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Transport, resealing, and re-poration dynamics of two-pulse electroporation-mediated molecular delivery

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ABSTRACT

Electroporation is of interest for many drug-delivery and gene-therapy applications. Prior studies have shown that a two-pulse-electroporation protocol consisting of a short-duration, high-voltage first pulse followed by a longer, low-voltage second pulse can increase delivery efficiency and preserve viability. In this work the effects of the field strength of the first and second pulses and the inter-pulse delay time on the delivery of two different-sized Fluorescein–Dextran (FD) conjugates are investigated. A series of two-pulse-electroporation experiments were performed on 3T3-mouse fibroblast cells, with an alternating-current first pulse to permeabilize the cell, followed by a direct-current second pulse. The protocols were rationally designed to best separate the mechanisms of permeabilization and electrophoretic transport. The results showed that the delivery of FD varied strongly with the strength of the first pulse and the size of the target molecule. The delivered FD concentration also decreased linearly with the logarithm of the inter-pulse delay. The data indicate that membrane resealing after electropermeabilization occurs rapidly, but that a non-negligible fraction of the pores can be reopened by the second pulse for delay times on the order of hundreds of seconds. The role of the second pulse is hypothesized to be more than just electrophoresis, with a minimum threshold field strength required to reopen nano-sized pores or defects remaining from the first pulse. These results suggest that membrane electroporation, sealing, and re-poration is a complex process that has both short-term and long-term components, which may in part explain the wide variation in membrane-resealing times reported in the literature.

1. Introduction

In the presence of suitably chosen external electric fields, the cell membrane increases its permeability to foreign molecules in the extra-cellular medium [1–5]. This phenomenon is termed electroporation, and has been used in various research and clinical applications for gene transport and protein or drug delivery [6–14]. Besides physiological [15–21] and cellular [22–25] variables, the electrical parameters clearly play an important role in the complex electroporation process [26,27]. Transient electropermeabilization of the membrane begins when the applied external field exceeds the critical transmembrane potential [28–32]. Maintaining the field strength over a critical threshold value expands the permeabilization of the membrane. Although other mechanisms such as endocytosis, diffusion (particularly for small molecules, *e.g.*, MW < 4 kDa), and membrane-DNA interactions may be involved in the delivery process, it has been recognized that the main molecular uptake of charged moderate and large molecules occurs through electrophoresis [18,19,33–41]. The direct current (DC) component of the applied field provides an electrophoretic force for delivering molecules into the cytoplasm [42]. Thus, in traditional electroporation, both permeabilization and delivery are controlled in large part by the parameters of the applied field, even though different physical mechanisms are involved for both. These electrical parameters, including field strength [37,43–49], pulse duration [35,44,47,50], number of pulses [34,51], and pulse shape [52–56], have been studied extensively over the past three decades.

Prior studies have shown that the combination of a short-duration, high-field-strength first pulse, together with a longer-duration, lower-field-strength second pulse (denoted by 'first pulse' and 'second pulse', respectively) can increase electroporation efficiency and preserve cell viability, especially when delivering larger molecules (MW > 4 kDa [47]) or DNA [33,34,37,39,42,57–66]. Pulsing parameters have been well studied, including the strength of the first pulse [33,37,58], the strength and duration of the second pulse [34,39,57–59], and the delay between pulses [34,42,57,58,66,67]. The two-pulse protocol has been reported to significantly increase delivery efficiency both *in vitro* in cell suspensions as well as *in vivo* in a variety of tissues [33,57] as compared to a single electroporation pulse.

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Ambiguities and inconsistencies, however, remain in the literature regarding three aspects of two-pulse electroporation: First, the effect of the delay time between the first and second pulses has been alternately reported to be a hindrance or advantageous to delivery efficiency. Sukharev et al. introduced the separation of the first pulse and second pulse with a well-defined time delay, and found that the *in vitro* cell transfection efficiency decreased monotonically with delay between the pulses [34]. Similar results were found by Wolf et al. [67]. However, *in vivo* studies of gene electrotransfer into tissues have shown no difference in transfection efficiency for delays between 20 ms and 5 s [42], or even *increased* transfection for 0.3 to 100 s delays as compared to the no-delay case [57]. Secondly, there is a wide variation in the membrane-resealing times reported in the literature between those estimated from two-pulse electroporation and other methods. Resealing times based on two-pulse experiments (inferred from the decline of molecular delivery with increasing delay times between pulses) tend to show that complete inhibition of delivery by a weaker second pulse takes minutes [34] to tens of minutes [67], while electrical measurements show that the majority of membrane resealing after electroporation occurs very rapidly, on the order of tens of milliseconds [50,68]. Finally, although the classical view is that two-pulse electroporation separates permeabilization and delivery, it has been recognized in many studies [33,34,39,42,60,65], that the DC, high-voltage first pulse alone can transport a considerable amount of the target molecule, thus co-mingling permeabilization and delivery. This confounds efforts to independently study cell permeabilization and molecular delivery/gene transfection based upon two-pulse electroporation.

The current work seeks to address these inconsistencies *via* a systematic study of two-pulse electroporation-mediated molecular delivery into 3T3 mouse fibroblasts. Distinct from prior studies, we use variable strengths of an alternating current (AC) electric field for the first pulse, and we use two target Fluorescein–Dextran (FD) conjugates of different sizes, 10 kDa and 70 kDa. The delay time between the pulses are also varied from milliseconds to minutes, spanning five orders of magnitude. Our experimental design flows from the following rationale: First, the pulse design is intended to best separate the effects of permeabilization and delivery. Most prior studies on multi-pulse electroporation have focused on DC fields, which combine permeabilization and delivery due to electrophoretic transport induced by the DC pulses. To overcome this limitation, we hypothesize and demonstrate that the high-frequency first pulse, due to changing polarity of the AC field, permeabilizes the membrane with minimal net delivery. Furthermore, we choose a DC second-pulse of lower strength and longer duration for delivery, so that by itself no significant permeabilization is achieved. Second, the large span of delay time between the pulses allows us to examine membrane-resealing dynamics in both the short (ms) and longer (minutes) ranges. Third, we use a non-binding assay (FD conjugates) to focus on delivery while avoiding complexities such as gene–membrane complex formation [62,66,69,70] in DNA delivery. The sizes of the FD conjugates are similar to those of small RNAs, peptides and drug molecules, and hence they serve as good model molecules. Previous studies [39] have shown that the delivery of these mid-sized molecules is primarily *via* electrophoresis. By design, therefore, we focus on studying permeabilization and transport, by temporarily decoupling from other confounding mechanisms. The two different molecular sizes employed also help shed light on the pore-size evolution. Overall, we seek to use these systematic measurements to make inferences regarding the dynamics of membrane electroporation, sealing, and re-poration *in vitro* two-pulse electroporation.

2. Materials and methods

In the current work, many experimental procedures are kept consistent with a recent previous study [39] to enable direct comparisons. The same cell line and membrane-impermeable fluorescent dye, 7-Aminoactinomycin D (7-AAD), for determining viability were used. The same delivery marker, 10 kDa Fluorescein–Dextran (FD) conjugates was used, as well as a new, larger 70 kDa FD. The data were collected with epifluorescence imaging, and by calibrating the observed fluorescence to known concentrations of the fluorophores (see Supplementary Material), fluorescence intensity was converted to intracellular concentration.

2.1. Cell culture

NIH 3T3 mouse fibroblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 1% L-glutamine (Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C and 5% CO_2. Approximately 3×10^6 cells were trypsinized at 70–80% confluency with 0.5% trypsin/EDTA (Sigma-Aldrich) followed by centrifugation for 2 min at 2000 rpm (Allegra X-21, Beckman Coulter, Brea, CA) in culture media, and then twice in electroporation buffer containing 0.4 mM MgCl2, 250 mM sucrose, and 10 mM HEPES (Sigma-Aldrich) (pH 7.4). The osmolarity was measured with an osmometer (3D3 Osmometer, Advanced Instruments, Norwood, MA) and adjusted to 310 mOsm/kg by adding either more sucrose solution or deionized water. The electrical conductivity was measured with conductivity meter (CON 6, Oakton Instruments, Vernon Hills, IL), and was adjusted to 100 μS/cm by adding either $MgCl_2$ solution or deionized water.

2.2. Electroporation protocol

The cells were suspended in the electroporation buffer including 100 μM of FD (Life Technologies, Grand Island, NY) and kept on ice for up to 15 min before applying the electric pulses. Two different FD conjugates (10 kDa and 70 kDa) were utilized to measure delivery. For each case, a 30 μL cell suspension was placed into an electroporation cuvette with a 1-mm electrode gap (VWR, Radnor, PA) and subjected to electric pulses under a sterile hood. The temperature of the pulsing buffer was consistently measured to be within 2° of 4 °C. Electric pulses were generated by a function generator (Tektronix AFG3022C, Melrose, MA) and amplified by a high-frequency, high-voltage amplifier (Trek Model PZD 350, Lockport, New York, NY). Applied pulses were measured with an oscilloscope (PicoScope 5203, Cambridgeshire, UK). As shown in Fig. 1, in most of the experiments, two consecutive different pulses were applied to the cells; an AC first pulse for cell permeabilization and a DC second pulse with some delay for FD delivery. The pulse parameters (shape, strength and duration) are given in Table 1. For each experiment, control cases were also studied in which cells were treated to every step of the experiment, but without exposure to the electroporation pulses. The delay times (Δt_{delay}) between two pulses were arranged by the function generator's Trigger Delay option between two channels, and precise delays ranging from no delay to milliseconds to seconds were obtained. Delay times longer than 80 s were timed with a stopwatch and applied manually. Immediately after pulsation, culture media was added to the cell suspension and incubated for 30 min on ice to allow for resealing. Then, the cell suspension was transferred to phosphate buffer saline (PBS) without Mg^{2+} and Ca^{2+} (Sigma-Aldrich) containing 2 μM of 7-AAD (MW = 1270.43 Da) (Life Technologies) solution and further incubated for 15–20 min on ice [39]. Under normal conditions, 7-AAD, which is used for fluorescently labeling nucleic acids, is a cell-membrane-impermeant dye. However, 7-AAD is able to enter the cells that were not able to recover and reseal after pulsation. Thus, cells which allowed 7-AAD entry after 30 min were considered to be dead cells. Similar to propidium iodide, 7-AAD serves as a reasonable assessment for membrane integrity and short-term cell viability [39,71–74]. The cell suspension was washed twice with PBS to remove free FD and unbound 7-AAD before imaging. All experiments were repeated at least 3 times, and for each experimental condition at least 100 cells were analyzed.

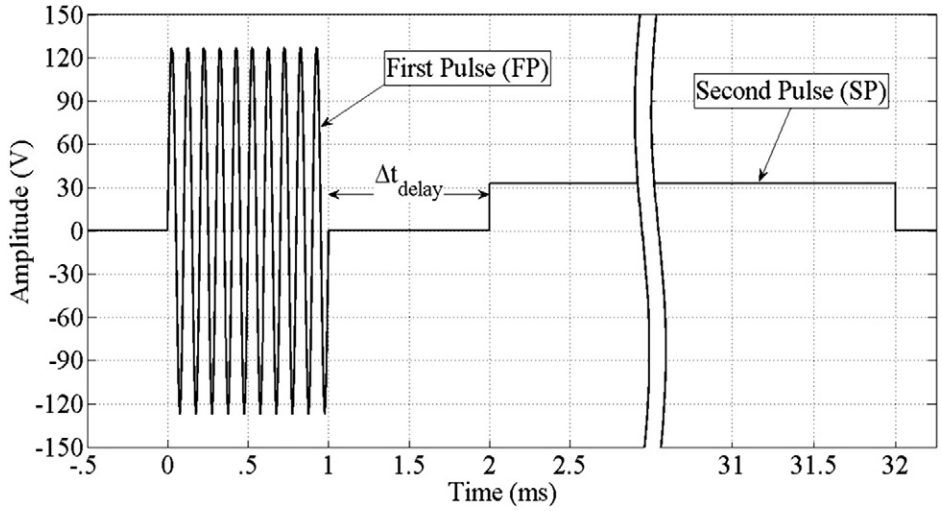

Fig. 1. Schematic of the AC first and DC second pulses applied to the cells with a specified delay.

2.3. Fluorescence imaging

Both FD and 7-AAD fluorescence intensity were acquired using fluorescence-imaging microscopy. Images were captured with a high-sensitivity camera (pco.edge sCMOS, PCO AG, Kelheim, Germany) attached to an inverted microscope (Olympus IX71, Center Valley, PA) with a 20× objective (Fig. 2A). Approximately 20 μL of cell solution was dropped on a glass slide, and a cover slip was placed on top of solution to obtain a standard thickness (approximate cell diameter: 15 μm) and avoid cell motion due to flow. Images of the cells were taken in two fluorescent channels (for FD ex: 470 nm, em: 525 nm, for 7-AAD ex: 545 nm, em: 605 nm). Additionally, one bright-field image was taken to measure the cell diameter and center coordinates for image processing. Fig. 2A shows an exemplary set of images which were taken in the bright-field, FD-fluorescence, and 7-AAD-fluorescence channels, from the top to bottom rows, respectively.

2.4. Image processing and data analysis

All images and data were analyzed with code written in MATLAB (The MathWorks, Natick, MA). The bright-field images (Fig. 2A) were used to compute a disk-shaped profile for each cell and record the centroid and diameter. Background noise was calculated from a control (no-pulse) case and subtracted for both the FD and 7-AAD channels to determine the two different fluorescence intensities for each individual cell. Fluorescence intensity per volume for each cell was calculated by integrating the signal within the disk shape area and normalizing by the cell volume, assuming spherical cell shape.

The scatter plot shown in Fig. 2B has FD intensity per volume (corresponding to the degree of delivery) as its abscissa, and 7-AAD intensity per volume (related to the viability of the cell) as its ordinate. A threshold intensity for viability was estimated as two standard deviations above the mean 7-AAD fluorescence intensity. For analysis of the delivery of FD, only alive cells were considered. Throughout the experiments, viability exceeded 60% and was above 80% in the majority of the cases.

Table 1
Applied pulse parameters for the first and second pulses.

	First pulse	Second pulse
Shape	AC sinusoidal (f = 1–500 kHz)	DC
E. field strength	E_1 = 0.60–0.90 kV/cm	E_2 = 0.33 kV/cm
Duration	t_1 = 1 ms	t_2 = 30 ms
Delay between first pulse and second pulse		1 ms–300 s

2.5. Calibration and confirmation of the fluorescence-imaging method

To convert the measured intensity to a concentration, serial dilutions of FD ranging between 0.312 and 50.0 μM in electroporation buffer were prepared. These samples were imaged at room temperature to generate calibration curves of FD intensity *versus* concentration for both the 10 kDa and 70 kDa FD conjugates (see Fig. S1 in the Supplementary Materials). The results derived from the fluorescence-imaging method described above were compared to data acquired *via* flow cytometry from our previous work on two-pulse DC electroporation [39]. For the same experimental conditions (E_1 = 1 kV/cm, t_1 = 1 ms and E_2 = 0.1–0.8 kV/cm, t_2 = 30 ms), the two approaches were consistent.

3. Results

In this section, we first describe how the pulse parameters were selected to (1) permeabilize the membrane with the first pulse with minimal delivery, and (2) deliver molecules with a second pulse that not sufficient to porate the cells by itself, in the absence of a porating first pulse. We then present our experimental results for (I) no delay between the pulses to evaluate permeability, and (II) various delay times ranging from 1 ms to 300 s to evaluate resealing dynamics.

3.1. Determining the pulse parameters

In the two-pulse-electroporation procedure, although the first pulse is considered in many studies to only permeabilize the cell membrane, a considerable amount of molecular delivery can occur, especially if a DC pulse is used. As seen in Fig. 3A, after a single DC pulse (E_1 = 0.90 kV/cm, t_1 = 1 ms), a significant amount of 10 kDa FD is delivered into the cell. On the other hand, an AC first pulse greatly reduces 10 kDa FD delivery due to the alternating polarity of the field. Increasing the frequency of the AC field further reduces electrophoretic delivery. A 10 kHz AC first pulse was judged to have negligible (<10%) delivery compared to that of the two-pulse case with no delay. The frequency of the first pulse (10 kHz) was also well below the ~100 kHz frequency at which the cell membrane would no longer have adequate time to charge and porate [75]. Therefore, to efficiently permeabilize with negligible delivery, we chose to employ a 10 kHz AC first pulse with E_1 = 0.90 kV/cm and t_1 = 1 ms.

To determine the appropriate field strength for the second pulse, we first studied single, 30 ms DC pulses with different field strengths. As seen in Fig. 3B, there is slight uptake of 10 kDa FD (around 0.25 μM) beginning at 0.40 kV/cm, which indicates the threshold DC field strength

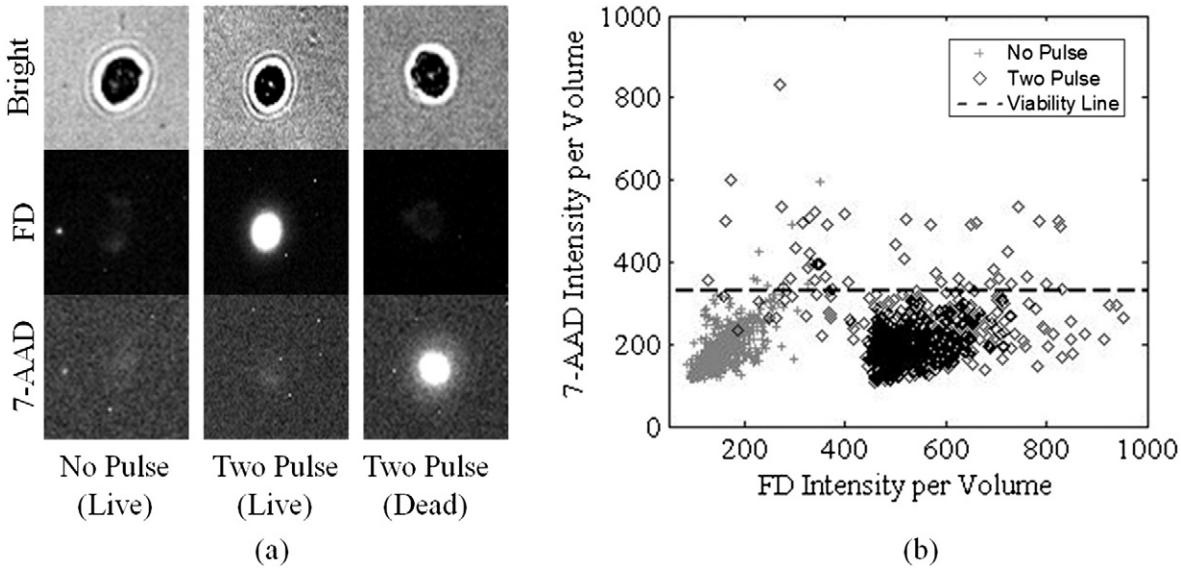

Fig. 2. Examples of (A) cell images taken for image processing (the contrast of these images has been adjusted for display purposes only), and (B) scatter plot showing FD and 7-AAD intensity per volume in a representative set of analyzed images.

that begins to permeabilize the 3T3 cells for 10 kDa FD. One of the objectives of this study was to study the effect of the delay time between pulses on membrane resealing and molecular delivery. As such, no new pores should be generated by the second pulse. Therefore, the second pulse's field strength was chosen to be 0.33 kV/cm, lower than threshold required for significant electroporation and delivery by a single DC pulse, but large enough to drive electrophoretic transport and delivery.

3.2. No delay between the first pulse and second pulse

We first consider a baseline two-pulse protocol consisting of an AC first pulse (10 kHz, $E_1 = 0.9$–0.6 kV/cm, $t_1 = 1$ ms) immediately followed by a DC second pulse ($E_2 = 0.33$ kV/cm, $t_2 = 30$ ms) that is not of sufficient strength to cause poration and delivery of 10 kDa FD by itself. Fig. 4A shows the delivered intra-cellular 10 kDa FD concentration for the first pulse-only and two-pulse cases. It can be seen that applying the first pulse only does not cause any significant FD delivery. The lack of delivery at the end of the first pulse is due to non-binding nature of FD molecules, since electrophoretically-driven FD molecules may enter into the cells during the pulsation, but also leave the cell due to alternating polarity of AC field. Therefore for first-pulse-only cases, the intra-cellular concentration remains almost the same in spite of increasing first pulse field strength, as seen in Fig. 4A. However, applying the DC second pulse with no delay achieves a relatively high molecular delivery, ranging from 1 μM to 4 μM for the 10 kDa FD depending on the strength of the first pulse (Fig. 4A). We note that this DC second pulse by itself would yield negligible delivery, as seen in Fig. 3B. Hence a clear separation of permeabilization and delivery is obtained by using an AC first pulse followed by a DC second pulse.

We next examined the degree of permeabilization by applying first pulses of different field strengths ($E_1 = 0.6$–0.9 kV/cm, $t_1 = 1$ ms). The field strength and duration of the second pulse are kept fixed ($E_2 = 0.33$ kV/cm, $t_2 = 30$ ms) for all cases. In this way, we correlated the amount of delivery obtained after the second pulse with the permeabilization level of the membrane achieved by the first pulse. As shown in Fig. 4, different first-pulse strengths between 0.6 and 0.9 kV/cm result in very different FD-delivery levels when followed by the same second

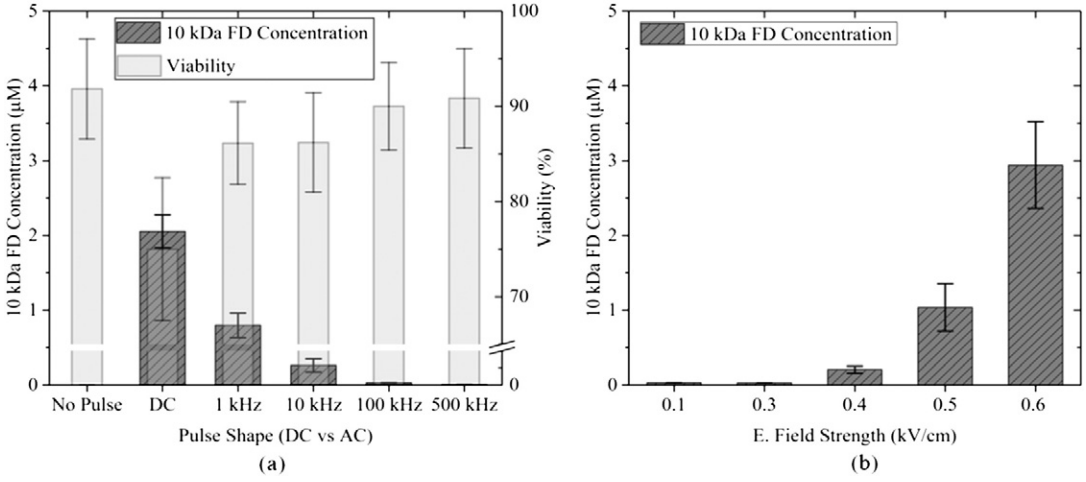

Fig. 3. (A) Intracellular 10 kDa FD concentrations and viability ratios for cells exposed to a single, 1 ms-long, 0.9 kV/cm-field-strength pulse of different shapes/frequencies. For the DC case, the pulse shape was rectangular with the given duration and strength. For AC pulses, frequencies varied from 1 kHz to 500 kHz with the same duration and strength. "No pulse" refers to a control experiment in which every step of the protocol was followed, except the exposure to the electroporation pulse. (B) Intracellular 10 kDa FD concentrations are shown for single 30-ms-long DC pulses of different field strengths.

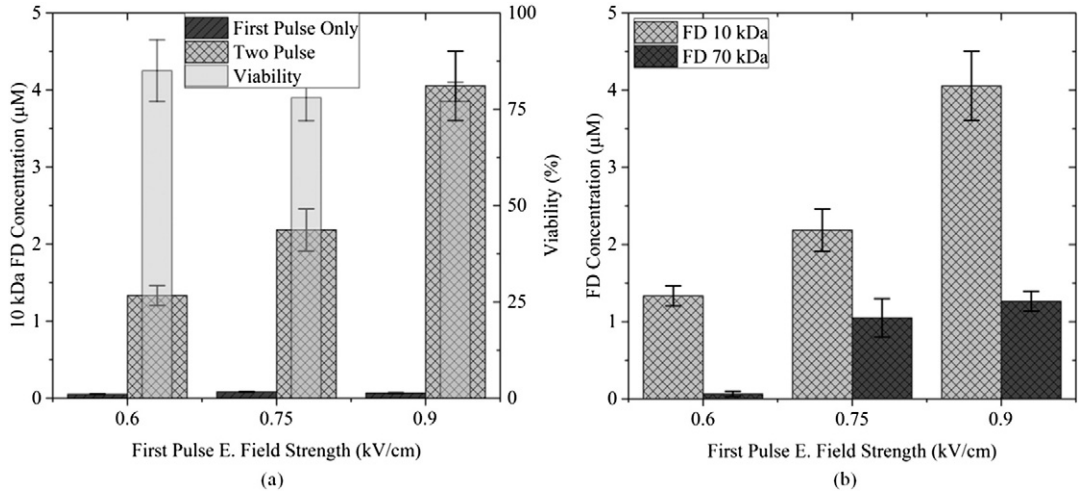

Fig. 4. (A) Intracellular 10 kDa FD concentrations for different AC first-pulse strengths. "First pulse only" shows the delivered concentration after cells were exposed to a single 1 ms-long AC pulse at the indicated field strengths. "Two pulse" represents the delivered concentration after cells were exposed to a 1 ms-long AC pulse at the indicated field strength, followed by a standard DC second pulse which was 30 ms-long and 0.33 kV/cm in field strength. Also shown are the viability ratios for the two-pulse cases. (B) Comparison of delivered concentrations for FD of two molecular weights (10 kDa and 70 kDa) for cells that were exposed to a 1 ms-long AC pulse at the indicated field strength, followed by a standard DC second pulse (30 ms duration and 0.33 kV/cm).

pulse. Increasing the field strength of the first pulse increases the delivered intracellular FD concentration, presumably due to increased permeabilization of the cell membrane. This result shows the importance of the first pulse and associated degree of permeabilization in the two-pulse electroporation protocol, since the final delivery amounts depend strongly on the first-pulse field strength, even though the first pulse itself achieves negligible delivery.

We next investigated the effect of molecular size on the delivery achieved by two-pulse electroporation. In Fig. 4B, we see that the intra-cellular concentrations of two different-sized FD molecules (10 kDa and 70 kDa) are very different under the same conditions. For example, with a 0.6 kV/cm first pulse immediately followed by our standard second pulse, there is considerable delivery of 10 kDa FD (around 1.25 μM). However, with the same pulsing parameters, there is essentially no detectable intra-cellular delivery of 70 kDa FD. This suggests that pores formed by the 0.6 kV/cm first pulse have a pore-size distribution that allows 10 kDa FD molecules to pass through, but very few 70 kDa FD molecules. We interpret this to mean that the distribution of electropores shifts to larger sizes with higher first-pulse field strength. Finally, with a 0.9 kV/cm-field-strength first pulse, the delivery of 10 kDa FD is almost doubled over that of the 0.75 kV/cm case, while the 70 kDa delivery only increases slightly. The data suggest that pulsing parameters (in particular the first-pulse strength) need to be adjusted for target-molecule size in two ways: First, the threshold first-pulse field strength required to porate and effectively deliver clearly depends on the size of the target molecules. However, for larger-sized molecules, increasing the field strength significantly beyond that threshold may not improve delivery, likely because of increased cell death.

3.3. Variable delay between the first pulse and second pulse

In reversible electroporation, pores eventually disappear and the cell membrane reseals. This resealing stage of reversible electroporation typically takes longer than permeabilization, *e.g.* electropermeabilization can occur in micro or nano-seconds with suitable pulsation, while the complete disappearance of the electropores has been reported to take seconds or minutes [76,77]. If long resealing times hold, post-pulsation diffusive delivery can significantly increase transfection efficiency, particularly for small molecules of high diffusivity. In the experiments described in this section, we took advantage of the separation between permeabilization and delivery offered by our AC + DC two-pulse design to study membrane sealing and re-poration dynamics. In particular, we delayed the second pulse between 1 ms and 300 s, and measured the total delivery to infer the permeability of the membrane to the target molecules. In this manner, we were able to probe the effect of delay time solely, without confounding permeabilization and delivery, and relate the delivery decrease with resealing and re-poration dynamics.

Fig. 5 shows the measured intra-cellular FD concentrations *versus* delay time for 10 kDa and 70 kDa FD. Consistent with Sukharev et al. [34], but different from *in vivo* work by others [57,58], the delivery of target molecules decreased monotonically with increasing delay time between pulses. Interestingly, an inverse linear correlation between the amount of delivery and the logarithm of delay time was observed. There is a nearly 50% drop from the no-delay case in delivered FD concentration with a 100 ms delay between first and second pulses. However, although it is reduced, delivery persists even when the second pulse is delayed by up to hundreds of seconds. Fig. 5A also indicates that the decrease in delivery with delay time is dependent on the field strength of the first pulse. For the 0.6 kV/cm first pulse, delivery of 10 kDa FD is observed until a delay of 100 s. However, for the 0.75 and 0.9 kV/cm first pulses, complete cessation of delivery by the second pulse occurs only after delays of thousands of seconds. Additionally, the slopes of fitted lines are greater when the intensity of the first pulse, and thus the degree of permeabilization, is larger. These results suggest that the efficiency of delivery in two-pulse electroporation is directly related to intensity of the permeabilizing first pulse. Furthermore, although complete elimination of delivery requires delay times of tens of minutes, a significant portion of pores reseal (and are not re-porated by the second pulse) within a very short time (~100 ms) after the porating first pulse.

In Fig. 5, it can be clearly seen that the delivery of both 10 kDa and 70 kDa FD decreases linearly with the logarithm of the delay time regardless of the molecule size. The effects of varying permeability *via* the strength of the first pulse can also be seen by comparing the different-sized FD. For instance, at a first pulse intensity of 0.75 kV/cm, very little 70 kDa FD is delivered following delays of 100 s or 300 s. However, the smaller 10 kDa molecule continues to be delivered after similar delay times. This suggests that, even though the initial permeabilization is the same, electropores shrink to different degrees during the delay between pulses. In addition, the rate of decrease of intra-cellular concentration varies more strongly with first-pulse strength for 10 kDa FD than for 70 kDa FD.

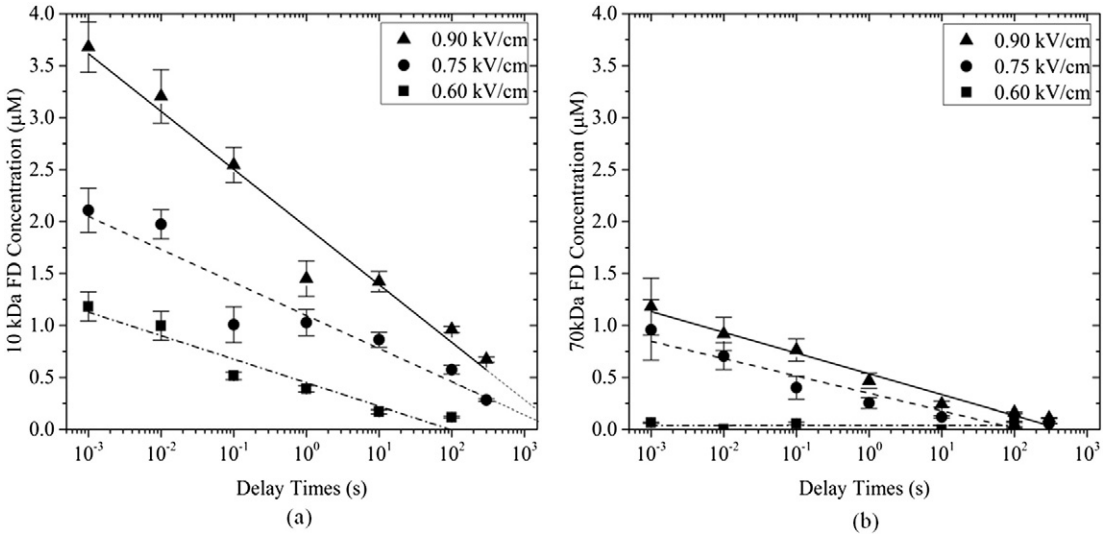

Fig. 5. Intracellular FD concentrations with different delay times (Δt_{delay}) ranging from 1 ms to 300 s. The cells were exposed to a 1 ms-long AC pulse at the indicated field strength, followed by a standard DC second pulse which was 30 ms-long at a field strength of 0.33 kV/cm. Two different-sized molecules were used: (A) 10 kDa FD and (B) 70 kDa FD.

4. Discussion

Experimentally, two techniques have been commonly used to determine the degree of cell-membrane permeabilization by electric pulses: (I) measuring the conductance or current change across the cell [37,50,68,78–80], and (II) measuring the transport of labeled molecules. For quantifying the size of pores, the second method is more attractive because different-sized molecules can be used to observe pore-size distribution [35,48]. However, this method is somewhat restricted because a sufficient amount of target molecule must be delivered to acquire signal, *i.e.*, it is actually sensitive to delivery and not permeabilization [7,46,48,81]. Therefore, membrane permeabilization is traditionally considered in tandem with molecular delivery, although the two phenomena are distinct and involve different physical mechanisms. In this work, we have performed two-pulse electroporation using a 10 kHz AC-field first pulse, which was shown to permeabilize the membrane with minimal net electrophoretic delivery of FD. The porating first pulse was followed by a second pulse of fixed field strength and duration, which was specifically chosen to not be able to permeabilize the membrane by itself, but only to provide electrophoretic force for molecular delivery. In this manner, we have sought to probe the degree of permeabilization remaining after various delays from the first pulse by measuring the total delivered amount.

It is shown in the two-pulse electroporation protocol that the first pulse has a crucial influence on the delivery, particularly for larger molecules. Our results, which showed that increasing the AC first-pulse field strength increases the degree of permeabilization, are in agreement with several previous studies [33,37,58,61,64]. In those studies, electroporation efficiency increased with the first-pulse strength until a critical point where the efficiency started to decrease due to reduced viability. Because of this, and in order to maintain viability ratios above 60%, we limited first-pulse field strengths to 0.6–0.9 kV/cm. Distinct from prior work, we have used two different-sized, non-binding FD molecules (10 kDa and 70 kDa) to infer the distribution of electropore sizes, while avoiding the complexities of DNA-membrane interactions. Our results are consistent with a recent experimental study by Saulis and Saulė using small molecules (MW < 4 kDa) to investigate electropore size. In that work, it was also shown that the size of electropores was extended by increasing the field strength and the duration of the pulse [48]. We studied the size distribution of larger electropores by using 10 kDa and 70 kDa FD, corresponding to approximate physical diameters of 4.6 nm and 12 nm, respectively [45]. With a 0.6 kV/cm field-strength first pulse, the largest electropore diameter was inferred to be smaller than the 70 kDa FD, since significant delivery was not observed, while first-pulse field strengths of 0.75 kV/cm or greater did result in delivery of 70 kDa FD. This result showed that there is a threshold first-pulse field strength for electroporation delivery, and that the threshold varies with molecular size. Furthermore, when the first-pulse strength is increased to 0.9 kV/cm, the delivery of the smaller (10 kDa) molecule increased significantly, while that of the larger (70 kDa) molecule only increased slightly. These experimental observations are consistent with the model of Krassowska and Filev, which suggests that increasing the field strength extends the pore size and increases the number of pores [82]. Their model also predicts that the rate of formation of large pores is lower than that of small pores. Therefore, when comparing highly and moderately permeabilized cells, it is possible that the number of large pores would be similar, but the number of small pores could be much higher in the highly permeabilized membrane. In light of these findings, we can speculate that the delivery of large genes, drugs or proteins requires a first-pulse strength above some critical threshold; however, too strong of a pulse may not help because it can decrease viability without significantly increasing delivery. We note that the optimum first-pulse electric-field strength is likely to be cell-type dependent, as well as molecule dependent.

As previously noted, there is a disagreement in the literature about the effect of delay time between pulses on the delivery efficiency. *In vitro* studies by Sukharev et al. and Wolf et al. [34,67] showed that the delivery decreased with increasing delay time between first pulse and second pulse, while some other *in vivo* electrotransfection studies showed that a delay could increase the transfection efficiency [57,58]. Our data clearly show that for our cells and conditions, the total delivered intracellular concentration decreases monotonically with the logarithm of delay time between pulses. We attribute the difference between our results and those of some of the *in vivo* studies in part to the difference in delivery between non-binding FD and DNA; the delivery of the latter is a much more complicated process involving the formation of DNA-membrane complexes. Furthermore, as discussed in [57], the delay between pulses *in vivo* may facilitate the redistribution of the DNA solution within the tissue, which would presumably have no beneficial effect *in vitro*. Our results unambiguously show that membrane resealing begins immediately after the first pulse, and that a delay between pulses in a two-pulse protocol decreases *in vitro* delivery efficiency.

The classical view of two-pulse electroporation dating back to Sukharev et al. is that the brief, strong first pulse primarily permeabilizes,

while the longer, weaker second pulse only drives molecular delivery into the cell [34]. This is because the strength of the second pulse is specifically chosen to be below the threshold for poration when applied to an unperturbed cell membrane (*cf.*, Fig. 3B).

However, our data suggest that the second pulse does not merely electrophoretically drive molecules into the cell, but also re-porates the weakened cell membrane. Fig. 6 shows the total delivered intracellular concentration of 10 kDa FD for varying second-pulse field strengths. If the role of the second pulse was merely electrophoretic, the delivered intracellular concentration would always be proportional to the second pulse field strength, and delivery through pores already opened by the first pulse would only disappear when the second-pulse field strength is zero. For second-pulse field strengths above a threshold of 0.3 kV/cm, the delivery of FD did indeed increase linearly with DC field strength, as would be expected for electrophoretic transport. However, as seen in Fig. 6, a threshold field strength is required for effective delivery by the second pulse. Regardless of whether the first pulse was AC or DC, no FD was delivered into the cell for second-pulse field strengths below 0.3 kV/cm. This suggests that a critical field strength is required for the second pulse to deliver target molecules into the cell, and that the second pulse actually reopens electropores in the membrane. The threshold field strength, however, is lower (0.3 kV/cm) for the second pulse than the field strength (0.4 kV/cm) originally required to porate the cell membrane (Fig. 3B). We speculate that this reduced critical field strength for the second pulse is because of the existence of nano-sized pores or defects remaining from the first pulse. Below this second critical field strength, the second pulse is unable to reopen electropores, and no electrophoretic delivery occurs. Thus, we hypothesize that the actual role of the second pulse in two-pulse electroporation is more complex than originally believed: The second pulse not only electrophoretically drives the target molecules into the cell, but actually re-porates the membrane.

Fig. 6 also shows that the separation of permeabilization and transport for the chosen conditions. The dotted lines show that there is negligible delivery for AC or DC pulses of the 1 ms duration and 1 kV/cm field strengths alone — delivery only occurs when 30-ms DC second pulses are included. The triangles (which replot the data shown in Fig. 3B) show that when only 30-ms DC pulses are used, there is also negligible delivery. Only when the 30-ms DC pulse is proceeded by a brief, intense poration pulse (1 ms, 1 kV/cm) does significant delivery occur.

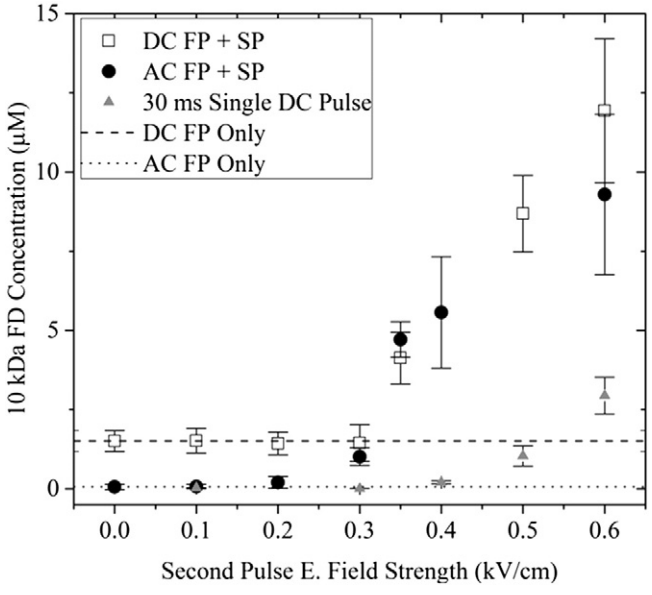

Fig. 6. Intracellular 10 kDa FD concentrations for two different electroporation protocols: (I) 1 kV/cm-field-strength DC first pulse of 1 ms duration and (II) 1 kV/cm-field-strength AC first pulse of 1 ms duration. In both cases, the field strength of the second pulse was varied between 0 and 0.6 kV/cm, while the duration was kept constant at 30 ms. Horizontal lines ("DC FP only" and "AC FP only") shows the delivered concentrations after only applying the 1-ms first pulse. "30 ms Single DC pulse" represents the delivered concentrations for a single 30-ms-long DC pulse at the indicated field strengths.

The hypothesized reopening of pores by the second pulse may explain in part the wide variation in membrane-resealing times reported in the literature. Researchers have measured membrane resealing times using various methods, with results ranging from milliseconds to minutes. Membrane-resealing times inferred from measuring the membrane conductance [30,50,68,83] tend to be shorter (short-term resealing) than the times suggested by uptake of small [5,47,48,84–86] or larger molecules [87,88] after pulsation (long-term resealing). For instance, Hibino et al. showed one of the first sub-second methods using a voltage-sensitive membrane dye [30], and later on He et al. [45] and Khine et al. [68] developed two sub-second methods to measure resealing times. All concluded that membrane resealing occurred on timescales on the order of milliseconds. On the other hand, studies using a delay between pulses in two-pulse electroporation tend to show much longer resealing times, on the order of minutes [34] to tens of minutes [67]. Our results suggest that apparently longer resealing times are in part due to the reopening of pores by the second pulse. Thus, electropores shrink rather quickly, but nano-sized pores or defects [89–91] tend to remain for a much longer time. According to our experimental results, resealing happens very quickly after the cessation of the first pulse (around 50% in the first 100 ms). However, the total delivered intracellular concentration decreases with the logarithm of the delay time, and complete resealing (such that the pores are not reopened by the weaker second pulse) takes tens of minutes after initial first-pulse poration. Thus, membrane electroporation, sealing, and re-poration are complex, inter-related processes that may have both short-term (initial electropore shrinkage) and long-term (complete resealing of nano-sized defects or pores against second pulse re-poration) components, which may in part explain the wide variation in membrane-resealing times reported in the literature.

5. Conclusion

In this work, we have conducted a series of two-pulse electroporation experiments, focusing on delivery efficiency of two different-sized molecules using different pulsing parameters. Distinct from prior work, we have employed an AC field to achieve permeabilization without significant delivery in the first pulse. When the second pulse was kept at a fixed strength (chosen to be below the critical permeabilization threshold), the delivery of 10 kDa and 70 kDa varied strongly with first-pulse field strength. In particular, at lower field strengths of 0.6 kV/cm, membrane pores generated by the AC first pulse were sufficiently large to allow transport of 10 kDa FD, but not 70 kDa FD. Increasing the field strength of the AC first pulse is hypothesized to increase the size and number of electropores on the membrane, allowing delivery of the larger molecule. We further systematically studied the effect of delay time between pulses on the delivery efficiency. Using the AC first pulse to porate the membrane, and a DC second pulse for transport, we are able to probe resealing dynamics over timescales ranging from milliseconds to minutes. We find for these cells and pulsing parameters that the electroporation-mediated delivery scales inversely with the logarithm of the delay time regardless of the molecule size.

Results from the current work help reconcile some of the discrepancies observed in the literature. Our data establishes convincingly that delay between pulses in a two-pulse protocol decreases delivery efficiency *in vitro*, consistent with the results on DNA delivery by Sukharev et al. [34]. The opposite trend observed *in vivo* [57,58] therefore may be attributed to mechanisms other than pore dynamics, *e.g.*, DNA-membrane interactions, complex formation, and molecular redistribution within the tissue. Furthermore, our results on resealing dynamics suggest that approximately 50% of resealing happens in the

first 100 ms after pulsation, but complete resealing takes hundreds of seconds or longer. The large variations in the resealing times observed previously therefore are possibly reflecting the sensitivity of different assays to different points within this wide range of times.

Last but not least, we observe that the role of the second pulse is more than just electrophoresis, in that a minimum threshold field-strength is required for the second pulse to re-porate nano-sized pores that remain from the first pulse. Thus, in contrast to the classical view that the first pulse and second pulse can be considered as independent porating and delivery pulses, we hypothesize that actual two-pulse electroporation involves complex, inter-related processes of initial poration, resealing, and re-poration, before electrophoretic transport takes place.

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Transparency document

The Transparency document associated with this article can be found in the online version.

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