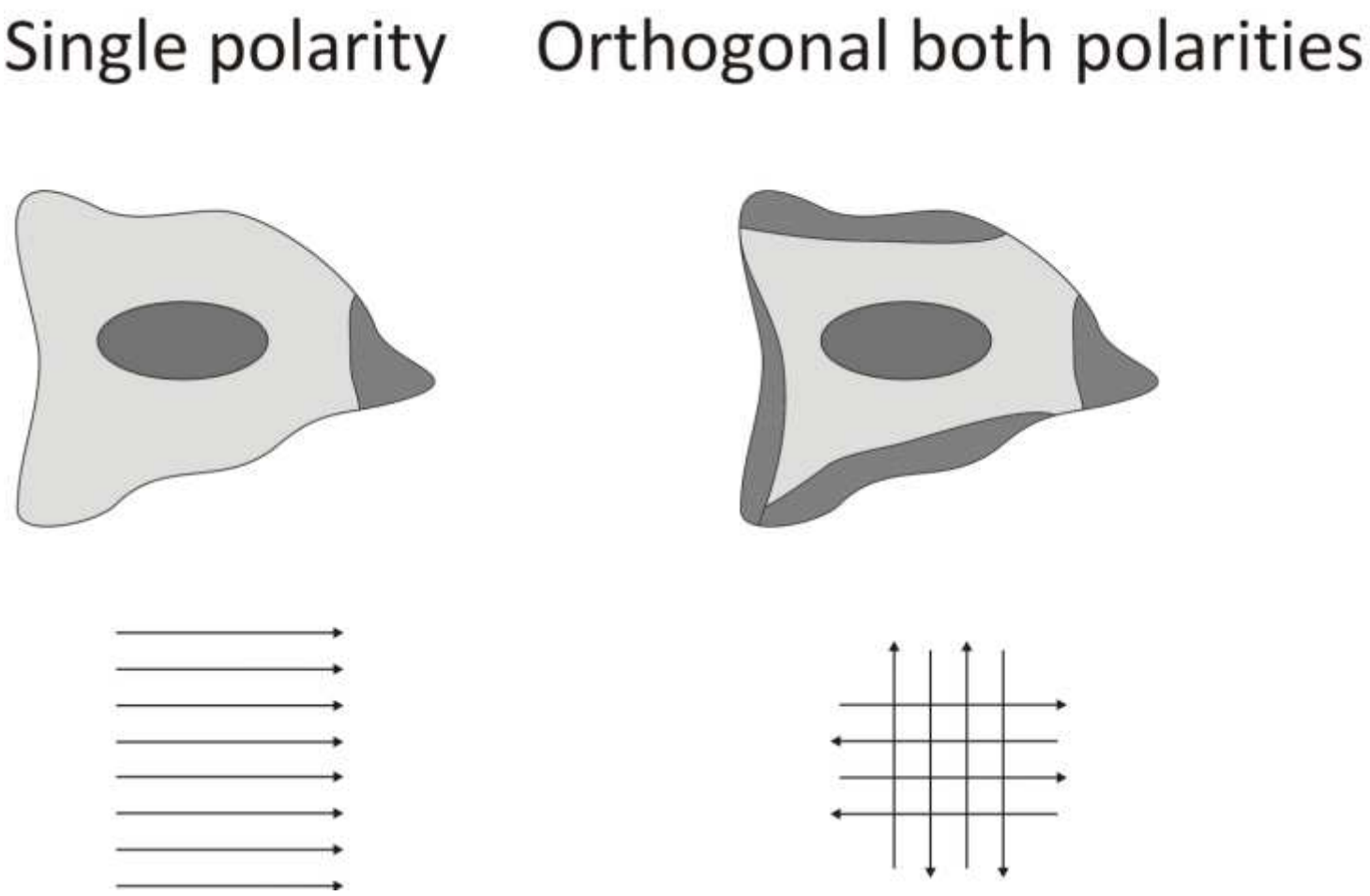

Figure 10

In single polarity (SP) electric pulses are applied between two opposite electrodes. While in orthogonal both polarities (OBP) electric pulses are applied between both opposite pairs of electrodes.