In-vitro bipolar nano- and microsecond electro-pulse bursts for irreversible electroporation therapies

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ABSTRACT

Under the influence of external electric fields, cells experience a rapid potential buildup across the cell membrane. Above a critical threshold of electric field strength, permanent cell damage can occur, resulting in cell death. Typical investigations of electroporation effects focus on two distinct regimes. The first uses sub-microsecond duration, high field strength pulses while the second uses longer (50 μs +) duration, but lower field strength pulses. Here we investigate the effects of pulses between these two extremes. The charging behavior of the cell membrane and nuclear envelope is evaluated numerically in response to bipolar pulses between 250 ns and 50 μs. Typical irreversible electroporation protocols expose cells to 90 monopolar pulses, each 100 μs in duration with a 1 second inter-pulse delay. Here, we replace each monopolar waveform with a burst of alternating polarity pulses, while keeping the total energized time (100 μs), burst number (80), and inter-burst delay (1 s) the same. We show that these bursts result in instantaneous and delayed cell death mechanisms and that there exists an inverse relationship between pulse-width and toxicity despite the delivery of equal quantities of energy. At 1500 V/cm only treatments with bursts containing 50 μs pulses (2×) resulted in viability below 10%. At 4000 V/cm, bursts with 1 μs (<100×), 2 μs (50×), 5 μs (20×), 10 μs (10×), and 50 μs (2×) duration pulses reduced viability below 10% while bursts with 500 ns (200×) and 250 ns (400×) pulses resulted in viabilities of 31% and 92%, respectively.

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1. Introduction

When cells are exposed to electric fields there is a resultant structural rearrangement of molecules in the lipid-bilayer that occurs due to increases in transmembrane potential. At low field intensities, nanoscale pores form in the cell membrane temporarily increasing the transport of molecules across the lipid-bilayer [1,2]. At high field intensities, the cell is unable to recover from the pore formation process, resulting in physical cell death [3,4]. This non-thermal mechanism has been adapted clinically as a focal ablation technique known as irreversible electroporation (IRE). IRE has shown to be safe and effective for treating a variety of cancerous pathologies [5,6], including tumors located in close proximity to major blood vessels that can rapidly remove heat out of the ablation zone, rendering thermally-mediated therapies ineffective [9,10]. Additionally, due to the targeted disruption of the plasma membrane, the treatment volume is visible in real-time on multiple imaging platforms [11].

When IRE is employed in-vivo the electric field is typically pulsed for durations of 50–100 μs to minimize joule heating effects which can damage the extracellular matrix. A typical treatment protocol involves delivering one pulse per second for 90 s [12,13] through needle electrodes with a voltage to distance ratio of 1000–2000 V/cm [5,7,8]. The electric field intensity required to induce IRE varies with tissue type, temperature, anisotropy and conductivity, cell size, as well as a number of other physiological factors, but is typically in the vicinity of 500 V/cm. In-vitro, this field intensity changes significantly between cells in suspension and those grown in a 3-D matrix and may be due to morphological changes that occur when they attach to the extracellular matrix [14].

The mechanisms of cell death due to pulsed electric field have been shown to vary with pulse length and amplitude. Microsecond and greater duration pulses typically result in immediate cell death due to irrecoverable damage to the cell membrane. In contrast, submicrosecond pulses typically lead to the induction of apoptotic cell death mechanisms [15,16] through caspase activation [17], calcium release from organelles [18], phosphatidylserine externalization [19], dissipation of mitochondrial membrane potential [20], and DNA damage [21]. An in depth analysis of these mechanisms can be found in [22]. Below 50 kV/cm, sub-microsecond pulses have been found to induce caspase-dependent apoptotic responses, while pulses at higher field intensities induce a non-caspase dependent cascade [22]. DNA damage due to sub-microsecond pulses has been demonstrated...
in vitro [23] and in vivo [24], though the role of this damage in the apoptotic cascade is not well understood.

The amplitude of the applied field required to induce electroporation effects has an inverse relation with pulse duration. Pucihar et al. found that the electric field required to electroporate seventy percent of cells in-vitro, using a single pulse, increased from approximately 400 V/cm to 10 kV/cm as the pulse duration was reduced from 1 ms to 150 ns, respectively [25]. In-vivo, Nucitelli et al. found that 100 pulses with duration of 300 ns were ineffective at reducing the size of melanomas when 10 kV/cm was applied. However, tumor volume reduced by 75% after eight days when the electric field intensity was increased to 20 kV/cm for 200 pulses of the same duration [26]. Interestingly, for very short duration pulses (60 ns), cell viability has been reported to be affected by media composition [27] indicating that molecular transport plays a significant role in the resulting cell death mechanisms. However, there is a clear inverse correlation between pulse length and the required dose [28] and protocols with higher pulse numbers require lower electric field intensities to induce cell death [29].

Bridging the gap between ultra-short and long duration pulses, Arena et al. recently showed that bursts of bipolar square waves with constitutive pulses of 1 and 2 μs can be used to ablate brain tissue [white/gray matter] without inducing muscle contractions [30]. In the high frequency irreversible electroporation (H-FIRE) protocol presented by Arena et al., bursts consisting of 50 bipolar square waves 2 μs in duration per phase did not induce muscle contractions when delivered directly into the motor cortex with a voltage to distance ratio of 4000 V/cm. In contrast, single monopolar pulses 200 μs in duration resulted in measurable muscle contractions with voltage to distance ratios as low as 500 V/cm. These bursts of short duration pulses have been theoretically shown to short through epithelial layers and produce more uniform treatment regions through heterogeneous tissues [31].

The ability to simultaneously achieve more predictable lesions in electrically complicated tissues without inducing muscle contractions by using bipolar bursts has important clinical implications. Lesion predictability directly influences treatment outcomes and is required to ensure adequate tumor coverage with a lethal electric field while minimizing damage to the surrounding healthy tissue. Eliminating muscle contractions obviates the need for neuromuscular blockade, which subsequently requires general anesthesia and monitoring of respiratory function. Thus, there is the potential to perform treatments outside of the operating room. The burst characteristics required to achieve electroporation within this intermediate range of pulse durations spanning 1 μs to 100 μs are still relatively unexplored [32], which serve as the basis of this paper.

Here we present the in-vitro effects of high frequency bipolar bursts, shown in Fig. 1. Individual pulses within the burst are separated by 2 μs and sequential pulses alternate in polarity. The bursts are repeated once per second for 80 s and each burst exposes cells to the applied voltages for 100 μs. This specific waveform serves three purposes which are focused on clinical applications. First, our bursts contain an equivalent 100 μs energized time to the monopolar pulses employed in clinical irreversible electroporation systems, allowing for direct comparison between our protocols with that of clinically viable electroporation systems [5,8,33]. Secondly, these bursts are delivered once per second to correspond to the approximate delivery rate of clinical systems which are synchronized with the patient’s heartbeat to minimize risks of tachycardia. Last, it has been shown that these waveforms effectively eliminate muscle contractions associated with irreversible electroporation therapies [30].

To demonstrate the effects of these pulses on the cell membrane and intracellular organelles, we present a finite element model of a cell including a nuclear envelope. The charging behavior of the cellular membrane and nuclear envelope is evaluated in response to pulses between 250 ns and 50 μs. A parametric analysis is conducted on the intra- and extracellular conductivity, nucleus-to-cytoplasm ratio, and pulse-to-pulse delay time. In-vitro experiments are presented to confirm the non-thermal nature of the protocol and demonstrate irreversible electroporation within this intermediate pulse-width range. These findings agree with previous work that shows there exists an inverse correlation between pulse-width and toxicity, and shows that this phenomenon remains despite delivery of equal quantities of energy within each burst.

2. Methods

2.1. Numerical modeling

A numerical model of a cell in suspension was created in COMSOL 4.2 using an impedance boundary condition scheme [34]. The solution domain consisted of a three dimensional cube with edge-lengths of 0.1 mm. At the center of this domain, two spheres were created representing the cytoplasm and nucleoplasm. Within the solution domain, the Electric Currents module was used to solve for following equations

\[ \nabla \cdot \mathbf{J} = 0 \left( \text{A/m}^3 \right) \]  
\[ \mathbf{J} = \left( \sigma + \varepsilon_0 \varepsilon_r \frac{\partial}{\partial t} \right) \mathbf{E} \left( \text{A/m}^2 \right) \]  
\[ \mathbf{E} = -\nabla U \left( \text{V/m} \right) \]
where \( U \) is the electric potential, \( E \) is the electric field, and \( J \) is the current density. One boundary was assigned as time dependent electrical potential

\[
U = U(t) / V.
\]

The opposing boundary was assigned as the relative ground

\[
U = 0 V.
\]

The remaining boundaries were defined as electrical insulation

\[
n \cdot J = 0 \quad (A/m)
\]

where \( n \) is the normal vector to the surface, and \( J \) is the electrical current density.

For each domain (media, cytoplasm, nucleoplasm), a separate Electric Currents module was used and the dependent electric potential variables \( U_{\text{media}}, U_{\text{cyto}}, \) and \( U_{\text{nuc}} \) for the media, cytoplasm, and nucleoplasm domains were defined, respectively. These variables were then defined to calculate the voltage across the cell membrane (\( U_m \)) and nuclear envelope (\( U_n \))

\[
U_m = U_{\text{media}} - U_{\text{cyto}} / V
\]

\[
U_n = U_{\text{cyto}} - U_{\text{nuc}} / V.
\]

In each Electric Currents module, the boundaries representing membranes were defined as impedance boundary conditions with reference potentials prescribed as the electric potential in the adjacent \( (U_{\text{media}}) \) domain

\[
n \cdot (J_i - J_f) = \frac{1}{d} \left( \sigma(U - U_{\text{ref}}) + \epsilon_0 \epsilon_m \frac{\partial}{\partial r} (U - U_{\text{ref}}) \right) \left( A/m^2 \right)
\]

where \( \sigma \) is the conductivity, \( \epsilon_0 \) is the permittivity of free space, \( \epsilon_m \) is the relative permittivity, and \( d \) is the thickness of the cell membrane or nuclear envelope. For example, in the Media domain, the boundary representing the cell membrane was defined as an impedance boundary with reference potential of \( U_{\text{cyto}} \). In the Cytoplasm domain, the same boundary representing the cell membrane was defined as an impedance boundary with a reference potential of \( U_{\text{media}} \). The boundary was defined as a ‘thin layer’ and the electrical conductivity, relative permittivity, and surface thickness were defined using the values presented in Table 1. The nuclear envelope consists of two individual lipid membranes separated by the perinuclear space. To limit the complexity of the model and avoid improperly assessing the electrical properties of these individual components, which are not readily available in the literature, we elected to lump these biological features into a single 40 nm membrane for which electrical properties representing their combined features are available.

The mesh was defined as a single free tetrahedral group with the elements between 1.8 and 10 μm on edge, resulting in 19,353 tetrahedral elements. In a preliminary study of this model, finer and coarser meshes were used. Simulation times were more than doubled between successive refinements. The average deviation between the mesh presented here and the next successive refinement was less than 2.0% and 5.5% for the cell membrane and nuclear envelope potentials, respectively. For each parameter, solutions were found in approximately 22 min on a quad core 3.0 GHz processor with 8 GB of RAM. Results of the numerical simulations, using the values in Table 1, were compared to those found using the analytical method presented by Kotnik and Miklavic [35]. When calculating the maximum/minimum potentials across the cell membrane and nuclear envelop, the error between the numerical and analytical solution was 0.15%/0.15% and 1.97%/0.89%, respectively. The values reported for \( U_m \) and \( U_n \) were derived from the geometric boundary point closest to the energized electrode (\( \theta = 0 \)). A backward differentiation formula (BDF) time stepping scheme was used for all simulations. The solver was allowed to freely define the time steps using an initial time step of 1 ns and a maximum time step of 1 μs. The simulation was solved for a duration equal to three times the pulse width plus the inter-pulse delay.

### 2.2. Cell preparation and experimentation

In all experiments, cells were suspended in a buffer consisting of a 5:5:1 ratio of culture media to low conductivity sucrose buffer (85 g sucrose, 3.0 g glucose, 7.25 mL RPMI, and 992.75 mL DI water) [36]. The electrical conductivity of the cell suspension was measured with a conductivity meter prior to experimentation (Horiba B-173, Cole-Parmer, Vernon Hills, IL) to ensure a final conductivity of 0.2 S/m. Clark et al. reported that the conductivity of pancreatic tissue varied between 0.097 and 0.44 S/m for frequencies between 1 kHz and 2 MHz, respectively [37]. A media conductivity of 0.2 S/m was chosen to minimize the current delivered through the sample while maintaining a conductivity value within the range of those found in in-vivo tissue. Due to limitations in our pulse generation system, higher conductivity buffers would drive the pulse delivery system outside of its safe operating region.

PPT8182 murine primary pancreatic tumor cells [38] were used in all experiments. These cells have been shown to replicate human pancreatic cancer in terms of histology, metastasis, and genetic alterations [38–41]. Cells were cultured in DMEM (supplemented with l-glutamine, ATCC, Manassas, VA) containing 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO) and 1% stock solution of penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in 5% CO2 in a humidified atmosphere. All cells were harvested for experiments by trypsinization at 80% confluence. Suspensions were centrifuged twice and resuspended in an exponential buffer at a concentration of \( 5 \times 10^5 \) cells/mL. 100 μL of cell suspension was injected into a 2 mm gap cuvette (Model 620, Harvard Apparatus, Holliston, MA) immediately prior to pulse delivery. A schematic of the experimental setup is shown in Fig. 1A.

The protocol for all experiments used the waveform presented in Fig. 1B. The schematic depicts an example burst which contains a repeated sequence of individual pulses. The burst begins with a positive polarity pulse followed by a 2 μs pause, then a negative polarity pulse followed by another 2 μs pause. This cycling is immediately repeated until the voltage has been delivered for a total of 100 μs (50 μs in each polarity). Eighty bursts were delivered with a frequency of 1 Hz. Within

### Table 1 Parameters used in numerical analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \epsilon_0 ) (m⁻³ kg⁻¹ s⁴ A²)</td>
<td>8.85 × 10⁻¹²</td>
</tr>
<tr>
<td>( \sigma_m ) (S/m⁻¹)</td>
<td>0.2</td>
</tr>
<tr>
<td>( \epsilon_m )</td>
<td>80ε₀</td>
</tr>
<tr>
<td>( d_{\text{mem}} ) (m)</td>
<td>5 × 10⁻⁹</td>
</tr>
<tr>
<td>( t_{\text{mem}} ) (μs⁻¹)</td>
<td>6.55 × 10⁻⁶</td>
</tr>
<tr>
<td>( \sigma_{\text{mem}} ) (S/m⁻¹)</td>
<td>3 × 10⁻⁷</td>
</tr>
<tr>
<td>( \epsilon_{\text{mem}} )</td>
<td>8.57ε₀</td>
</tr>
<tr>
<td>( \sigma_{\text{nuc}} ) (S/m⁻¹)</td>
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</tr>
<tr>
<td>( \epsilon_{\text{nuc}} )</td>
<td>154.4ε₀</td>
</tr>
<tr>
<td>NCR</td>
<td>0.8</td>
</tr>
<tr>
<td>( d_{\text{nuc}} ) (m)</td>
<td>40 × 10⁻⁹</td>
</tr>
<tr>
<td>( \sigma_{\text{nuc}} ) (S/m⁻¹)</td>
<td>6 × 10⁻³</td>
</tr>
<tr>
<td>( \epsilon_{\text{nuc}} )</td>
<td>28ε₀</td>
</tr>
<tr>
<td>( \epsilon_{\text{nuc}} )</td>
<td>1.35</td>
</tr>
<tr>
<td>( \epsilon_{\text{nuc}} )</td>
<td>52ε₀</td>
</tr>
</tbody>
</table>

Permittivity of free space \( \epsilon_0 \), media conductivity \( \sigma_m \), media permittivity \( \epsilon_m \), cell membrane thickness \( d_{\text{mem}} \), cell radius \( r_c \), cell membrane conductivity \( \sigma_{\text{mem}} \), cell membrane permittivity \( \epsilon_{\text{mem}} \), cytoplasm conductivity \( \sigma_{\text{cyto}} \), cytoplasm permittivity \( \epsilon_{\text{cyto}} \), nucleus-to-cytoplasm ratio (NCR), nuclear envelope thickness \( d_{\text{nuc}} \), nuclear envelope conductivity \( \sigma_{\text{nuc}} \), nuclear envelope permittivity \( \epsilon_{\text{nuc}} \), nucleoplasm conductivity \( \sigma_{\text{nuc}} \), nucleoplasm permittivity \( \epsilon_{\text{nuc}} \).
each burst, individual pulses had a single duration of 250 ns, 500 ns, 1, 2, 5, 10, or 50 μs and therefore bursts contained 400, 200, 100, 50, 20, 10, or 2 pulses, respectively to result in equivalent energized time. The 2 μs delay time was programmed between sequential opposite polarity pulses to protect the electronics from over-voltages due to ringing. Representative examples of the bursts are shown in Fig. 2. The cells were exposed to electric potentials with voltage-to-distance ratios (E) of 1500, 3000, and 4000 V/cm. The temperature change in the cell suspension due to pulsing was measured using fiber optic temperature probes (Lumxtron LOT Lab Kit, LumaSense Technologies, Santa Clara, CA) inserted directly into the cell suspension.

For the in vitro studies, each of the treatment groups was repeated a minimum of three times (n = 3) and experiments for each group were conducted on at least two different days. For each treatment, different experimental parameters, including sham exposure, were alternated in a random sequence. After treatment, samples were split into two equal 50 μL samples to be evaluated at 1 and 24 h time points. The samples were kept at room temperature for approximately 20–30 min prior to being placed on ice (1 hour group) or moved to the incubator (24 hour group) while the remaining experimental groups were completed. Approximately 1 h post exposure, viability was assessed using a trypan blue exclusion assay. Cells which had been irreversibly electroporated were unable to exclude the dye and were stained blue. Cells were counted visually using a hemocytometer and the percentage viability was determined as

$$Viability_{1\ hour} = \frac{N_{live}}{N_{total}} \times 100\ \%$$

$$r_{viability-1\ hour} = \frac{Viability_{1\ hour-treatment}}{Viability_{1\ hour-control}}$$

The average viability of sham control samples in the 1 hour time group was greater than 85%. Samples to be analyzed at 24 h were placed in separate wells in a 12-well plate containing a total of 1 mL of culture media and maintained at room temperature until the well plate was full (approximately 30 min). At this point the well plate was placed in an incubator at 37 °C and 5% CO2 for 24 h. Viability was then assessed using an Alamar blue metabolism assay (Life Technologies, Grand Island, NY) using the manufacturer’s recommended procedure. Briefly, 100 μL/mL stock Alamar blue solution was added to each well. After 4 h, the samples were read using a spectrophotometer at 570/600 nm wavelengths. For each sample, the absorbance was measured in three separate wells and averaged. Additional measurements were taken for sample media without cells and for control cell samples which were not exposed to an electric field. The percentage viability was determined as

$$r_{viability-24\ hour} = \frac{l_{sample} - l_{media}}{l_{control} - l_{media}}$$

where I is the relative intensity measurement from the spectrophotometer. In general, trypan blue analysis and metabolism assays complement each other quite well. Ihey et al. previously showed that metabolism assays mirrored those from trypan blue analysis after nano-second pulsed electric field exposure [28]. The Alamar blue assay used in this study is well established for measuring cytotoxicity in mammalian cells [42]. Reduction rates for cells seeded between 2.5 × 10³ and 2 × 10⁶ cells/mL were measured to ensure that the sham population did not completely reduce the Alamar blue solution (results not shown) and a 4 hour incubation time with 2.5 × 10³ cells/mL was determined to be optimal. Viability data for both the 1 hour and 24 hour groups were normalized to the sham control groups. Statistical analysis of the data was completed using JMP Pro V. 10.0 (SAS Institute Inc., Cary, NC).

2.3. Electronics

Waveforms were generated using an arbitrary function generator (AFG3021C, Tektronix Inc., Beaverton, Oregon), which were amplified by a custom built high voltage pulse generator capable of +/- 1000 V outputs through high impedance loads (Applied Energetics, Tucson, AZ, USA). Output waveforms were visualized using an oscilloscope (DP02002B, Tektronix Inc., Beaverton, Oregon) after the voltage was attenuated using a 50 MHz 1000 x high voltage probe (PS210A, Tektronix Inc., Beaverton, Oregon) and the current was measured using an active clamp on 50 MHz current probe (TCP305, Tektronix Inc., Beaverton, Oregon). Short circuit protection resistors on the output limited our maximum output voltage through the 2 mm cuvettes to approximately 800 V (4000 V/cm).

3. Results and discussion

3.1. Numerical modeling

As shown in Fig. 3, under the influence of a 1500 V/cm electric field, the potential drop across the cell membrane (Uₘ) and nuclear envelope (Uₚ) reaches maximums of 1.47 V and 0.28 V, respectively. Uₘ reaches 50% of the maximum value in 0.34 μs, 70.7% in 1.11 μs, and 99.99% maximum in 7.92 μs. Uₚ reaches 99.99% max in 145 ns and falls back below 70 mV in approximately 0.94 μs. This brief charging and
discharging of the nuclear envelope is due to current that flows within the cytoplasm as the cell membrane is charging. This transient current increases the potential across membranes surrounding the nucleus and organelles. These intracellular components are smaller than the cell and their exposure to currents is brief resulting in a smaller potential increase.

As the positive polarity pulse falls, the cell membrane begins to discharge resulting in a second current flow within the cytoplasm in the opposite direction, as compared to the rising pulse edge. This results in the formation of a negative potential across the nuclear envelope. This negative potential reaches a minimum of $-0.28\, V$ and falls below $-70\, mV$ in a similar 0.94 $\mu s$. The rising edge of the negative polarity pulse creates a similar decrease in $U_n$, creating an interesting double peak in the membrane potential of the nuclear envelope. This second peak reaches a value of $-0.29\, V$. Though this peak is only 10 mV different than the maximum achieved by the initial pulse, it suggests that optimization of the pulse length and delay time between pulses could result in an increased effect on intracellular membranes.

In this manuscript, we elected to disregard the effects of electroporation on the cell membrane to simplify our analysis. However, in the case of electroporation, current would be allowed to flow through the cytoplasm and a sustained potential would be induced across the intracellular membranes, thereby reducing the first negative peak in $U_n$.

3.2. Analysis of experimental parameters

Fig. 4 presents a parametric analysis of variables which can be controlled experimentally. The pulse duration, shown in Fig. 4A, directly impacts the maximum $U_m$ achieved and the duration that $U_m$ is elevated above the 1 V critical threshold. Pulses that are shorter than 1 $\mu s$ do not elevate the $U_m$ above this threshold. As pulse duration increases beyond 1 $\mu s$, $U_m$ saturates to a maximum value of 1.47 V. In contrast, because $U_n$ rises rapidly in comparison to the $U_m$, the effects on the nuclear envelope are minimally impacted by the pulse duration. Regardless of pulse width, the $U_n$ reaches a maximum value within 145 ns. For pulses 1 $\mu s$ or less,
the $U_n$ does not completely return to zero before the falling edge of the positive pulse, muting the negative $U_n$ response.

It has been observed that pore formation behavior occurs within 1 μs after $U_m$ is elevated above 1 V, quenching further increases in potential [43], after which new pore formation is limited and pore expansion takes over as the dominant phenomena [44,45]. At the field strengths presented here, pulses 1 μs in duration and shorter may not efficiently result in pore expansion within the cell membrane [46].

The conductivity of the sample media, Fig. 4B, contributes significantly to the charge–discharge behavior of the cell membrane and the nuclear envelope. At low media conductivities (0.01 S/m), the media presents a significant resistance to current flow and the cell membrane charges slowly. Similarly, this low conductivity media minimizes the current which can flow through the cytoplasm, muting the maximum $U_n$ achieved. As the media conductivity increases, the cell membrane charges more quickly, saturating as the conductivity is increased above 1 S/m. Based on these simulations, a media conductivity of 0.2 S/m used experimentally is a compromise between membrane charging times and current output required from the pulse generator. Increasing media conductivity may have resulted in slightly faster membrane charging times.

The delay between positive and negative polarity pulses, Fig. 4C, has a negligible effect on the transmembrane potential ($U_m$); though, it has a significant impact on the nuclear envelope ($U_n$). The falling edge of the positive pulse results in a negative potential build-up on the nuclear envelope. $U_n$ reaches a relative maximum approximately 140 ns after the falling edge of each pulse. For long delays between pulses, this potential decays back to zero. In contrast, as the delay is contracted, $U_n$ is compounded by the rising edge of the negative polarity pulse. Ultimately, as the delay is decreased to 140 ns or less, an effective doubling of the $U_n$ is achieved.

Typically, driver circuits for high voltage solid state switches employ a dead time between changes in polarity to avoid shoot-through (short circuiting the power supply to ground) or to protect the electronics from deleterious effects caused by ringing. This delay between changes in polarity is highly dependent on the topology of the pulse generation circuit and is typically between 100 and 500 ns. Based on these simulations, bursts with a 100 ns delay between changes in pulse polarity will continue to achieve a doubling of the potential across the nuclear envelop. An additional requirement to achieve this doubling in $U_n$ is that the potential across the nuclear envelope must be allowed to decay back to zero before the applied voltage is turned off. In this scenario, all pulses which are 0.94 μs in duration or longer resulted in approximately a 2× increase in $U_n$ versus the single pulse maximum.

The role of DNA damage in the pulsed electric field (PEF) apoptotic cascade is not fully understood and the nucleus is not typically the target for PEF therapy. However, intrinsic and extrinsic apoptotic cell death processes are associated with field strength dependent effects on mitochondria and the endoplasmic reticulum. If waveform optimization can be used to double the increase in the transmembrane potential of these organelles, as shown in Fig. 4C, then lower amplitude electric fields would be needed to induce the associated apoptotic cascades. Alternatively, by finely tuning the pulse widths and inter-pulse delays it may be possible to enhance DNA damage processes allowing for further study of this mechanism in the PEF apoptotic cascade. Unfortunately, experimental investigation of well controlled 100–500 ns inter-pulse delay scenarios was inhibited by ringing in the output voltages of our current system and is left as the subject of future work.

Fig. 5. Cell property parametric analysis: The voltage drop across the cell membrane, $U_m/V$, and nuclear envelop, $U_n/V$, are presented as a function of time, t/μs. (A) Nucleus–cytoplasm ratio, (B) cytoplasm conductivity, and (C) cell membrane permittivity. (D–E) The voltage drop across the cell membrane, $U_m/V$, and nuclear envelop, $U_n/V$, for a model of benign and cancerous cells. Note that the axes for $U_m$ and $U_n$ have different scales.
3.3. Analysis of cell electrical properties

Electrical properties for the cell membrane, nuclear envelope, cytoplasm, and nucleoplasm are readily available in the literature [47–52]. Subuncu et al. report a cytoplasmic conductivity between 0.3 and 0.6 S/m [53]. Labeed et al. report increases in conductivity from 0.28 S/m to 0.45 S/m as cells begin to undergo apoptosis [54]. Ron et al. report a conductivity of 0.724 S/m and 0.93 S/m for pre-osteoblast cells and normal canine kidney cells, respectively [55]. Mulhall et al. found cytoplasm conductivities of 0.71, 0.42, 0.26, and 0.25 S/m for normal keratinocytes, abnormal keratinocytes, and for two different malignant keratinocytes, respectively [56]. Additionally, Chen et al. show that drug resistant cells have a lower cytoplasmic conductivity than non-drug resistant cells [57]. These results provide evidence of decreasing cytoplasmic conductivity with cells transition from benign to malignant.

Yuan et al. show an increase in nucleus-to-cytoplasm (NCR) ratio from 0.45 to 0.49 and from 0.40 to 0.49 as cancer cells achieve drug resistance. Similarly, Helczynska et al. show histologically, that the NCR increases from 0.3 to 0.8 as a function of tumor grade, with higher NCRs for increasingly malignant cancers [58]. Salmanzadeh et al. showed that the specific membrane capacitance of a syngeneic cell line increased from 15.39 mF/m² to 26.42 mF/m² as the cells became successively more malignant [59]. This translates into an increase in relative membrane permittivity from 8.70 to 14.92.

A parametric analysis was conducted using cytoplasmic conductivity values of 0.7, 0.475, and 0.25 S/m, an NCR of 0.3, 0.55, and 0.8, and a membrane permittivity of 9, 12, and 15 to represent this transition from benign to intermediate to metastatic, respectively. We modeled the response of a ‘benign’ cell having cytoplasmic conductivity of 0.7 S/m, NCR of 0.3, and membrane permittivity of 8.7. A ‘metastatic’ cell was modeled as having cytoplasmic conductivity of 0.25 S/m, NCR of 0.8, and a membrane permittivity of 15.

A parametric analysis was conducted using an NCR of 0.3, 0.55, and 0.8, cytoplasmic conductivity values of 0.7, 0.475, and 0.25 S/m, and a membrane permittivity of 9, 12, and 15 to represent this transition from benign to intermediate to metastatic, respectively. The NCR, Fig. 5A, has a negligible effect on \( U_{m} \) and notable effect on \( U_{n} \). As expected from electromagnetic theory [60], the potential across an the nuclear envelope is related to the equation

\[
\Delta U = 1.5rE\cos\theta / V
\]

where \( r \) is the radius of the nucleus and \( E \) is the electric field to which the cell is exposed to. However, other dielectric properties of the nucleus may affect the membrane charging time [35,61]. As the NCR increases in Fig. 5A, \( U_{n} \) also increases. The cytoplasm conductivity, Fig. 5B, has a negligible impact on the maximum amplitude of \( U_{m} \) and \( U_{n} \). The permittivity of the cell membrane, Fig. 5C, impacts the charge and discharge of the cell membrane and the nuclear envelope. A higher permittivity causes the \( U_{n} \) to increase slightly slower than the lower permittivity cells. This slower charging time of the cell membrane results in the nuclear envelope reaching a slightly higher transmembrane potential.

From the numerical simulations, it is anticipated that cells with a larger NCR will achieve higher \( U_{n} \) amplitudes than cells of similar size with smaller NCR. A high NCR has been associated with the aggressiveness of malignant cells and is used as a parameter in grading cancers [65–68]. Additionally, it has been shown that an increase in invasiveness and metastatic potential has been correlated to cell membrane ruffling, which leads to higher membrane capacitances in aggressive cells [59,69,70]. We therefore modeled the response of a ‘benign’ cell having cytoplasmic conductivity of 0.7 S/m, NCR of 0.3, and membrane permittivity of 8.7. A ‘metastatic’ cell was modeled as having cytoplasmic conductivity of 0.25 S/m, NCR of 0.8, and a membrane permittivity of 15. All other values (Table 1) were held constant.

In numerical simulations (Fig. 5D), a normal cell model experiences a \( |U_{m}| \approx 0.14 \) V while a cancer cell model reaches \( |U_{m}| \approx 0.32 \) V. The nucleus in the cancer cell model reaches a potential approximately 2 times higher than the normal cell model as a result of changes in NCR. This effect is amplified further if the delay between pulses is reduced to 100 ns (Fig. 5E) where \( |U_{m}| \approx 0.6 \) V for the cancer cell.
model. It is anticipated that malignant cells will experience an increased response to bipolar pulses due the increase in cell membrane charging time, resulting from an increased membrane capacitance, coupled with increased NCR ratio. However, future work will be required to determine if these burst have an increased efficiency at targeting aggressive cells.

3.4. Numerical simulation of experimental pulses

The simulation results presented in Figs. 3 to 5 represent the response to a square wave with 10 ns rise and fall times. Experimentally, the waveforms exhibited ringing effects on the rising edge and after the falling edge as shown in Fig. 6A and C. Fig. 6B and D shows the cell transmembrane potential (U_m) and nuclear transmembrane potential (U_n) resulting from experimental 250 ns and 1 μs pulses, respectively. As in the square wave case, the falling edge of the pulses results in an increased U_n in the opposite polarity. The ringing in the output waveform causes an additional minor increase in U_m. At 1500 V/cm the first rising edge of a 250 ns pulse results in an U_m amplitude maximum of 0.21 V. The falling edge and ringing of the same pulse results in a maximum U_n amplitude of 0.25 V, a 19% increase.

For a 1 μs experimental pulse, |U_m| reaches a maximum of 1.12 V while |U_n| reaches a maximum of 0.32 V. The magnitude of U_m for this experimental pulse is approximately equal as in the case of ideal square wave, predicted in Fig. 4A (1.21 V). However, the magnitude of U_n for this experimental pulse (0.32 V) is greater than the value predicted in Fig. 4A (0.29 V). This is due to the ringing which occurs after the experimental pulses fall back to zero.

As the pulse length increases, the initial U_n response is allowed to fall back towards zero. The result is that for longer pulses, the negative going edge and subsequent ringing have an increased effect. For similar field strengths, a 5 μs pulse results in U_n amplitude change from 0.24 V to 0.36 V, a 50% increase (not shown). For these cases, the peak amplitude of the ringing is 46–52% that of the pulse amplitude and lasts for less than 200 ns.

3.5. Experimental results

Experiments were conducted with an initial sample temperature between 22 and 25 °C. At 4000 V/cm all experimental groups resulted in a temperature rise less than 3.5 °C. Representative temperature profiles for experiments with 50 μs and 250 ns constitutive pulses are shown in Fig. 7. The temperature increase for bursts with 250 ns pulses is similar to the increase for longer duration pulses. This is likely due to the delivery of an equivalent quantity of energy in each burst regardless of the duration of the constituent pulses. The starting temperature of the experiments ensured that the temperature never rose above 37 °C, mitigating the possibility of temperature as a confounding factor, affecting the viability of cells.

Fig. 8 shows the viability of the samples 1 and 24 h after treatment for field strengths of (Fig. 8A) 1500 V/cm, (Fig. 8B) 3000 V/cm, and (Fig. 8C) 4000 V/cm. There is a clear inverse relationship between constituent pulse length and viability, with longer duration pulses resulting in a lower viability for both the 1 and 24 hour viability studies.

Specifically, at 1500 V/cm, bursts containing 50 μs pulse (2×) resulted in a 1 h post-treatment viability of 31% which reduced to 3% after 24 h. The 1500 V/cm bursts containing pulses between 250 ns (400×) and 10 μs (10×) resulted in 1 h viability above 50% and notably, pulses 2 μs (5×) and shorter had viabilities of 85% or greater, similar to sham treatments. For this field strength, bursts containing 10 μs pulses had the largest change in viability over 24 h, 49%, while 250 and 500 ns pulses resulted in a negligible change in viability compared to controls. Significant changes in viability occurred between the 1 and 24 hour time points for bursts with pulses 2 μs and longer. It is interesting that 10 and 50 μs pulses resulted in delayed cell death, however, the mechanism of action is unclear.

Cell viability was significantly lower for 3000 V/cm versus 1500 V/cm bursts when the pulse duration was 1 μs or longer. After 24 h, the
viability for 2 to 50 μs pulses reduced to less than 5% at 3000 V/cm. Between 3000 V/cm and 4000 V/cm, the most significant impact on viability occurred for 500 ns pulses. For all field strengths, 250 ns pulses have a minimal impact on cell viability.

For bursts containing 250 ns pulses, the difference in viability after 1500, 3000, and 4000 V/cm treatments was not statistically significant (α ≤ 0.1). All other pulse-widths had a statistically significant difference between the 1500 V/cm and 3000 V/cm treatments at each time point (α ≤ 0.06). Between the 3000 and 4000 V/cm treatments, 5 μs (1 h), 500 ns (1 h), and 500 ns (24 h) groups had statistically different viabilities (α ≤ 0.03).

Interestingly, this study shows that viability is not directly correlated to the energy dose delivered. This conforms to the results presented by others that electropermeabilization [60] and lethal [28] effects of monopolar pulses of different pulse widths exhibit a complex relationship that cannot be correlated to the quantity of energy delivered alone. The inverse correlation between pulse length and toxicity presented may be related to the cell membrane charging time, calculated here as between 1.11 and 7.92 μs. Fig. 9 shows the effect of multiple pulses within each burst on the time in which U_m and U_n are elevated above critical thresholds. A single cycle of 1500 V/cm 250 ns pulses, one positive and one negative, increases U_m above 1 V for only 200 ns total. However, the cumulative effect of the full burst (400 total pulses) increases U_m above 1 V for approximately 40 μs. At 1500 V/cm (Fig. 9A–B) time above the 1 V threshold increases as constitutive pulse width increases. This protocol reaches a maximum of approximately 99.8 μs for bursts with 50 μs constitutive pulse widths, which only have one cycle. At 3000 and 4000 V/cm (Fig. 9E–F, I–J), bursts of shorter pulses elevate U_m above 1 V for a longer duration than those with longer pulse durations. This is additional time is due to the multiple charge/discharge cycles of the cell membrane which occur during a burst of shorter pulses, however, this appears to have a negligible impact on cell viability. Though not examined here, pulses energized for less than the membrane charging time may result in limited pore expansion, minimizing lethal effects.

At 1500 V/cm, none of the pulse durations elevated U_n above 0.5, 0.75, or 0.9 V (Fig. 9C–D). The voltages 0.5, 0.75 and 0.9 V were chosen rather arbitrarily, to demonstrate the induced voltage on the nuclear envelope for each set of pulse parameters used. 1.0 V threshold was not reached for any simulation. At 3000 V/cm (Fig. 9G–H), all pulse durations are able to increase U_n above the 0.5 V threshold. The cumulative impact of a full burst results in U_n increasing above 0.5 V for a substantially longer duration for shorter constitutive pulses. At 4000 V/cm (Fig. 9K–L), some pulse durations are able to elevate U_n above 0.75 and 0.9 V. Interestingly, 500 ns pulses result in greater cumulative time above all of the voltages (0.5, 0.75, and 0.9 V) than any other pulse durations. This may help explain why 500 ns bursts resulted in significant changes in viability between 1 and 24 h and Fig. 9.

Bursts have cumulative effect on the time membrane potentials which are above critical thresholds: The time, t/μs, for which the cell membrane or nuclear envelope is greater than a critical threshold is presented as a function of pulse width, Δt_p/μs. [A, B, E, F, I, J]. Time for which the cell membrane has a potential drop (U_m) greater than 1 V. [C, D, G, H, K, L]. Time for which the nuclear envelope has a potential drop (U_n) greater than 0.5, 0.75, or 0.9 V. Values were calculated using an idealized waveform with 10 ns rise and fall times. A single cycle [one positive and one negative pulse] may increase the transmembrane potentials only briefly, however, cumulative effect of having multiple pulses per burst substantially increases the duration that these cellular components experience an elevated potential.
250 ns did not. However, the exact mechanism cannot be determined from this study.

For all bursts containing pulses 1 µs in duration or longer, the viability at 3000 V/cm after 24 h is lower than the corresponding viability at 4000 V/cm after 1 h. This has interesting implications for in-vivo applications as it indicates that ablation sizes may grow over time and that immediate observation may be inadequate to predict the total volume treated. From the numerical simulations, it is anticipated that cells with a larger cytoplasm–nucleus ratio will achieve higher $U_h$ amplitudes than cells of similar size with a smaller ratio. A high nucleus–cytoplasmic ratio (NCR) has been associated with the aggressiveness of malignant cells and is used as a parameter in grading cancers [61–64]. Additionally, it has been shown that an increase in invasiveness and metastatic potential has been correlated to cell membrane ruffling, which leads to higher membrane capacitances in aggressive cells [59,65,66].

4. Conclusion

We found, through finite element simulations, that by reducing the delay between consecutive bipolar pulses, it would be possible to achieve a doubling of the nuclear transmembrane voltage, hence, possibly promoting intracellular effects induced by electric pulses. We also found that the NCR and cell membrane permittivity play a significant role in the charging characteristics of the nuclear envelope and that cancer cells may possess some properties which would result in more profound intracellular effects as compared to normal cells. However, our simplified model has some limitations. Cells were modeled as simple spheres to reflect the shape of the cells in their non-adhered state. Media composition, cell shape, adherence, temperature, repetition rate, waveform shape, and cell cycle are known to alter the response of cells to pulsed electric fields. In vivo, cells typically take on more complex, elongated, or spindled shapes which can alter the effects of pulsed electric fields on transmembrane potential. Cells in tissue are affected by local inhomogeneity and the responses of cells in their immediate vicinity which was not accounted for here.

NCR, cytoplasm conductivity, and cell membrane permittivity play a significant role in the charging characteristics of the nuclear envelope. Experimentally we found that bursts of bipolar square waves increased the media temperature less than 3.5 °C when the total energized time per burst was held constant at 100 µs and eighty bursts were delivered. The resulting cellular responses are therefore limited to those related directly to non-thermal phenomena. For the bursts of bipolar pulses presented, there exists an inverse correlation between pulse-width and toxicity despite the delivery of equal quantities of energy in each burst. The changes in cellular viability over the 24 h post treatment show presence of both instantaneous and delayed cell death processes, however, the exact mechanisms are unknown.

To the best of our knowledge, this is the first experimental parametric analysis on the effects of bipolar square wave bursts with pulses between 0.25 and 50 µs. In the 3000 V/cm treatment groups, cell viability was reduced to 4%, 0.5%, 0.3%, and 1.0% for bursts containing 2, 5, 10, and 50 µs pulses, respectively. In the 4000 V/cm treatment groups, cell viability was reduced to 3.8%, 1.4%, 0.9%, 0.8%, and 0.8% for bursts containing 2, 5, 10 and 50 µs pulses, respectively. Rubinsky et al. [67] showed that ten 100 µs monopolar pulses at 2000 V/cm resulted in a viability of 70%. In the same study, they showed that seventy-five 100 µs monopolar pulses at 250 V/cm resulted in a viability of 10–20% while ninety 100 µs monopolar pulses at 250 V/cm reduced viability to 0–10%.[14] showed that after eighty 100 µs monopolar pulses at 1500 V/cm, cell viability was approximately 8% and this protocols is consistent with those currently being employed successfully in clinical applications of irreversible electroporation in the prostate [68], pancreas [8], and liver [69]. The comparable level of toxicity resulting from the bipolar burst protocol presented here indicates that it may be advantageous in in-vivo therapies where muscle contractions due to longer duration monopolar pulses are undesirable.

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References


